The relationship between epithelial and stromal TRIM28 expression predicts survival in colorectal cancer patients

Running title: TRIM28 in Colorectal Cancer

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Abstract

Background and Aim: TRIM28 is a multi-domain nuclear protein with pleotropic effects in both normal and tumor cells. It is implicated in both tumor-promoting and tumor-suppressing functions in cancer. The aim of this study was to investigate the expression of TRIM28 in colorectal cancer and evaluated its role as a prognostic marker in colorectal cancer.

Methods: Immunohistological staining of TRIM28 was evaluated in tissue microarrays constructed from 137 colorectal cancer patients. Kaplan-Meier analysis and Cox proportional hazard modeling were used to assess overall survival (OS) and recurrence-free survival (RFS).

Results: Immunohistological staining revealed strong overexpression of TRIM28 in tumor epithelium in 57 out of 137 (42%) colorectal cancer cases. The overexpression was not associated with patient survival; however, it correlated significantly with p53 expression in matched cases (p=0.0168; Spearman rank test). A high epithelial to stromal TRIM28 expression ratio was associated with shorter OS (p=0.033; log-rank test) and RFS (p=0.043; log-rank test). Cox multivariate analysis showed that the epithelial to stromal TRIM28 expression ratio was an independent predictor of OS (hazard ratio (HR)=2.136; 95% confidence interval (CI) 1.015-4.498, p=0.046) and RFS (HR=2.100; CI 1.052-4.191, p=0.035).

Conclusion: Our results show a differential expression of TRIM28 in stromal fibroblasts and malignant epithelial cells in colorectal cancer. The epithelial to stromal TRIM28 expression ratio is an independent predictor of survival, suggesting that the pathophysiological role of TRIM28 in carcinogenesis is dependent on its expression levels and cell type within the tumor microenvironment.

Keywords: TRIM28; colorectal cancer; epithelium; stroma; prognosis, tumor antigen

Introduction

The interactions between tumor cells and the surrounding stroma play a significant role in the progression of cancer.[1] Tumor cells generally modulate their stromal microenvironment by producing stroma-modulating growth factors, which disrupt normal tissue homeostasis and activate surrounding stromal cell types, such as fibroblasts, smooth-muscle cells and adipocytes. Fibroblasts in particular can affect the stromal microenvironment leading to an increase in tumor aggressiveness.[2] Interestingly, TRIM28 forms part of a ternary complex with the fibroblast-specific protein (FSP1) and CArG box–binding factor–A (CBF-A) which controls the expression of a wide spectrum of epithelial-mesenchymal transition (EMT) responsive genes, enabling the transformation of epithelial cells into a spindle-shaped fibroblast morphology.[3, 4]

TRIM28, also known as KAP1 and TIF1β, is a universal co-repressor, mediating transcriptional control through interaction with Krüppel associated box (KRAB) zinc finger proteins.[5-7] TRIM28 is an essential partner in several multiple-protein complexes and is involved in a wide range of biological processes.[8, 9] It belongs to the Tripartite Motif (TRIM) family of proteins, which have been implicated in many pathological conditions, including developmental disorders, neurodegenerative diseases, viral infections, innate immunity and cancer.[10, 11] Importantly, the multi-domain structure of the protein infers a host of interactions through ring-finger domains, zinc binding motifs, and coiled-coil regions. [10, 12]

TRIM28 has both tumor-promoting and tumor-suppressing functions. The up-regulation of the TRIM28 gene has been shown in gastric cancer and is associated with poor prognosis.[13] The tumor-promoting role of TRIM28 is associated with p53-dependent apoptosis. The tumor-suppressor p53 has a major impact on carcinogenesis and it accumulates in cells in response to DNA damage, leading to DNA repair, cell cycle arrest or apoptosis.[14] These tumor-suppressor functions are inactivated by TRIM28, which interrupts the acetylation of key DNA-binding domains within the p53 protein.[15] TRIM28 mediates such p53 inactivation

through interactions with the oncogenic protein MDM2[16, 17], cancer testis antigens MAGE[18] and through the suppression of the transcription factor E2F1.[19]

Conversely, other studies suggest a role for TRIM28 as a tumor-suppressor.[20] Inactivation of TRIM28 has been shown to promote the formation of murine hepatocellular carcinoma.[21] In addition, mice lacking TRIM28 are defective in early post implantation development.[22] The tumor-suppressor activity of TRIM28 is mediated through its role in DNA repair mechanisms,[23-26] as well as through the silencing of retroviral DNA and epigenetic stability.[27, 28]

Although numerous studies have investigated the tumor-promoting and tumor-suppressor activity of TRIM28 in cancer, little is known about the expression of TRIM28 in the tumor microenvironment. The balance of TRIM28 expression in cancer epithelium and the surrounding stroma may be a critical determinant of the tumor-promoting or tumor-suppressing phenotype of the protein. By dissecting the effects of TRIM28 in stromal fibroblasts and epithelial tumor cells, we aim to elucidate the complex relationship between stromal and epithelial compartments in colorectal cancer (CRC) as we have previously shown that the TRIM28 gene is overexpressed in CRC.[29]

Methods

Study Design and Patient Cohort

A previous study carried out in this lab,[29] identified a novel antibody signature specific for colorectal cancer. Eighteen antigens associated with colorectal cancer and 4 antigens associated with the absence of the disease were identified and these markers were confirmed to have corresponding antibodies in sera of patients. Anti-TRIM28 antibodies were found in the sera of 8 out of 43 analyzed colorectal cancer patients (18.6%) and in one of 40 control sera (2.5%). Using quantitative reverse transcription-PCR methods, they also showed significantly elevated mRNA levels of the TRIM28 antigen in colorectal tumors compared with adjacent normal tissue. The purpose of this study was to investigate the expression of the TRIM28 antigen in colorectal cancer tissue.

