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SCREENING OF BREAST CANCER PATIENTS FOR KNOWN GENETIC VARIATIONS IN SELECTED CANDIDATE MICRO RNA GENES IN THE SELECTED AREA OF DISTRICT SWAT KP PAKISTAN

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Abstract

MicroRNAs (miRNAs), an important biological tool are strong regulators of gene expression; functioning in RNA silencing or controlling gene expression post-transcriptionally. It has been shown that besides the important role of miRNA in normal body functioning, abnormal expression of miRNA also has role in many serious diseases like cancer. The abnormal expression of miRNAs have been proposed to act as oncogene either by up-regulation of oncogenic mRNA or down-regulation of tumor suppressor genes in most malignancies such as in breast cancer (BC). Considering the important role of miRNAs, the present study was performed to screen the collected BC samples for known mutation in candidate miRNA genes and to establish co-relation of these genetic variations in miRNA genes with BC in the selected patients of District Swat, Pakistan. Blood samples from BC patients were collected at SINOR cancer hospital Swat and were then amplified with polymerase chain reaction (PCR) using two sets of newly designed primers. The PCR products were sequenced and screened for the presence of SNPs in hsa-mir-381, hsa-mir-487b and hsa-mir-126. This study reports the presence of SNP rs10083406 A>C, downstream to hsa-mir-381 and upstream to hsa-mir-487b while in hsa-mir-126, SNP rs4636297 A>G and SNPrs1140713 C>T were found. The resulted SNPs were analyzed statistically and found no association of these SNPs with BC. The negative association of the studied SNPs with BC may be due to small number of sequenced samples, however large number of samples may be sequenced to study the association of these SNPs with BC and analyze its frequency in the studied region of District Swat, Pakistan.

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INTRODUCTION:

Cancer is a fatal disease that arises when cells in the body start uncontrolled division and exhibit different function than normal [1]. Mostly, in cancer, a mass of cells called tumors are form in body tissues and are then named according to the affected part of the body. Of the different types of cancer, breast cancer (BC) is one of the devastating and life threatening disease in women. It is the most prevalent and fatal disease in women throughout the world [2]. It arises when a tumor originates from the milk ducts or lobules of the breast supplying milk to the ducts. Most women at certain stage of their life notice unwanted changes in their breast like changes in breast color, shape, and discharge from nipples or pain which is signs of BC [3]. It can be differentiated into two broad categories based on their morphological features: Invasive and noninvasive. In invasive cancer, the cancerous cells grow to other body parts through the bloodstream and lymph nodes while in Non-invasive cancer; the cancerous cells do not spread to surrounding tissue but remain to the affected region [3, 4]. BC is the most common malignancy in woman, causing about half million deaths every year in the world. It is considered to be the major cause of cancer-related deaths in women [6, 7]. It is estimated that every year about 1.38 million females suffer from BC with approximately 465,000 deaths per year [8, 9] and this value seems to increase day by day. On the basis of prevalence based information, it seems that each year about one million new cases of BC appear globally. In all types of reported cancers in women, about 25 percent is BC making it the most common type of cancer in females [10]. This disease is not only limited to females but can affect about 1% males also [11, 12]. According to world health organization, approximately 690,000 cases have been reported from developed and developing countries [13]. Over half of the reported cases are living in developing countries but its prevalence rate is increasing slightly in developed countries [14]. Like other countries, Pakistani women in rural and urban areas are suffering from different types of cancers [16] in which BC is the most common and has been reported to be the highest in Asia [17, 18]. One out of nine Pakistani women is the victim of this deadly disease [19, 20]. In Pakistan, it is among the top twenty causes of mortality [21]. The high prevalence rate of BC here is due to the lack of vigilance, remoteness to hospitals, high treatment cost and lack of acceptance of the disease due to fear [22]. For proper screening of different types of cancer, screening centers are lacking in Pakistan. Due to absence of facilities for early diagnosis and lack of appropriate diagnostic markers and tools, the mortality rate of women is at apex and about 40,000 women goes in the jaws of death by BC every year [23].

MATERIALS AND METHODS

Sample collection

Blood samples were collected from females with BC. These samples were taken from patients at SINOR cancer hospital Swat, Pakistan. In a similar way, blood samples were

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also taken from healthy females as a control. The blood at a volume of 3ml was taken for DNA extraction from anterior cubital vein from each female by using BD syringe, which was then transfer into EDIA tubes.

The collected blood samples were stored in the refrigerator at 4°c for DNA extraction and further processing.

Figure 3. 1: Grading of Breast cancer patients at various age groups

	BC Grading			
Age Groups	**Grade (I)**	**Grade (II)**	**Grade (III)**	**Grade (IV)**
20-40	4	16	16	1
40-60	2	29	26	1
60-80	3	8	12	0
80-100	0	0	0	0
Total	**9**	**53**	**54**	**2**

1.1. DNA extraction

Phenol chloroform protocol [24] was used to extract DNA from the collected human blood samples.

➢ Blood treatment with solution A

750 microliter (µL) blood was taken in 1.5 mL Eppendorf tube containing same amount of solution A. After mixing carefully, samples were kept at room temperature for 10 minutes. The tubes were then centrifuge for 1 min at 13000 rpm. Supernatant was discarded and the pellet was suspended in 400 µL of solution A.

➢ Treatment with solution B

After 1 min of centrifugation at 13000 rpm, the pellet was re-suspended in 8 µL of Proteinase k, 400 µL of solution B and 25 µL of 10 percent SDS.

➢ Overnight incubation

The solution was then incubated the whole night at 37·C or just for 3 hours at 65·C.

➢ Phase separation

After incubation, 500 µL of equal volume of solution C and D was added to that solution and then centrifuged at 13000rpm for 10 min. The resulted aqueous layer (supernatant) was then transfer into fresh 1.5 mL micro centrifuge tube.

➢ Washing

The collected supernatant/ aqueous phase was mixed with Solution D in the same volume and centrifuged for 10 min at 13000rpm. Again the resulted supernatant was poured into fresh micro-centrifuge tube.

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- **DNA Precipitation**

For this purpose, 55 μL of sodium acetate was added along with the same volume of isopropanol (kept at -20·C) as that of solution. The tubes were then inverted to precipitate the DNA.

- **Washing with Ethanol**

The resulted DNA was washed by adding 350 μL of 70 percent ethanol and after its complete removal by using vacuum dryer, the dried DNA pellet was dissolved in the suitable volume of DNA dissolving buffer.

