Basic Study

Alcohol promotes epithelial mesenchymal transformation-mediated premetastatic niche formation of colorectal cancer by activating interaction between laminin-γ2 and integrin-β1

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Abstract

BACKGROUND
Colorectal cancer (CRC) is a common malignant tumor. Alcohol consumption is positively correlated with CRC malignant metastasis; however, the mechanism is unclear. The interaction between laminin-γ2 (LAMC2) and integrin-β1 (ITGB1) plays a role in premetastatic niche signaling, which may induce epithelial mesenchymal transformation (EMT) and lead to metastasis.

AIM
To investigate the effects of alcohol on CRC metastasis from the molecular mechanism of the premetastatic niche.

METHODS
The interaction between LAMC2 and ITGB1 was measured by Duolink assay, and the expression levels of LAMC2, ITGB1 and focal adhesion kinase (FAK), snail, fibronectin, N-cadherin and special AT-rich sequence binding protein 1 (SATB1) were measured by quantitative real-time polymerase chain reaction, immunohistochemistry and western blotting. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 levels were measured *via* enzyme-linked immunosorbent assay, histopathological assessment *via* hematoxylin eosin staining, and determination of

aberrant crypt foci *via* methylene blue.

RESULTS
The lymph node metastasis rate was higher in the alcohol group than non-alcohol group. There was a significant increase in interaction signals between LAMC2 and ITGB1, and an increase in phosphorylate-FAK/FAK, snail, fibronectin, N-cadherin and SATB1, whereas E-cadherin was reduced in the alcohol group compared to the non-alcohol group in both animal and clinical samples. Serum IL-1β, TNF-α and IL-6 were higher in alcohol group than in non-alcohol group. Alcohol may promote CRC metastasis by influencing the molecular mechanism of the premeta-static niche.

CONCLUSION
Our study suggests that alcohol promotes EMT-mediated premetastatic niche formation of CRC by activating the early interaction between LAMC2 and ITGB1 and lead to CRC metastasis.

Key Words: Alcohol; Colorectal cancer; Premetastatic niche; Epithelial mesenchymal transformation; Metastasis

Core Tip: Our study indicated that alcohol promotes epithelial mesenchymal transformation-mediated premetastatic niche formation of colorectal cancer (CRC) by activating the early interaction between laminin-γ2 and integrin-β1 and lead to CRC metastasis.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy worldwide[1]. A majority of colorectal cancers are associated with the environmental factors such as lifestyle changes including diet, obesity, smoking and alcohol consumption[2,3]. It is reported that long-term alcohol consumption is associated with an increased risk of CRC metastasis[3]. In the presence of concomitant metastasis, the maximum survival rates after surgical intervention do not exceed 20%[4,5]. Therefore, the relationship between alcohol consumption and CRC metastasis should be investigated. However, the mechanism that the alcohol promotes CRC metastasis has not been clearly explained[6,7].

Accumulating evidence suggests that metastasis develops from disseminated cancer cells that can remain dormant, and the dissemination may occur during the early stage of tumor evolution[7,8]. The microenvironment around the early disseminated cancer cells, also known as the premetastatic niche[9,10], may play an important role during the proliferation and migration of disseminated cancer cells in the early stage of tumors[11-13]. The extracellular matrix (ECM) is an important part of the premeta-static niche[14-17].

As an important component of the ECM, laminins are important for adhesion, differentiation, migration, and resistance to apoptosis of various cells, including cancer cells[18-21]. Reports have indicated that laminins are involved in the formation of the premetastatic niche[22,23]. Laminin-γ2 (LAMC2), a subunit of laminin-332, has been reported to be an important component in the survival dependent environment of epithelial cells in CRC and plays a key role in the regulation of tumor cell motility in premetastatic niche formation[24-27]. The interaction between LAMC2 and integrin-β1 (ITGB1) may stimulate the formation of premetastatic focal contacts by activating the premetastatic niche signaling, which promotes the migration of cancer cells[28]. Focal adhesion kinase (FAK) is a premetastatic focal contact involved in LAMC2 and β1 integrin signal transduction[29]. Signaling cascades triggered by integrin-laminin interactions in cells phosphorylate FAK, which induced epithelial mesenchymal transformation (EMT). EMT is a key initiating step during the metastasis and invasion of cancer[30,31] and involved in the formation of the premetastatic niche[32,33].

Alcohol consumption may influence the ECM remodeling and thus induce EMT, which may be closely related to the premetastatic niche[34]. Therefore, we explore its effect on CRC metastasis from the view of the premetastatic niche.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were used: LAMC2 monoclonal antibody (YT5379, Immunoway, United States), ITGB1 monoclonal antibody (sc-9970, Santa Cruz, United States), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (ab125247, ABCAM, United Kingdom), phosphorylate-FAK (p-FAK)-Y397 (AP0302, ABclonal, Wuhan, China), FAK monoclonal antibody (A11131, ABclonal, Wuhan, China), snail monoclonal antibody (ab53519, ABCAM, United Kingdom), E-cadherin polyclonal antibody (ab1416, ABCAM, United Kingdom), N-cadherin polyclonal antibody (22018-1-AP, Proteintech, United States), fibronectin polyclonal antibody (A16678, ABclonal, Wuhan, China) and special AT-rich sequence binding protein 1 (SATB1) polyclonal antibody (A5800, ABclonal, Wuhan, China). Secondary antibodies were ab288151 and ab150113 (ABCAM, United Kingdom). Polyvinylidene difluoride membranes with a pore size of 0.45 mm (Millipore, United States) were used. RNAEX reagent (Accurate Biotechnology, Beijing, China), Evo M-MLV RT Premix (Accurate Biotechnology, Beijing, China), SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology, China). Interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits (Enzyme-linked Biotechnology Co., LTD, Shanghai, China) were used. Duolink proximity ligation assay (PLA) kits (DUO92012, Merck, United States), Duolink PLA Rabbit PLUS (DUO92002, Merck, United States), and PLA Mouse MINUS (DUO92004, Merck, United States) proximity probes were purchased from Sigma-Aldrich. All above the mentioned antibodies can be used in both in rats and in humans.

Patients and clinical data

A total of 63 patients (26-86 years old) at The First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China) from July 2019 to February 2020 was enrolled in this study. The baseline characteristics were collected, and data on alcohol consumption were collected at baseline using a direct interview method[35]. Participants were asked to answer whether they drank alcoholic beverages ("Yes", "Yes, but not now", or "No" were the answer options to the question, "Have you ever drunk alcohol?"), and for "ever drinkers", consumption frequency and average quantity of one serving over the past year by beverage type was recorded. CRC patients who never drank alcohol were added into the non-alcohol group, and CRC patients who drank alcohol over 3 times a week for more than 5 years were added into the alcohol group. And patients with smoking, family history, blood lipids, obesity, and chronic diseases were excluded. The present study was approved by the Ethics Committee of First Affiliated Hospital of Guangzhou University of Chinese Medicine [No. Y (2019) 172] and carried out in accordance with the "Helsinki Declaration". All CRC patients have sighed informed consent. Patients who received neoadjuvant chemotherapy or radiotherapy or had a history of malignancy were excluded. Clinicopathological data including pathological report, sex, age, macroscopic classification, tumor location, tumor size, tumor differentiation, lymphatic infiltration and invasion depth were collected *via* medical record. The American Joint Council on Cancer tumor, node and metastasis (TNM) staging system is used for the clinical staging of tumors[36].

Animals, induction of CRC and treatments

A total of 32 male Wistar rats, 7 wk old and weighing 200 ± 20 g, were obtained from the Experimental Animal Center of Southern Medical University [Certificate of quality: SCXK (Yue) 2016-0041]. All rats were housed in cages (25 cm × 30 cm × 30 cm, four rats per cage) under specified-pathogens free conditions at 25℃, 40%-60% relative humidity, and a 12-h light/dark cycle in the experimental animal center of Guangzhou University of Chinese Medicine. All rats had ad libitum access to standard rodent chow and filtered water and were acclimatized for 1 wk prior to the initiation of the experiment. The use of laboratory animals was checked by the "Institutional Animal Ethical Committee" and all procedures were approved by the Ethics Committee of Guangzhou University of Chinese Medicine, and performed according to the "Principles of Laboratory Animal Care" as well as specific national laws where applicable. All experimental protocols and handling of the animals followed the guide for the care and use of laboratory animals[37].

A total of 32 rats were randomly divided into two groups: the non-alcohol group and the alcohol group, with 16 rats in each group. Non-alcohol group: The rats were fed water (10 mL/kg b.wt.) every day and from the 6th week on, the rats were administered a subcutaneous injection of 1,2-dimethylhydrazine hydrochloride (DMH) at a dose of 30, 35, 25 or 20 mg/kg b.wt. once a week for 12 wk in order to induce CRC. Alcohol group (DMH + ethanol): The rats were fed 30% ethanol (10 mL/kg b.wt.) every day, and from the 6th week on, they were administered a subcutaneous injection of DMH at a dose of 30, 35, 25 or 20 mg/kg b.wt. once a week for 12 wk in order to induce CRC. The details of the specific modeling method of each group were shown in Figure 1. The blood samples were collected through the posterior orbital venous plexus. And the rats were sacrificed by cervical dislocation, then the colons were removed and used in further experiments.

