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Original Article

Association of polymorphisms in the 3'UTR of ErbB2 with the risk of hepatocellular carcinoma

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Abstract: ErbB2 can act as an oncogene and its related signaling server as essential roles in tumorigenesis. Polymorphisms in ErbB2 have been reported related to bad prognosis of with human cancer, however the association between SNP in 3'UTR of ErbB2 so far. In this study rs113054794 and rs76322625 located in the 3'UTR of ErbB2 was selected to evaluate its relationship with the risk of HCC among Chinese population. In this study, SNP of rs113054794 in the 3'-UTR of ErbB2 was involved as a risk factor in the occurrence of HCC. SNP rs113054794 could be regulated by miR-221-5p which caused an up-regulation of ErbB2 in patients. Furthermore, the carriers of CA and AA genotype in rs113054794 presented poor differentiation, a big tumor size as well as the high probability of metastasis. In conclusion, our findings have shown that the SNP rs113054794 in ErbB2 3'-UTR, through disrupting the regulatory role of miR-221-5p in ErbB2 expression, which might act as a promotion factor in the pathogenesis of HCC.

Keywords: SNP, ErbB2, HCC, miRNA

Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. Which is now the third leading cause of cancer deaths worldwide, with over 500,000 people affected? The endemic high prevalence of hepatitis B and hepatitis C strongly predisposes to the development of chronic liver disease and subsequent development of hepatocellular carcinoma [1, 2].

Several lines of evidence indicate that development of HCC is a multistep process affected by both inherited and acquired factors leading to the transformation of normal hepatocytes into malignant clones [3]. SNPs correspond to a modification of a DNA sequence due to the change of a single nucleotide; they account for >90% of allelic disparities scattered throughout the human genome [4, 5]. Although the vast majority of these modifications are situated in non-coding regions, some can modify gene-product expression and function, which may affect biological pathways. If the genes are involved in liver carcinogenesis, these modifications may partly explain the genetic heritability thought to influence individual susceptibility to HCC [6-8].

Receptor tyrosine-protein kinase erbB-2 is a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family. Amplification or overexpression of this oncogene has been shown to play an important role in the development and progression of certain aggressive malignancy [9, 10]. Up to 1994, erbb2 expression was analyzed within human HCC, 61.6% of candidate patients suffered by hepatocellular carcinoma was significantly raise of serum erbb2, 63.3% of liver cirrhosis and 43.2% of chronic hepatitis patients was also positive for serum erbb2, which suggested that serum c-erbB-2 protein levels are greatly influenced by liver dysfunction [11, 12]. Recently, significantly high expression of Erbb2 was observed within human HCC especially in those HBV infected HCC patients, mechanism studies revealed that ErbB-2/beta-catenin up-regulation contributes importantly to the mechanism of HBxAg mediated hepatocellular growth [13].

MiRNAs are small, non-coding RNA molecules of 19-25 nucleotides which have been reported to play important roles by regulating cell differentiation, proliferation, migration and apoptosis [14]. MiRNAs can not only negatively regulate their target genes expression at the post-transcription level through binding to 3' untranslated regions (UTRs) of their targets message RNAs, [15, 16]. Although several studies have revealed that ErbB2 can be suppressed by other miRNAs, few study has been reported describing loss regulation by the 3'UTR region SNP of ErbB2 by certain miRNAs.

In this study, we focused on the SNPs in the 3'UTR of ErbB2 which has rarely been reported before. By using the bioinformatics software (http://www.bioguo.org/miRNASNP/), we obtained all the SNPs which could regulate by different miRNAs as candidate SNPs and further investigated the allele distribution in a case-control study.

