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Cellular Accumulation of p53 Protein: an Independent Prognostic Factor in Stage II Breast Cancer

Marie Stenmark-Askmalm, Olle Stål, Siw Sullivan, Lilianne Ferraud, Xiao-Feng Sun, John Carstensen and Bo Nordenskjöld

The p53 gene product is a tumour suppressor protein, and alterations of the protein are common in human cancer. Previous studies have focused on nuclear accumulation of p53. To investigate if cytoplasmic accumulation of p53 strengthens the relationships to different pathobiological variables and distant recurrence-free survival in breast cancer, tumours from 164 stage II patients were examined with the monoclonal antibody PAb1801. Nine per cent of the tumours were nuclear positive and 21% were cytoplasmic positive. Cellular p53 accumulation, related to the nucleus or the cytoplasm or both, showed stronger associations with pathobiological variables than nuclear accumulation alone. Accumulation of p53 was significantly correlated to tumour size over 20 mm, negative oestrogen receptor (ER) status, DNA aneuploidy, high S-phase fraction and positive *erb*B-2 status. Cytoplasmic p53 was significantly correlated to distance recurrence-free survival in patients negative for nuclear p53 ($P < 0.0001$). Cellular p53 accumulation was an independent prognostic factor, in addition to lymph node status and ER content. We conclude that consideration of cytoplasmic staining enhances the clinical importance of p53.
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INTRODUCTION

A SMALL TUMOUR, absence of lymph node metastasis and well preserved differentation indicate a better prognosis in breast cancer. Despite the overall good prognosis in early stage breast cancer, a subgroup of node-negative patients has a poor prognosis, and the outcome of individual patients in the node-positive subgroup also varies. Further prognostic factors are, therefore, important. Among these are hormone receptor status, the DNA content of tumour stem cell populations (DNA index and ploidy), and the proportion of cells which actively divide (S-phase fraction) [1–3].

A new potential prognostic factor in breast cancer is p53 [4–6]. p53 is a nuclear tumour-suppressor protein, and is believed to transiently arrest the cell cycle at G_1, thus giving time for reparation of damaged DNA or apoptosis if reparation fails [7–9]. Mutated p53 tends to have much longer half-life than wild type p53, leading to accumulation of the protein [9, 10]. An accumulation of p53 in breast cancer is associated with a negative oestrogen receptor status [4–6, 11, 12], high histological grade [4, 5], DNA index > 1.3 [5], high S-phase fraction [5, 6] and positive *erb*B-2 status [5]. Nuclear p53 in breast cancer has been found to be an independant prognostic factor associated with poor survival [4, 6].

In breast cancer, cytoplasmic p53 immunostaining alone or together with nuclear staining has been observed [12–15], but most studies have focused on the accumulation of p53 in the nucleus. In colorectal cancer, the prognostic significance of cytoplasmic p53 has been shown [16]. In the present study, we have examined the prognostic value of both nuclear and cytoplasmic accumulation of p53, using immunohistochemistry on frozen sections from the tumours of 164 patients with stage II breast cancer.

Correspondence to O. Stål.
The authors are at the Department of Oncology, University Hospital, S-581 85 Linköping, Sweden.
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MATERIALS AND METHODS

Patients and tumours

The study included 164 patients with pathological stage II breast cancer according to the International Union Against Cancer staging system, i.e. patients without distant metastasis but with positive lymph nodes and/or a tumour diameter exceeding 20 mm. The patients were registered in the South-East Sweden Health Care Region, and diagnosed for breast cancer during 1985–1988. For all patients, data on DNA index, S-phase fraction and oestrogen receptor (ER) content were available. The follow-up period ended in October 1992. None of the patients received any pre-operative treatment. Patients were treated with breast conserving surgery plus 54 Gy breast irradiation or modified radical mastectomy. Patients with lymph node metastasis had 40 Gy chest wall and axillary irradiation. Ten per cent of the patients received adjuvant chemotherapy, usually CMF (cyclophosphamide/methotrexate/5-fluorouracil) and 70% tamoxifen. 43 patients relapsed with distant metastasis during the follow-up period.