Figure 3. 2: Use of different solutions/chemicals for DNA extraction

List of Chemicals	Concentration of chemicals
Solution-A	10 mM Tris-HCl at pH 7.5, MgCl_2, 1 percent v/v Triton X-100, 0.32 M sucrose
Solution-B	Tris-HCl (pH 7.5) 10mM, EDTA (pH 8.0) 2mM, 400mM Sodium chloride
Solution-C	Phenol in equilibrium with Tris-HCl having merceptoethanol and hydroxyqinoline
Solution-D	Isoamyl alcohol and chloroform in 1:24 ratio
Solution-K	made in 1 percent SDS as 50μl/ml
SDS 10%	1 Liter of SDS (100g/800ml) dissolved in dH_2O at pH 7.2
Sodium acetate (3M)	100mL (24.6102g sodium acetate/60ml of water) at pH 6
Ethanol	70%
TE Buffer	0.1 millimole EDTA, 10 millimole Tris-HCL at pH 8

For the confirmation of DNA, the extracted DNA was run on 1 percent Agarose gel having ethidium bromide as staining dye. For making 1 percent agarose gel, 1 gram of agarose was melt in 100 mL TBE Buffer and then 8 μL of ethidium bromide was added to it. This solution was poured into the gel tray and after when it became solidified put it in the Gel tank having TBE Buffer. After this, the electrophoresis apparatus was set at specific conditions which are 250 mA of current, 75 volts of voltage and for time 30 min. After completion of given time period, the gel was visualized under UV trans-illuminator.

1.2. Designing primers

For the miRNAs Hsa-miR-126 and Hsa-miR-381, primers were designed using bioinformatics tools. Then the primers of specific size were prepared commercially.

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Figure 3. 3: Primers used for amplification of Hsa-mir-381 and Hsa-mir-126 genes

Primers	Primer sequences	Size of PCR product
Has-miR-381-F Has-miR-381-R	ATAGTGAGGAACCTGCCCAGTG ATGGACGCCAATGTGATTCCC	790bp
Has-miR-126-F Has-miR-126-R	AACCCGACAGGTAAACAGCC TGCACTTCTTCCTTCATTGCAC	660bp

1.3. Polymerase chain reaction amplification

Polymerase chain reaction (PCR) was used to amplify the target genes. For this, micro centrifuge tubes (0.2mL) were used for a reaction volume of 50µl which consist of 25µl of Master mix, 16µl of water, 2µL of forward primer (10-20 pmol/µL), 2µL of reverse primer (10-20 pmol/µL) and 5µL of genomic DNA template. A thermal cycler adjusted with specific program was used for amplification of the target genes (Hsa-miR-126 and Hsa-mir-381). The amplified products were then run on the agarose gel for confirmation and were visualized on UV trans-illuminator.

Figure 3. 4: Details of 50 µl PCR reaction used during PCR

Reaction reagents	Volume
Master mix	25µl
Water	16µl
Primer (forward)	2µl
Primer (Reverse)	2µl
G. DNA template	5µl

1.3.1. Polymerase chain reaction amplification of Hsa-mir-381

Hsa-mir-381 was amplified with the initial denaturation at 95⁰C (5 min), followed by 40 cycles each with denaturation at 94⁰C (30 sec), annealing at 58⁰C (45 sec), extension at 72⁰C (1 min) and the final extension at 72⁰C (6 min).

Figure 3. 5: Thermal cycler conditions for Hsa-mir-381

Steps	Temperature	Time
Init. denat.	95 ⁰C	5 min
Denat.	94⁰C	30 sec
Anneal.	58⁰C	45 sec
Exten.	72⁰C	1 min
Final Exten.	72⁰C	6 min

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1.3.2. Polymerase chain reaction amplification of Hsa-mir-126

Hsa-mir-126 was amplified with the initial denaturation at 95 ºC (5 min), followed by 40 cycles each with denaturation at 94ºC (30 sec), annealing at 57ºC (45 sec), extension at 72ºC (1 min) and final extension step at 72ºC (6 min).

Figure 3. 6: Thermal cycler conditions for Hsa-mir-126

Steps	Temperature	Time
Init. denat.	95 ºC	5 min
Denat.	94ºC	30 sec
Anneal	57ºC	45 sec
Exten.	72ºC	1 min
Final Exten.	72ºC	6 min

1.4. Electrophoretic detection of PCR products

For Electrophoresis, 2 percent (w/v) Agarose gel was prepared from 60mg agarose melt in 30 mL 1×TBE buffer and 3µL ethidium bromide as a fluorescent dye. The resulted PCR product was then loaded into the wells of that gel and 100bp gene ruler was used for detecting the products size. The products were run for 1 hour at 100 volts and 250mA and then visualized through UV Trans-illuminator.

1.5. Sanger Sequencing

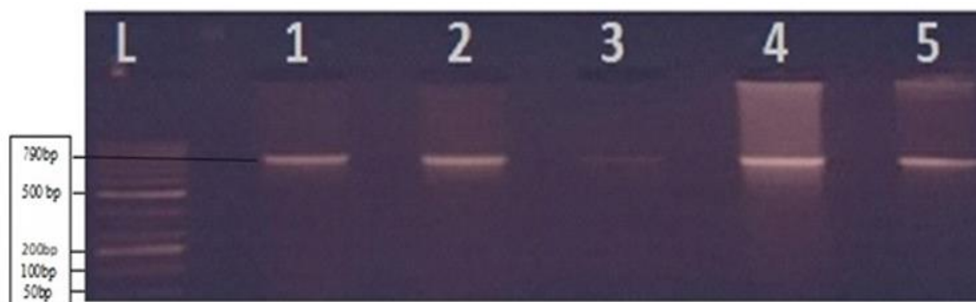
Direct Sanger sequencing (Macrogen, Korea) was performed for the sequencing of selected genes and the amplified data was analyzed.

CHAPTER-4

RESULTS

After DNA extraction from the collected blood samples of 118 BC patients and 15 healthy controls, primers has-mir-381 and has-mir-126 were used to amplify the DNA by thermal cycler PCR under specific conditions. After this, PCR products of randomly selected samples were sequenced.

Figure 4. 1: Gel picture of the amplified Hsa-mir-381 gene



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4.1. Direct Sanger sequencing of the selected genes

Selected samples of BC patients as well as healthy individuals were sequenced to investigate SNP in the selected miRNAs hsa-mir-381, hsa-mir-487b gene and its upstream and downstream regions. The resulted data were then examined by Vector NTI software. This study shows that SNP (rs10083406) A>C was found at a location which is downstream to hsa-mir-381 but upstream to hsa-mir-487b. At this locus, "A" has been replaced with "C".

