Iranian Biomedical Journal 5 (4): 127-132 (October 2001)

Immunohistochemical Evaluation of Human p53 Tumor Suppressor Protein Content in Ductal Carcinoma *in Situ* of the Breast

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ABSTRACT

The focus of this study was to determine if early detection of mutant p53 accumulation may be an early indicator of tumor aggressiveness and transformation to invasive breast cancer. For this purpose, the p53 content of 100 human breast biopsies classified as ductal carcinoma (DCIS), was evaluated by immunohistochemical method. All specimens were microscopically classified into histologic types of DCIS and nuclear grade. From this population, 15 specimens were immunopositive and six of them were converted to more invasive form. Most immunopositive specimens were classified as mixed histologic types. No relationship between nuclear grade and different histologic types of DCIS was noticed. Preliminary data indicated that the presence of immunopositive p53 may be a valid predictive indicator of the prognosis of individuals with DCIS and transformation to invasive breast cancer. *Iran. Biomed. J. 5 (4): 127-132, 2001*

Keywords: Immunohistochemical, Ductal carcinoma *in situ* (DCIS), Nuclear grade, Histologic types, p53

INTRODUCTION

The human p53 gene encompasses 16 to 20 kilobase (kb) of DNA on the short arm of chromosome 17 at position 17p 13.1 [1, 2]. The p53 gene codes for a homodimeric 393 amino acid nuclear phosphoprotein (with approximately 53 kDa MW). This gene has been conserved during evolution. In cross-species comparison, the p53 protein showed five highly conserved regions within the amino acid residues: 13-19, 117-142, 171-181, 234-258, and 270-286. These five evolutionary conserved domains within the coding regions are regarded as essential to the function of the p53 [3].

The p53 protein acts as a transcription factor and serves as a key regulator of the cell cycle [4, 5] and apoptosis [6]. Loss of normal p53 function can be reached in a variety of ways [5]. The most common change of p53 in human cancers is a point or missense mutation (73%) within the coding regions of the p53 gene resulting in an altered and a stable protein. The inactivation of the p53 by mutation disrupts the cell cycle, which in turn, sets the stage for tumor formation [5].

Evidence is accumulating that mutations in the p53 gene are present in 27-54% of primary invasive breast carcinomas [7]. However, studies of p53 expression in ductal carcinoma *in situ* (DCIS) have been limited [8]. DCIS is generally referred to as tumors arising from duct epithelium that are confined within the lumen of the ducts or lobules of the breast [9]. DCIS is heterogeneous in terms of its histopathologic growth pattern, cell type and biologic behavior. It is categorized into cribriform, micropapillary, papillary, and solid type. All types of DCIS may be found as "pure" or "mixed" forms [9]. This classification is based on the histologic growth pattern and considers comedo necrosis to be an independent feature of DCIS rather than a specific histologic type.

The present study will qualitatively evaluate p53 tumor suppressor protein expression in 100 breast tissue samples with DCIS by standard immuno-histochemistry techniques [10].

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MATERIALS AND METHODS

Source of tissue. Samples were acquired from women that had surgical removal of breast tumor/lumps for pathologic evaluation at St. Lawrence Local Hospital in E-Lansing (Michigan, USA). These samples were processed by routine procedures after immediate immersion in 10% Formalin saline. Preserved samples were then subjected to: dehydration and embedding in paraffin blocks. Five-micron (5μ) sections were cut and fixed on slides pretreated with poly-L-lysine to prevent detachment of tissues from the glass slides. The sections were first evaluated microscopically by a pathologist, after standard hematoxylin and eosin staining [12]. Tissue sections on glass slides were forwarded to our laboratory on specimens that had been classified as DCIS by initial pathologic evaluation. Along with these samples data on tumor histology and patients characteristics were obtained from the pathology reports.

Immunohistochemical technique.
Deparaffinized sections were first incubated with monoclonal antibody (Pb 1801, Ab-2, Oncogene science). The unbound antibody was removed by washing. Then, the secondary biotinylated anti-mouse was added followed by incubation with Avidin-Biotinylated horseradish peroxidase macromolecular complex (ABC). Substrate, Diaminobenzidine (DAB), was added and product was visualized under light microscopy as a brown precipitate [10].

