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ORIGINAL RESEARCH

Tumor SQSTM1 (p62) expression and T cells in colorectal cancer

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ABSTRACT

Evidence suggests that activation of autophagy in neoplastic cells potentiates antitumor immunity through cross-presentation of tumor-associated antigens to T cells and release of immune mediators. The SQSTM1 (sequestosome 1, p62) protein is degraded by activated autophagy, and might enhance immune response to tumor cells. We hypothesized that tumor SQSTM1 expression level might be inversely associated with T-cell densities in colorectal carcinoma tissue. We evaluated tumor SQSTM1 expression by immunohistochemistry in 601 rectal and colon cancer cases within the Nurses' Health Study and Health Professionals Follow-up Study. Ordinal logistic regression analyses were conducted to assess the association of tumor SQSTM1 expression with $CD3^+$, $CD8^+$, CD45RO $(PTPRC)^+$, or $FOXP3^+$ cell density in tumor tissue, controlling for potential confounders, including tumor status of microsatellite instability, CpG island methylator phenotype, long interspersed nucleotide element-1 methylation level, and *KRAS, BRAF*, and *PIK3CA* mutations. Tumor SQSTM1 expression level was inversely associated with $FOXP3^+$ cell density ($p_{trend} = 0.006$), but not with $CD3^+$, $CD8^+$, or $CD45RO^+$ cell density (with the adjusted α level of 0.01 for multiple hypothesis testing). For a unit increase in quartile categories of $FOXP3^+$ cell density, multivariable odds ratios were 0.66 [95% confidence interval (CI), 0.45–0.98] for intermediate-level SQSTM1 expression, and 0.55 (95% CI, 0.36–0.83) for high-level SQSTM1 expression, compared with low-level SQSTM1 expression. Tumor SQSTM1 expression is inversely associated with $FOXP3^+$ cell density in colorectal cancer tissue, suggesting a possible role of SQSTM1-expressing carcinoma cells on regulatory T cells in the tumor microenvironment.

Abbreviations: ATP, adenosine triphosphate; CI, confidence interval; CIMP, CpG island methylator phenotype; FFPE, formalin-fixed paraffin-embedded; LINE-1, long interspersed nucleotide element-1; MSI, microsatellite instability; MSS, microsatellite stable; OR, odds ratio; SD, standard deviation

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Introduction

Accumulating evidence attests a key role of T-cell-mediated adaptive immunity in inhibiting tumor evolution, and immunotherapy has emerged as a promising strategy to treat various cancers.[1-5] Autophagy is a homeostatic cellular recycling mechanism responsible for degrading cellular organelles and proteins. Autophagic activity in tumor cells may enhance extracellular release of immune mediators and cross-

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Use of standardized official symbols: We used HUGO (Human Genome Organization)-approved official symbols for genes and gene products, including BRAF, CACNA1G, CD274, CD3, CD8, CDKN2A, CRABP1, CTLA4, FOXP3, IGF2, KRAS, MLH1, NEUROG1, PDCD1, PIK3CA, PTPRC, RUNX3, SOCS1, and SQSTM1; all of which are described at www.genenames.org. Gene names are italicized, and gene product names are non-italicized.

presentation of tumor-associated antigens to T cells, thereby potentiating immune response to tumor cells.[6-19] Emerging evidence attests to a key role of tumor autophagic activity in modulating functions of T cells such as $CD8^+$ cytotoxic T cells and $FOXP3^+$ regulatory T cells.[17,20-22] The SQSTM1 (sequestosome 1, p62) protein is a ubiquitin-binding scaffold molecule that plays a key role in autophagic degradation of ubiquitinated proteins,[23-29] and the degradation of SQSTM1 with tumor-related antigen may promote T-cell-mediated immunity.[30-32]

Colorectal cancer represents a heterogeneous group of neoplasms resulting from genomic and epigenomic alterations, which influence and are influenced by tumor–host interactions.[33-36] A strong immune response to colorectal cancer manifested as high density of $CD3^+$, $CD8^+$, or CD45RO (PTPRC)$^+$ T cells has been consistently associated with better clinical outcome.[37-43] An enhanced infiltration of T cells in colorectal cancer tissue has been associated with specific tumor molecular status, including high-level microsatellite instability (MSI-high).[41-45] Studies have shown that SQSTM1 is overexpressed in colorectal cancer,[46,47] but significance of SQSTM1 expression in colorectal cancer needs to be investigated. We hypothesized that low-level tumor SQSTM1 expression (indicating high autophagic activity) might be associated with high T-cell densities in colorectal cancer tissue. Because the complexity of tumor–host immune interactions in human cancers cannot be exactly recapitulated by any *in vitro* or non-human models, analyses of tumor characteristics and immune cells in human cancer tissue are valuable.

