Original Article

P53 and *P63* as Associated Molecular Markers in Breast Cancer in Saudi Arabia Patients

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رابطة الواسمات الجزيئية مع الدرجات والمراحل المختلفة لسرطان الثدي. بالمملكة العربية السعودية

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الملخص العربي:

الهدف : هذه الدراسة تهدف الي التحقق و تقييم قيمة التعبير الوراثي في الجينات p63 و p53 في سرطان الثدي . وبالإضافة إلى ذلك، لتحديد رابطة هذه الواسمات الجزيئية مع الدرجات والمراحل المختلفة لسرطان الثدي. بالمملكة العربية السعودية

الطريقة :تم جمع عينات الأنسجة 26 الذين يعانون من سرطان الثدي .تم تنفيذ أسلوب المناعية الصبغيه للكشف عن البروتينp53 و p63. وكان التعبير الجيني عن P53 كان أيجابيا في 15.3٪ من مجموع العينات في حين أن التعبير الجيني عن p63

النتائج : كان ايجابيا في ٪ 19.1 من العينات. ووجدت الدراسة أن الجينات p63 و p53 كان إيجابيا في حالة سرطان الثدي ولا سيما في الفئة العمرية 30-60 سنة ومع المرحلة الثانيه والثالثه من المرض .

الخلاصة : الدراسه الحاليه تبين ان التعبير الجيني لـ p63 و P53 لا يرتبطان معا مما يوحي بـأن p63 يمكن أن يعمل بشكل غير مباشر على أنه الجين الورمي وتثبيط عمل P53 مما يفسر فرضية ارتباط p63 مع مؤشرات أخرى عديدة من المضاعفات الخطيرة.

ABSTRACT

Objectives:. The aim of the study is to evaluate and investigate the prognostic value of *p53* and *p63* genes in breast cancer in Saudi patients.

Method and Materials: A total of 26 tissue samples from men and women suffering from breast cancer were collected. Immunohistochemistry method was performed to detect *p53* and *p63*.

Results: The expression of *p53* was positive in15.3% of the total cases of samples while the expression of *p63* was positive in 19.1% of samples. The study found that the *p53* and *p63* genes was positive in case of breast cancer, especially within the age group 30-60 years old and with grade II, III.

Conclusion, *p53* is rarely co expressed with *p63*, suggesting that *p63* could act indirectly as an oncogene by inhibiting *p53*. This hypothesis could also explain why *p63* correlated with several other indicators of poor prognosis.

Keywords: *Breast cancer, p53, p63, immunohistochemistry, prognosis, Saudi Arabia.*

INTRODUCTION

The global burden of cancer has more than doubled in the past 30 years and breast cancer is the most common cancer among women worldwide (Lord, et al., 2007). It was reported by World Health Organization (WHO) that 636,000 and 514,000 incident cases occurred in developed and developing countries during 2008, respectively.

Breast cancer, one of the most frequent and deadly cancers in women, has been recognized as a heterogeneous disease in terms of natural history, genetic alteration, histopathological features, gene expression profile, and response to treatment in individual patients (Lakhani and Ashworth, 2001; Quackenbush, 2006; Sotiriou and Piccart, 2007; Stingl and Caldas, 2007).

Male breast cancer is a rare condition, accounting for only about 1% of all breast cancers. The American Cancer Society estimates that in 2008, about 1,990 new cases of breast cancer in men will diagnose and that breast cancer will cause approximately 480 deaths in men in comparison with more than 40,000 women die of breast cancer each year. Most cases of male breast cancer is detected in men between the ages of 60 and 70, although the condition can develop in men of any age (Fattaneh et al., 2003). The causes of the breast cancer in male are the same in female (Borgen et al., 1992; Fentiman et al., 2006).

