# Molecular study of p53 supressor gene and Bcl-2 oncogene by DNA In Situ Hybridization technique in breast cancer patients in Iraq\Wasit Province

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# دراسة جزيئية للجين الكابح للأورام p53 والجين المنشط للأورام BCl-2 باستخدام تقنية التهجين الموضعي للدنا لمريضات سرطان الثدي

## محافظة واسط-في العراق

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**المستخلص**

استخدمت تقنية التهجين الموضعي للدنا لتقيم التعبير الجيني للجينين (Bcl-2, p53) في مختلف الانواع النسيجية لسرطان الثدي في النساء العراقيات المصابات بسرطان الثدي وتعيين هذين المعلمين الحيويين مع مختلف المعايير المرضية والسريرية ( درجة الورم, مرحلة الورم, العمر, انتشار الورم). اجريت هذه الدراسة على 46 مريضة .تتراوح اعمارهن بين (20-77) سنة وكان المعدل العمري(08,46) للفترة بين (ايلول 2012 – ايار 2013 ). اظهرت النتائج ان نسبة التعبير الجين p53 كانت (63.04) بينما كانت نسبة تعبير الجين Bcl-2(52.17) ,وبفرق معنوي (p=0.001) لكلا الجينين.كما اوضحت النتائج ان هناك فرق معنوي بين تعبير كل من هذين المعلمين وبين درجة الورم ومرحلة الورم (p=0.05) .وكذلك فرق معنوي بين تعبير الجين p53 وبين عمر المريضات (p = 0.05).تؤيد نتائج هذه الدراسة الدور المهم لهذه الجينات في عملية التسرطن وامكانية استخدامها كمعلمات دالة في تشخيص الورم .

## Abstract

DNA In Situ Hybridization technique was used to estimate overexpression of p53 and Bcl-2 in different histological type of Iraqi female breast cancer patients and to assess whether these biomarkers are significantly correlated with clinicopathological parameters (tumor grade , stage , age and invasiveness ) of breast carcinoma .The study included forty six patients, their ages ranging between 20-77 years with a mean age of (46.08) years, between Septembers 2012 and May 2013. The results showed that p53 overexpression frequency was (63.04) and Bcl-2 was (52.17) in patients with significant differences (p=0.001). Also the results showed asignificant differences between the expression of each of these biomarkers with tumor grade and stage (p=0.05). As well as there was significant difference between the expression of p53 and patient age (p=0.05). The results of these study supported the importance role of these genes in carcinogenesis and ability to use both genes as a markers in diagnosis.

## Introduction

Globally, breast cancer (BC) is the most common cancer among women, comprising 23% of the 1.1 million female cancers that are newly diagnosed each year (1, 2) It is also the leading cause of cancer-related deaths worldwide, case mortality rates being highest in low resource countries (3) Approximately 4.4 million women diagnosed with breast cancer in the last 5 years are still alive, making breast cancer the most prevalent cancer worldwide (1) In Iraq, breast cancer is the commonest type of female malignancy, accounting for approximately one-third of the registered female cancers according to the latest Iraqi Cancer Registry (4). This shows that the breast is the leading cancer site among the Iraqi population in general, surpassing even bronchogenic cancer. As proposed by the World Health Organization, early detection and screening, especially when combined with adequate therapy, offer the most immediate hope for a reduction in breast cancer mortality (5)