To test our hypothesis, we examined tumor SQSTM1 expression in relation to $CD3^+$, $CD8^+$, $CD45RO^+$, or $FOXP3^+$ cell densities in cancer tissue of more than 600 human colorectal cancer cases within the two US-nationwide prospective cohort studies. A better understanding of the relationship between autophagy and immune cells in the tumor microenvironment may open new opportunities to target autophagy and immunity for colorectal cancer prevention and therapy.

Results

Tumor SQSTM1 (p62) expression in colorectal cancer

We examined immunohistochemical expression levels of the SQSTM1 protein in 601 cases of colorectal carcinoma within the two US-nationwide prospective cohort studies. Among the 601 colorectal cancer cases, 131 (22%), 271 (45%), and 199 (33%) tumors showed low-level, intermediate-level, and high-level SQSTM1 expression, respectively.

Clinical, pathological, and molecular characteristics according to the tumor SQSTM1 expression levels in colorectal cancer are summarized in Table 1. Tumor SQSTM1 expression level was not significantly associated with any of the characteristics examined ($p > 0.02$; with the adjusted α level of 0.003 for multiple hypothesis testing) (Table 1).

Association of tumor SQSTM1 expression with T-cell density in colorectal cancer

Table 2 shows the distribution of colorectal carcinoma cases according to the tumor SQSTM1 expression level and T-cell densities. Tumor SQSTM1 expression level was inversely correlated with $FOXP3^+$ cell density ($p = 0.001$, by Spearman correlation test) with the adjusted α level of 0.01. In our primary hypothesis testing, we conducted ordinal logistic regression analyses to assess the associations of the tumor SQSTM1 expression level (an ordinal predictor variable) with the density of $CD3^+$, $CD8^+$, $CD45RO^+$, or $FOXP3^+$ cells (an ordinal quartile outcome variable) in colorectal cancer tissue (Tables 3 and Table S1). Tumor SQSTM1 expression level was inversely associated with $FOXP3^+$ cell density in ordinal logistic regression analyses (all $p_{trend} \leq 0.006$; with the adjusted α level of 0.01). For a unit increase in quartile categories of $FOXP3^+$ cell density, the multivariable ORs were 0.66 [95% confidence interval (CI), 0.45–0.98] for cases with intermediate-level SQSTM1 expression and 0.55 (95% CI, 0.36–0.83) for those with high-level SQSTM1 expression, compared with those with low-level SQSTM1 expression. The tumor SQSTM1 expression level was not significantly associated with $CD3^+$, $CD8^+$, or $CD45RO^+$ cell density (all $p_{trend} > 0.05$; with the adjusted α level of 0.01).

Discussion

Using the database of the 601 colorectal cancer cases in the two US-nationwide prospective cohort studies, we found that higher tumor SQSTM1 expression was associated with lower density of $FOXP3^+$ cells in human colorectal cancer tissue. The association persisted after controlling for potential confounders, including the tumor statuses of MSI, CIMP and LINE-1 methylation level, which have been correlated with the abundance of tumor infiltrating T cells in colorectal cancer.[41-45] Although a replication in independent data sets is needed, our human population-based data suggest a possible role of autophagic activity of tumor cells in regulating host immunity in colorectal cancer microenvironment.

Colorectal cancer development is not only driven by genomic and epigenomic alterations of tumor cells but also influenced by tumor–host interactions.[48-53] The importance of analyses of human tumor characteristics and host immunity has been increasing.[37,54] In fact, higher densities of $CD3^+$, $CD8^+$, and $CD45RO^+$ cells in colorectal cancer tissue have been associated with better prognosis,[38-43,55] suggesting antitumor effects of these T cells in the tumor microenvironment. Therefore, there is a great need to identify potential molecular targets that can influence T-cell-mediated immune response to tumor.