P53, located at 17p13.1, was the first gene identified as a mutant in human tumors. Its normal protein product participates in regulation of the cell cycle and in apoptosis. Mutations of *p53* have occurred in 17–40% of sporadic breast cancers examined, (Coles et al., 1992) and most of them are missense mutations concentrated in a core region that encodes the sequence-specific DNA-binding. Mutant forms of *p53* protein interfere with the growth-suppressing effects of wild-type *p53*, indicating that the gene product is actually a tumor suppressor (dominant negative). Many investigators have examined mutations of *p53* in detail and have correlated them with the prognosis and with the sensitivity to anti-tumor drugs. A statistically significant association has been noted between *p53* mutations that occur in conserved domains, and poor prognosis. As *p53* mutations are found most frequently in advanced breast

cancers, it appears that aberrant *p53* is involved in the progression stages of such tumors (Nagahata et al., 2002).

P63 belongs to the family of transcription factors that also includes *p53* and *p73*. All members of the family have three highly conserved domains: a transactivation domain (TA), a DNA binding domain (DBD) and an oligomerization domain (OD), *p63* and p73 have an additional protein interacting domain at their c-terminus known as sterile alpha motif (SAM) (Graziano and De Laurenzi, 2011).

Several studies have attempted to correlate *p63* expression with prognosis but further work is required to obtain a clear picture of the value of this gene as a prognostic marker. A correlation of *p63* expression with increased features of poor prognosis has been reported (Garcia, et al., 2007), indeed expression increases from grade IIIa to grade IIIb–IV breast carcinomas (Ribeiro-Silva et al., 2003) and correlates with nuclear pleomorphism, a known feature of aggressive tumors (Thike et al., 2010). Moreover in one study it was reported that *p63* is negative in cancers smaller than 2 cm, but its expression increases with tumor size (Ribeiro-Silva et al., 2003). Conversely well differentiated tumors over expressing *p63* are associated

to a good prognosis (Hanker, et al., 2009). It is possible that expression of *p63* in cells of different origin has a different phenotype or more likely that the isoforms expressed by the different tumors are different and result in a different outcome. Indeed one could imagine that while basal like tumors express the oncogenic ΔN isoforms, more differentiated tumors express the tumor suppressor TA form. Hopefully future studies investigating differential isoform expression will clarify this point and allow the use of *p63* as a prognostic marker.

The use of immunohistochemical staining has been a major part of the routine diagnostic procedure in various malignancies, and recent studies have reported a relationship between immunohistochemistry (IHC) profiles of various types of breast carcinomas and molecular taxonomic classification (Ginestier, et al., 2002; Makretsov, et al., 2004; Nielsen, et al., 2004; Jacquemier, et al., 2005).

The current study was designed to identify the association of molecular markers (*p63* and *p53*) with breast cancer in different grades and stages in both men and women in Saudi Arabia.

MATERIAL AND METHODS
This study was performed at King Abdul-Aziz university hospital in histopathology laboratory. Samples were collected from Al-noor hospital, Alawi Tunsi hospital, King Abdul-Aziz hospital, Al Hada, and Al Taif military hospital. A total of 26 samples were collected. Samples studied belong to different age and sex. The questionnaire form included: file number, age, gender, diagnosis, stage and grade of cancer.

Samples preparation

Formalin-fixed, paraffin-embedded tissue blocks from 26 patients with breast carcinoma. The immunohistochemistry assay was performed on 4-5 μm sections were prepared and placed on positively charged slides where tumor sections were de waxed by placing in a glass jar containing xylene for 30 minutes with regular 10 minutes interval shaking. Slides were hydrated through a series of different ethanol concentrations (100%, 90% (v:v), and 70% (v:v)) one minute at each concentration. Slides were then rinsed in running tap water for 1 minute. All tissue sections were placed in a plastic microwavable container with 250 ml 10

µm citrate buffer (*pH* 6.0), which was heated at full power (750 watts) for 5 minutes. After 5 minutes, approximately 50 ml distilled water was added to substitute the evaporated water and continued heating for another 5 minutes at full power (750 watts). After the heating step, the container and tissue sections were placed at room temperature for 20 minutes to cool down in the same citrate buffer solution prior to rinsing in running tap water followed by transferring step where slides transferred to the DAKO Autostainer.

