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Evaluation of the microRNA-328 gene expression in some Iraqi breast cancer women

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Abstract:

Breast cancer remains one of worst threats to women's health, and so far there are no proven and appropriate methods for early diagnosis of breast cancer, Its count about 25% of the cancer rate in Iraq.

The aim of this study was to identify the expression of microRNA-328 molecules as a biomarker for early detection of breast tumors and detect its relation with clinicopathological characters of patients.

Fifty fresh tissue samples were collected from breast patients and 10 samples of normal tissue were collected as a control group, the age rang was 77 to 19 years for patients with benign and malignant breast tumors. Total RNA, including micro miRNAs, were extracted from tissue samples and converted to complementary DNA. The level of microRNA-328 gene expression was measured by the quantitative real time reaction technique (qRT-PCR).

The results showed that there were a significant differences in the expression of microRNA328 in benign and tumor breast patients compared with control group depending on tumor site (right) *P*=0.051. Significant differences in expression of miR-328 were found between malignant patients and control group (*P*=0.033) in age less than 50 years patients. Increase of gene expression miR-328 was associated with carcinomas Lobular patients (*P*=0.014) compared to control group but there were no significant differences depending on the tumor grade.

In conclusion: increase of miR-328 gene expression level in benign lesion and breast cancer can be used as a biomarker for early breast cancer detection.

Key words: Iraq, miR-328, Breast cancer, Gene expression, Benign.

INTRODUCTION:

Breast cancer is one of the most common diagnosed cancer in women and represent approximately 41% of all female cancers (Sturgeon *et al.*, 2018).Breast cancers are genetically heterogeneous, its detection and patients clinical outcomes are widely varied, the diagnostic methods and biomarkers that used for breast cancer early detection in routine work may be limited (Imani *et al.*, 2017). Genetic factors are considered the most important causes of breast cancer, the incidence of breast cancer due to the genetic factor is 80% (Siu, 2016). There is a necessity demand for investigate novel biomarker that may help in early detection of breast tumor as the early detection can help for long survival of breast cancer patients.

microRNAs possess most typical characteristics of biomarker, like analytical criteria and clinical advantage or benefit .It can be specific to the interest pathology, a reliable mark of the disease even before clinical symptoms can appear moreover sensitive to pathological changes or physiological (Detassis *et al.*, 2017).

Increasing attention in recent years, has been observed to the biological functions and pathological effects of miRNAs that involved in the regulation of many cellular processes, including development, cell proliferation , differentiation, motility, apoptosis, and cell cycle, which are all lead to carcinogenesis and cancer progression if there dysregulation in miRNAs expression (Ibrahim *et al.*, 2014,Hata and Lieberman, 2015).It's have important role are regulating expression of target gene by binding with its complementary nucleotides in the third end or fifth end of Un translated regions (3′ or 5′ UTRs) of the mRNAs target results in inhibiting translation or enhanced mRNA degradation (Bartel, 2004).

Several studies have been conducted to identify the miRNAs expression profile that are regulate the development of breast cancer and progress in various subtypes of breast cancer (Iorio *et al.*, 2005). Damvandi and his colleagues (2016) showed that gene expression was significantly reduced for miR-212 and miR-132 while increase for miR-22 in breast tumor samples, other miRNA molecules (miR-106b and miR-93) were both target single common target (phosphatase and tensin homolog (PTEN), that cause PI3K/AKT pathway activation, coding to promote tumor growth, proliferation, cellular migration, invasion in laboratory experiments (Li *et al.*, 2017).

Bishop *et al.*, investigate expression of the miR-328 as biomarker in cancer research, they found that high expression of miR-328 induce carcinogenesis and metastasis leading to secondary brain tumors in lung adenocarcinoma patients (Bishop *et al.*, 2010).Others reported that a new mechanism based on the miR-328 to induce central nervous system tumor (Gliomas) by effecting the inhibitory protein (Secrete frizzled-related protein1 SFRP1) which is a negative antibody to the Wnt pathway result in inactivation of this pathway(Paduano *et al*, 2012).

