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p53 Overexpression is a Predictor of Local Recurrence After Treatment for Both *in situ* and Invasive Ductal Carcinoma of the Breast

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Background. **Several biological markers have been related to prognosis in mammary ductal carcinoma. The aim of the study was to determine biological markers that could predict local recurrence following treatment for all stages of primary operable ductal carcinoma of the breast.**

Materials and methods. **A consecutive series of patients treated for pure ductal carcinoma in situ (DCIS, $n = 110$) and invasive ductal carcinoma (IDC, $n = 243$) was studied. Twenty-three patients with DCIS were excluded because of lack of original paraffin embedded tissue. All patients had been treated between July 1996 and December 2001. Median follow-up was 49.8 mo. From the original paraffin embedded tumors, tissue microarrays (TMAs) were constructed. On these TMAs, immunohistochemistry was performed for estrogen-receptor (ER), progesterone-receptor (PR), Her2/neu, p53, and cyclin D1. Main outcome was the event of LR. All analyses were stratified for diagnosis (DCIS or IDC) and pathological grade.**

Results. **In univariate analyses, Her2/neu overexpression (hazard ratio [HR] 3.1, 95% confidence interval [CI] 1.1–8.7, $P = 0.032$) and p53 overexpression (HR 3.5, 95% CI 1.3–9.3, $P = 0.014$) were associated with LR in patients treated for both DCIS and IDC. In multivariate analysis, p53 overexpression (HR 3.0, 95% CI 1.1–8.2, $P = 0.036$ and HR 4.4, 95% CI 1.5–12.9, $P = 0.008$) and adjuvant radiotherapy (HR 0.2, 95% CI 0.1–0.8, $P = 0.026$) were independent common predictors of LR in patients who had received treatment for both DCIS and IDC.**

Conclusions. **p53 overexpression is a common predictor of LR following treatment for all stages of primary operable ductal carcinoma of the breast. This marker may help in planning optimal treatment and follow-up.**

Key Words: **breast neoplasms; local neoplasm recurrence; ductal carcinoma in situ; immunohistochemistry; biological markers.**

INTRODUCTION

Local recurrence (LR) is an adverse event in the treatment of ductal carcinoma of the breast and, as a first event in follow-up, influences prognosis significantly after treatment for all primary operable stages of this disease. In ductal carcinoma in situ (DCIS) the 10-y disease specific survival decreases from almost a 100 to 92% in the case of LR [1, 2]. In primary operable invasive ductal carcinoma (IDC), the 5- and 10-y overall survival rate of patients with LR after breast conserving therapy for early breast cancer is 81% [3] and 39% [4], respectively, and the 5-y overall survival rate of patients with LR following mastectomy is 42% [5].

Established biological markers for prognosis that have been studied in both ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) are estrogen receptor (ER), progesterone receptor (PR), Her2/neu, p53, and cyclin D1 [6, 7]. There appear to be many parallels between DCIS and IDC with regard to the expression of these markers and their prognostic significance.

Since the incidence of LR reduces survival after treatment for all stages of primary operable ductal carcinoma of the breast, the aim of this study was to identify biological markers that could predict LR following treatment for all stages of this disease. There-

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fore, tissue microarrays (TMA) were constructed from a consecutive series of tumors from patients with DCIS and a consecutive series of tumors from patients with IDC followed by immunohistochemistry for the above mentioned biological markers. These markers were tested for their predictive power of LR. The analyses were stratified for diagnosis (DCIS or IDC) and pathological grade.

