

MicroRNAs *hsa-miR-222-3p*, *hsa-let-7b-5p*, and *hsa-let-7f-5p* Regulate Their Putative Target Genes *HMGAI* and *CDKN1B* in Pakistani Breast Cancer Patients

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ABSTRACT

MicroRNAs are the class of noncoding small RNAs that have the capability to control gene expression by degrading mRNA or repressing the post-transcriptional process. In the current era, role of microRNAs is one of the promising diagnostic, prognostic and therapeutic tools for early detection of cancer, which normally play their role in the regulation of post-transcriptional gene expression via a mechanism of complimentary sequences and repression of target RNAs. This study focused on the expression pattern of three miRNAs, *hsa-miR-222-3p*, *hsa-let-7b-5p*, *hsa-let-7f-5p* and effect on two of their putative target genes *HMGAI* and *CDKN1B* in human breast cancer samples. Qiagen miScript Primer Assay based expression analyses revealed the over-expression of all three miRNAs in most of the studied human breast cancer samples compared to normal breast tissue samples. Moreover, over-expression of *hsa-222-3p*, *hsa-let-7b-5p* and *hsa-let-7f-5p*, downregulated their target genes (*HMGAI* and *CDKN1B*) in breast cancer samples compared to normal tissue samples.

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Conceptualization and study design – MW and HMFY; Experiments and data collection – HMFY; Data analysis and interpretation – HMFY, RS and MW; Manuscript write-up, review and editing – HMFY, SF, ARA, MT, RS, MR and MW.

Key words

Expression profiling, miRNAs, Breast cancer, Human, Target genes

INTRODUCTION

Human breast cancer is one of the most common cancer in Pakistan and has been reported to have highest incidence in Asia with a survival rate of around 53% (Bhurgri *et al.*, 2006). Majority of Pakistani patients approximately (47.26%) are diagnosed in stage-2 and less than 4% with stage-1 (Soomro *et al.*, 2018). Therefore, early diagnosis of the cancer would be a good strategy to slow down the death trajectory and to increase the survival rate of the patients. Unfortunately, despite of histological and immune-histological diagnosis, the aggressiveness and

incidence rate of the breast tumors cannot be reduced so there was a dire need to think on the alternative diagnostics tools for early detection of cancers.

In the current era, role of microRNAs (miRNAs) is one of the promising diagnostic, prognostic and therapeutic tools for early detection of cancer, which normally play their role in the regulation of posttranscriptional gene expression via a mechanism of complimentary sequences and repression of target RNAs. Several studies have shown early diagnosis of the cancers where survival rate can be increased by starting the treatment modalities earlier and highlighted the occurrence of miRNAs' mutation clusters as a main cause of cancer development (Qadir and Faheem, 2017; Calin and Croce, 2006). Amplification of miRNAs in cancer prove their existence and oncogenic functions while at the same time they also perform as tumor suppressors genes when chromosomal deletion accrued in cancers. Abnormal expression level of miRNAs has been found in both solid and hematopoietic cancers (Iorio *et al.*, 2005; Di Fazio *et al.*, 2017). So, three miRNAs *hsa-let-7b-5p*, *hsa-let-7f-5p*, *hsa-miR-222-3p* were selected due to their tumor suppressor behavior, as loss of let-7 family miRNAs

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is seen as a major contributor in oncogenesis through increase of target oncogenes and stemness factors. It was further suggested that let-7 family take part in metastasis and influence down regulation of *CCR7* by targeting its 3' UTR that resulted in the down regulation of breast cancer cells capacity for invasion and migration. Other studies confirmed similar results as let-7a acted as a tumor suppressor in zebrafish embryo models via regulating the expression of *RAS* and *HMGA2* oncogenes. Furthermore, decreased let-7a levels were related to increased RAS levels in lung squamous Carcinoma as well (Cunningham *et al.*, 2010; Johnson *et al.*, 2005). Similarly, *hsa-miR-222-3p* miRNAs was also found overexpressed in patients with carcinoid lung tumors (Di Fazio *et al.*, 2017).

