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Invariant p53 immunostaining in primary and recurrent breast cancer

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Abstract

In animal models, acquired mutations of the *p53* gene that result in increased p53 protein expression are associated with tumour recurrence following chemotherapy. The aim of this study was to test the hypothesis that breast cancer recurrences following adjuvant therapy exhibit aberrant p53 expression. We therefore evaluated p53 expression in paired primary and recurrent breast tumours: 48% of primary and 32% of recurrent tumours had abnormally increased p53 expression. Of the paired samples, 84% showed no change in p53 expression between the primary tumour and the metastasis. In fact, in no case was low (normal) p53 expression in the primary tumour followed by the development of high (aberrant) p53 expression in the recurrence. These results show that increased p53 expression is not selected for in the malignant cells emerging following adjuvant therapy, suggesting that p53 expression is unlikely to play a central role in breast cancer recurrences.

Keywords: p53; p21; Breast cancer; Metastasis; Recurrence; Adjuvant chemotherapy

1. Introduction

The tumour suppressor gene product, p53, is involved in many cellular functions including cell-cycle regulation, apoptosis and maintenance of genomic stability. When examined by immunohistochemical staining (IHC), approximately 40% of invasive breast cancers exhibit high levels of stabilised, often mutant p53 protein [1,2]. Tumours with mutant p53 are thought to have a growth advantage because they are unable to eliminate cells with damaged DNA via apoptosis, and may therefore be therapy-resistant. Several studies, performed both *in vitro* [3,4] and *in vivo* [5–8], have found that mutations in tumour cell p53 correlate with resistance to cytotoxic therapies. However, the association between p53 status and susceptibility to cytotoxic treatment remains uncertain. Evidence also exists to support the hypothesis that tumours with aberrant p53 are actually more susceptible to certain chemotherapeutic regimens such as the antimicrotubule agents, which induce apoptosis through p53-independent mechanisms [8–10]. Still other studies have been unable to find any correlation between p53 status and sensitivity to chemotherapy [11–13], suggesting that p53 expression may not be a key regulator in the development of resistant clones of already established tumours, or that its role is tumour-type-specific. Instead, p53 may be required for early changes in the development of some cancers, although only a handful of studies have examined the natural history of p53 expression in tumours from individual patients over time.

The p21/Waf1 (*p21*) gene is a transcriptional target of p53 and its protein product is an inhibitor of cyclin-dependent kinases. Following DNA damage, p21 has been shown to be an important downstream effector in p53-regulated growth inhibitory pathways. However, p21 expression can also be induced by p53-independent pathways and therefore the inverse correlation between

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normal p53 function (decreased expression) and increased p21 expression is often altered resulting in many cancers having deregulated expression of p21 [30].

Because of the possible relationship between p53 function, p21 expression and malignant cell survival, we hypothesised that p53 and/or p21 protein expression might differ between the viable metastases that emerge months to years following treatment for early stage breast cancer, and the primary cancer. Therefore, we undertook a study of 25 paired primary and late recurrences in order to determine whether recurrent lesions do in fact exhibit altered p53 or p21 immunostaining when compared with the primary early-stage tumour.

2. Patients and methods

2.1. Case selection

Eligible subjects included patients with stage I, II or III breast cancer who were treated at the University of Chicago from 1984 to 1997. All patients except one received one or more adjuvant therapies and subsequently had a biopsy-proven breast cancer recurrence with tissue available from both the primary tumour and the asynchronous recurrence. 10 cases were excluded from the study due to an inadequate quantity or quality of archived paraffin-embedded metastatic tumour material as determined at the time of pathological review of haematoxylin and eosin (H and E)-stained slides. Information on tumour grade, size and nodal status was obtained from the original pathology reports and confirmed in consultation with our institution's breast pathologist.

2.2. Immunohistochemical analysis

All patient identifiers were removed from the biopsy samples and the pairing of samples was unknown to the investigators who scored the IHC staining. A 5-μm section was cut from each block and stained with H and E to ensure an adequate amount of tumour and fixation quality for the IHC analysis. Subsequent 5-μm sections were mounted on Fisher Superfrost®/plus slides, heated to 60 °C for 1 h, cooled, deparaffinised and hydrated through three changes of xylene and graded alcohols. Slides were then washed three times with phosphate-buffered saline (PBS), heated in a rice cooker in citric buffer for 20 min, cooled, rinsed with PBS, quenched in 0.3% H_2O_2 in PBS for 30 min and blocked with 10% horse normal serum for 30 min. Specimens were incubated overnight in either (1) anti-p53 monoclonal antibody DO-7, which stains for both wild-type and mutant p53 (Novocastra Laboratories Ltd., UK) using a stock concentration of 5 mg/ml at a 1:100 dilution or (2) monoclonal antibody against WAF-1 (p21) (Novocastra NCL-WAF1) at a 1:40 dilution. After rinsing with PBS, a secondary biotinylated anti-mouse IgG antibody at a dilution of 5 μg/ml (Vector Labs) was applied for 30 min, and slides were rinsed with PBS and exposed to diaminobenzidine tetrahydrochloride (DAB) chromogen to develop the colour reaction product. To avoid a colour reduction, slides were washed with tap water, counterstained in haematoxylin for 2 min washed, dehydrated in a series of ethanols, cleared in xylene and mounted. Negative controls for this study were performed for each sample using an isotype-specific mouse control antibody instead of the anti-p53 or anti-p21 antibody. Human normal colonic epithelial cells were used as a positive control for p21 expression, and a breast tumour determined previously to overexpress p53 was used as a positive control for p53 expression.

