Clinical significance of serum miR-21, CA153 and CEA in breast cancer

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ABSTRACT

One of the essential regulators of carcinogenesis is MicroRNA-21 (miR-21). Yet little light has been reported on its effectiveness as a tumor marker as compared with the conventional ones. The aim of the present study was to compare the diagnostic value of established tumor markers in breast cancer (BC) such as carcinoembryonic antigen (CEA) and CA153 with circulating level of miR-21. The study included 89 BC patients. Amplification of the circulating levels of miR-21 and miR-16 was done using real-time PCR qualitative detection, while electrochemiluminescence assays was used to detect the circulating levels of CEA and CA153. The diagnostic sensitivity for BC was compared between the three. The serum miR-21 levels of the BC patients were significantly high, as the latter had much higher levels (P<0.001). The CA153 and CEA sensitivities were 15.73 and 22.47%, respectively while miR-21 sensitivity and specificity were 87.6 and 87.3%. BC patients’ miR-21 exhibits far higher sensitivity for diagnoses than both CEA and CA153. Thus especially in the early stages of BC, miR-21 can become a potential indicator for diagnosis; although at the clinical stage, PR and ER statuses were not correlated in this study.

Key words: Real-time polymerase chain reaction (real-time PCR), breast cancer (BC), microRNA-21 (miR-21).

INTRODUCTION

Cancer is one of the illnesses where the stage at which the disease is diagnosed plays a crucial role in patients’ survival and quality of life (Iorio et al., 2005). A diagnostic indicator that can detect cancer in early stages is of great significance, especially for breast cancer (BC), as it is the most common cancer in woman (O’Hara et al., 2009). One field of interested is that of tumor markers, due to their noninvasive, rapid and simple nature (Chou et al., 2013). Despite their low sensitivity and specificity, the carcinoembryogenic antigen (CEA) and cancer antigen 153 (CA153) are the commonest markers used. MicroRNAs are found to have close ties with development and formation of tumors and are involved in regulating many cellular processes. They are a class of noncoding RNAs composed of 19-25 nucleotides (Zhang et al., 2007).

miR-21 is involved in oncogenic process and has been demonstrated to be an essential regulator, and due to its involvement in tumor formation, its level is raised in majority of human tumors. The overexpression of miR-21 in BC tissue was observed by Iorio et al. (2005) and suggested that it can be an effective marker; however, getting tissue is an invasive procedure. Easy monitoring, little invasiveness and simple collection are obvious advantages of serum sampling (Zheng et al., 2011; Samy et al., 2010). miR-21 expression was evaluated in 89 BC patients using SYBR-Green as a base and miR-16 as reference for the stem-loop real-time reverse transcription-polymerase chain reaction (RT- PCR) (Wu et al., 2010). Considering the hormone receptor status and disease stage, miR-21 expression levels were compared, and then its
### Materials and Methods

#### Subjects

The study was performed in Kurdistan Hospital and approved by its Ethic committee. All patients agreed to a written informed consent. The BC women confirmed by medical examination were aged between 28 and 60 years, with 50 as a median age and blood samples were collected from March 2011 to April 2011. All the patients had a confirmed diagnosis for primary BC by histology and were undergoing therapy at the time of study, aged between 29 and 40 years, with 36 as a median age. Samples were collected between March 2011 and May 2011.

#### miR-21 Detection

**Serum Samples and Total RNA Preparation:**

The samples were stored until processing at 80°C. Adhering to manufacturer’s instructions, the Trizol reagent from Invitrogen life technologies was used for extraction of total RNAs from serum.

**Reverse Transcription**

Each 10 mL RNA sample was mixed with 3 mL stem-loop RT primers of miR-16 and miR-21, and 4 mL of 5× RT Buffer, 1 mL of Moloney murine leukemia virus (M-MLV) reverse transcriptase [Promega (Beijing) Biotech Co., Ltd.], 0.5 mL of dNTPs [Tiangen Biotech (Beijing) Co., Ltd], 0.2 mL of RNasin [Tiangen Biotech (Beijing) Co., Ltd.], and 2 μL of 1 mol/L dithiothreitol [DTT; Tiangen Biotech (Beijing) Co., Ltd.] were added (Table 1). The final volume of the mixture was 20.7 L and incubated at various temperatures for different durations, at 61°C for 30 min, 73°C for 30 min and 170°C for 10 min, and lastly was held at 4°C.

#### Real-Time PCR

1.6 μL cDNA is the product of reverse transcription, and this product is mixed with 10 μL SYBR Green Master (Roche Co., Ltd.) and 1 μL PCR primers along with other PCR reagents (Table 2). The entire reaction was performed in the ABI 700 Fast PCR system. The conditions for the PCR was denaturing for 10 min at 95°C, after which 40 cycles of 95°C for 15 s is applied, followed by 60°C for 1 min.

#### CEA and CA153 Detection

Electrochemiluminescence assays were used to calculate CEA and CA153 levels and through Roche E170 MODULAR Immunoassay Analyzer, the reaction was carried out.

#### Statistical Analysis

Using relative change folds, normalization of circulating miR-21 expression was done. The characteristics of miR-21 relative expression levels are their range from 25th to 75th percentile and by their median. The connection of patient’s hormone receptor status with their miR-21 was analyzed with mann-whitney test, while the association with of their clinical stage with their miR-21 was calculated through Kruskal-Wallis test. Mann-whitney test was also used to measure miR-21’s expression between healthy and BC individuals. The receiver operating characteristic curve (ROC) was used to determine the cut-off value, which was used to identify the specificity and sensitivity values. SPSS 16.0 software was used for all statistical analysis and statistically significant threshold was set as P <0.05.

