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Association of autophagy status with amount of *Fusobacterium nucleatum* in colorectal cancer

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Author contributions statement

K.H., K.K., T.H. and T.S.T. contributed equally as co first authors. M.G., A.T.C., C.S.F., and S.O. contributed equally as co last authors. All authors contributed to review and revision. W.S.G., C.H., M.G., A.T.C., C.S.F., and S.O.: developed the main concept and designed the study. W.S.G., C.H., M.G., A.T.C., C.S.F. and S.O.: wrote grant applications. K.H., J.P.V., S.A.K., Y.M., Z.R.Q., K.M., Y.B., A.d.S., J.B., M.S., J.A.N., R.N., J.A.M., A.T.C., C.S.F., and S.O.: were responsible for collection of tumour tissue, and acquisition of epidemiologic, clinical and tumour tissue data, including histopathological and immunohistochemical characteristics. K.H., K.K., T.H., R.N., C.S.F., and S.O.: performed data analysis and interpretation. K.H., K.K., T.H., T.S.T., and S.O.: drafted the manuscript. J.P.V., S.A.K., Y.M., Z.R.Q., K.M., Y.B., A.d.S., J.B., K.A., K.F., M.C.L., P.L., C.G., Y.C., M.S., J.A.N., R.N., K.Y., X.Z., K.W., S.B., W.S.G., C.H., J.A.M., M.G., A.T.C., C.S.F., and S.O.: contributed to editing and critical revision for important intellectual contents.

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Use of Standardised official symbols

We use HUGO (Human Genome Organisation)-approved official symbols (or root symbols) for genes and gene products, including BECN1, BRAF, CACNA1G, CD3, CD4, CDKN2A, CDH1, CRABP1, CTSB, IGF2, KRAS, MAP1LC3A, MAP1LC3B, MLH1, NEUROG1, PIK3CA, RUNX3, SOCS1, SQSTM1, and TIGIT; 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 themselves with the official symbols for genes and gene products together with common colloquial names.

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Abstract

Fusobacterium nucleatum (*F. nucleatum*), which has been associated with colorectal carcinogenesis, can impair anti-tumour immunity and actively invade colon epithelial cells. Considering the critical role of autophagy in host defence against microorganisms, we hypothesised that autophagic activity of tumour cells might influence the amount of *F. nucleatum* in colorectal cancer tissue. Using 724 rectal and colon cancer cases within the Nurses' Health Study and the Health Professionals Follow-up Study, we evaluated autophagic activity of tumour cells by immunohistochemical analyses of *BECN1* (beclin 1), *MAP1LC3* (LC3), and *SQSTM1* (p62) expression. We measured the amount of *F. nucleatum* DNA in tumour tissue by quantitative PCR. We conducted multivariable ordinal logistic regression analyses to examine the association of tumour *BECN1*, *MAP1LC3*, and *SQSTM1* expression with the amount of *F. nucleatum*, adjusting for potential confounders, including microsatellite instability status, CpG island methylator phenotype, long-interspersed nucleotide element-1 methylation, and *KRAS*, *BRAF* and *PIK3CA* mutations. Compared with *BECN1*-low cases, *BECN1*-intermediate and *BECN1*-high cases were associated with lower amounts of *F. nucleatum* with odds ratios (for a unit increase in three ordinal categories of the amount of *F. nucleatum*) of 0.54 (95% confidence interval, 0.29–0.99) and 0.31 (95% confidence interval, 0.16–0.60), respectively ($P_{trend} < 0.001$ across ordinal *BECN1* categories). Tumour *MAP1LC3* and *SQSTM1* levels were not significantly associated with the amount of *F. nucleatum* ($P_{trend} > 0.06$). Tumour *BECN1*, *MAP1LC3*, and *SQSTM1* levels were not significantly associated with patient survival ($P_{trend} > 0.10$). In conclusion, tumour *BECN1* expression is inversely associated with the amount of *F. nucleatum* in colorectal cancer tissue, suggesting a possible role of autophagy in the elimination of invasive microorganisms.

Keywords

colorectal neoplasms; immunology; microbiology; microbiome; molecular pathological epidemiology; tumour microenvironment

Introduction

Accumulating evidence supports the importance of the gut microbiota in intestinal carcinogenesis through modulation of tumour immune microenvironment [1–5]. *Fusobacterium nucleatum* (*F. nucleatum*) has emerged as a potentially influential driver of colorectal carcinogenesis due to its contribution to the formation of proinflammatory condition [6–8]. Studies have shown an enrichment of *F. nucleatum* in colorectal cancer tissue compared with the adjacent normal colon [7–9], and revealed an association of high *F. nucleatum* levels with distinct clinical and tumour molecular features including proximal tumour location, high-level microsatellite instability (MSI), lower levels of tumour-infiltrating $CD3^+$ cells, and poor patient prognosis [9–12]. Given incomplete knowledge of the interaction between microbes and the host immune system, a better understanding of mechanism through which specific microbes can localise in colorectal cancer tissue would inform efforts to develop cancer prevention and treatment strategies [13–15].

Autophagy involves the lysosomal degradation and recycling of intracellular components. The *BECN1* (beclin 1) and *MAP1LC3* (LC3) proteins are key positive regulators of autophagy that initiate autophagosome formation, while the *SQSTM1* (p62) protein plays a crucial role in lysosomal degradation of ubiquitinated substrate [16–19]. This homeostatic process adjusts cellular biomass and function in response to diverse stimuli, including metabolic stress, inflammation, and pathogen infection. Autophagic activity of tumour cells may enhance anti-tumour immune response by presenting tumour antigens [16–18,20,21]. Autophagy also plays a key role in determining microbial composition in the microenvironment. Further evidence suggests that activated autophagic activity may eliminate intracellular microorganisms [22–28]. Therefore, we hypothesised that autophagic activity of tumour cells might influence tumour bacterial load including levels of *F. nucleatum* in colorectal cancer tissue.

