World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i13.3960

World J Gastroenterol 2015 April 7; 21(13): 3960-3969
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Methylation of *IRAK3* is a novel prognostic marker in hepatocellular carcinoma

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Supported by National Science Council, No. NSC 102-2320-B-016-016-MY3, Taiwan; and the Liver Disease Prevention and Treatment Research Foundation, Taiwan.
Ethics approval: The study was reviewed and approved by the Institutional Review Board of the Tri-Service General Hospital and TLCN User Committee.
Informed consent: Not applicable. This is a delinked tissue bank of Taiwan. Researchers can apply samples for study after the approval of TLCN User Committee and Institutional Review Board of the Tri-Service General Hospital.
Conflict-of-interest: A conflict-of-interest statement is included in the manuscript.
Data sharing: No additional data are available.

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Received: September 3, 2014
Peer-review started: September 4, 2014
First decision: September 27, 2014
Revised: November 7, 2014
Accepted: December 14, 2014
Article in press: December 16, 2014
Published online: April 7, 2015

Abstract

AIM: To examine the methylation levels of interleukin-1 receptor-associated kinase 3 (*IRAK3*) and *GLOXD1* and their potential clinical applications in hepatocellular carcinoma (HCC).

METHODS: mRNA expression and promoter methylation of *IRAK3* and *GLOXD1* in HCC cells were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and methylation-specific PCR (MSP), respectively. Using pyrosequencing results, we further established a quantitative MSP (Q-MSP) system for the evaluation of *IRAK3* and *GLOXD1* methylation in 29 normal controls and 160 paired HCC tissues and their adjacent nontumor tissues. We also calculated Kaplan-Meier survival curves to determine the applications of gene

methylation in the prognosis of HCC.

RESULTS: *IRAK3* and *GLOXD1* expression was partially restored in several HCC cell lines after treatment with 5-aza-2′-deoxycytidine (DNA methyltransferase inhibitor; 5DAC). A partial decrease in the methylated band was also observed in the HCC cell lines after 5DAC treatment. Using *GLOXD1* as an example, we found a significant correlation between the data obtained from the methylation array and from pyrosequencing. The methylation frequency of *IRAK3* and *GLOXD1* in HCC tissues was 46.9% and 63.8%, respectively. Methylation of *IRAK3* was statistically associated with tumor stage. Moreover, HCC patients with *IRAK3* methylation had a trend toward poor 3-year disease-free survival ($P < 0.05$).

CONCLUSION: *IRAK3* and *GLOXD1* were frequently methylated in HCC tissues compared to normal controls and nontumor tissues. *IRAK3* methylation was associated with tumor stage and poor prognosis of patients. These data suggest that *IRAK3* methylation is a novel prognostic marker in HCC.

Key words: *IRAK3*; *GLOXD1*; Hepatocellular carcinoma; DNA methylation biomarker; Quantitative methylation-specific polymerase chain reaction; Pyrosequencing

Core tip: The methylation biomarker is relatively stable in tissue samples and body fluids, suggesting that it is a good tool for the detection, diagnosis, prognosis, and even therapy of hepatocellular carcinoma (HCC). Our study not only demonstrated frequent methylation of interleukin-1 receptor-associated kinase 3 (*IRAK3*) and *GLOXD1* in HCC but also found that *IRAK3* methylation was positively associated with poor 3-year disease-free survival of patients. This indicates that *IRAK3* methylation could be used as a potential biomarker for prediction of prognosis in HCC.

Kuo CC, Shih YL, Su HY, Yan MD, Hsieh CB, Liu CY, Huang WT, Yu MH, Lin YW. Methylation of *IRAK3* is a novel prognostic marker in hepatocellular carcinoma. *World J Gastroenterol* 2015; 21(13): 3960-3969 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i13/3960.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i13.3960

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer deaths in the world[1]. HCC is a serious disease because it is difficult to detect in its early stages; this leads to a very poor prognosis and high mortality. It is believed that studying the molecular mechanisms of HCC development can help us to design better strategies for disease detection or prognosis prediction[2].

Aberrant changes in DNA methylation patterns, which alter gene expression and subsequently drive malignant transformation, are recognized as a common event during carcinogenesis[3] and are also found during the development of HCC[4,5]. Identification of these events not only allows for a detailed understanding of the hepatocarcinogenesis but also provides potential clinical applications in the diagnosis or prognosis of HCC[6]. Recently, technical advances in array systems have led to the development of higher-resolution genome-wide methods for DNA methylation analysis, such as Infinium assay[7]. It has also been successfully used in the study of HCC[8-17]. By using array-based platforms, researchers can simultaneously profile the DNA methylation of a large number of genes or the entire genome. Furthermore, by validating the results from the high-throughput screening approach, researchers can effectively discover more novel genes that may have potential applications in clinical practice.