Materials and methods

Study subjects

The hospital-based case-control study consists of 693 patients newly diagnosed with HCC and 752 cancer-free controls. All the subjects were genetically unrelated Han Chinese recruited from the First Affiliated Hospital of Nanjing Medical University (Nanjing China), January 2010 and May 2015. Patients with other hematological disorders, previous history of cancers, radiotherapy and chemotherapy were excluded. The cancer-free control subjects from the same geographic area showed no evidence of genetic relationship with the cases. The patients were classified according to World Health Organization classification. This study was approved by the Ethical Committee of the First Affiliated Hospital of Nanjing Medical University, and every patient had written informed consent.

Genotype

We extracted genomic DNA from peripheral whole blood of every validation subject by using QIAamp DNA blood mini kits (Qiagen, Germany) according to the manufacturer's instructions. Genotyping was performed with the TaqMan SNP Genotyping Assay. The PCR reactions were carried out in a total volume of 5 μL containing TaqMan Universal Master Mix, 80X SNP Genotyping AssayMix, Dnase-free water and 10-ng genomic DNA. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The 384-well ABI 7900HT Real Time PCR System

Real time PCR assay

Real time polymerase chain reaction (RT-PCR) was performed to determine whether the C/G to A mutation changed the expression level of ERBB2. The amplification conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 55°C for 40 s, and 72°C for 30 seconds, and finally 4°C for 30 minutes for cooling.

Cell lines and cell culture

HCC cell lines 97H and HepG2 were purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, USA) and grown in humidified 5% CO_2 at 37°C. MiR-221-5p and miR-3116 mimics and normal control were obtained from Genepharma (Shanghai, China). The transfection was conducted by using Lipofectamine 2000 (Invitrogen Corp, CA, USA).

Prediction of miRNAs binding to the SNP

Based on our bioinformatics analysis by using the bioinformatics software (http://www.bioguo.org/miRNASNP/) to predict the related SNPs in the 3'UTR of ERBB2 which could regulate by different miRNAs.

Construction of luciferase-based reporter plasmids

All the fragment of the 3'UTR containing the mutated alleles we desired were amplified and constructed. The PCR production was cloned into the pGL3-promoterless luciferase-based plasmid (Promega) at the cloning site between KpnI and XhoI. The amplified fragment was verified by DNA sequencing.

Dual-luciferase reporter assay

The 3'-UTR sequence of ERBB2 predicted to interact with miR-603 or a mutated sequence with the predicted target sites were inserted

Table 1. Frequency distributions of selected variables in patients and cancer-free controls

Variables	Cases (*n*=693)		Controls (*n*=752)		*P**
	N	%	*N*	%	
Age (years)					0.6359
≤50	347	50.07%	386	51.33%	
>50	346	49.93%	366	48.67%	
Gender					0.1707
Male	373	53.82%	377	50.13%	
Female	320	46.18%	375	49.87%	
Parental HBV infection status					<0.0001
Negative	442	63.78%	361	48.01%	
Positive	251	36.22%	391	51.99%	
Differentiation grade					
Well	318	36.43			
Moderate	253	28.98			
Poorly	302	34.59			
Tumor Size (cm)					
≤5 cm	317	45.74%			
>5 cm	376	54.26%			
Tumor Number					
Solid	402	58.01%			
Multiple	291	41.99%			
Metastasis					
Yes	278	40.12%			
No	415	59.88%			

*Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls.

Table 2. Association between SNPs and HCC

SNP	chr	3'UTR position	Associated miRNA	Allele
rs113054794	17	104-125	hsa-miR-221-5p	C/A
rs2910164	17	367-387	hsa-miR-146a-3p	G/C
rs185147690	17	178-199	hsa-miR-3116	G/A
rs192207383	17	182-203	hsa-miR-3622b-5p	C/A

Chr = chromosome; UTR = Untranslated Regions.

into the KpnI and XhoI sites of pGL3 promoter vector (Genscript, Nanjing, China). For reporter assay, cells were plated onto 24-well plates and transfected with 100 ng of pGL3-ERBB2 wild, pGL3-ErbB2 mutant, miRNA and their mimics, respectively by using Lipofectamine 2000 (Invitrogen Corp, CA, USA). A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected to normalize the differences in transfection efficiency. Transfection was repeated three times in triplicate.

