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The Clinical Value of Abnormal *TBX15* Hypermethylation in HCC

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Abstract

Background: HCC is one of the most common and invasive malignant tumors in the world. The objective of this research was to explore the methylation of *TBX15* and determine the clinical value of *TBX15* Hypermethylation in Hepatocellular Carcinoma (HCC).

Methods: The methylation status of the *TBX15* gene in 61 pairs of HCC patient tumor and paracancerous tissues was analyzed with bisulfite and methylation-specific PCR, and the association between *TBX15* Hypermethylation and specific clinical features was determined.

Results: *TBX15* Hypermethylation was associated with patient prognosis. Eighty percent of the tumor and associated paracancerous tissues were hypermethylated. The methylation levels of *TBX15* in all 61 tumors were higher than those in the paired paracancerous tissues. The difference between the two tissue types was statistically significant ($P<0.001$). The methylation level of *TBX15* was increased in tumor tissues with vascular invasion ($P>0.05$) and poor envelope integrity ($P<0.05$). Additionally, Hypermethylation of *TBX15* was statistically associated with advanced cancer stage ($P<0.05$). The Area Under the Curve (AUC) value (0.98) indicated that *TBX15* Hypermethylation had a high confidence value for diagnosing HCC. The Kaplan-Meier analysis suggested that *TBX15* Hypermethylation was associated with low relapse-free survival rates. The Cox proportional hazards regression analysis indicated that *TBX15* Hypermethylation was an independent factor affecting prognosis.

Conclusion: *TBX15* Hypermethylation was related to malignant behavior and was identified as a potential indicator for poor prognosis. Therefore, *TBX15* Hypermethylation can be used as a biomarker for the diagnosis and prognostication of HCCs.

Keywords: *TBX15*; DNA hypermethylation; Hepatocellular carcinoma; Biomarker; Hepatology

Introduction

Hepatocellular Carcinoma (HCC) is one of the most common and aggressive malignant tumors in the world, and it is also the third most common cause of death in the world [1,2]. The disease causes nearly 600,000 deaths worldwide every year, and its morbidity and mortality have been increasing in recent years. Although surgical treatment and medical management strategies for HCC have progressed, the overall prognosis of HCC patients is still not satisfactory because liver cancer patients undergoing treatment often suffer from terminal-stage cancer. The 5-year survival rate of HCC is approximately 17% [3,4]. Although the *Alpha-Fetoprotein*(*AFP*) tumor marker is routinely used in early screening for HCC, which has reduced the mortality of liver cancer, its sensitivity and specificity are still limited [5,6]. Therefore, discovering a valuable cellular biomarker specific for HCC and exploring new diagnosis and treatment strategies are crucial.

The alteration of DNA methylation is a key epigenetic event in cancer. Such alteration has been confirmed to play a vital role in cancer and other human diseases [7]. DNA methylation often occurs in Tumor Suppressor Genes (TSGs), and Hypermethylation can cause inappropriate transcriptional silencing [8,9], leading to the occurrence and development of cancer. Current research shows that the T-box gene family is a systematically conserved transcription factor family that regulates a variety of developmental processes [10] and the proliferation and differentiation of tissue-specific stem and progenitor cells [11,12]. The T-box gene family is divided into 5 subfamilies. The *TBX15* gene belongs to the *Tbx2* subfamily [13]. It is involved in a variety of biological processes, such as cell proliferation, differentiation, metastasis and apoptosis. Evidence shows that *TBX15* affects cell proliferation and apoptosis *via* antiapoptotic effects in cancer [14]. Therefore, it is considered a TSG. For example, in ovarian cancer, immunohistochemistry analysis showed a negative correlation between the *TBX15* methylation level and *TBX15* protein expression [15]. *TBX15* methylation is also significantly correlated with pathological stage, Gleason score and biochemical relapse in prostate cancer [16]. The studies of Gao [17], Zheng [18] and others have found that HCC tumor tissues display higher methylation of the *TBX15* gene promoter than paracancerous tissues. However, there is no research on the relationship between *TBX15* methylation and liver cancer clinical characteristics. Therefore, in this study, the methylation status of *TBX15* in HCC and adjacent paracancerous tissues was determined to study the relationship between the methylation status and clinical characteristics of HCC patients. Finally, an evaluation of its clinical value is provided.

