**Autoinducer-2 of gut microbiota, a potential novel marker for human colorectal cancer, is associated with the activation of TNFSF9 signaling in macrophages**

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ABSTRACT

**Objectives**: The interaction between the quorum sensing (QS) molecules of gut microbiota and the immunity of colorectal cancer (CRC) has not been investigated before.

**Methods**: We measured the concentration of autoinducer-2 (AI-2) in samples of stool, colorectal tissue, saliva and serum of CRC patients, and compared this to AI-2 levels in colorectal adenoma (AD) and normal colon mucosa (NC). To explore the activated signaling pathways involved, we utilized AI-2 extracted from *Fusobacterium nucleatum* to stimulate macrophages and validated these in vitro findings in human CRC tissues.

**Results**: The AI-2 concentration in both colorectal tissue and stool of CRC patients was significantly higher when compared to that in AD and NC (all P values < .01). The AI-2 concentration along with the progression of CRC in both tissues and stools was significantly increased (P = .0456P = .0003, respectively). After AI-2 stimulation, TNFSF9 was the most significantly increased protein in macrophage cells (P < .01). TNFSF9 expression was significantly higher in CRC tissues when compared to NCs (P < .0001), which was mainly derived from macrophages in the tumor microenvironment. Moreover, AI-2 level was positively associated with CD3 + T cell numbers (P = .0462), and negatively associated with CD4/CD8 ratio (P = .0113) within CRC tissues.

**Conclusions**: We demonstrated for the first time that AI-2 may serve as a novel marker for screening CRC in the clinic. AI-2 was associated with tumor immunity in CRCs through tumor-associated macrophages and CD4/CD8 ratio in a TNFSF9-dependent manner.

**Introduction**

Mammals have coevolved with microbiota for millions of years, therefore, microbiota profoundly influence the homeostasis of host physiology.1 In the gut of humans, roughly 100 billion microorganisms reside,2 which play a key role in establishing gut mucosal immunity.3 The imbalance of intestinal bacteria has been linked to various human diseases,4 including obesity,5 diabetes,6 atherosclerotic cardiovascular disease,7 neurogenic diseases like Alzheimer’s disease,8 and colorectal cancer (CRC).9

CRC has emerged as the third most common cancer worldwide.10 A growing body of literature has recognized the importance of microbiota in the development and progress of CRC.11 For example, *Fusobacterium nucleatum* (F. nucleatum) has recently been proposed to be associated with CRC,12 through two types of virulence mechanisms: colonization and induction of the host immune response.13 The dysbiosis of gut microbiota is closely related to CRC. However, most studies up to now have not fully elucidated the exact mechanism involved in the progress of dysbiosis of gut bacteria that contributes to CRC. The host immunity has received considerable attention in the development of CRC; however, the detailed mechanism of interaction between gut microbiota and mucosal immunity remains to be elucidated.14

The quorum sensing (QS) system, which is widely employed by bacteria, is an important mechanism of intercellular communication among bacterial species.15 The QS system enables bacteria to act as a group rather than an individual cell.16 Autoinducer-2 (AI-2), a major type of QS molecules that mediates the communication among interspecies,15 has the ability to regulate gene expression, virulence and biofilm formation of bacteria.17 The colon mucosal biofilm has been shown to be associated with an increased risk for sporadic CRC.18 Recent studies have revealed that AI-2 could potentially affect cytokine secretion of the host cells.19 Therefore, it is likely that AI-2 plays a role in the development and progression of CRC.

Coevolved with resident microbiota over millennia, the immune system of the gut may have developed the ability to respond to QS molecules, such as AI-2 of inhabited bacteria.20 Macrophages are immune cells that are vital for host defense, tissue repair, and inflammation.21 It is generally recognized

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that macrophages are responsible for detecting microbial signals, thereby playing a critical role in microbial sensing.\textsuperscript{22} Moreover, macrophages are the most abundant immune cells in the tumor microenvironment.\textsuperscript{23} Recent discoveries have shown that gut microbiota plays an essential role in the carcinogenesis of CRC, and macrophages play an important role in this progress.\textsuperscript{23} However, the exact role of macrophages in the interaction between gut microbiota and the immunity of CRC needs to be further investigated. Moreover, tumor-infiltrating lymphocytes (TILs) are important players in tumor immunosurveillance, and each subpopulation of TILs possesses a unique antitumor role.\textsuperscript{24} CD4 + T cells and CD8 + T cells have been demonstrated to play an important role in antitumor immunity.\textsuperscript{25,26} In addition, CD4/CD8 ratio is a significant predictor of overall survival in patients with CRC.\textsuperscript{27} Thus, macrophages or CD4/CD8 ratio in the tumor environment appear to be important in the anticancer immune response.