Hematoxylin and eosin staining and histological evaluation

All specimens were fixed in 4% paraformaldehyde solution for 24 h and embedded in paraffin and
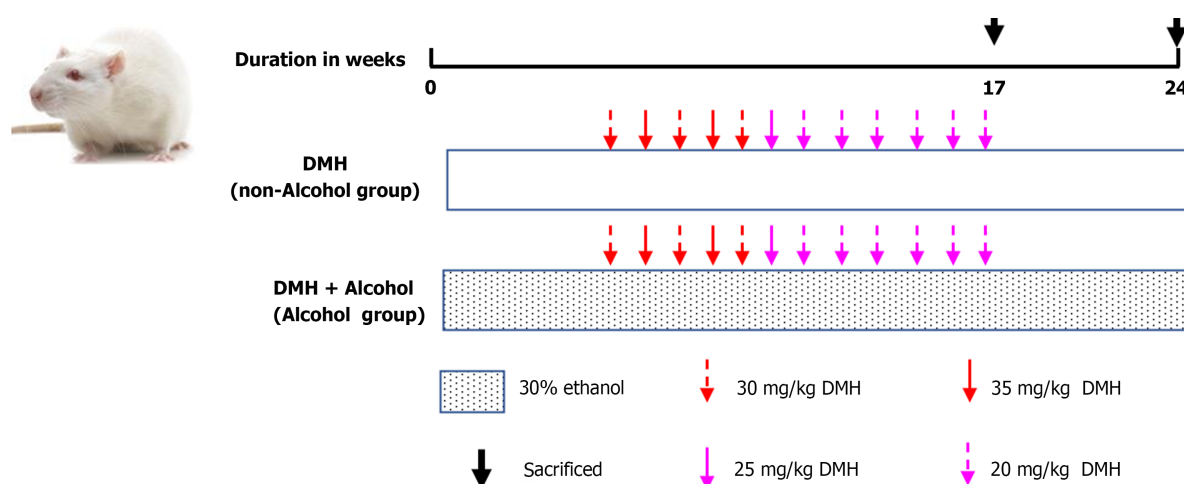
Figure 1 Schematic diagram of animal experimental modeling. DMH: 1,2-Dimethylhydrazine hydrochloride.

processed by standard histological processing techniques. Tissue sections (4-μm thick) were obtained from each sample. The hematoxylin and eosin results were observed under a light microscope at 40× magnifications (Olympus, BX51) to observe the colonic histology differences by the method of Martin B [38].

Determination of aberrant crypt foci

Aberrant crypt foci (ACF) were determined according to the method described by Bird *et al*[39]. Then, the formalin-fixed colon was stained with 0.2% methylene blue for 3 min and observed under light microscope. The diagnosis of colonic pathology and ACF was performed by two pathologists. The number of ACF was counted as described by Sivaranjani *et al*[40].

Cytokines detection by ELISA

Serum samples of rats were collected, and the inflammatory factors (TNF-α, IL-6 and IL-1β) were determined using ELISA kits according to the manufacturers' instructions. The optical density of the colorimetric reaction was measured at 450 nm with a flat microplate reader.

Western blotting

For western blotting, 50 μg of extracted protein was fractionated on a 10% SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane with a 0.45-mm pore size (Millipore, MA, United States). The membrane was blocked in 5% milk for 3 h at room temperature and probed with antibodies against acetylated LAMC2 (1:1000), integrinβ1 (1:1000), p-FAK (1:1000), FAK (1:1000), snail (1:1000), E-cadherin (1:1000), fibronectin (1:1000) and N-cadherin (1:1000) at 4℃ overnight. Horseradish peroxide-conjugated goat anti-rabbit and rabbit anti-mouse was used as the secondary antibodies. FAK and GAPDH (1:8000) were used as loading controls. Band intensity was visualized by ChemiDoc TMXRS+ (Bio-Rad, United States).

Immunohistochemical staining

The immunohistochemical (IHC) was performed by the method described in previous studies[41,42], all colonic tissue were fixed with 4% paraformaldehyde and paraffin embedded, then thin 4μm sections were obtained and deparaffinized in xylene and rehydrated through decreasing grades of alcohol. The sections were placed in a 95℃ antigen retrieval solution for 15 min. After cooling the retrieval solutions for 20 min at room temperature, the slides were treated with hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase activity. Then, slides were incubated with primary antibodies against ITGB1 (dilution 1:100), LAMC2 (dilution 1:500), p-FAK (dilution 1:500), snail (dilution 1:500), E-cadherin (dilution 1:100), N-cadherin (dilution 1:800), fibronectin (dilution 1:200), and SATB1 (dilution 1:200) overnight at 4℃. The next day, biotin-conjugated secondary antibody and streptavidin-biotin peroxidase were applied each for 20 min. 3,3'-Diaminobenzidine tetrahydrochloride was used as the substrate, and nuclear contrast was performed using hematoxylin counterstaining. The negative controls for IHC are performed with PBS instead of primary antibody. The results were observed under a light microscope at 40× magnifications (Olympus, BX51). For quantitative analyses, the IHC images were analyzed by Image-Pro Plus 6.0 to calculate the average optical density (AOD), and the following equation: AOD = integrated optical density/area.

Table 1 Primer sequences for quantitative real-time polymerase chain reaction

Gene name	Forward (5'>3')	Reverse (5'>3')
For human		
LAMC2	TTCTACAACGATCCGCACGAC	GAGTTAAAAGCAGCCCTGGT
ITGB1	CCTACTTCTGCACGATGTGATG	CCTTTGCTACGGTTGGTTACATT
FAK	GTCTGCCTTCGCTTCACG	GAATTTGTAACTGGAAGATGCAAG
Snail	CTAGCGAGTGGTTCTTCTGC	GTAGTTAGGCTTCCGATTGGG
E-cadherin	GACCGGTGCAATCTTCAAA	TTGACGCCGAGAGCTACAC
N-cadherin	AGCTTCTCACGGCATACACC	GTGCATGAAGGACAGCCTCT
Fibronectin	GCCTGGTACAGAATA TGTAGTG	ATCCCAGCTGATC AGTAGGCTGGTG
SATB1	GTGGAAGCCTTGGGAATCC	CTGACAGCTCTTCTTCTAGTT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
For rats		
LAMC2	GTGAAGCTTCCCTGCAAAAC	CATTTGGCCCCACGTAGT
ITGB1	TCGAAGGACCACTAGACCTGA	TTCCATGGGAACAAAAGGTAA
FAK	TTGGGTTGCAAACTATCTCTAGAAC	TGGTACAAAACTGGCACAGAA
Snail	TTCACATCCGAGTGGGTCTG	ACCCACACTGGTGAGAAGCC
E-cadherin	AAAGCAGGAAGAAAACACCACTC	AAAGGGCACGCTATCAACATTAG
N-cadherin	TCAGTGGCGGAGATCCTAC	GTGCTGAATTCCCTTGGCTA
Fibronectin	TGTCACCCACCACCTTGA	CTGATTGTTCTTCAGTGCGA
SATB1	AGATGCAGGGAGTGCCTTTA	TGCTCCTCCTTGCAATCATA
GAPDH	TGCCCTCATGTTCCTGATAAAT	CATTACATCACAGCTTTCCAGG

ITGB1: Integrin-β1; LAMC2: Laminin-γ2; SATB1: Special AT-rich sequence binding protein 1; FAK: Focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Number of rats with mucosal pathological tissue changes in different time periods

| Wk	Group	n	Normal	Atypical hyperplasia	Adenoma	Adenocarcinoma Mucosal carcinoma	Infiltrating carcinoma	With lymphatic metastasis Metastasis	Tumor metastasis rate
17	Non-alcohol	8	3	5	0	0	0	0	0.00%
	Alcohol	8	0	6	2	0	0	0	0.00%
24	Non-alcohol	8	0	0	0	6	2	0	0.00%
	Alcohol	8	0	0	0	2	6	3	37.5%

Duolink PLA

The Duolink PLA in situ was used in this study to detect the interaction between LAMC2 and ITGB1 in tissue samples, and it was performed as previously described by Bai *et al*[43]. Briefly, after washing, permeabilizing, and blocking as histological analysis, colon sections were incubated with primary antibodies against LAMC2 (1:200) and ITGB1 (1:400) overnight at 4℃. Then the slides were incubated with Duolink PLA Rabbit MINUS and PLA Mouse PLUS proximity probes for 1 h at 37℃. Ligation and amplification were conducted using the Duolink in situ detection reagent kit according to the protocol. Images were captured under the light microscope (Olympus, BX51).