Even the best experimental model could not recapitulate the complexity of human tumour microenvironment, which is influenced by genetic and epigenetic alterations, environmental exposures, gut microbiota, and host factors [29,30]. Thus, the importance of large-scale human studies on tumour molecular characteristics and microbiota cannot be overemphasised. To test our hypothesis, we utilised a molecular pathological epidemiology database of colorectal cancer cases within two large U.S. prospective cohort studies, with data on demographic, clinical, pathological, and tumour molecular characteristics. This comprehensive dataset enabled us to examine the association of autophagic activity of tumour cells with tumoural bacterial load including the amount of *F. nucleatum* as well as *Bifidobacterium* genus in the tumour tissue, taking into account the distinct features of microbial species.

Materials and methods

Study population

We collected data from two prospective cohort studies in the U.S., the Nurses' Health Study (NHS, 121,701 women aged 30–55 years followed since 1976) and the Health Professionals Follow-up Study (HPFS, 51,529 men aged 40–75 years followed since 1986) [31]. Study participants have been followed with biennial questionnaires on lifestyle factors and newly-diagnosed diseases including colorectal cancer. The response rate has been more than 90% for each follow-up questionnaire cycle in both cohort studies. The National Death Index was used to ascertain deaths of study participants and identify unreported lethal colorectal cancer cases. Participating physicians, who were blind to exposure data, reviewed medical records of identified colorectal cancer cases to confirm the disease diagnosis and to collect data on clinical characteristics including tumour size, tumour anatomical location, and disease stage based on the American Joint Committee on Cancer TNM classification.

Among 724 patients with available data on the amount of *F. nucleatum*, we analysed 628 patients with available data on tumour *BECN1* expression, 689 patients with available data on tumour *MAP1LC3* expression, and 674 patients with available data on tumour *SQSTM1* expression in colorectal cancer tissues diagnosed up to 2008. We included both colon and rectal carcinomas based on the colorectal continuum model [12,32]. Patients were followed until death or the end of follow-up (June 30, 2014 for the NHS; and January 1, 2014 for the HPFS), whichever came first. We collected formalin-fixed paraffin-embedded (FFPE) tumour tissue blocks from hospitals throughout the U.S. where colorectal cancer patients had undergone surgical resection. A single pathologist (S.O.), blinded to other data, reviewed haematoxylin and eosin-stained tissue sections from all collected blocks and recorded pathological features. Tumour differentiation was categorised into well/moderate vs. poor (> 50% vs. ≤ 50% gland formation, respectively).

Informed consent was obtained from all study participants at enrollment. This study was approved by the institutional review boards of Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health (Boston, MA, USA), and those of participating registries as required.

Immunohistochemical evaluation of *BECN1*, *MAP1LC3*, *SQSTM1*, and *CTSB*

We constructed tissue microarrays of colorectal cancer cases with sufficient tissue materials, including up to four tumour cores from each case in one tissue microarray block [33]. Immunohistochemical studies of *BECN1*, *MAP1LC3*, and *SQSTM1* were performed to evaluate autophagic activity of tumour cells. The *BECN1* and *MAP1LC3* expression have been positively associated with autophagic activity, while SQSTM1, degraded by autophagy, has been inversely associated with autophagic activity [16–18]. As secondary analysis, we evaluated tumour cell expression of *CTSB* (cathepsin B), which is one of the lysosomal enzymes involved in autophagic degradation and various other intracellular proteolytic processes [34].

For immunohistochemistry, deparaffinised tissue sections were heated in a microwave using a pressure cooker for 17 minutes in Antigen Retrieval Citra Solution, pH 6 (BioGenex

Laboratories, San Ramon, CA, USA). Tissue sections were incubated with a dual endogenous enzyme block (Dako, Glostrup, Denmark) for 30 minutes and then serum-free protein block (Dako) for 10 minutes. Slides were incubated for 16 hours at 4 °C with a primary antibody against *BECN1* [rabbit polyclonal antibody (ab55878), Abcam, Cambridge, MA, USA; dilution, 1:600], *MAP1LC3A* and *MAP1LC3B* [rabbit polyclonal antibody (ab58610), Abcam; dilution, 1:100], and *SQSTM1* [mouse monoclonal antibody, clone 2C11, Abnova, Taipei, Taiwan; dilution, 1:1500]. The EnVision HRP-labeled polymer (Dako) was then applied to the sections for 30 minutes, followed by visualization with 3,3-diaminobenzidine and counterstaining with haematoxylin. Sections processed with the replacement of the primary antibody with Tris-buffered saline were used as negative controls.

The cytoplasmic expression levels (intensity) of *BECN1*, *MAP1LC3*, and *SQSTM1* were recorded as low, intermediate, or high (Figure 1, Figure 2, and Figure 3). The immunostained slides were interpreted by the blinded study pathologists (S.A.K. for *BECN1*, Z.R.Q. for *MAP1LC3*, and Y.M. for *SQSTM1*). A subset of cases (N = 118 for *BECN1*, N = 111 for *MAP1LC3*, and N = 143 for *SQSTM1*) was scored independently by the second blinded pathologists (Y.M. for *BECN1* and *MAP1LC3*, and A.d.S. for *SQSTM1*). The weighted κ values for the ratings of the two observers were 0.76 for *BECN1*, 0.69 for *MAP1LC3*, and 0.63 for *SQSTM1* [20], indicating reasonably good interobserver agreement (all $P < 0.001$).

