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TP53 Mutations and Abnormal p53 Protein Staining in Breast Carcinomas Related to Prognosis

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Abnormalities in the *TP53* tumour suppressor gene were evaluated in 106 unselected breast carcinomas and compared to clinical outcome of the disease. Tumours were screened for p53 abnormalities using immunohistochemical staining and polymerase chain reaction–constant denaturant gel electrophoresis (PCR–CDGE) analysis, followed by PCR and direct sequencing. Allelic loss at the *TP53* locus was determined with polymorphic markers by comparing normal and tumour DNA. For approximately half of the patients, abnormal p53 protein expression in serum was determined by an ELISA assay. p53 abnormalities, detected as mutations and/or nuclear staining, were found in 37.6 (38/101) of cases. Nuclear staining for p53 protein could be identified in 33.7% of the tumours. Mutations in exons 5–8 were detected in 18.9% of the tumours, and an association was found between mutations and nuclear staining. Allelic loss in the *TP53* region on 17p was more frequent in tumours showing changes in the *TP53* gene (72.7%) compared to tumours with no mutation (45.8%). Serum levels of p53 antibodies showed no association with either *TP53* mutations or nuclear staining. Women with *TP53* mutations in their tumours had an elevated risk of dying during the study period (RR (relative risk) = 3.4, $P = 0.014$). The effects of p53 positive staining were similar (RR = 3.2, $P = 0.013$). Considering all abnormalities, mutation and/or staining, the relative risk of dying from breast cancer was 3.5 ($P = 0.008$).

Key words: breast cancer, p53, immunostaining, mutation analysis, CDGE, prognosis
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INTRODUCTION

ABNORMALITIES IN the *TP53* gene are among the most commonly reported genetic changes in malignant tissue, and are thought to play an important role in many types of cancer [1]. Overexpression of the p53 protein and/or mutation of the *TP53* tumour suppressor gene has been demonstrated in a variety of human malignancies, including cancer of the breast, colon and lung [2]. Germline changes in the *TP53* gene have been found in patients with the Li–Fraumeni cancer syndrome [3]. The human *TP53* gene is located on the short arm of chromosome 17 and encodes a 393 amino acid phosphoprotein, expressed in normal cells at a low level [4]. The p53 protein has been implicated in DNA repair and synthesis, cell differentiation and apoptosis. Recent studies have suggested that p53 may be involved in cell-cycle control, invoked by DNA damage. Cells with abnormal p53 do not show the normal G_1 arrest, necessary for repair, after exposure to ionising radiation [5].

The level of p53 protein is regulated both by RNA transcription control and a short protein half-life (10–30 min) [6]. Due to its short half-life, the wild-type p53 protein is not detected in tumours, but mutations in the *TP53* gene may result in a biologically altered protein, with increased stability and accumulation, which is detectable by immunohistochemical methods [6]. Other mechanisms may also alter the stability of the protein. Binding of the MDM2 protein and a number of viral proteins appear to stabilise p53 [7–10].

The *TP53* gene has five domains that are highly conserved throughout evolution [11]. *TP53* mutations in tumours tend to cluster in these conserved regions or "hot-spots" suggesting that tumorigenesis selects for expression of a mutant protein altered in a defined region [9]. Allelic loss on the short arm of chromosome 17 has been reported to occur in 50–70% of breast cancers,

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suggesting inactivation of a tumour suppressor gene. This is a much higher frequency than could be explained by the mutations found in the *TP53* gene (15–40%) [8]. Studies have indicated the presence of a second tumour suppressor gene that might be involved in breast cancer, more distal than the *TP53* gene, on 17p13.3, which might account for some of the allelic loss found [12].