As far as the putative targets of the Let-7 family and *hsa-miR-222-3p* miRNAs are concerned, expression level of high-mobility group A1 (*HMGA1*) and cyclin-dependent kinase inhibitor 1B, p27kip1 (*CDKN1B*) were also evaluated respectively. *HMGA1* is an architectural transcription factor and belongs to chromatin associated non-histone protein usually expressed during embryogenesis while downregulated in normally differentiated tissues. It is inversely correlated with let-7 family in liver cancer and upregulated in thyroid tissues (Di Fazio *et al.*, 2017; Marini *et al.*, 2011) therefore being investigated here to know its correlation with breast cancer patients. Similarly, *CDKN1B* is target gene of *hsa-miR-222-3p* in lungs and breast cancer studies (Li *et al.*, 2014) which inhibit the cyclin-dependent kinases to arrest the cell cycle and its ectopic expression may lead to pro-oncogenic functions in the cell.

The main objective of this study was to investigate the expression patterns of *hsa-miR-222-3p*, *hsa-let-7b-5p* and *hsa-let-7f-5p*. Moreover, the study aimed to explore the impact of these miRNAs on their potential target genes *HMGA1* and *CDKN1B* in the breast cancer patients from Pakistan.

MATERIALS AND METHODS

Sample collection

Human breast cancer and normal tissue samples (10 each) were collected from different hospitals in Lahore, Pakistan, with informed consent of the patients. All samples were preserved in absolute ethanol and 10% formalin solution for expression and histopathological analyses, respectively.

Histopathological examination

Histopathological examination was performed on formalin-fixed paraffin embedded (FFPE) cancerous tissues which were stored at room temperature. Formalin-

filled (10%) sample collection tubes were used to preserve the neoplastic tissues after grossing and isolation of core tumorous masses. The tissues were used for hematoxylin and eosin (H and E) staining to confirm the malignancy, grading and staging as described earlier (Yaqub *et al.*, 2023; Manzoor *et al.*, 2017).

Total RNA isolation and quantification

Total RNA was extracted from tumor and normal tissue samples, preserved in absolute ethanol and stored at -20°C , using RNeasy tissue mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Quantification of RNA was conducted by Nano Drop 2000 (Thermo Fisher Scientific, Pittsburg, PA, USA) spectrophotometer.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Complementary DNAs (cDNA) were amplified by using miScript Primer Assays (Qiagen, Hilden, Germany) and commercially available primers for *hsa-miR-222-3p* (MS00007609), *hsa-let-7b-5p* (MS00003122), *hsa-let-7f-5p* (MS00006489). *RNU6B* (MS00029204) was run as reference miRNA using miScript Primer Assays (Qiagen, Hilden, Germany). miScript II RT Kit (Qiagen, Hilden, Germany) was used to reverse transcribed miRNA-enriched RNA lysate. Primer 3 software was used to design primers for target genes (*HMGA1* and *CDKN1B*) of studied miRNAs. Sequences of these genes were taken from ENSEMBLE Genome Browser (<https://asia.ensembl.org/index.html>) and *GAPDH* was used as a reference gene. All experiments were performed on RotorGene-Q (5-plex) instrument (Qiagen).

