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PROGNOSTIC SIGNIFICANCE OF TP53 ACCUMULATION IN HUMAN PRIMARY BREAST CANCER: COMPARISON BETWEEN A RAPID QUANTITATIVE IMMUNOASSAY AND SSCP ANALYSIS

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TP53 accumulation in human primary breast carcinomas was studied by a quantitative luminometric immunoassay (LIA), and *TP53* gene alterations, exons 5–8, were examined by single-strand conformation polymorphism (SSCP) analysis. In 48 of 142 breast tumor samples, a *TP53* gene alteration was identified. In tumor samples without a *TP53* gene alteration, the median cytosolic TP53 protein level, as determined by LIA, was 0.4 ng/mg protein (range 0–70.8 ng/mg protein), whereas the median TP53 protein level for tumor samples with a *TP53* gene alteration was 10 times higher, *i.e.*, 4.1 ng/mg protein (range 0.1–176.0 ng/mg protein). Despite a significant correlation between the outcome of LIA and SSCP, a disagreement was found in 22% of cases analyzed. Significant correlations were found between TP53 protein accumulation and low estrogen receptor content, and with a shorter relapse-free as well as overall survival, with a median duration of follow-up of 100 months. Due to its rapid and easy performance on routinely prepared cytosols, the LIA for TP53 protein may be useful in evaluating the prognostic impact of TP53 protein accumulation in human primary breast cancer.
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Mutations in the tumor-suppressor gene *TP53* are the most common genetic alterations in human cancers (Hollstein *et al.*, 1991; Levine *et al.*, 1991). The human tumor-suppressor *TP53* gene is located on chromosome 17p13.1 and encodes for a 53-kDa nuclear phosphoprotein which is implicated in regulation of normal cell growth, division and apoptosis (Levine *et al.*, 1991; Lane, 1994). The 20-kb *TP53* gene consists of 11 exons, of which exons 2–11 code for a protein of 393 amino-acid residues (Lane, 1994). Exons 2–4 code for the acidic N-terminal domain of the TP53 protein, which is involved in the transcriptional control of other cellular genes, either directly or indirectly *via* interaction with other proteins (Lane, 1994). Exons 5–9 code for the central hydrophobic core of the protein, which is characterized by its conformational flexibility and ability to fold into a domain with sequence-specific DNA-binding activity (Cho *et al.*, 1994). Exons 10 and 11 specify the more basic C-terminus of the molecule, containing motifs involved in nuclear localization and in oligomerization (Sturzbecher and Deppert, 1994).

More than 90% of the *TP53* gene mutations in tumor cells appear to be confined to the phylogenetically conserved exons 5–8. Except for stop codon mutations and deletions, leading to the expression of a truncated protein, these mutations give rise to a conformationally altered protein, which is due to a prolonged half-life, often stably expressed at high levels (Hollstein *et al.*, 1991; Lane, 1994). The mutated TP53 protein forms oligomeric complexes with the wild-type protein and, as a consequence, can abrogate its normal function. Likewise, wild-type TP53 protein can be inactivated by binding to viral oncoproteins, such as adenovirus E1B and human papillomavirus E6 proteins. An additional mechanism for inactivation of wild-type *TP53* which occurs relatively common in breast cancer is loss of heterozygosity involving the *TP53* gene locus. Furthermore, the heterologous interaction of the murine double-minute protein MDM2 and heat shock protein HSP70 with wild-type TP53 protein can also result in loss of TP53 function (Calderwood, 1992; reviewed by Greenblatt *et al.*, 1994).

Numerous investigations have been performed to analyze either the relationship between TP53 protein accumulation and *TP53* gene mutation or the clinical significance of TP53 protein accumulation in many tumor types (Bodner *et al.*, 1992). In mammary cancers, *TP53* gene status has been assessed at the level of the gene by (constant) denaturant gel electrophoresis (CDGE/DGGE), single-strand conformation polymorphism (SSCP) analysis and DNA-sequencing techniques, whereas TP53 protein levels have been analyzed by immunohistochemical methods, quantitative immunoassays (ELISA) or Western blotting (Vojtesek *et al.*, 1992; Elledge and Allzed, 1994). In the present study, we examined TP53 protein accumulation in cytosols, prepared for steroid hormone receptor analysis, of human primary breast carcinomas using a recently developed quantitative luminometric immunoassay (LIA) (Borg *et al.*, 1995). We have correlated TP53 protein levels with *TP53* gene alteration as determined by SSCP analysis in the same tumor samples. TP53 protein levels were also correlated with clinical outcome to evaluate the prognostic significance of TP53 protein accumulation in human primary breast cancer.