Immunohistochemistry

For immunohistochemistry on frozen sections, we used the monoclonal antibody PAb1801 (Oncogene Science, Manhasset,

New York, U.S.A.), which recognises both wild type and mutant p53. As a negative control, we used IgG_1 antibody (Sigma Chemical Co, St Louis, Missouri, U.S.A.). Adjacent frozen sections for PAb1801 and for IgG_1 were placed on glass slides, air dried and stored at −20°C. The sections were fixed in acetone (4°C) for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 5 min. Normal rabbit serum (10%) was used for 10 min in order to block non-specific immunostaining. The sections were incubated with the primary antibodies at 4°C overnight (1 μg/ml). Then the sections were incubated with a peroxidase-conjugated rabbit immunoglobulin (1:50) and peroxidase mouse antiperoxidase (PAP) complex (1:100), 30 min for each step, staining with 3,3-diaminobenzidine tetrahydrochloride in phosphate-buffered saline with 0.036% hydrogen peroxidase for 8 min. Thereafter, the sections were counterstained with haematoxylin, dehydrated in a series of ethanols, cleared in xylene and mounted. After each step, except after incubation with normal rabbit serum, the sections were rinsed in phosphate-buffered saline with 0.1% bovine serum albumin. The same protocol was employed to detect overexpression of the *erb*B-2 protein. The monoclonal antibody used was c-*neu* (Ab-2, clone 9G6, Oncogene Science), and cases with clear membrane staining were regarded as positive for overexpression.

To investigate whether the staining pattern was influenced by the fixation procedure, six tumours with cytoplasmic staining were cut and fixed as follows. After cutting, the slides were kept in a freezing cabinet until section cutting was completed. They were thereafter immediately transported to a −70°C freezer. At the time of assay, they were air-dried for 30 min and then fixed with a 50:50 mixture of methanol and acetone, which had been kept at −20°C. After 5 min, the fixative was allowed to evaporate and the procedure continued as above. When cutting these tumours, new sections from the same piece of tumour were also cut. These were allowed to air-dry overnight, stored and fixed according to the first method described.

DNA flow cytometry

DNA flow cytometry was used to determine DNA index and S-phase fraction as described previously [17]. A suspension of isolated nuclei was prepared as described by Vindelöv and colleagues [18]. This procedure included treatment with a detergent (0.1% NP40), trypsin and RNAse, followed by filtering through a 41-μm nylon mesh. The suspension was finally stained with propidium iodide, and measured within 1 h with a Leitz MPV FLOW flow cytometer (Leitz, Wetzlar, Germany) interfaced to a Monroe OC8888 personal computer system (Litton Business, Morris Plains, New Jersey, U.S.A.). DNA indices (DI) were calculated after zero-point adjustment, using chicken and trout red blood cells as internal controls. Calculations of DNA index, and the percentages of cells in $G_{0/1}$, S and G_2/M phase were performed by the software, after selection of adequate peaks and S-phase interval by the user. Considering a rectangular S-phase distribution, the number of cells in S phase was estimated by the software by multiplying the number of channels between the $G_{0/1}$ and G_2/M peaks by the mean number of registrations per channel in the selected interval.

Hormone receptor analyses

The specimens were collected from fresh surgical resections, and stored below −70°C before the analysis. The majority of the tumours, diagnosed before 1988, were analysed for ER as described by Wrange and colleagues [19]. Cytosol was incubated with 5 nmol/l [^{3}H]oestradiol, and the receptors were isolated by isoelectric focusing in polyacrylamide gel. From the beginning of 1988, we used the Abbott enzyme immunoassay (EIA) assay. Receptor concentration was expressed as fmol receptor per μg DNA. A cut-off value of 0.1 fmol/μg DNA was used for receptor positivity.