2.3. IHC scoring

Slides were scored for p53 and p21 expression according to the percentage of tumour cells that stained positively above the background staining. The whole section was scanned at medium- and high-power and then independent observers analysed at least 3–5 high-power fields. At least 500 cells in the most densely stained areas were counted for the percentage of cells staining positively above the background. As in previously published studies [1,2,9,14,15], greater than 10% positively stained cells was used to represent positive p53 overexpression for this report. The level of p21 expression has been found previously to be negligible in normal breast epithelium by IHC and therefore p21 expression in greater than 5% of cells was scored as positive [2].

2.4. Statistical analysis

Fisher's exact test was used to determine the association between: p53 expression and grade (in primary tumours), p21 expression and grade (in primary tumours), and to compare p53 expression with p21 expression (in both primary and metastatic tumours). McNemar's test was used to compare expression of each protein (p53 and p21) in primary versus metastatic tumours. The association between p53 and p21 expression in metastatic tumours and time to recurrence was examined using the Wilcoxon's rank-sum test.

3. Results

Twenty-five pairs of primary and metastatic breast cancer biopsies from women diagnosed with a recurrence between January 1984 and October 1997 were evaluated in this study. The median age at the time of

Table 1
Summary of adjuvant therapy

Treatment regimen	*n*
Chemotherapy (chemo)	6
Chemo/tamoxifen (Tam)	4
Chemo/radiation therapy (RT)	4
Tam/RT	3
Chemo/Tam/RT	4
Tam	3
None	1

initial diagnosis was 48 years, with a range from 31 to 78 years of age. 11 of 25 (44%) patients were initially node-negative (Stage I or IIA), and 14 (56%) were node-positive (Stage IIB-IIIA). Interestingly, none of the primary tumours that went on to manifest recurrences was grade I; 13 were grade II and 12 were grade III. All except one patient received either systemic tamoxifen or chemotherapy; 11 patients also received adjuvant radiotherapy (Table 1). Thirteen of the recurrences were biopsied at distant sites including lung ($n=4$), brain ($n=3$), liver ($n=3$), ovary ($n=2$) and scalp ($n=1$); the remaining 12 recurrences occurred locally in the chest wall (n = 9), internal mammary lymph node ($n=2$) or supraclavicular lymph node ($n=1$). The median time to recurrence (interval between excision of the primary tumour and biopsy of the recurrence) was 43 months with a range from 9 to 142 months.

Overexpression of p53 protein (staining of >10% of the tumour cells) was observed in 12/25 (48%) of the primary tumours and 8/25 (32%) of the metastases (McNemar $P=0.12$). A distinct nuclear staining pattern characteristic of p53 was invariably observed. Primary and metastatic biopsies were concordant for p53 protein expression in 84% (21/25) of cases when a cut-off of 10% was used; interestingly, the percentage of cells staining for p53 in the primary versus the metastasis was found to be within 5% of each other in all 21 concordant pairs. Although 4 cases demonstrated a significant decrease in the percentage of cells expressing p53 protein in the metastasis compared with the primary, in no case was there an increase in p53 staining in the metastasis compared with the primary (Table 2). Furthermore, the 4 cases exhibiting a decrease in p53 expression in the metastases were not associated with a particular treatment regimen, metastatic location or time to progression.

Positive p21 expression (>5%) was found in 8/25 (32%) of primary tumours and 8/25 (32%) of metastatic sites (McNemar $P=1.0$). Concordant p21 expression between the primary and the metastatic tumour (positive versus negative) was observed in 19/25 (76%) of the paired specimens. Interestingly, no significant inverse correlation was found between p53 and p21 expression in either the primary ($P=0.20$) or the metastatic ($P=0.21$) tumour specimens (Table 3); this observation implies the existence of alternative mechanisms for p21 regulation that are likely to be independent of p53 function as a transcriptional activator of the *p21* gene.