MATERIAL AND METHODS

Patients and tumor samples

The present study includes 142 human primary breast tumor specimens, used for routine steroid hormone receptor analysis. All clinical data, including age, menopausal status, tumor size, lymph-node status, differentiation grade and steroid hormone receptor status, were available for 111 patients and are shown in Table I. Included were patients without signs of distant metastasis at diagnosis. Patients did not receive endocrine therapy, chemo- and/or radiotherapy before surgery. Median follow-up of patients was 100 (range 47–159) months. Tumor tissues were stored in liquid nitrogen, pulverized in the frozen state and homogenized in standard receptor buffer according to the revised standards of the EORTC Breast Cancer Cooperative Group (1980). Cytosols were aliquoted and stored at −80°C until further analysis.

SSCP analysis

DNA was isolated from an aliquot of the total tissue homogenate as described previously (Berns *et al.*, 1992) and

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TABLE I – CHARACTERISTICS OF PATIENTS, TUMORS AND TREATMENT (111 CASES)

Patients	Number of cases	TP53 over-expression (%)[1]	p value
Total	111	28	
Age (yr)			
Mean	57.9		NS[2]
Range	28–82		
Menopausal status			
Pre	39	31	NS
Post	72	26	
Tumours			
Size (cm)			
<2	29	17	NS
2–5	64	27	
>5	18	50	
Nodal status[3]			
N0	37	24	NS
N1-3	37	30	
N >3	35	29	
Differentiation grade[3]			
Well	2	—	NS
Moderately	27	19	
Poorly	65	31	
Hormone receptor status[3]			
ER–[4]	28	50	
ER+	83	20	0.03
PgR–[4]	33	46	
PgR+	73	21	NS
Treatment			
Surgery of primary tumours			
Breast conserving therapy	30		
Modified mastectomy	81		
Axillary dissection			
Yes	108		
No	3		
Radiotherapy			
Breast	52		
Axilla	53		
Other lymph-node areas	94		
Systemic adjuvant treatment			
Hormonal therapy	6		
Chemotherapy	20		

[1]Percentage of cases with TP53 protein expression above the cut-off point (>2.5 ng/mg protein).–[2]NS, not significant.–[3]Numbers do not always add up to 111 due to missing information.–[4]Cut-off points: 10 fmol/mg protein.

subsequently amplified by PCR using primer panels flanking exons 5–8 of the *TP53* gene (Clontech, Palo Alto, CA). SSCP analysis was performed as described by Orita *et al.* (1989). Mutations found by PCR-SSCP analysis were confirmed in an additional experiment with a second independent PCR product. Neutral polymorphism in PCR fragments from exon 6 (codon 213) was verified by PCR-SSCP and *Taq*I restriction analysis.

LIA

TP53 protein levels in cytosols were determined using a novel LIA from Sangtec Medical (Bromma, Sweden), as described by Borg *et al.* (1995). The assay was performed on an LIA-MAT S300 analyzer (Stratec, Dietzenbach, Germany), equipped with a pipetting station, incubator, washer and luminometer. In brief, either 100 μl standard (range 0–80 ng TP53 protein/ml) sample or control and 100 μl tracer (ABEI-conjugated monoclonal antibody [MAb] DO1 [21]) solution were pipetted into tubes coated with pAB1801 MAb (Banks *et al.,* 1986). After incubation for 18 hr at room temperature, tubes were washed 3 times with 2 ml 0.9% sodium chloride, and chemiluminescence was subsequently measured using an LIA-MAT starter service kit (Byk-Sangtec Diagnostica, Dietzenbach, Germany). All samples were diluted in kit diluent buffer to a final concentration of 0.5 mg/ml cytosol protein and analyzed in duplicate. The TP53 protein concentration was expressed in ng/mg protein. A representative standard curve of the TP53 LIA is shown in Figure 1. Cytosols of the human breast carcinoma cell line T-47D, which contains a mutation in exon 6 (codon 194) of the *TP53* gene (Nigro *et al.,* 1989), were used as control for TP53 protein over-expression. Cell line ZR-75.1, without a *TP53* gene mutation served as control for low TP53 protein expression.

