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ORIGINAL RESEARCH

A polymorphism at the microRNA binding site in the 3′ untranslated region of *RYR3* is associated with outcome in hepatocellular carcinoma

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Objective: MicroRNAs can bind to the 3′ untranslated regions (UTRs) of messenger RNAs, where they interfere with the translation of targeting genes, thereby regulating cell differentiation, apoptosis, and tumorigenesis. In this study, three microRNA binding site single nucleotide polymorphisms (SNPs) located in the 3′ UTR of *RYR3* (rs1044129), *C14orf101* (rs4901706), and *KIAA0423* (rs1053667) were genotyped to assess their relationships with the risks and outcomes of hepatocellular carcinoma (HCC).

Methods: The SNPs were genotyped with the ligation detection reaction method. Renilla luciferase reporter assays were used to measure the binding affinity between microRNA 367 and *RYR3.* Survival curves were calculated using the Kaplan–Meier method, and comparisons between the curves were made using the log-rank test. Multivariate survival analysis was performed using a Cox proportional hazards model.

Results: It was found that rs1044129 at the 3′ UTR of *RYR3* was related to postoperative survival in HCC, with the AA type associated with longer survival times as per the log-rank test. After adjusting with the Cox model, rs104419 was identified as an independent predictor of HCC survival (relative risk: 1.812; 95% confidence interval: 1.026–3.201; P=0.041). Luciferase analysis also indicated the different binding affinities between the SNPs of rs1044129 and microRNA 367.

Conclusion: The SNP in the microRNA binding site of *RYR3* can be used as a valuable biomarker when predicting HCC outcomes.

Keywords: SNP, rs1044129, *RYR3*, hepatocellular carcinoma, outcome

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer, and it is responsible for more than half a million deaths each year, making it the third leading cause of cancer-related death worldwide.[1] This disease is strongly associated with several risk factors, including chronic hepatitis B virus, chronic hepatitis C virus, and alcohol abuse.[2] Despite improved clinical detection methods and therapies, the prognosis of postoperative HCC patients is still poor due to a high recurrence rate. While the molecular mechanism of HCC carcinogenesis is still not fully understood, there are many prognostic factors and predictors of recurrence associated with the disease, including tumor size, tumor quantity, cell differentiation, venous invasion, and degree of inflammation.[3–7]

MicroRNAs (miRNAs) are ~22 nucleotide RNA molecules that act as posttranscriptional regulators of messenger RNA (mRNA) expression by base pairing to the 3′ untranslated region (UTR) of mRNAs to repress translation.[7–9] Specifically, miRNAs

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target nucleotides 2–8 at the 5′ end, which is known as the "seed region" of an mRNA's 3′ UTR. Perfect complementarity between the miRNA and its target mRNA sequence results in reduced protein levels due to RNA silencing.[10,11] Increasing evidence suggests that single nucleotide polymorphisms (SNPs) in the 3′ UTR targeted by miRNAs alter the expression of target gene, thus increasing an individual's risk for cancer.[12,13]

In this study, we genotyped three miRNA binding site SNPs located in the 3′ UTR of *RYR3* (rs1044129), *C14orf101* (rs4901706), and *KIAA0423* (rs1053667) in HCC patients to assess their relationships with cancer risk and outcomes.

Materials and methods

Tissue specimens and DNA extraction

Blood samples were collected from 88 HCC patients who underwent HCC resection at the Department of General Surgery of the Second Hospital of Hebei Medical University from 2008 to 2010. Blood samples were also collected from 90 healthy controls without a history of any cancer. Genomic DNA was extracted immediately with the Wizard Genomic DNA extraction kit (Promega Corporation, Madison, WI, USA). All procedures were supervised and approved by the hospital's Human Tissue Research Committee; informed consent was obtained from all the patients.

