

TGFBR2 and BAX Mononucleotide Tract Mutations, Microsatellite Instability, and Prognosis in 1072 Colorectal Cancers

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Abstract

Background: Mononucleotide tracts in the coding regions of the *TGFBR2* and *BAX* genes are commonly mutated in microsatellite instability-high (MSI-high) colon cancers. The receptor *TGFBR2* plays an important role in the TGF β 1 (transforming growth factor- β , TGF- β) signaling pathway, and *BAX* plays a key role in apoptosis. However, a role of *TGFBR2* or *BAX* mononucleotide mutation in colorectal cancer as a prognostic biomarker remains uncertain.

Methodology/Principal Findings: We utilized a database of 1072 rectal and colon cancers in two prospective cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study). Cox proportional hazards model was used to compute mortality hazard ratio (HR), adjusted for clinical, pathological and molecular features including the CpG island methylator phenotype (CIMP), LINE-1 methylation, and *KRAS*, *BRAF* and *PIK3CA* mutations. MSI-high was observed in 15% (162/1072) of all colorectal cancers. *TGFBR2* and *BAX* mononucleotide mutations were detected in 74% (117/159) and 30% (48/158) of MSI-high tumors, respectively. In Kaplan-Meier analysis as well as univariate and multivariate Cox regression analyses, compared to microsatellite stable (MSS)/MSI-low cases, MSI-high cases were associated with superior colorectal cancer-specific survival [adjusted HR, 0.34; 95% confidence interval (CI), 0.20–0.57] regardless of *TGFBR2* or *BAX* mutation status. Among MSI-high tumors, *TGFBR2* mononucleotide mutation was associated with CIMP-high independent of other variables [multivariate odds ratio, 3.57; 95% CI, 1.66–7.66; $p=0.0011$].

Conclusions: *TGFBR2* or *BAX* mononucleotide mutations are not associated with the patient survival outcome in MSI-high colorectal cancer. Our data do not support those mutations as prognostic biomarkers (beyond MSI) in colorectal carcinoma.

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Introduction

Colorectal cancer represents a group of molecularly heterogeneous diseases with different sets of epigenetic and genetic abnormalities. High degree of microsatellite instability (MSI-high) is caused by deficiency of DNA mismatch repair system, and observed in approximately 15% of colorectal cancers. MSI testing is widely used as screening for patients with Lynch syndrome/hereditary nonpolyposis colorectal cancer (HNPCC) [1,2,3]. In addition, MSI is generally accepted as a prognostic marker [4], and likely a predictive marker for resistance to 5-fluorouracil [5]. Since Markowitz et al. [6] discovered mutations in the coding mononucleotide repeats of *TGFBR2* in MSI-high colon cancer cells, similar mutations of coding mononucleotide repeats in many other genes (including *BAX*, *MSH3*, *MSH6*, *IGF2R* and *PTE*) have been found

in MSI-high colorectal cancers [3,7,8,9]. Among those genes, mononucleotide coding repeats of *TGFBR2* (A)₁₀ and *BAX* (G)₈ have frequent frameshift mutations resulting in the production of truncated, inactive form of the proteins [3,10]. TGF β 1 (transforming growth factor- β , TGF- β) and its receptor *TGFBR2* constitute a signaling pathway that regulates the transcription of many genes, and functions as a tumor suppressor [11,12,13,14] and an immune response regulator [15]. *BAX* generally promotes apoptosis and antagonizes the effect of *BCL2* [16,17,18]. Thus, inactivation of *TGFBR2* or *BAX* may contribute to tumor progression.

Several previous studies have examined the prognostic role of *TGFBR2* or *BAX* mononucleotide mutations in MSI-high colorectal cancers, yielding inconclusive results due to limited statistical power in most studies [19,20,21,22,23,24,25] (**Table 1**). All but one previous study [23] examined the prognostic role of

Table 1. Studies on prognostic significance of TGFB2 or BAX mononucleotide mutation in MSI-high colorectal cancer.

