Original paper

# Lymphocyte-activated gene-3 (LAG3) protein expressed in tumor-infiltrating lymphocytes of colorectal cancer

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Immunotherapy can reverse tumor immune escape by suppressing immune checkpoints. Lymphocyte activation gene 3 (LAG3) is an important checkpoint and its role in colorectal cancer is not clear. In this study, we investigated LAG3 protein expression and its correlation with clinicopathologic parameters. The expression of LAG3 protein was assessed in 150 surgically resected colorectal cancer tissue samples by immunohistochemistry. The relationship between LAG3 expression and clinicopathological parameters, MSI status and survival was statistically analyzed. LAG3 protein was not expressed in colorectal cancer cells, and was expressed on the tumor-infiltrating lymphocytes (TILs) in 31 out of 150 (20.7%) colorectal cancer samples. Positive expression of LAG3 in TILs is associated with lymph node metastasis ($p < 0.001$), TNM stage ($p = 0.024$) and MSI-H ($p = 0.035$). No significant relationship was found between LAG3 expression and gender, age, tumor location, tumor invasion depth, and differentiation. LAG3 expression is associated with longer overall survival ($p = 0.045$). Our data show LAG3 expression on TILs in parts of CRC tissue. Positive expression of LAG3 was associated with advanced tumor stage, MSI-H and a poor prognosis. We conclude that LAG3 is an important checkpoint gene in CRC and may be a potential marker for prognosis of CRC.

**Key words:** lymphocyte activation gene 3 (LAG3), tumor-infiltrating lymphocytes (TILs), colorectal cancer.

## Introduction

Colorectal cancer (CRC) is a common cause of cancer-related mortality worldwide, with over 144,000 new cases diagnosed in the United States [1]. In China, the incidence of colorectal cancer is increasing, and it is the leading cause of cancer-related death as well[2]. Although substantial progress has been made in surgical techniques and postoperative chemotherapy in recent years, the prognosis for colorectal cancer is still not satisfactory. The search for new molecular targets of early diagnosis, rational therapy, and prognosis is a current research hot spot. Immunotherapy using immune checkpoint inhibitors (programmed cell death protein 1/programmed death-ligand 1, PD1/PD-L1) has been rapidly developed for promising treatment of cancers [3, 4, 5, 6]. PD1 and PD-L1 are important immune checkpoint components that mainly regulate the function of tumor cells and tumor-infiltrating lymphocytes (TILs). PD1, a cell surface membrane protein expressed by various immune cells including T cells, is activated by its ligands PD-L1 and PD-L2, expressed by antigen-presenting cells such as dendritic cells, macro-

phages or B cells. After engagement by its ligands, PD1 attenuates lymphocyte activation and promotes T-regulatory cell development and function, allowing inhibition of the immune response. Cancer cells differently express PD-L1 and activation of PD1/PD-L1 represents a common mechanism of immune escape by cancer cells. Clinical trials of PD1/PD-L1 inhibitors showed very promising results with durable responses [3, 7, 8, 9, 10, 11, 12].

