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p53 Protein and Vimentin in Invasive Ductal NOS Breast Carcinoma—Relationship With Survival and Sites of Metastases

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p53 protein and vimentin status were available from immunocytochemical studies of 253 formalin-fixed paraffin-embedded invasive ductal not otherwise specified (NOS) carcinomas from patients for whom follow-up data was also on file. For the 127 node-negative patients, multivariate analysis showed a highly significant correlation between p53 and vimentin ($P < 0.001$), a strong correlation between vimentin and probability of survival to 90 months but only a weak association between p53 and survival to 90 months. p53 also never entered trees of prognostic indicators derived using stepwise regression with Kaplan–Meier statistics for node-negative and node-positive subgroups, while vimentin status dominated the node-negative trunk. In addition, p53 and vimentin status were analysed versus the site of the first distant metastasis for node-negative and node-positive patients. Analysis by p53 status showed no significant effect on visceral metastases. In contrast, vimentin-positive primaries metastasised twice (and in node-negative patients, 3.5 times) as often to lung, liver and brain as did the vimentin-negative primaries. Both p53-positive and vimentin-positive tumours showed a significantly lower tendency to metastasise to the bone than did their negative counterparts.

Key words: p53 protein, vimentin, breast carcinoma, metastases, prognosis
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INTRODUCTION

SIMPLE PATHOLOGICAL prognostic factors in breast cancer, such as tumour size, stage of disease and especially tumour grade, are of value in assigning women with breast cancer to different prognostic groups [1]. Some biological markers in breast cancer also seem to have prognostic value, and those related to proliferation of tumour cells may be particularly important [2].

The p53 gene product is thought to regulate cell proliferation. In its wild type form it suppresses cell growth whereas the mutated p53 gene acts as an oncogene [3]. Mutations in the p53 gene result in p53 protein stabilisation. The mutated protein accumulates in the nucleus, and can be detected by immunocytochemistry, as can the non-mutant stabilised protein [3]. p53 protein positivity in breast carcinomas has been associated with a number of poor prognostic indicators [4–6], including an increased number of tumour cells in S phase [7]. In studies of node-negative patients, where a sufficiently large number of cases have been studied, a significant association of p53 positivity with shortened survival [7–9] has been reported. In node-positive patients, p53 positivity has been related in one study to shortened [8] and in a second study to prolonged survival [10].

Vimentin is a second marker that may be related to proliferation. Vimentin is the intermediate filament protein normally associated with fibroblasts and other cells of mesenchymal origin [11], and is not present in the vast majority of normal epithelial cells, including those of the breast, which instead contain keratins. It has been shown that, while all breast carcinomas continued to express keratins, a substantial fraction (around 25%) also expressed vimentin in more than 10% of the tumour cells [12–15]. Vimentin was preferentially expressed in human breast carcinomas with low oestrogen receptor and high proliferative rate as measured by the Ki-67 growth fraction [15, 16]. We showed that expression of vimentin in more than 10% cancer cells of tumours from node-negative patients was associated with a significantly shortened overall survival at 5 years [17]. In addition, when cells in culture are considered, vimentin induction and proliferation have been linked in a variety of different cell types [18, 19] and, in particular, in human mammary luminal epithelial cells in culture [20]. Vimentin-positive human breast cell cancer lines have also been reported to show increased invasiveness in the Boyden chamber assay of invasiveness when compared to similar cell lines that are vimentin-negative [21]. Finally, vimentin was expressed during regeneration of tubular epithelia of the kidney [22]. There is thus a considerable amount of evidence relating vimentin expression to proliferation and to invasiveness both *in vivo* and *in vitro*.

In view of the current interest, particularly in p53, we have now determined the relative prognostic significance of p53 protein and of vimentin positivity in invasive ductal NOS breast carcinomas with emphasis on the node-negative patients. We have also examined the relationship of the two factors to each other and to classical prognostic factors, such as axillary node status, histological grade and size of the tumour. Each parameter has been related to probability of survival up to 90 months, and

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the data has been analysed by a variety of statistical methods. In addition, since the association between p53 protein and/or vimentin positivity of the primary tumour and the distribution of metastases in recurrent breast cancer was not known, we also looked for a possible relation between the p53 and/or vimentin protein status of the primary tumour and the subsequent incidence and distribution of metastases.

