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ORIGINAL RESEARCH

Single-nucleotide polymorphisms of microRNA processing machinery genes and risk of colorectal cancer

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Objective: MicroRNA (miRNA)-related single-nucleotide polymorphisms (miR-SNPs) in miRNA processing machinery genes can affect cancer risk, treatment efficacy, and patient prognosis. We genotyped 6 miR-SNPs of miRNA processing machinery genes including *XPO5* (rs11077), *RAN* (rs14035), *Dicer* (rs3742330), *TNRC6B* (rs9623117), *GEMIN3* (rs197412), and *GEMIN4* (rs2740348) in a case-control study to evaluate their impact on colorectal cancer (CRC) risk.

Materials and methods: miR-SNPs were genotyped using the polymerase chain reaction–ligase detection reaction. The χ^2 test was used to analyze dichotomous values, such as the presence or absence of any individual SNP in CRC patients and healthy controls.

Results: Two of these SNPs were identified for their association with cancer risk in the *Dicer* and *GEMIN3* genes. The AA allele of rs3742330 located in the *Dicer* gene exhibited a significantly increased risk of CRC (odds ratio, 2.11; 95% confidence interval: 1.33–3.34; P=0.001); the TT allele of rs197412 located in *GEMIN3* also exhibited a significantly increased risk of CRC (odds ratio, 1.68; 95% confidence interval: 1.07–2.65; P=0.024).

Conclusion: Our results suggest that the specific genetic variants in miRNA machinery genes may affect CRC susceptibility.

Keywords: miR-SNP, CRC, *GEMIN3*, *Dicer*

Introduction

Colorectal cancer (CRC) is the third most common cancer in males and the second in females, which make it the third most common cause of cancer-related mortality in both sexes worldwide.[1] It accounts for an estimated 1.2 million new cancer cases and over 630,000 cancer deaths per year.[2] The CRC incidence displayed a trend of rapid rise in a study involving Asian countries including the People's Republic of China, Japan, South Korea, and Singapore.[3] Dietary patterns, obesity, smoking, heavy alcohol consumption, physical inactivity, genetic and epigenetic have been identified as risk factors for CRC,[4–6] but the underlying mechanism of this cancer remains unknown.[7–10]

MicroRNAs (miRNAs) are RNA molecules that are ~22 nucleotides long and which play important roles in various biological processes, such as embryonic development, cell differentiation, proliferation, apoptosis, cancer development, and insulin secretion.[11,12] A growing body of studies suggests that miRNAs play important roles in cancer development through regulating the expressions of proto-oncogenes or tumor suppressor genes.[11,13,14] During miRNA processing, long primary transcripts of miRNAs (pri-miRNAs) are processed in the nucleus by the RNase III Drosha and are transported to the cytoplasm by the nuclear transport factor exportin-5 (*XPO5*) and *RAN*. In the cytoplasm, RNase III *Dicer* and transactivation-responsive RNA-binding

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protein (TRBP) mediate pre-miRNA processing to release a 21-bp miRNA, the RNA-induced silencing complex (RISC) including *GEMIN3* and *GEMIN4* will select one strand as the mature miRNA and guide mature miRNAs to their target mRNA sites.[11,15–19] miRNA-related single-nucleotide polymorphisms (miR-SNPs), defined as single-nucleotide polymorphisms (SNPs) in miRNA genes, miRNA binding site and miRNA processing machinery, are able to modulate miRNA and target gene expressions so as to influence cancer risk, treatment efficacy, and patient prognosis.[19–22]

It is reported that genetic variants in both miRNA processing pathway genes and miRNA genes might affect susceptibility to cancers such as bladder cancer, esophageal cancer, and renal cell carcinoma, but few studies focus on miR-SNPs of miRNA processing machinery genes and CRC risk.[23–25] In the present study, we genotyped six miR-SNPs of miRNA processing machinery genes including *XPO5* (rs11077), *RAN* (rs14035), *Dicer* (rs3742330), *TNRC6B* (rs9623117), *GEMIN3* (rs197412), and *GEMIN4* (rs2740348), which have shown susceptibility to carcinogenesis in a previous report, in a case-control study to evaluate the impact of these miR-SNPs on CRC risk.[19]

