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ORIGINAL ARTICLE

Common genetic variants in pre-microRNAs and risk of gallbladder cancer in North Indian population

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MicroRNAs (miRNAs) are small, non-coding RNA molecules that function as negative regulators of gene expression. Common genetic variants (single nucleotide polymorphisms, SNPs) in miRNA genes may alter their expression or maturation resulting in varied functional consequences. Present case–control study evaluated the potential association of three SNPs (rs2910164, rs11614913 and rs3746444) in pre-miRNAs with gallbladder cancer (GBC) risk in 230 GBC cases and 230 controls in a North Indian population. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated for the association of individual SNPs and their interactions with GBC. A non-significant increased risk was observed between carriers of variant genotypes of rs2910164, rs11614913 and rs3746444 (ORs=1.3, 1.3 and 1.1, respectively). This increased risk was more profound in GBC patients with gallstones (ORs=1.4, 1.6 and 1.1, respectively). To further evaluate the cumulative effects of the variant allele, we did a combined unfavorable genotype analysis, which showed a borderline statistical significance. In comparison with the low-risk group (0–2 variant alleles), the high-risk group (>2 variant alleles) had a 1.7-fold (95% CI=1.0–2.8) increased risk for GBC (P_{trend}=0.056). These findings suggest, for the first time, that common miRNA variants may not contribute to GBC susceptibility in North Indian population.
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Keywords: gallbladder cancer (GBC); microRNA; polymorphism

INTRODUCTION

Gallbladder cancer (GBC) is an aggressive malignancy and the most common biliary tract tumor in the world.[1] The worldwide variation in the incidence of GBC and the interindividual risks to those exposed to similar known risk factors suggests the involvement of major genetic and environmental factors. Identification and elimination of these factors may lead to early detection and prevention of this disease.[1–3]

MicroRNAs (miRNAs) are a class of endogenous, ~22 nucleotide long non-coding RNA molecules that function as negative regulators of gene expression.[4,5] miRNAs regulate the expression of roughly 10–30% of all human genes through post-transcriptional mechanisms and their abnormal expression have been linked with many human diseases including cancer.[6,7] Various single nucleotide polymorphisms (SNPs) are known to exist in miRNA genes, which can lead to variations in the quantity of miRNAs resulting in diverse functional consequences and, therefore, may represent ideal candidate biomarkers for cancer prognosis.[8] Various genetic association studies have explored the function of pre-miRNA polymorphisms in cancers including non-small cell lung cancer (NSCLC),[9] breast cancer,[10] hepatocellular carcinoma,[11] bladder cancer[12] and papillary thyroid carcinoma.[13] However, the function of genetic variants of miRNA genes in GBC is yet to be determined.

In this study, we hypothesized that SNPs in pre-miRNAs altering miRNA processing and expression could contribute to GBC susceptibility. To test this hypothesis, we selected three potentially functional polymorphisms in pre-miRNA [*hsa-miR-146a* (rs2910164), *hsa-mir-196a2* (rs11614913) and *hsa-mir-499* (rs3746444)] and evaluated their individual and joint effect on GBC susceptibility in a case–control study.

MATERIALS AND METHODS

Study population

The institutional ethical committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences approved the study protocol, and all participants provided written informed consent for the study. A total of 460 subjects between 37 and 80 years of age, including 230 GBC patients and 230 control subjects were enrolled in this study. GBC cases were consecutive newly diagnosed between June 2005 and July 2009. GBC diagnosis was confirmed for all cases by fine needle aspirated cell cytology and histopathology. Staging of cancer was documented according to the AJCC/UICC staging.[14,15] Control subjects were healthy adults without a history of cancer, who were randomly selected from general population and were frequency matched to cancer cases on age and gender. To test the possibility for population stratification, we used genomic control method as described by Devlin and Roeder.[16]

Genotyping

Genomic DNA was isolated from peripheral blood leukocytes by standard protocol. The genotypes were determined by the PCR-RFLP method. The PCR reaction conditions, primers used and restriction pattern used for studied

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polymorphisms were as described by Hu *et al*.[9] The restriction enzymes used for rs2910164, rs11614913 and rs3746444 polymorphisms were *SacI*, *BclI* and *MspI*, respectively (New England Biolabs, Inc., Beverly, MA, USA). As a negative control, PCR mix without DNA sample was used to ensure contamination-free PCR product. Samples that failed to genotype were scored as missing. Ten percent of samples from patients and controls including samples of each genotype were sequenced to evaluate the quality of genotyping, which showed 100% concordance. Genotyping was performed without knowledge of the case or control status.

