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Original Research

Prognostic association of *PTGS2* (COX-2) over-expression according to *BRAF* mutation status in colorectal cancer: Results from two prospective cohorts and CALGB 89803 (Alliance) trial

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Abbreviations: CALGB, Cancer and Leukemia Group B (now part of Alliance for Clinical Trials in Oncology); CI, confidence interval; CIMP, CpG island methylator phenotype; ECOG, Eastern Cooperative Oncology Group; FFPE, formalin-fixed paraffin-embedded; FU/LV, 5-fluorouracil and leucovorin; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; IFL, irinotecan 5-fluorouracil and leucovorin; MSI, microsatellite instability; NHS, Nurses' health study; PGE_2, prostaglandin E_2.

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Abstract ***Background:*** Prostaglandin-endoperoxide synthase 2 (*PTGS2*, cyclooxygenase-2, COX-2)-prostaglandin E_2 (PGE_2) pathway promotes tumour progression. Considering evidence suggesting increased PGE_2 synthesis by *BRAF* mutation in tumour cells, we hypothesised that the association of tumour *PTGS2* (COX-2) expression with colorectal cancer mortality might be stronger in *BRAF*-mutated tumours than in *BRAF*-wild-type tumours.
Methods: Using 1708 patients, including 1200 stage I-IV colorectal carcinoma cases in the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS) and 508 stage III colon cancer cases in a National Cancer Institute–sponsored randomised controlled trial of adjuvant therapy (CALGB/Alliance 89803), we evaluated tumour *PTGS2* (COX-2) expression status using immunohistochemistry. We examined the prognostic association of *PTGS2* (COX-2) expression in strata of *BRAF* mutation status by multivariable Cox proportional hazards regression models to adjust for potential confounders, including disease stage, tumour differentiation, microsatellite instability status and *KRAS* and *PIK3CA* mutations.
Results: In NHS and HPFS, the association of *PTGS2* (COX-2) expression with colorectal cancer-specific survival differed by *BRAF* mutation status ($P_{interaction} = 0.0005$); compared with *PTGS2* (COX-2)-negative/low carcinomas, the multivariable-adjusted hazard ratios for *PTGS2* (COX-2)-high carcinomas were 2.44 (95% confidence interval, 1.39–4.28) in *BRAF*-mutated cases and 0.82 (95% confidence interval, 0.65–1.04) in *BRAF*-wild-type cases. Differential prognostic associations of *PTGS2* (COX-2) expression in strata of *BRAF* mutation status were similarly observed in CALGB/Alliance 89803 trial ($P_{interaction} = 0.03$).
Conclusions: The association of tumour *PTGS2* (COX-2) expression with colorectal cancer mortality is stronger in *BRAF*-mutated tumours than in *BRAF*-wild-type tumours, supporting interactive roles of *PTGS2* (COX-2) expression and *BRAF* mutation statuses in prognostication of patients with colorectal cancer; ClinicalTrials.gov Identifier, NCT00003835.

1. Introduction

Prostaglandin-endoperoxide synthase 2 (*PTGS2*, cyclooxygenase-2, COX-2) regulates the synthesis of prostaglandin E_2 (PGE_2), which provokes chronic inflammation and plays important roles in the development of colorectal cancer [1–4]. Epidemiological studies have shown that regular use of the PTGS (COX) inhibitor aspirin is associated with lower colorectal cancer incidence and mortality [5–10]. Evidence has reinforced the theory that the *PTGS2* (COX-2)-PGE_2 pathway plays a critical role in suppression of anti-tumour immunity in the tumour microenvironment [11–16]. Our incomplete knowledge of the interactions between the immune system and cancer proves that there is a significant need for transdisciplinary integrated analyses of cancer and immunity [17–19].

Colon and rectal cancers consist of heterogeneous diseases with tumour cells possessing varying sets of genetic and epigenetic alterations [20], influenced by host-tumour interactions [21]. A mutation in *BRAF* is present in approximately 10–15% of colorectal cancers [22–24]. *BRAF* mutation in colorectal cancer is associated with high-level CpG island methylator phenotype

(CIMP), which is associated with microsatellite instability (MSI) [25]. Considering the association between *BRAF* mutation and worse clinical outcome in patients with colorectal cancer [26,27], further developments of effective treatment strategies are required for *BRAF*-mutated patients with colorectal cancer [23]. Emerging evidence indicates that upregulation of the *RAF-MAPK* pathway by *BRAF* mutation may activate *PTGS2* (COX-2) in tumour cells to increase the production of PGE_2 [28,29]. Therefore, we hypothesised that the association of tumour *PTGS2* (COX-2) expression with colorectal cancer mortality might be stronger in *BRAF*-mutated tumours than in *BRAF*-wild-type tumours.

To test this hypothesis, we used molecular pathological epidemiology databases of 1708 patients, including 1200 stage I-IV colorectal cancer cases in two large U.S. prospective cohort studies and 508 stage III colon cancer cases in a randomised controlled trial of adjuvant therapy.