As previously described [35], we conducted immunohistochemical study of *CTSB* using an antibody against *CTSB* [goat polyclonal antibody (S-12), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:1250 dilution]. The cytoplasmic expression of *CTSB* was recorded as absent, weak, moderate, or strong, as previously described [35].

Quantitative polymerase chain reaction (PCR) for *Fusobacterium nucleatum* and *Bifidobacterium* genus

Genomic DNA was extracted from archival FFPE tissue sections of colorectal cancer using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). As previously described [9,36], we performed a quantitative polymerase chain reaction (PCR) assay to measure the amount of *F. nucleatum* and *Bifidobacterium* genus DNA in the tumour tissue. Cases with any detectable *F. nucleatum* and *Bifidobacterium* genus DNA were categorised as low vs. high based on the median cut point amount of *F. nucleatum* or *Bifidobacterium* genus, while cases without detectable *F. nucleatum* and *Bifidobacterium* genus were categorised as negative.

Evaluation of tumour molecular characteristics

Tumour MSI status was analysed using PCR of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487), and MSI-high was defined as presence of instability in ≥ 30% of the markers, as previously described [32]. Using bisulfite-treated DNA, methylation statuses of eight CpG island methylator phenotype (CIMP)-specific promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) and long-interspersed nucleotide element-1

(LINE-1) was determined as previously described [32]. CIMP-high was defined as ≥ 6 methylated promoters of eight promoters, and CIMP-low/negative as 0–5 methylated promoters as previously described [32]. PCR and pyrosequencing were performed for *KRAS* (codons 12, 13, 61, and 146), *BRAF* (codon 600), and *PIK3CA* (exons 9 and 20), as previously described [32].

Statistical analysis

All statistical analyses were conducted using SAS software (version 9.4, SAS Institute, Cary, NC, USA), and all *P* values were two-sided. We used the two-sided α level of 0.005 [37]. Our primary hypothesis testing was an assessment of the association of tumour *BECN1*, *MAP1LC3*, and *SQSTM1* expression levels (low, intermediate, and high; as an ordinal predictor variable) with the amount of *F. nucleatum* DNA (negative, low, and high; as an ordinal outcome variable). All other tests, including analyses of *Bifidobacterium* genus, assessment of stratum-specific risk estimates, and survival analyses, represented secondary analyses.

We performed multivariable ordinal logistic regression analyses to control for potential confounders. The multivariable ordinal logistic model initially included sex (female vs. male), age at diagnosis (continuous), year of diagnosis (continuous), family history of colorectal cancer in any first-degree relative (present vs. absent), tumour location (proximal colon vs. distal colon vs. rectum), MSI status (MSI-high vs. non-MSI-high), CIMP status (high vs. low/negative), LINE-1 methylation level (continuous), *KRAS* mutation (mutant vs. wild-type), *BRAF* mutation (mutant vs. wild-type), and *PIK3CA* mutation (mutant vs. wild-type). A backward elimination was conducted with a threshold *P* of 0.05 to select variables for the final models. Cases with missing data [family history of colorectal cancer in a first-degree relative (1.1%), tumour location (0.3%), MSI (2.8%), CIMP (8.3%), *KRAS* (2.9%), *BRAF* (2.5%), and *PIK3CA* mutation (7.9%)] were included in the majority category of a given categorical covariate to limit the degrees of freedom of the models. For the cases with missing data on LINE-1 methylation (2.5%), we assigned a separate indicator variable. We confirmed that excluding the cases with missing information in any of the covariates did not substantially alter results (data not shown). We assessed the proportional odds assumption in an ordinal logistic regression model, which was generally satisfied ($P > 0.1$).

To assess the association between ordinal categories of the amount of *F. nucleatum* DNA and other categorical variables (except for tumour differentiation and pT stage, for which Fisher's exact test was performed), the chi-square test was performed. To compare continuous variables, an analysis of variance assuming equal variances was performed.

We estimated cumulative survival probabilities using the Kaplan-Meier method, and compared the differences between categories using the log-rank test. For analyses of colorectal cancer-specific mortality, deaths as a result from other causes were censored. To control for potential confounders, Cox proportional hazards regression analysis was performed and hazard ratio was calculated for mortality. The multivariable Cox proportional hazards regression models initially included tumour differentiation (well to moderate vs. poor), disease stage (I/II vs. III/IV) in addition to the same set of variables as in multivariable ordinal logistic regression analysis. A backward elimination was conducted

with a threshold *P* of 0.05 to select variables for the final models. In secondary analyses, we assessed the statistical interaction between *BECN1*. *MAP1LC3*, and *SQSTM1* expression (high vs. low/intermediate) and *F. nucleatum* status in colorectal cancer tissue (positive vs. negative) in relation to colorectal cancer-specific or overall mortality. We used the Wald test for the cross-product in multivariable-adjusted Cox proportional hazards regression models. We estimated hazard ratios for colorectal cancer and overall mortality comparing cases with high expression of *BECN1*, *MAP1LC3*, and *SQSTM1* to cases with low/intermediate expression in strata of *F. nucleatum* status using re-parameterization of the interaction term in a single regression model [38]. Cases with missing data on tumour differentiation (0.1%) and disease stage (7.7%) were included in the majority category, and the other covariates were dealt as in the multivariable ordinal logistic regression models. The assumption of proportional hazards was generally satisfied using the assessment of a time-varying covariate, which was the cross-product of log-transformed survival time and the level of *BECN1*, *MAP1LC3*, and *SQSTM1* expression in the whole population or in strata of *F. nucleatum* status ($P > 0.08$).