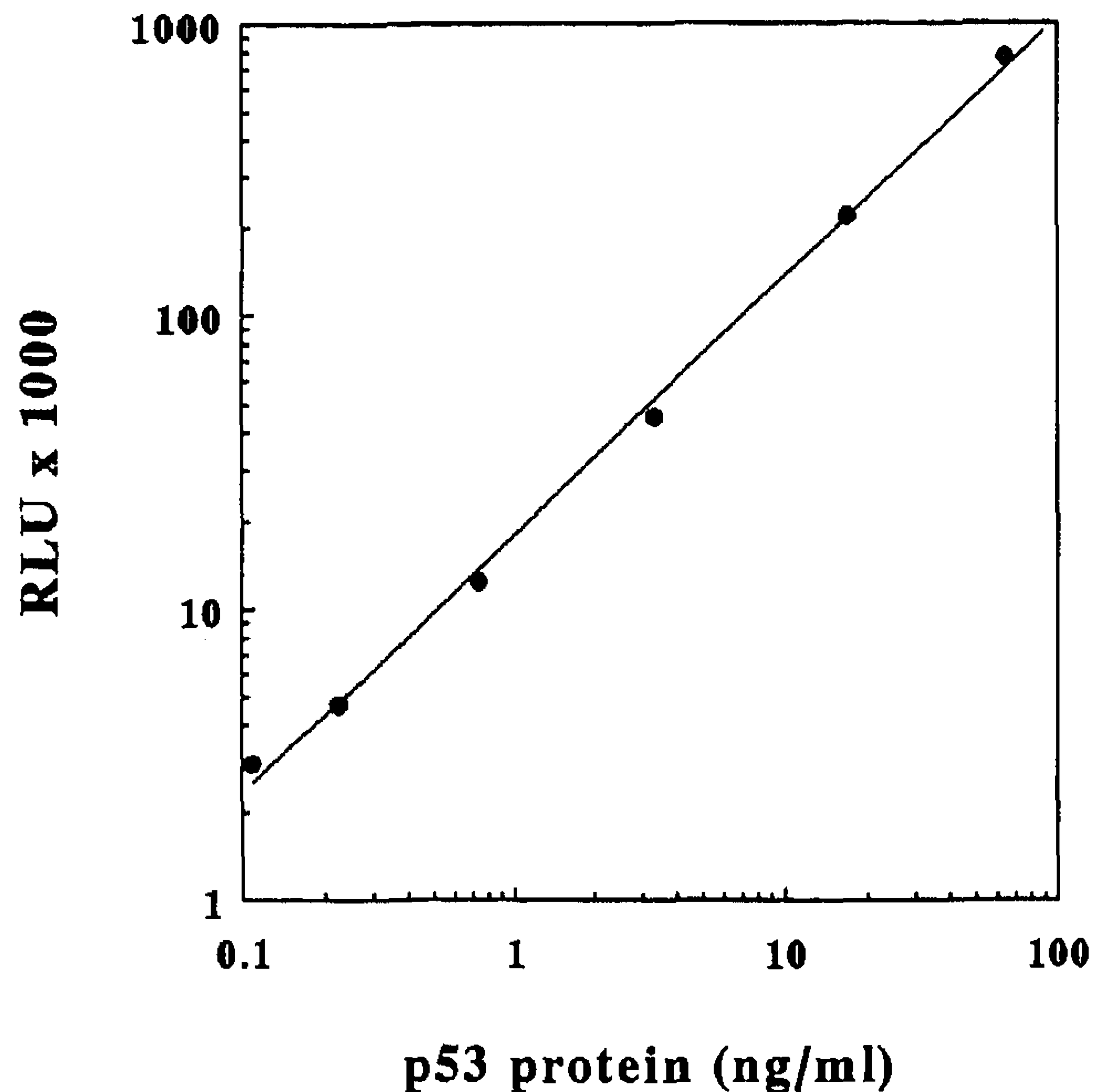

FIGURE 1 – Representative p53 LIA standard curve, using known amounts of recombinant human *TP53* protein. The standard points are the result of duplicate assays (coefficient of variation, below 10%). RLU, relative light units.

Statistical analysis

To study relations between patient and tumor characteristics and TP53 protein, we used Spearman rank correlations when variables were continuous and Kruskal-Wallis or Wilcoxon rank sum for categorized variables. Isotonic regression analysis was performed to determine the optimal cut-off level for TP53 expression (Barlow *et al.,* 1972). For comparison of the results of both PCR-SSCP and LIA, the 2-sample Wilcoxon rank sum test was applied. Overall and relapse-free survival probabilities were calculated by the actuarial method of Kaplan and Meier (1958). The Cox proportional hazard model was used for univariate and multi-variate survival analyses.