Immunohistochemistry

The current study used DakoCytomation immunohistochemistry which is refers to the Universal EnVision Doublestain System permits the simultaneous demonstration of two antigens within one specimen by double immunoenzymatic staining. IHC used to show whether or not the cancer cell has *p53-p63* proteins in it. Immunohistochemistry was then carried out using the DakoCytomation EnVision™ Detection Kit (DakoCytomation Ltd, US), according to the manufacturer's instructions using a semi-automated staining system (Autostainer, DakoCytomation Ltd., USA).

Immunohistochemical Procedure

Slides were initially rinsed with 2.5 µl tris buffered saline (TBS) (*pH* 7.6), followed by addition of the primary antibodies (Table -1-), which were diluted using antibody diluents solution (DAKO), incubated for 30 minutes, and rinsed with

2.5 µl TBS buffer. Endogenous peroxidase in the tissue sections were blocked by addition of 2.5 µl peroxidase blocking solution (DAKO) and incubated for 5 minutes. Slides were rinsed with 2.5 µl TBS buffer followed by addition of 2.5 µl Alkaline phosphatase labelled polymer, which was biotinylated goat anti-mouse and anti-rabbit immunoglobulin (ChemMate™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse, DakoCytomation, USA), incubated for 30 minutes before rinsed again with 2.5 µl TBS buffer. Two point five microliters of labelled polymer reagent, which is streptavidin peroxidase, HRP, was added to the tissue sections before incubation for 30 minutes. Slides were rinsed with 200 µl TBS buffer before 2.5 µl substrate-chromogen solution,(DAB) (ChemMate™ Detection Kit, DakoCytomation, USA) was added and incubated for 5-15 minutes. Slides were then removed from the Autostainer and rinsed in running tap water for 2 minutes followed by a counterstain step using haematoxylin (code S3309), by dipping the slides once in haematoxylin. Slides were rinsed in running tap water until the solution was clear. Slides were placed 10 times into a bath of 0.037 mol/L ammonia water , then rinsed in tap water, followed by dehydration through a series of different concentrations of ethanol (70% (v:v), 90% (v:v) and 100%) one minute for each concentration before immersed in xylene for three minutes. Slides then were mounted under cover slips with Glycergel and observed by light microscopy.

Table -1-: Breast cancer markers used in this study and their condition.

Protein	Catalog #	Isotype	Epitope	Company	Clone	Dilution factor
p63	sc-8431	mouse IgG$_{2a}$	1-205 (h)	Santa cruz biotechnology inc.	4A4	x100
p53	sc-47698	mouse IgG$_{2b}$	1-45 (h)	Santa cruz biotechnology inc.	DO-7	x100

RESULTS:

Our results are summarized in the Tables 2 and 3. The expression of *p63* was positive in 19.1% of the total cases of breast cancer and the expression of *p53* was positive in 15.3% while 34.4% of all cases expressed *p63* or *p53*.

The results were showed in Table 2 the relationship between the age and the percentage of expression on *p53* and *p63*

in breast cancer patients. The result confirmed the expression of *p53* and *p63* in age group 30-50 in men and women. The

results showed that expression of *p63* was found in 50-70 age groups.

Table -2- showed the relationship between the age and the percentage of expression on *p63 and p53* in breast cancer patients.

Age	% of expression *p63*	% of expression *p53*	Total %
Less than30	3.8	3.8	7.6
30-50	11.5	11.5	23
50-70	3.8	0	3.8
Total %	19.1	15.3	34.4

Table 3 explained that the expression of *p63* and *p53* in different types of breast cancer. The result showed that expression of *p63* and *p53* were found in grade II and III of breast cancers. Also, the result showed expression of *p53* was more than *p63* in grade II in breast cancer patients in Saudi Arabia.

Table -3- showed the relationship between the grade and the percentage of expression on *p53* and *p63* in breast cancer patients.