SNP genotyping of miRNA binding site SNPs

The miRNA binding site SNPs, including *RYR3* (rs1044129), *C14orf101* (rs4901706), and *KIAA0423* (rs1053667), were genotyped using the ligation detection reaction method, with forward and reverse primers to amplify the DNA fragments flanking the SNPs, based on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp/). Polymerase chain reaction (PCR) was performed using a PCR Master Mix Kit according to the manufacturer's instructions (Promega Corporation). The ligation was performed using different probes matched to the SNPs, and the ligated products were separated using the ABI PRISM Genetic Analyzer 3730XL (Thermo Fisher Scientific, Waltham, MA, USA). Polymorphisms were confirmed based on the difference in length of the ligated products. All sequences of primers and probes are listed in Table 1.

Renilla luciferase reporter assays

Four oligonucleotides containing from the 5′ to the 3′ end a XhoI stick end (5 bp), a fragment from the 3′ UTR of the *RYR3* gene containing the AA or GG genotype (rs1044129; 51 bp), and an NotI sticky end (2 bp) were synthesized: sense for AA (5′-TCGAGGTTTAGGTGAATCTCCTCAAATACAA TGAAGTGCCCACTGCAATAAAGTAAGC-3′);antisense for AA (5′-GGCCGCTTACTTTATTGCAGTGGGCAC TTCATTGTATTTGAGGAGATTCACCTAAACC-3′); sense for GG (5′-TCGAGGTTTAGGTGAATCTCCTCAAA TACAGTGAAGTGCCCACTGCAATAAAGTAAGC-3′); and antisense for GG (5′-GGCCGCTTACTTTATTGCAG TGGGCACTTCACTGTATTTGAGGAGATTCACCTA AACC-3′). The four oligonucleotides were first annealed with 1× NEBuffer 2 (New England Biolabs, Ipswich, MA, USA) in a heating block at 95°C for 5 minutes, followed by a gradual reduction of temperature to room temperature. The psiCheck2 vector (Promega Corporation) containing Renilla luciferase and controlled firefly luciferase genes was linearized by digestion with NotI and XhoI (New England Biolabs) and purified from an agarose gel. The annealed oligonucleotides were ligated in the linearized psiCheck2 vector into

Table 1 The primers and probes used in genotyping

Gene	rs NCBI	Primer		Probe	
		Forward	Reverse	S1 and S2	S3
RYR3	rs1044129	5′-ATGGAGTAATGC TTTATGGTC-3′	5′-CAGTCACAGAGT GGTTGTAGA-3′	S1: 5′-TTTAGGTGAATCTC CTCAAATACAA-3′ S2: 5′-TTTTTTAGGTGAAT CTCCTCAAATACAG-3′	5′-TGAAGTGCCCACTGC AATAAAGTAA-3′
C14orf101	rs4901706	5′-AAACTAAGTCA TCTCCCAGATA-3′	5′-GTCATCTGGTGA AAGACTGGA-3′	S1: 5′-TTTTTTTTTTAATGG GGTATTCAGTGACTAAGA-3′ S2: 5′-TTTTTTTTTTTTT AAT GGGGTATTCAGTGACTA AGG-3′	5′-TCTGCTATTTATGCAA AATTCTGTTTTTTTT-3′
KIAA0423	rs1053667	5′-CATGAAATCTG AGTCACATGG-3′	5′-GCTGAGAAATGA GACATACCA-3′	S1: 5′-TTTTTATTTTTGAGA AAAGTCCTGCTCAC-3′ S2: 5′-TTTTTTTTATTTTTGA GAAAAGTCCTGCTCAT-3′	5′-TTGCACTATTCTATAG AAACTACAATTT-3′

the NotI and XhoI cloning sites located downstream from the Renilla luciferase reporter gene with T4 DNA ligase (Promega Corporation). The ligated vectors were transformed in *Escherichia coli* competent cells, and positive clones were selected by sequencing.

The HeLa cell line was seeded in 48-well plates and transfected with 800 ng of the modified psiCheck2 vector containing either the AA or GG genotype. Then, the Renilla luciferase activity was measured with a luminometer (Lumat, Albuquerque, NM, USA) 48 hours after transfection with the Dual-Lucy Assay Kit (Vigorous Instrument, Beijing, People's Republic of China); the transfection efficiency was normalized with the firefly luciferase activities.