In our recent study[18], we found several aberrantly methylated genes in HCC by using the Infinium HumanMethylation27 BeadChip and then verified 34 genes by methylation-specific PCR (MSP). Of these genes, we further showed that frequent methylation of homeobox A9 (*HOXA9*) in HCC tissues and plasma samples from patients could be a helpful biomarker to assist in HCC detection. However, several novel genes in our array data were not further validated by quantitative MSP (QMSP), such as interleukin-1 receptor-associated kinase 3 (*IRAK3*) and 4-hydroxyphenylpyruvate dioxygenase-like (*HPDL*, also known as *GLOXD1*). IRAK3 plays an important role in alcohol-induced liver injury[19], and HPDL is an important enzyme in the catabolic pathway of tyrosine in the liver[20]. Moreover, there are no quantitative data about the methylation levels of *IRAK3* and *GLOXD1* in HCC. In this study, we aimed to examine the methylation levels of *IRAK3* and *GLOXD1* in HCC by QMSP and to further test whether these two genes have potential clinical applications in the diagnosis or prognosis of HCC.

MATERIALS AND METHODS

Cell lines and samples for methylation analysis

A normal liver cell line (THLE-3) and 6 HCC cell lines (HepG2, SK-HEP1, TONG, Mahlavu, PLC/PRF/5, and HuH6) were used in this study. THLE-3, HepG2, and SK-HEP1 cells were purchased from American Type Culture Collection. TONG, Mahlavu, PLC/PRF/5, and HuH6 cells were provided by Professor K.H. Lin (Chuang-Gung University, Taiwan). For 5-aza-2′-deoxycytidine (5DAC) treatment, HCC cells were prepared as previously described and harvested directly for reverse transcription-polymerase chain reaction (RT-PCR) and MSP[18]. THLE-3, 3 HCC cell lines (PLC/PRF/5, HepG2,

Table 1 Primer and probe sequences for reverse-transcription polymerase chain reaction, methylation-specific polymerase chain reaction, pyrosequencing, and quantitative methylation-specific polymerase chain reaction

Primer	Sequence (5'→3')	Amplicon (bp)
RT-PCR		
IRAK3-Forward	ATGCAGTGTAAGAAGCATTGGA	247
IRAK3-Reverse	GCAGGTAGTGAATGGCTTTGG	
GLOXD1-Forward	CCCTTCCTACCCGGCTTCA	122
GLOXD1-Reverse	TGGAACCAGCGCAAAAGTGT	
Pyrosequencing		
GLOXD1-Forward	GAAGGGAGGTTTAGTGTTTAAGGA	242
GLOXD1-Reverse	AGCTGGACATCACCTCCCACAACGCCACCCCAACCAAAAACA	
Universal primer	AGCTGGACATCACCTCCCACAACG-Biotin	
Sequencing primer	AGGTTTAGTGTTTAAGGAT	
MSP/Q-MSP		
IRAK3-Forward	AGGAGATCGTTTAGTCGTGGGGTAC	110
IRAK3-Reverse	ACCTCTACGATAAAAACGAAACTACCG	
IRAK3-Probe	CTACCGAAACAAACAAAATA	
GLOXD1-Forward	AGGATGTGATTAGGCGTGAGGTTC	122
GLOXD1-Reverse	AAAAAAACGAAACCCGTAACTCCG	
GLOXD1-Probe	FAM-CGCTACTCTTTCCCC	

Allele-specific primer sequences for MSP and Q-MSP are the same. RT-PCR: Reverse transcription-polymerase chain reaction; MSP: Methylation-specific polymerase chain reaction; Q-MSP: Quantitative MSP.

Table 2 Clinicopathological characteristics of hepatocellular carcinoma patients

Characteristic	Cases
Age, yr	59 ± 14
Mean ± SD	
Gender	
Female	94
Male	66
Hepatitis	
HBV-positive	68
HCV-positive	62
Double-negative	30
Cirrhosis	
No	77
Yes	80
Unknown	3
Tumor size, cm	
≤ 3	52
> 3	108
Nodule	
Solitary	98
Multiple	62
AFP level, ng/mL	
≤ 10	45
> 10	113
Unknown	2
Stage	
Ⅰ	60
Ⅱ	46
Ⅲ	47
Ⅳ	7
Invasion	
No	85
Yes	75
Recurrence	
No	58
Yes	36
Unknown	66
Survival	
No	71
Yes	27
Unknown	62

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein.

and HuH6), and 7 types of pooled tissues (each type was independently pooled with an equal amount of DNA from 5 tissues) were used as the samples for methylation analysis by pyrosequencing. The primer sequences of RT-PCR and MSP are summarized in Table 1.

Patients

The Taiwan Liver Cancer Network (TLCN) is funded by the National Science Council to provide researchers in Taiwan with primary liver cancer tissues and their associated clinical information. With the approval by the TLCN User Committee and the Institutional Review Board of the Tri-Service General Hospital (TSGH), 29 normal parts of liver hemangiomas (as normal controls) and a total of 160 HCC tissues and their paired adjacent nontumor tissues were used in this study. Among these samples, 40 HCC tissues and their paired adjacent nontumor tissues were obtained from TSGH; the others were obtained from TLCN. These specimens were obtained during surgery, frozen immediately in liquid nitrogen and preserved at -80 ℃ until DNA extraction. The diagnosis of HCC samples was confirmed by histology. The clinicopathological characteristics of the patients are summarized in Table 2.

