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Analyzing RASSF1A Promoter Methylation and MicroRNA Profiles in the Plasma of Breast Cancer Patients from Pakistan

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ABSTRACT

The current study assessed the promoter DNA methylation status of a tumor suppressor gene, namely Ras association domain family 1 isoform A (RASSF1A), and the expression levels of selected cancer-associated microRNAs (miR-10b and miR-34a) in plasma samples of breast cancer patients, with the aim of evaluating the diagnostic relevance of circulating nucleic acids. Circulating cell free DNA (cfDNA) was isolated from the plasma samples of 40 breast cancer patients, followed by bisulfite modification and methylation-specific PCR to determine RASSF1A promoter hypermethylation. Further, miR-specific real-time qRT-PCR approach was used to analyse the levels of microRNA employing DNA primers, instead of stem-loop/locked nucleic acid (LNA) primers. RASSF1A promoter hypermethylation was detected in 40% of patients. Circulating miR-10b levels showed a statistically significant difference between patients and healthy controls ($p < 0.001$). Receiver operating characteristic (ROC) curve analysis demonstrated a high discriminative performance of miR-10b, with an area under the curve (AUC) of 0.97 (95% CI: 0.90–0.99; SE = 0.16). The results point to the potential value of circulating microRNA profiling, especially miR-10b, as an affordable and invasive supplement to the current breast cancer detection techniques. However, more validation research in a larger cohort is required to prove their therapeutic value.

Keywords: biomarkers, blood-based diagnosis, breast cancer, circulating microRNAs, RASSF1A gene, real-time PCR

Highlights

- Approximately 40% of patients with breast cancer were found to have

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circulating RASSF1A promoter hypermethylation. Epigenetic changes were identified in the current investigation using cfDNA.

- The high diagnostic accuracy of the plasma levels of miR-10b (AUC=0.97) was observed with a significant difference in expression between patients and controls.
- The plasma microRNA analysis illustrates the potential of low-cost, less-invasive biomarkers for the detection of breast cancer.

1. INTRODUCTION

Independent research and hospital data consistently demonstrate a disturbing rise in the incidence and mortality rates of breast cancer among women in Pakistan, despite the absence of national data [1, 2]. This disturbing trend stands in sharp contrast to the situation in countries with a very high per capita income. For instance, over the past 20 years, the mortality rate due to breast cancer has fallen by 34% in the United States of America [3]. This marked reduction is mainly due to the extensive use of effective screening tools, such as mammography, ultrasound, and magnetic resonance imaging (MRI), which enable early evidence-based interventions and promote early detection. However, the benefit of early detection is not yet fully realized in Pakistan. One of the reasons is the lack of access to organized mammography services. Additionally, low level of public awareness, poor health education, associated stigma of cancer, fear of radiation exposure, and cost factors also act as deterrents for women to participate in preventive screening programs. Taken together, these factors contribute to poor health outcomes and late diagnosis. Further, these and similar factors restrict many women to discuss disease or to go for annual checkup/screening. As a result, a substantial portion of women present with advanced-stage breast carcinoma at the time of initial diagnosis, which adversely affects the prognosis and chances of survival. In this regard, the development of complementary, minimally invasive, radiation-free, and economical screening or diagnosis may be helpful in improving early detection, particularly in under-resourced settings.

The development of tumor, its progression, and metastasis are driven by distinct genetic, epigenetic, and/or molecular alterations. Furthermore, systematic characterization of these changes in tumor tissue samples have long been central to cancer diagnosis and classification [4, 5].

Accumulating evidence suggests that both normal and malignant cells release nucleic acid components into circulation. Notably, the concentration and molecular characteristics of circulating cell-free DNA (cfDNA) are altered in various malignancies, including breast cancer, offering opportunities for non-invasive cancer detection and disease monitoring [6–10]. In breast cancer, the analysis of aberrant promoter hypermethylation of tumor suppressor genes—such as RASSF1A, APC, DAP-K, and ITIH5—in cfDNA, along with the expression profiling of circulating microRNAs (small non-coding RNAs approximately 18-24 nucleotides in length), has emerged as a promising area of research [4, 6, 11–15]. Circulating biomarkers, whether assessed individually or alongside established tumor markers, hold promise as supportive tools for improving cancer detection.

