Association of *Fusobacterium nucleatum* with immunity and molecular alterations in colorectal cancer

Katsuhiko Nosho, Yasutaka Sukawa, Yasushi Adachi, Miki Ito, Kei Mitsuhashi, Hiroyoshi Kurihara, Shinichi Kanno, Itaru Yamamoto, Keisuke Ishigami, Hisayoshi Igarashi, Reo Maruyama, Kohzoh Imai, Hiroyuki Yamamoto, Yasuhisa Shinomura

Katsuhiko Nosho, Yasutaka Sukawa, Yasushi Adachi, Miki Ito, Kei Mitsuhashi, Hiroyoshi Kurihara, Shinichi Kanno, Itaru Yamamoto, Keisuke Ishigami, Hisayoshi Igarashi, Yasuhisa Shinomura, Department of Gastroenterology, Rheumatology and Clinical Immunology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

Reo Maruyama, Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

Kohzoh Imai, Division of Cancer Research, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

Hiroyuki Yamamoto, Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan

Author contributions: Nosho K designed the report, analyzed the data and wrote the manuscript; Adachi Y and Maruyama R analyzed the data; Ito M, Mitsuhashi K, Kurihara H, Kanno S, Yamamoto I, Ishigami K and Igarashi H performed experiments; Sukawa Y, Adachi Y, Imai K, Yamamoto H and Shinomura Y edited the manuscript.

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Correspondence to: Katsuhiko Nosho, MD, PhD, Department of Gastroenterology, Rheumatology and Clinical Immunology, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan. nosho@sapmed.ac.jp Telephone: +81-11-6112111 Fax: +81-11-6112282

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Abstract

The human intestinal microbiome plays a major role in human health and diseases, including colorectal cancer. Colorectal carcinogenesis represents a heterogeneous process with a differing set of somatic molecular alterations, influenced by diet, environmental and microbial exposures, and host immunity. *Fusobacterium* species are part of the human oral and intestinal microbiota. Metagenomic analyses have shown an enrichment of *Fusobacterium nucleatum* (F. nucleatum) in colorectal carcinoma tissue. Using 511 colorectal carcinomas from Japanese patients, we assessed the presence of *F. nucleatum*. Our results showed that the frequency of *F. nucleatum* positivity in the Japanese colorectal cancer was 8.6% (44/511), which was lower than that in United States cohort studies (13%). Similar to the United States studies, *F. nucleatum* positivity
INTRODUCTION

The human intestinal microbiome encompasses at least 100 trillion (10^{12}) microorganisms and plays a major role in human health and diseases, including colorectal cancer[1-3]. Colorectal carcinogenesis represents a heterogeneous process with a differing set of somatic molecular alterations, influenced by diet, environmental and microbial exposures, and host immunity[4,5]. Fusobacterium species (a group of non-spore-forming, anaerobic gram-negative bacteria) are a part of the normal human oral and intestinal microbiota. The species of the Fusobacterium genera are highly heterogeneous, and some of them have been recognized as opportunistic pathogens implicated not only in periodontitis[6-8], but also in inflammatory bowel disease (IBD)[9-11], pancreatic abscess[12,13], and hepatic abscesses[14,15]. Regarding gastrointestinal cancer, metagenomic analyses have shown an enrichment of Fusobacterium nucleatum (F. nucleatum) in colorectal carcinoma tissue, which has been confirmed by quantitative PCR for the 16S ribosomal RNA gene DNA sequence of F. nucleatum[16,17]. Studies have shown that a greater amount of F. nucleatum in colorectal carcinoma tissue is associated with high degrees of microsatellite instability (MSI-high) and CpG island methylator phenotype (CIMP)[18].

Accumulating evidence indicates that innate and adaptive immunity influences tumor evolution[19]. Attesting to an important role of T-cell-mediated adaptive immunity in inhibiting tumor progression, therapeutic antibodies against immune checkpoint molecules, including CTLA4, PD1 (programmed cell death 1; PD-1), and CD274 (programmed cell death 1 ligand 1; PD-L1), can effectively enhance antitumor T-cell activity in various malignancies[20,21]. Emerging evidence indicates that tumor genetic alterations and tumor-host interactions have complex roles in the effectiveness of T-cell-based immunotherapies[22-25]. Although these immunotherapies appeared to be less effective for colorectal cancer, high-level infiltrates of T-cells in colorectal cancer tissue have been associated with better patient survival[26-28], and a recent study has suggested a potential role for the immune checkpoint pathway in suppressing the antitumor immune response in a subset of colorectal cancers[29].