Sodium bisulfite treatment, pyrosequencing, and Q-MSP

Genomic DNA from tissue samples was extracted and prepared for sodium bisulfite treatment and methylation analysis as previously described[21]. Pyrosequencing for the methylation levels of 11 CpG sites in a *GLOXD1* promoter was carried out using PCR and sequencing primers, as previously described[22]. The primers for pyrosequencing were designed with PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany) to amplify and sequence bisulfite-treated DNA. PCR was carried out in a 20

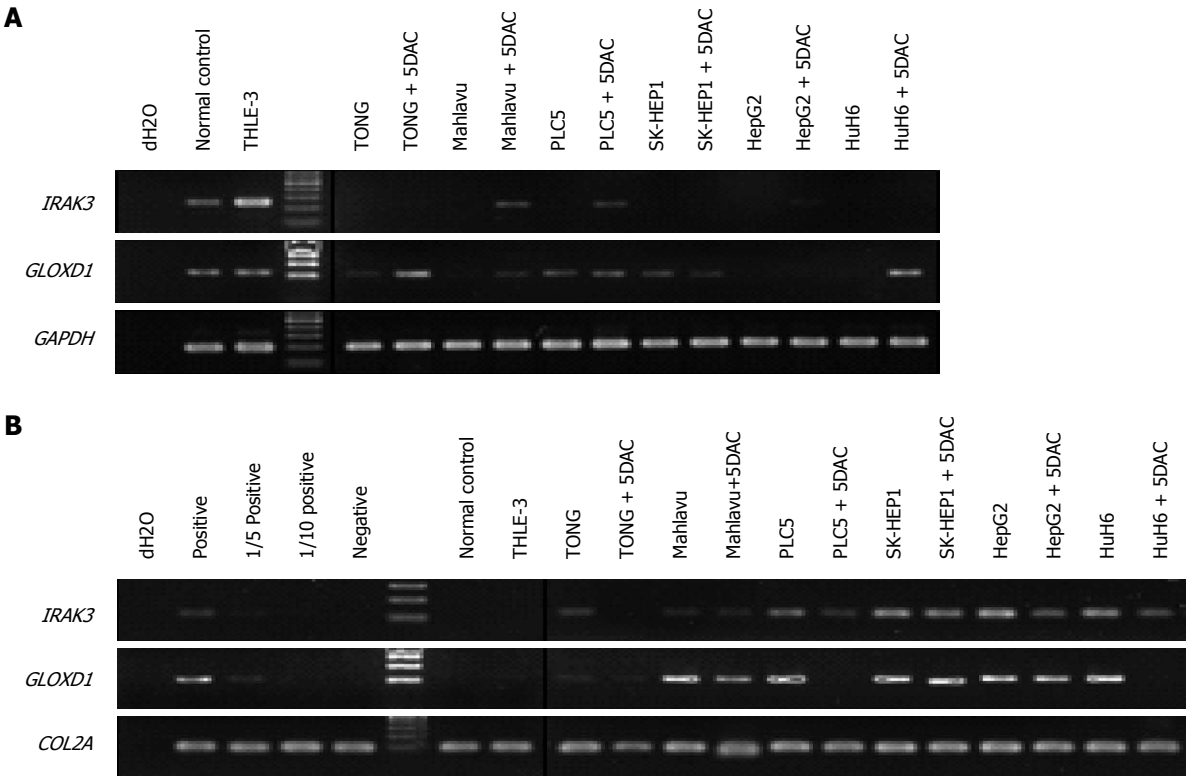

Figure 1 Gene expression and methylation analyses of *IRAK3* and *GLOXD1*. A: Gene expression levels of *IRAK3*, *GLOXD1*, and *GAPDH* (an internal reference gene) were analyzed by RT-PCR in normal controls, THLE-3 cells, 6 HCC cell lines, and HCC cell lines treated with 5DAC; B: Methylation status of *IRAK3*, *GLOXD1*, and *COL2A* (an internal reference gene) was analyzed by MS-PCR with methylated primers in normal controls, THLE-3 cells, 6 HCC cell lines, and HCC cell lines treated with 5DAC. Positive and negative are peripheral *blood* lymphocyte (*PBL*) DNA *in vitro* treated with or without CpG methyltransferase (*M.SssI*). 1/5 positive and 1/10 positive indicate 1:5 and 1:10 dilution of the positive control.

