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ORIGINAL RESEARCH

The EGFR Polymorphism Increased the Risk of Hepatocellular Carcinoma Through the miR-3196-Dependent Approach in Chinese Han Population

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Background: Previous studies have shown that epidermal growth factor receptor (EGFR) promotes cell proliferation through the PI3K-Akt-mTOR signaling pathway and participates in the occurrence and development of hepatocellular carcinoma (HCC). Here, we focused on the functional polymorphism of EGFR in the 3′-untranslated region (UTR), aiming to reveal the potential mechanisms by which functional polymorphism is associated with the risk and development of HCC in the Han Chinese population.

Methods: This study was a hospital-based case-control study. A total of 600 patients were enrolled, and another 600 healthy volunteers served as controls. The miR-associated SNPs in EGFR were screened, and genotyping was performed by TaqMan allele differential analysis. In this study, genotyping, real-time PCR, cell transfection and double luciferase reporter gene were used for subsequent analysis.

Results: HBV/HCV infection instead of alcohol exposure, smoking exposure, hypertension or diabetes mellitus was associated with an increased risk of HCC. Compared with TT genotypes, TG and GG genotypes of EGFR rs884225 were significantly associated with reduced HCC risk. The stratified analysis of association between rs884225 and HCC subgroup feature reveal a highly correlation with tumor size. Furthermore, qRT-PCR confirmed that EGFR rs884225, TG and GG genotypes were more likely to bind to miR-3196 and down-regulate EGFR level in cells, thereby inhibiting cell proliferation.

Conclusion: This study suggested that EGFR rs884225 is associated with a reduced risk of liver cancer and may be a developing biomarker.

Keywords: miR-3196, polymorphism, proliferation, hepatocellular carcinoma, HCC, epidermal growth factor receptor, EGFR

Introduction

Hepatocellular carcinoma (HCC) is one of the most common subtypes of liver cancer and one of the most common and deadliest cancers in the world.[1] HCC is characterized by delayed diagnosis and poor prognosis. At present, targeted therapies for liver cancer have gradually advanced from sorafenib, a small molecule inhibitor, to PD-1-based immunotherapy.[2,3] In China, most patients with liver cancer have a background of HBV or HCV infection.[4,5] Studies have shown that genetics plays an important role in the occurrence and development of HCC, including single nucleotide polymorphisms (SNPs) and mutation time, and in

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addition, abnormal expression of protein-coding genes has also been confirmed to be involved in the development of HCC;[6,7] However, the study of SNP in the 3′-untranslated region (UTR) of genes that are relatively key to the occurrence and development of HCC is still relatively rare.

Non-coding RNA (non-coding RNA) mainly includes miRNA (miRNA), long non-coding RNA (long non-coding RNA) and circular RNA (circRNA). Non-coding RNAs have been confirmed to play an important role in the occurrence and development of HCC, and may be involved in the interaction with protein–coding genes, leading to abnormal cell proliferation, invasion, differentiation, cell cycle change or apoptosis.[8–11] Current studies have shown that miRNA plays a negative regulatory role on target genes mainly through the combination of complementary pairing with the 3′-UTR of downstream target genes.[12,13] Current studies on genetic variation of HCC mainly focus on genotype changes in the exon regions of coding genes. Increasing evidence has proved that the miRNAs can participate in a polymorphism of gene transcription regulation, combined with the role of miRNAs main area for non-coding region, we put forward the idea, miRNAs may or with polymorphism in key genes that have different combination of ability, can alter the expression levels of genes and finally involved in disease development.[14]

Epidermal growth factor receptor (EGFR) is a tyrosine kinase transmembrane receptor, a member of the ERB family of receptors, mainly expressed on the surface of epithelial cells.[15] EGFR has been shown to be involved in the regulation of multiple processes of tumorigenesis, including timing of cell survival, cell cycle progression, tumor invasion, and angiogenesis.[16] Members of the EGFR family are involved in many cellular processes, such as cell proliferation and apoptosis, and play a central role in the development and progression of different types of cancer.[17,18] In particular, abnormal changes in EGFR signaling have been identified as a key driver in the development of glioblastoma. In addition, in HCC, researchers have paid attention to SNPs in EGFR itself, which is closely related to the risk of disease occurrence,[19] functional SNP located in the 3′UTR region of EGFR and the interaction with certain miRNA was poor investigated.

In this study, we first based on a public database miRNASNP (http://bioinfo.life.hust.edu.cn/miRNASNP/#!/) for EGFR may interact with miRNA in SNP is forecasted, finally we focus on EGFR 3 ’UTR of SNP (EGFR rs884225) site. Further validation was carried out with large samples to explore whether this SNP in EGFR was related to the occurrence of HCC, or whether it caused different binding to miRNA and thus affected the expression and signal activation of EGFR.