2. Methods

2.1. Study population

We used the database on colorectal cancer cases within two prospective cohort studies in the U.S.: the Nurses' Health Study (NHS, 121,701 women aged 30–55 years followed up since 1976) and the Health Professionals Follow-up Study (HPFS, 51,529 men aged 40–75 years followed up since 1986) [6]. Every two years, study participants have been sent follow-up questionnaires to collect information on lifestyle factors and medical history, including physician-confirmed diseases. The National Death Index was used to confirm deaths of study participants and to identify unreported lethal colorectal cancer cases. Participating physicians reviewed medical records to confirm diagnoses of colorectal cancer, record tumour characteristics [e.g. size, location and the American Joint Committee on Cancer tumour, node, and metastases (TNM) classification] and record causes of deaths for participants who died. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from hospitals where participants diagnosed with colorectal cancer underwent tumour resection. For this analysis, we included 1200 patients with available data on tumour *PTGS2* (COX-2) expression and *BRAF* mutation status. We included both colon and rectal cancers based on the colorectal continuum model [30]. Patients were followed up until death or the end of follow-up (January 1, 2014 for the HPFS; June 30, 2014 for the NHS), whichever occurred first. Written informed consent was obtained from all study participants. This study was approved by the institutional review boards of Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital (Boston, MA, USA).

As a validation set, we used 508 patients with stage III colon cancer with available data on tumour *PTGS2* (COX-2) expression and *BRAF* mutation status within Cancer and Leukemia Group B (CALGB) 89803 trial. CALGB is now part of the Alliance for Clinical Trials in Oncology. CALGB/Alliance 89803 (ClinicalTrials.gov NCT000038350) is a National Cancer Institute–sponsored adjuvant therapy trial for stage III colon cancer, comparing weekly 5-fluorouracil and leucovorin (FU/LV) and weekly irinotecan, 5-fluorouracil and leucovorin (IFL) [26]. Between April 1999 and April 2001, 1264 patients were enrolled in the treatment trial. The details of this study have been described elsewhere [26]. Data collection and statistical analyses were conducted by the Alliance Statistics and Data Center at Duke University Medical Center and Mayo Clinic. Data quality was ensured by review of data by the Alliance Statistics and Data Center. All analyses were based on the study database frozen on November 9, 2009. Written informed consent was obtained from all patients. This study was approved by the institutional review board of each institution.

In NHS and HPFS, a single pathologist (S.O.), who was unaware of other data, conducted a centralised review of haematoxylin and eosin–stained tissue sections of all colorectal cancer cases. Tumour differentiation was categorised as well to moderate or poor (>50% versus $\leq$ 50% glandular area, respectively).

2.2. Immunohistochemistry for PTGS2 (COX-2) expression

We constructed tissue microarrays that included up to four cores from colorectal cancer blocks and up to two cores from normal tissue blocks from the NHS and HPFS cohorts [5]. Immunohistochemistry for *PTGS2* (COX-2) was performed using an anti-*PTGS2* (COX-2) antibody (dilution 1:300; Cayman Chemical, Ann Arbor, MI, USA) [5]. We used whole-tissue sections for immunohistochemical analysis in the CALGB/Alliance 89803 set. Tumour *PTGS2* (COX-2) expression level, compared with adjacent normal colonic epithelium, was evaluated by a single pathologist (S.O.) and categorised as negative/low or high. A selected sample of 124 tumours was examined by a second pathologist (T.M.); concordance between the two observers was 0.85 ($\kappa = 0.69$) [7].

2.3. Analyses of microsatellite instability and KRAS, BRAF and PIK3CA mutations

DNA was extracted from archival FFPE tissue blocks using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Microsatellite instability (MSI) status [25,26] and mutation statuses for *KRAS* [26,30], *BRAF* [26,30] and *PIK3CA* [26,30] were determined.

Table 1
Characteristics of colorectal cancer cases according to *BRAF* mutation status in the Nurses' Health Study (NHS), Health Professionals Follow-up Study (HPFS) and CALGB/Alliance 89803 trial.