Pb-1801, Ab-2 is a mouse monoclonal antibody generated by immunizing BALB/c mice with p53 protein and spleen cells were fused with NS-1 mouse myeloma cells [13]. The mouse anti p53 Ab (p53 Ab-2) reacts with a denatured stable determinant of p53 [14]. Furthermore, the p53 Ab-2 recognizes the p53 of human cellular origin only and reacts with an epitope located near the amino end of all known forms of p53. Thus, it allows for the detection of accumulation of most forms of this protein [14].

Dilution of the monoclonal antibody Pb1801, Ab-2. IgG monoclonal antibody to human p53 protein used in the immunohistochemical procedure was 1/200 dilution of the stock protein produced by the manufacture. This dilution produced the highest degree of sensitivity and specificity, as determined by checkerboard analysis. When 1:50 dilution of antibody was used, cytoplasmic staining of normal breast tissue sections was noticed and the interpretation of nuclear reactivity was difficult. Positive results were considered only in conjunction with nuclear staining (Table 1).

Positive and negative controls. For each assay, formalin-fixed and paraffin-embedded tissue section of human colon cancer was used as positive control to ensure interassay consistency. Colon carcinoma tissue sections were known to have mutations of the p53 protein [15]. For the negative control, PBS (without antibody) and (purified mouse myeloma IgG, 1:200) were used instead of the primary antibody. Benign breast tissues were also studied for p53 immunoreactivity to role out the detection of the wild-type p53 by the primary antibody.

Grading system. The grading system was based on binding of the primary anti p53 antibody and subsequent evaluation of the horseradish peroxidase activity that generates a brown precipitate. The presence of brown precipitate in the nucleus of tumor cells was scored semiquantitatively for the proportion of the cells stained darkly within the tumor [16]. The staining was given scores from 0 to 3; (0, no staining; 1-30% = 1+, 31-60% = 2+, and > 60% = 3+).

Table 1. Checkerboard analysis for the determination of primary antibody concentration.

Primary Ab dilution	1:50	1:150	1:200	1:250	1:300
Positive breast tissue	3+	1+	2+	0.00	0.00
Negative breast tissue	Cytoplasmic	Cytoplasmic	0.00	0.00	0.00

Table 2. Histologic types of DCIS and p53 immunostaining

Histologic Patterns	P53 (+) Cases	Percent (%) P53 (+)/p53 (+) Total	P53 (-) Cases	Percent (%) p53 (-) /p53 (-)Total
Total	15	100.0	85	100.0
Mixed	10	66.6	44	51.8
Cribriform	2	13.3	11	12.9
Papillary	1	6.6	7	8.2
Solid	2	13.3	21	24.7
Micropapillary	0	00.0	2	2.4

Tumor grading. Each specimen was classified based on histologic presentation. Tumors were classified according to the predominant cellular architectural pattern: solid, cribriform, micro-papillary, and papillary types. This classification was only applied when such a pattern corresponded to more than 75% of the tumor. When no pattern predominated, the tumor was classified as mixed.

Nuclear grading was defined as grades 1-3 in order of increase pleomorphism with regard to the invasive carcinoma [17]. Pleomorphism of the nucleus and the presence of nucleoli were used as the criteria for grading. A nuclear grade was assigned in a semiquantitative manner by comparative evaluation of nuclei in adjacent normal breast duct as small (1-2x size), intermediate (3-4x size), or large (5x size or more).

RESULTS

Positive and negative control. Sections of colon carcinoma were used as the positive control [15]. These sections showed intense p53 staining (Fig. 1). Two negative controls were used with each assay, One control was section of colon carcinoma which had not been treated with primary antibody to the p53 (mouse myeloma IgG and secondary anti-mouse IgG and ABC reagent). The second control was benign breast tissue section that had been treated with standard staining procedure (Figs. 2 & 3).

Intense p53 staining in DCIS tissue samples have shown in Figures 4 & 5. These tissue sections were stained with avidin-biotin immunoperoxidase staining.

Histologic classification, nuclear grade, and p53 content. Fifteen out of 100 DCIS cases exhibited nuclear immunostaining for p53. Analysis of DCIS with regard to frequency of the predominant histologic pattern showed that mixed type was the most common pattern occurring in 54% of the cases. Solid pattern occurred in 23%, cribriform in 13%, papillary in 8%, and micropapillary occurred in 2% of the cases. Approximately 23% of all mixed forms of DCIS were p53 positive. In addition, comedo necrosis was present in all p53 positive DCIS cases.

