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ORIGINAL ARTICLE

Smoking induces the occurrence of colorectal cancer *via* changing the intestinal permeability

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Summary

Purpose: *Colorectal cancer (CRC) is the third most frequent cancer. Its occurrence is closely linked to lifestyle and diet habits, such as excessive intake of high-fat food, but their impact on CRC, however, remain unclear.*

Methods: *Eligible CRC patients were retrospectively analyzed. Overall survival (OS) and recurrence-free survival (RFS) in smokers and non-smokers of CRC patients were assessed. APC$^{min/+}$ mice were exposed to cigarette smoking, followed by detection of CRC growth and intestinal permeability.*

Results: *A total of 416 eligible CRC patients were recruited, involving 218 (52.4%) smokers and 198 (47.6%) non-smokers. OS was shorter in CRC smokers than in non-smokers (p=0.005), whereas smoking did not affect RFS in CRC patients (p=0.251). Cigarette smoking increased CRC tumor numbers of CRC in APC$^{min/+}$ mice. Proliferation and apoptosis of colorectal epithelial cells, and inflammatory response in mice were changed following smoking. Notably, the treatment of probiotics mixture VSL#3 decreased the number of CRC tissues and intestinal permeability in APC$^{min/+}$ mice exposed to cigarette smoking.*

Conclusions: *Smoking increases the susceptibility to CRC through damaging the intestinal permeability. Protecting the intestinal permeability significantly protects intestinal tracts.*

Key words: *CRC, smoking, intestinal permeability*

Introduction

Colorectal cancer (CRC) is the third most frequent tumor and the most-common cause of cancer death [1]. The occurrence of CRC is initiated by both internal and external stimuli [2]. Major changes have been made in the way we live. Nowadays, we are increasingly dependent on processed food, which is a high risk of CRC [3]. In a mouse model, high-fat diet is verified to trigger CRC [4]. In addition, a previous study has demonstrated the close relationship between alcohol drinking and CRC risk [5]. Smoking is identified to be a risk factor of CRC, and its pathogenic mechanism is unclear [6].

A great number of CRC-associated genes have been discovered with in-depth research. Mutation of APC is detected in over 90% of CRC tissues [7]. Moreover, familial adenomatous polyposis is directly resulting from APC mutation [8]. Later, it was found out that besides gene mutations, intestinal factors are also responsible for tumorigenesis. In recent years, the role of intestinal flora has been highlighted. Wong et al [9] suggested that detection of faecal Fusobacterium abundance contributes to improve the diagnostic efficacy of CRC. It was gradually recognized that the intes-

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tinal permeability is of significance in the occurrence of CRC. It is reported that the expression of tight junction-associated protein (TJP) is reduced in genetically mutated colorectal cancer epithelial cells, leading to dilated gap between cells. As a result, small molecules in the intestine can easily pass through epithelial cells and trigger immune response [10,11]. Inflammatory response following CRC also influences the intestinal microenvironment [12]. We believed that protecting the intestinal permeability is beneficial to decrease the incidence of CRC.

In this paper, we first analyzed survival in smokers and non-smokers CRC patients. Subsequently, the role of smoking in influencing pathological changes in CRC mice was mainly elucidated.

Methods

Subjects

Patients with medical history of drug treatment or chemotherapy for CRC, non-primary CRC cases and those with shorter than 5-year smoking history were excluded. Finally, 416 eligible CRC patients were recruited, involving 218 (52.4%) smokers and 198 (47.6%) non-smokers. This study was approved by the Ethics Committee of our hospital and complied with the Helsinki Declaration.

Animal experiment

Age- and gender-matched $APC^{min/+}$ or wild-type (WT) mice of four weeks old were habitated in a standard environment. They were daily exposed to the smoke of 5 cigarettes (3R4F research cigarettes; The Tobacco Research Institute, Lexington, Kentucky, USA) for 4, 8 or 12 weeks. Mice in the non-smoke group were exposed to ambient air. For detecting intestinal permeability, mice were exposed to cigarette smoking for 20 days. For therapeutic experiment, 15 mg probiotic mixture VSL#3 (Grifols, Barcelona, Spain) was dissolved in 200 μL of phosphate-buffered saline (PBS) and administered in each mouse by oral gavage. Controls were administered with the same volume of PBS. WT mice were administered with 0.4 mg/mL aspirin dissolved in drinking water for 20 days. The animal experiment was approved by the Animal Ethics Committee of our hospital.