Characteristic[a]	NHS			HPFS			CALGB/Alliance 89803 trial		
	All cases (N = 676)	*BRAF* mutation status		All cases (N = 524)	*BRAF* mutation status		All cases (N = 508)	*BRAF* mutation status	
		Wild-type (N = 542)	Mutant (N = 134)		Wild-type (N = 483)	Mutant (N = 41)		Wild-type (N = 433)	Mutant (N = 75)
Mean age ± SD (years)	66.4 ± 8.2	65.8 ± 8.3	68.9 ± 7.3	70.7 ± 8.8	70.6 ± 8.7	71.3 ± 9.6	59.9 ± 11.5	58.7 ± 11.6	66.5 ± 8.0
Sex									
Male	–	–	–	524	483	41	276 (54%)	247 (57%)	29 (39%)
Female	676	542	134	–	–	–	232 (46%)	186 (43%)	46 (61%)
Year of diagnosis									
1995 or before	249 (37%)	211 (39%)	38 (28%)	212 (40%)	198 (41%)	14 (34%)	–	–	–
1996–2000	240 (36%)	191 (35%)	49 (37%)	162 (31%)	147 (30%)	15 (37%)	345 (68%)	290 (67%)	55 (73%)
2001–2008	187 (28%)	140 (26%)	47 (35%)	150 (29%)	138 (29%)	12 (29%)	163 (32%)	143 (33%)	20 (27%)
Family history of colorectal cancer in first-degree relative(s)									
Absent	534 (79%)	429 (80%)	105 (78%)	421 (80%)	388 (80%)	33 (80%)	421 (84%)	363 (85%)	58 (77%)
Present	138 (21%)	109 (20%)	29 (22%)	102 (20%)	94 (20%)	8 (20%)	82 (16%)	65 (15%)	17 (23%)
Tumour location									
Caecum	99 (15%)	82 (15%)	17 (13%)	109 (21%)	101 (21%)	8 (20%)	127 (25%)	104 (24%)	23 (31%)
Ascending to transverse	233 (35%)	139 (26%)	94 (71%)	125 (24%)	100 (21%)	25 (61%)	162 (32%)	117 (27%)	45 (61%)
Descending to sigmoid	201 (30%)	184 (34%)	17 (13%)	169 (32%)	163 (34%)	6 (15%)	214 (43%)	208 (48%)	6 (8.1%)
Rectum	141 (21%)	136 (25%)	5 (3.8%)	119 (23%)	117 (24%)	2 (4.9%)	–	–	–
Tumour differentiation									
Well to moderate	592 (88%)	498 (92%)	94 (70%)	486 (93%)	458 (95%)	28 (68%)	381 (76%)	342 (79%)	39 (53%)
Poor	81 (12%)	41 (7.6%)	40 (30%)	35 (6.7%)	22 (4.6%)	13 (32%)	123 (24%)	89 (21%)	34 (47%)
pT stage (depth of tumour invasion)									
pT1 (submucosa)	71 (11%)	61 (12%)	10 (7.6%)	55 (12%)	54 (13%)	1 (2.7%)	12 (2.4%)	12 (2.8%)	0 (0%)
pT2 (muscularis propria)	112 (18%)	97 (19%)	15 (11%)	113 (24%)	106 (25%)	7 (19%)	45 (9.0%)	37 (8.6%)	8 (11%)
pT3 (subserosa)	410 (64%)	313 (62%)	97 (73%)	281 (60%)	256 (60%)	25 (68%)	412 (82%)	354 (83%)	58 (78%)
pT4 (serosa or other organs)	43 (6.8%)	33 (6.6%)	10 (7.6%)	18 (3.9%)	14 (3.3%)	4 (11%)	33 (6.6%)	25 (5.8%)	8 (11%)
pN stage (number of positive lymph nodes)									
pN0 (0)	377 (62%)	298 (61%)	79 (63%)	293 (65%)	269 (65%)	24 (67%)	–	–	–
pN1 (1–3)	140 (23%)	115 (24%)	25 (20%)	104 (23%)	96 (23%)	8 (22%)	319 (63%)	278 (65%)	41 (55%)
pN2 (≥4)	94 (15%)	72 (15%)	22 (17%)	55 (12%)	51 (12%)	4 (11%)	186 (37%)	153 (35%)	33 (45%)
AJCC disease stage									
I	147 (23%)	126 (25%)	21 (16%)	128 (28%)	121 (29%)	7 (18%)	–	–	–
II	209 (33%)	155 (31%)	54 (41%)	144 (31%)	128 (30%)	16 (40%)	–	–	–
III	186 (29%)	156 (31%)	30 (23%)	129 (28%)	120 (29%)	9 (23%)	508	433	75
IV	97 (15%)	71 (14%)	26 (20%)	59 (13%)	51 (12%)	8 (20%)	–	–	–
MSI status									
MSI-high	126 (19%)	52 (10%)	74 (56%)	58 (11%)	36 (7.6%)	22 (54%)	86 (17%)	53 (12%)	33 (44%)
Non-MSI-high	534 (81%)	476 (90%)	58 (44%)	457 (89%)	438 (92%)	19 (46%)	421 (83%)	379 (88%)	42 (56%)
KRAS mutation									
Wild-type	424 (64%)	296 (55%)	128 (96%)	285 (55%)	246 (52%)	39 (95%)	322 (64%)	248 (58%)	74 (99%)
Mutant	243 (36%)	238 (45%)	5 (3.8%)	232 (45%)	230 (48%)	2 (4.9%)	180 (36%)	179 (42%)	1 (1.3%)
PIK3CA mutation									
Wild-type	524 (86%)	414 (85%)	110 (88%)	403 (83%)	373 (83%)	30 (75%)	378 (88%)	318 (87%)	60 (92%)
Mutant	87 (14%)	72 (15%)	15 (12%)	85 (17%)	75 (17%)	10 (25%)	54 (13%)	49 (13%)	5 (7.6%)
PTGS2 (COX-2) expression									
Negative/low	260 (38%)	186 (34%)	74 (55%)	202 (39%)	180 (37%)	22 (54%)	337 (66%)	290 (67%)	47 (63%)
High	416 (62%)	356 (66%)	60 (45%)	322 (61%)	303 (63%)	19 (46%)	171 (34%)	143 (33%)	28 (37%)
Performance status (ECOG)[b]									
0	–	–	–	–	–	–	384 (76%)	333 (77%)	51 (69%)
1–2	–	–	–	–	–	–	120 (24%)	97 (23%)	23 (31%)
Treatment arm									
FU/LV	–	–	–	–	–	–	266 (52%)	233 (54%)	33 (44%)
IFL	–	–	–	–	–	–	242 (48%)	200 (46%)	42 (56%)