RESULTS

TP53 protein expression analysis by LIA

LIA for TP53 protein was performed on 142 primary breast tumor cytosols. TP53 protein concentrations ranged from 0 to 176.0 ng/mg protein, with a mean value of 5.8 ng/mg (SD 19.2 ng/mg) and a median value of 0.5 ng/mg cytosolic protein. Cox univariate regression analysis using logarithmically transformed TP53 protein values showed a significant ($p < 0.001$) positive association between TP53 protein concentrations and rate of relapse. This justified the search for cut-off points to classify tumors as TP53 protein–low and TP53 protein–high (accumulated). Different cut-off values were tested for their

TABLE II - ISOTONIC REGRESSION ANALYSIS OF TP53 PROTEIN LEVELS AND RELAPSE-FREE SURVIVAL

Range of TP53 protein levels	Number of patients	Number of relapses	Mean time to relapse (months)[1]
0.00–0.02	14	7	139.1
0.03–0.09	4	2	129.1
0.10–0.18	8	4	123.5
0.20–2.40	54	33	103.3
2.69–5.84	13	10	65.1
5.90–23.50	13	10	49.0
26.32–26.32	1	1	12.6
41.94–48.96	2	2	4.1
87.26–175.96	2	2	3.7

[1]Sum of times at risk divided by number of relapses during the full follow-up period.

ability to identify a subgroup of patients with TP53 accumulation and poor prognosis. Isotonic regression analysis was applied with relapse-free survival as the end-point for all tumor samples (Table II), and a cut-off point was found at 2.5 ng/mg protein to discriminate between TP53 protein–high (28%) and TP53 protein–low (72%) cases.

Comparison of LIA and SSCP

In 48 of 142 breast tumor samples (34%), a *TP53* gene alteration was identified by PCR-SSCP analysis. An autoradiogram of SSCP analysis of exon 8 of the *TP53* gene is shown in Figure 2. In 9 tumor samples, the *TP53* gene mutation was demonstrated to be confined to exon 5, in 6 samples a mutation in exon 6 was detected and another 9 samples showed a mutation in exon 8. Mutations were most frequently observed in exon 7 (20 samples; Table III). One breast tumor sample was shown to contain 2 mutations, 1 in exon 5 and the other in exon 6, whereas 3 other samples had alterations in both exons 6 and 8.

Mutations in the *TP53* gene were compared with TP53 protein levels as determined by LIA in the same tumor samples, using the cut-off point of 2.5 ng/mg protein. In 111 cases (78%), an agreement was found between the outcome of the LIA test and SSCP analysis: in 83 tumor samples without a *TP53* gene alteration, TP53 protein levels were below 2.5 ng/mg protein, and in 28 samples with a mutated *TP53* gene, elevated TP53 protein concentrations (>2.5 ng/mg protein) were measured. A different outcome was observed in 31 cases (22%); *i.e.,* 11 samples without a gene alteration had high levels of TP53 protein and 20 samples with a gene alteration were shown to contain low TP53 protein levels.

In the 94 tumor samples without a *TP53* gene alteration, TP53 protein levels ranged from 0 to 70.8 ng/mg protein (Table III). In samples with a mutation in the *TP53* gene, TP53 protein concentrations varied from 0.1 to 176.0 ng/mg protein and the median value was approximately 10 times higher, *i.e.,* 4.1 ng/mg cytosolic protein (Table III). Using the 2-sample Wilcoxon rank sum test, median TP53 protein concentrations differed in tumor samples with and without a *TP53* gene alteration ($p = 0.0001$).

TP53 protein expression vs. *affected exon*

Next, TP53 protein levels were investigated as a function of the mutated exon, analyses being confined to exons 5–8 (Table III). Cytosols from tumor tissues with a known alteration in exons 5–7 were quantitated to contain 2.0, 3.4 and 3.3 ng/mg protein (median values), respectively. Samples with a mutation in exon 8 contained the highest TP53 (median) protein levels, *i.e.,* 6.0 ng/mg protein (Table III). In the 4 samples with 2 independent mutations; median TP53 protein concentration was 1.2 ng/mg (range 1.2–5.9 ng/mg; data not shown). Except for exon 5, the differences between TP53 protein levels in samples with a mutation in a specific exon and without a *TP53* gene alteration were statistically significant ($p < 0.05$), as analyzed by the Wilcoxon rank sum test (p values corrected for multiple comparisons). High TP53 protein levels (65.0 ng/mg protein) were measured in the T-47D cell line, which contains a mutated *TP53* gene. In contrast, cytosol prepared from the ZR-75.1 cell line, with wild-type TP53, showed median cytosolic TP53 protein levels (0.4 ng/mg protein; data not shown).

