

Detection of mutant p53 protein in serum could be use to differentiated malignant from benign breast tumors.

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Abstract

The p53 wild type is a tumor suppressor gene involved in DNA gene transcription or DNA repair mechanisms. When damage to DNA is un-repairable, p53 induces programmed cell death (apoptosis). The mutant p53 gene is the most frequent molecular alteration in human cancer, including breast cancer. Here we analyzed the genetic alterations in p53 oncogene expression in 55 patients with breast cancer (51 with malignant tumors and 4 with benign lesions) and in 8 normal women. We measured by ELISA assay the serum levels of p53 mutant protein and also the protein expression of both p53 wild type and mutant using immunohistochemistry in breast tissue tumors. We found positive p53 mutant in the serum of 0/8 (0.0%) normal women, 0/4 (0%) in benign breast disease and 29/55 (52.72%) in breast carcinoma. The immunohistochemistry evaluation was positive in 29/55 (52.73%) for mammary carcinoma and 0/4 (0%) for benign breast disease. These data suggest that detection of mutated p53 could be a useful serological marker to differentiated the benign lesion from the malignant ones even before to perform the surgery.

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A variety of molecular genetic changes have been described in breast cancer. Oncogene and tumor suppressor gene alterations have been studied in an attempt to define the molecular correlation of prognosis and the clinical behavior of breast cancer phenotype (1). Among those, the p53 tumor suppressor gene has become the focus of intensive studies. The current and most powerful model of wild-type p53 function is one of which p53 monitors the genome for DNA damage (2). After treatment of cells with DNA damaging agents, p53 protein levels are increased by posttranslational stabilization and can trans-activate various genes that may be related to cell cycle arrest or apoptosis (3). Arrest of cell cycle progression following DNA damage is thought to represent a basic protective mechanism preventing replication of damaged template DNA. If damage is irreparable, the cell may be driven to apoptotic pathway, thus preventing replication of defective cells. Mutations in the p53 tumor suppressor gene are the most frequent known genetic alterations in all human cancers (4).

Most of the biologically significant mutations impair the ability of p53 to participate in the maintenance of genomic stability. Consequently, tumors lacking normal p53 might be expected to be prone to deleterious mutations and to be more aggressive clinically. Many studies have examined the association between breast cancer prognosis and p53 protein expression in tumor cells (5-13). The use of immunohistochemistry (IHC) was based on the fact that the missense mutations normally result in an increased half-life of the protein product and a consequent accumulation of the mutant p53 protein in the nucleus.

Another different methodology is the detection of p53-specific antibodies, which could be explaining the discrepant results for the p53 accumulation in the same tumor type (5, 8-12). Our purpose here was study the relationship between the p53 at protein and antibodies levels and IHC in 55 cases of human breast tumor at different stage of the disease. These results revealed that the level of p53 mutant protein in the serum of the breast cancer patients was directly correlated with the p53 accumulation and with the presence of mutations. These finding may be important in breast cancer detection and the analysis of p53 mutant protein could be use as a diagnostic tool and for differentiating the benign and malignant breast disease before the surgery.

MATERIALS AND METHODS

Patients: From August 1999 to August 2000, 55 patients with mammary pathology were analyzed from the Hospitals: Sanatorio Privado del Sur, Interzonal Dr. Jose Penna, Regional Español, Dr. Leonidas Lucero, Hospital de la Asociación Médica Bahía Blanca, Argentine. Serum and breast tissue of the patients with histological diagnosed of the breast were available for analysis.

Serum was collected preoperatively and was stored at -80°C, and the breast tissue was kept in liquid nitrogen until it was processed. Eight serums from normal patients without breast disease or any breast cancer history were utilized as negative control for the serologic assays. The median age of all patients was 55 from 28 to 91 years (Table I).

