RESEARCH ARTICLE

The Role of Genetic Polymorphisms in Nrf2 and P73 in Egyptian Women with Breast Cancer

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Abstract

Background: Breast cancer is the commonest cancer in Egyptian females. Nrf2 is involved in oxidative stress while P73 functions in response to DNA damage. This study aimed to assess the role of Nrf2 promoter and P73 G4C14 to A4T14 SNPs in breast cancer in Egypt. Patients: Eighty-five female patients with breast tumours (41 malignant, 44 benign) were included. Nrf2 (rs6721961) and p73 (G4A) SNPs were determined by PCR- CTPP assay. **Results:** Genotype frequencies of the Nrf2 promoter SNP were 34.2% and 37.9% for AA in benign and malignant groups respectively, and 43.9% and 40.5% for CC and, 21.9 % and 21.6% for CA. Genotype frequencies for the P73 G4A SNP were 52.9% and 44.7% for GA in benign and malignant groups respectively, and 47.1% and 55.3% for GG. **Discussion:** Nrf2 genotypes in pre - and post-menopausal patients, showed significantly different distributions in the 2 patient groups, the AA genotype being significantly more common in pre-menopausal patients. The P73 G4A SNP showed no relation to age of disease onset. **Conclusion:** The Nrf2 (rs6721961) AA genotype might be related to early breast cancer onset. In contrast the P73 G4A polymorphism showed no relation to either disease risk or age at presentation.

Keywords: Breast cancer- polymorphisms- Nrf2- P73- genotype

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Introduction

Breast cancer is the second cancer in mortality affecting mostly females. It is the most frequent malignancy with high morbidity and mortality among women worldwide (Gomes et al. ,2012). It accounts for 22.9% of all female cancers worldwide (Ferlay et al., 2010). Mortality in breast cancer patients is mostly caused by metastasis which is related to poor prognosis of breast cancer patients (Fang et al., 2013). In Egypt, breast cancer is the commonest site of cancer in females as it represents (38.8%) of all female cancers (Ibrahim et al., 2014). Pathogenesis and progression of breast cancer are multifactorial processes affected by genetic, biological, and environmental factors, as well as lifestyle (Porter et al., 2009). Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (hER-2) are the most important prognostic and predictive markers in breast cancer. Triple-negative breast cancer (TN), does not express ER, PR or hER-2. TN breast cancer cases are about 15–26% of all breast cancer cases. Women with TN breast cancer usually show poor prognosis (Pal et al., 2011). Nrf2 is a transcriptional regulator of cytoprotective gene involved in the cellular defence mechanisms against electrophilic and oxidative stress. Activation of Nrf2 defense response has been shown to protect against neurodegenerative diseases, aging, diabetes, photo-oxidative stress, cardiovascular disease, pulmonary fibrosis and cancer (Motohashi and Yamamoto, 2014; Jeong et al.,2006; Zhang, 2006; Kensler et al.,2007; LAU, at al.,2008). Nuclear NRF2 protein plays important roles in the proliferation and/or progression of breast carcinoma, and nuclear NRF2 immunoreactivity is therefore considered a potent prognostic factor in breast cancer patients (Onodera at al.,2013). However, accumulation of Nrf2 in cancer cells has been shown to create an environment helpful for cell growth and protects against oxidative stress, chemotherapeutic agents, and radiotherapy (LAU et al.,2008; Wang et al.,2008; Jarmillo and Zhang, 2013). Nrf2/HO-1 stress response mechanism is a promising target for anticancer treatment which is able to overcome resistance to therapies (Furfaro et al.,2016).

An association between Nrf2 accumulation and adverse outcome of the patients has been reported in the lung (Solis et al.,2010; Inoue et al., 2013), gallbladder (Wang et al., 2010) and ovarian (Konstantinopoulos et al.,2011) carcinomas. These findings suggest that Nrf2 is possibly involved in the growth and/or progression of these carcinomas. Many single nucleotide polymorphisms

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(SNP) have been identified in the Nrf2 gene. The 3 promoter SNPs rs35652124 (A→G) and rs6721961 (C→A) were found to reduce the transcription activity of Nrf-2, decreasing Nrf-2-dependent gene transcription (Shimoyama et al., 2014).

