

# Fusobacterium nucleatum Promotes Gastric Cancer Aggressiveness through Upregulation of Cell Mobility and Interferon Genes

**Yung-Yu Hsieh**

Chiayi Chang Gung Memorial Hospital

**Shui-Yi Tung**

Chiayi Chang Gung Memorial Hospital

**Hung-Yu Pan**

National Chiayi University

**Te-Sheng Chang**

Chiayi Chang Gung Memorial Hospital

**Kuo-Liang Wei**

Chiayi Chang Gung Memorial Hospital

**Wei-Ming Chen**

Chiayi Chang Gung Memorial Hospital

**Yi-Fang Deng**

Chiayi Chang Gung Memorial Hospital

**Chung-Kuang Lu**

Chiayi Chang Gung Memorial Hospital

**Yu-Hsuan Lai**

Chiayi Chang Gung Memorial Hospital

**Cheng-Shyong Wu**

Chiayi Chang Gung Memorial Hospital

**Chin Li (✉ [biocl@ccu.edu.tw](mailto:biocl@ccu.edu.tw))**

National Chung Cheng University <https://orcid.org/0000-0002-5457-9623>

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## Research

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# Abstract

**Background** *Fusobacterium nucleatum* was previously found to become a dominant species in the gastric cancer-associated microbiota of patients from Taiwan. However, the prevalence of *Fusobacterium nucleatum* infection in gastric cancer has not been examined in a larger patient cohort. In addition, whether *Fusobacterium nucleatum* elicits a cellular response in gastric cancer remains unknown.

**Methods** A study cohort of resected gastric cancer tissue specimens was examined using nested PCR to detect *Fusobacterium nucleatum*. In vitro coculture of *Fusobacterium nucleatum* was carried out to identify the alteration in the expression profile of patient-derived gastric cancer cell line.

**Results** approximately one-third of gastric cancer tissues are positive for *Fusobacterium nucleatum*. Statistical analysis showed that the risk for *Fusobacterium nucleatum* infection is increased in late-stage cancer tissue specimens and incurs poorer survival in *Helicobacter pylori*-positive patients. In vitro coculture experiment shows a drastic interferon response activated only by a high multiplicity of infection, and the response peaks within 24 hours and subsides after 72 hours of incubation. Another set of response genes is the continuous increase of actins and their regulators with prolonged time of incubation, activated by both low and high multiplicity of infection.

**Conclusions** Our data indicates that *Fusobacterium nucleatum* incites an inflammatory response from the cancer cells and promotes cell mobility, likely

## Introduction

Disruption of normal flora is often associated with pathogenic conditions. In the gastric environment, the extreme acidity and thick protective mucosa limit the bacterial growth and colonization. Therefore, the complexity and abundance of the microbiota in the stomach are generally much lower than in the lower alimentary tract and oral cavity, and most of the gastric bacteria remains in the gastric juice [29]. However, *H. pylori* penetrates the mucus layer in order to colonize the gastric mucosa. Once colonized, *H. pylori* produces urease and elevates the local pH level, allowing long-term infection [15]. In addition to changing the microenvironment of colonization niche, virulence factors produced by *H. pylori* facilitate the transformation of gastric mucosal cells and lead to a drastic increase in the risk of gastric cancer [15,35].

Although *H. pylori* infection has been identified as the strongest risk factor for gastric cancer, its abundance in gastric cancer-associated microbiota often decreases or even becomes undetectable [13]. Our hypothesis to account for the decline in the abundance of *H. pylori* in gastric cancer is microbial succession. Hence, *H. pylori* infection is an event that primes the gastric epithelium for further oncogenic changes. Once colonization takes place, *H. pylori* creates a niche microenvironment on the gastric epithelium that facilitates the colonization of secondary settler bacteria. It is reasonable to expect that predominance of *H. pylori* can be replaced by the predominance of other bacteria after prolonged

infection period. It is an often-considered possibility that these secondary gastric microbes could also participate in promoting the development of gastric cancer.

With advanced sequencing technology, it has become feasible to profile the microbiota without the need to isolate pure cultures. In a previous study employing such experimental approach, we identified *Fusobacterium nucleatum* as one of the bacteria enriched in gastric cancer-associated microbiota. Some studies have found an enrichment of *Fusobacterium nucleatum* in the colorectal cancer-associated microbiota [7,23]. Attachment of *Fusobacterium nucleatum* to the colorectal cancer cells is mediated through the interaction of bacterial lectin Fap2 with tumor-specific surface Gal-GalNAc [1]. *Fusobacterium nucleatum* infection is correlated with high microsatellite instability status of colon cancer [25]. This is likely mediated by the secondary metabolite of *Fusobacterium nucleatum*, which exhibits DNA cytotoxicity and causes single-nucleotide mutations, while disabling mismatch repair [36]. Hence, it appears that *Fusobacterium nucleatum* promotes oncogenesis by acting as a DNA damage agent.

The presence of *Fusobacterium nucleatum* in clinical specimens may facilitate cancer diagnosis. Detection of *Fusobacterium* DNA using polymerase chain reaction (PCR) increases the detection sensitivity of standard fecal immunochemical test [32], indicating its potential clinical application as a noninvasive diagnostic marker. On the other hand, current evidence also suggests a role of *Fusobacterium nucleatum* in promoting the growth and metastasis of colorectal cancer [11,27], and the level of *Fusobacterium nucleatum* in the colorectal cancer-associated microbiota is correlated with poor prognosis in the patients [10,25]. Hence, *Fusobacterium nucleatum* can be used as a prognosis biomarker for colorectal cancer as well.

Although its role in colorectal cancer has been intensively studied, it remains unclear whether *Fusobacterium nucleatum* exerts a similar oncogenic effect on gastric epithelium. In this study, we investigated the impact of *Fusobacterium nucleatum* on patient-derived gastric cancer cells using an in vitro coculture model. Our findings indicate a significant disturbance in the expression of microtubules. In addition, a nested-PCR-based detection method was developed to detect the presence of *Fusobacterium nucleatum* in gastric cancer tissue specimens. Statistical analysis shows that the risk of *Fusobacterium nucleatum* infection greatly increases in late-stage gastric cancer patients. Moreover, *Fusobacterium nucleatum* infection incurs a poorer prognosis.

## Materials And Methods

### Patient Cohort

Gastric biopsies were obtained from Chiayi Chang Gung Memorial Hospital Tissue Bank or collected from resected cancer tissues of patients who underwent gastrectomy. The acquisition and use of clinical specimens in this study were done in accordance with the Declaration of Helsinki, and all the patients participating in this study were clearly informed about the aim of the study and signed a written informed consent. This study was approved and overseen by the Institutional Review Board of Chiayi Chang Gung

Memorial Hospital (institutional review board approval numbers: 201700169A3, 201700172A3, and 201700173A3).

## DNA Extraction and Nested PCR

Biopsies were rinsed extensively in phosphate-buffered saline to remove the mucus. Rinsed specimens were immersed overnight in RNeasy lysis reagent (Qiagen, Crawfordsville, IN, USA) and stored at  $-80^{\circ}\text{C}$ . Biopsies were pulverized in TRIzol reagent (Thermo Fisher Scientific) and centrifuged to remove undissolved debris. Total DNA, including both cellular and microbial DNA, was extracted according to the manufacturer's protocol. The concentration of DNA was determined using fluorometric quantification.

The presence of *Fusobacterium nucleatum* in the specimens was determined by nested PCR. The forward and reverse primer sequences of the first-stage PCR are 5'-TGTTAGAGGAAAGCCCAAGAAG and 5'-CTTCTTCCATAGGAATAGGGTCAG. The cycling condition is denaturation at  $94^{\circ}\text{C}$  for five minutes, 36 amplification cycles at  $94^{\circ}\text{C}$  for 30 seconds,  $55^{\circ}\text{C}$  for 30 seconds, and  $72^{\circ}\text{C}$  for 30 seconds, and the final extension step at  $72^{\circ}\text{C}$  for five minutes. The product of the first-stage amplification was directly used in the second-stage PCR. The forward and reverse primer sequences of the second-stage PCR are 5'-GCTTGAAATGGAAGCTACAAGAG and 5'-GGTCAGAACCAACTCCTACAAA. The second-stage PCR sequences are identical to those of the first-stage PCR. The sequence of PCR product was determined by automatic sequencing to confirm that the product is indeed the bacterial target sequence. The PCR product was analyzed in nondenaturing 6% polyacrylamide gel electrophoresis and documented. Statistical analysis was carried out using SAS/STAT statistical analysis software v. 9.4.