The gene position of miRNA-328 molecules is 16q22.1,which encodes a small non-coding molecule of noncoding RNA (ncRNA) ranging from 20-24 nucleotides.Deregulation of miRNA328 molecules can serve as tumor suppressor genes or oncogenes in many human cancers Delic *et al.*, (2014).

The present study was designed to investigate the miRNA328 expression profiles in benign and malignant breast patients and its relation with clinicopathological character as biomarkers for early detection of breast cancer.

MATERIALS AND METHODS

Patient tissue specimens:

The samples were obtained from breast tumor patients who underwent the Medicine city Hospital in Baghdad and private laboratories from October 2017 to April 2018, the patients who received chemotherapy or radiotherapy were excluded. Fifty tissue samples (including 25 benign breast disease and 25 breast cancer samples, in addition 10 samples from normal tissue were used as control group) were collected and stored in deep freeze until RNA extraction. The research was approved by the Ethics Committee from Ministry of Health in Iraq and Baghdad University.

The miR328 Expression Analyses:

Total RNA was extracted from tissue samples using (miRNeasy Mini Kit (QIAgen, Germany)(Cat No. /ID: 217004) according to the manufacturer's procedure .The miScript II RT kit and HiSpec buffer (Qiagen Germany) (Cat No. /ID: 218161), were used to syntheses the complement DNA (cDNA), total volume of reaction was 10 µl including (2µl 5x miScript HiSpec Buffer , 1µl of 10x miScript Nucleics Mix , 4µl of RNase-free water RNase, 1µl of miScript Reverse Transcriptase Mix and 2µl of Template).The cDNA was stored in a -20°C freezer.

The master mix miScript SYBR Green PCR (Qiagen) kit(Cat No. /ID: 218073), was used to detect expression level of miR-328 genes by qRT-PCR technique. The U6 small nuclear (sn) RNA (as an internal control) .The primers that used for qRT-PCR reactions as described by Saberi *et al*., (2016) (Table 1) . All primers were purchased from (Alpha DNA Company, Canada) .Total volume of qRT-PCR reaction was 10 µl contained 5ul master mix ,1 µl for each fowerd and revers primers , 2 µl RNase free water and 1 µl of cDNa template.

Table 1**:** Sequence-specific primers of qRT-PCR reactions

Molecule	Squences
MicRNA-328 (Forward)	5'-GCTGGCCCTCTCTGCCC-3'
MicRNA-328 (Reverse)	5'-CTCGCTTCGGCAGCACA-3'
U6snRNA (Forward)	5'-CTCGCTTCGGCAGCACA-3'
U6snRNA (Reverse)	5'-AACGCTTCACGAATTTGCGT-3'

The reaction solutions were incubated at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds for denaturation then 20 seconds at 55°C for annealing ,finally 30 seconds at 72°C. Threshold cycle (Ct) is described as the cycle number at which the fluorescence level will pass into assumed threshold. To analysis miR-328 expression , the fold-change of gene expression between cancer tissues and normal breast tissue control was calculated by the $2^{-\Delta\Delta}$Ct method, in which ΔΔCt = (CtmiR-328 - CtU6 snRNA) mean tumor-(CtmiR-328 – CtU6 snRNA) mean normal .The relative expression levels of miRNAs in cancer compared to normal tissue (control group) were calculated using the method of $2^{-\Delta\Delta}$Ct (Livak and Schmittgen , 2001).

Statistical analysis:

Differences in mean between miR-328 expression in the sample of breast (benign and tumor) and normal tissue as well as clinical parameters were analyzed using $\chi 2$ and Student's t-test. The results were presented in an average ± standard error. All tests were performed using SPSS 16 and a $P<0.05$was suppose to be statistically significant

RESULTS:

The study group consist of 50 patients including 25 breast begnin tumor patients with an average age of 34 years range (19-57) years , 25 breast cancer patients with an average age of 50 years, range (35-77) years in addition to 10 normal samples with 38 years age mean. No significant difference was identified between the groups regarding age (P=0.549).