Statistical analyses

The sample size was calculated using QUANTO 1.1 program (http://hydra.usc.edu/gxe) considering the minor allele frequency of the *miRNA* gene polymorphisms in Caucasian population. The sample size of 230 cases and 230 controls was adequate to give us a power of 80% (probability of not making Type II error). The χ^2 analysis or two-sided Fisher's exact test was used to compare the differences in demographic variables, tobacco-smoking status and genotype distributions of miRNAs polymorphisms between cases and controls. Observed genotype frequencies for miRNAs polymorphisms in controls were examined for deviation from Hardy–Weinberg equilibrium (HWE) using a goodness-of-fit χ^2-test with one degree of freedom. Unconditional univariate and multivariate logistic regression analysis was used to estimate odds ratio (OR) and 95% confidence interval (CI) adjusted for age and gender to estimate the risk of GBC with miRNAs polymorphisms. Additional models with adjustment for gallstone status also were run to evaluate the risk of cancer independent of stones. Risk estimates were also calculated for a codominant genetic model using the most common homozygous genotype as reference. Tests of linear trend using an ordinal variable for the number of copies of the variant allele (0, 1 or 2) were conducted to assess potential dose–response effects of genetic variants on GBC risk.[17] In addition, cancer being a multifactorial disease, it is unlikely that any one single genetic polymorphism would affect the clinical outcome of cancer patients significantly, thus it is important to take a pathway-based analysis of multiple polymorphic genes. Thus, we also analyzed the number of total variant alleles in miRNAs polymorphisms studied. To further explore potential interaction between the miRNAs polymorphisms and tobacco smoking, we tested the null hypotheses of multiplicative gene-smoking interaction by evaluating departure from multiplicative interaction model including main-effect variables and their product terms in logistic regression model.[18] We also performed subgroup analyses stratifying GBC cases and controls on basis of various host characteristics. To explore the effect of gender, subjects were stratified as male and female. Stratification was also performed on the basis of gallstone status and analyzed separately. GBC patients were also stratified on the basis of age of onset. Patients up to 49 years of age were denoted as early onset (<50 years) and patients above 50 years were denoted as late onset patients ($\geqslant 50$ years). Power curves were constructed, and calculations were determined according to Campbell *et al*.[19] All tests were two sided by using SPSS software version 15.0 (SPSS, Chicago, IL, USA).

Multiple testing issues

Standard adjustments for multiple testing, such as Bonferroni's correction, are too conservative as they assume that tests are independent, which is usually not the case when multiple tests are applied on the same data set. We, therefore, applied the false-positive report probability statistical tool to evaluate noteworthiness of the associations by using the method as described by Wacholder *et al*.[20]

RESULTS

Population characteristics

Baseline characteristics of GBC patients and their age- and gender-matched controls are presented in Table 1. Of the 230 GBC cases and controls with complete clinical and successful genotyping for all the three miRNA polymorphisms, the mean age was 51.77 ± 6.826 years (range, 37–72 years) and 53.07 ± 10.107 years (range, 35–79 years), respectively. The mean age and gender distributions were not significantly different among cases and controls, suggesting that the frequency matching was adequate. Genomic control method ruled out the possibility of population stratification in our study. Gallstones were present in 51% of GBC patients and most of the GBC patients were in advanced stages of cancer (stage III and stage IV). Of the 230 GBC cases, 12 (5.3%) had stage II adenocarcinoma, 76 (33.0%) stage III and 142 (61.7%) stage IV. About 33.0% of the GBC patients were associated with tobacco usage in some form (smoking, chewing or both). All cancer patients were incident cases and none of the controls had family history of cancer. There were no significant differences in the distribution of the demographic variables between the patients in the test set and those in the validation set (180 cases in the original test set and 50 cases in the independent validation set). Therefore, the two sets were combined for the following analyses to increase the study power.

