Association of a let-7 *KRAS* rs712 polymorphism with the risk of breast cancer

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ABSTRACT. Breast cancer (BC) is a common malignancy affecting women, with increasing incidences of this disease in China every year. Recent studies have extensively investigated a single nucleotide polymorphism in the let-7 miRNA binding site of the 3'-untranslated region of *KRAS* mRNA. The aim of this study was to determine the genotype frequency of the *KRAS* rs712 polymorphism, and evaluate its effect on BC risk. This hospital-based case-control study comprised 228 patients with histologically confirmed BC and 251 healthy controls. The let-7a *KRAS* rs712 polymorphism was analyzed by polymerase chain reaction-restriction fragment length polymorphism. We observed no statistically significant association between BC risk and the let-7a *KRAS* rs712 polymorphism (GT *vs* GG, OR = 0.98, 95%CI = 0.66-1.46; TT *vs* GG, OR = 0.78, 95%CI =

0.28-2.21). However, the rs712 polymorphism was significantly associated with the N status of BC patients (GG *vs* GT/TT, OR = 0.52, 95%CI = 0.30-0.92; G allele *vs* T allele, OR = 0.60, 95%CI = 0.37-0.97). We found no association between the let-7 rs712 polymorphism and BC risk. However, the let-7 rs712 G/T polymorphism was discovered to play a potential role in BC tumor metastasis; therefore, it may be employed as a new biomarker or therapy targeted towards resistant tumor metastasis.

Key words: let-7; *KRAS*; Polymorphism; Breast cancer

INTRODUCTION

Breast cancer (BC) is currently recognized as the most common malignancy affecting women; breast cancer accounts for approximately 20% of all cancers in females in the Western countries. Approximately 1.2-1.4 million women have been reported to suffer from BC every year worldwide. The mortality rate of BC patients aged 40-50 is approximately 50% (McPherson et al., 2000). The incidence of BC has increased by 3-4% each year in China; in addition, research has indicated an increasing trend of BC developing in younger women (Linos et al., 2008). BC tumorigenesis is a complex process affected by several factors, including age, geographical variation, and lifestyle and genetic factors (Dunning et al., 1999). However, the exact etiology of BC tumorigenesis remains poorly understood.

Genetic biomarkers, which also function as cancer biomarkers, play a key role in preclinical and clinical oncology. These markers assist in predicting a high risk of cancer, as well as cancer diagnosis at an early stage. Therefore, these could be used to diagnose cancer, as well as to determine cancer prognosis and epidemiology (Savas et al., 2013). Several genetic biomarkers that affect various cancers, such as the epidermal growth factor receptor and Kit, have seen clinical application (Kaneda et al., 2013; Sicklick et al., 2013). Therefore, the discovery of genetic biomarkers specific for BC, which can be used to identify an at-risk population at an early age, has a lot of potential in a country comprising a fourth of the world population.

MicroRNA (miRNA) are short non-coding RNA that act as post-transcriptional regulators of gene expression by binding to the 3'-untranslated region (3'-UTR) of their target genes (Bartel, 2004). Recent evidence has shown that miRNA are differentially expressed in a number of human diseases, including tumors (Gottardo, 2007; Hu et al., 2008; Gramantieri et al., 2008); in addition, it has been reported that single nucleotide polymorphisms (SNPs) in miRNA binding sites could lead to differential regulation of target gene expression (Chin et al., 2008; Gao et al., 2009). The *KRAS* gene belongs to ras gene family, which plays a critical role in tumor pathogenesis. Polymorphisms in the let-7 binding site, locating in the 3'-UTR of the human *KRAS* gene, affects the binding of *KRAS* gene to the let-7 miRNA, thereby regulating KRAS activity (Johnson et al., 2005). Polymorphisms in the let-7 binding site are associated with increased risk of incidence of several cancers, including non-small-cell lung cancer and oral cancer (Chin et al., 2008; Christensen et al., 2009).

A polymorphism in the rs712 region of the let-7 binding site on the *KRAS* gene, which functions as an miRNA target site, is correlated with risk of oral squamous cell carcinoma, gastric cancer, and colorectal cancer (Wang et al., 2012; Li et al., 2013; Pan et al., 2014b). However, to our knowledge, there has been no study analyzing the association between rs712 polymorphism and BC risk in the Han population. Therefore, in this study, the genotype frequency of the rs712

polymorphism in the let-7 binding site was determined to interpret its potential effect on BC risk in a hospital-based case-control study in a Chinese Han population.