Nuclear grade 3 was seen in one of the positive DCIS cases (13%). Nuclear grade 2 was seen in 4 (27%), and nuclear grade 1 was seen in 10 (67%) cases (Tables 2 and 3).

Table 3. Nuclear grading of immunopositive p53 DCIS samples.

Histologic pattern	p53 Grade	Nuclear Grade
Mixed:		
Case #1	3+	1
Case #2	1+	2
Case #3	1+	3
Case #4	1+	1
Case #5	1+	1
Case #6	1+	1
Case #7	1+	1
Case #8	1+	1
Case #9	1+	1
Case #10	1+	1
Solid:		
Case #1	2+	1
Case #2	1+	2
Cribriform:		
Case #1	3+	1
Case #2	2+	2
Papillary		
Case#1	1+	2

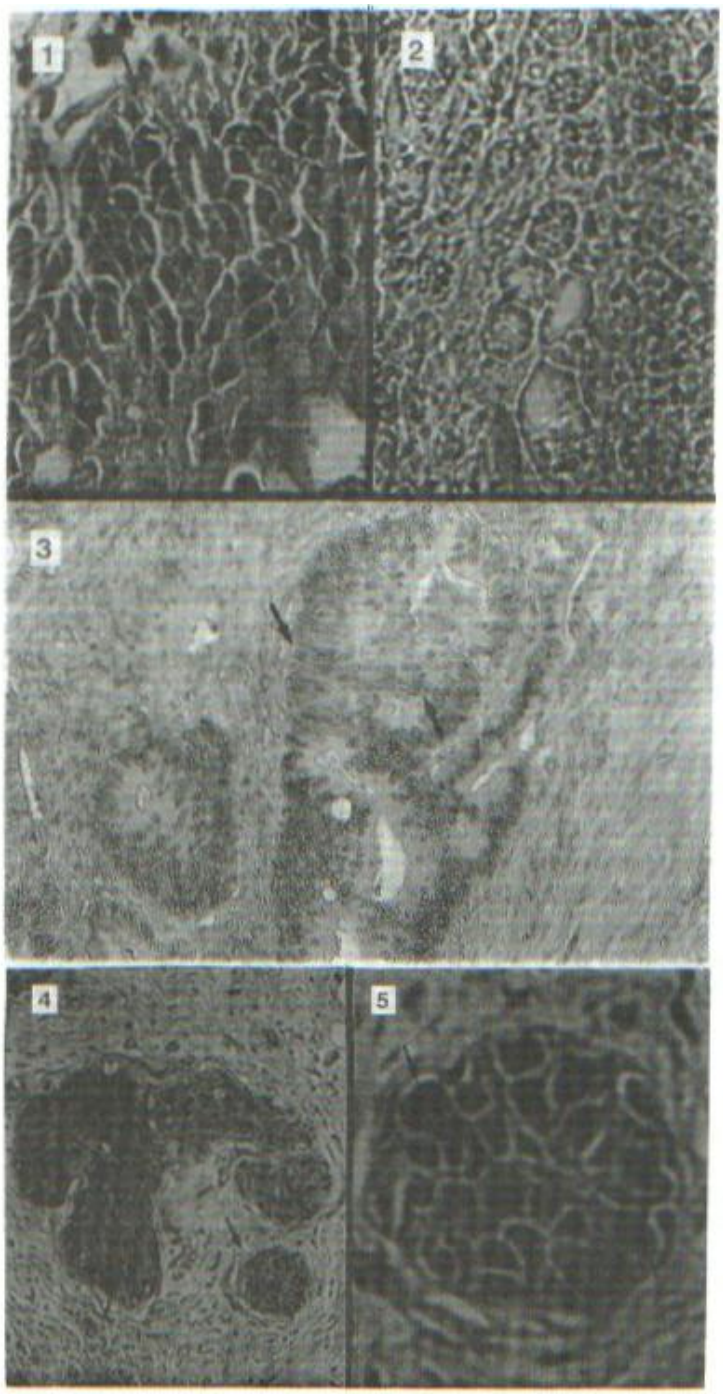

Fig 1. Detection of mutant p53 protein accumulation by immunohistochemistry. **(1)** p53 immunopositivity in colon tissue section. This section of tissue was stained using avidin-biotin immunoperoxidase staining methodology. Arrows indicate deposition of brown pigment showing a reactive positive result, magnification 100 × oil. **(2)** p53 immunonegativity in colon tissue section. Mouse myeloma IgG was used instead of the primary antibody (Pb1801, Ab-2). Arrows indicate no deposition of brown pigment showing a non- reactive result, magnification 100 × oil. **(3)** p53 immunonegativity in benign breast tissue section. This section of tissue was stained using avidin-biotin immunoperoxidase staining methodology. Arrows indicate no deposition of brown pigment showing a non-reactive result, magnification 40 ×. **(4)** p53 immunopositivity in DCIS breast tissue section. This section of tissue was stained using avidin-biotin immunoperoxidase staining methodology as well. Arrows indicate deposition of brown pigment showing a reactive positive result, magnification 40 ×. **(5)** Magnified section of section 4, indicating intense p53 immunopositivity in DCIS breast tissue section, magnification 100 × oil.

DISCUSSION

Despite the fact that a number of molecular and cellular markers have been proposed as prognostic indicators for breast cancer, p53 tumor suppressor protein appears to be valuable prognostic marker for many types of carcinomas including breast tumors [18]. Molina *et al.* [19] recently have observed p53 positiveness in 37.3% of 655 human breast carcinomas.

Many monoclonal antibodies have been developed to epitopes on both wild and mutant p53 species [11]. Depending on which monoclonal antibody is used different aspects of p53 can be evaluated [11]. Previous studies have found a concordance of monoclonal antibody pb1801 immunohistochemical nuclear p53 protein accumulation and point mutations within highly conserved regions of the p53 gene. These altered genes encode for p53 proteins with a higher level of stability [20] and thus intracellular accumulation of the protein that can be detected by standard immunohistochemical techniques.

From a clinical standpoint, this technique is a useful and practical method in detection of mutant forms of the p53 protein. It allows precise localization and identification of the cells that exhibit p53 alterations. Since the wild type p53 protein has an extremely short half-life, the detected intercellular concentration, is the mutant form of the p53 protein [5].