RNA extraction and quantitative real-time polymerase chain reaction

The total RNA was extracted from tissues using RNAEX reagent (Accurate Biotechnology, China). It
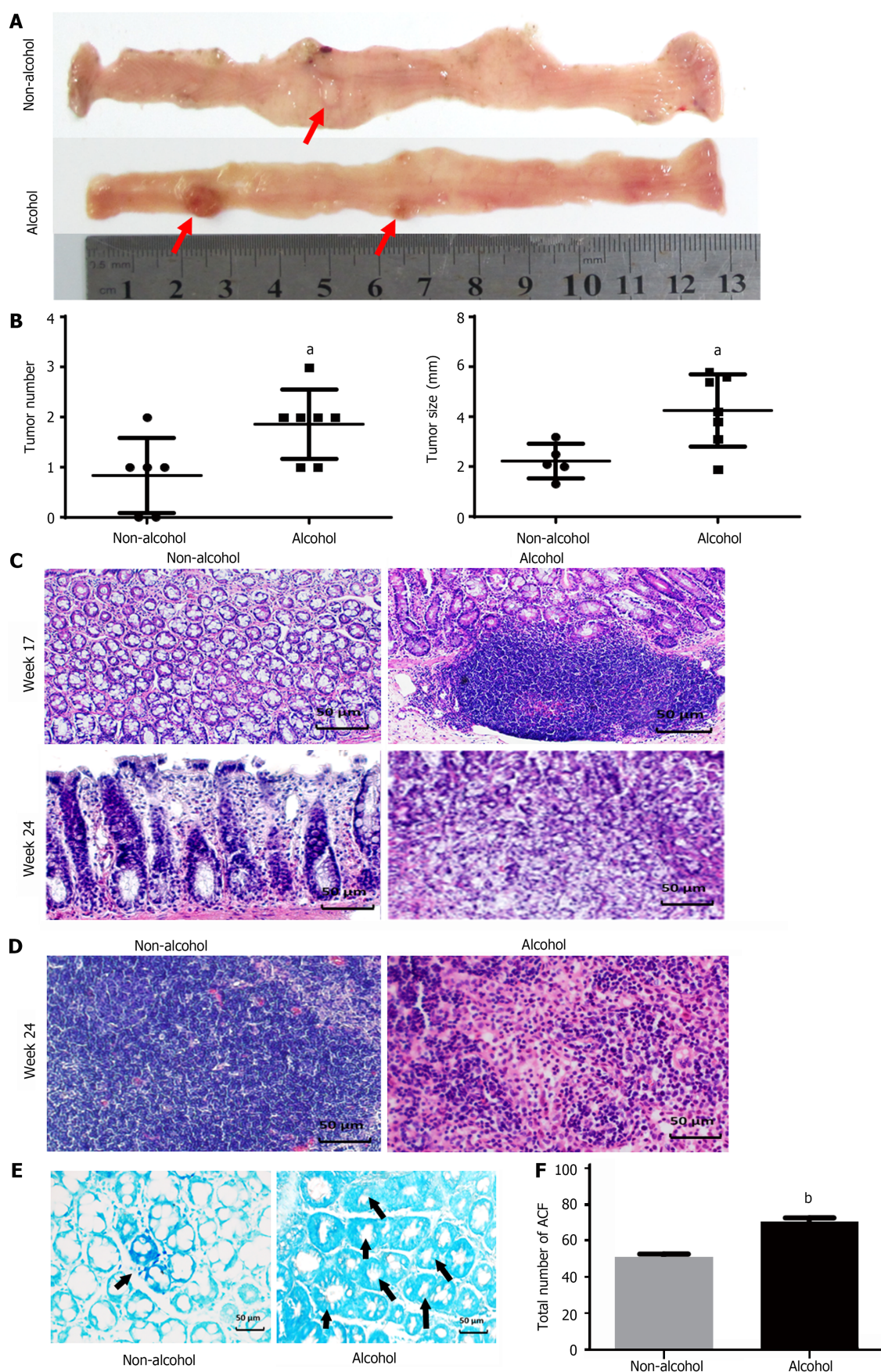
Figure 2 Alcohol consumption promotes colorectal cancer progression. A: Morphological view of colonic tumors; B: Average tumor number and tumor size in each group; C: Colon pathology of different groups (× 400); D: Pathology of colonic lymph node in different groups at the 24th week (× 400); E: Methylene blue staining for aberrant crypt foci (ACF) in the sectioned rat colon (× 400); F: Bar graph of ACF with different crypt multiplicity. The bars represent the mean ± SD (n = 8). Compared with the non-alcohol group: [b]$P < 0.01$, [a]$P < 0.05$. ACF: Aberrant crypt foci.

Table 3 Baseline characteristics of colorectal cancer patients			
Baseline characteristics	Alcohol group (*n* = 29)	Non-alcohol group (*n* = 34)	*P* value
Gender			
Male, *n* (%)	18 (62.1)	16 (47.1)	0.233
Female, *n* (%)	11 (37.9)	18 (52.9)	
Age (yr old)			
< 55, *n* (%)	10 (34.5)	14 (41.2)	0.586
≥ 55, *n* (%)	19 (65.5)	20 (58.8)	
Differentiation			
Medium, *n* (%)	24 (82.8)	30 (88.2)	0.536
Poor, *n* (%)	5 (17.2)	4 (11.8)	
Clinical stage			
Stage I-II, *n* (%)	12 (41.4)	24 (70.6)	0.020[a]
Stage III-IV, *n* (%)	17 (58.6)	10 (29.4)	
T stage			
T1-T2, *n* (%)	6 (20.7)	12 (35.3)	0.201
T3-T4, *n* (%)	23 (79.3)	22 (64.7)	
Tumor metastasis conditions			
Distant metastasis	12 (41.4)	23 (70.6)	0.046[a]
Lymphatic metastasis	9 (31)	8 (20.6)	
Non-metastasis	8 (27.6)	3 (8.8)	

[a]$P < 0.05$.

was reverse transcribed into cDNA by using Evo M-MLV RT premix (Accurate Biotechnology, China) and quantitative real-time polymerase chain reaction (RT-PCR) system (TaKaRa, Japan). Then a SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology, China) was used for PCR. The PCR liquid volume was 20 μL. Quantitative PCR was performed using an RT-PCR system (BioRad, Singapore). At 95℃, 40 cycles were amplified after 90 s of initial denaturation, 10 s desaturation at 95℃, and 34 s of extension at 60℃. GAPDH was used as the reference gene for calculation. And the primer sequences are shown in Table 1.

Statistical analysis

The data were described using the mean (standard deviation), median (range), or frequency (percentage). An independent-samples *t* test or the Mann-Whitney U test was applied for the comparison between two groups (non-alcohol group and alcohol group). The associations between the clinical parameters and immunohistochemical results were analyzed using the χ^2 test. Statistical analyses were conducted using SPSS version 25.0 for Windows (SPSS, Inc.). Statistical analysis was performed: [b]P < 0.01; [a]P < 0.05; [ns]P > 0.05.

RESULTS

Alcohol consumption promoted malignant progression in CRC rats

The total number of ACFs detected in the alcohol group was 70.33 ± 5.16 and 51.6 ± 4.5 in the non-alcohol group (Figure 2E and F), which implied that alcohol consumption may significantly increase considerably raise the risk of precancerous lesions of colorectal cancer ($P < 0.05$). Furthermore, CRC rats in alcohol group had more tumors and larger tumor size was observed in the alcohol group than those in the non-alcohol group (Figure 2A and B). Histopathological analysis revealed that at 17th week, 62.5% of rats in the non-alcohol group showed atypical hyperplasia, and the other 37.5% showed normal colonic tissues. Atypical hyperplasia was seen in 75% of the rats in the alcohol group; however, 25% of the rats in the alcohol group had many heteroepithelial cells in the colonic epithelium and one or more large nucleoli in the pleomorphic cystic nucleus, indicating a carcinoma adenoma. At the 24th week, 25%
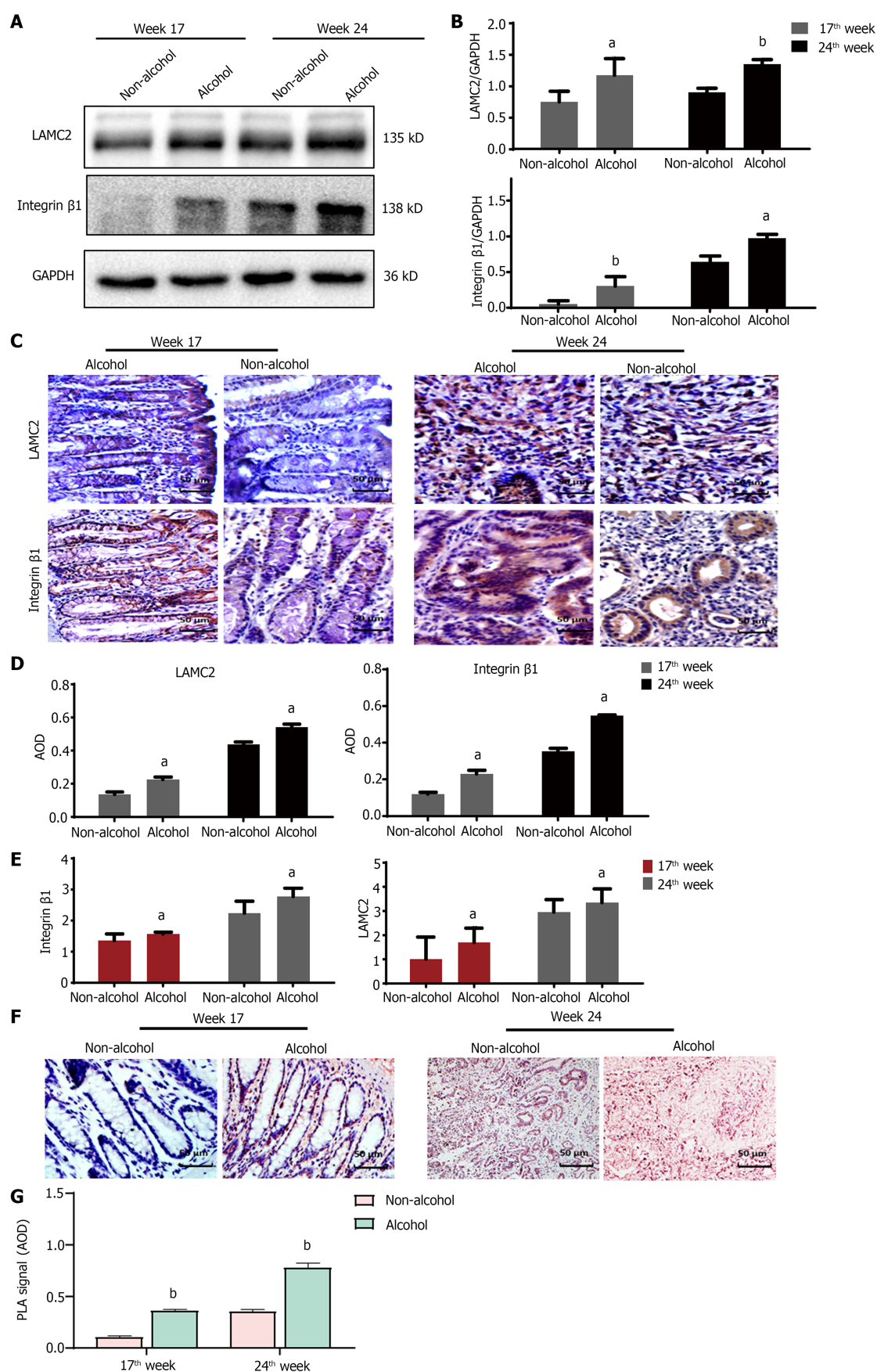
Figure 3 Alcohol consumption regulates the expression and interaction of integrin-β1 and laminin-γ2 in animal samples. The bars represent the mean ± SD (n = 8). A and B: Quantification of the protein levels of integrin-β1 (ITGB1) and laminin-γ2 (LAMC2) detected by western blotting in animal samples; C and D: Quantification of the protein levels of ITGB1 and LAMC2 by immunohistochemical (IHC) in animal samples; E: Quantification of the mRNA levels of ITGB1 and LAMC2 by quantitative real-time polymerase chain reaction in clinical samples; F: Statistical analysis of IHC was performed by counting the average optical density