This study examined the promoter methylation profile of the tumor suppressor gene RASSF1A and measured the plasma expression levels of two cancer-associated microRNAs, namely miR-10b and miR-34a, in patients diagnosed with breast cancer. Their potential use within the local patient population was the main aim. The results highlight the feasibility of using blood-based molecular tests. Although, further research is needed to better elucidate the potential use of such minimally invasive diagnostic tools.

2. MATERIALS AND METHODS

2.1. Ethical Approval and Study Population

A total of 60 participants were included, with 20 healthy, age-matched volunteers acting as control and 40 diagnosed with breast cancer. Plasma samples from the patients were collected from Jinnah Hospital and the Institute of Nuclear Medicine and Oncology, Lahore after obtaining the written informed consent of patients. Ethical clearance was obtained from the School of Biological Sciences' Ethical Review Board (Approval No. 873/12). All procedures were conducted in accordance with accepted standards.

2.2. Collecting Blood and Preparing Plasma

To ensure sufficient anticoagulation, 5 mL of peripheral blood was collected and inverted in vacutainer tubes coated with EDTA. Plasma was separated using a swing-bucket rotor centrifuged at 2,500×g for 5 minutes

at 4°C, in accordance with routine pre-analytical procedures. Each sample was visually inspected to ensure that there were no signs of hemolysis. To remove any remaining traces of cellular debris, the plasma supernatant was carefully transferred to sterile tubes and centrifuged for 5 minutes at 4,500×g. After clarification, the plasma was transferred to tubes that were free of RNase and DNase (500 L per tube). Further, it was either immediately processed for nucleic acid extraction or stored at -80°C.

2.3. Separation of Circulating DNA and Treatment with Bisulfite

Cell-free DNA (cfDNA) was purified from 500 L plasma samples, according to the manufacturer's protocol and using a circulating DNA purification kit that can be purchased (Norgen Biotek Corp., Canada). The purified cfDNA was then eluted in 30 µL buffer using a NanoDropTM 1000 spectrophotometer from Thermo Scientific, USA, which measured the effect of the elution of purified cfDNA and performed under denaturing conditions in the presence of pronase and proteinase K to ensure sufficient protein hydrolysis and inhibition of nuclease activity. To distinguish between methylated and unmethylated cytosines, bisulfite conversion was performed. Unmethylated cytosines were converted to uracil in this chemical conversion reaction, while methylated cytosines were left unchanged, making them detectable by methylation analysis. The CpGenomeTM DNA Modification Kit (EMD Millipore, USA) was used to perform conversion reactions, which involved the steps of sulphonation, desulphonation, and purification in a series of reactions. The eluted modified DNA was either analyzed immediately for methylation-specific PCR (MSP) or stored at -20°C.

2.4. Primer Design and Methylation-Specific PCR

Using the Meth Primer software, the promoter region of the tumor suppressor gene RASSF1A (RefSeq: NM_007182) was examined for CpG island regions. For the purpose of focused testing, a CpG island region within the promoter region between nucleotides 541 and 770 was selected. Primer pairs were designed to specifically amplify methylated and unmethylated DNA based on bisulfite-converted sequences (Table 1).

Methylation-specific PCR was performed using the CpG WIZ® RASSF1A amplification kit (Millipore, USA). Each 25 µL reaction mixture contained a universal MSP buffer, approximately 100 ng of bisulfite-treated DNA, and 0.5 µL (10 µM) each of forward and reverse

primers corresponding to either the methylated or unmethylated promoter sequence. Thermal cycling conditions included initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C, annealing at optimized temperatures [55°C (RP-F/R), 58°C (RASS-MF/MR), and 57°C (RASS-UF/UR)], and extension at 72°C for 30 seconds per step. For 15 minutes, a last extension was carried out at 72°C.