Serum levels of p53 antibodies are raised in a small percentage of cancer patients indicating a mutation in the *TP53* gene in the tumours. Autoantibodies against human p53 protein have been described in 9–15% of serum samples from breast cancer patients [13, 14]. A study by Caron de Fromentel and coworkers demonstrated circulating p53 antibodies in serum of children with a variety of cancers [15]. An association has been found between p53 antibodies in serum and the type of *TP53* mutation, p53 protein staining in tumours or poor prognosis [14]. It has been shown that mutation in the *TP53* gene is an independent marker of shortened survival and an indicator of poor prognosis in breast cancer patients [16–19]. The same has been shown for p53 protein abnormalities detected as increased nuclear staining [20, 21]. In order to examine the relationship between *TP53* mutations and p53 positive staining, we screened the same samples with both methods. We have previously shown a significant association between *TP53* mutations in breast tumours and shortened survival [16]. This is a follow-up study on the same material, extending the area analysed for mutations and adding protein staining data, as well as screening serum from the same patients for antibodies against the p53 protein. The abnormalities are compared to clinicopathological parameters including nodal status, tumour size, oestrogen and progesterone receptor status, age at diagnosis and survival.

PATIENTS AND METHODS

Patients

One hundred and six non-selected invasive breast carcinomas were collected over a 4 year period, 101 primary tumours and five metastases. Tumours were snap-frozen and homogenised, and DNA was extracted from tumour tissue and peripheral blood using standard phenol–chloroform methods. The mean age of patients was 58.9 years (range 33–94) compared to a mean age of 59.4 years for Icelandic breast cancer patients over a period of 30 years [22]. Information on clinical parameters for the group was collected from the Department of Pathology, University Hospital of Iceland and the Icelandic Cancer Registry, and used in multivariate analysis. Follow-up time was 3–64 months, mean 44 months, measured from date of diagnosis to end of study or death from breast cancer, whichever came first.

Immunohistochemical analysis

Immunohistochemical analysis was performed on 5 μm sections of formalin-fixed, paraffin-embedded tissue from the same patients. The tissue samples were fixed for 1–2 days in 10% formalin. Two antibodies were used; CM-1 and DO-1. CM-1 is a rabbit polyclonal antibody against human p53 protein [23]. The antibody was diluted 1:1000, the sections incubated with the antibody overnight at 4°C and detected using the peroxidase–antiperoxidase method. DO-1 is a monoclonal antibody that recognises the N-terminus of the p53 protein [24]. The antibody was used at a dilution of 1:30, incubated at 4°C overnight and an incubation with a second, biotinylated antibody was performed for 30 min. The reaction was then labelled with a streptavidin–biotin complex and detected by immunoperoxidase-labelling. A sample of breast carcinoma with a known *TP53* mutation and positive staining reaction with the monoclonal anti-p53 antibody PAb1801 served as positive control and surrounding normal mammary tissue was an internal negative control in each case. All slides were read independently by three investigators. Staining was scored as positive if > 5% of cancer cells showed clear nuclear staining and the staining intensity was indicated by + (weak) and ++ (strong). Cytoplasmic staining alone was not recorded as positive.

Mutational analysis

DNA from tumours was screened for mutations in the *TP53* gene by the polymerase chain reaction–constant denaturant gel electrophoresis (PCR–CDGE) method, and the exact nature of the mutation determined by PCR and direct sequencing [25]. Samples were screened for mutations in the conserved domains in exons 5–8 of the *TP53* gene. PCR conditions, constant denaturant electrophoresis and sequencing conditions were as described previously [16] except for exon 6 [26]. All samples showing aberrantly migrating bands in the CDGE gel for exon 6 were analysed for a neutral TaqI polymorphism in codon 213 [27] by PCR and restriction enzyme digestion.

Allelic loss

Loss of heterozygosity was examined by standard Southern blotting and hybridisation methods for four different markers on chromosome 17p [28] and three PCR polymorphic markers. Three of the probes map to 17p13.3: *pYNZ22 (D17S5)*, *pYNH37.3 (D17S28)* and *p144D6 (D17S34)*. Four markers map to the *TP53* locus at 17p13.1: an intragenic *TP53* probe *pBHTP53 (TP53*, [29]), two microsatellite markers at the *TP53* locus [30] and a PCR amplified *Acc*II polymorphism within the *TP53* gene [31]. The markers were informative for 55–90% of cases.

