Original Article

Evaluation of miRNA 34a Expression and its Relationship to molecular Subclassification with Androgen Receptor Status in a Prognosis of Breast Cancer Patients of Pakistan

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Abstract

Objective: The goal of our research was to determine the differential expression of miRNA 34a in breast cancer based on molecular subtypes and its relationship to other receptors.

Methodology: It was a retrospective study. The study included a total of 32 samples from the Islamabad Diagnostic Centre (IDC) and 4 samples of fibroadenoma, which were formalin fixed paraffin embedded tissue samples of clinically diagnosed breast cancer females for the past 5 years from University of Health Sciences (UHS). 5m tissue sections were cut for immunohistochemistry, and 20m sections for miRNA analysis were transported to laboratory, where they were stored at room temperature until further processing. Females who eligible for the study and had histologically diagnosed cases of breast cancer and fibroadenoma were included, but clinical history was excluded. As a control, we used 32 FFPE samples of breast cancer and 4 fibroadenoma samples. Luminal A made up 51% of the 32 cases tested for molecular subtyping for breast cancer heterogeneity; whereas 40% were triple negative and 9% had the HER-2/Nueenriched aggressive type clinically.

Results: 97% of invasive ductal carcinoma samples and 25% fibroadenoma samples were negative for androgen receptors. MiRNA34a analysis shows predominant down regulation in all molecular subtypes, 3 fibroadenoma samples were also downregulated. The sample with negative androgen receptors has shown normal value of miRNA 34a.

Conclusion: As a consequence, miRNA 34a can be used as a prognostic marker for assessing the progression of breast cancer, and future studies with bigger sample sizes on other types of breast cancer could be included to make a final conclusion and set a standard for miRNA research.

Keywords: Breast cancer; MicroRNAs; androgen receptor; molecular subclassification.

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Introduction

Cancer is the name of uncontrolled and abrupt cell division that eventually form a lump or mass called a tumour, which may be benign or malignant depending upon the type of mutation. Breast cancer is a multifaceted disease including clinical, morphological and molecular characteristics. It is a difficult task for clinical diagnosis and prediction for treatment due to the heterogeneity of this disease. [1] Although it is entirely a female disease, males were also reported. [2]

Sex steroid hormone receptors, including androgen receptor (AR), play an important role in the development and spread of breast cancer. It has been reported that 70–90% of breast cancer patients over express AR, and various studies have suggested that

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AR could be a predictive or prognostic factor as well as a therapeutic target in the disease . In almost 60% of breast cancer, the oncoprotein Her 2/neu and the AR are positive.[3]

MiRNAs are known to be over-expressed in cancer, with some working as tumor suppressors and others (oncomiRs) depending on which genes or pathways they regulate. [4] The miRNA-34a is a tumour suppressor miRNA which has shown to be downregulate in multiple cancers by the involvement of p53 network by targeting CDK6, c-MYC and c-MET by arresting proliferation and inducing apoptosis. [5] The literature has shown that BRAC1 induce the biogenesis of miRNA34a. [6]

Currently, the incidence of breast cancer has increased that every 8th woman will develop breast cancer in her life time. [7] In Asia, Pakistan has been reporting the highest number of breast cancer cases after Jews in Israel. The incidence of breast cancer is 2.5 times higher in Pakistan than in neighbouring countries like Iran and India. [8] The malignancy of breast cancer accounts for 34.6% of female cancers. [9] One in nine females in Pakistan develop breast cancer at any stage in their life, which is considered as highest ratio among Asian countries 10, and women death number increased to 40,000 every year. [9] The reason for the higher incidence of breast cancer in Pakistan is still unknown, although poor hygiene, life style, social-economic status, illiteracy and unfit medical facilities are considered. The goal of our research was to determine the differential expression of miRNA 34a in breast cancer based on molecular subtypes and its relationship to other receptors.

Methodology

This retrospective study was included total 32 samples of Islamabad Diagnostic Centre (IDC) and 4 samples of fibroadenoma which were formalin fixed paraffin embedded tissue samples of clinically diagnosed breast cancer females for the past 5 years from University of Health Sciences (UHS). 5m tissue sections were cut for immunohistochemistry, and 20m sections for miRNA analysis were transported to laboratory, where they were stored at room temperature until further processing.