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Materials and Methods

Tissue samples

Subjects were chosen from patients who underwent radical liver resection at the First Associated Hospital of Zhejiang University. The patients met the following criteria:

- The subject was undergoing radical resection for liver cancer.
- The subject was suffering from only HCC without any derived or secondary carcinomas.
- No radiotherapy or chemotherapy was administered before surgery.

All patients signed an informed consent form. The resected tumor tissue from the liver and its adjacent tissues were collected post-surgery. The paracancerous tissues were obtained from tissues surrounding the tumor within a radius of 1 cm. All tissues collected were stored in liquid nitrogen tanks at -80°C. The TNM classification method was used for clinical staging. Among the enrolled patients, 11 patients had stage I disease, 4 patients had stage II disease, 43 patients had stage III disease, and 3 patients had stage IV disease. In this study, the average age of the patients was 58.3 years with a standard deviation of 11.04. The follow-up time was from the end of the surgery to September 2020. The average follow-up time was 383.19 days with a standard deviation of 86.29. All but 3 patients kept in contact during the follow-up period. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University.

Experiments

DNA extraction: DNA was extracted from tissue samples, and a Nano drop 2000 (Thermo Fisher Scientific Co. Ltd) was used to test the quality of genomic DNA. The concentration of all samples was ≥ 20 ng/μL and the total amount was ≥ 1 μg. All DNA absorbance ratios at 260/280 nm were between 1.8 and 2.0. Agarose gel electrophoresis was used to detect the integrity of genomic DNA. The electrophoresis bands of all the samples were clearly visible without RNA contamination.

Bisulfite conversion and multiplex amplification: The DNA methylation level was analyzed with MethylTarget™ (Genesky Biotechnologies Inc., Shanghai, China), an NGS-based multiple-target CpG methylation analysis method. The CpG island between the 2K region upstream of Transcription Start Site (TSS) and the 1K region downstream of the first exon was analyzed with Gene CpG v1.0 software. The evaluation standards were as follows: observed/expected ratio>0.60, percent C^+ percent G>50.00% and length>200 bp. The search ultimately yielded two CpG island locations. Four targeted regions before the promoter region (ranging from TSS1500 to TSS200) were chosen and transformed into bisulfite-converted sequences. The design of primers for methylation experiments (Table 1) was performed using Methylation Fast Target V4.1 software. The primer standard characteristics were as follows: Tm: 58-62°C, primer length: 18-36 bp, product size range: 150-270, and minimum CpG site number: 10. Genomic DNA (400 ng) was subjected to sodium bisulfite treatment using the EZ DNA Methylation™-GOLD Kit (Zymo Research) according to the manufacturer's protocols. Multiplex PCR was performed with optimized primer combination sets. A 20 μL PCR mixture was prepared for each reaction and included 1x reaction buffer (Takara), 3 mM Mg^{2+}, 0.2 mM dNTPs, each primer (0.1 μM), 1U HotStarTaq polymerase (Takara) and 2 μL template DNA. The cycling program ran with the following conditions: 95°C for 2 min; 11 cycles of 94°C for 20s, 63°C for 40s with a 0.5°C decrease per cycle, and 72°C for 1 min; 24 cycles of 94°C for 20s, 65°C for 30s, and 72°C for 1 min and 72°C for 2 min.

Index PCR: PCR amplicons were diluted and amplified using indexed primers. Specifically, a 20 μL mixture was prepared for each reaction and included 1x reaction buffer (NEB Q5TM), 0.3 mM dNTPs, 0.3 μM F primer, 0.3 μM index primers, 1 U Q5TM DNA polymerase (NEB) and 1 μL diluted template. The cycling program ran with the following conditions: 98°C for 30s; 11 cycles of 98°C for 10s, 65°C for 30s, 72°C for 30s and 72°C for 5 min. PCR amplicons (170 bp-270 bp) are separated by Agarose electrophoresis and purified using the QIAquick Gel Extraction kit (QIAGEN).