Figure 4. 2: Picture showing mutation (A>C) in Hsa-mir-381 in BC samples and healthy controls

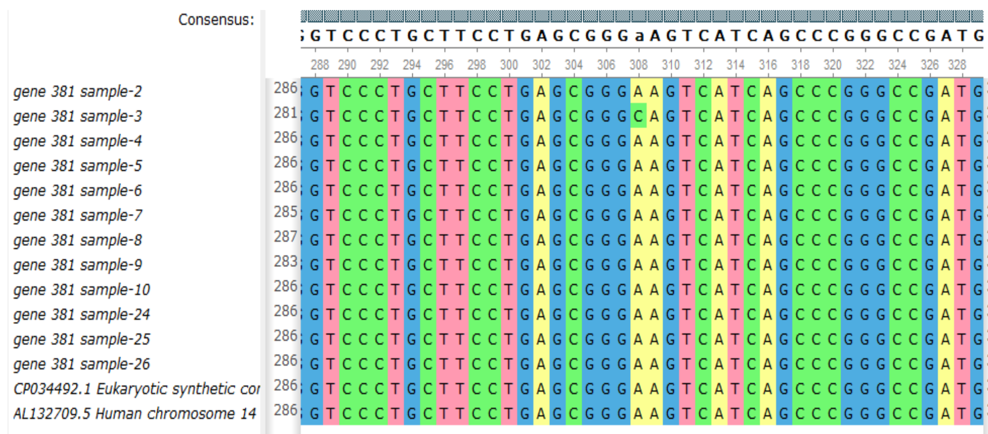

Figure 4. 3: Picture showing SNP (rs10083406) downstream to Hsa-mir-381 and upstream to Hsa-mir-487b

rs10083406 A>C: located downstream to hsa-mir-381 and upstream to hsa-mir-487b

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Figure 4. 4: Chromatogram representing Homozygous (AA) downstream to Hsa-mir-381 and upstream to Hsa-mir-487b in BC patients

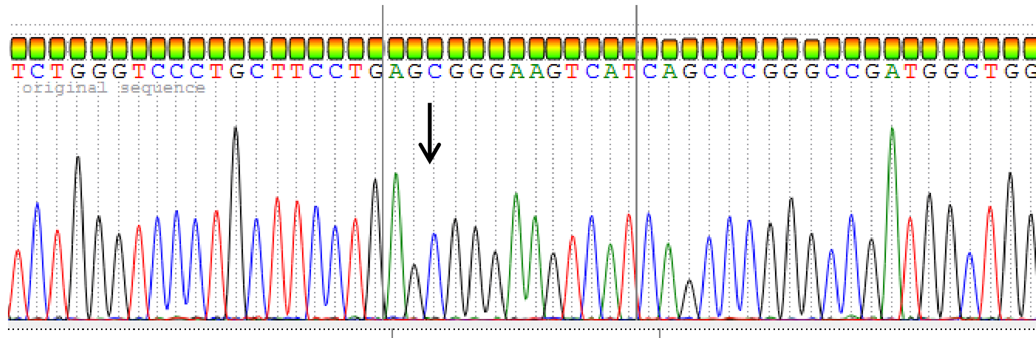

Figure 4. 5: Chromatogram representing Heterozygous (AC) downstream to Hsa-mir-381 and upstream to Hsa-mir-487b in BC patients

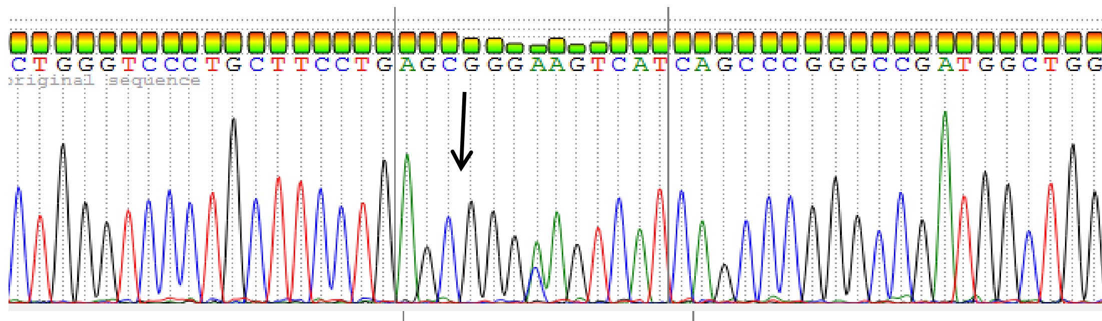

Figure 4. 6: Chromatogram representing Homozygous (CC) downstream to Hsa-mir-381 and upstream to Hsa-mir-487b in BC patients

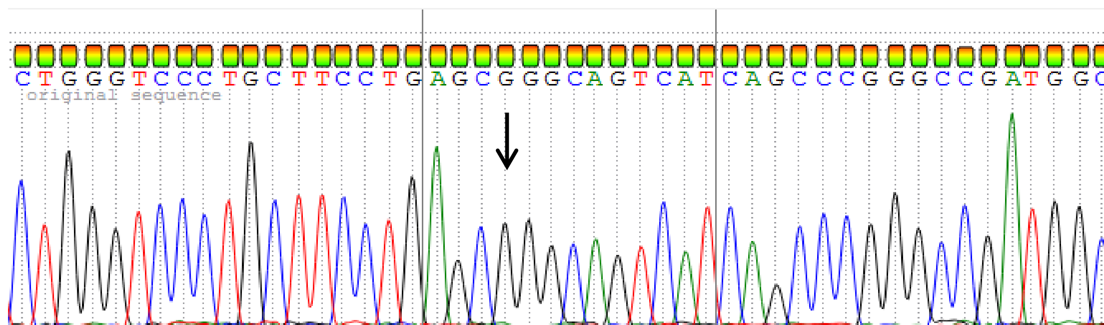

Figure 4.4 shows that there is homozygous AA at the location where SNP was located but in other cases there is presence of heterozygous AC condition at the same location as shown in figure 4.5 while figure 4.6 shows homozygous CC in other BC cases.