## Isolation of Gastric Cancer Cell Lines from Patients

The gastric cancer cell lines used in this study were isolated from recruited patients. Resected gastric cancer tissues were collected during surgery. To isolate gastric cancer cells, surgically resected cancer tissues were first extensively rinsed using Hank's balanced salt solution and were dissociated using Liberase TL in Hank's balanced salt solution at  $37^{\circ}\text{C}$  for one hour. After dissociation, the debris was removed, and isolated cells were extensively washed with phosphate buffered saline and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were allowed to attach to culture plates for 24 hours. Unattached cells were discarded, and the attached cells were cultured until expansion into single colonies. These colonies were then individually isolated and expanded. For this study, the gastric cancer cell line 008L-C2 derived from *Fusobacterium nucleatum*-infected patient was used in the in vitro coculture experiment.

## Coculturing of *Fusobacterium nucleatum* with Gastric Cancer Cells

The *Fusobacterium nucleatum* strain ATCC25586 [19] was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The bacteria were cultured on EG culture medium containing 2.5g Lab-Lemco powder, 10g proticasepeptone, 5g yeast extract, 4g glucose, 0.5g starch, 0.2g L-cystine, 0.5g L-cysteine HCl, 4g Na<sub>2</sub>HPO<sub>4</sub>, 15g Bacto-Agar, and 50 ml defibrinated horse blood per liter under anaerobic condition using BD GasPak system (Thermo Fisher). The bacteria were scrapped from the plate and resuspended in DMEM medium. The number of cells per millimeter in the resuspended medium was determined by microscopic observation. The correlation between the observed cell number and colony forming units was determined by reculturing of serial diluted bacteria. Cell line 008L-C2 was cultured using DMEM supplemented with 10% fetal bovine serum. The multiplicity of infection is 10 and 100. After 0, 24, and 72 hours of coculturing with *Fusobacterium nucleatum*, the cells were collected and washed twice with phosphate-buffered saline. Total RNA was extracted from washed cells using TRI reagent (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol.

## RNA Sequencing

The integrity and concentration of purified RNA samples were determined by capillary electrophoresis, using a TapeStation 2200 instrument (Agilent, Santa Clara, CA, USA), and by fluorometric quantification, using a Qubit Fluorometer (Invitrogen). The libraries for RNA sequencing analysis were prepared using SureSelect strand-specific mRNA library preparation kit (Agilent). The manufacturer's protocol was closely followed. Libraries were pooled and sequenced in a NextSeq sequencer. Quality filtration, mapping, annotation, and calculation of gene expression level were performed using CLC Genomic Workbench v.12.0.3 (Qiagen, Redwood City, CA, USA). The RNA level is expressed as transcripts per million (TPM). The sequencing data was deposited in Sequence Read Archive (SRA), National Center for Biotechnology Information, USA. The BioProject ID is PRJNA630089, and the BioSample accession numbers are SAMN14823957, SAMN14823958, SAMN14823959, SAMN14823960, and SAMN14823961.

## Results

In a previous metagenomic analysis, we identified colonization of potential pathogens, including *Fusobacterium nucleatum*, in gastric cancer-associated microbiota. Among the eleven cancer biopsies we collected in the study, we found that four specimens were colonized by *Fusobacterium nucleatum*, suggesting that *Fusobacterium nucleatum* infection is a frequent event in the gastric cancer patients of the southwestern region of Taiwan. To provide additional support to this observation, we obtained gastric cancer tissue specimens from the Tissue Bank of Chiayi Chang Kung Hospital. These specimens are resected gastric cancer tissues from patients who underwent partial or total gastrectomy. Patients with localized cancer undergo gastrectomy so that the main lesion can be effectively removed and this is expected to offer a better prognosis for the patients. As a result, the patient cohort in this study has a

better overall five-year survival rate than the survival rate reported by Cancer Registry Annual Report, Taiwan. Although this cohort represents a subgroup of gastric cancer patients, it is well suited to examine the frequency of *Fusobacterium nucleatum* infection.

In this study, we developed a nested PCR method in order to determine the presence of *Fusobacterium nucleatum* in the specimens. The purpose is to increase the sensitivity as well as the specificity of the detection assay. In previous studies using the conventional PCR method, *Fusobacterium nucleatum* infection in gastric cancer specimens was generally reported at low frequency [34], while our result obtained by metagenomic profiling suggests that the frequency is likely much higher than previously thought. There are two possibilities which could be the cause of this discrepancy. One possibility is that the frequency of *Fusobacterium nucleatum* infection is particularly high in this region in Taiwan, making it a unique phenomenon in this region. However, low frequency of detection may be due to the limitation of methodology, which subsequently leads to inability to detect low number of bacteria (*Fusobacterium nucleatum*) in the specimens. This is the likely scenario, especially when the specimens are removed from resected cancer tissues and contain little surface mucosa. By employing a nested PCR detection method, we hope to achieve higher detection sensitivity for identifying *Fusobacterium nucleatum* infection.

The detection target is highly conserved nusG gene of *Fusobacterium nucleatum* [17]. The size of the first- and second-stage PCR products is expected at 175 and 124 bp, respectively. The identity of amplified products was confirmed by Sanger's sequencing. We first tested the nested PCR detection method on DNA specimens from a previous study. The result showed that the nested PCR protocol is able to identify the majority of *Fusobacterium nucleatum*-positive specimens, except those specimens with very low percentage (data not shown). Although metagenomic analysis determines only the ratio but not the absolute number of bacteria (*Fusobacterium nucleatum*) in these specimens, we calculated the positive identification rate at approximately 90% while no specimen was falsely identified. We, subsequently, employed the nested PCR method followed to determine the presence of *Fusobacterium nucleatum* in the resected gastric cancer tissue specimens (Fig. 1A). The result showed that 19 of the 60 specimens examined are positively identified for *Fusobacterium nucleatum* infection. The other 41 specimens either are negative or show only marginally recognizable PCR products. All these specimens are grouped as negative for *Fusobacterium nucleatum*. The ratio of *Fusobacterium nucleatum*-positive patients is about one-third of the cohort, a result similar to that reported in our previous study using upper endoscopic-collected gastric biopsies.

The risk of *Fusobacterium nucleatum* infection was analyzed against clinical characteristics and cancer stage of the cohort (Fig.1B). The result of analysis indicates that older age ( $\geq 75$ ) does not change the risk of *Fusobacterium nucleatum* infection. However, *Helicobacter pylori* infection appears to be positively associated with a higher risk of *Fusobacterium nucleatum* infection. In addition, the risk increases in the late-stage gastric cancer patients, suggesting the microenvironment becoming more suitable for colonization and growth of *Fusobacterium nucleatum* during the progression of cancer. Interestingly, there is a significant decrease in the risk of *Fusobacterium nucleatum* infection in male

patients. We have no explanation for this observation at present, and it is necessary to examine larger or independent cohort to see if this phenomenon is also present in other cohorts.

We then analyzed the survival rate of patients who tested positive and negative for *Fusobacterium nucleatum*. The result showed that *Fusobacterium nucleatum*-infected patients appear to have a poorer outcome although a statistical significance ( $p < 0.05$ ) was not attained (Fig. 2A). When the stage I patients who have nearly 100% five-year survival rate are excluded in survival analysis, the result is still similar in that *Fusobacterium nucleatum*-positive patients seem to have a poorer outcome (Fig. 2A). As noted above, patients who underwent gastrectomy have an overall better treatment outcome, hence minimizing the effect of other unfavorable factors. Nevertheless, despite of the lack of statistical significance, our analysis showed that *Fusobacterium nucleatum* infection could have an adverse impact on the treatment outcome.