Materials and methods

Tissue specimens and DNA extraction

Blood samples were collected at the Fourth Hospital of Hebei Medical University, Shijiazhuang, People's Republic of China from 163 CRC patients who underwent tumor resection in the Department of Surgery. Blood samples were also collected from 142 age- and sex-matched health controls. The personal information about smoking, obesity, and alcohol consumption were reviewed in patients and controls. Total DNA was extracted using the Wizard Genomic DNA extraction kit (Promega Corporation, Fitchburg, WI, USA) and stored at −20°C. The program was approved by the Human Tissue Research Committee of the Fourth Hospital of Hebei Medical University. Written informed consent was obtained from all the patients for the collection of samples and subsequent analysis.

Genotyping of miR-SNPs

The miR-SNPs of the miRNA processing genes including *XPO5* (rs11077), *RAN* (rs14035), *Dicer* (rs3742330), *TNRC6B* (rs9623117), *GEMIN3* (rs197412), and *GEMIN4* (rs2740348) were genotyped with polymerase chain reaction-ligase detection reaction assay that amplify the DNA fragment flanking miR-SNPs basing on the sequence in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp/). All the primers and probes are listed in Table 1. The ligation was performed using the different probes matched to the miR-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 length difference of ligated products.

Measurement of *Dicer* levels in CRC tissue

Dicer immunochemistry was performed with CRC tissue. The tissue sections were incubated with an anti-*Dicer* antibody (Abcam, Cambridge, UK) at a dilution of 1:100 overnight

Table 1 Primers and probes used for genotyping of miR-SNPs

Gene	rs NCBI	Primer sequence	Probe sequence
XPO5	rs11077 (A/C)	F GAATCTGGTCACCTGATGGGA	S1 GTACCTCCAAGGACCAGGGCTGGGA
		R GTGCCTGAGTGGACCTTGAG	S2 TTTGTACCTCCAAGGACCAGGGCTGGGC
			S3 AGTCTTTAGTGCTAACATCCCCTTT
RAN	rs14035 (C/T)	F GCACTTGCTCAAAATCTGTGA	S1 TTTTAGTAATCATGTTTTAATGTAGAACC
		R TAACAGCAAGAATTCCCAACC	S2 TTTTTTTAGTAATCATGTTTTAATGTAGAACT
			S3 TCAAACAGGATGGAACATCAGTGGATTT
Dicer1	rs3742330 (A/G)	F AAAGGTATCAAGGTCTCAGTTTG	S1 TTTTTTTTTTCAATCTTGTGTAAAGGGATTAGA
		R CTGCAGAGGATCACTGGAATC	S2 TTTTTTTTTTTTTCAATCTTGTGTAAAGGGATTAGG
			S3 CACCCTAACAGAGCAAGATCCAATATTTTTT
TNRC6B	rs9623117 (C/T)	F TTTCTGTCTCCTCCTATCCAT	S1 TCTCCCTGTTACTCTTAAGTAGTGC
		R CATTAGTTTAGCCAACAAGGT	S2 TTTTCTCCCTGTTACTCTTAAGTAGTGT
			S3 CTCCTTTCCCCATCCACCCCATCTC
GEMIN3	rs197412 (T/C)	F TAGAGAAACCTGTGGAAATCA	S1 TTTTATGGTTTTGTGAGAAATAAAGTTAC
		R GAAGAGGTTCTTGAGCTGTAA	S2 TTTTTTTATGGTTTTGTGAGAAATAAAGTTAT
			S3 TGAACAGAGAGTCCCTGTGTTGGCATTT
GEMIN4	rs2740348 (G/C)	F TTGCCTCTGAGAAGAAGTGG	S1 TTTTTTTTGGGAGTAACAGGGCCCTCTTCCGAC
		R GACTCAGGGATGGCTCTGTC	S2 TTTTTTTTTTTTGGGAGTAACAGGGCCCTCTTCCGAG
			S3 AGCCAGACTTGGTGTTGAGGCTGCTTTTTTT

Abbreviation: miR-SNPs, microRNA-related single-nucleotide polymorphisms.

at 4°C followed by incubation with a biotinylated secondary anti-rabbit IgG antibody for 1 hour at room temperature. The sections were subsequently incubated with HRP-conjugated streptavidin and developed using 3,3-diaminobenzidine.