(AOD). In Duolink proximity ligation assay (PLA), the brown dot particles observed under the microscope represent the interactions between LAMC2 and ITGB1; G: The statistical analysis of PLA positive signals was performed by counting the AOD under a light microscope. Compared with the non-alcohol group: [b]$P < 0.01$, [a]$P < 0.05$. ITGB1: Integrin-β1; LAMC2: Laminin-γ2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; PLA: Proximity ligation assay; IHC: Immunohistochemical staining; AOD: Average optical density.

of rats in the alcohol group showed intramucosal carcinoma (mucosal carcinoma), which was characterized by less mucus secretion of colonic epithelial cells, compact and irregular arrangement of glandular ducts, and clear invasion of cancer cells into lamina propria in colons. The other 75% of rats in the alcohol group had invasive mucosal infiltrating carcinoma (infiltrating carcinoma), which manifested as abnormal tubular or mucinous structures invading through the muscularis mucosa or deep intestinal wall. In total, 75% and 25% of rats in the non-alcohol group showed mucosal carcinoma and infiltrating carcinoma, respectively. We found no lymphatic metastasis in the non-alcohol group, while cancer cell infiltration was found in the lymph nodes of the alcohol group and the lymph node metastasis rate was 37.5% (Figure 2C and D; Table 2). The abovementioned results indicated that alcohol consumption promoted CRC metastasis.

Alcohol consumption increased the expression of LAMC2, ITGB1 and FAK in CRC rats

The protein levels of LAMC2 and ITGB1 measured by western blotting (Figure 3A and B) and IHC (Figure 3C and D). The result showed that at both of 17th week and 24th week, the protein levels of LAMC2 and ITGB1 in the alcohol group were higher than those in the non-alcohol group ($P < 0.05$). Furthermore, the mRNA levels of LAMC2 and ITGB1 in alcohol group were higher than those in non-alcohol group at both of 17th week and 24th week ($P < 0.05$; Figure 3E). In addition, the protein level of FAK in the alcohol group was higher than that in the non-alcohol group at the 17th and 24th weeks (Figures 4 and 5, $P < 0.05$). However, there was no significant difference in the mRNA level of FAK between the alcohol and non-alcohol group at both of the 17th and 24th week ($P > 0.05$; Figure 6A).

Alcohol consumption enhanced the early interaction between LAMC2 and ITGB1 in CRC rats

Duolink PLA were used to detect the interaction between ITGB1 and LAMC2, and the red spots represent the interactions between LAMC2 and ITGB1. The results revealed that (Figure 3F and G) at 17th week, more early interacting signals between LAMC2 and ITGB1 were found in the alcohol group than that in non-alcohol group. At week 24, the results were consistent with that of week 17. The present study suggested that alcohol consumption may enhance the early interaction between LAMC2 and ITGB1 in CRC rats.

Alcohol consumption increased the expression of the CRC metastasis marker SATB1 in CRC rats

High expression level of SATB1 significantly influenced the malignant phenotypic characteristics of CRC. As shown in Figures 4 and 5, the results of IHC and western blotting indicated that at 17th week, the protein level of SATB1 in the alcohol group was higher than that in non-alcohol group, and the results of qRT-PCR (Figure 6A) showed that the mRNA level of SATB1 was higher than that in non-alcohol group.

Alcohol consumption regulates the protein and mRNA levels of EMT-associated markers in CRC rats

EMT is one of the key events that occur during the formation of premetastatic niche[44]. The results of western blotting (Figure 4A) and IHC (Figure 5) suggested that at the 17th week, long-term alcohol consumption resulted in an increase in the proteins involved in triggering the EMT process including p-FAK, N-cadherin, fibronectin and snail; however, there was a decrease in E-cadherin in the alcohol group. At week 24, the results were similar to those of week 17. In addition, the mRNA levels of FAK, E-cadherin, N-cadherin, fibronectin and snail were measured by qRT-PCR, and the results were consistent with those of protein levels (Figure 6A). The abovementioned results indicated that alcohol consumption may abnormally change the expression levels of EMT biomarkers in CRC rats in early stage.

Alcohol consumption increased the expression of proinflammatory factors associated with premetastatic niche in CRC rats

The results of ELISA (Figure 6B) showed that IL-1β, IL-6 and TNF-α levels in the alcohol group were higher than those in the non-alcohol group at week 17. The results at week 24 were consistent with those at week 17, indicating that the concentrations of IL-1β, IL-6, and TNF-α were higher in the alcohol group than in the non-alcohol group during precancerous lesions, which may be one of the reasons for lymph node metastasis in CRC patients in the alcohol group.

The obtained results showed that in early stages of CRC, the contents of IL-6, IL-1β and TNF-α in the alcohol group were higher than those in the non-alcohol group. This might be one of the explanations for the higher metastatic rate in the alcohol group.

A

B
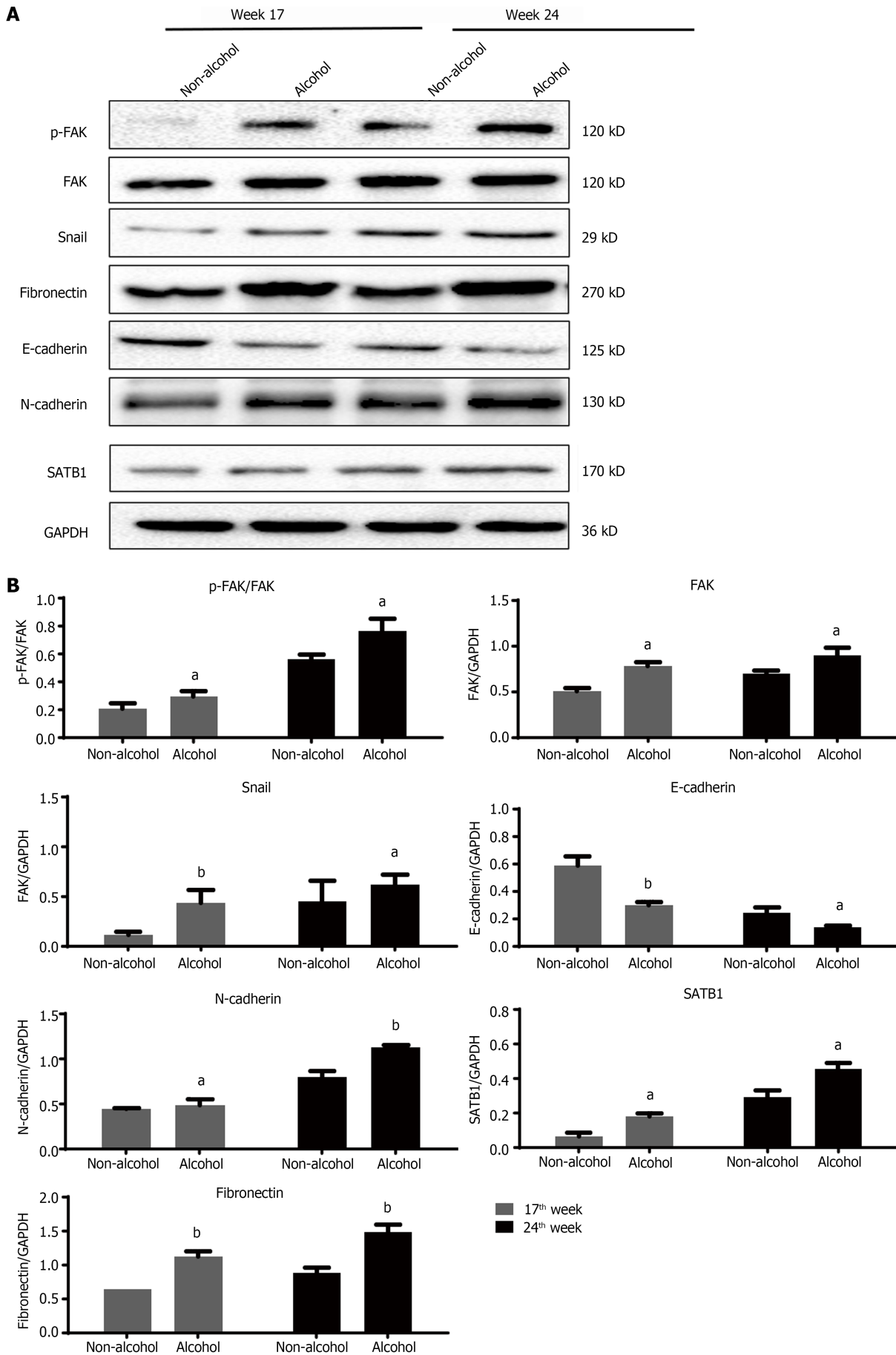
Figure 4 Quantification of the protein levels of epithelial mesenchymal transformation-related markers in colorectal cancer rats by using