Preparation of FFPE slides

The patient and control FFPE tissue blocks were placed on ice for 30 minutes to equalise temperature. It cut 5 micron slices at a predetermined gauge and floated them in a 600°C water bath. The sections were placed on pre-coated slides and numbered histopathologically. Hematoxylin and eosin staining was used to confirm the diagnosis by the senior supervisor. To ensure adequate section adhesion to slides, AR, negative and positive controls were collected and kept in a staining rack at 320°C overnight. All staining procedures were carried out at room temperature 25^{o}C.

Staining Procedure

For AR, positive and negative controls, the tissue slides were deparaffinised and rehydrated for 30 minutes at 600C. The slides were placed in a xylene bath for 5 minutes and then changed. The slides were cleaned in 70% ethanol for 5 minutes before being rinsed in 100% ethanol for 5 minutes. Heat-induced epitope recovery was used. The slides were placed in a recovery cushion containing AR receptor epitopes for an hour at 600C. Remove the container from the water shower and let it to cool. After cooling, the slides were placed in a staining rack with washing buffer for 5 minutes.

Incubation with Antibodies and other reagents

A 10% protein blocker solution was applied to the slides for 10 minutes at room temperature to minimise nonspecific protein limitation. The slides were washed with PBS several times before incubation with the AR immunizer. Keeping the staining racks damp kept the components from drying. After four PBS washes, the slides were incubated with biotinylated goat anti-polyvalent immunoglobulins for ten minutes. In order to simulate peroxidase action, the slides were repeatedly washed in PBS and then treated for ten minutes with DAB containing 0.03 percent H2O2. The slides were then dried at room temperature for 10 minutes before being rinsed.

Scoring system for ER-α, ER-β, ki-67and HER-2/neu

We used the Allerd scoring system per manufacturer's advice (Allerd at el 1998). It evaluates scores based on intensity and proportion. We used the Dako kit scoring system for HER-2/neu. Table I shows the manufacturer's instructions for the Ki-67 Dako kit.

This slide rack was placed in a 30°C oven. DPX was used to mount the slides, and sections were quickly covered with a cover slip. The consultant inspected the stained mounted slides using an Olympus microscope (CX41RF). The sections were scanned with a 10X objective, and the receptor status was evaluated in detail with a 40X objective.

Table I: Summary of antibodies used in Immunohistochemistry

Receptor	Company	Clone	Dilution	Working pH	Expression Nuclear/ Cytoplasmic
AR	ScyTek Laboratories by USA	Polyclonal Mouse anti human (Clone AR441 and DHRT/882)	1:50	8.0	Nuclear
Estrogen receptor α	Dako by Denmark	Monoclonal Rabbit anti human(EPI)	Ready to use	9.0	Nuclear
Estrogen receptor β	ScyTek laboratories USA	Monoclonal mouse anti human(ERb455)	1:100	6.0	Nuclear/cytoplasmic
HER-2/neu	Dako by Denmark	Polyclonal mouse anti human HER-2/neu antibody	1:250	9.0	Membranous
Ki-67	Dako by Denmark	MIB-1	Ready to use	8.0	Nuclear

Scoring System for AR:

The usual method of scoring AR receptor immunohistochemistry was used. This system grades ERs, PGRs, and ARs. As shown in Table II, the proportion score describes the fraction of positive receptors present in a cell.

Table II: Proportion Score

Proportion Score (PS)	% Positive Cells
0	0
1	<1%
2	1% to 10%
3	11% to 33%
4	34% to 66%
5	>67%

The positivity is rated from 0 to 3. 1+ is considered negative, while 3+ is considered positive.

Extraction Of miRNA from FFPE:

The 20m FFPE tissue squares were microtome blocked. We used an 80m eppendorf tube that held 2.0 ml. To dissolve the paraffin, the samples were immersed in 100% xylene for 3 minutes at 500°C. The samples were centrifuged for 2 minutes at room temperature. 1ml of 100% ethanol was used to rinse the pellet. Each example got digestion buffer and Protease. After an hour at 50°C in heat blocks, the samples were heated to 80°C for 15 minutes.

Nucleic Acid Isolation:

Each sample received a ready-to-use ethanol and isolation additive mixture. The 700 litre mixture was then centrifuged for 30 seconds at 10,000 rpm. The pellet was discarded. After that, the filter cartridge was filled with 700µl of buffer 1 and centrifuged at 10,000 rpm for 30 seconds. The stream was discarded once more, and 500 litres of wash support were added and centrifuged at 10,000 x g for 30 seconds.

