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Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001410
Article Type:	Research
Date Submitted by the Author:	03-May-2012
Complete List of Authors:	Xuan, Jim W; Lawson Heath Research Institute, Surgery
Primary Subject Heading:	Diagnostics
Secondary Subject Heading:	Diagnostics, Oncology, Pathology
Keywords:	Biochemistry < BASIC SCIENCES, HISTOPATHOLOGY, MOLECULAR BIOLOGY, Oncogenes < ONCOLOGY

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TRIM59, a novel multiple cancer biomarker for immunohistochemical detection of tumorigenesis

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Running title: TRIM59, a novel multiple-cancer marker for tumorigenesis

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List of abbreviations

Abbreviations: TRIM: TRIpartite Motif; CaP: prostate cancer; BPH: benign prostatic hyperplasia; PIN: prostate intraepithelial neoplasia; TMA: tissue microarray; RCC: renal cell carcinoma; SCC: squamous cell carcinoma; GI: gastrointestinal; GU: genitourinary; d.p.c: days post conception; GEM (genetically engineered mouse); H&E: haematoxylin and eosin; IHC: Immunohistochemistry; WD: well differentiated ; MD: moderately differentiated ; PD: poorly differentiated.

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Abstract

Objectives and design: We identified a novel TRIM59 gene, as an early signal transducer in two (SV40Tag and Ras) oncogene pathways in murine prostate cancer (CaP) models. We explore its clinical applications as a multi-tumor marker detecting early tumorigenesis by immunohistochemistry.

Setting and Participants: 88 CaP patients were from a tissue microarray (TMA) of radical prostatectomy specimen, 42 patients from a 35 multiple-tumor TMA, 75 patients with renal cell carcinoma (RCC) and 92 patients from 8 different tumor groups (breast, lung, parotid, gastrointestinal, female genital tract, bladder, kidney and prostate cancer).

Results: TRIM59 up-regulation specifically in tumor area was determined by immunohistochemistry in 291 cases of 37 tumor types. To demonstrate that TRIM59 up-regulation is "tumor-specific", we characterized a significant correlation of TRIM59 IHC signals with tumorigenesis and progression, while in control and normal area, TRIM59 IHC signal was all negative or significantly low. TRIM59 protein up-regulation in prostate and kidney cancers was detectable in both intensity and extent in early tumorigenesis of PIN (prostate intraepithelial neoplasia, $P<0.05$) and grade 1 of RCC ($P<0.05$), and stopped until high grades cancer. The results of the correlation in these two large cohorts of tumor types confirmed and repeated murine CaP model studies. Enhanced TRIM59 expression was identified in most of the 37 different tumors, while the highest intensities were in lung, breast, liver, skin, tongue and mouth (squamous cell cancer) and endometrial cancers. Multiple-tumor up-regulation was further confirmed by comparing relative scores of TRIM59 IHC signals in eight tumors with a larger patient population; and by a mouse whole mount embryo (14.5dpc) test on the origin of TRIM59 up-regulation in epithelial cells.

Conclusions: TRIM59 may be used a novel multiple-tumor marker for immunohistochemical detecting early tumorigenesis and could direct a novel strategy for molecular targeted diagnosis and therapy of cancer.

Keywords: TRIM59, Cancer biomarker, Immunohistochemistry, Prostate cancer, Multiple cancer marker, Ras signal pathway, SV40Tag oncogene, Tissue microarray, Confocal microscopy.

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Introduction

The TRIM (*TRI*partite *M*otif) family is an evolutionarily conserved gene family implicated in a number of critical processes including immunity [1-3], antiviral [4-8], proliferation [6, 9], transcriptional regulation [6, 10], neuro-development [11, 12], cell differentiation [12] and cancer [13] (reviewed in [1, 5, 14, 15]). However, the function of most TRIM family members is poorly understood and was surmised only based on computational analysis from their RBCC (*R*ING finger, *B*-box, Coiled-*C*oil) domains. The domain of RING (*R*eally *I*nteresting *N*ew *G*ene) is frequently involved in proteolysis acting as E3 ubiquitin ligases and the ubiquitin-proteasome system in the regulation of numerous cellular processes including cell cycle regulatory proteins, transcription factors, and signal transducers [6, 15]. Recent studies demonstrate that various TRIM-NHL proteins function as cofactor for the microRNA-induced silencing complex (miRISC) [12] [14, 16]. An ataxia-telangiectasia group D complementing gene (ATDC) was recently designated as TRIM29, which is elevated in most invasive pancreatic cancers in the Wnt/β-catenin signalling pathway [13].

In a previous report [17], we characterized the function of TRIM59, a novel TRIM family member, in SV40 Tag oncogene directed genetically engineered mouse (GEM) prostate cancer (CaP) models [18-21]. The TRIM59 gene was identified to be correlated with the SV40 Tag initiated tumorigenesis. TRIM59 protein up-regulation and hyper-phosphorylation started in the prostate cytoplasm in early tumorigenesis from PIN (prostate intraepithelial neoplasia) [17]. As a signal pathway effector, the p-Ser/Thr phosphorylated TRIM59 proteins correlate with tumorigenesis, while p-Tyr-TRIM59 proteins correlate with advanced CaP. The function of TRIM59 was identified by shRNA knockdown in human CaP cells resulted in S-phase arrest and cell growth retardation. Although TRIM59 is an effector gene with the SV40Tag oncogene, the

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initial functional targets of TRIM59 function were actually on the Ras signal pathway as an early and rapid signal transmitter. In a transgenic mouse test of TRIM59 up-regulation specifically in the prostate, TRIM59 demonstrated full oncogenic activity in directing tumorigenesis and progression to highest grade CaP. The signal pathway of TRIM59 may be possibly linked with two large oncogene routes: the Ras/Raf/MEK/ERK/P133K/AKT and the SV40 Tag/p53/pRB routes [(17)]. In this study we explored the clinical utility of the novel TRIM59 gene to serve as a biomarker for a panel of human cancers. To address the molecular biology basis, we combined results from both basic (animal model [(17)]) and human clinical studies.

Materials and Methods

Patient selection:

All patient samples were acquired as part of REB (Research Ethics Board) approved protocols at the University of Western Ontario (UWO) and Vancouver Prostate Center, University of British Columbia (UBC). Table 1 shows a complete list of 291 patients with 37 different tumor types examined in this study.

Prostate cancer Tissue Microarrays (TMA): 88 CaP patients between 2006 and 2008 who had no treatment prior to radical prostatectomy, were selected from the Vancouver General Hospital. Each patient block marked as containing benign tissue or cancer was sampled 2 times with a core diameter of 1 mm arrayed (176 cores) in a rectangular pattern with 0.7 mm between the centers of each core, creating a duplicate TMA layout using a manual tissue micro arrayer (Beecher Instruments, Silver Spring, MD). The TMA paraffin blocks, were sectioned into 0.5 micrometer sections and mounted on the positively charged slides.

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Table 1 Patient list selected in this study

Organ	Patient Number	Tumor type		Tumor grade	
35 tumor-TMA	42	Description in Table-2			
Prostate TMA	105	Adenocarcinoma		BPH	16
				PIN	4
				Gleason score 4	5
				Gleason score 6	31
				Gleason 7	15
				Gleason score 8	16
				Gleason score 9-10	9
				Stroma	3
				Absent cores	6
Kidney	75	Clear cell carcinoma	43	Grade 1	4
		Papillary RCC	11	Grade 2	38
		Chromophobe RCC	13	Grade 3	28
		Cystic RCC	6	Grade 4	5
		Sarcomatoid RCC	2		
Bladder	44	Urothelial carcinoma		Low grade	38
				High grade	6
Lung	4	Bronchoalveolar carcinoma	1	Grade 1	1
		Adenocarcinoma	1	Grade 2	1
		Large cell carcinoma	1	Grade 3	1
		Squamous cell carcinoma	1	Grade 4	1
Breast	3	Invasive lobular carcinoma	1	Grade 1	1
		Invasive mammary carcinoma	2	Grade 3	2
Female Genital tract	5	Endometrial carcinoma	4	Grade 1	4
		Ovary, Endometrioid carcinoma	1	Grade 2	1
Gatrointestinal tract	2	Colon carcinoma	1	Low grade	1
		Pancreas neuroendocrine carcinoma	1	high grade	1
Parotid	3	Mucoepidermoid carcinoma	1	low grade	1
		Metastatic SCC	1		
		Metastatic neuroendocrine Carcinoma	1	high grade	2
Mouth, tongue and larynx	4	Squamous cell carcinoma	4	Moderately differentiated	3
				Moderately to Poorly differentiated	1
Total	291				

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Automated image, acquisition and analysis on immunohistochemical staining of CaP-TMA: (UBC) Immunohistochemical staining was conducted by Ventana autostainer model Discover XT ™ (Ventana Medical System, Tuscan, Arizona) with enzyme labelled biotin streptavidin system and solvent resistant DAB Map kit. TMA was scanned by Bliss Digital imaging system using x20 objective, from Bacus Laboratories INC, Centre Valley PA, and stored in the Prostate Centre Saver (http//bliss.prostatecentre.com). A value on a four-point scale assigned to each core.

Multiple–tumor Tissue Microarray construction: Tissue samples form 42 patients that encompassed 35 distinct tumor subtypes were selected from London Laboratory Service Group, and the tumor bank in the Department of Pathology (UWO). TMA slides were constructed with triplicate cores for each sample following standard procedure as described [24]. 0.6 mm sections were prepared from TMA block and re-stained by H&E for each case to confirm the diagnosis.

Histopathologic analysis: All cases from 37 tumor types were graded according to standardized histopathology grading systems [25] by M.M (pathologist) and V. K (MD fellow).

Immunohistochemistry (IHC) and results evaluation: Standard ABC (Avidin Biotin Complex) protocol was performed as previously reported [21, 26, 27]. Two kinds of blocking reagents were used: Power Block (Universal Blocking Reagent, from BioGenex, San Ramon, CA) and Avidin-biotin blocking reagent kit (Vector Labs, Burlingame, CA). All B-Raf antibodies were from GenScript (Piscataway, NJ): B-Raf antibody (monoclonal antibody, used 1:500 dilution), Raf 1 (polyconal, 1:50) and Raf -1 -(polyclonal, Phospho-Ser^{259} 1:50).

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TRIM59 IHC staining signals were assessed by intensity for cytoplasmic staining and extent to assess percentage of nuclear staining as previously reported [28, 29]. Since in some tumors TRIM59 showed only cytoplasmic staining, for the purpose of comparing in different tumors, we used a combined relative score system based on both intensity and extent as following: score 0: 0/0 (intensity/ extent); score 1: weak cytoplasmic staining and/or $\leq 25\%$ nuclear staining; score 2: moderate cytoplasmic staining and/or $\leq 50\%$ nuclear staining; score 3: strong cytoplasmic staining and/or $\geq 50\%$ nuclear staining. All relative scores were accessed by two researchers independently M.M (pathologist) and V. K (MD fellow).

Construction and characterization of TRIM59 antibody:

Supplement Fig. 1 provided details, which combined Supplement materials of [17], and more informations. Supplement Fig. 1A showed that the antibody TRIM59#72 used for mouse and human studies was prepared from C-terminal 136 a.a. peptide (as recombinant GST- fusion protein, Suppl. Fig. B) of whole protein 403aa which is TRIM59 specific, while antibody TRIM59#71 was from mouse TRIM59 N-terminal sequence (163aa) containing mostly the common RBCC function domains of more than 70 members TRIM family [17]. As shown in Supplemental Fig. 1C, Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed multiple bands. We also demonstrated that N-terminal sequence specific antibody TRIM59#71 can recognize purified proteins from C-terminal sequence specific, TRIM59#72 Ab affinity column (the same 53kDa protein, Suppl. Fig. 1D). Since these two immunogenic sequences are not overlapped, we thus verified that they are detecting the same protein. We demonstrated that TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots (Suppl Fig. 1E).

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Confocal microscope imaging of immunostaining of mouse embryos 14.5 dpc (days post conception) for TRIM59: The mouse embryos were prepared according to our previous reports [18-21]. The anti-rabbit secondary antibody was conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA). Sections were double-stained with pan-cytokeratin antibody with FICT (Sigma), and the stained mouse embryo sections were visualized using a Carl Zeiss confocal microscope by the LSM Image program.

Statistical Analysis: Student's *t* tests and one-way ANOVA were used by programs of Microsoft Excel 2007 or SPSS 10 to analyze the data with $p < 0.05$ considered to be statistically significant.

Results and Discussion

TRIM59 up-regulation in human prostate cancer TMA (tissue microarray): correlation with tumorigenesis and tumor progression by TRIM59 intensity until high grade CaP:

We designated TRIM59 as one of the "tumorigenesis–associated" genes correlated with SV40 Tag oncogenesis in mouse prostate cancer (GEM-CaP) models [17]. SV40 Tag is essentially only required for the initiation of tumorigenesis, *i.e.* the "hit-and-run" effect, in GEM-CaP, but not for the tumor progression and metastasis directly. The "tumorigenesis–associated" effect, is due to the initial binding of the Tag oncogene with retinoblastoma (pRB), p53 proteins and several transcriptional co-activators. Once this process is initiated, the signal-transduction will continue on, even without the initiation effectors.

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In GEM-CaP models, the TRIM59 protein up-regulation correlation with tumorigenesis and progression, and down-regulated in the high grade CaP by immunohistochemistry (IHC) [17]. We assume this "tumorigenesis–associated" effect of TRIM59 may apply to human cancer studies.

We first characterized that TRIM59 antibody (#72) can cross react with and recognize specifically human TRIM59 counterpart (details see supplemental Fig. 1). We tested by IHC in a TMA of CaP patients (n=88, 176 cores). TRIM59 IHC signals detected in an automated digital image system were identified mostly in cytoplasm of luminal cells (Fig. 1A), which is different from rapid tumor progression mouse CaP models. The intensity (score= 2) in PIN (n=4, Fig. 1A) was higher than in non-tumor area (normal and BPH). Moderate to strong expression was observed in Gleason score 6 (3+3, n=25), 7 (4+3, n=15), and 8 (4+4, n=14). In high grade CaP (score 4+5, n=8, Fig. 1A), TRIM59-IHC signals were lower. As shown in a graph of Fig. 1B, TRIM59 protein IHC signals correlated significantly (P=0.014) with tumorigenesis and progression from PIN to -WDCaP (Gleason grade 1-2, scores 2-4) and MDCaP (Gleason grade 2-4, scores 4-8) (graph Fig 1B), and decreased in high grade CaP (Gleason score 9-10) with P=0.018, which is similar to GEMs [17].

TRIM59 up-regulation in human Renal Cell Carcinoma (RCC) patients: correlation of tumorigenesis and tumor progression with TRIM59 intensity until high grade RCC:

Next, we demonstrated that TRIM59 is not androgen-responsive and likely not prostate tissue-specific, indicating that the tumorigenesis role of TRIM59 may be applied to all or most of human cancers. Supplemental Fig. 2 showed this result in detail of IHC test by TRIM59 antibody#72 on a TMA of the LNCaP human CaP xenografts in nude/SCID castrated mice.

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We extended results from prostate cancer clinical samples to kidney cancer. We started 75 renal cell carcinoma (RCC) patients including all 5 different types of RCC tumors: 43 clear cell carcinoma (representative IHC figures shown in Fig. 2 A-D), 11 papillary renal cell carcinoma (Fig. 2 E-F), 13 chromophobe renal cell carcinoma (Fig. 2 G-H), 2 sarcomatoid renal cell carcinoma (Fig. 2J), and 6 cystic renal cell carcinoma (Fig. 2 K-L). RCC cases analyzed with Fuhrman grade 1-4 were 4, 38, 28, and 5 respectively. TRIM59-IHC staining in normal area including proximal tubules was negative (Fig. 2 last row). Background staining was eliminated by testing antibody dilutions (1:100, 200, 500, 1200 till 5000) while tumor specific signals of TRIM59 proteins were noticeable. Endogenous biotin signals were blocked and excluded by additional block reagents (avidin-biotin blocking reagent kit).

TRIM59 IHC staining in tumor areas in RCC (Fig. 2) was different from cases of CaP-TMA (mainly cytoplasmic). TRIM59 IHC staining was found in both cytoplasm and nucleus in all RCC grades and types (Fig. 2). We assessed TRIM59-IHC by visual scoring of both intensity (cytoplasmic staining) and extent (% nucleus staining) microscopically. Correlation of TRIM59 IHC signals by scoring the intensity in cytoplasm with grades of all five types of RCC is shown in Fig. 2M. TRIM59 IHC signals were increased with tumor progression from grade 1-3 ($p<0.05$). All grade 1 tumors (n=4) stained with weak TRIM59 IHC signals in cytoplasm, but with high extent of nuclear staining; while all grade 2 and 3 tumors (n=66) showed moderate to strong cytoplasmic staining intensity of TRIM59. All grade 4 tumors (n=5) showed weak to moderate intensity in cytoplasm of TRIM59 staining. No correlation between TRIM59 IHC staining in nucleus and tumor grade was found, although ~~in~~ low grade RCC showed higher nuclear staining.

Therefore by systematic IHC studies in CaP (88 patients, Fig. 1) and kidney cancer (75 patients, Fig. 2), we almost exactly repeated results from our mouse model studies on TRIM59

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[17]. We confirmed TRIM59 as an immunohistochemistry marker able to detect low grade tumor in early tumorigenesis. We also demonstrated the correlation of tumorigenesis and tumor progression with TRIM59 up-regulation until high grade tumor.

TMA analysis of TRIM59 protein expression demonstrates that TRIM59 is a multiple tumor marker:

In the basic research previously using animal GEM-CaP models [17], we have disclosed that TRIM59 up-regulation is involved in two oncogene families and two signal pathways of SV40Tag/pRB/p53 and Ras/Raf/MEK/ERK. TRIM59 may function as an early signal transducer in Ras signal pathway with bridging genes in two oncogene pathways [17].

While it was rarely reported that SV40 Tag oncogene induced human cancer, Ras mutations are among the most frequent alterations in human cancers (see review [22]). We assume that TRIM59 as an early Ras signal pathway effector may possibly act as a multiple tumor marker.

We therefore further extended TRIM59 IHC studies to 35- multiple cancer TMA sections (42 tumors, 126 cores, Table 1). We tested different dilutions (1/300, 1/600, 1/1200, 1/5000) of TRIM59 antibody (see Supplemental Fig. 3). To further confirm the specificity and reliability of TRIM59 antibody in IHC staining, we compared IHC staining in 35 different tumor-TMA sections with positive (TRIM59 antibody at 1:1200 and 1:5000 dilutions) and negative controls (no antibody added, details see Supplemental Fig. 4). As summarized in Table 2, TRIM59 expression was significantly and tissue-specifically up-regulated in most of these 35 tumors. When comparing the relative scores (both intensity and extent) in different tumors, the highest staining was observed in breast, lung, liver, skin (squamous cell carcinoma) and endometrial cancers.

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Further confirmation of TRIM59 as a tumor marker in patients with eight different tumors:

Since the 35 tumor-TMA contained only limited cases in each tumor type, we selected more cases (n=92) of eight different tumor types with different tumor grades, which all showed up-regulated expression of TRIM59. IHC staining of TRIM59 in eight tumors are shown: lung (n=4, Fig. 3A-C), breast (n=3, Fig. 3D-E), gastrointestinal (GI, n=2, Fig.3F-G), female genital tract (fGT, n=5, Fig.3H-J), bladder (n=44, Fig. 3K), prostate (n=27 from UWO, Fig. 3L), head and neck mucosal tumor (squamous cell carcinoma, SCC of mouth, tongue and larynx, n=4, Fig. 3M-O) and parotid gland (n=3, Fig. 3P-Q). Normal areas in lung, breast, colon, endometrial, bladder, larynx and parotid tissues showed very weak or completely negative staining (Fig. 3). Since some tumors (e.g. prostate) showed mostly cytoplasmic and no nuclear TRIM59-IHC staining, as a comparative study, we assessed their relative scores (combine both intensity and extent scores, see Materials and Methods). More tumors from kidney (RCC, n=75) and prostate cancer (n=27) were included as references and all were assessed by relative scores simultaneously, since we already analyzed a large cohort of these patients. Fig. 3R shows the comparison of the mean of IHC-TRIM59 relative scores. The highest relative scores were found in SCC of the parotid, mouth, larynx and tongue, followed by lung, breast and female genital tract cancers.

The comparison of relative scores on low and high grades separately was done (data not shown). Cases of grade 1 lung cancer (bronchoalveolar, adenocarcinoma, SCC and large cell carcinoma) and breast cancer (invasive lobular and invasive mammary carcinoma) all showed the strongest staining as compared with other tumors. In endometrial cancer, the TRIM59 relative scores were moderate in grade 1 and moderate to strong in grade 2. The three tumors of

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Table 2 Immunohistochemistry analysis of TRIM59 as multiple marker in 35 tumor TMA

Tumor Type	Patient number	Core number	Pathologic Grade	Cell Type	IHC staining	
					Cytoplasm staining (intensity)	Nuclear staining (extent)
Renal clear cell carcinoma,	2	6	2	Epithelial	moderate	-
			3		moderate	-
Adrenal gland cortical carcinoma	1	3	N/A	Epithelial	Moderate-strong	-
Squamous Cell Carcinoma, skin	2	6	WD	Epithelial	strong	-
			MD		moderate	-
Basal cell carcinoma, skin	2	6	N/A	Epithelial	moderate	-
					Moderate-strong	
Melanoma	1	3	N/A	Epithelial	weak	50% +
Endometroid adenocarcinoma	2	6	2	Epithelial	Moderate-strong	-
			1		Moderate-strong	-
Leiomyosarcoma	1	3	N/A	Mesenchymal	weak	-
Omentum serous adenocarcinoma,	1	3	WD	Epithelial	Weak-moderate	-
Ovary serous adenocarcinoma	1	3	N/A	Epithelial	Weak-moderate	-
Ovary clear cell carcinoma	1	3	PD	Epithelial	Moderate-strong	30% +
Cervix adenocarcinoma	1	3	WD-MD	Epithelial	Moderate-strong	-
Colon adenocarcinoma	1	3	Low grade	Epithelial	weak	-
Breast ductal adenocarcinoma	1	3	2/3	Epithelial	Moderate-strong	50% +
Bladder urothelial carcinoma	2	6	Low grade	Epithelial	weak	-
			2 (low grade)	Epithelial	moderate	-

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Stomach GIST	1	3		Epithelial	Weak-moderate	-
Esophagus adenocarcinoma	1	3	MD	Epithelial	0-weak	-
Thyroid, Papillary carcinoma	1	3	N/A	Epithelial	weak	-
Thyroid, medullary carcinoma	1	3	N/A	Epithelial	moderate	-
Pancreas adenocarcinoma,	2	6	2	Epithelial	Weak-moderate	-
			2		Moderate-strong	-
Pancreas endocrine tumor	1	3	N/A	Epithelial	strong	-
Lung SCC	1	3	PD	Epithelial	strong	-
Lung mesothelioma	1	3	MD-PD		strong	20% +
Lung adenocarcinoma	1	3	MD		moderate	50% +
Lung bronchoalveolar carcinoma	1	3	WD		Moderate-strong	50% +
Lung mesothelioma, biphasic	1	3	MD-PD		strong	20% +
Liver hepatocellular carcinoma (HCCa)	1	3	2/4	Epithelial	strong	-
Liver metastatic carcinoid	1	3	N/A	Epithelial	strong	-
Small bowel marginal zone lymphoma	1	3	N/A	Lymphocyte	0-weak	-
Lymph node, follicular lymphoma	1	3	1/3	Lymphocyte	0-weak	-
Lymph node, metastatic carcinoid	1	3	Low grade	Epithelial	strong	-
Spleen, Hodgkin's lymphoma	1	3	N/A	Lymphocyte	weak	-
Stomach, malt lymphoma	1	3	Low grade	Lymphocyte	0-weak	-
Thymus invasive thymoma	1	3	N/A	Epithelial	0-weak	-
Appendix, Goblet cell carcinoid	1	3	N/A	Epithelial	0	-

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squamous cell carcinoma (SCC) from mouth, tongue and larynx with different grades (Fig. 3M-O) also showed high relative scores (both intensity and extent).

As a comparative study (Fig. 3R, Table 2), we tested 44 bladder cancer cases with 38 low grade and 6 high grade tumors. The mean value of relative scores was 1.6, *i.e.* weak to moderate in bladder cancer cases . In 27 prostate cancer cases (from UWO only) tested, the relative scores of TRIM59 (cytoplasmic staining only) from PIN through Gleason scores of 10 were actually relatively weak (Fig. 3R), although in Gleason score 4, 6 and 8 were weak to moderate separately.

Thus far, we identified that TRIM59 up-regulation is "tumor-specific". First, we demonstrated the correlation of TRIM59 enhanced IHC signals with tumorigenesis and progression, which were statistically significant in m this report with 291 cases and 37 tumor types analyses. Second, although TRIM59 is a normal gene involved in CDC (cell cycle division) regulation from G1 to S-phase involved in DNA S-phase and cell growth [17], we demonstrated that in normal or non-tumor areas in all tested 37 different kinds of cancers, TRIM59 IHC staining signals were mostly negative or very low (Fig. 1, 2 3). By moderating antibody dilutions and testing various blocking reagents (Supplemental Fig. 4), we demonstrated that TRIM59 induces tumorigenesis/oncogenesis only when it is abnormally up-regulated.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Furthermore, we also confirmed that TRIM59 expression involved in multiple-tissue expression even in embryo development. We carried out IHC of mouse embryo sections by double-staining TRIM59. Cytokeratin (keratin), a family of proteins that are primarily found in epithelial cells was used as reference. Supplemental Fig. 5 illustrated confocal microscope

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images of IHC staining of TRIM59 in different organs/tissues of mouse embryo (14.5 d.p.c). TRIM59 was highly expressed in cytokeratin-expressing cells in the lung (first row), skin (second row), and kidney (not shown) of mouse embryos. TRIM59 staining in mouse embryos revealed the same pattern of the epithelium origin as in human tumors, which the TRIM59 gene were found highly up-regulated in those tumor types as well.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Given our previous experiments suggesting TRIM59 functions in the Ras pathway, we tested if TRIM59 up-regulation was correlated with the BRAF, an early signal effector Ras signal pathway (review see [22]). We selected 24 RCC patients, which previously were confirmed with up-regulation of TRIM59 expression. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing the total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing the activated phosphorylated B-Raf. Some of the serial slides were stained in parallel on each patient by different antibodies. As shown in Supplemental Fig. 6 A first two columns, in all 12 clear cell carcinoma of RCC samples, there were no or very weak B-Raf IHC signals in all three used Raf antibodies. In papillary RCC (Suppl Fig. 6 B) and chromophobe RCC samples (Suppl Fig. 6 C) (15 samples out of 24 samples or 62% of all RCC samples tested), there were higher IHC signals in all three antibodies used for staining in cancer areas specifically, showing higher intensity and extent than clear cell carcinoma (Suppl Fig. 6 A). Nuclear signals were found only by B-Raf P-Ser antibody in papillary and chromophobe tumors. Supplemental Table 1 summarizes the results. It is intriguing that in those TRIM59 up-regulated kidney cancers (RCC), neither total nor phosphorylated BRaf were detected in clear RCC (as a control), but were all highly positive in other two RCCs (papillary and

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chromophobe) tumors. We could not definitely confirm that TRIM59 was acting along the Ras pathway in all cases where it was detected.

Conclusions

This is the first report on a possible "ubiquitous" tumor marker. Ras mutations are among the most frequent alterations in human cancers that lead to approximately 30% of all human cancers with expression of constitutively active Ras proteins. In this report, we have demonstrated that there are more than mutation issues of the Ras signal pathway in tumorigenesis and progression, since TRIM59 is up-regulated as a novel proto-oncogene in a variety of human cancers.

Detection and treatment of cancer at the earliest stage is critical for patient survival. This investigation demonstrated a novel TRIM59 gene as a multiple tumor marker for early diagnosis of tumorigenesis. As a multiple biomarker associated with epithelium origin (very rarely arisen from mesenchymal tissue, see Table 2) TRIM59 maybe used as an EMT (epithelium – mesenchymal- transition) specific co-biomarker. The TRIM59 antibody may also possibly be used for molecular targeted imaging as a new diagnostic marker, for example in the context of targeted microbubble ultrasound destruction technology [23], before the protocol of a serum TRIM59 test can be established.

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Acknowledgements: This work was supported by grants from the Ontario Institute of Cancer Research (07NOV-52), the Canadian Institute of Health Research (MOP -77684), NIH-NCI (2 U01 CA084296-06) and the Terry Fox Foundation.

Legend of Figures:

Fig. 1 Correlation of TRIM59 Immunhistochemistry (IHC) staining in prostate cancer cases in TMA (Tissue Microarray) assessed by the Gleason Grading system. All panels in **A** were shown hematoxylin staining, x20. Non-tumor: weak or negative, PIN: TRIM59 is located in the cytoplasm of the luminal cells (Intensity=2). Gleason score 3+3, TRIM59 is located in the cytoplasm of tumor cells (Intensity=2). Gleason score 3+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+5, TRIM59 is located in the cytoplasm of the tumor cells (Intensity<1). Graph **B:** Correlation of TRIM59 protein levels by intensity with Gleason score in prostate cancer TMA analysis. Error Bars show mean (± SE).

Fig. 2 Immunohistochemistry analysis of TRIM59 expression in kidney cancer (RCC, Renal Cell Carcinoma) cases: correlation with tumor grade by intensity detecting early tumorigenesis. Five types of RCC with different grades were shown clear cell carcinoma (**A-D**), papillary RCC (**E-F**), chromophobe RCC (**G-I**), sarcomatoid RCC (**J**) and cystic RCC (**K-L**). **A**. Clear cell carcinoma, grade1, weak cytoplasmic staining in tumor cells, ≥ 50% nuclear staining, cytoplasmic staining in tumor cells (40X). **B.** Clear cell carcinoma, grade 2, moderate

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cytoplasmic staining in tumor cells, no nuclear staining (40X). **C.** Clear cell carcinoma, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **D.** Clear cell carcinoma, grade 4, moderate cytoplasmic staining and nuclear (%) staining (40X). **E.** Papillary RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **F.** Papillary RCC, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining, normal cortex tissue is visible adjacent to tumor area (40X). **G.** Chromophobe RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining. (40X). **H.** Chromophobe RCC, grade 3, strong cytoplasmic staining, no nuclear staining, (40X). **I.** Chromophobe RCC, grade 4, moderate cytoplasm staining and nuclear staining (40X). **J.** Sarcomatoid RCC, grade 4, weak cytoplasmic staining and nuclear staining (40X). **K.** Cystic RCC, grade 2, moderate cytoplasmic staining, no nuclear staining (40X). **L** Cystic RCC, grade 3, strong cytoplasmic staining, no nuclear staining (40X). Last two panels: normal kidney tissues (x20, x40). **M. Graph:** Correlation of TRIM59 protein levels by relative scores (both intensity and extent) with grade in RCC according to the Fuhrman nuclear grading system. Error Bars show mean (± SE).

Fig. 3 Comparison of TRIM59 expression as a multiple-cancer marker in eight types of tumor in breast, lung, parotid, gastrointestinal, female genital tract, bladder, head and neck mucosal tumor and prostate cancer. Negative TRIM59 staining in normal tissues was shown for each tumor respectively (20X). **(A)** Lung bronchoalveolar carcinoma, grade 1, strong cytoplasmic and nuclear staining of tumor cells, (40X). **(B)** Lung adenocarcinoma, grade 2, moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(C)** Lung large cell carcinoma, grade 4, moderate cytoplasmic and nuclear staining of tumor cells (40X). **(D)** Breast cancer, invasive lobular, low grade, moderate to strong cytoplasmic and nuclear staining of tumor cells (40X). **(E)** Breast cancer, invasive mammary (no specific type), high grade,

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moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(F)** Pancreas neuroendocrine carcinoma, poorly differentiated, strong cytoplasmic staining, no nuclear staining of tumor cells (40X). **(G)** Colon carcinoma, low grade, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(H)** Endometrial carcinoma, grade1, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(I)** Endometrial carcinoma, grade 2, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(J)** Ovary, Endometrioid carcinoma, grade 1, weak cytoplasmic staining, no nuclear staining (40X). **(K)** Bladder urothelial carcinoma, low grade, moderate cytoplasmic staining, no nuclear staining (40X). **(L)** Prostate adenocarcinoma, Gleason score 8, moderate cytoplasmic staining, no nuclear staining (40X). **(M)** Floor of mouth, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(N)** Tongue cancer, SCC moderately to poorly differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(O)** Larynx, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(P)** Parotid, mucoepidermoid carcinoma, low grade, strong cytoplasmic staining, no nuclear staining (40X). **(Q)** Parotid, metastatic SCC, poorly differentiated, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(R)** Graphic comparison of relative scores (both intensity and extent) of TRIM59 IHC signals in eight different tumor types. fGT: Female genital tract. Error Bars show means (± SE) of the relative scores in all grades analyzed and compared.

Legend of Supplemental data

Supplemental Fig. 1 Characterization of TRIM59 antibodies constructed and used for human tissue studies.

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Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

(A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows. **(B)**. 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). **(C-D)**. Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots. **(E)** Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145, LNCaP and human kidney cell HEK293. A unique 53 kDa band was shown exactly as in mouse cell and tissue lysates **(C)**.

Supplemental Fig. 2 From **Vancouver Prostate Center** The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP, TRIM59 is significantly up-regulated after

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10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.

Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Summary of results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 1:1200 and 1:5000 –(figures not shown but resemble to 1:1200). We assessed these IHC results with the negative control (with out TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3, 1:1200 dilution produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remained stronger than the normal non-tumor areas even at 1:300 dilution. In order to detect weak TRIM59-IHC staining signals, we tried 1:300 dilution and we compared the staining signal with negative controls (no antibody was added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining (data not shown), and similar results were obtained.

Legend of Figures: (A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular

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lymphoma, (20X), positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining.

Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry –(IHC) staining in human multiple tumor TMA —(tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody –(positive) and without any antibody –(negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some areas of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin –(squamous cell carcinoma); #4 Skin –(basal cell carcinoma); #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma; #13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma; #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma); #2 Adrenal gland (cortical carcinoma); #7 Omentum (serous adenocarcinoma); #9 Cervix adenocarcinoma.

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma); #6 Uterus (leiomyosarcoma); #8 Ovary, serous adenocarcinoma; #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma and # 25 Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid).

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Supplemental Fig. 5 Confocal microscope imaging of IHC analysis of TRIM59 expression in mouse embryo (14.5d.p.c). Patterns of multiple organ expression originated from epithelial cells were shown in the lung (first row) and skin (second row).

Supplemental Fig. 6 Test of Ras activation by B-Raf antibodies in kidney cancer tumors correlated with up-regulation of TRIM59. First column: Antibody Braf **(A)** in Clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (**B**), strong cytoplasmic staining, no nuclear staining, (40X); in Chromophobe RCC (**C**), strong cytoplasmic staining, no nuclear staining, (40X). In the second row: antibody to Raf 1, in clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (20X,40X), strong cytoplasmic staining, no nuclear staining; in chromophobe RCC, strong cytoplasmic staining, no nuclear staining (40X). Third row: Antibody to Raf 1 phosphor Ser ^{259}, in clear cell carcinoma, weak cytoplasmic and nuclear staining (40X); in Papillary RCC, strong cytoplasmic and nuclear staining (40X), in chromophobe RCC, strong cytoplasmic and nuclear staining, (40X). Arrows indicated nuclear staining.

Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

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Fig. 1

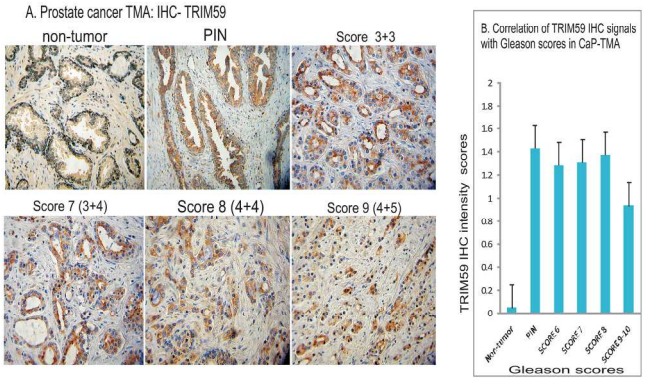

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Fig. 2 Immuno-staining of TRIM59 expression in five different types of kidney cancer (RCC)

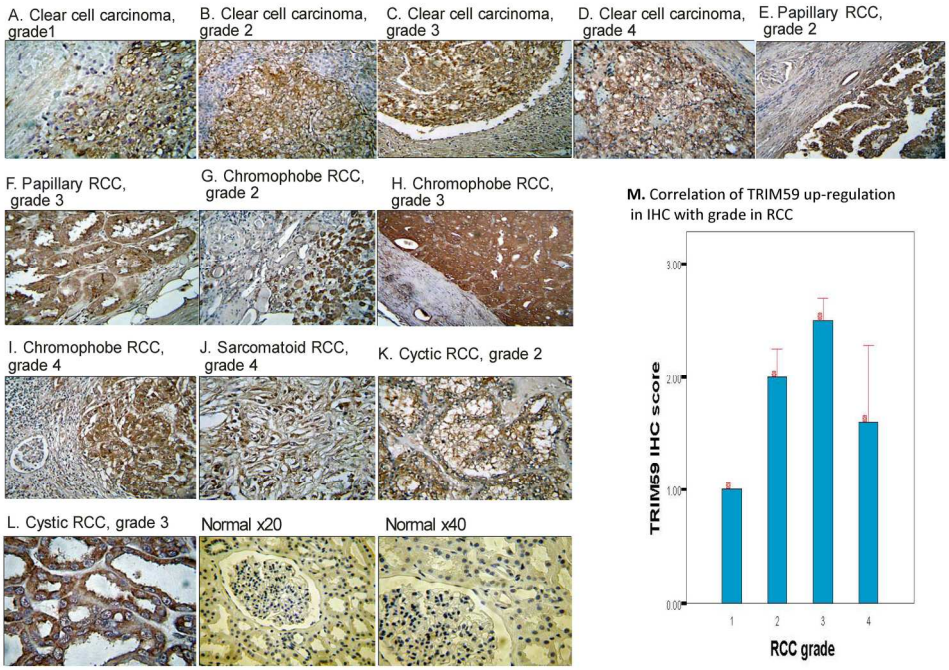

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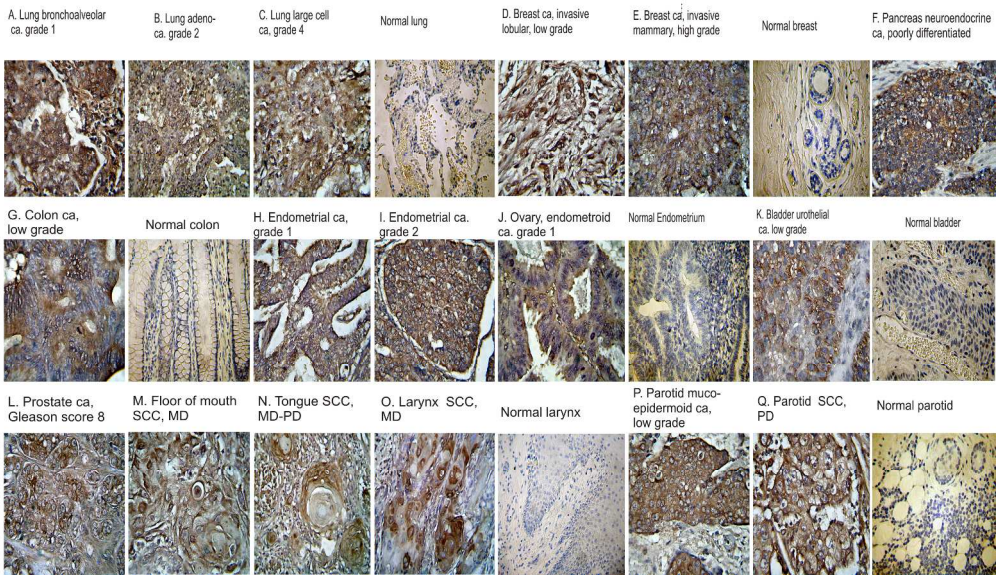
Fig. 3 Comparison of TRIM59 expression by immunohistochemistry in 8 different tumors



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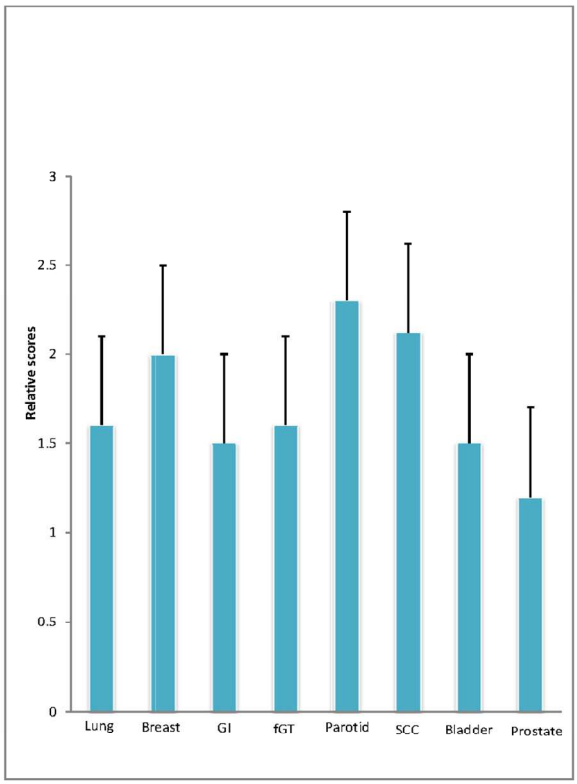
R. Comparison of relative scores of TRIM59 IHC staining in eight different tumor types



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Supplemental Fig. 1 Characterization of TRIM59 antibodies constructed and used for human tissue studies.

Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

Legend of Supplemental Fig. 1 (A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows.

(B). 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). The truncated forms of GST-TRIM59#71 may be due to bias of condon usage in the N-terminus. When immune to rabbits, we adjusted the doses by counting only the whole amount of all recombinant GST-TRIM59 proteins (1-1.5mg, GST protein was excluded).

(C-D). Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wildtype (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wildtype (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots.

(E). Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145 LnCaP and human kidney cell HEK293. An unique 53 kDa band was shown exactly as in mouse cell lyases **(C)**.

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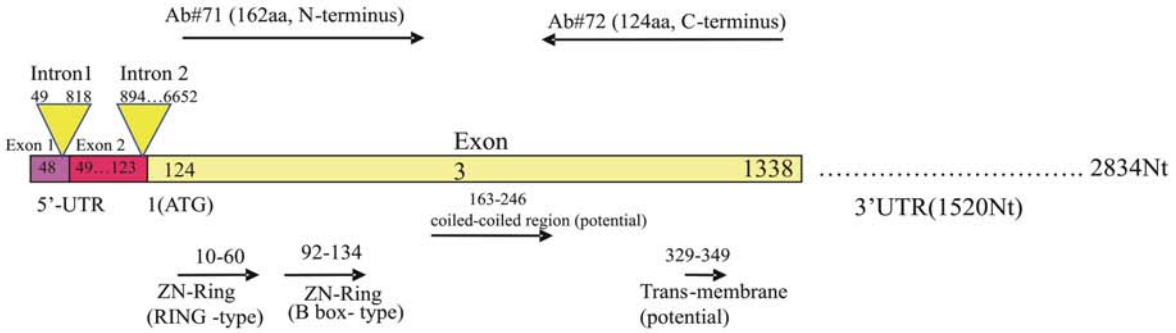
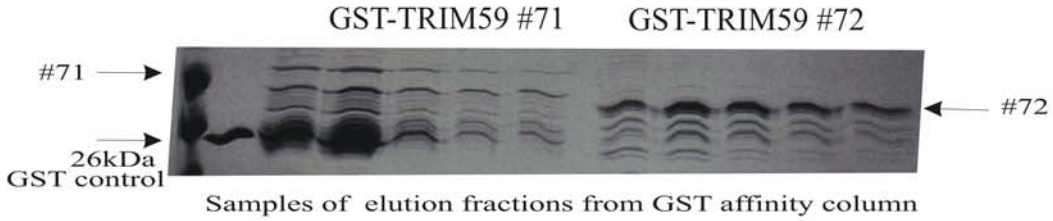
Supplemental Fig. 1

A. Structure, location of two antibodies to mouse TRIM59 GST fusion protein GST-TRIM59 #72 and /71

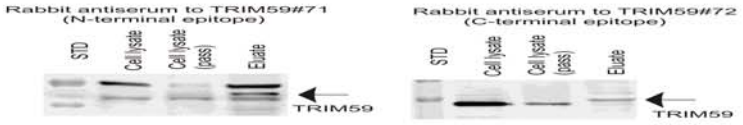


B. Purified GST-TRIM59 #72 and /71 recombinant proteins expressed in *E.coli* (10% SDS-PAGE)

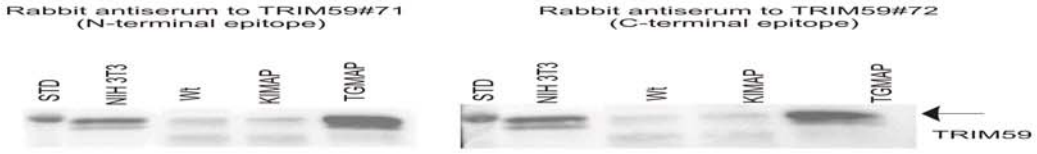


Characterization of TRIM59 antibodies
in mouse cells (C) and tissue lysates (D)

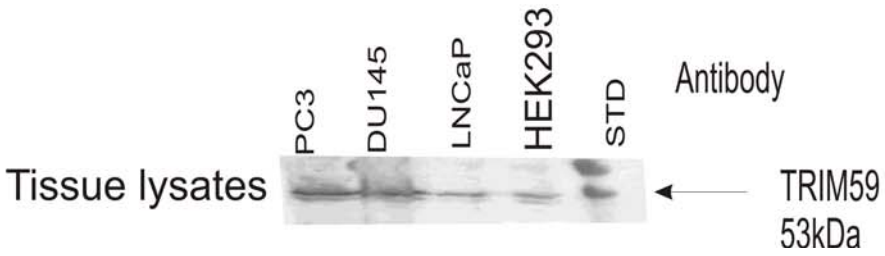
C. Total cellular phosphorylated proteins



D. Affinity purified proteins



E. Western blotting characterization of TRIM59/72 antibody in human cell lysates



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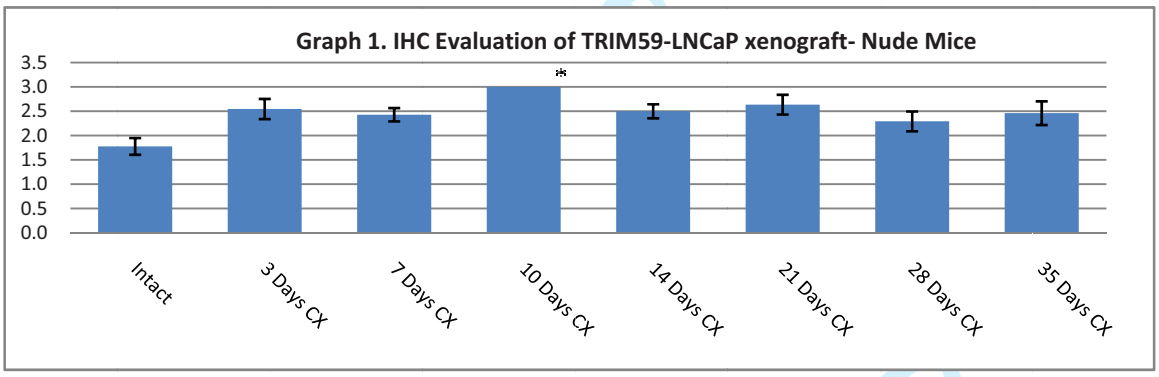
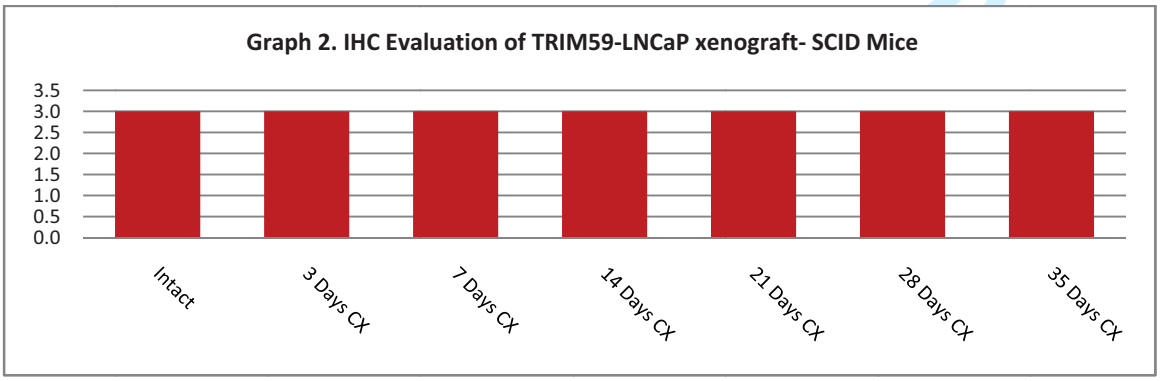
Supplemental Fig.2 **IHC evaluation of androgen responsiveness of TRIM59 in the LNCaP xenograft TMA**.

The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP , TRIM59 is significantly up-regulated after 10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.





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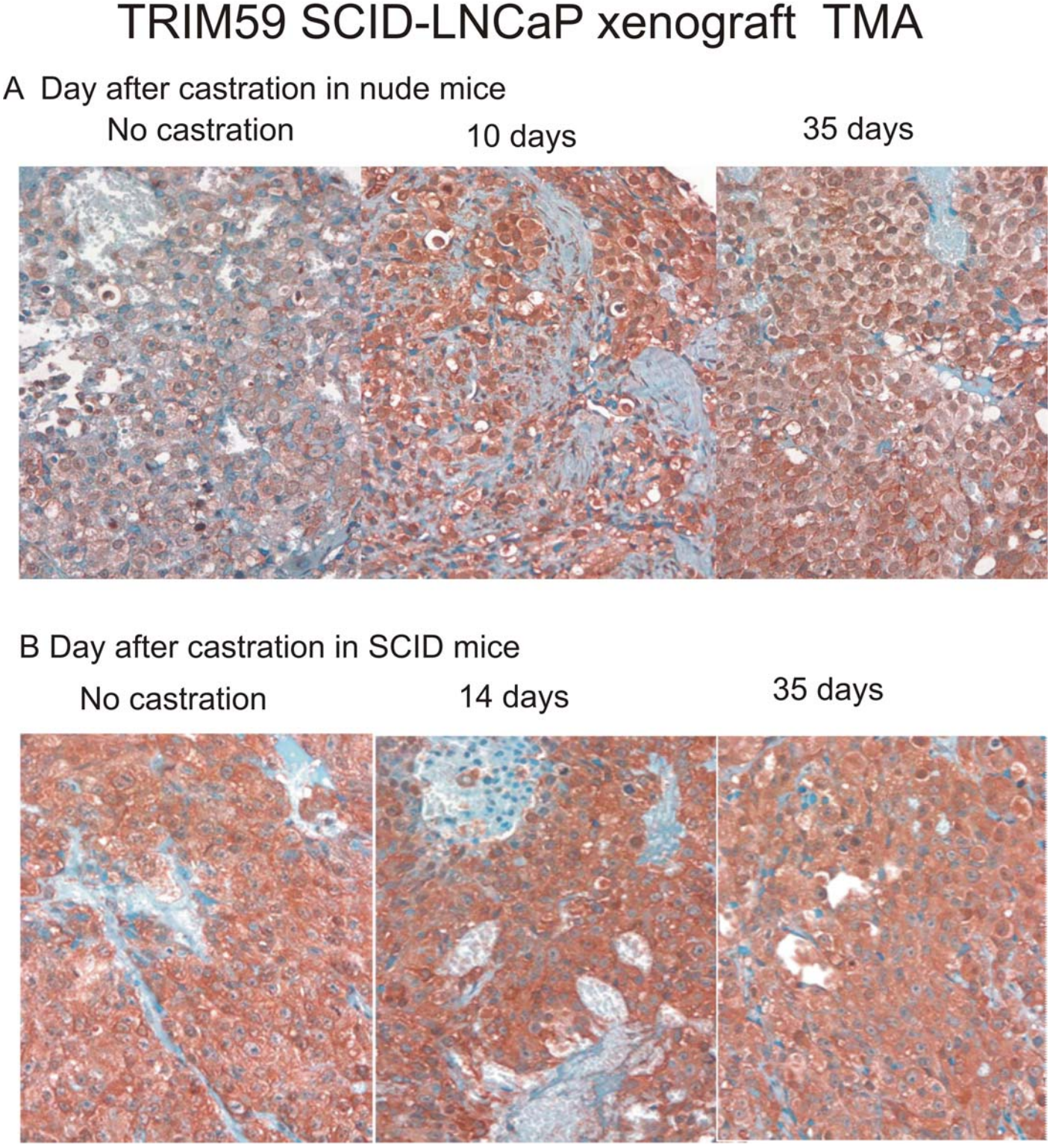
Suppl Fig. 2



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Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 and 1:1200. We assessed these IHC results with the negative control (without TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3. 1:1200 produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remain stronger than the normal non-tumor areas even at 1:300. In order to d detect weak TRIM59-IHC staining signals, we also tried 1:300 dilution and compared with negative controls (no antibody added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining, and similar results were obtained.