TP53 expression in uni- and multi-variate prognostic analyses

No significant associations of TP53 protein over-expression with age, menopausal and nodal status, tumor size or progesterone receptor status were observed. High TP53 protein levels were more frequently observed in tumors with low estrogen receptor content ($p = 0.03$).

In univariate analyses, both *TP53* gene alteration and TP53 protein over-expression were significantly associated with increased rates of relapse ($p = 0.013$ and $p < 0.001$, respectively; Fig. 3, top panels) and of death ($p = 0.026$ and $p < 0.0001$, respectively; Fig. 3, bottom panels). In this patient group, tumor size and low progesterone receptor content were also associated with an increased rate of relapse ($p = 0.001$ and $p = 0.03$, respectively). In multi-variate analyses, including age, menopausal status, tumor size, nodal status and estrogen and progesterone receptor status, TP53 protein over-expression (either dichotomized or as a continuous variable) was independently associated ($p < 0.01$) with poor prognosis (Table IV). Relative relapse and death rates (with 95% confidence limits) were 2.38 (1.34–4.23) and 2.99 (1.57–5.68), respectively.

DISCUSSION

In the present study TP53 protein expression levels, as measured with a quantitative immunoassay, were compared with the prevalence of *TP53* gene alterations, as detected by SSCP analysis, in the same 142 breast tumor samples. In 34% of tumor samples, a *TP53* gene alteration in exons 5–8 was identified. This value falls within a range of frequencies (17–46%) reported earlier for *TP53* mutations in primary breast carcinomas (Runnebaum *et al.,* 1991; Osborne *et al.,* 1991). Samples with a *TP53* gene mutation may give rise to a protein with a highly stabilized conformation and, consequently, an increased half-life as compared with wild-type TP53 protein (Levine *et al.,* 1991). Using a cut-off point of 2.5 ng/mg protein, high cytosolic levels of TP53 protein were observed in 28% of samples analyzed. Borg *et al.* (1995), who used the same LIA, found a lower cut-off value, but both studies showed TP53 protein over-expression in 30% of tumors. A significant correlation ($p = 0.0001$) was found between TP53 protein over-expression and the presence of a *TP53* gene alteration (exons 5–8). The highest (median) TP53 protein level was found for tumor samples harboring a mutation in exon 8 (but numbers are small). Interestingly, the high TP53 protein concentrations measured in cytosols prepared from the T47D cell line, which contains a mutated *TP53* gene, clearly reflect the elevated TP53 mRNA expression observed on Northern blots (not shown). The low levels observed in the cytosol from the ZR-75.1 cell line correspond to the presence of the wild-type *TP53* gene and, consequently, the low mRNA expression levels in the cells.

When dichotomized, a discrepancy was found between the results of both assays in 22% of the cases analyzed. The difference observed in 20 of 48 cases designated as being TP53 protein–low and *TP53* gene–altered by SSCP analysis could be explained by mutations that do not lead to either stabilization of the protein or accumulation in the cells. As suggested previously, the level of TP53 protein expression may be dependent on the type of mutation of the *TP53* gene (Bodner