| Ref. (year) | Authors (year) | No. of hospitals | Sample size for MSI determination | Disease stage | Chemotherapy | MSI-high cases | | Mononucleotide mutations in MSI-high colorectal cancer | | Other molecular covariates and notes | | | |
|-------------|---------------------------------|------------------|-----------------------------------|----------------|-------------------------|----------------|-----------|--|--|--|--|--|--|
| | | | | | | N | OS events | TGFB2 | BAX | | | | |
| [19] | Iacopetta et al. (1998) | 1 | 210 | Dukes' B and C | - | 37 | - | 32/37 | OS at the end point* 72% (vs. 60%) p=0.21 | 5-year survival value by log-rank test (vs. wild type cases) | Multivariate HR (95% CI) P value (vs. wild type cases) | Proximal colon cancer only | |
| [20] | Ionov et al. (2000) | ? | 508 | - | - | 67 | - | 31/36 | OS: p=0.55 | OS: inferior p<0.01 | - | - | |
| [21] | Watanabe et al. (2001) | many | 298 | II-III | Adjuvant chemo-therapy | 73 | - | 48/73 | OS: 74% (vs. 46%) p=0.04 | - | 22/60 | Multivariate model assessed effect of MSS vs. MSI-high and TGFB2 mutation. 18q LOH was also assessed. | |
| [22] | Samowitz et al. (2002) | many | 1427 | I-IV | - | 174 | - | 134/170 | OS: 72% (vs. 67%) NS | OS: 1.01 (0.54-1.88) NS | 63/160 | Cases from 8 county areas | |
| [23] | Fernández-Peralta et al. (2005) | 1 | 155 | Dukes' A-D | - | 16 | - | 13/16 | OS: superior p=0.04 | - | 6/16 | OS: superior p<0.001 | |
| [24] | Jung et al. (2006) | 4 | 172 | II | - | 48 | 11 | 35/45 | - | OS: NS (univariate HR only) | 29/47 | - | |
| [25] | Kim et al. (2007) | many | 542 | Dukes' B and C | Adjuvant chemo-therapy† | 98 | 26 | 54/98 | - | OS (stratified by stage and treatment): 1.26 (0.57-2.80) NS | - | National Surgical Adjuvant Breast and Bowel Project (NSABP) | |
| | Shima et al. (current study) | many | 1072 | I-IV | - | 162 | 59 | 20 | CS: 86% (vs. 90%), p=0.55 OS: 64% (vs. 60%), p=0.66 | CS: 1.18 (0.29-4.89), p=0.82 OS: 0.61 (0.32-1.15), p=0.12 | 48/158 | CS: 92% (vs. 85%), p=0.29 OS: 56% (vs. 65%), p=0.39 CS: 0.73 (0.22-2.41), p=0.60 OS: 1.46 (0.80-2.65), p=0.22 | Tumor molecular covariates include CIMP, LINE-1 methylation, and mutations in KRAS, BRAF and PIK3CA. |

CI, confidence interval; CIMP, CpG island methylator phenotype; CS, colorectal cancer-specific survival; HR, hazard ratio; LOH, loss of heterozygosity; MSI, microsatellite instability; MSS, microsatellite stable; NS, not significant; OS, overall survival;

*Including MSS/MSI-low cases.

†The authors (Kim et al. [25]) compared predictive effect of MSI for chemotherapy between the cases registered surgery alone and the cases registered chemotherapy. doi:10.1371/journal.pone.0025062.t001

TGFBR2 or *BAX* mononucleotide mutation in less than 100 MSI-high tumors (the number of MSI-high tumors ranging from 16 to 98) [19,20,21,22,23,24,25]. In addition, none of the previous studies [19,20,21,22,23,24,25] has comprehensively examined potential confounding effect of key molecular biomarkers in colorectal cancer, including the CpG island methylator phenotype (CIMP), and *KRAS*, *BRAF* and *PIK3CA* mutations. Thus, the prognostic role of *TGFBR2* or *BAX* mononucleotide mutation in MSI-high tumors still remains uncertain.

We conducted this study to test the hypothesis that *TGFBR2* or *BAX* mononucleotide mutations in colorectal cancer were associated with altered tumor behavior (beyond MSI), utilizing a database of 1072 stage I to IV colorectal cancers in two prospective cohort studies. Our current study represents the first study which utilized a database of prospective cohort studies to test the stated hypothesis. This fact increases generalizability of our study findings. Moreover, because we concurrently assessed clinical, pathologic and tumor molecular variables such as the CpG island methylator phenotype (CIMP), LINE-1 methylation, *KRAS*, *BRAF* and *PIK3CA* mutations, we could evaluate the effect of *TGFBR2* or *BAX* mutation independent of these potential confounders.

Methods

Study group

We utilized the database of two prospective cohort studies, the Nurses' Health Study (N = 121,701 women followed since 1976) and the Health Professionals Follow-up Study (N = 51,529 men followed since 1986) [26,27]. Participants have been sent biennial questionnaires to update information on potential risk factors and to identify newly diagnosed cancers in themselves and their first degree relatives. We collected paraffin-embedded tumor tissue blocks of incident colorectal cancers from hospitals throughout the U.S. where participants with colorectal cancer underwent tumor resection [26,27]. Clinical characteristics of the cases are described in **Table 2** (on the left, under the column heading "All cases"). There was no significant difference in demographic features between cases with tissue available and those without available tissue among our cohort studies [26]. A majority of cases have previously been characterized for statuses of *TGFBR2*, MSI, CIMP, *KRAS*, *BRAF*, *PIK3CA* and LINE-1 methylation [28,29,30,31]. However, none of our previous studies have analyzed the prognostic significance of mononucleotide mutation of *TGFBR2* or *BAX*. *BAX* mutation has not been analyzed in any of our previous studies. Thus, this study represents a new study utilizing a resource of the existing materials and database, analogous to novel studies using well-described cell lines (e.g., SW480 cell line) or mouse models (e.g., *Apc* min mouse model).

