

Association of miR-146a gene polymorphism and expression levels of its target protein RhoA in Pakistani breast cancer patients

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Abstract

Objective: To assess the association of miR-146a and its target protein RhoA expression levels in breast cancer.

Methods: The case-control study was conducted at Riphah International University, Islamabad, Pakistan, from March 2017 to November 2018, and comprised confirmed breast cancer cases and controls who were matched for age and ethnicity. Genotyping and expression profiling of archived samples was performed. Data was analysed using SPSS 22.

Results: Of the 590 subjects, 295(50%) each were cases and controls. Among the cases, there were 195(66%) Punjabis, 59(20%) Pathans and 41(14%) Kashmiris. The corresponding numbers among the controls were 198(67%), 58(19.7%) and 39(13.2%). The association between genotypes of the cases and controls was significant ($p < 0.05$). Strong association was seen in dominant, recessive and allelic models ($p = 0.05$). In Punjabi group the association was ($p < 0.00$) significant, but this association was not significant in Kashmiri and Pathan groups ($p > 0.05$). No association was found with the receptor status and miR-146a polymorphism.

Conclusion: The miR-146a gene polymorphism rs2910164 G/C was found to have increased susceptibility to breast cancer at genotype and allelic levels.

Keywords: Single nucleotide polymorphism, Breast cancer, miR-146a, RhoA. (JPMA 71: 686; 2021)

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Introduction

Universally, 611,600 women died from breast cancer in 2017, and from 2007 to 2017, a 27% increase in global breast cancer deaths were observed.¹ In Pakistan, approximately, 25.2/100,00 females die of breast cancer each year.² There are different ethnic groups in Pakistan, including Punjabis, Sindhis and Pathans.³ Micro-ribonucleic acids (miRNAs) are a few nucleotides long, endogenous, non-coding RNAs. They aim for the target sequences, base pair to the 3' untranslated (UTR) region of the target genes, and, after binding, regulate the expression of related genes either by repressing or breaking down messenger RNA (mRNA) after transcription.⁴ The miR-146a is located at chromosome 5q34,⁵ and acts as a tumour suppressor in various malignancies.⁶ The miRNA-146a is implicated in cell proliferation, tumour signalling pathways and it seems to affect metastasis in diverse tumours⁷ and plays a role in suppressing metastasis by inhibiting epidermal growth factor receptor.⁸

The rs2910164 G>C variation produces alteration in G to C in the stem region, affecting the expression of miRNA and leading to variations in the regulation of its target

mRNAs.⁹ One of its important target genes is RhoA etc.¹⁰ Studies have concluded that RhoA was up-regulated in a variety of cancers, including breast cancer, and its altered expression levels can affect the process of metastasis.⁵

The current study was planned to determine the relationship of miR-146a polymorphism along with the relative expression of its target gene RhoA in breast cancer in Pakistani population, and to assess data along ethnic lines.

Materials and Methods

The case-control study was conducted at the multidisciplinary lab of the Islamic International Medical College, Riphah International University, Islamabad, Pakistan, from March 2017 to November 2018. After approval from the institutional ethics review board, the sample size was calculated using the World Health Organisation (WHO) calculator with 95% confidence level, alpha error 5% and anticipated population proportion ranging 20-27% in aged women >50 years.¹¹ The sample was raised using non-probability convenient sampling technique from government hospitals, including Holy Family Hospital, Rawalpindi, the Arms Forces Institute of Pathology, Rawalpindi, and the Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Islamabad. Those included were confirmed breast cancer cases at various cancer stages and with different histological types with no pre-cancerous condition or any other chronic

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disease. Patients having any other accompanying malignancy either presently or in the past were excluded. Females with any first-degree relative with breast cancer were also excluded.

Also, healthy controls matched for age and ethnicity were enrolled from the general population.

After taking written informed consent from all the participants, clinico-pathological data was collected from the patient records, including breast cancer stage and receptor status.