Legend of Figures:

(A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular lymphoma, (20X),positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining

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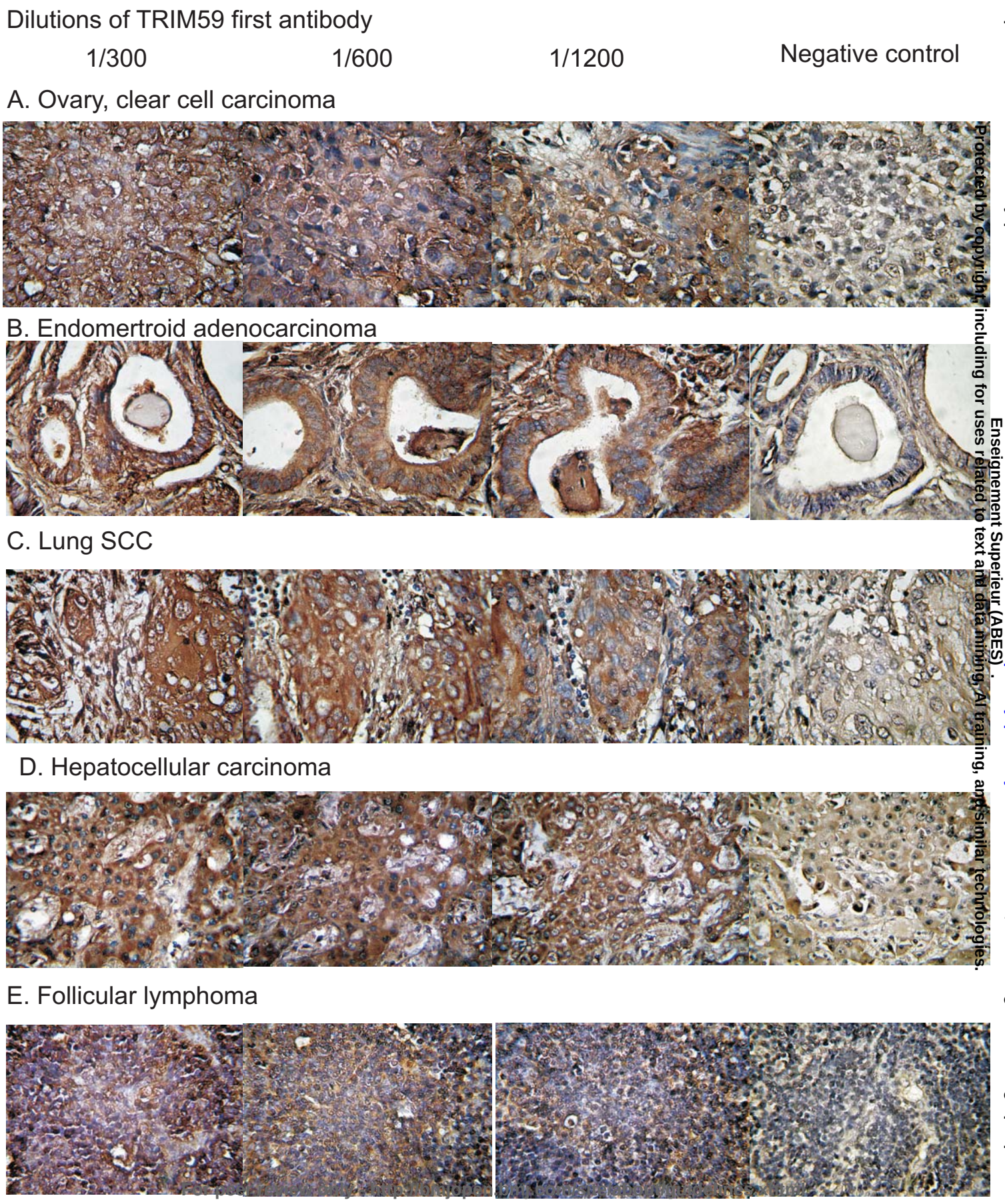
Supplemental Fig. 3 Titration of the diluted TRIM59 antibody in TMA (5 tumors) for moderate IHC staining



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Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry (IHC) staining in human multiple tumor TMA (tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody (positive) and without any antibody (negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some area of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin (squamous cell carcinoma), #4 Skin (basal cell carcinoma), #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma;#13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma, #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma), #2 Adrenal gland (cortical carcinoma), #7 Omentum (serous adenocarcinoma), #9 Cervix adenocarcinoma;

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma), #6 Uterus (leiomyosarcoma), #8 Ovary, serous adenocarcinoma, #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma;, and # 25. Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid)

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Legend of panels:

1. Kidney (Clear cell carcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

2. Adrenal gland (cortical carcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining with no nuclear staining; negative control in negative control, weak background staining in cytoplasmic of few cells (20X, 40X).

3. Skin (squamous cell carcinoma), positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

4. Skin (basal cell carcinoma), positive IHC for TRIM59 expression strong cytoplasmic staining with no nuclear staining; negative control no staining (20X, 40X).

5. Skin (melanoma), positive IHC for TRIM59 expression very weak cytoplasmic staining and nuclear staining; negative control no staining (20X, 40X).

6. Uterus (leiomyosarcoma), positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

7. Omentum (serous adenocarcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

8. Ovary (serous adenocarcinoma), positive IHC for TRIM59 expression weak to moderate cytoplasmic staining and no nuclear staining, negative control no staining (20X, 40X).

9. Cervix adenocarcinoma, positive IHC for TRIM59 expression moderate to strong cytoplasmic staining; negative control no staining (20X, 40X).

10. Breast ductal adenocarcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

11. Bladder (Urothelial carcinoma), positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

12. Thyroid medullary carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

13. Lung squamous cell carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

14. Lung bronchoalveolar carcinoma, positive IHC for TRIM59 expression strong cytoplasmic and nuclear staining; negative control no staining (20X, 40X).

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15. Lymph node metastatic carcinoid, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

16. Lung mesothelioma, positive IHC for TRIM59 expression strong cytoplasmic and nuclear staining; negative control no staining (20X, 40X).

17. Hepatocellular carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

18. Liver metastatic carcinoid, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

19. Endometrial carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

20. Small bowel, marginal zone lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

21. Spleen, Hodgkin lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

22. Stomach, malt lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining.

23.Thymoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

24. Appendix (Goblet cell carcinoid), IHC for TRIM59 expression no staining, negative control no staining (20X, 40X).

25. Follicular lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining, negative control no staining (20X, 40X).

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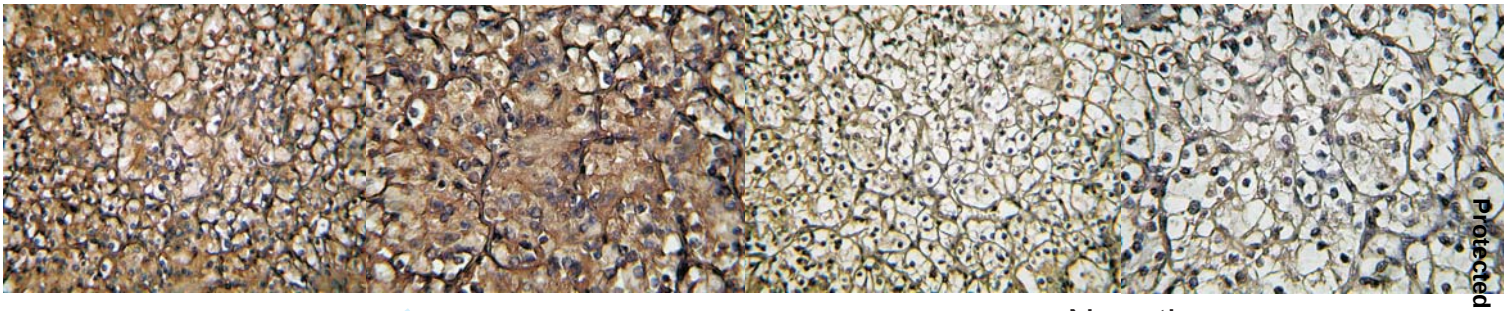
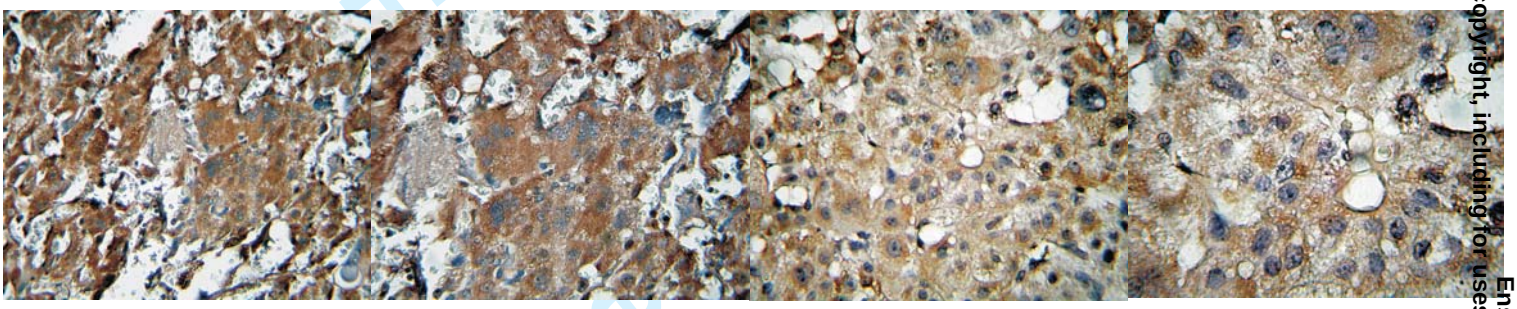
Suppl.Fig. 4 Positive and negative controls of immunohistochemistry (IHC) staining by TRIM59 antibody in 25 tumors listed from multiple-cancers TMA

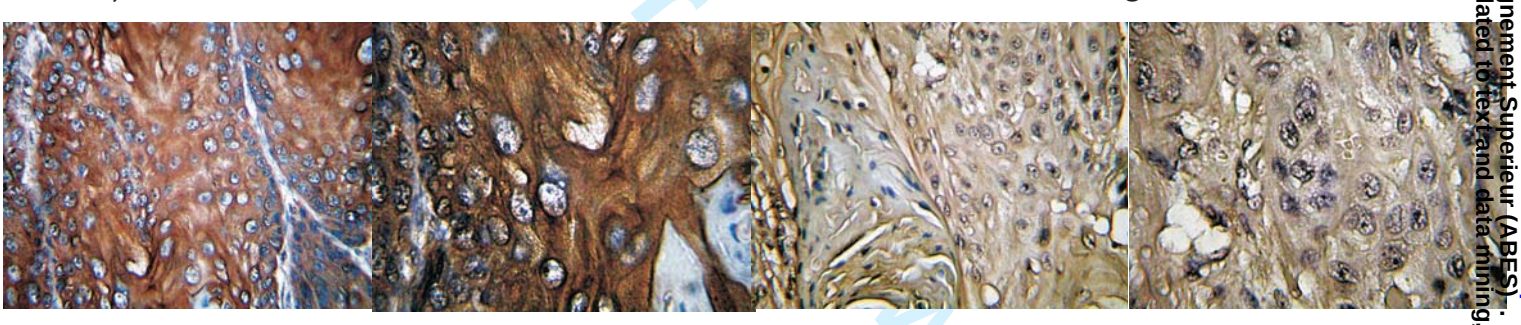
1-Kidney, clear cell carcinoma, positive (moderate) Negative



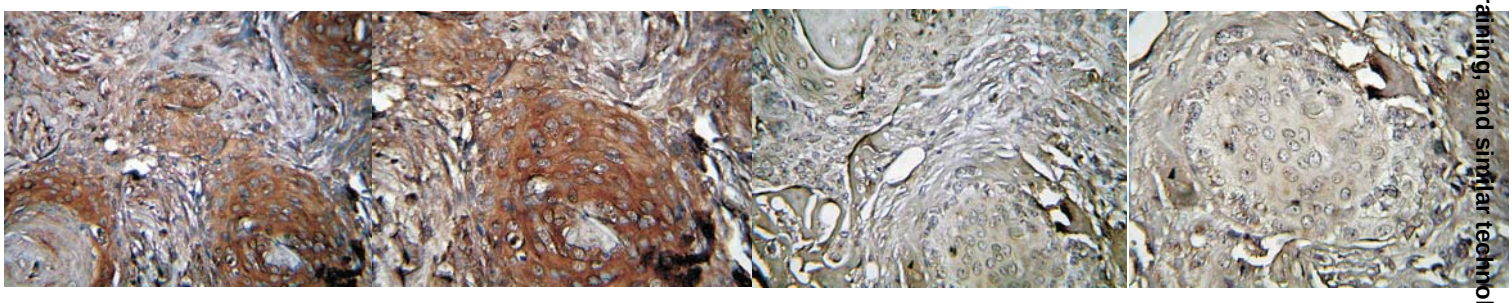
2-Adrenal gland, cortical carcinoma, positive (moderate) Negative



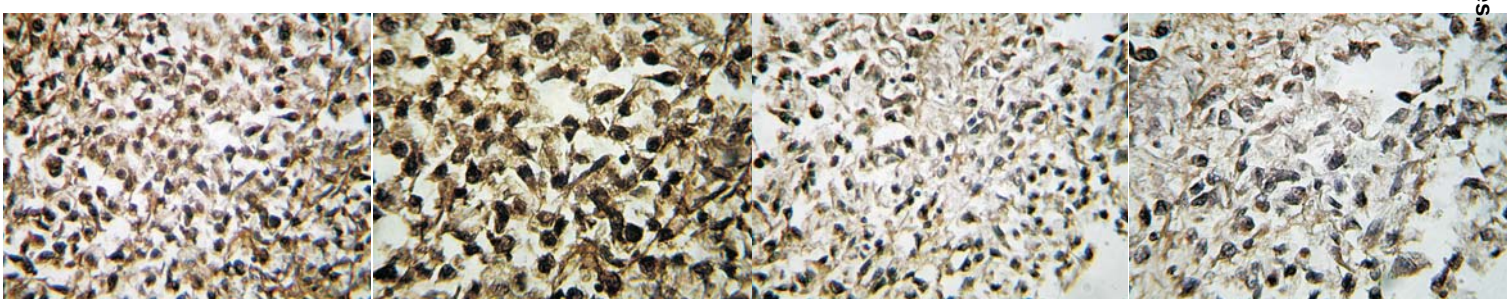
3-Skin, SCC, positive (strong) Negative



4- Skin, BCC, positive(strong) Negative



5-Skin, melanoma, positive (weak) Negative



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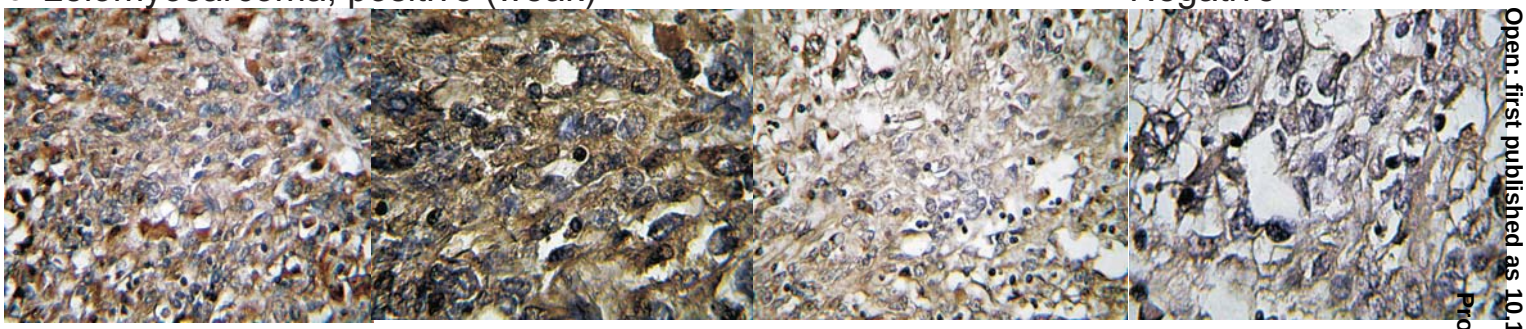
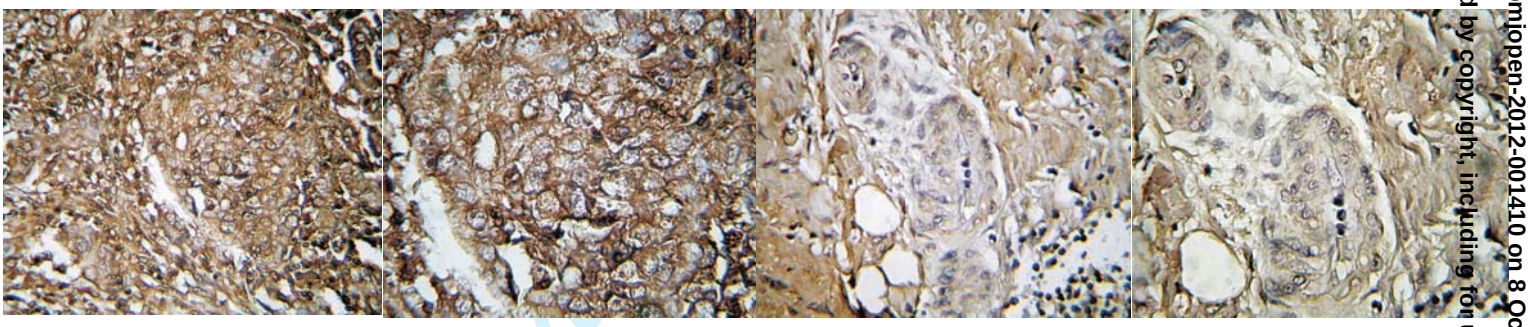
6- Leiomyosarcoma, positive (weak)

Negative



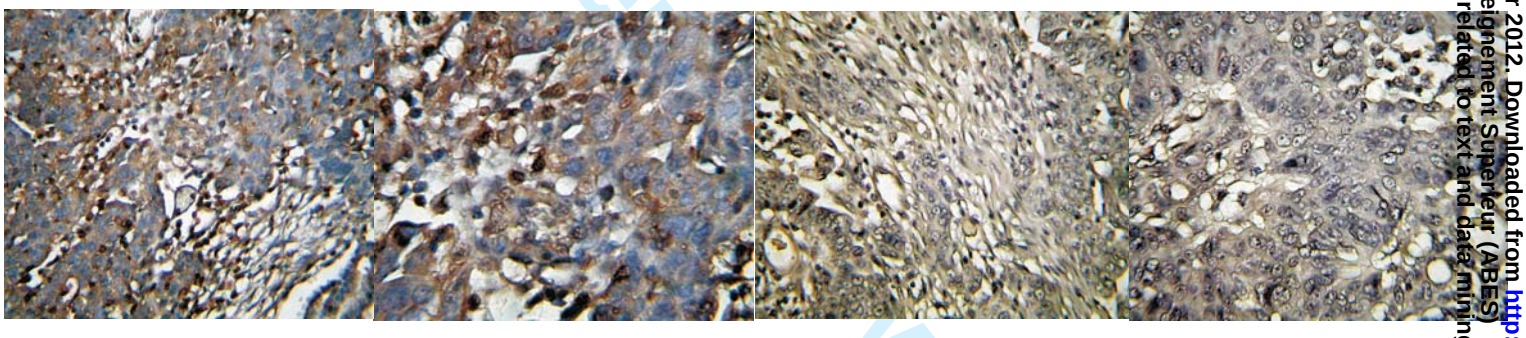
7- Omentum, serous adenocarcinoma, positive (moderate)

Negative



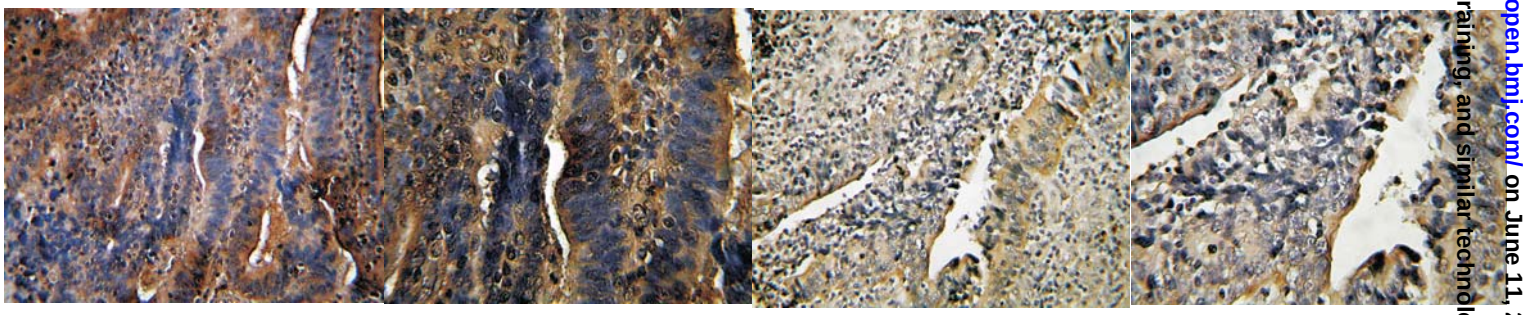
8- Ovary, serous adenocarcinoma, positive (weak)

Negative



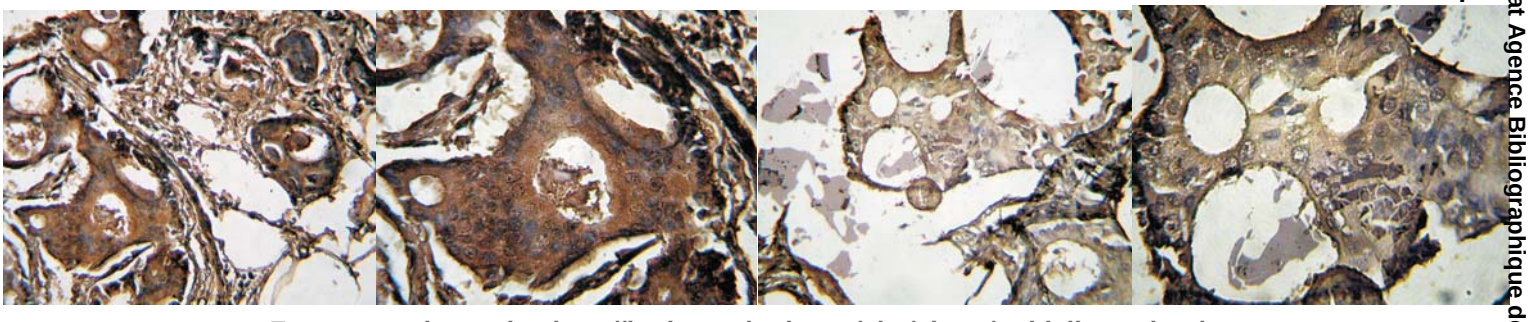
9- Cervix adenocarcinoma, positive (moderate)

Negative



10- Breast ductal adenocarcinoma, positive (strong)

Negative



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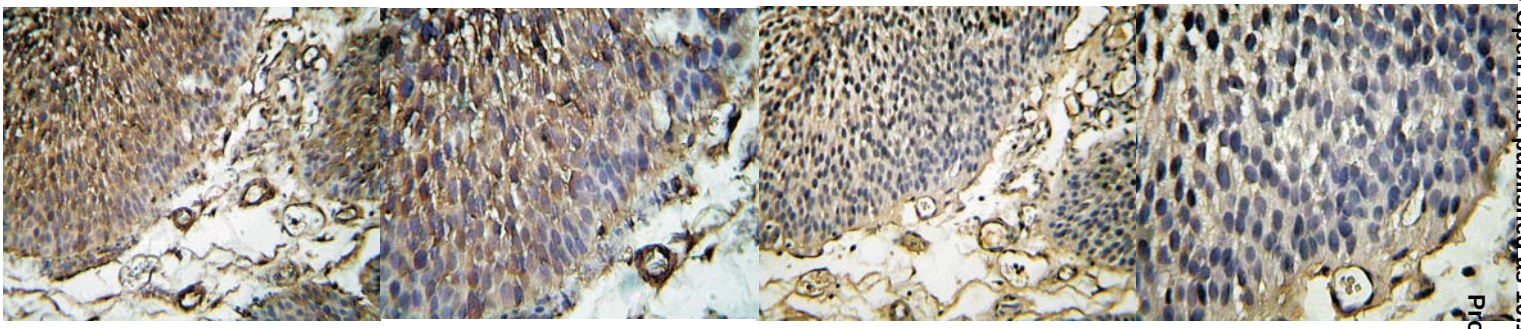
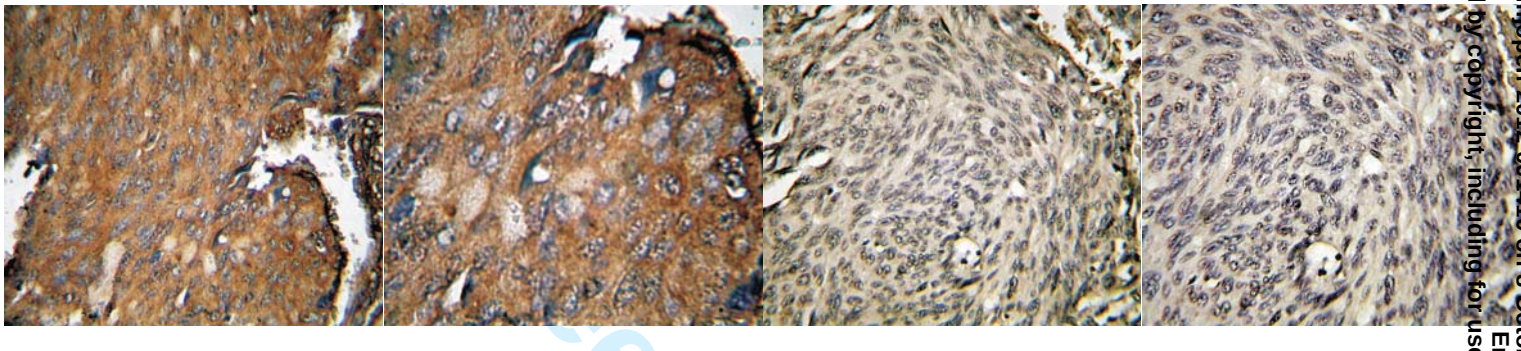
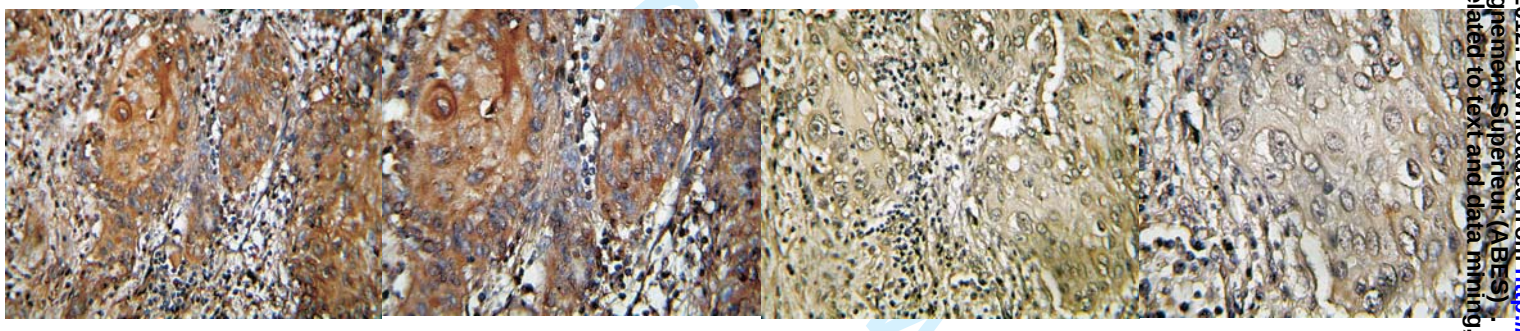
11- Bladder, Urothelial carcinoma, positive (weak) Negative



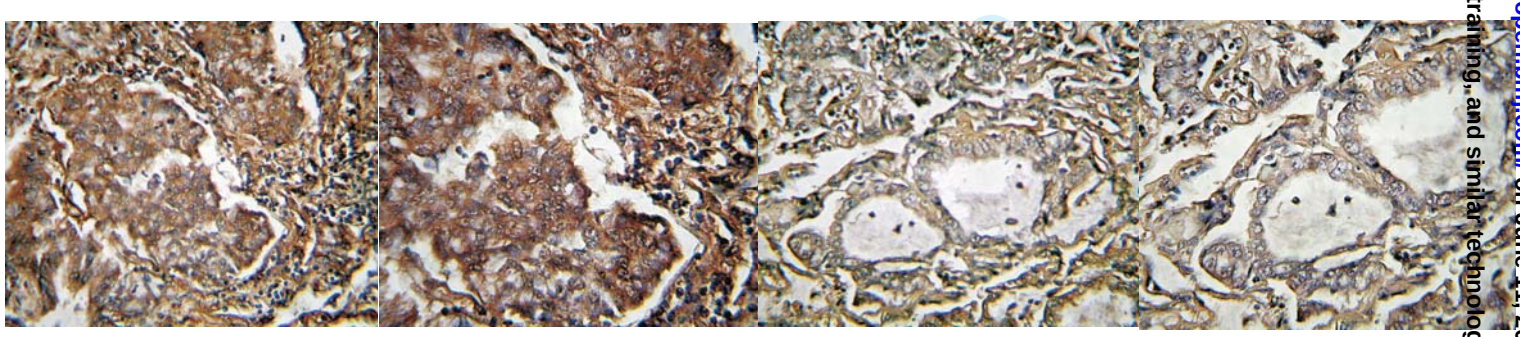
12-Thyroid, medulary carcinoma, positiv (strong) Negative



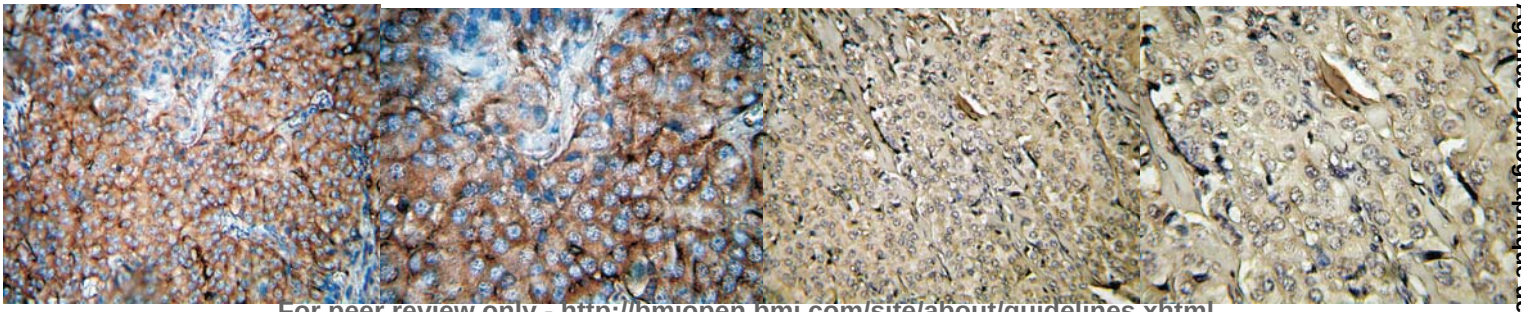
13-Lung SCC, positive (strong) Negative



14- Lung, bronchoalveolar carcinoma, positive (strong) Negative



15-Lymph node metastatic carcinoid, positive (strong) Negative



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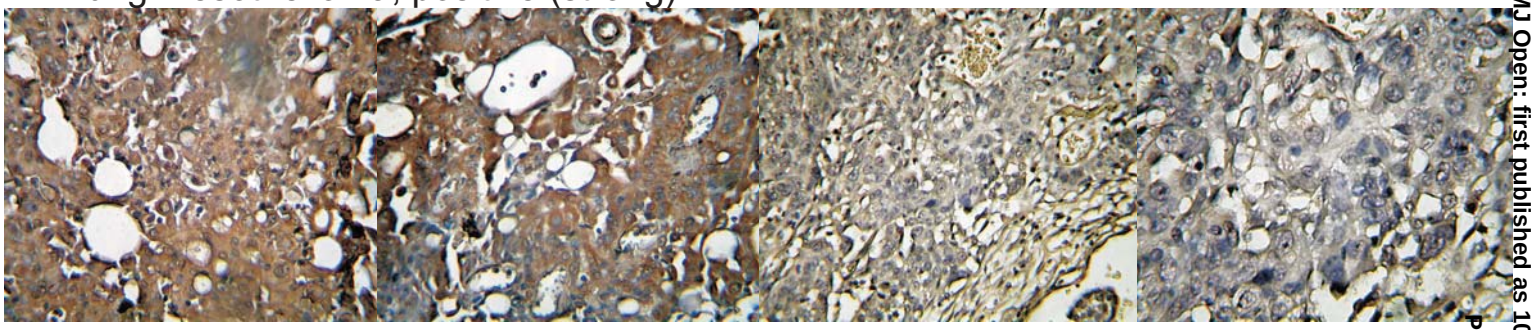
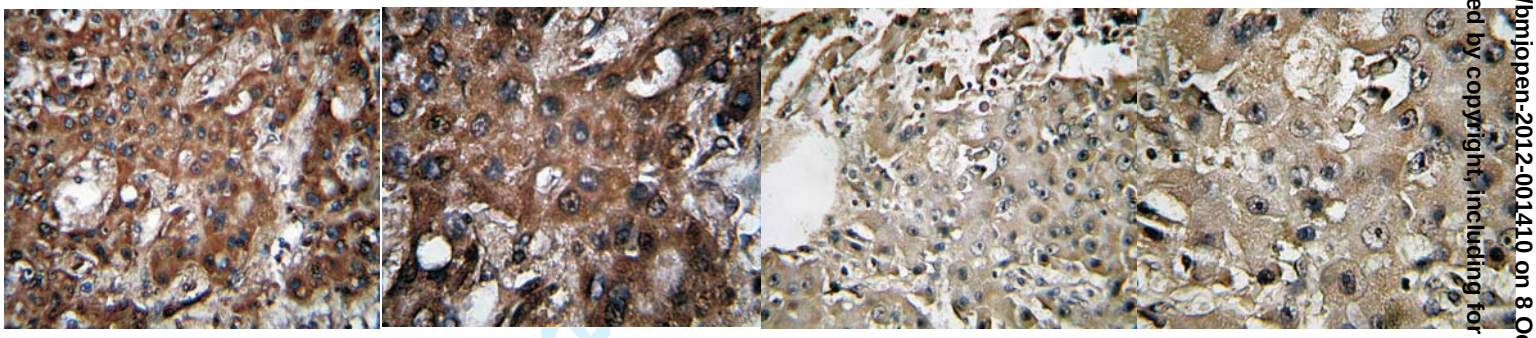
16- Lung mesothelioma, positive (strong)

Negative



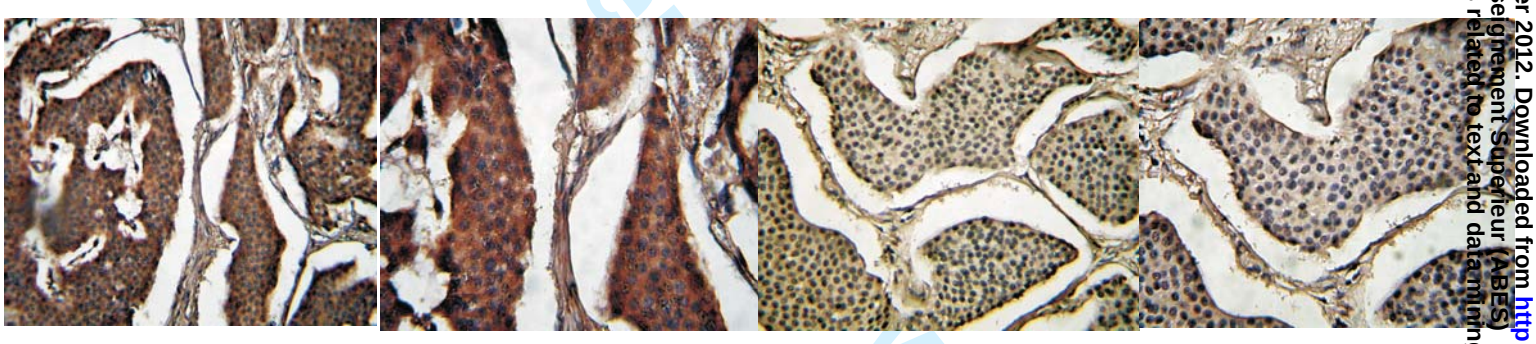
17- Hepatocell carcinoma, positive (strong)

Negative



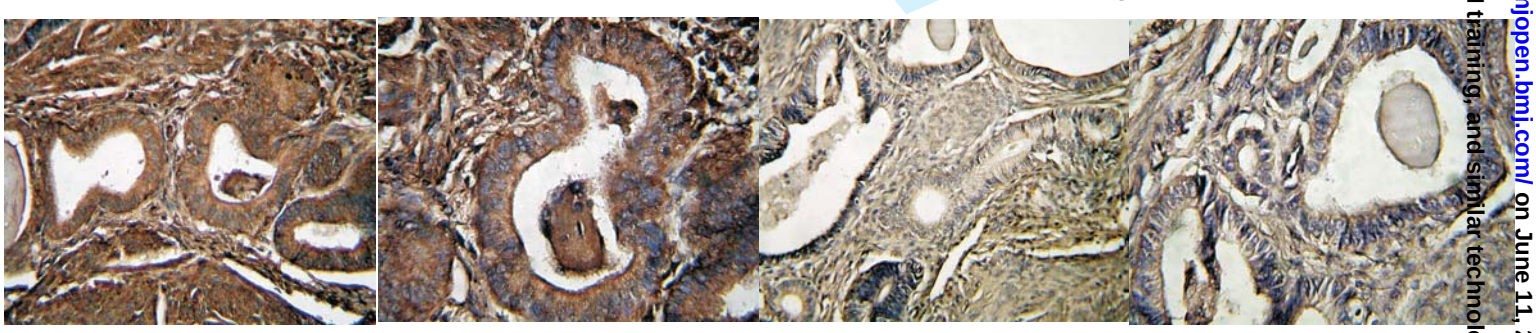
18- Liver metastatic carcinoid, positive (strong)

Negative



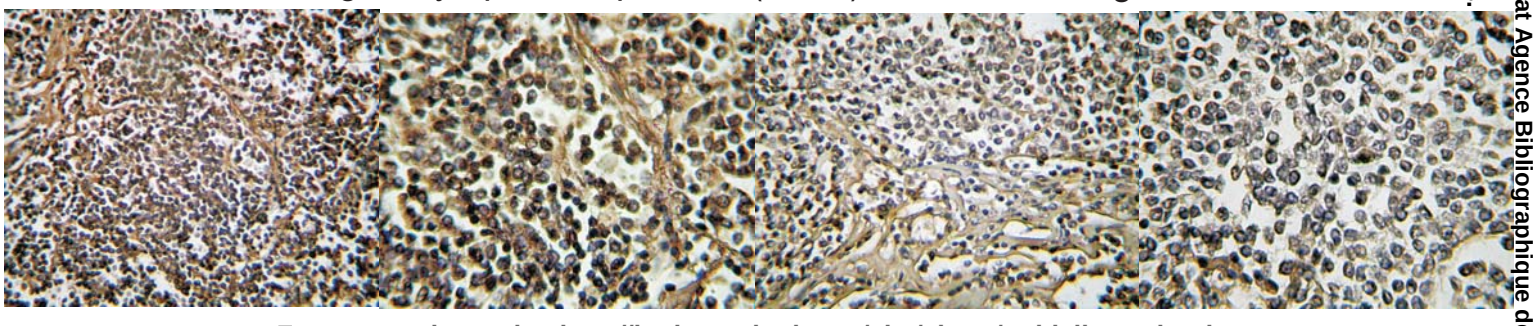
19- Endometrial adenocarcinoma, positive (strong)

Negative



20- Small bowel marginal lymphoma, positive (weak)

Negative



x20 x40 x20 x40

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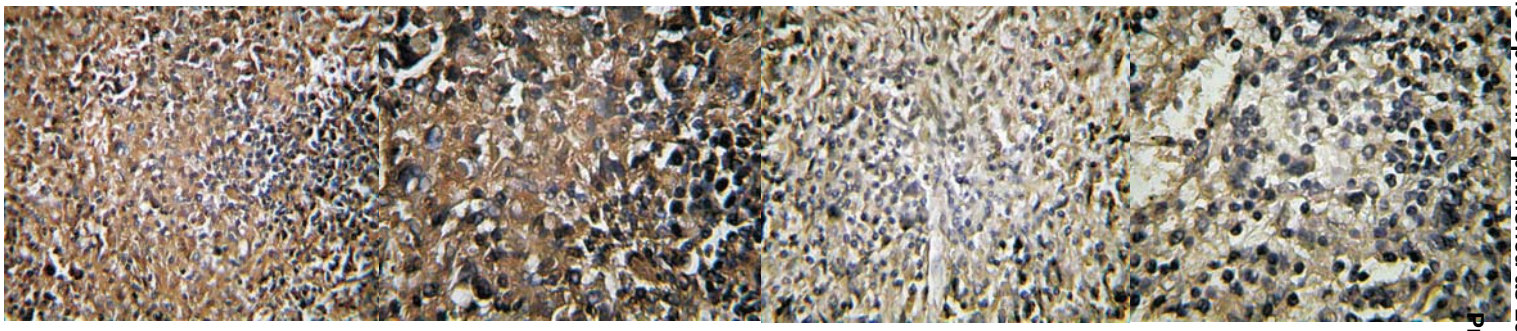
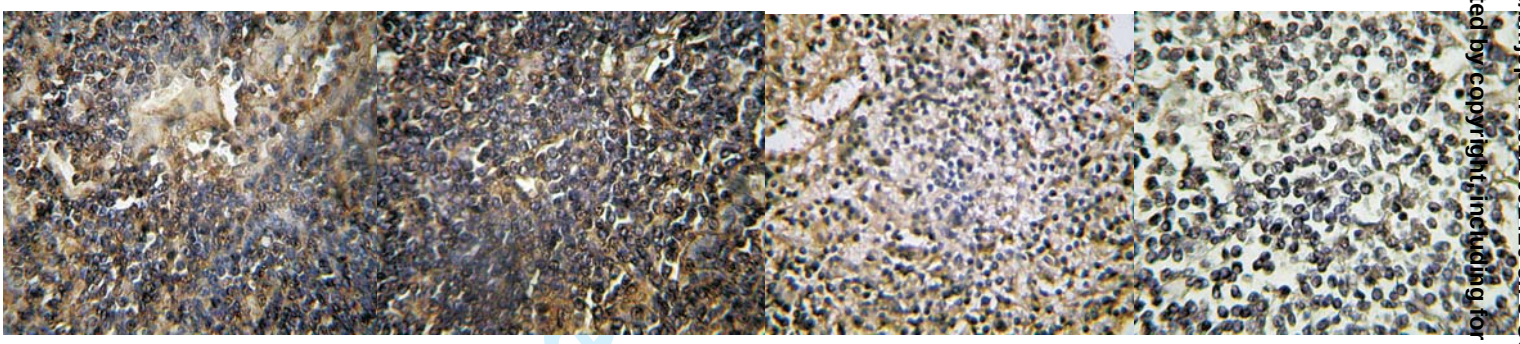
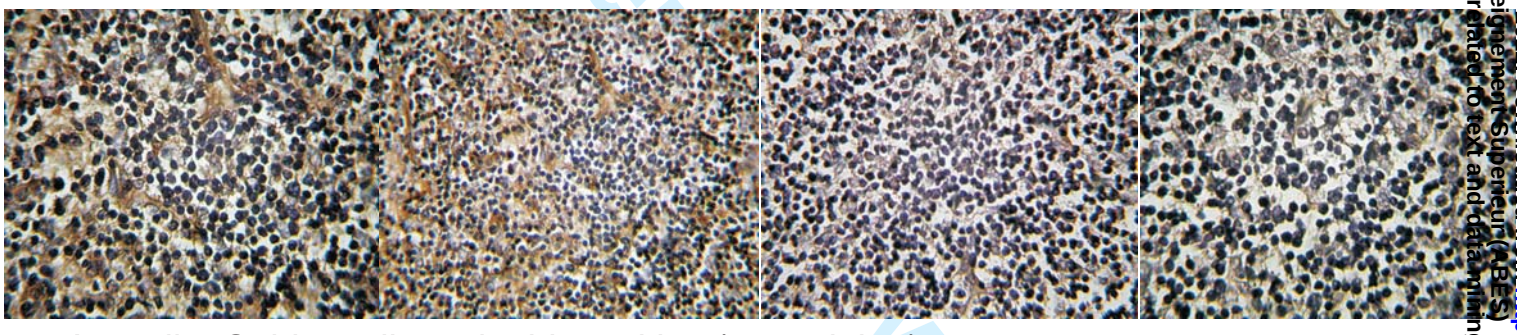
21- Spleen hodgkin lymphoma, positive (weak) Negative



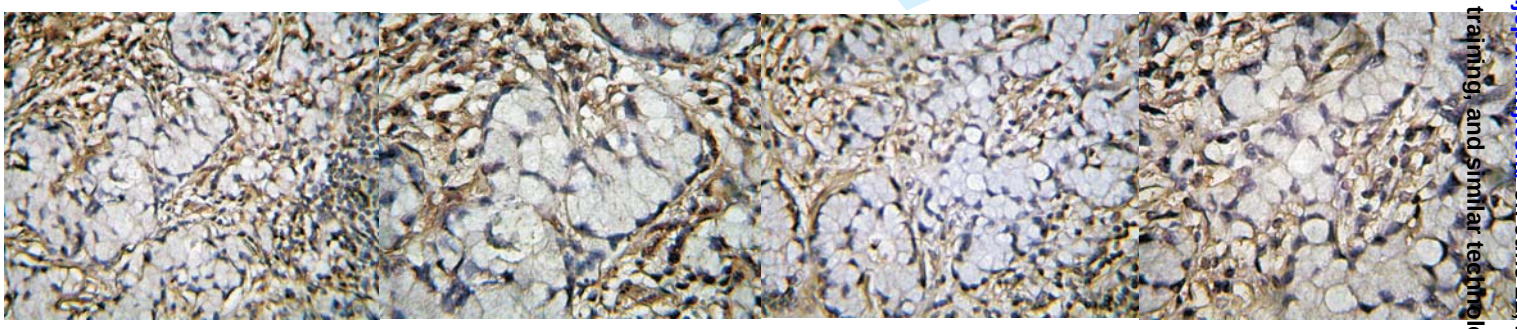
22- Stomach malt lymphoma, positive (weak) Negative



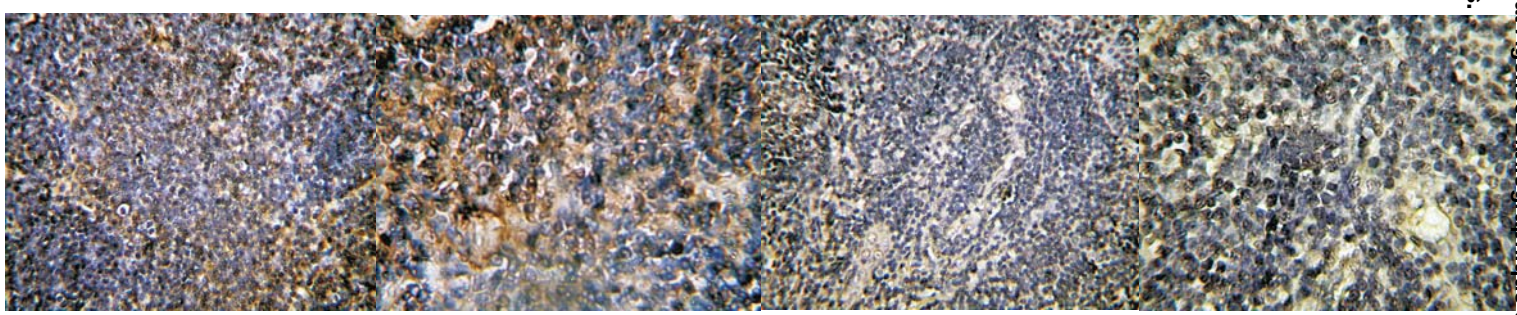
23- Thymoma, positive (weak) Negative



24- Apendix, Gablet cell carcinoid, positive (no staining) Negative



25- Follicular lymphoma, positive (weak) Negative



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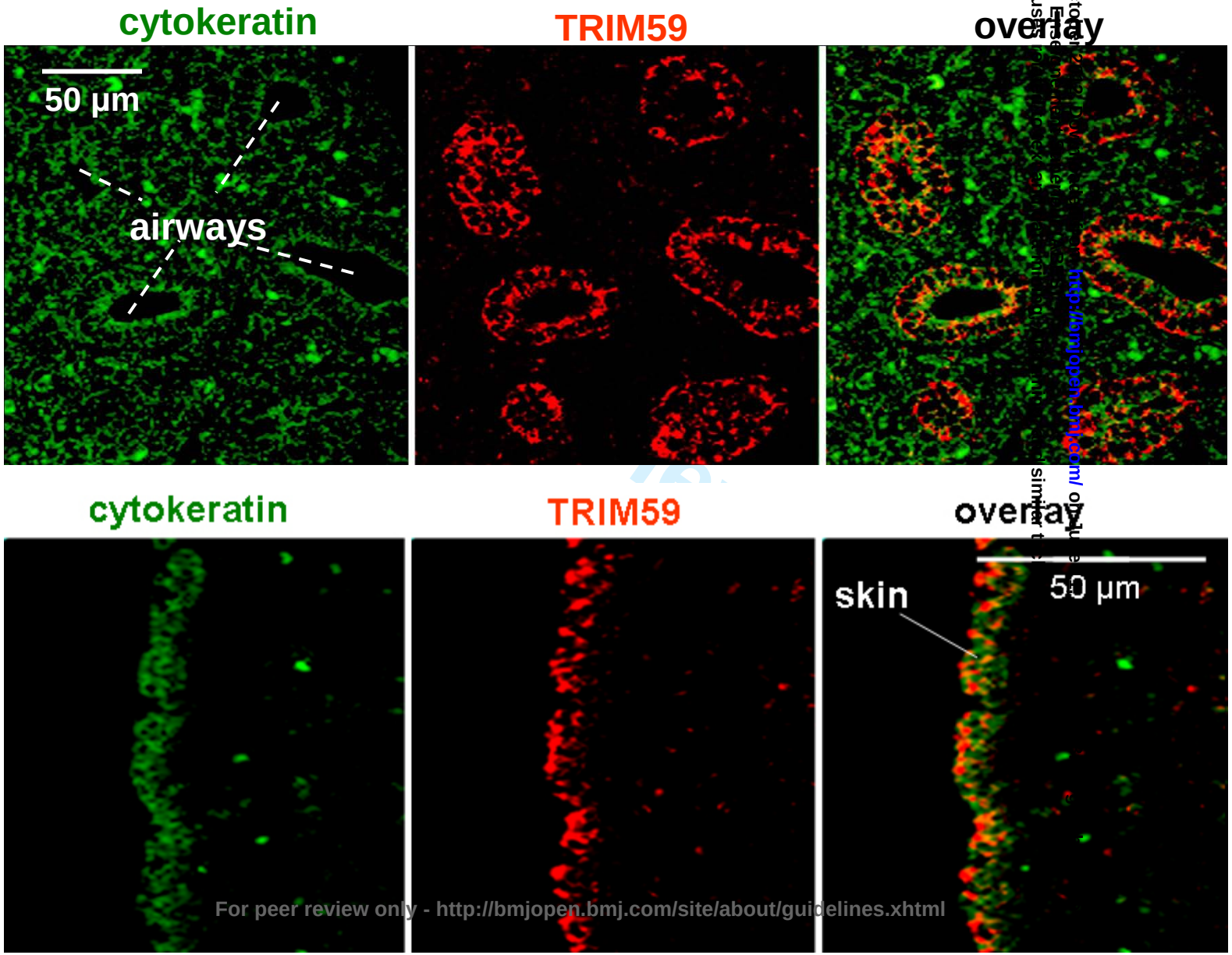
Supplemental Fig. 5

