Prognostic Value of TP53 Protein Accumulation in Human Primary Breast Cancer: An Analysis by Luminometric Immunoassay on 1491 Tumor Cytosols

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Abstract. The tumor suppressor gene TP53 is implicated in the regulation of normal cell growth and division, DNA repair and apoptosis. Mutations in this gene usually give rise to a conformationally altered protein which is stably expressed at high levels. We have studied TP53 protein accumulation in routinely prepared cytosols from 1491 human primary breast cancer specimens (median follow-up of patients alive, 66 months), using a quantitative luminometric immunoassay (LIA). The TP53-LIA values varied between 0 and 153.53 (median 0.20 ng/mg protein). Median TP53 levels were significantly higher in ER- and PgR-negative tumors. In Cox univariate regression analysis, when analyzed as a continuous variable, increasing TP53 levels were related with a poor relapse-free survival (p<0.01). In multivariate analysis for relapse-free survival, including age, menopausal status, tumor size, nodal status and steroid hormone receptor status, TP53 accumulation, when analyzed as a dichotomized variable, was an independent factor for predicting the rate of relapse with a relative relapse rate (95% confidence limits) of 1.39 (1.19-1.63). In conclusion, the LIA for the TP53 protein can easily be performed on cytosols routinely prepared for steroid hormone receptor analysis, it is a quantitative assay, and it may be useful in establishing the relation of TP53 accumulation and breast cancer prognosis.

The human tumor suppressor gene TP53 is located on chromosome band 17p13.1 and consists of eleven separate exons, of which the first one is noncoding. This phosphoprotein, with a molecular weight of 53 kDa, can act as a transcription factor. TP53 has been implicated in the regulation of normal cell growth and division, DNA repair and apoptosis. Inactivation of TP53 is one of the most common molecular genetic events described so far in the development of cancer. This inactivation may occur by binding to certain viral transforming proteins, to the cellular oncogene product of the murine double minute 2 protein or to heat shock protein 70, which all neutralize its activity. Another way to inactivate TP53 gene function is through gene mutation (reviewed by Greenblatt et al) (9). These mutations are mostly missense, resulting in a stably expressed conformationally altered protein which is detectable by immunohistochemical, ELISA or Western blotting techniques (3,12).

To study its prognostic significance, we examined TP53 protein accumulation in primary breast tumor cytosols, routinely prepared for steroid hormone receptor analysis, using a quantitative luminometric immunoassay (LIA). TP53 protein accumulation was related to patient and tumor characteristics, and with a clinical outcome of breast cancer patients.

Patients and Methods

In this study, primary breast tumor specimens, used for routine steroid hormone receptor analysis, from 1491 patients with known follow-up were included. All patients had primary invasive breast cancer with no signs of distant metastasis at time of surgery. The median age of the patients was 56 years (range: 24-93, mean 56 years). Forty-one percent of the patients were premenopausal, 59% postmenopausal; 46% had no involved lymph-nodes, and 42% of the tumors were smaller than 2cm (T1). Of these patients 23% received adjuvant treatment. Median follow-up of patients alive was 66 months. During follow-up, 686 patients experienced a relapse and 505 patients died.

Analysis of steroid hormone receptors. The breast tumor specimens were
Table I. Between assay variation of TP53 (n=15 assays).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>MIN</th>
<th>MAX</th>
<th>S.D.</th>
<th>% C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control “low”</td>
<td>0.21</td>
<td>0.18</td>
<td>0.23</td>
<td>0.02</td>
<td>9.5</td>
</tr>
<tr>
<td>Control “high”</td>
<td>8.44</td>
<td>7.49</td>
<td>9.47</td>
<td>0.47</td>
<td>5.6</td>
</tr>
<tr>
<td>Sample “reference”</td>
<td>2.96</td>
<td>2.49</td>
<td>3.39</td>
<td>0.27</td>
<td>9.1</td>
</tr>
<tr>
<td>Sample “cytosol pool”</td>
<td>0.89</td>
<td>0.78</td>
<td>1.06</td>
<td>0.10</td>
<td>11.2</td>
</tr>
</tbody>
</table>

stored in liquid nitrogen. The samples were pulverized in the frozen state and homogenized in a phosphate buffer according to procedures recommended by the EORTC for the preparation of cytosols for ER and PgR measurement (EORTC Breast Cancer Cooperative Group 1980) (7). ER and PgR were measured by ligand binding assay or with enzyme immunoassays (8).