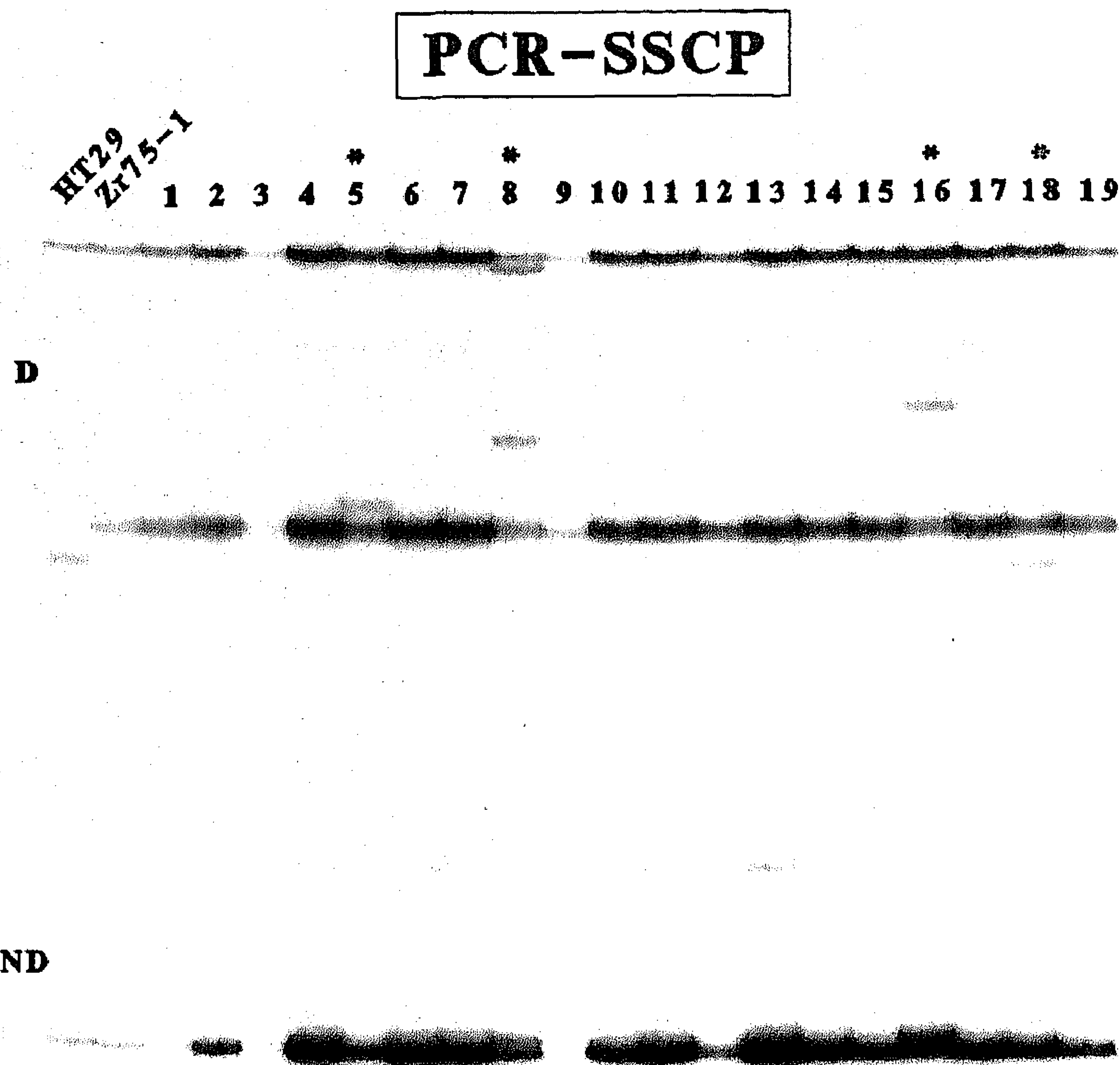

FIGURE 2 – Autoradiogram of PCR-SSCP analysis of exon 8 of the *TP53* gene. Lanes with altered migration patterns are indicated with an asterisk. HT29 serves as a positive control and ZR-75-1 serves as a negative control. D, denatured; ND, non-denatured bands.

TABLE III – RELATIONSHIP BETWEEN TP53 OVER-EXPRESSION, AS DETERMINED BY LIA, AND *TP53* GENE ALTERATIONS, AS ANALYZED BY PCR-SSCP

LIA/SSCP	Number[1]	Median	Range	Mean	SD	LIA-low[2]	LIA-high[2]	*p* value
Wild-type	94	0.4	0.0–70.8	2.0	7.9	83	11	0.0001
Mutant[3]	48	4.1	0.1–176.0	13.2	29.9	20	28	
Exon 5	9	2.0	0.1–41.9	7.0	13.3			
Exon 6	6	3.4	0.2–13.0	5.3	5.6			
Exon 7	20	3.3	0.3–176.0	22.9	19.0			
Exon 8	9	6.0	0.2–20.6	7.2	6.4			

[1]Number of cases.–[2]Cut-off point set at 2.5 ng/mg protein.–[3]Mutations in exons 5–8; 4 samples were shown to contain 2 mutations (see "Results").

et al., 1992), which is not disclosed by the SSCP analysis in this study. SSCP-positive tumors may include those with silent mutations as well as those with stop codons and deletions leading to expression of truncated protein, which is undetectable by immunological techniques (Greenblatt *et al.,* 1994). We have therefore sequenced DNA from 14 DNA samples available out of the 20 SSCP-positive/LIA-negative samples. Of these, 4 samples showed deletions or insertions leading to an altered protein product. All 4 samples had low levels of protein. The other 10 DNA samples showed a silent mutation. Of these, 1 sample showed an elevated LIA level, another sample showed an LIA level of 2.5 ng/mg protein (cut-off point) and the remaining 8 samples showed low LIA levels, meaning that 12 of 14 were concordant. So far, it is not clear from our data that LIA will detect mutations of particular prognostic interest according to the data of Bergh *et al.* (1995) or Borresen *et al.* (1995). Furthermore, proteins of viral origin, *e.g.,* E6 oncoprotein, may target the TP53 protein to more rapid catabolism (Greenblatt *et al.,* 1994), which leads to low TP53 protein levels.

