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

Vida Khatamianfar,1 Fatma Valiyeva,1 Paul S Rennie,2 Wei-yang Lu,3 Burton B Yang,4 Glenn S Bauman,5 Madeleine Moussa,6 Jim W Xuan1

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 multitumour marker detecting early tumorigenesis by immunohistochemistry (IHC).

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

Results: TRIM59 upregulation specifically in tumour area was determined by IHC in 291 cases of 37 tumour types. To demonstrate that TRIM59 upregulation is ‘tumour-specific’, we characterised 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 upregulation in prostate and kidney cancers was detectable in both intensity and extent in early tumorigenesis of 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 tumour types confirmed and repeated murine CaP model studies. Enhanced TRIM59 expression was identified in most of the 37 different tumours, while the highest intensities were in lung, breast, liver, skin, tongue and mouth (squamous cell cancer) and endometrial cancers. Multiple tumour upregulation was further confirmed by comparing relative scores of TRIM59 IHC signals in eight tumours with a larger patient population; and by a mouse whole-mount embryo (14.5 days post conception) test on the origin of TRIM59 upregulation in epithelial cells.

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

INTRODUCTION

The TRIM (TRIpartite Motif) family is an evolutionarily conserved gene family implicated in a number of critical processes including...
**STRENGTHS AND LIMITATIONS OF THIS STUDY**

- TRIM59 may be used as a novel multiple tumor marker for immunohistochemical detecting early tumorigenesis and could direct a novel strategy for molecular-targeted diagnosis and therapy of cancer.
- The work was mostly on human tumor samples by immunohistochemistry.

**MATERIALS AND METHODS**

**Patient selection**

All patient samples were acquired as part of REB (Research Ethics Board) approved protocols at the Western University (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**

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

**Automated image, acquisition and analysis on immunohistochemical staining of CaP-TMA**

Immunohistochemical staining was conducted by Ventana autostainer model Discover XT (Ventana Medical System, Tucson, Arizona, USA) with enzyme-labelled biotin streptavidin system and solvent-resistant DAB Map kit (Ventana Medical System). TMA was scanned by Bliss Digital imaging system using ×20 objective (Bacus Laboratories Inc., Centre Valley, Pennsylvania, USA) and stored in the Prostate Centre Saver (http://bliss.prostatecentre.com). A value on a four-point scale assigned to each core.

**Multiple tumour TMA construction**

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

**Histopathological analysis**

All cases from 37 tumour types were graded according to standardized histopathology grading systems by MM (pathologist) and VK (MD fellow).

**Immunohistochemistry and results evaluation**

Standard ABC (Avidin Biotin Complex) protocol was performed as previously reported. Two kinds of blocking reagents were used: Power Block (Universal Blocking Reagent; BioGenex, San Ramon, California, USA) and Avidin-biotin blocking reagent kit (Vector Labs, Burlingame, California, USA). All B-Raf antibodies were from GenScript (Piscataway, New Jersey, USA): B-Raf

---

**Reference**


---

**Note**

This is a sample text for a scientific paper discussing the use of TRIM59 as a novel multiple cancer marker. It describes the strengths and limitations of the study, materials and methods, and the clinical application of TRIM59 in prostate cancer research.
antibody (monoclonal antibody, used 1:500 dilution), Raf 1 (polyconal, 1:50) and Raf -1 (polyclonal, Phospho-Ser259 1:50).

TRIM59 IHC staining signals were assessed by intensity for cytoplasmic staining and extent to assess the percentage of nuclear staining as previously reported.\(^2\) \(^3\) Since in some tumours TRIM59 showed only cytoplasmic staining, for the purpose of comparing in different tumours, we used a combined relative score system based on both intensity and extent as follows: 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 MM (pathologist) and VK (MD fellow).

### Construction and characterisation of TRIM59 antibody

Online supplementary figure S1 provided details, which combined online supplementary materials of\(^1\) and more informations. Online supplementary figure S1A showed that the antibody TRIM59#72 used for mouse and human studies was prepared from C-terminal 136-amino acid peptide (as recombinant GST-fusion protein, see online supplementary figure S1B) 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 the TRIM family.\(^1\) As shown in online supplementary figure S1C, western blots on both mouse and human cell lysates, #72 recognised only one band, while #71 showed multiple bands. We also demonstrated that N-terminal sequence-specific antibody TRIM59#71 can recognise purified proteins from C-terminal sequence-specific, TRIM59#72 Ab affinity column (the same 53 kDa protein, see online supplementary figure S1D). 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 (IHC) and western blots (see online supplementary figure S1E).

