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Original Research

Circulatory miRNA-155, miRNA-21 target PTEN expression and activity as a factor in breast cancer development

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Abstract: Breast cancer is a complex disease with multiple factors involved in its pathophysiological development. genetic mutations of BRCA1, BRCA2 and p53 are among the most well-studied factors. The role of other genetic factors like altered expression profiles, SNPs in the regulatory regions of different genes or epigenetic factors like promoter methylation and histone modifications are also well studied but no solid understanding is available on distinct key players triggering malignancy in breast cancer, (Phosphatase and tensin homolog) PTEN is known to be a crucial tumor suppressor as it has been reported to be missing or abnormally expressed in many cancer cells. Here in this were studied how PTEN is expressed in malignant and benign cancer cells by investigating its expression profile and cellular location using Immuno-fluorescence microscopy. At the same time, quantitative studies of the circulatory mi-RNAs related to the downregulation of PTEN, namely mir-21 and mir-155 have studied also. Sixty biopsy samples, forty were diagnosed to be malignant and twenty were benign. It has been found that PTEN is normally expressed in benign samples and its normally localized in the cell membrane, while in malignant samples the expression level of PTEN is lower or absent and it is translocated to the cytoplasm. Interestingly the quantitative expression of circulatory mir-21 and mir-155 in the blood plasma of the corresponding patients showed a related pattern with higher expression in malignant samples, therefore can it's clear that PTEN is in the cross-talk of genetics and epigenetic regulation in regard of the development of malignant breast cancer. At the same time, this study confirms the importance of circulatory miRNAs as a biomarker for early breast cancer detection.

Key words: Breast cancer; Immuno-fluorescence assay; RNA Expression; PTEN; mir-21 and mir-155.

Introduction

Breast cancer is the second most popular malignancy after lung cancer in females (1), it is the leading cause of death among women worldwide, especially in developed countries which also occurs rarely among men in only about 1% (55). According to the WHO website, Breast cancer is the most frequent cancer among women, about 2.1 million women are diagnosed each year that causes the greatest number of cancer-related deaths among women. In 2018 about 627000 women died from breast cancer "approximately 15% of all cancer deaths among women". In addition, in 2020 an estimated 276,480 new cases of invasive breast cancer are expected to be diagnosed in women in the U.S.A, along with 48,530 new cases of non-invasive (in situ) breast cancer (2).

Cancer could be identified as cell proliferation which is abnormal and uncontrolled. Cancer cells often spread out to the neighboring tissues or metastasize through both the blood or lymphatic system to the distant organ (3). Cancer cells can occur in many cells in the body (4). Genetic mutations that arise in pro-oncogenic genes such as RAS (5) and MYC (6) or tumor suppressor genes such as BRCA1, BRCA2 and TP53 (7) are most well studied in breast cancer (8).

The cancerous cells appear from glandular milk duct or breast lobular epithelial cells, which may form tumors and metastasize to other tissue in later stages if not treated soon (9). Depending upon whether or not the tumor has begun growing outside the basal membrane, breast carcinoma is either non-invasive (in situ carcinoma) or invasive, (10)

PTEN was the first phosphatase reported as a tumor suppressor (11). It encodes 403 amino acids of 55 kDa protein phosphatase (EC 3.1.3.67) (12). which is part of the large family of tyrosine phosphatases. PTEN is mainly localized to the plasma membrane (13) which downregulates the PI3K pathway by dephosphorylating PIP3 to PIP2. Loss of PTEN function leads to excessive PIP3 accumulation at the plasma membrane and subsequent derepression of the PI3K/AKT pathway, which in turn stimulates cell growth, proliferation, survival, and other cellular processes (14).

A large variety of human tumors are concerned with mutations or declining expression of the PTEN gene (15) Cowden syndrome (CS), which is distinguished by an elevated breast cancer risk, is found to be a germline mutation in PTEN (16). Although the incidence and effect of PTEN-alterations in breast cancer have not been thoroughly explained in the previous studies, deletions or reduced expression of PTEN have been identified in

4% to 63% of cases of breast cancer (17, 18). Some studies suggest a correlation between the inactivation of PTEN and poor prognosis in breast cancer patients (19), While in other findings this could not be proven (20).