μL reaction mix containing 1 μL bisulfite-converted DNA, 2 × RBC SensiZyme HotStart Taq Mastermix (RBC Bioscience Corp., Taipei, Taiwan), and primers using the following program: 95 ℃ for 15 min, then 49 cycles of 95 ℃ for 30 s, 62 ℃ for 30 s and 72 ℃ for 30 s, with a final extension at 72 ℃ for 10 min. The biotinylated PCR product was purified by binding to streptavidin-sepharose beads, washed, and denatured. The sequencing primer was then added to the PCR products, and pyrosequencing was performed using the PyroMark Q24 system (Qiagen). Q-MSP was performed in the TaqMan probe system using the LightCycler 480 system (Roche Applied Science, Mannheim, Germany) and prepared as previously described[18]. The *COL2A* gene was used as an internal reference by amplifying non-CpG sequences. Results with cycle threshold values (Cq values) of *COL2A* > 38 were defined as detection failures. The DNA methylation level was determined as a methylation index using the following formula: 100 × 2 [(Cq of COL2A) - (Cq of target genes)][23]. Each set of amplifications included a positive control, a negative control, and a non-template control. The primer and probe sequences of pyrosequencing and Q-MSP are summarized in Table 1.

Statistical analysis

The prism software (version 4.03; Graphpad Software Inc, La Jolla, CA) was used for statistical analyses. The unpaired *t*-test and paired *t*-test were used to determine the difference of the methylation index between tissues with different disease status. Fisher's exact test, χ^2 test, and χ^2 test for trend were used to evaluate the association between gene methylation and clinical parameters. Pearson correlation was used to compare the consistency of different techniques. Receiver operating characteristic (ROC) curves were generated to determine the optimal cut-off point of gene methylation for discriminating tumors and normal controls. Kaplan-Meier curves were used to estimate survival fraction of patients for 3 years after treatment. Log-rank tests were used to *compare* the *survival* of patients with or without gene methylation.

RESULTS

Correlation between gene expression and promoter methylation of IRAK3 and GLOXD1 in cell lines

To confirm the results from the methylation array, we first analyzed the correlation between gene expression and promoter methylation of *IRAK3* and *GLOXD1* in cell