Regarding the association between the gut microbiome and immunity, a number of studies have shown that F. nucleatum has immunosuppressive activities via inhibiting human T-cell responses to mitogens and antigens[30-35]. Additionally, F. nucleatum inhibitory protein has been shown to arrest human T-cells in the G1 phase of the cell cycle[32]. Furthermore, F. nucleatum can induce apoptotic cell death in peripheral blood mononuclear cells and Jurkat T-cells[31]. This F. nucleatum-induced cell death is mediated through the aggregation of the immune cells, which might have important implications for the...
pathogenesis of this bacterial species\textsuperscript{[35]} These findings indicate that \textit{F. nucleatum} suppressively modulates the tumor-immune microenvironment.

Thus, the results of these studies suggest a complex link between the gut microbiome, immunity, and molecular alterations in colorectal tumorigenesis. A better understanding of the relationship between microorganisms and immune cells in the tumor microenvironment is needed in order to effectively target the microbiota and immunity for colorectal cancer prevention and therapy.

**AMOUNT OF \textit{F. nucleatum} IN COLORECTAL CARCINOMA TISSUE AND THE ASSOCIATION WITH MSI**

Using quantitative PCR, Mima \textit{et al.}\textsuperscript{[36]} have reported that \textit{F. nucleatum} was detected in 76 (13\%) of 598 colorectal carcinomas (stages I - IV) within the well-known United States cohort studies (the Nurses’ Health Study and the Health Professionals Follow-up Study) and in adjacent non-tumor tissue in 19 (3.4\%) of 558 cases analyzed. In the 558 pairs of colorectal carcinoma and adjacent non-tumor tissues, the amount of \textit{F. nucleatum} was higher in colorectal carcinoma tissue than in paired adjacent non-tumor tissue\textsuperscript{[36]}.

We also collected 511 colorectal carcinoma tissues (stages I - IV) from Japanese patients who underwent endoscopic resection or other surgical treatment and assessed the presence of \textit{F. nucleatum} via gene expression analysis. Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues. The amount of \textit{F. nucleatum} in colorectal carcinoma tissue was measured by quantitative PCR assay as previously described\textsuperscript{[36]}.

Considering the influence of contaminating stromal cells, we performed microdissection only in cases with \textit{F. nucleatum} positivity and conducted quantitative PCR again using the DNA extracted from the carcinoma component. Our current data demonstrated that \textit{F. nucleatum} positivity was detected in 44 (8.6\%) of the 511 Japanese patients with colorectal cancer (Table 1). The frequency of \textit{F. nucleatum} positivity in the Japanese patients was significantly lower than that in the United States cohort study\textsuperscript{[36]}.

Some cohort studies observed associations of highly enriched \textit{Fusobacterium} in colorectal cancer tissues with CIMP-high, MSI-high, and MLH1 methylation in patients with colorectal cancer\textsuperscript{[18,36,37]} Consistent with these reports, our current data using Japanese populations showed that high expression of \textit{F. nucleatum} in colorectal cancers was significantly associated with MSI-high status (Table 1). We also examined the relationship between the amount of \textit{F. nucleatum} and patient mortality; however \textit{F. nucleatum} status in colorectal cancers was not associated with cancer-specific survival. The role of \textit{F. nucleatum} in colorectal carcinogenesis remains uncertain. Recent studies showed that \textit{F. nucleatum} increases the production of reactive oxygen species (ROS) and inflammatory cytokines (\textit{e.g.,} IL-6 and TNF) in colorectal cancer\textsuperscript{[38]} Inflammation and ROS can reduce the enzymatic activity of mismatch repair (MMR) proteins and cause epigenetic silencing of the mismatch repair protein MLH1 leading to MSI\textsuperscript{[39]}.