Recently, a new family of RNAs defined as microRNAs (miRNAs) extended the classical definitions of oncogenes and tumor suppression genes. MiRNAs are a class non-coding single-stranded endogenous 22–24 nucleotides RNA molecules that post-transcriptionally regulate gene expressions. miRNAs could well affect various cellular processes such as proliferation, apoptosis, differentiation, angiogenesis, and development (21). They degrade their target mRNAs by identifying incomplete complementary sites, generally located throughout the target mRNAs ' 3 untranslated regions (3UTR), enabling miRNAs to regulate numerous biological functions. Gain or loss in the function of specific miRNAs could potentially lead to tumorigenesis and the development of cancer (22). The human genome involves > 700 miRNAs (23) and the tissue-specific expression of miRNAs (24). Every miRNA actively or passively targets about 200 transcripts (25). miRNA-expression deviant patterns were identified in human breast cancer (26). In silico study, several genetic variations in the development of breast cancer have been identified as aberrant targets of miRNAs in breast cancer (27).

PTEN is known to be targeted by some miRNAs for down-regulation. Both Mir-21 (28) and Mir-155 (29) are reported to be specific for PTEN targeting and their oncogenic roles are also reported. In this study, we investigated the abundance of the circulatory miR-21 and miR-155 in patients diagnosed with either benign or malignant breast cancer in parallel to the study of PTEN expression and its cellular location in surgically removed tissues from correspondent patients. based on the results, we tried to establish an understating of how PTEN expression is presented in malignant and benign breast tumors and how this may be related to the presence of miR-21 and miR-155 in patients' blood samples.

Therefore, this study aims to investigate the expression pattern of miR-21 and miR-155 and its target PTEN in breast cancer females.

Materials and Methods

Sample collection of molecular study

This study was performed at both Hiwa Hospital and the Department of Pathology in Shorsh General Hospital)- Sulaymaniyah- Iraq. 40 blocks (FFPE) (Formalin-Fixed Paraffin-Embedded**)** tissue of breast specimen diagnosed as breast cancer were collected from the archives of D. P. Shorsh G. H.) and 20 blocks diagnosed as benign were collected from the private laboratories in Erbil – Iraq, The samples are processed between November 2016 to April 2017 (The research was done within this time frame). The research work was performed in the Shorsh General Hospital laboratory. Blood samples were also taken from the corresponding patients (pre-chemotherapy) then sera were prepared and frozen at -80C°. So, Samples of patients are divided by breast cancer types and patient clinical characteristics, which include average age and grade.

All participants were given informed consent, and the study was accepted by the Local Ethics Committee. Approval number: 21Aug.2016/ 7299.

RNA extraction

The sera samples we used to isolate RNA using an extraction kit (Bioneer, ExiPrepTM total RNA kit, Korea) as instructed by the manufacture. Bio photometer (Eppendorf, Germany) used to quantify and qualify RNA concentration and its quality. Sample with (A260-A320)\ (A280-A320) ratio less than 1.7 and\or yields less than 0.5 µg total RNA were excluded from subsequent analysis.

Complementary DNA synthesis

Ipsogen RT Kit (Qiagen, GmbH, Hilden, Germany) was also used to turn isolated mRNA to evaluate the threshold of PTEN gene expression. In addition, the miScript II RT Kit (Qiagen GmbH, Hilden, Germany) was provided to collect isolated cDNA of non-coding RNA for mir-21 and miR-155 expression analysis. In thermal cycling processes, Master-cycler pro PCR System (Eppendorf, German) was used to produce cDNA. Also, 70% (v / v) ethanol was used to clean the workspace, and filter tips were used in all steps. Components of the Thaw system were placed on ice. To investigate the DNA interference in the samples, a control reaction with no reverse transcriptase is suggested. Different amounts of total RNA were used for any sample as the quality and quantity of total RNA were not equal.

Primer design

Pair of mRNA sequence primers were designed for PTEN / Exp. Online primer design program http://workbench.sdsc.edu was employed. Table 1 shows the sequence of the primers, the annealing temperature and the length of PCR products. For miRNA 21and miRNA 155 miScript Primer Assay was used (Qiagen, Germany).

The primers for gene expression should be to know how the gene is expressed anywhere in the coding regions. Due to the fact which the critical thing could be to code RNA product, the designed primers involved one or two exon-exon junction regions to prevent raising the number of copies of any product other than from the RNA product involved. Then maybe we couldn't sure if the product from which the number of copies rose was due to DNA or any other interference.

RT-PCR components

Real-Time PCR's response was based on the application of the IQ5 RT-qPCR (Biorad, USA) instrument.