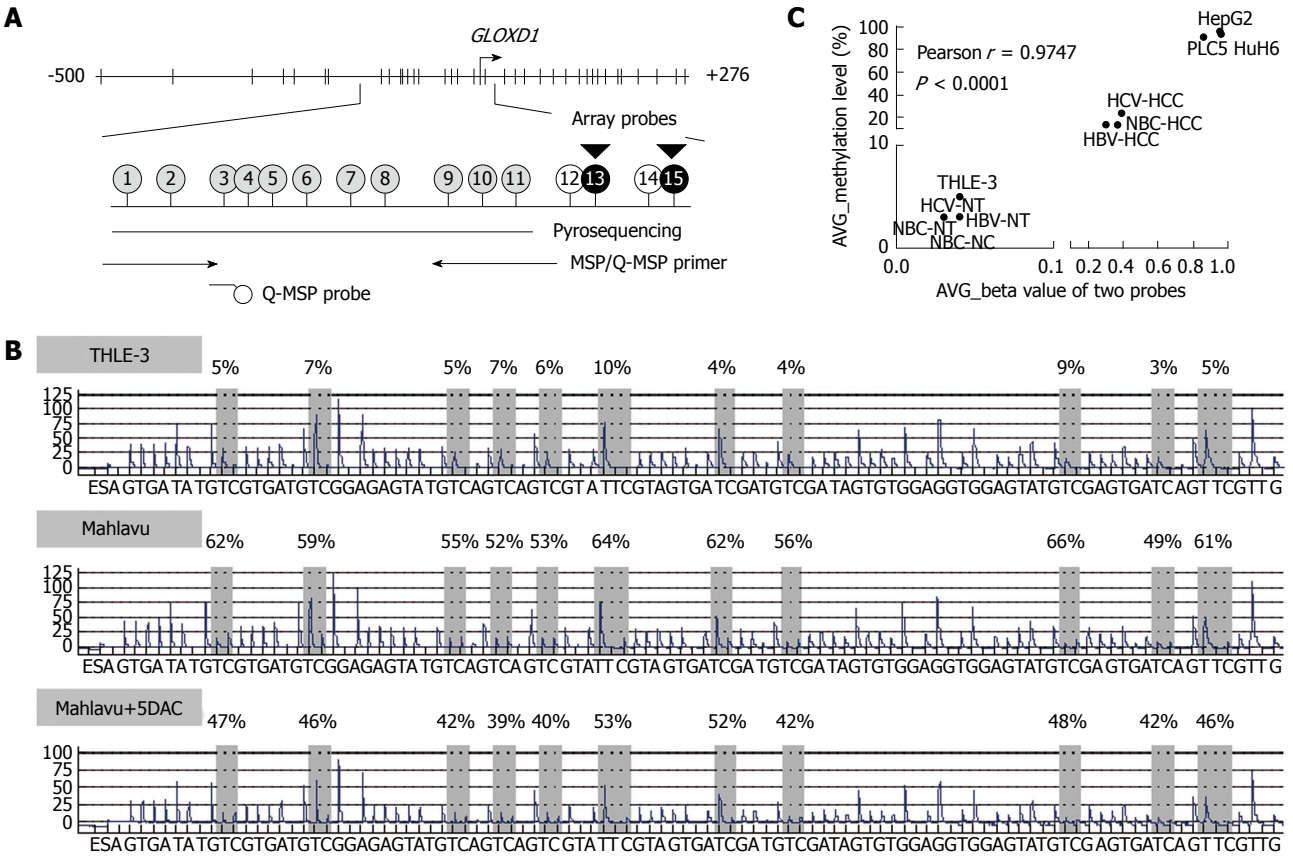

Figure 2 Map of the *GLOXD1* promoter region and representative methylation pattern determined by pyrosequencing. A: The 15 CpG sites within the *GLOXD1* promoter (-500/+276) were addressed using different techniques. Two black circles indicate the 2 CpG sites recognized by probes of the methylation array chip, respectively. Eleven gray circles indicate the 11 CpG sites addressed by pyrosequencing and the 6 CpG sites that MSP/Q-MSP primer set covered (5 CpG sites for allele-specific, *one CpG site for probe*); B: Methylation level of 11 CpG sites addressed by pyrosequencing in THLE-3 cells, Mahlavu cells, and Mahlavu cells treated with 5DAC; C: Pearson correlation was analyzed between the average β value of two array probes and average methylation levels of the 11 CpG sites assessed by pyrosequencing in samples for methylation array, including 4 cell lines and 7 types of liver tissues.

lines by RT-PCR and MSP (Figure 1). Expression analysis showed that *IRAK3* and *GLOXD1* were expressed in normal control and THLE-3 cells but down-regulated in several HCC cell lines (Figure 1A). In addition, the expression of *IRAK3* and *GLOXD1* was partially restored after treatment with 5DAC (a DNA methyltransferase inhibitor). Methylation analysis revealed that *IRAK3* and *GLOXD1* methylation was detected mainly in HCC cell lines, and a partial decrease in the methylated band was also observed in the HCC cell lines after 5DAC treatment (Figure 1B). These results implied that *IRAK3* and *GLOXD1* were down-regulated in HCC cell lines through promoter methylation.

Verification of gene methylation in cell lines and pooled samples by pyrosequencing

We then confirmed the methylation levels of *IRAK3* and *GLOXD1* in cell lines and pooled samples by pyrosequencing (Figure 2; *GLOXD1* as an example). Methylation levels of 11 CpG sites in *GLOXD1* promoter that is close to the two probe sites on array were examined (Figure 2A). It revealed that the *GLOXD1* methylation level was 3%-10% in THLE-3 cells and 49%-66% in Mahlavu cells (Figure 2B). Consistent with MSP, a partial decrease in the methylation level of the *GLOXD1* promoter was observed in Mahlavu cells after 5DAC treatment (39%-53%). Furthermore, the average β value for different array probes was significantly correlated to the average methylation level of the 11 CpG sites in the samples used in the methylation array (r = 0.9747, Figure 2C). In addition, *GLOXD1* methylation was much lower in THLE-3 cells, the pooled normal controls, and each type of pooled nontumor tissues compared to HCC cell lines and all types of pooled tumor tissues. Finally, we designed a primer and probe set based on the CpG methylation results of pyrosequencing to carry out Q-MSP analysis in larger clinical samples.

Methylation analysis of IRAK3 and GLOXD1 in HCC tissues by Q-MSP

To examine the methylation levels of *IRAK3* and *GLOXD1* in HCC, we analyzed 29 normal controls, 160 paired HCC tissues, and their adjacent nontumor tissues using Q-MSP (Figure 3). Promoter methylation of *IRAK3* and *GLOXD1* was both significantly increased in HCC tissues compared to normal controls and nontumor tissues (Figure 3A). Furthermore, to find