Serological Analysis: We have quantified the presence of p53 mutant protein from the serum of all the patients employing p53 ELISA Kit (Mutant Selective) Oncogene Research Products, Cambridge MA 02142, USA). We employed the control serum provided by the manufacturer. The results were expressed in O.D units and were categorized as negative or positive. We quantified the p53 mutant protein level from the serum using a sandwich enzyme linked immuno-absorbent assay (ELISA) with microtrite plates coated with recombinant human p53 protein expressed in Escherichia Coli (p53 ELISA Kit (Mutant Selective). The ELISA test was suitable for the quantitative determination of mutant p53 protein, the antibodies utilized in this assay kit reacted with an epitope

exposed on human and most mammalian mutant p53 proteins but not on wild-type p53, thus making the assay mutant-selective.

The results were expressed in O.D units and were categorized as negative or positive. Eight serum samples from normal women without any breast disease or any familiar cancer history were utilized as negative control.

Histology: Human breast tumor sections (5 µm) were cut from formalin-fixed, paraffin-embedded tissues from all the patients entered in this study. Nuclear grade was defined as grades I-III according to previously established criteria (13, 14). The histological classification and the nuclear grade were performed by medical pathologist. Each tissue was cut by duplicate for performing immunohistochemistry study of p53.

Immunohistochemistry: Tumor cell staining for p53 protein was performed using mouse monoclonal DO-1 antibody (Oncogene Research Products, CA). All sections were de-paraffinized in xylene, dehydrated through a graded series of alcohols, and washed in phosphate buffered saline. This buffer was used for all subsequent washes. IHC using the streptavidin-biotin-peroxidase method was performed on paraffin embedded tissues using the anti-p53 mouse monoclonal antibody DO-1 (diluted 1:100), which recognizes the N-terminus of the human p53 protein (amino acids 21 to 23). In addition, the antibody reacted with both wild type and many mutant p53 proteins. In order to do semi-quantitative assessment, the IHC results were scored, we established that the immunohistochemical staining for nuclear p53 in more than 10% of the tumor cells was interpreted as positive: (+) between 10 - 25 % of nucleus positives, (++) between 25- 50 % and (+++) between 50-100% of nuclear staining.

Statistical Analysis: The frequency of p53 values cut-off and the frequency of p53 values <cut-off were compared to the different parameters by a Chi-square test (T- test).

RESULTS

p53 protein expression in breast cancer patients

Using immunohistochemistry we analyzed the presence of p53 wild-type and/or mutant protein in 55 patients with different breast diseases and different stages of tumor and histology. We employed the mouse monoclonal DO-1 anti-p53 antibody HPR-conjugated (Oncogene Research Products, MA) that recognizes the amino-acids 21-25 in the amino-terminal domain of the protein. The antibody recognizes both the wild type and the mutant p53 forms. We used a dilution of 1:100 and incubated the sections overnight at 4°C.

Age-dependent p53 protein expression

Figure 1 shows the age-dependent curve and the expression of p53 in 55 patients with breast malignancies using the IHC and ELISA assay. In the Figure 1 red line represents the number of patients with mutant p53 protein in serum and yellow line represents the age-variation curve of positive cases with p53 expression by IHC. The age was group in 4 groups, <30, between 31-50, between 51-80 and between 81-100 years old (Figure 1).

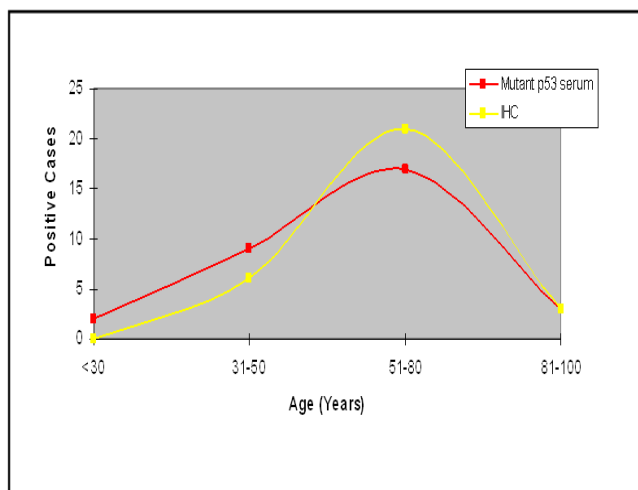


Figure 1: Age-dependent p53 analysis in breast cancer patients. The graphic represent the age-dependent p53 expression in the different assays performed. In the graphic, red line represent the positive cases with mutant p53 protein in serum, yellow line represents the p53 protein expression using the DO-1 antibody by IHC.

