Correlation of Clinicopathological Features and IL6 Expression in Tumor Budding of Colorectal Adenocarcinoma

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Abstract

**Background:** Interleukin-6 (IL6) is one of the main cytokines produced by cancer-associated fibroblasts (CAFs). IL6 is linked with cancer progression and poor prognosis by activating cancer cells and modifying the cancer microenvironment. However, little is known about the expression of IL6 in tumor budding (TB) and its association with TB in colorectal adenocarcinoma.

**Methods:** The clinicopathological and prognostic significance of IL6 in TB was examined using a tissue microarray consisting of 36 patient samples of TB in CA. IL6 mRNA was detected by RNAscope kit. Patients were stratified into negative and positive IL6 expression groups.

**Results:** IL6 expression was overwhelmingly observed in CAFs but was negligible in cancer cells. In the IL6-positive group in CAFs, TB grade was higher than in the IL6-negative group ($P=0.0161$). There was a significant difference in overall survival (OS) between CA cases in the IL6-positive group and the IL6-negative group (log rank test, $P=0.0367$). Cox proportional hazard regression model revealed that the IL6-negative group (OR = 0.25; 95% CI: 0.05–0.96; $P=0.0440$) had better OS for CA than the IL6-positive group.

**Conclusions:** TB may be affected by IL6 expression, and IL6 expression in CAFs at TB may make IL6 an important prognostic marker.

Background

Colorectal adenocarcinoma (CA) has increasing morbidity and mortality worldwide and is a global health problem [1]. Despite the high prevalence of colorectal cancer, the pathological mechanisms remain largely unknown [2]. However, many prognostic factors for colorectal cancer have been studied. In particular, the tumor budding (TB) region is a unique site and is known to be deeply involved in metastasis and invasion [3]. It has been demonstrated that TB is involved in EMT, which is known to be affected by the surrounding microenvironment of cancer [4, 5]. Cancer-associated fibroblasts (CAFs) have an important role in the cancer microenvironment, and interleukin-6 (IL6) produced by CAFs is involved in various processes [6]. We focused on the microenvironment in TB. IL6 is an important cytokine but has not been studied in TB. We investigated the clinicopathological characteristics of IL6 expression using RNAscope, a recently developed ISH technique with high sensitivity.

Methods

**Patients and Materials**

This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Shinshu University School of Medicine (approval no. 4088). Among 115 CA cases surgically resected at Shinshu University Hospital, Matsumoto, Japan between 2010 and 2012, stage I–III cases with TB were selected. Clinicopathological data were obtained from medical records. Materials used for evaluation were archived formalin-fixed paraffin-embedded tissues. According to the report of
Lugli et al., TB was classified into Bd1 (0–4 buds), Bd2 (5–9 buds), and Bd3 (≥ 10 buds) [3]. Furthermore, Bd1 and Bd2 were defined as low-grade TB and Bd3 was defined as high-grade TB. In the budding area, the score of inflammatory cell infiltration (tumor-infiltrating lymphocytes, TILs) was measured. According to the report of Ropponen et al., the TIL scores were: none, 0; mild, 1; moderate, 2; and marked, 3[7]. TIL scores were classified into low-grade scores 0 and 1 and high-grade scores 2 and 3.

**TMA construction**

A tissue microarray (TMA) was prepared from paraffin blocks containing sufficient tumor. The TMA was 3 mm in diameter and contained a fully analyzable TB region. The TB region was defined as an area with a single cell or a detached group of tumor cells consisting of five cells or fewer, and was selected based on the morphology of the hematoxylin and eosin-stained slide [8]. The generation of the TMA was in accordance with our previous report [9].

**IL6 RNA in situ hybridization**

*IL6* mRNA was detected using an RNAscope kit (Advanced Cell Diagnostics, Hayward, CA, USA), as previously described [9]. Intracellular brown dots indicated positive staining. *IL6* expression was measured according to a 5-grade scoring system recommended by the manufacturer's protocol. The 5-grade scoring system was determined under a 20× objective lens as follows: no staining, 0; 1–3 dots/cell, 1+; 4–10 dots/cell, 2+; 10–15 dots/cell, 3+; and > 15 dots/cell, defined as 4+. *IL6* mRNA expression was defined as negative expression in grade 0, 1+, and 2+, and positive expression in grade 3+ and 4+.

**Statistical analysis**

Pearson’s chi-squared test, log-rank test, and Cox proportional hazard regression analysis were analyzed by JMP Statistics software version 13 (JMP, Tokyo, Japan). A *P*-value less than 0.05 was considered significant.