Lymphocyte-activated gene-3 (LAG3, CD223) is another important checkpoint belonging to the immunoglobulin superfamily, which is upregulated on activated CD4+ and CD8+ T cells and a subset of natural killer cells. LAG3 structurally resembles the CD4 co-receptor and binds to MHC class II with a higher affinity than CD4 [13]. Increasing data suggest that modulating LAG3 can impact autoimmunity, cancer, chronic viral infection, and parasitic infection[14-20]. Deficiency in co-inhibitory receptor expression can promote autoimmunity. LAG3 deficiency alone does not predispose toward autoimmunity. Co-operative function of LAG3 with other co-inhibitor molecules can achieve optimal T cell regulation [13]. LAG3 expressing T cells are expanded in the tumor-infiltrating lymphocytes (TILs) of human lung cancer, breast cancer, gastric cancer, ovarian cancer and colon cancer [21, 22, 23, 24, 25]. Chen *et al.* analyzed LAG3 expression on CD4+CD25- T cells from non-small cell lung cancer patients. They found that LAG3 expression was significantly increased in CD4+ T cells. LAG3-expressing CD4+CD25- cells have higher levels of PD1 and TIM3 than in LAG3-nonexpressing CD4+CD25- cells. Interestingly, their data show that LAG3-expressing CD4+CD25- T cells infiltrating the resected tumors are more frequently found in metastasis than in primary tumors [24]. Burugu *et al.* found a statistically significant association between the presence of LAG3-expressing intraepithelial tumor-infiltrating lymphocytes and improved breast cancer specific survival outcomes [23]. Li *et al.* detected the soluble LAG3 level in peripheral serum of gastric cancer patients. They found that soluble LAG3 was poorly expressed in peripheral blood of gastric cancer and its expression was positively correlated with IL-12 and IFN-γ expression. High soluble LAG3 expression is related with a better prognosis in gastric cancer [22]. Huang *et al.* found that combinatorial blockade of LAG3 and PD1 pathways enhanced antitumor immunity in ovarian cancer [25]. Though few studies of LAG3 have been reported on colorectal cancer [21, 26, 27], the LAG3 expression and clinicopathological significance in colorectal cancer are still not well known. In this study, LAG3 expression was detected in a set of colorectal cancer tissues and the relationship between LAG3 expression and clinicopathological parameters was statistically analyzed.

## Material and methods

### Colorectal cancer tissue specimens

All the tissue specimens in our study were collected from 150 patients with colorectal carcinoma, as part of a study approved by the Research Ethics Board of the Jiangyin People's Hospital. These patients had undergone surgery in the Jiangyin People's Hospital between January, 2015 and December, 2017 without any preoperative therapy. These patients consisted of 93 males and 57 females, and the age range was from 29 to 85 years (median 67 years). Tumor staging (TNM) was evaluated based on the WHO classification of Tumors of the Digestive System (4th, 2010). In detail, the clinicopathological parameters of these 150 cases are listed in Table I.

**Table I.** Relationship between LAG3 expression and clinicopathological parameters

| Parameter | No. | LAG3 expression | | p-value |
|---|---|---|---|---|
| | | + | – | |
| | 150 | 31 | 119 | |
| Gender | | | | |
| Male | 93 | 18 | 75 | 0.679 |
| Female | 57 | 13 | 44 | |
| Age (years) | | | | |
| < 60 | 37 | 6 | 31 | 0.494 |
| ≥ 60 | 113 | 25 | 88 | |
| Location | | | | |
| Right | 32 | 10 | 22 | 0.137 |
| Left | 118 | 21 | 97 | |
| Invasion depth | | | | |
| Tis+T1+T2 | 28 | 4 | 24 | 0.455 |
| T3+T4 | 122 | 27 | 95 | |
| Lymph node | | | | |
| N0 | 83 | 15 | 68 | < 0.001 |
| N1 | 40 | 3 | 37 | |
| N2 | 27 | 13 | 14 | |
| Differentiation | | | | |
| Poor | 28 | 7 | 21 | 0.605 |
| Moderate + Well | 122 | 24 | 98 | |
| TNM stage | | | | |
| I + II | 87 | 12 | 75 | 0.024 |
| III+ IV | 63 | 19 | 44 | |
| MSI status | | | | |
| MSI-H | 20 | 8 | 12 | 0.035 |
| MSI-L/ MSS | 130 | 23 | 107 | |

### Immunohistochemistry

Formalin-fixed, paraffin-embedded samples used for immunohistochemistry were sectioned at 4 $\mu$m thickness. All the sections were deparaffinized using xylene, dehydrated by gradient ethanol, and then rehydrated with deionized water. Heat-mediated antigen retrieval was run by autoclave treatment (120ºC for 2 min in 1 mmol/l EDTA, pH 8.0) followed by cooling at room temperature. Incubation with a monoclonal antibody raised against the human LAG3 (dilution 1:200, Cell Signaling Technology, 3 Trask Lane, Danvers, MA 01923, USA) was performed overnight at 4ºC. After washing with phosphate-buffered saline (pH 7.4), the sections were then incubated with secondary antibody (Dako, UK) for 30 min at room temperature. Color development was performed with 3,3′-diaminobenzidine. Nuclei were counterstained with hematoxylin.