A large number of mutated genes play an important roles in the pathogenesis as well as breast cancer response to chemotherapy (6). Over the past few decades, a number of potential prognostic markers for breast cancer have been extensively investigated (7, 8, 9, 10). With the development of innovative techniques for gene expression profiling, novel molecular markers and biologic factors appear to have more important roles than more traditional prognostic factors (11, 12, 13 ,14). Mutate p53 gene or p53 overexpression has been observed in 20–50% of primary breast tumors (15). Several studies have found that mutation or overexpression of p53 is significantly associated with young or pre-menopausal patients (16, 17). The importance of some molecular markers in breast cancer has been of considerable interest during recent years, not only as prognostic markers, but also as predictors of response to therapy. p53 is the primary arbiter of the mammalian cells' response to stress. In its normal form, p53 can be involved in the induction of apoptosis and thus has a regulatory function over the cell cycle. In its mutant form, p53 inhibits apoptosis, loses control on cell cycle progression and thus helps tumor formation (18). Nuclear p53 accumulation which associates with p53 mutation is one of the most common events during breast carcinogenesis (19, 20, 21). The overexpression of bcl2 can prevent apoptosis in cells that are damaged. This can lead to the continued division of the mutated cells lines and eventually cancer. Also, overexpression of Bcl-2 can contribute to metastasis in certain cancers (22). Bcl-2 One of the main genes limiting apoptosis is paradoxically, its expression has been consistently associated with a bad prognosis of breast cancer patients (23). The bcl-2 anti-apoptotic gene is overexpressed in a majority of breast cancers, and is associated with a diminished apoptotic response and resistance to various antitumor agents (24).

## Materials and methods

### Patients and tissue sample

forty six patients with breast carcinoma, with an age ranged from (20 to 77) years, were included in this retrospective study, The patients' samples were collected from the archives of histopathology laboratories of Al-Karama Teaching Hospital and AL-Zahraa Teaching Hospital in Kut city between Septembers 2012 and May 2013. The diagnosis of these tissue blocks were primarily based on the obtained histopathological records of breast biopsy samples that had been accompanied in the hospital laboratory. Confirmatory histopathological re-evaluation of each obtained tissue blocks was done by specialist pathologist. In addition ten benign breast lesions the range of the age was the same as patients group. Formalin fixed paraffin embedded blocks tissue were sectioned (5μm) thickness, from each tissue

block, were mounted on charged slides to be used for *In situ hybridization* for the detection of p53, bcl2 gene.

**In Situ Hybridization procedure**

Serial tissue sections were cut (5µm) thick and were positioned on positive charged slides. The slides were backed by placed in oven at 60˚C overnight. The tissue sections were deparaffinized; the slides were dehydrated by graded alcohol concentration (100%, 95%, and 70%) and distal water. The slides were treated with proteinase K solution and dehydrated. One drop of the biotinylated DNA probe for human p53 and bcl2 (Maxim Biotech Cat. No.: IH-60001 (IHD-0050)). Hybridization/detection kit were used purchased from Maxim Biotech/USA Cat.Number IH-6001(IHD-0050) was placed on the tissue section in oven at 98˚C for 8-10 minutes. After that the slides were placed in a humid chamber and incubated over night at 37°C to allow hybridization of the probe with the target nucleic acid. The slides were soaked in 1X detergent washing buffer at 37˚C until the cover slips fall, and then treated with RNase A solution and streptavidin-AP-conjugate. One to two drops of 5-bromo-4-chloro-3-indolyl phosphartel/nitro blue tetrazolium substrate-chromogen solution (BCIP/NBT) conjugate were placed on tissue section at room temperature for about 30 minutes; the latter was monitored by viewing the slides under the microscope. A blue colored precipitate will form at the site of the probe in positive cells. Slides were then counterstained using nuclear fast red and sections were mounted with a permanent-mounting medium (DPX). Finally the examination and scoring were done under light microscope by a pathologist at power 400 according to the scoring system. (25)

**Statistical analysis**

Statistical analyses of all results were preceded by the help of SPSS program .Values were considered statistically significant when $p<0.05$, and use Chi-square test to Comparison between groups. Correlation and Fisher's exact test, Binary Logistics Regression analysis were also performed.