The 11 cases (of 39) assigned as TP53 protein–high and not altered according to SSCP (28%) could be explained by the accumulation of TP53 protein in cytosols due to stabilization of the wild-type protein when forming complexes with other cellular or viral molecules. In these cases, accumulated wild-type TP53 protein would also be detected by the immunoassay since the LIA test utilizes MAbs specific for denaturation-resistant epitopes of the NH_2-terminus of the protein, thus recognizing both mutant and wild-type TP53 proteins. Moreover, mutations may be present in other exons or introns or even in the regulatory gene sequences outside the regions screened by SSCP analysis, giving rise to high levels of TP53 expression without evidence of a mutation. However, only a small percentage (below 10%) of the reported mutations have been found outside of exons 5–8 (Greenblatt *et al.,* 1994). Another explanation could be accumulation of TP53 protein because of recent DNA damage from chemical and physical genotoxic agents (Greenblatt *et al.,* 1994). Obviously, the cut-off point itself, selected because of the correlation of relapse-free survival with TP53 protein over-expression and

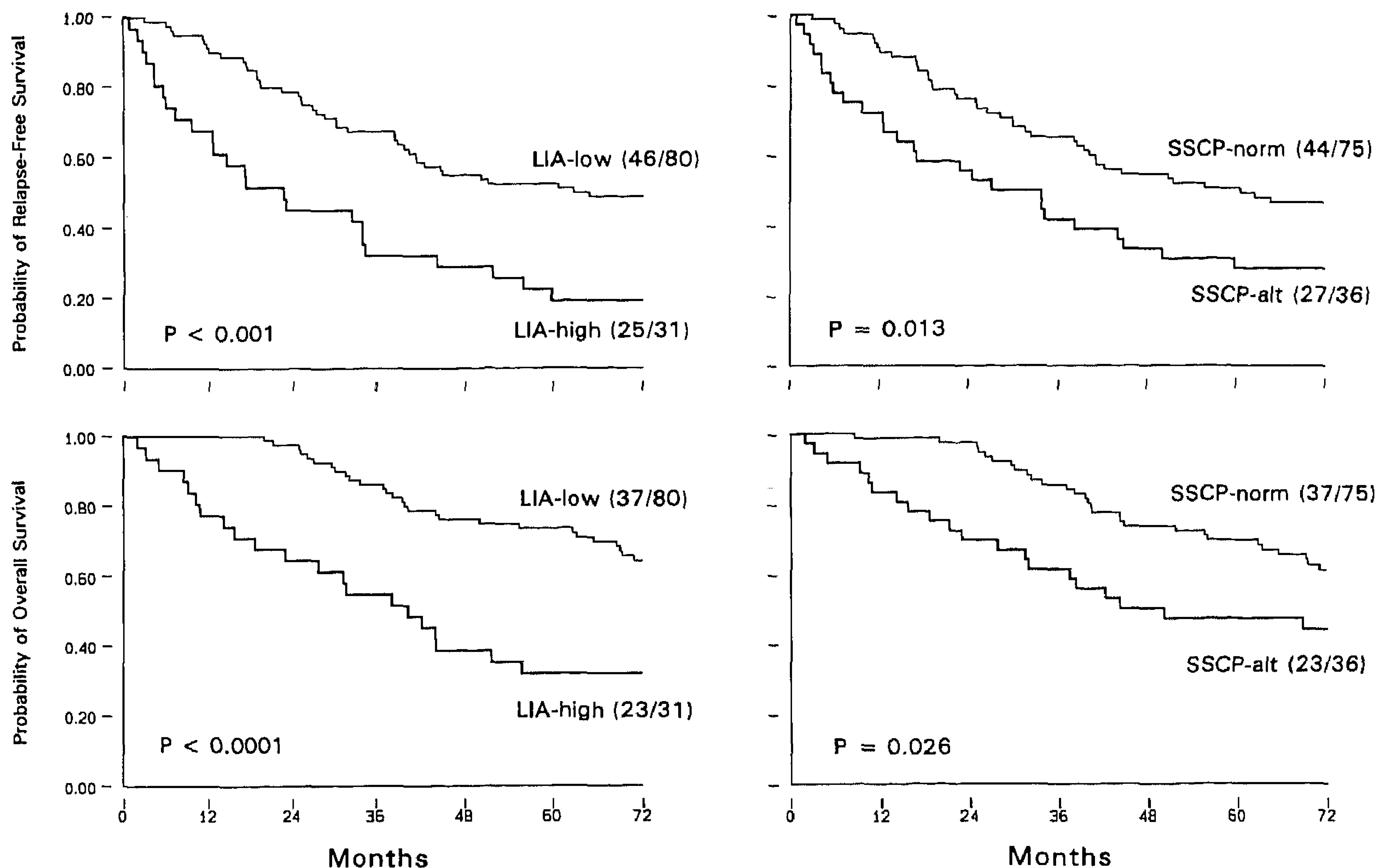

FIGURE 3 – Actuarial relapse-free and overall survival curves for 111 primary breast cancer patients as a function of *TP53* status. Relapse-free survival curves (upper panel) of patients with and without TP53 protein over-expression (left) and *TP53* gene alterations (right). Overall survival curves (lower panel) for patients with and without TP53 protein over-expression (left) and *TP53* gene alterations (right). SSCP-alt and SSCP-norm indicate tumor samples with and without *TP53* gene alterations in exons 5, 6, 7 or 8; LIA-low and LIA-high indicate TP53 protein expression below or above the cut-off point (2.5 ng/mg protein). Numbers in parentheses represent failures/total number of patients in each group.

TABLE IV – COX MULTIVARIATE ANALYSIS FOR RELAPSE-FREE AND OVERALL SURVIVAL IN 111 PRIMARY BREAST CANCER PATIENTS

Factor	Relapse-free survival		Overall survival	
	p value multivariate	RHR[1]	*p* value multivariate	RHR
Age/menopausal status	0.54		0.05	
Age/pre-menopausal				
Age/post-menopausal				
Menopausal status				
Tumor size[2]	0.09	1.30 (0.96–1.76)	0.54	
Lymph-node status[3]				
N1-3	0.03	2.12 (1.10–4.09)	0.24	