$FOXP3^+$ regulatory T cells have been considered as an immunosuppressive subset of T lymphocytes, and are functionally and phenotypically diverse with various functional profiles.[56] Accumulating evidence indicates that function of $FOXP3^+$ regulatory T cells can be tailored for differing immune milieu and contexts, and that their roles for cancer progression (tumor-promoting or tumor-suppressive roles) appear to depend on tumor site and progression stage, probably reflecting alterations of the tumor microenvironment.[39,56] Although functional roles of $FOXP3^+$ cells in various types of cancers remain unclear, high density of $FOXP3^+$ cells has been generally associated with favorable outcome in colorectal cancer patients.[39-43,55] Particularly, abundant infiltration of T cells with low-intensity FOXP3 expression may be associated with

Table 1. Clinical, pathological, and molecular features of colorectal cancers according to tumor SQSTM1 (p62) expression level.

Characteristic*	Total no. ($n = 601$)	Tumor SQSTM1 expression level			p value†
		Low ($n = 131$)	Intermediate ($n = 271$)	High ($n = 199$)	
Mean age ± SD (years)	67.2 ± 8.5	66.6 ± 8.3	68.0 ± 8.3	66.6 ± 8.8	0.13
Sex					0.12
Female (NHS)	386 (64%)	93 (71%)	164 (61%)	129 (65%)	
Male (HPFS)	215 (36%)	38 (29%)	107 (39%)	70 (35%)	
Year of diagnosis					0.16
Prior to 1996	252 (42%)	59 (45%)	101 (37%)	92 (46%)	
1996–2000	242 (40%)	47 (36%)	115 (43%)	80 (40%)	
2001–2008	107 (18%)	25 (19%)	55 (20%)	27 (14%)	
Family history of colorectal cancer in first-degree relative(s)					0.82
Absent	470 (79%)	104 (81%)	213 (79%)	153 (78%)	
Present	123 (21%)	24 (19%)	57 (21%)	42 (22%)	
Tumor location					0.027
Cecum	111 (19%)	15 (12%)	58 (22%)	38 (19%)	
Ascending to transverse colon	185 (31%)	40 (31%)	85 (32%)	60 (30%)	
Splenic flexure to sigmoid	182 (30%)	38 (29%)	86 (32%)	58 (29%)	
Rectosigmoid and rectum	119 (20%)	37 (28%)	39 (14%)	43 (22%)	
Tumor differentiation					0.66
Well to moderate	541 (90%)	115 (89%)	248 (92%)	178 (89%)	
Poor	58 (10%)	14 (11%)	23 (8%)	21 (11%)	
Disease stage					0.54
I	122 (22%)	30 (25%)	59 (23%)	33 (18%)	
II	190 (34%)	38 (32%)	87 (34%)	65 (34%)	
III	169 (30%)	30 (25%)	79 (31%)	60 (32%)	
IV	86 (15%)	21 (18%)	34 (13%)	31 (16%)	
MSI status					0.68
MSI-low/MSS	494 (84%)	103 (82%)	228 (85%)	163 (83%)	
MSI-high	96 (16%)	23 (18%)	40 (15%)	33 (17%)	
CIMP status					0.98
Low/negative	500 (85%)	108 (84%)	225 (85%)	167 (85%)	
High	89 (15%)	20 (16%)	40 (15%)	29 (15%)	
KRAS mutation					0.038
Wild-type	348 (59%)	82 (64%)	142 (53%)	124 (64%)	
Mutant	241 (41%)	47 (36%)	124 (47%)	70 (36%)	
BRAF mutation					0.50
Wild-type	502 (85%)	106 (82%)	229 (86%)	167 (86%)	
Mutant	90 (15%)	24 (18%)	38 (14%)	28 (14%)	
PIK3CA mutation					0.51
Wild-type	463 (86%)	99 (84%)	209 (85%)	155 (88%)	
Mutant	78 (14%)	19 (16%)	38 (15%)	21 (12%)	
Mean LINE-1 methylation level ± SD (%)	60.8 ± 9.5	61.5 ± 9.9	60.3 ± 9.4	61.1 ± 9.4	0.40
Fusobacterium nucleatum DNA					0.033
Negative	412 (88%)	90 (86%)	190 (92%)	132 (84%)	
Low	29 (6%)	10 (10%)	10 (5%)	9 (6%)	
High	28 (6%)	5 (5%)	7 (3%)	16 (10%)	
Crohn's-like lymphoid reaction					0.43
Absent/low	356 (74%)	77 (76%)	156 (72%)	123 (75%)	
Intermediate	89 (19%)	18 (18%)	46 (21%)	25 (15%)	
High	36 (7%)	6 (6%)	14 (6%)	16 (10%)	
Peritumoral lymphocytic reaction					0.69
Absent/low	57 (10%)	14 (11%)	25 (9%)	18 (9%)	
Intermediate	464 (78%)	104 (80%)	209 (78%)	151 (76%)	
High	74 (12%)	12 (9%)	33 (12%)	29 (15%)	
Intratumoral periglandular reaction					0.41
Absent/low	55 (9%)	15 (12%)	24 (10%)	16 (8%)	
Intermediate	472 (79%)	105 (81%)	213 (79%)	154 (78%)	
High	69 (12%)	10 (8%)	31 (12%)	28 (14%)	
Tumor-infiltrating lymphocytes					0.70
Absent/low	443 (74%)	100 (77%)	196 (73%)	147 (74%)	
Intermediate	85 (14%)	20 (15%)	38 (14%)	27 (14%)	
High	67 (11%)	10 (8%)	33 (12%)	24 (12%)	