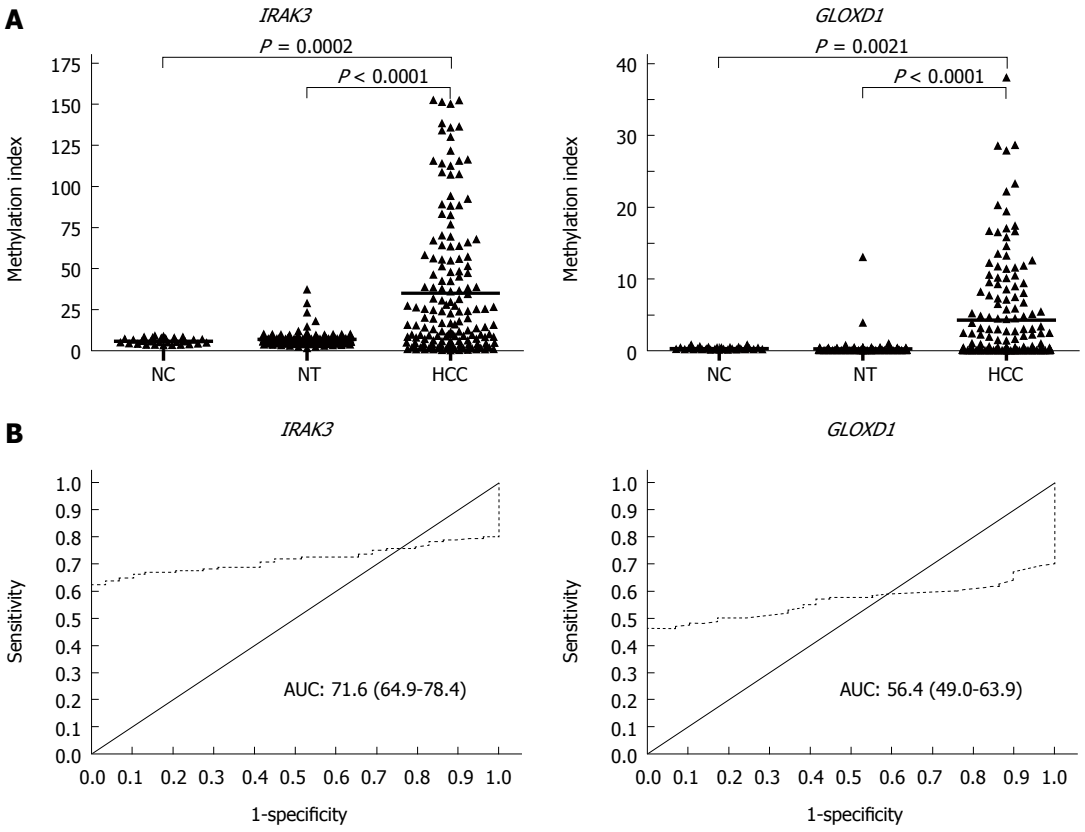

Figure 3 Methylation levels and receiver operating characteristic curve analysis of *IRAK3* and *GLOXD1* in liver tissues. A: Gene methylation was determined in 29 normal controls (NC) and 160 paired hepatocellular carcinoma (HCC) tissues and their adjacent notumor tissues (NT) by quantitative methylation-specific polymerase chain reaction. The results are represented as the difference in the methylation index. The black lines indicate the mean of the methylation index. (NC *vs* HCC, unpaired *t*-test; NT *vs* HCC, paired *t*-test); B: The area under the receiver operating characteristic curve (AUC) for each gene was calculated to discriminate 29 normal individuals and 160 HCC cases.

Table 3 Methylation frequency of *IRAK3* and *GLOXD1* in liver tissues

Symbol	M-Index[1]	No. of methylated cases/total			*P* value[2]
	cut-off value	Normal controls	Nontumor tissues	HCC tissues	
IRAK3	8.90	1/29 (3.4%)	23/160 (14.4%)	102/160 (63.8%)	< 0.0001
GLOXD1	0.60	2/29 (6.9%)	4/160 (2.5%)	75/160 (46.9%)	< 0.0001

[1]Methylation-Index: the best cut-off value to discriminate 29 normal controls and 160 hepatocellular carcinoma (HCC) tissues; [2]χ^2 test for trend.

a best cut-off value for defining methylated cases, ROC curve analysis of each gene was performed to discriminate normal controls and HCC tissues (Figure 3B). As summarized in Table 3, *IRAK3* and *GLOXD1* methylated cases were mainly present in HCC tissues (102/160, 63.8%; 75/160, 46.9%) compared to normal controls (1/29, 3.4%; 2/29, 6.9%).

Association between clinicopathologic parameters and gene methylation

To evaluate the association of gene methylation with clinicopathological characteristics, we analyzed a total of 160 HCC patients (Table 4 and Figure 4). As shown in Table 4, there was a statistically significant correlation between *IRAK3* methylation and tumor stage (P = 0.03), but no significant association was shown between *GLOXD1* methylation and clinicopathological parameters. As shown in Figure 4, HCC patients with *IRAK3* methylation was found to have a trend toward poor 3-year disease-free survival (P = 0.0386, log-rank test) but not in patients with or without *GLOXD1* methylation.

DISCUSSION

Recently, several high-resolution methods for genome-wide methylation analysis have been used in the study of HCC, such as methylated CpG island amplification microarray, bacterial artificial chromosome array-based methylated CpG island amplification, GlodenGate assay, and Infinium assay[8-17]. These results provide evidence that HCC tumors with specific DNA methylation patterns