western blotting. The bars represent the mean ± SD (*n* = 3). A: The western blotting images of phosphorylate-focal adhesion kinase (p-FAK), FAK, snail, fibronectin, E-cadherin, N-cadherin and special AT-rich sequence binding protein 1 (SATB1) in animal samples; B: Quantification of the protein levels of FAK, p-FAK/FAK, snail, fibronectin, E-cadherin, N-cadherin and SATB1 in animal samples. Compared with the non-alcohol group: [b]*P* < 0.01, [a]*P* < 0.05. SATB1: Special AT-rich sequence binding protein 1; FAK: Focal adhesion kinase; p-FAK: Phosphorylate focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
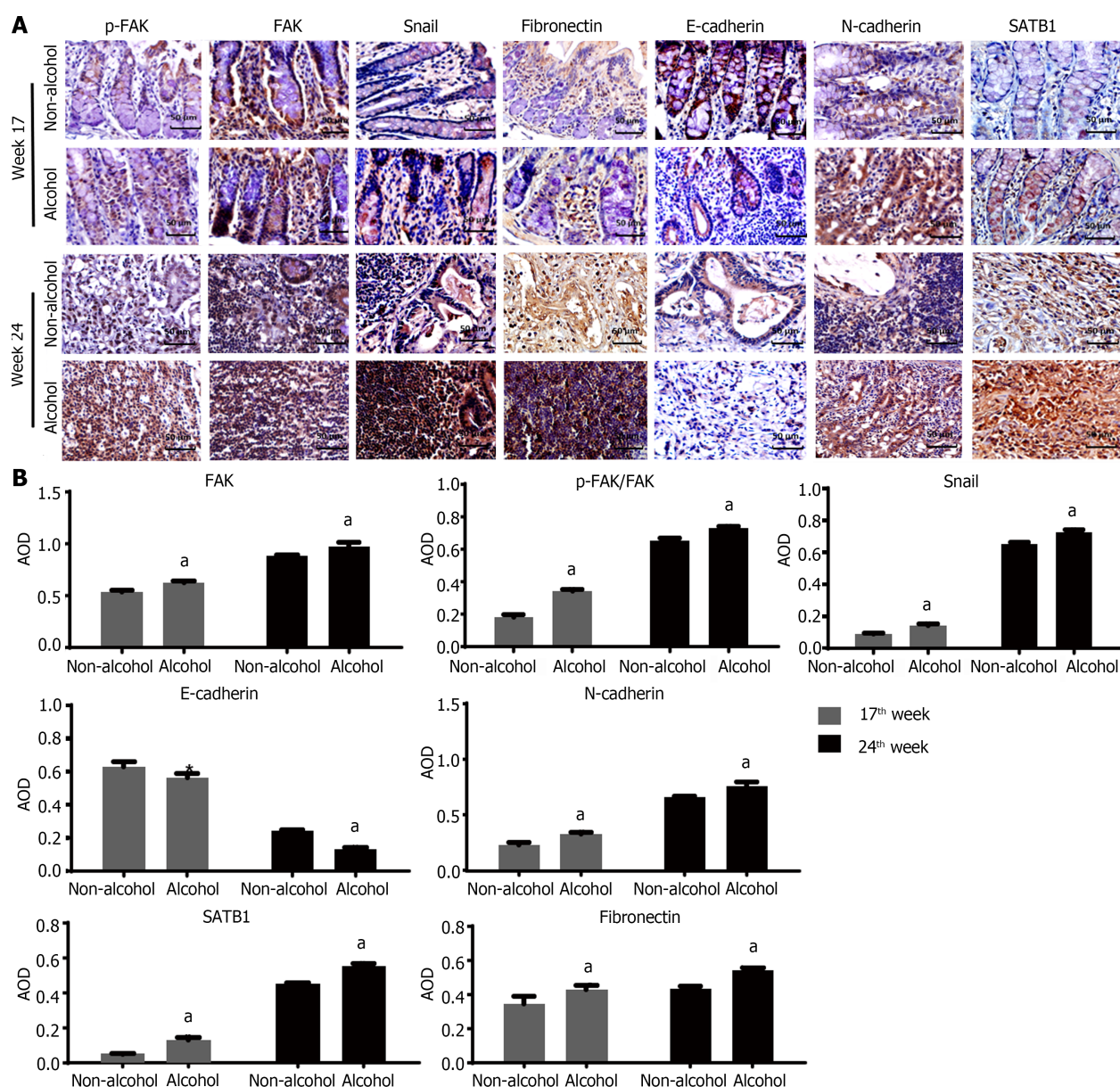
Figure 5 Immunohistochemical staining detected the protein level of epithelial mesenchymal transformation-related markers in colorectal cancer rats. The bars represent the mean ± SD (*n* = 3). A: The images of immunohistochemical (IHC) in animal samples; B: Quantification of the protein levels of focal adhesion kinase (FAK), phosphorylate-FAK (p-FAK)/FAK, snail, Fibronectin, E-cadherin, N-cadherin and special AT-rich sequence binding protein 1 detected by IHC counting the average optical density. Compared with the non-alcohol group: [b]*P* < 0.01, [a]*P* < 0.05. SATB1: Special AT-rich sequence binding protein 1; FAK: Focal adhesion kinase; p-FAK: Phosphorylate focal adhesion kinase; qRT-PCR: Quantitative real-time polymerase chain reaction; IHC: Immunohistochemical staining; AOD: Average optical density.

