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Evaluation of *hsa-miR-2355* MRE Site Sequence Within 3'-*UTR* of *ERCC1* Gene in Breast Cancer Clinical Samples by RFLP Method

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ABSTRACT

Aims: The involvement of deregulated miRNAs networks in breast cancer (BC) progression is validated previously. MiRNAs control genes by binding to their regulatory regions. A SNP within a miRNA binding site could change target mRNA level and protein. ERCC1 is involved in the Nucleotide excision repair (NER). This pathway is associated with BC. In present study, we genotyped 3'-UTR ERCC1 in BC patients.

Method: The 3'-UTR of ERCC1 were analysed for MRE sites using bioinformatics software. In present case-control study, we extracted genomic DNA from 34 BC clinical and control samples. These DNAs were used as template in PCR which bind to 3'-UTR ERCC1 gene. The nucleotide sequences of amplicons were evaluated by RFLP using Eco47I restriction endonuclease.

Results: Bioinformatics analysis showed that the restriction sites of Eco47I enzymes in the 2342 bp 3'-UTR of ERCC1 are related to MRE of miR-2355. The frequency of genotypes in present study was, 26.47% TT homozygote, 35.29% TG heterozygote and 38.23% GG mutant homozygote variant in patient group that was different with control group (OR=1.3465, 95% CI=0.7275 to 2.4923, p<0.05). The positive correlation between MRE change of miR-2355 (homozygote TT) with status of HER2 positive, BMI>22 and age>40 years were confirmed statistically (P=0.03).

Conclusion: In present study, we confirmed correlation between MRE nucleotide changes of miR-2355 within 3'-UTR ERCC1 with HER2 positive status. However, there is no association between miR-2355 MRE changes within 3'-UTRERCC1 with tumour-staging. Evaluation the genotype of aforementioned genomics position could be used as a validation test for HER2 status probably.

Key words: Breast cancer, *hsa-miR-2355*, *ERCC1*, *HER2* status

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INTRODUCTION

Breast cancer (BC) is one of the most common cancers among women, and is the second most common cancer in women. 1.38 million New cases of BC were estimated in 2008 [1]. It has been proven the association of BC with various changes in several genes. One of the pathways associated with BC is nucleotide excision repair (NER) pathway [1]. DNA repair genes are very important for the preservation of genomic integrity. Among the pathways of DNA repair, the NER system plays an important role in repairing of UV damage, thymidine dimers, cross-links, oxidative damage and alcoholic injuries. NER is a complex pathway and its mechanism has not yet been completely understood [2]. The Excision repair cross-complementing 1 (*ERCC1*)/*XPF* are a specific endonuclease involved in the NER and maintaining telomeres length. The shortening of the telomeres occurs due to incomplete DNA synthesis in the early stages of oncogenesis and leads to genomic instability, which has been studied as a factor in the prognosis of BC [3]. *ERCC1* protein is coded by the *ERCC1* (gene ID: 2067). This enzyme acts as a DNA repair component that is essential for the removal of DNA from the platinum, as well as for the detection and correction of DNA damage [4]. Single nucleotide polymorphism (SNPs) of DNA repair genes can affect the capacity of DNA damage in cancers [2]. Also it has been reported that SNPs in NER genes can alter and modify the function of involved genes [1].

MiRNAs, non-coding molecules range from about 20 to 24 nucleotides, regulate the expression of some genes at different levels of transcription and translation, by

interacting with their un-translated region (UTR) in these genes. Recent molecular studies have proven the role and importance of miRNAs molecules in relation to genetic changes in cancer. Today, the role of these molecules has been considered as a diagnostic cancer biomarker [5]. A SNP within a miRNA binding site or recognition element (MRE) could change mRNA level and protein of target gene such as *ERCC1*. Here, we aimed to evaluated changes in the regulatory region of *ERCC1* gene with demographic characteristics (including grade of tumor, BMI etc.) in breast cancer patients.

MATERIAL AND METHOD

Bioinformatics analysis

The 3'-*UTR* of *ERCC1* were analysed for MRE sites using bioinformatics software including miRwalk, UCSC, Targetscan, cancer-miner etc. Primer sequences were checked by *In-silico* PCR in UCSC and BLASTN to ensure they amplified specific region in human genome.