Materials and Methods

Clinical Samples

Six-hundred patients with a clinicopathologic diagnosis of HCC and an equal number of healthy controls were included in the study. All blood samples are taken from Liyang People’s Hospital during March 2009 to August 2019. Informed consent of patients was obtained before surgery, and relevant informed consent was signed. Blood samples were collected before operation and centrifuged within half an hour and frozen at −80°C. All peripheral blood samples were collected before the patient received any treatment. All patients in the control group were excluded from any other malignancy, genetic disease or autoimmune disease. This study has been approved by Liyang People’s Hospital System Review Committee. All studies were carried out in accordance with government policy and the Helsinki Declaration.

Genotyping and Real Time Polymerase Chain Reaction (RT-PCR)

Genomic DNA was extracted from peripheral blood leukocytes using QIAGEN kit. The Quantitative RT-PCR were employed to detect the expression of target in this study. Total RNA was extracted from plasma samples and tissue samples by Trizol method. We also used TaqMan method to detect EGFR expression. U6 was used as endogenous reference during the whole detection process. Detection primer as follows: 5′-AGGCACGAGTAACAAGCTCAC-3′ and 5′ ATGAGGACATAACCAGCCAC-3 ′. All genotypes were tested using the TaqMan probe and polymerase chain reaction (PCR) assay using a real-time PCR instrument according to ABI 7900HT company instructions.

Dual Luciferase Reporter Gene Assay

Firstly, the wild type of the 3 ‘UTR region of EGFR and the corresponding mutants at the binding site were cloned into the pGL4 promoter vector, respectively. The vectors obtained above, and miR-3196 mimics were transfected into HCC cell lines by the transfection method. In the transfection process, the Renal grass luciferase vector PRL-SV40 (5 ng) was transfected into the cell line as a reference, which was used as the background to test the standardized transfection

efficiency. Fluorescence intensity in each group was detected by chemiluminescence analyzer for data analysis.

CCK8 Assay

CCK8 cell proliferation assay was used to detect the proliferation ability of HCC cell lines. Cells transfected with miR-3196 mimics of different genotypes or EGFR-controls were inoculated on 96-well plates at a density of 1×10^4 cells/well and cultured for 48 h. The absorbance values measured at 450 nm represent the DNA synthesis rate for data analysis. All CCK8 experiments were carried out with three replicates and three independent replicates.

Statistical Analysis

In the absence of special metasomatism, all data in this study are expressed as mean ± standard deviation. Bilateral student *t* test was used to compare the differences between the two groups. SPSS10.0 was used for all statistical analyses. P<0.05 was considered statistically significant.

Results

Characteristics of Participants

According to the clinical information analysis of the patients included in this study, both the age and gender was matched between the control group and case group. The virus infection including HBV/HCV were significantly associated with HCC. However, alcohol exposure, smoking exposure, hypertension or diabetes mellitus presented without significant difference (Table 1).

Table 1 Clinical Characteristics of HCC Patients and Cancer-Free Controls

Variables	Cases (n = 600)		Controls (n = 600)		*P**
	N	%	N	%	
Age (years)					
≤ 60	350	58.3	330	55.0	0.244
> 60	250	41.7	270	45.0	
Gender					
Male	481	80.2	482	80.3	0.942
Female	119	19.8	118	19.7	
Virus infection					
HBV/HCV	477	79.5	35	5.8	<0.0001
Negative	123	20.5	565	94.2	
Alcohol exposure					
Positive	301	50.2	289	48.2	0.488
Negative	299	49.8	311	51.8	
Smoking exposure					
Positive	302	50.3	291	48.5	0.525
Negative	298	49.7	309	51.5	
Hypertension					
Positive	311	51.8	299	49.8	0.488
Negative	289	48.2	301	50.2	
Diabetes mellitus					
Positive	219	36.5	203	33.8	0.333
Negative	381	63.5	397	66.2	

Notes: *Two-sided chi-square test for analyzing the clinical characteristics of HCC patients and cancer-free controls.