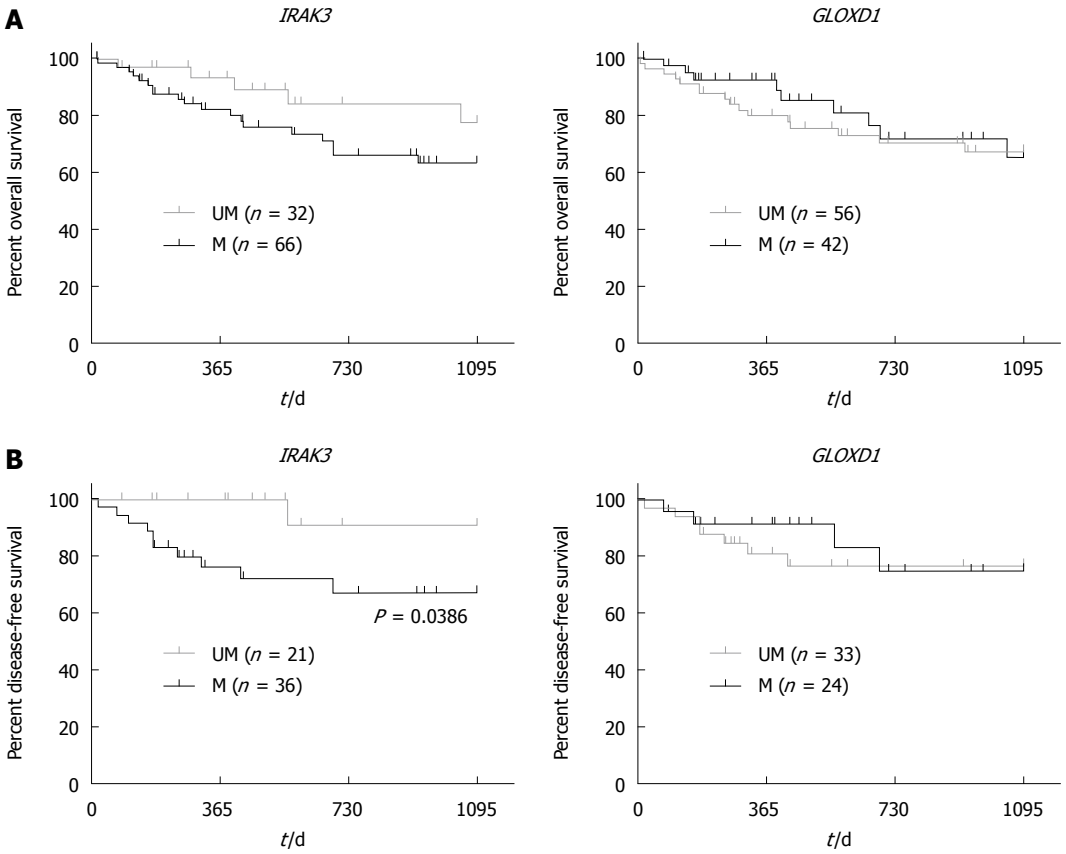

Figure 4 Correlation analyses between gene methylation and the survival of hepatocellular carcinoma patients. A: Survival was analyzed using Kaplan-Meier curves. The plots were made according to the patients with *IRAK3* and *GLOXD1* methylation and 3-year overall survival in 98 hepatocellular carcinoma (HCC) patients, respectively; B: Kaplan-Meier survival curves were made according to the cases with *GLOXD1* and *IRAK3* methylation and 3-year disease-free survival in 57 HCC patients (*P = 0.0386, log-rank test,* UM, unmethylated cases *vs* M, methylated cases).

associated with risk factors or progression of HCC have important clinical applications. In our recent study, we also used the Infinium HumanMethylation27 BeadChip to analyze DNA methylation signatures of HCC and found 1968 genes that were hypermethylated in non-tumor tissue and/or tumor tissue with different viral etiologies. Among 34 genes selected for verification, we further identified that methylation of the *HOXA9* gene could be a helpful biomarker to assist in HCC detection. In this study, we further identified that two novel genes, *IRAK3* and *GLOXD1,* were frequently methylated in HCC. However, both of these two genes were undetectable in plasma. Moreover, *IRAK3* methylation was statistically associated with tumor stage and poor 3-year disease-free survival of HCC patients.

IRAK3 encodes a member of the interleukin-1 receptor-associated kinase protein family that is an essential component of the Toll/IL-R immune signal transduction pathways. This gene is primarily expressed in monocytes and macrophages, and it is also detected in various adult human tissues including the liver[24]. It has been known that IRAK3 functions as a negative regulator in Toll-like receptor signaling and plays an important role in alcohol-induced liver injury[19,25]. In this study, we demonstrated that *IRAK3* was mainly methylated in HCC, and its methylation was positively associated with tumor stage and poor 3-year disease-free survival of patients. Furthermore, the inverse correlation between *IRAK3* expression and methylation status in HCC cell lines was also observed. Overall, our study indicates that *IRAK3* methylation is associated with tumor stage and *poor prognosis* of patients and also implies that IRAK3 might play an important role in the development of HCC. Confirmation of this hypothesis requires further investigation.