Hematoxylin-eosin (H&E) staining

CRC tissues collected from mice were fixed in 4% formalin for 24 h and subjected to H&E staining. Pathological lesions were evaluated by two pathologists independently.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA in tissues was extracted using TRIzol Reagent (Molecular Research Center Inc., Eugene, OR, USA). Complementary DNA (cDNA) was obtained from reverse transcription of total RNA using Transcriptor Reverse Transcriptase (Roche,Basel, Switzerland). RT-PCR was carried out using SYBR Green master mixture (Roche,Basel, Switzerland) on LightCycler 480 Instrument. Relative level of RNF6 was calculated by $2^{-\Delta\Delta Ct}$ method and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers sequence (5'-3'):IL-6: Forward: TCTATACCACTTCACAAGTCGGA, Reverse: GAATTGCCATTGCACAACTCTTT; IL-1β: Forward: TTCTTCGACACATGGGATAACG, Reverse: TGGAGAACACCACTTGTTGCT; Cox2:Forward: AAAAGCTGGGAAGCCTTCTC, Reverse: AAGTGCTGGGCAAAGAATGC; ZO-1:Forward: GCCGCTAAGAGCACAGCAA, Reverse: GCCCTCCTTTTAACACATCAGA; JAM-c:Forward: CTGCCTGACTTCTTCCTGCT, Reverse: ATGTACCACTGGGTTTCGGT; Occludin:Forward: TTGAAAGTCCACCTCCTTACAGA, Reverse: CCGGATAAAAAGAGTACGCTGG.

Western blot

Tissues were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses of grey values were finally conducted [11].

Ki-67 staining

Paraffin-embedded sections of mice colorectal tissues were stained with anti-Ki-67 antibody (ab833, Abcam,Cambridge, MA, USA). Ki-67-positive cells were calculated.

Intestinal permeability analysis

Mice were starved overnight and administered with 44 mg/kg FITC-dextran (Sigma-Aldrich, St. Louis, MO, USA) by oral gavage. Four h later, mice were sacrificed

Table 1. Clinical characteristics

Variables	*Number of patients (%)*
Age, years	
≥60.5	254 (61.0)
<60.5	162 (39.0)
Gender	
Male	268 (64.4)
Female	148 (35.6)
Tumor site	
Colon	221 (53.1)
Rectum	195 (46.9)
Differentiation	
High and moderate	277 (66.6)
Low	139 (33.4)
TNM stage	
I+II	235 (56.4)
III+IV	181 (43.6)
Smoking	
Yes	218 (52.4)
No	198 (47.6)

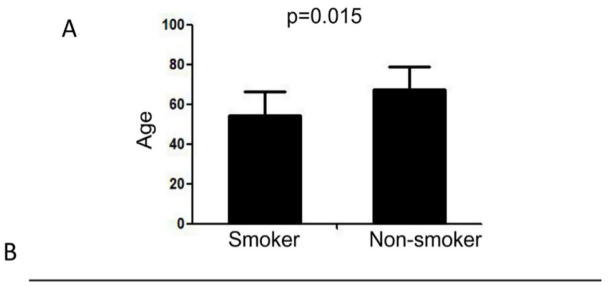
for collecting serum samples. Diluted serum in PBS was subjected to intestinal permeability by spectrophoto fluorometry (BioTek, Winooski, VT, USA) [13].

TdT-mediated dUTP Nick-End Labeling (TUNEL) staining

Paraffin-embedded sections of mice colorectal tissues were stained with TUNEL. TUNEL-positive cells were assessed by the DeadEnd Colorimetric TUNEL System (Promega,Madison, WI, USA).

Statistics

Data were expressed as means ± standard error of the mean (SEM) and analyzed by SPSS 22.0 software (IBM, Armonk, NY, USA) or GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA, USA). Kaplan-Meier method was used for analyzing overall survival (OS) and recurrence-free survival (RFS), followed by log-rank test for comparing differences between curves. Baseline characteristics in recruited smokers and non-smokers CRC patients were compared by x^2 test. Differences between groups were processed by the Student's *t*-test. Significant difference was set at $p<0.05$.



