Blood DNA methylation markers in potentially identified Chinese patients with hepatocellular carcinoma

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Abstract: To determine whether blood DNA methylation is associated with hepatocellular carcinoma (HCC) for Chinese patients, we used genome-wide DNA methylation detection to access the blood samples of Chinese patients by Illumina Human methylation 450K arrays. Sixty potentially gene locis which had different methylated levels significantly among tumor and adjacent normal tissues would be tested in this study. A previous study was conducted in China communities and followed with 7 years. The DNA from white blood cells (WBC) from 192 patients with HCC and 215 matched controls were assayed in this study. The χ 2 test was used to measure data to categorize variables and t -test was uesd to evaluate the different characteristics among groups. Besides, odds ratios (OR) and 95%CI was calculated for matching factors by conditional logistic regression models. We found that high methylation in WNK2 was related to increased risk of HCC, and high methylation in TPO were related to decreased risk of HCC. In our multivariable conditional logistic regression models, these results all exist. Those findings support the methylated changes of WNK2 and TPO may become a new detection index for HCC patients in clinical laboratory. However, the results should be replicated in additional prospective studies with lager samples.

Keywords: DNA methylation; White blood cell; Hepatocellular carcinoma; Epigenetics.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a kind of prevalent type of liver cancer (Ferlay, Shin *et al*., 2010). Several factors include alcoholism, hepatitis B, hepatitis C etc may promote the risk of HCC (Lin, Lei *et al*., 2014). However, about 15% of such diease could be removed completely by clinical operation. If not, patients with HCC would be dead within several months (Larson, 2014).

DNA methylation is one of the common mechanisms of epigenetic regulation. It is achieved via DNA methyltransferase (Dnmts), which utilizes methyl groups provided by S-Adenosyl methionine (SAM) to methylate the fifth carbon atom of cytosine, which is then converted to 5-methylcytosine (5mC) (Gold, Gefter *et al*., 1966). Previous studies revealed that HCC tumors are associated with several specific DNA methylation signatures, which regulate major risk factors and tumor progression (Lambert, Ancey *et al*., 2015, Lin, Wu *et al*., 2015). Epigenetic silencing of these genes such as AKR1B1, GRASP, MAP9etc may be associated with HCC. Besides, a Genome-wide DNA methylation study showed that there were more than two thousand CpG sites which had significantly different expression between different tissues(Yamada, Yasui *et al*., 2016). What's more, a study for Taiwan with sixteen years of follow up found high methylation in WNK2 was related to higher risk of HCC, whereas high methylation in both TPO and MYT1L were related with lower risk (Wu, Shen *et al*., 2016). Those specific mathylation changes in patients with HCC may be helpful for the measurement of HCC susceptibility via methylation biomarkers in WBCs.

Nevertheless, whether changes of DNA methylation in WBCs could be specific risk indicators for Chinese fatients with HCC is not clearly completely. Thus, in this study, we detected the methylated changes of sixty gene loci in WBC's DNA among 192 patients with HCC after enrollment in Pingyi County People's Hospital and 215 controls without cancer.

MATERIALS AND METHODS

Participants

192 patients with HCC and 215 controls were recruited from June 2010 to January 2016 in. Pingyi County People's Hospital Each participant need to finish a designed questionnaire so that we could get the information including basic demographic characteristics, drank history, smoking history, chronic disease and family history of HCC or other kinds of cancers. Each participant also need to donate a fasting blood sample for our study. This study was approved by the medical ethics committee in our hospital, and all the patients agreed and signed the informed consent. Detailed information was provided in previous studies (Wang, Hatch *et al*., 1996, Wu, Wang *et al*., 2012). In this study, we employed these blood samples from those participants with HCC from the beginning of the blood collection to the end of June 2016.

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The blood samples from 215 controls without cancer were also used in this study. Matched controls were picked out by gender, age (±5 years), living area and time of recruitment (±2 months). White blood cells were transported to Pingyi County People's Hospital to process.

Bisulfite conversion of DNA

Genomic DNA was extracted via a salting procedure in our lab. We treated DNA samples with bisulfite in DNA methylation detection kits (Am gen, China). Then resuspended the DNA solution into 20μL distilled water.

Methylation detection

Sixty CpG sites which were shown that methylation changes in HCC compared to matched round normal tissues previously were selected in the 450k array analysis (Shen, Wang *et al*., 2013). The target CpG sites were choosed from the methyalted sites. The selection criteria was shown as follows: (1) the most different methylation levels among tissues; (2) both CpG sites had hypo- and hyper-methylation which were accounted by 50%, respectively; and (3) each site for only each gene. DNA methylation were customized to detect the methylation changes among the CpG sites discovered in the 450k array. And the results of methylation scores were showed as beta values which ranges from zero to one.

STATISTICAL ANALYSIS

The χ 2 test was used as measurement data to categorize variables, and t -test was uesd to evaluate the different characteristics among groups. All analyses include logistic regression model were performed with SPSS 12 for Windows (SPSS Inc., Chicago, IL).