The study was approved by the Ethics (Medical) Research Committee at Beaumont Hospital, Dublin, Ireland and informed consent was obtained from all patients. Patients undergoing colonoscopy were screened prospectively, with the clinical notes of all patients attending for colonoscopies being reviewed daily by the clinical research nurses. Patients with a history of cancer or systemic inflammatory disease and patients taking immunosuppressive medication were excluded from the study. In total, 137 Caucasian patients with newly diagnosed CRC fulfilled the inclusion criteria. All of the cases were diagnosed between 2001 and 2007 and had a minimum of 5 years follow-up information. A pathologist sampled an area of invasive carcinoma from the tumor mass and an adjacent area of uninvolved colonic/rectal mucosa was also sampled. Representative sections of archived formalin-fixed and paraffin-embedded CRC tissue specimens for each of the patients in the cohort were retrieved for the study. Each block was sectioned and stained with haematoxylin and eosin (H&E) and graded by a pathologist (EWK) to confirm pathological stage and grade of the tumors and the relevant tumor areas were marked and used as the donor cores for TMA construction.

Tissue microarray construction

The tissue microarray (TMA) construction was performed as previously described[30, 31] using the Beecher Instruments Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA). Cores of 1.0 mm diameter were sampled in quadruplicate for each case. Of the 137 CRC cases, 126 were incorporated into the TMAs and a further 11 cases, that could not be included into the TMAs for various reasons, were investigated using whole sections of tissue. These 11 cases were also used to assess the homogeneity of the TRIM28 IHC staining across whole tumor sections. In addition, a TMA with 28 normal mucosa cases from surgical margins and further 10 normal whole sections from surgical margins were used to assess the TRIM28 staining in normal tissue.

Immunohistochemistry

Sections of 4 µm thickness were cut from all TMA blocks and the whole section blocks for the purpose of immunohistochemistry. Sections were immunostained with an anti-TRIM28 rabbit monoclonal antibody (mAb) (C42G12, Cell Signaling Technology Inc, Danvers, MA, USA) or anti-p53 mouse mAb (DO-7, Dako, Glostrup, Denmark) on an automated platform (Bond system – Leica Microsystems, Bannockburn, IL, USA). Briefly, cut sections were subjected to on-board dewaxing and the following conditions were applied: TRIM28 antigen retrieval in tri-sodium citrate buffer (Bond Epitope Retrieval 1 solution) for 20 mins and 1 : 50 antibody dilution; and p53 antigen retrieval in tri-sodium citrate buffer (Bond Epitope Retrieval 1 solution) for 30 mins and 1 : 100 antibody dilution. Detection of the antibody–antigen complex was achieved using a polymer-based kit (Bond Refine) with DAB as the chromogen. All sections were counterstained with haematoxylin. Negative controls were included for all sections by omitting the primary antibody and positive controls included bladder cancer for p53 and tonsil and colonic adenocarcinoma for TRIM28, as well as Western blots with a

recombinant human TRIM28 full-length protein (Supporting information, Supplementary Fig. 1).

Immunohistochemical assessment

Immunohistochemical evaluation was performed independently by two reviewers blinded to the clinicopathological details and clinical outcomes of the cohort. An intensity score was assigned to each core within the TMA and a representative score was assigned to each whole section based on the intensity of the staining in the majority of the positive cells. The degree of nuclear TRIM28 staining was evaluated for epithelial and stromal tissue and the intensity of the TRIM28 staining (negative = 0; weak = 1+; moderate = 2+; strong = 3+) was recorded for both epithelial and stromal tissue. This is a commonly used method for scoring the intensity of various types of immunohistochemical staining and has been used previously in our group.[32-34] The homogeneity of the staining between the four cores was assessed for each of the 126 cases within the TMA and across the additional 11 whole sections. The relationship between the epithelial and stromal intensity was calculated by determining the ratio of TRIM28 expression between the two compartments. A high TRIM28 expression ratio was defined as at least 2 units of difference in staining intensity (e.g. epithelium strong (3+) and stroma weak (1+), or epithelium moderate (2+) and stroma negative (0)). A low TRIM28 expression ratio was defined as 1 or 0 units of difference in staining intensity (e.g. epithelium moderate (2+) and stroma weak (1+), or epithelium weak (1+) and stroma weak (1+)). A TMA subset of 38 cases was used to examine the expression of p53. Nuclear p53 staining was evaluated and the intensity of expression was recorded (negative = 0; weak = 1+; moderate = 2+; strong = 3+). A previous study in our lab has shown that the inter-observer variability of immunohistochemistry scoring is as low as 7%,[35]. In cases where there were discrepancies between the scorers, a consensus was reached after a joint review using a multi-headed microscope.