Table 1. The primer used for amplification of PTEN region.

Gene name	Primer sequence	Optimal annealing temperature	PCR product Size
PTEN	F. Primer AACCTATGGGAAGCCAACGA R. Primer GGAGACAGTATGTCGTCGCA	56.1 °C	315 bp

Table 2. Components of Real-Time PCR reaction mix applied to measure PTEN expression profiling.

ID	Component	Quantity (ul)
1	RT² SYBR Green ROX FAST Mastermix	10.125 ul
2	Fw Primer (10 µM)	1 ul
3	Rv Primer (10 µM)	1 ul
4	RNAse/DNAse free water	14.875 ul
5	cDNA (50 ng)	3 ul
6	**Total**	30 ul

Table 3. Settings of Real-Time PCR to measure PTEN expression level.

ID	Step	Temperature (°C)	Time (sec)	Cycle
1	Enzyme activation	95	10 min	
2	Denaturation	95	15 Sec	
3	Primer annealing	56.1	30 Sec	
4	Extension	72	30 Sec	40

RT2 SYBR Green ROX FAST Mastermix (Qiagen GmbH, Hilden, Germany) was used as the master mix for the expression PTEN, MiRNA 21, and miRNA 155. According to Table 2, the Real-Time PCR master mix was prepared on ice. Eventually, it was blended and placed on ice. The Real-Time PCR master mix would include necessary components to evaluate the expression except for mRNA cDNA.

cDNA was added to each sample in tubes containing the Real-Time PCR master mix. Each tube was carefully mixed, briefly centrifuged and put back on ice. The saved program was run in the IQ5 RT-qPCR instrument (Biorad, USA) (Table 3).

Sample collection & preparation of immunohistochemistry study

A retrospective study was performed on 60 paraffin-embedded tissue blocks of 60 cases. These constituted of 40 blocks of breast specimen diagnosed as breast cancer, 20 blocks of benign tissues of the breast as a positive control. 20 of these samples were collected from the private laboratories in Erbil. And 40 of these samples were collected from the archives of the Department of Pathology in Shorsh General Hospital. Samples were collected from specimens processed between November 2016 to April 2017. The research work was performed in the Shorsh General Hospital laboratory, starting on the 5th of April and ended on the 2nd of December 2017. Information about the age, gender, staging and grading were obtained from the database available in the already mentioned Two sources from which the paraffin blocks were collected.

Two sections of 4 µm thickness were taken from each paraffin-embedded tissue block. The first section was placed on an ordinary slide for hematoxylin and Eosin stain while the second section was mounted on a positively charged slide then processed for immunohistochemical staining following the protocol that was supplied with the kit by DAKO.

Pre immunohistochemical staining steps

Slides were baked overnight in an oven at 56°C, Sections were deparaffinized and rehydrated to water in descending concentrations of ethanol: - Xylene for 5-10 minutes, absolute ethanol for 5 minutes, 90% of ethanol for 5 minutes and 70% ethanol for 5 minutes. The slides were washed in running tap water for 10 minutes. The slides were washed in Tris-buffer saline PH 7.0 and then placed in 2 changes of the same buffer, 5 minutes each. The retrieval solution (in coplin jar) was heated in a pressure cooker with an open lid until the temperature reached 95-98°C. The lid of the pressure cooker was closed and slides were left inside at 120 °C for 9 minutes, also the coplin jar with slides were removed from the pressure cooker and allowed to cool down for 20 minutes in running tap water at room temperature and the slides were washed twice with wash buffer before proceeding to step II. If step II was to be performed the next day, the slides are left in wash buffer overnight.

Immunohistochemistry Staining Steps

The slides were tapped off and the area around the specimen was wiped to remove any remaining liquid and the section was encircled with a Pap pen.

H&E (Hematoxylin and Eosin) stained slides interpretation

Staining of tissue sections mounted on ordinary slides was performed by conventional hematoxylin and eosin stain (H&E). The H&E stained slides were re-examined for further confirmation of the diagnoses and grading of malignant tissue samples.

Immunostained slides interpretation

We searched for any positively immunostained cells in all the sections with special emphasis on the intensity of the staining reaction and the locale of the staining (cytoplasmic and/or nuclear). We followed a scoring system for staining intensity that was adopted by Steven C. Cunningham et al. [225] According to the above, the intensity scoring is as follows: Negative scoring (score 0): No staining, score (1+): Moderate staining and score (2+): Intense staining.