Grade	% of expression *p63*	% of expression *p53*	Total %
I	0	0	0
II	3.8	7.6	11.4
III	3.8	3.8	7.6
Total %	7.6	11.4	19

Image analysis of biomarker expression showed that normal terminal-duct lobular unit with *p63* expressing myoepithelial cells, which served as positive internal control as shown in figure A. Figures B-F, Strong and diffuse expression of *p63* in metaplastic carcinomas. *P63* Expression in Invasive Carcinomas of the Breast, *p63* was expressed in the nuclei of myoepithelial cells of normal ducts and lobules adjacent to the carcinomas, which also served as internal positive controls in all cases. *P63* was negative in all the other invasive ductal, lobular and mixed ductal and lobular carcinoma as shown in Figure 1.
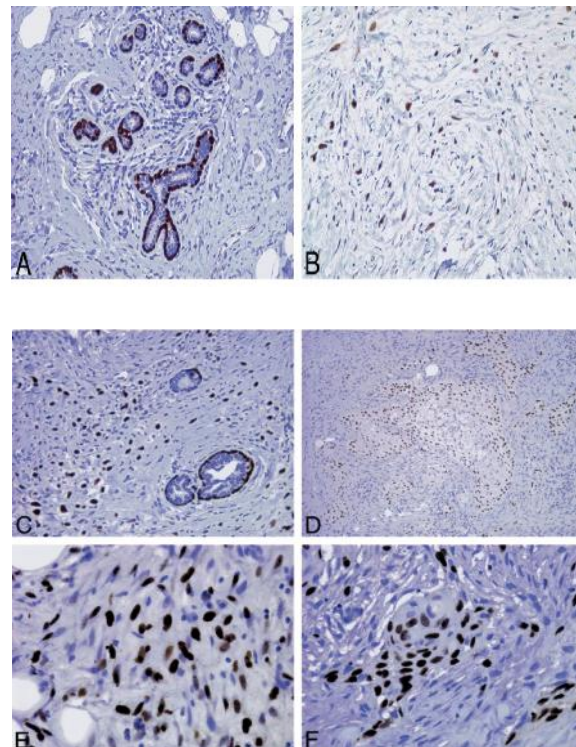

Figure -1-: (A) Normal terminal-duct lobular unit with *p63* expressing myoepithelial cells. (B-F) Strong expression of *p63* in metaplastic carcinomas. The image analysis also explained Basaloid squamous cell carcinoma. The *p63* immunohistochemical staining demonstrating diffuse nuclear staining as clarify in

Figure -2-

Figure -2-: *p63* immunohistochemical staining demonstrating diffuse nuclear staining. Basaloid squamous cell carcinoma.

The detection of mutation by using p53 biomarker showed that *p53* mutation is the most common genetic abnormality found so far in human cancer,

and in breast cancer *p53* mutation/alteration is seen in 15.3% of breast carcinomas Figure -3-.
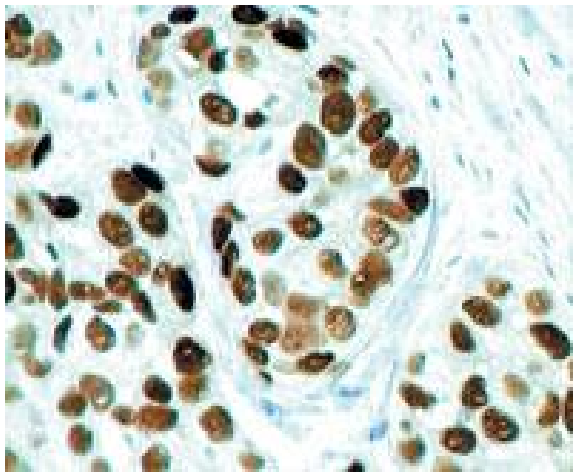
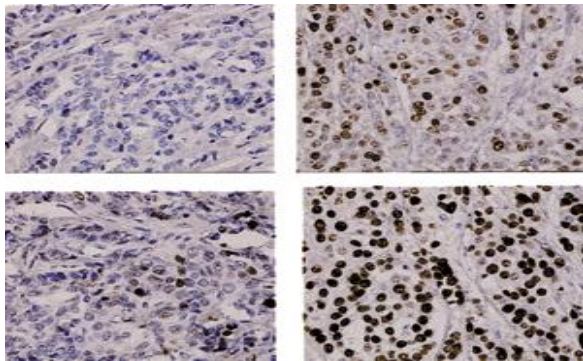

Figure -3-: *p53* mutation in breast cancer

Figure -4-: four sections of different breast cancers stained with DO-7 to detect *p53* protein.