Luminometric immunoassay. The TP53 protein levels of the breast tumor cytosols were measured using a quantitative luminometric immunoassay (LIA; AB Sangtec Medical, Bromma, Sweden) as described by Borg et al. (4). The LIA is based on a combination of two monoclonal antibodies, pAB1801 and D01, which detect both wild-type and mutant TP53 protein sandwich-type assay. The monoclonal antibody pAB1801, which is immobilized onto a solid phase (tube), is used for catching. Monoclonal antibody D01, labeled with a chemiluminescent compound (amino-butyrl-ethyl-isoluminol: ABEI), is used for detection. The immunoassay was performed by incubating either 100 μl of TP53 standard (range: 0-80 ng/ml), controls or tumor cytosols, together with 100 μl of the ABEI-conjugate in pre-coated tubes. After incubation for 18 hours at room temperature, unbound reagents were removed by washing the tubes three times with 2 ml 0.9% sodium chloride. The chemiluminescent reaction was initiated by the sequential addition of 300 μl alkaline hydrogen peroxide and 300 μl catalyst (microperoxidase) solution, immediately followed by measurement of the chemiluminescent counts in a luminometer. The TP53 protein contents of the samples were determined from the standard curve, plotting the chemiluminescent response (in relative light units: RLU) against the standard concentrations of the TP53 protein. A representative standard curve is shown in Figure 1. The detection limit is approximately 0.01 ng TP53 per ml sample. The concentration of TP53 protein in the tumor cytosols is expressed as ng/mg cytosolic protein (the protein concentration of all samples was brought to 0.5 mg/ml cytosol, with kit diluent buffer).

Statistical analysis. The relations between TP53 protein levels and patient- and tumor characteristics were studied with non-parametric tests: the Wilcoxon test (menopausal status, tumor size, ER, PgR), the Kruskall-Wallis test including a Wilcoxon-type test for trend for ordered variables (nodal status) and additionally the Spearman Rank correlation for continuous variables. Isotonic regression analysis was performed in order to determine the cut-off level for TP53 accumulation (2). Relapse-free survival probabilities were calculated by the actuarial method of Kaplan and Meier (10). The Cox proportional hazard model was used for uni- and multivariate survival analyses.

Results and Discussion

The TP53-LIA was performed on 1491 primary breast tumor cytosols. Fifteen separate experiments were required to obtain all the results. In each experiment the kit controls ("low" and "high"), a "reference" preparation and a tumor "cytosol pool" were included. The assays were highly reproducible and the between-assay variation ranged from 5.6% and 11.2% (shown in Table I). The TP53 protein values of the 1491 breast tumor cytosols ranged between 0 and 153.53 ng/mg cytosolic protein (median 0.20 ng/mg protein).
TP53 accumulation related with patient and tumor and characteristics. With regard to patient- and tumor characteristics there were no significant relationships between TP53 protein levels and menopausal status, nodal status or tumor size (Figure 2). The Spearman rank correlation coefficients between the levels of TP53 with ER and PgR were -0.06 and -0.04, respectively (not significant). However, in ER-negative and PgR-negative tumors, TP53 protein levels were significantly higher when compared to ER-positive and PgR-positive tumors (P=0.0001 and P=0.0002 respectively, shown in Figure 2). This is in agreement with studies by others (reviewed by Elledge and Allred, 1994).

TP53 accumulation and prognosis. Isotonic regression analysis was applied with relapse-free survival as the end point for all 1491 tumor samples, and 0.38 ng/mg protein was chosen as cut-off point to discriminate between high/accumulated TP53 protein (34%) and low TP53 protein levels (66%). This prevalence is comparable with the TP53 protein accumulation of 30% and 28%, observed in smaller series of breast tumors studied by Borg et al (4) and us (de Witte et al (5) respectively. Moreover, this is well in line with the percentage of TP53 gene alterations or protein overexpression, observed in 14-52% of 3000 human breast tumors using molecular or immunological techniques (reviewed by Elledge and Allred, 1994).

In univariate analysis TP53 protein accumulation was significantly associated with an increased relapse rate. In a multivariate analysis for relapse free survival, including age, menopausal status, tumor size, nodal status and steroid hormone receptor status, TP53 protein accumulation (analyzed as a dichotomized variable) was an independent factor for predicting the rate of relapse (Table II). The RHR, relative hazard rate, (with 95% CL, confidence limits) was 1.39 (1.19-1.63). This study supplements those of Thor et al (11), Andersen et al and Elledge et al (6), who also observed a relation between immunohistochemically assessed TP53 protein accumulation and shorter relapse-free survival, and confirms that of Borg et al (4) who employed the same assay as we used in this study.

In conclusion, the LIA for the TP53 protein can easily be performed on cytosols routinely prepared for steroid hormone receptor analysis, it is a quantitative assay, and it may be useful in establishing the relation of TP53 accumulation and breast cancer prognosis. However, of all mutations studied at the TP53 locus in breast cancer, about 16% are deletions, insertions or stopcodons, resulting in a deletion or truncation of the protein in the cell (Greenblatt et al (9). Unfortunately the latter alterations are not revealed with immunological techniques. Using Kaplan-Meier analyses we observed that both subsets of patients with either TP53 protein accumulation or very low expression do worse (not
shown). This implies that more studies will be needed to define cut-off points for the subset with very low expression.

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References


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