Statistical Analysis

Association between discrete variables was assessed using the χ^2 test. Correlation of TRIM28 and p53 expression was performed using the Spearman rank correlation test. 5-year survival and 5-year recurrence-free survival were analyzed for all 137 patients in our cohort. The survival curves were plotted according to the Kaplan-Meier method, and the generalized log-rank test was applied to compare the survival curves. Prognostic factors for 5-year survival and 5-year recurrence- free survival were evaluated by univariate and multivariate analyses for TNM stages, Age and Gender (Cox proportional hazard regression model). For both Kaplan-Meier and Cox regression analyses, patients who had follow-up information for more than 5-years, were censored at 5-years post-diagnosis. All tests were analyzed using SPSS 19.0 software (SPSS, Chicago, IL, USA) and the findings were considered statistically significant at $p<0.05$.

Results

Clinicopathological features

A total of 137 cases with a diagnosis of CRC met the inclusion criteria and were included in the study. The median age of the patients at the time of first diagnosis was 68 years (range 34-87 years). The cohort included 85 male and 52 female patients with a median follow up of 54 months (range 1-122 months). In total, 89 patients had colonic carcinoma, whereas 48 had rectal carcinoma. Table 1 shows the clinicopathological demographics of the patient cohort, along with the follow-up information.

TRIM28 is overexpressed in epithelial CRC tissue

The tissue sections were evaluated for immunohistochemical expression of the TRIM28 within the epithelium and the surrounding stroma, in both cancerous tissue and adjacent normal mucosa. There was distinct nuclear staining for TRIM28 and the intensity and staining distribution was usually homogenous within a case (Fig. 1). In both the normal and cancerous epithelial tissue, TRIM28 staining was confined to cell nuclei. In normal stromal tissue, TRIM28 expression was also nuclear and was predominantly found in lamina propria fibroblasts and occasionally in lymphoid cells in the germinal centers of lymphoid follicles. In tumor stromal tissue, the nuclear TRIM28 expression was present in fibroblasts and was occasionally present in lymphocytes.

The majority of normal epithelial and stromal tissue showed weak to moderate TRIM28 positivity (83.3% and 91.8%, respectively). However, markedly higher TRIM28 expression levels were found in the epithelium of CRC tissue, when compared to normal colorectal epithelium. A total of 57 out of 137 (42%) CRC cases showed strong epithelial TRIM28 staining (intensity 3+) in the nuclei of epithelial cells (Fig. 2*a*, *c*). The TRIM28 expression was independent of any clinicopathological features investigated, including survival and recurrence-free survival.

TRIM28 expression correlates with tumor suppressor p53

Tumor-suppressor p53 is a well-established marker in immunohistochemistry and its association with TRIM28 has been demonstrated in several previous studies.[15-19] We therefore evaluated the expression of the tumor-suppressor p53 in a TMA subset of 38 CRC cases and analyzed its co-expression with TRIM28. (Supporting information, Supplementary Fig. 2). Negative, weak and moderate nuclear p53 expression was found in 4 (11%), 5 (13%) and 10 (26%) cases, respectively. Strong p53 expression was found in 19 of the 38 patients (50%). When we compared the p53 expression pattern with TRIM28 in the same cohort and we found negative, weak and moderate TRIM28 expression in 1 (3%), 3 (8%) and 17 (45%) cases respectively. Strong TRIM28 expression was found in 17 of the 38 patients (45%). There was a significant positive correlation ($p = 0.0168$, Spearman rank test) between negative, weak, moderate and strong p53 and TRIM28 expression in matched tumor samples.

A high TRIM28 expression ratio is associated with shorter survival

The relationship between the epithelial and stromal tumor microenvironment was determined by ratios of TRIM28 expression between the two compartments (Fig. 2). In total, 103 cases had a low ratio of epithelial to stromal TRIM28 expression and 34 cases had a high ratio of epithelial to stromal TRIM28 expression (Supportive information, supplementary table 1). Kaplan-Meier curves for patients with colorectal carcinoma, categorized according to high or low TRIM28 expression ratios between the epithelium and patient-matched stromal tissue are shown in Fig. 3. We found that the ratio of the intensity of TRIM28 expression in patient-matched epithelial and stromal tissue had a significant prognostic value. Overall 5-year survival rates (OS) for patients with a high TRIM28 expression ratio ($\geq$2 units of difference) were significantly lower than those with a low TRIM28 expression ratio ($\leq$1 units of difference), ($p=0.033$). Five-year recurrence-free survival (RFS) was also significantly lower for patients with a high intensity ratio of TRIM28 expression ($\geq$2 units of difference) than

those with a low TRIM28 expression ratio (≤1 units of difference), (p=0.043). In addition, multivariate analysis showed that the epithelial to stromal TRIM28 expression ratio was an independent predictor of overall survival (p=0.046; Table 2) and recurrence-free survival (p=0.035; Table 2).

Discussion

An accumulating body of evidence suggests that the crosstalk between epithelial and stromal microenvironment plays a crucial role in tumor progression.[36] Fibroblasts and tumor cells act on each other and on other cellular components of the tumor microenvironment through the secretion of cytokines and growth factors.[37] Several studies have previously shown that altered protein expression in cells of the stromal tissue compartment, rather than tumor cells alone, can influence survival in lung, prostate and breast cancer.[38-40] These interactions, however, are complex, reciprocal and stage-dependent. Since the molecular and cellular basis of this crosstalk is not yet fully understood, it warrants further in-depth investigation.