Nuclease Digestion and Final Purification:

Purify DNA of impurities. A DNAs mixture was deposited in the centre of each filter cartridge and incubated for 30 minutes. Incubate 700µl wash buffer 1 for 30-60 seconds at room temperature. 500 litres of 2/3 wash solution were added to the flow. The flow was discarded after 30 seconds of 10,000 x g centrifugation. After another wash, the filter cartridge was moved to a fresh collection tube. Then fill the membrane with 60µl elution buffer. Incubation time was 1 minute at room temperature. The RNA was then centrifuged at maximum speed for 1 minute, and then eluted through the filter. The samples were treated at –20°C.

Quantification of RNA:

The absorbance at 260nm/280nm of the entire RNA samples was measured on a nano-drop ND-1000 Spectrophotometer. As demonstrated above, absolute RNA was extracted and converted to cDNA using the TaqMan® Advanced miRNA cDNA Synthesis Kit as directed by the manufacturer.

Poly (A) Tailing Reaction:

The Poly (A) reaction mix was vortexed vigorously before being centrifuged to remove air bubbles and spin down the contents.

Adaptor Ligation Reaction:

It was made in 1.5 mL micro centrifuge tube. The reaction plate was closed and the contents briefly vortexed to thoroughly mix. The response plate was then placed in a warm cycler and hatched using standard cycling. The PCR temperature was set to 16°C for an hour, then 4°C for the connector response.

Reverse Transcription (RT) Reaction:

The miR-Amp reaction was carried out right away, or the RT reaction product was stored at –20°C until it was processed further.

Mir-Amp Reaction:

The mixture was vortexed, centrifuged, and 45µL of miR-Amp Reaction Mix was added to each well.

Prepare PCR Reaction Plate

The assay reagents were thawed on ice, gently vortexed to combine, then centrifuged briefly to eliminate bubbles. Then add 5l diluted cDNA template to each PCR plate reaction well. 20 l total per reaction well. The PCR plate was sealed and put into the RT-PCR machine. For this experiment, we used the following settings.

Table III: PCR setup for qRT-PCR

Step	Temperature	Time	Cycles
Enzyme activation	95 °C	20 seconds	1
Denature	95 °C	1 seconds	50
Anneal/Extend	60 °C	20 seconds	

The data was analysed using SPSS, GraphPad Prism, and microsoft excel.

Results

Thirty-two patients were enrolled in this study, 10 patients having the range of age between 25 to 40. The 9 patients were between 41-55 and 10 were more than 55. Out of 32, 10 were with 1-3 positive axillary lymph nodes (31%), 5 were with 4-9 positive axillary lymph nodes (16%), 2 were ≥ to 10 positive lymph nodes (6%) and 9 were with unknown (28%).

Table IV: Clinical Pathological Characterization of Breast Carcinoma Cohort

Parameters		No of Cases	Percentage
Age (n=32)	25-40	10	31%
	41-55	9	28%
	>55	10	31%
	N/A	3	9%
Total Positive Lymph nodes (n=32)	0	6	19%
	1-3	10	31%
	4-9	5	16%
	≥ 10	2	6%
	N/A	9	28%
Pathological Stage (n=32)	pN 0	6	19%
	pN 1	8	25%
	pN 2	6	19%
	pN 3	3	9%
	N/A	9	28%
	pT2	12	38%
	pT3	9	28%
	pT4	8	25%
	N/A	3	9%
Tumour Grade	G1 + G2	13	41%
	G3	18	56%
	N/A	1	3%
DCIS (n=32)	Present	11	34%
	Absent	19	59%
	N/A	2	6%
ER-α (n=32)	Negative	22	69%
	Positive	10	31%
HER-2/neu (n=32)	Negative	21	66%
	Positive	11	34%

Each sample was assigned a TNM pathological stage. In the ipsilateral level I, II axillary lymph nodes, there were 6 samples with pN2 metastases fixed or matted, and 8 samples with pN1 metastases movable.

12 samples had pT2 tumours larger than 2cm but less than or equal to 5cm in size, 9 samples had pT3 tumours larger than 5cm in size, and 8 samples had pT4 tumours of any size with direct extension to chest wall. N/A for each pathogenic stage.