DISCUSSION

Cancers occur when a there is an increase of genetic mutations in critical genes—those that control cell growth and division or the repair of damaged DNA—allow cells to grow and divide uncontrollably to form a tumor. In most cases, these genetic changes are acquired during a person's lifetime and are present only in certain cells. These changes, which are called somatic mutations, are not inherited. Less commonly, gene mutations inherited from a parent increase the risk of developing cancer. In people with these inherited genetic changes, additional somatic mutations in other genes must occur for cancer to develop.

According to change in breast tissue; breast disease is originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Cancers originating from ducts known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. It may be benign, as in fibro adenoma, or it may be malignant, in which case it is termed breast cancer (Schuetz et al., 2006).

This study is to evaluate the prognostic value of the expression of *p63* and *p53* genes in breast cancer. In addition, to identify the association of molecular markers (*p63* and *p53*) with breast cancer in different grades and stages were performed on women and men attending in main hospitals in the western region of Saudi Arabia.

In the current study, molecular markers of *p63* and *p53* genes were evaluated in breast cancer in Saudi Arabia. In 26 samples we found *p63* was express in (19.1%) more than *p53* (15.3%) of breast cancer. In compared with other study in carcinomas, *p63* was expressed only in poorly differentiated ductal carcinomas (11.76%) of cases whereas *p53* was expressed in (21.17%) of carcinomas (Ribeiro-Silva et al., 2003). Since their initial identification *p53* homologues *p63* and *p73* have been expected to play a role in cancer development due to their close homology to *p53*, notoriously one of the most mutated genes in cancer. However soon after their discovery the awareness that these genes were rarely mutated in cancer seemed to indicate that they did not play a role in its development. However a large number of data collected in the following years indicated that altered expression rather than mutation could be found in different neoplasia and play a role in its biology. In particular *p63* due to its fundamental role in epithelial development seems to play a role in a number of tumors of epithelial origin (Graziano and De Laurenzi, 2011).

According to the results obtained, *p53* expressed in 3.8% axillaries lymph node, 3.8% of intraductal axillaries lymph node, 3.8% of ductal carcinoma, 7.6% of phyllodes tumor, 7.6% of invasive duct carcinoma (IDC), and 3.8% of DCIS. Comparing with the results published by Rudasa and others in 1997, they used 121 carcinomas and found 19% was lobular in situ carcinomas (LCIS), 61.2% was intra ductal carcinomas (DCIS) and 19.8% was minimal invasive carcinomas (Rudasa et al., 1997). The variation between their results and our results obtained may be due to the amount of samples size, as our samples size was 26 samples and they used 121 samples. Also it can be due to the types of carcinomas samples used.

The expression of *p63* was found in 3.8% of axillaries lymph node, 7.6% of invasive ductal carcinoma (IDC), 3.8% of ductal carcinoma in situ (DCIS), 3.8% of lobular carcinoma in situ (LCIS), and no phyllodes tumor and this agreements with previous study (Koker et al., 2004).

The expression of *p63* in myoepithelial cell which, positivity more than 80% and this agreements with previous studies (Hsiao et al., 2010).

The literature strongly suggests that *p63* is necessary for normal development of epithelial organs and may be essential for the maintenance of a stem cell population in various epithelial tissues, beings marker of reserve cells. *P63* expression in normal breast and in metaplastic carcinomas.

Conclusions

In conclusion the *p63* is a specific myoepithelial cell marker in normal breast tissue and is expressed in a minority of breast carcinomas, being seen only in grade III,II ductal carcinomas. In ductal carcinomas, malignant *p63*-positive cells have an immunophenotype similar to that of myoepithelial cells, suggesting that these cells originate from a primary progenitor cell that underwent divergent differentiation to ductal and myoepithelial cells during clonal expansion. Our study argues against a direct role in mammary tumorigenesis.

However, *p53* is rarely coexpressed with *p63*, suggesting that *p63* could act indirectly as an oncogene by inhibiting *p53*. This hypothesis could also explain why *p63* correlated with several other indicators of poor prognosis.

Acknowledgements

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