## Results

The study processed the malignant breast cancer and benign breast lesions (fibroadenoma). These tissues checked up for apoptotic genes p53 and Bcl-2, using In situ hybridization technique. This study involved 46 Iraqi females' patients with breast cancer; their mean age was 46 ± 1.50 years with a range of (20 to 77) years attending Al-Karama Teaching Hospital & AL-Zahraa Teaching Hospital, Wasit city between Septembers 2012 and May 2013 ,compared with 10 patient's control (with benign breast lesions: fibroadenoma). (Table 1)

**Table (1): Descriptive statistics of age of the studied breast cancer patients**

| | Range | Lower | Upper | Mean | Std. Error | Std. Deviation |
|---|---|---|---|---|---|---|
| **Age** | 57.00 | 20.00 | 77.00 | 46.0870 | 1.50163 | 10.18458 |

**p53 and Bcl-2 In Situ Hybridization**

The p53 overexpression was reported in 63.04% (n= 29) out of 46 cases of breast carcinoma and Bcl-2 overexpression was reported in 24(52.17%) out of 46 cases of breast cancer as shown in Table (2). We see highly significant differences between both genes expression in breast cancer and control breast tissues (p=0. 001, p=0.001) respectively. There correlation between p53 and Bcl-2 expression with clinicopathological variables of breast cancer patients histological type, grade, stage, age group and invasiveness. p53 overexpression was obvious in 29 cases, whereas negative was 17 cases. Analysis of overexpression p53 in relation to grade of tumor revealed that positive p53 was reported 2(6.9%)grade I, 23(79.3%)grade II, and 4(13.8%) grade III out of 29 cases. While Bcl-2 positive overexpression was reported, in 1 (4.2%) grade I, 19 (79.2%) grade II and 4 (16.7%) grade III. These results showed a highly correlation between both genes with grade (p=0.001 and p=0.002) respectively as shown as in Table(3). The same table demonstrated a positive correlation between p53 with stage and age group (p=0.003 and p=0.05) respectively. On the other hand Bcl-2 showed a highly positive correlation with stage (p=0.003). No correlation with other variables histological type and invasiveness. In all sections of control breast tissues (fibroadenoma), did not expressed neither p53 nor bcl2 (Fig 1 A, 2A), whereas tumor tissues did (Fig 1B, 2B).

**Table (2): Expression of p53 and bcl2 in 46 breast cancer patients and 10 control group**

| Cases | Marker | Positive frequency | Percent | Negative frequency | Percent | P-value |
|---|---|---|---|---|---|---|
| **Breast cancer** | P53 | 29 | 63.04% | 17 | 36.96% | o.oo1 |
| **Control group** | P53 | 0 | 0% | 0 | | |
| **Breast cancer** | Bcl2 | 24 | 52.17 | 22 | 47.83% | o.oo1 |
| **Control group** | Bcl2 | 0 | O% | 0 | 0% | |