Confocal microscope imaging of immunohistochemistry of TRIM59 protein in mouse embryo (14.5d.p.c)



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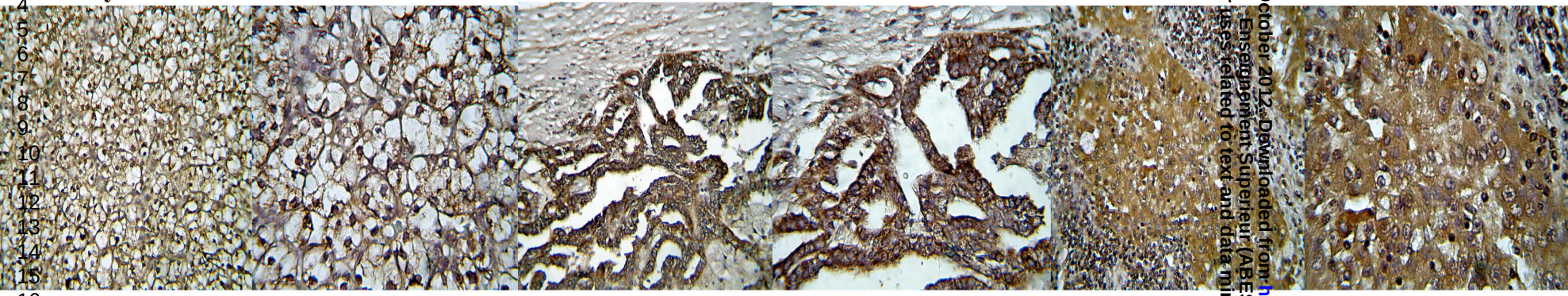
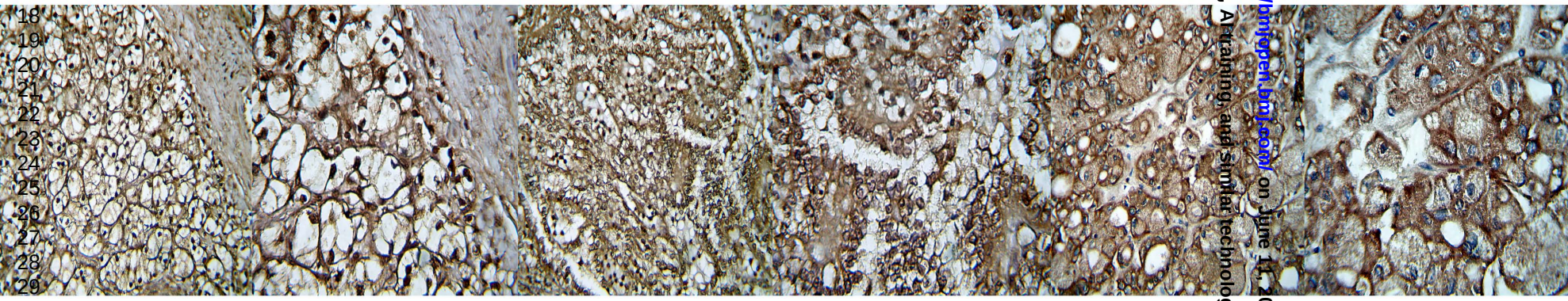
Test of Ras activation in kidney cancer patients correlated with up-regulation of TRIM59

A. Clear cell carcinoma B. Papillary RCC C. Chromophobe RCC

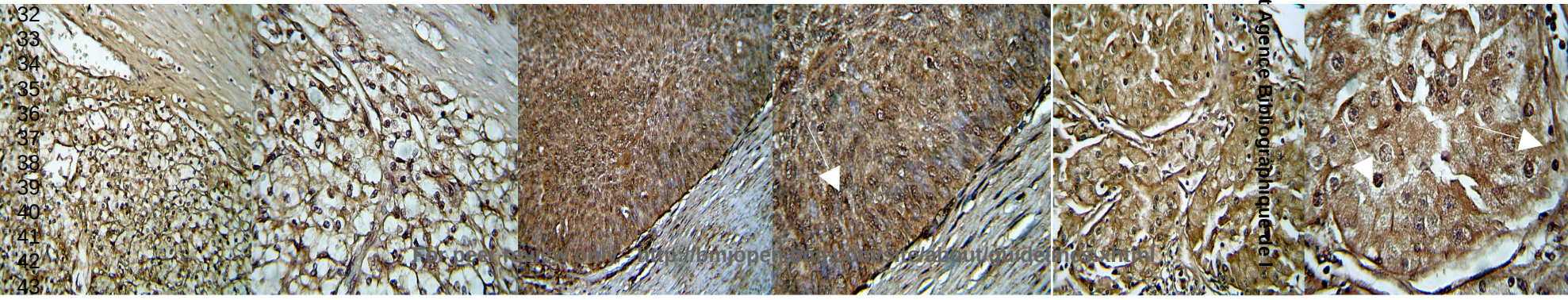
Antibody: B-Raf for total B-raf



Antibody: Raf 1 for total Raf



Antibody: Phosphor- Ser- 259 Raf 1



X20 X40 X20 X40 X20 X40

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Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

Antibody used	RCC types	Patient number	Intensity score	Nuclear staining
B-Raf (total B-raf)	Clear cell Carcinoma	6	0-1	No
	Chromophobe	3	2-3	No
	papillary	3	2-3	No
Raf-1 (total B-raf)	Clear cell Carcinoma	3	0	No
	Chromophobe	2	2-3	No
	papillary	1		No
B-Raf p-Ser ^{259} (phosphorylated B-Raf)	Clear cell Carcinoma	3	0-1	No
	Chromophobe	1	2-3	20%
	papillary	2	2-3	50%

Since BRAF is the most important early effector in the Ras signal pathway, and Raf mediate phosphorylation is the main effector recruited by GTP-bound Ras to activate the MEK-MAP kinase pathway, we performed IHC studies in these RCC sections by probing Raf activation /up-regulation and hyper-phosphorylation. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing for total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing activated phosphorylated B-Raf. Some of serial slides were stained parallelly on each patient by different antibodies.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7,8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*9*
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	9
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	9
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			9

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	6
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	17
Discussion			16
Key results	18	Summarise key results with reference to study objectives	
Limitations			18
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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TRIM59, a novel multiple cancer biomarker for immunohistochemical detection of tumorigenesis

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001410.R1
Article Type:	Research
Date Submitted by the Author:	06-Jul-2012
Complete List of Authors:	Xuan, Jim W; Lawson Heath Research Institute, Surgery
Primary Subject Heading:	Diagnostics
Secondary Subject Heading:	Diagnostics, Oncology, Pathology
Keywords:	Biochemistry < BASIC SCIENCES, HISTOPATHOLOGY, MOLECULAR BIOLOGY, Oncogenes < ONCOLOGY

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TRIM59, a novel multiple cancer biomarker for immunohistochemical detection of tumorigenesis

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Running title: TRIM59, a novel multiple-cancer marker for tumorigenesis

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E-mail: jim.xuan@lhsc.on.ca

List of abbreviations

Abbreviations: TRIM: TRIpartite Motif; CaP: prostate cancer; BPH: benign prostatic hyperplasia; PIN: prostate intraepithelial neoplasia; TMA: tissue microarray; RCC: renal cell carcinoma; SCC: squamous cell carcinoma; GI: gastrointestinal; GU: genitourinary; d.p.c: days post conception; GEM (genetically engineered mouse); H&E: haematoxylin and eosin; IHC: Immunohistochemistry; WD: well differentiated ; MD: moderately differentiated ; PD: poorly differentiated.

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Abstract

Objectives and design: We identified a novel TRIM59 gene, as an early signal transducer in two (SV40Tag and Ras) oncogene pathways in murine prostate cancer (CaP) models. We explore its clinical applications as a multi-tumor marker detecting early tumorigenesis by immunohistochemistry.

Setting and Participants: 88 CaP patients were from a tissue microarray (TMA) of radical prostatectomy specimen, 42 patients from a 35 multiple-tumor TMA, 75 patients with renal cell carcinoma (RCC) and 92 patients from 8 different tumor groups (breast, lung, parotid, gastrointestinal, female genital tract, bladder, kidney and prostate cancer).

Results: TRIM59 up-regulation specifically in tumor area was determined by immunohistochemistry in 291 cases of 37 tumor types. To demonstrate that TRIM59 up-regulation is "tumor-specific", we characterized a significant correlation of TRIM59 IHC signals with tumorigenesis and progression, while in control and normal area, TRIM59 IHC signal was all negative or significantly low. TRIM59 protein up-regulation in prostate and kidney cancers was detectable in both intensity and extent in early tumorigenesis of PIN (prostate intraepithelial neoplasia, $P<0.05$) and grade 1 of RCC ($P<0.05$), and stopped until high grades cancer. The results of the correlation in these two large cohorts of tumor types confirmed and repeated murine CaP model studies. Enhanced TRIM59 expression was identified in most of the 37 different tumors, while the highest intensities were in lung, breast, liver, skin, tongue and mouth (squamous cell cancer) and endometrial cancers. Multiple-tumor up-regulation was further confirmed by comparing relative scores of TRIM59 IHC signals in eight tumors with a larger patient population; and by a mouse whole mount embryo (14.5dpc) test on the origin of TRIM59 up-regulation in epithelial cells.

Conclusions: TRIM59 may be used a novel multiple-tumor marker for immunohistochemical detecting early tumorigenesis and could direct a novel strategy for molecular targeted diagnosis and therapy of cancer.

Keywords: TRIM59, Cancer biomarker, Immunohistochemistry, Prostate cancer, Multiple cancer marker, Ras signal pathway, SV40Tag oncogene, Tissue microarray, Confocal microscopy.

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Introduction

The TRIM (*TRI*partite *M*otif) family is an evolutionarily conserved gene family implicated in a number of critical processes including immunity [1-3], antiviral [4-8], proliferation [6, 9], transcriptional regulation [6, 10], neuro-development [11, 12], cell differentiation [12] and cancer [13] (reviewed in [1, 5, 14, 15]). However, the function of most TRIM family members is poorly understood and was surmised only based on computational analysis from their RBCC (*R*ING finger, *B*-box, Coiled-*C*oil) domains. The domain of RING (*R*eally *I*nteresting *N*ew *G*ene) is frequently involved in proteolysis acting as E3 ubiquitin ligases and the ubiquitin-proteasome system in the regulation of numerous cellular processes including cell cycle regulatory proteins, transcription factors, and signal transducers [6, 15]. Recent studies demonstrate that various TRIM-NHL proteins function as cofactor for the microRNA-induced silencing complex (miRISC) [12] [14, 16]. An ataxia-telangiectasia group D complementing gene (ATDC) was recently designated as TRIM29, which is elevated in most invasive pancreatic cancers in the Wnt/β-catenin signalling pathway [13].

In a previous report [17], we characterized the function of TRIM59, a novel TRIM family member, in SV40 Tag oncogene directed genetically engineered mouse (GEM) prostate cancer (CaP) models [18-21]. The TRIM59 gene was identified to be correlated with the SV40 Tag initiated tumorigenesis. TRIM59 protein up-regulation and hyper-phosphorylation started in the prostate cytoplasm in early tumorigenesis from PIN (prostate intraepithelial neoplasia) [17]. As a signal pathway effector, the p-Ser/Thr phosphorylated TRIM59 proteins correlate with tumorigenesis, while p-Tyr-TRIM59 proteins correlate with advanced CaP. The function of TRIM59 was identified by shRNA knockdown in human CaP cells resulted in S-phase arrest and cell growth retardation. Although TRIM59 is an effector gene with the SV40Tag oncogene, the

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initial functional targets of TRIM59 function were actually on the Ras signal pathway as an early and rapid signal transmitter. In a transgenic mouse test of TRIM59 up-regulation specifically in the prostate, TRIM59 demonstrated full oncogenic activity in directing tumorigenesis and progression to highest grade CaP. The signal pathway of TRIM59 may be possibly linked with two large oncogene routes: the Ras/Raf/MEK/ERK/P133K/AKT and the SV40 Tag/p53/pRB routes [(17)]. In this study we explored the clinical utility of the novel TRIM59 gene to serve as a biomarker for a panel of human cancers. To address the molecular biology basis, we combined results from both basic (animal model [(17)]) and human clinical studies.

Materials and Methods

Patient selection:

All patient samples were acquired as part of REB (Research Ethics Board) approved protocols at the University of Western Ontario (UWO) and Vancouver Prostate Center, University of British Columbia (UBC). Table 1 shows a complete list of 291 patients with 37 different tumor types examined in this study.

Prostate cancer Tissue Microarrays (TMA): 88 CaP patients between 2006 and 2008 who had no treatment prior to radical prostatectomy, were selected from the Vancouver General Hospital. Each patient block marked as containing benign tissue or cancer was sampled 2 times with a core diameter of 1 mm arrayed (176 cores) in a rectangular pattern with 0.7 mm between the centers of each core, creating a duplicate TMA layout using a manual tissue micro arrayer (Beecher Instruments, Silver Spring, MD). The TMA paraffin blocks, were sectioned into 0.5 micrometer sections and mounted on the positively charged slides.

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Table 1 Patient list selected in this study

Organ	Patient Number	Tumor type		Tumor grade	
35 tumor-TMA	42	Description in Table-2			
Prostate TMA	105	Adenocarcinoma		BPH	16
				PIN	4
				Gleason score 4	5
				Gleason score 6	31
				Gleason 7	15
				Gleason score 8	16
				Gleason score 9-10	9
				Stroma	3
				Absent cores	6
Kidney	75	Clear cell carcinoma	43	Grade 1	4
		Papillary RCC	11	Grade 2	38
		Chromophobe RCC	13	Grade 3	28
		Cystic RCC	6	Grade 4	5
		Sarcomatoid RCC	2		
Bladder	44	Urothelial carcinoma		Low grade	38
				High grade	6
Lung	4	Bronchoalveolar carcinoma	1	Grade 1	1
		Adenocarcinoma	1	Grade 2	1
		Large cell carcinoma	1	Grade 3	1
		Squamous cell carcinoma	1	Grade 4	1
Breast	3	Invasive lobular carcinoma	1	Grade 1	1
		Invasive mammary carcinoma	2	Grade 3	2
Female Genital tract	5	Endometrial carcinoma	4	Grade 1	4
		Ovary, Endometrioid carcinoma	1	Grade 2	1
Gatrointestinal tract	2	Colon carcinoma	1	Low grade	1
		Pancreas neuroendocrine carcinoma	1	high grade	1
Parotid	3	Mucoepidermoid carcinoma	1	low grade	1
		Metastatic SCC	1		
		Metastatic neuroendocrine Carcinoma	1	high grade	2
Mouth, tongue and larynx	4	Squamous cell carcinoma	4	Moderately differentiated	3
				Moderately to Poorly differentiated	1
Total	291				

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Automated image, acquisition and analysis on immunohistochemical staining of CaP-TMA: (UBC) Immunohistochemical staining was conducted by Ventana autostainer model Discover XT ™ (Ventana Medical System, Tuscan, Arizona) with enzyme labelled biotin streptavidin system and solvent resistant DAB Map kit. TMA was scanned by Bliss Digital imaging system using x20 objective, from Bacus Laboratories INC, Centre Valley PA, and stored in the Prostate Centre Saver (http//bliss.prostatecentre.com). A value on a four-point scale assigned to each core.

Multiple–tumor Tissue Microarray construction: Tissue samples form 42 patients that encompassed 35 distinct tumor subtypes were selected from London Laboratory Service Group, and the tumor bank in the Department of Pathology (UWO). TMA slides were constructed with triplicate cores for each sample following standard procedure as described [24]. 0.6 mm sections were prepared from TMA block and re-stained by H&E for each case to confirm the diagnosis.

Histopathologic analysis: All cases from 37 tumor types were graded according to standardized histopathology grading systems [25] by M.M (pathologist) and V. K (MD fellow).

Immunohistochemistry (IHC) and results evaluation: Standard ABC (Avidin Biotin Complex) protocol was performed as previously reported [21, 26, 27]. Two kinds of blocking reagents were used: Power Block (Universal Blocking Reagent, from BioGenex, San Ramon, CA) and Avidin-biotin blocking reagent kit (Vector Labs, Burlingame, CA). All B-Raf antibodies were from GenScript (Piscataway, NJ): B-Raf antibody (monoclonal antibody, used 1:500 dilution), Raf 1 (polyconal, 1:50) and Raf -1 -(polyclonal, Phospho-Ser^{259} 1:50).

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TRIM59 IHC staining signals were assessed by intensity for cytoplasmic staining and extent to assess percentage of nuclear staining as previously reported [28, 29]. Since in some tumors TRIM59 showed only cytoplasmic staining, for the purpose of comparing in different tumors, we used a combined relative score system based on both intensity and extent as following: score 0: 0/0 (intensity/ extent); score 1: weak cytoplasmic staining and/or $\leq 25\%$ nuclear staining; score 2: moderate cytoplasmic staining and/or $\leq 50\%$ nuclear staining; score 3: strong cytoplasmic staining and/or $\geq 50\%$ nuclear staining. All relative scores were accessed by two researchers independently M.M (pathologist) and V. K (MD fellow).

Construction and characterization of TRIM59 antibody:

Supplement Fig. 1 provided details, which combined Supplement materials of [17], and more informations. Supplement Fig. 1A showed that the antibody TRIM59#72 used for mouse and human studies was prepared from C-terminal 136 a.a. peptide (as recombinant GST- fusion protein, Suppl. Fig. B) of whole protein 403aa which is TRIM59 specific, while antibody TRIM59#71 was from mouse TRIM59 N-terminal sequence (163aa) containing mostly the common RBCC function domains of more than 70 members TRIM family [17]. As shown in Supplemental Fig. 1C, Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed multiple bands. We also demonstrated that N-terminal sequence specific antibody TRIM59#71 can recognize purified proteins from C-terminal sequence specific, TRIM59#72 Ab affinity column (the same 53kDa protein, Suppl. Fig. 1D). Since these two immunogenic sequences are not overlapped, we thus verified that they are detecting the same protein. We demonstrated that TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots (Suppl Fig. 1E).

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Confocal microscope imaging of immunostaining of mouse embryos 14.5 dpc (days post conception) for TRIM59: The mouse embryos were prepared according to our previous reports [18-21]. The anti-rabbit secondary antibody was conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA). Sections were double-stained with pan-cytokeratin antibody with FICT (Sigma), and the stained mouse embryo sections were visualized using a Carl Zeiss confocal microscope by the LSM Image program.

Statistical Analysis: Student's *t* tests and one-way ANOVA were used by programs of Microsoft Excel 2007 or SPSS 10 to analyze the data with $p < 0.05$ considered to be statistically significant.

Results and Discussion

TRIM59 up-regulation in human prostate cancer TMA (tissue microarray): correlation with tumorigenesis and tumor progression by TRIM59 intensity until high grade CaP:

We designated TRIM59 as one of the "tumorigenesis–associated" genes correlated with SV40 Tag oncogenesis in mouse prostate cancer (GEM-CaP) models [17]. SV40 Tag is essentially only required for the initiation of tumorigenesis, *i.e.* the "hit-and-run" effect, in GEM-CaP, but not for the tumor progression and metastasis directly. The "tumorigenesis–associated" effect, is due to the initial binding of the Tag oncogene with retinoblastoma (pRB), p53 proteins and several transcriptional co-activators. Once this process is initiated, the signal-transduction will continue on, even without the initiation effectors.

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In GEM-CaP models, the TRIM59 protein up-regulation correlation with tumorigenesis and progression, and down-regulated in the high grade CaP by immunohistochemistry (IHC) [17]. We assume this "tumorigenesis–associated" effect of TRIM59 may apply to human cancer studies.

We first characterized that TRIM59 antibody (#72) can cross react with and recognize specifically human TRIM59 counterpart (details see supplemental Fig. 1). We tested by IHC in a TMA of CaP patients (n=88, 176 cores). TRIM59 IHC signals detected in an automated digital image system were identified mostly in cytoplasm of luminal cells (Fig. 1A), which is different from rapid tumor progression mouse CaP models. The intensity (score= 2) in PIN (n=4, Fig. 1A) was higher than in non-tumor area (normal and BPH). Moderate to strong expression was observed in Gleason score 6 (3+3, n=25), 7 (4+3, n=15), and 8 (4+4, n=14). In high grade CaP (score 4+5, n=8, Fig. 1A), TRIM59-IHC signals were lower. As shown in a graph of Fig. 1B, TRIM59 protein IHC signals correlated significantly (P=0.014) with tumorigenesis and progression from PIN to -WDCaP (Gleason grade 1-2, scores 2-4) and MDCaP (Gleason grade 2-4, scores 4-8) (graph Fig 1B), and decreased in high grade CaP (Gleason score 9-10) with P=0.018, which is similar to GEMs [17].

TRIM59 up-regulation in human Renal Cell Carcinoma (RCC) patients: correlation of tumorigenesis and tumor progression with TRIM59 intensity until high grade RCC:

Next, we demonstrated that TRIM59 is not androgen-responsive and likely not prostate tissue-specific, indicating that the tumorigenesis role of TRIM59 may be applied to all or most of human cancers. Supplemental Fig. 2 showed this result in detail of IHC test by TRIM59 antibody#72 on a TMA of the LNCaP human CaP xenografts in nude/SCID castrated mice.

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We extended results from prostate cancer clinical samples to kidney cancer. We started 75 renal cell carcinoma (RCC) patients including all 5 different types of RCC tumors: 43 clear cell carcinoma (representative IHC figures shown in Fig. 2 A-D), 11 papillary renal cell carcinoma (Fig. 2 E-F), 13 chromophobe renal cell carcinoma (Fig. 2 G-H), 2 sarcomatoid renal cell carcinoma (Fig. 2J), and 6 cystic renal cell carcinoma (Fig. 2 K-L). RCC cases analyzed with Fuhrman grade 1-4 were 4, 38, 28, and 5 respectively. TRIM59-IHC staining in normal area including proximal tubules was negative (Fig. 2 last row). Background staining was eliminated by testing antibody dilutions (1:100, 200, 500, 1200 till 5000) while tumor specific signals of TRIM59 proteins were noticeable. Endogenous biotin signals were blocked and excluded by additional block reagents (avidin-biotin blocking reagent kit).

TRIM59 IHC staining in tumor areas in RCC (Fig. 2) was different from cases of CaP-TMA (mainly cytoplasmic). TRIM59 IHC staining was found in both cytoplasm and nucleus in all RCC grades and types (Fig. 2). We assessed TRIM59-IHC by visual scoring of both intensity (cytoplasmic staining) and extent (% nucleus staining) microscopically. Correlation of TRIM59 IHC signals by scoring the intensity in cytoplasm with grades of all five types of RCC is shown in Fig. 2M. TRIM59 IHC signals were increased with tumor progression from grade 1-3 ($p<0.05$). All grade 1 tumors (n=4) stained with weak TRIM59 IHC signals in cytoplasm, but with high extent of nuclear staining; while all grade 2 and 3 tumors (n=66) showed moderate to strong cytoplasmic staining intensity of TRIM59. All grade 4 tumors (n=5) showed weak to moderate intensity in cytoplasm of TRIM59 staining. No correlation between TRIM59 IHC staining in nucleus and tumor grade was found, although ~~in~~ low grade RCC showed higher nuclear staining.

Therefore by systematic IHC studies in CaP (88 patients, Fig. 1) and kidney cancer (75 patients, Fig. 2), we almost exactly repeated results from our mouse model studies on TRIM59

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[17]. We confirmed TRIM59 as an immunohistochemistry marker able to detect low grade tumor in early tumorigenesis. We also demonstrated the correlation of tumorigenesis and tumor progression with TRIM59 up-regulation until high grade tumor.

TMA analysis of TRIM59 protein expression demonstrates that TRIM59 is a multiple tumor marker:

In the basic research previously using animal GEM-CaP models [17], we have disclosed that TRIM59 up-regulation is involved in two oncogene families and two signal pathways of SV40Tag/pRB/p53 and Ras/Raf/MEK/ERK. TRIM59 may function as an early signal transducer in Ras signal pathway with bridging genes in two oncogene pathways [17].

While it was rarely reported that SV40 Tag oncogene induced human cancer, Ras mutations are among the most frequent alterations in human cancers (see review [22]). We assume that TRIM59 as an early Ras signal pathway effector may possibly act as a multiple tumor marker.

We therefore further extended TRIM59 IHC studies to 35- multiple cancer TMA sections (42 tumors, 126 cores, Table 1). We tested different dilutions (1/300, 1/600, 1/1200, 1/5000) of TRIM59 antibody (see Supplemental Fig. 3). To further confirm the specificity and reliability of TRIM59 antibody in IHC staining, we compared IHC staining in 35 different tumor-TMA sections with positive (TRIM59 antibody at 1:1200 and 1:5000 dilutions) and negative controls (no antibody added, details see Supplemental Fig. 4). As summarized in Table 2, TRIM59 expression was significantly and tissue-specifically up-regulated in most of these 35 tumors. When comparing the relative scores (both intensity and extent) in different tumors, the highest staining was observed in breast, lung, liver, skin (squamous cell carcinoma) and endometrial cancers.

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Further confirmation of TRIM59 as a tumor marker in patients with eight different tumors:

Since the 35 tumor-TMA contained only limited cases in each tumor type, we selected more cases (n=92) of eight different tumor types with different tumor grades, which all showed up-regulated expression of TRIM59. IHC staining of TRIM59 in eight tumors are shown: lung (n=4, Fig. 3A-C), breast (n=3, Fig. 3D-E), gastrointestinal (GI, n=2, Fig.3F-G), female genital tract (fGT, n=5, Fig.3H-J), bladder (n=44, Fig. 3K), prostate (n=27 from UWO, Fig. 3L), head and neck mucosal tumor (squamous cell carcinoma, SCC of mouth, tongue and larynx, n=4, Fig. 3M-O) and parotid gland (n=3, Fig. 3P-Q). Normal areas in lung, breast, colon, endometrial, bladder, larynx and parotid tissues showed very weak or completely negative staining (Fig. 3). Since some tumors (e.g. prostate) showed mostly cytoplasmic and no nuclear TRIM59-IHC staining, as a comparative study, we assessed their relative scores (combine both intensity and extent scores, see Materials and Methods). More tumors from kidney (RCC, n=75) and prostate cancer (n=27) were included as references and all were assessed by relative scores simultaneously, since we already analyzed a large cohort of these patients. Fig. 3R shows the comparison of the mean of IHC-TRIM59 relative scores. The highest relative scores were found in SCC of the parotid, mouth, larynx and tongue, followed by lung, breast and female genital tract cancers.

The comparison of relative scores on low and high grades separately was done (data not shown). Cases of grade 1 lung cancer (bronchoalveolar, adenocarcinoma, SCC and large cell carcinoma) and breast cancer (invasive lobular and invasive mammary carcinoma) all showed the strongest staining as compared with other tumors. In endometrial cancer, the TRIM59 relative scores were moderate in grade 1 and moderate to strong in grade 2. The three tumors of

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Table 2 Immunohistochemistry analysis of TRIM59 as multiple marker in 35 tumor TMA

Tumor Type	Patient number	Core number	Pathologic Grade	Cell Type	IHC staining	
					Cytoplasm staining (intensity)	Nuclear staining (extent)
Renal clear cell carcinoma,	2	6	2	Epithelial	moderate	-
			3		moderate	-
Adrenal gland cortical carcinoma	1	3	N/A	Epithelial	Moderate-strong	-
Squamous Cell Carcinoma, skin	2	6	WD	Epithelial	strong	-
			MD		moderate	-
Basal cell carcinoma, skin	2	6	N/A	Epithelial	moderate	-
					Moderate-strong	
Melanoma	1	3	N/A	Epithelial	weak	50% +
Endometroid adenocarcinoma	2	6	2	Epithelial	Moderate-strong	-
			1		Moderate-strong	-
Leiomyosarcoma	1	3	N/A	Mesenchymal	weak	-
Omentum serous adenocarcinoma,	1	3	WD	Epithelial	Weak-moderate	-
Ovary serous adenocarcinoma	1	3	N/A	Epithelial	Weak-moderate	-
Ovary clear cell carcinoma	1	3	PD	Epithelial	Moderate-strong	30% +
Cervix adenocarcinoma	1	3	WD-MD	Epithelial	Moderate-strong	-
Colon adenocarcinoma	1	3	Low grade	Epithelial	weak	-
Breast ductal adenocarcinoma	1	3	2/3	Epithelial	Moderate-strong	50% +
Bladder urothelial carcinoma	2	6	Low grade	Epithelial	weak	-
			2 (low grade)	Epithelial	moderate	-

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Stomach GIST	1	3		Epithelial	Weak-moderate	-
Esophagus adenocarcinoma	1	3	MD	Epithelial	0-weak	-
Thyroid, Papillary carcinoma	1	3	N/A	Epithelial	weak	-
Thyroid, medullary carcinoma	1	3	N/A	Epithelial	moderate	-
Pancreas adenocarcinoma,	2	6	2	Epithelial	Weak-moderate	-
			2		Moderate-strong	-
Pancreas endocrine tumor	1	3	N/A	Epithelial	strong	-
Lung SCC	1	3	PD	Epithelial	strong	-
Lung mesothelioma	1	3	MD-PD		strong	20% +
Lung adenocarcinoma	1	3	MD		moderate	50% +
Lung bronchoalveolar carcinoma	1	3	WD		Moderate-strong	50% +
Lung mesothelioma, biphasic	1	3	MD-PD		strong	20% +
Liver hepatocellular carcinoma (HCCa)	1	3	2/4	Epithelial	strong	-
Liver metastatic carcinoid	1	3	N/A	Epithelial	strong	-
Small bowel marginal zone lymphoma	1	3	N/A	Lymphocyte	0-weak	-
Lymph node, follicular lymphoma	1	3	1/3	Lymphocyte	0-weak	-
Lymph node, metastatic carcinoid	1	3	Low grade	Epithelial	strong	-
Spleen, Hodgkin's lymphoma	1	3	N/A	Lymphocyte	weak	-
Stomach, malt lymphoma	1	3	Low grade	Lymphocyte	0-weak	-
Thymus invasive thymoma	1	3	N/A	Epithelial	0-weak	-
Appendix, Goblet cell carcinoid	1	3	N/A	Epithelial	0	-

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squamous cell carcinoma (SCC) from mouth, tongue and larynx with different grades (Fig. 3M-O) also showed high relative scores (both intensity and extent).

As a comparative study (Fig. 3R, Table 2), we tested 44 bladder cancer cases with 38 low grade and 6 high grade tumors. The mean value of relative scores was 1.6, *i.e.* weak to moderate in bladder cancer cases . In 27 prostate cancer cases (from UWO only) tested, the relative scores of TRIM59 (cytoplasmic staining only) from PIN through Gleason scores of 10 were actually relatively weak (Fig. 3R), although in Gleason score 4, 6 and 8 were weak to moderate separately.

Thus far, we identified that TRIM59 up-regulation is "tumor-specific". First, we demonstrated the correlation of TRIM59 enhanced IHC signals with tumorigenesis and progression, which were statistically significant in m this report with 291 cases and 37 tumor types analyses. Second, although TRIM59 is a normal gene involved in CDC (cell cycle division) regulation from G1 to S-phase involved in DNA S-phase and cell growth [17], we demonstrated that in normal or non-tumor areas in all tested 37 different kinds of cancers, TRIM59 IHC staining signals were mostly negative or very low (Fig. 1, 2 3). By moderating antibody dilutions and testing various blocking reagents (Supplemental Fig. 4), we demonstrated that TRIM59 induces tumorigenesis/oncogenesis only when it is abnormally up-regulated.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Furthermore, we also confirmed that TRIM59 expression involved in multiple-tissue expression even in embryo development. We carried out IHC of mouse embryo sections by double-staining TRIM59. Cytokeratin (keratin), a family of proteins that are primarily found in epithelial cells was used as reference. Supplemental Fig. 5 illustrated confocal microscope

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images of IHC staining of TRIM59 in different organs/tissues of mouse embryo (14.5 d.p.c). TRIM59 was highly expressed in cytokeratin-expressing cells in the lung (first row), skin (second row), and kidney (not shown) of mouse embryos. TRIM59 staining in mouse embryos revealed the same pattern of the epithelium origin as in human tumors, which the TRIM59 gene were found highly up-regulated in those tumor types as well.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Given our previous experiments suggesting TRIM59 functions in the Ras pathway, we tested if TRIM59 up-regulation was correlated with the BRAF, an early signal effector Ras signal pathway (review see [22]). We selected 24 RCC patients, which previously were confirmed with up-regulation of TRIM59 expression. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing the total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing the activated phosphorylated B-Raf. Some of the serial slides were stained in parallel on each patient by different antibodies. As shown in Supplemental Fig. 6 A first two columns, in all 12 clear cell carcinoma of RCC samples, there were no or very weak B-Raf IHC signals in all three used Raf antibodies. In papillary RCC (Suppl Fig. 6 B) and chromophobe RCC samples (Suppl Fig. 6 C) (15 samples out of 24 samples or 62% of all RCC samples tested), there were higher IHC signals in all three antibodies used for staining in cancer areas specifically, showing higher intensity and extent than clear cell carcinoma (Suppl Fig. 6 A). Nuclear signals were found only by B-Raf P-Ser antibody in papillary and chromophobe tumors. Supplemental Table 1 summarizes the results. It is intriguing that in those TRIM59 up-regulated kidney cancers (RCC), neither total nor phosphorylated BRaf were detected in clear RCC (as a control), but were all highly positive in other two RCCs (papillary and

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chromophobe) tumors. We could not definitely confirm that TRIM59 was acting along the Ras pathway in all cases where it was detected.

Conclusions

This is the first report on a possible "ubiquitous" tumor marker. Ras mutations are among the most frequent alterations in human cancers that lead to approximately 30% of all human cancers with expression of constitutively active Ras proteins. In this report, we have demonstrated that there are more than mutation issues of the Ras signal pathway in tumorigenesis and progression, since TRIM59 is up-regulated as a novel proto-oncogene in a variety of human cancers.

Detection and treatment of cancer at the earliest stage is critical for patient survival. This investigation demonstrated a novel TRIM59 gene as a multiple tumor marker for early diagnosis of tumorigenesis. As a multiple biomarker associated with epithelium origin (very rarely arisen from mesenchymal tissue, see Table 2) TRIM59 maybe used as an EMT (epithelium – mesenchymal- transition) specific co-biomarker. The TRIM59 antibody may also possibly be used for molecular targeted imaging as a new diagnostic marker, for example in the context of targeted microbubble ultrasound destruction technology [23], before the protocol of a serum TRIM59 test can be established.

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Acknowledgements: This work was supported by grants from the Ontario Institute of Cancer Research (07NOV-52), the Canadian Institute of Health Research (MOP -77684), NIH-NCI (2 U01 CA084296-06) and the Terry Fox Foundation.

Legend of Figures:

Fig. 1 Correlation of TRIM59 Immunhistochemistry (IHC) staining in prostate cancer cases in TMA (Tissue Microarray) assessed by the Gleason Grading system. All panels in **A** were shown hematoxylin staining, x20. Non-tumor: weak or negative, PIN: TRIM59 is located in the cytoplasm of the luminal cells (Intensity=2). Gleason score 3+3, TRIM59 is located in the cytoplasm of tumor cells (Intensity=2). Gleason score 3+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+5, TRIM59 is located in the cytoplasm of the tumor cells (Intensity<1). Graph **B:** Correlation of TRIM59 protein levels by intensity with Gleason score in prostate cancer TMA analysis. Error Bars show mean (± SE).

Fig. 2 Immunohistochemistry analysis of TRIM59 expression in kidney cancer (RCC, Renal Cell Carcinoma) cases: correlation with tumor grade by intensity detecting early tumorigenesis. Five types of RCC with different grades were shown clear cell carcinoma (**A-D**), papillary RCC (**E-F**), chromophobe RCC (**G-I**), sarcomatoid RCC (**J**) and cystic RCC (**K-L**). **A**. Clear cell carcinoma, grade1, weak cytoplasmic staining in tumor cells, ≥ 50% nuclear staining, cytoplasmic staining in tumor cells (40X). **B.** Clear cell carcinoma, grade 2, moderate

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cytoplasmic staining in tumor cells, no nuclear staining (40X). **C.** Clear cell carcinoma, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **D.** Clear cell carcinoma, grade 4, moderate cytoplasmic staining and nuclear (%) staining (40X). **E.** Papillary RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **F.** Papillary RCC, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining, normal cortex tissue is visible adjacent to tumor area (40X). **G.** Chromophobe RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining. (40X). **H.** Chromophobe RCC, grade 3, strong cytoplasmic staining, no nuclear staining, (40X). **I.** Chromophobe RCC, grade 4, moderate cytoplasm staining and nuclear staining (40X). **J.** Sarcomatoid RCC, grade 4, weak cytoplasmic staining and nuclear staining (40X). **K.** Cystic RCC, grade 2, moderate cytoplasmic staining, no nuclear staining (40X). **L** Cystic RCC, grade 3, strong cytoplasmic staining, no nuclear staining (40X). Last two panels: normal kidney tissues (x20, x40). **M. Graph:** Correlation of TRIM59 protein levels by relative scores (both intensity and extent) with grade in RCC according to the Fuhrman nuclear grading system. Error Bars show mean (± SE).

Fig. 3 Comparison of TRIM59 expression as a multiple-cancer marker in eight types of tumor in breast, lung, parotid, gastrointestinal, female genital tract, bladder, head and neck mucosal tumor and prostate cancer. Negative TRIM59 staining in normal tissues was shown for each tumor respectively (20X). **(A)** Lung bronchoalveolar carcinoma, grade 1, strong cytoplasmic and nuclear staining of tumor cells, (40X). **(B)** Lung adenocarcinoma, grade 2, moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(C)** Lung large cell carcinoma, grade 4, moderate cytoplasmic and nuclear staining of tumor cells (40X). **(D)** Breast cancer, invasive lobular, low grade, moderate to strong cytoplasmic and nuclear staining of tumor cells (40X). **(E)** Breast cancer, invasive mammary (no specific type), high grade,

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moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(F)** Pancreas neuroendocrine carcinoma, poorly differentiated, strong cytoplasmic staining, no nuclear staining of tumor cells (40X). **(G)** Colon carcinoma, low grade, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(H)** Endometrial carcinoma, grade1, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(I)** Endometrial carcinoma, grade 2, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(J)** Ovary, Endometrioid carcinoma, grade 1, weak cytoplasmic staining, no nuclear staining (40X). **(K)** Bladder urothelial carcinoma, low grade, moderate cytoplasmic staining, no nuclear staining (40X). **(L)** Prostate adenocarcinoma, Gleason score 8, moderate cytoplasmic staining, no nuclear staining (40X). **(M)** Floor of mouth, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(N)** Tongue cancer, SCC moderately to poorly differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(O)** Larynx, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(P)** Parotid, mucoepidermoid carcinoma, low grade, strong cytoplasmic staining, no nuclear staining (40X). **(Q)** Parotid, metastatic SCC, poorly differentiated, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(R)** Graphic comparison of relative scores (both intensity and extent) of TRIM59 IHC signals in eight different tumor types. fGT: Female genital tract. Error Bars show means (± SE) of the relative scores in all grades analyzed and compared.

Legend of Supplemental data

Supplemental Fig. 1 Characterization of TRIM59 antibodies constructed and used for human tissue studies.

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Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

(A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows. **(B)**. 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). **(C-D)**. Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots. **(E)** Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145, LNCaP and human kidney cell HEK293. A unique 53 kDa band was shown exactly as in mouse cell and tissue lysates **(C)**.

Supplemental Fig. 2 From **Vancouver Prostate Center** The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP, TRIM59 is significantly up-regulated after

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10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.

Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Summary of results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 1:1200 and 1:5000 –(figures not shown but resemble to 1:1200). We assessed these IHC results with the negative control (with out TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3, 1:1200 dilution produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remained stronger than the normal non-tumor areas even at 1:300 dilution. In order to detect weak TRIM59-IHC staining signals, we tried 1:300 dilution and we compared the staining signal with negative controls (no antibody was added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining (data not shown), and similar results were obtained.

Legend of Figures: (A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular

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lymphoma, (20X), positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining.

Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry –(IHC) staining in human multiple tumor TMA —(tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody –(positive) and without any antibody –(negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some areas of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin –(squamous cell carcinoma); #4 Skin –(basal cell carcinoma); #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma; #13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma; #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma); #2 Adrenal gland (cortical carcinoma); #7 Omentum (serous adenocarcinoma); #9 Cervix adenocarcinoma.

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma); #6 Uterus (leiomyosarcoma); #8 Ovary, serous adenocarcinoma; #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma and # 25 Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid).

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Supplemental Fig. 5 Confocal microscope imaging of IHC analysis of TRIM59 expression in mouse embryo (14.5d.p.c). Patterns of multiple organ expression originated from epithelial cells were shown in the lung (first row) and skin (second row).

Supplemental Fig. 6 Test of Ras activation by B-Raf antibodies in kidney cancer tumors correlated with up-regulation of TRIM59. First column: Antibody Braf **(A)** in Clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (**B**), strong cytoplasmic staining, no nuclear staining, (40X); in Chromophobe RCC (**C**), strong cytoplasmic staining, no nuclear staining, (40X). In the second row: antibody to Raf 1, in clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (20X,40X), strong cytoplasmic staining, no nuclear staining; in chromophobe RCC, strong cytoplasmic staining, no nuclear staining (40X). Third row: Antibody to Raf 1 phosphor Ser ^{259}, in clear cell carcinoma, weak cytoplasmic and nuclear staining (40X); in Papillary RCC, strong cytoplasmic and nuclear staining (40X), in chromophobe RCC, strong cytoplasmic and nuclear staining, (40X). Arrows indicated nuclear staining.

Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

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Fig. 1

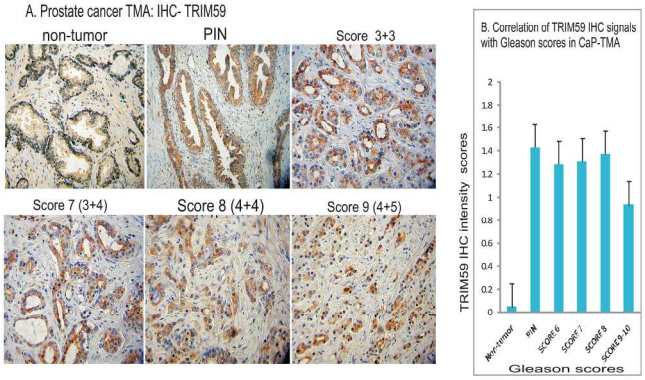

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Fig. 2 Immuno-staining of TRIM59 expression in five different types of kidney cancer (RCC)

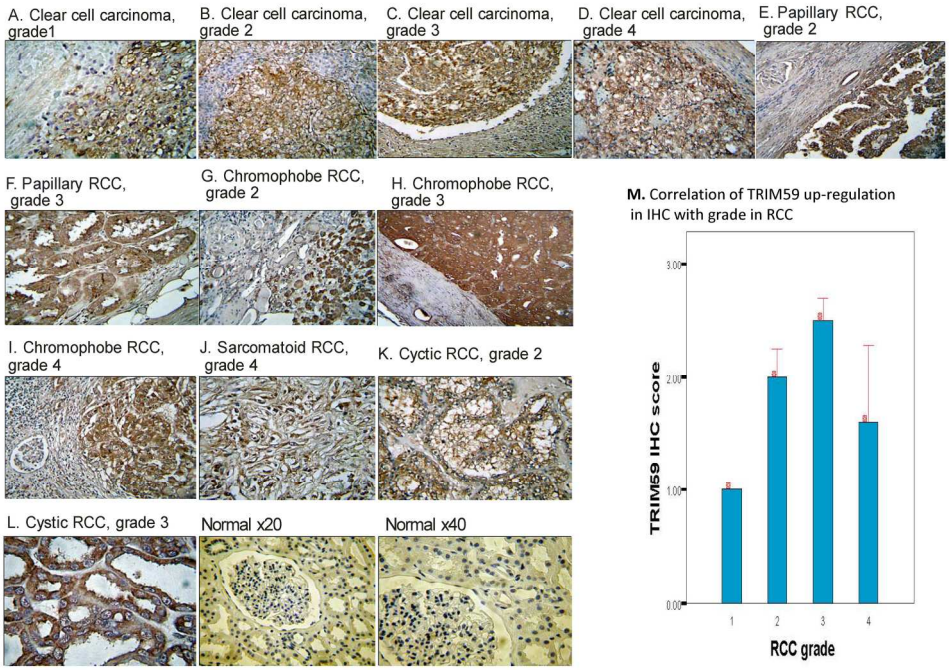

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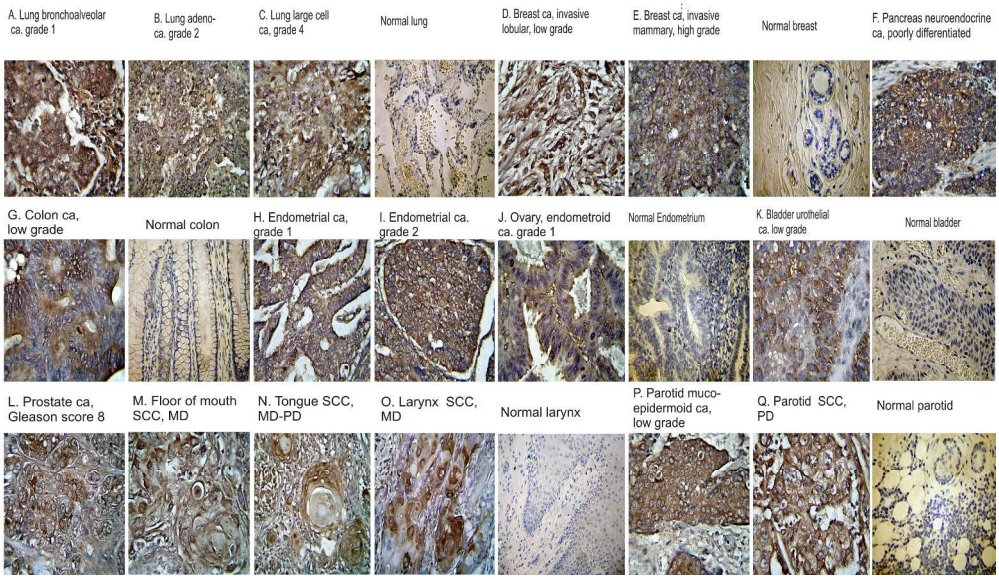
Fig. 3 Comparison of TRIM59 expression by immunohistochemistry in 8 different tumors



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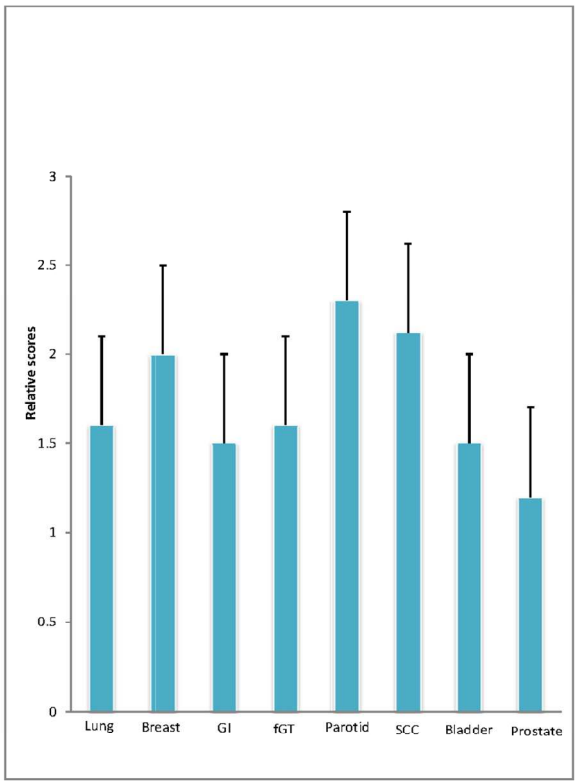
R. Comparison of relative scores of TRIM59 IHC staining in eight different tumor types



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Supplemental Fig. 1 Characterization of TRIM59 antibodies constructed and used for human tissue studies.

Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

Legend of Supplemental Fig. 1 (A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows.

(B). 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). The truncated forms of GST-TRIM59#71 may be due to bias of condon usage in the N-terminus. When immune to rabbits, we adjusted the doses by counting only the whole amount of all recombinant GST-TRIM59 proteins (1-1.5mg, GST protein was excluded).

(C-D). Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wildtype (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wildtype (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots.

(E). Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145 LnCaP and human kidney cell HEK293. An unique 53 kDa band was shown exactly as in mouse cell lyases **(C)**.