In ordinal logistic and Cox regression analyses, the inverse probability weighting (IPW) method was applied to reduce the potential bias due to the availability of tumour tissue [39–41]. Using the multivariable logistic regression model for the entire dataset of colorectal cancer cases (regardless of available tissue), we estimated the probability of the availability of tumour tissue, as previously described [36]. Each patient with complete data was weighted by the inverse probability. Weights greater than the 95th percentile were truncated and set to the value of the 95th percentile to reduce outlier effects [41]. We confirmed that results without weight truncation did not change substantially (data not shown). The logistic and Cox regression analyses without IPW yielded similar results to the IPW-adjusted model (Supplementary Table S2 and S8).

Results

Among the 724 colorectal cancer cases from the two prospective cohort studies, *F. nucleatum* DNA was detected using a quantitative PCR assay in 99 (14%) cases. Tumour *BECN1* (beclin 1) expression levels were low, intermediate, and high in 102 (16%), 289 (46%), and 237 (38%) cases, respectively. Tumour *MAP1LC3* expression levels were low, intermediate, and high in 201 (29%), 221 (32%), and 267 (39%) cases, respectively. Tumour *SQSTM1* (p62) expression levels were low, intermediate, and high in 158 (23%), 309 (46%), and 207 (31%) cases, respectively. Table 1 and Supplementary Table S1 show clinical, pathological, and molecular features of colorectal cancer cases according to the amount of *F. nucleatum* DNA and *Bifidobacterium* genus DNA, respectively. As previously reported [9,11], a greater amount of *F. nucleatum* DNA was associated with poor tumour differentiation, higher pT stage, MSI-high status, and CIMP-high status ($P < 0.005$; with the α level of 0.005). Tumour *BECN1* expression inversely correlated with the amount of *F. nucleatum* DNA ($P < 0.001$, by Spearman's correlation test with the α level of 0.005), while tumour *MAP1LC3* or *SQSTM1* expression was not significantly correlated with the amount of *F. nucleatum* DNA ($P > 0.061$, by Spearman's correlation test). There was no significant correlation between any of tumour *BECN1, MAP1LC3*, and *SQSTM1* expression and the amount of *Bifidobacterium* genus DNA ($P > 0.13$).

In our primary hypothesis testing, we used an ordinal logistic regression analysis to assess the association of tumour *BECN1*, *MAP1LC3*, or *SQSTM1* expression levels with the amount of *F. nucleatum* DNA (Table 2, and Supplementary Table S3, S4, and S5). In the multivariable analyses, compared with *BECN1*-low cases, multivariable odd ratios (ORs) for the amount of *F. nucleatum* DNA were 0.54 [95% confidence interval (CI), 0.29–0.99] for *BECN1*-intermediate cases and 0.31 (95% CI, 0.16–0.60) for *BECN1*-high cases (P_{trend} < 0.001; with the α level of 0.005). We did not observe a statistically significant association of tumour *MAP1LC3* or *SQSTM1* expression levels with the amount of *F. nucleatum* DNA in colorectal cancer (P_{trend} > 0.061; with the α level of 0.005).

In secondary analysis using a subset of cases with available *CTSB* data, we did not observe a statistically significant association of tumour *CTSB* expression with the amount of *F. nucleatum* DNA (P_{trend} = 0.74; with the α level of 0.005) (Supplementary Table S6 and S7).

As secondary analyses to assess the prognostic association of *BECN1*, *MAP1LC3*, and *SQSTM1* expression, we conducted Kaplan-Meier analyses and Cox regression analyses, and did not observe a significant association of *BECN1*, *MAP1LC3*, or *SQSTM1* levels with colorectal cancer-specific or overall mortality (P_{trend} > 0.10; with the α level of 0.005) (Table 3, Supplementary Table S8, and Supplementary Figure S1, S2, and S3). We further examined whether prognostic association of *BECN1*, *MAP1LC3*, and *SQSTM1* expression in strata of *F. nucleatum* status and did not observe a significant interaction between *BECN1*, *MAP1LC3*, or *SQSTM1* expression and of *F. nucleatum* status in relation to colorectal cancer-specific or overall mortality ($P_{interaction}$ > 0.25; with the α level of 0.005) (Supplementary Table S9).

Discussion

Using two large prospective cohort studies in the U.S., we tested the hypothesis that autophagic activity of tumour cells might influence the amount of *F. nucleatum* in colorectal cancer tissue. Notably, we found an inverse association of tumour *BECN1* expression level with the amount of *F. nucleatum*, but not with the amount of *Bifidobacterium* genus in colorectal cancer tissue. Our findings suggest a possible role of tumour autophagic activity in the elimination of a specific microorganism within the colorectal cancer microenvironment.

There is growing interest in the role of the gut microbiota in cancer biology, especially *F. nucleatum* in colorectal cancer. A translational study has shown that *F. nucleatum* is associated with colorectal cancer recurrence through the promotion of chemoresistance by engaging autophagic activity [42]. *F. nucleatum* binds host epithelial *CDH1* (E-cadherin) via the fusobacterial adhesin FadA and invades epithelial cells, resulting in the promotion of inflammation and tumour cell growth in transformed cells [6,43,44]. Moreover, invasive *F. nucleatum* is persistently associated with distant metastases from primary colorectal cancers [45]. In contrast, the members of *Bifidobacterium* genus have been considered to inhibit colorectal carcinogenesis through the prevention of enteropathogenic infection and the inhibition of secondary bile acid production [46–48]. Our previous population-based study has shown that intratumoural *Bifidobacterium* genus may reflect loss of intestinal barrier

function in poorly differentiated colorectal cancer [36]. These distinct characteristics of *F. nucleatum* and *Bifidobacterium* genus are supported by our results of differing associations with autophagic activity. Our finding suggests the autophagy may target only highly virulent bacteria which can cause stress to the tumour cells, while autophagy is not associated with the presence of *Bifidobacteria* genus. From an immunological point of view, *F. nucleatum* may suppress the adaptive immune responses and inhibit T-cell- and natural killer cell-mediated immune response against colorectal cancer through the immune cell receptor *TIGIT* [6,49]. In line with these studies, our previous population-based studies have shown an inverse association of *F. nucleatum* in colorectal cancer tissue with $CD3^+$ pan-T cell density and adaptive anti-tumour immune response in MSI-high tumours [9,50]. Hence, the elimination of *F. nucleatum* may potentiate the effect of current front-line immunotherapeutic treatments.