In this study, we used immunohistochemical analysis to demonstrate that TRIM28 is overexpressed in human colorectal cancer. We found that TRIM28 expression correlates with the expression of the tumor-suppressor p53 in colorectal cancer tissue and most importantly, that the epithelial to stromal TRIM28 expression ratio correlates significantly with patient survival. Furthermore, Cox regression analyses revealed that the high epithelial to stromal TRIM28 expression ratio is an independent prognostic factor for both, poor survival and poor recurrence-free survival. To our knowledge, this is the first study to examine the correlation between TRIM28 expression in CRC tissue and patient survival.

Based on these novel findings we propose that the pathophysiological role of TRIM28 in carcinogenesis is highly dependent on the expression of the protein in specific types of cells. TRIM28 may act on different pathways in stromal fibroblasts and tumor epithelial cells, resulting in an altered molecular outcome in each compartment. In addition, the balance of TRIM28 expression in cancer epithelium and the surrounding stroma may be a critical determinant of the tumor-promoting or tumor-suppressing phenotype of the protein. Recent studies from our group propose that during the epithelial to mesenchymal transformation (EMT) of tumor cells, a carcinoma cell can take on some characteristics of stromal fibroblasts.[41] EMT is thought to be required physiologically during embryogenesis, but its

persistence in tumor cells is suggested to play a role in the promotion of an invasive phenotype. By dissecting the effects of TRIM28 in stromal fibroblasts and epithelial tumor cells, we elucidate the complex relationship between stromal and epithelial compartments in CRC.

The tumor-suppressor p53 has previously been shown through immunohistochemical analysis to be accumulated in between 42-55% of CRC tissue samples.[42] In our patient cohort, we found that p53 was overexpressed in 50% of CRC samples, which is consistent with previous studies. In addition, we also showed that there was a significant positive correlation between TRIM28 and p53 expression levels in our cohort. Wang *et al*[17] demonstrated that TRIM28 does not interact with p53 on its own, but through its co-operation with MDM2, TRIM28 inhibits p53 acetylation, stimulates p53 ubiquitination and inhibits p53 transcription and apoptotic functions. The MDM2 protein regulates the stability of p53 and the abnormal accumulation of the MDM2 protein is observed in many tumors.[43] The observed TRIM28 up-regulation may occur in response to DNA damage in the absence of p53 DNA-damage repair functions.[23-26] Collectively, these results support a link between TRIM28 and the reduction of transcriptional activity of p53 by selectively negating apoptotic functions.

In conclusion, the pathophysiological role of TRIM28 in carcinogenesis may be contextual, depending on cell type of expression and the balance of expression levels between epithelial and stromal compartments, determining the tumor-promoting or tumor-suppressing phenotype. As multiple cellular processes including normal cell development, cell differentiation, neoplastic transformation, DNA repair and apoptosis, converge on this evolutionary conserved TRIM28 protein, it may emerge as a key player in the proliferation and differentiation of both normal and tumor cells. With this study demonstrating both, TRIM28 expression in the tumor microenvironment and potential as a prognostic marker, a combinatorial approach assessing the tumor cells as well as the the corresponding stromal cells may prove to be a more effective way of predicting survival in human cancers.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Sections from colorectal cancer tissue demonstrate epithelial and stromal staining for TRIM28. Panel (*a*) shows strong expression of TRIM28 in epithelial cells (black arrow) and moderate expression in stromal compartments, particularly in fibroblasts (red arrow). Panel (*b*) shows strong expression of TRIM28 in epithelial cells (black arrows) and weak expression in stromal compartments, fibroblasts (white arrow). (x400).

Figure 2. Epithelial to stromal TRIM28 expression ratios in colorectal cancer tissue. Negative (0), weak (1+), moderate (2+) or strong (3+) TRIM28 expression was found in cell nuclei of epithelial and stromal colorectal cancer tissue. Panel (*a*) shows strong expression of TRIM28 in epithelial cells and moderate expression in stromal compartments resulting in a low epithelial to stromal TRIM28 expression ratio. Panel (*b*) shows weak expression of TRIM28 in both, epithelial and stromal cells resulting in a low epithelial to stromal TRIM28 expression ratio. Panel (*c*) shows strong expression of TRIM28 in epithelial cells and weak expression in stromal compartments resulting in a high epithelial to stromal TRIM28 expression ratio. Panel (*d*) shows moderate expression of TRIM28 in epithelial cells and negative staining in stromal compartments resulting in a high epithelial to stromal TRIM28 expression ratio. (x200).

Figure 3. Epithelial to stromal TRIM28 expression ratios predict survival in colorectal cancer. (*a*) High TRIM28 expression ratio in colorectal cancer correlates with lower 5-year overall survival (OS). The Kaplan-Meier plot of colorectal cancer specimens (n = 137) demonstrates significantly lower (p=0.033; log-rank test) survival associated with high TRIM28 expression ratios. (*b*) High TRIM28 expression ratio in colorectal cancer correlates with lower 5-year recurrence-free survival (RFS). The Kaplan-Meier plot of colorectal cancer specimens (n = 137) demonstrates significantly lower (p=0.043; log-rank test) recurrence-free survival associated with high TRIM28 expression ratio.