Statistical analysis

The χ^2 test was used to analyze dichotomous values, such as the presence or absence of an individual SNP in patients and healthy controls. Survival curves were calculated using the Kaplan–Meier method, and comparisons between the curves were made using the log-rank test. Multivariate survival analysis was performed using a Cox proportional hazards model. The *t*-test was used to compare the different expression levels between genotypic groups with Renilla luciferase reporter assays. All statistical analyses were performed using the SPSS 18.0 software package (IBM Corporation, Armonk, NY, USA). A probability level less than 0.05 was used as the criterion for significance.

Results

Association of the RYR3 SNP with HCC outcomes

We genotyped miRNA binding site SNPs including *RYR3* (rs1044129), *C14orf101* (rs4901706), and *KIAA0423* (rs1053667) in a case-control study with 88 HCC patients and 90 healthy controls. None of these SNPs were associated with HCC cancer risk according to our analysis (data not shown).

The relationship between the data collected during the 3-year follow-up and patients' clinical characteristics was analyzed by the log-rank test. Sex, age, tumor size, and tumor quantity were not statistically significant predictors of postoperative survival times; however, tumor stage, child classification, and portal vein thrombosis were correlated with survival time in these patients (Table 2).

The three miRNA binding site SNPs of rs1044129, rs4901706, and rs1053667 were evaluated for their

Table 2 Univariate analysis of clinical characteristics and miR-SNPs with HCC survival

Characteristics	Number of cases	3-year survival rate (%)	*P*-value
RYR3			0.047
A/A	33	39.4	
A/G + G/G	55	25.5	
C14 or f101			0.573
G/G	48	27.1	
A/G + A/A	40	35.0	
KIAA0423			0.717
T/T	58	32.8	
C/T + C/C	30	26.7	
Sex			0.530
Male	79	31.6	
Female	9	22.2	
Age (years)			0.742
≤60	59	28.8	
>60	29	34.5	
Child classification			0.019
A	82	32.9	
B	6	0.0	
Portal vein thrombosis			0.027
No	80	32.5	
Yes	8	12.5	
Size of tumor (diameter/cm)			0.765
≤5	28	35.7	
>5	60	28.3	
TNM classification			0.017
0–I	30	46.7	
I–III	58	22.4	
Number of tumor			0.871
Single	68	30.9	
Multiple	20	30.0	

Abbreviations: miR-SNP, micro RNA-related single nucleotide polymorphism; HCC, hepatocellular carcinoma; TNM, tumor node metastasis.

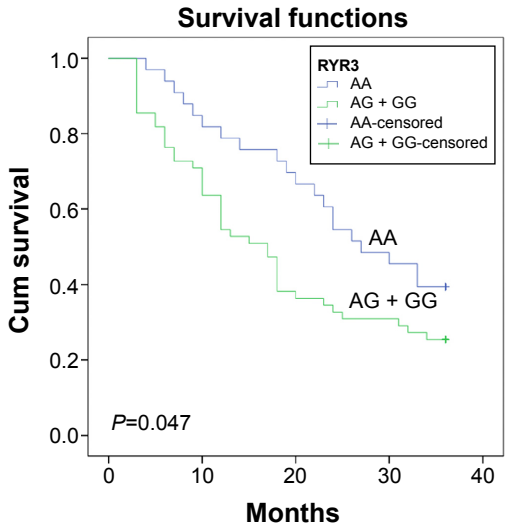

Figure 1 Genotypes of rs1044129 and its association with HCC survival.
Abbreviations: HCC, hepatocellular carcinoma; Cum, cumulative.