Relative expression of *RNU6B/GAPDH* normalized data of cancerous tissue (HMT) against normal tissue (HNS) for target miRNA/gene, was used to calculate ΔCt [ΔCt (Cancer) = Ct (miRNAs/genes) – Ct (*RNU6B/GAPDH*)]. Fold Change calculations were made on three technically repeats measurements averaged and normalized to the internal controls (*RNU6B/GAPDH*) as described earlier (Yaqub *et al.*, 2023; Mansha *et al.*, 2011). Log 2-fold change were calculated by using following statistics:

$$\Delta\text{Ct} (\text{Test}) = \text{Ct} (\text{Target}) - \text{Ct} (\text{Reference})$$

$$\Delta\text{Ct} (\text{Cancer}) = \text{Ct} (\text{miRNAs/genes}) - \text{Ct} (\text{RNU6B})$$

$$\Delta\text{Ct} (\text{Calibrator}) = \text{Ct} (\text{Target}) - \text{Ct} (\text{Reference})$$

$$\Delta\text{Ct} (\text{Normal}) = \text{Ct} (\text{miRNAs/genes}) - \text{Ct} (\text{RNU6B})$$

$$\Delta\Delta\text{Ct} = \Delta\text{Ct} (\text{Cancer}) - \Delta\text{Ct} (\text{Normal})$$

$$\text{Fold change} = 2^{-\Delta\Delta\text{Ct}}$$

Statistical analysis

Student t-test was applied on each of the targeted miRNAs/genes (cancer vs normal) data, which was normalized by *RNU6B/GAPDH*. *p*-value <0.05 was

considered as significant as described earlier (Yaquib *et al.*, 2023).

GraphPad prism analysis

GraphPad prism software (<https://www.graphpad.com/scientific-software/prism/>) was used to analyze the expression data of human breast cancer patient (miRNAs and Genes).

RESULTS

Histopathological examination

Histopathological analyses of the mammary tumors showed varied population of cells with enormous nucleus and prominent mitotic character (Fig. 1A). Numerous key regions showed the presence of fibroblast and collagen fibers (Fig. 1B).

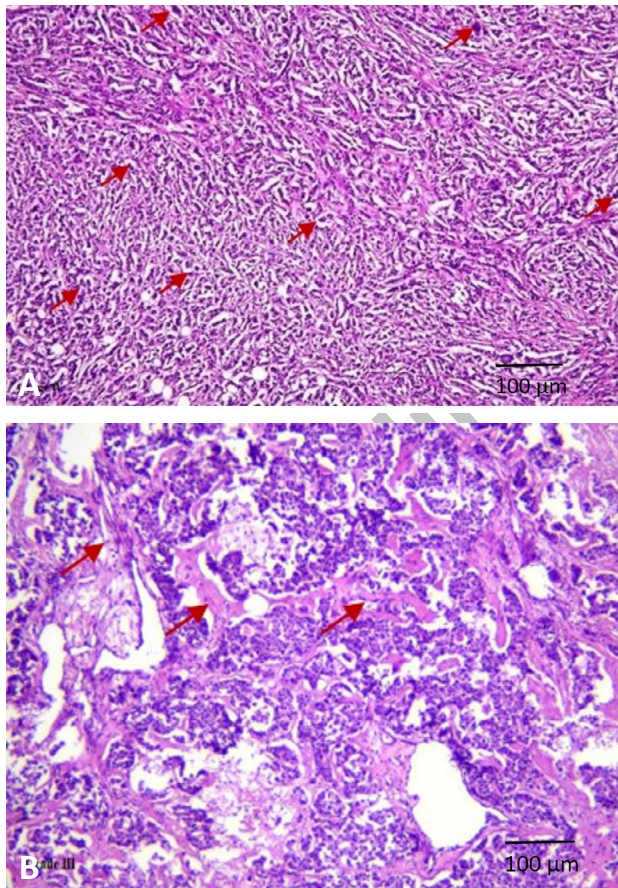


Fig. 1. Histopathological analysis of human mammary tumor. (A) Representative human mammary tumor showed varied population of cells with enormous nucleus and prominent mitotic characteristic. (B) Numerous key regions indicating the presence of fibroblasts.