Table 1. Clinicopathological details of patient cohort

	Number of patients (n = 137)	*%*
Age (years)		
Median	68	
Range	34-87	
Gender		
Female	52	38.0
Male	85	62.0
Tumor site		
Colon	89	65.0
Rectum	48	35.0
T-stage		
T1	9	6.6
T2	20	14.6
T3	87	63.5
T4	19	13.9
Not Stated †	2	1.5
N-stage		
N0	23	16.8
N1	75	54.7
N2	37	26.3
Not Stated †	2	2.2
M-stage		
M0	126	92.0
M1	11	8.0
Vascular Invasion		
Yes	30	21.9
No	105	76.6
Not Stated †	2	1.5
Differentiation		
Well	3	2.2
Moderately	119	86.9
Poorly	15	10.9
Follow Up (Months)		
Median	54.3	
Range	1-122	

Abbreviations: n = number of patients; T = tumor; N = node; M = metastasis; †These patients presented with terminal metastatic disease and only had biopsies taken; thus, their T and N stage could not be accurately determined.

Table 2. Cox uni- and multivariate analysis of relative risk of death from and recurrence of colorectal cancer within 5 years

	5-year Overall Survival				5-year Recurrence-Free Survival			
	Univariate		Multivariate		Univariate		Multivariate	
Variable	**Hazard Ratio (95% CI)**	**p Value**	**Hazard Ratio (95% CI)**	**p Value**	**Hazard Ratio (95% CI)**	**p Value**	**Hazard Ratio (95%CI)**	**p Value**
TRIM28 Ratio	2.070 (1.036-4.137)	0.039	2.136 (1.015-4.498)	0.046	1.944 (1.005-3.759)	0.048	2.100 (1.052-4.191)	0.035
Age <75>	2.398 (1.184-4.858)	0.015	4.119 (1.825-9.293)	0.001	2.084 (1.050-4.139)	0.036	2.979 (1.404-6.321)	0.004
Gender	1.094 (.542-2.211)	0.802	0.998 (0.441-2.260)	0.997	1.135 (.581-2.220)	0.710	1.297 (0.609-2.760)	0.500
T-Stage	2.092 (1.203-3.641)	0.009	2.628 (1.315-5.255)	0.006	1.709 (1.040-2.807)	0.034	2.267 (1.232-4.173)	0.009
N-Stage	2.390 (1.561-3.658)	0.000	2.243 (1.392-3.615)	0.001	2.053 (1.386-3.040)	0.000	2.055 (1.340-3.151)	0.001
M-Stage	8.091 (3.699-17.697)	0.000	3.879 (1.440-10.453)	0.007	N/A†		N/A†	

Abbreviations: CI = confidence interval; T = tumor; N = node; M = metastasis; †5-year Recurrence-Free Survival analysis not applicable for patients with metastatic disease

