MUTANT p53 PROTEIN EXPRESSION AND S PHASE FRACTION IN BREAST CANCER

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Abstract. The mutations of p53 gene are among the most prevalent alterations in human malignancies, including the breast cancer. A total of 330 samples of breast cancer cases were examined for mutant p53 overexpression using a simple enzyme-linked immunosorbent assay (ELISA), which fulfills the demands of a clinical assay in being quantitative and appropriate for the routine analysis in hospital laboratories. DNA flow cytometry was performed on frozen tissues from 162 primary breast carcinomas, of which the S phase fraction (SPF) was estimated in 120 (74%) cases. A correlation was observed between overexpression of mutant p53 protein and aneuploid SPF ($p = 0.047$). Mutant p53 protein positivity was also slightly related to diploid SPF ($p = 0.082$) and tumour size ($p = 0.095$), but not to the patient's age, lymph node status, TNM stage, histology, histologic grade, menopausal status, or DNA ploidy. Near-triploid range carcinomas (DI 1.40–1.60) were correlated with overexpression of mutant p53 ($p = 0.019$). DNA near-triploid tumours in breast cancer may have a more aggressive behaviour in comparison with the other DI classes. To further refine the prognostic assessment of breast cancer p53 overexpression and SPF should be used together with other established prognostic factors.

Key words: breast carcinoma, ELISA, DNA flow cytometry, mutant p53 protein, S phase fraction, prognostic factors.

INTRODUCTION

Breast cancer is the most common female malignancy in Estonia (Thomson et al., 1996). One of the primary objectives in breast cancer research is the identification of new biological markers of tumour behaviour for better

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prediction of recurrence and survival. For the identification of high risk breast cancer patients, a variety of prognostic factors have been evaluated. These factors include tumour involvement of the axillary lymph nodes, tumour size, histological grade, steroid hormone receptor status, DNA ploidy, proliferative index, growth factor receptors (McGuire et al., 1990; Younes et al., 1997). A variety of oncogene and tumour suppressor gene alterations have been studied as well. Alterations of p53 are among the most common abnormalities detected in primary breast cancer. These changes are usually caused by missense point mutations in the conserved regions of the gene and domains of the produced protein important for its function or structure. Mutant p53 protein usually undergoes conformational changes that prolong its normally very short half-life, resulting in its accumulation in the cell (Donehower & Bradley, 1993; Levine et al., 1994; Harris, 1996). Findings from several studies support the correlation between mutant p53 protein overexpression and aggressive breast cancer phenotype, and emphasize its clinical usefulness as an independent prognostic factor (Davidoff et al., 1991; Allred et al., 1993; Silvestrini et al., 1993; Borg et al., 1995; Kovach et al., 1996; Norberg et al., 1996; Eissa et al., 1997).

General molecular biology techniques involving the study of DNA using Southern blotting, polymerase chain reaction, and direct DNA sequencing are not easily performed in clinical laboratories (Voijtesek et al., 1993). A promising method for detecting genetic abnormalities is to study the mutant protein expression, which has been taken as a rough measurement of the presence of gene mutation (Chang et al., 1993; Lohmann et al., 1993; Tsuda & Hirohashi, 1994; Maass et al., 1997). An enzyme-linked immunosorbent assay (ELISA) for p53 protein has been developed and applied for the measurement of mutant p53 protein in tumour tissue extracts. ELISA has a number of distinct advantages over immunohistochemistry. In these procedures, in order to obtain a signal, p53 must bind to two different antibodies instead of the single p53-specific antibody used in immunohistochemical methods, which increased the specificity of the test. ELISA is also a sensitive, technically simple to perform, and easily automated test (Hassapoglidou et al., 1993; Diamandis & Levesque, 1995).

The purpose of the current study was to determine the levels of mutant p53 protein in cytosol extracts routinely prepared in our hospital for steroid hormone receptor assays and to compare the results with the other tumour variables, important for the treatment of patients.