Candidate Function SNP Located in the 3′UTR of EGFR

In this study, we focused on the relationship between SNPs in EGFR 3′-UTR and HCC risk. We first in GenBank of SNP database (https://www.ncbi.nlm.nih.gov/snp) to retrieve the EGFR all 3′–the SNP UTR region, and through the other two conditions for further filtering: 1) Satisfy the MAF interval (0.05–0.1); and 2) Validation Status by-1000 Genomes must be satisfied. Thus, potential SNP targets of EGFR function were obtained. And on this basis, according to the SNP further using bioinformatics software (http://bioinfo.life.hust.edu.cn/miRNASNP/#!/) predict functional miRNA may interact with EGFR. According to the rank score provided by miRNASNP database, we focused on the functional SNP rs884225 with the highest rank score. We further evaluated the genotype frequency distribution of SNP rs884225 in 600 HCC patients and 600 healthy controls. Chi-square test confirmed that rs884225 genotype showed Hardy–Weinberg balanced distribution in healthy control group (P = 0.41). As presented in Table 2, logistic regression analysis showed that TG and GG genotypes of EGFR rs884225 significantly reduced the risk of HCC compared with TT genotypes (OR: 0.45; 95% CI: TG genotype 0.21–0.66, OR: 0.38; 95% CI: GG genotype was 0.33–-0.58); A higher number of G alleles was also associated with a reduced risk of HCC (OR:0.41;95% confidence interval:0.25–0.61).

Hierarchical Analysis of Correlation Between rs884225 and HCC Subgroup Features

Next, we assessed the association of the EGFR rs884225 SNP with HCC subgroup feature. Patients with TG or GG

Table 2 The Genotype Distribution of the SNP rs884225 in HCC Patients and Cancer–Free Controls

Genotype	Cases (n =600)		Controls (n = 600)		OR (95% CI)[a]	*P* value[a]
	N	%	N	%		
TT	565	94.2	479	79.8	1.00	**0.002**
TG	21	3.5	71	11.8	0.45 (0.21–0.66)	
GG	14	2.3	50	8.4	0.38 (0.33–0.58)	
G carrier	35	5.8	121	20.2	0.41 (0.25–0.61)	**0.01**

Notes: [a]The ORs, 95% CIs and *P* value were calculated after adjusting for age, gender. Significant p value was labelled in bold.

genotype presented a lower tumor size comparing with HCC patients with TT genotype. No statistical difference was obtained in tumor differentiation grade, tumor number, tumor capsular or metastasis (Table 3).

EGFR rs884225 SNP Alteration of miR-3196 Binding

Thus far, we revealed that the EGFR rs884225 SNP was associated with risk and tumor size of HCC. According to the bioinformatics analysis, miR-3196 indicated the binding with MTHFR. Firstly, we detected the mRNA expression of EGFR in the HCC patients mentioned above, as presented in Figure 1A, we found a decreased level of EGFR in patients with TG or GG genotype. CCK8 assay also revealed a decreased cell proliferation in these groups (Figure 1B). Bioinformatical analysis reveal that miR-3196 might binding with the mutant type of EGFR (Figure 1C). We also found that patients with TT genotype presented a decreased level of miR-3196 comparing with TG or GG genotype (Figure 1D). The plasmid carried either the mutant type (GG genotype) or the wild type (TT genotype) was transfected into two HCC cells lines. We confirmed this binding by luciferase assay and found that the luciferase activity of the G-allele-specific PGL3 construct was significantly inhibited in the presence of miR-3196.In HepG2 and MHCC-97H cells, the luciferase

Table 3 The Stratified Analysis of Association Between rs884225 and HCC Risk

Feather	Genotype			TG vs TT *P* value*	GG vs TT *P* value*
	TT	TG	GG		
Differentiation grade					
Well	301	11	6	0.782	0.726
Moderate	156	7	5		
Poorly	108	3	3		
Tumor number					
Solitary	344	15	9	0.330	0.797
Multiple	221	6	5		
Tumor size (cm)					
≤5cm	168	17	13	**<0.001**	**0.007**
>5cm	397	4	1		
Tumor capsular					
Incomplete	182	7	5	0.914	0.782
Complete	383	14	9		
Metastasis					
Yes	188	7	4	0.996	0.712
No	377	14	10		

Notes: *Two-sided chi-square test for either genotype distributions or allele frequencies between cases and controls. Significant p value was labelled in bold.