We hypothesize that AI-2 produced by gut microbiota may play a role in the carcinogenesis of CRC through immune cells of the gut mucosa. To test our hypothesis, we measured the concentration of AI-2 in clinical samples (stool, colorectal tissues, saliva, and serum) of CRC patients, and compared this to colorectal adenoma (AD) and normal colon mucosa (NC). Next, we utilized AI-2 that was produced by \textit{F. nucleatum} to stimulate human macrophage cell line U-937, to explore the main AI-2-activated signaling pathway. Finally, we validated our \textit{in vitro} findings in human CRC tissues, focusing on tumor-infiltrating macrophages and TILs. To our knowledge, this is the first time in which the association of AI-2 with the immunity of CRC was explored.

\textbf{Materials and methods}

\textbf{Sample collection and preparation}

All samples were collected from patients who underwent colonoscopy or surgery in the Affiliated Hospital of North Sichuan Medical College (Nanchong, China) and the Affiliated Hospital of Southwest Medical University (Luzhou, China). A total of 312 samples (stool, tissue, saliva and serum samples) were collected and processed as described in previous studies.\textsuperscript{28,29} Clinicopathological data were acquired according to hospital records. Informed consent was obtained from all participants, and the study was approved by the review board of the Affiliated Hospital of North Sichuan Medical College (Nanchong, China).

\textbf{Bacterial strain and culture conditions}

\textit{Vibrio harveyi} (V. harveyi) strain BB170 was purchased from the Guangdong culture collection center and used to detect AI-2. Autoinducer bioassay (AB) media was prepared for culturing of cells and used for bioluminescence assays as described by Nilesh et al.\textsuperscript{30} \textit{F. nucleatum} strains F01 were isolated from CRC tissues and confirmed in our previous study.\textsuperscript{30}

\textbf{Analysis of physiological samples}

Standard solution of dihydroxy-2, 3-pentanedione (DPD) (Omm Scientific Inc. CAS: 710374-30-4, Dallas, TX, USA), the precursor of AI-2, was prepared as previously described.\textsuperscript{31} Ten microliters of each of the processed samples was added in triplicate to 90 µl of diluted \textit{V. harveyi} BB170 cultures in 96-well polystyrene microtiter plates (Costar, corning, NY, USA). The plates were incubated at 30°C and 160rpm in an orbital shaker, and bioluminescence was recorded by a TriStar LB 941 microplate reader (Berthold, Wildbad, Germany) and shown as a relative light unit (RLU). The concentration of AI-2 in each sample was calculated according to their bioluminescence value.\textsuperscript{32}

\textbf{Purification of \textit{F. nucleatum} (F01) AI-2}

Overnight cultured \textit{F. nucleatum} (F01) was diluted with fresh medium at a ratio of 1:10 and cultured until OD660 nm = 0.7, then the supernatant was collected by centrifugation at 13000 rpm for 10 min and 4°C. The supernatant was filtered through a 0.2-µm pore-size membrane filter and subsequently a Centricon YM-3 3-kDa exclusion filter. The filtrate was then chromatographed on a C18 Sep-Pak reverse-phase column (Waters Co, Milford, MA, USA) according to the manufacturer’s instructions.

\textbf{Cell culture and treatment}

U937 monocytes were purchased from BeNa culture collection (BNCC100498, Beijing, China), and RPMI medium 1640 supplemented with 10% FBS was used for cell culture purposes. For differentiation, U937 monocytes were cultured in 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h. Then, U937-derived macrophages were challenged with AI-2 at a concentration of 400 µmol/L for 6 h and 37°C. Differentiated cells without AI-2 treatment were used as controls. Three experimental replicates were performed for each condition. Then, the cells were harvested and stored at −80°C for further use.