In our cohort, approximately one third patients are tested positive for *Helicobacter pylori* infection. Our previous study suggested that *Fusobacterium nucleatum* is likely a secondary settler to the gastric microbiota after *Helicobacter pylori* colonizes gastric epithelium. It is possible that consecutive or simultaneous infection of *Helicobacter pylori* and *Fusobacterium nucleatum* could have synergistic effect to drive cancer progression. To examine this hypothesis, we analyzed the impact of *Fusobacterium nucleatum* infection to the survival of *Helicobacter pylori* positive patients. The result clearly demonstrated that patients infected by both *Helicobacter pylori* and *Fusobacterium nucleatum* have a poorer outcome than those infected with only *Helicobacter pylori* (Fig. 2B). Similar result is observed when stage I patients were excluded from the analysis (Fig. 2B). Our data provides an important clinical evidence indicating the impact of *Fusobacterium nucleatum* infection to the prognosis of gastric cancer patients.

The clinical data indicates that *Fusobacterium nucleatum* infection is frequent in patients with advanced-stage gastric cancer and may exert an unfavorable impact on the survival. We hypothesize that the interaction of *Fusobacterium nucleatum* with the host cells promotes cancer progression and leads to poorer outcome. In order to provide experimental support to this hypothesis, we investigated the pathogenic molecular effect of *Fusobacterium nucleatum* on the gastric cancer cells through an in vitro coculture system. For this study, we isolated a gastric cancer cell line derived from the resected gastric cancer tissue from a patient who was confirmed to have *Fusobacterium nucleatum* infection. *Fusobacterium nucleatum* strain ATCC25586 was cultured under anaerobic condition and harvested when the coculture experiment was performed. The bacteria was resuspended in DMEM and added to the cell culture with multiplicity of infection (MOI) at 10 and 100. The coculture was done at 37°C and 5% CO<sub>2</sub> for 24 and 72 hours. The cells were then extensively washed and collected for subsequent RNA expression analysis by next-generation sequencing. During the incubation period, an increase in the number of bacteria (*Fusobacterium nucleatum*) was observed under microscope although we did not determine the precise doubling time of *Fusobacterium nucleatum* during the assay.

The RNA sequencing analysis revealed that the presence of *Fusobacterium nucleatum* led to a change in the gene expression profile in a dosage- and time-dependent manner. After 24 hours of coculturing with *Fusobacterium nucleatum* at a low MOI (10), only a limited number of marginally expressed genes (TPM<10) showed more than fourfold change in expression level (Figure 3A). In contrast, expression of a specific set of genes was drastically unregulated by a high MOI (100) treatment (Figure 3B). After 72 hours of coculturing, low MOI treatment led to significantly a higher number of genes, displaying more than fourfold expression increase in expression level (Figure 3A). On the other hand, the number of drastically upregulated genes in the high MOI treated cells decreases after longer incubation, but there are an increased number of genes with more than fourfold decrease in expression level (Figure 3B). Taken together, our result indicates a rapid and drastic cellular response to a high number of bacteria (*Fusobacterium nucleatum*). Additionally, it appears that *Fusobacterium nucleatum* infection, regardless the MOI, elicits a chronic response from the gastric cancer cells.

Subsequent ontological analysis revealed that these genes with altered expression levels are participating in specific cellular functions. Most prominently is the drastic increase of the genes in response to the interferon response genes. These genes, including MX1, MX2, IFI35, IFI44, IFI44L, IFIT1, IFIT2, IFIT3, IFITM1, IFITM3, IFIH1, IRF7, IFI2, and IFI6 [28], were drastically upregulated by high MOI of *Fusobacterium nucleatum* infection (Fig.4A). After prolonged incubation (72 hours), the level of these genes, except IFI27 and IFI6, has decreased considerably, nearly to the unstimulated basal levels. A similar induction pattern is observed for TRIM14 [8,30], ISG15 [38], USP18 [4], CD317 [6], OAS1, OAS2, OAS3, and OASL [16,31], which are all interferon-dependent genes participating in the antiviral activity of the cells (Fig. 4B). The rapid decrease indicates that the activation and regulation of the interferon response genes triggered by high number of bacteria (*Fusobacterium nucleatum*) are an immediate and short-term response. Moreover, low MOI treatment did not elicit the activation of the interferon response. Although the interferon response is apparently induced by a high MOI of *Fusobacterium nucleatum*, we only found a slight change in interferon genes, including IFNA1, IFNB1, IFND1, IFND2, and IFNE, throughout the experiment. Together, it can be concluded that *Fusobacterium nucleatum* at high MOI is able to activate the innate antiviral response in the gastric cancer cells.

In addition to the interferon response genes, a simultaneously increased expression of interleukins and chemokines, including IL6, IL8, IL32, CXCL1, CXCL2, and CXCL6, was observed for the high MOI treated cells at 24 hours (Fig. 4C). Similarly, the expression of these inflammatory cytokines returned to unstimulated levels at 72 hours and is apparently a part of the immediate response to the *Fusobacterium nucleatum* infection. However, distinct from the short burst expression of the interleukins and chemokines, CCL2 remained largely unchanged at 24 hours but was drastically upregulated at 72 hours (Fig. 4C). CCL2 is a chemoattractant of macrophages, and upregulation of CCL2 is shown to promote cancer progression [21,33]. Since both low and high MOI treatments induce the expression of CCL2 with similar strength, this suggests that the activation of CCL2 by *Fusobacterium nucleatum* infection is possibly done through an independent mechanism.

In addition to the genes of immunological functions, our analysis also discovered that genes involved in cell mobility and adhesion were deregulated by *Fusobacterium nucleatum* infection. Among these genes are EPSTI1 [12,24] and ICAM1 [9], which are both involved in cell-matrix interaction; they were upregulated at 24 hours by high MOI treatment (Fig. 5). Since the expression of these two genes returned to unstimulated levels at 72 hours, it is possible that EPSTI1 and ICAM1 were regulated as part of the interferon response. On the other hand, TNXB [2], the gene encoding tenascin-X, is transiently downregulated at 24 hours and returned to its unstimulated level at 72 hours (Fig. 5). However, suppressed by both low and high MOI treatment, regulation of TNXB is not dosage dependent and is distinct from all the other genes that respond to the interferon signaling.

Other cytoskeleton and adhesion genes are activated in a distinct pattern. Specifically, the expression of ACTA2, ACTC1, and ACTG2 is continuously increased by both low and high MOI treatments (Fig. 5). In addition to actins, CNN1, EDN1, and CEMIP were upregulated as well (Fig. 5C). CNN1 [22,26] and EDN1 [20] are involved in the endothelin pathway that modulates smooth muscle regulation and thereby exerts multiple functions to promote cancer progression. Increasing the expression of these genes was time-dependent, but not dosage-dependent, indicating that chronic infection with a small number of bacteria (*Fusobacterium nucleatum*) could lead to long-term and drastic effects on the cytoskeleton dynamic and cell mobility.

As stated above, there are a significantly increased number of genes suppressed by both low and high MOI treatment after 72 hours of incubation. The most prominently suppressed genes, including EGR1, NR1D1, ARC, RRAD, FOS, BHLHE41, PER1, and HES1, have diverse cellular functions (Fig. 6). Among these genes are NR1D1 [5,18], [3,37], and PER1 [14], which participate in circadian molecular mechanism but are implicated in cancer progression as well. It is possible that suppression of these genes and activation of actins and other cell adhesion genes are done through a common *Fusobacterium nucleatum*-dependent regulation mechanism. This putative regulatory pathway remains to be investigated.

## Discussion

*Fusobacterium nucleatum* is a resident pathogen in the oral microbiota. It may be constantly passed to the gastrointestinal tract through food and water intake but it will not colonize the rest of the gastrointestinal tract under normal physiological condition. However, the growth of carcinoma cells creates a suitable microenvironment and subsequently allows a successful invasion of *Fusobacterium nucleatum*. Presence of *Fusobacterium nucleatum* in the cancer-associated microbiota was well demonstrated for colorectal cancer. Our previous study showed that *Fusobacterium nucleatum* can be found in gastric cancer-associated microbiota. Here, we employed nested PCR, a different experimental approach from 16S metagenomic analysis used in a previous study, to determine the presence of *Fusobacterium nucleatum* in resected gastric cancer tissues. The result confirms our previous finding that *Fusobacterium nucleatum* infection is present in approximately one-third of gastric cancer patients of

southwestern Taiwan. However, whether *Fusobacterium nucleatum* infection is a unique phenomenon in this region remains to be investigated.