Sequencing: Libraries from different samples were quantified and pooled together, followed by sequencing on the Illumina MiSeq platform according to the manufacturer's protocols. Sequencing was performed with the 2 × 300 bp paired-end mode.

Statistical analysis: Using R language (version 4.0.2) for statistical analysis, Student's t-test was used to analyze the methylation level differences between tumor tissues and paired paracancerous tissues. The associations between cancer tissue methylation status and clinicopathological characteristics were analyzed by Wilcoxon exact rank tests. A Receiver Operating Characteristic (ROC) curve was generated to determine the *TBX15* gene methylation threshold, and the area under the ROC curve (AUC) was determined to evaluate the diagnostic value in HCC. The determination of the ability of *TBX15* methylation in tumor tissues to predict relapse-free survival was performed *via* Kaplan-Meier survival analysis and log-rank tests. Cox proportional hazards regression analysis was performed to evaluate the prognostic utility of *TBX15* methylation in HCC. Results were determined significant with a standard of $P<0.05$.

Results

Differences in *TBX15* methylation levels between tumor tissues and paracancerous tissues

MethylTarget™ technology was used to analyze the methylation levels of tumor tissues and paired paracancerous tissues. Data are expressed in a "mean ± Standard Deviation (SD)" format. The methylation level was calculated as the number of methylated reads at the site divided by the total number of reads at the site. The methylation levels of four targeted regions within the *TBX15* promoter region were measured. The methylation levels of each region were higher in tumor tissues than in the respective paracancerous tissues ($P<0.01$), (Table 2). Additionally, 80% of tumor tissues displayed Hypermethylation, while the adjacent paracancerous tissues showed hypomethylation. In

Target	Length	Distance to TSS	Primer F	Primer R
TBX15_46	197	-523	AATTTTGGGGAGTTGGAAGTAGG	TCTACCTTATCTCCAACTTCTCTACTAAAACC
TBX15_47	211	-406	GATGTTAGTTTTTTGATATTTTGGTTGGA	AACCTCTAAAACRCCRAATTCTCTACC
TBX15_48	210	-882	GGATGAGAAAAGAGTTGGGAAAG	CATTCACTACTACCRAACTTCAACAA
TBX15_49	209	93	GATTTYGYGGATAGYGGTTTAGATATAGTT	CAAACTATAAACCCCCTAACCTAAACT

Table 1: Primers used in this study.

addition, the methylation levels of *TBX15* in all 61 tumor tissues were higher than those in the paired adjacent paracancerous tissues. The difference was statistically significant (P<0.001, (Figure 1).

Relationships between the *TBX15* methylation level and clinicopathological characteristics of patients with HCC

This study explored the association between the *TBX15* methylation level and clinicopathological characteristics of patients with HCC. Several factors including age, sex, *AFP* level, HBV status, TNM stage and tumor size, presence of thrombi and/or vascular invasion and envelope integrity were included in this study. As indicated in (Table 3), male patient *TBX15* methylation levels were significantly higher than those of female patients (P<0.05). *TBX15* methylation levels were increased in tumor tissues with vascular invasion (P>0.05) and tumor tissues with poor envelope integrity (P<0.05), which may indicate a poor prognosis outcome. In addition, Hypermethylation of *TBX15* was related to stage III and IV disease (P<0.05). There was no significant correlation between methylation and other clinicopathological characteristics (P>0.05).

Diagnostic value of *TBX15* methylation in HCC

ROC curve analysis was performed to determine the diagnostic

Target	Tumor tissues (mean ± SD)	Paracancerous tissues (mean ± SD)	P-value`
TBX15_46	74.05 ± 15.89	30.41 ± 10.92	P<0.01
TBX15_47	68.94 ± 19.33	20.55 ± 9.99	P<0.01
TBX15_48	70.87 ± 17.91	29.73 ± 11.83	P<0.01
TBX15_49	65.32 ± 18.91	16.60 ± 9.40	P<0.01
TBX15	70.12 ± 16.86	25.52 ± 9.68	P<0.01

Table 2: Methylation analysis of four target region in tumor tissues.