Sample preparation and DNA extraction

This case-control study was conducted in Khansari-nejad Hospital of Arak University of Medical Sciences in 2017. The clinical sample collection procedure was approved by the ethics committee, ethical approval: IR.ARAKMU.REC.1396.8. These samples have clinico-pathological data. For all samples, a questionnaire including age, sex, duration of illness, type of drug was collected. Patient characteristics are presented in Table 1. In present case-control study 34 patients with BC with an average age of 49 years. Also, 44 normal individuals were evaluated. The peripheral blood was collected in a tube containing EDTA. Genomic DNA from blood samples of normal and patients were extracted using PZP molecular IVD kit (Cat No. s120796, Iran) according to its in structure. Tubes containing DNA templates were stored at -20°C.

DNA quality and quantity was measured based on electrophoresis on 1% agarose gel and the absorption ratio of A260/A280.

Table 1: Relationship between clinic-pathological features of patient group and studied genotype in present study

Clinic-pathological features	Detail	Digested (GG & TG)	(GG & TG) from total samples	Non-digested (TT genotype)	(TT) from total samples
Age	<40	83.88	58.82	40	11.76
	>40	16.66	11.76	60	17.64
BMI	>22	75	52.94	70	20.58
	<22	25	17.64	30	8.82
ER, PR status	+ +	29.16	20.58	40	11.76
	- -	70.83	50	60	17.64
HER2 status	+	45.83	32.35	80	23.52
	-	54.16	38.23	10	5.88
Metastasis	+	16.66	11.76	40	11.76
	-	83.33	58.82	60	17.64
Tumor grade	high	16.66	11.76	40	11.76
	low	83.33	58.82	60	17.64

PCR-RFLP and sequencing

Amplification reaction including 50 ng genomics DNA, 12.5 µl red master-mix (Amplicon, UK) and 10 pmol of each primer (Table 2) in total volume 25 µl. Temperature program in each amplication tube was consisted of an initial denaturation for 5 min at 94°C followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min and 1 cycle of final extension at 72°C for 8 min. Digestion reaction was performed by *Eco47I* restriction enzyme for 3 hours at 37°C followed by enzyme inactivation at 65°C for 20 min. These fragments on 1.3% agarose gel (gene fanavaran, Iran) were evaluated using gel doc system (Quantum ST4, Germany).

Table 2: The sequence of used primers in this study

Primer ID	Sequence (5'-3')
F 3*utr ercc*1	ACCCCAAAGCCATGTCCACCTC
R 3*utr ercc*1	CCTCTGCCCTCCCTGTTTCTCT

In order to validation of RFLP method, the PCR products were sent for sequencing by ABI Apply biosystem 3730 xl to Macrogene company (South Korea).

Statistics analysis

In present study, difference between groups was analyzed by unpaired t test GraphPad prism 7.00, SPSS 16.0 and Microsoft Excel 2013 software. Also P values of less than 0.05 were considered as statistically significant.

RESULTS

Bioinformatics analysis showed that the restriction sites of some endonucleases in the 2342 bp 3'-*UTR* of *ERCC1* are related to the binding region of miRNAs (MRE) including *miR-2355*. These bindings have negative ΔΔG (-0.07 and -0.002) (Figure 1).

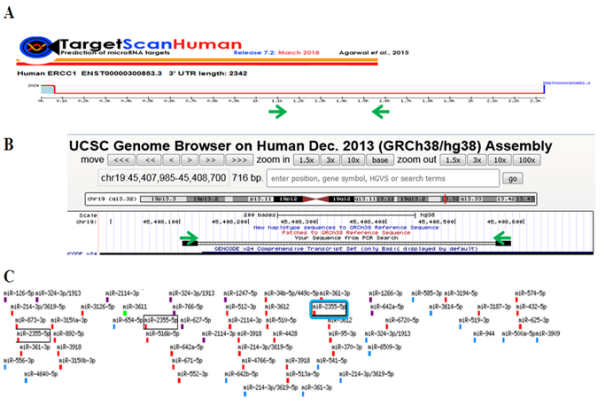

Figure 1: (A) Schematics view of Targetscan software and *ERCC1-UTR*, (B) Schematics view of UCSC *In-Silico* PCR and binding site of primers at *ERCC1-UTR*. The green arrows showed binding site of primers, (C) MRE sites of *miR-2355* in Targetscan, PCR product length is from 1094 to 1570 of *ERCC1-UTR* 2342 bp

The results of electrophoresis (Figure 2) indicated the accuracy of the genomic purification process, the PCR and enzymatic digestion reaction.