GLOXD1 (the official gene symbol is *HPDL*) encodes a protein that may function like 4-hydroxyphenylpyruvate dioxygenase. Although the function of GLOXD1 is still unclear, 4-hydroxyphenylpyruvate dioxygenase is known as an important enzyme in the catabolic pathway of tyrosine in the liver, and defects in this gene will cause diseases such as tyrosinemia type 3[20]. Till now, there are no data regarding the *GLOXD1* methylation in any cancer, even in HCC. We showed that *GLOXD1* expression was down-regulated in HCC cell lines, which was inversely correlated with its methylation status, and *GLOXD1* was frequently

Table 4 Association between gene methylation and clinicopathological characteristics of 160 hepatocellular carcinoma patients

Methylation status	*IRAK3*, M-Index		*P* value	*GLOXD1*, M-Index		*P* value
Characteristic	≤ 8.90	> 8.90		≤ 0.60	> 0.60	
Cases	58	102		85	75	
Age, yr						
≤ 59	27	47	1.00	45	29	0.08
> 59	31	55		40	46	
Gender						
Female	25	41	0.74	39	27	0.26
Male	33	61		46	48	
Hepatitis						
HBV-positive	21	47	0.32	38	30	0.22
HCV-positive	23	39		28	34	
Double-negative	14	16		19	11	
Cirrhosis						
No	33	44	0.07	46	31	0.15
Yes	23	57		38	42	
Unknown	2	1		1	2	
Tumor size, cm						
≤ 3	16	36	0.38	25	27	0.40
> 3	42	66		60	48	
Nodule						
Solitary	37	61	0.74	53	45	0.87
Multiple	21	41		32	30	
AFP level, ng/mL						
≤ 10	14	31	0.58	20	25	0.16
> 10	42	71		65	48	
Unknown	2	0		0	2	
Stage						
Ⅰ	22	38	0.03	28	32	0.66
Ⅱ	23	23		26	20	
Ⅲ	13	34		27	20	
Ⅳ	0	7		4	3	
Invasion						
No	30	55	0.87	39	46	0.06
Yes	28	47		46	29	
Recurrence						
No	22	36	0.38	34	24	0.83
Yes	10	25		19	16	
Unknown	26	41		32	35	
Survival						
No	25	46	0.47	38	33	0.26
Yes	7	20		18	9	
Unknown	26	36		29	33	

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFP: Alpha-fetoprotein.

methylated in HCC tissues. All these results suggest that *GLOXD1* expression might be down-regulated in HCC through the promoter methylation. However, the role of GLOXD1 in the development of HCC requires further investigation.

In this study, we used pyrosequencing to verify the actual methylation pattern of CpG sites within the promoter of the target genes, similar to previous studies. Then, we used the results of pyrosequencing to design a Q-MSP system for validation in a large clinical cohort. Therefore, we easily determined the methylation frequency of the target genes in 349 tissue samples, including 29 normal controls and 160 HCC tissues and their paired adjacent nontumor tissues. According to these results, our data indicate that this quantitative methylation analysis workflow is an efficient and economical approach to verify initially and validate further the data from high-throughput screening.

In summary, our data demonstrated that *IRAK3* and *GLOXD1* were frequently methylated in HCC tissues. Furthermore, *IRAK3* methylation was statistically associated with tumor stage and a poor 3-year disease-free survival rate of HCC patients. This indicated that detection of IRAK3 methylation would be helpful in the prediction of patients' survival as well as the follow-up of patients. Taken together, these findings reveal that methylation of *IRAK3* and *GLOXD1* has a potential clinical application.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a serious disease because it is difficult to detect and therefore leads to a very poor prognosis and high mortality rates. Studying the molecular *mechanisms* of HCC development can help us to design better strategies for disease detection or prognosis prediction.

Research frontiers

Aberrant DNA methylation is associated with the development of HCC, suggesting that gene methylation could provide potential clinical applications in the diagnosis or prognosis of HCC. The authors' previously identified that *IRAK3* and *GLOXD1* were frequently methylated in HCC using a methylation array. However, there are no quantitative data about the methylation level of two novel genes in HCC.

Innovations and breakthroughs

This study demonstrated frequent methylation of two novel genes [interleukin-1 receptor-associated kinase 3 (*IRAK3*) and *GLOXD1*] in HCC and further showed the potential value of *IRAK3* methylation as a biomarker in the prognosis of HCC.

Applications

IRAK3 methylation would be helpful in prediction of patients' survival as well as the follow-up of patients.

Terminology

DNA methylation is a common epigenetic event that alters gene expression. Identification of DNA methylation pattern not only allows for a detailed understanding of the hepatocarcinogenesis but also provides potential clinical applications in the diagnosis or prognosis of HCC.

Peer-review

In this study, the authors demonstrated that *IRAK3* and *GLOXD1* gene expression was down-regulated in HCC cell lines and that it was partially restored after treatment with 5DAC. Importantly, they also found that *IRAK3* methylation was statistically associated with tumor stage and with a trend of poor 3-year disease-free survival in HCC samples. Data are very interesting.

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P- Reviewer: Alisi A, Lakatos PL, Sacco R **S- Editor**: Ma YJ
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ISSN 1007-9327