Expression of miRNAs

The expression levels of *hsa-miR-222-3p*, *hsa-let-7b-5p*, and *hsa-let-7f-5p* miRNAs were analyzed in human breast cancer tissue samples obtained from both normal and cancer patients. The graphical representation of *hsa-miR-222-3p* expression is depicted in Figure 2A, and GraphPad prism analysis is shown in Figure 2B. Overall, it was observed that the expression of *hsa-miR-222-3p* was significantly upregulated in all tumor tissue samples (HMT) compared to normal tissue samples (HNS). However, the degree of upregulation varied among patients depending on the severity of the disease. Three tumor samples (HMT 02, 05, and 08) exhibited a more than ~2500-fold change (FC) in expression compared to normal tissue samples, while samples 01 and 09 showed moderate expression ranging from 1500-2000 FC. The remaining four tumor samples showed a slightly upregulated expression ranging from 100-300 FC, as shown in Figure 2A and 2B with a significant p-value of 3.51×10^{-6} .

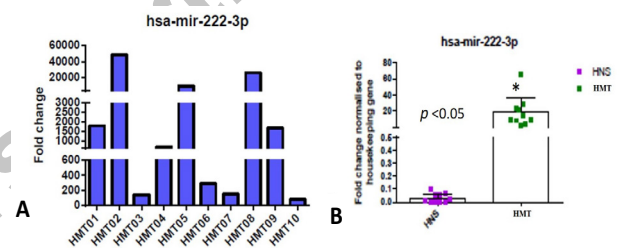


Fig. 2. Expression level of *hsa-miR-222-3p* miRNA in human mammary tumor was measured using RT-qPCR (A) Data from three technically replicated measurements were averaged and normalized to the internal *RNU6B* control. Log 2-fold change values were calculated for 10 human mammary tumor samples (HMT). (B) Expression data of *hsa-miR-222-3p* miRNA (fold change), in human tumor (HMT) and normal mammary tissue (HNS) samples, was analyzed using GraphPad Prism software.

Similarly, the expression of *hsa-let-7b-5p* was also upregulated in all tumor tissue samples (HMT), with the degree of upregulation depending on the severity of the disease, as depicted in Figure 3A and 3B. Samples HMT03, 04, and 09 showed higher expression, while samples HMT02 and 10 exhibited moderate upregulation. The remaining five samples (HMT01, 05, 06, 07, and 08) showed slightly significant upregulation, with a p-value of 1.59×10^{-5} .

Furthermore, the expression of *hsa-let-7f-5p* was also analyzed, as shown in Figure 4A and 4B. Samples HMT03, 04, and 07 exhibited highly upregulated expression, while samples HMT02, 09, and 10 showed moderate upregulation. The remaining samples (HMT01, 05, 06, and 08) showed slightly significant upregulation,

with a p -value of 1.12×10^{-05} .

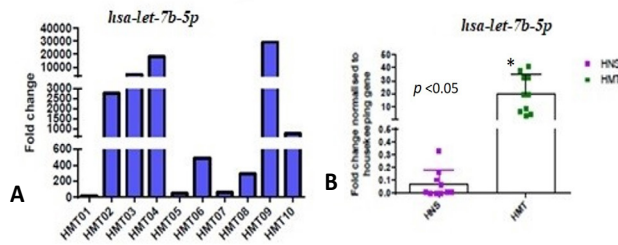


Fig. 3. Expression level of *hsa-let-7b-5p* miRNA in human mammary tumor was measured using RT-qPCR (A) Data from three technically replicated measurements were averaged and normalized to the internal *RNU6B* control. Log 2-fold change values were calculated for 10 human mammary tumor samples (HMT). (B) Expression data of *hsa-let-7b-5p* miRNA (fold change), in human tumor (HMT) and normal mammary tissue (HNS) samples, was analyzed using GraphPad Prism software.