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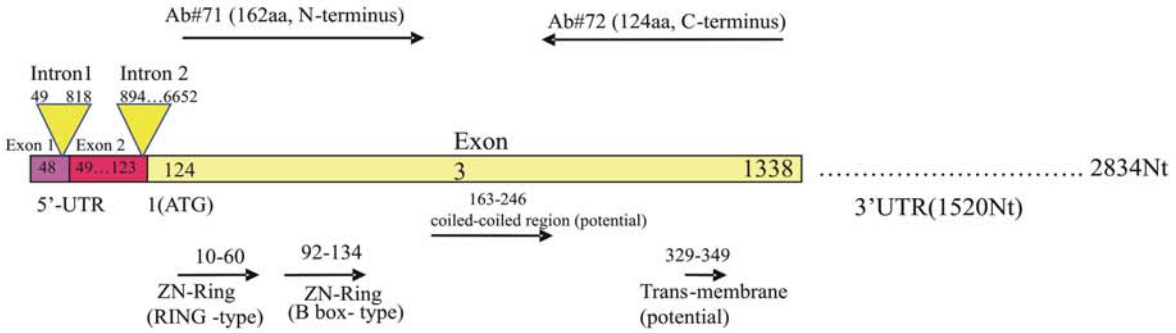
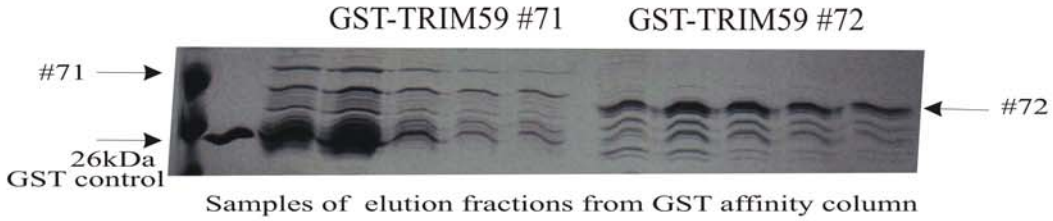
Supplemental Fig. 1

A. Structure, location of two antibodies to mouse TRIM59 GST fusion protein GST-TRIM59 #72 and /71

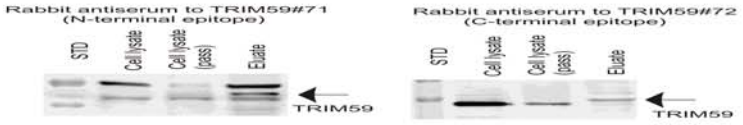


B. Purified GST-TRIM59 #72 and /71 recombinant proteins expressed in *E.coli* (10% SDS-PAGE)

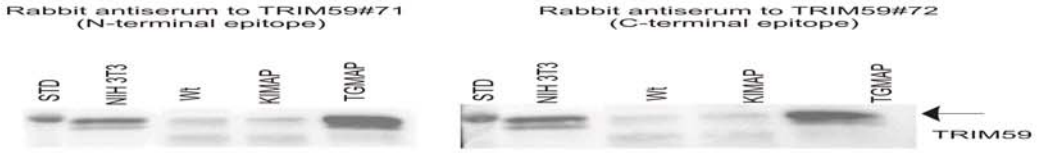


Characterization of TRIM59 antibodies
in mouse cells (C) and tissue lysates (D)

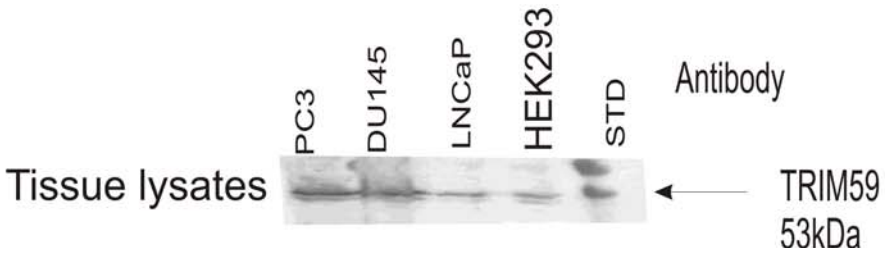
C. Total cellular phosphorylated proteins



D. Affinity purified proteins



E. Western blotting characterization of TRIM59/72 antibody in human cell lysates



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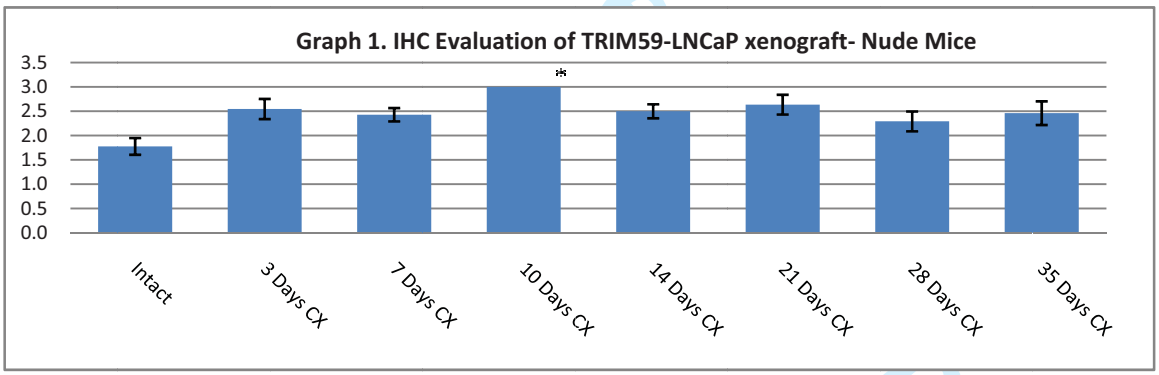
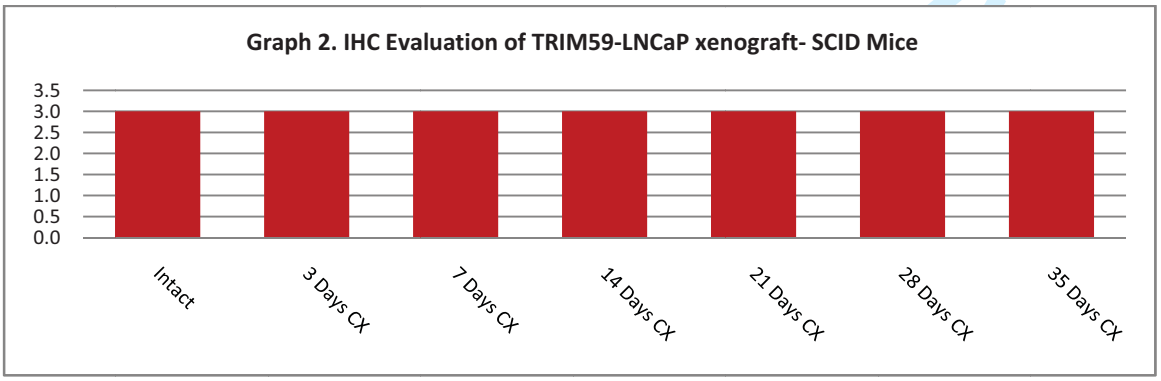
Supplemental Fig.2 **IHC evaluation of androgen responsiveness of TRIM59 in the LNCaP xenograft TMA**.

The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP , TRIM59 is significantly up-regulated after 10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.





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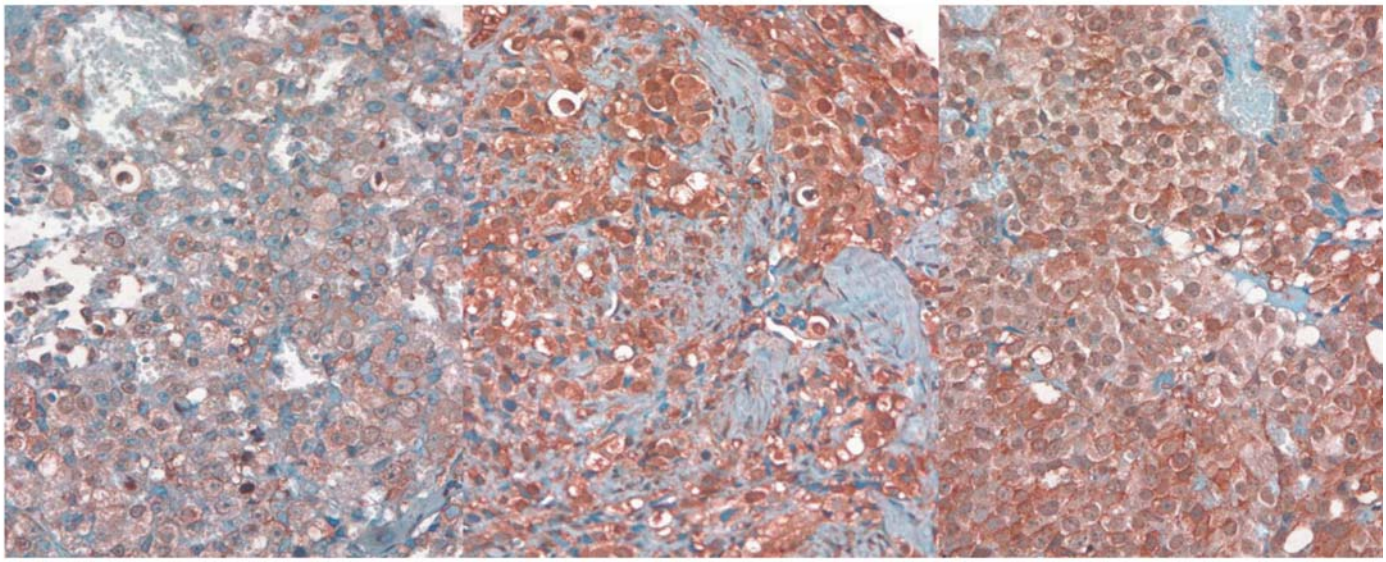
Suppl Fig. 2

TRIM59 SCID-LNCaP xenograft TMA

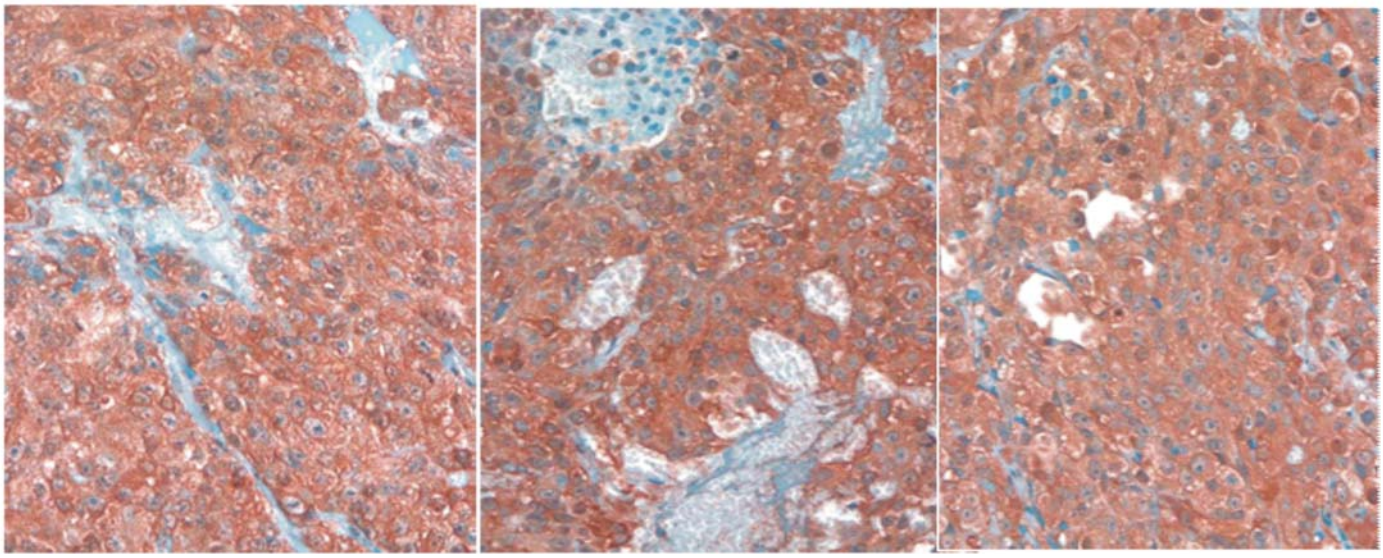
A Day after castration in nude mice

No castration 10 days 35 days



B Day after castration in SCID mice

No castration 14 days 35 days



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Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 and 1:1200. We assessed these IHC results with the negative control (without TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3. 1:1200 produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remain stronger than the normal non-tumor areas even at 1:300. In order to d detect weak TRIM59-IHC staining signals, we also tried 1:300 dilution and compared with negative controls (no antibody added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining, and similar results were obtained.

Legend of Figures:

(A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular lymphoma, (20X),positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining

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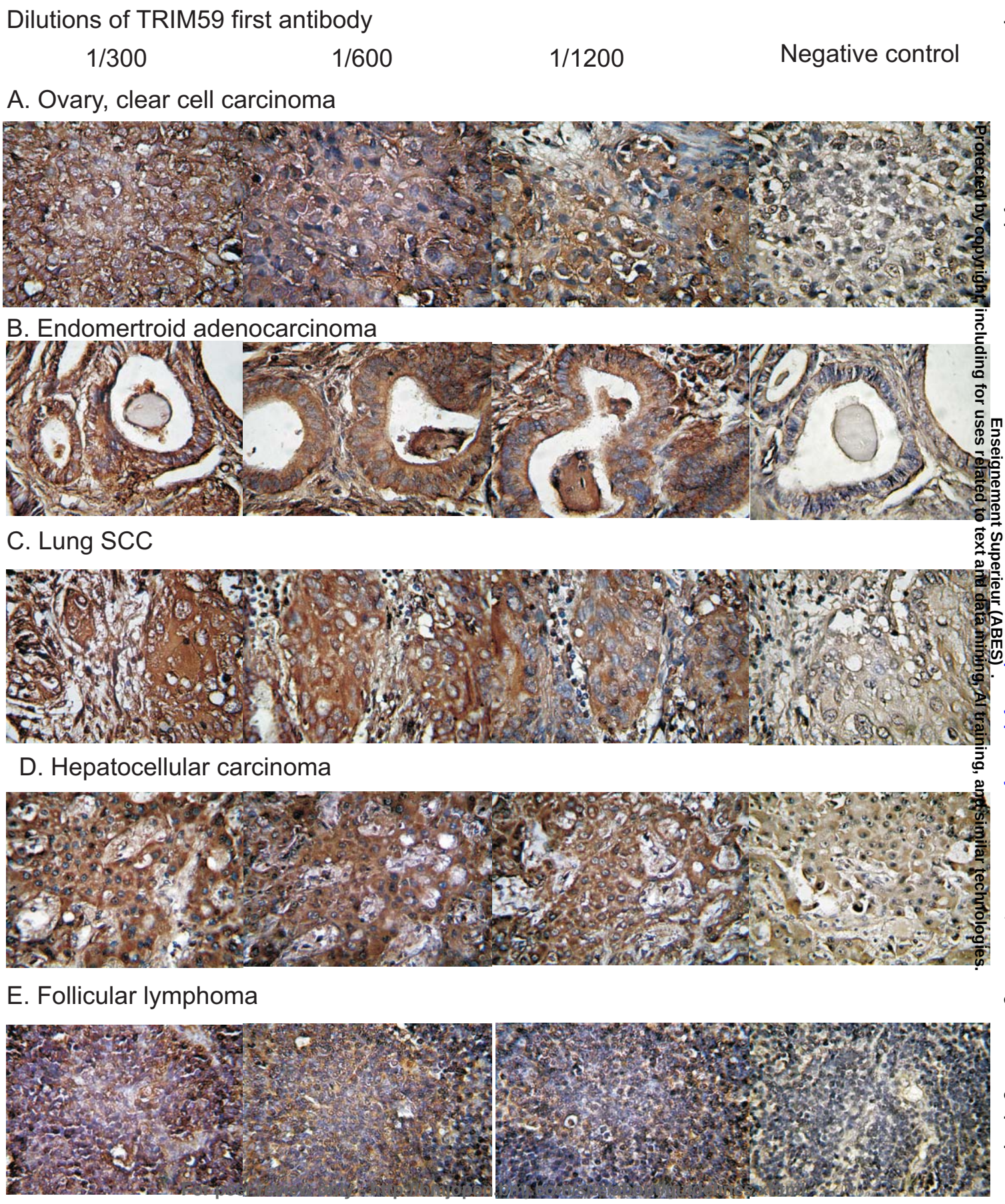
Supplemental Fig. 3 Titration of the diluted TRIM59 antibody in TMA (5 tumors) for moderate IHC staining



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Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry (IHC) staining in human multiple tumor TMA (tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody (positive) and without any antibody (negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some area of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin (squamous cell carcinoma), #4 Skin (basal cell carcinoma), #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma;#13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma, #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma), #2 Adrenal gland (cortical carcinoma), #7 Omentum (serous adenocarcinoma), #9 Cervix adenocarcinoma;

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma), #6 Uterus (leiomyosarcoma), #8 Ovary, serous adenocarcinoma, #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma;, and # 25. Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid)

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Legend of panels:

1. Kidney (Clear cell carcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

2. Adrenal gland (cortical carcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining with no nuclear staining; negative control in negative control, weak background staining in cytoplasmic of few cells (20X, 40X).

3. Skin (squamous cell carcinoma), positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

4. Skin (basal cell carcinoma), positive IHC for TRIM59 expression strong cytoplasmic staining with no nuclear staining; negative control no staining (20X, 40X).

5. Skin (melanoma), positive IHC for TRIM59 expression very weak cytoplasmic staining and nuclear staining; negative control no staining (20X, 40X).

6. Uterus (leiomyosarcoma), positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

7. Omentum (serous adenocarcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

8. Ovary (serous adenocarcinoma), positive IHC for TRIM59 expression weak to moderate cytoplasmic staining and no nuclear staining, negative control no staining (20X, 40X).

9. Cervix adenocarcinoma, positive IHC for TRIM59 expression moderate to strong cytoplasmic staining; negative control no staining (20X, 40X).

10. Breast ductal adenocarcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

11. Bladder (Urothelial carcinoma), positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

12. Thyroid medullary carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

13. Lung squamous cell carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

14. Lung bronchoalveolar carcinoma, positive IHC for TRIM59 expression strong cytoplasmic and nuclear staining; negative control no staining (20X, 40X).

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15. Lymph node metastatic carcinoid, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

16. Lung mesothelioma, positive IHC for TRIM59 expression strong cytoplasmic and nuclear staining; negative control no staining (20X, 40X).

17. Hepatocellular carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

18. Liver metastatic carcinoid, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

19. Endometrial carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

20. Small bowel, marginal zone lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

21. Spleen, Hodgkin lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

22. Stomach, malt lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining.

23.Thymoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

24. Appendix (Goblet cell carcinoid), IHC for TRIM59 expression no staining, negative control no staining (20X, 40X).

25. Follicular lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining, negative control no staining (20X, 40X).

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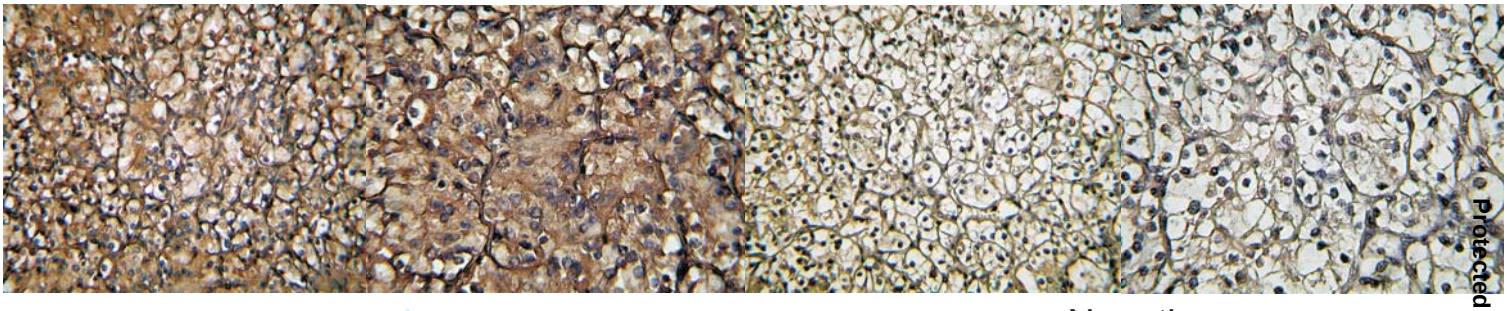
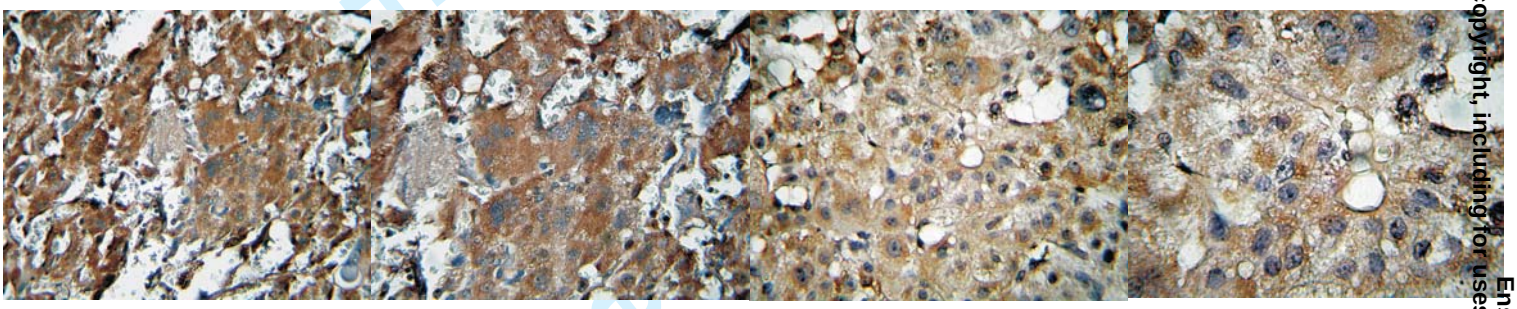
Suppl.Fig. 4 Positive and negative controls of immunohistochemistry (IHC) staining by TRIM59 antibody in 25 tumors listed from multiple-cancers TMA

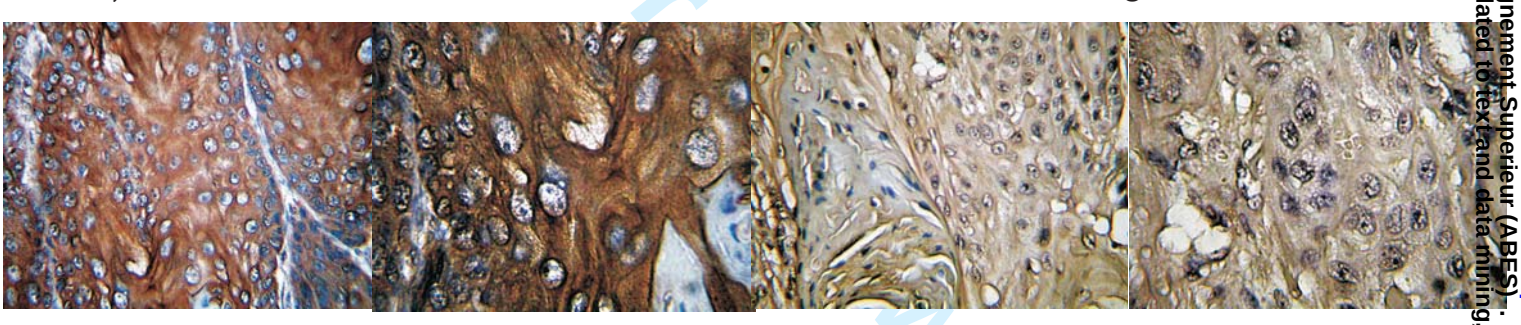
1-Kidney, clear cell carcinoma, positive (moderate) Negative



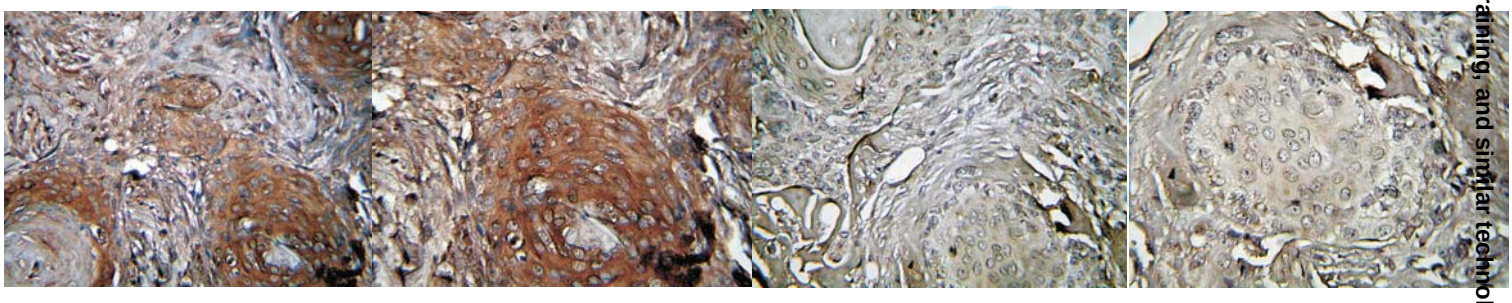
2-Adrenal gland, cortical carcinoma, positive (moderate) Negative



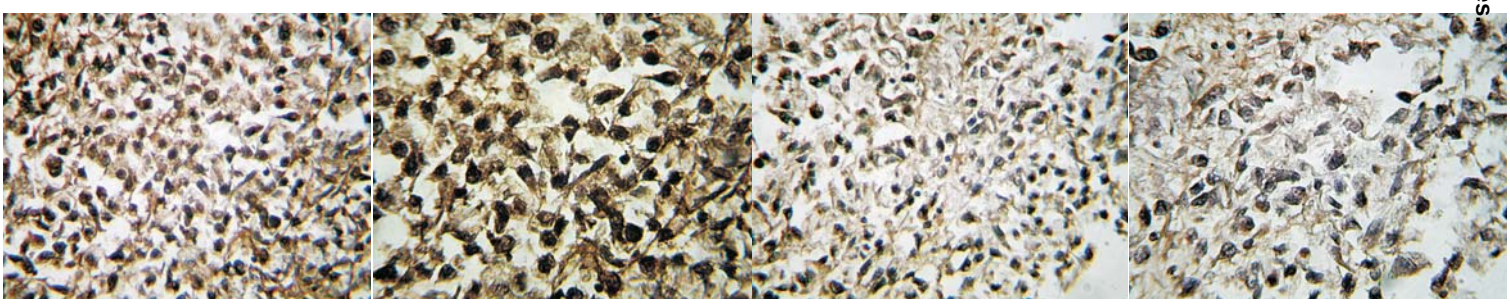
3-Skin, SCC, positive (strong) Negative



4- Skin, BCC, positive(strong) Negative



5-Skin, melanoma, positive (weak) Negative



x20 x40 x20 x40

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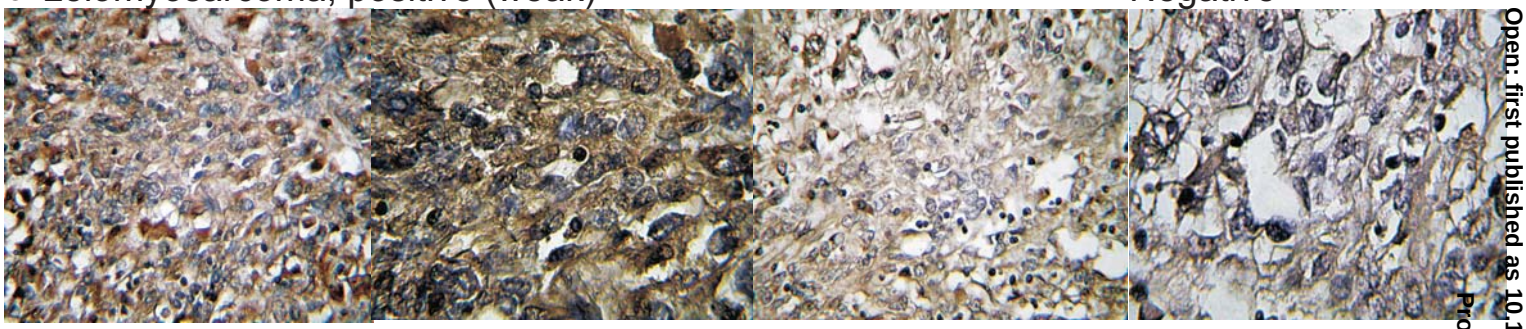
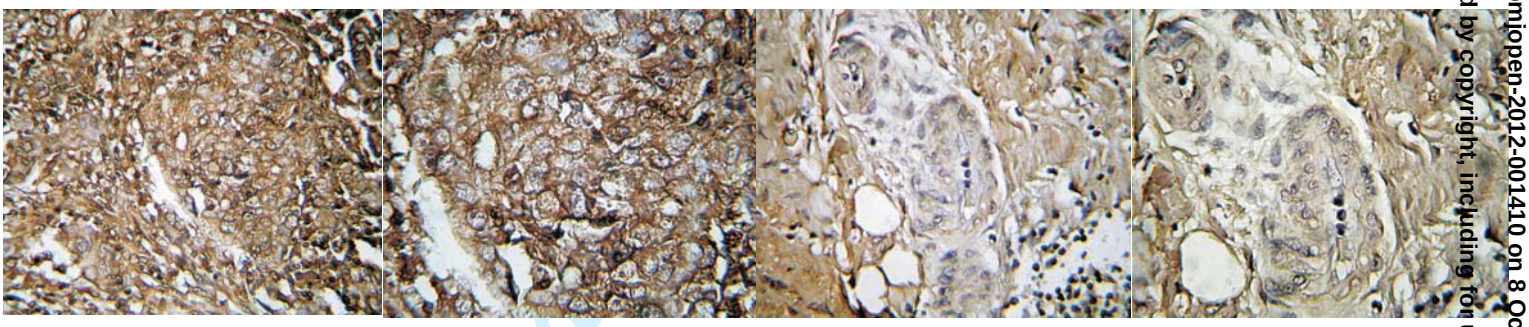
6- Leiomyosarcoma, positive (weak)

Negative



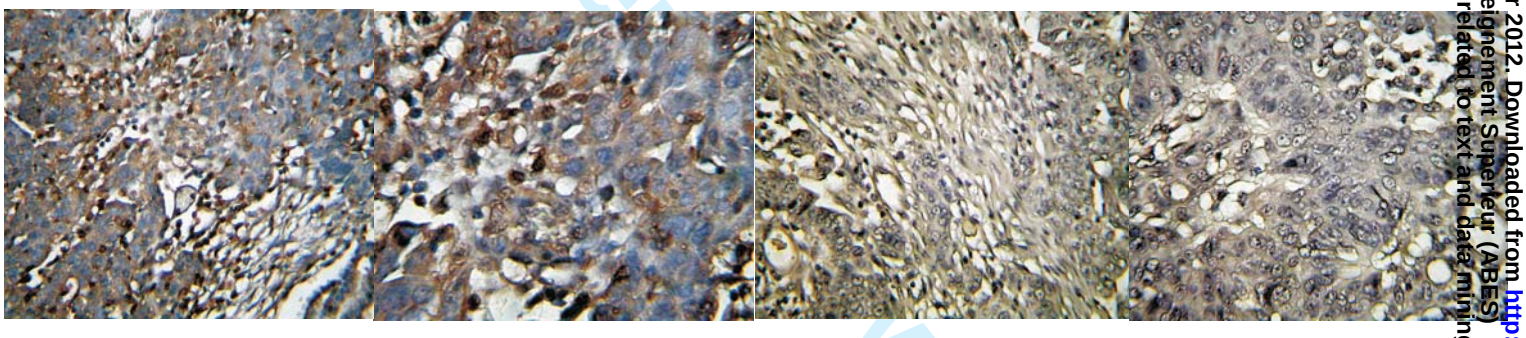
7- Omentum, serous adenocarcinoma, positive (moderate)

Negative



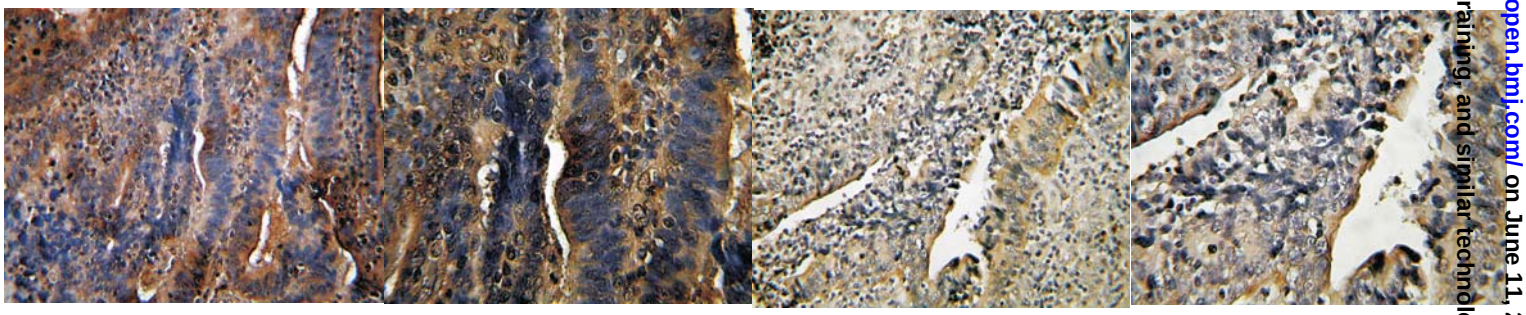
8- Ovary, serous adenocarcinoma, positive (weak)

Negative



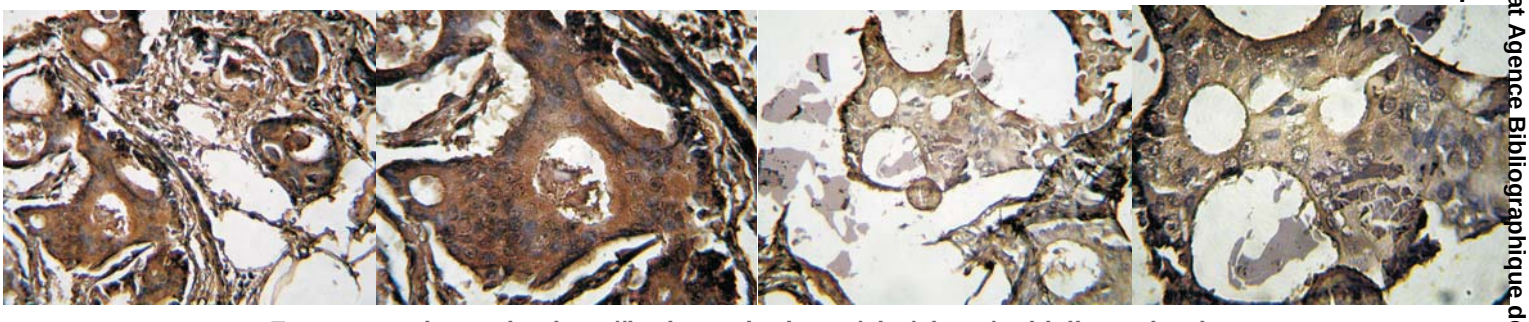
9- Cervix adenocarcinoma, positive (moderate)

Negative



10- Breast ductal adenocarcinoma, positive (strong)

Negative



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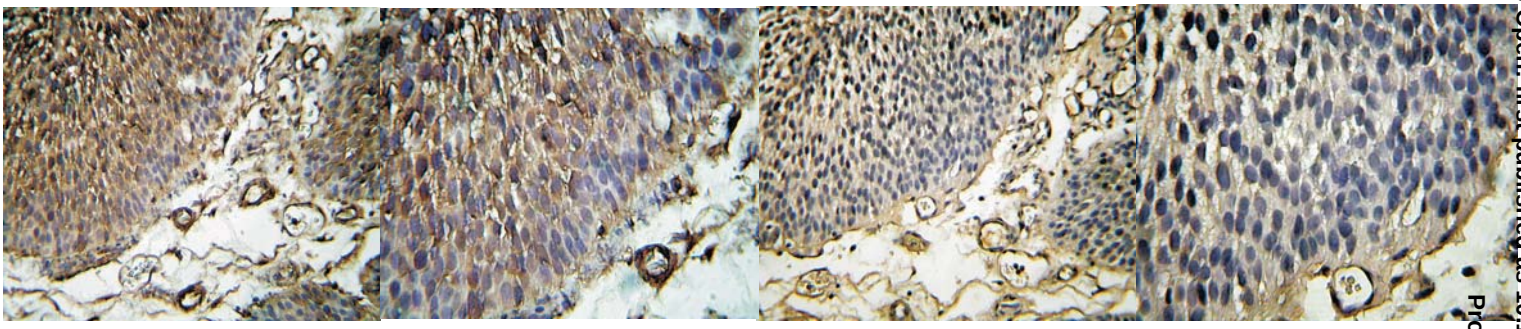
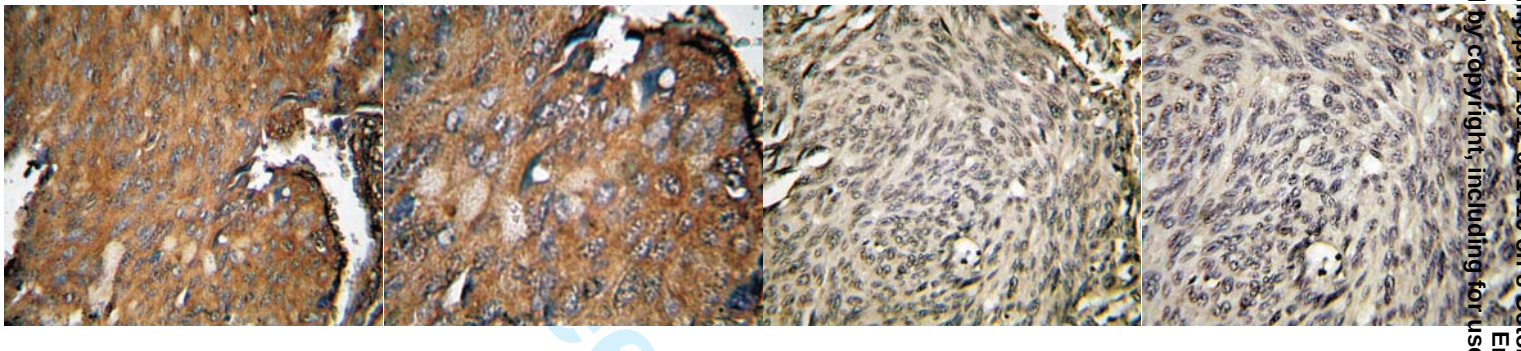
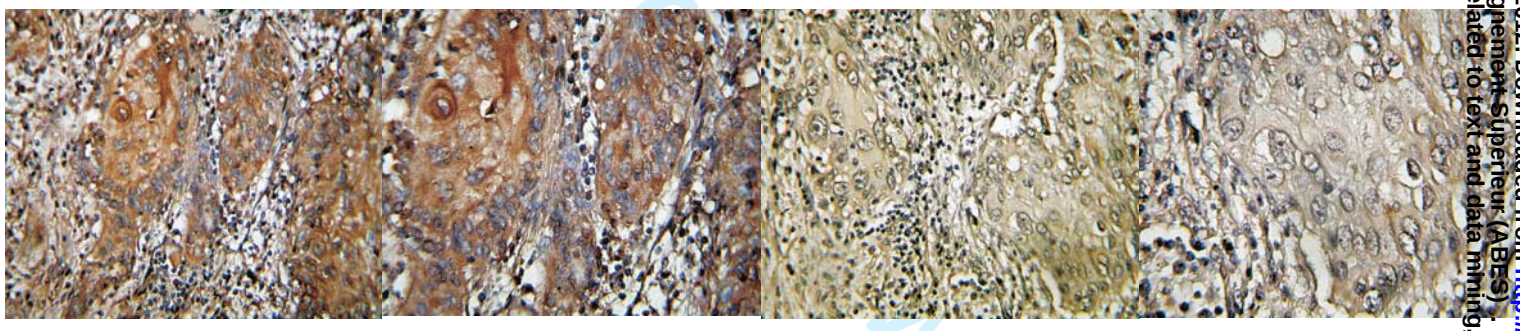
11- Bladder, Urothelial carcinoma, positive (weak) Negative



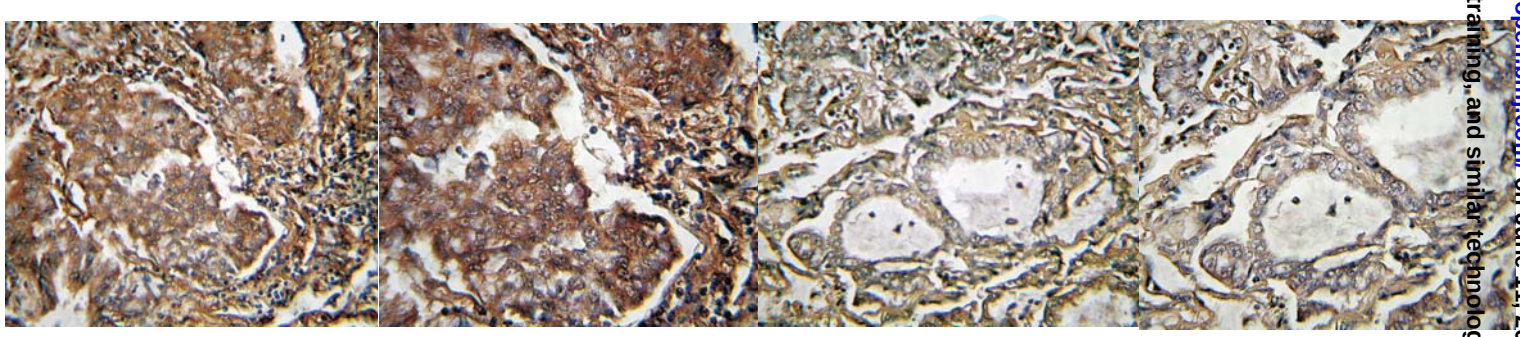
12-Thyroid, medulary carcinoma, positiv (strong) Negative



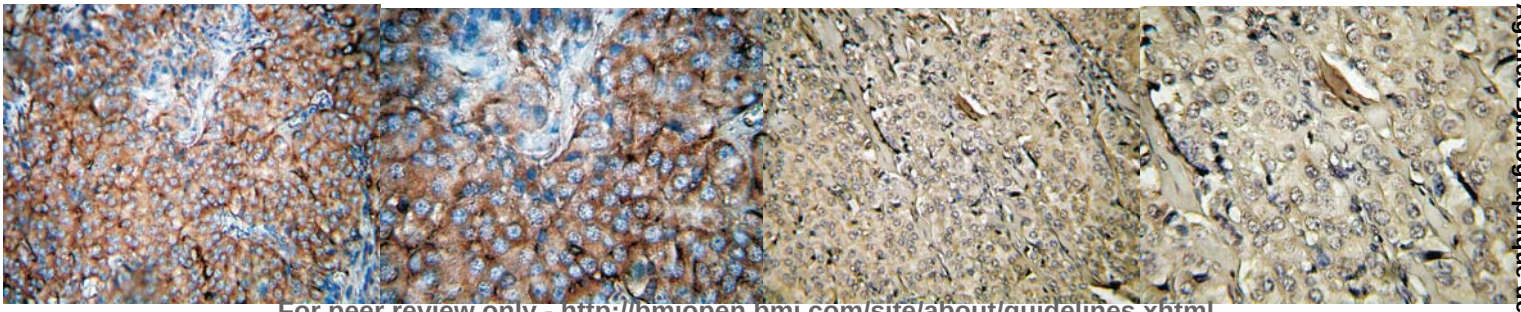
13-Lung SCC, positive (strong) Negative



14- Lung, bronchoalveolar carcinoma, positive (strong) Negative



15-Lymph node metastatic carcinoid, positive (strong) Negative



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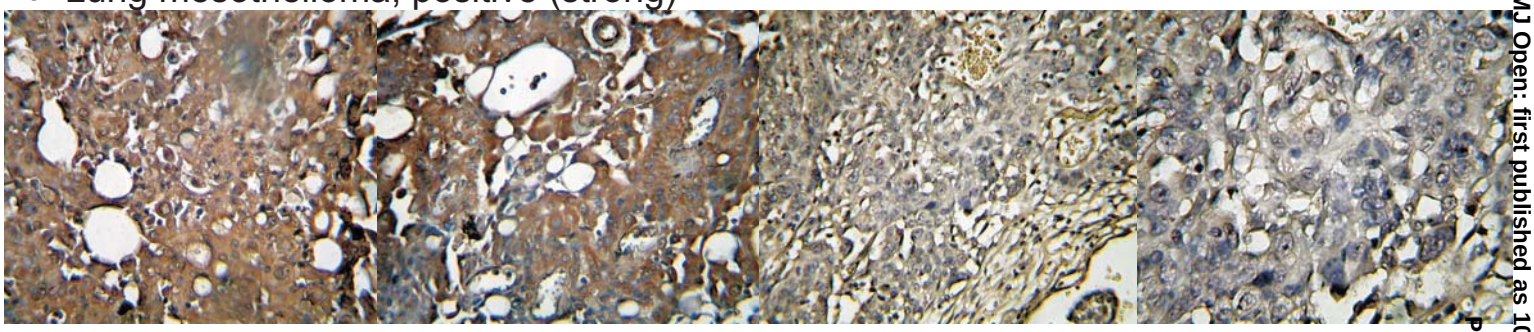
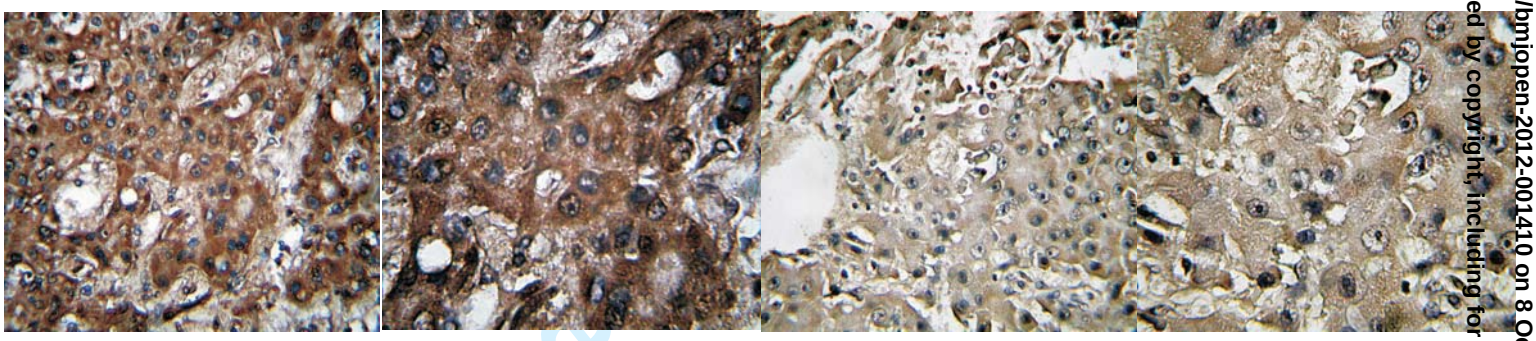
16- Lung mesothelioma, positive (strong)

Negative



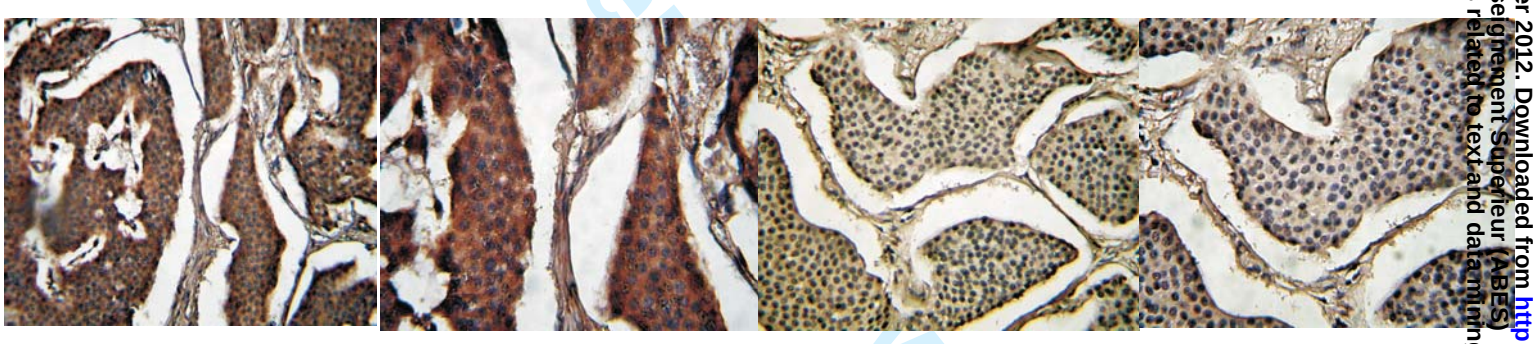
17- Hepatocell carcinoma, positive (strong)

Negative



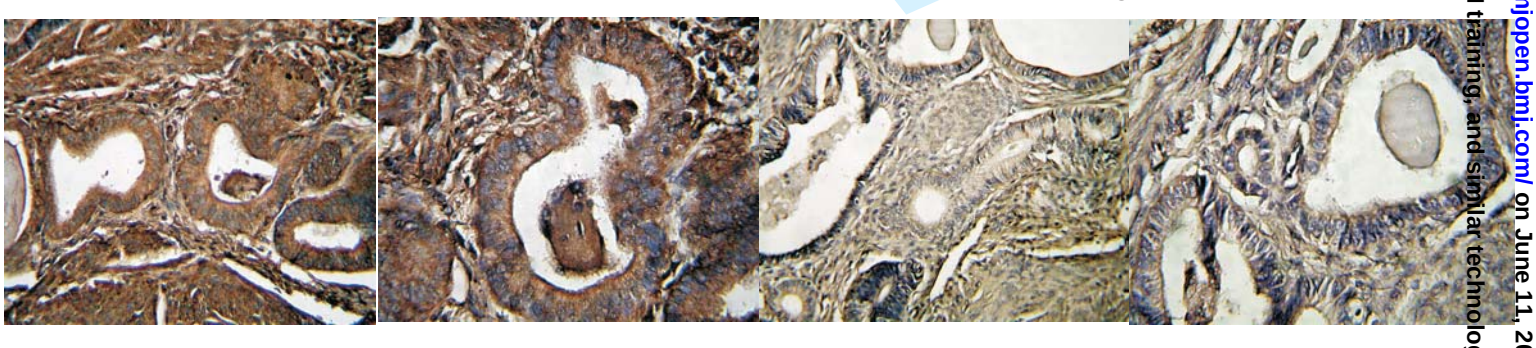
18- Liver metastatic carcinoid, positive (strong)

Negative



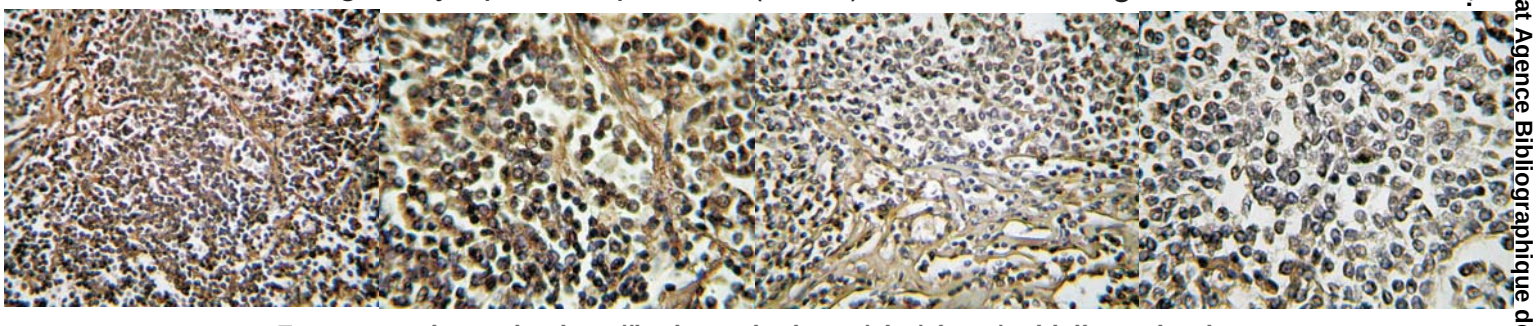
19- Endometrial adenocarcinoma, positive (strong)

Negative



20- Small bowel marginal lymphoma, positive (weak)