Each PCR cycle included RT-minus controls to assess non-specific amplification and no-template controls to identify possible contamination. In order to verify the fragment size, PCR products were resolved on 2% agarose gels with a 50 bp DNA ladder.

Table 1. Sequence of the Designed Primers Used in this Study ["M" and "U" Represent Methylated and Unmethylated Primer Sets, respectively].

Oligo Name	Sequence (5'→3')
Primers used in the analysis of *RASSF1A* promoter methylation	
RP	F: GACCCTCTTCCTCTAGCACA
	R: CTGCACCCAGGTTTCCATTGCGC
RASS	MF: GGGTTTTGCGAGAGCGCG
	MR: GCTAACAAACGCGAACCG
	UF: GGTTTTGTGAGAGTGTGTTTG
	UR: CACTAACAAACACAAACCAG
miR-specific DNA primers used in microRNA analysis	
miR-10b	F: CAGTACCCTGTAGAACCGAATT
	R: AGGTCCAGTTTTTTTTTTTTTTCACA
miR-34a	F: GTGGCAGTGTCTTAGCTGG
	R: CAGGTCCAGTTTTTTTTTTTTTTACAA

2.5. Extraction of Circulating RNA and microRNA Analysis

Total RNA, including small RNA species, was isolated from 500 µL plasma samples using TRIzol® LS reagent (Life Technologies, USA). RNA concentration was determined spectrophotometrically. For microRNA analysis, 2 µg of total RNA was subjected to polyadenylation at the 3′ end using poly(A) polymerase in a reaction containing PAP buffer, $MnCl_2$, ATP, and enzyme, followed by incubation at 37°C for 60 minutes. The reaction was terminated by heating at 65°C. Complementary DNA (cDNA) synthesis was performed using the ImProm-II™ reverse

transcription system (Promega, USA). Polyadenylated RNA was primed with a universal oligo(dT) adapter primer and reverse-transcribed at 42°C for 1 hour, followed by heat inactivation at 65°C.

Quantitative PCR was, thereafter, performed using VeriQuest™ Fast SYBR® Green qPCR Master Mix (Affymetrix, USA) on a Bio-Rad CFX96™ real-time PCR system. Each 10 µL reaction contained 1 µL of cDNA template, 250 nM of miRNA-specific forward and reverse primers (Table 2), and 2× SYBR Green master mix. Cycling conditions consisted of initial denaturation at 95°C for 5 minutes, followed by 50 amplification cycles at 95°C for 10 seconds and at 55°C for 20 seconds.

To compensate for variations in reverse transcription efficiency and RNA extraction quality, an artificial Caenorhabditis elegans miR-39 was employed as an external spike-in control. This control enabled more reliable normalization among samples. Following amplification, a 65-95°C melt curve analysis was performed to exclude non-specific amplification and primer-dimer formation and to verify the specificity of PCR products. For maximum analytical precision, all reactions were performed in technical replicates and included both positive controls and blank controls. The relative expression values were quantified by taking threshold cycle (CT) values. To compensate for variations in the process, normalization was achieved by subtracting the cel-miR-39 CT value from those of the target microRNAs, as there was no known authentic endogenous reference miRNA**.**

3. RESULTS AND DISCUSSION

3.1. Clinical Features of Study Subjects

The clinical and pathological features of the subjects are presented below in Table 2. The age of the patients ranged from 30 to 65 years, with a median age of 42 years. Approximately 20% of female patients were postmenopausal at the time of diagnosis. Based on the histopathological analysis, the majority of the patients (87%) had invasive ductal carcinoma (IDC). The rest of the patients had invasive lobular carcinoma (ILC). Only one patient had hypertension and none of the patients had diabetes.