Baseline characteristics of CRC patients

A total of 63 patients with a preliminary diagnosis of colorectal cancer participated in the trial. According to the baseline characteristics and data on alcohol consumption of 63 CRC patients, 29 CRC patients who had been drinking alcohol over 3 times each week for more than 5 years were classified in alcohol group, while the remaining 34 CRC patients who have never drunk alcohol before were classified in non-alcohol group. As shown in Table 3, there are significant differences in clinical stage and tumor metastasis (*P* < 0.05) between alcohol group and non-alcohol group. There were no significant differences in sex, age, degree of differentiation or T stage (*P* > 0.05). The experimental
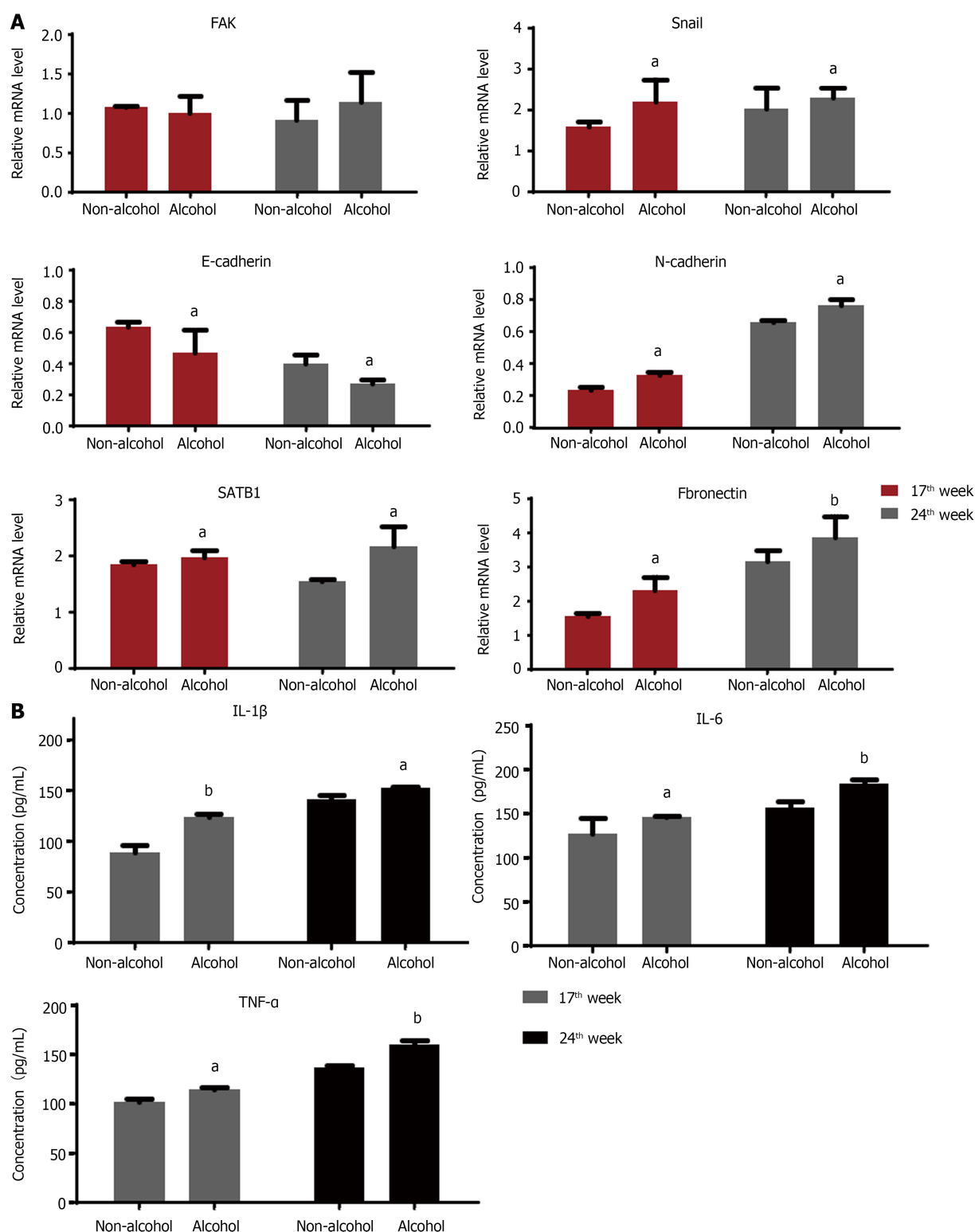
Figure 6 Alcohol consumption regulated the mRNA expression of epithelial mesenchymal transformation-related markers and proinflammatory factors in colorectal cancer rats. The bars represent the mean ± SD (n = 8). A: Quantification of the mRNA levels of snail, focal adhesion kinase, fibronectin, N-cadherin, E-cadherin and special AT-rich sequence binding protein 1 in each group analyzed by quantitative real-time polymerase chain reaction; B: Tumor necrosis factor-α, interleukin-6 (IL-6) and IL-1β levels in each group. Compared with the non-alcohol group: [b]P < 0.01, [a]P < 0.05. SATB1: Special AT-rich sequence binding protein 1; FAK: Focal adhesion kinase; IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α.

results were consistent with the results of animal experiments. The degree of tumor deterioration in CRC patients in the alcohol group was significantly higher than that in non-alcohol group.

Alcohol consumption increased the expression and interaction of LAMC2 and ITGB1 in CRC patients

The expression of LAMC2 and ITGB1 in clinical samples determined by IHC (shown in Figure 7C and D). In cancer tissues, the mRNA and protein levels of LAMC2 and ITGB1 in alcohol group were significantly higher than those in non-alcohol group (Figure 7B). In addition, according to the survival analysis of 392 CRC patient samples from the GSE39582 dataset, we explored the prognostic significance of LAMC2 and ITGB1. As shown in Figure 7A, the overexpression of LAMC2 and ITGB1 was closely related to poor CRC prognosis. The abovementioned results were consistent with the results of animal experiments. Alcohol consumption may increase the expression levels and the interaction of LAMC2 and ITGB1 in CRC patients.

The interactions of LAMC2-ITGB1 are pivotal in CRC progression. Animal experiments revealed that more interactions between LAMC2 and ITGB1 were found in the alcohol group than in the non-alcohol group in the early stage of CRC. And it has been validated in clinical samples. According to the results of duolink PLA (Figure 7E and F), more interacting signals between LAMC2 and ITGB1 were found in alcohol group than those in non-alcohol group.

Alcohol consumption influenced the protein and mRNA expression of EMT-associated markers in CRC patients

In the animal experiment, we found that alcohol may abnormally change the expression levels of EMT-associated biomarkers in CRC rats in the early stage. To verify the clinical implications of this finding, the levels of EMT biomarkers were detected in clinical samples. The results of IHC (Figure 8A and B) showed that there were significant differences in EMT-associated biomarkers ($P < 0.01$) in cancer tissues between the alcohol and non-alcohol group. In cancer tissues, the protein levels of p-FAK, N-cadherin, fibronectin and snail were significantly higher in alcohol group was significantly higher than that in non-alcohol group; however, the expression of E-cadherin in alcohol group was significantly lower in alcohol group than that in non-alcohol group. The qRT-PCR results (Figure 8C) also showed that the mRNA levels of N-cadherin, fibronectin and snail in the cancer tissues of alcohol group was higher than that in non-alcohol group. The abovementioned findings were consistent with the results of animal experiments. The abnormal expression of EMT biomarkers in CRC patients was different from that in alcohol group, and the abnormal expression of LAMC2 and ITGB1 was more severe in alcohol group.
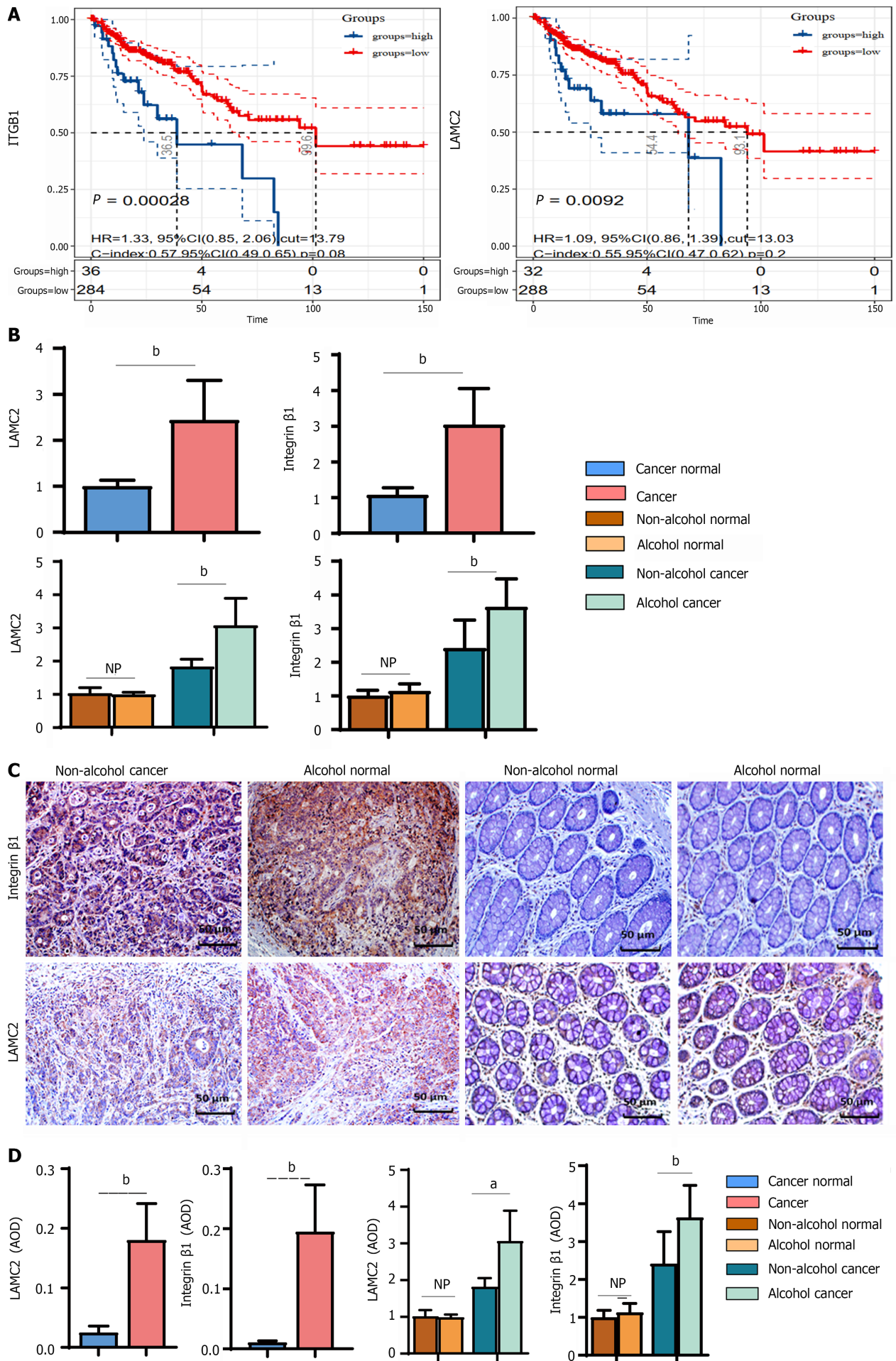
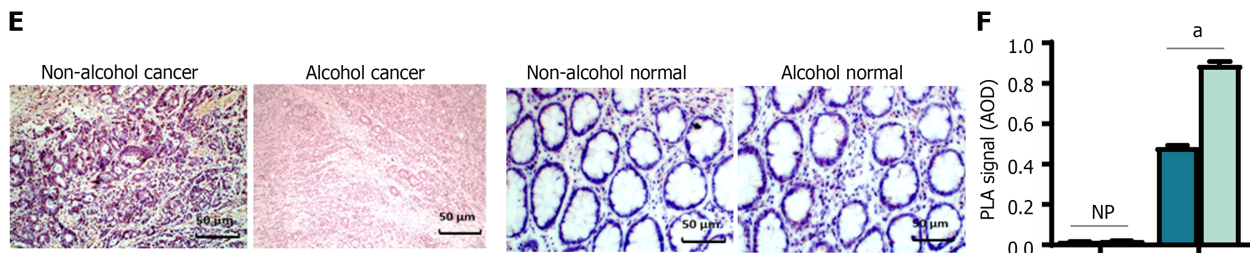### Alcohol consumption increased the expression of the CRC metastasis marker SATB1 in CRC patients