MATERIAL AND METHODS
Material

We studied altogether 376 women who attended at Estonian Cancer Centre in Tallinn during the time period 1994–97 and who had received surgical treatment at the Department of Surgery for benign ($n = 46$) or malignant ($n = 330$) breast

tumours. Surgically removed tissues were divided into three parts, the first was formaline fixed and paraffin embedded for histopathological diagnosis, the second and third were frozen at –20°C for flow cytometric DNA analysis and for the extraction of mutant p53 protein and estrogen receptor (ER). Frozen tissues were processed within 1–2 weeks. For all cases histological classification and grading was carried out by histopathologists.

Methods

Preparation of cytosol

Samples were obtained at the time of surgery. The samples were freed from the surrounding fat and connective tissue, cut to convenient size (approximately 300 mg), and placed immediately in vials with liquid nitrogen. All samples were pulverized manually at –80°C to an extra fine powder. All tools required for homogenization and transfer were cooled in liquid nitrogen as well. The dry tumour powder was transferred to a test tube and treated with 2 mL of 4°C Tris-buffer, pH 7.4, containing 1.9 mM EDTA (Sigma), 0.5 mM dithiothreitol (Sigma), and 100 mL/L glycerol (Sigma). The samples were centrifuged at 7500 rpm for 60 min with constant cooling at 4°C. The upper phase was stored at –20°C and analysed within 1–2 weeks. For longer storage the cytosols were kept at –70°C.

To verify the specificity of ELISA, 25 samples of tumour free noninflamed breast tissue were homogenized and determined by ELISA as well, defining a positive elevation of the cytosol mutant p53 protein as any value greater than 2 standard deviations above the mean of the controls (>0.14 ng/mg).

ER, total protein

The ER content was determined with the dextran coated charcoal assay. A cut-off level of 10 fmol/mg protein was used to classify tumours as receptor positive or negative. Total protein measurements were carried out according to Lowry in order to compare the mutant p53 protein fraction with the amount of total protein.

ELISA was performed using the mutant p53 ELISA-kit (Oncogene Research Product, USA). ELISA readings were carried out with an automatic ELISA microplate-reader (Multiscan MCC/340, Labsystems, Finland) at 405 nm.

DNA analysis

Fresh frozen tumour material was used. Cell suspension was prepared using the scraping method. Staining with propidium iodide was performed with staining solutions listed in Table 1, as described by Vindeløv & Christensen

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Table 1. Preparation of solutions for DNA analysis

Stock solution

$C_6H_5Na_3O_7 \cdot 2H_2O$	1000 mg (3.4 mM)
Nonidet P40	1000 µL (0.1% v/v)
Spermine tetrahydrochloride	522 mg (1.5 mM)
Tris	61 mg (0.5 mM)
Distilled water is added to a total volume of	1000 mL

Solution A, pH = 7.6

Stock solution	1000 mL
Trypsin	30 mg

Solution B, pH = 7.6

Stock solution	1000 mL
Trypsin inhibitor	500 mg
Ribonuclease A	100 mg

Solution C, pH = 7.6

Stock solution	1000 mL
Propidium iodide	416 mg
Spermine tetrahydrochloride	1160 mg

(1990). Briefly, 300 µL of solution A was added to 100 µL of cell suspension in phosphate-buffered saline, after 5 min at room temperature 250 µL of solution B was added, and 10 min later 250 µL of ice-cold solution C was added. The sample was filtered through a 30 µm nylon mesh and analysed after 25 min. The DNA was analysed on a flow cytometer (Coulter XL), using chicken and rainbow trout erythrocytes as an external and internal standard (Vindeløv et al., 1983).

The DNA index and S phase fraction (SPF) were calculated using the program Multicycle Advanced Version 3.0 (Phoenix Flow Systems, San Diego, CA). DNA aneuploidy was defined as any population with a distinct peak corresponding to a DNA index (DI) > 1.05 or < 0.95 or the presence of a tetraploid population greater than 15%. Background correction was applied with the no sliced nuclei option. This appeared to provide optimal debris correction for fresh frozen tumour tissue. Zero order S phase was chosen and the G_2/G_1 ratio was not fixed. Median coefficient of variation for tumour G_0/G_1 peaks was 3.1% with a range between 1.6 and 7.4%.