The study was conducted to investigate the miR328 expression levels in breast benign and malignant specimens that was compared with normal breast tissues, quantitative real-time analysis was conducted . (Figure 1, A) indicate to narrow peak for melting-curves of miR-328 referred to that homogeneous PCR and pure products were produced, (Figure 1,B) represented cycling curves of qRT-PCR technique.

Patients clinical characteristics:

Clinical characterist of breast cancer patients showed in (Table 2),depend on age there were 52% of female less than fifty years patients were participate in this study 80% (20/25%) were with Invasive.

According to tumor grade 36%, and 28% were grade 1 and grade 2 respectively while 44% of female patient were with Moderately differentiated . No significant difference was identified between the groups regarding age (P=0.549).

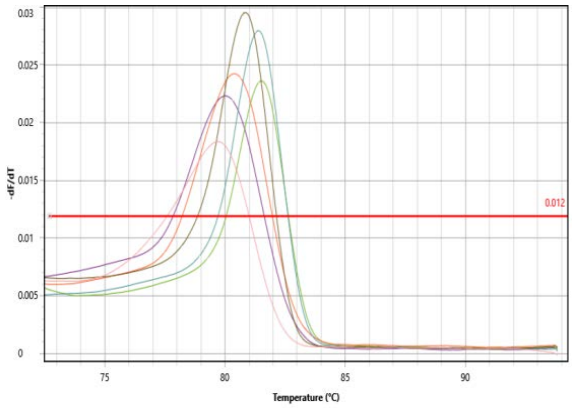
Expression level of miR-328 in breast benign disease:

The PCR products were pure and homogeneous as Indicating by melting curves of miR-328,they are showed as sharply defined melting curves with narrow peaks (Figure 1: A, B). Expression of miR-328 in 25 breast benign patients were detected, the result showed increase of fold expression of miR-328 in those patients. The mean of fold expression was 6.9 compared with 1.6 mean for normal tissue with standard error (SE 1.6±0.77) no significance differences were found depending on age (p=0.605) in breast lesion .

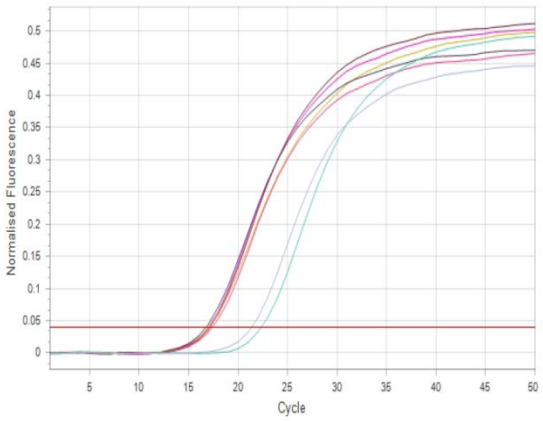
There were high expression level of miR-328 in right site, the mean of fold expression for (8/25) of patients were 9.923 (SE 9.923±3.798) while the expression of miR-328 in left site breast benign patients was 3.129 (SE 3.129± 1.267) in (13/25) of patients, the results indicate to significant differences in right breast benign comparable with fold expression level of miR-328 in normal tissues (p=0.051), also there were significant differences between right and left site of breast benign tumor compared to control group (P=0.30) (Figure 2:A,B).