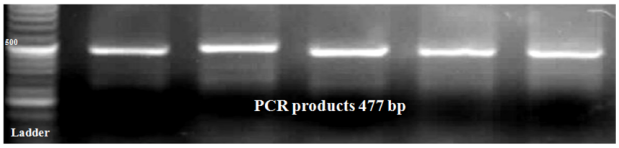

Figure 2: Electrophoresis result of 477 bp related to *ERCC1-UTR*

The length of amplicons were 477 pb, which after digestion with *Eco471* enzyme, in the presence of restriction site, produces 252 and 225 bp fragment. 73.52% of the studied patients and 88.62% of the control samples with *Eco471* enzymes were digested (Figure 3).

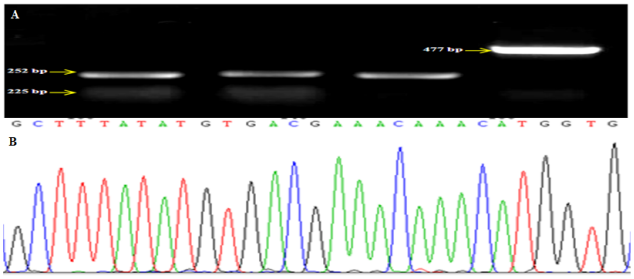

Figure 3: (A) The enzymatic digestion was created different patterns, (B) Sequencing results of amplicons

In present study, the one of MRE site of *miR-2355* genotypes at *ERCC1* regulatory region. TT homozygote, TG heterozygote and GG mutant homozygote variant in patient group had differences with control group (OR=1.3465, 95% CI=0.7275 to 2.4923, P=0.03) (Table 3). All of these alterations were validated by sequencing method as the golden standard which was accordance with RFLP-PCR findings.

Table 3: The patterns of RFLP results and related genotypes

Row	Pattern from digestion reaction	Size of band (bp)	Genotype	Patients (%)	Control (%)	P value
				n=34	n= 44	
1	Template A	477	TT homozygote	26.47	11.36	0.033
2	Template B	477, 252, 225	TG heterozygote	35.29	6.81	0.04
3	Template C	252, 225	GG homozygote (WT)	38.23	81.81	0.05

Also, the positive correlation of MRE change of *miR-2355* (homozygote TT) with *HER2* positive status, BMI>22 and age>40 were confirmed statistically (95% CI, P=0.033, Pearson r=0.397).

55.88% of non-metastatic samples have GG genotypes which digest with *Eco47I*. Among of patient samples that have TT genotype (non-digest), 70% and 60% have higher BMI and age. Also among patient samples that have TT genotype, 80% were *HER2* (human epidermal growth factor receptor 2) positive.

DISCUSSION

Despite the advances in detection and management of cancer, the most common cause of cancer deaths in countries of the world is BC. Only 2% of the patients live within five years of being diagnosed with the disease, so a better understanding of BC tumor and the identification of new biomarkers are essential for early diagnosis [6].

The defect in the DNA repair pathway enables cancer cells to accumulate genomic changes that contribute to their invasive phenotype. However, tumors rely on the capacity to this repair to prevent DNA created damage by genotoxic stress. Different DNA repair pathways are commonly used as compensating machine of defects [7].

One of the pathways for DNA repair by UV wave and chemical damage caused by platinum-based chemotherapy agents is NER pathway. In this pathway, *ERCC1* provides protein interactions and endonuclease activity of the XPF. Also, *ERCC1* is considered as a predictable biomarker of response to treatment. The *ERCC1* expression was assessed as a prognostic factor for post-treatment survival in NSCLC patients, in 2002 [2].