Thirty-two samples were graded as G1+G2 (low to moderately differentiate) and G3 (poorly differentiated and highly graded).

11 patients had ductal carcinoma (milk ducts). The same lab detected 10 positive ER-receptors in breast cancer samples. 11 samples had HER-2/neu.

Figure 1(A) shows the benign tumour fibroadenoma. It has epithelial and fibroblastic components. The fibroblastic component is cancerous, while the epithelial component is reactive.

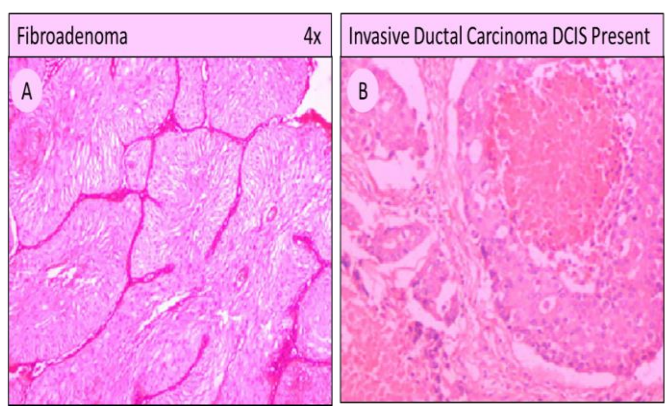

Figure 1: (A) Fibroadenoma B) Invasive ductal carcinoma

The image shows a nodular and encapsulated fibroadenoma. Unusually large terminal ducts with epithelial development suggest epithelial growth. Figure 1(B) shows invasive ductal carcinoma, a cancer that starts inside ducts and spreads to fatty tissues of the breast.

The Figure 2 has shown the pi graph of total percentage of specimens with molecu4lar subtyping. 32 samples subjected for Molecular subtyping for breast cancer heterogeneity were compromised 35 % Luminal A type, 56% Triple negative breast type, 9% HER-2/neu type and no luminal B type was observed.

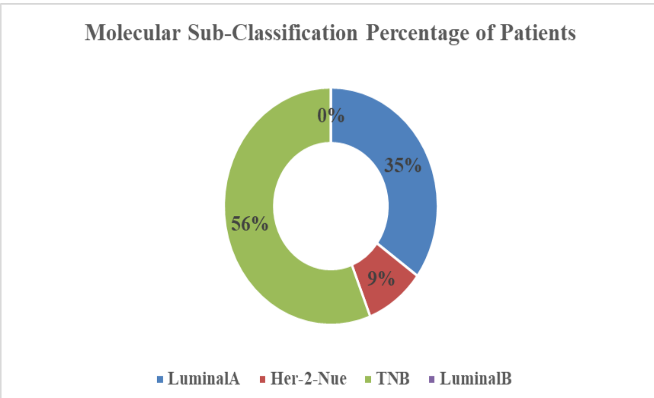

Figure 3: Percentage of specimens with molecular subtyping

Luminal A

Luminal 35 percent of the 32 FFPE slides tested for estrogen positive and HER-2/neu negative immunohistochemistry had a breast cancer.

ER expression is mostly in the nucleus, with some in the cytoplasm. ER- was only found in ER-negative breast cancer tissues. The HER-2/neu status of 32 patient samples was analysed, with a 9% positive result.

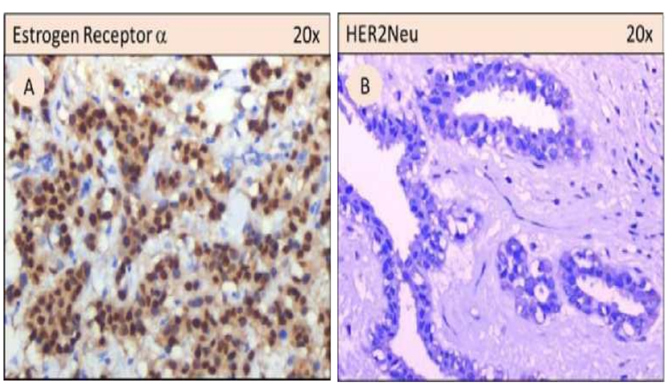

Figure 4: (A) Estrogen receptor α positive IHC staining (B) Her-2-Nue negative IHC staining.