The role of p53 protein in initiation and progression of cancer is unclear. However, studies provided information indicating a relationship between high level of p53 and clinically advanced, highly aggressive forms of the breast tumors [8, 21]. In breast carcinoma, proposed previously [21], p53 mutation may occur relatively early at the development of DCIS and may have a growth advantage that would result in clonal expansion of the neoplastic population.

The objective of this study was to screen tumor samples from 100 patients diagnosed with DCIS for p53 content. Then, to determine what percentage of the DCIS cases convert to invasive form of breast carcinoma and correlate that to the initial p53 content. Data presented here indicate that accumulation of p53 protein, as assessed by immunohistochemistry using monoclonal antibody Pb1801, (Ab-2), occurs at low frequency (15%) in DCIS. The p53 immunostaining often was seen in mixed histologic type of the DCIS. These findings are in accordance with the results of obtained by Lennington *et al.* [17], that showed evidence regarding the frequency of mixed patterns in DCIS. In this study, the predominant pattern seen in p53 immunopositive mixed cases was solid followed by cribriform type of DCIS. Furthermore, all p53 immunopositive DCIS specimens exhibit comedo necrosis features. This is especially true in specimens with pure cribriform and papillary types of DCIS.

Bellamy *et al*. [22] emphasized the significance of nuclear grade for classifying DCIS into high and low grade of malignancy. In their experiments, they found that conversion to the invasive form occurred only in high nuclear grade DCIS, regardless of the histologic pattern. This study showed no relationship between DCIS histologic patterns, p53 immuno-staining, and nuclear grade. Preliminary data, related to the long-term evaluation of the patient population are available on a limited basis. At present, no specimen with immunonegative staining for p53 has converted to invasive form. However, six out of fifteen immunopositive samples were converted to the more invasive form. Interestingly, one case presented with a cribriform pattern and a nuclear grade of 2. This may indicate the clinically more aggressiveness from.

The purpose of this study was to establish a database for the long-term evaluation of patients that will convert to the invasive carcinoma. To refine this database, studies on the population are needed using molecular techniques such as DNA sequencing and/or probe analyses. These techniques may identify correlation between mutations and immuno-positive p53 accumulations that rule out the occurrence of the false positive and the false negative results.

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