Invasion of bacterial pathogens into epithelial cells triggers various innate immune responses, such as proinflammatory signaling and cell-autonomous restriction of bacterial growth. Among these host defence mechanisms, autophagy plays a critical role in the targeting and degradation of intracellular bacteria [24,28,51]. The autophagic machinery targets intracellular bacterium and promotes protective immune and stress responses, as the invasion of bacterium triggers the rapid induction of the intracellular starvation state [52]. There are three main autophagic mechanisms that provide a series of barriers against invading microorganisms. The first anti-microbial function is xenophagy, which is the uptake of intracellular microorganisms into double membrane autophagosomes regulated by *BECN1* and *MAP1LC3* [24,25]. The second is *MAP1LC3*-associated phagocytosis which promotes phagosome fusion with lysosomes [26]. Finally, a group of autophagic adaptors, known as *SQSTM1*-like receptors, are involved in the elimination of microorganisms from the cytoplasm [27]. In this study, tumour *BECN1* expression level was inversely associated with the amount of *F. nucleatum* and tumour *MAP1LC3* expression had the same trend as that of *BECN1*, however there was no correlation of tumour *SQSTM1* expression with the amount of *F. nucleatum*. Potential reason for the difference might be caused by distinct features of autophagy markers. *BECN1* and *MAP1LC3* are involved in the same phase of autophagy (i.e. autophagosome formation), while *SQSTM1* contributes to a different phase and also regulates transcriptional factors, suggesting that *F. nucleatum* might be eliminated through xenophagy and *MAP1LC3*-phagocytosis [17,18]. Autophagic machineries are not only required to eliminate microorganisms but also contribute to major histocompatibility complex class II presentation of their derived antigens that stimulate the $CD4^+$ T-cell adaptive response [22,23]. Moreover, autophagy is likely to influence the efficacy of immune checkpoint blockade therapy as well as gut microbiota [4,5,16,53]. Collectively, a better understanding of autophagy in relation to gut microbiota would have considerable therapeutic implications by its connection to tumour cells, host cells, and microorganisms in the tumour microenvironment. We did not observe significant association of tumour *BECN1*, *MAP1LC3*, and *SQSTM1* expression levels with colorectal cancer mortality. Prognostic roles of tumour *BECN1*, *MAP1LC3*, and *SQSTM1* in colorectal cancer remain controversial, possibly due to differences in study populations, designs and methods as well as a multifaceted nature of autophagic process [54–61].

We acknowledge potential limitations of the current study. First, we designed a cross-sectional study, by which we cannot exclude the possibility of reverse causation. While there is a possibility that *F. nucleatum* might modify autophagic activity of tumour cells, our specific hypothesis was based on several lines of experimental evidence indicating that autophagic activity eliminates microorganisms [22–28]. Second, our study only concentrated on a limited number of microbial species and genus. Accumulating evidence suggests that various species of microbiota and dysregulated microbial communities are involved in carcinogenesis and anti-tumour immune response [1,3–5]. Although *F. nucleatum* has garnered considerable attention as a potential cancer promoting microbe, other bacteria, such as *Bacteroides fragilis*, colibactin-producing *Escherichia coli*, and *Peptostreptococcus stomatis*, have been reported to be enriched in colorectal cancer and to be associated with colorecral cancer carcinogenesis [62,63]. Therefore, more comprehensive analyses including 16S rRNA gene sequencing and whole metagenomic shotgun sequencing would provide further insight into the relationship between microbiota and autophagy. Third, we evaluated the autophagic activity using a limited number of autophagic markers. Autophagy is a complex multistep process where more than 30 autophagy-related genes have been identified to make up the core machinery [16–19]. Although *BECN1*, *MAP1LC3*, and *SQSTM1* have been widely used as immunohistochemistry-based autophagy markers in human FFPE tissues [54–61], a simultaneous multimarker evaluation of other components of the autophagic pathway might further enlighten the interactions between autophagy and microbiota. Finally, we used the quantitative PCR assay for *F. nucleatum* and *Bifidobacterium* genus in FFPE tissue specimens; therefore, the detection rates might have been influenced by histopathology procedures and storage conditions. However, our previous validation study using the quantitative PCR assay showed a good concordance in detection of *F. nucleatum* in paired FFPE and frozen tissue specimens [9]. We also observed high linearity and reproducibility of *F. nucleatum* and *Bifidobacterium* genus measurements in FFPE tissue specimens [9,36].

The current study has notable strengths, including the utilisation of a molecular pathological epidemiology database [14,15,64] derived from two U.S.-based large prospective cohort studies. The integrated data on autophagic activity, tumour molecular characteristics, pathological findings, and microbial features enabled us to comprehensively examine possible roles of autophagy in relation to microorganisms in colorectal cancers. Importantly, our study population was derived from a large number of hospitals located throughout the U.S., which increases the generalisability of our findings. Nevertheless, our findings need to be validated in independent studies.