Statistical methods

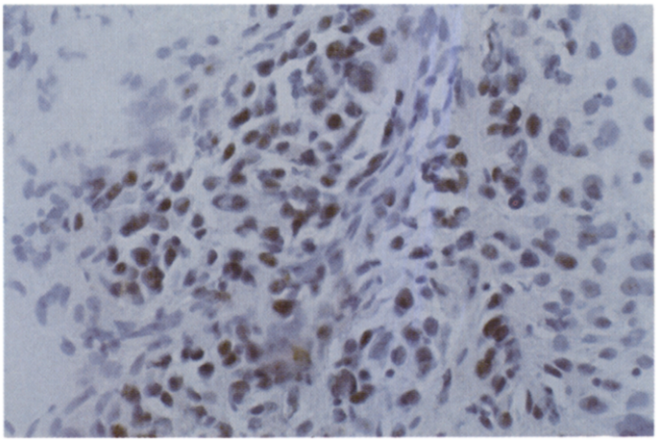
For testing the significance of differences in p53 accumulation patterns in relation to pathobiological variables, we used χ^2 tests for contingency tables [20]. Survival curves were computed with the product limit method presented by Kaplan and Meier [21]. Cox's proportional hazards model [22] was used to estimate and test the relation of p53 accumulation and other prognostic variables to distant recurrence. P values less than or equal to 0.05 were regarded as significant.

RESULTS

p53 accumulation patterns

The slides were examined and scored by three independent examiners, and thereafter the results were compared. Positivity with PAb1801 was seen as clearly stained tumour cells either in the nucleus, cytoplasm or both. If, irrespective of its location, p53 immunostaining was present in the tumour cells, it was regarded as cellular p53 accumulation. Examples of nuclear and cytoplasmic staining are shown in Fig. 1. In 6 cases, the negative control was stained with IgG_1. These cases were scored positive

(a)



(b)

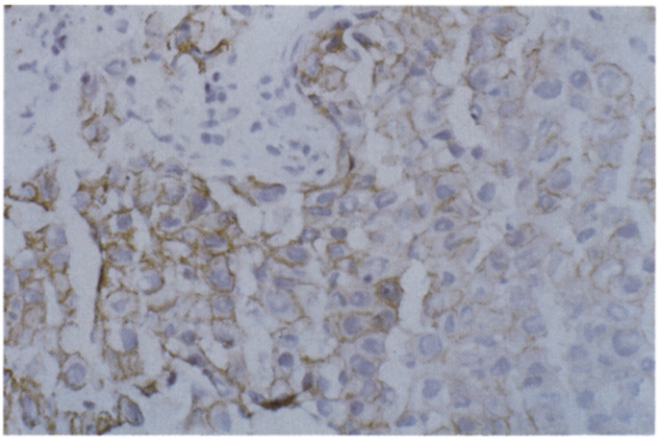


Fig. 1. p53 immunoreactivity in breast cancer with PAb1801. The sections were counterstained with haematoxylin. (a) Positive nucleus and negative cytoplasm. (b) Positive cytoplasm and negative nucleus.

if they were more strongly stained than the controls (3 cases), otherwise negative.

Among the 164 breast cancers studied, 37 cases showed positive staining. 3 cases were positive in the nucleus alone (1.8%), 22 (13.4%) were positive in the cytoplasm alone, and 12 (7.3%) were positive both in the cytoplasm and the nucleus. The remaining 127 cases were scored as negative.

Comparing the two methods of storing and fixation of the sections, none of the six tumours with cytoplasmic p53 had an altered accumulation pattern. 1 case was positive in the cytoplasm alone, and the remaining 5 cases were positive both in the nucleus and the cytoplasm. Among these, there were cases where cells from the same tumour showed staining either in the nucleus alone, or in the cytoplasm alone or in both locations. All controls were negative.

p53 accumulation patterns and pathobiological variables

The association of p53 accumulation patterns with pathobiological variables in breast cancer is shown in Table 1. Tumours in patients younger than 50 years showed accumulated p53 more often than older patients, but the difference did not reach statistical significance. Tumours larger than 20 mm were p53 positive more often than smaller ones, while the number of lymph node metastases showed no clear correlation to p53.