(*continued on next page*)

Table 1 (*continued*)

Characteristic[a]	NHS			HPFS			CALGB/Alliance 89803 trial		
	All cases (N = 676)	*BRAF* mutation status		All cases (N = 524)	*BRAF* mutation status		All cases (N = 508)	*BRAF* mutation status	
		Wild-type (N = 542)	Mutant (N = 134)		Wild-type (N = 483)	Mutant (N = 41)		Wild-type (N = 433)	Mutant (N = 75)
Clinical bowel perforation or obstruction									
Absent	–	–	–	–	–	–	384 (76%)	328 (76%)	56 (75%)
Present	–	–	–	–	–	–	124 (24%)	105 (24%)	19 (25%)

Abbreviations: AJCC, American Joint Committee on Cancer; ECOG, Eastern Cooperative Oncology Group; FU/LV, 5-fluorouracil and leucovorin; HPFS, Health Professionals Follow-up Study; IFL, irinotecan, 5-fluorouracil and leucovorin; MSI, microsatellite instability; NHS, Nurses' Health Study; SD, standard deviation.

[a] Percentage indicates the proportion of patients with a specific clinical, pathologic or molecular characteristic among all patients or in strata of *BRAF* mutation status within each cohort.

[b] Definition of performance status (ECOG): 0, fully active, able to carry on all pre-disease performance without restriction; 1, restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature; 2, ambulatory and capable of all self-care but unable to carry out any work activities (up and about more than 50% of waking hours).

2.4. Statistical analysis

Descriptive statistics of patient clinical features were presented according to dichotomised *BRAF* status for categorical variables or mean and standard deviation for continuous variables. Our primary hypothesis testing focused on the assessment of a statistical interaction (using the Wald test on the cross-product) between tumour *PTGS2* (COX-2) expression (negative/low versus high) and *BRAF* mutation (mutant versus wild-type) in the Cox proportional hazards regression model for colorectal cancer-specific survival. We also estimated the hazard ratios (HRs) for *PTGS2* (COX-2)-high versus *PTGS2* (COX-2)-negative/low cases in strata of *BRAF* mutation status using a re-parameterisation of the interaction term in a single regression model. All other analyses, including evaluation of individual HR estimates, represent secondary analyses. We used the two-sided α level of 0.005 for our primary hypothesis testing on new discovery [31]. To account for the multiple hypothesis testing in secondary analyses, we interpreted the results of our secondary analyses conservatively. All statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA), and all P values were two-sided. The authors had access to the study data and had reviewed and approved the final manuscript.

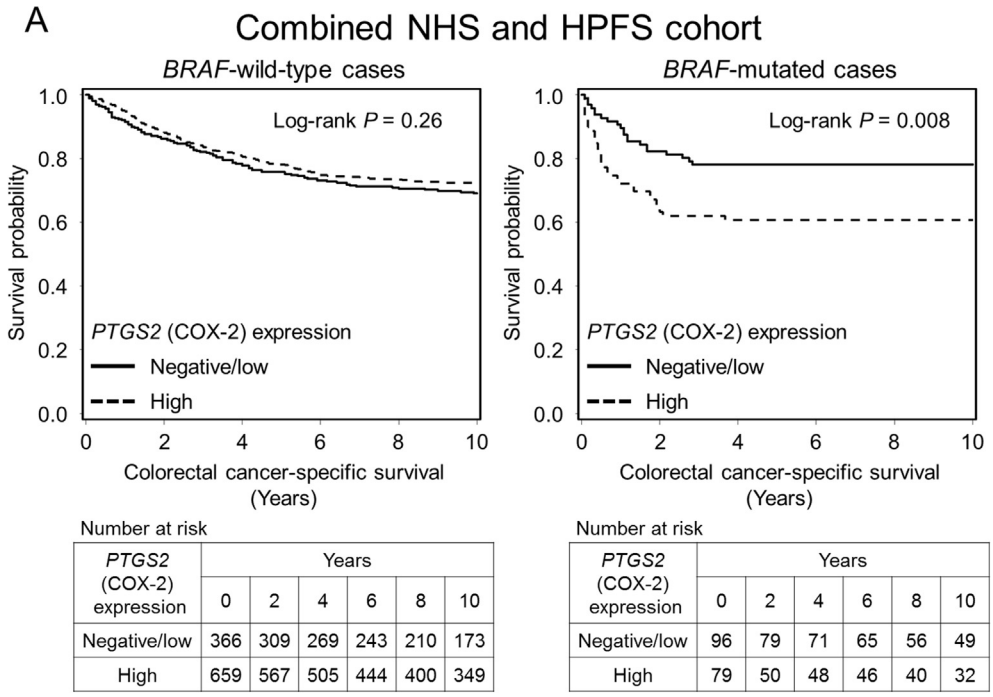
In NHS and HPFS, survival time was defined as the period from colorectal cancer diagnosis to death or the end of follow-up. For analyses of colorectal cancer-specific survival, participants who died from other causes were censored at the time of death. In CALGB/Alliance 89803, the definitions of survival time were as follows: (i) colorectal cancer-specific survival, defined as the time from study enrolment to death from the primary colon cancer; (ii) recurrence-free survival, defined as the time from study enrolment to tumour recurrence or occurrence of the new primary colon tumour; (iii) disease-free survival, defined as the time from study enrolment to tumour recurrence, occurrence of the new primary colon tumour or death from any cause and (iv) overall survival, defined as the time from study enrolment to death from any cause [26]. For recurrence-free survival, patients who died without known tumour recurrence were censored at the last documented evaluation by a treating provider.