PATIENTS AND METHODS

Patients and Tumors

One hundred ten consecutive patients treated for DCIS and 243 consecutive patients treated for a primary operable IDC were identified between July 1996 and December 2001. Patients and tumors were selected on the availability of the original pathological slides and sufficient paraffin embedded tissue. Eighty-seven DCIS tumors and all IDC tumors ($n = 243$) were included in the study. Patient and tumor characteristics and data on follow-up were obtained retrospectively from hospital records and are summarized in Table 1. Pathological grade for DCIS was classified according to the European Pathologists Working Group (EPWG) [8] and pathological grade for IDC was scored according to Elston and Ellis [9]. The median follow-up was 49.8 mo. Follow-up was performed according to the regional follow-up guidelines (http://www.ikcnet.nl/page.php?id=97). LR was classified as ipsilateral recurrence near the site of the original tumor in the breast in the case of breast conserving therapy and near the scar tissue on the thoracic wall in the case of a mastectomy. Axillary recurrence was not regarded as LR. During follow-up, 18 patients developed LR after a median follow-up of 27.8 mo. Thirty-four patients developed distant metastasis after a median follow-up of 29.7 mo.

TABLE 1

Clinico-Pathological and Biological Parameters of the Study Population

Clinico-pathological characteristics	DCIS ($n = 87$)	IDC ($n = 243$)
Age (median)	57.7 (36.8–77.5)	57.9 (27–89)
Screening detected	52	70
Palpable lesion	12	198
Surgery		
Breast conserving	39	98
Mastectomy	48	145
Axillary staging		
Yes	—	238
No	87	5
Pathological size		
≤2 cm	37	135
>2 cm	50	101
Unknown	—	7
Pathological grade*		
1	12	57
2	44	110
3	31	76
Positive nodes		
None	—	131
1–4	—	71
>4	—	36
Unknown	—	5
Adjuvant therapy		
Radiotherapy	21	145
Chemotherapy	—	61
Hormonal therapy	—	87
Follow-up		
No problems	79	198
Local recurrence	7	11
Axillary recurrence	—	1
Metastasis	1	33

DCIS = ductal carcinoma in situ; IDC = invasive ductal carcinoma.

* Pathological grade for DCIS was classified according to the European Pathologists Working Group [8] classification and pathological grade for IDC was scored according to Elston and Ellis [9].

Tissue Microarray Construction

Slides from all blocks were evaluated for representative areas with DCIS or IDC and TMAs were prepared as described earlier [10]. In brief, the most representative area of tumor was marked on the original hematoxylin and eosin (H&E) stained section. With this marked section as an orientation, three 0.6 mm punches were taken from the selected area in the donor blocks and mounted in a recipient block containing approximately 110 biopsies, using a manual tissue microarray device (Beecher Instruments, Silver Springs, MD).

Immunohistochemistry

Immunohistochemistry for ER, PR, Her2/neu, cyclin D1, and p53 was performed on these sections. The antibodies and antigen retrieval methods are summarized in Table 2. The immunostaining protocol was as follows: sections were deparaffinized in pure xylene, rehydrated in decreasing concentrations of ethanol, and washed in distilled water. Antigen retrieval was performed. The endogenous peroxidase reaction was blocked by incubating in 3% perhydrol for 30 min. The primary antibody diluted in PBS containing 1% bovine serum albumin was incubated for 1 h, after which the secondary (1:100 diluted in PBS containing 1% BSA and 1% AB-serum) and tertiary (1:100 diluted in PBS containing 1% BSA and 1% AB-serum) were incubated for 30 min each. Visualization was performed using the diaminobenzidine tetrahydrochloride/peroxidase reaction. Counterstaining was performed using hematoxylin. Sections were dehydrated using rising concentrations of alcohol and were mounted.

Evaluation of Immunohistochemistry

Scoring of the stainings was performed by two well-trained residents (BvdV and MdR). The scoring was randomly verified by an experienced pathologist (JW). ER, PR, p53, and cyclin D1 were graded based on the percentage of tumor cells showing positive nuclear staining. ER, PR, and cyclin D1 were considered positive if nuclear staining was present in ≥10% of the cells, and p53 was considered positive (overexpression) in case of a substantial percentage of positively stained nuclei (>30%). Her2/neu expression was graded as recommended by the manufacturer's scoring guidelines; 0: no staining at all or membrane staining in <10% of the tumor cells; 1+: a faint/barely perceptible partial membrane staining in >10% of the tumor cells; 2+: weak to moderate complete membrane staining in >10% of the tumor cells; 3+: strong complete membrane staining in >10%. Her2/neu was considered positive if the score was 3+.