Table 1. Association of p53 accumulation patterns and pathobiological variables in breast cancer

	n	p53N+* *n* = 15 (%)	*P* value	p53+† *n* = 37 (%)	*P* value
Age					
< 50 years	48	10.4	0.72	31.2	0.087
≥ 50 years	116	8.6		19.0	
Tumour size					
≤ 20 mm	54	1.8	0.023	13.0	0.040
> 20 mm	110	12.7		27.3	
Number of positive nodes					
0	37	16.2		21.6	
1–3	86	7.0	0.19‡	20.9	0.57‡
> 3	41	7.3		26.8	
Oestrogen receptor status					
Negative	45	20.0	0.0030	53.3	< 0.0001
Positive	119	5.0		10.9	
DNA ploidy type					
Diploid	46	0.0	0.011	2.2	0.0001
Aneuploid	118	12.7		30.5	
DNA index					
< 0.94	5	20.0		20.0	
0.95–1.04	41	0.0		2.4	
1.05–1.29	19	0.0	0.019	0.0	< 0.0001
1.30–1.79	60	15.0		40.0	
1.80–2.19	33	9.1		24.2	
≥ 2.20	6	33.3		50.0	
S-phase fraction					
0–4.9%	28	0.0		3.6	
5–9.9%	55	3.6	0.0032‡	7.3	< 0.0001
≥ 10%	81	16.0		39.5	
*erb*B-2 (*n* = 160)					
Negative	124	8.1	0.29	13.7	< 0.0001
Positive	36	13.9		55.6	

*p53N+, nuclear accumulation regardless of cytoplasmic staining. †p53+, cellular accumulation related to the nucleus or the cytoplasm or both. ‡Test for trend. The categories were coded 1, 2, 3.

Negative oestrogen receptor status was significantly correlated to p53 accumulation, irrespective of its location. The proportion of tumours with accumulated p53 was significantly higher in DNA aneuploid cases compared with diploids. This was most evident for hypertetraploid (DI > 2.1) tumours and for those with a DNA index between 1.3 and 1.8 (Fig. 2). Among the 60 tumours with a diploid or near hyperdiploid DNA content (DI 0.95–1.29), only two showed accumulation of p53. A high S-phase fraction as well as positive *erb*B-2 status were strongly associated with cellular p53 accumulation. Among the 16 tumours, which were both *erb*B-2 positive and oestrogen receptor negative, 14 (88%) were positive for p53.

p53 accumulation patterns in relation to distant recurrence-free survival

The estimated distant recurrence-free survival after 5 years was 68% for those lacking nuclear accumulation compared with 58% for those with nuclear accumulation (Fig. 3a). The recurrence rate ratio was 2.0 according to Cox regression analysis ($P = 0.12$). The prognostic significance of cytoplasmic expression was analysed separately in cases without nuclear accumulation (Fig. 3b). While the 5-year recurrence-free survival rate was 74% for completely negative cases, it was only 37% for those with cytoplasmic accumulation alone. The difference was highly significant with a rate ratio of 5.2 ($P < 0.0001$). In addition, nuclear positivity as related to the overall negative subgroup, significantly predicted recurrence (rate ratio 2.7, $P = 0.028$).

In univariate Cox analysis of survival of all the 164 patients, cellular accumulation of p53 was associated with a poor prognosis, as were the occurrence of more than three lymph node metastases, the absence of oestrogen receptors, a high S-phase fraction and age under 50 years (Table 2). A higher recurrence rate was also related to large tumour size, DNA aneuploidy and *erb*B-2 positivity. However, these relationships did not reach statistical significance. In multivariate analysis, accumulation of p53, lymph node metastases and the lack of oestrogen receptors remained independently predictive of a higher risk of distant recurrence (Table 2). The prognostic significance of age and S-phase fraction was lost in the multivariate analysis.

DISCUSSION

Previous studies have reported the importance of p53 as a prognostic factor in breast cancer [4–6]. Our results show

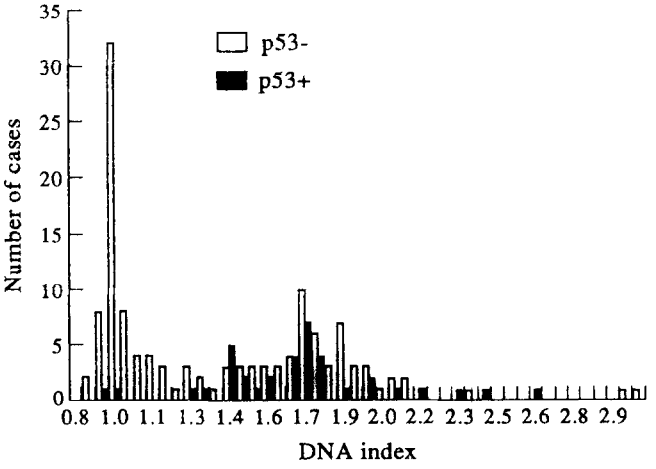


Fig. 2. DNA index distribution related to cellular p53 status.