MATERIAL AND METHODS

Study population

A hospital-based case-control study was designed to assess the effect of the let-7 *KRAS* rs712 polymorphism on the risk of BC. Two hundred and twenty eight patients with histologically-confirmed BC and two hundred and fifty one healthy controls were included in this study. All study subjects were recruited from the Han ethnic group and were selected from People's Hospital of Leshan between February 2010 and December 2013. Clinical information, including the age, age at menarche, immunohistochemical features, and TNM status, was collected from the medical records of the patients. Patients with recurring BC, or with BC combined with other cancers, were excluded from the study. Genetically unrelated controls were recruited from healthy volunteers who visited to the hospital for a routine physical examination during the same period. Individuals with a history of cancer and inflammatory diseases were excluded from this study. The control subjects were age- (± 2 years) and ethnicity-matched to the patients. Written informed consent was obtained from each participant prior to the study; the study protocol was approval by the Ethics Committee of the hospital.

Genotyping

Genomic DNA was extracted from venous blood using a standard DNA isolation kit (Bioteke, Beijing, China) according to the instructions provided by the manufacturer. Polymorphisms in the let-7a *KRAS* rs712 gene were determined using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Previously described primer sequences and reaction conditions were used in this assay (Li et al., 2013). Genotyping accuracy was confirmed by sequencing randomly-selected PCR products; the results were discovered to be completely identical.

Statistical analysis

The data were analyzed using the SPSS software (v.16.0; SPSS Inc., Chicago, IL, USA). The observed genotype frequencies were compared between the controls and cases by determining the Hardy-Weinberg equilibrium (HWE) using the chi-square test. Two-sided χ^2 tests were used to evaluate the differences in the genotype and allele frequencies of the let-7 *KRAS* rs712 polymorphisms between cases and controls. The effect of the *KRAS* rs712 polymorphism on BC risk and the differential clinical status of BC was expressed as the odds ratio (OR) and 95% confidence interval (CI). P value <0.05 was considered to be statistically significant.

RESULTS

Characteristics of study subjects

Table 1 summarizes the clinical characteristics of the cases and controls. The average age

of the 228 BC patients was 50.4 years and 251 healthy control subjects was 48.3 years. The cases and controls were suggested to be adequately matched in terms of the age and age at menarche. More than half of the BC patients expressed the estrogen receptor (62.7%) or the progesterone receptor (54.4%). An analysis of the TNM status revealed that 78.1% of the BC patients were at the T1 and T2 stages of tumor; a majority of the remaining patients (21.9%) were at stages T3 and T4. The clinical records also revealed that 42.5% of the patients were at the N0 stage of tumor, while the remaining 57.5% of the patients were at the N1, N2, or N3 stages. Nearly all of the patients were at the M0 stage (99.1%).

Table 1. Characteristics of the study population.

Variables	BC patients (N = 228)	Controls (N = 251)
Age (years)	50.4 ± 9.4	48.3 ± 11.8
Age at menarche (years)	14.1 ± 1.5	14.1 ± 1.6
Estrogen receptor (%)		
Positive	143 (62.7)	
Negative	85 (37.3)	
Progesterone receptor (%)		
Positive	124 (54.4)	
Negative	104 (45.6)	
TNM grades		
T status (%)		
T1-2	178 (78.1)	
T3-4	50 (21.9)	
N status (%)		
N0	97 (42.5)	
N1-3	131 (57.5)	
M status (%)		
M0	226 (99.1)	
M1	2 (0.9)	

BC = breast cancer.

