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LncRNA-GAS5 and its Promoter Region Polymorphism Associate with Helper Th17 Polarization and Predict Postoperative Pain and the Prognosis of the Patients with Hepatocellular Carcinoma Undergoing Hepatectomy

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ABSTRACT

Background: The relationship between genetic polymorphism and postoperative pain and the prognosis of patients with hepatocellular carcinoma (HCC) undergoing hepatectomy is not fully understood.
Objective: To examine whether lncRNA-GAS5 and its promoter region rs145204276 polymorphism can predict postoperative pain and prognosis of the patients with HCC undergoing hepatectomy.
Methods: Seventy patients with HCC undergoing hepatectomy were enrolled. The lncRNA-GAS5 levels in CD4+ T cells from peripheral blood mononuclear cells (PBMC-CD4+ T cells) and tumor tissues were measured by qRT-PCR. Genotyping analysis of rs145204276 was performed using the TaqMan platform. PBMC-CD4+ T cells were isolated and the cytokine levels in helper T (Th) cells were determined by flow cytometry. Patients with Ins/Ins genotype carrying the rs145204276 polymorphism were allocated into the Ins group, and others were allocated into the Del group.
Results: The lncRNA-GAS5 level decreased significantly in PBMC-CD4+ T cells and tumor tissues compared with the healthy controls and corresponding adjacent non-tumor tissues. The patients with Del/Del genotype showed significantly higher lncRNA-GAS5 expression in PBMC-CD4+ T cells, lower postoperative pain scores, and better overall survival. LncRNA-GAS5 expression in PBMC-CD4+ T cells was negatively associated with IL-6, IL-17, and the RORγT/CD3 ratio (an indicator of TH17 polarization).
Conclusion: LncRNA-GAS5 expression and its promoter region rs145204276 polymorphism are prognostic biomarkers that can predict postoperative pain of patients with HCC undergoing hepatectomy.

Keywords: Hepatocellular Carcinoma, Polymorphism, Postoperative Pain, Prognosis

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INTRODUCTION

Hepatectomy is generally recognized as the first line of therapy for most hepatocellular carcinoma (HCC) patients [1-2]. There is an evolving opinion that some perioperative factors, including surgical stress and pain, can affect the prognosis of the HCC [3]. It is now believed that postoperative pain is defined by cross-talk between the immune system and central and peripheral nervous systems and neoplastic cells [4]. However, the regulation of inflammatory factors and immune cell functions, as well as their contribution to postoperative pain, is still unknown [3].

Helper T cells (Th), which include Th1, Th2, and Th17 subtypes, are the main mediators of tumor immunity and postoperative pain [5-7]. A recent study has demonstrated that posttranscriptional regulation by long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) is crucial in T cell polarization [8]. Several lncRNAs have been identified to bind to miRNA sites as competing endogenous RNAs (ceRNAs), thereby affecting the levels of target miRNAs [9]. Newly-identified lncRNA-GAS5 has been reported to be involved in T cell polarization by acting ceRNAs [10]. Studies have also pointed out that lncRNA-GAS5 is a carcinostatic lncRNA in a variety of tumor types [11, 12].

Tao et al. have reported the presence of a 5-base pair indel polymorphism (rs145204276) in the promoter region of GAS5 and demonstrated that the deletion of an allele of rs145204276 significantly increased the risk of the HCC [13]. Subsequent studies by other investigators showed that rs145204276 can affect the susceptibility of individuals to a variety of cancers [14, 15]. However, it is not yet clear whether this polymorphism is related to inflammation-associated postoperative pain in the HCC patients. Here, we examined the rs145204276 polymorphism, together with the expression of lncRNA-GAS5, to demonstrate the effects of lncRNA-GAS5 on postoperative pain and prognosis in the HCC patients.