Hematoxylin and eosin stained tissue sections from all colorectal cancer cases were reviewed by a pathologist (S.O.) unaware of other data. Tumor differentiation was categorized as well-moderate vs. poor (>50% vs. ≤50% glandular areas). We excluded cases which were preoperatively treated. Based on the availability of adequate follow-up and tumor tissue data, 1072 stage I-IV colorectal cancer cases diagnosed up to 2004 were included in the current study (**Figure 1**). Patients were observed until death or June 30 2009, whichever came first. Death of a participant was confirmed by the National Death Index. Returning questionnaire indicated informed consent from all study subjects. Informed consent was obtained from all study subjects. Tissue collection and analyses were approved by the Human Subjects Committees at Harvard School of Public Health and Brigham and Women's Hospital.

Microsatellite instability (MSI) analysis and detection of *TGFBR2* and *BAX* mononucleotide tract mutations

DNA was extracted from paraffin embedded tissue. MSI analysis was performed using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487) [30]. MSI-high was defined as the presence of instability in ≥30% of the markers, and MSI-low/microsatellite stable (MSS) as instability in 0–29% of markers [30]. Mononucleotide tract mutations of *TGFBR2* and *BAX* were examined in MSI-high tumors. Primers and PCR conditions for *TGFBR2* were previously described [28]. Primer sequences for *BAX* were; 5'-(FAM) ATCCAGGATC-GAGCAGGGCG-3' and 5'-ACTCGCTCAGCTTCTTGGTG-3'. PCR condition was preheat at 95°C for 5 min, 45 cycles (at 94–55–72°C for 30–30–30 sec), and extension at 72°C for 2 min. PCR products were electrophoresed and analyzed by ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Pyrosequencing of *KRAS*, *BRAF* and *PIK3CA*

PCR and Pyrosequencing targeted for *KRAS* (codons 12 and 13) [32], *BRAF* (codon 600) [33] and *PIK3CA* (exons 9 and 20) were performed as previously described [29].

Methylation analyses for CpG islands and LINE-1

Sodium bisulfite treatment and subsequent real-time PCR (MethylLight [34]) were previously validated [35], and performed to quantify promoter methylation in eight CpG islands (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*) [36,37,38]. CIMP-high was defined as the presence of ≥6/8 methylated markers, CIMP-low as the presence of 1/8 to 5/8 methylated markers, and CIMP-0 as the absence (0/8) of methylated markers [37,39]. LINE-1 methylation levels were quantified by PCR-Pyrosequencing [40,41].

Statistical analysis

We used SAS program (Version 9.1, SAS Institute, Cary, NC) for all statistical analyses. All p values were two-sided. When we perform multiple hypothesis testing (i.e., analyses of molecular correlates and interactions), a p value for statistical significance was adjusted to $p = 0.0038$ ($= 0.05/13$) by Bonferroni correction. The chi-square test (or Fisher's exact test) was performed for categorical variables. The t test assuming unequal variances was done to compare mean age and mean LINE-1 methylation level. For survival analysis, the Kaplan-Meier method and log-rank test were used. For analyses of colorectal cancer-specific mortality, deaths as a result of causes other than colorectal cancer were censored. To control for confounding, we used multivariate stage-matched (stratified) Cox proportional hazards model to compute hazard ratio (HR) of death. To avoid residual confounding and overfitting, disease stage (I, II, III, IV, unknown) was used as a stratifying variable, utilizing the "strata" option in the SAS "proc phreg" command. The multivariate model initially included age at diagnosis (continuous), sex, year of diagnosis (continuous), body mass index (BMI; <30 vs. ≥30 kg/m²), family history of colorectal cancer in any first degree relative (present vs. absent), tumor location (proximal vs. distal), tumor differentiation (well-moderate vs. poor), CIMP (high vs. low/CIMP-0), LINE-1 methylation (continuous), *KRAS*, *BRAF* and *PIK3CA*. A backward elimination method with a threshold of $p = 0.20$ was used to limit the number of variables in the final model and avoid overfitting. For cases with missing information in any of the categorical variables [BMI (0.1%), tumor location (1.0%), tumor grade (0.6%), CIMP (2.5%), *KRAS* (0.5%), *BRAF* (0.7%) and *PIK3CA* (8.8%)], we included those cases in a majority category of a given covariate to

Table 2. MSI status and TGFBR2 or BAX mononucleotide tract mutation in colorectal cancer.

