RESEARCH ARTICLE

Association between Microrna 146a and Microrna 196a2 Genes Polymorphism and Breast Cancer Risk in North Indian Women

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Abstract

Background: Micro RNAs (miRNAs) are small, noncoding RNA molecules. They can function as either oncogenes or tumor suppressor genes. Single nucleotide polymorphisms (SNP) present in the pre-miRNA region could affect the processing of miRNA and thus alter mature miRNA expression. The studies done so far had shown conflicting results regarding association of two common polymorphisms i.e.hsa-miR-146 rs2910164 and hsa-miR-196a2 rs11614913 with breast cancer. OBJECTIVE: In the study, we examined the hsa-miR-146 rs2910164 and hsa-miR-196a2 rs11614913 SNP association with breast cancer patients in north Indian women. **Materials and Methods:** This study included 100 breast cancer patients and 100 controls and was done over a period of two years. Genotypes of the hsa-miR-146 (rs2910164 G>C) and hsa-miR-196a2 (rs11614913 C>T) were identified by polymerase chain reaction – restriction length polymorphism (PCR-RFLP) technique in peripheral blood DNA samples. **Statistical analysis:** We assessed the strength of association of miRNA polymorphisms with breast cancer using Odds ratio (OR) along with 95% confidence intervals. **Results:** Heterozygous genotypes of hsa-miR-196a2 rs11614913 and combined hsa-miR-146 rs2910164 & hsa-miR-196a2 polymorphism were associated with significantly increased risk of breast cancer (OR-1.7, 95% CI–1.00-3.18) and (OR-1.9, 95% CI-0.85-4.46) respectively. **Conclusion:** Our study suggests that rs2910164 GC and rs11614913 CT genotypes may contribute to breast cancer susceptibility in north Indian women.

Keywords: Breast cancer- miRNA- SNP- PCR

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Introduction

Breast cancer is the second most common cancer in the world and by far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers) (Ferlay et al., 2013). In India, 90,659 deaths occurred due to breast cancer in 2010. Breast cancer accounts for 22.2% of all new cancer diagnoses and 17.2% of all cancer deaths among women in India. Breast cancer is the most common cancer for women in metropolitan cities (Delhi, Mumbai, Chennai, Kolkata, and Bangalore). Breast cancer in urban areas of India is three times higher than in rural parts. The majority of new cases are advanced stage - locally advanced or higher stage at the time of diagnosis. (National Cancer Registry Programme, 2010) Life style factors such as later age at marriage, age at first birth, reduced breastfeeding, westernization of diet and physical activity patterns may be associated with increasing trend of breast cancer. (Moore, 2010). Although research continues on known genes to further understand breast cancer development, epigenetics and gene regulation has become new areas of interest. The discovery of micro RNAs (miRNAs) has been one of the recent progresses in understanding the mechanisms of gene regulation (Wightman et al., 1993)

Micro RNAs are short, single-stranded RNAs of ~22 nucleotides that do not code for proteins themselves. micro RNAs alter gene expression at a post-transcriptional level through base pairing with messenger RNAs (mRNA) at the 3'-untranslated region, resulting in translational repression or at the open reading frame, resulting in cleavage and degradation of the mRNA. (Verghese et al., 2008). Single-nucleotide polymorphisms (SNPs) or mutations may change the function of miRNAs through changing miRNA expression and/or maturation. (Duan et al., 2007) Small alterations in micro RNAs may have an effect on thousands of mRNAs and thus they are considered to be ideal candidates for cancer predisposition loci. However, the role of genetic variants in miRNAs is largely unknown. (Huang et al., 2012)

Single nucleotide polymorphisms that disrupt miRNA gene sequences have been associated with different types of cancer risk. Inherited mutations or rare SNPs in the primary transcripts of miRNA-15a and miRNA-16 have been linked to familial chronic lymphocytic leukemia and familial breast cancer (Kminkova et al., 2014).