The immunostaining results were evaluated independently by two pathologists. The different results were unified by consensus. The score of LAG3 expression was made semiquantitatively by assessing the percentage of stained cells and the staining intensity in tumor-infiltrating lymphocytes according to scoring system used in the previous CRC studies [16]. Briefly, the staining intensity was graded as follows: 0, no staining; 1, equivocal staining; 2, moderate staining; and 3, strong staining. The immunohistochemical (IHC) expression in tumor-infiltrating lymphocytes of LAG3 was defined as positive when a moderate-to-strong intensity was observed in more than 5% of the tumor-infiltrating lymphocytes.

### DNA extraction

HE staining of tumor tissue slides was performed before DNA extraction and slides were reviewed by a pathologist. The specimens with > 80% tumor component were subjected to DNA extraction and gene mutation analysis. Genomic DNA from tumor tissue and normal mucosa was extracted with a QIAamp DNA FFPE tissue extraction kit (QIAGEN Strasse 1 40724 Hilden, Germany) according to the protocol from manufacturer. Briefly, 8 sections at 10 $\mu$m thick were deparaffinized with xylene and ethanol. The pellet was lysed by adding proteinase K and incubated at 56°C for 1 h. DNA was isolated with the QIAamp MinElute spin column. gDNA concentration was measured with a NanoDrop 2000 instrument.

### MSI analysis

The MSI analysis system (Yqbiomed, Shanghai, China) contains five mononucleotide markers (BAT-25, BAT-26, D5S346, D2S123, and D17S250). The assay includes fluorescently labeled primers for PCR amplification of FFPE-extracted DNA followed by capillary electrophoreses (Applied Biosystems 3500 Genetic Analyzers, 5791 Van Allen Way in Carlsbad, USA). In this study, per case FFPE tissue sections of 5-10 $\mu$m from tumor and normal mucosa were used. MSI assay was carried out according to the manufacturer's protocol. Briefly, PCR amplification conditions were as follows: 42°C, 5 min, 94°C, 5 min; then 94°C, 15 s, 55°C, 25 s, 72°C, 50 s for 40 cycles; 72°C, for 10 min. After PCR amplification, 1 $\mu$m of PCR product was mixed with 0.5 $\mu$m of LIZ 500 and 10 $\mu$m of HIDI, 95°C denatured for 5 min, then 4°C for 5 min. The PCR products were loaded on Applied Biosystems 3500 Genetic Analyzers for capillary electrophoreses. MSI-H (high) is defined as instability of 2 or more loci, MSI-L (low) as instability of only 1 locus, and MSS no locus instability.

### Survival analysis

Follow-up data were retrieved from medical records and confirmed by direct phone interviews with the patients' family members. Overall survival (OS) was defined as the interval between surgery and date of death. At the end of this study, the median follow-up time for all patients was 27 months (range from 13 to 48 months).

### Statistical analysis

The $\chi^2$ test was adopted to determine differences among intergroup variables by use of SPSS 15.0. software (SPSS, Chicago, IL, USA). Kaplan-Meier survival analysis was used to examine the relationship between LAG3 expression and overall survival (OS) for univariate analysis. All the tests were two-sided. A p-value of 0.05 was considered statistically significant.

## Results

### Expression of LAG3 in tumor-infiltrating lymphocytes of CRC

Expression of LAG3 was assessed in 150 tumor specimens of CRC. LAG3 was only detected in tumor-infiltrating lymphocytes, not in tumor cells (Fig. 1). LAG3 was expressed in the cytoplasm of the lymphoid cells. LAG3 was positively expressed in 31 out of 150 (20.7%) cases of tumor-infiltrating lymphocytes of CRC.