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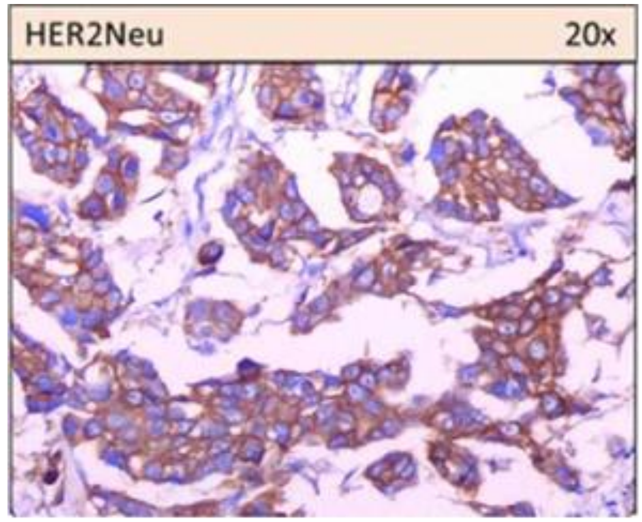

Figure 2: Her-2/neu Positive

Table V: HER-2-Enrichcd

HER-2/neu	No of cases	Percentage
Negative	29	91
Positive	3	9

Thirteen of 32 FFPE samples were triple negative. Histopathologically, lymphocytes infiltrate tumours with a broad central acellular zone. All triple negative breast tumours have apocrine differentiation. Age, tumour size, grade, and lymph node were not statistically different.

Estrogen Receptor and Androgen Receptors Expression:

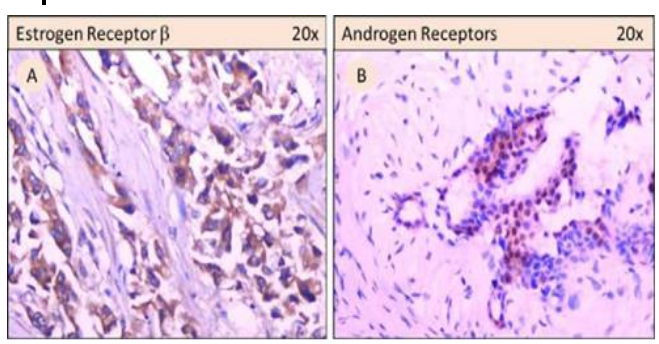

Figure 5: A) Estrogen receptor beta positive IHC B) Androgen receptor positive IHC

Table VI: E.R& AR Expression in IDC

Receptor	Status	No of cases	Percentage
ER-	Negative	8	25
	Positive	24	75
AR	Negative	31	97
	Positive	1	3

A total of 32 IDC samples were tested for ER, with 75% of results positive and 25% negative. Similarly, just 3% of 32 instances tested positive for AR, while 97% tested negative.

Table VII: ER & AR Expression in Fibroadenoma

Receptor	Status	No of cases	Percentage
ER-□	Negative	0	0
	Positive	4	100
AR	Negative	0	0
	Positive	4	100

Four fibroadenoma samples tested positive for ER-beta. Similarly, four cases tested positive for AR.

Table VIII: ER / ER Ratio and its correlation with AR expression in IDC

ER		No. of Patient	%	AR	%
Groups	Ratio				
Group 1	<1	22	69.75		
Group 2	1-1.5	3	9.38		
Group 3	>1.5	7	21.88	1	3.13

Out of 32 IDC instances, 22 (69%) had higher ER than ER scores (ER/ER ratio 1). Group 2 consisted of 3 (9%) patients with ER/ER score ratios between 1 and 1.5, while group 3 consisted of 7 (21.5%) patients with ratios >1.5. Only one IDC sample tested positive for AR.

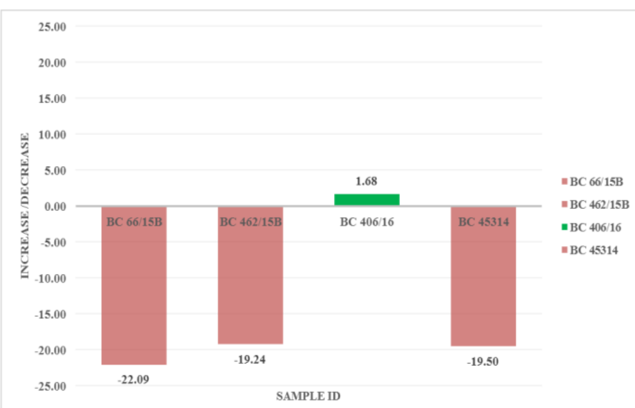

Figure 7: miRNA 34a expression profile in fibroadenoma

U6 value used to normalize samples of fibroadenoma. 3 samples were showing down regulation and one was at normal level.