\textbf{Quantitative proteomics analysis}

Proteins were isolated and digested from AI-2-treated cells and control. After digestion, peptides were desalted and reconstituted in 0.5 M triethylammonium bicarbonate (TEAB) and processed using 6-plex TMT kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Subsequently, the tryptic peptides were fractionated and analyzed as previously described.\textsuperscript{32}

\textbf{Immunofluorescence analysis}

Colorectal tissue samples were embedded in paraffin and sectioned at 5-µm thickness. Sections were blocked for 30 min and 37°C by 10% goat serum and incubated with primary antibodies: CD3 (1:100, R&D Systems, MN, USA); CD4 (1:100, Santa Cruz, CA, USA); CD8-α (1:100, Santa Cruz, CA, USA); CD68 (1:100, Santa Cruz, CA, USA); TNFSF9 (1:100, Bioworld, MN, USA) for 18 h at 4°C. Subsequently, each section was incubated with appropriate secondary antibodies for 1 h at 37°C. DAPI (Beyotime, China) was used for nuclear counterstain at room temperature for 10 min. Images were captured using a microscope (BX53; Olympus, Tokyo, Japan).
Quantification of immune cells

Immunofluorescence for immune cell markers (CD3, CD4, CD8, and CD68) was determined from captured pictures. As described in our previous study, the cell density of T cells and macrophages was determined by CD3+ or CD68+, respectively. Subpopulations of T cells were determined by CD4+ or CD8+. A total of five random fields at 400× magnification were captured for each sample, and positive cells were presented as an average number of positive cells per square millimeter.

Statistical analysis

Data are presented as the mean ± standard deviation for continuous variables and proportions for categorical variables. Data were analyzed using one-way analysis of variance (ANOVA with Bonferroni correction) and unpaired t-test for parametric variables, and Kruskal–Wallis for nonparametric variables, as appropriate. For categorical variables, differences were determined by χ2 or Fisher exact tests. Pearson and Spearman correlation analysis were used for correlational analyses. All statistical calculations were performed using SPSS software Version 13.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant if P < .05.

Results

The AI-2 concentration in stools and colorectal tissues of patients with CRC was significantly higher when compared to that in adenomas and healthy controls

First, we measured the concentration of AI-2 in colorectal tissue, stool, saliva, and serum samples of patients with CRC, and compared this to AD and NC. The clinicopathological findings of patients are presented in Supplementary Table S1-S4. Among the three histological types, there were no significant differences in patient gender or age. The AI-2 concentration in both colorectal tissue and stool of CRC patients was significantly higher than that in ADs and NCs (all P< .01) (Figure 1(a,b)). In addition, the AI-2 concentration in stool samples of AD patients was significantly higher when compared to that of NCs (P < .01) (Figure 1(b)). In saliva samples, no significant differences were observed among CRC, AD and NC groups (P > .05) (Figure 1(c)). The AI-2 concentration in saliva of CRC patients was higher compared to that of ADs and NCs but did not reach statistical significance (P> .05). In serum samples, the concentration of AI-2 did not reach the minimum detection limit of the whole-cell biosensing system.

The AI-2 levels were increased with the progression of CRC staging in tumor tissues and stool samples

In this study, we investigated whether the AI-2 concentration was associated with CRC TNM staging. The clinicopathological findings of patients with different stages of CRC are presented in Supplementary Table S5, S6. In tissues and stool samples, no significant difference was found in patient age, sex, and tumor differentiation among different stages of CRC. The AI-2 concentration was significantly increased along with the progression of CRC in both tumor tissues and stool samples (P = .045 , P = .0003, respectively) (Figure 2(a,b)). In addition, when compared to proximal CRC, the AI-2 levels were significantly higher in distal CRC in both tumor tissues and stool samples (P < .0001, P = .0101, respectively) (Figure 2(c,d)).