### Table 1 Patient list selected in this study

<table>
<thead>
<tr>
<th>Organ</th>
<th>Patient number</th>
<th>Tumour type</th>
<th>Tumour grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 Tumour-TMA</td>
<td>42</td>
<td>Description in table 2</td>
<td></td>
</tr>
<tr>
<td>Prostate TMA</td>
<td>105</td>
<td>Adenocarcinoma</td>
<td>BPH 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PIN 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gleason score 4 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gleason score 6 31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gleason score 7 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gleason score 8 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gleason score 9–10 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stroma 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absent cores 6</td>
</tr>
<tr>
<td>Kidney</td>
<td>75</td>
<td>Clear cell carcinoma 43</td>
<td>Grade 1 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Papillary RCC 11</td>
<td>Grade 2 38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromophobe RCC 13</td>
<td>Grade 3 28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cystic RCC 6</td>
<td>Grade 4 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sarcomatoid RCC 2</td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>44</td>
<td>Urothelial carcinoma</td>
<td>Low grade 38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High grade 6</td>
</tr>
<tr>
<td>Lung</td>
<td>4</td>
<td>Bronchoalveolar carcinoma 1</td>
<td>Grade 1 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenocarcinoma 1</td>
<td>Grade 2 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large cell carcinoma 1</td>
<td>Grade 3 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squamous cell carcinoma 1</td>
<td>Grade 4 1</td>
</tr>
<tr>
<td>Breast</td>
<td>3</td>
<td>Invasive lobular carcinoma 1</td>
<td>Grade 1 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Invasive mammary carcinoma 2</td>
<td>Grade 3 2</td>
</tr>
<tr>
<td>Female genital tract</td>
<td>5</td>
<td>Endometrial carcinoma 4</td>
<td>Grade 1 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovary, endometrioid carcinoma 1</td>
<td>Grade 2 1</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>2</td>
<td>Colon carcinoma 1</td>
<td>Low grade 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pancreas neuroendocrine carcinoma 1</td>
<td>High grade 1</td>
</tr>
<tr>
<td>Parotid</td>
<td>3</td>
<td>Mucoepidermoid carcinoma 1</td>
<td>Low grade 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metastatic SCC 1</td>
<td>High grade 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metastatic neuroendocrine carcinoma 1</td>
<td></td>
</tr>
<tr>
<td>Mouth, tongue and larynx</td>
<td>4</td>
<td>Squamous cell carcinoma 4</td>
<td>Moderately differentiated 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Moderately to poorly differentiated 1</td>
</tr>
<tr>
<td>Total</td>
<td>291</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RCC, renal cell carcinoma; SCC, squamous cell carcinoma; TMA, tissue microarray.
Confocal microscope imaging of immunostaining of mouse embryos 14.5 days post conception for TRIM59

The mouse embryos were prepared according to our previous reports.\textsuperscript{19} 20 The anti-rabbit secondary antibody was conjugated to Cy3 (1:200, Jackson Immuno Research, West Grove, Pennsylvania, USA). Sections were double-stained with pan-cytokeratin antibody with FITC (Sigma, St. Louis, Missouri, USA), and the stained mouse embryo sections were visualised using a Carl Zeiss (Oberkochen, Germany) confocal microscope by the LSM Image program.