Statistical analysis

The association between rs113054794 and rs185147690 genotypes and the risk of HCC was evaluated by calculating the odds ratios (ORs) and their 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. Stratification analysis was performed according to the clinical characteristic and risk classification to determine the genotype distribution in cases and controls as well as their association with the risk of HCC. The difference of the expression levels of ERBB2 with three genotypes and the difference of the relative luciferase activities between the wild and mutant genotype were evaluated by independent-sample t test. All statistical tests were two-sided and P<0.05 was considered statistically significant. Statistical analysis was performed with SPSS 13.0 (SPSS Ltd.) and SAS software (version 9.1.3; SAS Institute, Cary, NC, USA). The graphs were generated by Graphpad Prism 5.0 (Graphpad Software, Inc.).

Results

Subject characteristics

The characteristics of the 693 HCC patients and 752 healthy controls are summarized. No statistically significant differences were observed between cases and controls in terms of sex and age (both P>0.05). This indicates that the frequency matching was adequate. Patients suffering from heritage of HBV infection indicated to be the susceptible population by comparing with controls (**Table 1**).

The miRSNPs in the ErbB2 gene 3'-UTR

In order to investigate the miRNA associated SNPs in the 3'UTR of ERBB2, we first found all

Table 3. Genotype frequencies of the ErbB2 at rs113054794 and rs185147690 polymorphism among HCC cases and controls

Genotype	Cases (*n*=693)		Controls (*n*=752)		OR (95% CI)[a]	*P* Value[a]
	N	%	N	%		
rs113054794						
CC	265	38.24%	578	81.29%	1.00	<0.0001
CA	217	31.31%	121	17.02%	3.91 (1.34-1.93)	
AA	211	30.45%	54	6.19%	8.52 (1.27-1.79)	
A Carrier	428	61.76%	175	23.20%	5.33 (1.55-1.98)	<0.0001
rs185147690						
GG	237	34.20%	418	55.59%	1.00	<0.0001
GA	255	36.80%	201	26.73%	2.23 (1.45-1.79)	
AA	201	29.00%	134	17.82%	2.64 (1.36-1.79)	
A Carrier	456	65.80%	335	44.55%	2.40 (1.05-1.85)	<0.0001

[a]The ORs, 95% CIs and *P* value were calculated after adjusting for age, gender, parental HBV infection history and family cancer history.

the SNPs from the SNP databases NCBI db SNP BUILED 129 and ENSEMBL v58 in the 3'-UTR of ERBB2 gene with the minor allele frequency (MAF) >0.05. We then used bioinformatics software Diana-Micro, RNA hybrid to predict miRNAs that can bind to the ERBB2 3'-UTR. The miRNASNP database was also applied to explore the miRNAs which could also bind to the 3'-UTR of patients harbored the SNP. As shown in **Table 2**. Finally, we obtained 4 SNPs in the 3'UTR which could be regulated by four different miRNAs (hsa-miR-221-5p, hsa-miR-146a-3p, hsa-miR-3116 and hsa-miR-3622b-5p). The positions of the SNPs in 3'UTR of ERBB2 as well as the variants were listed. Further genotyping was performed to detect the distribution of allele gene of the 2 SNPs in our research.