**Results**

**IL6 expression in cancer stroma**

In the TB region, *IL6*-expressing cells were mainly identified in cancer stroma. These *IL6*-expressing cells were spindle-shaped and were considered as CAFs (Fig. 1A and 1C). In four cases, *IL6* expression could not be detected in the cancer stroma. Thirteen cases could be recognized as the *IL6*-high expression group. There was no tendency in the distribution of expressing cells in the stroma. However, there was almost no *IL6* expression in the cancer cells in the TB region. Cancer cells throughout the TMA core also had little *IL6* expression. Thirty cases were completely negative for *IL6* expression in cancer cells. *IL6* expression in the cancer cells was faint and had no characteristic distribution (Fig. 1B and 1D). No cases could be recognized as *IL6*-positive.

**Association between IL6 expression and clinicopathological characteristics**
As presented in Table 1, the clinicopathological characteristics of patients with CA are described in Table 1. In the IL6-positive group, TB grade was higher than in the IL6-negative group ($P = 0.0161$). There was no significant difference between the IL6-positive group and the IL6-negative group in terms of age, sex, vascular invasion, histological grade, TILs, or TNM stage.

**Table 1**

IL6 expression and clinicopathological characteristics in CA.

| Factors         | n | Positive (n = 13) | Negative (n = 23) | $P$-value |
|-----------------|---|------------------|-------------------|-----------|
| Age             |   |                  |                   | 0.9231    |
| >70 years       | 17| 6                | 11                |           |
| ≤70 years       | 19| 7                | 12                |           |
| Sex             |   |                  |                   | 0.8767    |
| Male            | 16| 6                | 10                |           |
| Female          | 20| 7                | 13                |           |
| TILs            |   |                  |                   | 0.9685    |
| High            | 22| 8                | 14                |           |
| Low             | 14| 5                | 9                 |           |
| Histological grade |   |                  |                   | 0.587     |
| High            | 20| 8                | 12                |           |
| Low             | 16| 5                | 11                |           |
| Vascular invasion |   |                  |                   | 0.7286    |
| High            | 18| 7                | 11                |           |
| Low             | 18| 6                | 12                |           |
| Tumor budding grade |   |                  |                   | 0.0161*   |
| High            | 3 | 3                | 0                 |           |
| Low             | 33| 10               | 23                |           |
| TNM stage       |   |                  |                   | 0.587     |
| I–II            | 20| 8                | 12                |           |
| III             | 16| 5                | 11                |           |

*Asterisk (*) indicates a significant difference between groups ($P < 0.05$).*
**IL6 negativity predicts better prognosis of CA**

To clarify the impact of *IL6* expression, Kaplan-Meier analysis with log-rank test was used to evaluate the association between *IL6* expression and OS in CA (Fig. 2). The *IL6*-negative group (median OS, 1980 (range, 1771–2531) days) had significantly better OS than the *IL6*-positive group (median OS, 1556 (range; 1212–2377.5) days) (log-rank test, *P* = 0.0367).

A Cox proportional hazard regression model revealed the relationship between clinicopathological factors and OS (Table 2). These results revealed that the *IL6*-negative group (OR = 0.25; 95% CI: 0.05–0.96; *P* = 0.0440) had better OS for CA than the *IL6*-positive group.

### Table 2
Univariate analyses for prognostic factors of CA.

| Factors                                  | Univariate analysis |
|------------------------------------------|---------------------|
|                                          | OR (95% CI)         | *P*-value         |
| Age: >70 years vs ≤ 70 years              | 2.82 (0.74–13.38)   | 0.1291            |
| Sex: male vs female                       | 3.36 (0.88–15.95)   | 0.0753            |
| Histological grade: low vs high           | 0.33 (0.05–1.37)    | 0.135             |
| TILs: low vs high                         | 3.53 (0.93–16.77)   | 0.0638            |
| Vascular invasion: absent vs present      | 0.822 (0.20–3.11)   | 0.7701            |
| Tumor budding grade: low vs high          | 0.79 (0.14–14.58)   | 0.8253            |
| TNM stage: I–II vs III                    | 1.01 (0.27–4.08)    | 0.9883            |
| *IL6* expression: negative vs positive    | 0.25 (0.05–0.96)    | 0.044*            |

* Asterisk (*) indicates a significant difference between groups (*P* < 0.05).