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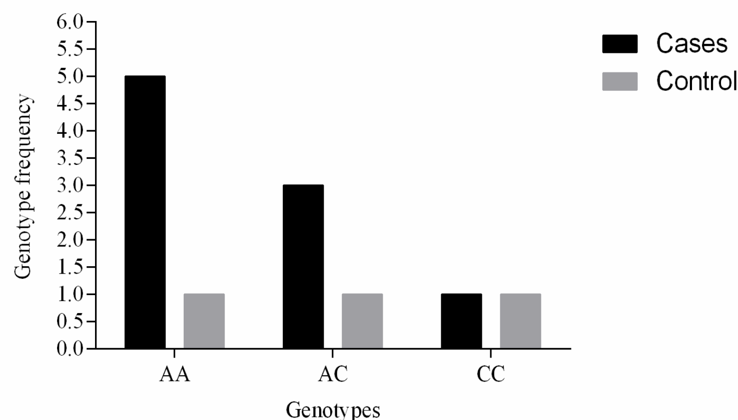
Table 4. 1: Percentage of Genotype frequency of Hsa-mir-381 in BC patients and healthy controls

MIR381 (rs10083406)	Genotype		
	AA%	AC %	CC %
Cases (n=10)	5(55%)	3 (33%)	1 (11%)
Controls (n=3)	1 (33.3%)	1(33.3%)	1(33.3%)

Table 4. 2: Genotype frequency of Hsa-mir-381 in BC patients and healthy controls

Table Analyzed	Data 1
Chi-square	
Chi-square, df	0.8889, 2
P value	0.6412
P value summary	ns
One- or two-sided	NA
Statistically significant? (alpha<0.05)	No
Data analyzed	
Number of rows	3
Number of columns	2

Figure 4. 7: Graph showing genotypic frequency of BC patients (cases) and healthy controls for Hsa-mir-381



The sequenced data was statistically analyzed applying Chi-square test for estimating genotype frequency. Here, we found no significant association of rs10083406 with increased risk of BC. In the sequenced BC samples, at rs10083406 locus, we have 55% AA, 33% AC and 11% CC. The p-value (P=0.6412) for genotype frequency from genetic

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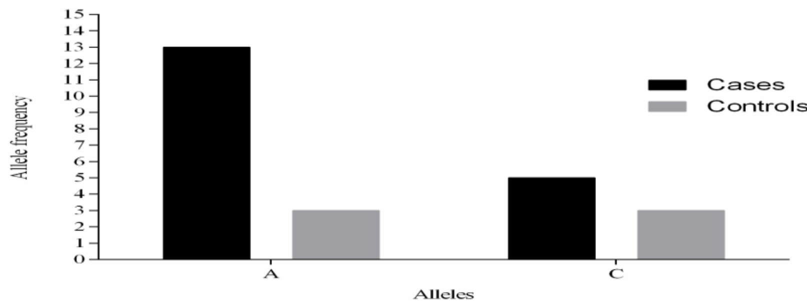
variation (AA, AC, CC) in BC cases for rs10083406 within hsa-mir-381 and hsa-mir-487b represent no co-relation with BC.

Table 4. 3: Allelic frequency of Hsa-mir-381 in BC patients and healthy controls

Table Analyzed	Data 1
Fisher's exact test	
P value	0.3618
P value summary	ns
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	No
Strength of association	
Odds ratio	2.600
95% confidence interval	0.3872 to 17.46

Figure 4. 8: Graph showing allelic frequency of BC patients (cases) and healthy controls for Hsa-mir-381



We have statistically analyzed the sequenced data by applying Fisher's exact test for assessing the allele frequency of AA, AC, CC in the SNP (rs10083406) locus within hsa-mir-381 and hsa-mir-487b but did not find any significant association with increased risk of BC as the p-value (P=0.3618) for allele frequency from genetic variation in BC cases for hsa-mir-381 and hsa-mir-487b represent no co-relation with BC. Furthermore, no study has been carried out for the analysis of rs10083406 SNP association with increased risk of BC till now.

Figure 4. 9: Gel picture of the amplified Hsa-mir-126 gene

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4.2. Sanger sequencing of Hsa-mir-126 and its flanking regions

Direct Sanger sequencing were performed for selected samples of BC patients as well as for healthy individuals for the analysis of single nucleotide polymorphism (SNP) in hsa-mir-126 gene and in its upstream and downstream regions. The sequenced data were then analyzed with the help of Vector NTI software. This study reports the presence of SNP (rs4636297) in the downstream region of hsa-mir-126 for the first time in BC patients in Pakistani population as per my knowledge. This SNP was analyzed 12 base pairs (bp) downstream to the sequence of that miRNA gene where G replaced A in BC patients as shown in figure 4.10. Beside this, another SNP (rs1140713) was also detected downstream to hsa-mir-126 gene as C was replaced by T shown in figure 4.11.

Table 4. 4: SNPs found in the gene Mir-126 in BC blood samples

Variant id	Chr:bp	Alleles	Class
rs4636297	chr9: 136670602-136670686 [+]	A>G	SNP
rs1140713	chr9: 136670602-136670686 [+]	C>T	SNP