Analysis of p53 antibodies in serum

Serum levels of the p53 protein antibody were measured for 47 of the patients where serum was available, using the ELISA assay according to the manufacturer's instructions, in samples taken on the day of surgery. Samples for testing were selected on the basis of the results already obtained with mutational analysis and immunohistochemistry to include representative samples from each of the following four categories: mutation present and staining reaction positive; mutation absent and staining reaction positive; mutation present and staining reaction negative; and mutation absent and staining reaction negative.

Statistical methods

Chi-square test and Fisher's exact test were used to test the significance of difference between two proportions. All *P*-values used were two-sided, using 5% confidence limits. Multivariate evaluation of the survival data was performed using the Cox proportional hazards model [32]. The risk of dying was estimated after taking into account the effects of *TP53* mutation, p53 positive staining, nodal status, tumour size, and age at the time of diagnosis. Data from 86 of the patients with primary carcinoma filled the criteria for the multivariate analysis. Covariates were selected in a stepwise manner, using the maximum likelihood ratio and the statistical package BMDP (BMDP Statistical Software, Los Angeles, California, U.S.A.).

RESULTS

One hundred and one primary tumours were analysed for abnormal p53 protein expression. Increased protein expression,

with one or both antibodies used, was seen in 34 tumours (33.7%). Positive staining was found more often with the polyclonal antibody CM-1. Staining with DO-1 was usually weaker in intensity but easier to interpret because this antibody gave no cytoplasmic staining (Table 1, Figure 1). Heterogeneity was seen in many tumours, i.e. an area of the tumour would be strongly stained for p53 protein while another, clearly malignant, would be negative. In some cases, the latter areas appeared to be better differentiated. Cytoplasmic staining was found in few samples only with the polyclonal antibody, and never found exclusively in malignant cells. Cells were only scored as positive if specific staining was found in the nucleus.

One hundred and six tumours were analysed for *TP53* mutations, 101 primary tumours and 5 metastases or recurrences. We have previously published results of mutation analysis in exons 5, 7 and 8 in this set of tumours [16]. In the present study, we screened through exon 6 with CDGE and found mutations in two additional samples. A total of twenty mutations (18.9%) were detected. A wide range of mutations was found including four transversions, 11 transitions and five deletions. The mutations found by CDGE could all be confirmed by sequencing. All cases were also anslysed for germ line changes but none were found. Table 2 shows the results of mutational analysis, protein staining and results of the ELISA assay.

The 14 tumours with missense mutations, leading to an amino acid change, all showed positive nuclear staining. The five tumours with a nonsense or frameshift mutation showed no protein expression. No tissue was available for protein staining from one case. The association between *TP53* mutation and p53 positive staining was highly significant ($P < 0.0001$) (Table 2). Of 101 samples analysed for both mutations and staining, 38 were shown to have p53 abnormalities (37.6%).

In nineteen of the tumours with p53 positive staining, no mutation was detected in exons 5–8. In four of these tumours, high levels of p53 expression were seen with both antibodies. The remaining 15 tumours had detectable staining but in a smaller percentage of cancer cells. In some cases, only a fraction of the tumour cells showed staining; in other cases faint staining was detected in most cells. In 9 of the 15 samples, staining was only detectable with the polyclonal antibody.