Al-2 treatment greatly increased TNFSF9 expression in macrophages in vitro

Macrophages in the gut mucosa play an important role in resisting bacterial pathogens. Therefore, we wondered whether macrophages would respond to bacterial AI-2. After AI-2 treatment of U973-derived macrophages, a total of 5317 proteins were identified and quantified by comparing AI-2-treated U973-derived macrophages with non-treated controls using quantitative proteomics. In this study, a total of nine proteins showed an abundance change of at least twofold in AI-2 treated U973-derived macrophages when compared with controls: nine proteins were over-expressed whereas no proteins were down-regulated (Table 1). The results showed that TNFSF9 was the most significantly increased protein after AI-2 stimulation when compared to controls (P < .01). The findings suggested that TNFSF9 signaling may possibly be the main pathway in the response of macrophages to bacterial AI-2.

TNFSF9 expression positively associated with AI-2 levels and human CRC progression

Next, we explored whether the expression of TNFSF9 was upregulated in human CRC tissues. TCGA and GTEx projects
provided a large number of RNA sequencing data from tumor samples and normal tissues. GEPIA (Gene Expression Profiling Interactive Analysis) is a visualization online website based on TCGA and GTEx data. We first explored the mRNA expression of TNFSF9 in CRC patients by using the GEPIA database, and found that the mRNA expression of TNFSF9 was significantly higher in CRC samples (n = 275) when compared to NCs (n = 41) ($P < .05$, Figure 3(b)). Immunofluorescence analysis showed that TNFSF9 protein expression was significantly higher in CRC tissues when compared to NCs ($P < .0001$, Figure 3(c)). Moreover, the expression of TNFSF9 increased with the progression of CRC ($P < .0001$) ($P < .0001$, **Figure 3(d,e)**). In addition, a positive correlation was found between TNFSF9 expression and AI-2 concentration within CRC tissues (Spearman correlation coefficient: 0.4218, 95% confidence interval [CI]: 0.0613 to 0.6849, $P = .0203$) (Figure 3(f)).

Table 1. Proteins with at least twofold change in abundance in AI-2 treated U973-derived macrophages compared with control.

| Protein accession | Protein description           | Gene name | AI-2/control ratio | Regulated type | AI-2/control P value |
|-------------------|------------------------------|-----------|--------------------|----------------|---------------------|
| O15492            | Regulator of G-protein signaling 16 | OS = Homo sapiens Ox = 9606 GN = RGS16 | RGS16 | 2.007 | Up | 0.00091878 |
| P01584            | Interleukin-1 beta | OS = Homo sapiens Ox = 9606 GN = IL1B | IL1B | 3.045 | Up | 0.00063738 |
| P05121            | Plasminogen activator inhibitor 1 | OS = Homo sapiens Ox = 9606 GN = SERPINE1 | SERPINE1 | 2.282 | Up | 0.0014379 |
| P10147            | C-C motif chemokine 3 | OS = Homo sapiens Ox = 9606 GN = CCL3 | CCL3 | 2.166 | Up | 0.0079787 |
| P35354            | Prostaglandin G/H synthase 2 | OS = Homo sapiens Ox = 9606 GN = PTGS2 | PTGS2 | 3.551 | Up | 0.00014238 |
| P41273            | Tumor necrosis factor ligand superfamily member 9 | OS = Homo sapiens Ox = 9606 GN = TNFSF9 | TNFSF9 | 3.621 | Up | 0.0019838 |
| P78556            | C-C motif chemokine 20 | OS = Homo sapiens Ox = 9606 GN = CCL20 | CCL20 | 2.168 | Up | 0.0001382 |
| Q02487            | Desmocollin-2 | OS = Homo sapiens Ox = 9606 GN = DSC2 | DSC2 | 2.045 | Up | 0.0096407 |
| Q702N8            | Xin actin-binding repeat-containing protein 1 | OS = Homo sapiens Ox = 9606 GN = XIRP1 | XIRP1 | 2.307 | Up | 0.010498 |

Three experimental replicates were performed for each condition. Data were analyzed using the unpaired t-test.