In NHS and HPFS, the multivariable Cox proportional hazards regression models initially included sex, age at diagnosis (continuous), year of diagnosis (continuous), family history of colorectal cancer in any first-degree relative (present versus absent), tumour location (proximal colon versus distal colon versus rectum), tumour differentiation (well/moderate versus poor), disease stage (I-II versus III-IV), MSI status (high versus non-high), *KRAS* (mutant versus wild-type) and *PIK3CA* (mutant versus wild-type). In CALGB/Alliance 89803, the multivariable Cox model initially included sex, age at diagnosis (continuous), year of diagnosis (continuous), family history of colorectal cancer in any first-degree relative (present versus absent), tumour location (proximal colon versus distal colon), tumour differentiation (well/moderate versus poor), pT stage (T1 versus T2 versus T3 versus T4), pN stage (N1 versus N2), treatment arm (FU/LV versus IFL), Eastern Cooperative Oncology Group (ECOG) performance status (0 versus 1–2), obstruction or perforation (present versus absent), MSI status (high versus non-high), *KRAS* (mutant versus wild-type) and *PIK3CA* (mutant versus wild-type). A backward elimination was conducted with a threshold of $P = 0.05$ to select variables for the final models. Cases with missing data were included in the majority category of a given categorical covariate to avoid excluding patients with missing data (Supplementary Table S1). For cases with missing information on *PIK3CA* mutation in CALGB/Alliance 89803, because the missing percentage is higher (15%), we assigned a separate missing indicator variable. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown). The assumption of proportional hazards was satisfied using the assessment of a time-

varying covariate, i.e. the cross-product of tumour *PTGS2* (COX-2) expression and survival time in strata of *BRAF* mutation status ($P > 0.12$). The Kaplan–Meier method was used to describe the distribution of colorectal cancer-specific survival, and the log-rank test was used to compare survival probabilities across *PTGS2* (COX-2) expression status.

3. Results

We included 1200 patients with colorectal cancer in NHS and HPFS (Table 1). During the median follow-up time of 15.8 years (interquartile range, 12.0–19.0 years) for all censored patients, there were 745 all-cause deaths, including 352 colorectal cancer-specific deaths.

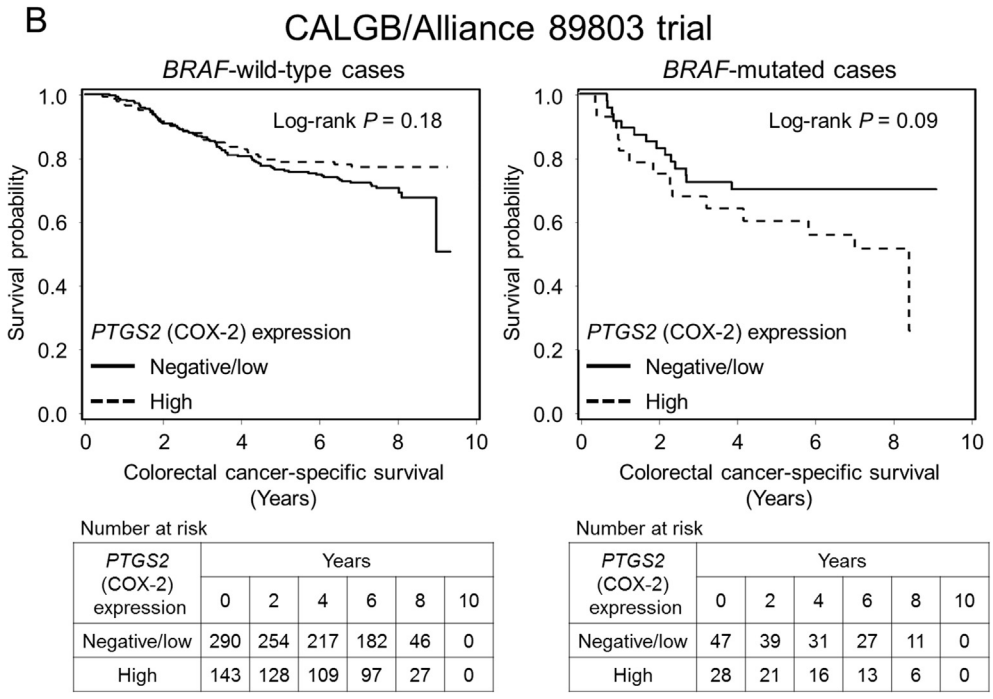


Number at risk

PTGS2 (COX-2) expression	Years					
	0	2	4	6	8	10
Negative/low	366	309	269	243	210	173
High	659	567	505	444	400	349

Number at risk

PTGS2 (COX-2) expression	Years					
	0	2	4	6	8	10
Negative/low	96	79	71	65	56	49
High	79	50	48	46	40	32



Number at risk

PTGS2 (COX-2) expression	Years					
	0	2	4	6	8	10
Negative/low	290	254	217	182	46	0
High	143	128	109	97	27	0

Number at risk

PTGS2 (COX-2) expression	Years					
	0	2	4	6	8	10
Negative/low	47	39	31	27	11	0
High	28	21	16	13	6	0

Fig. 1. Kaplan–Meier analysis of colorectal cancer-specific survival according to tumour *PTGS2* (COX-2) expression status in strata of *BRAF* mutation status. A, Combined Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS) cohort; B, CALGB/Alliance 89803 trial. *P* values were calculated using the log-rank test (two-sided).