Table 2: Clinical characteristics of patients with breast malignances

Characteristics of patients with cancer	Number	Percentage
Total number of patients	25	100
Average age 50		
<50	13	52
> 50	12	48
sex		
Male	0	0
Female	25	100
Type of tumor		
Lobular carcinomas	2	8
Ductal carcinomas	3	12
Invasive carcinomas	20	80
Grade		
Grade I	12	48
Grade II	9	36
Grade III	4	8
Differentiation		
Moderately	21	84
Well	4	16
Poorly	0	0
Tumor site		
Left	10	40
Right	10	40
Un known	5	20

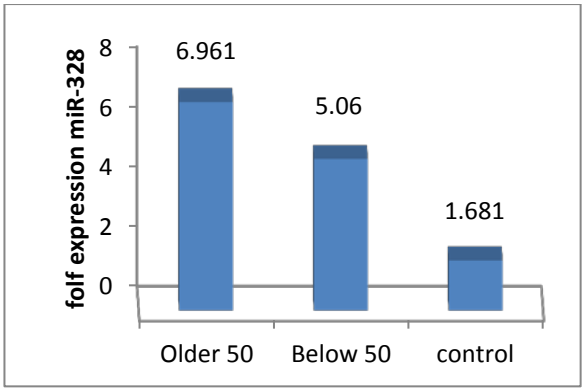


A

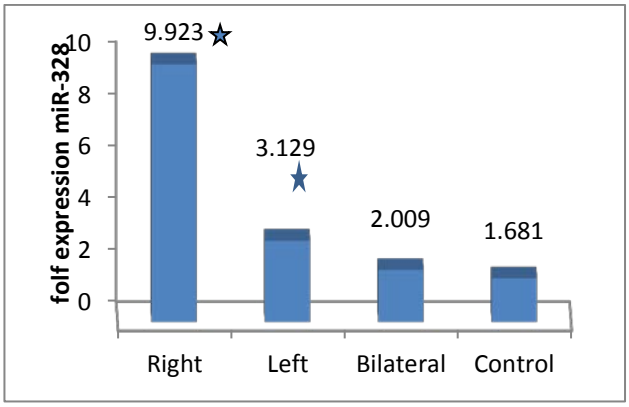


B

Figure 1: A: Melting Curves, B: Cycling Curves of miR-328.



A



B

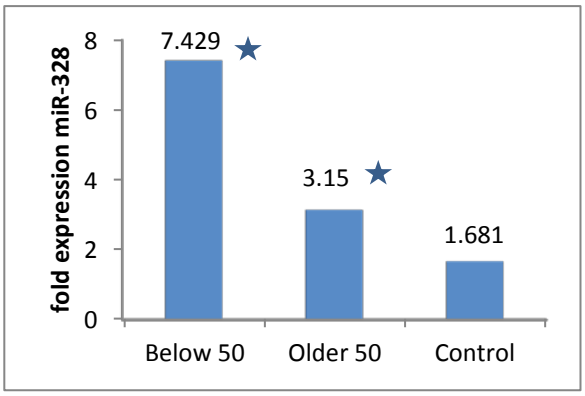
Figure 2: Gene expression of miR-328 in benign tumors A: Depending on age. B: Depending on benign breast disease location(*). Significant difference ($p \leq 0.005$).

Expression level of miR-328 in breast cancer:

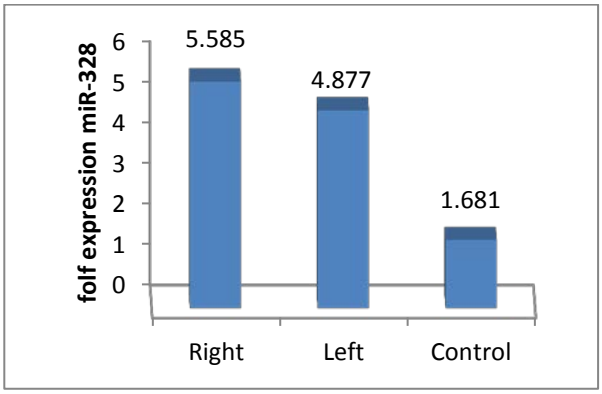
The miRNA-328expression level was significantly elevated in breast cancer tissue compared with normal tissue. The mean level of miR-328 expression in tumor patients was seven-folds higher than the mean level of the control group depend on ages less than 50 years, it was **(** SE 7.429± 1.702) in 13/25 of breast cancer patients while the fold expression was (SE 3.15 ± 0.65) in 12/25 of patients older than 50 years compared to (1.68±0.779) for control group, significant associated depend on age P=0.033 and P=0.024 in younger and older ages respectively (Figure 3, A). Depend on tumor site the results referred to increase fold expression of miR-328 (5.58± 1.8) in 10/ 25 of breast cancer patients in right side with no statistically significantly relation (P = 0.5) (Figure 3, B).