Immunofluorescence staining of PTEN (IF)

For this purpose, 3 – 4 µm thick tissue sections were made from FFPE blocks using a microtome. The sections were placed onto charged histological slides, sections were deparaffinized and rehydrated in water in descending concentrations of

ethanol from 100 to 70% ethanol then the slides are rinsed with deionized H2O and let rehydrate the slides with wash buffer for 10 minutes. The sections were surrounded by a hydrophobic barrier using a barrier pen for the next treatments. The samples were Permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature, then washed twice with 1X PBS for 5 minutes each. The sections then blocked with 1X BSA solution for 30 minutes applying gentle shaking to block unspecific binding of the antibodies. After washing the blocking buffer with 1X PBS the sections were incubated in the diluted primary antibody (FITC labeled anti-PTEN antibody for 1 hour at room temperature. The unbound antibodies were washed with PBST (1X PBS + 0.1% Tween) three times, each time 5 minutes with gentle shaking. To mount the slides, mounting medium added to the sections and coverslips were carefully put on top of the sections on the slides. To prevent drying and movement the coverslips were sealed. The slides store in dark at 4 C until investigation with a fluorescent microscope using 488m filter (green filter) to visualize FITC labeled anti-Pten antibody.

Statistical analysis

The data obtained from our observation were analyzed, ANOVA, Student t-test, and test Chi-Square using SPSS software.

Results and Discussion

PTEN is normally expressed in breast tissue especially in the location where sub-segmental ducts connect terminal ducts to the segmental ducts. Here we show that benign tumors exhibit normal PTEN expression in segmental and sub-segmental ducts (Figure 1a), located mostly on the membrane cytoskeletal of the cells, which is considered to be normal for PTEN localization site in normal cells (17). The mechanism of PTEN recruitment to the membrane is still the matter of debate, non the less C2-helix of the C2-domain of PTEN is reported to be necessary for the membrane localization via stable electrostatic interactions (30). In addition, other investigations showed that phosphatase catalytic site, the calcium-binding region 3 (CBR3) loop, the C2-loop and the C-terminal tail phosphorylation site are all important for PTEN recruitment to the cell membrane (31). The Inner luminal epithelial layer and an Outer myoepithelial contractile layer of the ducts (Figure 1b) are clearly active in the expression of PTEN, while the surrounding stromal cells significantly produce lower or no PTEN. Therefore, PTEN seems to be crucial to keep the integrity of segmental and sub-segmental ducts, as it may be involved in the regulation of the duct's formation in normal breast and also in the benign tumors. So, fare, very little is known about benign tumors in regard of PTEN expression and function. In this work through numerous immunofluorescent staining of benign tumors, we can clearly see that PTEN is not involved in the formation of such tumors. Unfortunately, and because that this study was done on human samples, we could not get normal breast tissues to see differences between a benign tumor and healthy tissue, but resources like Human Protein atlas show that PTEN expresses in breast tissue within the normal average range. in addition, studies mainly done in mice show PTEN is crucial for normal development and physiology of different tissues (32).

On the other hand, in the majority of malignant samples we stained with anti-PTEN antibody, we cannot see regular segmental and sub-segmental ducts (Figure 2) with PTEN being partially or totally lost or even differentially expressed among different malignant tumor samples. Most of the malignant samples show no PTEN expression (Figure 2a), few samples show slight expression in the cells of the stromal tissue (Figure 2b), which is not the case in benign tumors and within the samples where PTEN is expressed, the cellular localization of PTEN also varies in between cytoskeletal location and cytoplasmic locations (Figure 2c and d). As mentioned above, binding to the plasma membrane is a critical regulatory step for PTEN function which is antagonizing the PI3K signaling pathway. Although PTEN is also shown to localize in cytoplasm and nucleus too (8), cytoplasmic PTEN reported to down-regulates phosphorylation of Akt and up-regulates p27kip1, whereas nuclear PTEN down-regulates cyclin D1 and prevents the phosphorylation of MAPK (33). In agreement with the loose of PTEN in malignant samples and metastasis; The effects of PTEN on the adhesion, migration and invasion of cells were reported (11). The loss of PTEN expression is relatively frequent and is associated signi-