Table 1 Selected characteristics of subjects by case–control status

Characteristic	*Controls,* N *(%)*	*Gallbladder cancer,* N *(%)*
Total	230	230
Gender		
Male	78 (33.9)	79 (34.3)
Female	152 (66.1)	151 (65.7)
Age at interview (years)		
$\leqslant 54$	147 (63.9)	140 (60.9)
55–64	71 (30.9)	75 (32.6)
$\geqslant 65$	12 (5.2)	15 (6.5)
Stages		
0, I		None
II		12 (5.3)
III		76 (33.0)
IV		142 (61.7)
Gallstone present	None	117 (51.0)
Gallstone absent	All	113 (49.0)
Tobacco users	—	76 (33.0)

The symbol (—) denotes that no information is available for tobacco consumption in control group.

Association of miRNA polymorphisms with GBC

The distribution of hsa-mir-196a2 C>T, hsa-mir-499 T>C and hsa-mir-146 G>C genotypes are shown in Table 2. The distribution of genotypes of all the miRNA polymorphisms in controls was in agreement with HWE (P=0.216 for *hsa-mir-146a*, 0.190 for *hsa-mir-196a2* and 0.848 for *hsa-mir-499*). Table 2 shows the GBC risk related to the three miRNA polymorphisms. Owing to missing statistics on BMI and alcohol information for 112 cases and 84 controls, we did all our analyses with or without BMI and alcohol adjustment and found that the ORs and 95% CI were comparable in both situations. Therefore, the results presented here were risk estimates without BMI and alcohol adjustment for all the GBC patients. In the single-locus analyses, none of the three miRNA polymorphisms reached significant difference in the genotype distributions between GBC cases and controls. Multivariate logistic regression analyses revealed an increased risk of GBC associated with the variant containing dominant model of the hsa-mir-196a2 C>T, hsa-mir-499 T>C and hsa-mir-146a G>C polymorphisms (ORs=1.3, 1.1

Table 2 Case–control distribution of genotypes of polymorphisms analyzed

Genotype	Controls, n (%)	Cases, n (%)	OR (95% CI)[a]	P-value	Type II error
hsa-mir-196a2 C>T (rs11614913)					
CC	136 (59.1)	119 (51.7)	Reference	—	
CT	75 (32.6)	95 (41.3)	1.4 (0.9–2.1)	0.077	0.04
TT	19 (8.3)	16 (7.0)	0.9 (0.5–1.9)	0.864	0.06
CT+TT	94 (40.9)	111 (48.3)	1.3 (0.9–1.9)	0.136	0.01
P_{trend}[b]			0.303		
hsa-mir-499 T>C (rs3746444)					
TT	121 (52.6)	112 (48.7)	Reference	—	
TC	94 (40.9)	97 (42.2)	1.1 (0.7–1.6)	0.676	0.001
CC	15 (6.5)	21 (9.1)	1.5 (0.7–3.1)	0.257	0.22
TC+CC	109 (47.4)	118 (51.3)	1.1 (0.8–1.6)	0.473	0.001
P_{trend}[b]			0.269		
hsa-mir-146a G>C (rs2910164)					
GG	138 (61.6)	129 (56.1)	Reference	—	
GC	81 (36.2)	90 (39.1)	1.2 (0.8–1.8)	0.361	0.007
CC	5 (2.2)	11 (4.8)	2.4 (0.8–7.0)	0.119	0.63
GC+CC	86 (38.4)	101 (43.9)	1.3 (0.9–1.8)	0.219	0.005
P_{trend}[b]			0.127		
Combined effect of minor alleles					
0–2 variant alleles	201 (87.4)	185 (80.4)	Reference	—	
>2 variant alleles	29 (12.6)	45 (19.6)	1.7 (1.0–2.8)	0.051	0.26

Abbreviations: CI, confidence interval; OR, odds ratio.
Note: The total number of subjects in some columns may vary because of missing values.
[a]Adjusted for age, gallstone status and gender.
[b]Tests for trend of odds were two sided.