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**Figure 2.** The level of AI-2 increased along with the progression of colorectal cancer in tumor tissues and stool samples and differed between proximal and distal colorectal cancer. (a) AI-2 concentration increased along with the progression of CRC in tumor tissues ($P = .045$). Stage I (n = 10), stage II (n = 12), stage III (n = 13), stage IV (n = 10). (b) AI-2 concentration increased along with the progress of CRC in stool samples ($P = .0003$). Stage I (n = 12), stage II (n = 14), stage III (n = 13), stage IV (n = 14). (c) The AI-2 concentration was significantly higher in distal CRC (n = 29) when compared to proximal CRC (n = 21) in tumor tissues ($P < .0001$). (d) AI-2 concentration was significantly higher in distal CRC (n = 32) when compared to proximal CRC (n = 21) in stool samples ($P = .0101$). **$P < .05$** compared to CRC stage I, *$P < .05$* compared to CRC stage II, **$P < .05$** compared to CRC stage III, Kruskal–Wallis test. **$P < .0001$**. **$P < .05$**, unpaired t-test. CRC, colorectal cancer.
**TNFSF9 was mainly derived from macrophages in the human CRC microenvironment**

To delineate the cell source of high abundance of TNFSF9 in CRC microenvironment, multi-immunofluorescence was performed to analyze the co-staining of immune cells (CD3, CD68) and TNFSF9 in 30 CRCs and 15 paired NCs (Figure 4(a–c)). The co-staining of immune cells (CD68+ macrophages, CD3+ T cells) and TNFSF9 protein in CRC tissues were significantly higher when compared to that in NCs (P < .0001, respectively) (Figure 4(a)). In addition, the frequency of co-staining for CD68+ macrophages and TNFSF9 protein in CRCs was significantly higher when compared to that for CD3+ T cells and TNFSF9 (P = .0002) (Figure 4(a)). Together, these findings suggested that increased TNFSF9 expression in human CRC tissues was mainly derived from macrophages in the tumor microenvironment.

**AI-2 concentration and TNFSF9 expression negatively associated with the CD4/CD8 ratio within the human CRC microenvironment**

We further analyzed the T cell subpopulations (CD4+ T cells, CD8+ T cells) of TILs correlating to AI-2 concentration and TNFSF9 expression. We found a significantly positive correlation between CD3+ T cell numbers and AI-2 concentration (P = .0462), and between CD3+, CD8+ T cell numbers and TNFSF9 expression in CRCs (P = .0159, P = .0025, respectively) (Figure 5(b,c)). TILs in CRCs had a reversed CD4/CD8 ratio when compared to that for CD3+ T cells and TNFSF9 (P = .0002) (Figure 5(a)). Moreover, the CD4/CD8 ratio negatively associated with AI-2 concentration (Spearman correlation coefficient: −0.4561, 95% CI: −0.7068 to −0.1036, P = .0113) and TNFSF9 expression (Spearman correlation coefficient: −0.5672, 95% CI: −0.7746 to −0.2495, P = .0011) within CRC tissues (Figure 5(c,f)). Combined, these results suggested that AI-2 and TNFSF9 may be associated with increased T cell numbers and decreased CD4/CD8 ratio within the CRC microenvironment.

**Discussion**

In a number of studies, it has been suggested that gut microbiota plays a critical role in regulating host immunity and metabolism, while the imbalance of gut microbiota was believed to lead to intestinal disorders by disturbing the immune system of the mucosa. In a previous study, it was shown that in the mammalian gastrointestinal tract, AI-2 mediated communication among bacteria, thereby shaping the structure of the microbial community. We demonstrated, for the first time, that the AI-2 concentration was significantly increased in cancer tissues and stool samples of CRC patients when compared to ADs and NCs. Another interesting finding was that the AI-2 concentration increased along with the progression of CRC. These findings indicated that AI-2 may play a role in the development and progression of CRC, possibly through influencing the microbial community in the human gut.

In addition, the concentration of AI-2 in distal CRC was significantly higher when compared to proximal CRC. This discrepancy may result from the different structures of microbiota in different subsites of the colon in CRC. Importantly, these findings suggested that AI-2 may be a novel marker for screening CRC, especially for distal CRC. AI-2, as a QS molecule of gut microbiota, may serve as a prospective marker for screening CRC from a new visual angle that is different from the traditional method, which is based on the biological behavior of the tumor itself. Moreover, the detection of AI-2 in stool samples of patients would be a simple and feasible method for screening of CRC in the clinic.