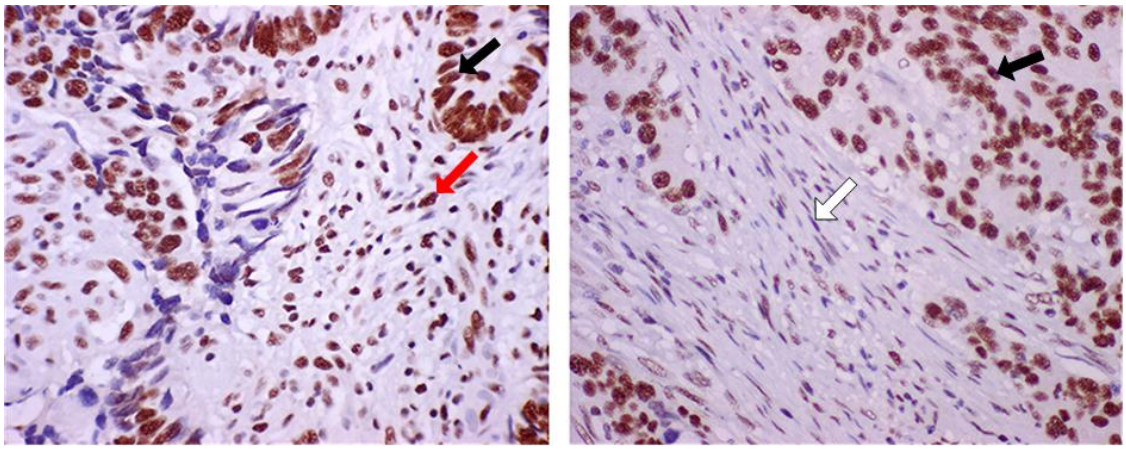


Figure 1

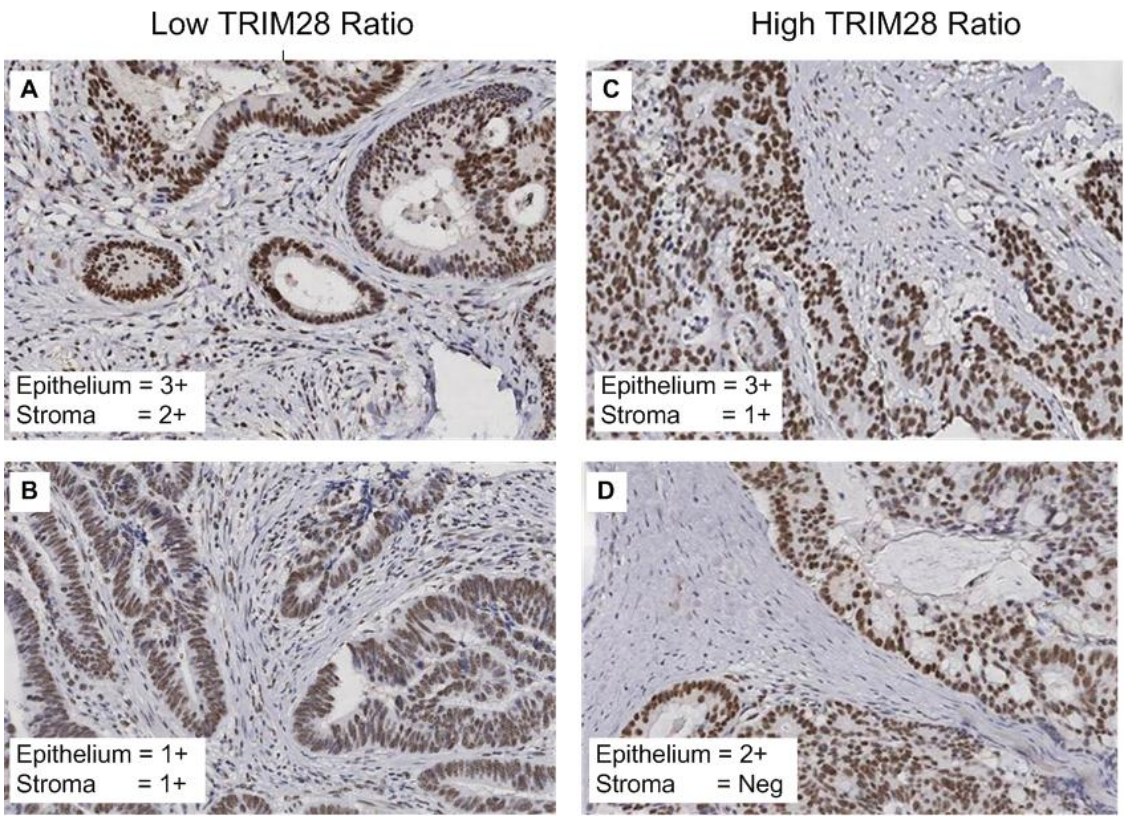

Low TRIM28 Ratio
High TRIM28 Ratio
A
Epithelium = 3+
Stroma = 2+
C
Epithelium = 3+
Stroma = 1+
B
Epithelium = 1+
Stroma = 1+
D
Epithelium = 2+
Stroma = Neg