Genomic deoxyribonucleic acid (DNA) from blood was extracted by 5-7% chelex (Bio-Rad). The genotyping of genetic variant rs2910164 G/C of miR-146a gene was done as described previously through simple and cost-effective allele-specific tetra-primer amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR) technique.¹² Four sets of primers, two outer forward and reverse (FO- 5'-GGCCTGGTCTCTCCAGATGTTTAT 3') and (RO-5'-ATACCTTCAGAGCCTGAGACTCTGCC 3') and two inner allele-specific forward and reverse (G-allele -FI-5'-GATATCCCAGCTGAAGAACTGAATTGAC 3') and (C-allele RI-5'-ATGGGTTGTGTCACTGTGACGTC 3') were used for the amplification of G and C alleles. Complementary DNA (cDNA) synthesised was used for the measurement of RhoA gene expression, and after quantification by SYBR green assay (MiniOpticon real-time PCR detection system with CFX Manager™ software- Bio Rad). The mRNA expression levels were normalised to that of β -actin. Primers for RhoA were Forward 5'-TTTGGAGGTGGCATAGCCTT-3' and Reverse 5'-ATGTTTAGTCAGCTGGAGAGAAGAG-3'. Primers for β -actin were Forward 5'-TCGACAACGGCTCCGGCAT3 and reverse 5'-AAGGTGTGGTCCAGATTTTC3. Immunohistochemical (IHC) staining for RhoA protein was done on formalin-fixed paraffin-embedded (FFPE) breast tissues using standard protocol. For RhoA, Rabbit antibody to Rho-related GTP binding protein RhoA was used (BIOCYC GmbH & Co-distributed by Quartett).

Data was statistically analysed using SPSS 22. Frequencies and percentages were calculated using descriptive statistics. Clinico-pathological data, receptor status, allele frequency and RhoA protein expression of the cases was analysed using chi-square test. Genotype association was studied by using chi-square and odds ratio (OR) along with 95% confidence interval (CI). Univariate and multivariate logistic regression models were applied to estimate OR and 95% CI. $P < 0.05$ was taken as significant.

Results

Of the 590 subjects, 295(50%) each were cases and

Table-1: Association of miR-146a genotype and allelic frequencies in breast cancer cases and control groups.

SNP 146 G/C	Cases N=295 n (%)	Controls N=295 n (%)	OR (95% CI)	p-value
Genotype				
GG	240 (81.4)	265 (89.8)	Ref 1	
GC	34 (11.4)	20 (6.8)	1.87 (1.05-3.35)	0.03
CC	21 (7.1)	10 (3.4)	2.31 (1.07-5.02)	0.02
Dominant Model				
GG	240 (81.4)	265 (89.8)	--	
GC+CC	55 (18.6)	30 (10.1)	2.02 (1.25-3.26)	0.00
Recessive Model				
GG+GC	274 (92.8)	295 (100)	--	
CC	21 (7.1)	10 (3.4)	2.26 (1.04-4.88)	0.03
Alleles				
G	514 (87.1)	550 (93.2)	--	
C	76 (12.8)	40 (6.7)	2.03 (1.36-3.03)	0.00

SNP: Single nucleotide polymorphism.

Table-2: Association of miR-146a genotype with ethnicity in breast cancer cases and control groups.

Ethnicity/ genotype	Cases N=295 n(%)	Controls N=295 n(%)	OR (95% CI)	p-value
Punjabi				
GG	n=195 (%)	n=198 (%)	GG vs GC+CC	
GC	157 (80.5)	188 (94.9)	4.55 (2.19-9.42)	<0.00
GC+CC	38 (19.4)	10 (5.05)		
Kashmiri				
GG	n=41(%)	n=39(%)		
GC	34 (82.9)	33 (84.6)	1.13 (0.34-3.72)	0.84
GC+CC	7 (17)	6 (15.3)		
Pathans				
GG	n=59(%)	n=58(%)		
GC	49 (83)	44 (75.9)	0.64 (0.25-1.59)	0.33
GC+CC	10 (16.9)	14 (24.1)		

controls. Among the cases, there were 195(66%) Punjabis, 59(20%) Pathans and 41(14%) Kashmiris. The corresponding numbers among the controls were 198(67%), 58(19.7%) and 39(13.2%). Among the patients age ($p = 0.02$), tumour size ($p = 0.00$), grade ($p = 0.00$), and tumour stage ($p = 0.00$) were significantly associated with genotype. No significant correlation of family history, side, type and hormone receptor status was found with genotypes of miRNA-146a gene.