Feature	n	Methylation level	P-value
Age			
>50	16	68.28 ± 19.21	0.64
≤ 50	45	70.78 ± 16.32	
Sex			
Male	48	79.43 ± 17.56	0.0046
Female	13	67.60 ± 16.11	
AFP			
>20	32	72.49 ± 16.96	0.27
≤ 20	29	67.51 ± 16.95	
HBV status			
HBV (+)	49	70.72 ± 17.28	0.64
HBV (-)	12	68.60 ± 17.05	
Vascular invasion			
Yes	29	70.95 ± 18.60	0.63
No	32	69.37 ± 15.67	
Envelope integrity			
Intact	11	63.20 ± 16.03	0.02
Lost	50	73.67 ± 16.68	
Tumor size			
>5 cm	35	66.85 ± 17.27	0.12
≤ 5 cm	26	72.90 ± 16.52	
Thrombus			
Yes	12	71.33 ± 18.67	0.8
No	49	69.83 ± 16.76	
TNM stage			
I+II	15	60.83 ± 17.76	0.02
III+IV	46	73.15 ± 15.77	

Table 3: Relationship between the *TBX15* methylation level and clinicopathological characteristics of patients with HCC.

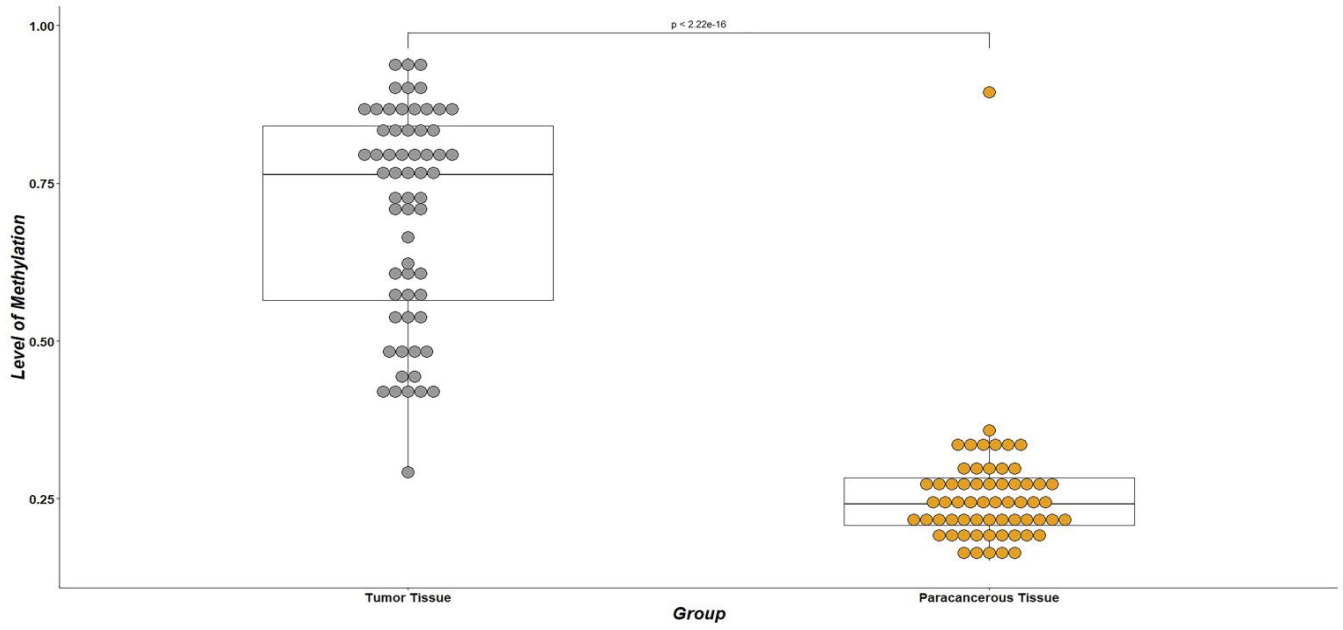

Figure 1: Comparison of *TBX15* methylation levels in tumor tissues and the respective paracancerous tissues.

potential of *TBX15* methylation. When the threshold was 38% for the methylation level, the Youden index was the largest, with a sensitivity of 96%, specificity of 84% and AUC of 0.98 (CI=0.952-0.998, P<0.01), (Figure 2). The diagnostic concordance rate was 90%.