**ASSOCIATION BETWEEN IMMUNE CELL AND CLINICAL OUTCOME IN COLORECTAL CANCER**

The abundance of tumor-infiltrating T-cells has been associated with improved clinical outcomes in colorectal cancer patients\textsuperscript{[18,46,47]} Although the exact mechanism remains uncertain, the adaptive immune system may play an important role in suppressing tumor progression\textsuperscript{[27,41]} Tumor-infiltrating T-cells may be an indicator of a host immune response to tumors and are attractive targets for immunotherapy\textsuperscript{[42-45]} Tumor-infiltrating lymphocytes may also reflect specific molecular alterations associated with indolent tumor behavior. Previous studies have shown that lymphocytic infiltration is associated with MSI in colorectal cancer\textsuperscript{[40,46-48]} Truncated peptides produced by frame-shift mutations due to MSI may be immunogenic and contribute to host immune response\textsuperscript{[41,43,49]} However, little is known about the relationship between tumor-infiltrating T-cells and other tumor molecular features, including the CIMP status, and KRAS, BRAF and PIK3CA mutations.

We previously utilized a database of clinically and molecularly annotated colorectal carcinoma cases (\textit{n} = 768; stages I - IV) in the United States cohort studies\textsuperscript{[38]} Using tissue microarray and automated Ariol image analysis system, we quantified densities of CD3\textsuperscript{+}, CD8\textsuperscript{+}, CD45RO\textsuperscript{+}, and FOXP3\textsuperscript{+} T-cells within neoplastic epithelial areas. Our data demonstrated that tumor-infiltrating CD45RO\textsuperscript{+} T-cell density is significantly associated with longer survival of colorectal cancer patients, independent of clinical, pathological, and molecular features (\textit{i.e.,} MSI, CIMP, and KRAS, BRAF and PIK3CA mutations). In addition, MSI-high is an independent predictor of CD45RO\textsuperscript{+} T-cell density. The strong association between MSI and CD45RO\textsuperscript{+} T-cell density supports the hypothesis that truncated peptides produced by MSI and frameshift mutations may elicit a host immune response and recruit CD45RO\textsuperscript{+} T-cells\textsuperscript{[41,49]}

In most studies, MSI in colon cancer has been associated with improved survival\textsuperscript{[27,41,50,51]} although the mechanism underlying this association is largely unknown. Similar to these reports from United States and Western countries\textsuperscript{[27,41,50,51]}, our current Japanese population-based study showed a significantly lower
Table 1 Clinical and molecular features in Japanese patients with colorectal cancer according to *Fusobacterium nucleatum* status

| Clinical and molecular features | Total (n) | *F. nucleatum* expression |
|--------------------------------|-----------|--------------------------|
|                                |           | Negative (n) | Positive (n) | P-value |
| All cases                      | 511       | 467 (91)     | 44 (8.6)     |         |
| Gender                         |           |              |              |         |
| Male                           | 286 (56)  | 267 (57)     | 19 (43)      | 0.075   |
| Female                         | 225 (44)  | 200 (43)     | 25 (57)      |         |
| Age (mean ± SD)                | 67.1 ± 11.8| 67.3 ± 11.7  | 65.0 ± 12.1  | 0.220   |
| Tumor size (mm) (mean ± SD)    | 49.8 ± 24.1| 49.2 ± 24.5  | 56.4 ± 19.6  | 0.063   |
| Tumor location                 |           |              |              |         |
| Rectum                         | 207 (41)  | 194 (42)     | 13 (30)      |         |
| Distal colon                   | 133 (26)  | 121 (26)     | 12 (27)      | 0.240   |
| (Sigmoid colon to splenic flexure) |       |              |              |         |
| Proximal colon                 | 171 (33)  | 152 (33)     | 19 (43)      |         |
| Disease stage                  |           |              |              |         |
| I                              | 56 (11)   | 53 (11)      | 3 (6.8)      | 0.470   |
| II                             | 160 (31)  | 142 (30)     | 18 (41)      |         |
| III                            | 235 (46)  | 216 (46)     | 19 (43)      |         |
| IV                             | 60 (12)   | 56 (12)      | 4 (9.0)      |         |
| KRAS mutation (codon 12/13/61/146) |         |              |              |         |
| Wild-type                      | 354 (69)  | 324 (69)     | 30 (68)      | 0.870   |
| Mutant                         | 157 (31)  | 143 (31)     | 14 (32)      |         |
| BRAF mutation (codon 600)      |           |              |              |         |
| Wild-type                      | 483 (95)  | 445 (95)     | 38 (86)      | 0.031   |
| Mutant                         | 28 (5.5)  | 22 (4.7)     | 6 (14)       |         |
| PIK3CA mutation (exon 9/20)    |           |              |              |         |
| Wild-type                      | 451 (88)  | 414 (89)     | 37 (84)      | 0.390   |
| Mutant                         | 60 (12)   | 53 (11)      | 7 (16)       |         |
| MSI status                     |           |              |              |         |
| MSS/MSI-low                    | 470 (92)  | 435 (93)     | 35 (80)      | 0.0059  |
| MSI-high                       | 41 (8.0)  | 32 (6.9)     | 9 (20)       |         |