Abbreviations: CIMP, CpG island methylator phenotype; LINE-1, long interspersed nucleotide element-1; MSI, microsatellite instability; MSS, microsatellite stable; SD, standard deviation.

*Percentage (%) indicates the proportion of cases with a specific clinical, pathological, or molecular feature in colorectal cancer cases with each tumor SQSTM1 expression level.

†To assess associations between the ordinal categories of tumor SQSTM1 expression level and categorical data, the χ^2 test was performed. To compare mean age and mean LINE-1 methylation level, an analysis of variance was performed. We adjusted two-sided α level to 0.003 (= 0.05/18) by simple Bonferroni correction for multiple hypothesis testing.

Table 2. Distribution of colorectal cancer cases according to tumor SQSTM1 expression level and the density of T cells.

		Tumor SQSTM1 expression level			
	Total no.	Low	Intermediate	High	*p* value*
CD3$^+$ cell density ($n = 579$)					0.97
Quartile 1 (lowest)	144 (25%)	32 (25%)	66 (26%)	46 (23%)	
Quartile 2	145 (25%)	27 (21%)	64 (25%)	54 (28%)	
Quartile 3	145 (25%)	33 (26%)	62 (24%)	50 (26%)	
Quartile 4 (highest)	145 (25%)	34 (27%)	65 (25%)	46 (23%)	
CD8$^+$ cell density ($n = 573$)					0.07
Quartile 1 (lowest)	143 (25%)	34 (28%)	72 (28%)	37 (19%)	
Quartile 2	143 (25%)	30 (25%)	63 (24%)	50 (26%)	
Quartile 3	143 (25%)	31 (26%)	60 (23%)	52 (27%)	
Quartile 4 (highest)	144 (25%)	26 (21%)	64 (25%)	54 (28%)	
CD45RO$^+$ cell density ($n = 586$)					0.45
Quartile 1 (lowest)	147 (25%)	33 (27%)	62 (23%)	52 (27%)	
Quartile 2	146 (25%)	36 (29%)	67 (25%)	43 (22%)	
Quartile 3	146 (25%)	26 (21%)	72 (27%)	48 (24%)	
Quartile 4 (highest)	147 (25%)	28 (23%)	66 (25%)	53 (27%)	
FOXP3$^+$ cell density ($n = 557$)					0.001
Quartile 1 (lowest)	140 (25%)	25 (21%)	57 (23%)	58 (31%)	
Quartile 2	138 (25%)	24 (20%)	68 (27%)	46 (25%)	
Quartile 3	140 (25%)	28 (24%)	71 (28%)	41 (22%)	
Quartile 4 (highest)	139 (25%)	41 (35%)	56 (22%)	42 (22%)	

*p value was calculated by Spearman correlation test between the tumor SQSTM1 expression score (ranging from low to high) and the density of CD3$^+$, CD8$^+$, CD45RO (PTPRC)$^+$, or FOXP3$^+$ T cells (cells/mm^2; as continuous variables). Because we assessed four primary outcome variables, we adjusted the two-sided α level to 0.01 (= 0.05/4) by simple Bonferroni correction.

better outcome.[57] Ladoire et al. have proposed that FOXP3$^+$ regulatory cells have a crucial role in inhibiting tumor-promoting inflammatory responses to gut microbiota, which may explain favorable prognosis associated with abundant FOXP3$^+$ cells in colorectal cancer.[58] Taken together, it seems to be plausible that FOXP3$^+$ regulatory T cells may have a role in suppressing colorectal tumor progression through regulating tumor-promoting inflammation.