Figure 4. 10: Picture showing mutation A>G in Hsa-mir-126 in BC samples

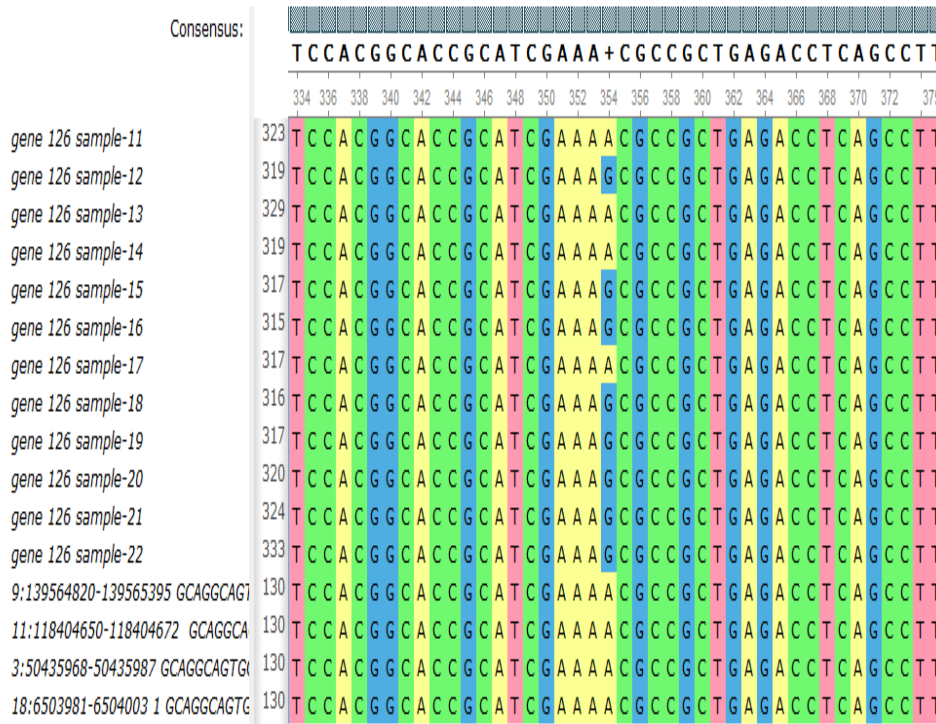

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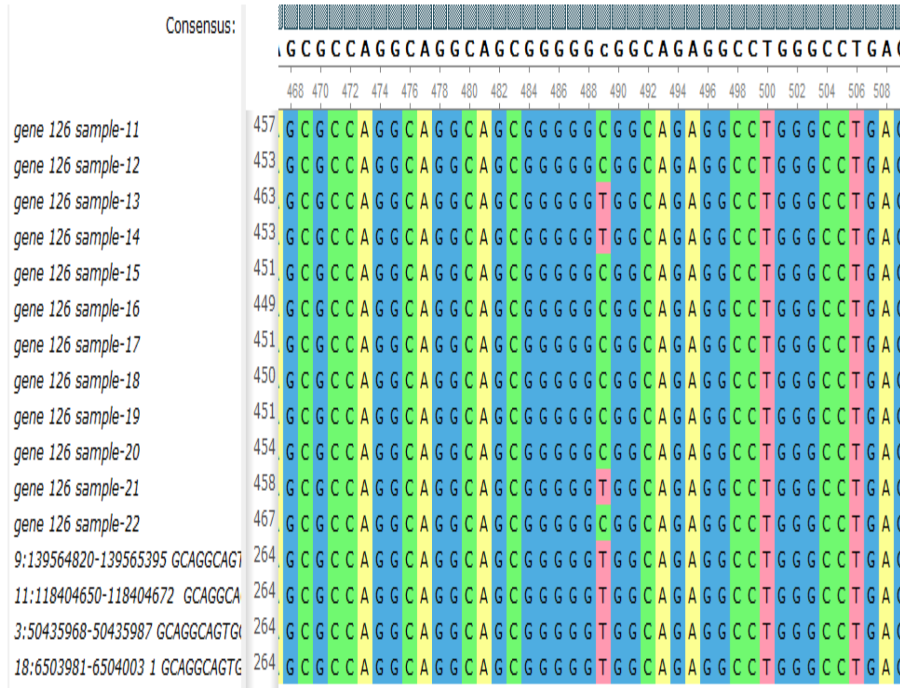
Figure 4. 11: Picture showing mutation C>T in Hsa-mir-126 in BC samples



SNP rs4636297 (A>G) TCGAAAACGCCGC

Located 12 base pairs downstream to Hsa-mir-126

Figure 4. 12: Chromatogram representing Homozygous (GG) in the genomic sequence of Hsa-mir-126 in BC patients

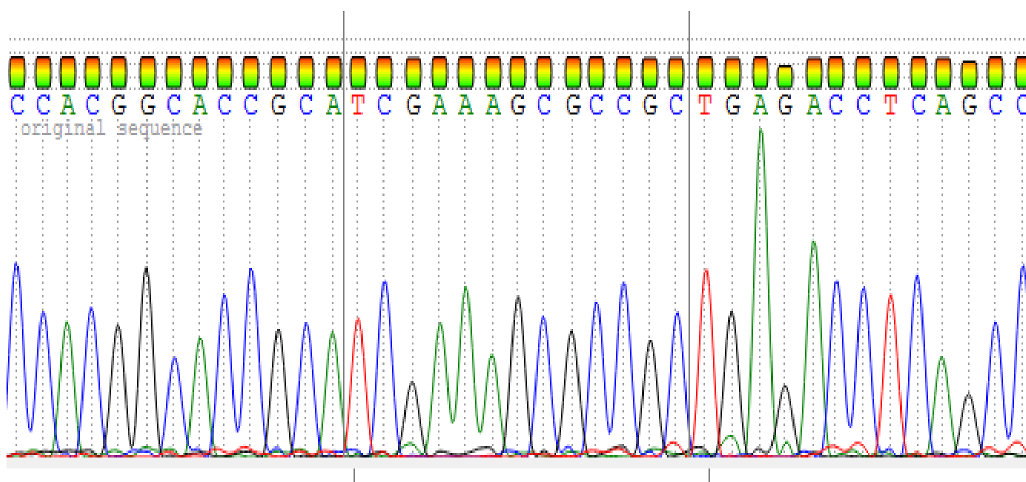


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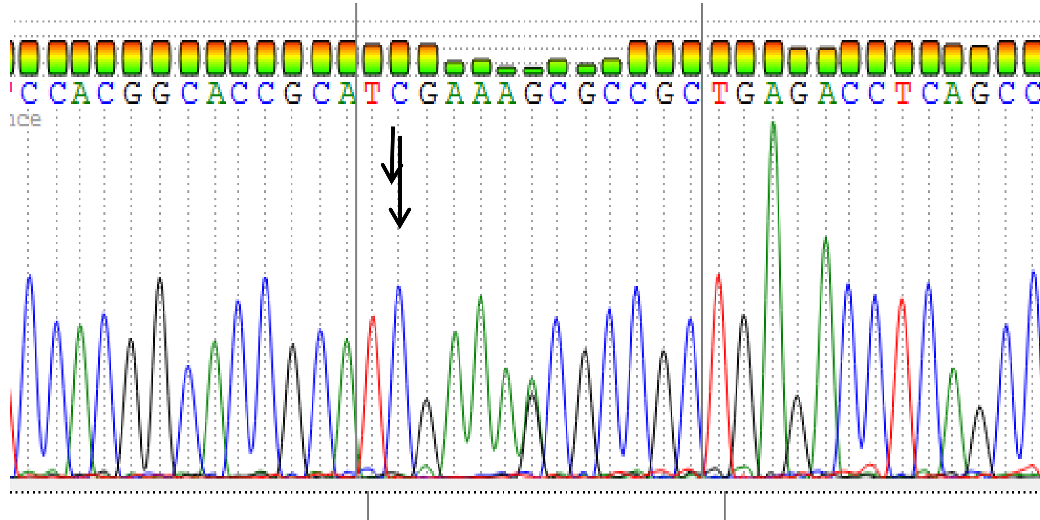
Figure 4. 13: Chromatogram representing Heterozygous (AG) in the genomic sequence of Hsa-mir-126 in BC patients



The above figure 4.12 shows homozygous (GG) condition at a location, 12bp downstream to hsa-mir-126, indicates variation as A is replaced by G and figure 4.13 shows that there may be the chances of heterozygous (AG) condition at the same location in BC patients.