Significant p< 0.05

**Table (3): Correlation between P53 and Bcl2 genes expression and clinicopathological variables**

| Variables | P53 expression | | Total | P value | Bcl2 expression | | Total | P value |
|---|---|---|---|---|---|---|---|---|
| | Positive | negative | | | Positive | negative | | |
| **Histological type** | | | | | | | | |
| **Ductal** | **26(89.8)** | | **43(93.5)** | **0.597** | **21(87.5)** | **22(100%)** | **43(93.5)** | |
| **Lobular** | **17(100%)** | | **1(2.2)** | | **1(4.16)** | **0(0%)** | **1(2.2)** | **0.597`** |
| **Medullary** | **1(3.4)** | | **1(2.2)** | | **1(4.16)** | **0(0%)** | **1(2.2)** | |
| **Papillary** | **0(0%)** | | **1(2.2)** | | **1(4.16)** | **0(0%)** | **1(2.2)** | |
| | **1(3.4)** | **0(0%)** | | | | | | |
| | **1(3.4)** | **0(0%)** | | | | | | |
| **Tumor grade** | | | | | | | | |
| **Grade 1** | **2(6.9)** | **9(52.9)** | **11(23.9)** | | **1(4.2)** | | **11(23.9)** | **0.002** |
| **Grade 2** | **23(79.3)** | | **31(67.4)** | **0.001** | **10(45.5)** | | **31(67.4)** | |
| **Grade 3** | **8(47.1)** | | **4(8.7)** | | **19(79.2)** | | **4(8.7)** | |
| | **4(13.8)** | **0(0%)** | | | **12(54.5)** | | | |
| | | | | | **4(16.7)** | | | |
| | | | | | **0(0%)** | | | |
| **Tumor stage** | | | | | | | | |
| **Stage 1** | **2(6.9)** | **8(47.1)** | **10(21.7)** | | **1(4.2)** | **9(40.9)** | **10(21.7)** | |
| **Stage 2** | **13(44.9)** | **8(47.1)** | **21(45.7)** | **0.003** | **11(45.8)** | **10(45.5)** | **21(45.7)** | **0.003** |
| **Stage 3** | **7(24.1)** | **1(6)** | **8(17.4)** | | **5(20.8)** | **3(13.6)** | **8(17.4)** | |
| **Stage 4** | **7(24.1)** | **0(0%)** | **7(15.2)** | | **7(29.2)** | **0(0%)** | **7(15.2)** | |
| **Age group** | | | | | | | | |
| **0-25** | **2(6.9)** | **0(0%)** | **2(4.3)** | | **2(8.3)** | | **2(4.3)** | |
| **26-50** | **20(69.0)** | | **36(78.3)** | **0.049** | **0(0%)** | | **36(78.3)** | |
| **51-75** | **16(94.1)** | | **7(15.2)** | | **17(70.8)** | | **7(15.2)** | **0.229** |
| **75-100** | **7(24.1)** | **0(0%)** | **1(2.2)** | | **19(86.4)** | | **1(2.2)** | |
| | **0(0%)** | **1(5.9)** | | | **5(20.8)** | | | |
| | | | | | **2(9.1)** | | | |
| | | | | | **0(0%)** | | | |
| | | | | | **1(4.5)** | | | |
| **Invasiveness** | | | | | | | | |
| **Invasive** | **26(89.7)** | **14(82.4)** | **40(87.0)** | **0.478** | **22(91.7)** | **18(81.8)** | **40(87.0)** | **0.322** |
| **Noninvasive** | **3(10.3)** | **3(17.6)** | **6(13.0)** | | **2(8.3)** | **4(18.2)** | **6(13.0)** | |

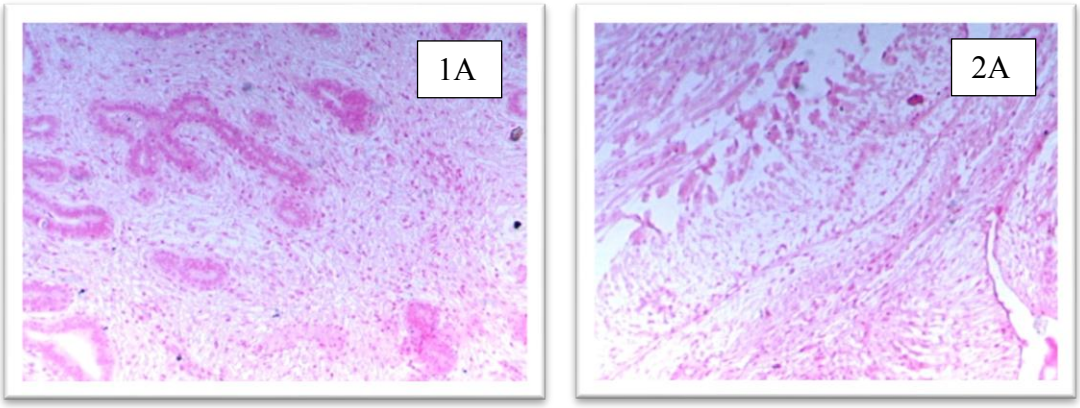


**Figure (1):**(1A) ISH of p53 in benign breast lesion by nuclear fast red stain 40x
(2A) ISH of bcl2 in benign breast lesion by nuclear fast red stain 40X