	I+II	III+IV	P-value
Smoker	125/218(57.3%)	93/218(42.1)	0.332
Non-smoker	110/198 (55.6%)	98/198 (44.4%)	

Figure 1. Smoking influenced the occurrence, rather than the progression of CRC. **A:** Age in smokers and non-smokers CRC patients. **B:** The number of smokers and non-smokers in stage I+II and stage III+IV CRC patients.

Results

Smoking influenced the occurrence, rather than the progression of CRC

Clinical data of 416 eligible CRC patients treated in our center from 2005 and 2010 were recorded and retrospectively analyzed (Table 1). Among them, 218 (52.4%) were smokers and 198 (47.6%) non-smokers. Smokers in CRC patients were significantly younger than non-smokers (54.3±12.1 years *vs.* 67.5±11.5 years, p=0.015) (Figure 1A). In smokers, there were 125/218 (57.3%) stage I+II cases and 93/218 (42.7%) stage III+IV cases. In non-smokers, 110/198 (55.6%) CRC patients had stage I+II and 98/198 (44.4%) III+IV (p=0.332) (Figure 1B). These results imply that smoking could influence the occurrence of CRC, rather than its progression.

Smoking influenced the survival in CRC patients

By analyzing 5-year follow-up data, it was found that OS was worse in smokers of CRC patients than that in non-smokers (p=0.002) (Figure 2A). Nevertheless, RFS in CRC patients was not affected by smoking (p=0.126) (Figure 2B).

Smoking increased tumor numbers in $APC^{Min/+}$ mice

Subsequently, we constructed a cigarette smoking model in $APC^{Min/+}$ mice. The number of CRC tissues in $APC^{Min/+}$ mice was much higher in smoke group compared with that of non-smoke group (p=0.003) (Figure 3A). However, we did not find significant difference in tumor staging between groups, which was consistent with the findings in clinical subjects (p=0.572) (Figure 3B). Worse survival was observed in smoking $APC^{Min/+}$ mice than that of non-smokers (p=0.004) (Figure 3C). To compare tumor incidence between smoke group and non-smoke group, mice were sacrificed at 4 weeks, 8 weeks, and 12 weeks, respective-

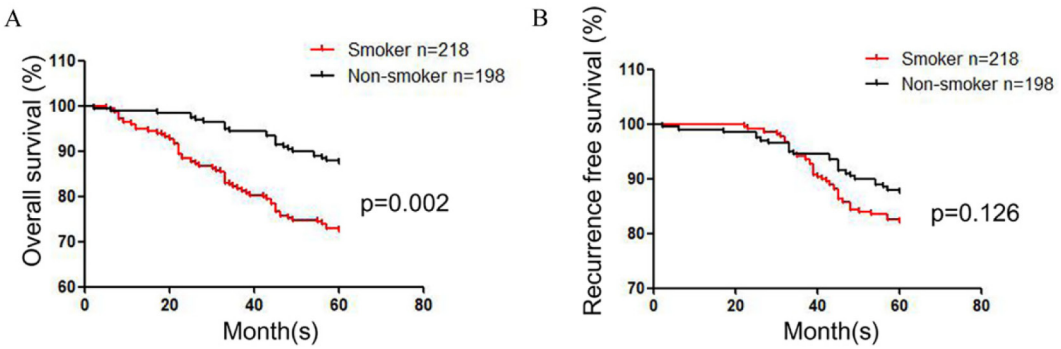
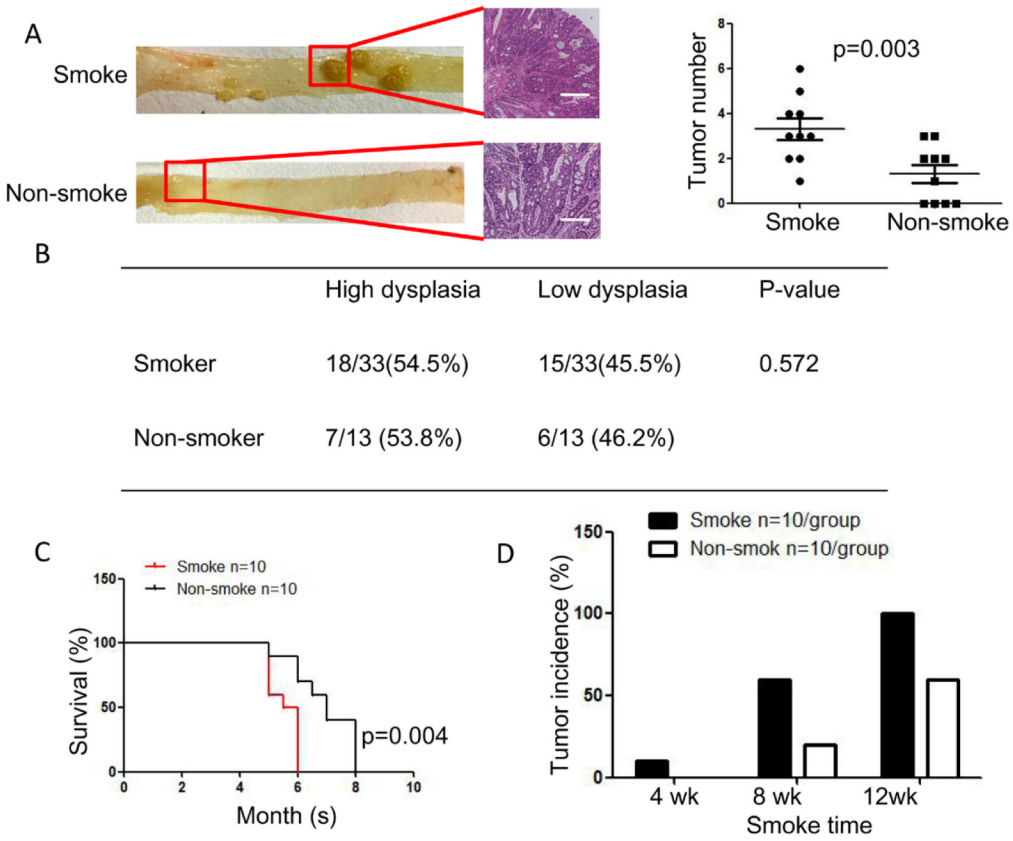