Statistical Analysis

Univariate logistic regression analyses, investigating the effect of biological markers on local recurrence, were performed using Cox regression models. All analyses were adjusted for diagnosis and pathological grade to obtain diagnosis and grade independent predictors. Variables with a P value ≤0.10 were included in a multivariate logistic regression analysis that was also performed with a Cox

TABLE 2

Antigen Retrieval Methods and Antibodies

Antibody	Clone	Supplier	Dilution	Antigen retrieval	Secondary antibody	Supplier	Tertiary antibody	Supplier
ER	6F11	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 98° C microwave	RAMBIO	Dako	SARBIO	Dako
PR	1A6	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 98° C microwave	RAMBIO	Dako	SARBIO	Dako
Her2/Neu	CB11	Ventana	*	Tris/HCL 0.1M (pH 9.5) 30' 98° C microwave	RAMBIO	Dako	SARBIO	Dako
p53	BP-53-12-1	Biogenix	1:800	Tris/HCL 0.1M (pH 9.5) 30' 98° C microwave	RAMBIO	Dako	SARBIO	Dako
Cyclin D1	SP4	Neomarkers	1:50	Tris/HCL 0.1M (pH 9.5) 30' 98° C microwave	RAMBIO	Dako	SARBIO	Dako

ER = estrogen receptor; PR = progesteron receptor; * = prediluted by supplier; RAMBIO = rabbit antimouse biotin; SARBIO = swine antirabbit biotin.

regression model. The selection of variables in a stepwise manner identified the statistically significant clinico-pathological and biological parameters. Finally, clinico-pathological variables known to be related to prognosis were entered in a multivariate Cox regression model evaluating the predictive power of p53. P values of ≤ 0.05 were considered significant. All calculations were performed with SPSS 12.01 (SPSS Inc., Chicago, IL).

RESULTS

From the 87 DCIS cases, representative tissue cores were obtained in 80 cases (92%) and acceptable immunohistochemistry (at least one of the three cores was stained sufficiently) was achieved in 69 (79%) cases for p53-, 70 (80%) cases for cyclin D1- and PR-staining, 73 cases (84%) for ER-staining, and 80 cases (92%) for Her2/neu staining. Out of the 243 IDC cases, the tissue cores of 237 cases (98%) were adequately represented in the TMA. Immunohistochemistry could be evaluated in all cases (100%, $n = 237$) for p53 and D1, in 235 cases (99%) for Her2/neu, in 232 cases (98%) for ER, and in 230 cases (97%) for PR.

The relation of the biological markers with local recurrence in univariate analysis is outlined in Table 3. Her2/neu overexpression (hazard ratio [HR] 3.1, 95% confidence interval [CI] 1.1–8.7, $P = 0.032$) and p53 overexpression (HR 3.5, 95% CI 1.3–9.3, $P = 0.014$) were associated with LR in patients treated for both DCIS and IDC (stratification for diagnosis).

In multivariate analysis (including Her2/neu- and p53 overexpression), p53 positive immunoreactivity (HR 3.0, 95% CI 1.1–8.2, $P = 0.036$) was an independent common predictor of LR in patients that had received treatment for both DCIS and IDC (Table 4).

To evaluate the power of prediction of Her2neu and p53 in relation to clinico-pathological parameters that are known for their association with prognosis Her2/neu overexpression and p53 overexpression were evaluated in a multivariate analysis model including these clinico-pathological parameters. In this analysis adjuvant radiotherapy (HR 0.2, 95% CI 0.1–0.8, $P = 0.026$) and p53 overexpression (HR 4.4, 95% CI 1.5–12.9, $P = 0.008$) were both independent predictors of LR irrespective of DCIS or IDC (Table 5).