MATERIALS AND METHODS

Patients

Survival of 262 unselected patients who developed invasive ductal NOS breast carcinoma between 1980–1987 was determined. Clinical follow-up was available for at least 60 months and representative archival paraffin blocks were available for each patient. 9 patients who developed a second primary invasive malignant tumour during the study were excluded, leaving 253 patients in the database. There were 136 lymph node-negative and 117 lymph node-positive patients. Lymph node-negative patients underwent either only mastectomy with removal of the axillary lymph nodes (58 patients) or mastectomy with removal of the nodes followed by either radiation therapy (22 patients), chemotherapy (29 patients), hormone therapy (4 patients) or combinations of radiation, chemo- and hormone therapy (19 patients). 4 patients underwent quadrantectomy with removal of axillary nodes followed by radiation therapy. Lymph node-positive patients underwent either only mastectomy with removal of the axillary lymph nodes (9 patients) or mastectomy with removal of the nodes followed by either radiation therapy (41 patients), chemotherapy (10 patients), radiation and chemotherapy (30 patients), hormone therapy (2 patients) or combinations of radiation, chemo- and hormone therapy (22 patients). 3 patients were treated by quadrantectomy with removal of the axillary nodes plus radiotherapy. Formalin-fixed and paraffin-embedded biopsies were retrieved from the files of the Department of Oncology, Medical Academy of Lodz, Poland. Histological typing was performed as in [23] and histological grading according to Bloom and Richardson [24]. The computerised database contained the age of the patient, the number of positive lymph nodes, the size of the tumour, histological type and grade, the stage of the disease at diagnosis, and the treatment protocol. In addition, the site and date of relapse and the date of the last checkup or of death were available for 235 patients. For 18 patients who had died detailed information on site and date of metastasis was not available and these patients were, therefore, not included in the part of the study dealing with metastasis. The sites of recurrence were divided into the following categories: distant metastases subdivided into visceral (includes liver, lung, brain) and bones, local recurrence (symptomatic or progressive metastases in mastectomy flaps), regional recurrence (metastases to supraclavicular nodes) or recurrence in the contralateral breast. Distant metastases were confirmed by clinical examination and, where appropriate, by X-rays, liver ultrasonography, liver or brain scans, sputum cytology or fine needle aspiration biopsy. The other recurrences were confirmed either by fine needle aspiration biopsy or histologically. Some patients analysed in this study were included in previous studies: 195 on vimentin expression and survival [17], and 173 on p53 expression in different histological types of breast carcinomas [25]. Thus far, we have not reported on p53 expression and survival. Correlation statistics and survival was reported for all patients in a particular category, e.g. 253 for whom vimentin, grade, age and size were known, and 227 for whom p53 and vimentin status were available.

Immunohistochemistry

Tumours were assayed for p53 positivity with the rabbit polyclonal p53 anti-serum CM 1 diluted 1:70 (Medac, Hamburg, F.R.G.), which recognises both wild type and mutant p53 protein [26]. In 21/227 cases tested with polyclonal p53 antibody, nuclear p53 staining could not be assessed unequivocally because cytoplasmic staining was sufficiently strong so that the nuclei could not be scored. The tumours were retested with the monoclonal p53 antibody DO1 [27] (Ab-6 from Oncogene Science, Uniondale N.Y. via Dianova, Hamburg, F.R.G.), which became commercially available during this study. Using the DO1 antibody, the 21 cases could be assigned to either positive or negative categories since cytoplasmic staining was not present. Additional cases in which the p53 tests with the CM-1 anti-serum were unequivocal were also tested with the monoclonal p53 antibody DO1 with 100% agreement in the results. Vimentin positivity was determined with the mouse monoclonal V9 antibody used as an undiluted hybridoma supernatant [11]. This antibody is commercialy available. Sections were deparaffinised, incubated with the appropriate antibodies, washed and reacted with biotinylated rabbit anti mouse antibody and streptavidin peroxidase (Histostain-SP Kit, Zymed Laboratories Inc., San Francisco, U.S.A.). The sections were slightly counterstained with haematoxylin.