Table 2
Tumour *PTGS2* (COX-2) expression and colorectal cancer survival according to *BRAF* mutation status in the Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS).

	No. of cases	Colorectal cancer-specific survival			Overall survival		
		No. of events	Univariable HR (95% CI)	Multivariable HR (95% CI)[a]	No. of events	Univariable HR (95% CI)	Multivariable HR (95% CI)[a]
Combined NHS and HPFS cohort							
All cases							
PTGS2 (COX-2) expression							
Negative/low	462	134	1 (referent)	1 (referent)	288	1 (referent)	1 (referent)
High	738	218	1.01 (0.81–1.25)	0.97 (0.77–1.21)	457	0.94 (0.81–1.09)	0.98 (0.84–1.14)
BRAF-wild-type							
PTGS2 (COX-2) expression							
Negative/low	366	113	1 (referent)	1 (referent)	231	1 (referent)	1 (referent)
High	659	187	0.89 (0.71–1.13)	0.82 (0.65–1.04)	401	0.89 (0.76–1.05)	0.91 (0.77–1.07)
BRAF-mutant							
PTGS2 (COX-2) expression							
Negative/low	96	21	1 (referent)	1 (referent)	57	1 (referent)	1 (referent)
High	79	31	2.16 (1.24–3.76)	2.44 (1.39–4.28)	56	1.42 (0.98–2.05)	1.45 (1.00–2.11)
$P_{interaction}$[b]			0.004	0.0005		0.02	0.02
NHS							
All cases							
PTGS2 (COX-2) expression							
Negative/low	260	79	1 (referent)	1 (referent)	157	1 (referent)	1 (referent)
High	416	130	1.03 (0.78–1.36)	0.97 (0.72–1.30)	242	0.93 (0.76–1.14)	0.97 (0.79–1.19)
BRAF-wild-type							
PTGS2 (COX-2) expression							
Negative/low	186	64	1 (referent)	1 (referent)	118	1 (referent)	1 (referent)
High	356	107	0.85 (0.63–1.16)	0.78 (0.57–1.07)	199	0.82 (0.65–1.03)	0.84 (0.67–1.06)
BRAF-mutant							
PTGS2 (COX-2) expression							
Negative/low	74	15	1 (referent)	1 (referent)	39	1 (referent)	1 (referent)
High	60	23	2.23 (1.16–4.28)	2.42 (1.24–4.72)	43	1.70 (1.10–2.63)	1.55 (1.00–2.40)
$P_{interaction}$[b]			0.009	0.003		0.004	0.02
HPFS							
All cases							
PTGS2 (COX-2) expression							
Negative/low	202	55	1 (referent)	1 (referent)	131	1 (referent)	1 (referent)
High	322	88	0.98 (0.70–1.37)	0.96 (0.68–1.35)	215	0.94 (0.76–1.17)	1.02 (0.82–1.28)
BRAF-wild-type							
PTGS2 (COX-2) expression							
Negative/low	180	49	1 (referent)	1 (referent)	113	1 (referent)	1 (referent)
High	303	80	0.94 (0.66–1.34)	0.88 (0.61–1.26)	202	0.98 (0.78–1.23)	1.00 (0.80–1.27)

BRAF-mutant							
PTGS2 (COX-2) expression							
Negative/low	22	6	1 (referent)	1 (referent)	18	1 (referent)	1 (referent)
High	19	8	1.92 (0.66–5.54)	2.20 (0.71–6.81)	13	0.77 (0.38–1.58)	1.22 (0.58–2.57)
$P_{interaction}$ [b]			0.21	0.13		0.54	0.62

Abbreviations: CI, confidence interval; HR, hazard ratio; HPFS, Health Professionals Follow-up Study; NHS, Nurses' Health Study.

[a] The initial multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumour location, tumour differentiation, disease stage, microsatellite instability and *KRAS*, *BRAF* (except for *BRAF*-stratified analyses) and *PIK3CA* mutations. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models. The variables that remained in the final models are shown in Supplementary Table S2.

[b] $P_{interaction}$ (two-sided) was calculated using the Wald test on the cross-product term of *PTGS2* (COX-2) expression (negative/low versus high) and *BRAF* mutation (wild-type versus mutant) in the Cox regression model.