Negative



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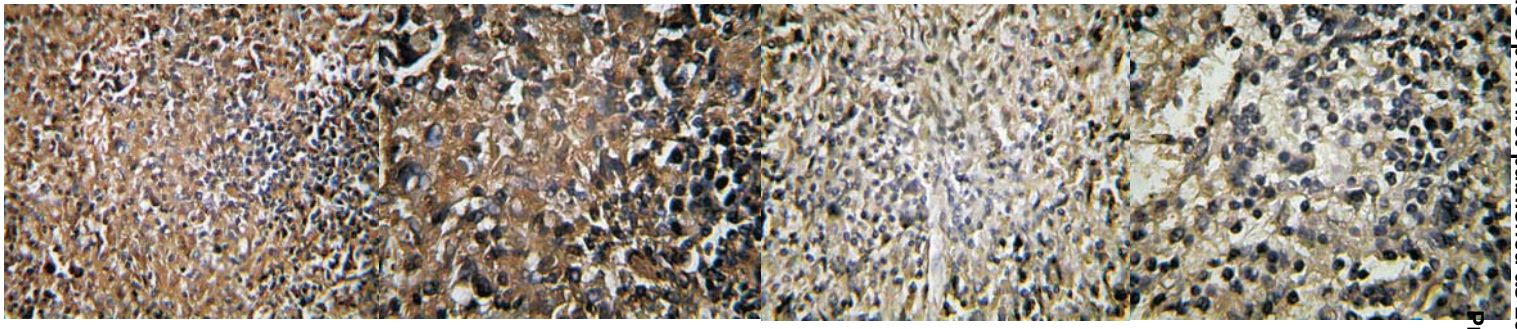
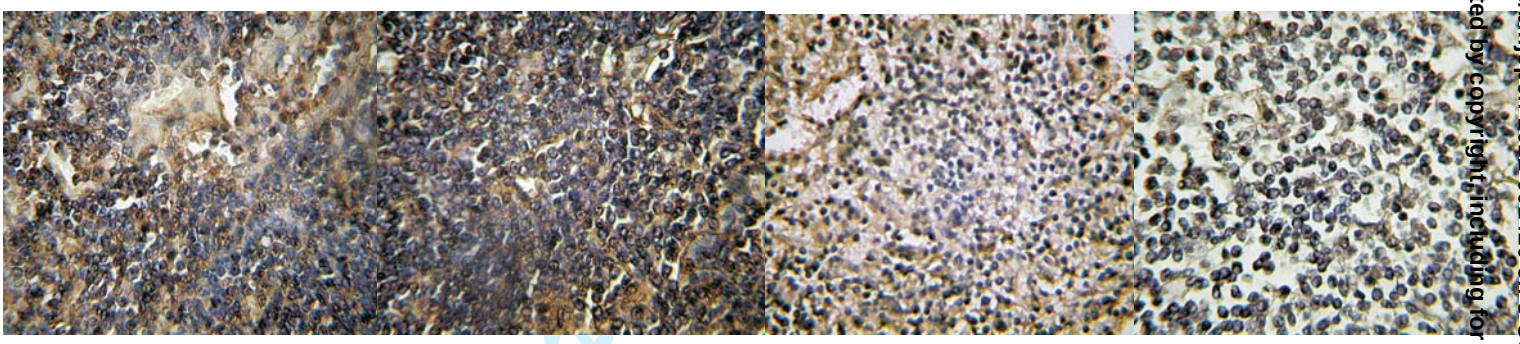
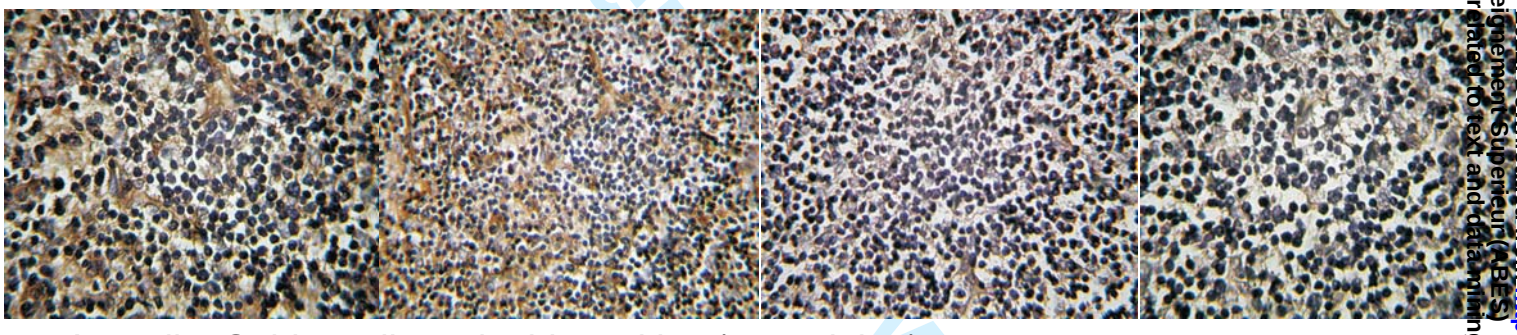
21- Spleen hodgkin lymphoma, positive (weak) Negative



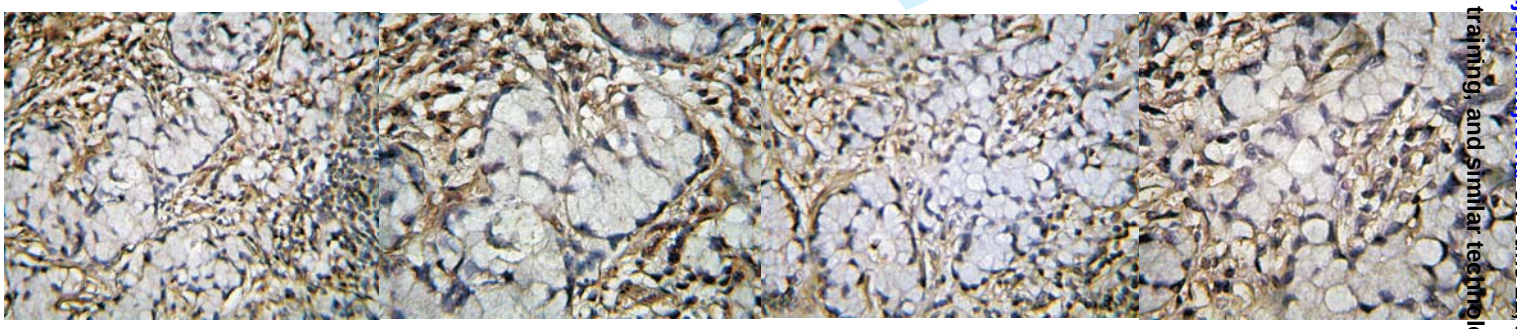
22- Stomach malt lymphoma, positive (weak) Negative



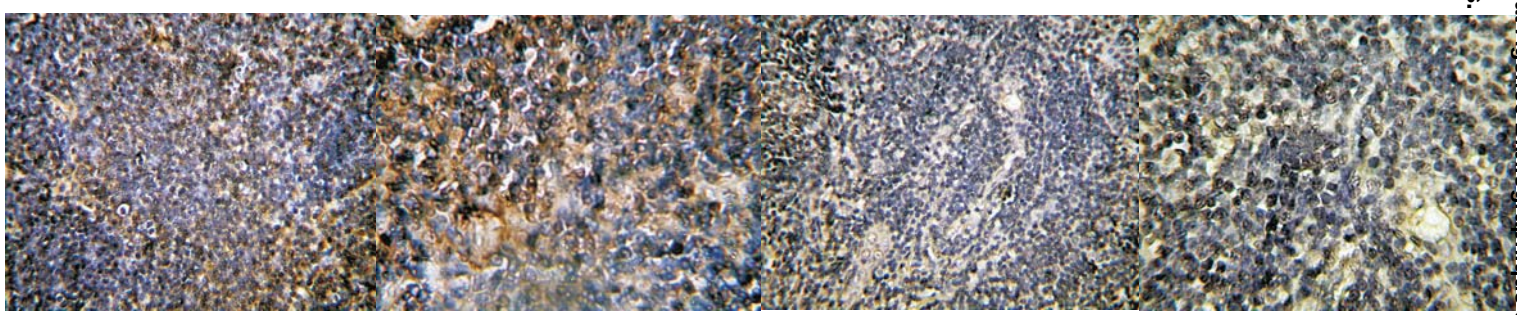
23- Thymoma, positive (weak) Negative



24- Apendix, Gablet cell carcinoid, positive (no staining) Negative



25- Follicular lymphoma, positive (weak) Negative



x20 x40 x20 x40

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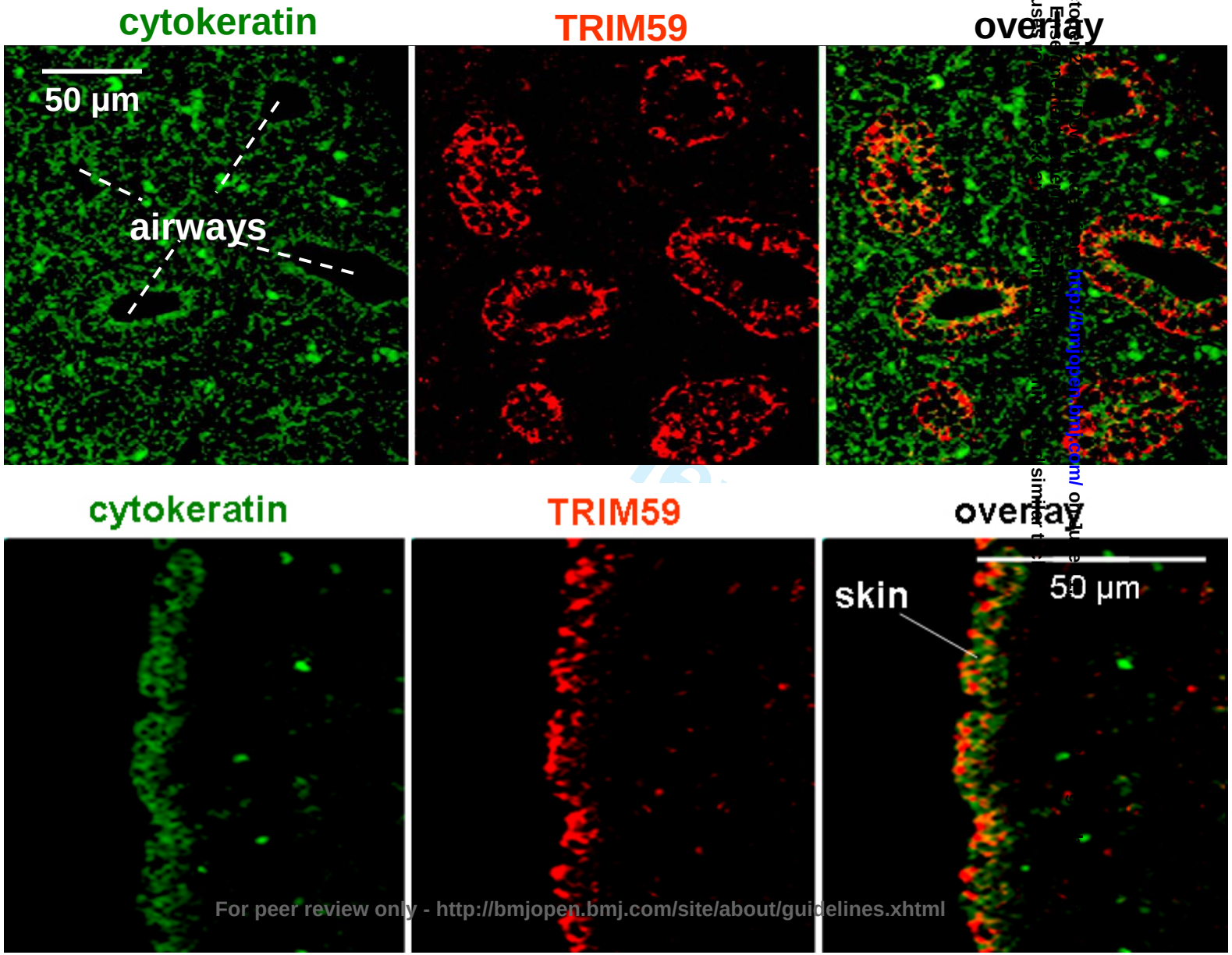
Supplemental Fig. 5

Confocal microscope imaging of immunohistochemistry of TRIM59 protein in mouse embryo (14.5d.p.c)



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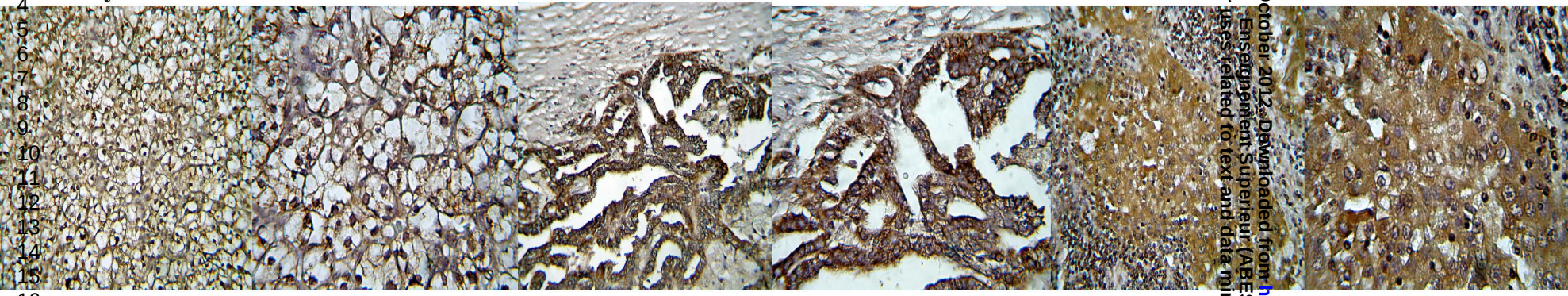
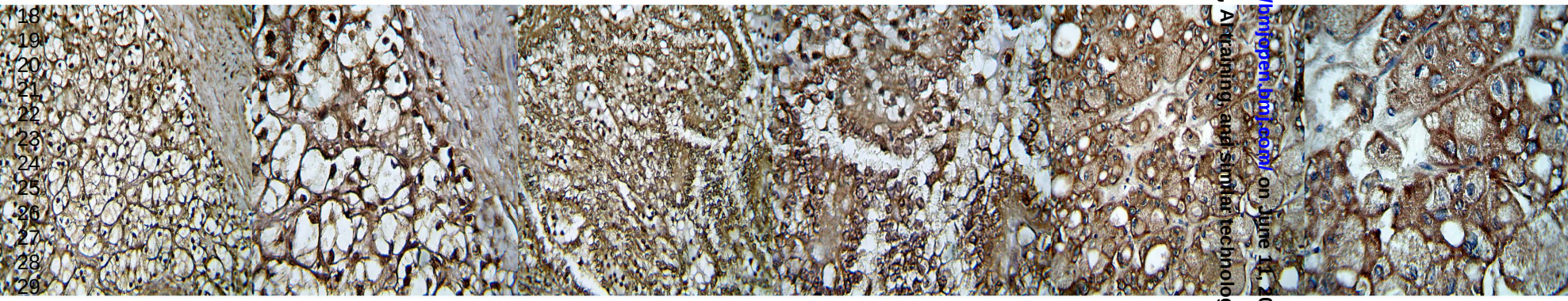
Test of Ras activation in kidney cancer patients correlated with up-regulation of TRIM59

A. Clear cell carcinoma B. Papillary RCC C. Chromophobe RCC

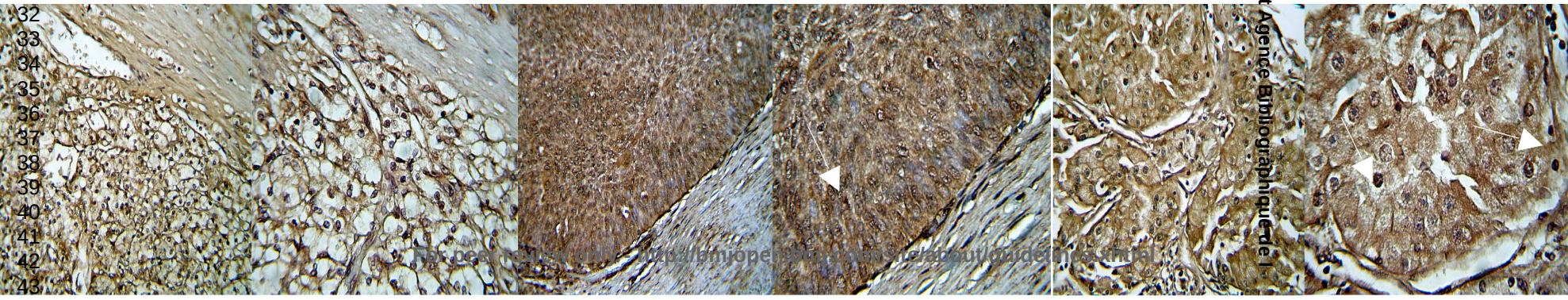
Antibody: B-Raf for total B-raf



Antibody: Raf 1 for total Raf



Antibody: Phosphor- Ser- 259 Raf 1



X20 X40 X20 X40 X20 X40

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Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

Antibody used	RCC types	Patient number	Intensity score	Nuclear staining
B-Raf (total B-raf)	Clear cell Carcinoma	6	0-1	No
	Chromophobe	3	2-3	No
	papillary	3	2-3	No
Raf-1 (total B-raf)	Clear cell Carcinoma	3	0	No
	Chromophobe	2	2-3	No
	papillary	1		No
B-Raf p-Ser ^{259} (phosphorylated B-Raf)	Clear cell Carcinoma	3	0-1	No
	Chromophobe	1	2-3	20%
	papillary	2	2-3	50%

Since BRAF is the most important early effector in the Ras signal pathway, and Raf mediate phosphorylation is the main effector recruited by GTP-bound Ras to activate the MEK-MAP kinase pathway, we performed IHC studies in these RCC sections by probing Raf activation /up-regulation and hyper-phosphorylation. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing for total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing activated phosphorylated B-Raf. Some of serial slides were stained parallelly on each patient by different antibodies.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7,8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*9*
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	9
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	9
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			9

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	6
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	17
Discussion			16
Key results	18	Summarise key results with reference to study objectives	
Limitations			18
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

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TRIM59, a novel multiple cancer biomarker for immunohistochemical detection of tumorigenesis

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Running title: TRIM59, a novel multiple-cancer marker for tumorigenesis

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List of abbreviations

Abbreviations: TRIM: TRIpartite Motif; CaP: prostate cancer; BPH: benign prostatic hyperplasia; PIN: prostate intraepithelial neoplasia; TMA: tissue microarray; RCC: renal cell carcinoma; SCC: squamous cell carcinoma; GI: gastrointestinal; GU: genitourinary; d.p.c: days post conception; GEM (genetically engineered mouse); H&E: haematoxylin and eosin; IHC: Immunohistochemistry; WD: well differentiated ; MD: moderately differentiated ; PD: poorly differentiated.

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Abstract

Objectives and design: We identified a novel TRIM59 gene, as an early signal transducer in two (SV40Tag and Ras) oncogene pathways in murine prostate cancer (CaP) models. We explore its clinical applications as a multi-tumor marker detecting early tumorigenesis by immunohistochemistry.

Setting and Participants: 88 CaP patients were from a tissue microarray (TMA) of radical prostatectomy specimen, 42 patients from a 35 multiple-tumor TMA, 75 patients with renal cell carcinoma (RCC) and 92 patients from 8 different tumor groups (breast, lung, parotid, gastrointestinal, female genital tract, bladder, kidney and prostate cancer).

Results: TRIM59 up-regulation specifically in tumor area was determined by immunohistochemistry in 291 cases of 37 tumor types. To demonstrate that TRIM59 up-regulation is "tumor-specific", we characterized a significant correlation of TRIM59 IHC signals with tumorigenesis and progression, while in control and normal area, TRIM59 IHC signal was all negative or significantly low. TRIM59 protein up-regulation in prostate and kidney cancers was detectable in both intensity and extent in early tumorigenesis of PIN (prostate intraepithelial neoplasia, $P<0.05$) and grade 1 of RCC ($P<0.05$), and stopped until high grades cancer. The results of the correlation in these two large cohorts of tumor types confirmed and repeated murine CaP model studies. Enhanced TRIM59 expression was identified in most of the 37 different tumors, while the highest intensities were in lung, breast, liver, skin, tongue and mouth (squamous cell cancer) and endometrial cancers. Multiple-tumor up-regulation was further confirmed by comparing relative scores of TRIM59 IHC signals in eight tumors with a larger patient population; and by a mouse whole mount embryo (14.5dpc) test on the origin of TRIM59 up-regulation in epithelial cells.

Conclusions: TRIM59 may be used a novel multiple-tumor marker for immunohistochemical detecting early tumorigenesis and could direct a novel strategy for molecular targeted diagnosis and therapy of cancer.

Keywords: TRIM59, Cancer biomarker, Immunohistochemistry, Prostate cancer, Multiple cancer marker, Ras signal pathway, SV40Tag oncogene, Tissue microarray, Confocal microscopy.

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Introduction

The TRIM (*TRI*partite *M*otif) family is an evolutionarily conserved gene family implicated in a number of critical processes including immunity [1-3], antiviral [4-8], proliferation [6, 9], transcriptional regulation [6, 10], neuro-development [11, 12], cell differentiation [12] and cancer [13] (reviewed in [1, 5, 14, 15]). However, the function of most TRIM family members is poorly understood and was surmised only based on computational analysis from their RBCC (*R*ING finger, *B*-box, Coiled-*C*oil) domains. The domain of RING (*R*eally *I*nteresting *N*ew *G*ene) is frequently involved in proteolysis acting as E3 ubiquitin ligases and the ubiquitin-proteasome system in the regulation of numerous cellular processes including cell cycle regulatory proteins, transcription factors, and signal transducers [6, 15]. Recent studies demonstrate that various TRIM-NHL proteins function as cofactor for the microRNA-induced silencing complex (miRISC) [12] [14, 16]. An ataxia-telangiectasia group D complementing gene (ATDC) was recently designated as TRIM29, which is elevated in most invasive pancreatic cancers in the Wnt/β-catenin signalling pathway [13].

In a previous report [17], we characterized the function of TRIM59, a novel TRIM family member, in SV40 Tag oncogene directed genetically engineered mouse (GEM) prostate cancer (CaP) models [18-21]. The TRIM59 gene was identified to be correlated with the SV40 Tag initiated tumorigenesis. TRIM59 protein up-regulation and hyper-phosphorylation started in the prostate cytoplasm in early tumorigenesis from PIN (prostate intraepithelial neoplasia) [17]. As a signal pathway effector, the p-Ser/Thr phosphorylated TRIM59 proteins correlate with tumorigenesis, while p-Tyr-TRIM59 proteins correlate with advanced CaP. The function of TRIM59 was identified by shRNA knockdown in human CaP cells resulted in S-phase arrest and cell growth retardation. Although TRIM59 is an effector gene with the SV40Tag oncogene, the

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initial functional targets of TRIM59 function were actually on the Ras signal pathway as an early and rapid signal transmitter. In a transgenic mouse test of TRIM59 up-regulation specifically in the prostate, TRIM59 demonstrated full oncogenic activity in directing tumorigenesis and progression to highest grade CaP. The signal pathway of TRIM59 may be possibly linked with two large oncogene routes: the Ras/Raf/MEK/ERK/P133K/AKT and the SV40 Tag/p53/pRB routes [(17)]. In this study we explored the clinical utility of the novel TRIM59 gene to serve as a biomarker for a panel of human cancers. To address the molecular biology basis, we combined results from both basic (animal model [(17)]) and human clinical studies.

Materials and Methods

Patient selection:

All patient samples were acquired as part of REB (Research Ethics Board) approved protocols at the University of Western Ontario (UWO) and Vancouver Prostate Center, University of British Columbia (UBC). Table 1 shows a complete list of 291 patients with 37 different tumor types examined in this study.

Prostate cancer Tissue Microarrays (TMA): 88 CaP patients between 2006 and 2008 who had no treatment prior to radical prostatectomy, were selected from the Vancouver General Hospital. Each patient block marked as containing benign tissue or cancer was sampled 2 times with a core diameter of 1 mm arrayed (176 cores) in a rectangular pattern with 0.7 mm between the centers of each core, creating a duplicate TMA layout using a manual tissue micro arrayer (Beecher Instruments, Silver Spring, MD). The TMA paraffin blocks, were sectioned into 0.5 micrometer sections and mounted on the positively charged slides.

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Table 1 Patient list selected in this study

Organ	Patient Number	Tumor type		Tumor grade	
35 tumor-TMA	42	Description in Table-2			
Prostate TMA	105	Adenocarcinoma		BPH	16
				PIN	4
				Gleason score 4	5
				Gleason score 6	31
				Gleason 7	15
				Gleason score 8	16
				Gleason score 9-10	9
				Stroma	3
				Absent cores	6
Kidney	75	Clear cell carcinoma	43	Grade 1	4
		Papillary RCC	11	Grade 2	38
		Chromophobe RCC	13	Grade 3	28
		Cystic RCC	6	Grade 4	5
		Sarcomatoid RCC	2		
Bladder	44	Urothelial carcinoma		Low grade	38
				High grade	6
Lung	4	Bronchoalveolar carcinoma	1	Grade 1	1
		Adenocarcinoma	1	Grade 2	1
		Large cell carcinoma	1	Grade 3	1
		Squamous cell carcinoma	1	Grade 4	1
Breast	3	Invasive lobular carcinoma	1	Grade 1	1
		Invasive mammary carcinoma	2	Grade 3	2
Female Genital tract	5	Endometrial carcinoma	4	Grade 1	4
		Ovary, Endometrioid carcinoma	1	Grade 2	1
Gatrointestinal tract	2	Colon carcinoma	1	Low grade	1
		Pancreas neuroendocrine carcinoma	1	high grade	1
Parotid	3	Mucoepidermoid carcinoma	1	low grade	1
		Metastatic SCC	1		
		Metastatic neuroendocrine Carcinoma	1	high grade	2
Mouth, tongue and larynx	4	Squamous cell carcinoma	4	Moderately differentiated	3
				Moderately to Poorly differentiated	1
Total	291				

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Automated image, acquisition and analysis on immunohistochemical staining of CaP-TMA: (UBC) Immunohistochemical staining was conducted by Ventana autostainer model Discover XT ™ (Ventana Medical System, Tuscan, Arizona) with enzyme labelled biotin streptavidin system and solvent resistant DAB Map kit. TMA was scanned by Bliss Digital imaging system using x20 objective, from Bacus Laboratories INC, Centre Valley PA, and stored in the Prostate Centre Saver (http//bliss.prostatecentre.com). A value on a four-point scale assigned to each core.

Multiple–tumor Tissue Microarray construction: Tissue samples form 42 patients that encompassed 35 distinct tumor subtypes were selected from London Laboratory Service Group, and the tumor bank in the Department of Pathology (UWO). TMA slides were constructed with triplicate cores for each sample following standard procedure as described [24]. 0.6 mm sections were prepared from TMA block and re-stained by H&E for each case to confirm the diagnosis.

Histopathologic analysis: All cases from 37 tumor types were graded according to standardized histopathology grading systems [25] by M.M (pathologist) and V. K (MD fellow).

Immunohistochemistry (IHC) and results evaluation: Standard ABC (Avidin Biotin Complex) protocol was performed as previously reported [21, 26, 27]. Two kinds of blocking reagents were used: Power Block (Universal Blocking Reagent, from BioGenex, San Ramon, CA) and Avidin-biotin blocking reagent kit (Vector Labs, Burlingame, CA). All B-Raf antibodies were from GenScript (Piscataway, NJ): B-Raf antibody (monoclonal antibody, used 1:500 dilution), Raf 1 (polyconal, 1:50) and Raf -1 -(polyclonal, Phospho-Ser^{259} 1:50).

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TRIM59 IHC staining signals were assessed by intensity for cytoplasmic staining and extent to assess percentage of nuclear staining as previously reported [28, 29]. Since in some tumors TRIM59 showed only cytoplasmic staining, for the purpose of comparing in different tumors, we used a combined relative score system based on both intensity and extent as following: score 0: 0/0 (intensity/ extent); score 1: weak cytoplasmic staining and/or $\leq 25\%$ nuclear staining; score 2: moderate cytoplasmic staining and/or $\leq 50\%$ nuclear staining; score 3: strong cytoplasmic staining and/or $\geq 50\%$ nuclear staining. All relative scores were accessed by two researchers independently M.M (pathologist) and V. K (MD fellow).

Construction and characterization of TRIM59 antibody:

Supplement Fig. 1 provided details, which combined Supplement materials of [17], and more informations. Supplement Fig. 1A showed that the antibody TRIM59#72 used for mouse and human studies was prepared from C-terminal 136 a.a. peptide (as recombinant GST- fusion protein, Suppl. Fig. B) of whole protein 403aa which is TRIM59 specific, while antibody TRIM59#71 was from mouse TRIM59 N-terminal sequence (163aa) containing mostly the common RBCC function domains of more than 70 members TRIM family [17]. As shown in Supplemental Fig. 1C, Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed multiple bands. We also demonstrated that N-terminal sequence specific antibody TRIM59#71 can recognize purified proteins from C-terminal sequence specific, TRIM59#72 Ab affinity column (the same 53kDa protein, Suppl. Fig. 1D). Since these two immunogenic sequences are not overlapped, we thus verified that they are detecting the same protein. We demonstrated that TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots (Suppl Fig. 1E).

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Confocal microscope imaging of immunostaining of mouse embryos 14.5 dpc (days post conception) for TRIM59: The mouse embryos were prepared according to our previous reports [18-21]. The anti-rabbit secondary antibody was conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA). Sections were double-stained with pan-cytokeratin antibody with FICT (Sigma), and the stained mouse embryo sections were visualized using a Carl Zeiss confocal microscope by the LSM Image program.

Statistical Analysis: Student's *t* tests and one-way ANOVA were used by programs of Microsoft Excel 2007 or SPSS 10 to analyze the data with $p < 0.05$ considered to be statistically significant.

Results and Discussion

TRIM59 up-regulation in human prostate cancer TMA (tissue microarray): correlation with tumorigenesis and tumor progression by TRIM59 intensity until high grade CaP:

We designated TRIM59 as one of the "tumorigenesis–associated" genes correlated with SV40 Tag oncogenesis in mouse prostate cancer (GEM-CaP) models [17]. SV40 Tag is essentially only required for the initiation of tumorigenesis, *i.e.* the "hit-and-run" effect, in GEM-CaP, but not for the tumor progression and metastasis directly. The "tumorigenesis–associated" effect, is due to the initial binding of the Tag oncogene with retinoblastoma (pRB), p53 proteins and several transcriptional co-activators. Once this process is initiated, the signal-transduction will continue on, even without the initiation effectors.

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In GEM-CaP models, the TRIM59 protein up-regulation correlation with tumorigenesis and progression, and down-regulated in the high grade CaP by immunohistochemistry (IHC) [17]. We assume this "tumorigenesis–associated" effect of TRIM59 may apply to human cancer studies.

We first characterized that TRIM59 antibody (#72) can cross react with and recognize specifically human TRIM59 counterpart (details see supplemental Fig. 1). We tested by IHC in a TMA of CaP patients (n=88, 176 cores). TRIM59 IHC signals detected in an automated digital image system were identified mostly in cytoplasm of luminal cells (Fig. 1A), which is different from rapid tumor progression mouse CaP models. The intensity (score= 2) in PIN (n=4, Fig. 1A) was higher than in non-tumor area (normal and BPH). Moderate to strong expression was observed in Gleason score 6 (3+3, n=25), 7 (4+3, n=15), and 8 (4+4, n=14). In high grade CaP (score 4+5, n=8, Fig. 1A), TRIM59-IHC signals were lower. As shown in a graph of Fig. 1B, TRIM59 protein IHC signals correlated significantly (P=0.014) with tumorigenesis and progression from PIN to -WDCaP (Gleason grade 1-2, scores 2-4) and MDCaP (Gleason grade 2-4, scores 4-8) (graph Fig 1B), and decreased in high grade CaP (Gleason score 9-10) with P=0.018, which is similar to GEMs [17].

TRIM59 up-regulation in human Renal Cell Carcinoma (RCC) patients: correlation of tumorigenesis and tumor progression with TRIM59 intensity until high grade RCC:

Next, we demonstrated that TRIM59 is not androgen-responsive and likely not prostate tissue-specific, indicating that the tumorigenesis role of TRIM59 may be applied to all or most of human cancers. Supplemental Fig. 2 showed this result in detail of IHC test by TRIM59 antibody#72 on a TMA of the LNCaP human CaP xenografts in nude/SCID castrated mice.

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We extended results from prostate cancer clinical samples to kidney cancer. We started 75 renal cell carcinoma (RCC) patients including all 5 different types of RCC tumors: 43 clear cell carcinoma (representative IHC figures shown in Fig. 2 A-D), 11 papillary renal cell carcinoma (Fig. 2 E-F), 13 chromophobe renal cell carcinoma (Fig. 2 G-H), 2 sarcomatoid renal cell carcinoma (Fig. 2J), and 6 cystic renal cell carcinoma (Fig. 2 K-L). RCC cases analyzed with Fuhrman grade 1-4 were 4, 38, 28, and 5 respectively. TRIM59-IHC staining in normal area including proximal tubules was negative (Fig. 2 last row). Background staining was eliminated by testing antibody dilutions (1:100, 200, 500, 1200 till 5000) while tumor specific signals of TRIM59 proteins were noticeable. Endogenous biotin signals were blocked and excluded by additional block reagents (avidin-biotin blocking reagent kit).

TRIM59 IHC staining in tumor areas in RCC (Fig. 2) was different from cases of CaP-TMA (mainly cytoplasmic). TRIM59 IHC staining was found in both cytoplasm and nucleus in all RCC grades and types (Fig. 2). We assessed TRIM59-IHC by visual scoring of both intensity (cytoplasmic staining) and extent (% nucleus staining) microscopically. Correlation of TRIM59 IHC signals by scoring the intensity in cytoplasm with grades of all five types of RCC is shown in Fig. 2M. TRIM59 IHC signals were increased with tumor progression from grade 1-3 ($p<0.05$). All grade 1 tumors (n=4) stained with weak TRIM59 IHC signals in cytoplasm, but with high extent of nuclear staining; while all grade 2 and 3 tumors (n=66) showed moderate to strong cytoplasmic staining intensity of TRIM59. All grade 4 tumors (n=5) showed weak to moderate intensity in cytoplasm of TRIM59 staining. No correlation between TRIM59 IHC staining in nucleus and tumor grade was found, although ~~in~~ low grade RCC showed higher nuclear staining.

Therefore by systematic IHC studies in CaP (88 patients, Fig. 1) and kidney cancer (75 patients, Fig. 2), we almost exactly repeated results from our mouse model studies on TRIM59

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[17]. We confirmed TRIM59 as an immunohistochemistry marker able to detect low grade tumor in early tumorigenesis. We also demonstrated the correlation of tumorigenesis and tumor progression with TRIM59 up-regulation until high grade tumor.

TMA analysis of TRIM59 protein expression demonstrates that TRIM59 is a multiple tumor marker:

In the basic research previously using animal GEM-CaP models [17], we have disclosed that TRIM59 up-regulation is involved in two oncogene families and two signal pathways of SV40Tag/pRB/p53 and Ras/Raf/MEK/ERK. TRIM59 may function as an early signal transducer in Ras signal pathway with bridging genes in two oncogene pathways [17].

While it was rarely reported that SV40 Tag oncogene induced human cancer, Ras mutations are among the most frequent alterations in human cancers (see review [22]). We assume that TRIM59 as an early Ras signal pathway effector may possibly act as a multiple tumor marker.

We therefore further extended TRIM59 IHC studies to 35- multiple cancer TMA sections (42 tumors, 126 cores, Table 1). We tested different dilutions (1/300, 1/600, 1/1200, 1/5000) of TRIM59 antibody (see Supplemental Fig. 3). To further confirm the specificity and reliability of TRIM59 antibody in IHC staining, we compared IHC staining in 35 different tumor-TMA sections with positive (TRIM59 antibody at 1:1200 and 1:5000 dilutions) and negative controls (no antibody added, details see Supplemental Fig. 4). As summarized in Table 2, TRIM59 expression was significant and tissue-specifically up-regulated in most of these 35 tumors. When comparing the relative scores (both intensity and extent) in different tumors, the highest staining was observed in breast, lung, liver, skin (squamous cell carcinoma) and endometrial cancers.

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Further confirmation of TRIM59 as a tumor marker in patients with eight different tumors:

Since the 35 tumor-TMA contained only limited cases in each tumor type, we selected more cases (n=92) of eight different tumor types with different tumor grades, which all showed up-regulated expression of TRIM59. IHC staining of TRIM59 in eight tumors are shown: lung (n=4, Fig. 3A-C), breast (n=3, Fig. 3D-E), gastrointestinal (GI, n=2, Fig.3F-G), female genital tract (fGT, n=5, Fig.3H-J), bladder (n=44, Fig. 3K), prostate (n=27 from UWO, Fig. 3L), head and neck mucosal tumor (squamous cell carcinoma, SCC of mouth, tongue and larynx, n=4, Fig. 3M-O) and parotid gland (n=3, Fig. 3P-Q). Normal areas in lung, breast, colon, endometrial, bladder, larynx and parotid tissues showed very weak or completely negative staining (Fig. 3). Since some tumors (e.g. prostate) showed mostly cytoplasmic and no nuclear TRIM59-IHC staining, as a comparative study, we assessed their relative scores (combine both intensity and extent scores, see Materials and Methods). More tumors from kidney (RCC, n=75) and prostate cancer (n=27) were included as references and all were assessed by relative scores simultaneously, since we already analyzed a large cohort of these patients. Fig. 3R shows the comparison of the mean of IHC-TRIM59 relative scores. The highest relative scores were found in SCC of the parotid, mouth, larynx and tongue, followed by lung, breast and female genital tract cancers.

The comparison of relative scores on low and high grades separately was done (data not shown). Cases of grade 1 lung cancer (bronchoalveolar, adenocarcinoma, SCC and large cell carcinoma) and breast cancer (invasive lobular and invasive mammary carcinoma) all showed the strongest staining as compared with other tumors. In endometrial cancer, the TRIM59 relative scores were moderate in grade 1 and moderate to strong in grade 2. The three tumors of

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Table 2 Immunohistochemistry analysis of TRIM59 as multiple marker in 35 tumor TMA

Tumor Type	Patient number	Core number	Pathologic Grade	Cell Type	IHC staining	
					Cytoplasm staining (intensity)	Nuclear staining (extent)
Renal clear cell carcinoma,	2	6	2	Epithelial	moderate	-
			3		moderate	-
Adrenal gland cortical carcinoma	1	3	N/A	Epithelial	Moderate-strong	-
Squamous Cell Carcinoma, skin	2	6	WD	Epithelial	strong	-
			MD		moderate	-
Basal cell carcinoma, skin	2	6	N/A	Epithelial	moderate	-
					Moderate-strong	
Melanoma	1	3	N/A	Epithelial	weak	50% +
Endometroid adenocarcinoma	2	6	2	Epithelial	Moderate-strong	-
			1		Moderate-strong	-
Leiomyosarcoma	1	3	N/A	Mesenchymal	weak	-
Omentum serous adenocarcinoma,	1	3	WD	Epithelial	Weak-moderate	-
Ovary serous adenocarcinoma	1	3	N/A	Epithelial	Weak-moderate	-
Ovary clear cell carcinoma	1	3	PD	Epithelial	Moderate-strong	30% +
Cervix adenocarcinoma	1	3	WD-MD	Epithelial	Moderate-strong	-
Colon adenocarcinoma	1	3	Low grade	Epithelial	weak	-
Breast ductal adenocarcinoma	1	3	2/3	Epithelial	Moderate-strong	50% +
Bladder urothelial carcinoma	2	6	Low grade	Epithelial	weak	-
			2 (low grade)	Epithelial	moderate	-

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Stomach GIST	1	3		Epithelial	Weak-moderate	-
Esophagus adenocarcinoma	1	3	MD	Epithelial	0-weak	-
Thyroid, Papillary carcinoma	1	3	N/A	Epithelial	weak	-
Thyroid, medullary carcinoma	1	3	N/A	Epithelial	moderate	-
Pancreas adenocarcinoma,	2	6	2	Epithelial	Weak-moderate	-
			2		Moderate-strong	-
Pancreas endocrine tumor	1	3	N/A	Epithelial	strong	-
Lung SCC	1	3	PD	Epithelial	strong	-
Lung mesothelioma	1	3	MD-PD		strong	20% +
Lung adenocarcinoma	1	3	MD		moderate	50% +
Lung bronchoalveolar carcinoma	1	3	WD		Moderate-strong	50% +
Lung mesothelioma, biphasic	1	3	MD-PD		strong	20% +
Liver hepatocellular carcinoma (HCCa)	1	3	2/4	Epithelial	strong	-
Liver metastatic carcinoid	1	3	N/A	Epithelial	strong	-
Small bowel marginal zone lymphoma	1	3	N/A	Lymphocyte	0-weak	-
Lymph node, follicular lymphoma	1	3	1/3	Lymphocyte	0-weak	-
Lymph node, metastatic carcinoid	1	3	Low grade	Epithelial	strong	-
Spleen, Hodgkin's lymphoma	1	3	N/A	Lymphocyte	weak	-
Stomach, malt lymphoma	1	3	Low grade	Lymphocyte	0-weak	-
Thymus invasive thymoma	1	3	N/A	Epithelial	0-weak	-
Appendix, Goblet cell carcinoid	1	3	N/A	Epithelial	0	-

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squamous cell carcinoma (SCC) from mouth, tongue and larynx with different grades (Fig. 3M-O) also showed high relative scores (both intensity and extent).

As a comparative study (Fig. 3R, Table 2), we tested 44 bladder cancer cases with 38 low grade and 6 high grade tumors. The mean value of relative scores was 1.6, *i.e.* weak to moderate in bladder cancer cases . In 27 prostate cancer cases (from UWO only) tested, the relative scores of TRIM59 (cytoplasmic staining only) from PIN through Gleason scores of 10 were actually relatively weak (Fig. 3R), although in Gleason score 4, 6 and 8 were weak to moderate separately.

Thus far, we identified that TRIM59 up-regulation is "tumor-specific". First, we demonstrated the correlation of TRIM59 enhanced IHC signals with tumorigenesis and progression, which were statistically significant in this report with 291 cases and 37 tumor type analyses. Second, although TRIM59 is a normal gene involved in CDC (cell cycle division) regulation from G1 to S-phase involved in DNA S-phase and cell growth [17], we demonstrated that in normal or non-tumor areas in all tested 37 different kinds of cancers, TRIM59 IHC staining signals were mostly negative or very low (Fig. 1, 2 3). By moderating antibody dilutions and testing various blocking reagents (Supplemental Fig. 4), we demonstrated that TRIM59 induces tumorigenesis/oncogenesis only when it is abnormally up-regulated.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Furthermore, we also confirmed that TRIM59 expression involved in multiple-tissue expression even in embryo development. We carried out IHC of mouse embryo sections by double-staining TRIM59. Cytokeratin (keratin), a family of proteins that are primarily found in epithelial cells was used as reference. Supplemental Fig. 5 illustrated confocal microscope

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images of IHC staining of TRIM59 in different organs/tissues of mouse embryo (14.5 d.p.c). TRIM59 was highly expressed in cytokeratin-expressing cells in the lung (first row), skin (second row), and kidney (not shown) of mouse embryos. TRIM59 staining in mouse embryos revealed the same pattern of the epithelium origin as in human tumors, which the TRIM59 gene were found highly up-regulated in those tumor types as well.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Given our previous experiments suggesting TRIM59 functions in the Ras pathway, we tested if TRIM59 up-regulation was correlated with the BRAF, an early signal effector Ras signal pathway (review see [22]). We selected 24 RCC patients, which previously were confirmed with up-regulation of TRIM59 expression. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing the total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing the activated phosphorylated B-Raf. Some of the serial slides were stained in parallel on each patient by different antibodies. As shown in Supplemental Fig. 6 A first two columns, in all 12 clear cell carcinoma of RCC samples, there were no or very weak B-Raf IHC signals in all three used Raf antibodies. In papillary RCC (Suppl Fig. 6 B) and chromophobe RCC samples (Suppl Fig. 6 C) (15 samples out of 24 samples or 62% of all RCC samples tested), there were higher IHC signals in all three antibodies used for staining in cancer areas specifically, showing higher intensity and extent than clear cell carcinoma (Suppl Fig. 6 A). Nuclear signals were found only by B-Raf P-Ser antibody in papillary and chromophobe tumors. Supplemental Table 1 summarizes the results. It is intriguing that in those TRIM59 up-regulated kidney cancers (RCC), neither total nor phosphorylated BRaf were detected in clear RCC (as a control), but were all highly positive in other two RCCs (papillary and

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chromophobe) tumors. We could not definitely confirm that TRIM59 was acting along the Ras pathway in all cases where it was detected.

Conclusions

This is the first report on a possible "ubiquitous" tumor marker. Ras mutations are among the most frequent alterations in human cancers that lead to approximately 30% of all human cancers with expression of constitutively active Ras proteins (review see [30]). In this report, we have demonstrated that there are more than mutation issues of the Ras signal pathway in tumorigenesis and progression, since TRIM59 is up-regulated as a novel proto-oncogene in a variety of human cancers.

Detection and treatment of cancer at the earliest stage is critical for patient survival. This investigation demonstrated a novel TRIM59 gene as a multiple tumor marker for early diagnosis of tumorigenesis. As a multiple biomarker associated with epithelium origin (very rarely arisen from mesenchymal tissue, see Table 2) TRIM59 may be used as an EMT (epithelium – mesenchymal- transition) specific co-biomarker. The TRIM59 antibody may also be used for molecular targeted imaging as a new diagnostic marker, for example in the context of targeted microbubble ultrasound destruction technology [23], before the protocol of a serum TRIM59 test can be established.

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Acknowledgements: This work was supported by grants from the Ontario Institute of Cancer Research (07NOV-52), the Canadian Institute of Health Research (MOP -77684), NIH-NCI (2 U01 CA084296-06) and the Terry Fox Foundation.

Legend of Figures:

Fig. 1 Correlation of TRIM59 Immunhistochemistry (IHC) staining in prostate cancer cases in TMA (Tissue Microarray) assessed by the Gleason Grading system. All panels in **A** were shown hematoxylin staining, x20. Non-tumor: weak or negative, PIN: TRIM59 is located in the cytoplasm of the luminal cells (Intensity=2). Gleason score 3+3, TRIM59 is located in the cytoplasm of tumor cells (Intensity=2). Gleason score 3+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+5, TRIM59 is located in the cytoplasm of the tumor cells (Intensity<1). Graph **B:** Correlation of TRIM59 protein levels by intensity with Gleason score in prostate cancer TMA analysis. Error Bars show mean (± SE).

Fig. 2 Immunohistochemistry analysis of TRIM59 expression in kidney cancer (RCC, Renal Cell Carcinoma) cases: correlation with tumor grade by intensity detecting early tumorigenesis. Five types of RCC with different grades were shown clear cell carcinoma (**A-D**), papillary RCC (**E-F**), chromophobe RCC (**G-I**), sarcomatoid RCC (**J**) and cystic RCC (**K-L**). **A**. Clear cell carcinoma, grade1, weak cytoplasmic staining in tumor cells, ≥ 50% nuclear staining, cytoplasmic staining in tumor cells (40X). **B.** Clear cell carcinoma, grade 2, moderate

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cytoplasmic staining in tumor cells, no nuclear staining (40X). **C.** Clear cell carcinoma, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **D.** Clear cell carcinoma, grade 4, moderate cytoplasmic staining and nuclear (%) staining (40X). **E.** Papillary RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **F.** Papillary RCC, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining, normal cortex tissue is visible adjacent to tumor area (40X). **G.** Chromophobe RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining. (40X). **H.** Chromophobe RCC, grade 3, strong cytoplasmic staining, no nuclear staining, (40X). **I.** Chromophobe RCC, grade 4, moderate cytoplasm staining and nuclear staining (40X). **J.** Sarcomatoid RCC, grade 4, weak cytoplasmic staining and nuclear staining (40X). **K.** Cystic RCC, grade 2, moderate cytoplasmic staining, no nuclear staining (40X). **L** Cystic RCC, grade 3, strong cytoplasmic staining, no nuclear staining (40X). Last two panels: normal kidney tissues (x20, x40). **M. Graph:** Correlation of TRIM59 protein levels by relative scores (both intensity and extent) with grade in RCC according to the Fuhrman nuclear grading system. Error Bars show mean (± SE).

Fig. 3 Comparison of TRIM59 expression as a multiple-cancer marker in eight types of tumors in breast, lung, parotid, gastrointestinal, female genital tract, bladder, head and neck mucosal tumor and prostate cancer. Negative TRIM59 staining in normal tissues was shown for each tumor respectively (20X). **(A)** Lung bronchoalveolar carcinoma, grade 1, strong cytoplasmic and nuclear staining of tumor cells, (40X). **(B)** Lung adenocarcinoma, grade 2, moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(C)** Lung large cell carcinoma, grade 4, moderate cytoplasmic and nuclear staining of tumor cells (40X). **(D)** Breast cancer, invasive lobular, low grade, moderate to strong cytoplasmic and nuclear staining of tumor cells (40X). **(E)** Breast cancer, invasive mammary (no specific type), high grade,

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moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(F)** Pancreas neuroendocrine carcinoma, poorly differentiated, strong cytoplasmic staining, no nuclear staining of tumor cells (40X). **(G)** Colon carcinoma, low grade, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(H)** Endometrial carcinoma, grade1, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(I)** Endometrial carcinoma, grade 2, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(J)** Ovary, Endometrioid carcinoma, grade 1, weak cytoplasmic staining, no nuclear staining (40X). **(K)** Bladder urothelial carcinoma, low grade, moderate cytoplasmic staining, no nuclear staining (40X). **(L)** Prostate adenocarcinoma, Gleason score 8, moderate cytoplasmic staining, no nuclear staining (40X). **(M)** Floor of mouth, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(N)** Tongue cancer, SCC moderately to poorly differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(O)** Larynx, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(P)** Parotid, mucoepidermoid carcinoma, low grade, strong cytoplasmic staining, no nuclear staining (40X). **(Q)** Parotid, metastatic SCC, poorly differentiated, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(R)** Graphic comparison of relative scores (both intensity and extent) of TRIM59 IHC signals in eight different tumor types. fGT: Female genital tract. Error Bars show means (± SE) of the relative scores in all grades analyzed and compared.

Legend of Supplemental data

Supplemental Fig. 1 Characterization of TRIM59 antibodies constructed and used for human tissue studies.

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Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

(A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows. **(B)**. 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). **(C-D)**. Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots. **(E)** Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145, LNCaP and human kidney cell HEK293. A unique 53 kDa band was shown exactly as in mouse cell and tissue lysates **(C)**.

Supplemental Fig. 2 From **Vancouver Prostate Center** The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP, TRIM59 is significantly up-regulated after

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10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.

Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Summary of results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 1:1200 and 1:5000 –(figures not shown but resemble to 1:1200). We assessed these IHC results with the negative control (with out TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3, 1:1200 dilution produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remained stronger than the normal non-tumor areas even at 1:300 dilution. In order to detect weak TRIM59-IHC staining signals, we tried 1:300 dilution and we compared the staining signal with negative controls (no antibody was added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining (data not shown), and similar results were obtained.

Legend of Figures: (A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular

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lymphoma, (20X), positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining.

Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry –(IHC) staining in human multiple tumor TMA —(tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody –(positive) and without any antibody –(negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some areas of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin –(squamous cell carcinoma); #4 Skin –(basal cell carcinoma); #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma; #13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma; #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma); #2 Adrenal gland (cortical carcinoma); #7 Omentum (serous adenocarcinoma); #9 Cervix adenocarcinoma.

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma); #6 Uterus (leiomyosarcoma); #8 Ovary, serous adenocarcinoma; #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma and # 25 Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid).

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Supplemental Fig. 5 Confocal microscope imaging of IHC analysis of TRIM59 expression in mouse embryo (14.5d.p.c). Patterns of multiple organ expression originated from epithelial cells were shown in the lung (first row) and skin (second row).