Statistical analysis

Follow-ups of the 253 patients were conducted until May 1993. 111 patients died of cancer between 1 and 109 months after initial surgery (mean 37.8 months), while 142 patients were alive at the last follow-up up to 132 months after surgery (mean 87.1 months). Using the product-limit (PL) estimator P(t), the probability of survival for all or any subset of patients at any time in this interval, the mean lifetimes of these patients limited to the end of the interval, and the variances of these statistics could be calculated [28]. At any point in time, only the patients still at risk determined the survival probabilities. This permits survival graphs and corresponding statistics for times up to 90 months. To test the significance of the Kaplan–Meier survival plots, χ^2 values were calculated by log-rank statistics (generalised Savage test). Multivariate analysis was performed by calculation of the coefficients of correlation in certain subgroups. The significance of correlation was determined by the t statistical test. Correlation coefficients were calculated between all available prognostic variables. In addition, partial correlations [29] of some intercorrelated variables were calculated to be able to assess the effect on survival of one of these, eliminating the effect of the other. Stepwise regression tree analysis [30], using Kaplan–Meier statistics, was used to compare the prognostic significance of several variables affecting prognosis of breast carcinomas. A stepwise regression was carried out, using a data file containing histological grade, nodal status, size, vimentin and p53 protein status along with survival data of the 227 patients for whom all these factors were known. As a root node (first step), one of the factors with very high significance ($P < 0.001$), namely grade, nodal status (0 or ≥ 1) or number of affected nodes (<3, ≥ 3) was selected. At each further step of the tree, the most significant factor that remained was chosen to further subdivide the patient group, stopping when none of these factors were at all significant ($P < 0.1$) by Kaplan–Meier statistics. A programme for producing such trees, permitting control of choice at each node, was written in Fortran for a Vax 9000 computer.

RESULTS

p53 positivity

Tumours were considered positive for p53 if more than 10% of tumour cells showed strong staining of nuclei (cf [25]). Sixty eight of 227 (30%) of the invasive ductal NOS breast carcinomas were p53 positive using this criterion (Table 1). In the node-negative subset, 44 of 127 (35%), and in the node-positive subset, 24 of 100 (24%), were p53 protein-positive. The percentage of p53-positive cell nuclei was estimated semi-quantitatively for each tumour. In 20 tumours >75%, in 19 tumours 50–75%, in 25 tumours 25–50% and in 4 tumours 10–25% of tumour cells had p53-positive nuclei. 159 tumours were scored as negative for p53. In 130 of these tumours, no nuclei were stained, in 22 tumours <1% tumour cells were stained, and in 16 tumours between 1 and 10% of tumour cells showed positive staining of cell nuclei with the p53 antibodies. Nuclei of benign cells were negative.

Vimentin positivity

Tumours were scored as vimentin positive when there was cytoplasmic staining in >10% of tumour cells assessed semiquantitatively (cf [17]). Of the 253 tumours in this study, 56 were vimentin positive (22%) and 197 were vimentin negative (78%). In the node-negative subset, 40/136 (29%), and in the node-positive subset, 16/117 (14%), were vimentin positive.

Although the vimentin V9 antibody, which was isolated in our laboratory [11], is somewhat formaldehyde sensitive, there was no difficulty scoring tumours as positive or negative. Negative staining of benign epithelial cells and positive staining of fibroblasts, macrophages and lymphocytes served as built-in positive and negative controls for each tumour. When divided by year, approximately equal percentages of the formaldehyde-fixed paraffin-embedded breast carcinomas in our database were vimentin positive. Actual percentages were, for 1981, 19% (12/64); 1982, 15% (7/47); 1983, 23% (12/52); 1984, 22% (11/50); 1985, 16% (5/31), and for 1989, 21% (17/81). Thus, there was no apparent decrease in the percentage of tumours that were vimentin positive with time under our assay conditions. The 22% of tumours that were vimentin positive in this study, using formaldehyde-fixed and paraffin-embedded material, concurs with other incidences in the literature using non-paraffin-embedded material. Thus, Cattoretti and colleagues [13] found that 25% of breast carcinomas were vimentin positive when frozen sections were used, while we found that 22% of ductal NOS breast carcinomas were vimentin positive when fine needle aspirates were examined [15].

Table 1. p53 protein accumulation related to other clinical factors and survival

	Total *n*	p53 positive *n* (%)	90-month survival (%) p53+	90-month survival (%) Total
All patients				
Total	227	68 (30)	53	56
Grade				
I and II	118	20 (17)	65	64
III	109	48 (44)	48	48
Vimentin				
Negative	171	36 (21)	67	60
Positive	56	32 (57)	38	45
Nodes				
Negative	127	44 (35)	57	66
Positive	100	24 (24)	46	44
Size				
≤ 3 cm	76	24 (32)	63	70
> 3 cm	151	44 (29)	48	50
Age				
< 50 years	73	24 (33)	54	60
≥ 50 years	154	44 (29)	52	55
Node-negative patients				
Total	127	44 (35)	57	66
Grade				
I and II	67	14 (21)	64	73
III	60	30 (50)	53	58
Vimentin				
Negative	87	19 (22)	79	75
Positive	40	25 (63)	40	48
Size				
≤ 3 cm	50	15 (30)	67	80
> 3 cm	77	29 (38)	52	57
Age				
< 50 years	43	17 (40)	53	67
≥ 50 years	84	27 (32)	59	65