| Clinical, pathologic or molecular feature | Total N | MSS/MSI-low | MSI-high | TGFBR2 mononucleotide mutation in MSI-high tumors | | | BAX mononucleotide mutation in MSI-high tumors | | |
|---|-----------|-------------|-----------|---|-----------|---------|--|----------|---------|
| | | | | (-) | (+) | P value | (-) | (+) | P value |
| All cases | 1072 | 910 | 162 | 42 | 117 | | 110 | 48 | |
| Sex | | | | | | 0.45 | | | 0.51 |
| Female (NHS) | 603 (56%) | 495 (54%) | 108 (67%) | 26 (62%) | 80 (68%) | | 72 (65%) | 34 (71%) | |
| Male (HPFS) | 469 (44%) | 415 (46%) | 54 (33%) | 16 (38%) | 37 (32%) | | 38 (35%) | 14 (29%) | |
| Mean age ± SD | 67.5±8.5 | 67.2±8.6 | 69.5±7.3 | 68.3±7.6 | 69.7±7.0 | 0.26 | 68.7±7.4 | 70.6±6.4 | 0.13 |
| Body mass index | | | | | | 0.34 | | | 0.62 |
| <30 kg/m ² | 871 (81%) | 740 (81%) | 131 (81%) | 32 (76%) | 97 (83%) | | 88 (80%) | 40 (83%) | |
| ≥30 kg/m ² | 200 (19%) | 169 (19%) | 31 (19%) | 10 (24%) | 20 (17%) | | 22 (20%) | 8 (17%) | |
| Family history of colorectal cancer | | | | | | 0.68 | | | 0.56 |
| Absent | 866 (81%) | 743 (82%) | 123 (76%) | 31 (74%) | 90 (77%) | | 85 (77%) | 35 (73%) | |
| Present | 206 (19%) | 167 (18%) | 39 (24%) | 11 (26%) | 27 (23%) | | 25 (23%) | 13 (27%) | |
| Year of diagnosis | | | | | | 0.28 | | | 0.68 |
| Prior to 1995 | 390 (36%) | 347 (38%) | 43 (26%) | 14 (33%) | 29 (25%) | | 31 (28%) | 12 (25%) | |
| 1995 to 2004 | 682 (64%) | 563 (62%) | 119 (73%) | 28 (67%) | 88 (75%) | | 79 (72%) | 36 (75%) | |
| Tumor location | | | | | | 0.12 | | | 0.51 |
| Proximal colon (cecum to transverse) | 492 (47%) | 351 (39%) | 141 (87%) | 35 (83%) | 105 (90%) | | 95 (86%) | 44 (92%) | |
| Distal colon | 337 (32%) | 321 (36%) | 16 (9.9%) | 4 (10%) | 11 (9.4%) | | 11 (10%) | 4 (8.3%) | |
| Rectum | 228 (22%) | 223 (25%) | 5 (3.1%) | 3 (7.1%) | 1 (0.9%) | | 4 (3.6%) | 0 | |
| Disease stage | | | | | | 0.045 | | | 0.014 |
| I | 256 (24%) | 224 (25%) | 32 (20%) | 15 (36%) | 17 (15%) | | 26 (24%) | 6 (13%) | |
| II | 308 (29%) | 221 (24%) | 87 (54%) | 18 (43%) | 66 (56%) | | 48 (44%) | 35 (73%) | |
| III | 282(26%) | 256 (28%) | 26 (16%) | 4 (9.5%) | 22 (19%) | | 21 (19%) | 5 (10%) | |
| IV | 146 (14%) | 136 (15%) | 10 (6.2%) | 3 (7.1%) | 7 (6.0%) | | 8 (7.3%) | 2 (4.2%) | |
| unknown | 80 (7.5%) | 73 (8.0%) | 7 (4.3%) | 2 (4.8%) | 5 (4.3%) | | 7 (6.4%) | 0 | |
| Tumor grade | | | | | | 0.86 | | | 0.19 |
| Low | 962 (90%) | 851 (94%) | 111 (69%) | 29 (69%) | 79 (68%) | | 78 (71%) | 29 (60%) | |
| High | 104 (10%) | 53 (5.9%) | 51 (31%) | 13 (31%) | 38 (32%) | | 32 (29%) | 19 (40%) | |
| CIMP status | | | | | | 0.0010 | | | 0.52 |
| CIMP-0 | 462 (44%) | 450 (51%) | 12 (7.5%) | 3 (7.3%) | 9 (7.8%) | | 9 (8.3%) | 3 (6.3%) | |
| CIMP-low | 411 (39%) | 382 (43%) | 29 (18%) | 15 (37%) | 12 (10%) | | 21 (19%) | 6 (13%) | |
| CIMP-high | 172 (16%) | 53 (6.0%) | 119 (74%) | 23 (56%) | 95 (82%) | | 78 (72%) | 39 (81%) | |
| KRAS mutation | | | | | | 0.016 | | | 0.81 |
| (-) | 684 (64%) | 546 (60%) | 138 (86%) | 31 (76%) | 105 (91%) | | 93 (86%) | 42 (88%) | |
| (+) | 383 (36%) | 361 (40%) | 22 (14%) | 10 (24%) | 11 (9.5%) | | 15 (14%) | 6 (13%) | |
| BRAF mutation | | | | | | 0.0049 | | | 0.59 |
| (-) | 915 (86%) | 835 (92%) | 80 (50%) | 28 (68%) | 50 (43%) | | 55 (50%) | 22 (46%) | |
| (+) | 150 (14%) | 69 (7.6%) | 81 (50%) | 13 (32%) | 67 (57%) | | 54 (49%) | 26 (54%) | |
| PIK3CA mutation | | | | | | 0.53 | | | 0.079 |
| (-) | 789 (81%) | 679 (82%) | 110 (76%) | 26 (72%) | 82 (77%) | | 71 (72%) | 37 (86%) | |
| (+) | 189 (19%) | 154 (18%) | 35 (24%) | 10 (28%) | 24 (23%) | | 27 (28%) | 6 (14%) | |
| Mean LINE-1 methylation (%) ± SD | 62.0±9.4 | 61.2±9.3 | 66.1±8.5 | 66.2±9.8 | 65.9±8.0 | 0.83 | 66.0±8.6 | 66.0±8.4 | 0.96 |