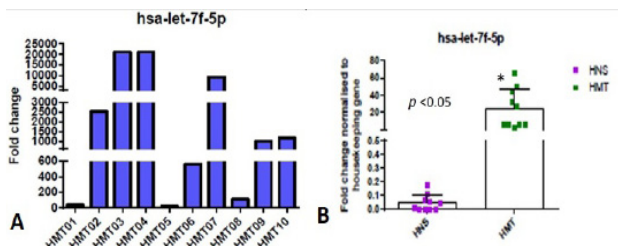


Fig. 4. Expression level of *hsa-let-7f-5p* miRNA in human mammary tumor was measured using RT-qPCR (A) Data from three technically replicated measurements were averaged and normalized to the internal *RNU6B* control. Log 2-fold change values were calculated for 10 human mammary tumor samples (HMT). (B) Expression data of *hsa-let-7f-5p* miRNA (fold change), in human tumor (HMT) and normal mammary tissue (HNS) samples, was analyzed using GraphPad Prism software.

Comparative miRNAs expression analysis

Figure 5A and 5B demonstrate the expression of three miRNAs (*hsa-miR-222-3p*, *has-let-7b-5p* and *hsa-let-7f-5p*) in both human normal (HNS) and mammary tumor samples (HMT), individually as well as cumulatively. Upon comparing the overall expression levels of these miRNAs, it was observed that all three were significantly up-regulated in all tumor samples. The intensity of up-regulation for these miRNAs is provided in Table I.

HMGA1 and *CDKN1B* expression in human mammary tumors

The study investigated the expression of *HMGA1* and *CDKN1B* genes in human normal tissue samples (HNS)

and human tumor samples (HMT) (Table II). The analysis showed that expression of *HMGA1* gene was down-regulated in tested tumor samples (HMT), as evidenced by significant p -value of 7.35×10^{-07} (Fig. 6A). Similarly, the expression of *CDKN1B* gene was also down-regulated in tested human mammary tumor samples (HMT) and had a very significant p -value of 2.49×10^{-07} (Fig. 6B). To better illustrate the data displayed in Figure 7, the GraphPad Prism software was used to combine the representation of HNS and HMT samples.

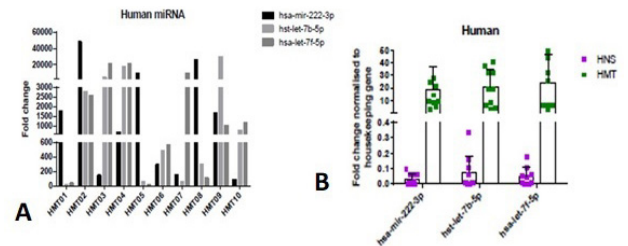


Fig. 5. Comparison of *hsa-miR-222-3p*, *hsa-let-7b-5p* and *hsa-let-7f-5p* expression (fold change) in human mammary tumor samples (HMT): (A) using RT-qPCR, (B) using GraphPad Prism software.

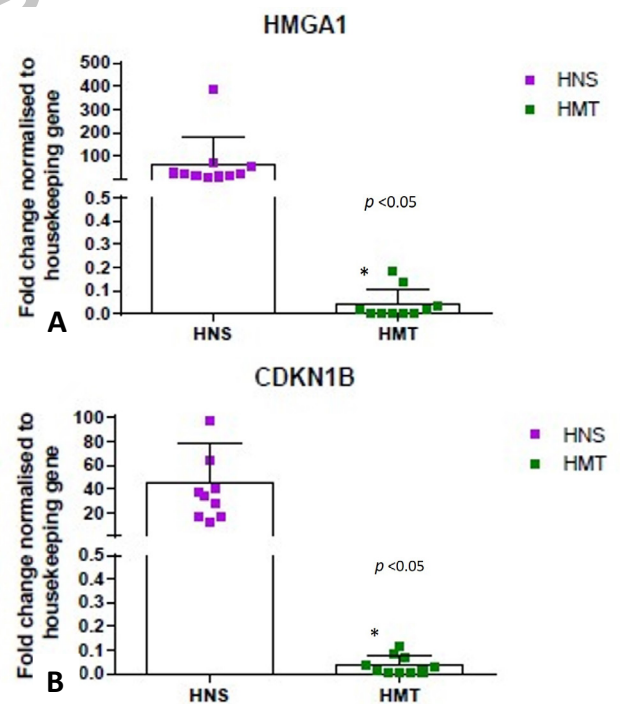


Fig. 6. Expression data of *HMGA1* (fold change) (A) and *CDKN1B* (fold change) (B), in human tumor (HMT) and normal mammary tissue (HNS) samples, was analyzed using GraphPad Prism software.