p53 positivity and vimentin expression

The results in Tables 1 and 2 show that p53 expression was associated with vimentin expression and with high grade. The incidence of tumours that were positive for p53 was significantly greater in the vimentin-positive subgroup than in the vimentin-negative subgroup, both in node-negative patients (63 versus 22%; $P < 0.001$), and also in the whole cohort of patients (57 versus 21%; $P < 0.001$). For node-positive patients, the trend was present (44% vs. 20%) but, because of the low number of patients in the vimentin-positive subgroup, was not significant. Table 1 shows the strong association of p53 status with grade (44% of grade III versus only 17% of grade I and II tumours were p53-positive). Similar results for vimentin have been previously presented [13]. Conversely, the percentage of tumours that were positive for vimentin was greater in the p53-positive subgroup than in the p53-negative subgroup, both in node-negative patients (57 versus 18%) and in node-positive patients (29 versus 12%). Overall, 47% of p53-positive tumours were also vimentin-positive.

Comparison of p53 status and vimentin status to other clinicopathological characteristics

Table 2 correlates data on p53, vimentin status and other clinicopathological characteristics of the carcinomas in this study by multivariate analysis. In the whole cohort of patients, p53 accumulation correlated significantly with vimentin ($P < 0.001$) and high histological grade ($P < 0.001$). Vimentin was also correlated with high histological grade ($P < 0.001$), p53 protein accumulation ($P < 0.001$) and, in addition, with negative axillary nodes ($P < 0.01$).

In node-negative patients, a strong, significant correlation was seen between p53 accumulation and vimentin expression ($P < 0.001$) and between p53 accumulation and high histological grade ($P < 0.001$). Vimentin positivity was associated with high histological grade ($P < 0.001$), as well as p53 accumulation ($P < 0.001$).

In node-positive patients, there was a significant correlation only between p53 accumulation and a high histological grade ($P < 0.01$). Vimentin was associated only with the number of positive lymph nodes ($P < 0.05$) (data not shown).

p53 vimentin and survival to 90 months

The results shown in Table 2 indicate that classical prognostic factors, such as tumour size, involved lymph nodes or histologi-

Table 2. Correlation coefficients between survival at 90 months, p53, vimentin status and other clinicopathological variables

	Survival	Vimentin	p53	Grade	Nodes	Age	Size
All patients							
n	253	253	227	253	253	253	253
Survival	—	−0.16	NS	**−0.21**	**−0.28**	NS	**−0.32**
Vimentin	−0.16	—	**0.33**	**0.23**	−0.19	NS	NS
p53	NS	**0.33**	—	**0.29**	NS	NS	NS
Grade	**−0.21**	**0.23**	**0.29**	—	NS	NS	NS
Nodes	**−0.28**	−0.19	NS	NS	—	NS	**0.27**
Age	NS	NS	NS	NS	NS	—	NS
Size	**−0.32**	NS	NS	NS	**0.27**	NS	—
Node-negative patients							
n	136	136	127	136		136	136
Survival	—	**−0.35**	−0.18	NS		NS	−0.23
Vimentin	**−0.35**	—	**0.39**	**0.37**		NS	NS
p53	−0.18	**0.39**	—	**0.30**		NS	NS
Grade	NS	**0.37**	**0.30**	—		NS	NS
Age	NS	NS	NS	NS		—	NS
Size	−0.23	NS	NS	NS		NS	—

Only significant coefficients of correlation are printed ($P < 0.05$), those with $P < 0.001$ are in bold. Negative numbers indicate inverse correlation, e.g. survival is less when the size of the tumour is greater. NS, non-significant.

cal grade were better predictors of survival than p53 in the entire cohort of patients. A similar result was obtained for the node positive subset (data not shown). Only in the node-negative group was there a weak negative correlation of p53 positivity with survival at 90 months (coefficient of correlation −0.18; $P < 0.05$, i.e. less patients with p53-positive tumours survive). However, an even stronger negative correlation of vimentin expression and survival was seen (−0.35; $P < 0.001$) (Table 2). The partial correlation of p53 and survival, once the effect of vimentin status was eliminated, was essentially zero (−0.05).