Table 3 ORs and 95% CIs for GBC in relation to miRNA polymorphisms by gallstone status

Genotype	Controls n (%)	Gallbladder cancer patients n (%)	OR (95% CI)[a]	P-value
hsa-mir-196a2 C>T (rs11614913)				
Stones				
CC	136 (59.1)	55 (47.0)	Reference	—
CT+TT	94 (40.9)	62 (53.0)	1.6 (1.0–2.4)	0.057
No stones				
CC	136 (59.1)	64 (56.6)	Reference	—
CT+TT	94 (40.9)	49 (43.4)	1.1 (0.7–1.8)	0.594
hsa-mir-499 T>C (rs3746444)				
Stones				
TT	121 (52.6)	59 (50.4)	Reference	—
TC+CC	109 (47.4)	58 (49.6)	1.1 (0.7–1.7)	0.728
No stones				
TT	121 (52.6)	53 (46.9)	Reference	—
TC+CC	109 (47.4)	60 (53.1)	1.2 (0.8–1.9)	0.375
hsa-mir-146a G>C (rs2910164)				
Stones				
GG	138 (61.6)	62 (53.0)	Reference	—
GC+CC	86 (38.4)	55 (47.0)	1.4 (0.9–2.2)	0.157
No stones				
GG	138 (61.6)	67 (59.3)	Reference	—
GC+CC	86 (38.4)	46 (40.7)	1.2 (0.7–1.9)	0.501

Abbreviations: CI, confidence interval; GBC, gallbladder cancer; miRNA, microRNA; OR, odds ratio.
Note: The total number of subjects in some columns may vary because of missing values.
[a]Adjusted for age, gallstone status and gender in logistic regression model.

and 1.3, respectively), compared with their wild-type genotypes (Table 2). To look for the combined effect of these polymorphisms, we took the three miRNA loci into consideration together and found that patients carrying >2 unfavorable loci had a borderline significantly increased risk of GBC than those with 0–2 unfavorable loci (adjusted OR=1.7; 95% CI=1.0–1.8; P=0.051) (Table 2). Additional models, adjusted for other variables, gave qualitatively equivalent results (data not shown).

To further explore whether the effects of genetic variations in miRNAs were modified by epidemiologic factors, GBC patients and controls were stratified on the basis of various host characteristics. First, we examined differences between the GBC cases and controls on the basis of gender, based on the statistical interaction tests using the likelihood ratio test, and found no statistically significant interaction (data not shown).

miRNA polymorphisms and modulation of risk in the presence of gallstone

To explore the modulation of GBC risk by the gallstone status, GBC patients were further stratified on the basis of the presence or absence of gallstones and compared separately with controls in relation to the *hsa-mir-146a* G>C, *hsa-mir-196a2* C>T and *hsa-mir-499* T>C markers (Table 3). After adjustment for age and gender, a borderline statistically significant association was observed between *hsa-mir-196a2* CT+TT genotype and GBC cases with gallstones (OR=1.6; P=0.057). We did not observe statistically significant interactions for gallstone status with any of the other miRNA polymorphisms (Table 3).

miRNA polymorphisms and interaction with tobacco usages

Interactions of tobacco usages with the genotypes of *hsa-mir-146a*, *hsa-mir-196a2* and *hsa-mir-499* polymorphisms were analyzed in a case-only analysis to explore the modulation of risk for GBC. None of the genotypes of the polymorphisms studied reached statistical significance for developing GBC (data not shown).

DISCUSSION

In this population-based case–control study of GBC in North India, we found that the genetic polymorphism in the *hsa-miR-146a* (rs2910164), *hsa-mir-196a2* (rs11614913) and *hsa-mir-499* (rs3746444) genes were associated with increased overall risk of developing GBC albeit the associations were non-significant. This is the first study exploring the function of common SNPs in miRNA genes as candidate biomarkers for GBC susceptibility.