SNP rs1140713 (C>T) GGGGGCGGCAG

Located downstream to mir-126

Figure 4. 14: Chromatogram representing Homozygous (CC) in the genome sequence of Hsa-mir-126 in BC patients

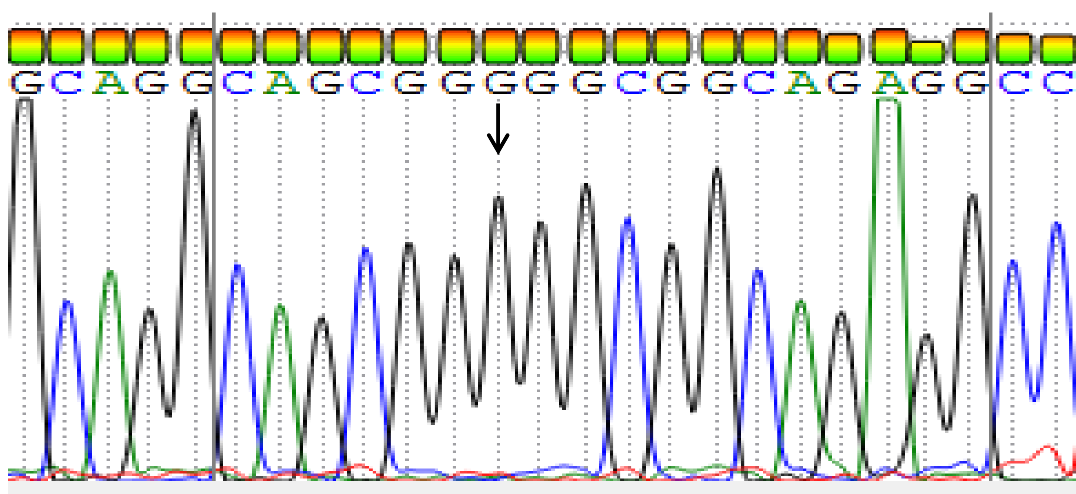


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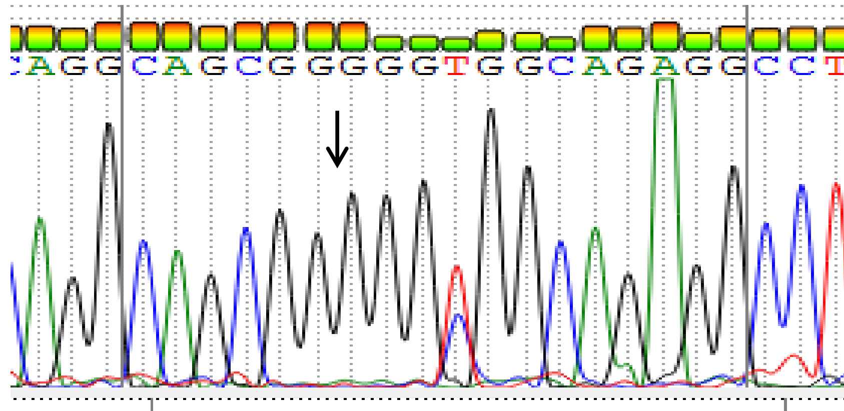
Figure 4. 15: Chromatogram representing Heterozygous (CT) in the genomic structure of Hsa-mir-126 in BC patients



The above figure 4.14 shows the presence of homozygous CC downstream to hsa-mir-126 gene in the genomic sequence of BC individuals but on the other hand figure 4.15 indicates that C is replaced by T at the same position.

Table 4. 5: Percentage of Genotype frequency of the SNP rs4636297 in BC patients and healthy controls

MIR126 (rs4636297)	Genotype		
	AA%	AG %	GG %
Cases (n=10)	0(0%)	4 (40%)	6 (60%)
Controls (n=2)	0 (0%)	1(50%)	1(50%)

Table 4. 6: Genotype frequency of the SNP rs4636297 in Hsa-mir-381 in BC patients and healthy controls

Table Analyzed	Data 1		
Chi-square			
Chi-square, df	0.06857, 1		
Z	0.2619		
P value	0.7934		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant? (alpha<0.05)	No		
Data analyzed	**AG**	**GG**	**Total**
Case	4	6	10
Control	1	1	2
Total	5	7	12

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Figure 4. 16: Graph showing genotypic frequency of BC patients (cases) and healthy controls for the SNP rs4636297 in Hsa-mir-126

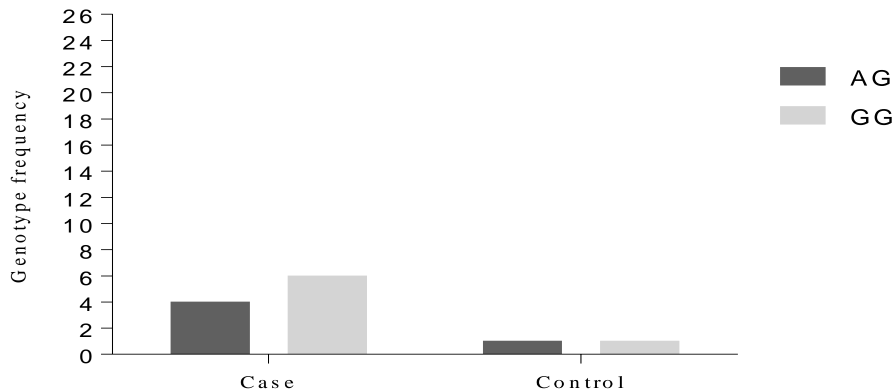

Table 4. 7: Percentage of Genotype frequency of the SNP rs1140713 in BC patients and healthy controls

MIR126 (rs1140713)	Genotype		
	CC%	CT %	TT %
Cases (n=10)	8(80%)	2 (20%)	0 (0%)
Controls (n=2)	1 (50%)	1(50%)	0(0%)

Table 4. 8: Genotype frequency of the SNP rs1140713 in Hsa-mir-126 in BC patients and healthy controls

Table Analyzed	Data 1		
Chi-square			
Chi-square, df	0.8000, 1		
Z	0.8944		
P value	0.3711		
P value summary	ns		
One- or two-sided	Two-sided		
Statistically significant? (alpha<0.05)	No		
Data analyzed	CC	CT	Total
Case	8	2	10
Control	1	1	2
Total	9	3	12