Supplemental Fig. 6 Test of Ras activation by B-Raf antibodies in kidney cancer tumors correlated with up-regulation of TRIM59. First column: Antibody Braf **(A)** in Clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (**B**), strong cytoplasmic staining, no nuclear staining, (40X); in Chromophobe RCC (**C**), strong cytoplasmic staining, no nuclear staining, (40X). In the second row: antibody to Raf 1, in clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (20X,40X), strong cytoplasmic staining, no nuclear staining; in chromophobe RCC, strong cytoplasmic staining, no nuclear staining (40X). Third row: Antibody to Raf 1 phosphor Ser ^{259}, in clear cell carcinoma, weak cytoplasmic and nuclear staining (40X); in Papillary RCC, strong cytoplasmic and nuclear staining (40X), in chromophobe RCC, strong cytoplasmic and nuclear staining, (40X). Arrows indicated nuclear staining.

Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

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Immunohistochemical analysis of TRIM59: A novel multiple cancer biomarker

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2012-001410.R2
Article Type:	Research
Date Submitted by the Author:	12-Jul-2012
Complete List of Authors:	Khatamianfar, Vida; Lawson Heath Research Institute, Surgery Valiyeva, Fatma; Lawson Heath Research Institute, Surgery Rennie, Paul; University of British Columbia, Surgery Lu, Weiyang; Western University, Robarts Research Institute Yang, Burton B.; University of Toronto, Sunnybrook Research Institute Bauman, Glenn; Western University, London Regional Cancer Program Moussa, Madeleine; Western University, Pathology Xuan, Jim W; Lawson Heath Research Institute, Surgery
Primary Subject Heading:	Diagnostics
Secondary Subject Heading:	Diagnostics, Oncology, Pathology
Keywords:	Biochemistry < BASIC SCIENCES, HISTOPATHOLOGY, MOLECULAR BIOLOGY, Oncogenes < ONCOLOGY

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TRIM59, a novel multiple cancer biomarker for immunohistochemical detection of tumorigenesis

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Running title: TRIM59, a novel multiple-cancer marker for tumorigenesis

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List of abbreviations

Abbreviations: TRIM: TRIpartite Motif; CaP: prostate cancer; BPH: benign prostatic hyperplasia; PIN: prostate intraepithelial neoplasia; TMA: tissue microarray; RCC: renal cell carcinoma; SCC: squamous cell carcinoma; GI: gastrointestinal; GU: genitourinary; d.p.c: days post conception; GEM (genetically engineered mouse); H&E: haematoxylin and eosin; IHC: Immunohistochemistry; WD: well differentiated ; MD: moderately differentiated ; PD: poorly differentiated.

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Abstract

Objectives and design: We identified a novel TRIM59 gene, as an early signal transducer in two (SV40Tag and Ras) oncogene pathways in murine prostate cancer (CaP) models. We explore its clinical applications as a multi-tumor marker detecting early tumorigenesis by immunohistochemistry.

Setting and Participants: 88 CaP patients were from a tissue microarray (TMA) of radical prostatectomy specimen, 42 patients from a 35 multiple-tumor TMA, 75 patients with renal cell carcinoma (RCC) and 92 patients from 8 different tumor groups (breast, lung, parotid, gastrointestinal, female genital tract, bladder, kidney and prostate cancer).

Results: TRIM59 up-regulation specifically in tumor area was determined by immunohistochemistry in 291 cases of 37 tumor types. To demonstrate that TRIM59 up-regulation is "tumor-specific", we characterized a significant correlation of TRIM59 IHC signals with tumorigenesis and progression, while in control and normal area, TRIM59 IHC signal was all negative or significantly low. TRIM59 protein up-regulation in prostate and kidney cancers was detectable in both intensity and extent in early tumorigenesis of PIN (prostate intraepithelial neoplasia, $P<0.05$) and grade 1 of RCC ($P<0.05$), and stopped until high grades cancer. The results of the correlation in these two large cohorts of tumor types confirmed and repeated murine CaP model studies. Enhanced TRIM59 expression was identified in most of the 37 different tumors, while the highest intensities were in lung, breast, liver, skin, tongue and mouth (squamous cell cancer) and endometrial cancers. Multiple-tumor up-regulation was further confirmed by comparing relative scores of TRIM59 IHC signals in eight tumors with a larger patient population; and by a mouse whole mount embryo (14.5dpc) test on the origin of TRIM59 up-regulation in epithelial cells.

Conclusions: TRIM59 may be used a novel multiple-tumor marker for immunohistochemical detecting early tumorigenesis and could direct a novel strategy for molecular targeted diagnosis and therapy of cancer.

Keywords: TRIM59, Cancer biomarker, Immunohistochemistry, Prostate cancer, Multiple cancer marker, Ras signal pathway, SV40Tag oncogene, Tissue microarray, Confocal microscopy.

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Introduction

The TRIM (*TRI*partite *M*otif) family is an evolutionarily conserved gene family implicated in a number of critical processes including immunity [1-3], antiviral [4-8], proliferation [6, 9], transcriptional regulation [6, 10], neuro-development [11, 12], cell differentiation [12] and cancer [13] (reviewed in [1, 5, 14, 15]). However, the function of most TRIM family members is poorly understood and was surmised only based on computational analysis from their RBCC (*R*ING finger, *B*-box, Coiled-*C*oil) domains. The domain of RING (*R*eally *I*nteresting *N*ew *G*ene) is frequently involved in proteolysis acting as E3 ubiquitin ligases and the ubiquitin-proteasome system in the regulation of numerous cellular processes including cell cycle regulatory proteins, transcription factors, and signal transducers [6, 15]. Recent studies demonstrate that various TRIM-NHL proteins function as cofactor for the microRNA-induced silencing complex (miRISC) [12] [14, 16]. An ataxia-telangiectasia group D complementing gene (ATDC) was recently designated as TRIM29, which is elevated in most invasive pancreatic cancers in the Wnt/β-catenin signalling pathway [13].

In a previous report [17], we characterized the function of TRIM59, a novel TRIM family member, in SV40 Tag oncogene directed genetically engineered mouse (GEM) prostate cancer (CaP) models [18-21]. The TRIM59 gene was identified to be correlated with the SV40 Tag initiated tumorigenesis. TRIM59 protein up-regulation and hyper-phosphorylation started in the prostate cytoplasm in early tumorigenesis from PIN (prostate intraepithelial neoplasia) [17]. As a signal pathway effector, the p-Ser/Thr phosphorylated TRIM59 proteins correlate with tumorigenesis, while p-Tyr-TRIM59 proteins correlate with advanced CaP. The function of TRIM59 was identified by shRNA knockdown in human CaP cells resulted in S-phase arrest and cell growth retardation. Although TRIM59 is an effector gene with the SV40Tag oncogene, the

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initial functional targets of TRIM59 function were actually on the Ras signal pathway as an early and rapid signal transmitter (see review [22]). In a transgenic mouse test of TRIM59 up-regulation specifically in the prostate, TRIM59 demonstrated full oncogenic activity in directing tumorigenesis and progression to highest grade CaP [23]. The signal pathway of TRIM59 may be possibly linked with two large oncogene routes: the Ras/Raf/MEK/ERK/PI3K/AKT and the SV40 Tag/p53/pRB routes [17]. In this study we explored the clinical utility of the novel TRIM59 gene to serve as a biomarker for a panel of human cancers. To address the molecular biology basis, we combined results from both basic (animal model [17]) and human clinical studies.

Materials and Methods

Patient selection:

All patient samples were acquired as part of REB (Research Ethics Board) approved protocols at the University of Western Ontario (UWO) and Vancouver Prostate Center, University of British Columbia (UBC). Table 1 shows a complete list of 291 patients with 37 different tumor types examined in this study.

Prostate cancer Tissue Microarrays (TMA): 88 CaP patients between 2006 and 2008 who had no treatment prior to radical prostatectomy, were selected from the Vancouver General Hospital. Each patient block marked as containing benign tissue or cancer was sampled 2 times with a core diameter of 1 mm arrayed (176 cores) in a rectangular pattern with 0.7 mm between the centers of each core, creating a duplicate TMA layout using a manual tissue micro arrayer (Beecher Instruments, Silver Spring, MD). The TMA paraffin blocks, were sectioned into 0.5 micrometer sections and mounted on the positively charged slides.

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Organ	Patient Number	Tumor type		Tumor grade	
35 tumor-TMA	42	Description in Table-2			
Prostate TMA	105	Adenocarcinoma		BPH	16
				PIN	4
				Gleason score 4	5
				Gleason score 6	31
				Gleason 7	15
				Gleason score 8	16
				Gleason score 9-10	9
				Stroma	3
				Absent cores	6
Kidney	75	Clear cell carcinoma	43	Grade 1	4
		Papillary RCC	11	Grade 2	38
		Chromophobe RCC	13	Grade 3	28
		Cystic RCC	6	Grade 4	5
		Sarcomatoid RCC	2		
Bladder	44	Urothelial carcinoma		Low grade	38
				High grade	6
Lung	4	Bronchoalveolar carcinoma	1	Grade 1	1
		Adenocarcinoma	1	Grade 2	1
		Large cell carcinoma	1	Grade 3	1
		Squamous cell carcinoma	1	Grade 4	1
Breast	3	Invasive lobular carcinoma	1	Grade 1	1
		Invasive mammary carcinoma	2	Grade 3	2
Female Genital tract	5	Endometrial carcinoma	4	Grade 1	4
		Ovary, Endometrioid carcinoma	1	Grade 2	1
Gatrointestinal tract	2	Colon carcinoma	1	Low grade	1
		Pancreas neuroendocrine carcinoma	1	high grade	1
Parotid	3	Mucoepidermoid carcinoma	1	low grade	1
		Metastatic SCC	1		
		Metastatic neuroendocrine Carcinoma	1	high grade	2
Mouth, tongue and larynx	4	Squamous cell carcinoma	4	Moderately differentiated	3
				Moderately to Poorly differentiated	1

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Table 1 Patient list selected in this study

Total	291		

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Automated image, acquisition and analysis on immunohistochemical staining of CaP-TMA: (UBC) Immunohistochemical staining was conducted by Ventana autostainer model Discover XT ™ (Ventana Medical System, Tuscan, Arizona) with enzyme labelled biotin streptavidin system and solvent resistant DAB Map kit. TMA was scanned by Bliss Digital imaging system using x20 objective, from Bacus Laboratories INC, Centre Valley PA, and stored in the Prostate Centre Saver (http//bliss.prostatecentre.com). A value on a four-point scale assigned to each core.

Multiple–tumor Tissue Microarray construction: Tissue samples from 42 patients that encompassed 35 distinct tumor subtypes were selected from London Laboratory Service Group, and the tumor bank in the Department of Pathology (UWO). TMA slides were constructed with triplicate cores for each sample following standard procedure as described [24]. 0.6 mm sections were prepared from TMA block and re-stained by H&E for each case to confirm the diagnosis.

Histopathologic analysis: All cases from 37 tumor types were graded according to standardized histopathology grading systems [25] by M.M (pathologist) and V. K (MD fellow).

Immunohistochemistry (IHC) and results evaluation: Standard ABC (Avidin Biotin Complex) protocol was performed as previously reported [21, 26, 27]. Two kinds of blocking reagents were used: Power Block (Universal Blocking Reagent, from BioGenex, San Ramon, CA) and Avidin-biotin blocking reagent kit (Vector Labs, Burlingame, CA). All B-Raf antibodies were from GenScript (Piscataway, NJ): B-Raf antibody (monoclonal antibody, used 1:500 dilution), Raf 1 (polyconal, 1:50) and Raf -1 -(polyclonal, Phospho-Ser^{259} 1:50).

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TRIM59 IHC staining signals were assessed by intensity for cytoplasmic staining and extent to assess percentage of nuclear staining as previously reported [28, 29]. Since in some tumors TRIM59 showed only cytoplasmic staining, for the purpose of comparing in different tumors, we used a combined relative score system based on both intensity and extent as following: score 0: 0/0 (intensity/ extent); score 1: weak cytoplasmic staining and/or $\leq 25\%$ nuclear staining; score 2: moderate cytoplasmic staining and/or $\leq 50\%$ nuclear staining; score 3: strong cytoplasmic staining and/or $\geq 50\%$ nuclear staining. All relative scores were accessed by two researchers independently M.M (pathologist) and V. K (MD fellow).

Construction and characterization of TRIM59 antibody:

Supplement Fig. 1 provided details, which combined Supplement materials of [17], and more informations. Supplement Fig. 1A showed that the antibody TRIM59#72 used for mouse and human studies was prepared from C-terminal 136 a.a. peptide (as recombinant GST- fusion protein, Suppl. Fig. B) of whole protein 403aa which is TRIM59 specific, while antibody TRIM59#71 was from mouse TRIM59 N-terminal sequence (163aa) containing mostly the common RBCC function domains of more than 70 members TRIM family [17]. As shown in Supplemental Fig. 1C, Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed multiple bands. We also demonstrated that N-terminal sequence specific antibody TRIM59#71 can recognize purified proteins from C-terminal sequence specific, TRIM59#72 Ab affinity column (the same 53kDa protein, Suppl. Fig. 1D). Since these two immunogenic sequences are not overlapped, we thus verified that they are detecting the same protein. We demonstrated that TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots (Suppl Fig. 1E).

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Confocal microscope imaging of immunostaining of mouse embryos 14.5 dpc (days post conception) for TRIM59: The mouse embryos were prepared according to our previous reports [18-21]. The anti-rabbit secondary antibody was conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA). Sections were double-stained with pan-cytokeratin antibody with FICT (Sigma), and the stained mouse embryo sections were visualized using a Carl Zeiss confocal microscope by the LSM Image program.

Statistical Analysis: Student's *t* tests and one-way ANOVA were used by programs of Microsoft Excel 2007 or SPSS 10 to analyze the data with $p < 0.05$ considered to be statistically significant.

Results and Discussion

TRIM59 up-regulation in human prostate cancer TMA (tissue microarray): correlation with tumorigenesis and tumor progression by TRIM59 intensity until high grade CaP:

We designated TRIM59 as one of the "tumorigenesis–associated" genes correlated with SV40 Tag oncogenesis in mouse prostate cancer (GEM-CaP) models [17]. SV40 Tag is essentially only required for the initiation of tumorigenesis, *i.e.* the "hit-and-run" effect, in GEM-CaP, but not for the tumor progression and metastasis directly. The "tumorigenesis–associated" effect, is due to the initial binding of the Tag oncogene with retinoblastoma (pRB), p53 proteins and several transcriptional co-activators. Once this process is initiated, the signal-transduction will continue on, even without the initiation effectors.

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In GEM-CaP models, the TRIM59 protein up-regulation correlation with tumorigenesis and progression, and down-regulated in the high grade CaP by immunohistochemistry (IHC) [17]. We assume this "tumorigenesis–associated" effect of TRIM59 may apply to human cancer studies.

We first characterized that TRIM59 antibody (#72) can cross react with and recognize specifically human TRIM59 counterpart (details see supplemental Fig. 1). We tested by IHC in a TMA of CaP patients (n=88, 176 cores). TRIM59 IHC signals detected in an automated digital image system were identified mostly in cytoplasm of luminal cells (Fig. 1A), which is different from rapid tumor progression mouse CaP models. The intensity (score= 2) in PIN (n=4, Fig. 1A) was higher than in non-tumor area (normal and BPH). Moderate to strong expression was observed in Gleason score 6 (3+3, n=25), 7 (4+3, n=15), and 8 (4+4, n=14). In high grade CaP (score 4+5, n=8, Fig. 1A), TRIM59-IHC signals were lower. As shown in a graph of Fig. 1B, TRIM59 protein IHC signals correlated significantly (P=0.014) with tumorigenesis and progression from PIN to -WDCaP (Gleason grade 1-2, scores 2-4) and MDCaP (Gleason grade 2-4, scores 4-8) (graph Fig 1B), and decreased in high grade CaP (Gleason score 9-10) with P=0.018, which is similar to GEMs [17].

TRIM59 up-regulation in human Renal Cell Carcinoma (RCC) patients: correlation of tumorigenesis and tumor progression with TRIM59 intensity until high grade RCC:

Next, we demonstrated that TRIM59 is not androgen-responsive and likely not prostate tissue-specific, indicating that the tumorigenesis role of TRIM59 may be applied to all or most of human cancers. Supplemental Fig. 2 showed this result in detail of IHC test by TRIM59 antibody#72 on a TMA of the LNCaP human CaP xenografts in nude/SCID castrated mice.

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We extended results from prostate cancer clinical samples to kidney cancer. We started 75 renal cell carcinoma (RCC) patients including all 5 different types of RCC tumors: 43 clear cell carcinoma (representative IHC figures shown in Fig. 2 A-D), 11 papillary renal cell carcinoma (Fig. 2 E-F), 13 chromophobe renal cell carcinoma (Fig. 2 G-H), 2 sarcomatoid renal cell carcinoma (Fig. 2J), and 6 cystic renal cell carcinoma (Fig. 2 K-L). RCC cases analyzed with Fuhrman grade 1-4 were 4, 38, 28, and 5 respectively. TRIM59-IHC staining in normal area including proximal tubules was negative (Fig. 2 last row). Background staining was eliminated by testing antibody dilutions (1:100, 200, 500, 1200 till 5000) while tumor specific signals of TRIM59 proteins were noticeable. Endogenous biotin signals were blocked and excluded by additional block reagents (avidin-biotin blocking reagent kit).

TRIM59 IHC staining in tumor areas in RCC (Fig. 2) was different from cases of CaP-TMA (mainly cytoplasmic). TRIM59 IHC staining was found in both cytoplasm and nucleus in all RCC grades and types (Fig. 2). We assessed TRIM59-IHC by visual scoring of both intensity (cytoplasmic staining) and extent (% nucleus staining) microscopically. Correlation of TRIM59 IHC signals by scoring the intensity in cytoplasm with grades of all five types of RCC is shown in Fig. 2M. TRIM59 IHC signals were increased with tumor progression from grade 1-3 ($p<0.05$). All grade 1 tumors (n=4) stained with weak TRIM59 IHC signals in cytoplasm, but with high extent of nuclear staining; while all grade 2 and 3 tumors (n=66) showed moderate to strong cytoplasmic staining intensity of TRIM59. All grade 4 tumors (n=5) showed weak to moderate intensity in cytoplasm of TRIM59 staining. No correlation between TRIM59 IHC staining in nucleus and tumor grade was found, although ~~in~~ low grade RCC showed higher nuclear staining.

Therefore by systematic IHC studies in CaP (88 patients, Fig. 1) and kidney cancer (75 patients, Fig. 2), we almost exactly repeated results from our mouse model studies on TRIM59

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[17]. We confirmed TRIM59 as an immunohistochemistry marker able to detect low grade tumor in early tumorigenesis. We also demonstrated the correlation of tumorigenesis and tumor progression with TRIM59 up-regulation until high grade tumor.

TMA analysis of TRIM59 protein expression demonstrates that TRIM59 is a multiple tumor marker:

In the basic research previously using animal GEM-CaP models [17], we have disclosed that TRIM59 up-regulation is involved in two oncogene families and two signal pathways of SV40Tag/pRB/p53 and Ras/Raf/MEK/ERK. TRIM59 may function as an early signal transducer in Ras signal pathway with bridging genes in two oncogene pathways [17].

While it was rarely reported that SV40 Tag oncogene induced human cancer, Ras mutations are among the most frequent alterations in human cancers (see review [22]). We assume that TRIM59 as an early Ras signal pathway effector may possibly act as a multiple tumor marker.

We therefore further extended TRIM59 IHC studies to 35- multiple cancer TMA sections (42 tumors, 126 cores, Table 1). We tested different dilutions (1/300, 1/600, 1/1200, 1/5000) of TRIM59 antibody (see Supplemental Fig. 3). To further confirm the specificity and reliability of TRIM59 antibody in IHC staining, we compared IHC staining in 35 different tumor-TMA sections with positive (TRIM59 antibody at 1:1200 and 1:5000 dilutions) and negative controls (no antibody added, details see Supplemental Fig. 4). As summarized in Table 2, TRIM59 expression was significant and tissue-specifically up-regulated in most of these 35 tumors. When comparing the relative scores (both intensity and extent) in different tumors, the highest staining was observed in breast, lung, liver, skin (squamous cell carcinoma) and endometrial cancers.

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Further confirmation of TRIM59 as a tumor marker in patients with eight different tumors:

Since the 35 tumor-TMA contained only limited cases in each tumor type, we selected more cases (n=92) of eight different tumor types with different tumor grades, which all showed up-regulated expression of TRIM59. IHC staining of TRIM59 in eight tumors are shown: lung (n=4, Fig. 3A-C), breast (n=3, Fig. 3D-E), gastrointestinal (GI, n=2, Fig.3F-G), female genital tract (fGT, n=5, Fig.3H-J), bladder (n=44, Fig. 3K), prostate (n=27 from UWO, Fig. 3L), head and neck mucosal tumor (squamous cell carcinoma, SCC of mouth, tongue and larynx, n=4, Fig. 3M-O) and parotid gland (n=3, Fig. 3P-Q). Normal areas in lung, breast, colon, endometrial, bladder, larynx and parotid tissues showed very weak or completely negative staining (Fig. 3). Since some tumors (e.g. prostate) showed mostly cytoplasmic and no nuclear TRIM59-IHC staining, as a comparative study, we assessed their relative scores (combine both intensity and extent scores, see Materials and Methods). More tumors from kidney (RCC, n=75) and prostate cancer (n=27) were included as references and all were assessed by relative scores simultaneously, since we already analyzed a large cohort of these patients. Fig. 3R shows the comparison of the mean of IHC-TRIM59 relative scores. The highest relative scores were found in SCC of the parotid, mouth, larynx and tongue, followed by lung, breast and female genital tract cancers.

The comparison of relative scores on low and high grades separately was done (data not shown). Cases of grade 1 lung cancer (bronchoalveolar, adenocarcinoma, SCC and large cell carcinoma) and breast cancer (invasive lobular and invasive mammary carcinoma) all showed the strongest staining as compared with other tumors. In endometrial cancer, the TRIM59 relative scores were moderate in grade 1 and moderate to strong in grade 2. The three tumors of

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Table 2 Immunohistochemistry analysis of TRIM59 as multiple marker in 35 tumor TMA

Tumor Type	Patient number	Core number	Pathologic Grade	Cell Type	IHC staining	
					Cytoplasm staining (intensity)	Nuclear staining (extent)
Renal clear cell carcinoma,	2	6	2	Epithelial	moderate	-
			3		moderate	-
Adrenal gland cortical carcinoma	1	3	N/A	Epithelial	Moderate-strong	-
Squamous Cell Carcinoma, skin	2	6	WD	Epithelial	strong	-
			MD		moderate	-
Basal cell carcinoma, skin	2	6	N/A	Epithelial	moderate	-
					Moderate-strong	
Melanoma	1	3	N/A	Epithelial	weak	50% +
Endometroid adenocarcinoma	2	6	2	Epithelial	Moderate-strong	-
			1		Moderate-strong	-
Leiomyosarcoma	1	3	N/A	Mesenchymal	weak	-
Omentum serous adenocarcinoma,	1	3	WD	Epithelial	Weak-moderate	-
Ovary serous adenocarcinoma	1	3	N/A	Epithelial	Weak-moderate	-
Ovary clear cell carcinoma	1	3	PD	Epithelial	Moderate-strong	30% +
Cervix adenocarcinoma	1	3	WD-MD	Epithelial	Moderate-strong	-
Colon adenocarcinoma	1	3	Low grade	Epithelial	weak	-
Breast ductal adenocarcinoma	1	3	2/3	Epithelial	Moderate-strong	50% +
Bladder urothelial carcinoma	2	6	Low grade	Epithelial	weak	-
			2 (low grade)	Epithelial	moderate	-

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Stomach GIST	1	3		Epithelial	Weak-moderate	-
Esophagus adenocarcinoma	1	3	MD	Epithelial	0-weak	-
Thyroid, Papillary carcinoma	1	3	N/A	Epithelial	weak	-
Thyroid, medullary carcinoma	1	3	N/A	Epithelial	moderate	-
Pancreas adenocarcinoma,	2	6	2	Epithelial	Weak-moderate	-
			2		Moderate-strong	-
Pancreas endocrine tumor	1	3	N/A	Epithelial	strong	-
Lung SCC	1	3	PD	Epithelial	strong	-
Lung mesothelioma	1	3	MD-PD		strong	20% +
Lung adenocarcinoma	1	3	MD		moderate	50% +
Lung bronchoalveolar carcinoma	1	3	WD		Moderate-strong	50% +
Lung mesothelioma, biphasic	1	3	MD-PD		strong	20% +
Liver hepatocellular carcinoma (HCCa)	1	3	2/4	Epithelial	strong	-
Liver metastatic carcinoid	1	3	N/A	Epithelial	strong	-
Small bowel marginal zone lymphoma	1	3	N/A	Lymphocyte	0-weak	-
Lymph node, follicular lymphoma	1	3	1/3	Lymphocyte	0-weak	-
Lymph node, metastatic carcinoid	1	3	Low grade	Epithelial	strong	-
Spleen, Hodgkin's lymphoma	1	3	N/A	Lymphocyte	weak	-
Stomach, malt lymphoma	1	3	Low grade	Lymphocyte	0-weak	-
Thymus invasive thymoma	1	3	N/A	Epithelial	0-weak	-
Appendix, Goblet cell carcinoid	1	3	N/A	Epithelial	0	-

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squamous cell carcinoma (SCC) from mouth, tongue and larynx with different grades (Fig. 3M-O) also showed high relative scores (both intensity and extent).

As a comparative study (Fig. 3R, Table 2), we tested 44 bladder cancer cases with 38 low grade and 6 high grade tumors. The mean value of relative scores was 1.6, *i.e.* weak to moderate in bladder cancer cases . In 27 prostate cancer cases (from UWO only) tested, the relative scores of TRIM59 (cytoplasmic staining only) from PIN through Gleason scores of 10 were actually relatively weak (Fig. 3R), although in Gleason score 4, 6 and 8 were weak to moderate separately.

Thus far, we identified that TRIM59 up-regulation is "tumor-specific". First, we demonstrated the correlation of TRIM59 enhanced IHC signals with tumorigenesis and progression, which were statistically significant in this report with 291 cases and 37 tumor type analyses. Second, although TRIM59 is a normal gene involved in CDC (cell cycle division) regulation from G1 to S-phase and involved in DNA S-phase and cell growth [17], we demonstrated that in normal or non-tumor areas in all tested 37 different kinds of cancers, TRIM59 IHC staining signals were mostly negative or very low (Fig. 1, 2 3). By moderating antibody dilutions and testing various blocking reagents (Supplemental Fig. 4), we demonstrated that TRIM59 induces tumorigenesis/oncogenesis only when it is abnormally up-regulated.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Furthermore, we also confirmed that TRIM59 expression involved in multiple-tissue expression even in embryo development. We carried out IHC of mouse embryo sections by double-staining TRIM59. Cytokeratin (keratin), a family of proteins that are primarily found in epithelial cells was used as reference. Supplemental Fig. 5 illustrated confocal microscope

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images of IHC staining of TRIM59 in different organs/tissues of mouse embryo (14.5 d.p.c). TRIM59 was highly expressed in cytokeratin-expressing cells in the lung (first row), skin (second row), and kidney (not shown) of mouse embryos. TRIM59 staining in mouse embryos revealed the same pattern of the epithelium origin as in human tumors, which the TRIM59 gene were found highly up-regulated in those tumor types as well.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Given our previous experiments suggesting TRIM59 functions in the Ras pathway, we tested if TRIM59 up-regulation was correlated with the BRAF, an early signal effector Ras signal pathway (review see [22]). We selected 24 RCC patients, which previously were confirmed with up-regulation of TRIM59 expression. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing the total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing the activated phosphorylated B-Raf. Some of the serial slides were stained in parallel on each patient by different antibodies. As shown in Supplemental Fig. 6 A first two columns, in all 12 clear cell carcinoma of RCC samples, there were no or very weak B-Raf IHC signals in all three used Raf antibodies. In papillary RCC (Suppl Fig. 6 B) and chromophobe RCC samples (Suppl Fig. 6 C) (15 samples out of 24 samples or 62% of all RCC samples tested), there were higher IHC signals in all three antibodies used for staining in cancer areas specifically, showing higher intensity and extent than clear cell carcinoma (Suppl Fig. 6 A). Nuclear signals were found only by B-Raf P-Ser antibody in papillary and chromophobe tumors. Supplemental Table 1 summarizes the results. It is intriguing that in those TRIM59 up-regulated kidney cancers (RCC), neither total nor phosphorylated BRaf were detected in clear RCC (as a control), but were all highly positive in other two RCCs (papillary and

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chromophobe) tumors. We could not definitely confirm that TRIM59 was acting along the Ras pathway in all cases where it was detected.

Conclusions

This is the first report on a possible "ubiquitous" tumor marker. Ras mutations are among the most frequent alterations in human cancers that lead to approximately 30% of all human cancers with expression of constitutively active Ras proteins (review see [30, 31]). In this report, we have demonstrated that there are more than mutation issues of the Ras signal pathway in tumorigenesis and progression, since TRIM59 is up-regulated as a novel proto-oncogene in a variety of human cancers.

Detection and treatment of cancer at the earliest stage is critical for patient survival. This investigation demonstrated a novel TRIM59 gene as a multiple tumor marker for early diagnosis of tumorigenesis. As a multiple biomarker associated with epithelium origin (very rarely arisen from mesenchymal tissue, see Table 2) TRIM59 may be used as an EMT (epithelium – mesenchymal- transition) specific co-biomarker. The TRIM59 antibody may also be used for molecular targeted imaging as a new diagnostic marker, for example in the context of targeted microbubble ultrasound destruction technology [23], before the protocol of a serum TRIM59 test can be established.

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Acknowledgements: This work was supported by grants from the Ontario Institute of Cancer Research (07NOV-52), the Canadian Institute of Health Research (MOP -77684), NIH-NCI (2 U01 CA084296-06) and the Terry Fox Foundation.

Legend of Figures:

Fig. 1 Correlation of TRIM59 Immunhistochemistry (IHC) staining in prostate cancer cases in TMA (Tissue Microarray) assessed by the Gleason Grading system. All panels in **A** were shown hematoxylin staining, x20. Non-tumor: weak or negative, PIN: TRIM59 is located in the cytoplasm of the luminal cells (Intensity=2). Gleason score 3+3, TRIM59 is located in the cytoplasm of tumor cells (Intensity=2). Gleason score 3+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+5, TRIM59 is located in the cytoplasm of the tumor cells (Intensity<1). Graph **B:** Correlation of TRIM59 protein levels by intensity with Gleason score in prostate cancer TMA analysis. Error Bars show mean (± SE).

Fig. 2 Immunohistochemistry analysis of TRIM59 expression in kidney cancer (RCC, Renal Cell Carcinoma) cases: correlation with tumor grade by intensity detecting early tumorigenesis. Five types of RCC with different grades were shown clear cell carcinoma (**A-D)**, papillary RCC (**E-F)**, chromophobe RCC **(G-I)**, sarcomatoid RCC **(J)** and cystic RCC **(K-L)**. **A**. Clear cell carcinoma, grade1, weak cytoplasmic staining in tumor cells, ≥ 50% nuclear staining, cytoplasmic staining in tumor cells (40X). **B.** Clear cell carcinoma, grade 2, moderate

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cytoplasmic staining in tumor cells, no nuclear staining (40X). **C.** Clear cell carcinoma, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **D.** Clear cell carcinoma, grade 4, moderate cytoplasmic staining and nuclear (%) staining (40X). **E.** Papillary RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **F.** Papillary RCC, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining, normal cortex tissue is visible adjacent to tumor area (40X). **G.** Chromophobe RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining. (40X). **H.** Chromophobe RCC, grade 3, strong cytoplasmic staining, no nuclear staining, (40X). **I.** Chromophobe RCC, grade 4, moderate cytoplasm staining and nuclear staining (40X). **J.** Sarcomatoid RCC, grade 4, weak cytoplasmic staining and nuclear staining (40X). **K.** Cystic RCC, grade 2, moderate cytoplasmic staining, no nuclear staining (40X). **L** Cystic RCC, grade 3, strong cytoplasmic staining, no nuclear staining (40X). Last two panels: normal kidney tissues (x20, x40). **M. Graph:** Correlation of TRIM59 protein levels by relative scores (both intensity and extent) with grade in RCC according to the Fuhrman nuclear grading system. Error Bars show mean (± SE).

Fig. 3 Comparison of TRIM59 expression as a multiple-cancer marker in eight types of tumors in breast, lung, parotid, gastrointestinal, female genital tract, bladder, head and neck mucosal tumor and prostate cancer. Negative TRIM59 staining in normal tissues was shown for each tumor respectively (20X). **(A)** Lung bronchoalveolar carcinoma, grade 1, strong cytoplasmic and nuclear staining of tumor cells, (40X). **(B)** Lung adenocarcinoma, grade 2, moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(C)** Lung large cell carcinoma, grade 4, moderate cytoplasmic and nuclear staining of tumor cells (40X). **(D)** Breast cancer, invasive lobular, low grade, moderate to strong cytoplasmic and nuclear staining of tumor cells (40X). **(E)** Breast cancer, invasive mammary (no specific type), high grade,

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moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(F)** Pancreas neuroendocrine carcinoma, poorly differentiated, strong cytoplasmic staining, no nuclear staining of tumor cells (40X). **(G)** Colon carcinoma, low grade, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(H)** Endometrial carcinoma, grade1, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(I)** Endometrial carcinoma, grade 2, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(J)** Ovary, Endometrioid carcinoma, grade 1, weak cytoplasmic staining, no nuclear staining (40X). **(K)** Bladder urothelial carcinoma, low grade, moderate cytoplasmic staining, no nuclear staining (40X). **(L)** Prostate adenocarcinoma, Gleason score 8, moderate cytoplasmic staining, no nuclear staining (40X). **(M)** Floor of mouth, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(N)** Tongue cancer, SCC moderately to poorly differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(O)** Larynx, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(P)** Parotid, mucoepidermoid carcinoma, low grade, strong cytoplasmic staining, no nuclear staining (40X). **(Q)** Parotid, metastatic SCC, poorly differentiated, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(R)** Graphic comparison of relative scores (both intensity and extent) of TRIM59 IHC signals in eight different tumor types. fGT: Female genital tract. Error Bars show means (± SE) of the relative scores in all grades analyzed and compared.

Legend of Supplemental data

Supplemental Fig. 1 Characterization of TRIM59 antibodies constructed and used for human tissue studies.

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Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

(A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows. **(B)**. 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). **(C-D)**. Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots. **(E)** Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145, LNCaP and human kidney cell HEK293. A unique 53 kDa band was shown exactly as in mouse cell and tissue lysates **(C)**.

Supplemental Fig. 2 From **Vancouver Prostate Center** The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP, TRIM59 is significantly up-regulated after

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10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.

Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Summary of results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 1:1200 and 1:5000 –(figures not shown but resemble to 1:1200). We assessed these IHC results with the negative control (with out TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3, 1:1200 dilution produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remained stronger than the normal non-tumor areas even at 1:300 dilution. In order to detect weak TRIM59-IHC staining signals, we tried 1:300 dilution and we compared the staining signal with negative controls (no antibody was added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining (data not shown), and similar results were obtained.

Legend of Figures: (A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular

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lymphoma, (20X), positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining.

Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry –(IHC) staining in human multiple tumor TMA –(tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody –(positive) and without any antibody –(negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some areas of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin –(squamous cell carcinoma); #4 Skin –(basal cell carcinoma); #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma; #13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma; #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma); #2 Adrenal gland (cortical carcinoma); #7 Omentum (serous adenocarcinoma); #9 Cervix adenocarcinoma.

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma); #6 Uterus (leiomyosarcoma); #8 Ovary, serous adenocarcinoma; #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma and # 25 Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid).

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Supplemental Fig. 5 Confocal microscope imaging of IHC analysis of TRIM59 expression in mouse embryo (14.5d.p.c). Patterns of multiple organ expression originated from epithelial cells were shown in the lung (first row) and skin (second row).

Supplemental Fig. 6 Test of Ras activation by B-Raf antibodies in kidney cancer tumors correlated with up-regulation of TRIM59. First column: Antibody Braf **(A)** in Clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (**B**), strong cytoplasmic staining, no nuclear staining, (40X); in Chromophobe RCC (**C**), strong cytoplasmic staining, no nuclear staining, (40X). In the second row: antibody to Raf 1, in clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (20X,40X), strong cytoplasmic staining, no nuclear staining; in chromophobe RCC, strong cytoplasmic staining, no nuclear staining (40X). Third row: Antibody to Raf 1 phosphor Ser ^{259}, in clear cell carcinoma, weak cytoplasmic and nuclear staining (40X); in Papillary RCC, strong cytoplasmic and nuclear staining (40X), in chromophobe RCC, strong cytoplasmic and nuclear staining, (40X). Arrows indicated nuclear staining.

Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

Contributorship Statement

All authors havecontributed significantly to the conception and design of the manuscript,interpretation of data, drafting the article and revisions for importantintellectual content and final approval of the version to be published. MM (pathologist), VK and FV worked on dataacquisition, analysis and interpretation of human tumor samples. JWX conceived and designed the study. JWX is the guarantor of the study.

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Fig. 1

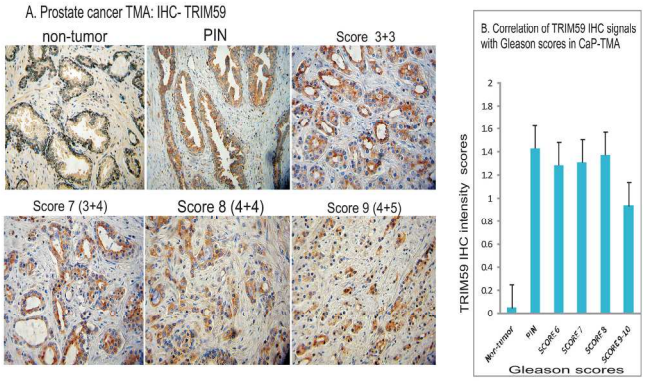

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Fig. 2 Immuno-staining of TRIM59 expression in five different types of kidney cancer (RCC)

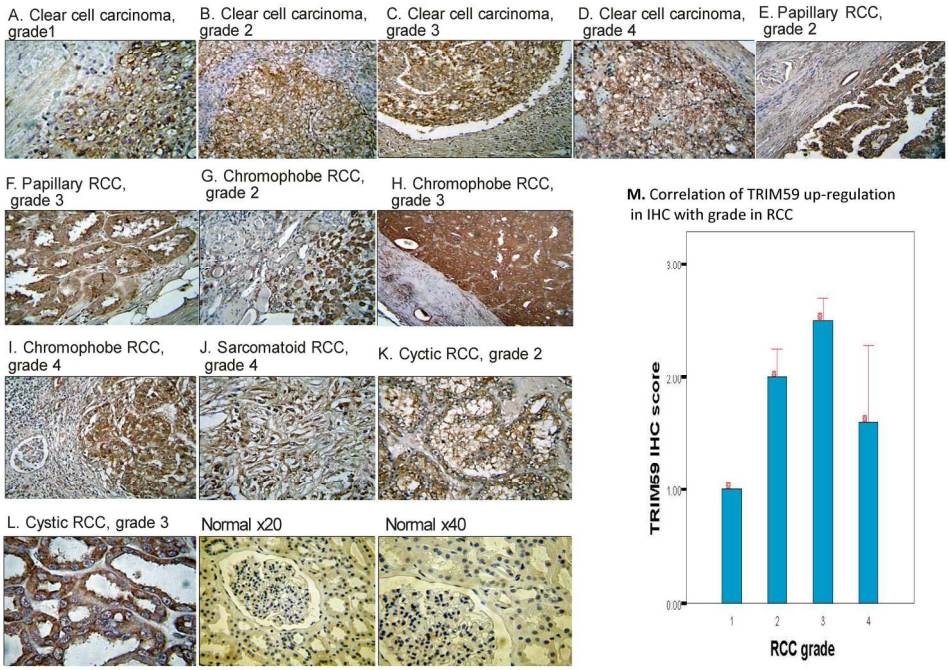

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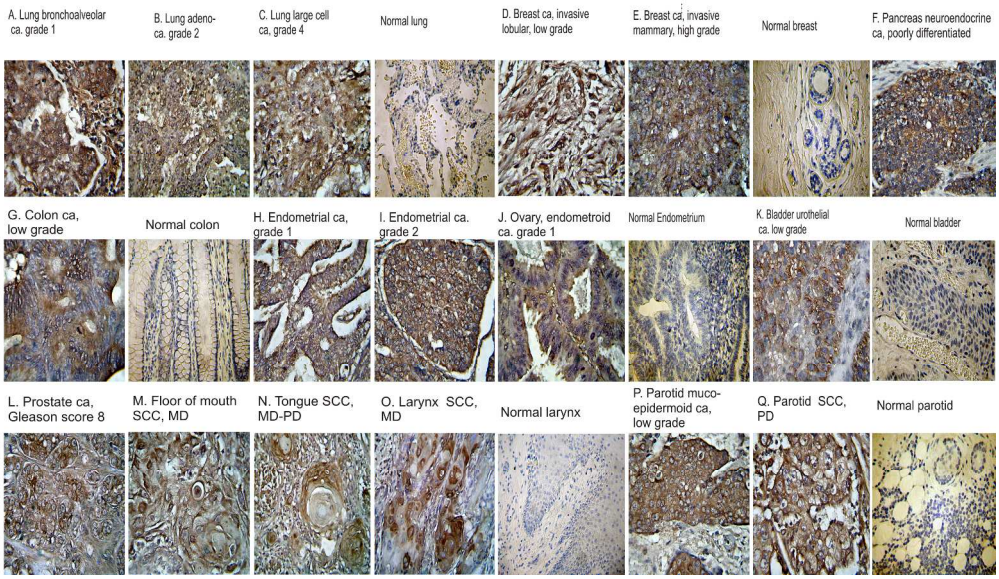
Fig. 3 Comparison of TRIM59 expression by immunohistochemistry in 8 different tumors



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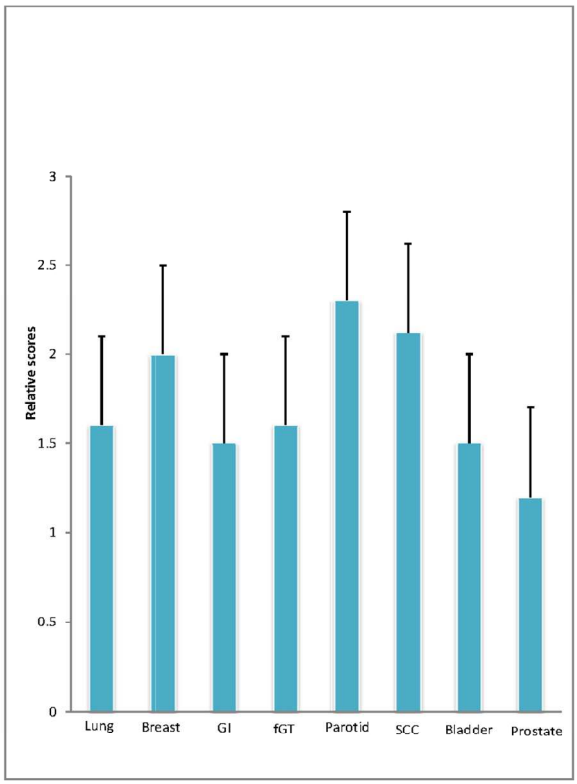
R. Comparison of relative scores of TRIM59 IHC staining in eight different tumor types



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Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

Legend of Supplemental Fig. 1 (A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows.

(B). 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). The truncated forms of GST-TRIM59#71 may be due to bias of condon usage in the N-terminus. When immune to rabbits, we adjusted the doses by counting only the whole amount of all recombinant GST-TRIM59 proteins (1-1.5mg, GST protein was excluded).

(C-D). Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wildtype (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wildtype (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots.

(E). Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145 LnCaP and human kidney cell HEK293. An unique 53 kDa band was shown exactly as in mouse cell lyases **(C)**.

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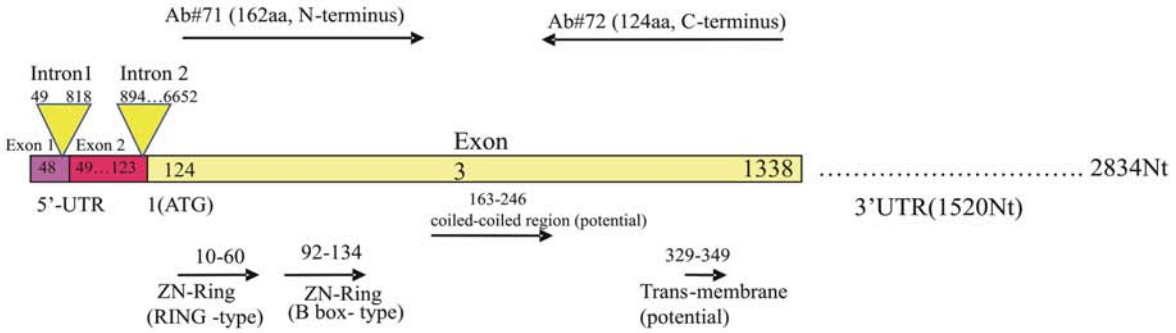
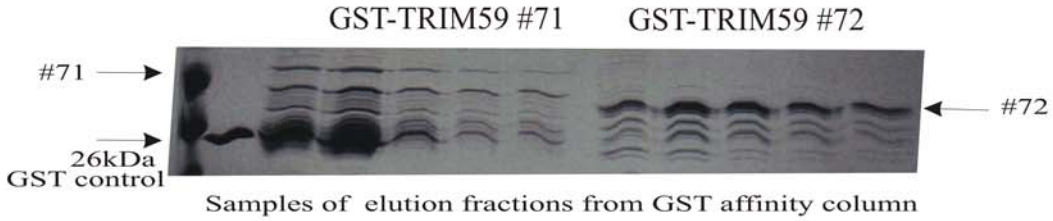
Supplemental Fig. 1

A. Structure, location of two antibodies to mouse TRIM59 GST fusion protein GST-TRIM59 #72 and /71

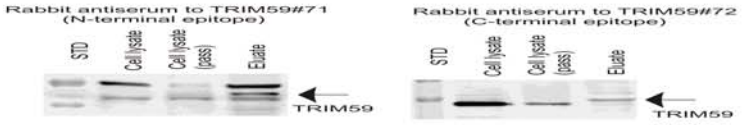


B. Purified GST-TRIM59 #72 and /71 recombinant proteins expressed in *E.coli* (10% SDS-PAGE)

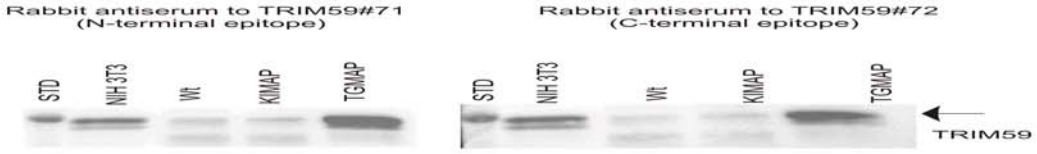


Characterization of TRIM59 antibodies
in mouse cells (C) and tissue lysates (D)

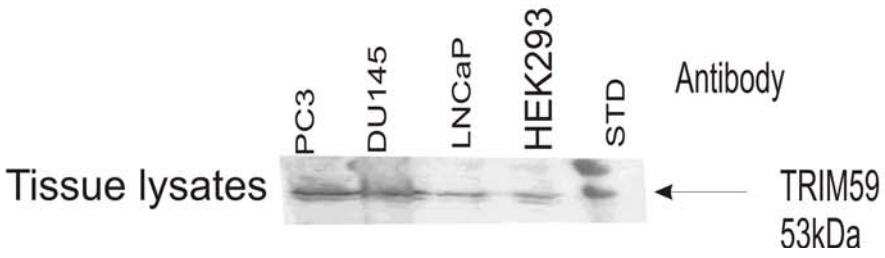
C. Total cellular phosphorylated proteins



D. Affinity purified proteins



E. Western blotting characterization of TRIM59/72 antibody in human cell lysates



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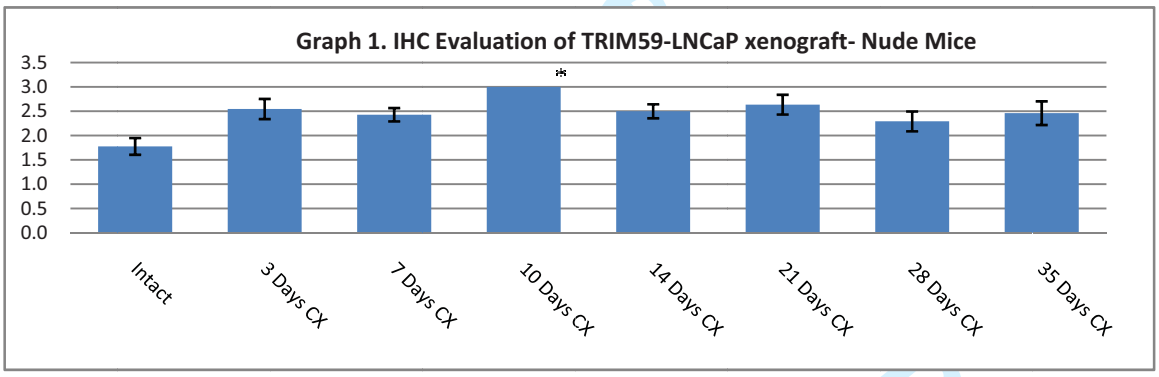
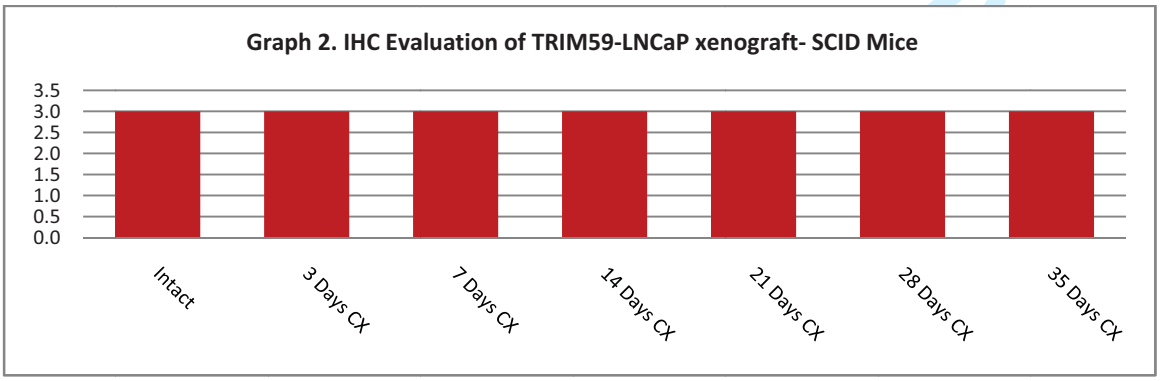
Supplemental Fig.2 **IHC evaluation of androgen responsiveness of TRIM59 in the LNCaP xenograft TMA**.

The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP , TRIM59 is significantly up-regulated after 10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.