Statistics

Statistical analysis was performed using SPSS/WIN statistical software. Logistic regression analysis was used to find the relationship between the mutant

p53 overexpression and the patient's age and menstrual status, tumour histology, size, histologic grade, stage, and estrogen receptors.

The rates of p53 overexpression in different tumour or patients' subgroups (diploid SPF, aneuploid SPF) were compared with Pearson's chi-square analysis. Mann–Whitney U test was used to assess the difference between cancer and benign groups.

RESULTS

Three hundred and thirty breast cancer samples were analysed for mutant p53 protein (Table 2). The patients' mean age was 56 years, range 23–82. Out of the 330 patients 167 had axillary lymph node metastasis. The cytosol total protein concentration was in the range 0.95–11.0 mg/mL, the mean was 3.04 mg/mL and the median 3.05 mg/mL. Mutant p53 protein was detected in 260 (79%) of the samples (range 0.01–48.0 ng/mg protein, mean 0.619 ng/mg protein, median 0.090 ng/mg protein). Using a cut-off value of 0.14 ng/mg protein, 109 (33%) of the tumours were classified as manifesting mutant p53 protein overexpression (p53+). Of 162 histograms, 61 (38%) were DNA diploid and 101 (62%) were DNA aneuploid. The SPF was estimated in 120 tumours (74%). If SPF of aneuploid population was used, only 58% of the cases were interpreted, the others had multiple overlapping cell cycles or there was only another aneuploid cell population with no cell cycle.

For comparison 46 samples from patients with benign breast diseases (fibroadenoma, fibrocystic disease) were analysed and 2 of them overexpressed mutant p53 protein (4%). The mean mutant p53 protein value in this benign group was 0.135 ng/mg protein, median 0.010 ng/mg. The difference between cancer and benign group was significant ($p < 0.0001$).

There was an association between the overexpression of mutant p53 protein and aneuploid SPF ($p = 0.047$). The p53 overexpression was also related to the tumour size ($p = 0.095$) and diploid SPF ($p = 0.082$), but this correlation was statistically not significant. No relationship was found with the patient's age, lymph node status, TNM stage, histology, histologic grade, menopausal status, or DNA ploidy. A weak negative correlation existed between ER and p53, but it remained statistically insignificant.

Grouping the tumours according to their DI values, we observed a correlation of the near-triploid range carcinomas ($n = 15$; DI 1.40–1.60) with overexpression of p53 ($p = 0.019$). Three of these near-triploid carcinomas had multiple (three) cell populations (20%) compared with other ploidy groups, where there were only 4 multiple cell populations out of 147 (2.7%). No correlation was established between the near-triploid carcinomas and tumour grade or lymph nodes involvement.

Table 2. Relationship between mutant p53 protein overexpression and other clinicopathologic characteristics in primary breast cancer

Variable	No.	p53+	%	p-Value
All patients	330	109	33	
Age				0.224*
<40	23	7	30	
40–55	138	40	29	
>55	169	62	37	
Tumour size				0.095*
≤2.0 cm	140	36	26	
>2.0 cm	190	73	38	
TNM stage				0.411*
I	71	18	25	
II	158	58	37	
III–IV	101	33	33	
Histologic grade				0.294*
1	33	7	21	
2	123	42	34	
3	124	45	36	
Unknown	50	15	30	
Histology				0.585*
Ductal	249	84	34	
Lobular	15	4	27	
Medullary	22	10	45	
Other	44	11	25	
Menopausal status				0.626*
Pre	95	30	32	
Post	235	79	34	
ER status				0.549*
<10 fmol/mg protein	126	45	36	
≥10 fmol/mg protein	204	64	31	
DNA ploidy				0.290**
Diploid	61	15	25	
Aneuploid	101	34	34	
S phase fraction				
Diploid SPF				0.082**
<4.0%	45	8	18	
≥4.0%	16	7	44	
Aneuploid SPF				0.047**
<11.0%	30	7	23	
≥11.0%	29	15	52	

* calculated by logistic regression model;
** calculated by chi-square test.