Figure 2

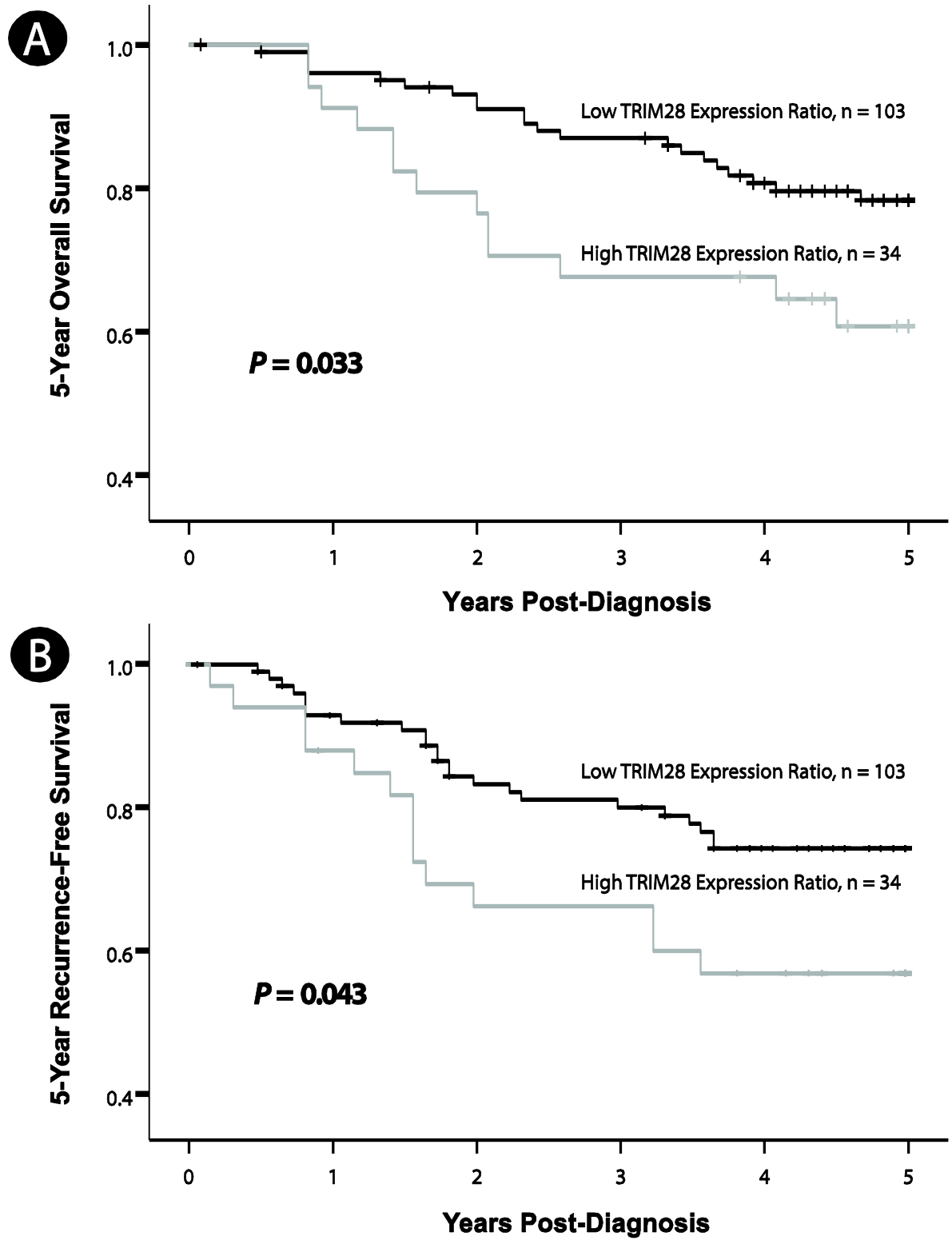

A
5-Year Overall Survival
1.0
0.8
0.6
0.4
Low TRIM28 Expression Ratio, n = 103
High TRIM28 Expression Ratio, n = 34
P = 0.033
0
1
2
3
4
5
Years Post-Diagnosis
B
5-Year Recurrence-Free Survival
1.0
0.8
0.6
0.4
Low TRIM28 Expression Ratio, n = 103
High TRIM28 Expression Ratio, n = 34
P = 0.043
0
1
2
3
4
5
Years Post-Diagnosis

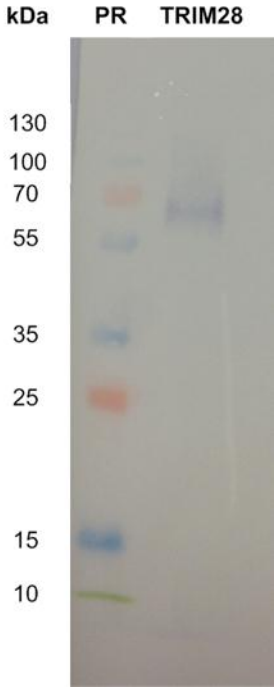
Figure 3

Supplementary Table 1. TRIM28 Epithelial and Stromal Intensity and Corresponding Ratio

Patient No:	TRIM28 Epithelial Intensity Score	TRIM28 Stromal Intensity Score	TRIM28 Expression Ratio
Patient 001	3	2	Low
Patient 002	2	1	Low
Patient 003	3	0	High
Patient 004	2	1	Low
Patient 005	2	2	Low
Patient 006	1	0	Low
Patient 007	2	2	Low
Patient 008	2	1	Low
Patient 009	2	1	Low
Patient 010	3	2	Low
Patient 011	2	0	High
Patient 012	2	2	Low
Patient 013	3	1	High
Patient 014	3	2	Low
Patient 015	1	1	Low
Patient 016	2	1	Low
Patient 017	3	1	High
Patient 018	3	1	High
Patient 019	2	2	Low
Patient 020	2	1	Low
Patient 021	2	1	Low
Patient 022	3	2	Low
Patient 023	3	1	High
Patient 024	3	1	High
Patient 025	0	2	Low
Patient 026	2	2	Low
Patient 027	3	1	High
Patient 028	2	1	Low
Patient 029	3	2	Low
Patient 030	3	2	Low
Patient 031	2	2	Low
Patient 032	3	1	High
Patient 033	2	1	Low
Patient 034	3	2	Low
Patient 035	2	1	Low
Patient 036	3	1	High
Patient 037	1	1	Low
Patient 038	3	1	High
Patient 039	2	1	Low
Patient 040	3	1	High
Patient 041	2	1	Low
Patient 042	3	1	High
Patient 043	0	1	Low
Patient 044	2	2	Low
Patient 045	3	1	High
Patient 046	1	0	Low
Patient 047	1	1	Low
Patient 048	2	2	Low
Patient 049	2	1	Low
Patient 050	2	1	Low
Patient 051	2	1	Low
Patient 052	3	1	High
Patient 053	2	1	Low