Effect of the rs712 polymorphism on BC risk

The genotype and allele frequencies of the let-7 *KRAS* rs712 polymorphism are summarized in Table 2. The genotype frequencies of rs712 were in agreement with the HWE in both cases and controls. The GG, GT, and TT genotype frequencies of the let-7 *KRAS* rs712 polymorphism were 68.0, 28.5, and 3.5% respectively, in the members of the BC group; the genotype frequencies were similar in the control subjects (68.9, 28.3, and 2.8%, respectively). The GT and TT genotypes were not significantly associated with BC risk, compared to the GG genotype (GT *vs* GG, OR = 0.98, 95%CI = 0.66-1.46; TT *vs* GG, OR = 0.78, 95%CI = 0.28-2.21). Similarly, the allele distribution did not differ significantly among the cases and controls (OR = 0.94, 95%CI = 0.68-1.32). This implied that the rs712 polymorphism at the let-7 binding site may not be correlated with BC risk.

Effect of the rs712 polymorphism on the clinical features of BC patients

The effect of the rs712 polymorphism on the clinical features of BC patients is summarized in Table 3. The rs712 polymorphism was not associated with the status of the estrogen and progesterone receptors in BC patients. On the other hand, the rs712 polymorphism was significantly associated with the N-stage (TNM staging) of BC patients (GG *vs* GT/TT, OR = 0.52, 95%CI = 0.30-0.92; G allele *vs* T allele, OR = 0.60, 95%CI = 0.37-0.97). This suggested that the

rs712 TT polymorphism and T allele was more likely associated with decreased risk of lymph node metastasis in BC patients.

Table 2. Genotype and allele frequencies of the rs712 polymorphism in breast cancer (BC) patients and controls.

Polymorphism	Patients [N = 228 (%)]	Controls [N = 251 (%)]	OR (95%CI)	P value
GG	155 (68.0)	173 (68.9)	1.00 (Ref)	
GT	65 (28.5)	71 (28.3)	0.98 (0.66-1.46)	0.92
TT	8 (3.5)	7 (2.8)	0.78 (0.28-2.21)	0.65
G	375 (82.2)	417 (83.1)	1.00 (Ref)	
T	81 (17.8)	85 (16.9)	0.94 (0.68-1.32)	0.73

OR = odds ratio; CI = confidence interval.

Table 3. Association between the rs712 polymorphism and clinical features of breast cancer (BC) patients.

Clinical features	Genotype frequency		OR (95%CI)	P value
	N (%)	N (%)		
Estrogen receptor	Positive	Negative		
GG	100 (69.9)	55 (64.7)	1.00 (Ref)	
GT/TT	43 (30.1)	30 (35.3)	1.27 (0.72-2.25)	0.41
G	237 (82.9)	138 (81.2)	1.00 (Ref)	
T	49 (17.1)	32 (18.8)	1.12 (0.69-1.84)	0.65
Progesterone receptor	Positive	Negative		
GG	87 (70.2)	68 (65.4)	1.00 (Ref)	
GT/TT	37 (29.8)	36 (34.6)	1.25 (0.71-2.18)	0.44
G	207 (83.5)	168 (80.8)	1.00 (Ref)	
T	41 (16.5)	40 (19.2)	1.20 (0.74-1.94)	0.45
T status	T1-2	T3-4		
GG	120 (67.4)	35 (70.0)	1.00 (Ref)	
GT/TT	58 (32.6)	15 (30.0)	0.89 (0.45-1.75)	0.73
G	293 (82.3)	82 (82.0)	1.00 (Ref)	
T	63 (17.7)	18 (18.0)	1.02 (0.57-1.82)	0.94
N status	N0	N1-3		
GG	58 (59.8)	97 (74.0)	1.00 (Ref)	
GT/TT	39 (40.2)	34 (26.0)	0.52 (0.30-0.92)	0.02
G	151 (77.8)	224 (85.5)	1.00 (Ref)	
T	43 (22.2)	38 (14.5)	0.60 (0.37-0.97)	0.03

OR = odds ratio; CI = confidence interval.

DISCUSSION

In this study, the let-7 *KRAS* rs712 polymorphism was investigated in 228 BC patients and 251 healthy control subjects. We did not find any significant association between the rs712 polymorphism at the let-7 binding site in the *KRAS* 3'-UTR and BC risk. However, the rs712 TT polymorphism and T allele was found to be more likely associated with decreased risk of lymph node metastasis in BC patients.