The immunostaining results for all receptors were semi-quantified by two investigators using the HSCORE as described previously.[26] Briefly, the score was calculated by the percentage of positively stained CRC tissue with five intensity categories (0, 1+, 2+, 3+, and 4+). The HSCORE represents the sum of each of the percentages multiplied by the weighted intensity of staining as follows:

$$\text{HSCORE} = (i+1)\,\pi, \quad (1)$$

where i =1, 2, 3, and 4 and π varies from 0% to 100% for the percentage of positive stained area. A score >100% was defined as high expression and ≤100% as low expression

Statistical analysis

The χ^2 test was used to analyze dichotomous values, such as the presence or absence of any individual SNP in CRC patients and healthy controls. Statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). For all the statistical tests, $P<0.05$ was considered to indicate a statistically significant difference.

Results

A total of 163 CRC patients were enrolled in this study. The control group consisted of 142 people without any history of hereditary or malignant disease by physical and imaging examinations. No distribution difference existed between patients and controls referring to age and sex. All patients and controls were the same nationality (Han Chinese) and recruited from Shijiazhuang and surrounding areas in North China. The clinical characteristics of the CRC patients and healthy controls are listed in Table 2.

We genotyped the six miR-SNPs of miRNA processing machinery genes including *XPO5* (rs11077), *RAN* (rs14035), *Dicer* (rs3742330), *TNRC6B* (rs9623117), *GEMIN3* (rs197412), and *GEMIN4* (rs2740348) in 163 CRC patients and 142 healthy controls and evaluated the impact of these miR-SNPs on CRC risk. The genotype distributions and allele frequencies of the SNPs are shown in Table 3. Two SNPs of these miR-SNPs were identified for their association with CRC risk by χ^2 test. For the rs3742330 located in *Dicer* genes, the frequencies of genotype AA and AG + GG were 56.4% and 43.6% in patients but 38.0% and 62.0% in the controls. The AA genotype carrier of rs3742330 was associated with a 2.11-fold increased risk when compared with AG + GG genotype carrier (odds ratio, 2.11; 95% confidence interval: 1.33–3.34; P=0.001). As for the rs197412 located in *GEMIN3*, the frequencies of genotype TT and CT + CC were 55.2% and 44.8% in the patients and 42.3% and 57.7% in controls. The TT genotype carrier showed a significantly increased risk for CRC compared with CT + CC carrier (odds ratio, 1.68; 95% confidence interval: 1.07–2.65; P=0.024).

Table 2 Association of clinical characteristics with cancer risk in CRC patients

	CRC (n=163)	Control (n=142)	*P*-value
Age (years)			
≤60	79	66	
>60	84	76	0.729
Sex			
Male	93	93	
Female	70	49	0.132
Smoking			
Yes	32	36	
No	131	106	0.231
Obesity			
Yes	12	18	
No	151	124	0.120
Alcohol consumption			
Yes	27	30	
No	136	112	0.308
Tumor stage, TNM			
I	9		
II	82		
III	53		
IV	19		

Abbreviation: CRC, colorectal cancer.

One hundred and sixty-four CRC patients including 89 rs3742330 AA carriers and 75 rs3742330 AG + GG carriers with cancer tissue available were used for *Dicer* protein measurement by immunostaining. The AA type (35 high *Dicer* expression and 54 low *Dicer* expression) displayed a great trend toward association with low *Dicer* expression at borderline statistical level (P=0.073) compared with that of AG + GG (40 high *Dicer* expression and 35 low *Dicer* expression).

Discussion

The identification of predictive markers from miR-SNPs for cancer risk is a new field in cancer research. We evaluated the potential associations of six miR-SNPs in miRNA processing machinery genes including *XPO5* (rs11077), *RAN* (rs14035), *Dicer* (rs3742330), *TNRC6B* (rs9623117), *GEMIN3* (rs197412), and *GEMIN4* (rs2740348) with the risk of CRC. Two miR-SNPs in *Dicer* (rs3742330) and *GEMIN3* (rs197412) were found to be associated with CRC risk.