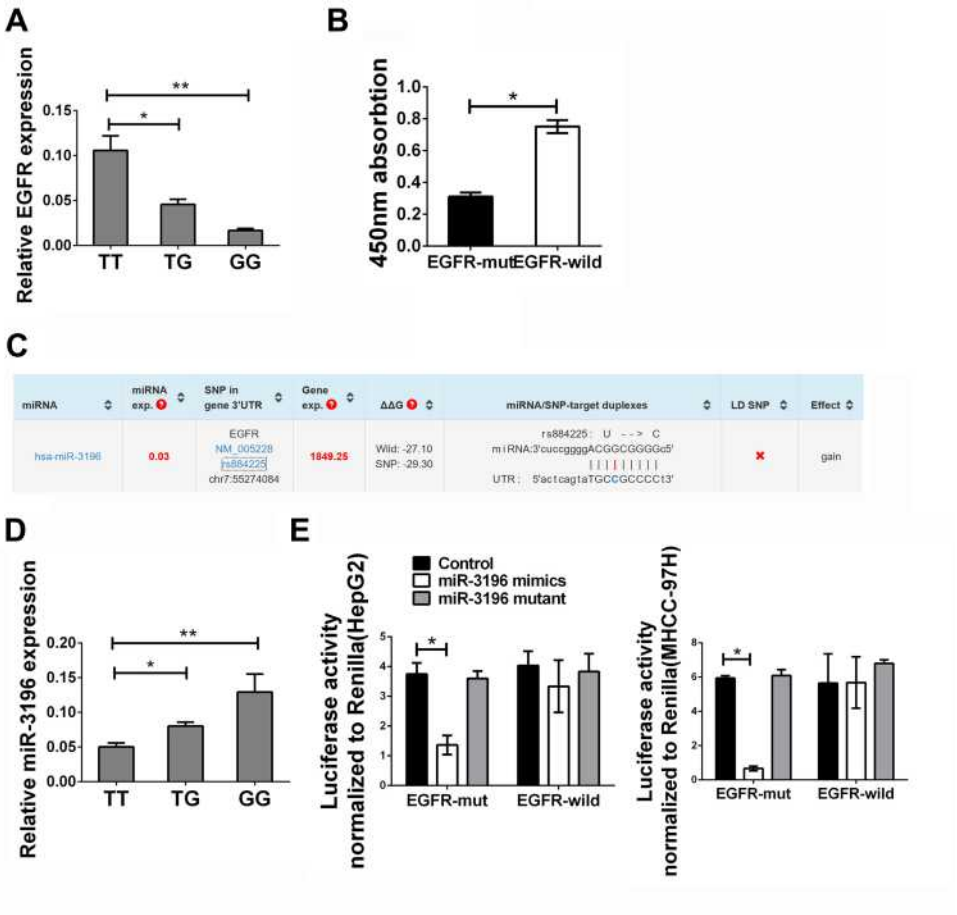

Figure 1 EGFR rs884225 SNP could bind with miR-3196 causing the suppression of EGFR and cell proliferation. (**A**) Expression of EGFR in HCC patients with TT, TG or GG genotype. (**B**) The CCK8 assay in HepG2 cell line treated with EGFR with different genotypes under miR-3196 stimulation. (**C**) Schematic diagram of specific binding sites of miRNA-3196 and EGFR. (**D**) Expression of miR-3196 in HCC patients with TT, TG or GG genotype. (**E**) Dual luciferase reporter gene assay in HepG2 and MHCC-97H cell lines. The data were presented as the mean ± SD. **Indicates $P<0.01$. *Indicates $P<0.05$. (left panel) HepG2 cell line, (right panel) MHCC-97H cell line.

activity of the A allele-specific PGL3 construct did not change after transfection with miR-3196, besides, the mutant type of miR-3196 presented no binding ability on EGFR (Figure 1E).

Based on the stratified analysis of association between rs884225 and patients' characteristics, we found that rs884225 had correlation with tumor size, and no correlation with tumor differentiation grade, tumor number, tumor capsular and metastasis. Thus, according to your suggestion, we thought it is necessary to detect the function of miR-3196 on HCC cell's proliferation and the expression of EGFR related factors including Ki67 and phosphorylation of ERK/AKT. We found that overexpression of miR-3196 will reduced the cell proliferation only when cells was overexpressed with EGFR mutant type, the expression of Ki67 was also decreased with the suppression of ERK/AKT phosphorylation (Figure 2A and B).

Discussion

In this study, we found that polymorphism at EGFR (rs884225) suggested a lower risk of HCC. Further functional analysis suggested that this polymorphic locus and miRNA might interact with each other, enabling miR-3196 to have a stronger affinity with EGFR, thus binding to the 3′UTR region of EGFR, thereby inhibiting EGFR expression level and further inhibiting cell proliferation.