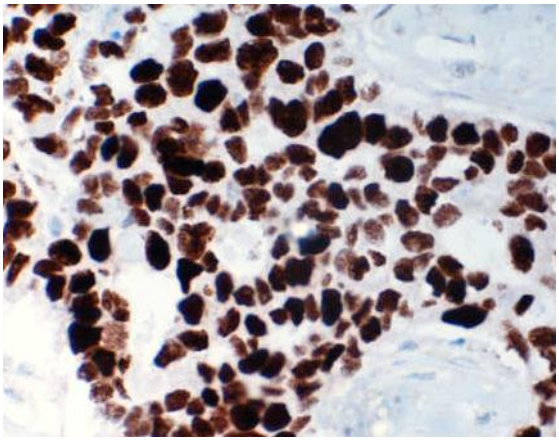
Patient 054	2	2	Low
Patient 055	3	1	High
Patient 056	3	1	High
Patient 057	3	1	High
Patient 058	3	1	High
Patient 059	3	1	High
Patient 060	2	1	Low
Patient 061	1	2	Low
Patient 062	2	1	Low
Patient 063	3	2	Low
Patient 064	3	2	Low
Patient 065	2	1	Low
Patient 066	3	2	Low
Patient 067	2	2	Low
Patient 068	2	2	Low
Patient 069	2	2	Low
Patient 070	2	1	Low
Patient 071	1	2	Low
Patient 072	2	1	Low
Patient 073	1	0	Low
Patient 074	2	2	Low
Patient 075	1	1	Low
Patient 076	2	1	Low
Patient 077	2	1	Low
Patient 078	1	1	Low
Patient 079	3	1	High
Patient 080	2	2	Low
Patient 081	2	1	Low
Patient 082	2	2	Low
Patient 083	2	1	Low
Patient 084	3	1	High
Patient 085	3	2	Low
Patient 086	2	1	Low
Patient 087	3	1	High
Patient 088	1	1	Low
Patient 089	2	1	Low
Patient 090	2	2	Low
Patient 091	2	2	Low
Patient 092	3	1	High
Patient 093	3	2	Low
Patient 094	1	1	Low
Patient 095	3	2	Low
Patient 096	2	1	Low
Patient 097	2	1	Low
Patient 098	2	2	Low
Patient 099	2	2	Low
Patient 100	2	1	Low
Patient 101	3	2	Low
Patient 102	2	1	Low
Patient 103	3	2	Low
Patient 104	3	2	Low
Patient 105	3	2	Low
Patient 106	2	2	Low
Patient 107	3	1	High
Patient 108	3	1	High
Patient 109	3	2	Low
Patient 110	3	1	High
Patient 111	2	1	Low

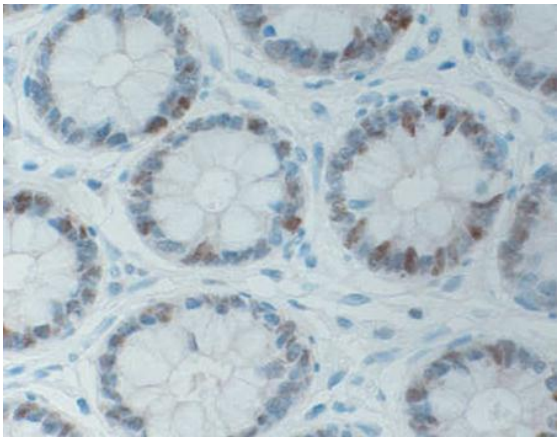
Patient 112	3	2	Low
Patient 113	3	2	Low
Patient 114	3	2	Low
Patient 115	2	2	Low
Patient 116	3	1	High
Patient 117	2	1	Low
Patient 118	3	2	Low
Patient 119	3	1	High
Patient 120	2	2	Low
Patient 121	2	2	Low
Patient 122	3	1	High
Patient 123	3	1	High
Patient 124	3	1	High
Patient 125	2	1	Low
Patient 126	3	1	High
Patient 127	2	2	Low
Patient 128	2	2	Low
Patient 129	3	1	High
Patient 130	2	1	Low
Patient 131	3	3	Low
Patient 132	2	2	Low
Patient 133	1	0	Low
Patient 134	2	1	Low
Patient 135	3	2	Low
Patient 136	2	1	Low
Patient 137	1	2	Low

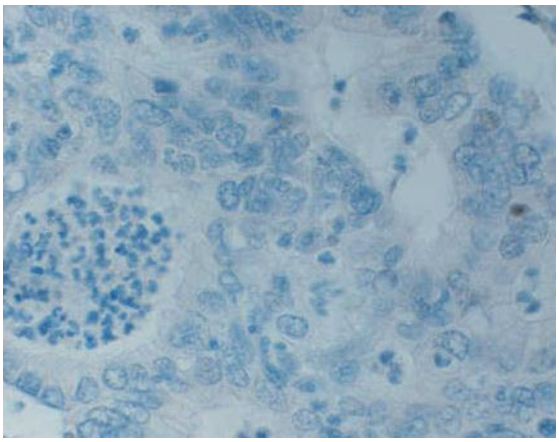


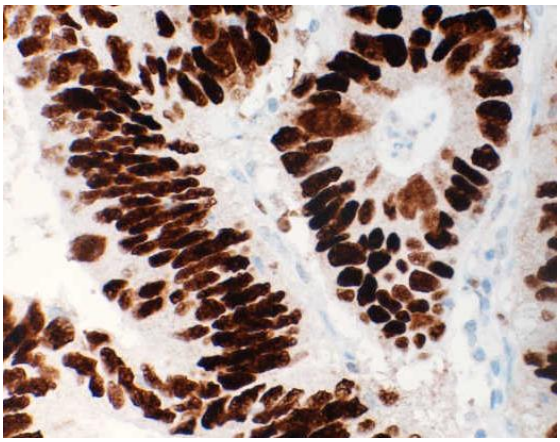
Supplementary Figure 1

A B









C D

Supplementary Figure 2 p53 staining using a mouse monoclonal antibody DO-7. (A) Positive control, bladder carcinoma, strong nuclear staining. (B) Normal colon epithelium, weak nuclear staining. (C) Colorectal cancer, negative staining. (D) Colorectal cancer, strong nuclear staining. (x400).