Table I. Overview of three miRNAs and their fold

changes in human mammary tumor samples.

microRNAs	Tested samples	Fold change	Up-regulation intensity
hsa-mir-222-3p	HMT 01	1782.89	
	HMT 02	47864.44	
	HMT 03	114.01	
	HMT 04	670.92	
	HMT 05	9173.99	
	HMT 06	288.68	
	HMT 07	152.57	
	HMT 08	25828.33	
	HMT 09	1663.49	
	HMT 10	80.45	
hsa-let-7b-5p	HMT 01	20.02	
	HMT 02	2771.91	
	HMT 03	4309.59	
	HMT 04	18095.37	
	HMT 05	53.45	
	HMT 06	487.75	
	HMT 07	56.62	
	HMT 08	291.36	
	HMT 09	29328.18	
	HMT 10	756.57	
hsa-let-7f-5p	HMT 01	35.67	
	HMT 02	2562.49	
	HMT 03	21173.91	
	HMT 04	21125.04	
	HMT 05	21.41	
	HMT 06	562.87	
	HMT 07	9280.58	
	HMT 08	105.90	
	HMT 09	1009.90	
	HMT 10	1181.72	

■ Highly up-regulated: FC >25000; ■ Moderately up-regulated: FC >9000 and <25000; ■ slightly up-regulated: FC >1 and <9000.

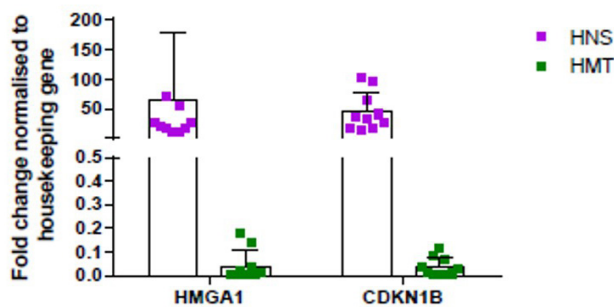


Fig. 7. Comparison of expression data of *HMGA1* and *CDKN 1B* (fold change), in human tumor (HMT) and normal mammary tissue (HNS) samples, was analyzed using GraphPad Prism software.

Table II. Comparison of down-regulation expression

of *HGMA 1* and *CDKN 1B* genes in human mammary tumors.

Genes	Samples	Fold change	Down-regulation intensity
HGMA1	HMT01	0.00003	
	HMT02	0.00229	
	HMT03	0.00019	
	HMT04	0.00691	
	HMT05	0.00005	
	HMT06	0.00005	
	HMT02	0.00145	
	HMT08	0.00694	
	HMT09	0.00016	
	HMT10	0.00018	
CDKN1B	HMT01	0.00251	
	HMT02	0.00009	
	HMT03	0.00027	
	HMT04	0.00004	
	HMT05	0.00234	
	HMT06	0.00031	
	HMT07	0.00663	
	HMT08	0.00027	
	HMT09	0.00015	
	HMT10	0.00317	

■ highly down-regulated: FC>40; ■ Moderately down-regulated: FC>10<40; ■ slightly down-regulated: FC>0<10.