Interestingly, there were no instances of stage 0 illness at the time of presentation. While, 27 patients (68%) had a diagnosis of late-stage (stage III–IV) breast cancer, 13 patients (32%) had early-stage (stage I–II) breast cancer. According to the data from Pakistan that has already been

reported, this trend indicates a significant burden of late-stage diagnosis [1, 16]. Although precise epidemiological evaluation is limited by the lack of a comprehensive national tumor registry, previous research and the current cohort show that delayed diagnosis still remains a major determinant of the population's worse clinical outcomes and low rates of five-year survival.

Table 2. Clinicopathological Information and Patient History for Enrolled Patients with Breast Cancer

Breast Cancer Patients	40
Age at diagnosis	
Median	42 (32-65 years)
≤50	28
≥50	12
Histological type	
Invasive ductal carcinoma	35
Invasive lobular carcinoma	05
Distant metastasis	
Yes	25
No	15
Estrogen receptor	
Positive	23
Negative	10
Unknown	07
Progesterone receptor	
Positive	22
Negative	11
Unknown	07
Her2 status	
Positive	16
Negative	12
Unknown	12
Therapy (chemo/radio)	
Not received	37
Received	03

3.2. RASSF1A Promoter Methylation in Circulating DNA

Normal gene transcription is made feasible by recognizing that CpG

islands in the promoter regions of tumor suppressor genes are usually unmethylated. On the other hand, one known epigenetic modification linked to carcinogenesis is aberrant promoter hypermethylation that triggers transcriptional silence. In this study, the methylation status of RASSF1A promoter was evaluated in cfDNA using a methylation-specific PCR approach.

Prior to bisulfite-based analysis, the amplification of a 212 bp fragment spanning the CpG-rich region of RASSF1A promoter confirmed the presence and integrity of the target sequence in all cfDNA samples (Figure 1A), indicating its suitability for downstream methylation analysis. MSP analysis demonstrated the absence of RASSF1A promoter methylation in all healthy controls, in agreement with the established association between tumor suppressor gene hypermethylation and malignant states.