P73, which is a member of P53 family of transcription factors, has similar cellular activities to those of P53, including binding and transactivation of P53-responsive genes and induction of apoptosis and cell cycle arrest, wherefore; P73 has tumor-suppressive activities (Dotsch et al., 2010). Also P73 plays unique roles in neuronal development and differentiation, metabolic control, and spermatogenesis and maintenance of male fertility (Dotsch et al., 2010; Cutruzzola et al., 2013; Inoue et al., 2014). P73 have several isoforms with different actions. Several SNPs of P73 were found to be related to cancer and could help in predicting cancer risk and chemotherapeutic outcome (Chen et al., 2008). P73 SNPs include G4C14-to-A4T14 which is a functional dinucleotide polymorphism at positions 4 (G→A) and 14 of the 50- untranslated region (50-UTR) of exon 2 of the P73 gene (C→T) (G4C14-to-A4T14, simply designed as G4A hereafter)(Gali et al., 2009; Lee, 2010).

The aim of this work is to assess the role of Nrf-2 promoter and P73 G4C14-to-A4T14 polymorphisms in breast cancer and the potential relation to the onset of the disease.

Materials and Methods

Ethical approval

The project and data forms were approved by the Regional Research and Ethics Committee at the National Cancer Institute (NCI), Cairo University, Egypt. Written informed consent was obtained from all participants involved in our study.

Subjects

This study included 85 female patients with breast tumor, they were admitted at the Department of Surgery in National Cancer Institute, Cairo University, where they were divided into two groups, first group includes 41 patients with malignant breast tumors, their age ranged from 28 to 78 (49.77±12.84), and 44 age matched patients with benign breast tumors.

Methods

Five ml blood sample was collected after overnight fasting and divided into 2 tubes; one for DNA extraction and the other tube for serum separation for biochemical parameters assay using standard laboratory methods. ER, PR and hER-2 were done on 10% formalin-fixed paraffin embedded blocks for each patient. DNA extraction was done by commercially available kit (KAPA Express Extract kit, cat. KK 7101, USA).

Genotyping of SNP of Nrf2 and P73: Genotyping was performed using (PCR-CTPP) PCR with confronting 2 -pair primers assay (Hamajima et al., 2000) using KAPA2G fast PCR kit (KAPA2G Fast PCR kit, cat. # KK 5008, USA) following the manufacturer instructions with some modifications.

1- Nrf2 promoter polymorphism: In this assay, the genotyping of Nrf2 (rs6721961) was performed using confronting pairs of primers (Wang et al., 2010) as shown in table (1) Region containing the polymorphism of Nrf2 was amplified by PCR with those primers with the initial denature at 95°C for 10 min followed by 30 cycles at 95°C for 1 min, at 58°C for 1 min, at 72°C for 1 min and additionally at 72°C for 5 min. PCR products were visualized on a 2% agarose gel with ethidium bromide staining. Genotyping was performed as follows; 282, 113 bp for CC genotype, 282, 205, 113 bp for CA genotype, and 282, 205 bp for AA genotype.

2- P73 exon 2 polymorphism: In this assay, the genotyping of P73 G4A polymorphism was performed using confronting pairs of primers(Ibrahim et al., 2014) as shown in table (1). For amplification, an initial denaturation step at 95°C for 10 min was followed by 35 cycles of 95°C for 1 min, 62°C for 45 seconds, and 72°C for 1 min, and a final extension step at 72°C for 5 min. The amplified DNA was visualized on a 2% agarose gel with ethidium bromide staining. 5 The P73 G4A polymorphism was genotyped as a 193 base pair band for the G allele, a 270 base pair band for the A allele, and a 428 base pair common band.

Statistical analysis

Data were assessed with Graph Pad prism software using Student t- test and Z- test. Fisher exact test was used to calculate the significance between genotype distributions in different studied group. Results were expressed as means ± standard deviation and p value less than 0.05 was considered statistically significant.