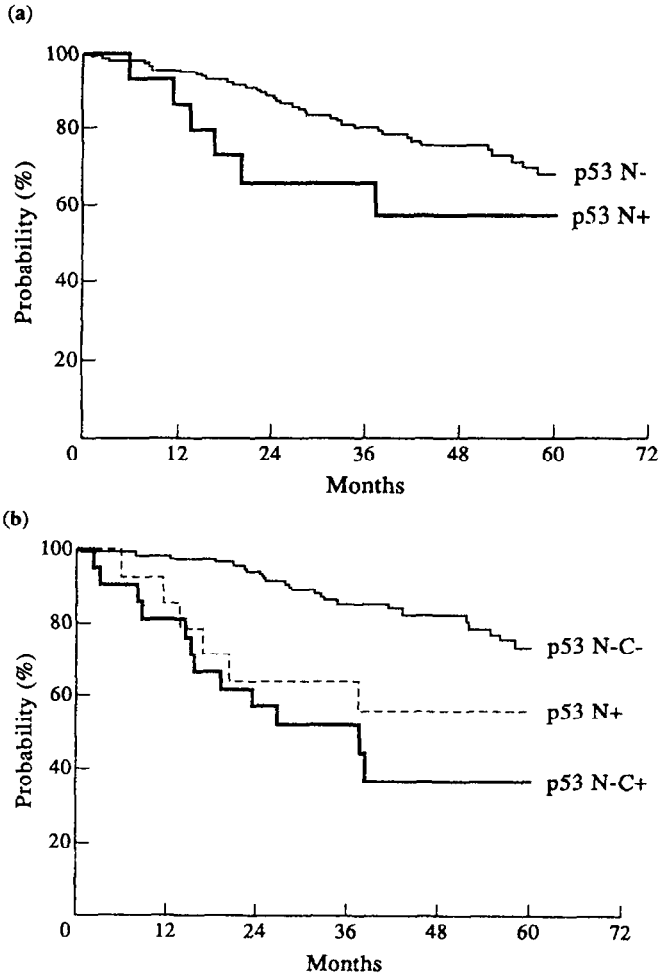


Fig. 3. Distant recurrence-free survival related to p53 immunoreactivity. (a) N−, negative nucleus (n = 149); and N+, positive nucleus (n = 15), regardless of cytoplasmic staining. (b) N−C−, negative (n = 127), N+, positive nucleus (n = 15), N−C+, positive cytoplasm alone (n = 22).

that there is a strong correlation between several different pathobiological variables and the accumulation of p53, not only in the cell nucleus but also in the cytoplasm of the breast tumour cells.