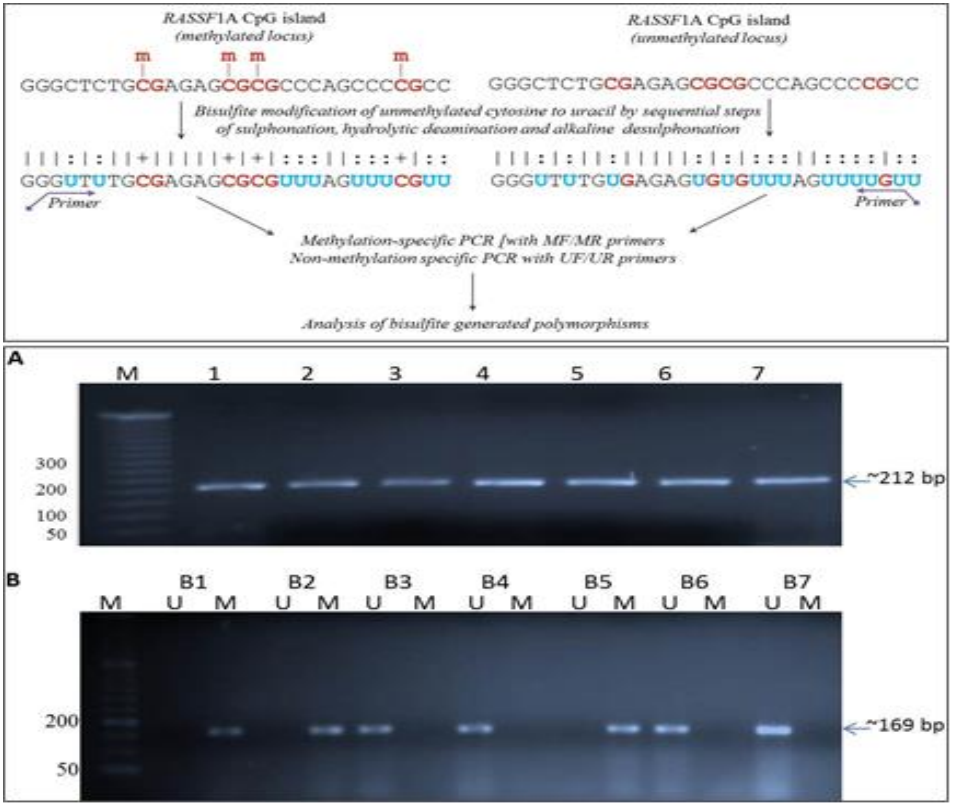

Figure 1. Top Panel: Schematic Representation of The Steps Involved in The Analysis of RASSF1A Promoter DNA Hypermethylation in Breast

Cancer Patients.

Methylated cytosines in the CpG island are shown as “m-C”. The unmethylated cytosines, which convert to uracil following bisulfite conversion, are denoted by colon “ : ”, while the unmodified m-C are represented by plus “ + ” symbol. 1A) PCR with RP-F/R primers to assess the intactness of RASSF1A promoter region in extracted cfDNA samples. Representative samples (Lanes 1-7) were resolved on 2% agarose gel. M represents 50 bp DNA ladder. Arrow indicates the position of amplicon (~212 bp). 1B). Analysis of promoter hypermethylation in representative breast cancer patients [B1 to B7] by methylation-specific PCR. “M” and “U” represent the MSP analysis using methylated and unmethylated primers, respectively. While, the arrow on right side indicates the size of amplicon (~169bp).

In 16 out of 40 breast cancer patients (40%), promoter hypermethylation of RASSF1A was detected; representative results are shown in Figure 1B. When stratified by disease stage, methylation was observed in 30% (4/13) of early-stage cases and 44% (12/27) of late-stage cases.

Furthermore, distant metastases were reported in 75% of patients with positive promoter methylation tests, suggesting an association between RASSF1A silencing and accelerated disease development. With promoter hypermethylation documented at different phases of the disease, the RASSF1A gene is one of the most studied epigenetically altered tumor suppressors in breast cancer [4]. Through epigenetic dysregulation, a number of other genes, such as RARβ2, HIC1, APC, ITIH5, DKK3, SOX17, and CST6, have also been linked to breast carcinogenesis [6, 7, 11, 12].

The specificity of RASSF1A promoter methylation as a blood-based biomarker, however, has been shown to vary widely from one study to another, ranging from approximately 15% to 85% [4, 6, 17–20]. The fact that the detection rate in the current study remains intermediate suggests that it is important to validate epigenetic biomarkers in specific populations before they can be used as part of the standard diagnostic workup.

3.3. Expression of Circulating miR-10b and miR-34a

The analysis of circulating RNA isolated from 500 L plasma samples showed greater median values in breast cancer patients (7.9 g; range, 2.7-13.5 g), as compared with healthy controls (5.5 g; range, 2.7-10.4 g). Although it is known that these substances are normally secreted into circulation in various physiological conditions, increased rates of circulating nucleic acid in cancer patients have been attributed to increased cellular turnover, apoptosis, necrosis, and total tumor burden [3]. The stability of microRNAs in RNase-rich bodily fluids makes them particularly attractive as circulating biomarkers. These include miR-10b and miR-34a. These small non-coding RNAs play a crucial role in maintaining cellular homeostasis by regulating post-transcriptional gene expression.

Tumor initiation, progression, and metastasis have all been linked to the dysregulation of microRNA networks, supporting their use as diagnostic and prognostic biomarkers [21–26]. In the current study, plasma levels of miR-10b (an oncogenic microRNA) and miR-34a (a tumor suppressor microRNA) were quantified using a miR-specific qRT-PCR approach (Figure 2). To minimize technical variability, equal amounts of RNA were subjected to polyadenylation and reverse transcription, while Caenorhabditis elegans miR-39 was used as an external spike-in control. Further, miR-10b expression in breast cancer patients exhibited markedly lower CT values (35–40 cycles), as compared to healthy controls with minimal or undetectable amplification (Figure 3). Statistical analysis revealed a highly significant difference between the two groups ($p < 0.001$).