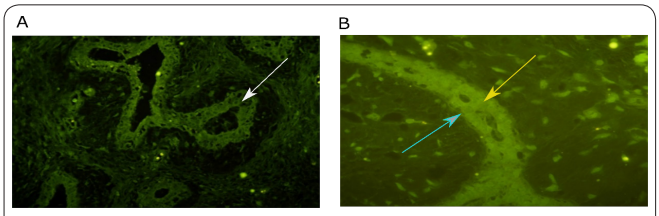

Figure 1. Immunohistofluorescence staining of paraffin-embedded benign diagnosed breast tumor isolated from patients. Showing sub-segmental duct (A: 40x magnification – White arrow head) and Inner luminal epithelial layer (blue arrow head) and an Outer myoepithelial contractile layer of a given duct (yellow arrowhead) (B: 60x magnification). The sections are stained with mouse primary antibody Fitc labeled anti-PTEN antibody.

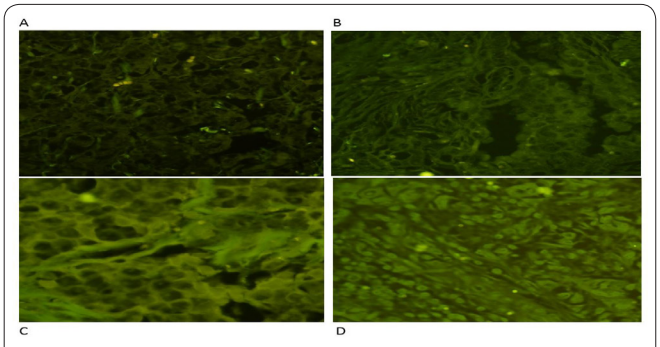

Figure 2. Immunohistofluorescence staining of paraffin-embedded malignant diagnoses malignant breast tumor samples isolated from patients. No regular segmental and sub-segmental ducts can be recognized in all samples A. No PTEN expression B, slight expression in the cells of the stromal tissue C. PTEN is localized to the cytoskeleton compartment and D. PTEN is expressed in cytoplasmic locations. The sections are stained with mouse primary antibody Fitc labeled anti-PTEN antibody.

ficantly with metastasis (34). This influence of PTEN may be partly due to the crucial role of PTEN in actin cytoskeleton regulation (35). On the molecular level, ROCK phosphorylates and activates PTEN which intern inactivates Akt, within the Akt family AKt3 seems to be tightly involved in cancer development and induction of metastasis (36).

Inactivation of the PI3K/AKT pathway with PTEN overexpression, results in inhibited cancer cell motility, at the same time inhibition of the PI3K pathway by treatment with wortmannin (Akt inhibitor) markedly suppresses experimental metastasis in mice (37). The main physiological substrate of PTEN is membrane-bound PIP3, and PTEN is activated when recruited to the plasma membrane. At this location, PTEN converted the lipid secondary messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to PIP2, as a negative regulator of phosphoinositide 3-kinase (PI3K) signaling. Thus the balance between PTEN and PI3K controls the basal levels of PIP3 in the plasma membrane, which intern regulates cell survival and proliferation.

The fact that miRNAs are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) means such types of RNA are relatively stable when they secreted out to the blood circulation. miRNAs are shown to be stable at room temperature for one hour after blood collection, but if the samples were frozen at -80C and frequent freeze-though cycles were avoided, the stability of this type RNAs are enormously extended as long as few years (38). This biophysical characteristics of miRNAs allow to use them as a novel class of blood-based cancer biomarkers, although this field has been extensively studied with various approaches like Next Generation Sequencing (NGS) and miRNA microarrays to fins unique miRNAs in circulation to be used to detect specific cancer types with confident precision. Unfortunately, this is yet to be established as a diagnostic tool, but in academic research, this field is growing very fast to make such an approach reliably possible. Many challenges exist to establish this approach including technical constraint for the isolation and purification of samples, the source of the sample, the method which is used to measure miRNAs and normalization of the data with reliable internal miRNA controls (39). Each of these factors is counted to the challenges exposed to get final results that can be translated into trustworthy readouts in any experiments. As far as is this a growing field, each of these restrictions is becoming narrower each day with extra knowledge published and experiences collected.