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Human miRNA-146a resides in the LOC285628 gene on human chromosome 5. (Taganov et al., 2006). A G→C polymorphism (rs2910164) is located within the sequence of miR-146a precursor, which leads to a change from a G: U pair to a C: U mismatch in its stem region. The predicted miR-146a target genes include BRCA1 and BRCA2, which are key breast and ovarian cancer genes. (Shen et al., 2008). Up to now, a few studies have investigated the association between the miR-146a rs2910164 polymorphism and cancers risk like papillary thyroid cancer, gastric cancer, hepatocellular cancer and breast cancer. (Jazdzewski et al., 2008; Zeng et al., 2010; Akkiz et al.,2011; Xu et al., 2011). Jazdzewski et al. (2008) found that the miR-146a rs2910164 CC genotype in pre-miR-146a was associated with decreased mature miR-146a expression and increased influence on genes, including genes involved in the Toll-like receptor and cytokine signaling pathway.

Iorio et al., (2005) observed that many miRNAs, including miR-196a have been aberrantly expressed in breast cancer tissue compared with normal breast tissue. The hsa-miR-196a2 rs11614913 polymorphism, located on chromosome 12 (12q13.13), appears to regulate the Hox gene in a subgroup of the homeobox genes (Chen et al., 2011). The SNP rs11614913 C>T in has-miR-196a2 has been implicated in lung cancer susceptibility in a study by Tian et al., (2009). Two studies have also recently reported that this variant might be linked to breast cancer susceptibility (Hoffman et al., 2009; Hu et al., 2009). These two case control studies demonstrated that SNP rs11614913 of miRNA196a2 was significantly associated with increased breast cancer risk in both a Chinese population (Hu et al., 2009; Tian et al., 2009) and a US population (Hoffman et al., 2009). Whereas a recent case–control study showed that the hsa-miR-196a2 rs11614913 genotype was not associated with increased risk of breast cancer in Italian and German women (Catucci et al., 2010).

To build upon these findings, this case control study investigated whether there is an association between hsa-miR-146 (rs2910164) and hsa-miR-196a2 (rs11614913) polymorphism and breast cancer susceptibility in North Indian women.

Materials and Methods

The study group consisted of 98 breast cancer patients and 99 healthy controls . Criteria for the patient selection included cases that were microscopically diagnosed by histopathological examination of needle biopsy, lumpectomy and mastectomy specimens or with microscopic diagnosis of breast cancer on cytological examination (i.e. FNAC or nipple discharge). Informed consent of the participants (cases and controls) was taken. They were interviewed to collect demographic data and clinical information. Ethnic origin and race for cases and controls were similar. The inclusion criteria for the controls (only women) were absence of any prior history of cancer or pre-cancerous lesions.

DNA extraction and genotyping

Five ml of venous blood was collected from each patient and control after informed consent in sterile EDTA vials. Genomic DNA was isolated from peripheral blood samples by Proteinase K and phenol chloroform extraction procedure. The genotyping of miRNA-146a and miRNA-196a2 was performed through PCR-RFLP using specific primer sequences and restriction enzymes. The following pairs of primers were used to generate a 147-bp product for miRNA146a: forward 5′-CAT GGG TTG TGT CAG TGT CAG AGC T-3′ and reverse 5′-TGC CTT CTG TCT CCA GTC TTC CAA-3′ and 149-bp product for miRNA 196a2: forward 5′-CCC CTT CCC TTC TCC TCC AGA TA-3′ and reverse 5′-CGA AAA CCG ACT GAT GTA ACT CCG-3’.

The PCR reaction was performed in 25µl of reaction mixture containing PCR buffer, 1.5 mM $MgCl_2$, 0.5 µM of both primer, 200 µM of each dNTP’s, 100 µg/ml bovine serum albumin (BSA), 1U Taq polymerase and approximately 300 ng DNA. After confirmation of successful PCR amplification by 1.5% agarose gel electrophoresis, each PCR product was digested overnight with 5U of SacI and MspI (New England Biolabs, Ipswich, MA, USA) for miR-146 and miR196a2 respectively at 37°C overnight. For the miRNA-146a (rs2910164) polymorphism, PCR product of 147bp was digested into two fragments of 122bp and 25bp products for mutant genotype (CC) whereas three fragments of 147bp, 122bp and 25bp represented the heterozygous (GC) genotype and an undigested 147bp band was indicative of the wild type genotype (GG). In case of miRNA-196a2 (rs11614913) polymorphism, PCR product of 149bp was digested into fragments of 125bp and 24bp which was indicative of wild type genotype (CC), whereas the presence of three bands of 149bp, 125bp and 24bp represented the heterozygous (CT) genotype and an undigested 149-bp band represented the mutant genotype (TT). To ensure quality control, genotyping was performed without knowledge of the subjects’ case/control status and a 15% random sample of cases and controls was genotyped twice by different persons; reproducibility was 100%. For visualization Poly acryl amide gel electrophoresis (PAGE) was performed with silver staining for permanent record.