Histology-dependent curve and p53 protein expression

In Figure 2 we indicated the number of positive cases for the presence of mutant p53 protein in

serum (red line) or presence of p53 protein by IHC (green line) from normal women, patients with benign disease, carcinoma in situ and patients with invasive carcinoma in stage I, II and III. We analyzed serum for the mutant p53 protein from 8 normal women, negative results, meaning 0% of normal patients had positive detection of mutant p53 in their serum (Figure 2)..

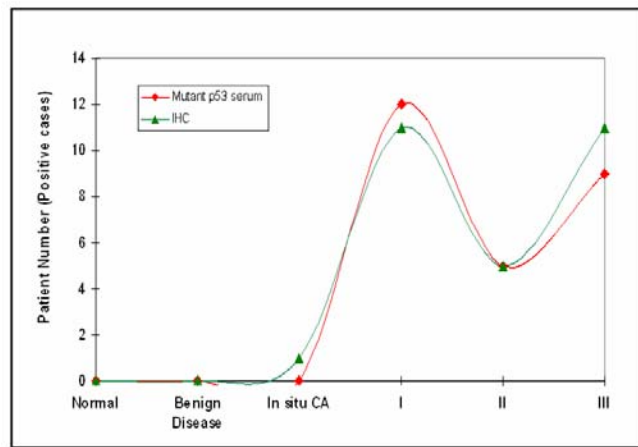


Figure 2: p53 protein expression Tumor type-dependent curve. The graphic represents the number of positive cases for the p53 protein expression by ELISA (red line) and by IHC (green line) in normal, benign, carcinoma *in situ* and Invasive carcinomas stages I, II and III.

We did not detect the mutant p53 protein in serum of patients with benign disease (total 5 patients) (Figure 2), also the p53 protein expression by IHC was negative in those patients. Means 0% of patients with benign disease had positive assay.

We detected the presence of p53 protein by IHC in 1 out of 2 patients with in situ carcinoma (Figure 2); meaning that 50% of in situ carcinoma patients showed positive expression of p53 by IHC, but none of them show the presence of mutant p53 protein in their serum (Figure 2).. We also analyzed the p53 expression in 43 patients with invasive carcinoma (I.C.) (Figure 2).. 26 patient out of 43 (60.45%) showed presence of mutant p53 protein in their serum and 62.79% (27/43) were found positive results by IHC for the presence of p53 in the tissue.

Tumor differentiation grade and p53 expression in breast cancer patients

In order to determine which assay for the p53 expression was more sensible base upon tumor differentiation grade, we quantified the number of positive patients in each assay at low, semi and

well-differentiation level. We found 7/13 (63.64%) positive for the presence of mutant p53 protein in serum and 8/13 (72.73%) positive for p53 expression by IHC at poor-differentiation level (Figure 3).. At semi-differentiation level, 8 out of 24 patients (33.33%) showed the presence of p53-auto-antibodies, 13/24 (54.17%) showed positive for mutant p53 assay and 14/24 (58.33%) have been show positive presence of p53 protein by IHC (Figure 3).. In tumors at well-differentiated level we found, 5/8 (62.50%) patients showed mutant p53 protein expression by ELISA in serum and by IHC in breast tumor tissue (Figure 3)..

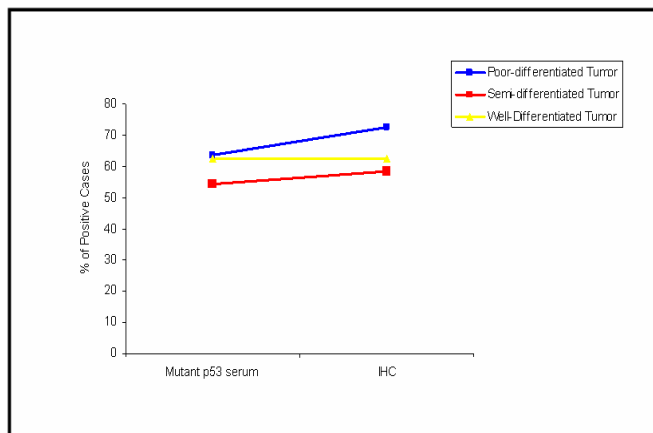


Figure 3: p53 expression and Tumor differentiation level-dependent curve in breast cancer patients. The graphic shows the % of positive cases detected in all the assays at different grade of tumor differentiation, meaning poor, semi and well differentiation. The table behind the figure shows the % of the positive cases in each differentiation grade in each assay.