**Statistical analysis**

Student’s t tests and one-way analysis of variance were used by programs of Microsoft Excel 2007 or SPSS 10 to

---

**Table 2** Immunohistochemistry (IHC) analysis of TRIM59 as multiple marker in 35 tumour tissue microarray

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Patient number</th>
<th>Core number</th>
<th>Pathological grade</th>
<th>Cell type</th>
<th>IHC staining</th>
<th>Cytoplasm staining (intensity)</th>
<th>Nuclear staining (extent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal clear cell carcinoma,</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>Epithelial</td>
<td>moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adrenal gland cortical carcinoma (SCC), skin</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>2</td>
<td>6</td>
<td>WD</td>
<td>Epithelial</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Basal cell carcinoma, skin</td>
<td>2</td>
<td>6</td>
<td>MD</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>Weak</td>
<td>50%+</td>
<td>–</td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Mesenchymal</td>
<td>Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Omentum serous adenocarcinoma</td>
<td>1</td>
<td>3</td>
<td>WD</td>
<td>Epithelial</td>
<td>Weak–moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ovary clear cell carcinoma</td>
<td>1</td>
<td>3</td>
<td>PD</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>30%+</td>
<td>–</td>
</tr>
<tr>
<td>Cervix adenocarcinoma</td>
<td>1</td>
<td>3</td>
<td>WD-MD</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>1</td>
<td>3</td>
<td>Low grade</td>
<td>Epithelial</td>
<td>Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Breast ductal adenocarcinoma</td>
<td>1</td>
<td>3</td>
<td>2/3</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>50%+</td>
<td>–</td>
</tr>
<tr>
<td>Bladder urothelial carcinoma</td>
<td>2</td>
<td>6</td>
<td>Low grade</td>
<td>Epithelial</td>
<td>Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stomach GIST</td>
<td>1</td>
<td>3</td>
<td>MD</td>
<td>Epithelial</td>
<td>Weak–moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Esophagus adenocarcinoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thyroid, papillary carcinoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thyroid, medullary carcinoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pancreas adenocarcinoma</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>Epithelial</td>
<td>Weak–moderate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pancreas endocrine tumour</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lung SCC</td>
<td>1</td>
<td>3</td>
<td>PD</td>
<td>Epithelial</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lung mesothelioma</td>
<td>1</td>
<td>3</td>
<td>MD-PD</td>
<td>Epithelial</td>
<td>Strong</td>
<td>20%+</td>
<td>–</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>1</td>
<td>3</td>
<td>MD</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>50%+</td>
<td>–</td>
</tr>
<tr>
<td>Lung bronchoalveolar carcinoma</td>
<td>1</td>
<td>3</td>
<td>WD</td>
<td>Epithelial</td>
<td>Moderate</td>
<td>50%+</td>
<td>–</td>
</tr>
<tr>
<td>Lung mesothelioma, biphasic</td>
<td>1</td>
<td>3</td>
<td>MD-PD</td>
<td>Epithelial</td>
<td>Strong</td>
<td>20%+</td>
<td>–</td>
</tr>
<tr>
<td>Liver hepatocellular carcinoma</td>
<td>1</td>
<td>3</td>
<td>2/4</td>
<td>Epithelial</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Liver metastatic carcinoid</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Small bowel marginal zone lymphoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Lymphocyte</td>
<td>0–Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lymph node, follicular lymphoma</td>
<td>1</td>
<td>3</td>
<td>1/3</td>
<td>Lymphocyte</td>
<td>0–Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lymph node, metastatic lymphoid</td>
<td>1</td>
<td>3</td>
<td>Low grade</td>
<td>Lymphocyte</td>
<td>Strong</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Spleen, Hodgkin’s lymphoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Lymphocyte</td>
<td>Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stomach, malt lymphoma</td>
<td>1</td>
<td>3</td>
<td>Low grade</td>
<td>Lymphocyte</td>
<td>0–Weak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thymus invasive thymoma</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>0–Vweak</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Appendix, goblet cell carcinoid</td>
<td>1</td>
<td>3</td>
<td>N/A</td>
<td>Epithelial</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

GIST, gastrointestinal stromal tumour.
RESULTS AND DISCUSSION

TRIM59 upregulation in human prostate cancer TMA: 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. SV40 Tag is essentially only required for the initiation of tumorigenesis, that is, the ‘hit-and-run’ effect, in GEM-CaP, but not for the tumour 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 coactivators. Once this process is initiated, the signal transduction will continue on, even without the initiation effectors.

In GEM-CaP models, the TRIM59 protein upregulation correlated with tumorigenesis and progression, and downregulated in the high-grade CaP by IHC. We assume this ‘tumorigenesis-associated’ effect of TRIM59 may apply to human cancer studies.