MATERIALS AND METHODS

Clinical Subjects

This perspective study was performed according to the Helsinki Declaration. Informed consent was obtained from all the patients before participation. Each author confirmed that they had no conflict of interest. Ethical approval was obtained from the Ethics Committee of Shandong Provincial Third Hospital.

Seventy HCC patients, together with 70 individuals free of intestinal diseases (the healthy controls, the HCs), were included All the HCC patients underwent hepatectomy in our hospital, and tumor tissues were obtained. Blood samples were collected in EDTA tubes from all the participants before surgery.

For anesthesia, the patients were injected with 0.1-g phenobarbital sodium and 0.5-mg atropine 30 min before anesthesia induction. General anesthesia was induced with sufentanil (0.3 mg/kg), midazolam (0.04 mg/kg), and Propofol (2 mg/kg). Anesthesia was maintained with Sevoflurane (2–3% end-tidal concentration) or with Propofol (4-6 mg/kg•h) in combination with Sufentanil (0.15-0.35 μg/kg•h) during surgery. After surgery, all the patients received patient controlled intravenous analgesia (PCIA) with sufentanil. The pain intensity was scored using the Visual Analog Scale (VAS).

CD4+ Cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated and stained for dead cells according to a previous report [16]. CD4+ T cells (Dynabeads FlowComp™ Human CD4, ThermoFisher), CD8+ T cells (Dynabeads FlowComp™ Human CD8), and natural killer (NK) cells (Dynabeads Untouched Human NK Cells) were sorted using the Dynabead Separation Kit. CD4+ cells, CD8+ T cells, and NK cells were used for determining the lncRNA-GAS5 levels (defined as PBMC-CD4+ samples, PBMC-CD8+ samples, and PBMC-NK samples, respectively). The values of T-bet/CD3, the GATA3/CD3, and RORγT/CD3 in PBMC-CD4+ T cells were determined by flow

cytometry according to a previous report [16]. Briefly, sorted PBMC-CD4+ T cells were surface stained for anti-CD3. Subsequently, cells were fixed and stained for intracellular markers, including anti-T-bet, anti-GATA3, and anti-RORγT. Samples were analyzed by BD LSR II flow cytometer (Becton Dickinson).

Quantitative Reverse-transcription Polymerase Chain Reaction (qRT-PCR)

RNAs were reverse transcribed into cDNAs using a cDNA Synthesis Kit (TaKaRa, Osaka, Japan), and cDNAs were amplified using the SYBR Premix Ex Taq II Kit (TaKaRa, Osaka, Japan). LncRNA-GAS5 levels were measured using the LightCycle®96 Real-time PCR System (Roche, Basel, Switzerland). Primers for lncRNA-GAS5 were 5′-CTTCTGGGCTCAAGTGATCCT-3′ (forward) and 5′-TTGTGCCATGAGACTCC ATCAG-3′ (reverse).

Genotyping

The lncRNA-GAS5 rs145204276 polymorphism was identified using the TaqMan platform (Thermo Fisher Scientific, Waltham, MA) according to previous reports [13-15]. Patients with the rs145204276 Ins/Ins genotype were allocated to the Ins group, and Ins/Del or Del/Del genotype patients were allocated to the Del group.

Statistical Analysis

Results were expressed as an average±SD, and the differences between the two groups were determined with a Student t-test or paired t-test using SPSS software. The Chi-square test or Fisher exact test was used to analyze the relationship between the rs145204276 polymorphism and clinicopathological features. Linear correlation analysis was used to determine the relationship between lncRNA-GAS5 expression and specific marker or cytokine levels. Receiver operating curve (ROC) analysis was used to determine the diagnostic performance of the lncRNA-GAS5 level in the HCC patients. Kaplan–Meier curves were used to assess the differences in overall survival (OS) and disease-free survival (DFS) of patients with the rs145204276 polymorphism.