Relationship between LAG3 expression and clinicopathological parameters

The relationship between LAG3 expression in tumor-infiltrating lymphocytes and clinicopathological parameters of CRC was statistically analyzed. As shown in Table I, LAG3 expression was associated with lymph node metastasis ($p < 0.001$) and TNM stage ($p = 0.024$). No significant rela-

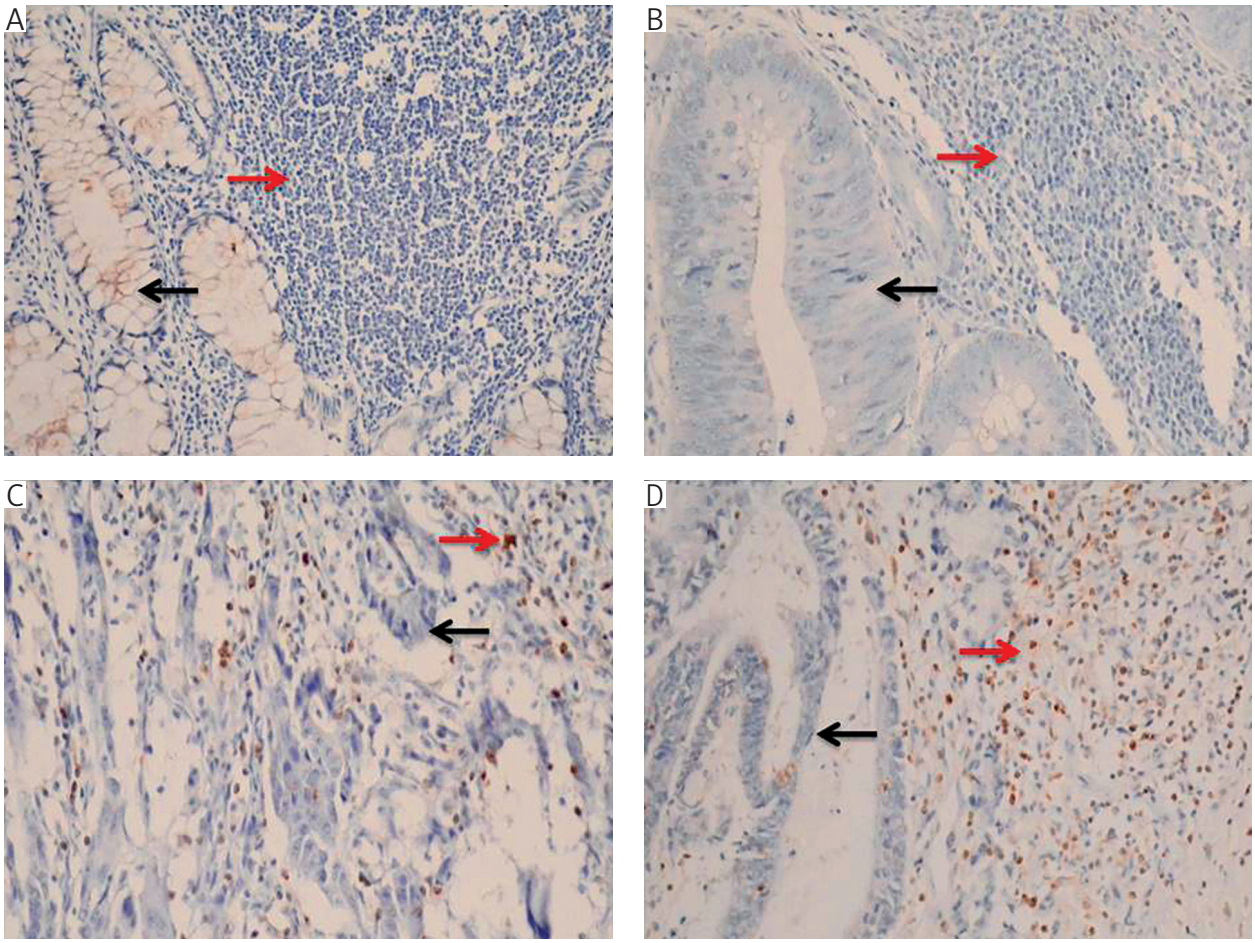


**Fig. 1.** LAG3 expression in tumor-infiltrating lymphocytes of CRC. A) LAG3 was negative in immune cells of normal mucosa. (Red arrow indicates lymphocytes, black one shows normal mucosa). B) LAG3 was negative in colorectal cancer cells. (Red arrow indicates lymphocytes, black one shows tumor.) C) LAG3 was detected in less than 5% of tumor-infiltrating lymphocytes. (Red arrow indicates lymphocytes, black one shows tumor) D) LAG3 was detected in more than 5% of tumor-infiltrating lymphocytes. (Red arrow indicates lymphocytes, black one shows tumor; ×400)

tionship was found between LAG3 expression and gender, age, tumor location, tumor depth, and differentiation.

### Positive expression of LAG3 related to MSI-H

MSI status was analyzed in 150 cases of CRC samples by the PCR amplification method (Fig. 2). In 20 out of 150 (13.3%) samples MSI-H was detected, and in 107 out of 150 (86.7%) MSI-L or MSS was detected. MSI-H was more often detected in samples that show positive expression of LAG3 ($p = 0.035$).

### Effects on survival

Overall survival (OS) was calculated from the date of surgery to death from any cause. Data were censored if patients were free of recurrence or alive at the last follow-up. LAG3 expression was significantly associated with a longer OS in a univariate analysis ($p = 0.045$, Fig. 3).

## Discussion

Anti-PD1 monoclonal antibodies ipilimumab, pembrolizumab, and nivolumab yield objective responses in a significant proportion of patients with a broad spectrum of tumor types. However, the majority of patients do not respond. Nivolumab demonstrates robust efficacy and a manageable safety profile across multiple tumor types, and was approved in the USA, EU and Japan for the treatment of advanced NSCLC in the second line. Montana *et al.* retrospectively evaluated efficacy and safety of nivolumab in 98 patients with non-small cell lung cancer. After four doses of nivolumab, 4.1% of patients showed a partial response, 32.7% a stable disease and 63.2% had a progressive disease [28]. A retrospective cohort study of patients with NSCLC in Japan was conducted by Hirai *et al.* to evaluate the efficacy and safety of nivolumab. Their results showed that the response rates were 20% [29].