Correlation of rs113054794 and rs185147690 with HCC

Interestingly, two SNPs (rs113054794 and rs185147690) indicated a significant difference in HCC patients respectively; as listed in **Table 3**. The results showed that the genotypes of both rs113054794 and rs185147690 were in Hardy--Weinberg equilibrium distribution pattern in the healthy control group (P<0.0001). Further, logistic regression analysis results revealed that the HCC genotype GG genotype in SNP rs113054794 presented a significant decreased risk of HCC as compared with the A carrier genotype (CA and AA) (P<0.0001). Similarly, rs185147690 presented a significant decreased risk of HCC as compared with the A carrier genotype (GA and AA) (P<0.0001). Logistic regression analyses indicated that individuals with the rs113054794 GA and AA genotype was significantly associated with HCC risk (OR=3.91, 95% CI=1.34-1.93 for CA; OR=8.52, 95% CI=1.27-1.79 for AA; and OR=5.33; 95% CI=1.55-1.98 for A carrier). Similarly, the rs185147690 GA and AA genotype was also significantly associated with HCC risk (OR=2.23, 95% CI=1.45-1.79 for GA; OR=2.64, 95% CI=1.36-1.79 for AA; and OR=2.40; 95% CI=1.05-1.85 for A carrier). All ORs were adjusted for sex, age, and smoking status, drinking history or family cancer history.

The effect of rs113054794 and rs185147690 on the regulatory role of miRNAs in ErbB2 expression

Since the SNP rs113054794 and rs185147690 were predicted to locate in the binding site of miR-221-5p and miR-3116 respectively. We hypothesized that the expression of ErbB2 might be regulated by these microRNAs, which can be impacted by rs113054794 and rs185147690. To test whether or not the inhibitory role of these miRNAs impacted by the two SNPs, we first detected the expression level of ErbB2 expression level in patients harbored the CC, CA and AA genotypes as well as the patients with GG, GA and AA genotypes. We found that patients with CA or AA genotypes presented a significantly higher level of ERBB2 by comparing with the patients with CC genotype in SNP rs113054794 (**Figure 1A**); however, on the contrary, in rs185147690 of ErbB2 3'UTR, the patients with genotype of GA, AA also has no significantly increased lever of ErbB2 than those with GG genotypes (**Figure 2A**). We then constructed pGL3 vectors including the allele-specific binding sequences of both SNPs (**Figures 1B** and **2B**), and then co-transfected it with miR-221-5p and miR-3116 as well as the controls into HCC cell lines including 97H and HepG2. As presented in **Figure 1C**, **1D**, a-allele-specific of both pGL3 construct was not significantly suppressed by miR-221-5p, while results