RESULTS

LncRNA-GAS5 Expression Decreased in Tumor Tissue Samples and PBMC-CD4+ Samples from the HCC Patients

Detailed demographics for all participants are listed in Table 1. The indicators of tumor

Table 1. Demographics and clinical features of the HCC patients and the HC cases.

		HCs (n=70)	HCCs (n=70)	P value
Gender	Male	41	44	0.885
	Female	23	26	
Age	>60	33	28	0.394
	≤60	37	42	
rs145204276 polymorphism	Ins	33	29	0.496
	Del	37	41	
ASA class	I	---	46	---
	II	---	24	
TNM stage	I/II	---	33	---
	III/IV	---	37	
Lymph node metastasis	No	---	32	---
	Yes	---	38	
Duration of surgery (Minutes)		---	132.2±57.6	---
Blood loss (mL)		---	233.5±167.3	---
Weight (kg)		64.4±10.8	62.1±11.3	0.220
Height (cm)		168.0±7.7	167.2±7.9	0.545

ASA: American Society of Anaesthesiologists

development, including TNM stage and lymph node metastasis, together with the characteristics of hepatectomy, including blood loss and operation time, were recorded for each HCC patient. Using quantitative PCR analysis, we found that lncRNA-GAS5 levels were lower in tumor tissues than that in adjacent tissues from the HCC patients (Figure 1A). Subsequently, we isolated several important immune cell subsets from PBMCs, including CD4+ T cells (PBMC-CD4+), CD8+ T cells (PBMC-CD8+), and NK cells (PBMC-NK), and observed that the HCC patients had significantly lower lncRNA-GAS5 expression in the PBMC-CD4+ subset compared with HCs (Figure 1B), whereas no significant differences were found in the other subsets (data not shown).

Next, the ROC curve of lncRNA-GAS5 expression in the PBMC-CD4+ subset was constructed to investigate their diagnostic value. The lncRNA-GAS5 level discriminated the HCC patients from HCs with an AUC of 0.8292 (95% CI=0.7406–0.9179) (Figure 1C).

Association of the rs145204276 Polymorphism with lncRNA-GAS5 Level and the HCC Prognosis

Of the 70 HCC-WP patients, 29 cases showed the Ins/Ins genotype (Ins group) and 41 cases showed the Ins/Del or Del/Del genotype (Del group). There was no significant difference in the genotype distribution concerning the rs145204276 polymorphism between the HCC patients and HCs (Table 1). In the HCC cases, the Del group showed significantly higher lncRNA-GAS5 levels in the PBMC-CD4+ subset (Figure 2A).