(%) indicates the proportion of cases with a specific clinical, pathologic or molecular feature among all cases, MSS/MSI-Low, TGFBR2 mutated or BAX mutated cases. CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; MSI, microsatellite instability; MSS, microsatellite stable, NHS, Nurses' Health Study; SD, standard deviation.

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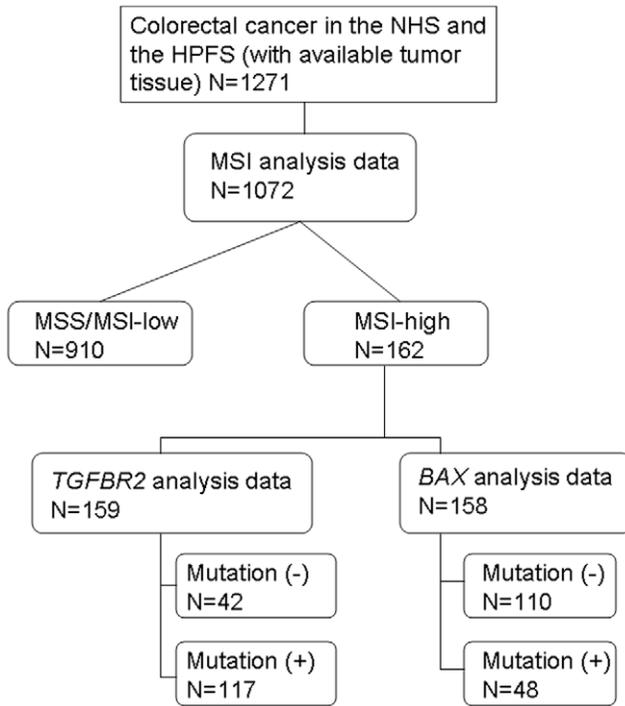


Figure 1. Flow diagram of the current study. Based on the availability of adequate follow-up and tumor molecular data among incident colorectal cancers identified in the Nurses’ Health Study (NHS; N = 121,701) and the Health Professionals Follow-up Study (HPFS; N = 51,529), a total of 1072 stage I–IV colorectal cancer cases diagnosed up to 2004 were included. MSI, microsatellite instability; MSS, microsatellite stable. doi:10.1371/journal.pone.0025062.g001

avoid overfitting. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown).

A multivariate logistic regression analysis was performed to examine an independent relationship of each covariate with *TGFBR2* mutation (as an outcome variable). The multivariate model initially included a similar, but not the same set of the covariates as the initial

Cox model, considering possible cause-effect relationship with *TGFBR2* mutation. Specifically, disease stage and tumor differentiation were likely consequences (rather than causes) of *TGFBR2* mutation. Thus, those variables were not included in the logistic regression model. A backward elimination with a threshold of $p = 0.10$ was used to select variables in the final model and avoid overfitting.

Results

Mononucleotide mutations of *TGFBR2* and *BAX* in MSI-high colorectal cancers

Among 1072 colorectal cancers in the two prospective cohort studies, MSI-high was observed in 162 (15%) tumors. *TGFBR2* and *BAX* mononucleotide tract mutations were detected in 117 (of 159, 74%) and 48 (of 158, 30%) MSI-high tumors, respectively. Among MSI-high tumors, *TGFBR2* mutation was significantly associated with CIMP-high ($p = 0.0010$) (Table 2).