In conclusion, we have shown an inverse association of tumour *BECN1* expression level with the amount of *F. nucleatum* in colorectal cancer tissue allowing us to derive a possible role of autophagy in the elimination of invasive microorganisms such as *F. nucleatum* from the tumour microenvironment. Our population-based data, if validated, may inform future translational research on anti-microorganism therapy including treatment strategies targeting autophagy in colorectal cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AJCC	American Joint Committee on Cancer
CI	confidence interval
CIMP	CpG island methylator phenotype
FFPE	formalin-fixed paraffin-embedded
HPFS	Health Professionals Follow-up Study
IPW	inverse probability weighting
LINE-1	long-interspersed nucleotide element-1
MSI	microsatellite instability
NHS	Nurses' Health Study
OR	odds ratio
PCR	polymerase chain reaction
SD	standard deviation

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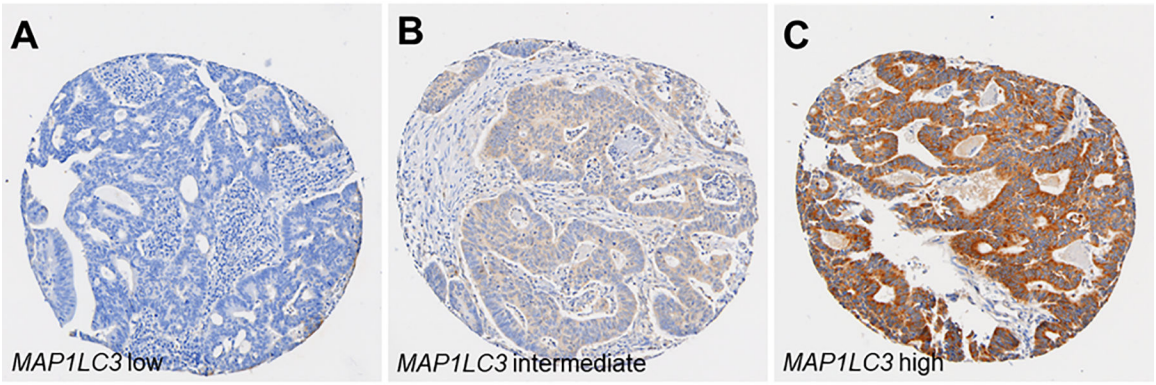

Figure 1.

Tumour *BECN1* (beclin 1) expression in colorectal cancer. Tumour *BECN1* expression was scored as low (A), intermediate (B), or high (C), according to cytoplasmic expression level of *BECN1*.

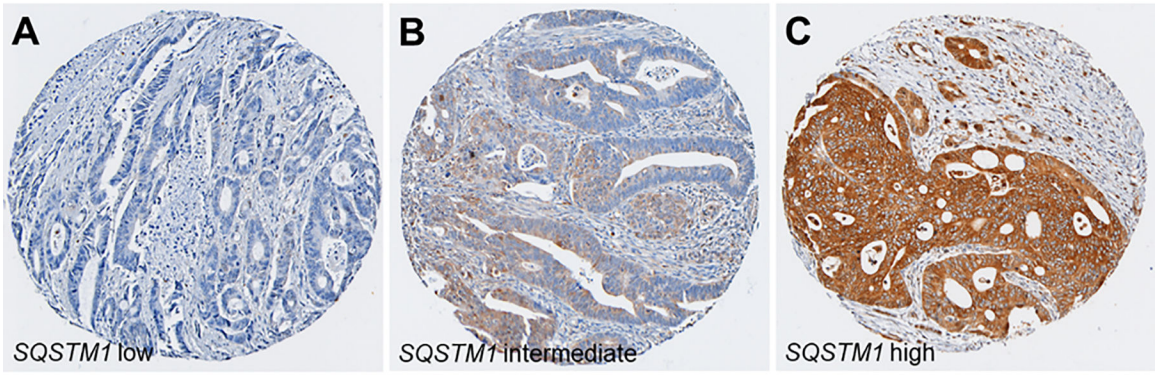

Figure 2.

Tumour *MAP1LC3* (LC3) expression in colorectal cancer. Tumour *MAP1LC3* expression was scored as low (A), intermediate (B), or high (C), according to cytoplasmic expression level of *MAP1LC3*.

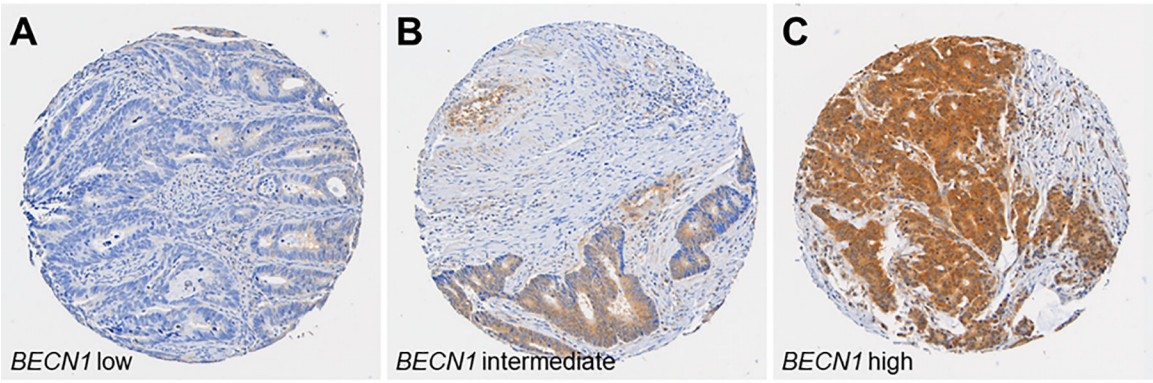

Figure 3.