We first characterised that TRIM59 antibody (#72) can cross-react with and recognise specifically human TRIM59 counterpart (for details see online supplementary figure S1). 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 (figure 1A), which is different from rapid tumour progression mouse CaP models. The intensity (score=2) in PIN (n=4, figure 1A) was higher than in non-tumour area (normal and benign prostatic hyperplasia). 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, figure 1A), TRIM59-IHC signals were lower. As shown in a graph of figure 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 figure 1B), and decreased in high-grade CaP (Gleason score 9–10) with p=0.018, which is similar to GEMs.

TRIM59 upregulation in human renal cell carcinoma patients: correlation of tumorigenesis and tumour 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. Online supplementary figure S2 showed this result in detail of IHC.

Figure 1 Correlation of TRIM59 immunohistochemistry (IHC) staining in prostate cancer cases in tissue microarray (TMA) assessed by the Gleason grading system. All panels in (A) were shown haematoxylin staining, ×20. Non-tumour: weak or negative, prostate intraepithelial neoplasia (PIN): TRIM59 is located in the cytoplasm of the luminal cells (intensity = 2). Gleason score 3 + 3, TRIM59 is located in the cytoplasm of tumour cells (intensity = 2). Gleason score 3 + 4, TRIM59 is located in the cytoplasm of the tumour cells (intensity = 2). Gleason score 4 + 4, TRIM59 is located in the cytoplasm of the tumour cells (intensity = 2). Gleason score 4 + 5, TRIM59 is located in the cytoplasm of the tumour 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).
test by TRIM59 antibody #72 on a TMA of the LNCaP human CaP xenografts in nude/severe combined immuno deficiency castrated mice.

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 tumours: 43 clear cell carcinoma (representative IHC figures shown in figure 2A–D), 11 papillary RCC (figure 2E,F), 13 chromophobe RCC (figure 2G–I), 2 sarcomatoid RCC (figure 2J), and 6 cystic RCC (figure 2K,L). RCC cases analysed with Fuhrman grade 1–4 were 4, 38, 28 and 5, respectively. TRIM59-IHC staining in normal area including proximal tubules was negative (figure 2, last row). Background staining was eliminated by testing antibody dilutions (1 : 100, 200, 500, 1200 till 5000) while tumour-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 tumour areas in RCC (figure 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 (figure 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 figure 2M. TRIM59 IHC signals were increased with tumour progression from grade 1–5.

![Figure 2](image-url) Immunohistochemistry analysis of TRIM59 expression in kidney cancer (RCC, renal cell carcinoma) cases: correlation with tumour grade by intensity detecting early tumorigenesis. Five types of RCC with different grades were shown clear cell carcinoma (A–D), papillary RCC (E and F), chromophobe RCC (G–I), sarcomatoid RCC (J) and cystic RCC (K and L). (A) Clear cell carcinoma, grade 1, weak cytoplasmic staining in tumour cells, ≥50% nuclear staining, cytoplasmic staining in tumour cells (×40). (B) Clear cell carcinoma, grade 2, moderate cytoplasmic staining in tumour cells, no nuclear staining (×40). (C) Clear cell carcinoma, grade 3, strong cytoplasmic staining in tumour cells, no nuclear staining (×40). (D) Clear cell carcinoma, grade 4, moderate cytoplasmic staining and nuclear (% ) staining (×40). (E) Papillary RCC, grade 2, strong cytoplasmic staining in tumour cells, no nuclear staining (×40). (F) Papillary RCC, grade 3, strong cytoplasmic staining in tumour cells, no nuclear staining, normal cortex tissue is visible adjacent to tumour area (×40). (G) Chromophobe RCC, grade 2, strong cytoplasmic staining in tumour cells, no nuclear staining. (×40). (H) Chromophobe RCC, grade 3, strong cytoplasmic staining, no nuclear staining, (×40). (I) Chromophobe RCC, grade 4, moderate cytoplasmic staining and nuclear staining (×40). (J) Sarcomatoid RCC, grade 4, weak cytoplasmic staining and nuclear staining (×40). (K) Cystic RCC, grade 2, moderate cytoplasmic staining, no nuclear staining (×40). (L) Cystic RCC, grade 3, strong cytoplasmic staining, no nuclear staining (×40). 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).
TRIM59, a novel multiple cancer marker for tumorigenesis

We have disclosed that TRIM59 upregulation is correlated with tumorigenesis. We also demonstrated the correlation of TRIM59 with tumorigenesis and tumour progression with TRIM59 upregulation until high-grade tumour.