ROC curve analysis further demonstrated appreciable discriminatory performance, with an AUC of 0.97 (95% *CI*: 0.91-0.99; *SE* = 0.016) (Figure 3). Although this value differs from those reported previously [27], it is consistent with several other studies documenting elevated circulating miR-10b levels in breast cancer patients [28, 29]. Furthermore, miR-10b promotes invasion and metastasis through the suppression of the HOXD10 signaling pathway, providing a biological rationale for its increased expression in advanced disease [30]. The findings are in accordance with recent studies that demonstrated a strong correlation between elevated miR-10b levels and tumor size, grade, and metastasis [31, 32].

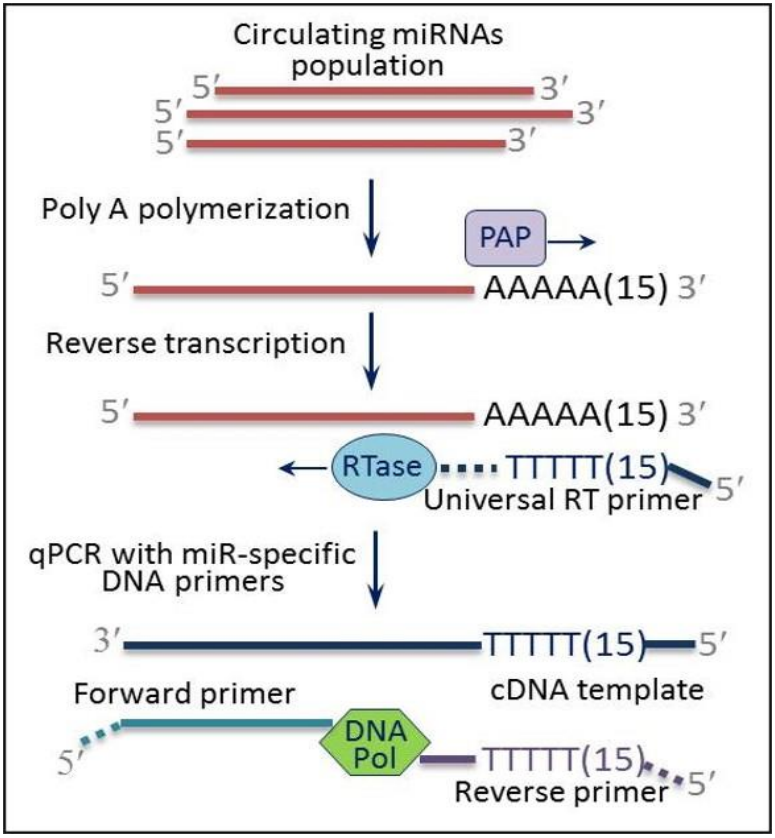

Figure 2. Schematic Representation of the Steps Involved in miR-specific qRT-PCR Approach. The Abbreviations PAP, RTase, and DNA Pol Represent polyA Polymerase, Reverse Transcriptase, and DNA Polymerase, Respectively.

Compared to miR-10b, miR-34a expression showed overlapping CT values between patients and controls, resulting in limited diagnostic discrimination (AUC = 0.64). Moreover, miR-34a is a well-characterized tumor suppressor microRNA regulated by p53 and involved in the control of apoptosis and oncogene expression, including BCL-2 and MYCN [33-35]. Its relatively higher expression in healthy individuals is, therefore, consistent with its established biological function.