In animal experiments, alcohol consumption was found to increase the expression of SATB1 in CRC rats. IHC and qRT-PCR were used to evaluate the protein and mRNA levels of SATB1 in clinical samples (Figure 8A-C). The obtained results showed that protein and mRNA levels of SATB1 in cancer tissues were significantly higher in alcohol group than that in non-Alcohol group. Our results indicate that the expression of SATB1 is higher in alcohol group than those in non-Alcohol group, which is consistent with the higher metastasis rate of CRC patients who drink alcohol as described by TNM staging. The above results suggest that alcohol consumption may up-regulate the expression of SATB1, thereby induce the early EMT event and leads to malignant metastasis of the tumor[45].

DISCUSSION

Metastasis is the prominent cause of death in CRC[7]. Chronic alcohol intake has been extensively reported to enhance CRC metastasis; nevertheless, the specific mechanism remains unknown. In the present study, the results of TNM stage, pathological grade and other clinicopathological parameters suggested that the tumor deterioration rate of patients in alcohol group was higher than that in non-alcohol group, and the results were consistent with previous reports[6,7]. The present study demonstrated that the metastasis rate in alcohol group was higher than that in non-alcohol group. Similar to the clinical results, in animal experiments, the number of ACFs in the alcohol group was significantly higher than that in the non-alcohol group, and the pathological evaluation results were similar to the ACF screening results, suggesting that the alcohol group had a higher risk of precolonic lesions in CRC rats. And the number and size of tumors in the alcohol group were higher than those in the non-alcohol group. Lymph node metastasis was found in 37.5% of patients in the alcohol group, while no metastasis was found in the non-alcohol group. The present study indicated a higher risk of precancerous lesions and malignant metastasis of CRC were found in alcohol group compared with the non-alcohol group. Thus, we hypothesized that alcohol may promote the metastasis of CRC, however, the molecular mechanism is still unclear.

A key step in the formation of metastasis is the extravasation of circulating tumor cells to distant organs and their adaptation to the new environment[46,47]. Thus, the interaction between disseminated cancer cells and the microenvironment of the premetastatic niche is necessary for metastasis[10]. Primary tumor cells regulate the metastasis by secreting a variety of molecules that promote the mobilization and recruitment of various cells to the premetastatic niche and alter ECM protein expression and ECM properties in secondary organs[25,48]. The ECM constitutes a scaffold that

Figure 7 Alcohol consumption increased the expression and interaction of laminin-γ2 and integrin-β1 in colorectal cancer rats. The bars represent the mean ± SD (*n* = 8). A: Survival analysis of integrin-β1 (ITGB1) and laminin-γ2 (LAMC2) in colorectal cancer; B: The mRNA levels of ITGB1 and LAMC2 determined by quantitative real-time polymerase chain reaction in clinical samples; C: Representative immunohistochemical (IHC) images of ITGB1 and LAMC2 in clinical samples (× 400); D: The quantifications of the protein levels of ITGB1 and LAMC2 determined by IHC in clinical samples; E and F: Duolink proximity ligation assay (PLA) verified the interactions between ITGB1 and LAMC2 in clinical samples. The statistical analysis of PLA positive signals was performed by counting the average optical density under a light microscope. Compared with the non-alcohol group: [b]$P < 0.01$, [a]$P < 0.05$. LAMC2: Laminin-γ2; ITGB1: Integrin-β1; qRT-PCR: Quantitative real-time polymerase chain reaction; PLA: Proximity ligation assay; IHC: Immunohistochemical staining; AOD: Average optical density. Cancer normal group: cancer adjacent tissue group. NP: No statistical difference.

supports the attachment and thus reactivation of survival signaling in cancer cells[16]. And the remodeling of ECM is essential during formation of premetastatic niche, which may support cancer cell attachment, metabolism and migration of recruited cells[17,18]. Laminin, as an important component of the ECM and the main structural component of basement membrane, is involved in the formation of pre-metastasis niche[49]. Among them, LAMC2 is an important component of the survival-dependent environment of colorectal cancer epithelial cells and plays a key role in the regulation of tumor cell motility by premetastatic niche formation[50]. LAMC2 plays a role in premetastatic niche signaling by stimulating the formation of premetastatic local contacts and promoting the migration of cancer cells through its interaction with ITGB1[51]. It has been reported that the interaction of ITGB1 and LAMC2 may promote the tumor budding of CRC cells, which leads to malignant metastasis of CRC, and the focal adhesion formed by the interaction of LAMC2 and ITGB1 plays an important role in the prognosis of CRC. This adhesion is not only the basis of cancer cell survival but is also closely related to the migration and metastasis of cancer cells[52]. FAK is involved in LAMC2 and ITGB1 signal transduction, and the overexpression of FAK was found to play a key regulatory role in ITGB1-mediated signal transduction. As the phosphorylated FAK is activated, it transmits signals to the transcription factor snail, thereby inducing EMT[32,33]. The interaction of LAMC2 and ITGB1 and the overexpression of FAK were seen as markers of premetastatic niche formation, which may lead to malignant metastasis of CRC[52]. According to the results of our study, more interacting signals between LAMC2 and ITGB1, and higher level of FAK were found in the alcohol group than those in the non-alcohol group at early stage. In addition, the higher ratio of p-FAK/FAK in the alcohol group in the early stage indicates a higher degree of FAK activation, which may induce early EMT events and promote CRC metastasis.

CRC metastasis depends on EMT, during EMT, epithelial cells lose the connectivity and top-to-base polarity, resulting in a mesenchymal phenotype capability of migration and invasion[32]. There is evidence that some cancer cells may acquire the ability to spread and metastasize before they are fully malignant, which suggests that EMT may be an early event involving in the metastasis[53]. In addition, SATB1 has been found to be associated with early EMT events and can be used as a marker of CRC metastasis[54]. SATB1 was found to be a driver of the malignant phenotype in CRC, and its expression was shown to be positively associated with invasion depth, poor degree of differentiation and advanced TNM stage of CRC. And SATB1 organizes chromatin into spatial rings that provide the necessary "docking sites" for further binding of transcription factors and chromatin modifying enzymes[45]. SATB1 was found to regulate entire genes, even those located on distant chromosomes[55]. The results of the animal experiment suggested that the expression of SATB1 was significantly higher in the alcohol group than that in the non-alcohol group during precancerous lesions, which may predict the early EMT events.