13 samples showed significant down-regulation, 7 samples showed up-regulation, and 12 samples showed no significant change. 50% of patients had decreased miRNA34a expression in Luminal A subtype while the rest patients had no change. Thirteen individuals were triple negative, with 38% down-regulated, 46% up-regulated, and 15% up-regulated miRNA34a. Out of the three HER-2/neu enriched patients, one showed down-regulation (33%), and two showed slight up-regulation.

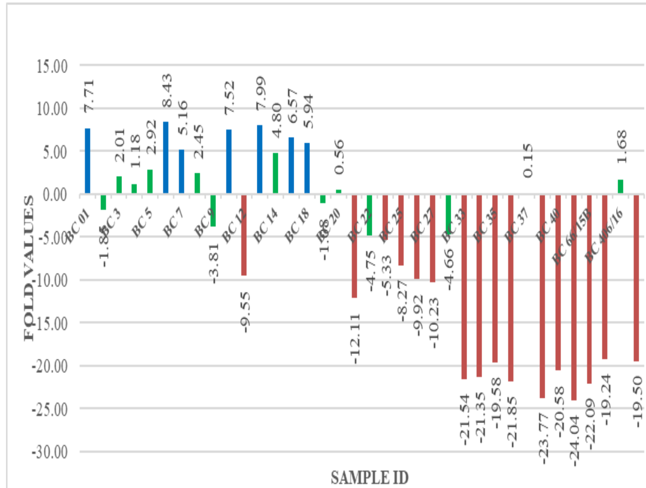

Figure 6: MiRNA 34a expression profile in IDC Patients

Table IX: miRNA 34a correlation with ER-β positive patients in IDC

Parameters		No. of Cases	MiRNA 34a Expression in IDC					
			Down	%	Up	%	Normal	%
ER-β	Positive	24	10	4 2	5	2 1	9	3 7
	Negative	8	3	9	2	6	3	9

24 samples were ER-positive. MiRNA 34a expression was normal in 42% of ER-positive tissues and down regulated in 21%.

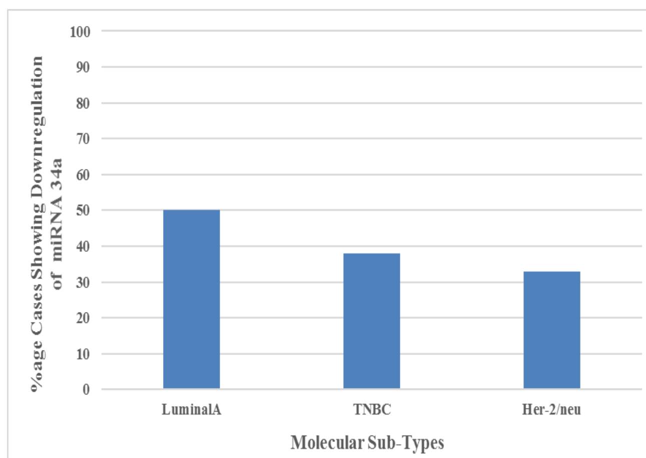

Figure 8: miRNA 34a Expression Profile in Molecular Sub-Type of Breast Cancer

MiRNA 34a was found at normal level in androgen receptors positive patient that was only one in our study.

Table X: miRNA 34a Down Expression with Increase in Tumour Grade (IDC)

Grade	Down Expression	
	Cases	Percentage
I	0	0
II	2	15
III	11	61

Table shows down expression of mir34a with Tumour grade. Tumor size, pleomorphism, chromatin, and orientation are used to grade it. Our data show decreased mir34a in high grade tumors.