Kaplan–Meier survival curves were calculated, dividing all tumours and the node-negative subgroup either according to p53 status (Figures 1a, b), or according to vimentin status

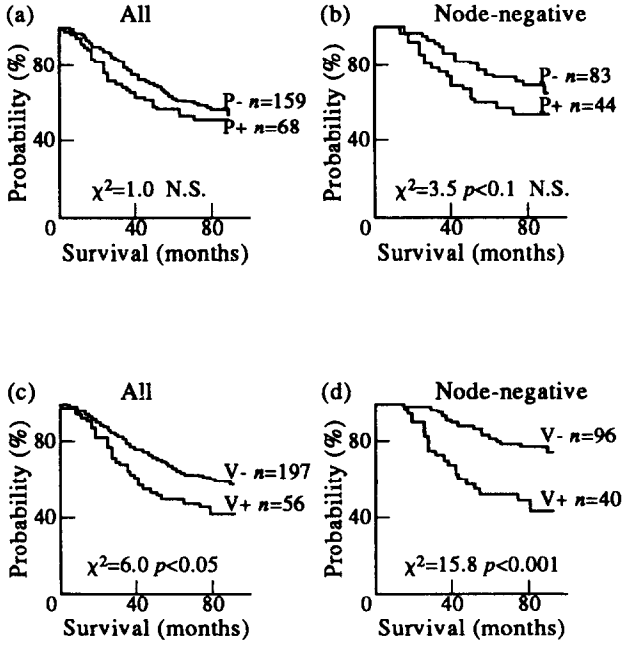

Figure 1. Effect of p53 protein accumulation (a,b) and vimentin expression (c,d) on survival curves of total (a,c) and of node-negative patients (b,d) with invasive ductal NOS breast carcinomas. *n* = number of patients at risk; P−, p53 negative; P+, p53 positive. V−, vimentin negative; VT, vimentin positive.

(Figures 1c, d). For patients in the node-negative group, those with p53-negative tumours appeared to have a better prognosis than those with p53-positive tumours (Figure 1b). However, the trend was non-significant. When patients were divided according to vimentin status, there was a significant prognostic difference for all patients, (Figure 1c) and a highly significant difference for the node-negative subgroup ($P < 0.01$) (Figure 1d). Furthermore, when the node-negative group of patients was divided by vimentin status and survival curves plotted by p53 status, there was no difference in survival for p53 positivity (data not shown). Thus, the prognostic effect of p53 seems to be derived solely from the high correlation between p53 and vimentin, and the strong prognostic power of vimentin in the node-negative group. This suggestion was further supported by the greatly reduced partial correlation of p53 and survival at constant vimentin (see above).

The Kaplan–Meier plots for the node-positive group and for the whole cohort of breast cancer patients did not show any association between p53 protein accumulation and survival (data not shown). Similarly, no association between vimentin and survival was found in the node-positive group (data not shown).

Regression tree analysis [30] using Kaplan–Meier survival statistics at 90 months was used to assign patients to distinct survival groups. Results of these analyses are summarised in Figure 2. Trees were grown to test the interrelation of the various available covariates of survival. At each step, only the population still characterised by the present branchpoint was tested, and the most significant remaining variable was chosen. This variable need not have been significant at the previous step, as is the case with histological grade, which was prognostically neutral for all node-negative, vimentin-negative patients, but prognostically favourable once tumours were divided by size. This process was continued along each branch until no significance was detected. Our initial variables were nodal status (negative or positive), histological grade (I and II taken together versus III), size of tumour (≤3 versus >3 cm), p53 status, vimentin expression and age (<50 versus ≥50 years). The root (initial branch point), which had the greatest significance ($P < 0.001$) for all patients was nodal status. There was hardly any difference between the trees for survival at 60 months (data

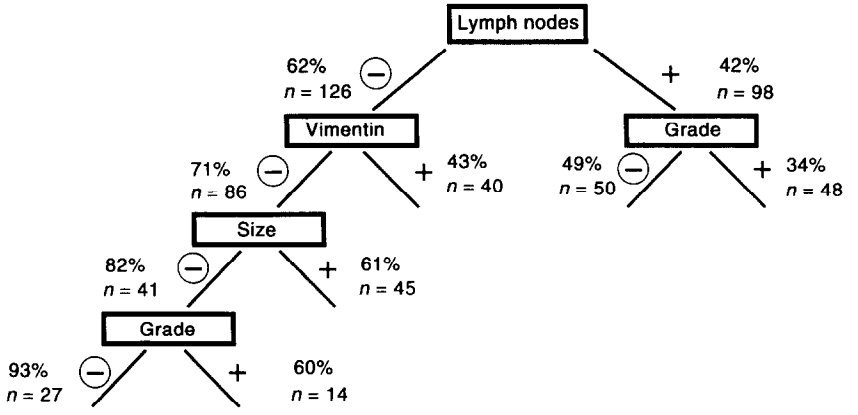

Figure 2. Regression tree for 90-month survival of patients with invasive ductal carcinoma. At each decision node, the number of patients and their survival probability (%) is shown. The prognostically favourable path is circled.