**Discussion**

In the present study, we demonstrated that *IL6* expression in TB had significant effects on OS. Recently, it has been shown that CAFs, which account for the majority of the tumor stroma, have an important role in producing factors involved in invasion and metastasis. In CA, CAFs are known to be involved in prognostic factors such as invasion and metastasis [10] [11], and there are some reports of *IL6* expression in CAFs [12] [13]. Hugo et al. reported that cancer cells cause an inflammatory response in fibroblasts and promote *IL6* expression [14]. In our study, no association was found between *IL6* and inflammation expressed as TILs, possibly because of the method of evaluation and the number of cases. However, there are no reports of *IL6* expression in CAFs in CA. Nonetheless, there are reports of *IL6*
expression from CAFs in several other carcinomas [15] [16]. Qiao et al. reported that IL6 expression from CAFs is associated with poor prognosis in esophageal squamous cell carcinoma [16]. This is the first report on IL6 expression from CAFs in the TB region, and indicates that IL6 expression is a poor prognostic factor.

TB grade was previously reported to be associated with prognosis [17]. In our study, TB grade was not related to prognosis, possibly because of the small number of samples. The TB region strongly affects metastasis and invasion. Although the mechanism of TB involvement in prognosis is unclear, the involvement of EMT has been reported in recent years [5]. TB in CA has been shown to upregulate mesenchymal markers and known inducers of EMT, such as the transcription factors ZEB1 and ZEB2 [18]. However, another report revealed that TB shows downregulation of E-cadherin but does not share other regulatory changes common to EMT, suggesting that TB formation may occur by other mechanisms [19] [20]. Yamada et al. reported that ZEB1, an EMT protein, is highly expressed in stroma near TB [20]. Our study demonstrates that IL6 expression is correlated with TB grade. As mentioned above, its involvement of TB and EMT is speculated [20]. EMT and IL6 expression in the cancer stroma are known to be involved in miR-34A suppression [21]. This fact proves an indirect link between TB and IL6. However, IL6-affected TB may be directly involved in EMT IL6-affected TB may be directly involved in EMT.

There are several studies of IL6 in CA, but these mostly focused on IL6 expression in cancer cells [22] [23]. Although many reports indicate that IL6 expression in cancer cells is associated with poor prognosis [24] [25], one report demonstrated that IL6 expression at other sites confers a favorable prognosis [26]. Meanwhile, Nagasaki et al. reported that IL6 expression is higher in CAFs than in cancer cells when comparing cancer cells and CAFs isolated from human CA [12]. In our study, IL6 expression has been largely identified in the stroma corresponding to CAFs, and IL6 expression in cancer cells is negligible. Therefore, although IL6 produced by CAFs seems to have a strong effect on prognosis, further investigation is necessary. Many reports have examined IL6 expression by immunostaining [24] [25] [26], but there may be many nonspecific reactions. Thus, RNA in situ measurement may provide more accurate information.

There are several limitations of our study. An increased number of cases would enable more accurate information to be obtained. In addition, expression analysis of IL6 receptor in cancer cells in the TB area should be performed.

Taken together, inhibition of IL6 expression may be a potential therapeutic strategy for the treatment of cancers in which IL6 from CAFs may have important effects.

**Conclusion**

Our results reveal the relationship between IL6 expression of CAFs and TB in CA. A further study is warranted to confirm these findings.
Abbreviations
CA, colon adenocarcinoma; TB, tumor budding; IL6, interleukin-6; CAFs, cancer-associated fibroblasts; TIL, tumor-infiltrating lymphocyte; TMA, tissue microarray

Declarations

Ethics approval and consent to participate
The study protocol conformed to the Declaration of Helsinki and was approved by the ethic committee of the Ethics Committee of Shinshu University School of Medicine (Approval Code: 4088), with a waiver of the need for informed consent because of the retrospective study design.

Consent for publication
Not applicable.

Availability of data and materials
All data generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
TU participated in the design of the study, performed the pathological analysis, and drafted the manuscript. MI and HO helped with the pathological analysis. TU and TN performed statistical analysis. KS, TN, and YT conducted immunohistochemistry. TN and YM examined the clinical data of cases. HO critically revised the draft for important intellectual content. All authors have read and approved the manuscript.

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Figures
Figure 1

Representative features of IL6 expression. Representative features of IL6 expression in CAFs (A and C). High levels of IL6 expression (arrow) were determined as IL6-positive. Representative features of IL6 expression in cancer cells at TB (B and D). Faint IL6 expression in cancer cells (arrow) was determined as IL6-negative. (A and B: HE; C and D: IL6 RNAscope)
Figure 2

Prognostic value of IL6 in CA by Kaplan-Meier analysis. There was a significant difference in OS between CA cases in the IL6-positive group (median OS, 1556 (range, 1212–2377.5) days) and the IL6-negative group (median OS, 1980 (range, 1771–2531) days) (log rank test, P=0.0367).