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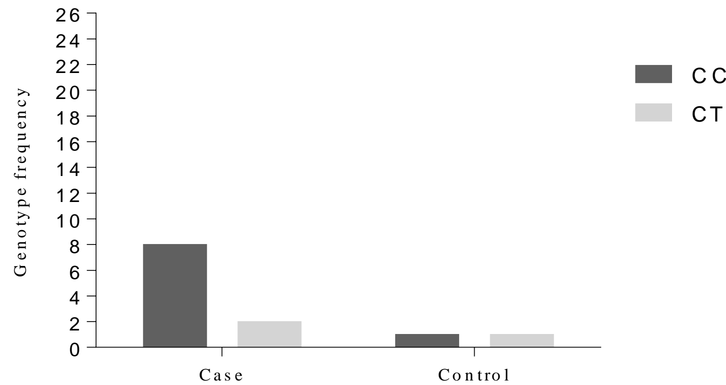
Figure 4. 17: Graph showing genotypic frequency of BC patients (cases) and healthy controls for the SNP rs1140713 in Hsa-mir-126



The sequenced data was statistically analyzed by applying Chi-square test for estimating Genotype frequency for the SNPs in hsa-mir-126. Here, we found no significant association of rs4636297 with increased risk of BC as the P=0.79342. In the sequenced BC samples, at rs4636297 locus, we have 0% AA, 40% AG and 60% GG. Similarly, the p-value (P=0.3711) for genotype frequency from genetic variation (CC, CT and TT) in BC cases for the SNP rs1140713 represents no significant association with BC. At this locus, the percentage of genotype frequency is 80%, 20% and 0% for CC, CT and TT respectively.

Figure 4. 18: Picture showing SNP (rs4636297) downstream to Hsa-mir-126

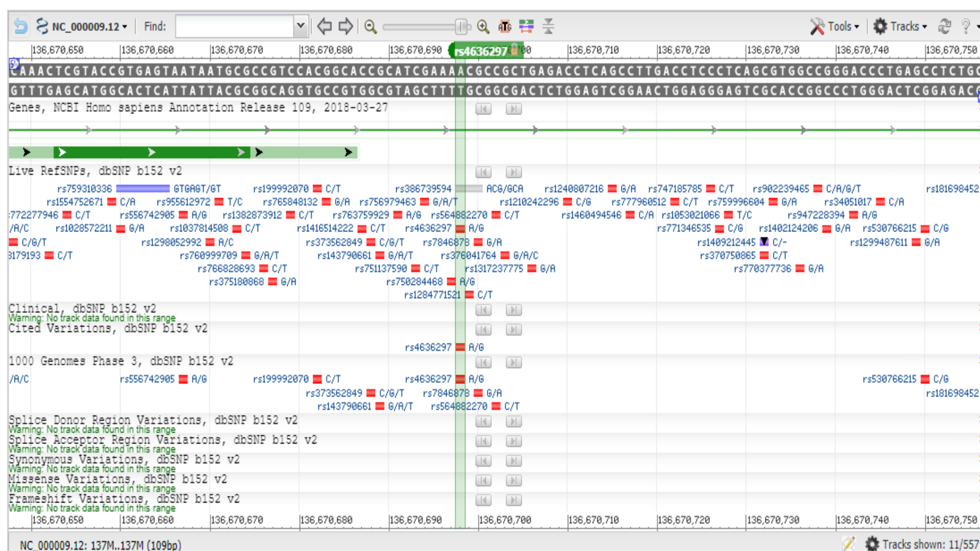


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Figure 4. 19: Picture showing SNP (rs1140713) downstream to Hsa-mir-126

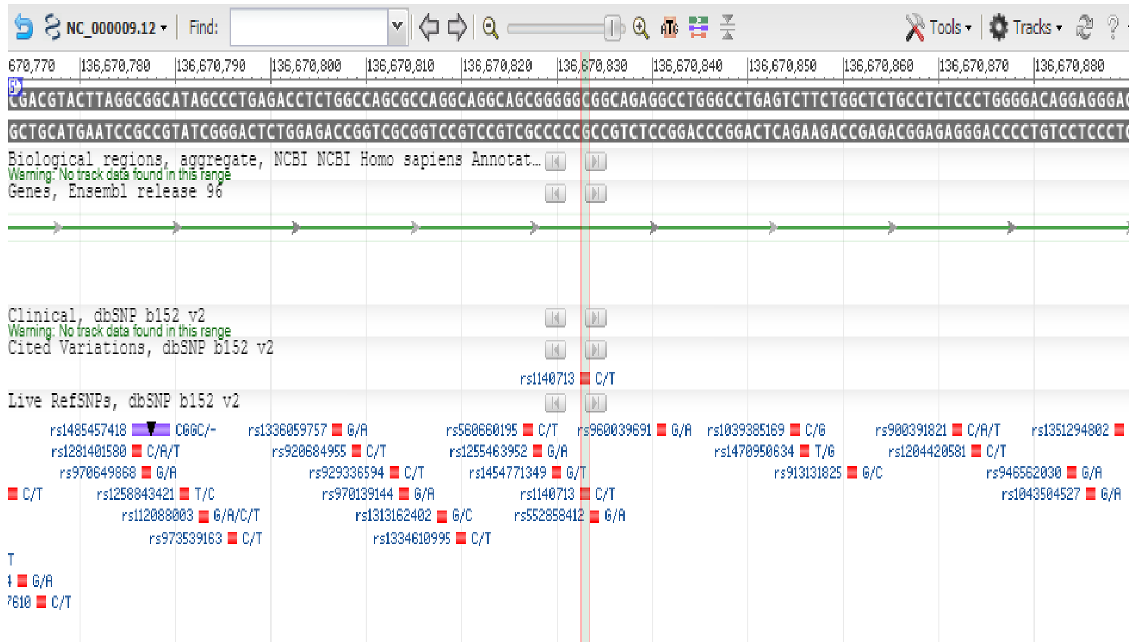

Table 4. 9: Detailed information about hsa-mir-381, hsa-mir-487b and hsa-mir-126

Hsa-mir-381					
1	Genomic Sequence	Coordinates (GRCh38)			**Resulted changes**
			Upstream region		No
			chr14: 101045920-101045994 [+]	TACTTAAAGCGAGGTTGCC CTTTGTATATTCGGTTTATT GACATGGAATATACAAGGG CAAGCTCTCTGTGAGTA	No
			Downstream region		A>C
	RNA Sequence	Stem-loop	hsa-mir-381 MI0000789	UACUUAAAGCGAGGUUGCC CUUUGUAUAUUCGGUUUAU UGACAUGGAAUAUACAAGG GCAAGCUCUCUGUGAGUA	No
		Mature	hsa-miR-381-5p MIMAT0022862	AGCGAGGUUGCCCUUUGU AUAU	No
			hsa-miR-381-3p MIMAT0000736	UAUACAAGGGCAAGCUCUC UGU	No
Hsa-mir-487b					
2	Genomic sequence	Coordinates (GRCh38)			**Resulted changes**
			Upstream region		A>C