Autophagy is an evolutionarily conserved catabolic process by which cellular components are sequestered into a double-membrane vesicle (autophagosome) and delivered to the lysosome for terminal degradation and recycling.[16] Accumulating evidence suggests that autophagy plays a critical role in the regulation of immune response,[19,59] in particular, antitumor immunity that may influence response to immunotherapies.[16] Autophagy in neoplastic cells appears to increase the emission

Table 3. Ordinal logistic regression analysis to assess the association of tumor SQSTM1 expression level (predictor) with the density of T cells (outcome).

		Univariable OR (95% CI)	Multivariable OR (95% CI)*
Model for CD3$^+$ cell density ($n = 579$, as an ordinal quartile outcome variable)			
Tumor SQSTM1 expression level	Low	1 (referent)	1 (referent)
	Intermediate)	0.91 (0.62–1.33)	0.85 (0.58–1.25)
	High	0.91 (0.61–1.36)	0.89 (0.59–1.32)
	p_{trend}†	0.68	0.63
Model for CD8$^+$ cell density ($n = 573$, as an ordinal quartile outcome variable)			
Tumor SQSTM1 expression level	Low	1 (referent)	1 (referent)
	Intermediate	1.07 (0.73–1.57)	1.11 (0.75–1.64)
	High	1.44 (0.96–2.17)	1.46 (0.97–2.20)
	p_{trend}†	0.06	0.06
Model for CD45RO$^+$ cell density ($n = 586$, as an ordinal quartile outcome variable)			
Tumor SQSTM1 expression level	Low	1 (referent)	1 (referent)
	Intermediate	1.24 (0.84–1.81)	1.35 (0.92–1.99)
	High	1.22 (0.81–1.82)	1.28 (0.85–1.92)
	p_{trend}†	0.40	0.31
Model for FOXP3$^+$ cell density ($n = 557$, as an ordinal quartile outcome variable)			
Tumor SQSTM1 expression level	Low	1 (referent)	1 (referent)
	Intermediate	0.69 (0.47–1.02)	0.66 (0.45–0.98)
	High	0.55 (0.36–0.83)	0.55 (0.36–0.83)
	p_{trend}†	0.005	0.006

Abbreviations: CI, confidence interval; OR, odds ratio.

*The multivariable ordinal logistic regression model initially included age, sex, year of diagnosis, family history of colorectal cancer in any parent or sibling, tumor location, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF*, and *PIK3CA* mutations, and LINE-1 methylation level. A backward stepwise elimination with a threshold of $p = 0.05$ was used to select variables in the final models.

†p_{trend} value was calculated by the linear trend across the ordinal categories of tumor SQSTM1 expression level (low, intermediate, and high) in the ordinal logistic regression model for the density of CD3$^+$, CD8$^+$, CD45RO (PTPRC)$^+$, or FOXP3$^+$ cells (an ordinal quartile outcome variable). Because we assessed four primary outcome variables, we adjusted two-sided α level to 0.01 (= 0.05/4) by simple Bonferroni correction.

of potent chemotactic factors including adenosine triphosphate (ATP) and lysophosphatidylcholine, which in turn attracts immune cells to the tumor bed.[11-15] Emerging evidence provides that autophagic activity in tumor cells is essential for promoting cross-presentation of tumor-associated antigens to T cells, indicating a positive link between autophagy and adaptive immunity.[8-11] On the other hand, autophagy may has an immunosuppressive role,[60] while keeping inverse balance with $FOXP3^+$ cells. These lines of evidence together with our current findings suggest that tumors may choose one or the other for immune evasion.