Other tumor characteristics such as tumor type and tumor stage are summarized in (Figure 3,C and D) . High rate of breast cancer tissue 80% (20/25) were invasive carcinoma as tumor type that up regulated of miR-328 gene expression (SE 5.09 ±0.99) more than control group with positive significant association. Results were showed no significantly association was observed in miR-328 expression with regard to clinical stage, (P= 0.5) although there were high level of fold expression (5.35 and 5.61) in stage 1 and 2 respectively compared to control group , also there is a statistically significant relationship between miR-328 expression level and well differentiated breast

cancer patients , the expression was increase 10 fold (SE 10.18±2.99) compared to control group (SE 1.68± 0.77) .



A



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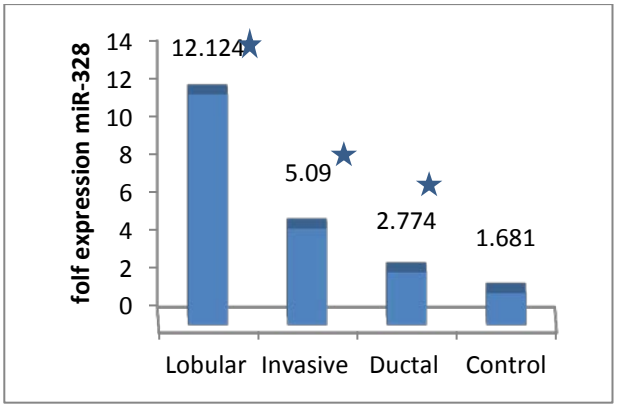

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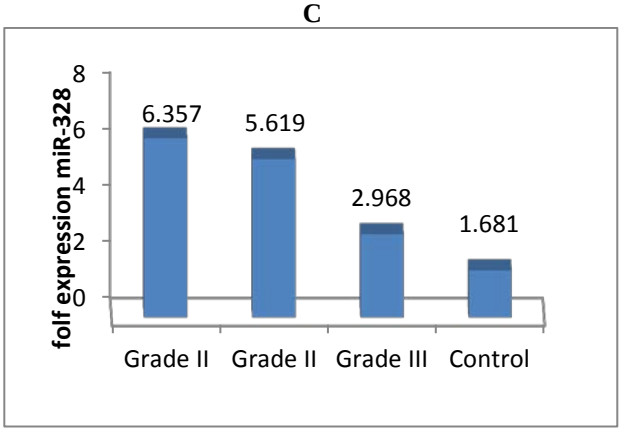

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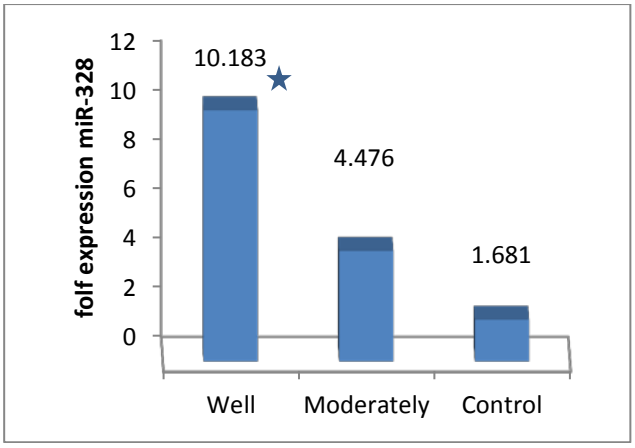

E

Figure 3: Gene expression of miR-328 in tumor patients:
A: Depending on age, (*) Significant differences.
B: Depending on tumor location.
C: Depending on type of tumor, (*).
D: Depending on Grade of tumor.
E: Depending on differentiation, (*).