Results

The genotype frequencies of Nrf2 promoter SNP were 34.2% and 37.9% for AA in benign and malignant groups respectively, 43.9% and 40.5% for CC in benign and malignant groups respectively, 21.9 % and 21.6% for CA in benign and malignant group respectively (Figure 1).

Genotype frequencies for P73 G4A SNP were 52.94% and 44.73% for GA in benign and malignant groups respectively, 47.06% and 55.26% for GG in benign and malignant group respectively. AA genotype was not found in any case (Figure 2).

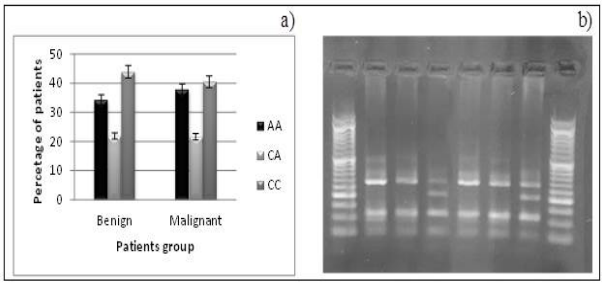

Figure 1. a) Nrf2 Promotor Genotype Distribution among Patients with benign and Malignant Breast Tumors. b) Gel Showing Genotype for Promotor SNP of Nrf2 Gene. Lanes (Left to Right) 3, 6 CA Genotype (282, 205, 113 bp), and Lanes 1, 2, 4, 5 CC Genotype (282, 113 bp).

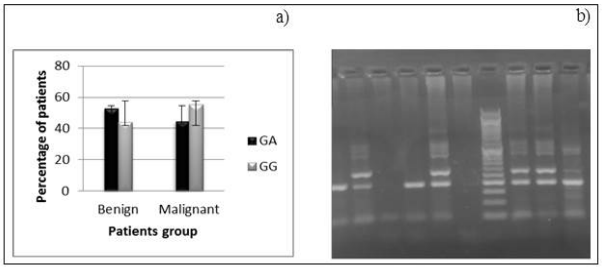

Figure 2. a) P73 G4A Genotype Distribution among Patients with benign and Malignant Breast Tumors. b) Gel Showing Genotype for G4C14-to-A4T14 SNP of P73 Gene. Lanes (Left to Right) 2,5, 8, 9 GA Genotype (428, 270, 193 bp), Lanes 1,4, 10 GG Genotype (428, 193 bp).

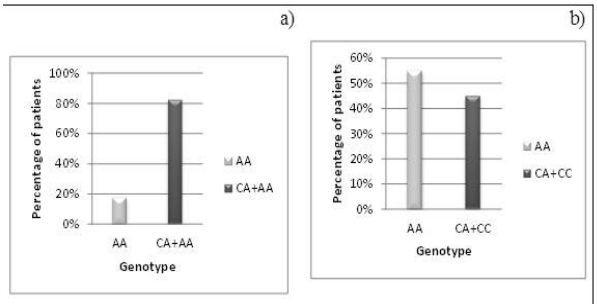

Figure 3. Genotype Distribution of Nrf2 rs6721961 SNP in: a) Post-Menopausal Patients with Malignant Breast Cancer. b) Pre-Menopausal Patients with Malignant Breast Cancer.

Table 1. Nrf2 Genotype Distribution in Post and Pre-Menopausal with Malignant Breast Cancer Patients.

Gene	SNP	Genotype	Post- menopause	Pre- menopause	P
			(%)	(%)	
Nrf2	rs6721961	CC	41.2	40	< 0.05
		CA	41.2	5	
		AA	17.6	55	
P73	G4A	GG	50	57.9	
		GA	50	42.1	

Regarding the disease onset, the three Nrf2 genotypes in pre - and post-menopausal patients, showed that the distribution differ significantly in the 2 patient's groups ($p < 0.05$). Nrf2 rs6721961 SNP shows different genotype distribution between pre- and post- menopausal breast cancer patients. CA genotype is significantly higher in post-menopausal patients compared to pre-menopausal patients ($p < 0.05$). P73 G4A SNP showed no significant difference in genotype frequencies or distribution in pre- and post-menopausal breast cancer patients (Table 1). Nrf2 genotype distribution showed significant difference ($p < 0.05$) in post and pre-menopausal with malignant breast cancer patients.