N >3	0.01	2.25 (1.18–4.31)	0.04	2.10 (1.04–4.26)
Receptor status[4]				
Estrogen (ER)	0.65		0.89	
Progesterone (PgR)	0.06	0.55 (0.30–1.02)	0.004	0.36 (0.18–0.72)
TP53 LIA result[5]	0.003	2.38 (1.34–4.23)	<0.001	2.99 (1.57–5.68)

[1]Relative hazard rates with 95% confidence limits (values in parentheses) are presented for the variables with $p < 0.10$ in the multivariate analysis.–[2]Tumour size scored as T1–T4.–[3]As compared to node-negative.–[4]Receptor-positive as compared with receptor-negative (cut-off points, 10 fmol/mg protein).–[5]TP53 protein expression above 2.5 ng/mg protein as compared with TP53 protein levels below the cut-off value.

not with prevalence of *TP53* gene mutations, also affects the agreement between the results of both assays. Nevertheless, the overall correlation between the outcome of both assays is highly significant ($p = 0.0001$).

With respect to prognosis, TP53 protein accumulation appeared to be an independent predictor for both relapse-free survival and death. These findings complement the results of both DNA-based and immunohistochemical investigations, which showed a strong association between either *TP53* gene alteration or TP53 protein accumulation and poor prognosis (Elledge *et al.*, 1994; Andersen *et al.*, 1993; Boer *et al.*, 1995). Immunohistochemical analyses evaluate tumor tissue sections by estimating staining intensity and the percentage of stained cells. These results should be interpreted as semiquantitative

(Greenblatt *et al.,* 1994). In contrast, SSCP, CGGE and DGGE analyses have a sensitivity and specificity of approximately 90% (Greenblatt *et al.,* 1994) but are time-consuming and labor-intensive. The immunoassay applied in this study is easier and faster to perform and can be carried out on breast tumor cytosols routinely prepared for steroid hormone receptor assays. Moreover, the immunoassay objectively quantitates TP53 protein expression, TP53 protein levels apparently being a reasonably good representation of both mutant and wild-type *TP53* DNAs. Quantitative analysis of the TP53 protein status in cytosols routinely prepared for steroid hormone receptor analysis may contribute to the selection of patients who, depending on the relationship of *TP53* gene status with response to a specific type of systemic adjuvant therapy, could benefit from adjuvant therapy.

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