Cytoplasmic staining in breast cancer has been shown in several studies [12–15]. p53 expressed in the cytoplasm has also been shown in cancers of the colon, ovary and lung [13, 16, 23]. Chang and colleagues [13] found, among 11 positive cases with PAb1801, 10 with both nuclear and cytoplasmic expression of p53, 1 with only cytoplasmic expression, and no cases of nuclear accumulation alone. Borg and colleagues [12] showed, in their study of 147 cases of breast cancer, that 92 cases were positive for p53 in the cytoplasm with antibody CM1. Strong cytoplasmic p53 staining in 32% of lobular breast carcinomas was reported by Domgala and colleagues [14]. Different methods might partly explain different staining patterns. Thor and colleagues [4] noticed cytoplasmic staining when using more concentrated antibody of PAb1801. Bartek and colleagues [24] suggested that the cytoplasmic staining they observed could be due to inappropriate fixation. In our study, we tested two different methods of storing and fixing sections, from tumours which earlier had exhibited cytoplasmic staining. No difference in the staining pattern was found. For some tumours, cells in the same section showed staining either in the cytoplasm or in the nucleus alone or in both compartments. The location of p53 may reflect different forms of p53. Zerrahn and colleagues [25] investigated p53 and its subcellular location in mouse-derived cell lines, with normal to maximally transformed cells, and found mutated p53 both in the cytoplasm and in the nucleus. Cytoplasmic p53 in inflammatory breast cancer was found by Moll and colleagues [15]. Further analysis revealed mainly wild type p53. In the same study, a case of normal lactating breast tissue also exhibited cytoplasmic p53 accumulation. Moll and colleagues [15] suggested that oestrogen-mediated cell division of the normal lactating breast could involve inactivation of p53 protein via exclusion from the cell nucleus. Suzuki and colleagues [26] showed that growing human breast cancer MCF-7 cells had p53 localised in the cytoplasm and in the nuclei, if the cells were growth arrested by serum starvation. Some mutant forms of p53 are also known to be temperature-sensitive. Gannon and colleagues [27] showed mutant p53 in the nucleus of arrested cells at 32°C and in the cytoplasm in growing cells at 37°C. Furthermore, some mutant forms of p53 are known to bind to cytoplasmic heat shock proteins [28]. Other factors which can explain different nuclear and cytoplasmic p53 patterns remain to be found.

We believe that the inclusion of cytoplasmic accumulation of p53 contributed to the strong correlations we observed between p53 accumulation and different variables which, except for tumour size, showed a stronger association with total cellular p53 accumulation compared with only nuclear accumulation.

Our finding that lymph node status was not significantly correlated to p53 accumulation corresponds to what was found in other studies [4, 11, 12]. In contrast, larger tumours were more frequently associated with p53 expression. Isola and colleagues [5], and Thor and colleagues [4] found similar trends. Borg and colleagues [12], who compared tumours divided into three size categories, noted a significant association.

In our series, the majority of tumours with accumulation of p53 were negative for oestrogen receptors. Similar correlations have been shown in several studies [4–6, 11, 12]. Overexpression of *erb*B-2, was strongly correlated to cytoplasmic p53. p53 was nearly always found in tumours which were both ER-negative and *erb*B-2-positive. Isola and colleagues [5], and Chang and colleagues [13] reported a strong correlation between positive *erb*B-2 status and p53 accumulation.

In accordance with Allred and colleagues [6], accumulation of p53 was an independent prognostic factor when the relationship to distant recurrence was examined. The same was true in the study of Thor and colleagues [4], although in their study, S-phase fraction was not analysed. When S-phase fraction was included in the analysis of node-negative breast cancer presented by Isola and colleagues [5], the prognostic value of p53 did not reach statistical significance. We found that accumulation of p53 in the cytoplasm was associated with early distant recurrence and contributed prognostic information in addition to that of nuclear p53. Furthermore, Sun and colleagues [16] recently reported the prognostic significance of cytoplasmatic p53 in colorectal cancer.

One function of wild-type p53 is to inhibit the cell cycle in case of DNA damage [7]. It is thus reasonable to assume that tumour cells with loss of functional p53 are genetically instable, and that these cells will accumulate alterations. This would correspond to our results which showed that many of the aneuploid tumours, with a high S-phase fraction and a DNA index between 1.3 and 1.8, accumulated p53. A cytogenetic study by Dutrillaux and colleagues [29] has demonstrated that

Table 2. Cox regression analysis of distant recurrence related to cellular p53 accumulation and other prognostic variables