Macrophages play a critical role in microbial sensing. We hypothesized that AI-2 may be sensed and responded to by the gut immune system, especially macrophages in the gut mucosa. Using quantitative proteomics analysis, the most significant increase of...
TNFSF9 was found in macrophages that were treated with AI-2 extracts from *F. nucleatum*. In previous studies, we demonstrated that *F. nucleatum* plays an essential role in the progression of CRC. Furthermore, we observed that the expression of TNFSF9 protein was significantly higher in human CRC tissue when compared to adjacent normal tissue, and a positive correlation was found between AI-2 concentration and TNFSF9 expression. Our findings were consistent with previous studies, in which it was revealed that TNFSF9 expression was upregulated in CRC tissues. Our results demonstrated that AI-2 may be associated with the development and progression of CRC through macrophages involving TNFSF9 signaling.

Interestingly, it has recently been reported that mammalian epithelial cells of the tumor produced AI-2 mimics, which have AI-2 activity. Communication between host cells and bacteria occurs by generating AI-2 mimics that affect gene expression of bacteria through the AI-2 pathway in return. Thus, AI-2, a small molecule generated by both bacteria and the host, may act as a hub for communication between gut microbiota and immunity of host colon mucosa. Moreover, AI-2 produced by gut microbiota could be sensed by the gut immune system, thereby contributing to the development of microbiota-related diseases, such as CRC.

TNFSF9 expression was mainly found on several antigen-presenting cells, such as macrophages. In our study, TNFSF9 in the microenvironment of CRC was mainly derived from tumor-associated macrophages (TAMs). It is reported that high macrophage infiltration correlated with improved survival for CRC patients. Furthermore, TNFSF9 signaling has been proven to have potential effects on T cells, including both CD4+ and CD8+ T cells. Infiltration of CD8+ T-cells within cancer tissues has been demonstrated to contribute to a better survival of CRC patients. Moreover, CRC patients with low CD4/CD8 ratios had favorable overall survival. In this study, we found a positive correlation between CD3+ T cell numbers and AI-2/
Figure 5. The CD4/CD8 ratio was decreased within the human CRC microenvironment, and negatively associated with AI-2 concentration and TNFSF9 expression. (a) Representative images of subpopulations of tumor infiltration lymphocytes (CD4⁺ T cells and CD8⁺ T cells) in NC and CRC tissues. (b) Positive correlation between CD3⁺ T cell numbers and AI-2 concentration in CRCs (r = 0.3668, P = .0462, Pearson correlation analysis). (c) Positive correlation between CD8⁺ T cell numbers and TNFSF9 expression in CRCs (r = 0.5310, P = .0025, Spearman correlation analysis). (d) A reversed CD4/CD8 ratio was found in CRC when compared to NC (P < .0001, unpaired t-test). (e) Negative correlation between CD4/CD8 ratio and AI-2 concentration in CRC tissues (n = 30) (r = −0.4561, P = .0113, Spearman correlation analysis). (f) Negative correlation between CD4/CD8 ratio and TNFSF9 expression in CRC tissues (n = 30) (r = −0.5672, P = .0011, Spearman correlation analysis). CRC, colorectal cancer; NC, normal colon.
TNFSF9 levels, and between CD8+ T cell numbers and TNFSF9 expression in CRCs, suggesting that AI-2/TNFSF9 levels were associated with anti-tumor immunity in CRC patients. Moreover, our results showed that the CD4/CD8 ratio negatively associated with AI-2 concentration within CRC tissues. The association of AI-2 level in CRC patients with overall survival needs to be investigated in future studies. Taken together, these findings demonstrated that AI-2 produced by gut microbiota was associated with tumor immunity in CRC patients through TAMs and CD4/CD8 ratio in a TNFSF9-dependent manner.

Disclosure of interest
The authors report no conflict of interest.

Funding
This work was supported by Natural Science Foundation of Sichuan Science and Technology, Chengdu City, China, under Award No. 2018JY0167.

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