Figure 2. Smoking influenced the survival in CRC patients. **A,B:** Overall survival and recurrence-free survival in smokers and non-smokers CRC patients.



	High dysplasia	Low dysplasia	P-value
Smoker	18/33(54.5%)	15/33(45.5%)	0.572
Non-smoker	7/13 (53.8%)	6/13 (46.2%)	

Figure 3. Smoking increased tumor numbers in APC$^{Min/+}$ mice. **A:** Tumor number of CRC in smoking and non-smoking APC$^{Min/+}$ mice (magnification 400×, scale bar = 50 μM). **B:** The number of smoking and non-smoking APC$^{Min/+}$ mice with high or low dysplasia. **C:** Survival in smoking and non-smoking APC$^{Min/+}$ mice. **D:** The incidence of CRC in smoking and non-smoking APC$^{Min/+}$ mice at 4, 8 and 12 weeks.

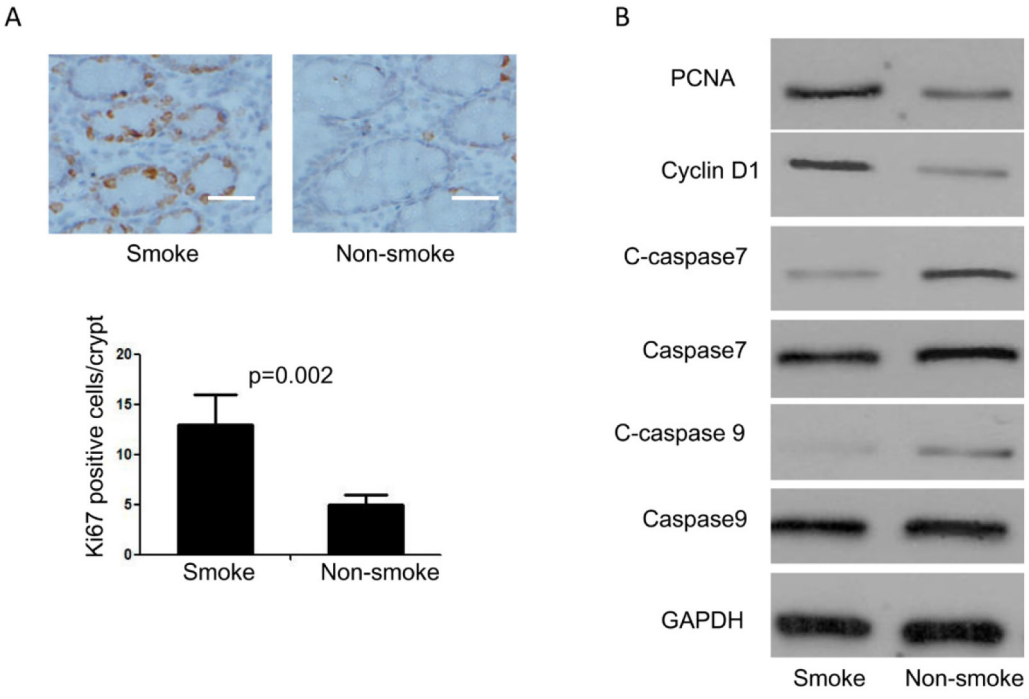


Figure 4. Smoking promoted proliferative ability in colorectal epithelial cells. **A:** Ki-67-positive colorectal epithelial cells in smoking and non-smoking wild-type mice (magnification 400×, scale bar=50 μM). **B:** Protein levels of PCNA, Cyclin D1, C-caspase7, Caspase7, C-caspase9 and Caspase9 in smoking and non-smoking wild-type mice.

ly, which revealed that the incidence of CRC in smoke group and non-smoke group was 10% *vs.* 0%, 60% *vs.* 20% and 100% *vs.* 60%, respectively (Figure 3D). The incidence of CRC was prior in smoke group than that of non-smoke group, which supported our previous finding that CRC smokers patients were younger than non-smokers.

Smoking promoted proliferative ability in colorectal epithelial cells

WT mice were subjected to cigarette smoking or ambient air. Three months later, mice colorectal tissues were harvested. Higher Ki-67-positive cell number was identified in smoking mice than non-smokers (Figure 4A). Identically, protein levels of proliferation-associated genes (PCNA and cyclin D1) were upregulated, whereas apoptosis-associated genes (cleaved-caspase 7 and cleaved-caspase 9) were downregulated in smoking mice (Figure 4B). Collectively, smoking promoted proliferative ability and inhibited apoptosis in colorectal epithelial cells.

Smoking increased the intestinal permeability

FITC-dextran concentration was much higher in WT mice exposed to smoking than in non-smokers, suggesting that smoking remarkably increased the intestinal permeability (p=0.005) (Figure 5A). Intestinal permeability responds to intestinal inflammation. Here, inflammatory factor levels, including IL-6, IL-1β and COX-2 increased in smoking mice (Figure 5B), and the inflammatory response was markedly alleviated by aspirin administration (Figure 5D). In addition, both mRNA and protein levels of TJP, including ZO-1, JAM-c and Occlaudin, were downregulated in smoking mice than in controls (Figure 5C).

Protection of intestinal permeability decreased the number of CRC tissues in $APC^{Min/+}$ mice

To elucidate the role of intestinal permeability in influencing the occurrence of CRC, $APC^{Min/+}$ mice were administered probiotic mixture VSL#3 after smoking exposure. Interestingly,