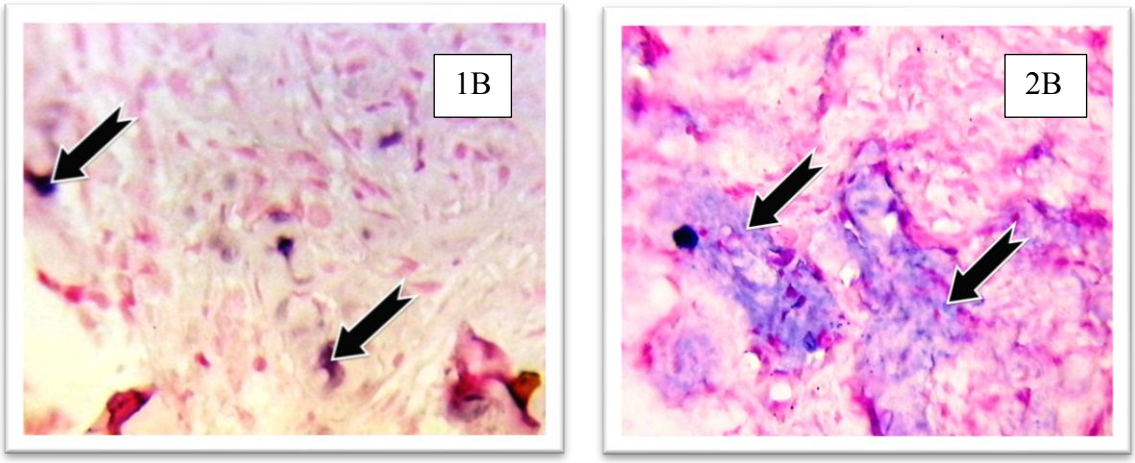


**Figure (2** ):(1B) ISH of P53 in breast cancer patients by nuclear fast red stain 40x
(2B) ISH of Bcl2 in breast cancer patients by nuclear fast red stain 40x

## Discussion

The breast cancer is the most common tumor in females; constituting 14.3% of all malignant tumors and 30% of registered Iraqi female cancers (26). Our study was designed to assess the p53 and bcl-2 expression in a series of Iraqi women with breast cancer. According to the previous studies most of the Iraqi patients are diagnosed in young age groups with late stage at presentation and a prevalence of more aggressive tumors(27,28) .The biology of breast carcinoma remains poorly understood as the knowledge about individual prognostic factors provides limited information **(**29**)**. A wide variety of morphology-based and molecular-based prognostic factors and tumor markers had been studied according to their potentials to predict the outcome in breast cancer. Verifying molecular abnormalities in breast cancer is an important strategy for its early detection, assessment of prognosis, and treatment selection **(**30**)..**One major goal of this study was to choose an appropriate and reliable prescreening method for p53, bcl2 mutation analysis. We performed In situ hybridization analysis targeting p53, bcl2 gene in tumor

cells. p53 gene is predicted to accumulate in nucleus when the mutation is occurring. Up to our knowledge this is the first study in our Province in this field. In the current study, the mean age of patients was (46.08±1.50) with a range of (20 – 77 years). The peak age frequency was 26-50 years which constituted (78.26%) our result agree with Ibraheem Yassen Hachim (2005)**(**31), Enas A. M.R. (2005)(32), Ban Jamal (2006)(33), Estabraq Abd Al-Rsool (2007)(34), Hassanien Ghassan(2009)(35). Accordance with age range our results agree with Al-Anbary S. S.(2009)(36), Manwar Abdulelah(2009)( 37 ). The peak age frequency was agreed with Al-Anbary S. S.(2009)(36), Alwan N.A.S.(2010) (38). These findings are one decade lower than United States (average age at diagnosis is 64 years) (39). Life style, environmental factors and genetics are important contributing factors in such differences (40). Although there have been many reports on p53 and bcl2 expression in human breast cancer carried out by multiple molecular genetic technique, there are only a few published studies involving application of the p53 and bcl2 expression using ISH technique.

The current study demonstrated that although completely absent p53 and bcl2 expression in benign breast lesion, other study found Bcl-2 was expressed in less than 1% of normal cells (41), conversely there was a significant overexpression of p53, bcl2 among the 46 investigated breast carcinoma (P value < 0.001) (Table. 2).

The results have clarified that 63.04% cases of breast cancer were expressing p53 in situ hybridization in their histological sections. The result of p53 expression our results disagree with Hiroko (2006)(42).