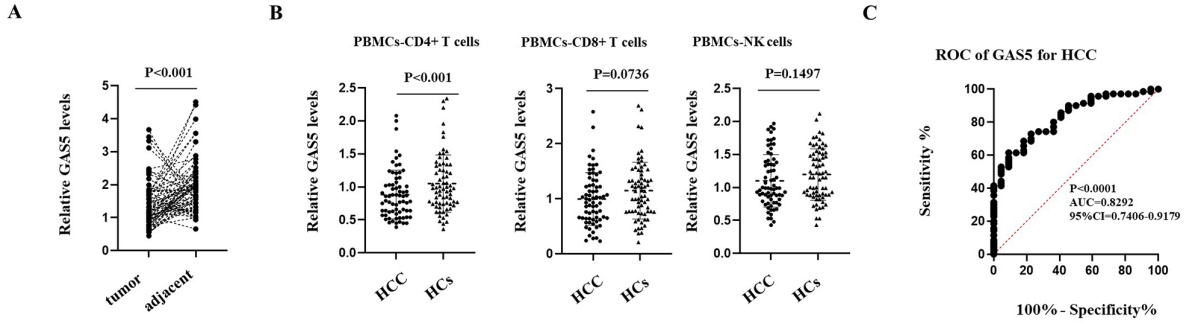

Figure 1. LncRNA-GAS5 was decreased in tumor tissue samples and PBMC samples from HCC patients. (A) qPCR results comparing lncRNA-GAS5 levels between tumor tissue samples and adjacent non-tumor samples in 70 cases of HCC. Paired t-test was used to determine the statistical difference. (B) qPCR results comparing lncRNA-GAS5 levels in PBMCs-CD4+ T cells (left), PBMCs-CD8+ T cells (middle), and NK cells (right) between 70 cases of HCC and 70 HCs. Student's t-test was used to determine the statistical difference. (C) The ROC curve for lncRNA-GAS5 levels in PBMCs-CD4+ T cells concerning 70 cases of HCC. GAS5: lncRNA-GAS5.

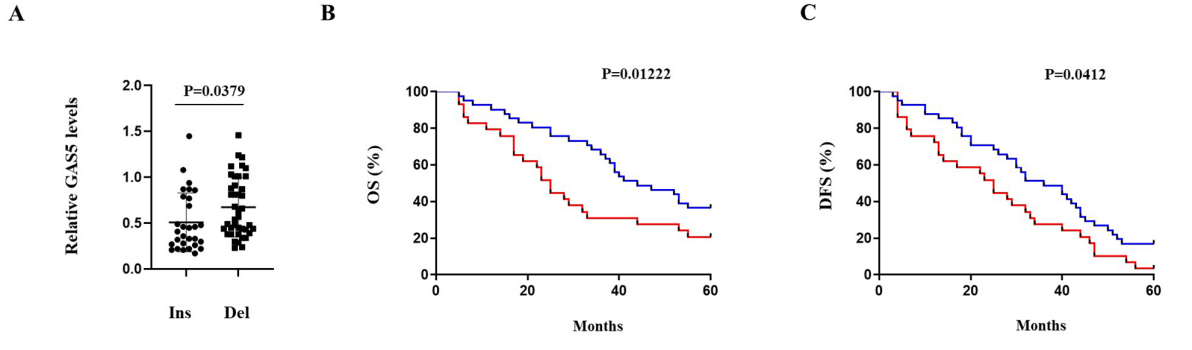

Figure 2. Association of lncRNA-GAS5 expression and the rs145204276 polymorphism with the prognosis of HCC patients. (A) qPCR results comparing lncRNA-GAS5 levels in PBMCs-CD4+ T cells between 29 cases of the Ins group and 41 cases of the Del group. Student's t-test was used to determine the statistical difference. (B, C) Kaplan–Meier curves for time to (B) the overall survival (OS) rate and (C) the disease-free survival (DFS) rate in 29 cases of the Ins group and 41 cases of the Del group. Blue line: Del group; red line: Ins group.

Furthermore, the rs145204276 polymorphism was associated with TNM stage and lymph node metastasis in the HCC patients (Table 2). VAS scores in the Del group were significantly lower than those in the Ins group at 6 and 24 hrs., but not at 48 hrs. after surgery. PCIA sufentanil consumption in the Del group was also significantly lower (Table 3). Furthermore, the HCC patients in the Del group had better OS and DFS than those in the Ins group (Figures 2B, C).

Association of lncRNA-GAS5 Expression and the rs145204276 Polymorphism with PBMC-CD4+ T Cell Cytokine Levels in the HCC Patients

Various immune cell subtypes and their secreted cytokines are highly associated with the HCC development as well as postoperative pain [17]. There were no significant correlations between lncRNA-GAS5 expression and CD4+ T cell ratios, CD8+ T cell ratios, and NK cell ratios in the HCC patients (data not shown). Interestingly, in the HCC patients, lncRNA-GAS5 expression was negatively correlated with IL-6 and IL-17 levels in PBMC-CD4+ T cells (Figures 3A, B). In addition, patients in the Del group showed lower IL-6 and IL-17 levels in PBMC-CD4+ T cells than those in the Ins group (Figures 3C, D).