Table XI: Down Expression of mir34a In Different Stages of IDC

Parameters	MiRNA 34a Down Expression	
	Cases	Percentage
pT2	5	41
pT3	6	66
pT4	2	25
pN0	2	33
pN1	4	50
pN2	4	67
pN3	1	33

MiRN 34a expression was reduced in 5 (41%) of IDCs at T2 stage. Similarly, it was seen in 6 (66%) T3 patients and 2 (25%) T4 individuals. Similarly, downregulation of miRNA 34a was seen in 2 (33%) patients at N0, 4 (50%) patients at N1, 4 (67%) patients at N2, and only 1 (33%) patient at N3.

Discussion

Breast cancer causes rapid cell division. Delay in diagnosis and poor prognosis have increased mortality. Breast cancer has high tumour grade, metastasis, invasion, and recurrence features that impact treatment outcome. Thyroid cancer is treated with surgery, chemotherapy, and radiotherapy.

To start, we used FFPE embedded human breast cancer tissues (IDC n=32 and fibroadenoma n=4) to express new ER and AR. Molecular subtyping was also used to clarify the link with ER and AR. ER expression was identified in 75% (24/32) of IDC cases. Breast cancer samples express both ER subtypes. Valerie Speirs et al. (2004) conducted a similar study and discovered that 50% of breast tumours examined co-expressed ER- and ER-.The co-expression of ER subtypes within the same breast tumour suggests that ER- and ER-proteins may interact, resulting in differential responses to oestrogen and anti-estrogens.

We analysed 32 patients for AR and 3% were positive, while 97% were negative. These results are in contrast to the study done by Luis O Gonzales and co-workers, which showed AR reactivity of 74.8% in invasive ductal carcinoma tissues.[12] Four fibroadenoma samples tested positive for androgen receptors. This shows that benign tumours express androgen receptors more than invasive breast cancer. The data show that androgen receptor expression is low in IDC samples, which may be related to patient age, regional dispersion, or tissue fixation.[13]

In the second part, 32 samples were subjected to qRT-PCR for the expression of miRNA 34a, 7(21%) samples were upregulated in expression whereas 13 (40%) samples were down regulated. 3 out of 4 Fibroadenoma samples showed downregulated expression of miRNA 34a. The present study has shown the one AR positive sample with normal expression of miRNA 34a. Quite a few studies presented that low expression of miR-34a in primary tumour associates with the presence of metastases in cancer patients, suggesting that miR-34ahas a role in tumour suppression.[14]

Li et al. studied breast cancer tissue to understand the mechanism of miRNA 34a's inhibitory role in proliferation and migration via Bcl-2 and SIRT 1 downregulation. All the breast cancer tissues were found down-regulated in expression of miRNA 34a as compared to breast cell lines and normal tissues.[15] Furthermore, miRNA-34a has been shown to suppress cancer stem cells (CSCs), implying that miRNA-34a may function as an anti-oncogene by inhibiting self-renewal and invasion while increasing chemo- and radiation sensitivity. [16]

In 89 breast cancer patients, Roth *et al*. (2010) found that higher miR-34a was substantially related to metastases. Furthermore, during the evolution of liver cancer in a rat model, an up-regulation of circulating miR-34a in serum was found.[17] For estrogen-receptor-alpha-positive (ERα+) breast tumours, miR-34a has been shown to decrease tumour growth.18 Andliena *et al*., (2018) found the same pattern for downregulation of miRNAs and as well detected in benign tumours when compared to expression levels in normal tissue samples in a study. These studies highlighted the similarities between benign and malignant tumours. In the study, MiR-34a was found to be down-regulated in both benign and malignant breast tissues.[19]

Many miRNAs thought to be "cancer-related" are actually markers of proliferation and growth rather than malignancy. They were deregulated in benign tumours as well as in breast carcinomas. This could suggest that early malignancy-related miRNA expression dysregulation can occur in benign tumours as well. The recent work adds to our understanding of miRNAs in breast cancer.

Conclusions

Apart from miRNA 34a, multiple miRNA play an important role in receptor expression or downregulation, so apart from miRNA 34a we have to determine a miRNA signature in order to develop miRNAs as a tool for management in breast cancer treatment. Our results suggest that miRNA 34a can be used to target androgen receptors for cancer therapeutics. MiR-34a also gives information regarding a patient's prognosis: patients with high levels of miR-34a are thought to be more aggressive and have a worse prognosis. To validate the diagnostic relevance of miR-34a, more research should be done with a larger sample size and prospective studies on larger cohorts of patients.

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