Correlation of Clinicopathological Features and *IL6* Expression in Tumor Budding of Colon Adenocarcinoma

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Research article

Keywords: interleukin-6; tumor budding; RNA in situ hybridization; colon adenocarcinoma

Posted Date: May 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-26146/v1

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 colon adenocarcinoma (CA).

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 cancer (CRC) 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 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 colon adenocarcinoma (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 (H&E)-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, 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. 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.

		***IL6* expression**		
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 ($P < 005$).				

*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 ($P < 005$).		

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 CRC, 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 CRC. 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 CRC 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 CRC, 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 CRC [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.

Conclusions

Our results reveal the relationship between *IL6* expression of CAFs and TB in CA. A further study is warranted to confirm these findings.

Abbreviations

IL6, interleukin-6; CAFs, cancer-associated fibroblasts; TB, tumor budding; CA, colon adenocarcinoma

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Shinshu University School of Medicine (Approval Code: 4088). The requirement of informed consent was waived, and an opt-out method was used due to the retrospective design of the study. The investigation was conducted in compliance with the Helsinki Declaration.

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.

Funding

This study was partially supported by the Hokuto Foundation for Bioscience (grant awarded to T.U.). The funding body had no role in the study design, collection, analysis, or interpretation of data, or manuscript writing. The authors declare no conflicts of interest.

Authors' contributions

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

Acknowledgements

We are grateful to Yukihoro Kobayashi, Masanobu Momose, Yasuyo Shimojo, Naoko Ogiwara, Akiko Inamura, Chitoshi Arai, Yasuhiro Kinugawa, Marina Nuno, Kanade Wakabayashi, Naoko Yamaoka, and Tomoya Hachisu at Shinshu University Hospital for their excellent technical assistance. We thank H. Nikki March, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