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			chr14: 101046455-101046538 [+]	TTGGTACTTGGAGAGTGGTTATCCCTGTCCTGTTCGTTTTGCTCATGTCGAATCGTACAGGGTCATCCAC	No
			Downstream region		No
	RNA sequence	Stem-loop	hsa-mir-487b MI0003530	UUGGUACUUGGAGAGUGGUUAUCCCUGUCCUGUUCGUUUUGCUCAUGUCGAAUCGUACAGGGUCAUCCACUUUUUCAGUAUCAA	No
		Mature	hsa-miR-487b-5p MIMAT0026614	GUGGUUAUCCCUGUCCUGUUCG	No
			hsa-miR-487b-3p MIMAT0003180	AAUCGUACAGGGUCAUCCACUU	No
Hsa-mir-126					
3	Genomic Sequence	Coordinates (GRCh38)			**Resulted changes**
			Upstream region		No
			chr9: 136670602-136670686 [+]	CGCTGGCGACGGGACATTATTACTTTTGGTACGCGCTGTGACACTTCAAACTCGTACCGTGAGTAATAATGCGCCGTCCACGGCA	No
			Downstream region		A>G, C>T
	RNA Sequence	Stem-loop	hsa-mir-126 MI0000471	CGCUGGCGACGGGACAUUAUUACUUUUGGUACGCGCUGUGACACUUCAAACUCGUACCGUGAGUAAUAAUGCGCCGUCCACGGCA	No
		Mature	hsa-miR-126-5p MIMAT0000444	CAUUAUUACUUUUGGUACGCG	No
			hsa-miR-126-3p MIMAT0000445	UCGUACCGUGAGUAAUAAUGCG	No

DISCUSSION

MicroRNAs (miRNAs), an important biological tools are involved in mRNA degradation or transcriptional inhibition by binding to 3' untranslated region (3'UTR) of the target mRNA [25,26]. miRNA has specific region of about 2-8 nucleotides in length in its 5' terminal called seed sequence which is important for to bind with the target mRNA molecules. Sometime there is complete or nearly complete binding with mRNA, leads to gene

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silencing. However, mostly there is incomplete binding and just leads to translational suppression, resulting to decreased level of protein. It is predicted that every miRNA has ability to control 200 mRNAs, hence responsible to regulate about one-third of mRNAs in human [27, and 28]. Above 50 percent of miRNA genes exist in cancer associated chromosomal regions . miRNA is very important for regulating several biological processes but its dysregulation also affects various process including cell differentiation, cell proliferation and apoptosis resulting into many diseases i.e. cancer [29-30]. Various miRNAs have been found as tumor suppressors or oncogenes in BC by regulating tumor initiation, metastasis and chemo-resistance. SNPs in miRNA genes make genetic variation in their mRNA, representing new cause of BC.

Hsa-mir-381 is found to be involved in BC by inhibiting tumor development, invasion and metastasis . Cx43, a member of Cx family (Gap junction proteins) significantly express in mammary glands and contribute to cell migration and also tumor metastasis but is controlled by multiple miRNAs including miR-381. During BC, due to the low expression of miR-381, the Cx43 perform major role in tumor cell metastasis [31-32].

The other selected miRNA, hsa-mir-487b has been suggested to inhibit cell proliferation, cell invasion and colony formation and play an important role of tumor suppressor or anti-oncomir. Up to now, no report has been performed to examine the association between rs10083406 within hsa-mir-381 and BC; however this gene has been reported to suppress expression of cx43 during BC [33].

In our focused study, SNP rs10083406 A>C polymorphism at Chromosome 14 was located downstream to hsa-mir-381 and upstream to hsa-mir-487b in the genomic structure of BC patients belonging to selected area of District Swat, Pakistan. As per our understanding from the present study, hsa-mir-381 rs10083406 A>C polymorphism may not play any role in the progression of BC as we didn't notice any association of this variant with BC. However, the association between rs10083406 A>C polymorphism within hsa-mir-381 gene and BC risk remains unclear. Other miRNA binding SNPs have been reported to be significantly associated with BC incidence [34].

This study also analyzed the possibility for association of hsa-mir-126 with increased risk of BC. It has been shown by previous studies that miR-126 act as tumor suppressor by targeting several genes being involved in i.e. EGFL730 [35], CXCR431 VEGF12 [36, PIK3R2 [37] and so on. Beside this, overexpression of hsa-mir-126 directly targets the key molecule, ADAM9 involved in BC progression and metastasis [38]. Normally, miR-126 suppresses metastasis and angiogenesis but becomes down-regulated in cancer Analyzing hsa-mir-126 in the present study, alteration rs4636297 A>G was detected in the downstream region of the selected miRNA gene in BC patients. . Our findings are in contrast to findings of [39] where no such alteration (A>G) was reported. However, our work is consistent with the work of [40-41] where no association was found between rs4636297 A>G and BC. Yang et al., (2011) investigated genotype and allele frequencies of about 2600 patients suffering from BC but could not find any significant results [42. It

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means that the studied SNPs were not linked or associated with BC. Further investigation is needed for confirmation and functional analysis on large number of BC samples. Our results of getting no association of A>G with BC is in concordance with reports of [43] where no significant association of genotype and allele frequency of microRNA-126 SNP/variant (A>G, rs4636297) was found in patients and control of BC. Similarly our results of reporting rs1140713 C>T polymorphism is in line with findings of [44] and [45] where such alteration was observed in connection of miRNA 126 to coronary disease and BC respectively.

CONCLUSION

It is concluded that the resulted SNPs, SNP rs10083406 A>C downstream to hsa-mir-381 and upstream to hsa-mir-487b as well as SNP rs4636297 A>G and SNPrs1140713 C>T in hsa-mir-126 showed no association with BC. The negative association of the studied SNPs with BC may be due to small number of sequenced samples, however large number of samples from multiple genes may be sequenced to study the association of these SNPs with BC which may be provide useful information about BC risks and its prevalence etc in the studied region.

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