Statistical Analysis

Statistical analysis was done. Odds Ratio (OR) and its 95% confidence interval (CI) were calculated. All those p values which were less than 0.05 were taken to be significant. The differences in the distribution between cases and controls were tested using the χ^2, and student’s t test where appropriate. OR and 95% CI were used to describe the strength of association between genotypes and breast cancer.

Results

The characteristics of study population are shown in Table 1. The mean age (±SD) of breast cancer patients and controls was 51.83±12.15 and 46.25±11.45 years, respectively. The most common histopathological pattern of breast cancer was Intra Ductal Carcinoma (IDC) seen

Table 1. Showing Demographic Characteristics of Both Cancer Cases and Controls Group

Variable	Cancer Cases	Controls	P Value
Mean Age	51.8±12.1	46.2±11.4	0.0009
Mean age of menarche	14.1±0.8	14.1±0.7	0.592
Patients at Menopause	69%	55%	
Family history of Breast cancer	9%	-	
Axillary lymph node involvement	54%	-	
Nulliparity	7%	2%	

in 87 (87%) patients. Fifty four patients had metastasis in lymph nodes from breast cancer. Sixty nine (69%) were postmenopausal in breast cancer group whereas 55 (55%) were postmenopausal in control group. Genotyping was done by PCR-RFLP. The frequency of miRNA146a (rs2910164) and miRNA196a2 genotype distributions in the breast cancer cases and controls group is shown in (Table 2) as that 46.9% breast cancer patients were heterozygous for miR-146a as compared to 39.39% controls (OR=1.36. CI = (0.77-2.39)

In case of miR-196a2, heterozygous genotype was seen in 49.5% breast cancer patients and 35.35% controls. (OR= 1.7. CI= (1.00-3.18).However, no mutant genotype was found in both.

We also tried to study association between other breast cancer characteristics and genotypes of miR-146 and miR196a2. Only Intra Lobular Carcinoma (ILC) cases showed slightly higher association with heterozygous genotype of miRNA146a gene. Significant association was found between heterozygous genotype of miRNA146 (OR – 2.57, 95%CI – 0.95- 6.95) and miRNA 196a2 (OR – 4.6, 95%CI – 1.62 -13.0) genes and patients aged >54 years.

We also studied the association of combined miRNA196a2 C/T (rs11614913) and miRNA146a G/C (rs2910164) functional polymorphisms and breast cancer risk. Table 3 shows pooled ORs of the miRNA196a2 C/T (rs11614913) and miRNa146a G/C (rs2910164) functional polymorphisms on breast cancer risk.

On carrying out the association analysis between the individuals who had variant genotype for the gene and breast cancer risk, an increased odds of 1.95, 95% CI, 0.85-4.46 and p=0.11 was found. Thus, from this data we concluded that due to joint effect of both the polymorphisms, risk for breast cancer is elevated almost twice.

Table 2. Showing miRNA146a (rs2910164) and miRNA 196a2 Polymorphism Rate among the Cancer Cases and Control Group

Genotype	Cancer cases n=98(%)	Controls n=99(%)	OR(95% CI)	p value
hsa-miR-146a				
GG	52(53.1%)	60(60.61%)	1	
GC	46(46.9%)	39(39.39%)	1.36(0.77-2.39)	0.285
CC	0	0		
	n=95(%)	n=99(%)		
hsa-miR-196a2				
CC	48(50.5%)	64(64.6%)	1	
CT	47(49.5%)	35(35.35%)	1.7(1.00-3.18)	0.04
TT	0	0		