miR-21 is known to target PTEN and cause its down-regulation (28), but most of the studies are done intracellularly, meaning within the same cell, where the cell produces miRNA and targets its transcript. The oncomeric effect (cancer-associated miRNAs) of mir-21 is well studied in colorectal cancer (39), prostate cancer (40), Pancreatic cancer (41) and Breast cancer (6) and others to show that when PTEN is lost or low expressed, miR-21 is to be associated with it. Even though PTEN is not the only known target of mir-21 but it stays one of the most prominent targets of mir-21. miR-21 has been found to suppressed cell proliferation and metastasis in breast cancer patients with the level of this miRNA increased in plasma of the patients, interestingly the level

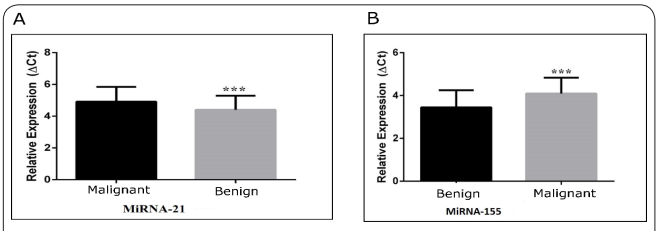

Figure 3. Circulatory miRNA level is known to target PTEN expression in patients with malignant and benign tumors. A. miRNA-21 (p=0,001). The mean average of benign samples = 4.918 and malignant = 4.403.and **B.** miRNA-155 level measured with qRT-PCR. (p=0,0001). The mean average of benign samples = 3.448 and malignant = 4.095.

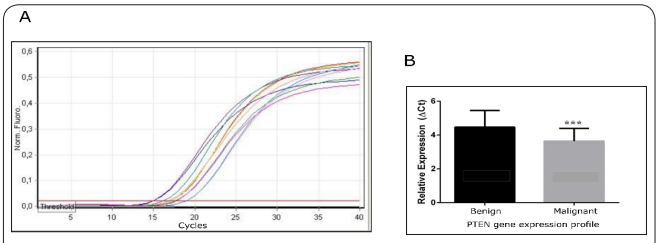

Figure 4. PTEN expression profile in breast cancer patients. A. qRT-PCR analysis. The highest curve indicated between 20th and 26th cycles in Samples. **B.** statically analyzed mRNA abundance of PTEN amplified with q-RT-PCR of the 40 malignant and 20 benign sera samples collected from patients. (p = 0.0001, T-test; p > 0,05). The mean average of control samples = 4.473 and tumor = 3.645.

of the miRNA is decreased after surgical removal of the tumors, which indicated that the source of the miRNA is the tumor itself.

We show that mir-21 upregulation in the plasma samples collected from malignant breast cancer patients (Figure 3a) is linearly correlated with the down-regulation of PTEN in the same samples (Figure 3b) indicating a direct link between mir-21 and PTEN as its target molecule. The same observation is true for mir-155 which we studied (Figure 3b). The higher expression profile of mir-155 is in relative relation with PTEN down-regulation, which highlights the negative effect of miR155 on PTEN expression. miR-155 is well characterized, conserved and multifunctional miRNA which is mainly characterized by overexpression in multiple diseases including malignant tumors (7). This sort of miRNA is also reported to be multifunctional with its involvement in various physiological and pathological processes such as hematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases and DNA viruses infections (42). Mir-155 is shown to target PTEN (43), which seems to promote hepatocellular carcinoma progression via suppressing PTEN. In a recent study, miR-9 and miR-155 were identified to be overexpressed in metastatic triple-negative breast cancer cells which they also showed that these two miRNAs target PTEN and DUSP14 tumor suppressor genes (43, 44).

In this thesis, we showed a pattern between PTEN lost or low expression in malignant breast tumors correlated with the circulatory mir-21 and mir-155 higher expression in corresponding patient's serum samples. This observation cannot be found in benign tumors in which PTEN displays normal expression pattern and the studied miRNAs are lower compared to the malignant samples. Our study also showed that malignant tumors

have normal tubular ducts which PTEN seems to be part of keeping the integrity of their structure as no obvious expression of PTEN to be seen in the surrounding stromal cells. The malignant tumors in the other hand have no regular histological forms with PTEN been lost or low expressed in irregular cells which seems to be stromal cells. Our study highlights the importance of PTEN as a crucial histological marker that can make a clear difference between benign and malignant tumors. At the same time elevated levels of mir-21 and mir-155 could be the causing factor of PTEN down-regulation and promising circulatory miRNAs to be used to identify malignancy in breast tumors. The genetic and epigenetic regulation of PTEN in breast cancer highlights the impotence of this protein for further investigation and possible therapeutic approaches.

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