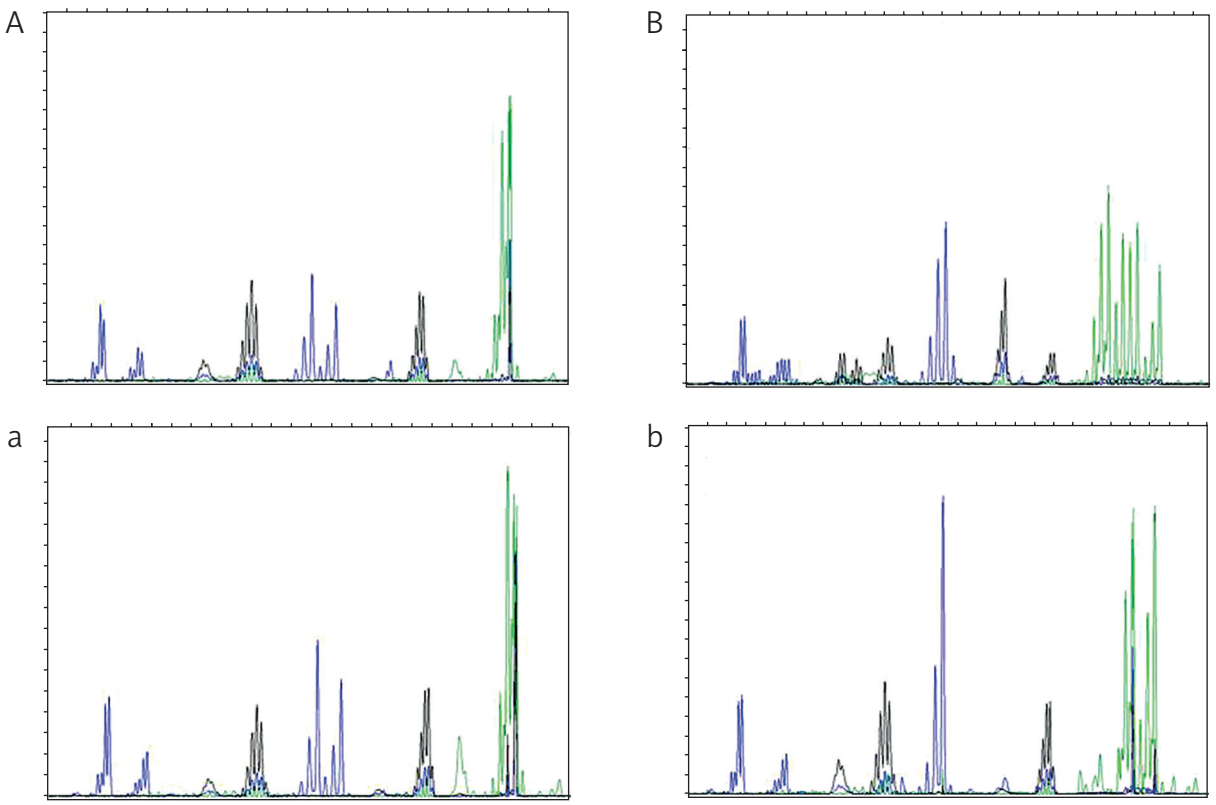


**Fig. 2.** Representative examples of MSI analysis in CRC samples. A) (tumor) and a (normal mucosa from same patient) show MSS after analysis of mononucleotide markers (BAT-25, BAT-26, D5S346, D2S123, and D17S250) in CRC. B) (tumor) and b (normal mucosa from same patient) show MSI-H after analysis of mononucleotide markers (BAT-25, BAT-26, D5S346, D2S123, and D17S250) in CRC