Association of lncRNA-GAS5 Expression and the rs145204276 Polymorphism with Th17 Polarization in PBMC-CD4+ T Cells from HCC-WP Patients

Next, we examined the association of lncRNA-GAS5 expression and thers145204276 polymorphism with PBMC-CD4+ T cell polarization. A previous study used T-bet/

Table 2. Genotype distributions of lncRNA-GAS5 rs145204276 polymorphism in HCC patients.

Parameters		HCC patients		
		Ins (n=29)	Del (n=41)	P value
Gender	Male	22	22	0.058
	Female	7	19	
Age	>60	11	17	0.767
	≤60	18	24	
TNM stage	I/II	6	27	0.001
	III/IV	23	14	
Lymph node metastasis	Yes	19	13	0.005
	No	10	28	
ASA class	I	22	24	0.133
	II	7	17	
Weight (kg)		61.3±10.3	62.6±11.7	0.632
Height (cm)		166.5±7.4	167.8±7.8	0.485

ASA: American Society of Anaesthesiologists

Table 3. Association of VAS scores after surgery with the genotype distributions of lncRNA-GAS5 rs145204276 polymorphism in HCC patients.

Parameters		HCC patients		
		Ins (n=29)	Del (n=41)	P value
VAS at the time	6 h After Surgery	3.34±0.71	2.61±0.83	<0.001
	24 h After Surgery	2.44±1.02	1.83±0.66	0.003
	48 h After Surgery	1.52±0.86	1.30±0.58	0.205
PCIA Sufentanil Consumption (μg)		114.50±15.17	103.32±17.44	0.005
Duration of surgery (Minutes)		134.82±55.56	130.44±58.61	0.754
Blood loss (mL)		240.12±144.88	227.49±157.63	0.734