Tumour *SQSTM1* (p62) expression in colorectal cancer. Tumour *SQSTM1* expression was scored as low (A), intermediate (B), or high (C), according to cytoplasmic expression level of *SQSTM1*.

Table 1.

Clinical, pathological, and molecular characteristics of colorectal cancer cases according to the amount of *Fusobacterium nucleatum* (*F. nucleatum*) DNA in tumour tissue

	Amount of *F. nucleatum* DNA in colorectal cancer tissue				
Characteristic*	**All cases (N = 724)**	**Negative (N = 625)**	**Low (N = 50)**	**High (N = 49)**	***P* value†**
Sex					0.14
Female (NHS)	423 (58%)	361 (58%)	27 (54%)	35 (71%)	
Male (HPFS)	301 (42%)	264 (42%)	23 (46%)	14 (29%)	
Mean age ± SD (years)	69.3 ± 8.9	69.3 ± 8.9	70.5 ± 8.7	68.0 ± 8.5	0.38
Year of diagnosis					0.17
1995 or before	223 (31%)	200 (32%)	8 (16%)	15 (31%)	
1996–2000	222 (31%)	191 (31%)	16 (32%)	15 (31%)	
2001–2008	279 (39%)	234 (37%)	26 (52%)	19 (39%)	
Family history of colorectal cancer in first-degree relative(s)					0.37
Absent	574 (80%)	492 (79%)	43 (88%)	39 (81%)	
Present	142 (20%)	127 (21%)	6 (12%)	9 (19%)	
Tumour location					0.020
Cecum	129 (18%)	102 (16%)	11 (22%)	16 (33%)	
Ascending to transverse colon	244 (34%)	209 (34%)	18 (36%)	17 (35%)	
Descending to sigmoid colon	197 (27%)	180 (29%)	7 (14%)	10 (20%)	
Rectum	152 (21%)	132 (21%)	14 (28%)	6 (12%)	
Tumour differentiation					<0.001
Well to moderate	660 (91%)	581 (93%)	42 (86%)	37 (76%)	
Poor	63 (8.7%)	44 (7.0%)	7 (14%)	12 (24%)	
pT stage					0.003
pT1	55 (8.2%)	52 (9.0%)	1 (2.1%)	2 (4.3%)	
pT2	135 (20%)	124 (22%)	9 (19%)	2 (4.3%)	
pT3	443 (66%)	373 (65%)	33 (70%)	37 (79%)	
pT4	37 (5.5%)	27 (4.7%)	4 (8.5%)	6 (13%)	
pN stage					0.29
pN0	396 (61%)	349 (62%)	23 (51%)	24 (53%)	
pN1	158 (24%)	132 (24%)	15 (33%)	11 (24%)	
pN2	95 (15%)	78 (14%)	7 (16%)	10 (22%)	
M stage					0.11
M0	572 (86%)	493 (86%)	43 (93%)	36 (78%)	
M1	96 (14%)	83 (14%)	3 (6.5%)	10 (22%)	
AJCC disease stage					0.008
I	150 (22%)	141 (24%)	6 (13%)	3 (6.5%)	
II	223 (33%)	187 (32%)	16 (35%)	20 (43%)	
III	199 (30%)	165 (29%)	21 (46%)	13 (28%)	
IV	96 (14%)	83 (14%)	3 (6.5%)	10 (22%)	

Characteristic*	All cases (N = 724)	Amount of *F. nucleatum* DNA in colorectal cancer tissue: Negative (N = 625)	Low (N = 50)	High (N = 49)	*P* value†
MSI status					< 0.001
Non-MSI-high	588 (84%)	527 (87%)	32 (67%)	29 (59%)	
MSI-high	116 (16%)	80 (13%)	16 (33%)	20 (41%)	
CIMP status					< 0.001
Low/negative	546 (82%)	484 (84%)	35 (78%)	27 (59%)	
High	118 (18%)	89 (16%)	10 (22%)	19 (41%)	
Mean LINE-1 methylation level ± SD (%)	62.4 ± 9.6	62.1 ± 9.6	62.7 ± 10.0	65.0 ± 9.2	0.12
KRAS mutation					0.16
Wild-type	414 (59%)	363 (60%)	21 (46%)	30 (62%)	
Mutant	289 (41%)	246 (40%)	25 (54%)	18 (38%)	
BRAF mutation					0.009
Wild-type	597 (85%)	523 (86%)	40 (83%)	34 (69%)	
Mutant	109 (15%)	86 (14%)	8 (17%)	15 (31%)	
PIK3CA mutation					0.66
Wild-type	566 (85%)	493 (85%)	36 (84%)	37 (80%)	
Mutant	101 (15%)	85 (15%)	7 (16%)	9 (20%)	
BECN1 (beclin 1) expression‡					< 0.001
Low	102 (16%)	77 (14%)	14 (31%)	11 (28%)	
Intermediate	289 (46%)	251 (46%)	20 (44%)	18 (46%)	
High	237 (38%)	216 (40%)	11 (24%)	10 (26%)	
MAP1LC3 (LC3) expression‡					0.062
Low	201 (29%)	163 (27%)	17 (36%)	21 (46%)	
Intermediate	221 (32%)	199 (33%)	12 (26%)	10 (22%)	
High	267 (39%)	234 (39%)	18 (38%)	15 (33%)	
SQSTM1 (p62) expression‡					0.83
Low	158 (23%)	131 (22%)	15 (33%)	12 (28%)	
Intermediate	309 (46%)	278 (48%)	18 (39%)	13 (30%)	
High	207 (31%)	176 (30%)	13 (28%)	18 (42%)	

*Percentage indicates the proportion of patients with a specific clinical, pathologic, or molecular characteristic among all patients or in strata of the amount of *F. nucleatum* DNA.