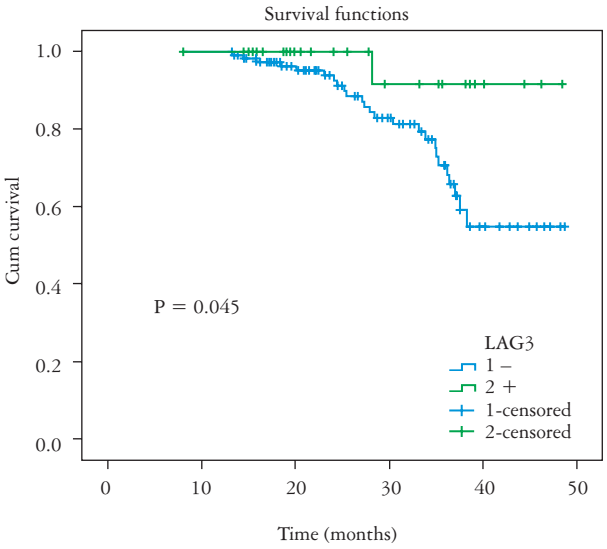


**Fig. 3.** LAG3 expression was significantly associated with a longer OS in a univariate analysis (p = 0.045)

Despite the impressive impact of PD1 and PD-L1 targeted cancer immunotherapy, a large proportion of patients with many tumor types fail to respond. It highlighted the necessity of further investigation of other checkpoints. Increasing evidence suggests that LAG3 and PD1 synergistically contribute to autoimmunity and tumor evasion. Huang *et al.* found that combinatorial blockade of LAG3 and PD1 pathways enhances antitumor immunity in ovarian cancer [25]. They found that approximately 15% of tumor-infiltrating T lymphocytes of murine ovarian cancer in C57BL/6 mice expressed PD1 and about 2-10% of these TILs also expressed LAG3. Their results showed that treatment with dual anti-LAG3/PD1 antibodies significantly delayed tumor growth. The study by Wierz *et al.* demonstrated that dual targeting of immune checkpoints PD1 and LAG3 successfully controlled CLL (chronic lymphocytic leukemia) development in preclinical mouse models, and they inferred that it could represent an effective treatment to restore a functional antitumor immune response [3]. Regulatory T (Treg) cells are critical suppressors to anticancer responses and associated with poor prognosis. Ma *et al.* examined the circulating Treg cells in CRC patients. They found that CRC patients presented elevated levels of LAG3 and TIM3 expression in $CD4^{+}CD^{25+/hi}$ $Foxp3^{+}$Treg cells. Furthermore, LAG3+TIM3+ Treg cells could suppress the proinflammatory activation of macrophages [21]. Increasing evidence shows that colorectal cancers with mismatch repair (MMR) deficiency are sensitive

to immune checkpoint blockade with anti-PD1 antibodies [30, 31]. Zhou *et al.* compared intra-tumor expression of multiple inhibitory molecules including LAG3, PD-1, TIM3 and CTLA4 among mismatch repair-proficient liver metastases of colorectal cancer (LM-CRC), peritoneal metastasis of colorectal cancer (PM-CRC) and primary CRC [26]. The effects of blocking inhibitory pathways on tumor-infiltrating T-cell responses were studied. They found that inhibitory receptors were more highly expressed on CD8+ T-cells, CD4+ T-helper and/or regulatory T-cells in LM-CRC tumors. Antibody blockade of LAG3 or PD-L1 increased proliferation and effector cytokine production of intra-tumoral T-cells isolated from LM-CRC in response to both polyclonal and autologous tumor-specific stimulations. They concluded that mismatch repair-proficient LM-CRC may be more sensitive to immune checkpoint inhibitors than primary CRC. Blocking LAG3 enhances tumor-infiltrating T-cell responses of mismatch repair-proficient LM-CRC. They concluded that LAG3 may be a promising new immunotherapeutic target for LM-CRC.

Nair *et al.* assessed mRNA expression of various immune checkpoints including PD-1, CTLA-4, TIM-3, LAG3, TIGIT and PD-L1 in specimens from 14 colorectal cancer cases containing tumor tissues and adjacent non-cancerous normal tissues [27]. Their results showed that LAG3 was slightly up-regulated in tumor tissues compared to normal tissues. Their data could not indicate exactly what kind of cells the LAG3 expressed, because qPCR detected the LAG3 mRNA expression level of tumor cells and tumor-infiltrating lymphocytes. Lee *et al.* detected immune checkpoint protein (PD-1, PD-L1, CTLA4, IDO1 and LAG3) expression in 89 cases of colon cancer with MSI-H. They reported that LAG3 was expressed in 13.5% of tumor-infiltrating immune cells (TIICs) in CRC tissues. Their results showed that co-expression of PD1, LAG3 and IDO1 in TIICs was associated with better disease-free survival (DFS). Moreover, in a multivariate survival analysis, LAG3 expression in TIICs remained an independent prognostic factor for a better DFS in colon cancer with MSI-H [16]. In this study, we examined LAG3 expression in a set of colorectal cancer tissues and statistically analyzed the relationship between LAG3 expression and clinicopathologic parameters, MSI status and overall survival. LAG3 expression levels in 150 cases of CRC tissues were checked using immunohistochemistry. We found that LAG3 protein was not expressed in colorectal cancer cells, but was positively expressed in the tumor-infiltrating lymphocytes (TILs) in 20.7% of colorectal cancer samples. Positive expression of LAG3 in TILs is associated with lymph node metastasis ($p < 0.001$), TNM stage ($p = 0.024$), MSI-H ($p = 0.035$) and a longer overall survival ($p = 0.045$). Our data suggest that LAG3 is an important checkpoint gene in CRC and may be a potential marker for prognosis of CRC.

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