DISCUSSION

The development of molecular markers is needed to improve the diagnosis and assessment of tumor progression in breast cancer patients. Mutations of the p53 tumor suppressor gene, as well as over-expression of serum p53 antibodies and of p53 protein in tumor tissue, have been encountered in a variety of human malignancies (17).

p53-Ab was originally described in 1982, by Crawford et al. (16) in the serum of 9% of breast cancer patients using a Western blotting method. Using ELISA, more than 15 studies have been recently performed by Soussi, T. et al in breast cancer (17). The frequency of p53-Abs in breast cancer range from 15 to 20% but the majority of these studies were performed either in Europe or in US. No study has been performed in South America and specifically in Argentina where the frequency of breast cancer is lower than those countries.

The overexpression of mutant p53 protein in breast cancer patients has been usually evaluated in tumor tissue with immunohistochemical staining, however the serum assay for p53 oncoproteins with ELISA can be easily and repeatedly performed because of its minimal invasiveness compared with assays using tissue materials (18, 19). In the current study, the median serum level of mutant p53 protein showed significant difference compared to controls ($p < 0.001$), 60.45% (26/43) of positively with invasive carcinoma which demonstrated positive elevation of mutant p53 protein in serum.

Our results are very well correlated with studies investigated the serum level of mutant p53 in cervical carcinoma patients done by Sobti et al. (20, 22), who reported that 61.5 % of patients with invasive cervical carcinoma showed positive elevation of mutant p53 in serum. However there are very few results in breast cancer, Micelli, G et al have been demonstrated the presence of mutant p53 protein in serum in 23% of breast cancer patients without lymph node involvement and they show that the specificity of mutant p53 assay evaluated in 20 healthy controls was 100% (21).

The presence of mutant p53 protein in serum was predominantly found in invasive carcinomas at early stages (I or II) (63.15%, 12/19 at stage I, $p < 0.0001$ and 83.33 %, 5/6 at stage II, $p < 0.0001$) rather than late stages (stage III, 50% 8/16). Similar results were found for the p53-accumulation, meaning that at early stages of invasive carcinoma we detected higher expression, than at late stages. The specificity of mutant p53 protein detection was 100%, because was negative in the normal control serum, and also was negative in the patient with benign diseases (0/6, 0%).

The presence of mutant p53 protein in serum and the p53 accumulation in the tissue was correlated with poor-differentiation grade in invasive carcinomas (63.64% and 72.73 % for both assays, $p < 0.005$) rather than higher-differentiation level. Several publications have been demonstrated the presence of p53-accumulated protein by IHC in breast cancer. Al-Moundhri et al (23) have been found p53 over expression in 41.7% of the breast tumors examined with statistically significant. They also have published that the p53-accumulation are related with poor tumor

differentiation in human breast cancer (23).

In conclusion, serum mutant p53 protein was elevated in invasive breast carcinomas with strong correlation with p53-accumulation detected by IHC. This data strongly indicated that the detection of mutant p53 in serum and the p53-accumulation are correlated each other and both test are sensitive and specific for invasive breast carcinomas.

Although a prospective study with large sample size is warranted, the presence of mutant p53 protein in serum could have potential usefulness as a biological marker of breast carcinoma especially for prediction of prognosis and follow-up after treatment.

Our findings indicate that mutant p53 in serum appears to be a promising new parameter to evaluate the cellular biology and prognosis of breast cancer from blood samples without surgery. The presence of mutant p53 protein can be an important tool in order to differentiate a benign disease before performed a surgery.

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