Comparison between the miR-328 expressions in Breast benign disease and tumor breast tissues:

The miR-328 was up-regulated in 62 % (29/50) of benign and malignant breast tissues compared with normal tissue, with an average increase of 5.3 fold expression ($P = 0.05$) (Figure 4).

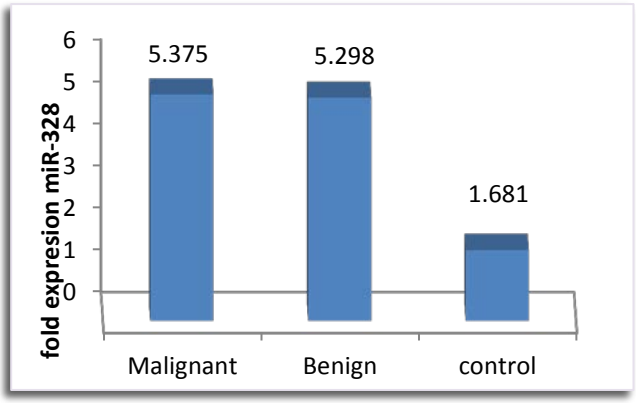

Figure 4: comparison of miR-328 gene expression between benign, malignant and normal tissues. .

DISCUSSION:

The two past decades have witnessed great advance on microRNA studies, MicroRNAs also showed high sensitivity and specificity in diagnosis of cancer, furthermore confirmed their potential function as biomarkers (Xiong, *et al.*,2017, Zhang, *et al.*, 2015). Consequently, investigation of new biomarkers with high sensitivity and specificity for early breast benign and malignant tumors detection is urgently needed. Several studies have published that miRNAs play important roles in the initiation, development and progression of breast cancer and many certain miRNAs can serve as potential

biomarkers diagnostic for this disease (Imani, *et al.*, 2017; Arabkheradmand, *et al*., 2016; Gao, *et al*.,2016).

A few studies have investigated the level of gene expression of the miR-328 molecule in benign and malignant tumors , especially in Iraq, as well as its relationship with the ciinical characteristics of patients as preliminary data that helps to determine the extent to which it can act as a biomarker of tumor detection and prognosis of patients. The aim of this study was to investigate any possible dysregulation in the miR-328 expression in both benign (lesion) and malignant breast tissues as the benign cells lead to high risk of breast carcinoma. The gene expression was detected using real-time RT-PCR technique , the results were analyzed with applying the ($2^{-\Delta\Delta}$ Ct eq.) represented fold expression.

The mean age of patients with benign breast was 34 years also 88% (22/25) of breast malignant patients were younger women less than fifty years , these results keep on with Iranian patients with breast cancer (Mollainezhad, *et al*.,2016) but different from other country like USA or China with high rate of mean ages in breast cancer patients Fan, *et al*.,(2014).The impact of war has a role in the exacerbation of the low mean age as well as other important factors, notably genetic factors, which accounts for (10%) of cases, moreover consumption of hormones (AL-Hasnawi , 2015).

Based on the our results, in all 25 benign tumor samples the fold expression level of miR-328 was up regulated six time compared to control group without significant association were observed depend on ages that may due to small number of patients who were participate in this study. High fold expression was found in benign samples with right site ,the results revealed there was a positive association depend on location of benign tumor ($P \leq 0.005$) compared with control group. The alteration of miR-328 gene expression in benign tumor patients give important sight to change of miRNA328 gene which cause change profile of miR-328 gene expression. We investigated the role of miR-328 in 25 breast cancer patients and its relation with clinicopathological characteristic and prognostic value of miR-328 expression pattern dysregulation in breast cancer. In current study, miR-328 up regulation was significantly related to age less than fifty years , tumor type (invasive carcinoma) and moderately differentiated .There was no significant differentiation depend on tumor location (right site) although high fold expression was observed when compared with normal breast tissues. These results indicated that upregulated of miR-328 can act as oncomiRNA in breast cancer, increased expression of miR-328 may be responsible for the development of benign tumor breast and progression of breast cancer.