Multivariate analysis to assess independent relations with *TGFBR2* mutation

We performed multivariate logistic regression analysis to examine whether *TGFBR2* mutation was independently associated with any clinical, pathologic and other molecular variables. In MSI-high tumors, *TGFBR2* mutation was independently associated with CIMP-high [multivariate odds ratio (OR), 3.57; 95% confidence interval (CI), 1.66–7.66; $p = 0.0011$].

Mononucleotide mutations of *TGFBR2* and *BAX* and colorectal cancer prognosis

During adequate follow-up (11.6 years of median follow-up of censored cases), there were 505 deaths including 302 colorectal cancer-specific deaths. Among all cases, MSI-high was significantly associated with longer colorectal cancer-specific survival compared to MSS/MSI-low cancers by log-rank test ($p < 0.0001$), univariate and multivariate Cox regression analysis (adjusted HR, 0.34; 95% CI, 0.20–0.57; $p < 0.0001$) (Table 3). When we separately examined *TGFBR2*-mutated MSI-high cases and *TGFBR2*-wildtype MSI-high cases, both groups showed significantly longer colorectal cancer-specific survival compared to MSS/MSI-low cases (Figure 2, Table 3). When we separately examined *BAX*-mutated MSI-high cases and *BAX*-wildtype MSI-high cases, both groups

Table 3. MSI status, *TGFBR2* and *BAX* mononucleotide tract mutation and survival of colorectal cancer patients.

| | Total N | Colorectal cancer-specific mortality | | | Overall mortality | | |
|----------------------------|---------|--------------------------------------|------------------------|--|----------------------|------------------------|--|
| | | Deaths/ person-years | Univariate HR (95% CI) | Multivariate stage-matched HR (95% CI) | Deaths/ person-years | Univariate HR (95% CI) | Multivariate stage-matched HR (95% CI) |
| MSS/MSI-low tumors | 910 | 282/8015 | 1 (referent) | 1 (referent) | 445/8015 | 1 (referent) | 1 (referent) |
| MSI-high tumors | 162 | 20/1468 | 0.37 (0.24–0.58) | 0.34 (0.20–0.57) | 60/1468 | 0.72 (0.55–0.94) | 0.64 (0.47–0.89) |
| MSI-high tumors | | | | | | | |
| <i>TGFBR2</i> mutation (–) | 42 | 4/390 | 0.30 (0.11–0.79) | 0.29 (0.10–0.83) | 17/390 | 0.79 (0.48–1.28) | 0.79 (0.47–1.34) |
| <i>TGFBR2</i> mutation (+) | 117 | 16/1060 | 0.41 (0.25–0.67) | 0.35 (0.20–0.62) | 42/1060 | 0.69 (0.50–0.95) | 0.59 (0.41–0.86) |
| <i>BAX</i> mutation (–) | 110 | 16/999 | 0.44 (0.27–0.73) | 0.36 (0.20–0.62) | 38/999 | 0.67 (0.48–0.93) | 0.57 (0.39–0.84) |
| <i>BAX</i> mutation (+) | 48 | 4/443 | 0.25 (0.09–0.66) | 0.30 (0.11–0.83) | 21/443 | 0.84 (0.54–1.30) | 0.82 (0.50–1.32) |

The multivariate, stage-matched (stratified) Cox regression model initially included the *TGFBR2* mutation or *BAX* mutation variable, sex, age at diagnosis, year of diagnosis, tumor location, body mass index, family history of colorectal cancer, tumor grade, CIMP, *KRAS*, *BRAF*, *PIK3CA* and LINE-1 methylation. A backward elimination with a threshold of $p = 0.20$ was used to select variables in the final models. Stage adjustment (I, II, III, IV, unknown) was done using the “strata” option in the SAS “proc phreg” command.

CI, confidence interval; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.

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showed significantly longer colorectal cancer-specific survival compared to MSS/MSI-low cases (Figure 2, Table 3). In overall mortality analyses, although somewhat attenuated, results showed similar trends (Table 3). Among MSI-high cases, patient survival did not significantly differ by TGFBR2 or BAX mutation status.

We compared colorectal cancer specific and overall survival between TGFBR2-mutated MSI-high cases and TGFBR2-wildtype MSI-high cases (or between BAX-mutated MSI-high cases and BAX-wild type MSI-high cases). There was no significant difference between the two groups (Table 1).