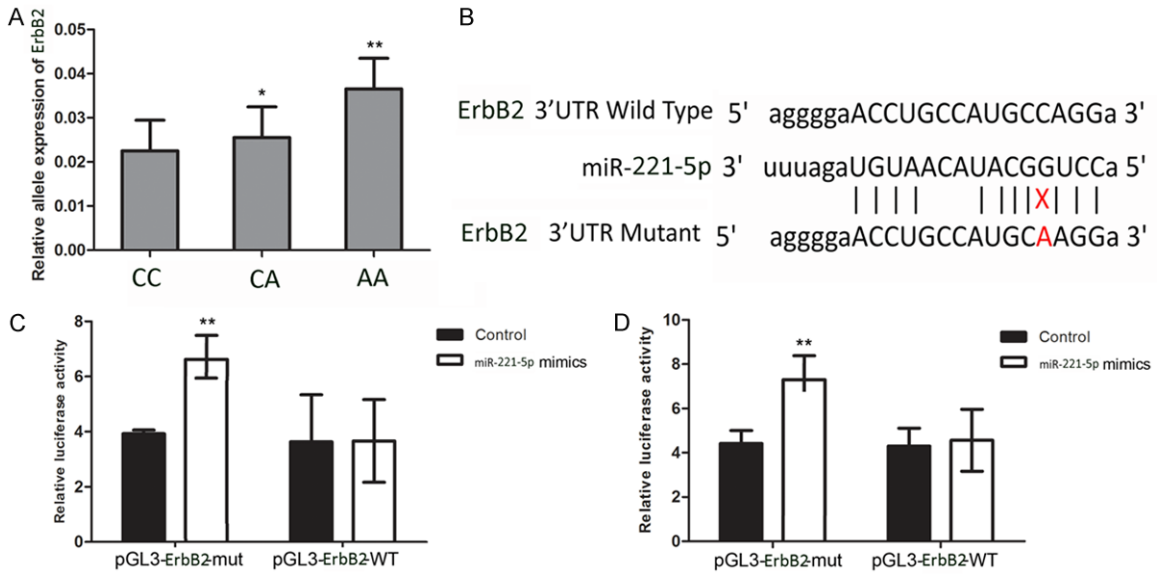

Figure 1. SNP rs113054794 in 3'UTR of HCC patients with CA/AA genotype can up-regulate of ErbB2 transcription by deregulated by miR-221-5p. A: The expression level of ErbB2 was determined by RT-PCR in patients with CC, CA and AA genotypes. B: Bioinformatics predicted the binding site between the miR-221-5p with ErbB2 and the mutation types were conducted into the pGL3 plasmid as presented. C, D: Cells were co-transfected with miR-221-5p mimics or control, Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the 97H cell line (left panel) while the right indicated HepG2 cell line (right panel). Data were presented as the mean ± SEM. *Indicates a significant difference ($P<0.05$).

was reversed in rs185147690. These find might suggested that rs113054794 was regulated by miR-221-5p while rs185147690 was not.

Stratified analyses of association between ErbB2 polymorphism and HCC risk

Then, we did stratified analysis of the association of the rs113054794 genotypes with the clinicopathological parameters of HCC (**Table 4**). We found a significant association of the rs113054794 genotypes with the tumor size, differentiation and metastasis as well as. Compared with the CC homozygote, the carriers of A genotype presented significant large tumor size, poor differentiation as well as the high potential of metastasis.

Discussion

In the present study, we investigated the relationship between miRSNPs within the 3'-UTR of ERBB2 gene and the risk of HCC. We observed that ERBB2 rs113054794 with either CA, AA genotype were associated with significantly increased tumor size, poor differentiation and metastasis of HCC. We further found that the SNP rs113054794, locating in the binding sites of miR-221-5p, can disrupt their inhibitory role on ERBB2 expression, played an important role in the development of HCC.

ErbB2 was one of member of EGFR family which can transduce growth signaling intracellular through various of signaling including PI3K/AKT, MAPK, PKC etc. [10, 17]. Several studies have revealed that ErbB2 was overexpressed within human hepatocellular carcinoma [11-13]. High levels of soluble human ErbB2 were found in chronic HCV hepatitis and related HCC. plus with Child-Pugh score in those patients with LC and with tumor size in those patients with HCC, confirming that this protein may be used as a predictor of liver damage and of inflammatory process leading to fibrosis, cirrhosis, and subsequently to cancer. The expression of also related to HBV infection. HBx expression increased HER2 protein level via enhancing its mRNA stability. The induction of RNA-binding protein HuR expression by HBx mediated the HER2 mRNA stabilization [13].

ErbB2 SNP was previously reported to in many human malignancies including breast cancer [18], colon cancer [19], gastric cancer [20],