not shown) and 90 months (Figure 2). p53 status never entered into these trees. The node-negative trunk was dominated by vimentin (always $P > 0.01$), while the node-positive side was dominated by histological grade.

p53, vimentin and site of relapse

Of the 235 patients for whom accurate recurrence information was available, 125 were free of relapse. Of the remaining 110, 12 had only local or regional recurrence which were scored, but no distant metastasis. The other 98 patients had distant metastases, the site of the first being scored, and for these patients, local or regional recurrence, if they had occurred, were not included in Tables 3 and 4. Distant metastases were scored as visceral (liver, lung, or brain) or skeletal, and only the first distant metastasis was included in the data in Tables 3 and 4. There were 44 patients with a first distant metastasis to viscera, and 48 patients where the first distant metastasis was to bone. In 6 patients, visceral and bone matastases were found simultaneously, giving a total of 50 visceral and 54 skeletal metastases.

Table 3. Vimentin status and site of first metastasis

		Probabilities (%)			
	n	All	V−	V+	*P*
All patients	235				
No metastases	125	53	56	42	NS
Local and regional	12	5	4	10	NS
Bone*	54	23	26	12	< 0.05
Visceral*	50	21	16	38	< 0.001
Node-negative patients	127				
No metastases	79	62	70	43	< 0.01
Local and regional	6	5	3	8	NS
Bone*	21	17	18	14	NS
Visceral*	24	19	11	38	< 0.001
Node-positive patients	108				
No metastases	46	43	43	40	NS
Local and regional	6	6	4	13	NS
Bone*	33	31	34	7	< 0.05
Visceral*	26	24	22	40	NS

The number of relapses and their probability is given for all cases and for vimentin positive (V+) and negative (V−) tumours. First occurrence is taken for distant metastases, in which case local and regional occurrences are ignored. *The total number is greater than the number of patients as 6 patients (3 node-negative, 3 node-positive) had visceral and bone metastases that were diagnosed simultaneously. NS, non-significant.

Patients were subdivided by site of metastasis and then according to node status. Table 3 shows the total number of patients in each of the categories, and the probabilities with which vimentin-negative and vimentin-positive tumours metastasised in each category. Vimentin status was determined only on the original primary tumour not on the metastases. Probabilities were calculated for all tumours in each category and then for vimentin-negative and vimentin-positive subgroups. Where these probabilities differed significantly between the vimentin-negative and vimentin-positive subgroups, the *P* value is given. Table 4 provides the same data divided according to p53 status.

For node-negative patients, the probability of remaining relapse-free was 70% for those with vimentin-negative versus 43% for those with vimentin-positive tumours (Table 3). Thus, node-negative patients with vimentin-positive tumours had a significantly lower probability of remaining relapse-free ($P < 0.01$). In contrast, for node-positive patients, the probabilities of relapse for patients with vimentin-negative and vimentin-positive tumours were similar (43 versus 40%). p53 did not play a significant role in either case (Table 4).

If node-negative and node-positive patients were compared, a significant association between vimentin status and incidence of distant metastases was found only in the node-negative group. The probability of distant metastases was 49% for patients with vimentin-positive tumours and only 27% for those with vimentin-negative tumours. However, patients with vimentin-positive tumours incurred distant metastases with a similar probability regardless of whether they were node-negative or node-positive (49 versus 47%). In contrast, patients with vimentin-negative tumours incurred distant metastases only half as often in the node-negative subgroup than in the node-positive subgroup (27 versus 53%). Hence, with regard to haematogenous spread, it was the vimentin-negative tumours that accounted for the difference in probability seen for the lymph node-negative and node-positive subgroups.