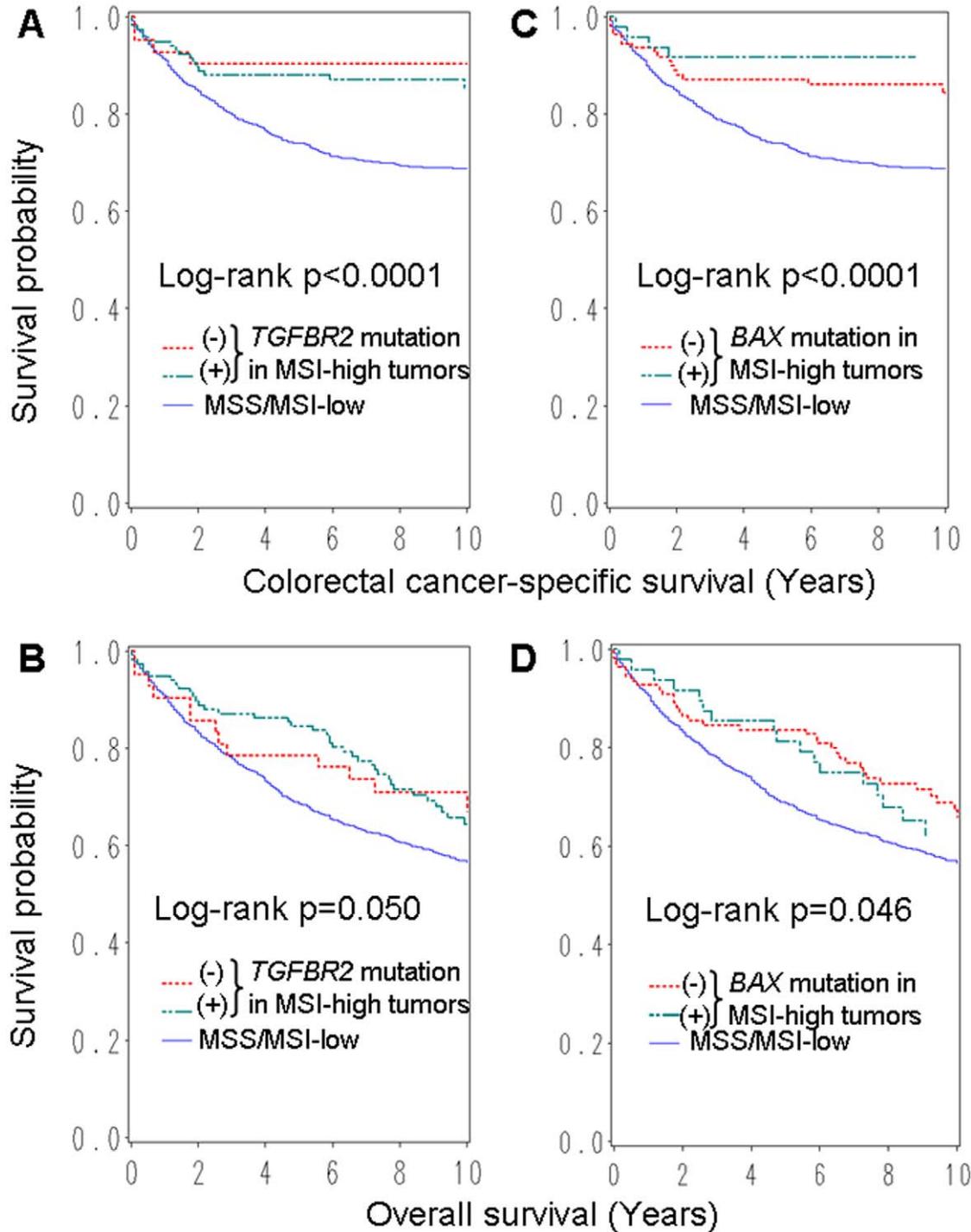


Figure 2. Kaplan-Meier curves according to MSI status and TGFBR2 or BAX mononucleotide mutation in colorectal cancer. Kaplan-Meier curves for colorectal cancer-specific survival (A) and overall survival (B), according to TGFBR2 mononucleotide mutation status. Regardless of TGFBR2 status, MSI-high cases were associated with longer survival. Kaplan-Meier curves for colorectal cancer-specific survival (C) and overall survival (D), according to BAX mononucleotide mutation status. Regardless of BAX status, MSI-high cases were associated with longer survival. MSI, microsatellite instability; MSS, microsatellite stable. doi:10.1371/journal.pone.0025062.g002

Discussion

We conducted this study to examine the prognostic significance of mononucleotide tract mutations in the coding regions of *TGFBR2* or *BAX* in MSI-high colorectal cancers. We utilized two prospective cohort studies with a large number of clinically and molecularly well-annotated colorectal cancer cases with adequate follow-up. Our result showed that MSI-high tumors were associated with indolent tumor behavior regardless of *TGFBR2* or *BAX* mononucleotide mutation status, independent of CIMP and other key tumor molecular biomarkers. Nonetheless, it may be of interest to examine interactions between these molecular alterations and dietary and lifestyle factors if there is a hypothesis in evolving science of molecular pathological epidemiology [42,43].