VAS: Visual Analog Scale; PCIA: patient controlled intravenous analgesia

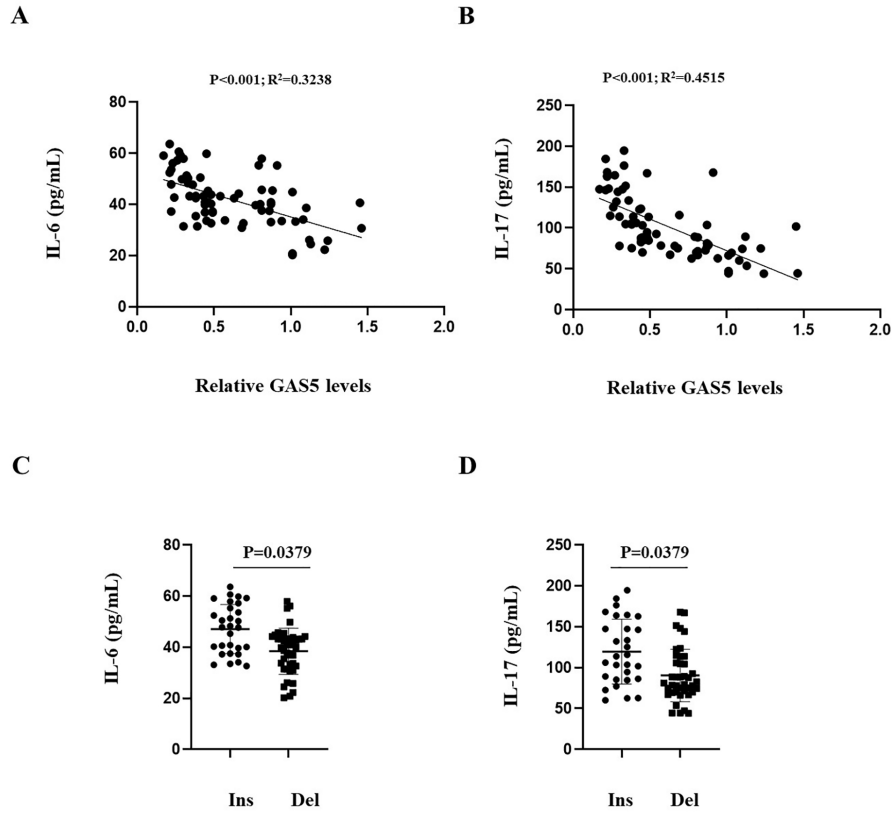

Figure 3. Association of lncRNA-GAS5 expression and the rs145204276 polymorphism with cytokine levels in PBMCs-CD4+ T cells from HCC-WP patients. (A, B) Linear correlation analysis determined the correlation between (A) IL-6 and (B) IL-17 levels and lncRNA-GAS5 levels in PBMCs-CD4+ T cells from 70 cases of HCC. (C, D) Determining the (C) IL-6 and (D) IL-17 levels in PBMCs-CD4+ T cells between 29 cases of the Ins group and 41 cases of the Del group. Student's t-test was used to determine the statistical difference.

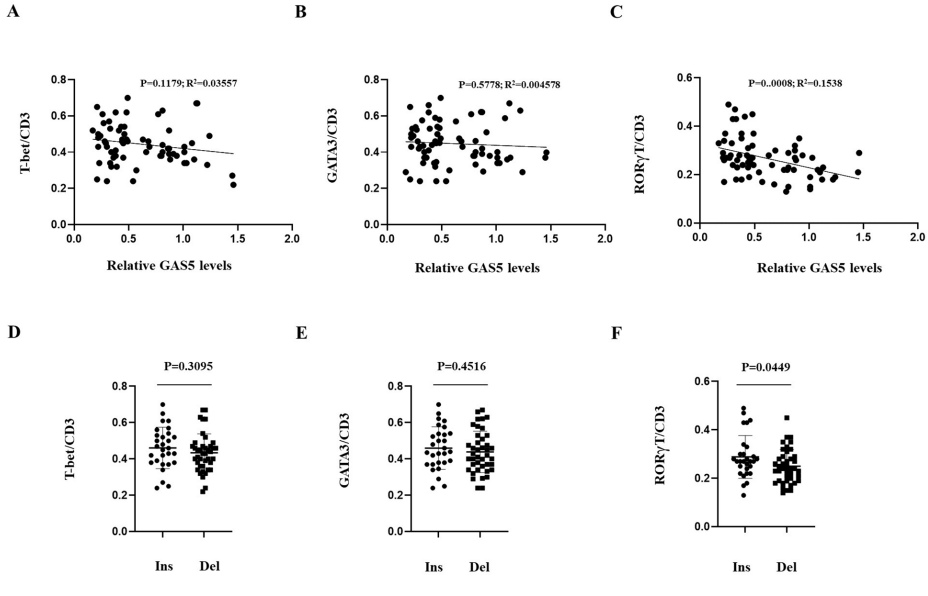

Figure 4. Association of lncRNA-GAS5 expression and the rs145204276 polymorphism with PBMCs-CD4+ T cell polarizations from HCC patients. (A–C) Linear correlation analysis determining the correlation between (A) the T-bet/CD3 ratio; (B) GATA3/CD3 ratio; (C) RORγT/CD3 ratio, and lncRNA-GAS5 levels in PBMCs-CD4+ T cells in HCC patients. (D–F) Determining the (D) T-bet/CD3 ratio; (E) the GATA3/CD3 ratio; and (F) RORγT/CD3 ratio in PBMCs-CD4+ T cells between 29 cases of the Ins group and 41 cases of the Del group. Student's t-test was used to determine the statistical difference.

CD3, GATA3/CD3 and RORγT/CD3 ratios to assess Th1, Th2, and Th17 patterns [16]. In the HCC patients, lncRNA-GAS5 expression was negatively correlated to the RORγT/CD3 ratio, which represents the Th17 ratio in PBMC-CD4+ T cells, whereas it was not correlated with Th1 and Th2 markers (Figures 4A–C). Meanwhile, the RORγT/CD3 ratio, a T17 marker, was lower in PBMC-CD4+ T cells in the Del group. In addition, there were no significant differences between the rs145204276 polymorphism and Th1 and Th2 markers (Figures 4D–F).