RESULTS

As we could see in table 1, no significant difference in matching indexes including age, sex etc among groups. Smoking was similar between these two groups as well, while the percentage of drank history of control group (16.7%) was lower than in case group (35.4%).

Table 2 shows the contributions of the 60 methylation biomarkers for such cancer. The mean methylation values of twenty-four biomarkers were below 10% in these two groups, whereas thirteen DNA methylation markers had this values above ninety percent. About 23 DNA methylation markers had the levels vary from ten to ninety percent. The mean levels of these two methylation markers: cg10272601 and cg12680131, which had significant difference between cases and controls.

Table 3 showed our logistic regression model. In couclusion, HBsAg (+) was related with higher HCC risk (OR=4.80, 95%CI: 3.31-7.25). The alcohol factor had a higher risk of HCC (OR=2.03, 95%CI: 1.12-4.28). For cg10272601, cg12680131 hypermethylation, the ORs (95%CI) were 2.32(1.39-3.71) and 0.61(0.42-0.89).

DISCUSSION

In this study, we found WNK2 (cg10272601) and TPO (cg12680131) were realted with the risk of HCC. WNK2

Table 1: Sociodemographic characteristics of hepatocellular carcinoma cases and matched controls

Variable	Cases (%)	Controls (%)	P
n	192	215	
Age (yr, mean, SD)	51.7(10.2)	52.3(11.3)	0.68
BMI (mean, SD)	23.2(3.5)	23.9(3.6)	0.21
Gender			
Female	90(46.9)	105(48.8)	0.75
Male	102(53.1)	110(51.2)	
HBsAg			
Negative	81(42.2)	98(45.6)	< 0.0001
Positive	111(57.8)	117(54.4)	
Missing	0	0	
Anti-HCV			
Negative	112(58.3)	168(78.1)	< 0.0001
Positive	49(25.5)	23(10.7)	
Missing	31(14.4)	24(11.2)	
Smoking			
Never	88(45.8)	173(80.5)	0.52
Ever	104(54.2)	42(19.5)	
Alcohol			
Never	124(64.6)	179(83.3)	0.037

Table 2: Distribution of DNA methylation by hepatocellular carcinoma status

Locus	Gene	HCC cases		Controls		P
		Mean	SD	Mean	SD	
cg00028598	GABRA5	0.87	0.06	0.87	0.1	0.79
cg00108164	ACP1	0.03	0.04	0.03	0.04	0.53
cg00753478	LDHB	0.11	0.1	0.11	0.09	0.1
cg00817367	GRASP	0.12	0.06	0.12	0.04	0.21
cg01530024	STK32B	0.04	0.11	0.05	0.1	0.77
cg01860297	BASP1	0.89	0.06	0.89	0.11	0.47
cg02527669	OBSL1	0.99	0.05	0.99	0.08	0.51
cg02553663	SECTM1	0.05	0.07	0.05	0.06	0.63
cg02736548	FAM109B	0.06	0.12	0.06	0.12	0.44
cg03396005	APCDD1	0.11	0.07	0.11	0.09	0.97
cg03621881	BRUNOL6	0.95	0.08	0.95	0.07	0.68
cg05328339	PTPRN2	0.07	0.12	0.06	0.13	0.53
cg05661282	ZNF154	0.92	0.08	0.92	0.11	0.73
cg05970721	HS3ST2	0.06	0.13	0.06	0.13	0.47
cg06382344	TBR1	0.93	0.06	0.93	0.08	0.11
cg06445348	ILDR2	0.05	0.09	0.05	0.04	0.22
cg06641285	TIMP2	0.04	0.05	0.04	0.08	0.72
cg07061738	SMOC2	0.96	0.11	0.96	0.14	0.73
cg07759394	GLB1L2	0.03	0.06	0.03	0.05	0.42
cg07765706	KCNQ3	0.97	0.06	0.97	0.11	0.1
cg09210956	SNTG2	0.69	0.11	0.69	0.15	0.91
cg09433131	KCNB2	0.96	0.08	0.96	0.13	0.41
cg09901035	PLEKHG4B	0.89	0.08	0.89	0.11	0.64
cg10272601	WNK2	0.32	0.09	0.32	0.11	0.02
cg10342963	IGF1R	0.83	0.15	0.82	0.18	0.05
cg11686528	ABR	0.03	0.09	0.04	0.09	0.58
cg12610564	SLC39A12	0.89	0.03	1	0.1	0.07
cg12680131	TPO	0.82	0.11	0.82	0.14	0.16
cg12852139	MYO10	0.98	0.04	0.98	0.09	0.68
cg13204512	RNF135	0.03	0.08	0.04	0.05	0.21
cg13517866	SMOC2	0.91	0.13	0.92	0.13	0.56
cg13564825	PPP1R14A	0.03	0.07	0.04	0.05	0.96
cg13604246		0.13	0.1	0.14	0.12	0.66
cg13895235	PRKAR1B	0.03	0.03	0.04	0.06	0.37
cg14486338	KCNS2	0.14	0.09	0.15	0.1	0.51
cg14644001	PRRT1	0.06	0.05	0.07	0.08	0.61
cg14645545	SLC11A1	0.22	0.14	0.22	0.15	0.81
cg14715697	HRNBP3	0.72	0.1	0.74	0.11	0.18
cg14866200	SHISA3	0.04	0.09	0.05	0.09	0.72
cg14988503	CDKL2	0.04	0.05	0.05	0.06	0.83
cg15167871	TCERG1L	0.94	0.12	0.95	0.14	0.96
cg17497608	FZD1	0.85	0.13	0.87	0.15	0.41
cg18537730	IZUMO1	0.18	0.09	0.19	0.11	0.61
cg19429281	ZNF702P	0.04	0.03	0.05	0.06	0.38
cg19464917	ISL2	0.08	0.06	0.08	0.06	0.15
cg20399616	BCAT1	0.07	0.1	0.07	0.11	0.38
cg21385746	LOC150568	0.98	0.12	0.98	0.14	0.78
cg21472506	OTX1	0.03	0.06	0.04	0.07	0.96
cg21790626	ZNF154	0.06	0.06	0.08	0.08	0.3