Therefore, by systematic IHC studies in CaP (88 patients, figure 1) and kidney cancer (75 patients, figure 2), we almost exactly repeated results from our mouse model studies on TRIM59. We confirmed TRIM59 as an IHC marker able to detect low-grade tumour in early tumorigenesis. We also demonstrated the correlation of tumorigenesis and tumour progression with TRIM59 upregulation until high-grade tumour.

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

In the basic research previously using animal GEM-CaP models, we have disclosed that TRIM59 upregulation 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.

While it was rarely reported that SV40 Tag oncogene induced human cancer, Ras mutations are among the most frequent alterations in human cancers (for a review see ref. 28). We assume that TRIM59 as an early Ras signal pathway effector may possibly act as a multiple tumour marker.

We therefore further extended TRIM59 IHC studies to 35 multiple cancer TMA sections (42 tumours, 126 cores, table 1). We tested different dilutions (1/300, 1/600, 1/1200 and 1/5000) of TRIM59 antibody (see online supplementary figure S3). To further confirm the specificity and reliability of TRIM59 antibody in IHC staining, we compared IHC staining in 35 different tumour-TMA sections with positive (TRIM59 antibody at 1:1200 and 1:5000 dilutions) and negative controls (no antibody added, for details see online supplementary figure S4).

As summarised in table 2, TRIM59 expression was significant and tissue-specifically upregulated in most of these 35 tumours. When comparing the relative scores (both intensity and extent) in different tumours, the highest staining was observed in breast, lung, liver, skin (squamous cell carcinoma) and endometrial cancers.

Further confirmation of TRIM59 as a tumour marker in patients with eight different tumours

Since the 35 tumour-TMA contained only limited cases in each tumour type, we selected more cases (n=2) of eight different tumour types with different tumour grades, which all showed upregulated expression of TRIM59. IHC staining of TRIM59 in eight tumours are shown: lung (n=4, figure 3A–C), breast (n=3, figure 3D,E), gastrointestinal (n=2, figure 3FG), female genital tract (n=5, figure 3H–J), bladder (n=4, figure 3K), prostate (n=27 from UWO, figure 3L), head and neck mucosal tumour (squamous cell carcinoma, SCC of mouth, tongue and larynx, n=4, figure 3M–O) and parotid gland (n=3, figure 3PQ). Normal areas in lung, breast, colon, endometrial, bladder, larynx and parotid tissues showed very weak or completely negative staining (figure 3).

Since some tumours (eg, 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 tumours 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 analysed a large cohort of these patients. Figure 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, squamous cell carcinoma (SCC) and large cell carcinoma) and breast cancer (invasive lobular and invasive mammary carcinoma) all showed the strongest staining as compared with other tumours. In endometrial cancer, the TRIM59 relative scores were moderate in grade 1 and moderate to strong in grade 2. The three tumours of SCC from mouth, tongue and larynx with different grades (figure 3M–O) also showed high relative scores (both intensity and extent).

As a comparative study (figure 3R, table 2), we tested 44 bladder cancer cases with 38 low-grade and 6 high-grade tumours. The mean value of relative scores was 1.6, that is, 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 (figure 3R), although in Gleason score 4, 6 and 8 were weak to moderate separately.

So far, we identified that TRIM59 upregulation is ‘tumour-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 tumour 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, we demonstrated that in normal or non-tumour areas in all tested 37 different kinds of cancers, TRIM59 IHC staining signals were mostly negative or very low (figures 1–3). By moderating antibody dilutions and testing various blocking reagents (see online supplementary figure S4), we demonstrated that TRIM59 induces tumorigenesis/oncogenesis only when it is abnormally upregulated.
In most of human cancers tested in this clinical IHC study, strong TRIM59 expression in tumour were identified in epithelial cancers: lung, breast, skin, which all associated with epithelium originations (very rare from mesenchymal tumours, 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. Online supplementary figure S5 illustrated confocal microscope images of IHC staining of TRIM59 in different organs/tissues of mouse embryo (14.5 days postconception). TRIM59 was highly expressed in cytotkeratin-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 tumours, which the TRIM59 gene were found highly upregulated in those tumour types as well (see table 2).