In addition, there is an increasing evidence that proinflammatory cytokines play an important role in the formation of premetastatic niches. Previous studies have shown that the high expression of IL-6 and IL-1β may activate ITGB1[56,57] to stimulate the adhesion of bone marrow cells to endothelial cells, thereby promote the formation of premetastatic niche, and inducing EMT to promote metastasis[58]. Pal *et al*[55] found that TNF-α induces EMT in human HCT116 cells and promotes colorectal cancer invasion and metastasis. According to our study, the levels of the proinflammatory cytokines IL-1β, TNF-α and IL-6 in CRC rats in the alcohol group were significantly increased at the early stage of precancerous lesions, which may serve as paracrine signals to enhance the proliferation and invasion of early disseminated cancer cells. It may help cancer cells change their phenotypes through EMT to enhance metastasis.
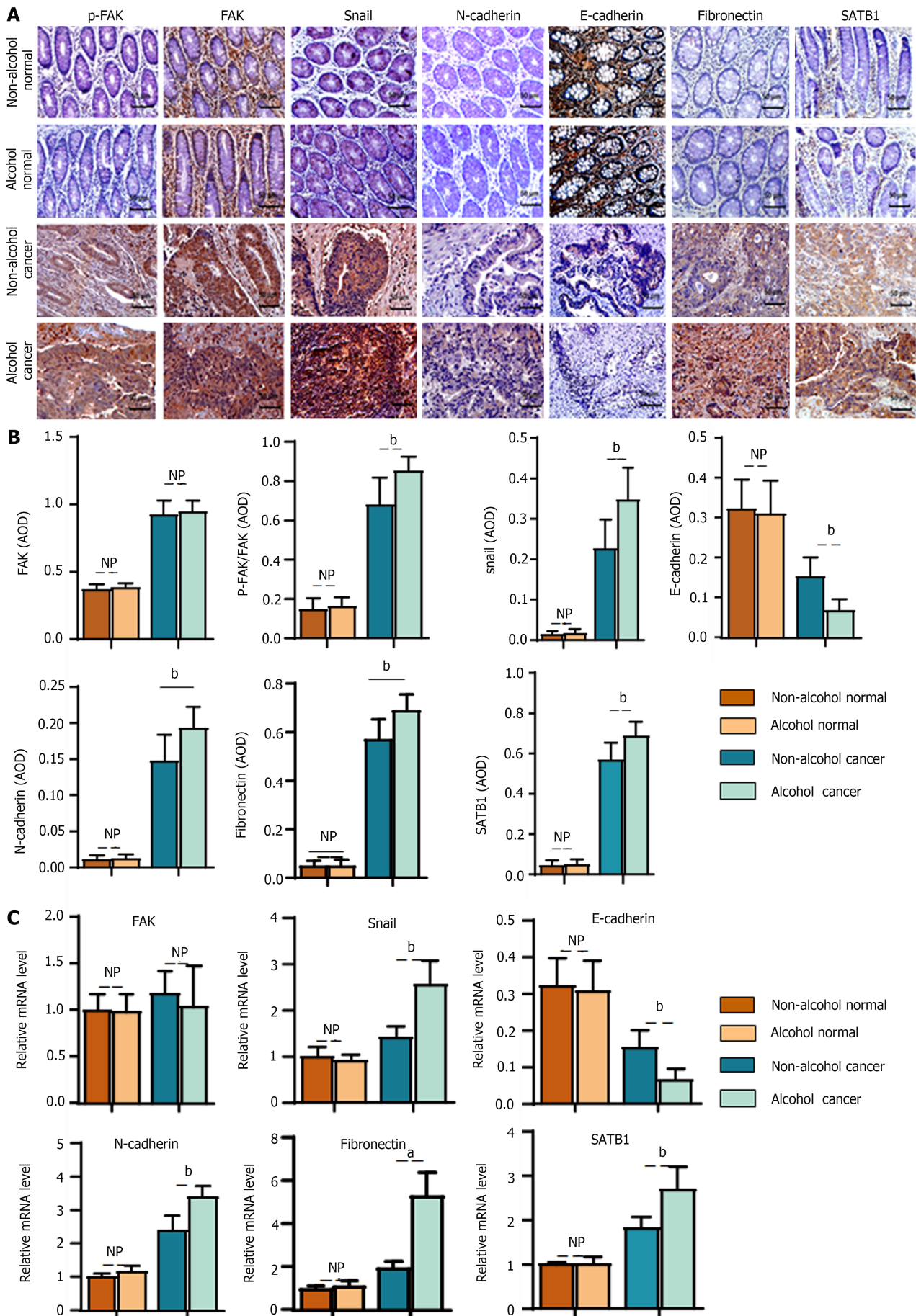
Figure 8 Alcohol consumption influenced the protein and mRNA levels of epithelial mesenchymal transformation -associated markers in

colorectal cancer patients. The bars represent the mean ± SD (n = 8). A: Representative immunohistochemical images of phosphorylate-focal adhesion kinase (p-FAK), FAK, snail, fibronectin, E-cadherin, N-cadherin and special AT-rich sequence binding protein 1 (SATB1) in different groups in clinical samples (× 400); B: Quantification of the protein levels of FAK, p-FAK/FAK, snail, fibronectin, E-cadherin, N-cadherin and SATB1 by counting the average optical density in clinical samples; C: Quantification of the mRNA levels of FAK, snail, fibronectin, E-cadherin, N-cadherin and SATB1 by quantitative real-time polymerase chain reaction in clinical samples. Compared with the non-alcohol group: [b]P < 0.01, [a]P < 0.05. SATB1: Special AT-rich sequence binding protein 1; FAK: Focal adhesion kinase; p-FAK: Phosphorylate focal adhesion kinase; AOD: Average optical density. NP: No statistical difference.
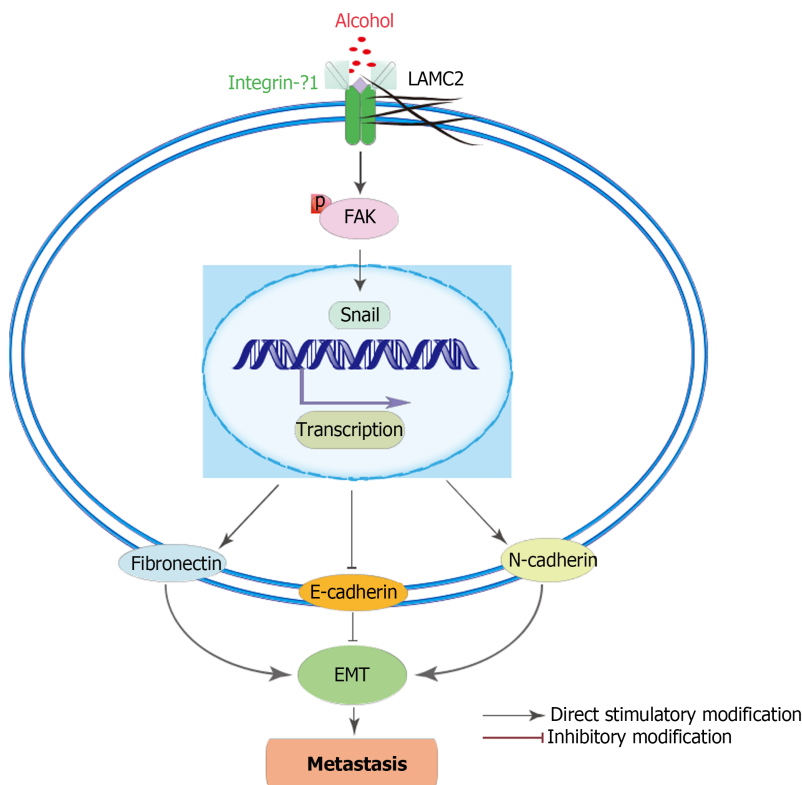
Figure 9 Schematic diagram of the possible signaling pathway regulated by alcohol. EMT: Epithelial mesenchymal transformation; LAMC2: Laminin-γ2; ITGB1: Integrin-β1; FAK: Focal adhesion kinase; p-FAK: Phosphorylate focal adhesion kinase.

The EMT event is also a key event involving in the formation of the premetastatic niche[32,33], which is almost featuring the loss of E-cadherin expression and overexpression of snail, N-cadherin and fibronectin[56,59]. The results of animal experiments suggested that long-term alcohol consumption (shown in Figure 9), as a risk factor of CRC, may further activate p-FAK by enhancing the interaction between LAMC2 and ITGB1 and forming a premetastatic niche, thus upregulating the expression of snail in precancerous lesions. Thus, the expression levels of N-cadherin and fibronectin were increased and the expression level of E-cadherin was downregulated, leading to the mesenchymal phenotype of cancer cells and colorectal cancer metastasis. This may be one of the reasons for the higher metastasis rate of CRC rats in the alcohol group. We found the similar results in clinical samples. The main limitation of the clinical study is the relatively small sample size. Larger-scale functional studies are required to improve our understanding of the effects of alcohol on the premetastatic niche in CRC. However, the obtained results provide some interesting suggestions for future studies with an increased sample size. Based on the abovementioned results, the present study demonstrated that alcohol may promote the formation of premetastatic niche *via* enhancing the EMT event mediated by the LAMC2-ITGB1 interaction, leading to the high incidence of CRC metastasis in the alcohol group.

CONCLUSION

In summary, the present study attempted to examine the nonspecific effect of alcohol on the premetastatic niche of colorectal cancer. Our study suggests that alcohol has an effect on the colorectal carcinogenesis paradigm and may accelerate the malignant metastasis of CRC. The molecular mechanism may involve the effect of alcohol on the early EMT-mediated premetastatic niche of colorectal cancer induced by LAMC2 and ITGB1 interactions.

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ARTICLE HIGHLIGHTS

Research background
Colorectal cancer (CRC) is a common malignant tumor.

Research motivation
Alcohol consumption is positively correlated with CRC malignant metastasis; however, the mechanism is unclear.

Research objectives
To investigate the effects of alcohol on CRC metastasis

Research methods
The interaction between laminin-γ2 (LAMC2) and integrin-β1 (ITGB1) was measured by Duolink assay, and the expression levels of LAMC2, ITGB1 and focal adhesion kinase, snail, fibronectin, N-cadherin and special AT-rich sequence binding protein 1 were measured by quantitative real-time polymerase chain reaction, immunohistochemistry and western blotting. Interleukin-1β (IL-1β), tumor necrosis factor-α and IL-6 levels were measured *via* enzyme-linked immunosorbent assay, histopathological assessment *via* hematoxylin eosin staining, and determination of aberrant crypt foci *via* methylene blue.

Research results
Alcohol may promote CRC metastasis by influencing the molecular mechanism of the premetastatic niche.

Research conclusions
Our study suggests that alcohol promotes epithelial mesenchymal transformation-mediated premetastatic niche formation of CRC by activating the early interaction between LAMC2 and ITGB1 and lead to CRC metastasis.

Research perspectives
To explore the effect of alcohol on colorectal cancer metastasis.

FOOTNOTES

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