Table 3. Distribution of Combined Polymorphisms of miRNA-196a2 (rs11614913) and miRNA-146a (rs2910164) Gene and Their Association with Breast Cancer

miRNA-196a2 and 146a Gene	Cancer Cases	Controls	Odds Ratio (95% CI)	p value
Wild	24 (55.8%)	42 (71.2%)	1	
Heterozygous	19 (44.2%)	17 (28.8%)	1.95 (0.85-4.46)	0.11
Total	43 (100%)	59 (100%)	-	-

Discussion

It has been estimated that miRNAs can regulate at least 50% of human genes, including tumor suppressor genes such as BRCA1, BRCA2, p53 and PTEN. Because miRNA targets a number of functionally important protein-encoding genes, so the genetic variations in miRNA genes might represent a new mechanism of cancer predisposition (Shen et al., 2008)

The growing interest in evaluating the association between the hsa-miR-196a2, hsa-miR-146 SNP and development of breast cancer were the foundations for this study. The current study was undertaken to find the association between SNPs of miRNA 146a, 196a2 genes with breast cancer risk in North Indian population, to study genotypes of these miRNAs and their association with different clinicopathological features of breast cancer.

Many associations between miRNAs and occurrence of breast cancer have been demonstrated experimentally. The understanding of the mechanisms involved in these associations could establish the use of these markers (miRNAs) as powerful tools for the prevention and treatment of breast cancer (Shen et al., 2008).

Recently, a strong link between miRNAs that are altered, either structurally or in the number of mature miRNAs, and cancer has been demonstrated. Mutations, polymorphisms, misexpression, or altered mature miRNA processing are likely to be pleiotropic and may contribute to cancer susceptibility and progression. Inherited mutations or are SNPs in the primary transcripts of has-mir-15a and has-mir-16- 1 have been linked to familial chronic lymphocytic leukemia and familial breast cancer which was further supported in chronic lymphocytic leukemia. It has also been shown that miRNA expression patterns have relevance to the biological and clinical behavior of human solid tumors. For example, hsa-miR196a was highly expressed in pancreatic and breast cancers compared with normal tissues, and the elevated expression was associated with significantly reduced survival for pancreatic cancer (Alshatwi et al., 2012).

To the best of our knowledge, this is the first study in north India to provide evidence that common SNPs in miRNAs might play an important role in breast cancer prediction.

In our study, miR146 variant allele was found to be marginally associated with increased breast cancer risk.

Others (Hu et al., 2009; Catucci et al., 2010; Alshatwi et al., 2012) have also reported no significant association of miRNA146 gene with breast cancer risk. So our findings are consistent with other studies. Whereas miRNA 196 gene was found to be associated with increased risk of breast cancer as suggested by other studies (Hu et al., 2009; Alshatwi et al., 2012). However, Hoffman et al. (2009); Jedlinski et al. (2011) and Catucci et al. (2010) do not support miRNA196a2 gene SNP association with breast cancer risk in their studies.

Gao et al., (2011) conducted a meta-analysis regarding the hsa-miR-196a2 distribution and reported that women with the CC polymorphic genotype had an increased risk of breast cancer with OR of 1.30 (1.01-1.68). Conversely, Catucci et al., (2010) did not identify statistical correlations between the presence of hsa-miR-196a2 rs11614913 in women with breast cancer and controls in German and Italian populations .Thus the results from these studies remain conflicting due to different genetic background and other population related factors.

The results of our study showed that heterozygous genotype of miR196a2 and combined polymorphism of miR-146 and miR196a2 genes were associated with increased risk of breast cancer which suggests that miRNA polymorphism can contribute to breast cancer susceptibility.

In conclusion, individual and combined genotypes of micro RNA processing pathway genes may influence breast cancer susceptibility. As miRNA are potentially useful in the diagnosis, prognosis and the monitoring of treatment response so they can be used as biomarkers.

The present study indicates that both miRNA146 and miRNA196a2 single nucleotide polymorphisms could contribute to increased breast cancer risk. To the best of our knowledge, this is the first genetic study in north Indian population in breast cancer which demonstrated association of miRNA polymorphisms with breast cancer.

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