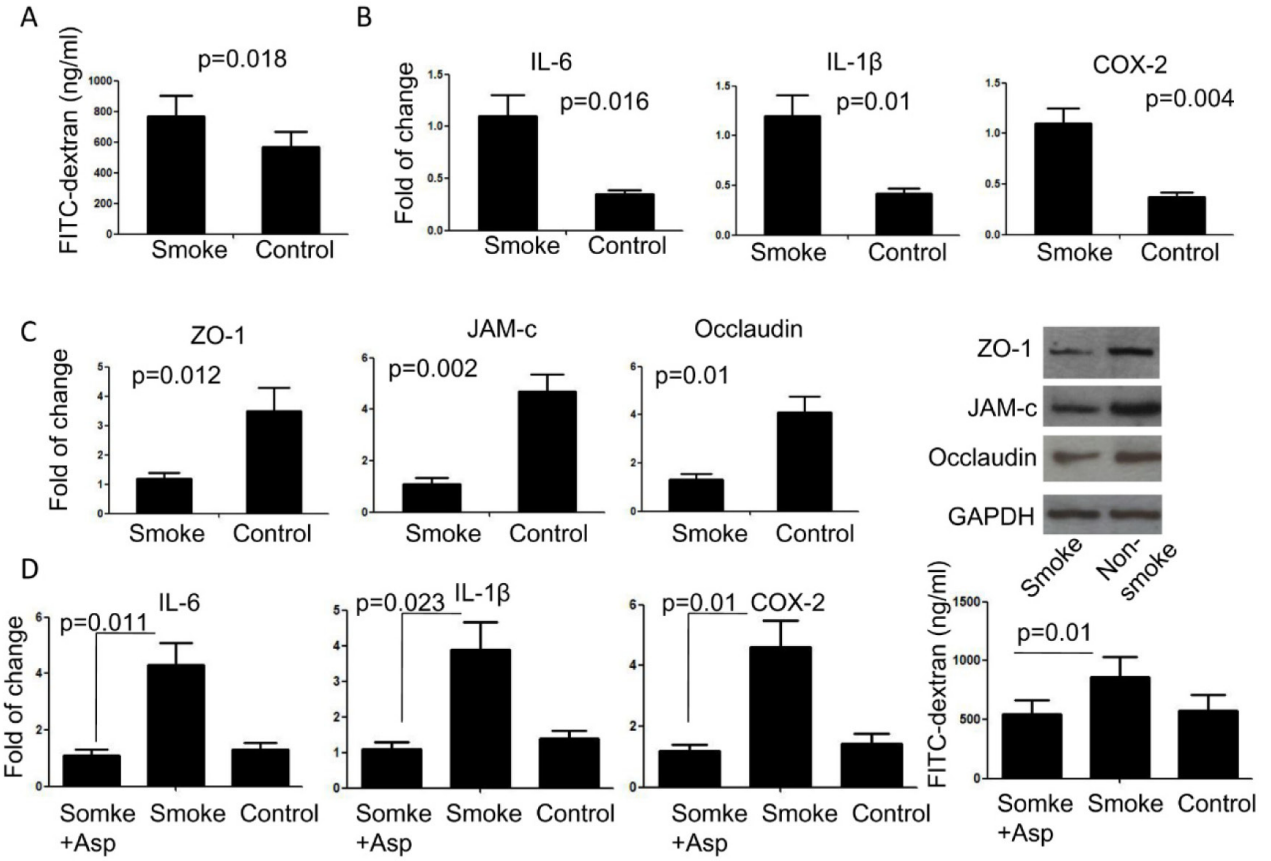


Figure 5. Smoking increased the intestinal permeability. **A:** FITC-dextran concentration in smoking mice and control mice. **B:** Relative levels of IL-6, IL-1β and COX-2 in smoking mice and control mice. **C:** Relative levels of ZO-1, JAM-c and Occlaudin in smoking mice and control mice. **D:** Relative levels of IL-6, IL-1β and COX-2, and FITC-dextran concentration in smoking mice treated by aspirin, smoking mice and control mice.