Dicer is an endonuclease in the RNase III family that specially cleaves double-stranded RNAs to produce miRNA and

Table 3 Associations of the six SNPs with colorectal cancer risk

Gene	SNP	Genotype	Number (%) of patients with each genotype		Odds ratio	95% CI	*P*-value
			Case	Control			
XPO5	rs11077	AA	143 (12.3)	123 (86.6)	1.10	0.56–2.16	0.772
		AC + CC	20 (87.7)	19 (13.4)			
RAN	rs14035	CC	113 (69.3)	107 (75.4)	0.74	0.45–1.23	0.242
		CT + TT	50 (30.7)	35 (24.6)			
Dicer	rs3742330	AA	92 (56.4)	54 (38.0)	2.11	1.33–3.34	0.001
		AG + GG	71 (43.6)	88 (62.0)			
TNRC6B	rs9623117	TT	149 (91.4)	128 (90.1)	1.16	0.54–2.53	0.702
		CT + CC	14 (8.6)	14 (9.9)			
GEMIN3	rs197412	TT	90 (55.2)	60 (42.3)	1.69	1.07–2.65	0.024
		CT + CC	73 (44.8)	82 (57.7)			
GEMIN4	rs2740348	GG	128 (78.5)	114 (80.3)	0.89	0.51–1.57	0.706
		GC + CC	35 (21.5)	28 (19.7)			

Abbreviations: CI, confidence interval; SNP, single-nucleotide polymorphism.

small interfering RNA so as to repress gene expression.[27,28] The miR-SNP of rs3742330 of *Dicer* has been identified for its association with the cancer outcome of T-cell lymphoma as well as the risk of oral premalignant lesions and renal cell carcinoma.[25,29,30] The mechanism by which this SNP modifies the CRC risk remains unclear. rs3742330 located in the 3′-untranslated region of *Dicer* might potentially influence the gene stability and expression. Our immunostaining data show that this SNP seems to associate with different *Dicer* expression levels. Moreover, the altered *Dicer* expression may further affect miRNA expression profiles, thereby mediates the CRC carcinogenesis, deregulation of *Dicer* are strongly associated with the adverse development of CRC.[31]

GEMIN proteins in miRNA ribonucleoprotein particles is involved in the processing of miRNA precursors through their interaction with the key components of the RNA-induced silencing complex.[25,32,33] rs197412 located in exon 11 of *GEMIN3* gene could induce Ile to Thr substitution at 636 amino acid position through the T to C transition. This SNP showed a trend toward susceptibility to the risk of renal cell carcinoma.[25] This amino acid substitution might change mRNA stability or protein function, thereby influencing miRNA expression profiles to modify the CRC carcinogenesis. In addition, *GEMIN3* can form a complex with p53 and EBNA3C through the C-terminal domain (amino acid 546-825) to block p53-mediated apoptosis.[34] The amino acid substitution of this miR-SNP, which is located in the C-terminal of *GEMIN3*, might alter binding affinity to p53, thereby modifying the apoptosis process of CRC cells.

The relationships of the frequency distribution of these two SNPs and metastasis status were compared; no association exists by our analysis (data not shown). These six miR-SNPs were analyzed for their association with postoperative survival in 55 patients with 5-year follow-up data available. rs14035 and rs3742330 showed association with survival at borderline statistical level (Fan et al, unpublished data, 2014), these findings should be validated in a larger sample size.

To our knowledge, this is the first study investigating the associations between SNPs of miRNA processing machinery genes and CRC susceptibility. miR-SNPs emerged as new promising markers for disease prediction in cancer. Although miR-SNP studies for miRNA processing machinery genes are at an early stage, our results are encouraging because they indicate that miR-SNPs may have an effect on cancer susceptibility. However, further laboratory-based functional studies in other populations are warranted to validate these results.

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Disclosure

The authors report no conflicts of interest in this work.

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