Accumulating evidence attests to a key role of tumor autophagic activity on not only $CD8^+$ cytotoxic T cells but also $FOXP3^+$ regulatory T cells in the tumor microenvironment although detailed mechanisms have not been discovered.[17,20-22] In this study, tumor SQSTM1 expression level was inversely associated with $FOXP3^+$ cell density in tumor tissue, but not density of $CD3^+$, $CD8^+$, or $CD45RO^+$ cells. Potential reasons for the difference may include difficulty in accurately measuring cellular autophagic activity, which might have caused false negative findings.

In our secondary analyses, we observed trends toward positive associations of tumor SQSTM1 expression with cecal tumor location, wild-type *KRAS*, and a higher level *Fusobacterium nucleatum* DNA in tumor tissue. One previous study[46] has reported that tumor SQSTM1 expression is not associated with tumor location, but the sample size was much smaller ($n = 178$) than our current study. In an *in vitro* experimental study,[61] *KRAS* mutation in colorectal cancer appeared to attenuate tumor SQSTM1 expression level, and we observed the similar trend. In another *in vitro* experimental study,[62] *F. nucleatum* appeared to induce impairment of autophagic activity of host cells; however, our current data do not support such negative effects of *F. nucleatum* on autophagic activity of tumor cells. Further studies are needed to determine the associations of tumor SQSTM1 expression with clinical, pathological and molecular characteristics of colorectal cancer.

One limitation of our current study is its cross-sectional nature. Hence, we cannot exclude the possibility of reverse causation. It is possible that $FOXP3^+$ cells might alter the tumor autophagy status. However, our specific hypothesis was based on several lines of experimental evidence indicating that autophagic activity in tumor cells promotes antitumor immune response.[8-16] Another limitation is that our study used SQSTM1 (but no other biomarkers) to quantify autophagic activity. Future studies should evaluate other autophagic markers including MAP1LC3 (LC3). Strengths of this study include the use of our molecular pathological epidemiology[42,63] database of more than 600 colorectal cancer cases in the two US-nationwide prospective cohort studies, which integrate epidemiologic exposures, clinicopathological features, tumor molecular features, and immune reaction status in colorectal cancer tissue. This population-based colorectal cancer database enabled us to rigorously examine the association of tumor SQSTM1 expression level with the T-cell density, controlling for potential confounders. In addition, our colorectal cancer specimens were derived from a large number of hospitals in diverse settings across the United States (but not based on a limited number of hospitals), which increases the generalizability of our findings. Last, we also used robust laboratory assays including tissue image analysis that could objectively quantify specific T-cell populations in tumor tissue.

In summary, tumor SQSTM1 expression level is inversely associated with $FOXP3^+$ cell density in colorectal cancer tissue. Our population-based data suggest a possible effect of tumor SQSTM1 expression and autophagic activity on regulatory T cells in colorectal cancer microenvironment, and can promote further translational research on the associations of autophagy with host immunity in colorectal cancer.

Patients and methods

Study population

We used two independent US-nationwide prospective cohort studies: the Nurses' Health Study (121,701 women followed since 1976) and the Health Professionals Follow-up Study (51,529 men followed since 1986).[64-66] Every 2 y, study staff had sent follow-up questionnaires to the participants, to update information on diet and lifestyle factors and to identify newly diagnosed cancer and other diseases. In addition, we identified deaths of cohort participants in the National Death Index, to find fatal colorectal cancer cases that had not been reported. Study physicians reviewed medical records to gain information on tumor location and disease stage, and determined cause of deaths for deceased individuals. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from hospitals across the United States, where participants with colorectal cancer had undergone tumor resection. We included both colon and rectal carcinoma cases, considering the colorectal continuum model.[67] The study pathologist (S.O.) blinded to other data conducted centralized pathology review of all colorectal carcinoma cases, and recorded pathological features including tumor differentiation, and four patterns of histological lymphocytic reaction [Crohn's-like lymphoid reaction, peritumoral lymphocytic reaction, intratumoral periglandular reaction, and tumor-infiltrating lymphocytes (TILs)].[44,68] Tumor differentiation was categorized as well to moderate ($> 50\%$ glandular area) or poor ($\leq 50\%$ glandular area). Based on availability of data on tumor SQSTM1 expression and T-cell densities, a total of 601 colorectal cancer cases diagnosed up to 2008 were included in this study. Written informed consent was obtained from all study participants. Tissue collection and analyses were approved by the institutional review boards at the Harvard T.H. Chan School of Public Health and the Brigham and Women's Hospital (Boston, MA, USA).