Allelic loss with chromosome 17p markers was detected in 55% of informative tumours. Of the tumours positive for *TP53* mutation, 72.7% showed loss of heterozygosity for at least one of the four *TP53* markers tested as compared to 45.8% of the non-mutated tumours. This difference was also seen when staining data were included (71.4% and 38.8%, respectively). We also detected increased allelic loss at the more distal marker on chromosome 17p in p53 abnormal tumours. Allelic loss at the

Table 1. *Results of staining with monoclonal antibody DO-1 and polyclonal antibody CM-1*

	n (%)
DO-1 (n = 96)	
Negative staining	76 (79.2)
Positive staining	20 (20.8)
CM-1 (n = 101)	
Negative staining	68 (67.3)
Positive staining	33 (32.7)
Total positive (n = 101)	34 (33.7)

Figure 1. Immunostaining of breast carcinomas with antibodies to the p53 protein. The sections were counterstained with haematoxylin. (a) invasive ductal carcinoma showing strong nuclear staining with polyclonal antibody CM-1. Mutation in codon 273 in the *TP53* gene. (b) Invasive breast carcinoma showing strong nuclear staining with monoclonal antibody DO-1. Mutation in codon 241 in the *TP53* gene. (c) Invasive ductal carcinoma with fewer stained nuclei. Mutation in codon 273 in the *TP53* gene. Using the antibody CM-1, staining was detected in some cells but others, clearly malignant, are negative.

Table 2. Comparison of TP53 *mutations and protein staining data*

Sample no.	Exon no.	Codon no.	Base change	Amino acid change	Immunostaining	p53 serum antibodies*
11	5	144	CAG > TAG	gln− > *stop*	−	ND
31	5	151–152	5C > 4C	frameshift, 1 base del.	−	0.29
103	5	163	TAC > TGC	tyr− > cys	++	0.34
44	5	173	GTG > TTG	val− > leu	+	ND
6	5	175	CGC > CAC	arg− > his	++	0.41
107	5	182	TGC > −GC	frameshift, 1 base del.	−	0.25
43	6	205	TAT > TCT	tyr− > ser	+	0.31
84	6	210	AAC > AA−	frameshift, 1 base del.	−	1.17
63	7	237	ATG > ATA	met− > ile	+	0.42
83	7	241	TCC > TTC	ser− > phe	++	0.38
41	7	248	CGG > CAG	arg− > gln	+	0.27
48	7	248	CGG > CAG	arg− > gln	++	0.32
70	7	248	CGG > CAG	arg− > gln	+	0.45
1	7	249	AGG > AGT	arg− > ser	++	ND
42	8	262	GGT > − −	frameshift, 14 bases del.	−	0.61
36	8	273	CGT > TGT	arg− > cys	++	0.14
95	8	273	CGT > TGT	arg− > cys	++	0.79
20	8	282	CGG > TGG	arg− > trp	++	1.48
102†	5	141	TGC > TTC	cys− > phe	+	0.21
55†	7	251	ATC > A−C	frameshift, 1 base del.	ND	0.30

ND = not done. *Average of duplicates measured by ELISA as ng/ml; †Samples 102 and 55 are from recurrent breast carcinomas.

more distal markers was 61.5% for the mutated tumours as compared to 41.0% loss in the non-mutated tumours.

We used a mutant-selective ELISA assay to measure the amount of circulating p53 antibodies in serum from the same patients. Serum levels of p53 protein antibodies ranged from 0.08 to 2.80 ng/ml (mean 0.50 ng/ml). No association could be detected between serum levels and either positive protein expression or the presence of *TP53* gene mutation in the tumour (Table 2).

p53 abnormalities were compared to clinical factors such as tumour type, tumour size, node status and hormonal receptor status. There was a strong association between *TP53* mutations and lack of oestrogen receptors ($P < 0.001$) and the same was true for the group of tumours with p53 positive staining ($P < 0.01$). No apparent association was found with the other clinical factors analysed (Table 3).