The differences in marker expression were due to sample size, samples are all fresh and newly diagnosed not receive any hormonal therapy, chemotherapy or radiotherapy and might be due to different selection criteria of the studied population. Breast cancer has a highly variable prognosis and benefit from available therapies is unpredictable for the individual patient. Key factors such as tumour size, histological grade, vascular invasion, and nodal status are helpful, but increasing attention is being paid to the molecular features of the tumour (43) indeed, not all mutations yield a stable protein and some mutations lead to a truncated protein not detected by IHC. On the other hand, wild-type p53 may accumulate in some tumours as a result of a response to DNA damage or by binding to other cellular proteins, giving a positive IHC result. Breast tumours expressing a high amount of p53 (as measured by IHC). They are also associated with a high proliferation rate, high histological and nuclear grades, aneuploidy, and poorer survival. The bcl2 antiapoptotic gene is overexpressed in a majority of breast cancers, and is associated with a diminished apoptotic response and resistance to various antitumor agents (24). A study made by Dema et al. showed that on a group of post-menopausal women with breast carcinomas, bcl-2 protein was expressed more by the small sized, well and moderately differentiated, hormone-dependent and with low proliferative activity tumors. Regarding the Bcl-2 protein/gene (44), ( Grace, et al.) the one would predict that aberrations of the Bcl family of proteins might be prevalent in breast cancer given that impaired apoptosis is a crucial step in neoplastic progression and that the p53/Rb signaling pathway is dysregulated in most tumors. Bcl-2 belongs to the Bcl family of proteins that regulate apoptosis; whether a cell undergoes apoptosis or survives depends on the relative expression and dimerization status of the proapoptotic (Bax, $Bcl_{Xs}$, Bas, Bik/Nbk, Bid, and Bag-1) and antiapoptotic (Bcl-2, $Bcl_{Xl}$, Bcl-w, A1, and Mcl-1) proteins. An increase in Bcl-2 shifts the balance in favor of cell survival (45). Like the result in our study, Al-Joudi, et al., show that the overall reported expression of p53 in breast cancer ranged from 9% to 69% (46). In the present work, p53 was detected in 63.04% of the cases. These results were comparable with previous reports especially regarding the correlations with histologic grading.

The relatively high expression of p53 may be attributed to genetic and environmental factors that dictate the p53 mutation type. Mutant p53 may itself be a candidate for tumor therapy since the down regulation of p53 can result in reduction in tumor aggressiveness. p53 detection was significantly associated with higher grades of tumors, and may thus serve in directing clinical decisions regarding diagnosis, therapy, and prognosis.

We further investigated the relationship of cDNA p53 and bcl2 gene expression with clinicopathological features of breast tumor (Table 3), our results found a highly positive significant correlation between p53 expression and grade (p=0.001) and stage (p=0.003) and age group (p=0.05), No significant relationship of p53 expression with histological type nor invasiveness was observed. Regarding of bcl2 expression the finding referred to a positive significant correlation between bcl2 overexpresssion and grade (p=0.002) and stage (p=0.003), no significant correlation with other pathological variables. This suggested that p53 and bcl2 might play an important role in breast cancer progression. Indeed, several other studies show that p53 and bcl2 overexpression correlates with clinicopathological variables for breast carcinoma. Other studies have reported that Bcl2 is associated with histological prognostic parameters and patient prognosis in prostate cancer (47,48).

## Conclusion

1- Positive overexpression of both genes (p53, bcl2) associated significantly with grade and stage of tumour.
2- p53, bcl2 overexpression play an important role in carcinogenesis of breast cancer evolution, as their positivity associated with biologically aggressive of tumours , so incorporation of these biomarkers with other parameters as grade, stage, age group, and invasiveness into a prognostic index will more accurately predict clinical outcome .
3- The criteria of patients with breast cancer at first presentation including environmental risk factors; and the low age, the excess of high-grade, the advanced stage, may suggest a role of genetic predisposition in developing the cancer in this population.

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