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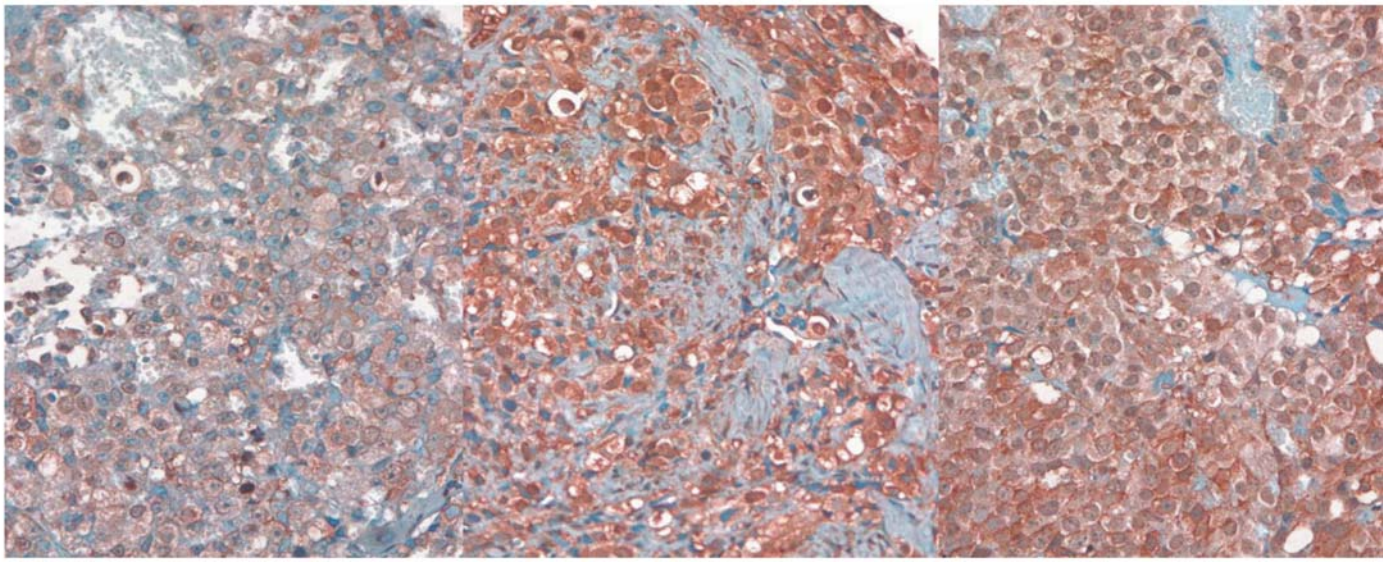
Suppl Fig. 2

TRIM59 SCID-LNCaP xenograft TMA

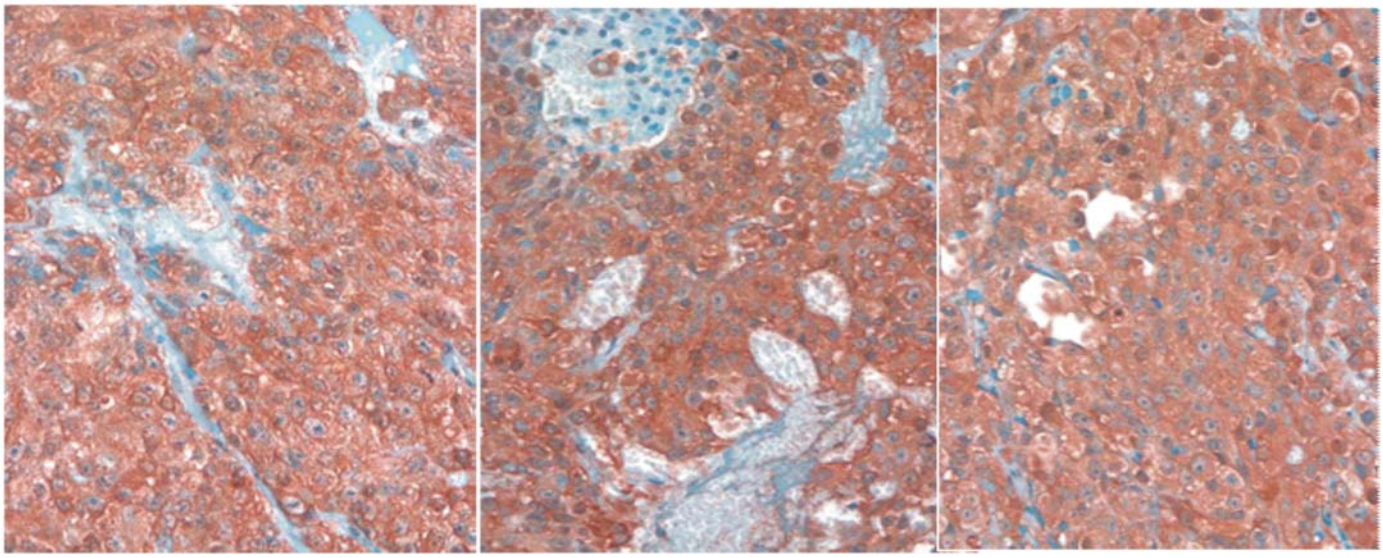
A Day after castration in nude mice

No castration 10 days 35 days



B Day after castration in SCID mice

No castration 14 days 35 days



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Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 and 1:1200. We assessed these IHC results with the negative control (without TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3. 1:1200 produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remain stronger than the normal non-tumor areas even at 1:300. In order to d detect weak TRIM59-IHC staining signals, we also tried 1:300 dilution and compared with negative controls (no antibody added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining, and similar results were obtained.

Legend of Figures:

(A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular lymphoma, (20X),positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining

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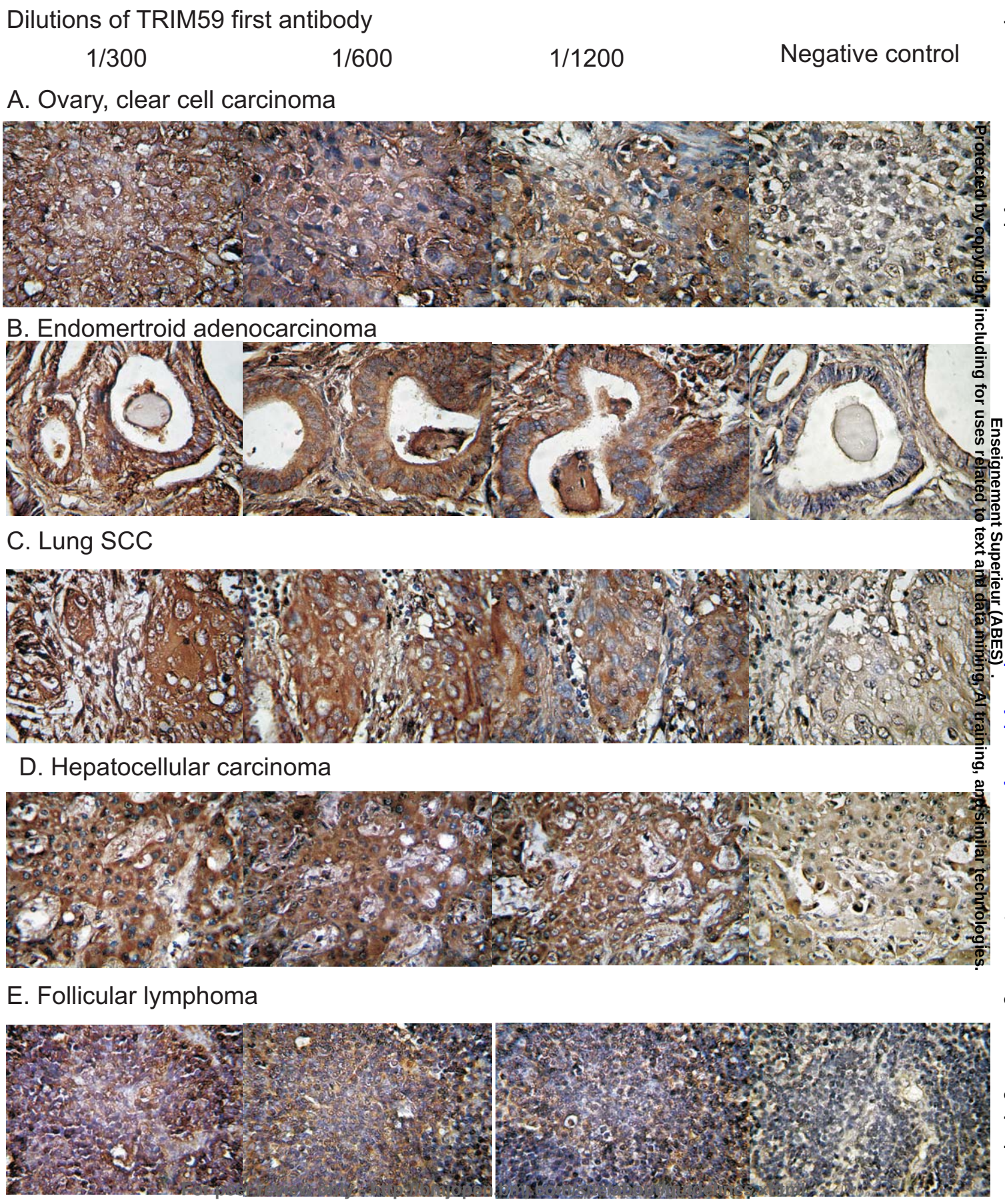
Supplemental Fig. 3 Titration of the diluted TRIM59 antibody in TMA (5 tumors) for moderate IHC staining



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Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry (IHC) staining in human multiple tumor TMA (tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody (positive) and without any antibody (negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some area of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin (squamous cell carcinoma), #4 Skin (basal cell carcinoma), #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma;#13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma, #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma), #2 Adrenal gland (cortical carcinoma), #7 Omentum (serous adenocarcinoma), #9 Cervix adenocarcinoma;

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma), #6 Uterus (leiomyosarcoma), #8 Ovary, serous adenocarcinoma, #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma;, and # 25. Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid)

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Legend of panels:

1. Kidney (Clear cell carcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

2. Adrenal gland (cortical carcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining with no nuclear staining; negative control in negative control, weak background staining in cytoplasmic of few cells (20X, 40X).

3. Skin (squamous cell carcinoma), positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

4. Skin (basal cell carcinoma), positive IHC for TRIM59 expression strong cytoplasmic staining with no nuclear staining; negative control no staining (20X, 40X).

5. Skin (melanoma), positive IHC for TRIM59 expression very weak cytoplasmic staining and nuclear staining; negative control no staining (20X, 40X).

6. Uterus (leiomyosarcoma), positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

7. Omentum (serous adenocarcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

8. Ovary (serous adenocarcinoma), positive IHC for TRIM59 expression weak to moderate cytoplasmic staining and no nuclear staining, negative control no staining (20X, 40X).

9. Cervix adenocarcinoma, positive IHC for TRIM59 expression moderate to strong cytoplasmic staining; negative control no staining (20X, 40X).

10. Breast ductal adenocarcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

11. Bladder (Urothelial carcinoma), positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

12. Thyroid medullary carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

13. Lung squamous cell carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

14. Lung bronchoalveolar carcinoma, positive IHC for TRIM59 expression strong cytoplasmic and nuclear staining; negative control no staining (20X, 40X).

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15. Lymph node metastatic carcinoid, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

16. Lung mesothelioma, positive IHC for TRIM59 expression strong cytoplasmic and nuclear staining; negative control no staining (20X, 40X).

17. Hepatocellular carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

18. Liver metastatic carcinoid, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

19. Endometrial carcinoma, positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

20. Small bowel, marginal zone lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

21. Spleen, Hodgkin lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

22. Stomach, malt lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining.

23.Thymoma, positive IHC for TRIM59 expression weak cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).

24. Appendix (Goblet cell carcinoid), IHC for TRIM59 expression no staining, negative control no staining (20X, 40X).

25. Follicular lymphoma, positive IHC for TRIM59 expression weak cytoplasmic staining, negative control no staining (20X, 40X).

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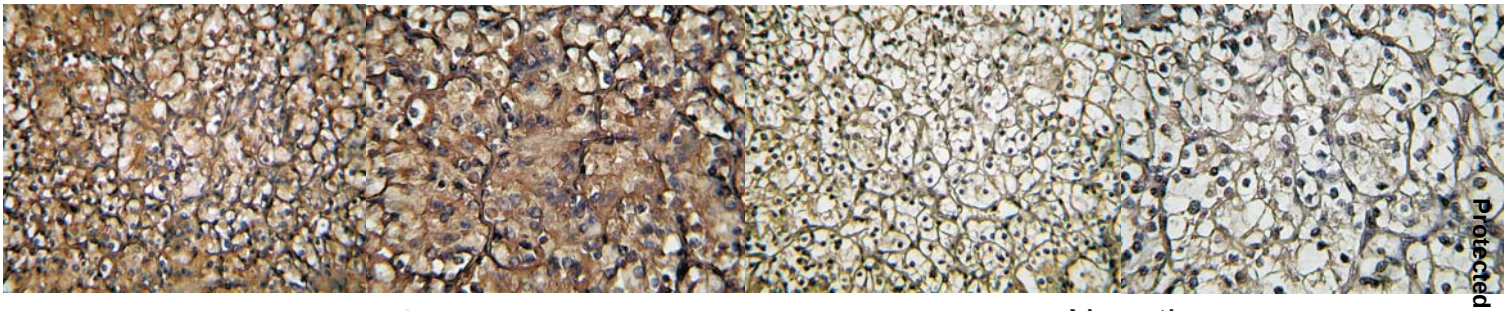
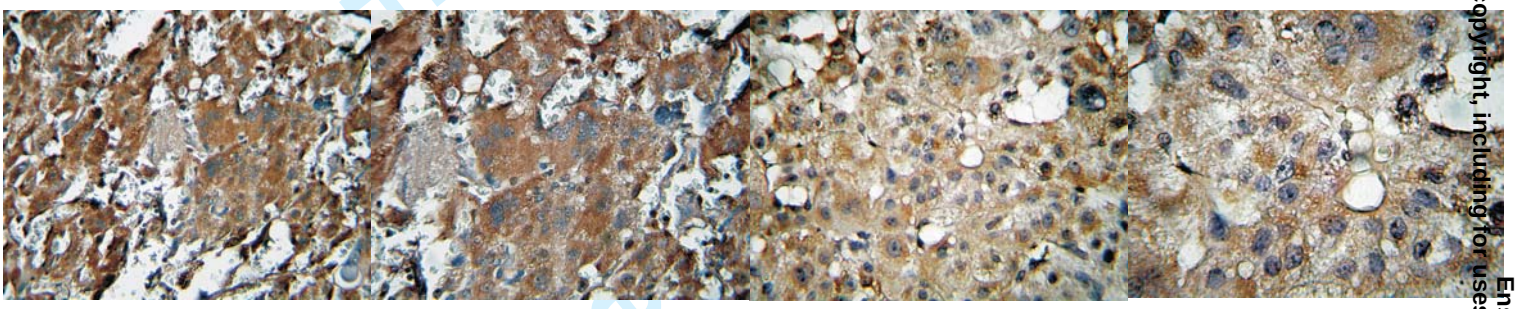
Suppl.Fig. 4 Positive and negative controls of immunohistochemistry (IHC) staining by TRIM59 antibody in 25 tumors listed from multiple-cancers TMA

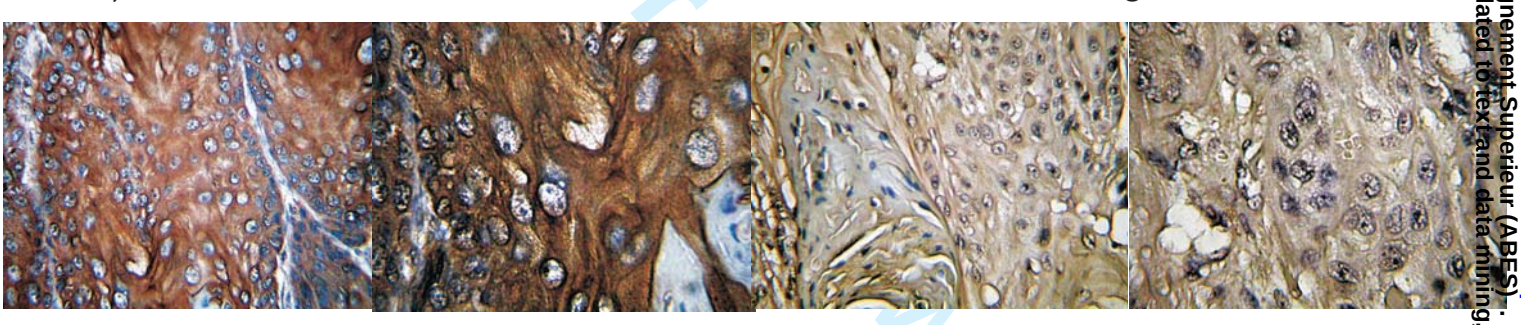
1-Kidney, clear cell carcinoma, positive (moderate) Negative



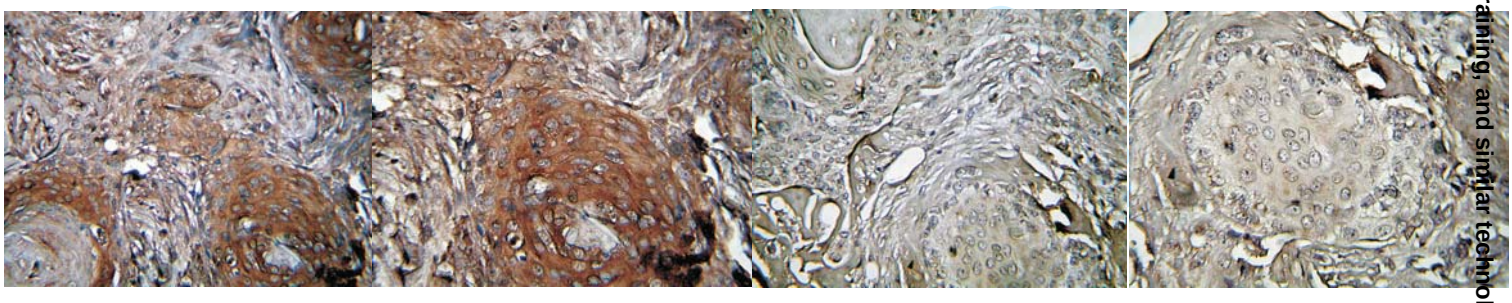
2-Adrenal gland, cortical carcinoma, positive (moderate) Negative



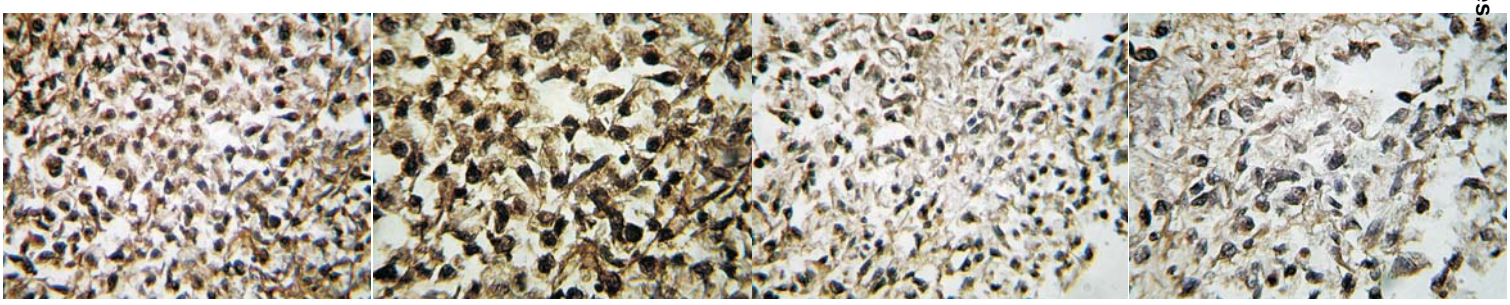
3-Skin, SCC, positive (strong) Negative



4- Skin, BCC, positive(strong) Negative



5-Skin, melanoma, positive (weak) Negative



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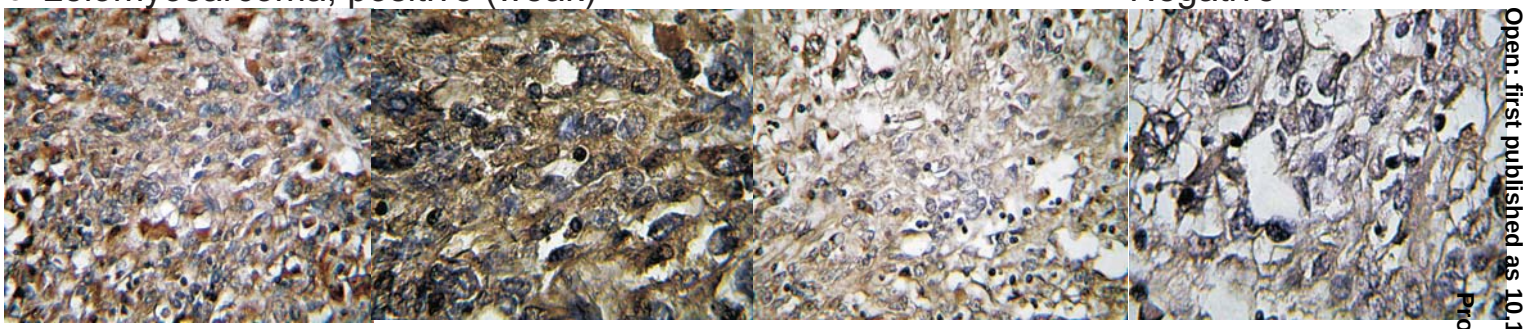
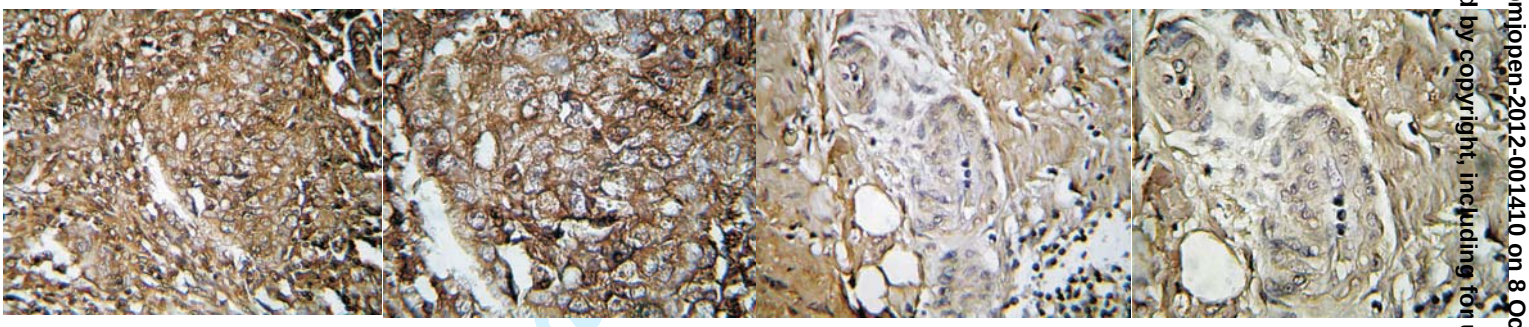
6- Leiomyosarcoma, positive (weak)

Negative



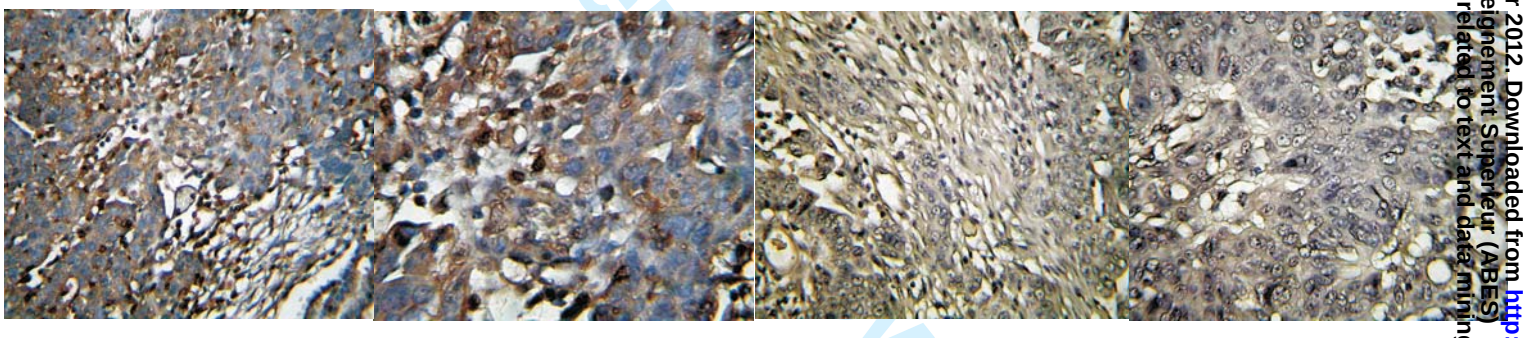
7- Omentum, serous adenocarcinoma, positive (moderate)

Negative



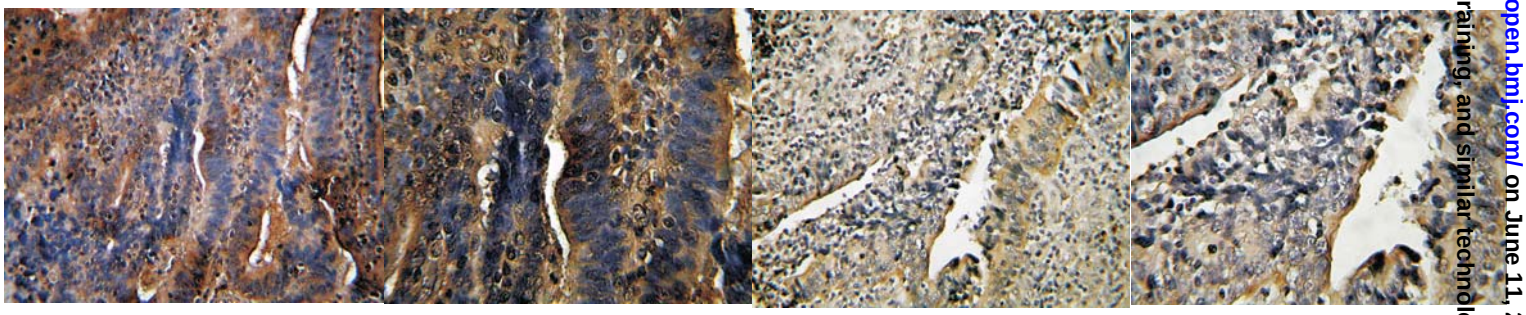
8- Ovary, serous adenocarcinoma, positive (weak)

Negative



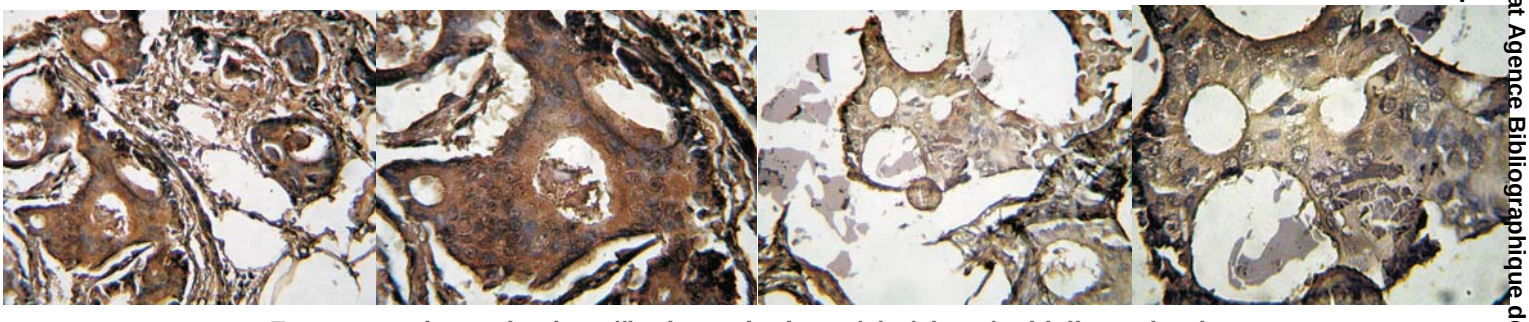
9- Cervix adenocarcinoma, positive (moderate)

Negative



10- Breast ductal adenocarcinoma, positive (strong)

Negative



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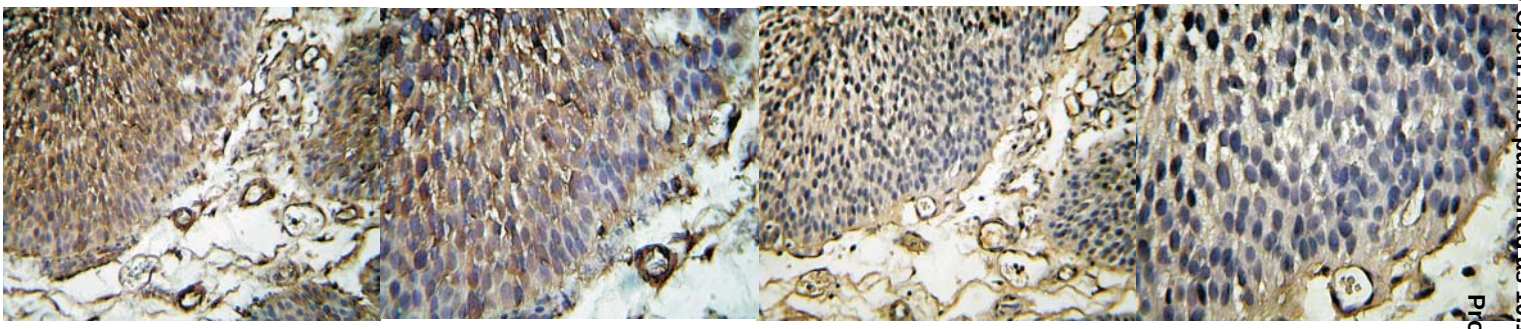
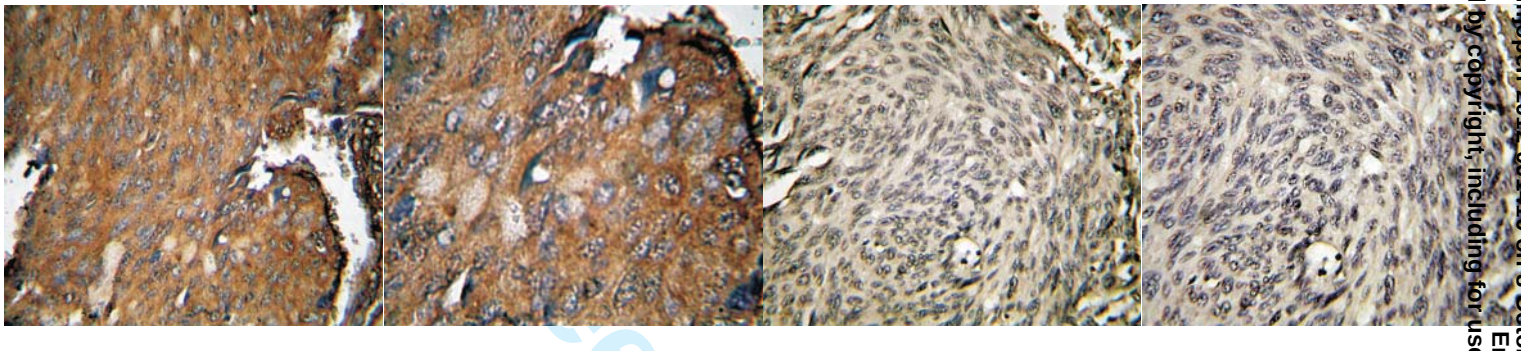
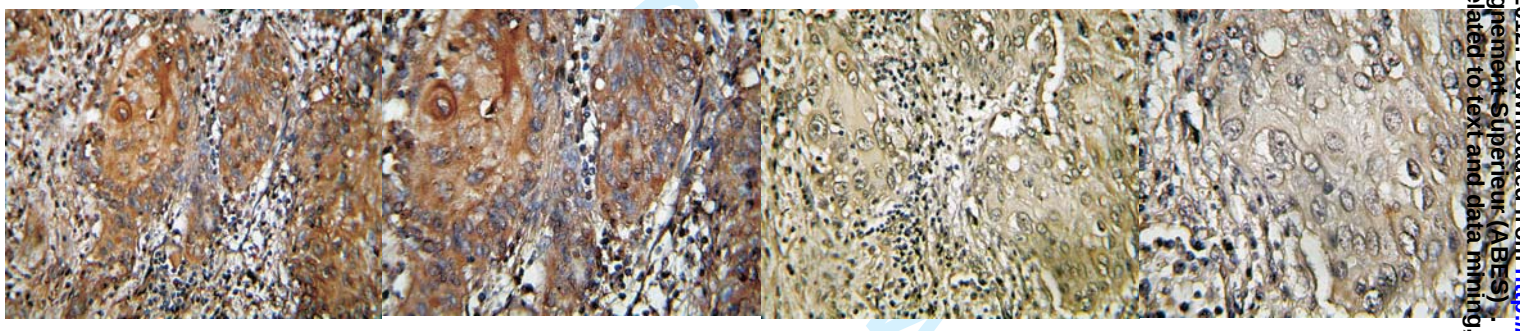
11- Bladder, Urothelial carcinoma, positive (weak) Negative



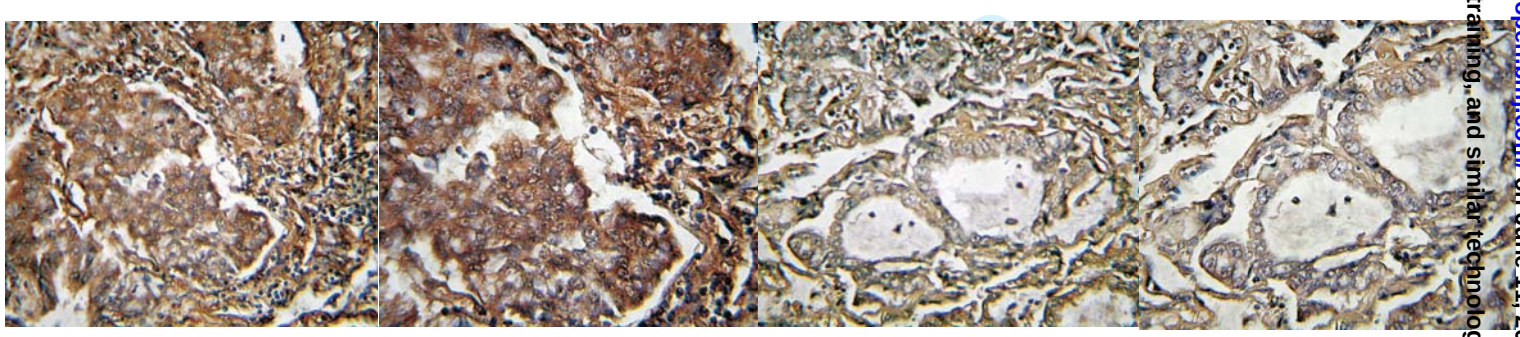
12-Thyroid, medulary carcinoma, positiv (strong) Negative



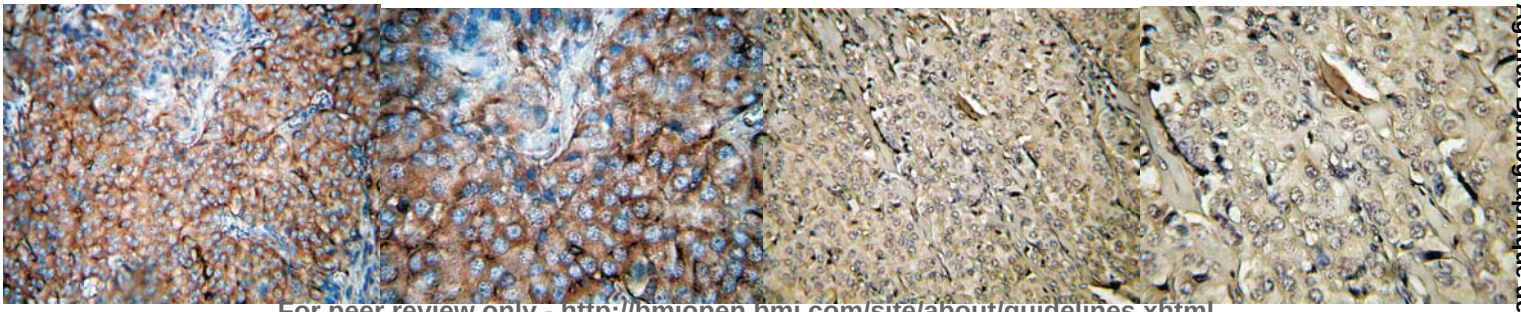
13-Lung SCC, positive (strong) Negative



14- Lung, bronchoalveolar carcinoma, positive (strong) Negative



15-Lymph node metastatic carcinoid, positive (strong) Negative



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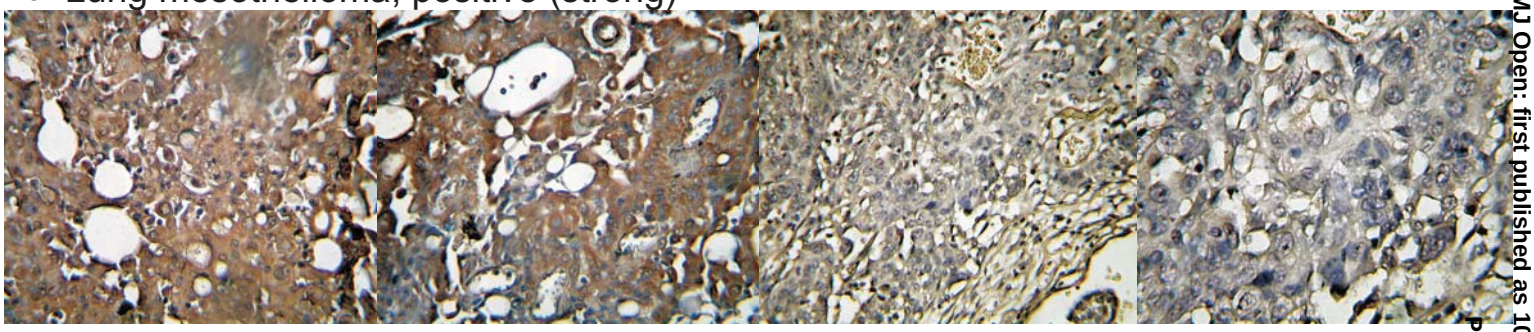
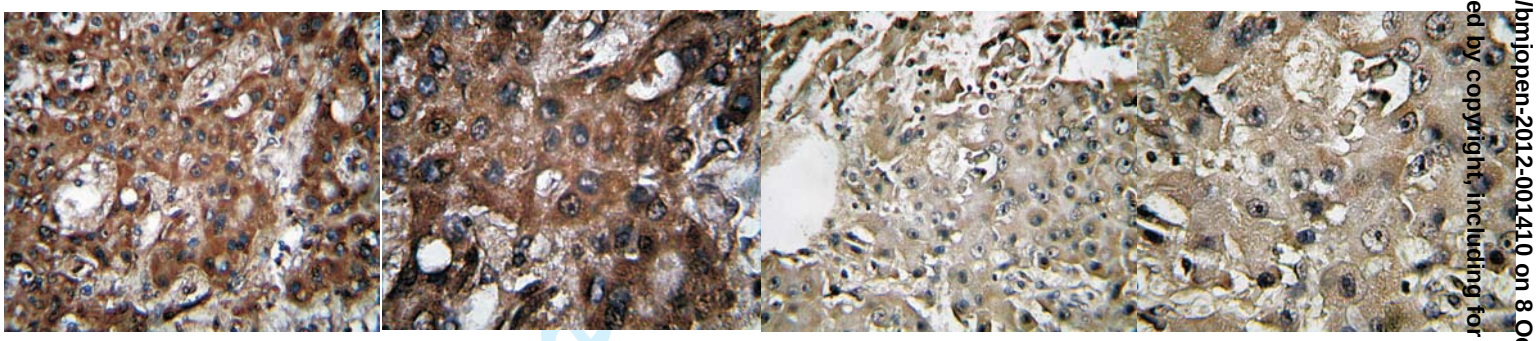
16- Lung mesothelioma, positive (strong)

Negative



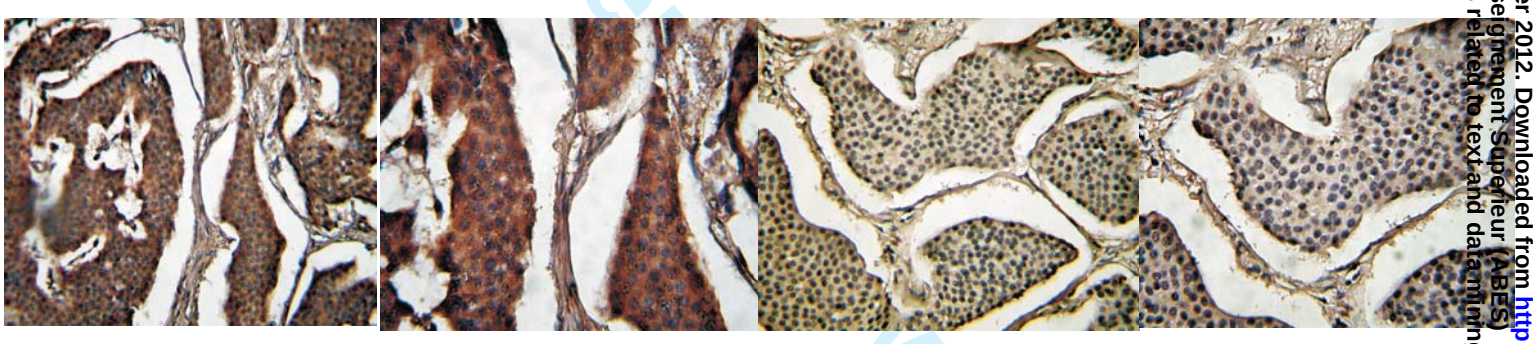
17- Hepatocell carcinoma, positive (strong)

Negative



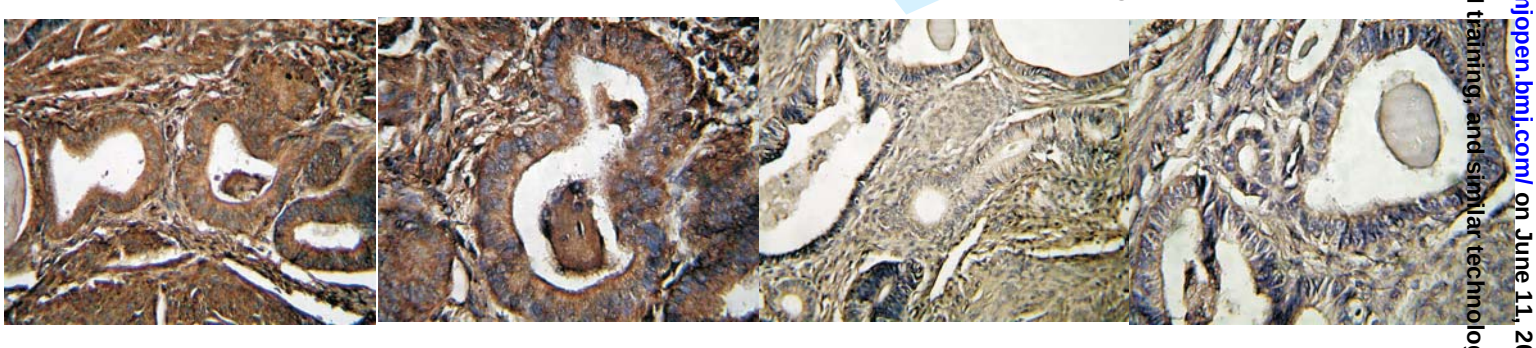
18- Liver metastatic carcinoid, positive (strong)

Negative



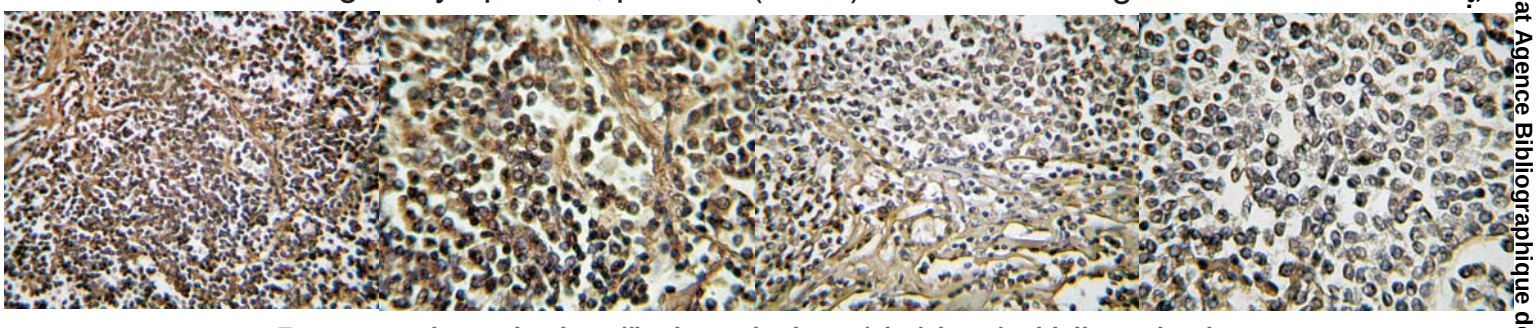
19- Endometrial adenocarcinoma, positive (strong)

Negative



20- Small bowel marginal lymphoma, positive (weak)

Negative



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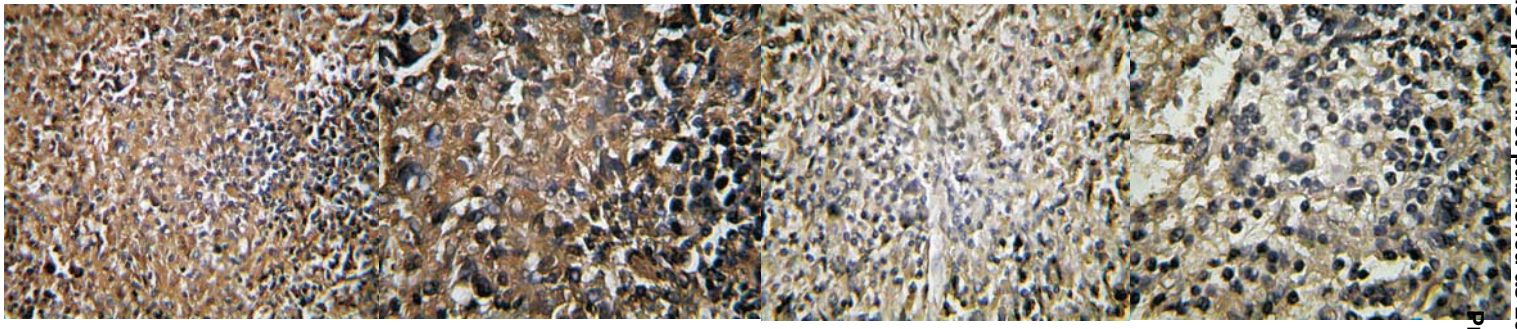
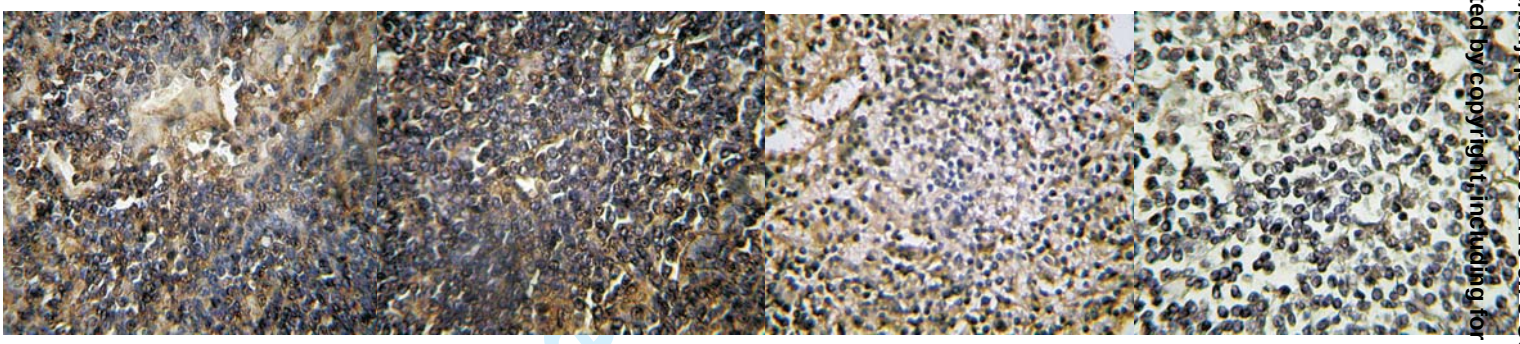
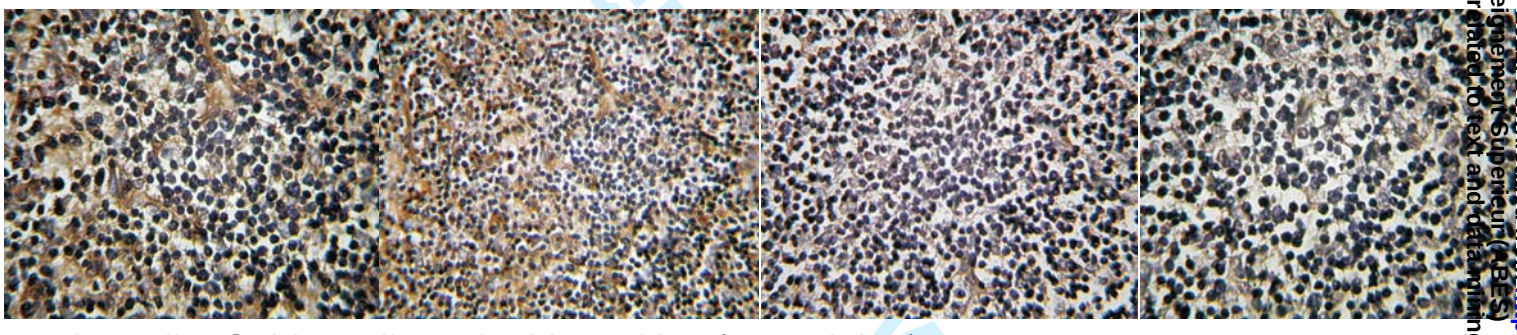
21- Spleen hodgkin lymphoma, positive (weak) Negative



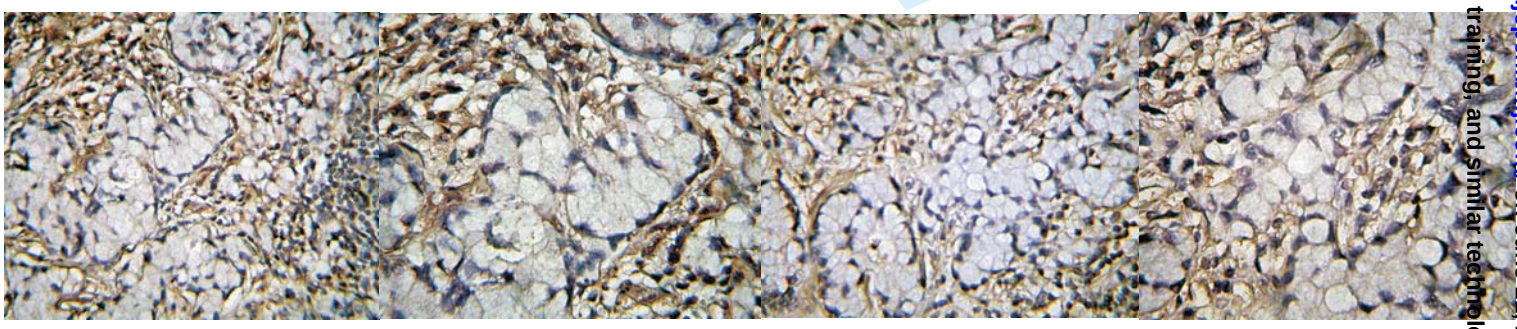
22- Stomach malt lymphoma, positive (weak) Negative