DISCUSSION

LncRNA-GAS5 is a newly identified tumor-suppressing lncRNA, which can inhibit the proliferation of tumor cells by interacting with its targets [12]. Our study found that lower lncRNA-GAS5 levels existed in both PBMC-CD4+ T cells and tumor tissues than in non-tumor controls, which is consistent with a previous study [18]. The lncRNA-GAS5 promoter site polymorphism, rs145204276, is associated with immunity dysregulation and cancer development [13-15]. Herein, we found that the rs145204276 polymorphism is associated with the indicators for tumor development such as TNM stage and lymph node metastasis. These results are consistent with those reported by other groups [13-15].

Inadequately controlled postoperative pain can adversely affect patients, resulting in unnecessary physical, psychological, and emotional manifestations [19]. Furthermore, pain can affect the prognosis of the HCC patients [3]. Sufentanil, a synthetic opioid analgesic, exerts its effects primarily on the μ–opioid receptor. The analgesic activity of sufentanil is better than that of fentanyl, and it shows fewer side effects. For example, its lipophilicity is about twice that of fentanyl, and it easily passes through the blood-brain barrier. It not only has greater analgesic activity, but its effects last longer (about twice as long as fentanyl). Therefore, sufentanil is often used to maintain anesthesia. However, there are still personalized differences in its clinical effect [20]. Our new finding is that lncRNA-GAS5 expression and the rs145204276 polymorphism are involved in the regulation of postoperative pain. Under comparable operation time and bleeding volume, patients in the Del group had significantly lower VAS scores. In addition, they needed less sufentanil after surgery, suggesting that patients in the Del group perceived weak pain after surgery.

A previous study has proposed that inflammation is the driving force of pain [5]. Indeed, inflammation is highly associated with the HCC progression [3]. It has been reported that inflammatory cytokine secretion is correlated with postoperative VAS scores and sufentanil consumption [21]. Therefore, we speculated that the rs145204276 polymorphism may affect postoperative pain by regulating the secretion of certain cytokines.

We focused on the cytokines secreted by PBMC-CD4+ T cells because they mediate a close host immune response in the HCC [10]. We found that the Del group showed higher lncRNA-GAS5 expression in PBMC-CD4+ T cells, and lncRNA-GAS5 expression was negatively correlated with the RORγT/CD3 ratio and IL-6 and IL-17 levels in PBMC-CD4+ T cells, which are markers for Th17 polarization. Peng et al. reported that sufentanil partly reduces the increased Th17 cell polarization [22]. The same target cells involved in lncRNA-GAS5 expression and sufentanil consumption indicated that lncRNA-GAS5 may have a synergetic effect on sufentanil, which might explain why lncRNA-GAS5 expression was negatively correlated with postoperative pain and sufentanil consumption.

Importantly, T-bet/CD3 and GATA3/CD3 ratios, which are indicators of Th1 and Th2 polarization, were not associated with lncRNA-GAS5 expression, and were not significantly different between the Del and Ins groups, indicating that lncRNA-

GAS5 expression and the rs145204276 polymorphism specifically impact Th17 polarization. In other words, they can regulate the balance between Th17/Th1 and Th17/Th2, which are important factors for the HCC progression [23]. Consistent with these results, we found that the rs145204276 polymorphism was associated with both OS and DFS of the HCC patients.

In conclusion, our findings showed that the rs145204276 polymorphism was associated with postoperative pain and the HCC prognosis through Th17 regulation. However, this conclusion should be verified in the future with a larger sample size. With pain treatment becoming more and more individualized, additional studies investigating the association of the rs145204276 polymorphism with pain should be carried out.

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Declaration of interest: None.

Conflict of Interest: None declared.

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