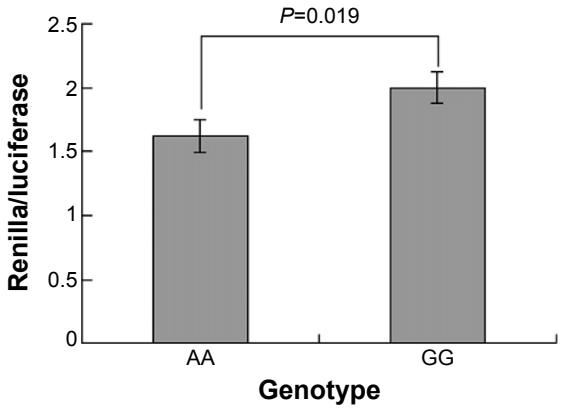

Figure 2 Renilla luciferase assay at 48 hours with modified psiCheck2 vector transfected in HeLa cell lines.
Note: The Renilla luciferase activities are defined as the ratio of Renilla luciferase activities versus firefly luciferase activities.

association with the postoperative survival of HCC patients, as shown in Table 2 and Figure 1; only rs1044129 at the 3′ UTR of *RYR3* appeared to be related to HCC survival, with the AA type associated with longer survival times (P=0.047). We performed a multivariate analysis with the Cox proportional hazards model for these predictive factors, as shown in Table 3. The rs1044129 was identified as an independent predictor of HCC survival (relative risk: 1.812; 95% confidence interval: 1.026–3.201; P=0.041).

The SNP in rs1044129 affects protein translation

To analyze the functional effect of rs1044129 on *RYR3* expression, we constructed a vector containing the AA or GG genotype of rs1044129 in the 3′ UTR region of the Renilla luciferase gene and transfected them in HeLa cells. A dramatic reduction in Renilla luciferase activity was observed in the AA genotype (Figure 2). These results indicated that the rs1044129 SNP in the 3′ UTR of *RYR3* changed its binding affinity with miRNAs, thus affecting its expression.

Table 3 Multivariate analysis of prognostic factors associated with overall survival in HCC patients with Cox proportional hazards model

Factors	Relative risk	95% CI	*P*-value
RYR3	1.812	1.026–3.201	0.041
Child classification	2.464	1.020–5.951	0.045
TNM classification	1.922	1.037–3.562	0.038
Portal vein thrombosis	1.571	0.664–3.719	0.304

Abbreviations: HCC, hepatocellular carcinoma; CI, confidence interval; TNM, tumor node metastasis.

Discussion

The SNPs of miRNA binding sites, including *RYR3* (rs1044129), *C14orf101* (rs4901706), and *KIAA0423* (rs1053667), were assessed for their predictive value in cancer risk and outcomes in HCC, and rs1044129 of the *RYR3* gene was identified as a predictor of HCC outcomes. This was the first report to assess the miRNA binding site SNPs at the 3′ UTR of *RYR3* and their predictive ability in HCC outcomes. The SNPs within the miRNA binding site, as well as polymorphisms in the miRNAs themselves, are key factors in disease phenotypes.[14,15] The association between SNPs and cancer risk was identified by Yu et al[16] in their investigation of many kinds of cancers as part of their entire research objective.

RYR3, the third isoform of the RYR family, is a Ca^{2+}-induced/Ca^{2+} release channel protein located in the sarcoplasmic reticulum that mediates cytosolic calcium levels.[17,18] rs1044129, located in the 3′ UTR seed region of RYR3 where miR 367 binds, was found to be related to the outcomes of colonic cancer and breast cancer.[19,20] Consistent with a previous report on breast cancer cells, we confirmed that the A to G transition at rs1044129 reduced the binding affinity between miR 367 and *RYR3*, thereby inducing increased RYR3 expression in HeLa cells. We performed RYR3 immunostaining in 30 HCC patients with the available HCC tissue. The AA types displayed a trend of increased RYR3 expression when compared with GG (P=0.07); however, the results need to be validated with more HCC patients. In addition to the underlying mechanism found in breast cancer, the expression variation of *RYR3* appears to mediate calcium levels so as to modify the proliferation and migration of HCC.[20] Furthermore, vitamin D deficiency

was also confirmed to be associated with cancer risk and outcomes in HCC.[21,22]

Conclusion

In conclusion, a SNP in the *RYR3* miRNA binding site was found to be a biomarker for HCC outcomes. The results from this study require validation in other populations and in laboratory-based functional studies.

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Disclosure

The authors report no conflicts of interest in this work.

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