After determining the prevalence of *Fusobacterium nucleatum* infection in gastric cancer patients, we next sought to determine the clinical impact of such finding. All the patients enrolled in this study underwent gastrectomy and presumably have the main cancerous lesions completely removed. It is conceivable that gastrectomy removes cancer-associated microbiota as well, hence minimizing the negative impact of pathogenic microbes on the subsequent treatment. The average five-year survival in our study cohort is approximately 60%, indicating the effectiveness of the overall treatment. Under this background, survival analysis shows that *Fusobacterium nucleatum* infection negatively impacts the survival in the subgroup of *Helicobacter pylori*-positive patients. It is clear that *Fusobacterium nucleatum* further promotes gastric cancer progression by consecutively or synergistically working with *Helicobacter pylori*. This finding indicates the complex interaction between multiple pathogens and the gastric cancer cells. Our data also suggests that *Fusobacterium nucleatum* infection could serve as a prognosis biomarker for *Helicobacter pylori*-positive patients.

The analysis of the *in vitro* coculture experiment indicates that *Fusobacterium nucleatum* invokes two distinct cellular responses. Based on the dosage dependence and expression pattern, these two responses are likely activated through independent signaling pathways. One is an immediate response that peaks at 24 to 48 hours and declines to near unstimulated level after 72 hours. This immediate response is activated only by a high number of bacteria (*Fusobacterium nucleatum*) and exemplified by the interferon response genes, antiviral genes, cytokines, and chemokines. Although the immediate response appears to be transient, it is conceivable that the response may be repeatedly activated if *Fusobacterium nucleatum* is constantly present in the microenvironment at a sufficient number. Other inflammatory pathogens, such as *Helicobacter pylori*, may collaborate with *Fusobacterium nucleatum* to sustain the activation of these genes. The other response consists of actins and genes functioning in regulating cell mobility. Both low and high MOI treatments are able to activate a continuous increase in expression level during the incubation time. High expression of these gene products are shown to have multiple effects in promoting cancer progression, especially in increasing mobility and distant metastasis. Similar dosage-independent and time-dependent manners were observed for the suppression of genes in circadian mechanism and cell growth as well. Due to the same regulation manner, these genes, either activated or suppressed, were likely controlled by a common mechanism dependent from the short-term activation of the interferon response.

Overall, our experiment demonstrated that colonization of *Fusobacterium nucleatum* activates specific signaling pathways and potentially promotes the aggressiveness of the gastric cancer cells. Although the pathogenic microbiota is removed through gastrectomy and hence ceases to affect the outcome of subsequent treatment, it may still impact the prognosis of patients that have not undergone surgery. Whether *Fusobacterium nucleatum* infection possesses a clinical significance in these patients remains to be investigated.

## Conclusions

In this study, we demonstrate that the prevalence of *Fusobacterium nucleatum* infection in gastric cancer is much higher than previously thought. *In vitro*, *Fusobacterium nucleatum* incites an inflammatory response from the cancer cells and promotes cell mobility. More importantly, co-infection of *Fusobacterium nucleatum* with *Helicobacter pylori* leads to poor survival of gastric cancer patients. Together, our study supports the conclusion that secondary infection of *Fusobacterium nucleatum* to the microenvironment created by *Helicobacter pylori* increases the aggressiveness of the gastric cancer cells.

## Declarations

### Ethics Approval and Consent to Participate

Acquisition and use of clinical specimens in this study was in accordance with the Declaration of Helsinki. Resurrected gastric cancer specimens, comprising the lesion and adjacent normal tissues, were obtained from Tissue Bank, Department of Medical Research, Chang Gung Memorial Hospital at Chiayi, and stomach biopsies were collected during endoscopic examination. All the patients participating in this study were clearly informed about of the study and signed a written informed consent.

### Consent for publication

All authors approved the final manuscript and consent for publication.

### Availability of supporting data

The sequencing data was deposited in Sequence Read Archive (SRA), National Center for Biotechnology Information, USA. The BioProject ID is PRJNA630089, and the BioSample accession numbers are SAMN14823957, SAMN14823958, SAMN14823959, SAMN14823960, and SAMN14823961.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

Y.Y.H., S.Y.T., T.S.C., K.L.W., W.M.C., and C.S.W. recruited patients for the study and collected biopsies; Y.F.D. performed *in vitro* experiments and performed sequencing experiments; Y.H.L. and H.Y.P. performed data and statistical analyses; C.S.W. and C.L. designed and oversaw the study. C.L. wrote the manuscript.

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## References

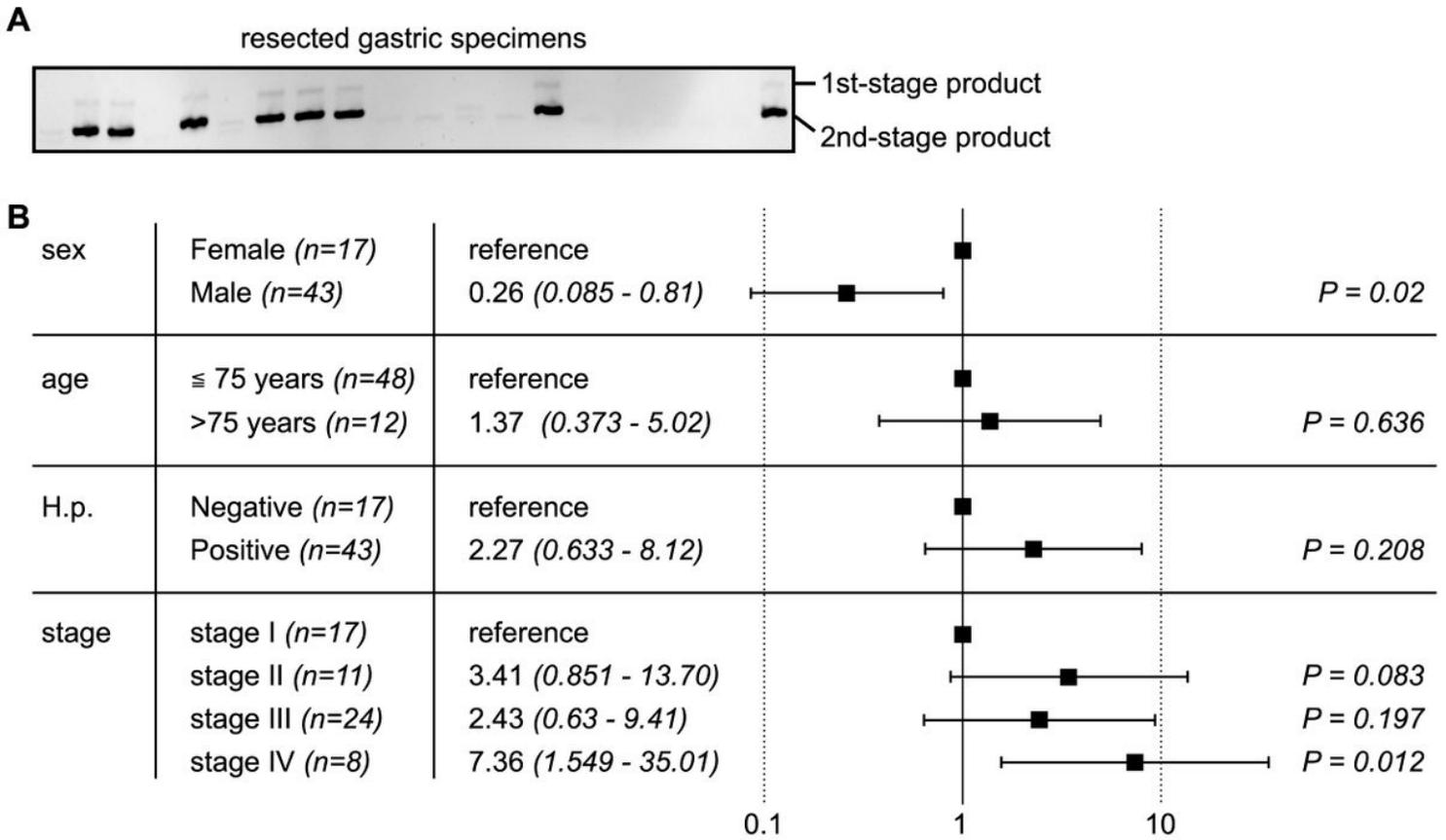
1. Abed J., Emgard J.E., Zamir G., Faroja M., Almogy G., Grenov A., Sol A., Naor R., Pikarsky E., Atlan K.A., Mellul A., Chaushu S., Manson A.L., Earl A.M., Ou N., Brennan C.A., Garrett W.S. and Bachrach G. Fap2 Mediates *Fusobacterium nucleatum* colorectal adenocarcinoma enrichment by binding to tumor-expressed Gal-GalNAc. *Cell Host Microbe* 20(2):215-225, 2016.
2. Aktar R., Peiris M., Fikree A., Eaton S., Kritas S., Kentish S.J., Araujo E.J.A., Bacarin C., Page A.J., Voermans N.C., Aziz Q. and Blackshaw L.A. A novel role for the extracellular matrix glycoprotein-Tenascin-X in gastric function. *J Physiol* 597(6):1503-1515, 2019.
3. Asanoma K., Liu G., Yamane T., Miyanari Y., Takao T., Yagi H., Ohgami T., Ichinoe A., Sonoda K., Wake N. and Kato K. Regulation of the Mechanism of TWIST1 Transcription by BHLHE40 and BHLHE41 in cancer cells. *Mol Cell Biol* 35(24):4096-4109, 2015.
4. Basters A., Knobeloch K.P. and Fritz G. USP18 - a multifunctional component in the interferon response. *Biosci Rep* 38(6), 2018.
5. Basti A., Fior R., Yalin M., Pova V., Astaburuaga R., Li Y., Naderi J., Godinho Ferreira M. and Religio A. The core-clock gene NR1D1 impacts cell motility *in vitro* and invasiveness in a zebrafish xenograft colon cancer model. *Cancers (Basel)* 12(4), 2020.
6. Billcliff P.G., Rollason R., Prior I., Owen D.M., Gaus K. and Banting G. CD317/tetherin is an organiser of membrane microdomains. *J Cell Sci* 126(Pt 7):1553-1564, 2013.