One of the mechanisms of tumor resistance to cisplatin chemotherapy is the increase in NER activity as a result of an increase in the level of *ERCC1*. In addition, the expression of *ERCC1* is a marker for the effectiveness of cisplatin [8]. For example in ovarian cancer cell lines, *ERCC1* over-expression is related to cisplatin resistance [9]. In another study, a positive correlation was found between the expression level of *ERCC1* and the amount of cisplatin administration and decreased sensitivity to this drug. However, the relationship between expression of *ERCC1* and clinic-pathologic variables with survival data in patients with metastatic BC was not found [10].

According to obtained results in our study, changes in the regulatory region of *ERCC1* gene are associated with some of the clinical features of breast cancer (Table 1).

MiRNAs are small, non-encoding RNAs that regulate the expression of the genes by binding to 3'-*UTR* in the target mRNA. Many studies have shown that miRNAs play important role in processes such as cell proliferation, apoptosis, and tumorigenicity [11]. Many evidences have shown that miRNAs are stable biomarkers in cancers. In some genes, such as *PSMC3*, mutagen agents often affect 5'-*UTR* in BC, ovarian cancer, and tumor of the fallopian tube which modified miRNA binding sites (MREs) of miRNAs [12].

In our study, the 3'-*UTR* region of the *ERCC1* gene was investigated in aspect of the MREs, by using the bioinformatics analysis. Data showed there are 3 MREs of *Hsa-miR-2355* at 3'-*UTR* of *ERCC1* (nucleotide sequences of 86-93, 626-633 and 1341-1347 bp). Positions of used primers surrounded one of conserved aforementioned MREs (1341-1347 bp). One of these miRNAs is *Hsa-miR-2355*.

Hsa-miR-2355 gene is over-expressed in BC patients and correlates with stage and molecular subtype [13,14]. In a study in 2016, on 23 patients with BC, were presented a list of miRNAs associated with BC, which among them *hsa-miR-2355* had a significant association with BC [6]. In 2014, expression analysis of *miRNom* in BC showed that miRNAs that probably regulated pathways are highly relevant to cancer progression. It has been determined that many miRNAs including *hsa-miR-2355-5p* are differentially expressed in BC compared to normal breast tissue [3]. Also in 2013, the clinical significance of *ERCC1* expression in BC was investigated and the over-expression of *ERCC1* was correlated with a smaller tumor size (P=0.007) and positive estrogen receptors status (P=0.04), but did not correlate with other clinical features of the disease [15].

In accordance with aforementioned studies, in our study, it was also determined that the change in the regulatory region of the *ERCC1* gene, which certainly changes the expression level of this gene, has a direct correlation with the *miR-2355* binding site (TT genotype) with age and BMI and its positive *HER2* levels, but are not related with other clinical features. In our study, number of patients that have TT genotype and metastasis to another organs and higher grade of tumor were lower than non-metastatic with TT genotype.

One of the Effective factors in management of cancers is early diagnosis [16]. Over-expression of *ERBB2* or *HER2* receptors called *HER2*-positive which is one of experiments for BC detection. There are different drugs specifically for *HER2*-positive BCs. Determination of *HER2* status test is critical for treatment and respond to the drugs such as Herceptin that target this receptor in *HER2* positive BCs. Because there is a different test for *HER2* status including IHC, FISH, SPoT-Light *HER2* CISH test, probably *HER2* status result test results may be wrong. Our data probably help to oncologist to validation of *HER2* status test.

CONCLUSION

A better understanding of the miRNAs targeting MREs has vital diagnostic and therapeutic purposes in cancers. We confirmed MRE nucleotide changes of *miR-2355* within 3'-*UTR ERCC1* is related to *HER2* positive status, therefore, could be used as a validation test of *HER2* status probably. Although should be analysed larger population of BC patients.

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AUTHORS CONTRIBUTION

The first two authors have equal role to this study; AA: Designed study, performed the experiments, analyzed data, interpreted data and manuscript preparation, and approved final manuscript. MA: Designed study, interpretation of data, manuscript preparation; RA: Clinical sample preparation; MS and MM: Performed the experiments.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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