The variables used for the multivariate survival analysis were: age at diagnosis, nodal status, tumour size, *TP53* mutation and p53 positive staining. Information on 86 patients with primary breast cancer fulfilled the criteria set by the programme. At the end of the study time, 19 of these had died from breast cancer. Follow-up time was 3–64 months, mean 44 months. Patients with tumours larger than 2 cm had a significantly increased risk of dying from breast cancer ($P = 0.004$; RR = 5.8). There was, however, no association between tumour size and either *TP53* mutation or p53 positive staining ($P = 0.68$ and $P = 0.86$, respectively; Table 3). Women with *TP53* mutations had an elevated risk of dying during the study period ($P = 0.014$) showing a relative risk of dying of breast cancer of 3.4. We also found a significant association between p53 protein staining and survival ($P = 0.013$ and RR = 3.2). This was even more significant when looking at all patients with p53 abnormalities, i.e. either *TP53* mutation in the tumour or positive staining ($P = 0.008$ and RR = 3.5). The Kaplan–Meier survival curves further support the importance of p53 abnormalities in predicting survival (Figure 2).

DISCUSSION

In this study, we compared the results of immunostaining and mutation analysis for the assessment of p53 abnormalities in breast tumours. We found a strong association between *TP53* mutation and positive p53 staining, although both methods missed some abnormalities.

We used the CDGE method for mutation analysis and found mutations in 18.9% of tumours by screening through the four exons of the *TP53* gene where the majority of mutations in

Table 3. Association between clinical factors and p53 abnormalities

	Oestrogen receptor		Progesterone receptor		Node status		Tumour size	
	Positive ($n = 79$)	Negative ($n = 22$)	Positive ($n = 75$)	Negative ($n = 25$)	Positive ($n = 56$)	Negative ($n = 34$)	≤ 2 cm ($n = 34$)	> 2 cm ($n = 64$)
TP53 mutation	9	10*	11	8	13	6	5	13
p53 positive staining	21	13†	22	12	20	13	11	21

*$P < 0.001$; †$P < 0.01$.

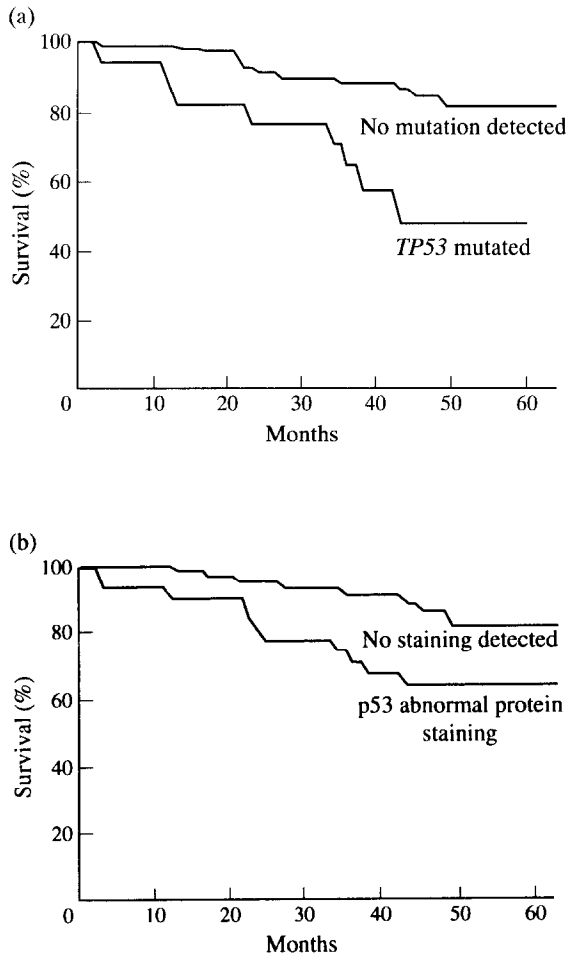

Figure 2. Survival curves. (a) Relationship between *TP53* mutation and breast cancer survival. (b) Relationship between abnormal p53 staining and breast cancer survival.