DISCUSSION

Although breast cancer remains one of the most extensively researched cancers and there is vast amount of literature available, further understanding of the molecular changes associated with the disease is necessary to ultimately achieve the genomic medicine solutions. The expression profiles of miRNAs offer a non-invasive approach to record molecular phenotypes in cells (Cortez *et al.*, 2012), making circulating miRNAs a promising potential genetic marker for human cancers (Calin and Croce, 2006; Qu *et al.*, 2011), especially for the study of breast cancer dynamics in humans. Prior to implementing this method in humans, several cross-species studies were carried out to investigate the gene expression profiles of circulating miRNAs as potential biomarker for detecting tumors in dogs and mice (Kim *et al.*, 2020; Cunningham *et al.*, 2010).

This study aimed to understand the role of miRNAs *hsa-let-7b-5p*, *hsa-let-7f-5p* and *hsa-miR-222-3p* in human breast cancer and their use as potential biomarker for early diagnosis of breast cancer in humans. Hence, the

expression of *hsa-let-7b-5p*, *hsa-let-7f-5p* and *hsa-miR-222-3p* miRNAs and their putative target genes *HMGA1* and *CDKN1B* was analyzed in human breast cancer samples. In our breast cancer samples, we observed an increase in the expression of *has-miR-222-3p*. This is consistent with previous findings in human lung, thyroid colorectal cancers and atherosclerosis, where *has-miR-222-3p* was identified as a potential diagnostic marker (Kara *et al.*, 2015). In the same vein, *hsa-let-7b-5p* and *hsa-let-7f-5p* miRNAs also exhibited increased expression, albeit not to the extent of *hsa-miR-222-3p*, which showed a more than 2500 fold change in expression in three breast cancer samples. The up-regulation of *hsa-let-7b-5p* and *hsa-let-7f-5p* was observed in only one tumor sample with same fold change value. This discrepancy could be attributed to the cancer stage and micro-evolutionary phase of tumor samples, which is consistent with previous research (Di Fazio *et al.*, 2017).

Regarding the expression of two target genes of studied miRNAs, *HMGA1* and *CDKN1B*, it has been found that this has implications for the use of miRNAs as new markers for genetic diagnosis, prognosis, treatment efficacy and tracking in breast cancer. This is because the miRNAs being studied have a direct impact on the expression of these target genes, making them a double-edged factor to consider. The expression of *HMGA1* and *CDKN1B* genes have been decreased, but at a moderate rate with the maximum down-regulated FC value of 0.0003. However, in the case of the *CDKN1B* gene, the maximum down-regulated expression was observed with an FC value of 0.0004. This finding provides evidence of an inverse correlation between miRNA markers and their target genes, which is in agreement with previous studies (Di Fazio *et al.*, 2017).

The results of the current study are correlated to our previous study (Yaqub *et al.*, 2023) in which same three miRNAs were profiled in canine mammary tumor samples and up-regulated expression was observed in most of the studied samples, whereas their same target genes of *HMGA1* and *CDKN1B* showed down-regulation, consequently, inversely correlated in canine mammary tumor samples as we report here in human breast cancer.

CONCLUSION

The objective of this study was to investigate the expression patterns of three miRNAs (*has-miR-222-3p*, *has-let-7b-5p*, *has-let-7f-5p*) and their two putative target genes (*HMGA1* and *CDKN1B*) in human breast cancer samples. Using the Qiagen miScript Primer Assay we discovered that the majority of breast cancer samples showed higher expression levels of all three miRNAs

compared to normal breast tissue samples. Furthermore, the over-expression of these miRNAs was found to down-regulate their putative target genes in breast cancer samples compared to normal samples. In this end, this research may contribute to improving the identification and comprehension of molecular phenotypes of cancerous breast tissues, which may enhance the diagnosis of human breast cancer.

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IRB approval

The study was approved by Advanced Studies and Research Board (ASRB), UVAS (DAS/575-06.03.2019).

Ethical statement

During the samples collection, animals were handled according to the approved guidelines provided by Ethical Institutional Review Board of University of Veterinary and Animal Sciences, Lahore.

Data availability

There is no data submitted to any database and no supplementary files available. All figures and tables are available in the manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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