EGFR is a known transmembrane tyrosine kinase receptor. Existing studies have shown that the function of EGFR is mainly involved in the signal transduction of DNA repair, tumor cell survival and cell proliferation, and plays its role through a variety of signal pathways such as PI3K-PTEN-Akt and Ras-RAF-MAPK signaling cascades.[20] Genetic variation of EGFR gene is also a research hotspot at present. Studies have confirmed that EGFR specific mutation exists in most cancer patients, and

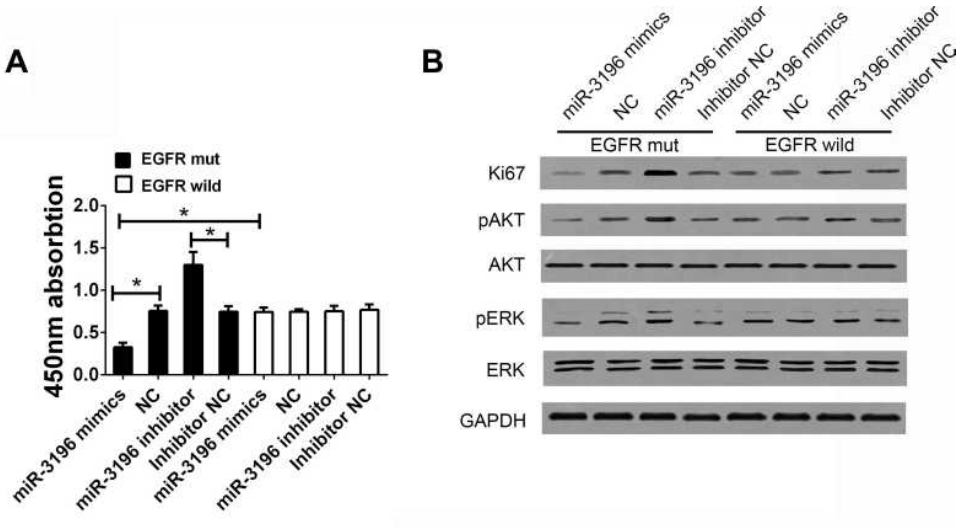

Figure 2 EGFR rs884225 SNP reduced the cell proliferation through the miR-3196-dependent approach. (**A**) CCK8 assay was detected in HCC cells treated with different expression of miR-3196 with EGFR mutant type or wild type. (**B**) The expression of Ki67, phosphorylation of ERK and AKT in HCC cells. *Indicates $P<0.05$.

this type of mutation is closely related to DNA amplification and allele copy number variation.[21,22]

Through a searchable database, we found that in The Cancer Genome Atlas (TCGA) in the league has included information about EGFR somatic mutations, in human tumors, six mutation of EGFR have been proved in human cancers, their site distributed throughout the whole structure of EGFR gene mutation, both including the extracellular and intracellular domain of EGFR, indicating that EGFR mutations may be involved in the process of the whole signal to include with the combination of the ligands and the downstream signal transduction.[23,24] In the study of HCC, we have found that abnormal activation of EGF/EGFR signaling pathway is one of the key factors in the development of HCC.[25] In accordance with this, in the studies on the occurrence and development of EGFR polymorphism in liver cancer, rs4947986 polymorphism was highly correlated with the susceptibility to liver cancer. The study found that rs4947986 polymorphism was located 47 base from the boundary of exon 7, which was the key site for the dimerization of EGFR.[26]

Among the studies on the polymorphism related effects of miRNAs with specific target genes, some researchers have found that some miRNAs can bind to some polymorphic target genes 3′UTR. For example, in irritable bowel syndrome, miR-16 and miR-103 have been proved to have a high affinity with the specific receptor signals of 5-HT, affecting downstream signal transduction.[27] In addition, similar reports have been reported in human cancer diseases. In prostate cancer patients, miR-502 can bind to the 3′UTR polymorphism of SETD8 gene and specifically inhibit the expression of SETD8, increasing the risk of prostate cancer.[28] In gastrointestinal tumors, there are also relevant reports on functional SNPs, especially in the Han population. As for gastrointestinal tumors, in the Chinese Han population, the BSG gene 3′-UTR has functional polymorphic sites that can specifically bind to miR-483-5p, thus affecting the occurrence of esophageal cancer.[29] In addition, in HCC, it has also been reported that miR-214 regulates U/G SNP rs111904020 in STAT3 3′UTR, which promotes the development of HCC in Chinese population. Similarly, FOXO has similar functional SNPs that bind to specific miRNAs and are highly associated with the risk of oncogenesis.[30]

Conclusions

To sum up, this study found closely related to liver cancer of EGFR gene 3 ‘UTR region exists specificity of polymorphism loci is closely related to the occurrence of liver cancer risk, at the same time, the polymorphism and miR-3196 has a relatively strong affinity, and then under the function of miR-3196 inhibits the expression of EGFR, at the same time reduce the proliferation of tumor cells. Of course, this study also has some defects, because it is a hospital-based case-control study with certain selection bias, and large-scale verification in a larger sample is needed in the later stage.

Disclosure

The authors declare that they have no conflicts of interest.

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