tumours have been detected. Immunohistochemical staining revealed changes in p53 protein in 33.7% of these same tumours. Others have reported p53 abnormalities, mutations and/or protein staining in 15–45% of breast tumours [20, 25, 33–34]. In our study, all tumours with *TP53* mutations, leading to an amino acid change, showed positive staining with antibodies against the p53 protein. As would be expected, no staining was detected in tumours with nonsense of frameshift mutations. In 19 of the tumours that showed positive staining, no mutation could be detected. This might be due to mutations in codons outside the amplified regions or in exons outside the screening area. Approximately 10% of mutations in the *TP53* gene have been found in exons 1–4 or 9–11 [10]. In addition, mutations in splice sites or control regions cannot be detected by exon amplification. Mutations in other genes, coding for proteins involved in the *TP53* pathway, could also stabilise the p53 protein [35]. At present, immunohistochemistry is the most widely used method for screening for p53 alterations in cancer. Tumours that do not stain for p53 are not necessarily non-mutated. As mentioned above, not all *TP53* mutations lead to protein accumulation. In addition to lack of protein staining in tumours with nonsense and frameshift mutations, it has been shown that protein staining is less common in higher grade tumours. This may be due to a loss of both copies of the *TP53* gene in more advanced tumours [36].

Only four of the 19 *TP53* mutation negative tumours showed strong nuclear staining with p53 antibody. The remaining 15 tumours stained weakly and often only with the polyclonal antibody. Tumours of the breast have a very heterogeneous cell population. In many tumours, clear positive staining was seen in only an area of the tumour cell population while the rest was negative. This pattern has also been noted by others [34, 36]. In our material, the tumour sections used for protein staining were obtained from paraffin-embedded tissue whereas the DNA for mutation analysis came from fresh tumour samples. It is, therefore, possible that the tumour cell populations studied for protein staining and mutation analysis were not exactly the same.

In this study, we detected frequent allelic loss on the short arm of chromosome 17 in the tumours. The loss was most commonly found with markers close to the *TP53* gene in p53 abnormal tumours. This is in accordance with the theory that *TP53* is a tumour suppressor gene, i.e. inactivation of one allele of the gene by mutation and loss of the wild-type allele by chromosomal deletion is needed for tumorigenicity. Interestingly, we also detected a high frequency of deletions with 17p13.3 markers. In only four of our cases, informative for markers in both regions, was allelic loss restricted to 17p13.3. None of these tumours had a *TP53* mutation, indicating a different target for this allelic loss. Most of our *TP53* mutant samples with allelic loss at 17p13.3, also showed loss in the *TP53* area.

We could not find any association between levels of p53 antibody in serum and either protein expression in tumour tissue or mutations. Other studies have found raised p53 antibodies in serum from cancer patients, but only in a low percentage of patients [13–15]. Measuring p53 antibody in serum by ELISA, therefore, does not appear to be sensitive enough to be of use in clinical screening.

Examining clinical parameters, we found an association between p53 abnormalities and lack of oestrogen receptor in the tumours. The presence of oestrogen receptors has previously been related to a more favourable prognosis [16, 19, 37]. Patients with p53 abnormalities in their tumour have a 3.5 times higher risk of dying of the disease than those with normal p53. We also found an association between tumour size and survival, but p53 abnormalities were not associated with tumour size. The effect of p53 on survival can, therefore, not be explained as an association with large and more advanced tumours.

It has been suggested that cells with non-functional p53 have a selective growth advantage and that this will enhance clonal expansion of these tumour cells. This may result in a clone of more aggressive tumour cells, leading to decreased disease-free survival and lower overall survival rates.

Abnormalities in the *TP53* gene are the most frequent changes found in human cancers, and it is becoming increasingly clear that these changes are of prognostic significance. It is, therefore, essential that screening methods are accurate and efficient. Even though staining detects additional cases that are not detected by mutation analysis, it misses an important fraction of p53 abnormalities. It is worth noting that at the end of the study period, only 2 of the 6 patients with deletions or stop codon mutations are alive or disease-free (3 patients have died from breast cancer and one has metastatic disease). Of the 19 mutation negative, staining positive cases, 12 are alive. Of these cases, 3 of the 4 who had strong staining have died of the disease. These are, as yet, too few cases for us to draw any firm conclusions but the results indeed show an important trend.