It also noted that the frequency of AA genotype is significantly lower in post-menopausal breast cancer cases compared to the C allele carrier genotypes (CC & CA) ($P< 0.05$). On the other 6 hand, the genotype AA is significantly related to premenopausal cases when compared with genotypes CC+CA ($p<0.05$) (Figure 3).

Lymph nodes involvement showed no significant association with genotype distribution of both Nrf -2 and P73 G4A. Regarding triple negative breast cancer patients, they were all of homozygous Nrf2 genotype (5o% were CC, 50% were AA). The heterozygous genotype (CA) is absent in this group. TN group shows marked (although statistically non-significant) predominance of the heterozygous genotype of P73 G4A (80% were GA).

Discussion

Breast cancer is one of the most malignant threats against women worldwide. Patient phenotype is closely associated with tumor behaviour, progression and treatment response (von Minckwitz et al., 2010). In Egyptian women, breast cancer is a challenging health problem coming on top of all malignancies with poor outcome compared to international figures (Ferlay et al., 2010). It was shown that age at diagnosis of breast cancer in Arab countries is a decade younger than that in Western countries (El Saghir et al., 2006; El Saghir et al., 2007). Defective Nrf2 signaling pathway may increase cancer susceptibility. Targeting Nrf2 is shown to effectively enhance chemotherapeutic agent in suppression of tumor growth in several animal models (Manandhar et al., 2012). The genetic polymorphism in the human Nrf2gene is considered as one of prognosis markers for cancer therapy (Ishikawa, 2014).

Genetic polymorphisms of Nrf2 on several SNPs (including rs6721961) were associated with breast cancer risk (Hartikainen et al., 2012). In the current study, Nrf2 rs6721961 genotype CC was found in more than 40% of Egyptian patients with benign or malignant breast tumours. Different ethnicities might have different genotype distribution, for example, it is reported that CC is the least common Nrf2 rs6721961 genotype in Japanese people in general (Shimoyama et al., 2014). 7 Nrf2 promoter rs6721961 (C→A) polymorphism was found to reduce the transcription activity of Nrf2, possibly resulting in decreased Nrf2-dependent gene transcription. This promoter polymorphism was shown to have functional significance affecting basal Nrf2 expression and function (Marzec et al., 2007). Nrf2 gene transcription activity was significantly high in rs6721961 C wild-type compared to rs6721961 A variant. Decreased Nrf2 transcription is shown to be related to some types of cancer, and Nrf2 inducers show cancer preventive effect. Thus, the presence of the A allele might explain the occurrence of breast cancer earlier (premenopausal) in the Nrf2 rs6721961 AA patients in the current study. In the current study, TN patients were all homozygous to Nrf-2 (50% were CC, 50% were AA). The heterozygous genotype is absent in this group. Two SNPs at position 4 (G to A) and 14

(C to T) in P73 gene have been identified. Functional analysis implies that this common p73 polymorphism may contribute to cancer development and progression (Li et al., 2006). In the current study, no patient had the AA genotype, which is the least common genotype in P73 G4A polymorphisms (Li et al., 2006). The current study results showed no statistically significant difference in P73 G4A between malignant and benign breast tumor patients groups. This suggests that the A allele might be not related to breast cancer risk. A previous study reported that the A allele isn't related to gastric cancer risk (Liu et al., 2014). Further study is required to assess a potential effect of ethnicity on P73 G4A relation to breast cancer risk in Egyptians. Such effect was reported in Caucasians (Wang et al., 2012). TN group shows marked (although statistically non-significant) predominance of the A allele carriers genotype of P73 G4A (80% were GA). It was reported that the GG genotype is related to increased risk of TN breast cancer (Zhou and Wu, 2012). This point requires further investigations. 8 In conclusion, Nrf2 (rs6721961) AA genotype might be related to early breast cancer onset. P73 G4A polymorphism shows no relation to both disease risk and disease onset. Therefore, Nrf2 (rs6721961) promoter genotyping could be used to predict the risk of pre-menopausal breast cancer.

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