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Figures

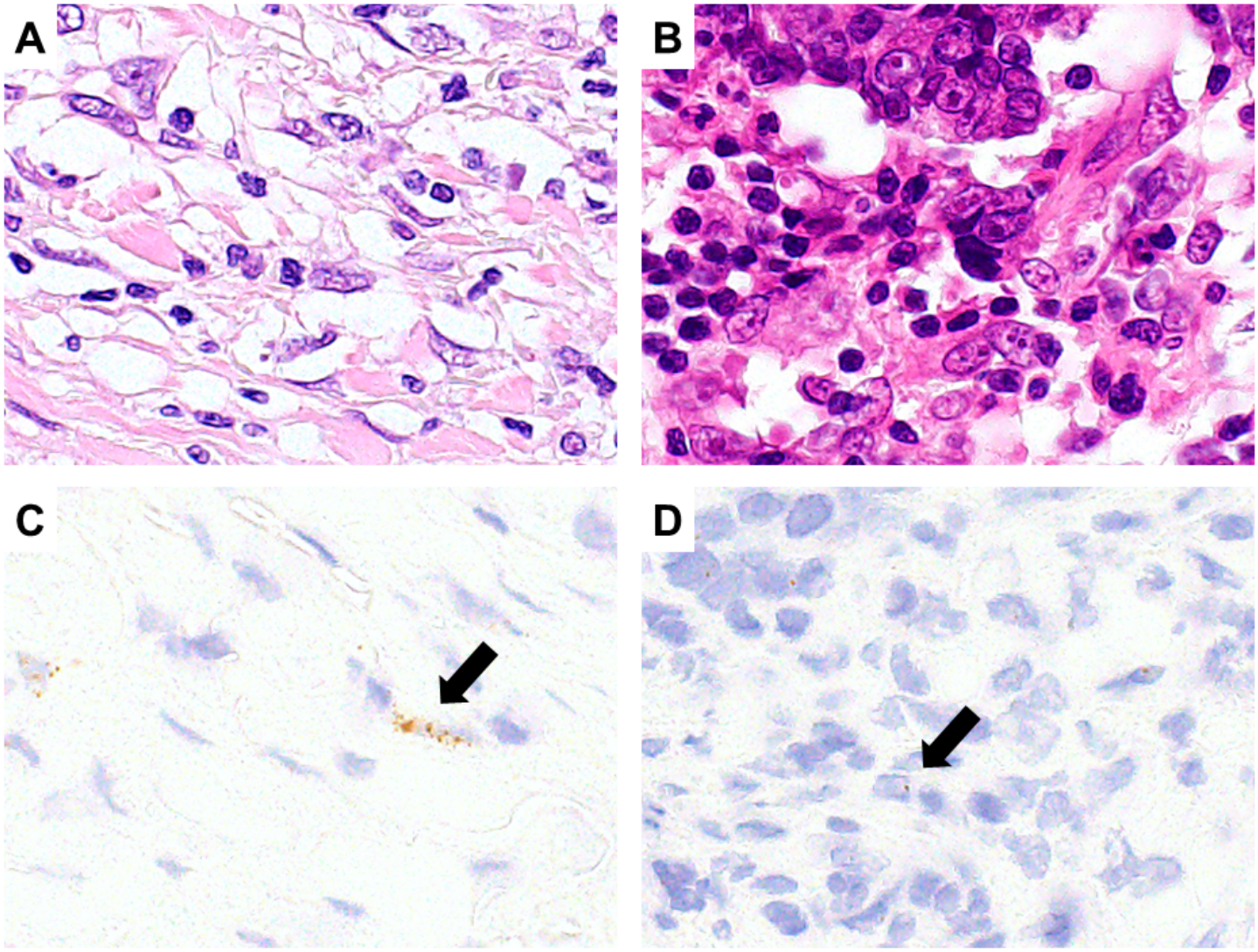

Figure 1

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)

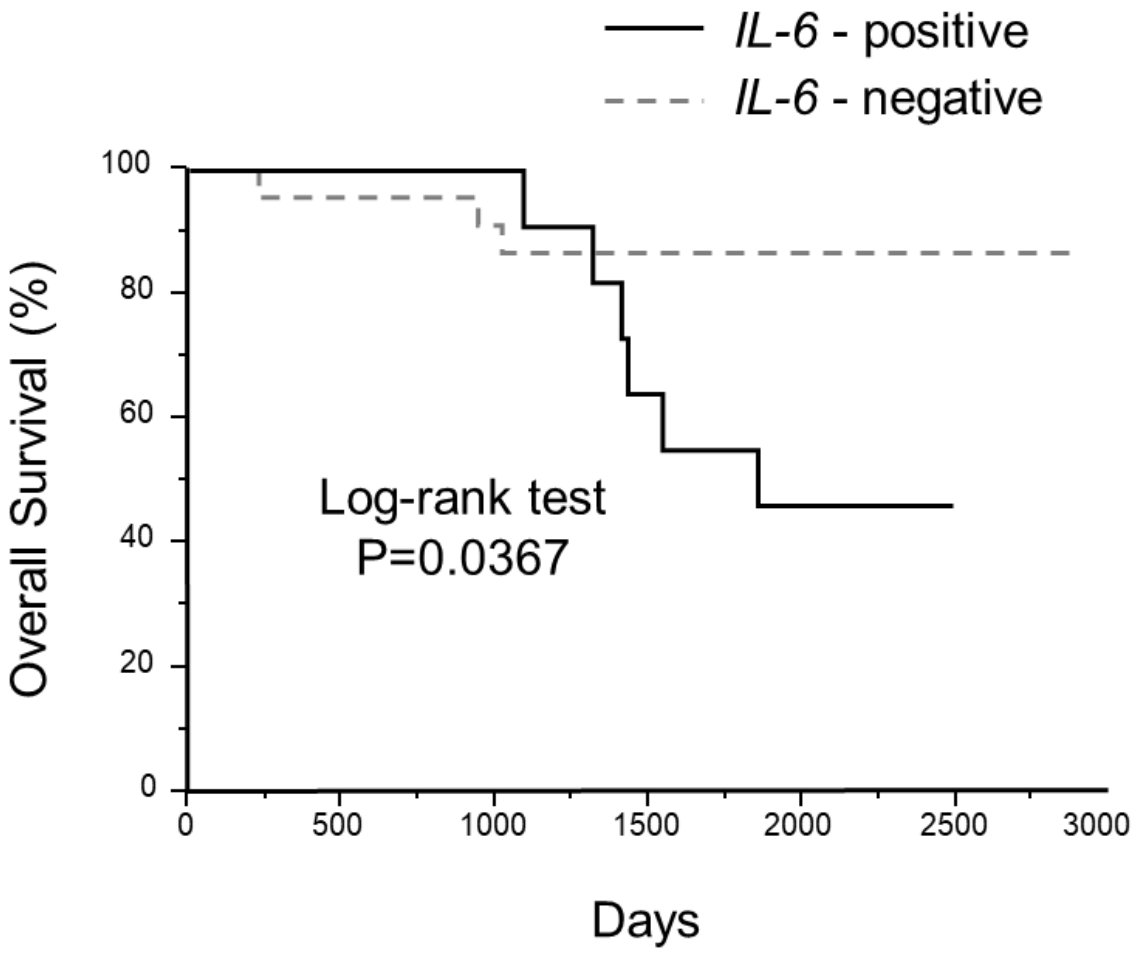

Figure 2

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).