7. Castellarin M., Warren R.L., Freeman J.D., Dreolini L., Krzywinski M., Strauss J., Barnes R., Watson P., Allen-Vercoe E., Moore R.A. and Holt R.A. *Fusobacterium nucleatum* infection is prevalent in human colorectal carcinoma. *Genome Res* 22(2):299-306, 2012.
8. Chen M., Meng Q., Qin Y., Liang P., Tan P., He L., Zhou Y., Chen Y., Huang J., Wang R.F. and Cui J. TRIM14 inhibits cGAS degradation mediated by selective autophagy receptor p62 to promote innate immune responses. *Mol Cell* 64(1):105-119, 2016.
9. Chen S., Pan S., Wu H., Zhou J., Huang Y., Wang S. and Liu A. ICAM1 regulates the development of gastric cancer and may be a potential biomarker for the early diagnosis and prognosis of gastric cancer. *Cancer Manag Res* 12:1523-1534, 2020.
10. Chen Y., Lu Y., Ke Y. and Li Y. Prognostic impact of the *Fusobacterium nucleatum* status in colorectal cancers. *Medicine (Baltimore)* 98(39):e17221, 2019.
11. Chen Y., Peng Y., Yu J., Chen T., Wu Y., Shi L., Li Q., Wu J. and Fu X. Invasive *Fusobacterium nucleatum* activates beta-catenin signaling in colorectal cancer via a TLR4/P-PAK1 cascade. *Oncotarget* 8(19):31802-31814, 2017.
12. de Neergaard M., Kim J., Villadsen R., Fridriksdottir A.J., Rank F., Timmermans-Wielenga V., Langerod A., Borresen-Dale A.L., Petersen O.W. and Ronnov-Jessen L. Epithelial-stromal interaction 1 (EPSTI1) substitutes for peritumoral fibroblasts in the tumor microenvironment. *Am J Pathol* 176(3):1229-1240, 2010.
13. Espinoza J.L., Matsumoto A., Tanaka H. and Matsumura I. Gastric microbiota: An emerging player in *Helicobacter pylori*-induced gastric malignancies. *Cancer Lett* 414:147-152, 2018.
14. Hernandez-Rosas F., Hernandez-Oliveras A., Flores-Peredo L., Rodriguez G., Zarain-Herzberg A., Caba M. and Santiago-Garcia J. Histone deacetylase inhibitors induce the expression of tumor suppressor genes *Per1* and *Per2* in human gastric cancer cells. *Oncol Lett* 16(2):1981-1990, 2018.
15. Holleczeck B., Schottker B. and Brenner H. *Helicobacter pylori* infection, chronic atrophic gastritis and risk of stomach and esophagus cancer: Results from the prospective population-based ESTHER cohort study. *Int J Cancer*, 2019.
16. Hornung V., Hartmann R., Ablasser A. and Hopfner K.P. OAS proteins and cGAS: unifying concepts in sensing and responding to cytosolic nucleic acids. *Nat Rev Immunol* 14(8):521-528, 2014.
17. Huang S., Yang Z., Zou D., Dong D., Liu A., Liu W. and Huang L. Rapid detection of *nusG* and *fadA* in *Fusobacterium nucleatum* by loop-mediated isothermal amplification. *J Med Microbiol* 65(8):760-769, 2016.
18. Ka N.L., Na T.Y., Na H., Lee M.H., Park H.S., Hwang S., Kim I.Y., Seong J.K. and Lee M.O. NR1D1 Recruitment to sites of DNA damage inhibits repair and is associated with chemosensitivity of breast cancer. *Cancer Res* 77(9):2453-2463, 2017.
19. Kapatral V., Anderson I., Ivanova N., Reznik G., Los T., Lykidis A., Bhattacharyya A., Bartman A., Gardner W., Grechkin G., Zhu L., Vasieva O., Chu L., Kogan Y., Chaga O., Goltsman E., Bernal A., Larsen N., D'Souza M., Walunas T., Pusch G., Haselkorn R., Fonstein M., Kyrpides N. and Overbeek R.

- Genome sequence and analysis of the oral bacterium *Fusobacterium nucleatum* strain ATCC 25586. *J Bacteriol* 184(7):2005-2018, 2002.
20. Kim T.H., Xiong H., Zhang Z. and Ren B. beta-Catenin activates the growth factor endothelin-1 in colon cancer cells. *Oncogene* 24(4):597-604, 2005.
  21. Liu Q., Song J., Pan Y., Shi D., Yang C., Wang S. and Xiong B. Wnt5a/CaMKII/ERK/CCL2 axis is required for tumor-associated macrophages to promote colorectal cancer progression. *Int J Biol Sci* 16(6):1023-1034, 2020.
  22. Liu Y., Wu X., Wang G., Hu S., Zhang Y. and Zhao S. CALD1, CNN1, and TAGLN identified as potential prognostic molecular markers of bladder cancer by bioinformatics analysis. *Medicine (Baltimore)* 98(2):e13847, 2019.
  23. Mima K., Nishihara R., Qian Z.R., Cao Y., Sukawa Y., Nowak J.A., Yang J., Dou R., Masugi Y., Song M., Kostic A.D., Giannakis M., Bullman S., Milner D.A., Baba H., Giovannucci E.L., Garraway L.A., Freeman G.J., Dranoff G., Garrett W.S., Huttenhower C., Meyerson M., Meyerhardt J.A., Chan A.T., Fuchs C.S. and Ogino S. *Fusobacterium nucleatum* in colorectal carcinoma tissue and patient prognosis. *Gut* 65(12):1973-1980, 2016.
  24. Nielsen H.L., Ronnov-Jessen L., Villadsen R. and Petersen O.W. Identification of EPSTI1, a novel gene induced by epithelial-stromal interaction in human breast cancer. *Genomics* 79(5):703-710, 2002.
  25. Oh H.J., Kim J.H., Bae J.M., Kim H.J., Cho N.Y. and Kang G.H. Prognostic impact of *Fusobacterium nucleatum* depends on combined tumor location and microsatellite instability status in stage II/III colorectal cancers treated with adjuvant chemotherapy. *J Pathol Transl Med* 53(1):40-49, 2019.
  26. Qiu X., Cheng S.H., Xu F., Yin J.W., Wang L.Y. and Zhang X.Y. Weighted gene co-expression network analysis identified MYL9 and CNN1 are associated with recurrence in colorectal cancer. *J Cancer* 11(8):2348-2359, 2020.
  27. Rubinstein M.R., Baik J.E., Lagana S.M., Han R.P., Raab W.J., Sahoo D., Dalerba P., Wang T.C. and Han Y.W. *Fusobacterium nucleatum* promotes colorectal cancer by inducing Wnt/beta-catenin modulator Annexin A1. *EMBO Rep* 20(4), 2019.
  28. Schoggins J.W. Interferon-Stimulated Genes: What Do They All Do? *Annu Rev Virol* 6(1):567-584, 2019.
  29. Shah M.A. Gastric cancer: The gastric microbiota - bacterial diversity and implications. *Nat Rev Gastroenterol Hepatol* 14(12):692-693, 2017.
  30. Tan P., He L., Cui J., Qian C., Cao X., Lin M., Zhu Q., Li Y., Xing C., Yu X., Wang H.Y. and Wang R.F. Assembly of the WHIP-TRIM14-PPP6C mitochondrial complex promotes RIG-I-mediated antiviral signaling. *Mol Cell* 68(2):293-307 e295, 2017.
  31. Wang Y., Holleufer A., Gad H.H. and Hartmann R. Length dependent activation of OAS proteins by dsRNA. *Cytokine* 126:154867, 2020.
  32. Wong S.H., Kwong T.N.Y., Chow T.C., Luk A.K.C., Dai R.Z.W., Nakatsu G., Lam T.Y.T., Zhang L., Wu J.C.Y., Chan F.K.L., Ng S.S.M., Wong M.C.S., Ng S.C., Wu W.K.K., Yu J. and Sung J.J.Y. Quantitation of