When patients with distant metastases were subdivided further into those with skeletal or visceral metastases, a striking and highly significant correlation between the vimentin status of the primary tumours and the probability of visceral metastases was seen (Table 3). Whereas for all patients, the probability of

Table 4. p53 status and site of first metastasis

	n	Probabilities in % All	p53−	p53+	P
All patients	210				
No metastases	110	52	52	52	NS
Local and regional	12	6	3	11	< 0.05
Bone*	48	23	27	14	< 0.05
Visceral*	45	21	19	26	NS
Node-negative patients	118				
No metastases	70	59	61	56	NS
Local and regional	6	5	4	7	NS
Bone*	21	18	18	17	NS
Visceral*	24	20	18	24	NS
Node-positive patients	92				
No metastases	40	43	43	46	NS
Local and regional	6	7	3	17	< 0.05
Bone	27	29	37	8	< 0.01
Visceral	21	23	21	29	NS

The number of relapses and their probability is given for all cases in which p53 status was available and for p53-positive and -negative tumours. First occurrence is taken for distant metastases in which case non-distant recurrences are ignored. *The total number is greater than the number of patients as 5 patients (3 node-negative, 2 node-positive) had visceral and bone metastases that were diagnosed simultaneously. NS, non-significant.

visceral metastasis was 21%, it was 38% for patients with vimentin-positive primaries and only 16% for those with vimentin-negative breast cancers ($P < 0.001$). The difference was even greater if only node-negative patients were considered. For this group, the probability of visceral metastasis for patients with vimentin positive primaries was 38%, versus 11% for those with vimentin-negative primaries. Thus, patients with vimentin-positive primaries appeared to have a significantly higher probability of visceral relapse. In contrast, Table 4 shows that there was no significant association between p53 status and visceral metastasis, neither for all patients nor node-negative or node-positive subgroups.

However, as shown in Table 3, vimentin-positive primaries seemed less likely to metastasise to the skeleton (12%) than vimentin-negative primaries (26%, $P < 0.05$). This tendency not to invade the bones came primarily from the node-positive patients (7% for vimentin-positive versus 34% for vimentin-negative primaries). As shown in Table 4, when the node-positive group was divided by p53 status, the patients with p53-positive tumours had a decreased probability of skeletal metastasis (8%) compared to patients with p53-negative tumours, whose probability was much higher (37%).

In Tables 3 and 4, the 6 patients for whom visceral and bone metastases were diagnosed simultaneously were included in both groups. If all (and not only the first) occurrences of visceral and bone metastases were included, there were 14 patients who had distant metastases to both bone and viscera (data not shown). The two categories were statistically independent. Under these conditions for node-negative patients, the probability of incurring a visceral metastasis for those with a vimentin-positive tumour was 43% versus 11% for those with vimentin-negative primaries ($P < 0.0001$).

The site of initial recurrence was an important determinant for predicting survival after the first relapse. Mean survival after relapse for patients with bone metastases was 17.1 ($\sigma = 2.3$) months versus 8.5 ($\sigma = 1.6$) months for those with visceral metastases.

DISCUSSION

In the current study of node-negative patients, the difference in survival between vimentin-positive and -negative groups was three times that seen when the same group was divided into positive and negative groups by p53 status.

Studies of prognostic indicators generally are compared by the statistical strength of the association between the cofactor and survival. This P value depends on the difference in survival between patients with and without the marker and strongly on the number of patients in the study. In this study of 127 node-negative patients the probability of survival at 90 months was 65% for patients with p53-negative tumours, but only 55% for patients with p53-positive tumours (non-significant, $P < 0.1$, Figure 1b). Thus, there was a difference of 10% in survival between the p53-negative and -positive groups. An approximately similar difference was noted by Thor and colleagues [8] at 10 years and by Allred and associates [7] at 80 months. The P value given in the study of Thor and colleagues [8] was 0.057, while that in the study of Allred and colleagues [7] was much greater ($P = 0.003$), since they looked at 700 patients. In addition, Bosari and associates [31] found a 5% difference in survival between the p53-positive and -negative groups at 12 years, while at 9 years the difference was about 9%. Thus, in the current study, and in the other studies cited, p53 positivity seems to have a small negative effect on survival for node-negative breast cancer patients.

Alternatively, when the same group of node-negative patients was divided by vimentin rather than p53 status, the probability of survival at 90 months was 75% for patients with vimentin-negative tumours but only 42% for patients with vimentin-positive tumours ($P < 0.001$; Figure 1d). Thus, for vimentin, there was a 30% difference in survival between patients with vimentin-negative versus those with vimentin-positive tumours, as reported previously [17].

The weak association between p53 and survival seemed to depend on the strong correlation between p53 positivity and vimentin expression (Table 2), since p53 positivity ceased to be

a significant variable after vimentin expression was taken into account in the successive regression model (Fig. 2), in partial correlation, and in the Kaplan–Meier survival curves.