Genotypic frequencies of homozygous GG, heterozygous GC and homozygous CC genotypes showed significant association in the cases compared with the controls ($p < 0.05$). OR and probabilities for genotype homozygous GG was taken as reference 1, showing heterozygous GC OR 1.87 (95% CI: 1.05-3.35; $p = 0.03$) and homozygous CC OR 2.31 (95% CI: 1.07-5.02; $p = 0.02$). In dominant model, the combined GC and CC genotypes in cases and controls

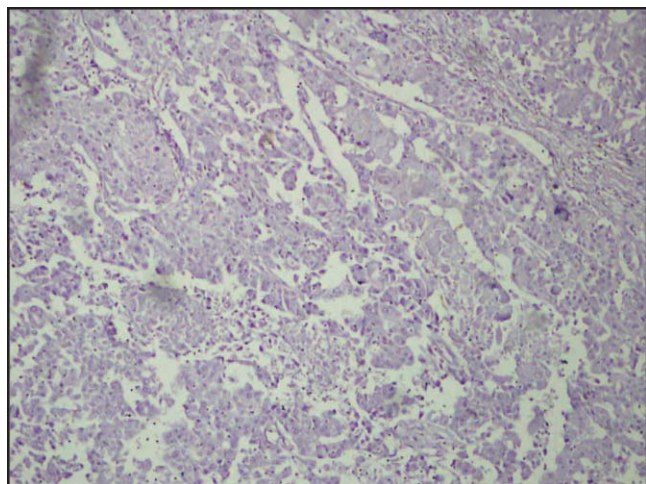


Figure-1: immunohistochemistry (IHC) negative for RhoA protein in malignant breast cancer tissue.

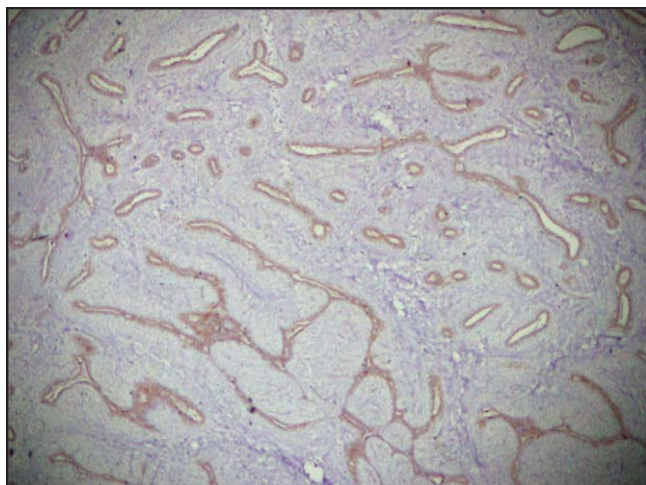


Figure-2: Positive for RhoA protein in ductal epithelial cells in non-malignant tissue.

were 18.6% and 10.1% respectively, OR2.02 (95% CI: 1.25-3.26; $p=0.001$). The recessive model was also significant, with combined GG and CC genotypes in cases and controls being 92.8% and 100%, OR 2.26 (95% CI: 1.04-4.88; $p=0.03$). Significant association with C allele variants was observed in both groups with OR 2.03 (95% CI: 1.36-3.03; $p=0.001$) (Table-1).

In terms of ethnicity, the dominant allele was G, and Punjabi group had significant association with OR 4.55 (95% CI: 2.19-9.42); $p<0.001$) (Table-2).

Significant parameters found at univariate level were put in binary logistic regression modelling, with genotype GG OR 0.47, (95% CI: 0.22-1.00; $p=0.05$) being less likely to develop breast cancer.

In multinomial logistic model, HER2 had a protective response with OR 0.09 (95% CI: 0.01-0.67; $p=0.01$). ER and PR had no association at multivariate logistic level.