DISCUSSION

In this study, established biological markers were evaluated for their predictive capacities for LR following treatment for DCIS and all stages of primary op-

TABLE 3

Biological Markers and Local Recurrence, Univariate Analysis, Adjusted for DCIS or IDC and Pathological Grade (Cox Regression)

Biological marker	Study population	HR	95% CI	P value
Her2/neu				
Positive	55	3.1	1.1-8.7	0.032
Negative	263	1		
$n = 318$				
ER				
Positive	235	1		
Negative	74	1.8	0.6-5.9	0.298
$n = 309$				
PR				
Positive	181	1		
Negative	122	1.1	0.4-3.0	0.895
$n = 303$				
p53				
Positive	56	3.5	1.3-9.3	0.014
Negative	254	1		
$n = 310$				
Cyclin D1				
Positive	208	1		
Negative	98	1.5	0.6-4.1	0.402
$n = 316$				

HR = hazard ratio, adjusted for pathological diagnosis (DCIS or IDC) and grade; 95% CI = 95% confidence interval; ER = estrogen receptor; PR = progesterone receptor.

TABLE 4

Biological Predictors of Local Recurrence, Multivariate Analysis, Adjusted for DCIS or IDC and Pathological Grade (Cox Regression)

Biological marker	HR	95% CI	*P* value
Her2/neu over expression			
Positive	2.3	0.8-6.5	0.132
Negative	1		
p53			
Positive	3.0	1.1-8.2	0.036
Negative	1		

Note. Regression analysis. HR adjusted for pathological diagnosis (DCIS or IDC) and grade.

erable IDC of the breast. p53 overexpression proved to be an independent predictor of LR in patients irrespective of treatment for in situ or node-negative or -positive invasive ductal carcinoma (HR 4.4, 95% CI 1.5–12.9, $P = 0.008$). Adjuvant radiotherapy (HR 0.2, 95% CI 0.1–0.8, $P = 0.026$) was another independent predictor of LR. In univariate logistic regression analysis Her2/neu overexpression was also related with LR (HR 3.1, 95% CI 1.1–8.7, $P = 0.032$).

The p53 gene is a tumor suppressor gene located on the short arm of chromosome 17 at the position 17p13.1. It encodes a 53kD nuclear phosphoprotein, p53, which maintains genomic integrity by inducing cell cycle arrest or apoptosis in case of acquired DNA damage [11]. Mutations in one allele of the p53 gene can result in inactivation or alteration of its function. Such mutations occur in many human cancers including in approximately 20% of the breast carcinomas [12]. Normal p53 protein is practically undetectable by immunohistochemistry because of the short half-life of the protein and the low amount of p53 protein present in the cell. Overexpression of p53 detected by immunohistochemistry indicates mutation of the p53 gene, which leads to a stabilized form and nonfunctional form of p53 [12].

Most studies on p53 overexpression and prognosis in patients with resectable node-positive and node-negative breast cancer have observed a shortened relapse-free and/or overall survival in case of tumors with a positive immunoreactivity for p53 [13, 14]. There are, however, other reports that have not found such an effect on prognosis [15, 16]. Discrepant findings may be explained by the diversity in the different antibodies for p53 and scoring methods for p53 immunoreactivity.

Fewer studies have investigated the relation of p53 protein expression with LR. After evaluation of several clinico-pathological factors and biomarkers in a case-control study of 66 patients with LR following breast conserving therapy, p53 was found to be an independent predictor of LR [17]. Similar results have been obtained in the study by Turner *et al*. [18] In DCIS, there are not many studies that describe the relation between p53 immunoreactivity and LR. Two groups of investigators, however, have shown that p53 positive expression is also associated with LR in DCIS [19, 20].

In this present series, the majority of patients was treated with mastectomy (DCIS 55.2% and IDC 59.7%). Chest wall recurrence is not very common and was not present in patients treated for DCIS. In patients treated for IDC, however, chest wall recurrence was present in eight cases. Zellars *et al*. [21] have demonstrated the prognostic value of p53 overexpression for local failure in 1530 mastectomy treated patients.