Furthermore, while p53 status had no effect on the probability of visceral metastasis in node-negative patients, vimentin-positive primaries metastasised 3.5 times as often to the lungs, liver and brain compared with vimentin-negative tumours (Table 3). These findings concur with data showing that oestrogen receptor (ER)-negative primaries tend to metastasise to viscera [32–35], while ER-positive primaries tend to metastasise to the skeleton [32–37], since vimentin was expressed preferentially in breast carcinomas with low ER and high Ki67 growth fraction [13, 15, 16]. Overexpression of c-*erb*B2 protein, another marker associated with low ER and high S-fraction, has also been associated with a 3-fold higher incidence of visceral metastases in a study comparing c-*erb*B2-positive and -negative tumours [38].

In the node-positive subset of breast cancer patients, we did not find a significant relationship between vimentin status nor p53 protein status with survival. Thor and colleagues [8] reported a strong association between p53 and reduced overall survival, and between p53 and metastasis-free survival in the node-positive group. In contrast, a recent report [10] showed an association between intense p53 overexpression with long relapse-free survival.

When all patients were considered together (Figure 1a) a small but statistically non-significant difference in survival was seen, while Ostrowski and colleagues [39] found a weak but non-significant correlation. In contrast, a significant association between p53 positivity and poor survival of the whole cohort of breast cancer patients was found by Thor and colleagues [8], Iwaya and colleagues [40] and Lipponen and associates [10].

In node-positive patients, there was no significant association between p53 status and visceral metastasis. However, p53-positive tumours as well as vimentin-positive tumours appeared less likely to metastasise to bones.

Carcinomas from node-negative and node-positive patients differed with respect to p53 protein and vimentin positivity. First, vimentin positivity was twice as frequent in tumours taken from node-negative than from node-positive patients (29 versus 14%). The difference in p53 positivity between node-negative and node-positive groups was smaller (35 versus 24%). Second, while there was a strong correlation between vimentin and p53 in tumours from node-negative patients ($P < 0.001$), no such association was found for node-positive patients. Third, a significant correlation between vimentin status and incidence of visceral metastases was seen in node-negative patients while no difference was seen for p53, irrespective of node involvement. Fourth, in node-positive but not in node-negative patients, vimentin-positive and also p53-positive tumours were less likely to metastasise to bones. These differences may be a reflection of more fundamental differences between tumours from node-negative versus node-positive patients, the molecular nature of which is not understood.

The differences in survival seen for the node-negative group, when divided according to vimentin status, is striking. Patients in the vimentin-positive group had a 42% probability of survival at 90 months versus 75% for those in the vimentin-negative group. This difference is much greater than that seen when node-negative patients are divided according to p53 status. Thus, vimentin appears to be a more useful prognostic marker for the node-negative group than p53. In contrast, our results show no prognostic difference when node-positive patients were divided by either vimentin or p53 status. For this group of patients, only histological grade seems of importance.

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In vitro Effect of Lonidamine on the Cytotoxicity of Mitomycin C and BCNU in Human Colon Adenocarcinoma Cells

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The ability of lonidamine (LND), an energolytic derivative of indazole carboxylic acid, to modulate the cytotoxic activity of mitomycin C (MMC) and 1,3,bis(2-chloroethyl)-1-nitrosourea (BCNU) was investigated in two human adenocarcinoma cell lines (LoVo and HT29) expressing different sensitivity profiles to the drugs. After a 1-h treatment with MMC or BCNU, cells were postincubated for 24 h with 150–225 μM LND. In the LoVo cells, a synergistic interaction between LND and MMC or BCNU was observed at both LND concentrations. In HT29 cells, only additive effects of the drugs given in sequence were seen. Flow cytometric analysis indicated that LND was generally able to stabilise the cell cycle perturbations induced by MMC and BCNU in the two cell lines. The ability of LND to potentiate anticancer drug activity, and the consideration that LND causes side-effects different from those of conventional antitumour drugs, make this compound an attractive candidate for multidrug combination therapy in colon cancer.

Key words: lonidamine, mitomycin C, BCNU, colon adenocarcinoma cells
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INTRODUCTION

COLON CANCER is one of the human tumour histotypes most refractory to systemic treatment [1] due to the inherent resistance of cells to conventional drugs [2, 3]. Thus there is great interest in new drugs with different molecular targets, other than DNA, for clinical control of this malignancy.

LND, [1-(2,4-dichlorophenyl)methyl]-1H-indazole-3-carboxylic acid, is a non-conventional anticancer drug which interferes with the energy metabolism of tumour cells. In fact, it reduces oxygen consumption in both normal and neoplastic cells [4] and increases aerobic glycolysis of normal cells and inhibits that of tumour cells [5]. Such a selective action has been ascribed