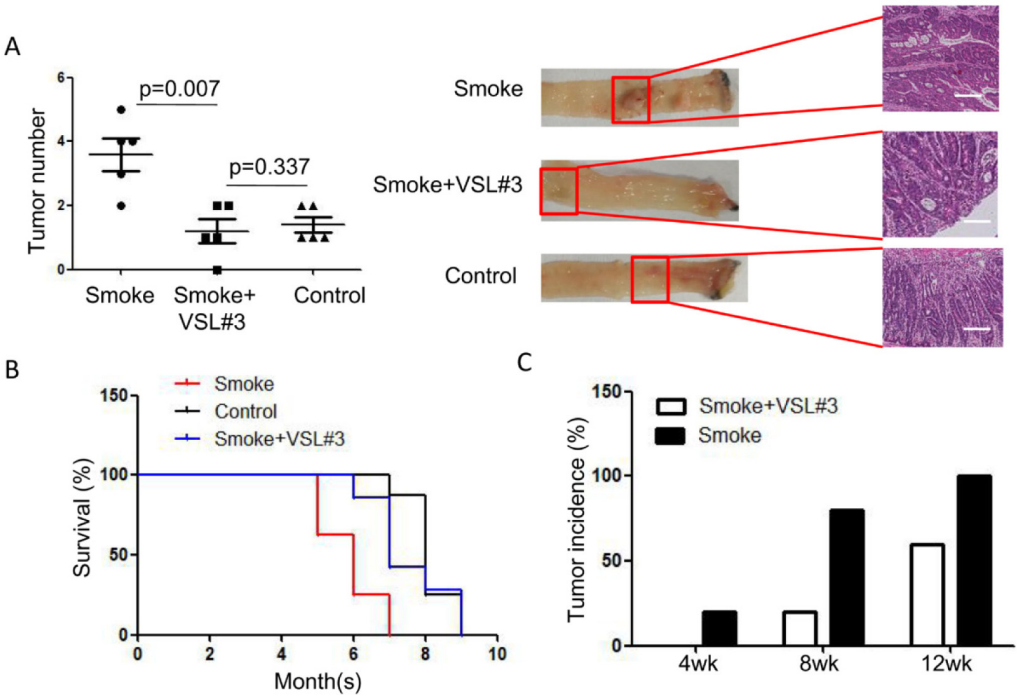


Figure 6. Protection of intestinal permeability decreased the number of CRC tissues in APC$^{Min/+}$ mice. **A:** The incidence of CRC in smoking mice, smoking mice treated with VSL#3 and control mice (magnification 400×, scale bar=50 μM). **B:** Survival in smoking mice, smoking mice treated with VSL#3 and control mice (magnification 400×, scale bar=50 μM). **C:** The incidence of CRC in smoking mice and smoking mice treated with VSL#3 at 4, 8 and 12 weeks.

VSL#3 treatment decreased tumor numbers in APC$^{Min/+}$ mice exposed to smoking (p=0.007), while no significant difference in tumor number was identified between VSL#3-treated smoking mice and mice exposed to ambient air (p=0.337) (Figure 6A). This could imply that protecting intestinal permeability remarkably decreased the number of CRC tissues. Moreover, VSL#3 treatment improved survival in smoking APC$^{Min/+}$ mice (p=0.004) (Figure 6B). The incidence of CRC in VSL#3-treated smoking and non-treated smoking mice was 0% *vs.* 20%, 20% *vs.* 80% and 60% *vs.* 100% at 4, 8 and 12 weeks, respectively (Figure 6C).

Discussion

CRC has become a prevalent tumor in the world [1]. Excessive intake of high-fat food, drinking and bad lifestyle increase the susceptibility to CRC [2]. In the modern society, the number of smokers has sharply increased. The potential influence of smoking on the occurrence of CRC remains largely unclear. In this paper, we found that CRC smokers were younger than non-smokers, indicating that smoking may be a risk factor for CRC. Consistently, the occurrence of CRC in smoking mice was prior to that in non-smoking mice. Although smoking indeed affected the occurrence of CRC, it did not influence the progression of CRC. No significant difference in tumor staging was identified between smokers and non-smokers in both CRC patients and mice. Therefore, smoking displayed a huge impact on the early tumorigenesis of CRC, rather than its malignant progression.

Intestinal permeability exerts a vital function in protecting the intestine. Once it is damaged, microorganisms in the intestine, including bacteria, and some small-molecular metabolites will enter the intestine, triggering an immune response [10, 11]. Here, higher intestinal permeability and more pronounced inflammatory response were detected in smoking mice. Inhibition of inflammatory response markedly protected the intestinal permeability. A previous study has shown that inflammation damages intestinal permeability [14]. We therefore speculated that smoking triggers certain substances in the circulating blood into colorectal epithelium, where initial inflammatory response is stimulated and thus damages intestinal permeability. It is well known that enteritis is a risk factor for CRC, which explains the younger onset of CRC in smokers. In the following experiments, we confirmed that protecting intestinal perme-

ability effectively reduced tumor number of CRC and more importantly, delayed the occurrence of CRC.

Experimental evidence from clinical subjects and animals has shown that smoking remarkably shortened OS, while it did not affect RFS. We speculated that the effect of smoking on CRC survival may be caused by its damage on other organs. Specific mechanisms need to be further studied.

Conclusions

Smoking increases the susceptibility to CRC through damaging the intestinal permeability. Protecting the intestinal permeability significantly protects intestinal tracts.

Conflict of interests

The authors declare no conflict of interests.

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