Association between *TBX15* methylation and the prognosis of HCC patients

Kaplan-Meier survival analysis according to the relapse-free survival rate was performed to evaluate the prognostic potential of *TBX15* methylation. Based on the methylation level threshold obtained by the ROC curve analysis, HCC patients were divided into a Hypermethylation group (n=49) and a hypomethylation group (n=12). During the follow-up, 21 people experienced relapse in the Hypermethylation group, with two dying from cancer. Four people had a relapse in the hypomethylation group, with two dying from cancer. During the follow-up period, 3 patients in the Hypermethylation group lost contact with the research team. The relapse-free survival time of the Hypermethylation group was significantly lower than that of the hypomethylation group (P<0.01), (Figure 3). Cox proportional hazards regression model analysis was used to further study the prognostic value of *TBX15* methylation in HCC patients. The results showed that the risk of relapse in the hypomethylation group was 5.6% lower than that in the Hypermethylation group, (P<0.01) (Figure 4 and Table 4).

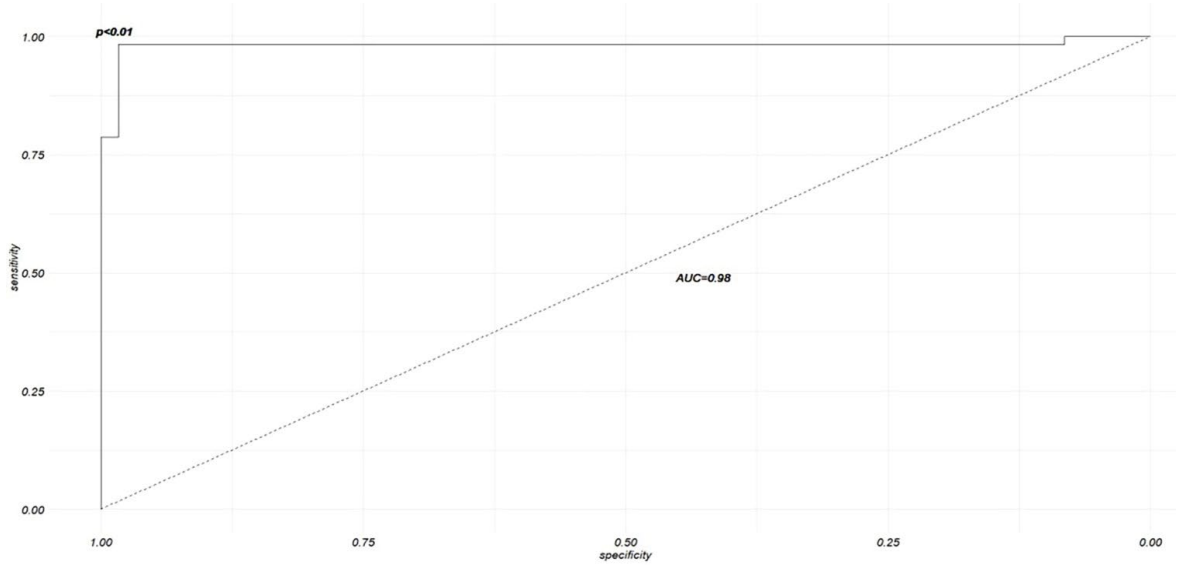

Figure 2: Receiver Operating Characteristic (ROC) curve analysis.