The exact relation of mutant p53 with LR remains unclear, however. The short median follow-up in which local recurrences developed (27.8 mo) in this study suggests a clonogenic origin of these recurrences. The fact that p53 overexpression is a predictor of LR in

TABLE 5

Multivariate Analysis Including Clinico-Pathological Parameters Known to be Related with Prognosis Investigating the Relation with Local Recurrence in Order to Test the Predictive Power of p53 Expression

Characteristics	HR	95% CI	*P* value
Surgical procedure			
Lumpectomy	3.1	0.7-12.4	0.092
Mastectomy	1		
Margins			
Positive	3.2	0.7-13.5	0.118
Negative	1		
Pathological size			
≤2 cm	1.1	0.4-3.0	0.900
>2 cm	1		
Pathological grade*			
1	0.9	0.2-5.1	0.979
2	0.4	0.1-1.5	0.181
3	1		
Axillary status			
Positive	2.6	0.4-18.6	0.351
Negative	1		
Adjuvant radiotherapy			
Yes	0.2	0.1-0.8	0.026
No	1		
Adjuvant chemotherapy			
Yes	0.3	0.1-2.1	0.231
No	1		
Her-2/neu overexpression			
Positive	2.1	0.7-6.4	0.195
Negative	1		
p53			
Positive	4.4	1.5-12.9	0.008
Negative	1		

Note. Regression analysis. HR adjusted for pathological diagnosis (DCIS or IDC). Pathologists Working Group [8] classification and pathological grade for IDC was scored according to Elston and Ellis [9].

* Pathological grade for DCIS was classified according to the European.

patients treated for all operable stages of ductal carcinoma suggests the presence of a biologically more aggressive subgroup throughout all stages of breast cancer progression. An explanation for the relation of p53 overexpressing tumors with LR may be a more aggressive biological profile and behavior, but could also be a consequence of p53-related treatment resistance [22]. In this retrospective study with a consecutive series of patients, all being treated according to existing guidelines, including adjuvant endocrine- or chemotherapy and radiotherapy, it is very difficult to entirely separate prognostic and predictive characteristics. Adjuvant radiotherapy was an independent predictor of LR (HR 0.2, 95% CI 0.1–0.8, $P = 0.026$). However, there was no relation between LR and adjuvant chemotherapy (HR 0.4, 95% CI 0.1–2.0; $P = 0.461$).

The identification of a predictive marker for LR throughout the progression of ductal carcinoma may help in guiding the optimal treatment for every stage of this disease. p53 expression may particularly influence choice of therapy in patients with pure DCIS because mastectomy results in a survival rate of almost 100%. This marker may also be used for therapeutic considerations in more progressed stages of ductal carcinoma. In a study by Mieog *et al*. [23], p53 overexpression was an independent predictor of clinical tumor response after anthracycline based neoadjuvant chemotherapy for patients with operable breast cancer. In contrast with the findings of Elledge and Allred [22], Silvestrini *et al*. demonstrated that radiation therapy seems to prevent LR in patients with tumors that express elevated levels of p53 [24].

We also found a relation of Her2/neu overexpression with LR (HR 3.1, 95% CI 1.1–8.7, $P = 0.032$). Other studies concerning Her2/neu overexpression and prognosis in invasive cancer have indicated a relation with chest wall recurrence after mastectomy [25] and with disease recurrence following BCT [26]. In the last study [26], a relation with LR could not be demonstrated. In DCIS, Her2/neu overexpression is associated with high pathological grade [27] and with LR [28].

Cyclin D1 did not show any effect on LR in our series (negative staining; HR 1.5, 95% CI 0.6–4.1, $P = 0.402$). Turner *et al*. have investigated the influence of cyclin D1 protein expression on breast cancer recurrence in a case-matched study in 49 patients and found that low levels of immunohistochemically detected cyclin D1 protein correlated with LR [29]. Also, in DCIS, low levels of cyclin D1 protein expression were associated with local recurrence [30]. As has been suggested before, these different findings from our study may be explained by different antibodies for cyclin D1 and methods of detection.

In conclusion, p53 overexpression is an independent predictor of LR in patients treated throughout all stages of primary operable ductal carcinoma of the breast including in situ ductal carcinoma. This marker may help in planning optimal treatment and follow-up.

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