The *KRAS* gene encodes a protein that is a member of the small GTPase superfamily, and plays a critical role in the pathogenesis of various tumors by activating the RAF/MEK/MAPK pathway (Kranenburg, 2005; Kent et al., 2012). Previous studies have shown that mutations in the *KRAS* gene, which leads to a change in KRAS sensitivity or elevated KRAS expression, are correlated with the development of various human cancers (van Grieken et al., 2013; Zhang et al., 2013). miRNA are trans-acting factors that function as regulators of cancer development, progression, and metastasis by binding to specific target genes, including *KRAS*. Members of

the let-7 family, one of the earliest discovered miRNA, function as tumor suppressors in various malignant tumors, including BC (Yu et al., 2007; Chang et al., 2008; Liang et al., 2011). Targeting and down-regulation of RAS, and binding to specific sites in the 3'-UTR of the *KRAS* mRNA activates the tumor suppressing function of let-7 (Johnson et al., 2005). Yu et al. (2007) discovered that let-7 was highly expressed in breast cancer stem cells, and suppressed tumor cell amplification in mice. In addition, let-7 miRNA was suggested to play a role in the growth of BC cells.

The regulatory mechanism of let-7 miRNA on target genes was explained by analyzing the various polymorphisms in the target gene. Functional SNPs in the miRNA binding site of let-7 (in the 3'-UTR of *KRAS* mRNA) mainly focus on the LCS6 polymorphism (rs61764370); these are associated with the risk of several types of cancer, including colorectal cancer, ovarian cancer, and breast cancer (Paranjape et al., 2011; Ratner et al., 2012; Sebio et al., 2013). Hollestelle et al. (2011) reported that the LCS6 variant allele in the *KRAS* mRNA was associated with an increased risk of breast cancer and triple-negative breast cancer in families with the *BRCA*1 mutation (Hollestelle et al., 2011; Paranjape et al., 2011). Several recent studies focusing on the rs712 polymorphism in the *KRAS* let-7 region have indicated its association with increased risk of oral squamous cell carcinoma, gastric cancer, and colorectal cancer (Wang et al., 2012; Li et al., 2013; Pan et al., 2014b). The let-7 *KRAS* rs712 polymorphism has been suggested to be a new biomarker of tumor genesis. However, in this study, we found no significant correlation between the let-7 *KRAS* rs712 polymorphism and BC risk. The let-7 miRNA was suggested to regulate the *KRAS* gene through the *KRAS* LCS6 mutation, or other pathways, which must be further analyzed for multiple gene function. Alternatively, the *KRAS* rs712 polymorphism may be responsible for a selective risk of tumor development. For example, Pan et al. (2014a) failed to determine an association between this polymorphism and nasopharyngeal carcinoma. Furthermore, a very small sample size may affect the correlation between genetic biomarkers and cancer risk because of the limitation exerted by population distribution. Therefore, further studies must be performed with larger sample sizes to test this hypothesis.

However, further correlation analyses revealed that the rs712 TT polymorphism and T allele was associated with decreased risk of lymph node metastasis in the BC group. This could contribute to a greater understanding of the negative regulation of BC risk and tumor stem cell amplification. A previous study has also shown that let-7 miRNA might regulate self-renewal and tumorigenicity in BC, and even decrease potential tumor metastasis (Yu et al., 2007). The discovery that the rs712 TT polymorphism is associated with BC lymph node metastasis could help determine the potential effect of *KRAS* target regulation on BC metastasis. Further studies must be performed to reinforce this finding, and to determine if the let-7 rs712 polymorphism could be employed as a new biomarker for the treatment of tumor metastasis.

This study has some limitations. The patients enrolled to this study were recruited from only one hospital, while the members of the control group were recruited from a nearby area; this may not be expressive of the general Han population. This study also analyzed a very small sample size, which is another limitation of this study. Further studies with a larger sample size, and with samples recruited from different hospitals and regions, must be performed to confirm the findings of this study.

CONCLUSION

In this hospital-based case-control study, we did not find any association between the let-7

rs712 polymorphism and BC risk. However, the rs712 G/T polymorphism was determined to be a potential regulated target indicating the effect of *KRAS* on BC metastasis. Therefore, it might be employed as a new biomarker in the treatment of resistant tumor metastasis, if confirmed by further studies employing larger sample sizes.

Conflicts of interest

The authors declare no conflict of interest.

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