Our results, using multivariate survival analysis, show that

TP53 mutations, or p53 staining in tumours are important prognostic markers for breast cancer. We conclude that the patients with a *TP53* mutation and/or positive p53 staining in their tumours are at higher risk of death from breast cancer than patients without p53 abnormalities. The results of this study confirm our previous findings that p53 alterations are associated with poor prognosis in breast cancer.

1. Hollstein M, Sidransky D, Vogelstein B, Harris CC. *p53* mutations in human cancer. *Science* 1991, **253**, 49–53.
2. Nigro JM, Baker SJ, Preisinger AC, *et al.* Mutation in the *p53* gene occur in diverse human tumour types. *Nature* 1989, **342**, 705–708.
3. Malkin D, Li FP, Strong LC, *et al.* Germ-line *p53* mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. *Science* 1990, **250**, 1233–1238.
4. Levine AJ, Momand J, Finlay CA. The *p53* tumour suppressor gene. *Nature* 1991, **351**, 453–456.
5. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci USA* 1992, **89**, 7491–7495.
6. Lane DP, Benchimol S. *p53*: Oncogene or anti-oncogene. *Genes Dev* 1990, **4**, 1–8.
7. Oliner JD, Kinzler KW, Meltzer PS, Georges DL, Vogelstein B. Amplification of a gene encoding a p53 associated protein in human sarcomas. *Nature* 1992, **358**, 80–83.
8. Crook T, Wrede D, Tidy JA, Mason WP, Evans DJ, Vousden KH. Clonal *p53* mutation in primary cervical cancer: association with human-papillomavirus-negative tumours. *Lancet* 1992, **339**, 1070–1073.
9. Levine AJ, Perry ME, Chang A, *et al.* The 1993 Walter Hubert lecture: the role of the *p53* tumour suppressor gene in tumorigenesis. *Br J Cancer* 1994, **69**, 409–416.
10. Soussi T, Legros Y, Lubin R, Ory K, Schlichtholz B. Multifactorial analysis of p53 alteration in human cancer: a review. *Int J Cancer* 1994, **57**, 1–9.
11. Soussi T, Caron de Fromentel C, May P. Structural aspects of the p53 protein in relation to gene evolution. *Oncogene* 1990, **5**, 945–952.
12. Cornelis RS, vanVliet M, Vos CBJ, *et al.* Evidence for a gene on 17p13.3, distal to *TP53*, as a target for allele loss in breast tumors without *p53* mutations. *Cancer Res* 1994, **54**, 4200–4206.
13. Crawford LV, Pim DC, Bulbrook RD. Detection of antibodies against the cellular protein *p53* in sera from patients with breast cancer. *Int J Cancer* 1982, **30**, 403–408.
14. Schlichtholz B, Legros Y, Gillet D, *et al.* The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hotspots. *Cancer Res* 1992, **52**, 6380–6384.
15. Caron de Fromentel C, May-Levin F, Mouriesse H, Lemerle J, Chandrasekaran K, May P. Presence of circulating antibodies against cellular protein p53 in a notable proportion of children with B-cell lymphoma. *Int J Cancer* 1987, **39**, 185–189.
16. Thorlacius S, Börresen A-L, Eyfjörd JE. Somatic *p53* mutations in human breast carcinomas in an Icelandic population: a prognostic factor. *Cancer Res* 1993, **53**, 1637–1641.
17. Andersen TI, Holm R, Nesland JM, Heimdal KR, Ottestad L, Börresen A-L. Prognostic significance of TP53 alterations in breast carcinoma. *Br J Cancer* 1993, **68**, 540–548.
18. Saitoh S, Cunningham J, DeVries EMG, *et al.* *p53* gene mutations in breast cancers in midwestern US women: null as well as missense-type mutations are associated with poor prognosis. *Oncogene* 1993, **9**, 2869–2875.