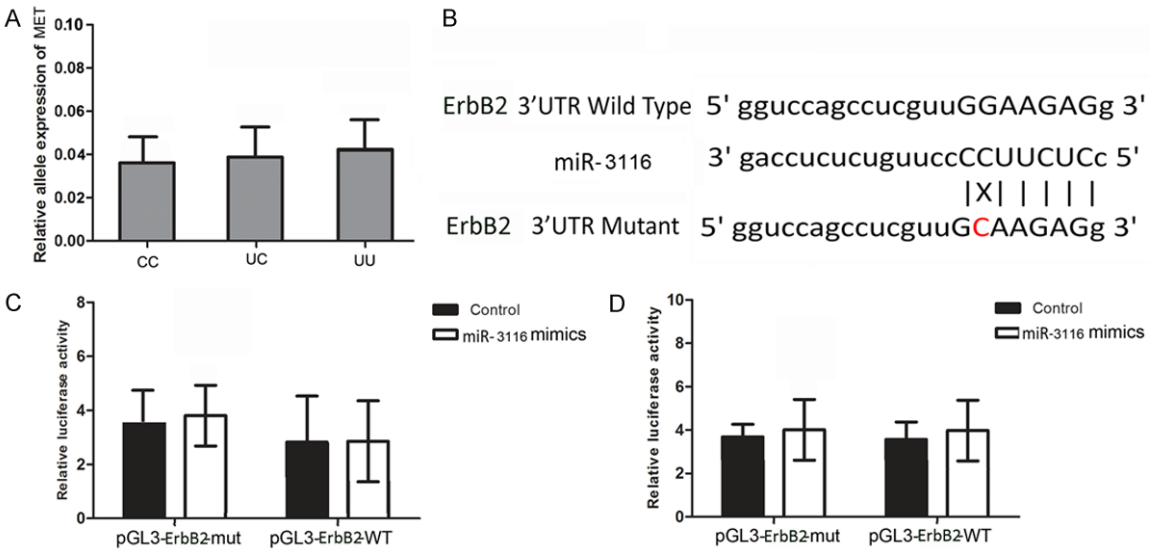

Figure 2. SNP rs185147690 in 3'UTR of HCC patients with GA/AA genotype cannot up-regulate of ErbB2 transcription by deregulated by miR-3116. A: The expression level of ERBB2 was determined by RT-PCR in patients with GG, GA and AA genotypes. B: Bioinformatics predicted the binding site between the miR-3116 with ErbB2 and the mutation types were conducted into the pGL3 plasmid as presented. C, D: Cells were co-transfected with miR-3116 mimics or control, Renilla luciferase vector pRL-SV40 for 48 h. Both firefly and Renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with Renilla luciferase signals. Left panel indicated the 97H cell line (left panel) while the right indicated HepG2 cell line (right panel). Data were presented as the mean ± SEM. *Indicates a significant difference ($P<0.05$).

Table 4. Stratified analysis of rs113054794 genotype with clinicopathological parameters of HCC

Feather	Genotype				CA vs. CC	AA vs. CC	A carrier vs. GG
	CC	CA	AA	A carrier	*P* Value*	*P* Value*	*P* Value*
Age (years)							
≤50	133	113	101	214	0.621	0.712	0.937
>50	132	104	110	214			
Gender							
Male	126	103	144	247	0.986	0.538	0.679
Female	139	114	176	290			
Differentiation grade							
Well	142	123	53	318	0.017	<0.0001	<0.0001
Moderate	113	74	66	253			
Poorly	10	20	71	101			
Tumor Size (cm)							
≤5 cm	143	109	65	174	0.414	<0.0001	0.0006
>5 cm	122	108	146	254			
Tumor Number							
Solitary	142	122	138	260	0.563	0.0093	0.0634
Multiple	123	95	73	168			
Metastasis							
Yes	249	249	249	249	<0.0001	<0.0001	<0.0001
No	179	179	179	179			

*Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls.

gastroesophageal adenocarcinoma [21], especially in their metastatic sites. However, SNP in 3'UTR of ErbB2 which related to gain or loss function was rarely reported and recently con-

sidered as another genetically factors in heterogeneityindividually. In present study, we found four potentially SNP in 3'UTR region of ErbB2 in which one of them was really impact on the regulation by miRNA through loss of blocking effect of binding.

In summary, we reported the first evidence that the SNP rs113054794 in ERBB2 3'-UTR was involved in the occurrence of HCC by acting as a promotion factor, and this result can also serve as one of the reasons of overexpression of ErbB2 in human HCC. SNP rs113054794 could be regulated by miR-221-5p which caused an up-regulation of ERBB2 in patients. This SNP was also found to be related to the clinicopathological features of HCC, suggesting it may have important roles in promoting tumor development. Our results support the hypothesis that genetic variants interrupting miRNAs mediated regulation tumor suppressors would be involved in HCC etiology.

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Disclosure of conflict of interest

None.

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