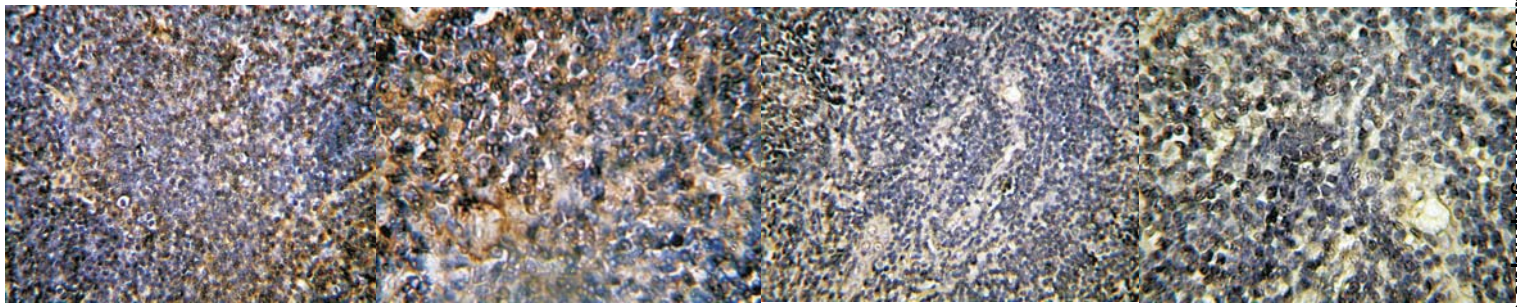
23- Thymoma, positive (weak) Negative



24- Apendix, Gablet cell carcinoid, positive (no staining) Negative



25- Follicular lymphoma, positive (weak) Negative



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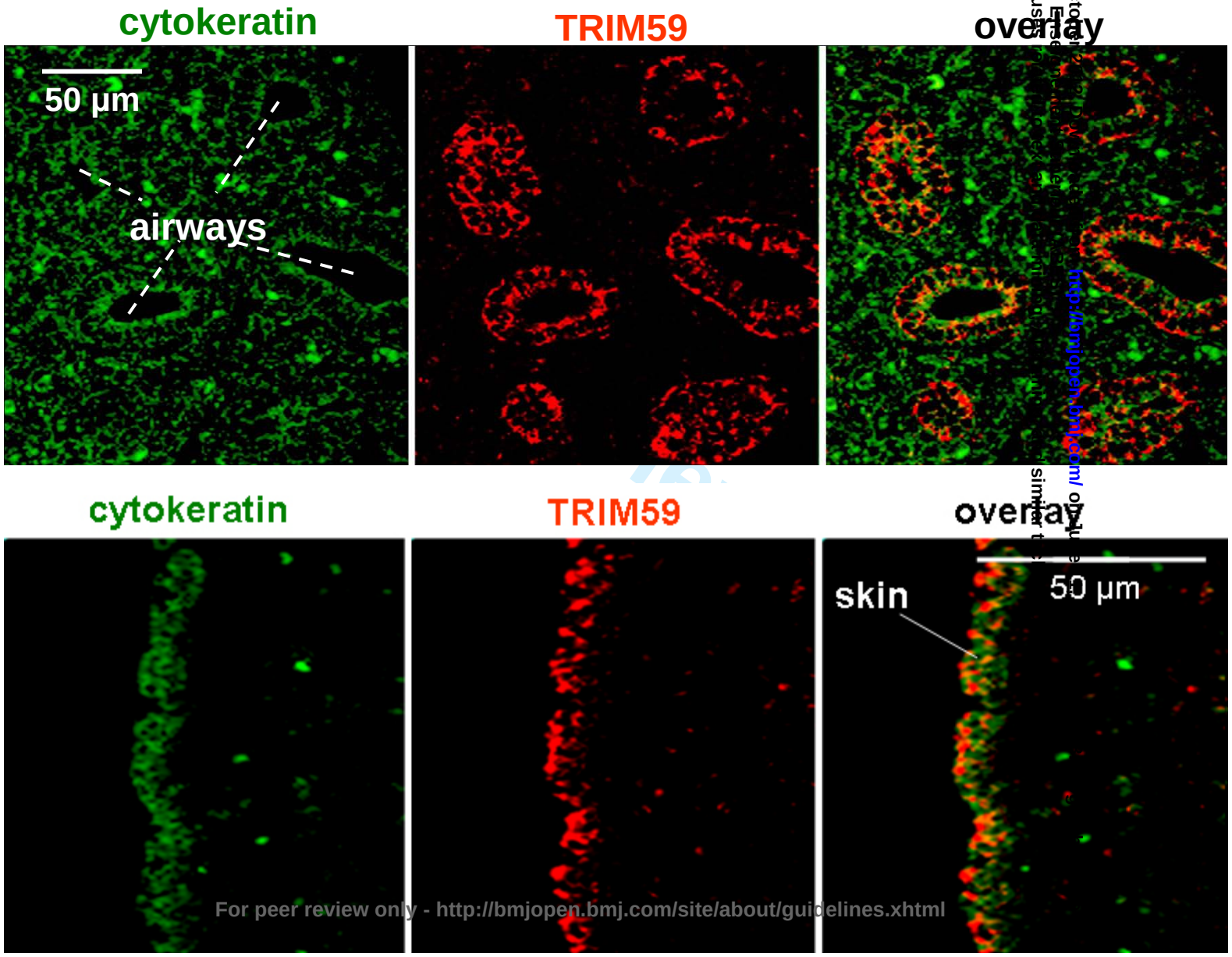
Supplemental Fig. 5

Confocal microscope imaging of immunohistochemistry of TRIM59 protein in mouse embryo (14.5d.p.c)



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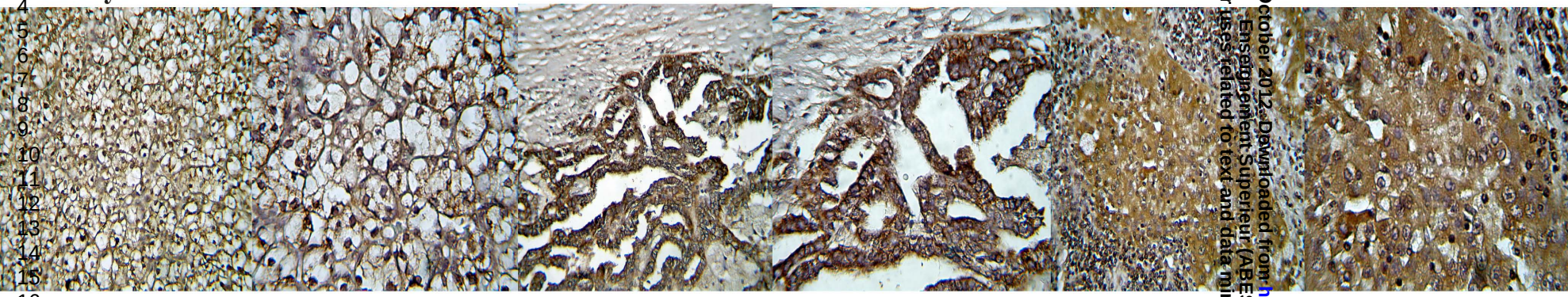
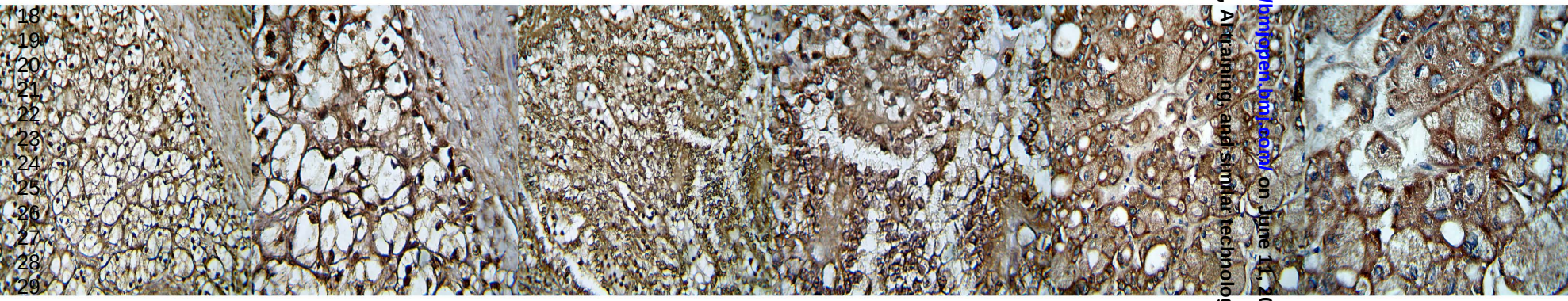
Test of Ras activation in kidney cancer patients correlated with up-regulation of TRIM59

A. Clear cell carcinoma B. Papillary RCC C. Chromophobe RCC

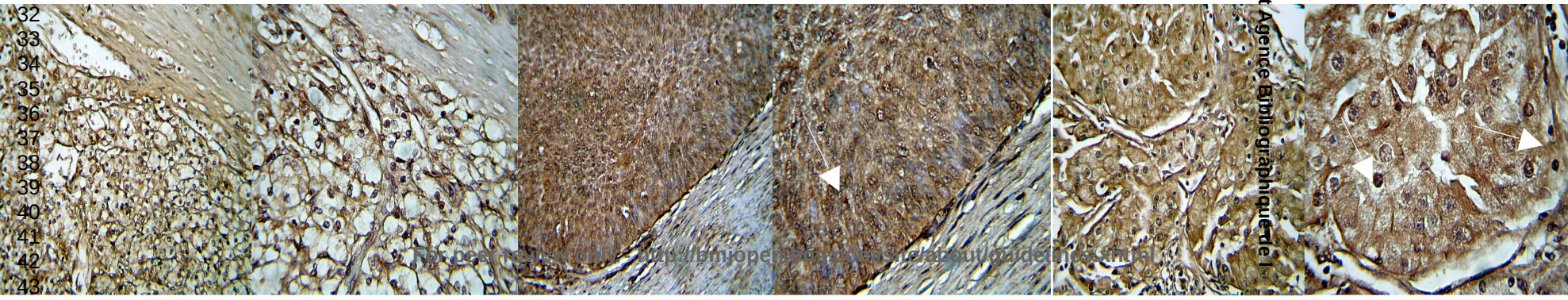
Antibody: B-Raf for total B-raf



Antibody: Raf 1 for total Raf



Antibody: Phosphor- Ser- 259 Raf 1



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Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

Antibody used	RCC types	Patient number	Intensity score	Nuclear staining
B-Raf (total B-raf)	Clear cell Carcinoma	6	0-1	No
	Chromophobe	3	2-3	No
	papillary	3	2-3	No
Raf-1 (total B-raf)	Clear cell Carcinoma	3	0	No
	Chromophobe	2	2-3	No
	papillary	1		No
B-Raf p-Ser ^{259} (phosphorylated B-Raf)	Clear cell Carcinoma	3	0-1	No
	Chromophobe	1	2-3	20%
	papillary	2	2-3	50%

Since BRAF is the most important early effector in the Ras signal pathway, and Raf mediate phosphorylation is the main effector recruited by GTP-bound Ras to activate the MEK-MAP kinase pathway, we performed IHC studies in these RCC sections by probing Raf activation /up-regulation and hyper-phosphorylation. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing for total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing activated phosphorylated B-Raf. Some of serial slides were stained parallelly on each patient by different antibodies.

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	3
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7,8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*9*
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	9
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	9
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			9

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	6
Outcome data	15*	Report numbers of outcome events or summary measures over time	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	17
Discussion			16
Key results	18	Summarise key results with reference to study objectives	
Limitations			18
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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TRIM59, a novel multiple cancer biomarker for immunohistochemical detection of tumorigenesis

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Running title: TRIM59, a novel multiple-cancer marker for tumorigenesis

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E-mail: jim.xuan@lhsc.on.ca

List of abbreviations

Abbreviations: TRIM: TRIpartite Motif; CaP: prostate cancer; BPH: benign prostatic hyperplasia; PIN: prostate intraepithelial neoplasia; TMA: tissue microarray; RCC: renal cell carcinoma; SCC: squamous cell carcinoma; GI: gastrointestinal; GU: genitourinary; d.p.c: days post conception; GEM (genetically engineered mouse); H&E: haematoxylin and eosin; IHC: Immunohistochemistry; WD: well differentiated ; MD: moderately differentiated ; PD: poorly differentiated.

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Abstract

Objectives and design: We identified a novel TRIM59 gene, as an early signal transducer in two (SV40Tag and Ras) oncogene pathways in murine prostate cancer (CaP) models. We explore its clinical applications as a multi-tumor marker detecting early tumorigenesis by immunohistochemistry.

Setting and Participants: 88 CaP patients were from a tissue microarray (TMA) of radical prostatectomy specimen, 42 patients from a 35 multiple-tumor TMA, 75 patients with renal cell carcinoma (RCC) and 92 patients from 8 different tumor groups (breast, lung, parotid, gastrointestinal, female genital tract, bladder, kidney and prostate cancer).

Results: TRIM59 up-regulation specifically in tumor area was determined by immunohistochemistry in 291 cases of 37 tumor types. To demonstrate that TRIM59 up-regulation is "tumor-specific", we characterized a significant correlation of TRIM59 IHC signals with tumorigenesis and progression, while in control and normal area, TRIM59 IHC signal was all negative or significantly low. TRIM59 protein up-regulation in prostate and kidney cancers was detectable in both intensity and extent in early tumorigenesis of PIN (prostate intraepithelial neoplasia, $P<0.05$) and grade 1 of RCC ($P<0.05$), and stopped until high grades cancer. The results of the correlation in these two large cohorts of tumor types confirmed and repeated murine CaP model studies. Enhanced TRIM59 expression was identified in most of the 37 different tumors, while the highest intensities were in lung, breast, liver, skin, tongue and mouth (squamous cell cancer) and endometrial cancers. Multiple-tumor up-regulation was further confirmed by comparing relative scores of TRIM59 IHC signals in eight tumors with a larger patient population; and by a mouse whole mount embryo (14.5dpc) test on the origin of TRIM59 up-regulation in epithelial cells.

Conclusions: TRIM59 may be used a novel multiple-tumor marker for immunohistochemical detecting early tumorigenesis and could direct a novel strategy for molecular targeted diagnosis and therapy of cancer.

Keywords: TRIM59, Cancer biomarker, Immunohistochemistry, Prostate cancer, Multiple cancer marker, Ras signal pathway, SV40Tag oncogene, Tissue microarray, Confocal microscopy.

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Introduction

The TRIM (*TRI*partite *M*otif) family is an evolutionarily conserved gene family implicated in a number of critical processes including immunity [1-3], antiviral [4-8], proliferation [6, 9], transcriptional regulation [6, 10], neuro-development [11, 12], cell differentiation [12] and cancer [13] (reviewed in [1, 5, 14, 15]). However, the function of most TRIM family members is poorly understood and was surmised only based on computational analysis from their RBCC (*R*ING finger, *B*-box, Coiled-*C*oil) domains. The domain of RING (*R*eally *I*nteresting *N*ew *G*ene) is frequently involved in proteolysis acting as E3 ubiquitin ligases and the ubiquitin-proteasome system in the regulation of numerous cellular processes including cell cycle regulatory proteins, transcription factors, and signal transducers [6, 15]. Recent studies demonstrate that various TRIM-NHL proteins function as cofactor for the microRNA-induced silencing complex (miRISC) [12] [14, 16]. An ataxia-telangiectasia group D complementing gene (ATDC) was recently designated as TRIM29, which is elevated in most invasive pancreatic cancers in the Wnt/β-catenin signalling pathway [13].

In a previous report [17], we characterized the function of TRIM59, a novel TRIM family member, in SV40 Tag oncogene directed genetically engineered mouse (GEM) prostate cancer (CaP) models [18-21]. The TRIM59 gene was identified to be correlated with the SV40 Tag initiated tumorigenesis. TRIM59 protein up-regulation and hyper-phosphorylation started in the prostate cytoplasm in early tumorigenesis from PIN (prostate intraepithelial neoplasia) [17]. As a signal pathway effector, the p-Ser/Thr phosphorylated TRIM59 proteins correlate with tumorigenesis, while p-Tyr-TRIM59 proteins correlate with advanced CaP. The function of TRIM59 was identified by shRNA knockdown in human CaP cells resulted in S-phase arrest and cell growth retardation. Although TRIM59 is an effector gene with the SV40Tag oncogene, the

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initial functional targets of TRIM59 function were actually on the Ras signal pathway as an early and rapid signal transmitter (see review [22]). In a transgenic mouse test of TRIM59 up-regulation specifically in the prostate, TRIM59 demonstrated full oncogenic activity in directing tumorigenesis and progression to highest grade CaP [23]. The signal pathway of TRIM59 may be possibly linked with two large oncogene routes: the Ras/Raf/MEK/ERK/PI3K/AKT and the SV40 Tag/p53/pRB routes [17]. In this study we explored the clinical utility of the novel TRIM59 gene to serve as a biomarker for a panel of human cancers. To address the molecular biology basis, we combined results from both basic (animal model [17]) and human clinical studies.

Materials and Methods

Patient selection:

All patient samples were acquired as part of REB (Research Ethics Board) approved protocols at the University of Western Ontario (UWO) and Vancouver Prostate Center, University of British Columbia (UBC). Table 1 shows a complete list of 291 patients with 37 different tumor types examined in this study.

Prostate cancer Tissue Microarrays (TMA): 88 CaP patients between 2006 and 2008 who had no treatment prior to radical prostatectomy, were selected from the Vancouver General Hospital. Each patient block marked as containing benign tissue or cancer was sampled 2 times with a core diameter of 1 mm arrayed (176 cores) in a rectangular pattern with 0.7 mm between the centers of each core, creating a duplicate TMA layout using a manual tissue micro arrayer (Beecher Instruments, Silver Spring, MD). The TMA paraffin blocks, were sectioned into 0.5 micrometer sections and mounted on the positively charged slides.

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Organ	Patient Number	Tumor type		Tumor grade	
35 tumor-TMA	42	Description in Table-2			
Prostate TMA	105	Adenocarcinoma		BPH	16
				PIN	4
				Gleason score 4	5
				Gleason score 6	31
				Gleason 7	15
				Gleason score 8	16
				Gleason score 9-10	9
				Stroma	3
				Absent cores	6
Kidney	75	Clear cell carcinoma	43	Grade 1	4
		Papillary RCC	11	Grade 2	38
		Chromophobe RCC	13	Grade 3	28
		Cystic RCC	6	Grade 4	5
		Sarcomatoid RCC	2		
Bladder	44	Urothelial carcinoma		Low grade	38
				High grade	6
Lung	4	Bronchoalveolar carcinoma	1	Grade 1	1
		Adenocarcinoma	1	Grade 2	1
		Large cell carcinoma	1	Grade 3	1
		Squamous cell carcinoma	1	Grade 4	1
Breast	3	Invasive lobular carcinoma	1	Grade 1	1
		Invasive mammary carcinoma	2	Grade 3	2
Female Genital tract	5	Endometrial carcinoma	4	Grade 1	4
		Ovary, Endometrioid carcinoma	1	Grade 2	1
Gatrointestinal tract	2	Colon carcinoma	1	Low grade	1
		Pancreas neuroendocrine carcinoma	1	high grade	1
Parotid	3	Mucoepidermoid carcinoma	1	low grade	1
		Metastatic SCC	1		
		Metastatic neuroendocrine Carcinoma	1	high grade	2
Mouth, tongue and larynx	4	Squamous cell carcinoma	4	Moderately differentiated	3
				Moderately to Poorly differentiated	1

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Table 1 Patient list selected in this study

Total	291		

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Automated image, acquisition and analysis on immunohistochemical staining of CaP-TMA: (UBC) Immunohistochemical staining was conducted by Ventana autostainer model Discover XT ™ (Ventana Medical System, Tuscan, Arizona) with enzyme labelled biotin streptavidin system and solvent resistant DAB Map kit. TMA was scanned by Bliss Digital imaging system using x20 objective, from Bacus Laboratories INC, Centre Valley PA, and stored in the Prostate Centre Saver (http//bliss.prostatecentre.com). A value on a four-point scale assigned to each core.

Multiple–tumor Tissue Microarray construction: Tissue samples from 42 patients that encompassed 35 distinct tumor subtypes were selected from London Laboratory Service Group, and the tumor bank in the Department of Pathology (UWO). TMA slides were constructed with triplicate cores for each sample following standard procedure as described [24]. 0.6 mm sections were prepared from TMA block and re-stained by H&E for each case to confirm the diagnosis.

Histopathologic analysis: All cases from 37 tumor types were graded according to standardized histopathology grading systems [25] by M.M (pathologist) and V. K (MD fellow).

Immunohistochemistry (IHC) and results evaluation: Standard ABC (Avidin Biotin Complex) protocol was performed as previously reported [21, 26, 27]. Two kinds of blocking reagents were used: Power Block (Universal Blocking Reagent, from BioGenex, San Ramon, CA) and Avidin-biotin blocking reagent kit (Vector Labs, Burlingame, CA). All B-Raf antibodies were from GenScript (Piscataway, NJ): B-Raf antibody (monoclonal antibody, used 1:500 dilution), Raf 1 (polyconal, 1:50) and Raf -1 -(polyclonal, Phospho-Ser^{259} 1:50).

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TRIM59 IHC staining signals were assessed by intensity for cytoplasmic staining and extent to assess percentage of nuclear staining as previously reported [28, 29]. Since in some tumors TRIM59 showed only cytoplasmic staining, for the purpose of comparing in different tumors, we used a combined relative score system based on both intensity and extent as following: score 0: 0/0 (intensity/ extent); score 1: weak cytoplasmic staining and/or $\leq 25\%$ nuclear staining; score 2: moderate cytoplasmic staining and/or $\leq 50\%$ nuclear staining; score 3: strong cytoplasmic staining and/or $\geq 50\%$ nuclear staining. All relative scores were accessed by two researchers independently M.M (pathologist) and V. K (MD fellow).

Construction and characterization of TRIM59 antibody:

Supplement Fig. 1 provided details, which combined Supplement materials of [17], and more informations. Supplement Fig. 1A showed that the antibody TRIM59#72 used for mouse and human studies was prepared from C-terminal 136 a.a. peptide (as recombinant GST- fusion protein, Suppl. Fig. B) of whole protein 403aa which is TRIM59 specific, while antibody TRIM59#71 was from mouse TRIM59 N-terminal sequence (163aa) containing mostly the common RBCC function domains of more than 70 members TRIM family [17]. As shown in Supplemental Fig. 1C, Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed multiple bands. We also demonstrated that N-terminal sequence specific antibody TRIM59#71 can recognize purified proteins from C-terminal sequence specific, TRIM59#72 Ab affinity column (the same 53kDa protein, Suppl. Fig. 1D). Since these two immunogenic sequences are not overlapped, we thus verified that they are detecting the same protein. We demonstrated that TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots (Suppl Fig. 1E).

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Confocal microscope imaging of immunostaining of mouse embryos 14.5 dpc (days post conception) for TRIM59: The mouse embryos were prepared according to our previous reports [18-21]. The anti-rabbit secondary antibody was conjugated to Cy3 (1:200, Jackson ImmunoResearch, West Grove, PA). Sections were double-stained with pan-cytokeratin antibody with FICT (Sigma), and the stained mouse embryo sections were visualized using a Carl Zeiss confocal microscope by the LSM Image program.

Statistical Analysis: Student's *t* tests and one-way ANOVA were used by programs of Microsoft Excel 2007 or SPSS 10 to analyze the data with $p < 0.05$ considered to be statistically significant.

Results and Discussion

TRIM59 up-regulation in human prostate cancer TMA (tissue microarray): correlation with tumorigenesis and tumor progression by TRIM59 intensity until high grade CaP:

We designated TRIM59 as one of the "tumorigenesis–associated" genes correlated with SV40 Tag oncogenesis in mouse prostate cancer (GEM-CaP) models [17]. SV40 Tag is essentially only required for the initiation of tumorigenesis, *i.e.* the "hit-and-run" effect, in GEM-CaP, but not for the tumor progression and metastasis directly. The "tumorigenesis–associated" effect, is due to the initial binding of the Tag oncogene with retinoblastoma (pRB), p53 proteins and several transcriptional co-activators. Once this process is initiated, the signal-transduction will continue on, even without the initiation effectors.

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In GEM-CaP models, the TRIM59 protein up-regulation correlation with tumorigenesis and progression, and down-regulated in the high grade CaP by immunohistochemistry (IHC) [17]. We assume this "tumorigenesis–associated" effect of TRIM59 may apply to human cancer studies.

We first characterized that TRIM59 antibody (#72) can cross react with and recognize specifically human TRIM59 counterpart (details see supplemental Fig. 1). We tested by IHC in a TMA of CaP patients (n=88, 176 cores). TRIM59 IHC signals detected in an automated digital image system were identified mostly in cytoplasm of luminal cells (Fig. 1A), which is different from rapid tumor progression mouse CaP models. The intensity (score= 2) in PIN (n=4, Fig. 1A) was higher than in non-tumor area (normal and BPH). Moderate to strong expression was observed in Gleason score 6 (3+3, n=25), 7 (4+3, n=15), and 8 (4+4, n=14). In high grade CaP (score 4+5, n=8, Fig. 1A), TRIM59-IHC signals were lower. As shown in a graph of Fig. 1B, TRIM59 protein IHC signals correlated significantly (P=0.014) with tumorigenesis and progression from PIN to -WDCaP (Gleason grade 1-2, scores 2-4) and MDCaP (Gleason grade 2-4, scores 4-8) (graph Fig 1B), and decreased in high grade CaP (Gleason score 9-10) with P=0.018, which is similar to GEMs [17].

TRIM59 up-regulation in human Renal Cell Carcinoma (RCC) patients: correlation of tumorigenesis and tumor progression with TRIM59 intensity until high grade RCC:

Next, we demonstrated that TRIM59 is not androgen-responsive and likely not prostate tissue-specific, indicating that the tumorigenesis role of TRIM59 may be applied to all or most of human cancers. Supplemental Fig. 2 showed this result in detail of IHC test by TRIM59 antibody#72 on a TMA of the LNCaP human CaP xenografts in nude/SCID castrated mice.

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We extended results from prostate cancer clinical samples to kidney cancer. We started 75 renal cell carcinoma (RCC) patients including all 5 different types of RCC tumors: 43 clear cell carcinoma (representative IHC figures shown in Fig. 2 A-D), 11 papillary renal cell carcinoma (Fig. 2 E-F), 13 chromophobe renal cell carcinoma (Fig. 2 G-H), 2 sarcomatoid renal cell carcinoma (Fig. 2J), and 6 cystic renal cell carcinoma (Fig. 2 K-L). RCC cases analyzed with Fuhrman grade 1-4 were 4, 38, 28, and 5 respectively. TRIM59-IHC staining in normal area including proximal tubules was negative (Fig. 2 last row). Background staining was eliminated by testing antibody dilutions (1:100, 200, 500, 1200 till 5000) while tumor specific signals of TRIM59 proteins were noticeable. Endogenous biotin signals were blocked and excluded by additional block reagents (avidin-biotin blocking reagent kit).

TRIM59 IHC staining in tumor areas in RCC (Fig. 2) was different from cases of CaP-TMA (mainly cytoplasmic). TRIM59 IHC staining was found in both cytoplasm and nucleus in all RCC grades and types (Fig. 2). We assessed TRIM59-IHC by visual scoring of both intensity (cytoplasmic staining) and extent (% nucleus staining) microscopically. Correlation of TRIM59 IHC signals by scoring the intensity in cytoplasm with grades of all five types of RCC is shown in Fig. 2M. TRIM59 IHC signals were increased with tumor progression from grade 1-3 ($p<0.05$). All grade 1 tumors (n=4) stained with weak TRIM59 IHC signals in cytoplasm, but with high extent of nuclear staining; while all grade 2 and 3 tumors (n=66) showed moderate to strong cytoplasmic staining intensity of TRIM59. All grade 4 tumors (n=5) showed weak to moderate intensity in cytoplasm of TRIM59 staining. No correlation between TRIM59 IHC staining in nucleus and tumor grade was found, although ~~in~~ low grade RCC showed higher nuclear staining.

Therefore by systematic IHC studies in CaP (88 patients, Fig. 1) and kidney cancer (75 patients, Fig. 2), we almost exactly repeated results from our mouse model studies on TRIM59

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[17]. We confirmed TRIM59 as an immunohistochemistry marker able to detect low grade tumor in early tumorigenesis. We also demonstrated the correlation of tumorigenesis and tumor progression with TRIM59 up-regulation until high grade tumor.

TMA analysis of TRIM59 protein expression demonstrates that TRIM59 is a multiple tumor marker:

In the basic research previously using animal GEM-CaP models [17], we have disclosed that TRIM59 up-regulation is involved in two oncogene families and two signal pathways of SV40Tag/pRB/p53 and Ras/Raf/MEK/ERK. TRIM59 may function as an early signal transducer in Ras signal pathway with bridging genes in two oncogene pathways [17].

While it was rarely reported that SV40 Tag oncogene induced human cancer, Ras mutations are among the most frequent alterations in human cancers (see review [22]). We assume that TRIM59 as an early Ras signal pathway effector may possibly act as a multiple tumor marker.

We therefore further extended TRIM59 IHC studies to 35- multiple cancer TMA sections (42 tumors, 126 cores, Table 1). We tested different dilutions (1/300, 1/600, 1/1200, 1/5000) of TRIM59 antibody (see Supplemental Fig. 3). To further confirm the specificity and reliability of TRIM59 antibody in IHC staining, we compared IHC staining in 35 different tumor-TMA sections with positive (TRIM59 antibody at 1:1200 and 1:5000 dilutions) and negative controls (no antibody added, details see Supplemental Fig. 4). As summarized in Table 2, TRIM59 expression was significant and tissue-specifically up-regulated in most of these 35 tumors. When comparing the relative scores (both intensity and extent) in different tumors, the highest staining was observed in breast, lung, liver, skin (squamous cell carcinoma) and endometrial cancers.

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Further confirmation of TRIM59 as a tumor marker in patients with eight different tumors:

Since the 35 tumor-TMA contained only limited cases in each tumor type, we selected more cases (n=92) of eight different tumor types with different tumor grades, which all showed up-regulated expression of TRIM59. IHC staining of TRIM59 in eight tumors are shown: lung (n=4, Fig. 3A-C), breast (n=3, Fig. 3D-E), gastrointestinal (GI, n=2, Fig.3F-G), female genital tract (fGT, n=5, Fig.3H-J), bladder (n=44, Fig. 3K), prostate (n=27 from UWO, Fig. 3L), head and neck mucosal tumor (squamous cell carcinoma, SCC of mouth, tongue and larynx, n=4, Fig. 3M-O) and parotid gland (n=3, Fig. 3P-Q). Normal areas in lung, breast, colon, endometrial, bladder, larynx and parotid tissues showed very weak or completely negative staining (Fig. 3). Since some tumors (e.g. prostate) showed mostly cytoplasmic and no nuclear TRIM59-IHC staining, as a comparative study, we assessed their relative scores (combine both intensity and extent scores, see Materials and Methods). More tumors from kidney (RCC, n=75) and prostate cancer (n=27) were included as references and all were assessed by relative scores simultaneously, since we already analyzed a large cohort of these patients. Fig. 3R shows the comparison of the mean of IHC-TRIM59 relative scores. The highest relative scores were found in SCC of the parotid, mouth, larynx and tongue, followed by lung, breast and female genital tract cancers.

The comparison of relative scores on low and high grades separately was done (data not shown). Cases of grade 1 lung cancer (bronchoalveolar, adenocarcinoma, SCC and large cell carcinoma) and breast cancer (invasive lobular and invasive mammary carcinoma) all showed the strongest staining as compared with other tumors. In endometrial cancer, the TRIM59 relative scores were moderate in grade 1 and moderate to strong in grade 2. The three tumors of

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Table 2 Immunohistochemistry analysis of TRIM59 as multiple marker in 35 tumor TMA

Tumor Type	Patient number	Core number	Pathologic Grade	Cell Type	IHC staining	
					Cytoplasm staining (intensity)	Nuclear staining (extent)
Renal clear cell carcinoma,	2	6	2	Epithelial	moderate	-
			3		moderate	-
Adrenal gland cortical carcinoma	1	3	N/A	Epithelial	Moderate-strong	-
Squamous Cell Carcinoma, skin	2	6	WD	Epithelial	strong	-
			MD		moderate	-
Basal cell carcinoma, skin	2	6	N/A	Epithelial	moderate	-
					Moderate-strong	
Melanoma	1	3	N/A	Epithelial	weak	50% +
Endometroid adenocarcinoma	2	6	2	Epithelial	Moderate-strong	-
			1		Moderate-strong	-
Leiomyosarcoma	1	3	N/A	Mesenchymal	weak	-
Omentum serous adenocarcinoma,	1	3	WD	Epithelial	Weak-moderate	-
Ovary serous adenocarcinoma	1	3	N/A	Epithelial	Weak-moderate	-
Ovary clear cell carcinoma	1	3	PD	Epithelial	Moderate-strong	30% +
Cervix adenocarcinoma	1	3	WD-MD	Epithelial	Moderate-strong	-
Colon adenocarcinoma	1	3	Low grade	Epithelial	weak	-
Breast ductal adenocarcinoma	1	3	2/3	Epithelial	Moderate-strong	50% +
Bladder urothelial carcinoma	2	6	Low grade	Epithelial	weak	-
			2 (low grade)	Epithelial	moderate	-

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Stomach GIST	1	3		Epithelial	Weak-moderate	-
Esophagus adenocarcinoma	1	3	MD	Epithelial	0-weak	-
Thyroid, Papillary carcinoma	1	3	N/A	Epithelial	weak	-
Thyroid, medullary carcinoma	1	3	N/A	Epithelial	moderate	-
Pancreas adenocarcinoma,	2	6	2	Epithelial	Weak-moderate	-
			2		Moderate-strong	-
Pancreas endocrine tumor	1	3	N/A	Epithelial	strong	-
Lung SCC	1	3	PD	Epithelial	strong	-
Lung mesothelioma	1	3	MD-PD		strong	20% +
Lung adenocarcinoma	1	3	MD		moderate	50% +
Lung bronchoalveolar carcinoma	1	3	WD		Moderate-strong	50% +
Lung mesothelioma, biphasic	1	3	MD-PD		strong	20% +
Liver hepatocellular carcinoma (HCCa)	1	3	2/4	Epithelial	strong	-
Liver metastatic carcinoid	1	3	N/A	Epithelial	strong	-
Small bowel marginal zone lymphoma	1	3	N/A	Lymphocyte	0-weak	-
Lymph node, follicular lymphoma	1	3	1/3	Lymphocyte	0-weak	-
Lymph node, metastatic carcinoid	1	3	Low grade	Epithelial	strong	-
Spleen, Hodgkin's lymphoma	1	3	N/A	Lymphocyte	weak	-
Stomach, malt lymphoma	1	3	Low grade	Lymphocyte	0-weak	-
Thymus invasive thymoma	1	3	N/A	Epithelial	0-weak	-
Appendix, Goblet cell carcinoid	1	3	N/A	Epithelial	0	-

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squamous cell carcinoma (SCC) from mouth, tongue and larynx with different grades (Fig. 3M-O) also showed high relative scores (both intensity and extent).

As a comparative study (Fig. 3R, Table 2), we tested 44 bladder cancer cases with 38 low grade and 6 high grade tumors. The mean value of relative scores was 1.6, *i.e.* weak to moderate in bladder cancer cases . In 27 prostate cancer cases (from UWO only) tested, the relative scores of TRIM59 (cytoplasmic staining only) from PIN through Gleason scores of 10 were actually relatively weak (Fig. 3R), although in Gleason score 4, 6 and 8 were weak to moderate separately.

Thus far, we identified that TRIM59 up-regulation is "tumor-specific". First, we demonstrated the correlation of TRIM59 enhanced IHC signals with tumorigenesis and progression, which were statistically significant in this report with 291 cases and 37 tumor type analyses. Second, although TRIM59 is a normal gene involved in CDC (cell cycle division) regulation from G1 to S-phase and involved in DNA S-phase and cell growth [17], we demonstrated that in normal or non-tumor areas in all tested 37 different kinds of cancers, TRIM59 IHC staining signals were mostly negative or very low (Fig. 1, 2 3). By moderating antibody dilutions and testing various blocking reagents (Supplemental Fig. 4), we demonstrated that TRIM59 induces tumorigenesis/oncogenesis only when it is abnormally up-regulated.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Furthermore, we also confirmed that TRIM59 expression involved in multiple-tissue expression even in embryo development. We carried out IHC of mouse embryo sections by double-staining TRIM59. Cytokeratin (keratin), a family of proteins that are primarily found in epithelial cells was used as reference. Supplemental Fig. 5 illustrated confocal microscope

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images of IHC staining of TRIM59 in different organs/tissues of mouse embryo (14.5 d.p.c). TRIM59 was highly expressed in cytokeratin-expressing cells in the lung (first row), skin (second row), and kidney (not shown) of mouse embryos. TRIM59 staining in mouse embryos revealed the same pattern of the epithelium origin as in human tumors, which the TRIM59 gene were found highly up-regulated in those tumor types as well.

In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumor were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumors, for details see Table 2).

Given our previous experiments suggesting TRIM59 functions in the Ras pathway, we tested if TRIM59 up-regulation was correlated with the BRAF, an early signal effector Ras signal pathway (review see [22]). We selected 24 RCC patients, which previously were confirmed with up-regulation of TRIM59 expression. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing the total Raf protein and Raf-l Antibody (Phospho-Ser^{259} pAb) testing the activated phosphorylated B-Raf. Some of the serial slides were stained in parallel on each patient by different antibodies. As shown in Supplemental Fig. 6 A first two columns, in all 12 clear cell carcinoma of RCC samples, there were no or very weak B-Raf IHC signals in all three used Raf antibodies. In papillary RCC (Suppl Fig. 6 B) and chromophobe RCC samples (Suppl Fig. 6 C) (15 samples out of 24 samples or 62% of all RCC samples tested), there were higher IHC signals in all three antibodies used for staining in cancer areas specifically, showing higher intensity and extent than clear cell carcinoma (Suppl Fig. 6 A). Nuclear signals were found only by B-Raf P-Ser antibody in papillary and chromophobe tumors. Supplemental Table 1 summarizes the results. It is intriguing that in those TRIM59 up-regulated kidney cancers (RCC), neither total nor phosphorylated BRaf were detected in clear RCC (as a control), but were all highly positive in other two RCCs (papillary and

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chromophobe) tumors. We could not definitely confirm that TRIM59 was acting along the Ras pathway in all cases where it was detected.

Conclusions

This is the first report on a possible "ubiquitous" tumor marker. Ras mutations are among the most frequent alterations in human cancers that lead to approximately 30% of all human cancers with expression of constitutively active Ras proteins (review see [30, 31]). In this report, we have demonstrated that there are more than mutation issues of the Ras signal pathway in tumorigenesis and progression, since TRIM59 is up-regulated as a novel proto-oncogene in a variety of human cancers.

Detection and treatment of cancer at the earliest stage is critical for patient survival. This investigation demonstrated a novel TRIM59 gene as a multiple tumor marker for early diagnosis of tumorigenesis. As a multiple biomarker associated with epithelium origin (very rarely arisen from mesenchymal tissue, see Table 2) TRIM59 may be used as an EMT (epithelium – mesenchymal- transition) specific co-biomarker. The TRIM59 antibody may also be used for molecular targeted imaging as a new diagnostic marker, for example in the context of targeted microbubble ultrasound destruction technology [23], before the protocol of a serum TRIM59 test can be established.

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Acknowledgements: This work was supported by grants from the Ontario Institute of Cancer Research (07NOV-52), the Canadian Institute of Health Research (MOP -77684), NIH-NCI (2 U01 CA084296-06) and the Terry Fox Foundation.

Legend of Figures:

Fig. 1 Correlation of TRIM59 Immunhistochemistry (IHC) staining in prostate cancer cases in TMA (Tissue Microarray) assessed by the Gleason Grading system. All panels in **A** were shown hematoxylin staining, x20. Non-tumor: weak or negative, PIN: TRIM59 is located in the cytoplasm of the luminal cells (Intensity=2). Gleason score 3+3, TRIM59 is located in the cytoplasm of tumor cells (Intensity=2). Gleason score 3+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+4, TRIM59 is located in the cytoplasm of the tumor cells (Intensity=2). Gleason score 4+5, TRIM59 is located in the cytoplasm of the tumor cells (Intensity<1). Graph **B:** Correlation of TRIM59 protein levels by intensity with Gleason score in prostate cancer TMA analysis. Error Bars show mean (± SE).

Fig. 2 Immunohistochemistry analysis of TRIM59 expression in kidney cancer (RCC, Renal Cell Carcinoma) cases: correlation with tumor grade by intensity detecting early tumorigenesis. Five types of RCC with different grades were shown clear cell carcinoma (**A-D**), papillary RCC (**E-F**), chromophobe RCC (**G-I**), sarcomatoid RCC (**J**) and cystic RCC (**K-L**). **A**. Clear cell carcinoma, grade1, weak cytoplasmic staining in tumor cells, ≥ 50% nuclear staining, cytoplasmic staining in tumor cells (40X). **B.** Clear cell carcinoma, grade 2, moderate

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cytoplasmic staining in tumor cells, no nuclear staining (40X). **C.** Clear cell carcinoma, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **D.** Clear cell carcinoma, grade 4, moderate cytoplasmic staining and nuclear (%) staining (40X). **E.** Papillary RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining (40X). **F.** Papillary RCC, grade 3, strong cytoplasmic staining in tumor cells, no nuclear staining, normal cortex tissue is visible adjacent to tumor area (40X). **G.** Chromophobe RCC, grade 2, strong cytoplasmic staining in tumor cells, no nuclear staining. (40X). **H.** Chromophobe RCC, grade 3, strong cytoplasmic staining, no nuclear staining, (40X). **I.** Chromophobe RCC, grade 4, moderate cytoplasm staining and nuclear staining (40X). **J.** Sarcomatoid RCC, grade 4, weak cytoplasmic staining and nuclear staining (40X). **K.** Cystic RCC, grade 2, moderate cytoplasmic staining, no nuclear staining (40X). **L** Cystic RCC, grade 3, strong cytoplasmic staining, no nuclear staining (40X). Last two panels: normal kidney tissues (x20, x40). **M. Graph:** Correlation of TRIM59 protein levels by relative scores (both intensity and extent) with grade in RCC according to the Fuhrman nuclear grading system. Error Bars show mean (± SE).

Fig. 3 Comparison of TRIM59 expression as a multiple-cancer marker in eight types of tumors in breast, lung, parotid, gastrointestinal, female genital tract, bladder, head and neck mucosal tumor and prostate cancer. Negative TRIM59 staining in normal tissues was shown for each tumor respectively (20X). **(A)** Lung bronchoalveolar carcinoma, grade 1, strong cytoplasmic and nuclear staining of tumor cells, (40X). **(B)** Lung adenocarcinoma, grade 2, moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(C)** Lung large cell carcinoma, grade 4, moderate cytoplasmic and nuclear staining of tumor cells (40X). **(D)** Breast cancer, invasive lobular, low grade, moderate to strong cytoplasmic and nuclear staining of tumor cells (40X). **(E)** Breast cancer, invasive mammary (no specific type), high grade,

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moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(F)** Pancreas neuroendocrine carcinoma, poorly differentiated, strong cytoplasmic staining, no nuclear staining of tumor cells (40X). **(G)** Colon carcinoma, low grade, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(H)** Endometrial carcinoma, grade1, weak to moderate cytoplasmic staining of tumor cells, no nuclear staining (40X). **(I)** Endometrial carcinoma, grade 2, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(J)** Ovary, Endometrioid carcinoma, grade 1, weak cytoplasmic staining, no nuclear staining (40X). **(K)** Bladder urothelial carcinoma, low grade, moderate cytoplasmic staining, no nuclear staining (40X). **(L)** Prostate adenocarcinoma, Gleason score 8, moderate cytoplasmic staining, no nuclear staining (40X). **(M)** Floor of mouth, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(N)** Tongue cancer, SCC moderately to poorly differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(O)** Larynx, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (40X). **(P)** Parotid, mucoepidermoid carcinoma, low grade, strong cytoplasmic staining, no nuclear staining (40X). **(Q)** Parotid, metastatic SCC, poorly differentiated, moderate to strong cytoplasmic staining, no nuclear staining (40X). **(R)** Graphic comparison of relative scores (both intensity and extent) of TRIM59 IHC signals in eight different tumor types. fGT: Female genital tract. Error Bars show means (± SE) of the relative scores in all grades analyzed and compared.

Legend of Supplemental data

Supplemental Fig. 1 Characterization of TRIM59 antibodies constructed and used for human tissue studies.

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Summary of results: The antibody TRIM59#72 used for mouse and human studies were from C-terminal 150 a.a. peptide, which is TRIM59 specific, while N-terminal sequence of TRIM59 contains mostly the common function domains of the 70 member TRIM59 family. In Western blots on both mouse and human cell lysates, #72 recognized only one band, while #71 showed more bands. We demonstrated TRIM59#72 can be used for human tissues for immunohistochemistry and Western blots.

(A). Diagram of a novel TRIM family member TRIM59 showing the gene structure (upper line), functional domains of RBCC family. Two antibodies (TRIM59#71 and #72) are shown by arrows. **(B)**. 10 % SDS-PAGE showing purified GST-TRIM59 protein #71 and #72 protein (shown by arrows). GST protein showed as a control (26kda, by arrow). **(C-D)**. Characterization of polyclonal antibodies (#71 and #72) of mouse TRIM59 in mouse cell and tissue lysates. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. TRIM59 protein was shown by an arrow. (D) Characterization of polyclonal antibodies against mouse TRIM59. Similar identical Western blots prepared for total cellular IMAC column purified proteins and TRIM59#72 antibody-affinity column purification products from NIH3T3 cells, prostate tissue of wild type (WT), KIMAP and TGMAP prostate tumor samples. Using a TRIM59#72 affinity column purified TRIM59 protein, both TRIM59#71 and TRIM59#72 recognized the same major band (53kDa) in Western blots. **(E)** Western blots test of levels of TRIM59 protein in human prostate cancer cultured cells PC3, DU145, LNCaP and human kidney cell HEK293. A unique 53 kDa band was shown exactly as in mouse cell and tissue lysates **(C)**.

Supplemental Fig. 2 From **Vancouver Prostate Center** The LNCaP xenograft TMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immuno Deficiency) mice. The TMA is a tumor sample array comprising samples of time points (from day 1 to day35) of the xenografts taken before and after castration (CX) of the implanted LNCaP tumors in nude (SCID) mice.

Summary of results: In the nude mice implanted with xenograft induced by androgen-dependent human prostate cancer cell line LNCaP, TRIM59 is significantly up-regulated after

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10 days castration. After 35 days after the castration resistance stage, TRIM59 expression does up-regulated but not significantly (shown in graph 1, Fig. A). Similarly, in the SCID mice TRIM59 shows strong immunoreactivity but does not change in the all time points of castration (shown in graph 1, Fig. B).

Conclusion: TRIM59 is not androgen –responsive in castration test in TMA of human prostate cancer xenograft in nude and SCID mice test.

Supplemental Fig. 3 Test of the diluted TRIM59 antibody for moderate IHC staining

Summary of results: The following IHC staining showed results of the moderated TRIM59 IHC signals, when the first antibody TRIM59 diluted to 1:300, 1:600 1:1200 and 1:5000 –(figures not shown but resemble to 1:1200). We assessed these IHC results with the negative control (with out TRIM59 antibodies added) in five different tissues: ovary clear cell carcinoma, endometroid adenocarcinoma, lung squamous cell carcinoma, hepatocellular carcinoma and follicular lymphoma.

When comparing the first three columns with the negative control in Fig. s-3, 1:1200 dilution produced less background than concentrated TRIM59 antibodies. However, the TRIM59 IHC staining still remained stronger than the normal non-tumor areas even at 1:300 dilution. In order to detect weak TRIM59-IHC staining signals, we tried 1:300 dilution and we compared the staining signal with negative controls (no antibody was added).

We also tested additional avidin/biotin blocking agent (from Vector Lab) to decrease the background staining (data not shown), and similar results were obtained.

Legend of Figures: (A). Ovary, clear cell carcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1:300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining. **(B).** Endometroid adenocarcinoma, (20X), positive, moderate to strong cytoplasmic staining in 1/300 dilution, weak to moderate cytoplasmic staining in 1:600 and 1:1200 dilution of first antibody, negative control, no staining **(C).** Lung squamous cell carcinoma (20X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(D).** Hepatocellular carcinoma, (40X), positive, strong cytoplasmic staining in 1/300 and 1:600 dilution, moderate to strong cytoplasmic staining in 1:1200 dilution of first antibody, negative control, no staining. **(E).**Follicular

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lymphoma, (20X), positive, weak cytoplasmic staining in 1:300, very weak cytoplasmic staining in 1:600 dilution , no staining with 1:1200 dilution, negative control, no staining.

Supplemental Fig. 4 Comparison of TRIM59 protein levels in immunohistochemistry –(IHC) staining in human multiple tumor TMA —(tissue microarray) with negative controls

Summary of results:

We performed IHC staining of 35 tumors TMA with both TRIM59 antibody –(positive) and without any antibody –(negative) in serial consecutive slides for each tissue and in all three cores for each patient. The comparison was performed histologically by cell and tissue type, since there were some differences even in serial slides in some areas of TMA, tissues in each core, which were not identical, and in some cores tissues were missing during IHC process.

The following results in most (25 panels marked as #) of 35 tumor TMA confirmed results of Table 2 of the text on TRIM59-TMA.

Strong cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#3 Skin –(squamous cell carcinoma); #4 Skin –(basal cell carcinoma); #10 Breast ductal adenocarcinoma; #12 Thyroid medullary carcinoma; #13 Lung squamous cell carcinoma; #14. Lung bronchoalveolar carcinoma; #15 Lymph node with metastatic carcinoid; #16 Lung mesothelioma; #17 Hepatocellular carcinoma; #18 Metastatic carcinoid liver; #19 Endometrial carcinoma.

Moderate cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#1 Kidney (Clear cell carcinoma); #2 Adrenal gland (cortical carcinoma); #7 Omentum (serous adenocarcinoma); #9 Cervix adenocarcinoma.

Weak cytoplasmic /nuclear staining by TRIM59 antibody in tumor area featured less different with controls:

#5 Skin (melanoma); #6 Uterus (leiomyosarcoma); #8 Ovary, serous adenocarcinoma; #11 Bladder (urothelial carcinoma); #20 Small bowel, marginal zone lymphoma; #21 Spleen, Hodgkin's lymphoma; #22 Stomach, malt cell lymphoma; #23 Thymoma and # 25 Lymph node follicular lymphoma.

Negative cytoplasmic /nuclear staining by TRIM59 antibody in tumor area:

#24 Appendix (goblet cell carcinoid).

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Supplemental Fig. 5 Confocal microscope imaging of IHC analysis of TRIM59 expression in mouse embryo (14.5d.p.c). Patterns of multiple organ expression originated from epithelial cells were shown in the lung (first row) and skin (second row).

Supplemental Fig. 6 Test of Ras activation by B-Raf antibodies in kidney cancer tumors correlated with up-regulation of TRIM59. First column: Antibody Braf **(A)** in Clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (**B**), strong cytoplasmic staining, no nuclear staining, (40X); in Chromophobe RCC (**C**), strong cytoplasmic staining, no nuclear staining, (40X). In the second row: antibody to Raf 1, in clear cell carcinoma, weak cytoplasmic staining, no nuclear staining (40X); in Papillary RCC (20X,40X), strong cytoplasmic staining, no nuclear staining; in chromophobe RCC, strong cytoplasmic staining, no nuclear staining (40X). Third row: Antibody to Raf 1 phosphor Ser ^{259}, in clear cell carcinoma, weak cytoplasmic and nuclear staining (40X); in Papillary RCC, strong cytoplasmic and nuclear staining (40X), in chromophobe RCC, strong cytoplasmic and nuclear staining, (40X). Arrows indicated nuclear staining.

Supplemental Table 1 Correlation of TRIM59 up-regulation with B-Raf hyper-phosphorylation

Contributorship Statement

All authors havecontributed significantly to the conception and design of the manuscript,interpretation of data, drafting the article and revisions for importantintellectual content and final approval of the version to be published. MM (pathologist), VK and FV worked on dataacquisition, analysis and interpretation of human tumor samples. JWX conceived and designed the study. JWX is the guarantor of the study.

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Funding Statement : this research received no specific funding.

Competing Interest: there are no competing interests.

Data Sharing Statement:

Extra data is available by emailing correspondent author Dr. Jim W Xuan.

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