The miRNAs are 20- to 23-nucleotide long non-coding RNA molecules that post-transcriptionally regulate gene expression, thus having an imperative function in diverse cellular processes such as proliferation, differentiation, apoptosis and various diseases including cancer.[7,21]

Conservative predictions indicate that up to 30% of the genes in human beings may be regulated by miRNAs, acting as endogenous repressors of target genes including tumor suppressor genes such as

BRCA1-2, p53 and PTEN.[22] Besides gallstones, chronic infection and subsequent inflammation of the gallbladder with *Salmonella typhi* and *Helicobacter species* have been associated with increased risk of GBC.[1,23,24] Recent evidence suggests that a subset of miRNAs is devoted for the perception of pathogen-associated molecular patterns present on the surface of bacteria and viruses.[25] These miRNAs in turn regulate the development and differentiation of immune cells, antibody production and innate immunity regulation.[26] Repression of these critical miRNA pathways could potentially affect miRNA transcription, biogenesis or activity affecting expression of genes that regulate cell proliferation and apoptosis. This study was aimed to evaluate the function of SNPs in pre-miRNAs, which might represent a newly described mechanism of GBC predisposition.

The *hsa-miR-146a* G>C polymorphism (rs2910164) results in a change from a G:U pair to a C:U mismatch in the stem region of miR-146a precursor, which results in elevated expression of mature miR-146a.[27] Overexpression of miR-146a corresponds to loss of various tumor suppressor genes and receptors associated with signaling pathways that has vital functions in cell growth and immune recognition.[28] Overexpression of miR-146a has been reported as a signature in various cancers.[29] The *hsa-mir-196a2* C>T (rs11614913) polymorphism is located in the pre-miRNA hairpin loop affecting the miRNA biogenesis. Recently, this SNP was found to be associated with survival in individuals with NSCLC and was shown to affect the binding of miR-196a to its target mRNA resulting in a significant increase in mature miR-196a levels.[9] This polymorphism was also associated with a follow-up case–control study in Chinese women with breast cancer.[10] The *hsa-mir-499* T>C (rs3746444) polymorphism was associated with a significantly increased risk of breast cancer susceptibility in Chinese women.[10] One of the targets of miR-499 is PDCD4 (Programmed cell death 4), which is a neoplastic transformation inhibitor. Variations in the miR-499 gene could modify the PDCD4 expression levels, which can subsequently progress to gallbladder epithelium transformation.

In this study, patients carrying >2 variant alleles were showing borderline significance. But as borderline significance results should be interpreted with caution, this study with negative results in individual SNPs restrict us to draw any positive conclusion.

This study in GBC failed to replicate the recent association of miRNA gene variants in various other cancers.[9–13] It is possible that association of miRNA polymorphisms may be cancer specific. However, the non-significant results of our study could be explained because of inherent difficulties in validating and replicating association studies with complex traits such as cancer and/or as a consequence of ethnic variation. Moreover, to limit potential population bias in our study, we used genomic control method as described by Devlin and Roeder,[16] so our failure to replicate the initial findings also does not emerge out to be a consequence of population stratification.

Our study has several strengths, as all our control subjects were under HWE, all our cases were histopathologically confirmed and severe quality control for genotyping was used. This study with 230 GBC cases and controls had >80% power to detect an OR of 2.0 or greater at a 5% significance level. However, the lack of significant associations suggested that the sample size of our study may be insufficient.

In conclusion, we analyzed the association between *hsa-mir-196a2* C>T, *hsa-mir-499* T>C and *hsa-mir-146a* G>C polymorphisms and the susceptibility to develop GBC in North Indian population. This study attempted to find out the potential contribution of miRNA SNPs to GBC etiology and progression and also for screening high-risk individuals for prevention and early detection of GBC. Even though our study was unable to reproduce the earlier findings, the data may be useful for a meta-analytical evaluation of the association of miRNA gene variants and GBC risk. To further evaluate these polymorphisms and GBC risk, similar studies in diverse ethnic groups with a much larger sample size along with functional and expressional characterizations of the pre-miRNA SNPs and their target mRNAs in GBC are required. To the best of our knowledge, this is the first study evaluating the effects of genetic polymorphisms in pre-miRNA genes on the risk of GBC.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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