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DISCUSSION

One of the important features of breast cancers is that they are heterogeneous in the way they behave biologically and clinically. It is, therefore, essential that the nature of individual tumours be characterized and used to plan the most appropriate therapy.

In the present study a simple ELISA for the measurements of mutant p53 protein was used, which meets the demands of a clinical assay in being quantitative and appropriate for the routine analysis in hospital laboratories. Out of the 330 tumour extracts 109 (33%) were positive for mutant p53 protein, which is in agreement with the previous reports (Hassapoglidou et al., 1993; Silvestrini et al., 1993; Voijtesek et al., 1993; Borg et al., 1995). Two samples (4%) from patients with benign breast disease also overexpressed mutant p53 protein, so the overexpression of mutant p53 protein should not be used as exclusive evidence of malignancy, which supports the findings of some other investigators (Koutselini et al., 1991; Younes et al., 1995). Our results indicate that in breast cancer the p53 protein overexpression is more often seen in medullary and ductal tumours, and this may be related to the intense proliferative activity observed in these tumours (Marchetti et al., 1993; Rudas et al., 1997).

An association was observed between tumour size and p53 overexpression, with an increase in p53 positivity for large tumours. Silvestrini et al. (1997) reported the same in their study.

A correlation of p53 overexpression with aneuploid high S phase was observed. We also found a correlation of near-triploid range carcinomas with the overexpression of p53. This correlation was described by Leonardi et al. (1997). They also found associations between near-triploid carcinomas and high tumoral grade and presence of axillary metastases. Our results did not confirm the latter. Although the DNA near-triploid carcinoma group was small in our study, these data suggest that this breast cancer group may have a more aggressive behaviour in comparison with other DI classes. This remains to be confirmed in a study with more patients.

Beyond the potential of p53 overexpression and high SPF as prognostic factors, the detection of these factors will allow studying the interaction of these parameters with treatment, in particular chemotherapy and radiotherapy, for which some data have been published. These preliminary data show that high S phase is considered an indicator of chemoresponsiveness and that chemoresistance or chemosensitivity in cases of mutated p53 depend on the respective chemo-therapeutic regimen and/or anticancer drug applied (Stål et al., 1994; Bergh et al., 1995; Hietanen et al., 1995; Mueller & Eppenberger, 1996; Lesec et al., 1997; Remvikos et al., 1997). Further investigation with longer follow-up data is needed to identify prognostic factors that will provide better prediction of the disease course for breast cancer patients. Mutant p53 protein overexpression and SPF could be used with other established prognostic factors to identify high-risk breast cancer groups.

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MUTANTSE p53 VALGU ÜLEPRODUKTSIOON JA S-FAASI SUURUS RINNAVÄHIHAIGEIL

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Mutantset p53 valku määrati ELISA-meetodil ja S-faasi suurust läbivoolu-tsütomeetria meetodil. Nimetatud valgu üleproduktsiooni täheldati 330-st uuritud rinnavähihaigest 33%-l, mis on vastavuses kirjanduse andmetega. Mutantset p53 valku ei saa käsitleda kui pahaloomulisuse markerit, sest kahel haigel (4%), kellel eemaldati healoomuline kasvaja, märgati kasvajakoes samuti p53 üleproduktsiooni. Leiti tugev seos p53 üleproduktsiooni ja DNA aneuploidsete kasvajate S-faasi suuruse vahel. Nagu viimased uuringud on tõestanud, ei ole mõlemal näitajal mitte ainult prognostiline, vaid ka ravi tulemust etteütlev väärtus. ELISA-test ja läbivoolutsütomeetria meetod on rakendatavad rutiinses kliinilises praktikas ja annavad lisateavet riskirühmade paremaks eristamiseks rinnavähihaigete seas.