Immunohistochemistry for $CD3^+$, $CD8^+$, CD45RO, FOXP3, and SQSMT1

We constructed tissue microarray from colorectal cancer blocks,[69] and conducted immunohistochemistry. Immunohistochemical analyses were performed for $CD3^+$, $CD8^+$, CD45RO (one isoform of the PTPRC protein), and FOXP3, as described previously.[43] We measured the densities of $CD3^+$, $CD8^+$, $CD45RO^+$, and $FOXP3^+$ cells in tumor tissue by using an automated scanning microscope and the Ariol image analysis system (Genetix). We evaluated up to four tissue microarray

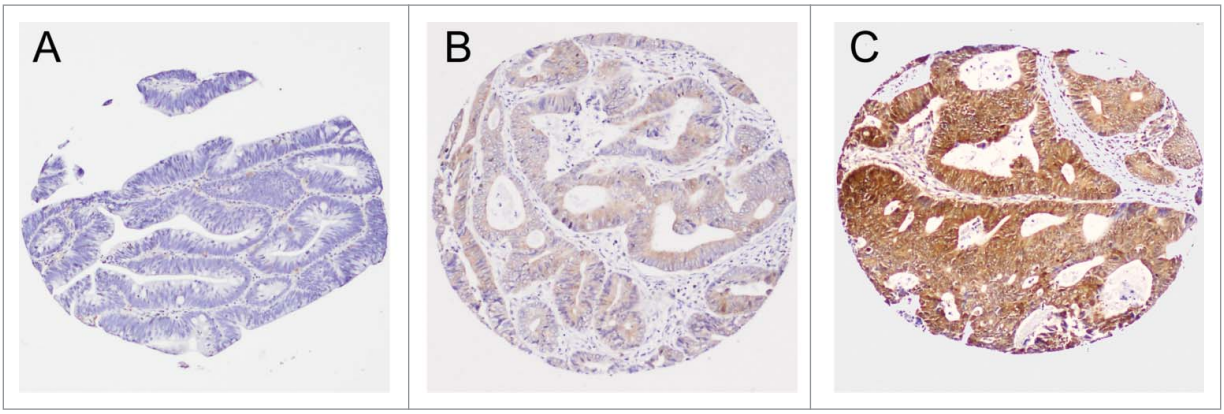

Figure 1. Tumor SQSTM1 (p62) expression in colorectal cancer. Tumor SQSTM1 expression was scored as low (A), intermediate (B), or high (C), according to cytoplasmic expression level of SQSTM1 in tumor cells.

cores from each tumor, and calculated the average density (cells/mm^2) of each T-cell population.[43]

We performed immunohistochemistry for SQSTM1 (p62) as an autophagy-related marker. Since SQSTM1 is degraded by autophagy, autophagic activity has been inversely associated with SQSTM1 expression.[6,23-25,70] For immunohistochemistry, deparaffinized tissue sections were heated in a microwave using a pressure cooker for 17 min in Antigen Retrieval Citra Solution, pH 6 (BioGenex Laboratories). Tissue sections were incubated with a dual endogenous enzyme block (Dako) for 30 min and then serum-free protein block (Dako) for 10 min. Slides were incubated for 16 h at 4°C with a primary antibody against SQSTM1 (mouse monoclonal antibody, clone 2C11, Abnova; dilution, 1:1,500). Then, the EnVision HRP-labeled polymer (Dako) was applied to the sections for 30 min, followed by visualization with 3,3-diaminobenzidine and counterstaining with hematoxylin. Sections processed with the replacement of the primary antibody with Tris-buffered saline were used as negative controls. The cytoplasmic expression level (intensity) of SQSTM1 was recorded as low, intermediate, or high (Fig. 1). Tumor SQSTM1 expression was interpreted by a single pathologist (Y.M.) unaware of other data. A sample of 143 tumors was examined by a second pathologist (A.dS.). The weighted κ value for agreement between the two pathologists for SQSTM1 was 0.63, indicating reasonably good interobserver agreement ($p < 0.0001$ by Spearman's correlation test).