†To compare categorical data between subgroups classified by the amount of *F. nucleatum* DNA (except for tumour differentiation and pT stage, for which the Fisher's exact test was performed), the chi-square test was performed, unless otherwise noted. To compare continuous variables, an analysis of variance was performed.

‡To assess associations between the amount of *F. nucleatum* DNA in colorectal cancer tissue (negative, low, and high) and tumour *BECN1*, *MAP1LC3*, and *SQSTM1* expression level (low, intermediate, and high) and, the Spearman's correlation test was performed.

Abbreviations: AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; LINE-1, long-interspersed nucleotide element-1; MSI, microsatellite instability; NHS, Nurses' Health Study; SD, standard deviation.

Table 2.

Inverse probability weighting (IPW)-adjusted ordinal logistic regression analysis to assess the association of tumour *BECN1* (beclin 1), *MAP1LC3* (LC3), and *SQSTM1* (p62) expression level (Predictor) with the amount of *Fusobacterium nucleatum* (*F. nucleatum*) DNA (Outcome)

	Amount of *F. nucleatum* DNA in colorectal cancer tissue	
	Univariable OR (95% CI)*	**Multivariable OR (95% CI)*,†**
Tumour *BECN1* expression (N = 628)		
Low	1 (referent)	1 (referent)
Intermediate	0.48 (0.26–0.88)	0.54 (0.29–0.99)
High	0.27 (0.14–0.52)	0.31 (0.16–0.60)
P_{trend}‡	< 0.001	< 0.001
Tumour *MAP1LC3* expression (N = 689)		
Low	1 (referent)	1 (referent)
Intermediate	0.46 (0.25–0.83)	0.46 (0.25–0.85)
High	0.55 (0.32–0.95)	0.57 (0.33–0.99)
P_{trend}‡	0.043	0.061
Tumour *SQSTM1* expression (N = 674)		
Low	1 (referent)	1 (referent)
Intermediate	0.54 (0.30–0.97)	0.55 (0.30–1.01)
High	0.88 (0.48–1.60)	0.89 (0.48–1.65)
P_{trend}‡	0.75	0.79

*IPW was applied to reduce a bias due to the availability of tumour tissue after cancer diagnosis (see “Statistical analysis” subsection for details).

†The multivariable ordinal logistic regression model initially included age, sex, year of diagnosis, family history of colorectal cancer, tumour location, microsatellite instability, CpG island methylator phenotype, long-interspersed nucleotide element-1 methylation level, and *KRAS*, *BRAF*, and *PIK3CA* mutations. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final model. The variables which remained in the final models are shown in Supplementary Table S3, S4, and S5.

‡P_{trend} was calculated by the linear trend across the ordinal categories of tumour *BECN1*, *MAP1LC3*, and *SQSTM1* expression level (low, intermediate, and high, as an ordinal predictor variable) in the IPW-adjusted ordinal logistic regression model for the amount of *F. nucleatum* DNA (negative, low, and high, as an ordinal outcome variable).

Abbreviations: CI, confidence interval; IPW, inverse probability weighting; OR, odds ratio.

Table 3.

Tumour *BECN1* (beclin 1), *MAP1LC3* (LC3), and *SQSTM1* (p62) expression level and patient survival with inverse probability weighting (IPW)

		Colorectal cancer-specific survival			Overall survival		
	No. of cases	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†	No. of events	Univariable HR (95% CI)*	Multivariable HR (95% CI)*,†
Tumour *BECN1* expression							
Low	98	29	1 (referent)	1 (referent)	60	1 (referent)	1 (referent)
Intermediate	283	79	0.96 (0.61–1.51)	0.80 (0.50–1.27)	163	0.94 (0.66–1.34)	0.89 (0.62–1.26)
High	233	66	0.99 (0.63–1.57)	0.74 (0.45–1.20)	143	0.95 (0.67–1.36)	0.85 (0.59–1.22)
P_{trend}‡			0.99	0.26		0.85	0.39
Tumour *MAP1LC3* expression							
Low	196	47	1 (referent)	1 (referent)	103	1 (referent)	1 (referent)
Intermediate	218	65	1.33 (0.89–1.99)	1.49 (1.00–2.22)	136	1.18 (0.88–1.57)	1.19 (0.90–1.58)
High	261	77	1.30 (0.89–1.91)	1.07 (0.72–1.58)	164	1.18 (0.91–1.53)	1.11 (0.84–1.45)
P_{trend}‡			0.20	0.94		0.25	0.63
Tumour *SQSTM1* expression							
Low	152	50	1 (referent)	1 (referent)	98	1 (referent)	1 (referent)
Intermediate	304	83	0.80 (0.55–1.16)	0.78 (0.54–1.12)	183	0.97 (0.73–1.29)	0.94 (0.71–1.23)
High	204	54	0.82 (0.55–1.24)	0.71 (0.47–1.08)	119	0.94 (0.70–1.26)	0.86 (0.63–1.17)
P_{trend}‡			0.38	0.10		0.66	0.32

*IPW was applied to reduce a bias due to the availability of tumour tissue after cancer diagnosis (see “Statistical analysis” subsection for details).

†The multivariable Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer, tumour location, tumour differentiation, disease stage, microsatellite instability, CpG island methylator phenotype, *KRAS, BRAF*, and *PIK3CA* mutations, and long-interspersed nucleotide element-1 methylation level. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models.

‡P_{trend} value was calculated across the ordinal categories of tumour *BECN1, MAP1LC3*, and *SQSTM1* expression levels (low, intermediate, and high) in the IPW-adjusted Cox regression model.

Abbreviations: CI, confidence interval; HR, hazard ratio; IPW, inverse probability weighting.