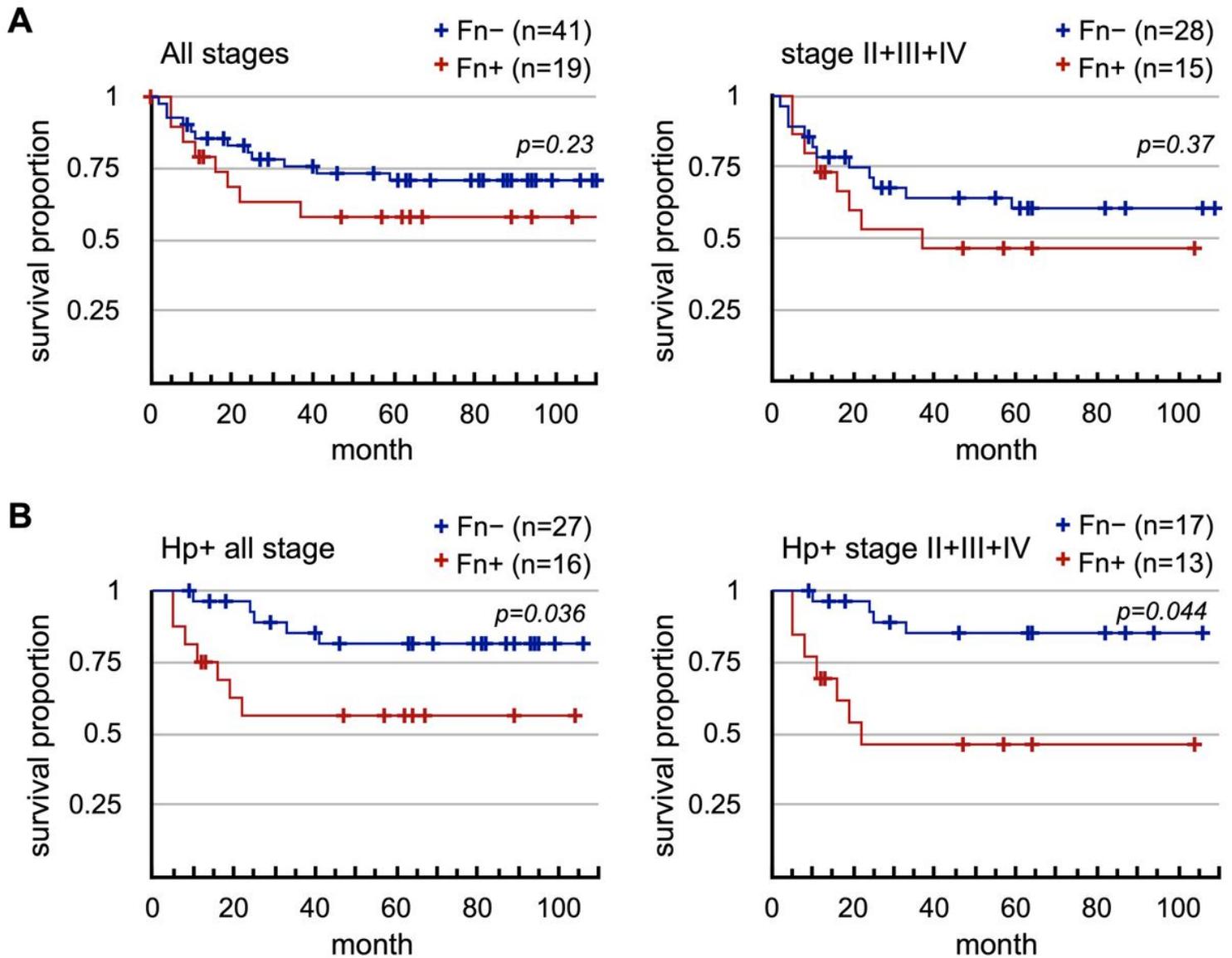
- faecal *Fusobacterium* improves faecal immunochemical test in detecting advanced colorectal neoplasia. *Gut* 66(8):1441-1448, 2017.
33. Xu W., Wei Q., Han M., Zhou B., Wang H., Zhang J., Wang Q., Sun J., Feng L., Wang S., Ye Y., Wang X., Zhou J. and Jin H. CCL2-SQSTM1 positive feedback loop suppresses autophagy to promote chemoresistance in gastric cancer. *Int J Biol Sci* 14(9):1054-1066, 2018.
  34. Yamamura K., Baba Y., Miyake K., Nakamura K., Shigaki H., Mima K., Kurashige J., Ishimoto T., Iwatsuki M., Sakamoto Y., Yamashita Y., Yoshida N., Watanabe M. and Baba H. *Fusobacterium nucleatum* in gastroenterological cancer: Evaluation of measurement methods using quantitative polymerase chain reaction and a literature review. *Oncol Lett* 14(6):6373-6378, 2017.
  35. Yong X., Tang B., Li B.S., Xie R., Hu C.J., Luo G., Qin Y., Dong H. and Yang S.M. *Helicobacter pylori* virulence factor CagA promotes tumorigenesis of gastric cancer via multiple signaling pathways. *Cell Commun Signal* 13:30, 2015.
  36. Yoshida Y., Suwabe K., Nagano K., Kezuka Y., Kato H. and Yoshimura F. Identification and enzymic analysis of a novel protein associated with production of hydrogen sulfide and L-serine from L-cysteine in *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586. *Microbiology* 157(Pt 7):2164-2171, 2011.
  37. Zhang D., Zheng Q., Wang C., Zhao N., Liu Y. and Wang E. BHLHE41 suppresses MCF-7 cell invasion via MAPK/JNK pathway. *J Cell Mol Med* 24(7):4001-4010, 2020.
  38. Zhao C., Collins M.N., Hsiang T.Y. and Krug R.M. Interferon-induced ISG15 pathway: an ongoing virus-host battle. *Trends Microbiol* 21(4):181-186, 2013.