Given our previous experiments suggesting TRIM59 functions in the Ras pathway, we tested if TRIM59 upregulation was correlated with the BRAF, an early signal effector Ras signal pathway (for a review see ref. 22). We selected 24 RCC patients, which previously were confirmed with upregulation of TRIM59 expression. Three antibodies were used: B-Raf Antibody (mAb) and Raf-l Antibody (Ab-259) testing the total Raf protein and

Figure 3  Comparison of TRIM59 expression as a multiple-cancer marker in eight types of tumours in breast, lung, parotid, gastrointestinal, female genital tract, bladder, head and neck mucosal tumour and prostate cancer. Negative TRIM59 staining in normal tissues was shown for each tumour, respectively (×20). (A) Lung bronchoalveolar carcinoma, grade 1, strong cytoplasmic and nuclear staining of tumour cells, (×40). (B) Lung adenocarcinoma, grade 2, moderate cytoplasmic staining of tumour cells, no nuclear staining (×40). (C) Lung large cell carcinoma, grade 4, moderate cytoplasmic and nuclear staining of tumour cells (×40). (D) Breast cancer, invasive lobular, low-grade, moderate to strong cytoplasmic and nuclear staining of tumour cells (×40). (E) Breast cancer, invasive mammary (no specific type), high-grade, moderate cytoplasmic staining of tumour cells, no nuclear staining (×40). (F) Pancreas neuroendocrine carcinoma, poorly differentiated, strong cytoplasmic staining, no nuclear staining of tumour cells (×40). (G) Colon carcinoma, low-grade, weak-to-moderate cytoplasmic staining of tumour cells, no nuclear staining (×40). (H) Endometrial carcinoma, grade 1, weak-to-moderate cytoplasmic staining of tumour cells, no nuclear staining (×40). (I) Endometrial carcinoma, grade 2, moderate-to-strong cytoplasmic staining, no nuclear staining (×40). (J) Ovary, endometrioid carcinoma, grade 1, weak cytoplasmic staining, no nuclear staining (×40). (K) Bladder urothelial carcinoma, low-grade, moderate cytoplasmic staining, no nuclear staining (×40). (L) Prostate adenocarcinoma, Gleason score 8, moderate cytoplasmic staining, no nuclear staining (×40). (M) Floor of mouth, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (×40). (N) Tongue cancer, SCC moderately to poorly differentiated, strong cytoplasmic staining, no nuclear staining (×40). (O) Larynx, SCC moderately differentiated, strong cytoplasmic staining, no nuclear staining (×40). (P) Parotid, mucoepidermoid carcinoma, low-grade, strong cytoplasmic staining, no nuclear staining (×40). (Q) Parotid, metastatic SCC, poorly differentiated, moderate-to-strong cytoplasmic staining, no nuclear staining (×40). (R) Graphic comparison of relative scores (both intensity and extent) of TRIM59 IHC signals in eight different tumour types. IGT, female genital tract. Error bars show means (±SE) of the relative scores in all grades analysed and compared.
Raf-1 Antibody (Phospho-Ser259 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 online supplementary figure S6A 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 (see online supplementary figure S6B) and chromophobe RCC samples (see online supplementary figure S6C) (15 samples 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 (see online supplementary figure S6A). Nuclear signals were found only by B-Raf P-Ser antibody in papillary and chromophobe tumours. Online supplementary table S1 summarises the results.

It is intriguing that in those TRIM59 upregulated 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 chromophobe) tumours. 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’ tumour 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 (for a review see refs. 22–30). 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 upregulated as a novel proto-oncogene in a variety of human cancers.

Detection and treatment of cancer at the earliest stage are critical for patient survival. This investigation demonstrated a novel TRIM59 gene as a multiple tumour 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 cobiomarker. 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, before the protocol of a serum TRIM59 test can be established.