#### Results

**Target Gene Amplification**

Pure homogenous products of miR-21 and miR-16 from PCR was obtained (Figure 1), as indicated from their melting curves (Figure 2) with narrow peak and sharply defined curves.
The expression of miR-21 in BC

miR-21 expression was evaluated in 89 BC diagnosed patients. The patients showed high level of miR-21 which was significantly high among BC patients (30.82), resulting in ($P < 0.001$) with a ratio of 3.39 (Figure 3).

miR-21 ROC curve

It was found that 13.22 was the best designated cut-off value, and 92.9% was determined to be the area under ROC curve (ROC-AUC) (95% confidence interval: 88.3%, 97.4%), while 87.6 and 87.3% were the sensitivity and specificity.
values respectively, as shown in Figure 4.

Clinical and pathological feature's association with miR021 expression levels

According to status of hormone receptors and clinical stage of the patients, they were classified into groups and the miR-21 median expression level is shown in Table 3. However, no correlation was observed between patient's clinical stage and hormone receptor statuses against their miR-21(P>0.05).

miR-21 comparison with traditional tumor markers CEA and CA153

Patients are grouped into different classes according to
their clinical stage, and their CEA, CA153 and miR-21 median expression levels are listed (Table 4). Significant difference was detected as the overall sensitivity of CEA and CA153 were merely 15.73 and 22.47%, respectively while miR-21’s overall sensitivity was 87.64%. Particularly in the early stage (stage I), the sensitivity of CEA and CA153 was only 4.76%, while miR-21 boasts a marked diagnostic sensitivity of 95.24% (Table 4).

DISCUSSION

The molecule of miRNAs is made up of 19-25 nucleotides, small indeed, but various biological signaling pathways are regulated by them (Song, 2007). The close relationship between the many characteristics of tumors including their development, invasion and metastasis and miRNA has been demonstrated in many studies, and in cancer therapy, this could be a bases for entirely new strategies to fight cancer (Slaby et al., 2008). Many malignant tumors have shown increased miRNA serum expression (Fujita et al., 2008). And as a diagnostic and prognostic marker, it has been gaining increasingly more attention.

It is to no surprise that an early diagnostic indicator for the commonest cancer among woman, that is BC, is of great value, affecting prognosis. Due to their simple and less invasive nature, serum markers are the focus of interest. Despite their low sensitivity and specificity, especially in early stages (Slaby et al., 2008), CEA and CA153 are still commonly used in BC patients monitoring, since relapse is associated with high levels of these markers. Furthermore, CEA cannot be used in diagnosis of early stages of BC, since it has a lower positive rate, and it is a nonspecific tumor marker.

Differential expression of some miRNA in normal and BC tissues has been demonstrated recently, such as let-7a(8), miR-21, and miR-145 (Schetter et al., 2008). In BC cells, they regulate biological process, and play various roles in apoptosis and proliferation. The possibility of miRNA acting as a BC tumor marker for diagnostic and therapeutic purposes has recently been reported (Yanaihara et al., 2006).

miR-21 has independent transcriptional units (Fujita et al., 2008) and is located on 17q23.2. It has been shown to play significant role in colon cancer development (Fujita et al., 2008; Slaby et al., 2008), lung cancer development (Schetter et al., 2008; Yanaihara et al., 2006), as well as stomach cancer (Volinia et al., 2006) and finally BC (Yanaihara et al., 2006), as it involves in the expression and regulation of numerous tumor suppressor genes. Many studies have used various methods such as Northern blotting, in situ hybridization (ISH), microarray, the profiling method of flow cytometric miRNA expression that is bead based, and finally RT-PCR (20-23) to prove that in BC, miR-21 is up-regulated both in in vitro and in vivo. A preliminary study about miR-21 overexpression in BC tissue has been conducted (1,21,24-26). The conclusion of the study is that miR-21 expression correlated with pathological and clinical variables in BC tissues, and its expression was higher than normal breast tissues (Schetter et al., 2008). Yet the study avoided the discussion of practical value of this finding in diagnosis of BC. In addition to the fact, that breast tissue was the center of previous studies, and little was mentioned about miR-21 serum levels, which is the focus of our study. miR-21 serum expression levels in BC patients was evaluated using stem-loop real-time RT-PCR which is based on SYBR-Green, and addressed the application of miR-21 as a diagnostic and monitoring marker in BC patients. The miR-21 expression level was (3.39) times higher in BC patients, which is statistically significant (P<0.001). Moreover, in the diagnosis of BC, the miR-21 demonstrated sensitivity and specificity of 87.6 and 87.3%, respectively which shines as compared with the traditional marker’s sensitivity of CEA and CA153, that were merely 15.73% and 22.47%. The present study also showed that there was no correlation between clinical stages and miR-21 serum expression, as well as no correlation between hormone receptor statuses (Progesterone receptor and Estrogen receptor) and miR-21 expression. Similar results have been reported (Slaby et al., 2008).

In conclusion, the new serum marker miR-21 topples traditional serum markers such as CEA and CA153 in sensitivity, which can improve prognosis of BC by allowing earlier diagnostic sensitivity. miR-21 can be addressed as a potential early stage BC serum tumor marker. We will have a follow up study to this preliminary study, where we will have more in-depth analysis and increase the sample size, hopefully becoming a good basis for miR-21 as a diagnostic tool in BC (Si et al., 2013).

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