## Figures



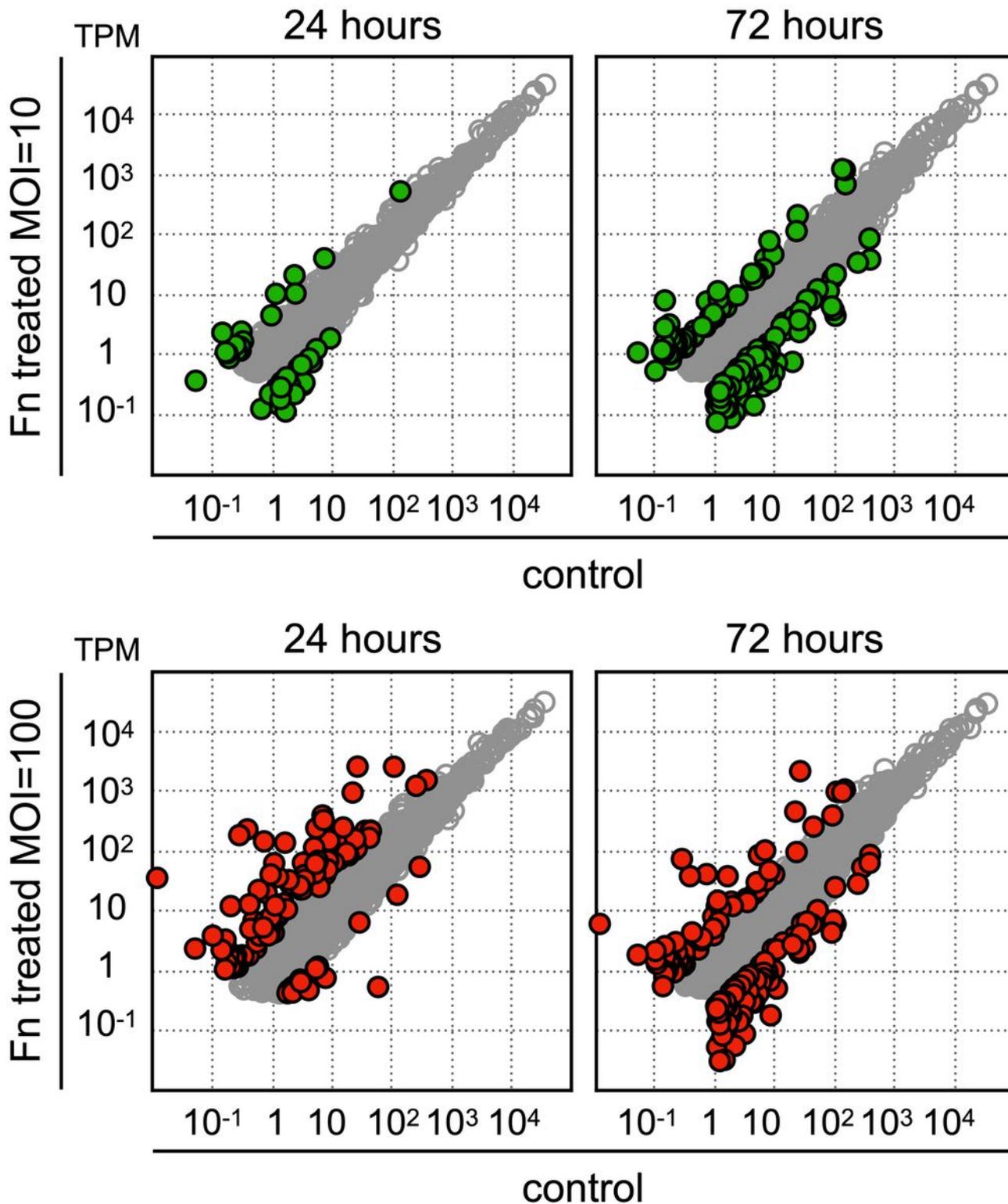
**Figure 1**

Identification of *Fusobacterium nucleatum* in the resected gastric cancer tissues. (A) *Fusobacterium nucleatum* was detected by amplification of the conserved NusG sequence using nested PCR. The end point product was analyzed by 6% nondenaturing polyacrylamide gel electrophoresis. (B) The relative risk of *Fusobacterium nucleatum* infection was analyzed against the sex, age, status of *Helicobacter pylori* infection, and cancer stage and was shown as Forest plot.



**Figure 2**

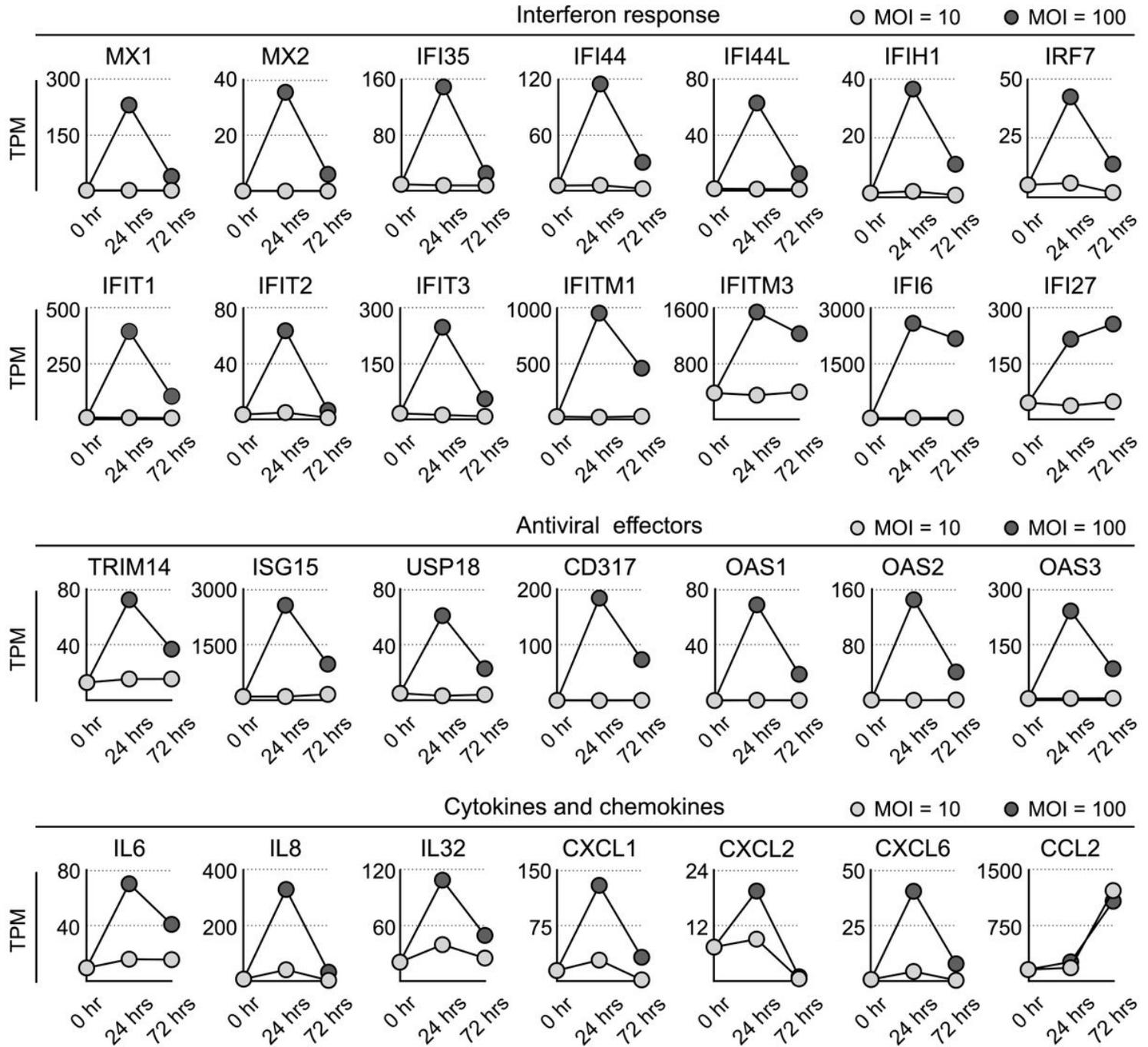
Analysis of *Fusobacterium nucleatum* infection in gastric cancer patients. (A) The study cohort was divided into positive and negative groups according to the result of nested-PCR analysis. Survival probability was calculated using Kaplan-Meier analysis in all cancer patients with at least one-year follow-up. (B) Survival probability was calculated in stages II, III, and IV cancer patients.



**Figure 3**

Expression profile change in patient-derived cancer cell line cocultured with *Fusobacterium nucleatum*. *Fusobacterium nucleatum* was added to the cell culture at MOI 10 and 100 and incubated under normal cell culture condition. After 24 and 72 hours, the cells were collected and analyzed using RNA sequencing. The cells collected at time 0 serves as the control. Sequencing reads were trimmed and mapped to hg19 using CLC genomic workbench v.12.0.3. The colored dots (green for MOI=10 and red for MOI=100) are

genes showing more than fourfold change at respective incubation time. Genes with TPM<1 in all datasets and having less than a fourfold change are not shown here.



**Figure 4**

Identification and ontological analysis of those genes with drastically changed expression level by coculture with *Fusobacterium nucleatum*. (A) The expression dynamic of those genes belonging to the interferon response is depicted here. (B) The expression dynamic of the antiviral genes. (C) The expression change of the cytokines and chemokines.