Our work may be considered as the first research to investigate the miR-328 expression level depending SYBR-Green quantitative real- time RT-PCR technique in Iraqi breast benign and malignant patients . Rare studies that related the alteration of miR-328 pattern expression in breast cancer were found in an extensive literature review,Saberi with his colleagues reported that the expression level was increased in breast tumor tissues without statistically significant association between the miR-328 median expression level and prognostic factors (Saberi *et al*.,2016).

The miR-328 oncogenic or tumor suppressor role has been proved in other types of cancers . Study conducted by Arora *et al*., (2011) was aimed to detected the miR-328 expression in 13 non-small cell lung cancer (NCLC) patients, they found that miR-328 profile expression was significantly higher in NCLC with brain metastasis patients than those without brain metastasis .

Fifty five percent of the hepatocellular carcinoma patients were showed increasing miR-328 expression and significantly associated with cancer progression of those patients, its appear that miR-328 molecules may serve as an oncogene that induced the hepatocellular carcinoma cell invasion in vitro by increasing of cell migration (Luo *et al*., 2015). Other research was measured the miR328 expression in colorectal cancer suggesting a great role of the miR328 in maintaining cancer stem-like side population phenotype and may be benefit as potential target for effective therapy in colorectal cancer patients (Xu *et al.,* 2012).In contrast to this context, previous study suggested that miR-328 expression pattern was decreased with increasing malignancy stage
in gliomas (Malzkorn *et al*., 2010).

Delic *et al*.,(2013) were compared the miR-328 expression between low-grade and recurrent high-grade gliomas of individual patients, their experiment results showed decreasing of miR-328 in higher-grade gliomas but the increasing of miR-328 expression in lower-grade gliomas appears contribute to Wnt pathway activation and tumor cell infiltration at early stages of glioma progression.Tay with colleagues showed that treated macrophages neutrophils with ant-328 increased expression of the lysosomal enzyme, Cathepsin D significantly improved the macrophages clearance of infectious bacteria (Tay *et al*.,2015).These results may give proving data for that upregulation of miR328 in cancers may cause immune suppression which leading to progress malignancy.

The miR-328 direct effects have been discussed in a wide range of cancer cells. The miR-328 target in breast cancer cells is an efflux transporter protein responsible for cellular drug transfer called breast cancer resistance protein G member 2(BCRP/ABCG2) and influence the drug disposition (Pan *et al.,* 2009).In a context of cancer microenvironment, increased infiltrated macrophages in tumor stroma are found to suppress miR-328 expression level through reactive oxygen species (ROS) production, and then induced CD44 expression in gastric cancer cells for regulating tumor progression (Ishimoto *et al.,* 2015).

The CD44 marker represent other target of miR-328 which is a cell surface glycoprotein mediated a variety of normal activities of cell survival like cell proliferation, angiogenesis cell differentiation and tumor metastasis (Naor *et al*., 2002) It's also related to wound healing, inflammation and adhesion migration (Lin *et al.,* 2018). It was found that miR-328 regulates zonation morphogenesis glycoprotein by targeting CD44 expression in human cells. In the human epithelial carcinoma cell line A431, miR-328

was found overexpression lead to reduced cell adhesion, aggregation, and capillary formation by silencing of CD44 (Wang *et al*., 2008).

In conclusion: The present study suggested that miR-328 may serve as an onco-miRNA and its association with breast benign and malignant tumor stages can be beneficial as a diagnostic biomarker. More studies are needed with large number of patients to support the current results and evaluating new effective biomarker tool against breast cancer.

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