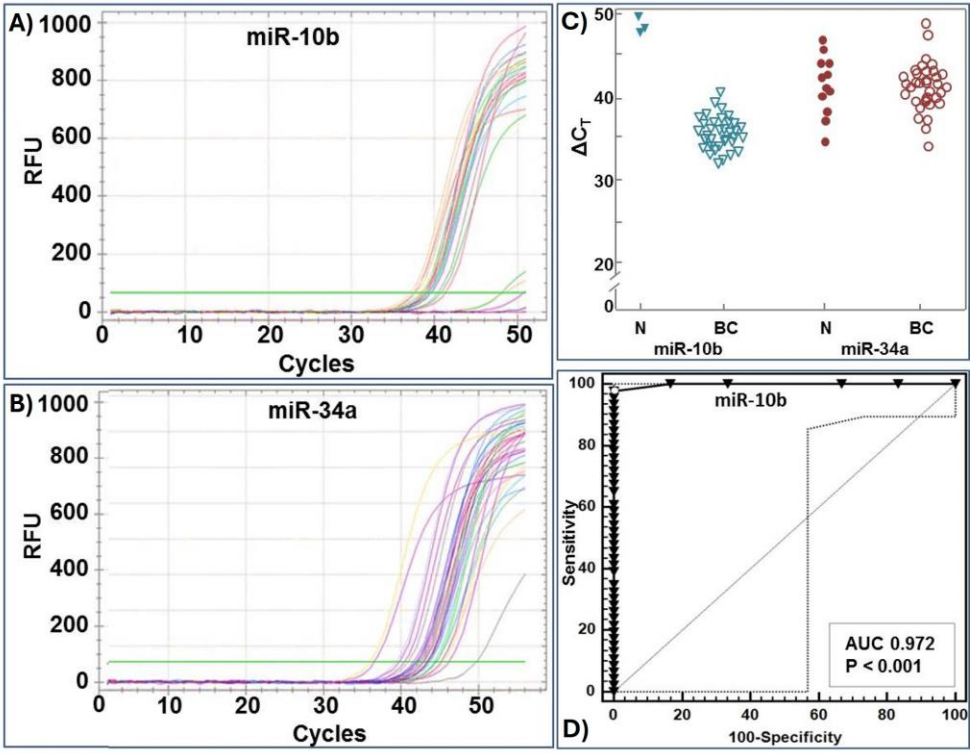

Figure 3. Analysis of Expression Levels of (A) Circulating miR-10b and (B) miR-34a in Patient and Control Groups by miR-specific qRT-PCR. C) and D): Assessing the Diagnostic Potential of miR-10b in the Plasma Of Breast Cancer Patients by ROC Curve Analysis. AUC: Area Under the Curve

3.4. Conclusion, Limitations and Implications

According to the findings, circulating miR-10b levels fluctuate between breast cancer patients in Pakistan and the healthy control group. This suggests that miR-10b may be used as a selective biomarker. Despite the poor sensitivity of RASSF1A promoter methylation alone, its concurrent assessment with circulating miRNAs may provide additional information. However, this is only preliminary observation and validation requires larger, multi-centered sample cohort studies.

Moreover, the current study has a number of limitations, such as a small number of participants and a cohort selected for advanced-stage disease, which restricts the use of ROC curves for particular stages of

performance evaluation. Furthermore, circulating miRNA normalization did not incorporate a proven endogenous reference; instead, it depended on an exogenous spike-in control. Before assessing the diagnostic potential and usefulness of these biomarkers, larger, prospectively conducted trials with universally accepted endogenous reference miRNA and standardized pre-analytical controls would be necessary.

Author Contribution

Jawaria Shaheen: data curation, investigation, methodology, formal analysis, writing - original draft. **Safia Firdous:** resources, software, methodology, visualization. **Khulod Ibraheem Hassan:** visualization, writing-review & editing. **Saima Sadaf:** conceptualization, funding acquisition, project administration, supervision.

Conflict of Interest

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement

Data supporting the findings of this study will be made available by the corresponding author upon request.

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Generative AI Disclosure Statement

The authors did not use any type of generative artificial intelligence software for this research.

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