Percentage (%) indicates the proportion of cases with a specific clinical or molecular feature within a given category of *F. nucleatum* detection by quantitative PCR. The P-values were calculated using t-test for age and tumor size and by means of the χ² test or Fisher’s exact test for all other variables. *F. nucleatum*: *Fusobacterium nucleatum*; MSI: Microsatellite instability; MSS: Microsatellite stable.

Mortality rate (log-rank test: P = 0.048) in the MSI-high group than in the MSS/MSI-low group using the Kaplan-Meier method (data not shown). These results suggest one explanation that a host immune is stimulated in response to MSI-high colorectal cancer.

ASSOCIATION BETWEEN *F. NUCLEATUM* AND IMMUNE RESPONSE IN COLORECTAL CANCER

Myeloid-derived immune cells can inhibit T-cell proliferation and induce T-cell apoptosis⁵². Recently, Kostic et al.⁶ reported that *F. nucleatum* selectively expands myeloid-derived immune cells in colorectal cancer. In particular, myeloid-derived immune cells were enriched in *F. nucleatum*-fed mice vs controls. Myeloid-derived immune cells have been proposed to be myeloid cells present in the bone marrow, spleen, or tumor microenvironment that are able to suppress T-cell responses⁵³. During tumor progression, reactive myeloid cells might mediate immunosuppression either by the self-limiting mechanism of T helper type (Th)1 inflammation resolution, such as ROS and IL-10 production, or by switching to a wound repair and angiogenic protumor Th2 inflammation with the expression of arginase, TGF-β, and IL-10⁵⁴. These results indicate that *F. nucleatum* suppressively modulates the tumor-immune microenvironment because T-cell-mediated adaptive immunity plays an important role in preventing the development of tumors and inhibiting tumor progression⁵⁵. Thus, immunosuppression by *F. nucleatum* may affect patient mortality in colorectal cancer. Additionally, the data in the United States cohort studies along with these lines of experimental evidence revealed that the amount of tissue *F. nucleatum* is inversely associated with CD3+ T-cell density in colorectal carcinoma tissue⁵⁶.  

ASSOCIATION BETWEEN MICRONORNA EXPRESSION AND IMMUNITY IN COLORECTAL CANCER

MicroRNAs constitute a class of small non-coding RNA molecules that function as post-transcriptional gene regulators and have been increasingly recognized as biomarkers of various human cancers⁵⁶–⁷¹. Regarding colorectal cancer, we recently discovered...
that microRNA-31 (miR-31) expression is significantly up-regulated in BRAF-mutated cancers compared with that in wild-type cancers using microRNA array analysis. Moreover, associations were identified between miR-31 expression and poor prognosis for colorectal cancers.

Certain microRNAs are induced during the macrophage inflammatory response and have the ability to regulate host-cell responses to pathogens. In addition, pathogens themselves may regulate microRNA expression. Among the various microRNAs, microRNA-21 (miR-21) has been shown to play roles in immunity and colorectal carcinogenesis. In fact, high-level miR-21 expression in colorectal cancer tissue has been associated with worse clinical outcome, suggesting that miR-21 could act as a prognostic tumor biomarker. Studies have shown that miR-21 increases the levels of IL-10 and PGE2, which suppress antitumor T-cell-mediated adaptive immunity through the inhibition of the antigen-presenting capacities of dendritic cells and T-cell proliferation and through the recruitment of myeloid-derived suppressor cells into the tumor microenvironment.