19. Elledge RM, Fuqua SAW, Clark GM, Pujol P, Allred DC, McGuire WL. Prognostic significance of *p53* gene alterations in node-negative breast cancer. *Breast Cancer Res Treat* 1993, **26**, 225–235.
20. Thor AD, Moore II DH, Edgerton SM, *et al.* Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst* 1992, **84**, 845–855.
21. Visscher DW, Castellani R, Wykes SM, Sarkar FH, Hussain ME. Concurrent abnormal expression of *ERBB-2, EGFR,* and *p53* genes and clinical disease progression of breast carcinoma. *Breast Cancer Res Treat* 1993, **28**, 261–266.
22. Tulinius H, Bjarnason O, Sigvaldason H, Bjarnadottir G, Olafsdottir G. Tumours in Iceland. Malignant tumours of the female breast. *APMIS* 1988, **96**, 229–238.
23. Midgley CA, Fisher CJ, Bártek J, Vojtesek B, Lane D, Barnes DM. Analysis of p53 expression in human tumours: an antibody raised against human p53 expressed in *Escherichia coli*. *J Cell Sci* 1992, **101**, 183–189.
24. Vojtesek B, Bartek J, Midgley CA, Lane DP. An immunochemical analysis of the human nuclear phosphoprotein p53: new monoclonal antibodies and epitope mapping using recombinant p53. *J Immunol Meth* 1992, **151**, 237–244.
25. Börresen A-L, Hovig E, Smith-Sörensen B, *et al.* Constant denaturant gel electrophoresis (CDGE) as a rapid screening technique for p53 mutations. *Proc Natl Acad Sci USA* 1991, **88**, 8405–8409.
26. Smith-Sörensen B, Gebhardt MC, Kloen P, *et al.* Screening for TP53 mutations in osteosarcomas using constant denaturant gel electrophoresis (CDGE). *Hum Mutat* 1993, **2**, 274–285.
27. Carbone D, Chiba I, Mitsudomi T. Polymorphism at codon 213 within the p53 gene. *Oncogene* 1991, **6**, 1691–1692.
28. Thorlacius S, Jonasdottir O, Eyfjörd JE. Loss of heterozygosity at selective sites on chromosomes 13 and 17 in human breast carcinoma. *Anticancer Res* 1991, **11**, 1501–1508.
29. Hoyheim B, Nakamura Y, White R. A *Bam*HI-polymorphism is detected by a genomic p53-clone (pBHP53). *Nucl Acids Res* 1989, **17**, 88–98.
30. Jones MH, Nakamura Y. Detection of loss of heterozygosity at the human TP53 locus using a dinucleotide repeat polymorphism. *Genes Chrom Cancer* 1992, **5**, 89–90.
31. Ara S, Lee PSY, Hansen MF, Saya H. Codon 72 polymorphism of the TP53 gene. *Nucl Acids Res* 1990, **18**, 4961.
32. Cox DR. Regression models and life-tables. *JR Stat Soc* 1972, **34**, 187–220.
33. Davidoff AM, Humphrey PA, Iglehart JD, Marks JR. Genetic basis for overexpression in human breast cancer. *Proc Natl Acad Sci USA* 1991, **88**, 5006–5010.
34. Osborne RJ, Merlo GR, Mitsudomi T, *et al.* Mutations in the *p53* gene in primary human breast cancers. *Cancer Res* 1991, **51**, 6194–6198.
35. Momand J, Zambetti GP, Olson DC, Georege D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated trans activation. *Cell* 1992, **69**, 1237–1245.
36. Fisher CJ, Gillett CE, Vojtesek B, Barnes DM, Millis RR. Problems with p53 immunohistochemical staining: the effect of fixation and variation in the methods of evaluation. *Br J Cancer* 1994, **69**, 26–31.
37. Norden T, Lindgren A, Bergström R, Holmberg L. Defining a high mortality risk group among women with primary breast cancer. *Br J Cancer* 1994, **69**, 520–524.

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