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Locus	Gene	HCC cases		Controls		P
		Mean	SD	Mean	SD	
cg22403469	RIMBP2	0.85	0.07	0.86	0.11	0.61
cg22511877	MYT1L	0.49	0.16	0.51	0.15	0.15
cg22655988	CRMP1	0.98	0.1	0.99	0.13	0.75
cg22789900	MIXL1	0.02	0.03	0.04	0.07	0.53
cg23004031	MGMT	0.61	0.33	0.61	0.35	0.39
cg23498518	POM121L12	0.81	0.09	0.83	0.13	0.34
cg23864180	ADARB2	0.92	0.08	0.92	0.1	0.24
cg24432073	CDKL2	0.04	0.05	0.04	0.07	0.82
cg24563094	FAM59B	0.12	0.06	0.12	0.08	0.53
cg25577023	AMN	0.11	0.11	0.11	0.12	0.8
cg26841013	WNT3A	0.05	0.04	0.05	0.06	0.43

P value for student's t-test.

Table 3: Multiple variables model for DNA methylation and hepatocellular carcinoma

Gene	Locus	OR (95%CI)	P
WNK2	cg10272601	2.32(1.39-3.71)	0.0012
TPO	cg12680131	0.61(0.42-0.89)	0.02
HBsAg	(positive vs negative)	4.80(3.31-7.25)	<0.0001
Alcohol	(yes vs no)	2.03(1.12-4.28)	0.01

HBsAg: Hepatitis B virus surface antigen.

gene could encode WNK2 enzyme, which also acts by interfering with the activity of PAK1 by controlling the balance of the activity of upstream regulators of PAK1 activity (Moniz, Verissimo *et al*., 2007). Such kind of serine/threonine kinase could regulate electrolyte homeostasis, cell signaling, survival, and proliferation.

Thyroid per oxidase (TPO), a kind of hemoprotein enzyme, mainly expressed in the thyroid (Kimura, Kotani *et al*., 1987). However, the role of TPO for HCC has not shed on light. Besides, previous studies also reported that myelin transcription factor 1-like (MYT1L) which is a significant member of MYT family also related with HCC (Wu, Shen *et al*., 2016). However, our study didn't find such result. Thus, futher study need to probe into the role of TPO hypomethylation in hepatocarcinogenesis and whether MYT1L involved in such diease from lager samples

Nowadays, the clinical course of HCC are highly variable due to the fact that it is may affected by complex environmental factors. Growing evidence has confirmed the role of epigenetic process in HCC, especially epigenetic regulation of genes related to several signaling pathways like insulin-like growth factor (IGF) signaling pathway in the promoters of HCC-associated genes (Chen, Zhang *et al*., 2015, El Tayebi and Abdelaziz, 2016, Kanda, Sugimoto *et al*., 2015). Those all stress the significance of epigenetic modulation in HCC development. Although studies in methylated pathogenesis of HCC were not very clearly and the therapeutic strategies were not perfect, there is a high recurrence of HCC after surgical tumor removal. Whether DNA methylation might solve this problem still requires to be further explored.

What's more, in recent studies, aberrant DNA methylation and demethylation has served as a novel biomarker in cancers' therapy like HCC, especially several changes in methylation or demethylation are specific in HCC (Hardy, Zeybel *et al*., 2016, Messier, Boyd *et al*., 2016, Wu, Sun *et al*., 2016). The methylated changes of WNK2 and TPO may become a new detection index for HCC patients in clinical laboratory. Besides, our study still need to adjust due to the limited sample size. We should do this in further data analysis when we haver lager samples in the future. Characterization of other epigenetic factors could be probably uesd in treated studies in the future. This field also need extra efforts to probe that whether epigenetic factors that can be potentially used for diagnostic and prognostic purposes.

In conclusion, we probed the evidence between DNA methylation in WBC and the risk of cancer, especially HCC. The study paid attention to the relationship of HCC differently methylated loci observed in tumor tissues with HCC risk by using information analysis. These findings could lead to a new detection method of blood measurement of DNA methylation to detect the risk of HCC.

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