Analyses of MSI, DNA methylation, and KRAS, BRAF, and PIK3CA mutations

DNA was extracted from archival colorectal cancer tissue blocks. MSI status was analyzed with the use of 10 microsatellite markers (BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, D18S55, D18S56, D18S67, and D18S487), as described previously.[66,71] We defined MSI-high as the presence of instability in $\geq$ 30% of the markers, and MSI-low/microsatellite stability (MSS) as instability in <30% of the markers.[66,71] Methylation analyses of long interspersed nucleotide element-1 (LINE-1) and eight promoters specific to CpG island methylator phenotype (CIMP) (*CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3*, and *SOCS1*) were performed.[66,72] CIMP-high was defined as $\geq$ 6/8 methylated promoters, while CIMP-low/negative as 0/8 to 5/8 methylated promoters, as described previously.[66,72] PCR reaction and pyrosequencing were performed for *KRAS* (codons 12, 13, 61, and 146),[73] *BRAF* (codon 600),[71] and *PIK3CA* (exons 9 and 20).[66,74]

Analysis of the amount of F. nucleatum DNA

We extracted DNA from colorectal cancer FFPE tissue sections, and performed a quantitative PCR assay to measure the amount of tissue *F. nucleatum*DNA.[75] We categorized colorectal carcinoma cases with detectable *F. nucleatum* DNA as low or high in relation to the median of *F. nucleatum* DNA amounts among *F. nucleatum* detectable cases.[75,76]

Statistical analysis

All statistical analyses were conducted using SAS (version 9.4, SAS Institute, Cary, NC, USA), and all *p* values were two-sided. Our primary hypothesis testing was assessment of the association of the tumor SQSTM1 expression level (an ordinal predictor variable) with the density of CD3$^+$, CD8$^+$, CD45RO$^+$, or FOXP3$^+$ cells in colorectal cancer tissue (an ordinal quartile outcome variable). Because we tested four primary outcome variables (CD3$^+$ cells, CD8$^+$ cells, CD45RO$^+$ cells, and FOXP3$^+$ cells), we adjusted a two-sided significance level to 0.01 (= 0.05/4) based on the Bonferroni correction. All other analyses, including evaluation of individual odds ratio (OR) estimates, were secondary analyses.

To control for potential confounding, we performed multivariable ordinal logistic regression analysis where each T-cell density variable (CD3$^+$, CD8$^+$, CD45RO, and FOXP3) was used as an ordinal quartile outcome variable, and SQSTM1 expression level as the ordinal predictor variable of our primary interest. In the regression model, we initially included age (continuous), sex (female vs. male), year of diagnosis (continuous), family history of colorectal cancer in a first-degree relative (present vs. absent vs. missing), tumor location (proximal colon vs. distal colon vs. rectum vs. missing), MSI status (MSI-high vs. MSI-low/MSS vs. missing), CIMP status (high vs. low/negative vs. missing), *KRAS* mutation (mutant vs. wild-type vs. missing), *BRAF* mutation (mutant vs. wild-type vs. missing), *PIK3CA* mutation (mutant vs. wild-type vs. missing), and

LINE-1 methylation level (continuous, with a missing indicator variable). Then, we performed a backward elimination with a threshold of $p = 0.05$ to select variables for the final model. In the final multivariable ordinal logistic regression model, for cases with missing information in any of the selected categorical variables, we included those cases in the majority category of a given covariate to limit the degrees of freedom and avoid overfitting of the model. We assessed the proportional odds assumption in the ordinal logistic regression model, which was generally satisfied ($p > 0.05$).

To assess the associations of SQSTM1 expression level with other categorical variables, the chi-square test was performed. To compare mean age and mean LINE-1 methylation levels, an analysis of variance assuming equal variances was performed. All of the cross-sectional univariable analyses for clinical, pathological, and molecular associations (with variables listed in Table 1) were secondary exploratory analyses, and we adjusted two-sided sinificance level to 0.003 (= 0.05/18) by the Bonferroni correction for multiple hypothesis testing.

Disclosure of potential conflict of interest

A.T.C. previously served as a consultant for Bayer Healthcare, Millennium Pharmaceuticals, Pozen, Inc., and Pfizer, Inc. This study was not funded by Bayer Healthcare, Millennium Pharmaceuticals, Pozen, Inc., or Pfizer, Inc. The other authors declare that they have no conflicts of interest.

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