## Mobility and adhesion

○ MOI = 10      ● MOI = 100

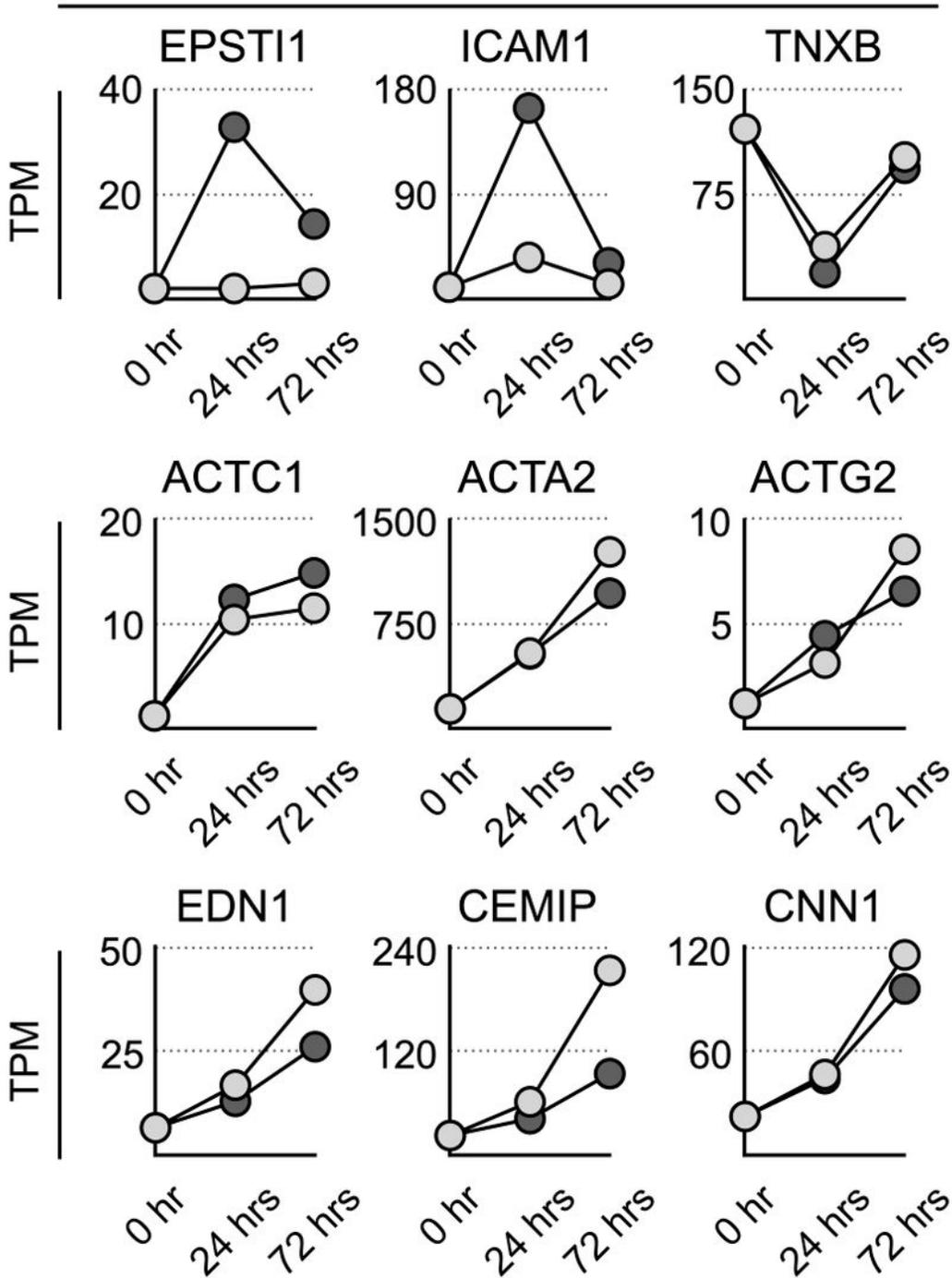


Figure 5

The expression dynamics of actins and their regulators as well as genes involved in cell cohesion was drastically deregulated by coculture with *Fusobacterium nucleatum*.

## Downregulated genes

○ MOI = 10      ● MOI = 100

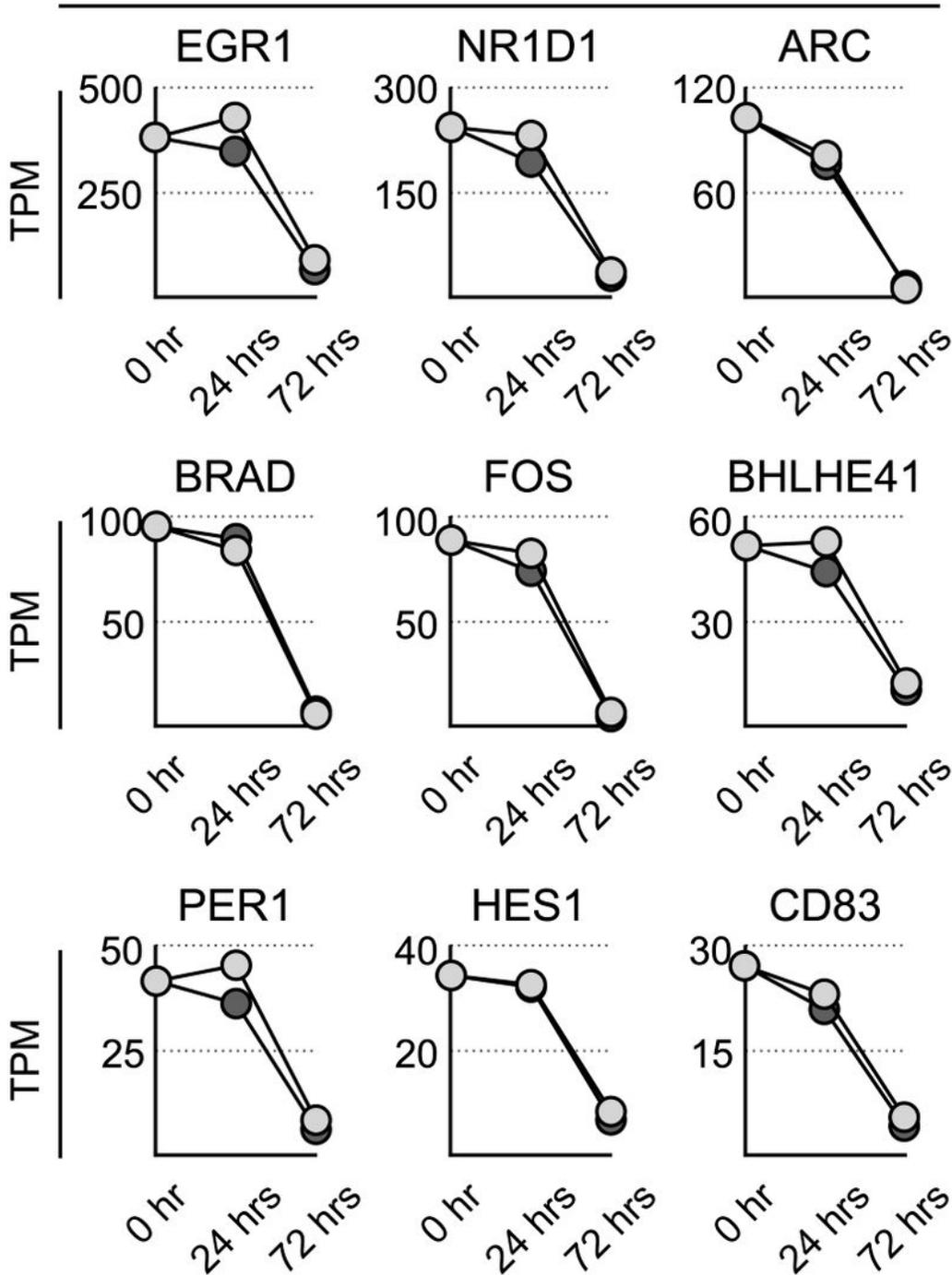


Figure 6

Genes showing dosage-independent and time-dependent suppression by *Fusobacterium* treatment.