IHC of 100 FFPE cases showed loss of expression in 93% cases and positive expression was seen only in 7% (Figures-1,2). RhoA expression showed significant association with stage and HER2 receptor status only ($p=0.001$). It was not significantly associated with age, grade, type and size ($p>0.05$). Negative expression in GG genotype was 75.3%, GC 18.3% and CC 6.5% ($p=0.074$)

Average mean calculated after normalizing with reference gene β -actin showed mean levels of 33.86 ± 1.73 (range: 31.00-38.91), and RhoA had mean levels of 33.78 ± 1.76 (range: 30.65-39.60).

Discussion

Definitive role of miRNA-146a rs2910164 G>C has been established in various cancers, like lung, hepatocellular and colorectal cancers,¹³ but so far no studies in Pakistan have been conducted especially for breast cancer. Studies have been conducted in various populations, like Arabs¹⁴ Jews¹⁵ and Europeans,¹⁶ to assess the risk association of miRNA-146a rs2910164 G>C polymorphism with breast cancer. Predominant genotype in the current study was GG 81.4%, in comparison to the rare allele, which was 7.1%. In different Pakistani ethnicities, G allele was the dominant one, followed by C allele. Data from 1000 genomes project and HapMap show that C is the common allele with a frequency of 60%, followed by G 40% in Asians.¹⁷ A meta-analysis for single nucleotide polymorphism (SNP) rs2910164 observed C-allele as the dominant one in Asian (0.489) and Caucasian (0.246) controls compared to 0.246 in Asians.¹⁸ In Pakistani population the percentage of C-allele was 7.1% compared to 3.4% in controls. Logistic regression model for the genotypes confirmed increased susceptibility to breast cancer with CC genotype of miR-146a in Pakistani population.

Significant association was found with Punjabi ethnic group and miR-146a genotypes. This ethnic group is present in Pakistan and India, and their haplo-group shows a strong association with the Europeans,¹⁹ and increasing association of this SNP was noted in the Europeans.²⁰ This shows that different ethnic backgrounds may have different genotypic frequency and it could depend upon different factors, like migration and environmental factors.

IHC analysis also exhibited negative expression of RhoA protein in breast cancer tissue samples. Significant association was observed only with stage of the disease, with the rest of the clinico-pathological features having no

significant relationship. Positive association with grade was observed earlier, and different stages of cancer were regulated by immune cells, which release soluble bioactive molecules as a result of adaptive immune response.²¹ In miR-146a rs2910164 G/C polymorphism negative expressions of RhoA protein levels can be explained by the fact that the activity of RhoA is controlled through some other mechanism rather than by NF- κ B signalling pathway, because in both the genotypes, negative expression levels of RhoA protein were seen. This aspect needs further in-depth study concerning different genotypes of miR-146a, because complementary binding with GG genotype (wild type) causes decrease expression levels of RhoA, but the mechanism with CC genotype needs further elucidation. A study demonstrated that increased binding capacity of the minor allele C with BRCA 1 and BRCA2, which are also target genes of miR-146a, leads to increased expression levels of miR-146a.²² It suggests that base pairing sequences are disrupted by G/C polymorphism. A study demonstrated that miR-146a directly targets the 3' UTR of RhoA mRNA.⁵

In a study on miRNA target recognition supplementary seed pairing evaluation observed that supplemental 3' pairing does play a role in the recognition of a particular target, and such pairing to the 3' UTR of miRNA will supplement the 7-8mer match, and it will also balance or recompense for any polymorphism or mismatch in the seed region.²³

In Pakistan, data related to the incidence of breast cancer is deficient, and, due to limited resources, it was not possible to include all ethnicities present across the and. As such, only three ethnicities were included in the current study.

Conclusion

Genotypes of miR-146a polymorphism and its relationship to breast cancer susceptibility showed that miR-146a gene polymorphism rs2910164 G/C had increased susceptibility to breast cancer at genotype and allelic levels in Pakistani female population, and its target protein RhoA had negative expression levels in breast cancer.

Disclaimer: The text is based on a research thesis for PhD (Biochemistry) done at Riphah University, Islamabad.

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