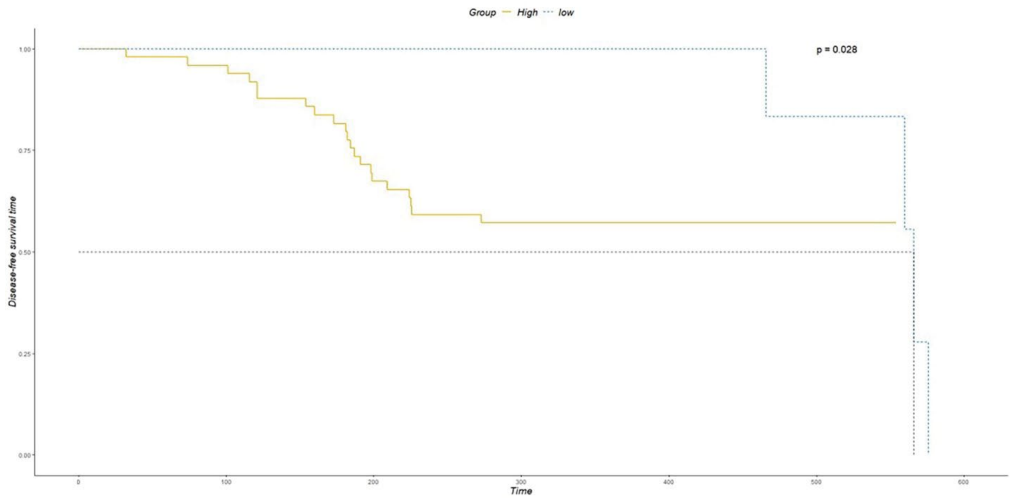

Figure 3: Disease-free survival analysis.

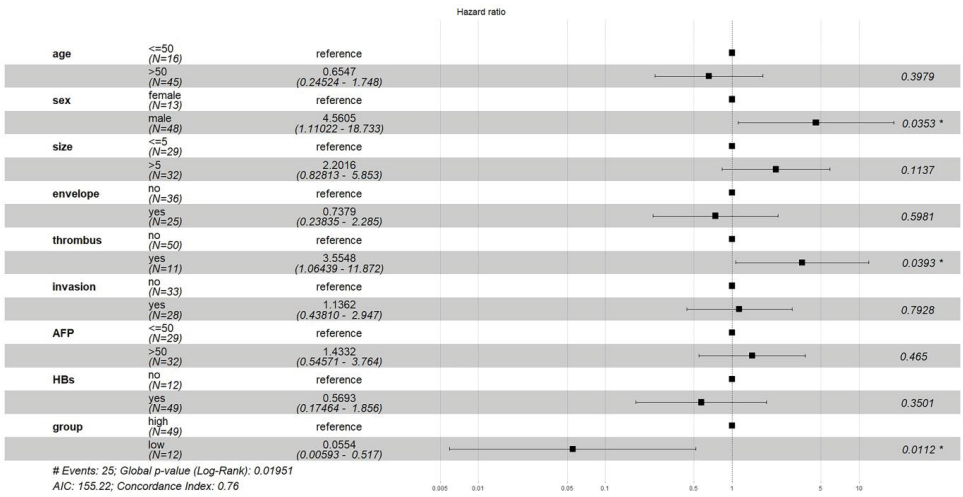

Figure 4: Cox proportional hazards regression analysis.

Variables	Multivariate model			
	Number of patients	HR	95% CI of HR	P value
Age				
≤ 50	16	1	0.25-1.75	0.4
>50	45	0.65		
Sex				
Female	13	1	1.11-18.73	0.035
Male	48	4.56		
Size				
≤ 50	29	1	0.83-5.96	0.11
>50	32	2.2		
Envelope integrity				
Lost	36	1	0.24-2.29	
Intact	25	0.74		0.6
Thrombus				
No	50	1	1.06-11.9	0.04
Yes	11	3.55		
Invasion				
No	33	1	0.44-2.95	0.8
Yes	28	1.14		
AFP				
≤ 50	29	1	0.55-3.77	0.47
>50	32	1.43		
HBs				
No	12	1	0.17-1.86	0.35
Yes	49	0.57		
Methylation				
High	49	1	0.0059-0.52	0.01
Low	12	0.05		

Table 4: Multivariate cox regression analysis data.