CONCLUSION
The association between highly enriched F. nucleatum in colorectal carcinoma tissues and MSI-high status was observed in both the United States cohort studies and Japanese population-based study. Previous studies have reported that the frequency of colorectal cancers with MSI-high status in Japan (less than 10%) tend to be lower than those in the United States and Western countries (approximately 15%). Therefore, the low rate of MSI-high colorectal cancer in Japan might be due to the amount of F. nucleatum in carcinoma tissues because our current data showed that the rate of F. nucleatum positivity in Japanese patients was significantly lower than that in the United States cohorts. MSI-high status in colorectal cancer has been associated with high levels of infiltrating T-cells, as mismatch repair defects in MSI-high tumors cause numerous frameshift mutations and truncated proteins, which elicit antitumor T-cell-mediated adaptive immunity. However, MSI status is not the sole determinant of the immune response to colorectal cancer because the amounts of tumor-infiltrating T-cells considerably overlap between MSI-high and microsatellite stable (MSS) colorectal tumors. Hence, there must be other factors that influence the antitumor immune response to colorectal cancer.

T-cell-mediated adaptive immunity plays an important role in regulating tumor evolution and in inhibiting tumor progression. The immunity includes multiple steps involving the clonal selection of antigen-specific cells, their activation and proliferation in secondary lymphoid tissues, and their recruitment into the tumor microenvironment. In a mouse model, F. nucleatum recruits myeloid-derived suppressor cells into the tumor microenvironment. Myeloid-derived suppressor cells can inhibit T-cell proliferation and...
induce T-cell apoptosis\textsuperscript{[52]}, Virulence factors derived from \textit{F. nucleatum} also inhibit T-cell proliferation\textsuperscript{[33,94]}. The experimental evidence may be consistent with a recent finding that a higher abundance of \textit{F. nucleatum} in colorectal carcinoma tissue was associated with a lower density of T-cells, as measured by CD3 in the tumor microenvironment\textsuperscript{[36]}. These findings support a role of \textit{F. nucleatum} in down-regulating antitumor T-cell-mediated adaptive immunity.

Both tumor molecular and immunity analyses are increasingly important in cancer research and clinical practice. MicroRNAs play roles in carcinogenesis and immunity and can be potential biomarkers or therapeutic targets. MicroRNA-targeting therapies for human disease, including cancer, are currently being investigated\textsuperscript{[69,95,96]}. Accumulating evidence suggest miR-21 increases the levels of IL-10 and PGE2 in the tumor microenvironment, which can lead to the suppression of antitumor T-cell-mediated adaptive immunity\textsuperscript{[84-86]}. In light of these findings, it would be intriguing for future research to explore a potential strategy for inhibiting miR-21 and its immunosuppressive effect in immunotherapy and immunoprevention for colorectal cancer. In contrast, no study has reported whether \textit{F. nucleatum} regulates microRNA expressions, including miR-21. Therefore, functional analysis and/or human population-based study are expected to identify the association between \textit{F. nucleatum} and miR-21 expression in colorectal cancer.

We have summarized the hypothesis of the potential mechanism underlying the association of \textit{F. nucleatum} in colorectal cancer with immune cells and molecular alterations in Figure 1. \textit{F. nucleatum} increases the production of ROS and inflammatory cytokines in colorectal cancer. Inflammation and ROS can cause epigenetic silencing of the mismatch repair protein MLH1 leading to MSI. \textit{F. nucleatum} possesses immunosuppressive activities by inhibiting human T-cell responses and modulates tumor-immune microenvironment suppressively. miR-21 increases the levels of IL-10 and PGE2, which suppress antitumor T-cell-mediated adaptive immunity in the tumor microenvironment.

Thus, emerging evidence may provide insights for strategies to target microbiota, immune cells, and tumor molecular alterations for colorectal cancer prevention and treatment. Further investigation is needed to clarify the association of \textit{Fusobacterium} with T-cells and microRNA expressions in colorectal cancer.

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