		Univariate analysis			Multivariate analysis		
	n	Rate ratio	95% CI	*P* value	Rate ratio	95% CI	*P* value
Cellular p53 status							
Negative	127	1.0		<0.001	1.0		0.002
Positive	37	3.8	(2.1–7.2)		3.6	(1.6–7.8)	
Age							
< 50 years	48	1.0		0.0099	1.0		0.078
≥ 50 years	116	0.44	(0.24–0.82)		0.55	(0.28–1.1)	
Lymph node status							
0	37	1.0			1.0		
1–3	86	0.9	(0.3–2.1)	<0.001*	1.3	(0.5–3.3)	<0.001*
> 3	41	3.3	(1.4–8.0)		6.4	(2.4–16.5)	
Tumour size							
≤ 20 mm	54	1.0		0.069			
> 20 mm	110	1.9	(0.9–3.9)				
Oestrogen receptor status							
Negative	45	1.0		<0.001	1.0		0.009
Positive	119	0.29	(0.16–0.54)		0.42	(0.20–0.86)	
DNA ploidy type							
Diploid	46	1.0		0.36			
Aneupliod	118	1.4	(0.7–2.8)				
S-phase fraction							
0–4.9%	28	1.0			1.0		
5–9.9%	55	1.8	(0.6–5.7)	0.027*	1.4	(0.4–4.5)	0.87
≥ 10%	81	2.8	(1.0–8.0)		1.2	(0.4–3.9)	
*erb*B-2 (*n* = 160)							
Negative	124	1.0		0.31			
Positive	36	1.4	(0.7–2.9)				

*Test for trend. The categories were coded 1, 2, 3. CI, confidence interval.

tumours with DNA indices in this range belong to those with the highest number of chromosomal rearrangements. Cattoretti and colleagues [11], and Allred and colleagues [6] found that aneuploidy was associated with accumulation of p53, and Isola and colleagues [5] showed that DNA index > 1.3 was related to p53. A similar relationship was also observed in colorectal tumours [30]. In contrast, Borg and colleagues [12] did not obtain a significant correlation between p53, high S-phase or aneuploidy.

From the present results, we conclude that consideration of cytoplasmic staining enhances the clinical importance of p53, and that cellular p53 is an independent prognostic factor in stage II breast cancer.

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Genetic Linkage in Muir-Torre Syndrome to the Same Chromosomal Region as Cancer Family Syndrome

Nigel R. Hall, Victoria A. Murday, Pamela Chapman, M. Angela T. Williams, John Burn, Paul J. Finan and D. Timothy Bishop

The Muir-Torre syndrome, in which sebaceous gland tumours occur in association with internal malignancy, is inherited as an autosomal dominant disorder. Many features of the syndrome are similar to those of the Lynch II cancer family syndrome, and thus the two disorders might share a common genetic basis. We typed two large families with DNA markers on chromosome 2p around D2S123, a site recently shown to be linked to the Lynch II syndrome. LOD scores at this locus demonstrated significant and tight linkage to D2S123, suggesting that defects in the same gene might give rise to both syndromes.

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INTRODUCTION

MUIR-TORRE syndrome is a genodermatosis (McKusick number 158320) [1] characterised by the presence of sebaceous gland tumours (adenoma, epithelioma or carcinoma) associated with one or more of a wide variety of internal malignancies, particularly colorectal, endometrial, urological and upper gastrointestinal tumours, as well as other skin tumours, notably keratoacanthomas and basal cell carcinomas [2]. Affected members have a high incidence of synchronous and metachronous tumours yet, despite this, survival is frequently prolonged [1]. Many cases show a strong family history consistent with autosomal dominant inheritance, and it has been proposed that the syndrome is an unusual phenotypic variant of the Lynch II cancer family syndrome [3], also known as hereditary non-polyposis colorectal cancer. Following the recent report of genetic linkage to the D2S123 locus on chromosome 2p for the Lynch II syndrome [4], we wished to see if the Muir-Torre syndrome gene also mapped to the same locus, thus testing the hypothesis that both syndromes share a common genetic basis.

Correspondence to D.T. Bishop.
N.R. Hall, M.A.T. Williams and D. T. Bishop are at the Imperial Cancer Research Fund Genetic Epidemiology Laboratory, Ashley Wing, St James's University Hospital, Beckett Street, Leeds LS9 7TF; N.R. Hall is also at and P.J. Finan is at the Department of Surgery, The General Infirmary at Leeds, Great George Street, Leeds LS1 3EX; V.A. Murday is at the Department of Clinical Genetics, Jenner Wing, St George's Hospital, Cranmer Terrace, London SW17 0RE; and P. Chapman and J. Burn are at the Department of Human Genetics 19/20 Claremont Place, University of Newcastle upon Tyne, NE2 4AA, U.K.
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