It should be noted that small studies are more prone to “publication bias” than large studies [44]. This phenomenon of publication bias occurs because studies with null findings have a higher likelihood of being unwritten and unpublished compared to those with significant results. Compared to small studies (e.g., studies with a sample size of <200 cancers) with null data, large studies with null data are more likely published. As a result, large studies are less prone to publication bias than small studies. Furthermore, academic pressures might force investigators to design small studies which are easy to complete and get data for haste publications, which might contribute to bias [45,46,47]. Therefore, we should weigh more on large-scale studies when we evaluate the published literature on prognostic significance of any biomarker such as *TGFBR2* or *BAX* mononucleotide mutation. Publishing null data in well-powered studies [44,48,49,50,51,52] are important because publishing significant results in small underpowered studies leads to publication bias.

Our data are generally consistent with some of previous studies [19,22,24,25] (Table 1). Watanabe et al. [21] used stage II and III cases that underwent adjuvant chemotherapy, and reported that, *TGFBR2* mutation was associated with improved 5-year overall survival among 73 MSI-high tumors. In another study [20], among 67 MSI-high tumors, *BAX* mutation was associated with poor prognosis. In an underpowered study by Fernández-Peralta et al. [23], among 16 MSI-high tumors, both *TGFBR2* mutation and *BAX* mutation were associated with better prognosis. The largest study (total N = 1427; 170 MSI-high cancers) by Samowitz et al. [22] showed no prognostic role of *TGFBR2* or *BAX* mutations among MSI-high colorectal cancer cases, in agreement with our current study - the second largest study to date and the only study which examined other key tumor molecular biomarkers such as CIMP, LINE-1 methylation and *KRAS*, *BRAF* and *PIK3CA* mutations.

Studying somatic molecular changes and molecular correlates is important in cancer research towards personalized medicine [53,54,55,56]. The CpG island methylator phenotype (CIMP) has been established as an epigenomic molecular classifier of colorectal cancer [57,58,59,60,61,62,63,64,65,66,67,68,69,70,71]. In the past, Iacopetta et al. [20] showed no significant association between *KRAS* mutation and *TGFBR2* mutation. We assessed the association between tumor molecular variables (CIMP, LINE-1, *KRAS*, *BRAF* and *PIK3CA*) and *TGFBR2* mutation and did not find significant relation between *TGFBR2* mutation and *KRAS* or *BRAF*

mutation. Interestingly, we have found that, among MSI-high tumors, *TGFBR2* mutation was associated with CIMP-high, independent of clinical and other molecular features. A recent study [72] has reported that genetic variants in the TGFBI pathway related genes (*MAPK1*, *RUNX1* and *RUNX2*) are associated with CIMP-high colon cancer. Further studies are needed to elucidate the exact mechanism of the relationship between CIMP and the TGFBI pathway.

There are limitations in this study. For example, data on cancer treatment were limited. Nonetheless, it is unlikely that chemotherapy use substantially differed according to *TGFBR2* or *BAX* mutation status in tumor, since such data were typically unavailable for treatment decision making. As another limitation, beyond cause of mortality, data on cancer recurrences were unavailable in these cohort studies. Nonetheless, given median follow-up of over 11 years for censored cases, colorectal cancer-specific survival might be a reasonable surrogate of colorectal cancer-specific outcome.

There are advantages in utilizing the database of the two prospective cohort studies, the Nurses’ Health Study and the Health Professionals Follow-up Study, to examine prognostic significance of tumor biomarkers. Anthropometric measurements, family history, cancer staging, and other clinical, pathologic, and tumor molecular data were prospectively collected, blinded to patient outcome [26]. Cohort participants who developed cancer were treated at hospitals throughout the U.S., and thus more representative colorectal cancers in the U.S. population than patients in one to a few academic hospitals. There were no demographic difference between cases with tumor tissue analyzed and those without tumor tissue analyzed [26]. Finally, our rich tumor database enabled us to simultaneously assess pathologic and tumor molecular correlates and control for potential confounding by the tumor molecular features.

In conclusion, our large tumor database has shown that, compared to MSS/MSI-low cases, MSI-high colorectal cancer is associated with longer cancer-specific survival, regardless of *TGFBR2* or *BAX* mononucleotide tract mutation status. The importance of large-scale studies cannot be overemphasized because, compared to large studies, small studies are much more prone to publication bias, which can mislead clinical practice.

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Author Contributions

Conceived and designed the experiments: SO. Performed the experiments: KS TM MY YI XL JAM CSF SO. Analyzed the data: KS TM AK JAM CSF SO. Wrote the paper: KS TM MY SO.

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