In the combined NHS and HPFS cohort, we examined the prognostic association of tumour *PTGS2* (COX-2) expression status in strata of *BRAF* mutation status. In Kaplan–Meier survival analyses, tumour *PTGS2* (COX-2) expression was associated with shorter colorectal cancer-specific survival in *BRAF*-mutated cases, but not in *BRAF*-wild-type cases (Fig. 1A). In our primary hypothesis testing using Cox regression analysis, we observed a statistically significant interaction between tumour *PTGS2* (COX-2) expression and *BRAF* mutation status in colorectal cancer-specific survival analysis ($P_{interaction}$ = 0.0005; Table 2 and Supplementary Table S2). After adjustment for potentially prognostic factors, high tumour *PTGS2* (COX-2) expression was significantly associated with shorter colorectal cancer-specific survival in *BRAF*-mutated tumours [multivariable HR, 2.44; 95% confidence interval (CI), 1.39–4.28], but not in *BRAF*-wild-type tumours (multivariable HR, 0.82; 95% CI, 0.65–1.04). These interactive associations between *PTGS2* (COX-2) expression and *BRAF* mutation status in colorectal cancer survival were observed in both the NHS and HPFS cohorts when examined separately, although statistical power was limited for cohort-specific analyses (Table 2 and Supplementary Fig. S1).

In analyses limited to patients with stage I-III colorectal cancer, a similar differential prognostic association of tumour *PTGS2* (COX-2) expression by *BRAF* mutation status was observed, although statistical power was limited (Supplementary Table S3).

We validated our findings using an independent cohort of 508 patients with stage III colon cancer in CALGB/Alliance 89803 (Table 1). The median age was 59.9 years, 46% were women and 76% had a performance status (ECOG) of 0. During the median follow-up time of 7.6 years (interquartile range, 7.1–8.0 years) for all censored patients, there were 159 all-cause deaths, including 140 colon cancer-specific deaths. The multivariable HR for colorectal cancer-specific survival for *PTGS2* (COX-2)-high cases compared to *PTGS2* (COX-2)-negative/low cases was higher in the *BRAF*-mutated group (multivariable HR, 1.85; 95% CI, 0.88–3.88) than in the *BRAF*-wild-type group (multivariable HR, 0.74; 95% CI, 0.49–1.12; $P_{interaction}$ = 0.03; Table 3 and Supplementary Table S4). Similar differential survival association was observed for recurrence-free survival ($P_{interaction}$ = 0.005; Fig. 1B) and disease-free survival ($P_{interaction}$ = 0.006).

4. Discussion

To test our hypothesis that the association of tumour *PTGS2* (COX-2) expression with colorectal cancer mortality might be stronger in *BRAF*-mutated tumours than in *BRAF*-wild-type tumours, we conducted this study using the two U.S. prospective cohort studies and

Table 3
Tumour *PTGS2* (COX-2) expression and colorectal cancer survival according to *BRAF* mutation status in CALGB/Alliance 89803 trial.

	No. of cases	Colorectal cancer-specific survival			Recurrence-free survival			Disease-free survival			Overall survival		
		No. of events	HR (95% CI) Univariable	HR (95% CI) Multivariable[a]	No. of events	HR (95% CI) Univariable	HR (95% CI) Multivariable[a]	No. of events	HR (95% CI) Univariable	HR (95% CI) Multivariable[a]	No. of events	HR (95% CI) Univariable	HR (95% CI) Multivariable[a]
All cases													
PTGS2 (COX-2) expression													
Negative/low	337	95	1 (referent)	1 (referent)	128	1 (referent)	1 (referent)	141	1 (referent)	1 (referent)	108	1 (referent)	1 (referent)
High	171	45	0.93 (0.65–1.33)	0.88 (0.61–1.25)	54	0.82 (0.60–1.13)	0.78 (0.57–1.07)	60	0.82 (0.61–1.11)	0.79 (0.58–1.07)	51	0.93 (0.67–1.30)	0.87 (0.62–1.22)
BRAF-wild-type													
PTGS2 (COX-2) expression													
Negative/low	290	81	1 (referent)	1 (referent)	113	1 (referent)	1 (referent)	123	1 (referent)	1 (referent)	91	1 (referent)	1 (referent)
High	143	31	0.76 (0.50–1.14)	0.74 (0.49–1.12)	39	0.66 (0.46–0.95)	0.64 (0.44–0.92)	44	0.68 (0.48–0.96)	0.67 (0.48–0.95)	36	0.78 (0.53–1.15)	0.79 (0.53–1.16)
BRAF-mutant													
PTGS2 (COX-2) expression													
Negative/low	47	14	1 (referent)	1 (referent)	15	1 (referent)	1 (referent)	18	1 (referent)	1 (referent)	17	1 (referent)	1 (referent)
High	28	14	1.93 (0.92–4.04)	1.85 (0.88–3.88)	15	2.11 (1.03–4.32)	2.04 (0.98–4.21)	16	1.90 (0.97–3.72)	1.96 (0.99–3.87)	15	1.71 (0.85–3.42)	1.43 (0.71–2.89)
$P_{interaction}$[b]			0.03	0.03		0.005	0.005		0.008	0.006		0.05	0.15

Abbreviations: CI, confidence interval; HR, hazard ratio.

[a] The initial multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumour location, tumour differentiation, pT stage, pN stage, treatment arm, performance status, perforation or obstruction, microsatellite instability and *KRAS, BRAF* (except for *BRAF*-stratified analyses) and *PIK3CA* mutations. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models. The variables that remained in the final models are shown in Supplementary Table S4.