Discussion

Research shows that the annual incidence of HCC increases by approximately 3.1% per year [19]. Seventy-five percent of patients in this study diagnosed with HCC had stage III or IV disease. The overall prognosis of HCC patients after treatment is poor. Clinically, the most important thing is to identify HCC patients and provide active treatment measures. In addition, patients must also be monitored after treatment for relapse to reduce mortality. Therefore, it is imperative to find biomarkers that can be used to diagnose HCC and predict the prognosis of HCC patients. DNA methylation is the most common and widely studied epigenetic modification and is an important mechanism related to the occurrence and development of cancers; thus, it is a promising factor to consider when attempting identifying biomarkers.

TBX15 is an important transcription factor that can regulate the proliferation and differentiation of tissue-specific stem cells and progenitor cells [11,12]. The expression of *TBX15* can inhibit tumor cell growth and invasion. *TBX15* can also change the ratio of pro-apoptotic proteins to antiapoptotic proteins. *TBX15* can negatively affect tumor regulation [20]. DNA Hypermethylation can cause *TBX15* expression to be down regulated. *TBX15* is hypermethylated and down regulated in ovarian cancer [21], pancreatic cancer [22] and esophageal cancer [23]. In this study, we confirmed that the methylation levels of *TBX15* in HCC tissues were higher than those in paired paracancerous tissues. The methylation of *TBX15* may lead to the down regulation of its expression and the development of tumors. In addition, the association between *TBX15* Hypermethylation and the clinicopathological characteristics of HCC patients was also explored. The results showed that *TBX15* methylation levels were increased in incomplete capsules ($P<0.05$). The *TBX15* methylation level was significantly increased in patients with advanced-stage cancer, suggesting that *TBX15* methylation is associated with malignant behavior.

To determine the *TBX15* methylation cutoff value, ROC curve analysis was used, and the AUC value of *TBX15* methylation was calculated to be 0.98 ($P<0.01$). The ROC curve analysis indicated that *TBX15* methylation can be used as a diagnostic biomarker for HCC. The Cox proportional hazards regression model showed that *TBX15* methylation was an independent factor affecting the prognosis of HCC. To verify the impact of *TBX15* methylation on the prognosis of HCC patients, the disease-free survival rate of HCC patients with different *TBX15* methylation statuses was studied. Patients with hypermethylated *TBX15* had significantly worse prognosis than patients with hypomethylated *TBX15*. Patients with hypomethylated *TBX15* were more likely to experience relapse and had a shorter relapse-

free survival time than those with hypermethylated *TBX15*. Therefore, patients with hypermethylated *TBX15* can receive earlier intervention to achieve better curative effects. Due to the limited follow-up time, the relationship between *TBX15* methylation and overall survival was not evaluated. In the future, more patients who complete follow-up can be studied for a longer duration than was used in this study to obtain more meaningful results regarding the correlation between *TBX15* and the overall survival rate.

Conclusion

The results of research on DNA methylation have been applied clinically. Some reports have shown that gene methylation can be detected in body fluids such as urine, plasma, and serum and tissues. In addition, gene silencing caused by TSG methylation is reversible. Demethylating reagents such as 5-azacytidine can re-express previously silenced genes in cancer cells. Clinically, Demethylating reagents such as decitabine or azacytidine have been used to treat solid carcinomas. Therefore, using gene methylation as a biomarker for tumor diagnosis and treatment is a very promising research strategy. However, more clinical trials are needed to further determine the clinical value of *TBX15* methylation.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Rui-kun Zhang and Jia-lin Liu conceived of the study design and determined appropriate surveys and survey items for the study. Zhu Hai collected the data. Rui-kun Zhang and Zhu Hai performed the data analysis. Hai-yang Xie and Jia-lin Liu interpreted analysis results and participated in drafts and revisions of this manuscript. All authors reviewed and provided approval for the final version of the manuscript.

Ethics Approval and Consent to Participate

This research has been approved by the Ethics Committee of the First Associated Hospital of Zhejiang University. All subjects signed an informed consent and all methods are done according to the Declaration of Helsinki together with relevant rules and standards.

Patient Consent for Publication

All patients who participated also consented for the publication of this manuscript.

Competing Interests

Not applicable..

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