**Author affiliations**

1Department of Surgery, Lawson Health Research Institute, Western University, London, Ontario, Canada
2Department of Surgery, University of British Columbia, Vancouver, British Columbia, Canada
3Department of Physiology, Robarts Research Institute, Western University, London, Ontario, Canada
4Department of Laboratory Medicine and Pathobiology, Sunnybrook Research Institute, University of Toronto, Toronto, Ontario, Canada
5London Regional Cancer Program, Western University, London, Ontario, Canada
6Department of Pathology, Western University, London, Ontario, Canada

**Acknowledgements** This work was supported by grants from the Ontario Institute of Cancer Research (O7NOV-52), the Canadian Institute of Health.
TRIM59, a novel multiple cancer marker for tumorigenesis

Research (MOP-77684), NIH-NCI (2 U01 CA084296-06) and the Terry Fox Foundation.

Contributor All authors have contributed significantly to the conception and design of the manuscript, interpretation of data, drafting the article and revisions for important intellectual content and final approval of the version to be published. MM (pathologist), VK and FV worked on data acquisition, analysis and interpretation of human tumour samples. JWX conceived and designed the study. JWX is the guarantor of the study.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

Data Sharing Statement Extra data are available by emailing correspondent author JWX.

REFERENCES

Supplementary Fig.2 **IHC evaluation of androgen responsiveness of TRIM59 in the LNCaP xenograft TTMA.**

The LNCaP xenograft TMMA (tissue microarray) were prepared from xenografts generated by an androgen-dependent human prostate cancer cell line LNCaP in nude SCID (Severe Combined Immunodeficiency) mice. The TMAA is a tumor sample array comprising samples of time points (from day 1 to day 35) 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. BB).

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

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 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

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

Dilutions of TRIM59 first antibody

<table>
<thead>
<tr>
<th>1/600</th>
<th>1/1200</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/300</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A. Ovary, clear cell carcinoma
B. Endometroid adenocarcinoma

C. Lung SCC

D. Hepatocellular carcinoma

E. Follicular lymphoma

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)

**Legend of panels:**

1. 1. Kidney (Clear cell carcinoma), positive IHC for TRIM59 expression moderate cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).
2. 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. 3. Skin (squamous cell carcinoma), positive IHC for TRIM59 expression strong cytoplasmic staining and no nuclear staining; negative control no staining (20X, 40X).
4. 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).

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).
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
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
21- Spleen Hodgkin lymphoma, positive (weak)  
Negative

22- Stomach malt lymphoma, positive (weak)  
Negative

23- Thymoma, positive (weak)  
Negative

24- Appendix, Goblet cell carcinoid, positive (no staining)  
Negative

25- Follicular lymphoma, positive (weak)  
Negative
Confocal microscope imaging of immunohistochemistry of TRIM59 protein in mouse embryo (14.5d.p.c).

Fig. 5

- **Lung**
  - Cytokeratin
  - TRIM59

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

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

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

Antibody: B-Raf for total B-raf

Antibody: Raf 1 for total Raf

Antibody: Phosphor- Ser- 259 Raf 1

X20  X40  X20  X40
<table>
<thead>
<tr>
<th>Antibody used</th>
<th>RCC types</th>
<th>Patient number</th>
<th>Intensity score</th>
<th>Nuclear staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Raf (total B-raf)</td>
<td>Clear cell Carcinoma</td>
<td>6</td>
<td>0-1</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Chromophobe</td>
<td>3</td>
<td>2-3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>papillary</td>
<td>3</td>
<td>2-3</td>
<td>No</td>
</tr>
<tr>
<td>Raf-1 (total B-raf)</td>
<td>Clear cell Carcinoma</td>
<td>3</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Chromophobe</td>
<td>2</td>
<td>2-3</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>papillary</td>
<td>1</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>B-Raf p-Ser 259</td>
<td>Clear cell Carcinoma</td>
<td>3</td>
<td>0-1</td>
<td>No</td>
</tr>
<tr>
<td>(phosphorylated B-Raf)</td>
<td>Chromophobe</td>
<td>1</td>
<td>2-3</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>papillary</td>
<td>2</td>
<td>2-3</td>
<td>50%</td>
</tr>
</tbody>
</table>

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 MEKMAP 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-1 Antibody (Ab-259) testing for total Raf protein and Raf-1 Antibody (Phospho-Ser 259 pAb) testing activated phosphorylated B-Raf. Some of serial slides were stained parallelly on each patient by different antibodies.
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.

(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).