[b] $P_{interaction}$ (two-sided) was calculated using the Wald test on the cross-product term of *PTGS2* (COX-2) expression (negative/low versus high) and *BRAF* mutation (wild-type versus mutant) in the Cox regression model.

the randomised controlled trial. We observed a differential prognostic association of tumour *PTGS2* (COX-2) expression in strata of *BRAF* mutation status.

The *PTGS2* (COX-2)-PGE_2 pathway plays key roles in tumour progression in a variety of tumour types, including colorectal cancer [1,2]. Evidence indicates that PGE_2 overproduction may enable tumour cells to evade host immune surveillance mechanisms through accumulation of myeloid-derived suppressor cells, suppression of dendritic cells and evasion of the T cell–mediated anti-tumour immune response [12–14,32]. Considering that the immunomodulatory effect by PGE_2 inhibition can synergise with immune checkpoint blockade therapies targeting *PDCD1* (programmed cell death 1, PD-1) or *CD274* (*PDCD1* ligand 1, PD-L1) in various cancer types [12,33,34], a better understanding of the roles of tumour *PTGS2* (COX-2) expression in the context of tumour-immune interactions would have considerable clinical implications [35].

Gain-of-function *BRAF* mutation leads to accelerated production and activity of a number of critical cellular substrates involved in cell proliferation and survival through phosphorylation of the *MAPK* kinases [23,24]. Studies indicate that *BRAF* mutation has been associated with high-level CIMP and worse clinical outcomes in colorectal cancer [22–27,36,37]. Evidence suggests that *BRAF* mutation may increase microRNA *MIR21* (miR-21) expression level through the activation of the *MAPK* and *STAT3* signalling pathways [28,29,38]. Given that *MIR21* increases local levels of PGE_2 by suppressing PGE_2 degradation [29,38,39], the prognostic association of *PTGS2* (COX-2) expression might be especially pronounced in *BRAF*-mutated colorectal cancer. Overall, increased synthesis of PGE_2 resulting from *BRAF* mutation and heightened *PTGS2* (COX-2) activity may serve as one possible pathway through which the survival of patients with colorectal cancer with this combination is affected.

We acknowledge limitations in our study. Data on cancer recurrence were unavailable in NHS and HPFS. However, colorectal cancer-specific survival can be considered a reasonable cancer-specific outcome in a population-based study with long-term follow-up because median survival for recurrent (metastatic) colorectal cancer was approximately 10–20 months during the period of this study. Moreover, we found that the association of tumour *PTGS2* (COX-2) expression with recurrence-free survival and disease-free survival stratified by *BRAF* mutation status remained consistent in the validation set of CALGB/Alliance 89803. Data on cancer treatment were also limited in the NHS and HPFS cohorts. However, the decision to undergo chemotherapy and the specific regimen utilised would be unlikely to differ substantially according to tumour *PTGS2* (COX-2) expression in resected specimens as these data were not available to treating physicians. We also recognise another limitation that the present study is an observational cohort study, not an intervention trial such as a randomised controlled trial using aspirin and/or *BRAF* inhibitor. Therefore, we cannot conclude that inhibiting *PTGS2* (COX-2) in *BRAF*-mutated colorectal cancer is an effective therapeutic strategy. In the present study, we certainly observed that the association of tumour *PTGS2* (COX-2) expression with colorectal cancer mortality is stronger in *BRAF*-mutated tumours than in *BRAF*-wild-type tumours, and further research is warranted to investigate the therapeutic roles of *BRAF* and *PTGS2* (COX-2) inhibitors in patients with this malignancy.

A major strength of this study is utilisation of a molecular pathological epidemiology database of rectal and colon cancer cases from the two large U.S. prospective cohort studies [40], which integrates clinicopathologic features, long-term survival data and tumour molecular features. This population-based colorectal cancer database enabled us to rigorously examine the interactive prognostic association of tumour *PTGS2* (COX-2) expression and *BRAF* mutation status while controlling for potential confounders. Use of the randomised controlled trial as a validation set was another significant strength of this study. The data of patients with colorectal cancer in our study were derived from a large number of hospitals from diverse locations within the U.S., which adds greatly to the generalisability of our findings.

In conclusion, we found a stronger association of tumour *PTGS2* (COX-2) expression with colorectal cancer mortality in *BRAF*-mutated tumours than in *BRAF*-wild-type tumours. Our population-based data suggest the potential of tumour *PTGS2* (COX-2) expression status as a prognostic biomarker in patients with *BRAF*-mutated colorectal cancer.

Conflicts of interest statement

A.T.C. previously served as a consultant for Bayer Healthcare and Pfizer Inc. for areas unrelated to this research. K.N. has participated in an advisory board for Bayer. No other conflict of interest exists. The other authors declare that they have no conflicts of interest.

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Use of standardised official symbols

We use Human Genome Organisation (HUGO)–approved official symbols (or root symbols) for genes, gene products and gene families, including BRAF, CD274, KRAS, MAPK, MIR21, NFKB, PDCD1, PIK3CA, PTGS2, RAF, STAT3 and WNT, all of which are described at www.genenames.org. The official symbols are italicised to differentiate from non-italicised colloquial names that are used along with the official symbols. This format enables readers to familiarise the official symbols for genes and gene products together with common colloquial names.

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The authors assume full responsibility for analyses and interpretation of these data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.01.022.

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