In addition, no significant correlation was found between p53 ($P=0.43$) or p21 ($P=0.67$) expression and the histological grade of the primary tumour or between p53 ($P=0.84$) or p21 ($P=0.22$) expression in the primary or metastatic tumour and the time to recurrence. Analyses of these data treating p53 and p21 as ordinal variables led to similar results. Taken together, these data suggest that within this subset of metastasising breast cancers, p53 and p21 expression levels do not associate with traditional measurements of tumour virulence.

4. Discussion

We originally hypothesised that tumour recurrences several months to years (median time to recurrence in

Table 2
Change in p53 and p21 status between primary and metastatic breast cancer

Initial Stage[a]	*n*	Change in p53 status[b]/ total tumours	Change in p21 status[c]/ total tumours
I	3	0/3	0/3
IIA	8	2/8	2/8
IIB	9	1/9	3/9
IIIA	5	1/5	1/5
Total	25	[b]4/25	[c]6/25

[a] Stage is based on the American Joint Committee on Cancer (AJCC) [24].
[b] 4/4 tumours with a change in p53 expression exhibited a decrease in p53 expression.
[c] 3/6 tumours with a change in p21 expression exhibited an increase and 3/6 a decrease in p21 expression.

Table 3
p53 and p21 expression in (a) primary and (b) metastatic tumours

		p53 expression	
(a) Primary tumors			
		+	–
p21 expression	+	2	6
	–	10	7
$P=0.20$			
(b) Metastatic tumors			
p21 expression	+	1	7
	–	7	10
$P=0.21$			

our study was 43 months) following adjuvant treatment of the primary breast cancer would consist predominantly of cancers with abnormal (elevated) p53 expression. The aim of the current study was to test this hypothesis by comparing the expression of p53 and p21 in primary breast cancers and late-occurring breast cancer metastases from the same patients. However, our results show that increased p53 expression is not observed in the metastases compared with the matched primary tumours. These findings suggest that aberrant p53 expression does not impart a survival advantage for breast cancer cells *in vivo*.

It is clear that one cannot directly extrapolate between abnormal (elevated) p53 protein expression and the presence of p53 mutations because misclassification can occur when p53 protein accumulation is used as a surrogate marker for a mutation in the *p53* gene. For example, one report using the anti-p53 monoclonal antibody, 1801, noted as high as a 33% false-negative and 30% false-positive rate of detection of mutant p53 for breast cancer specimens if the p53 expression level is used as a surrogate for mutant protein expression [16]. However, a more recent analysis, using the DO-7 antibody that we used in our study, found only a 13% misclassification rate of head and neck cancer specimens (two of 15 tumours containing a *p53* deletion mutation were scored as negative (normal) for p53 protein) [17]. In our study, the striking similarity in percentages of tumour cells expressing p53 in the primary tumour and the subsequent metastasis suggest that the acquisition of a mutation preventing normal p53 degradation is not a common occurrence in the selection of cells within the primary tumour that survive adjuvant chemotherapy and become a viable metastasis.

Two earlier studies found concordant p53 expression in paired breast primary and local lymph node metastases that were sampled together at the time of the patient's initial surgery for early stage breast cancer [18,19]. These studies suggested that p53 is not a marker for metastasis from the breast to the local lymph node. More relevant to the question examined in this study, that of whether chemoresistance is associated with p53 expression, is a report of concordant p53 expression in breast cancer biopsies performed before neoadjuvant chemotherapy and in the tumour remaining after therapy [20]. More recently, two groups have reported the results of p53 immunostaining of primary breast cancers and asynchronous metastases [21,22]. In the first, Shimizu and colleagues [22] reported consistent p53 expression in 100% of 21 matched asynchronous primary and metastatic breast tissue specimens; however, seven patients had not received adjuvant therapy and only three patients had distant metastases. In the second study, Tsutsui and colleagues [22] examined a group of 18 asynchronous metastases from breast cancer patients; most (67%) of the cases also showed concordance of p53 protein expression. Our current study extends these findings to a group of patients with a relatively long disease-free interval (range 9–142 months) and predominantly distant, rather than local recurrences.

Another finding from our study is that the pattern of p53 and p21 staining found does not suggest that wild-type p53 (low level of expression) consistently induces p21 (high level of expression). We did note that six tumours were discordant in p21 status between the primary and metastatic lesion; three went from positive to negative and three went from negative to positive. Only two of these six tumours exhibited an accompanying change in p53 expression (both showed detectable p53/p21 staining in the primary and were negative for p53/p21 expression in the metastases). The significance of the change in p21 expression between the primary and metastatic lesion remains uncertain because the alterations were infrequent and not consistent in the direction of the change; the lack of an accompanying inverse correlation with p53 staining suggests that p53 did not mediate the change in p21 expression.

In summary, recurrent breast cancers arising despite adjuvant therapy do not commonly exhibit altered p53 or p21 expression when compared with the primary tumour. While the molecular events that converge to allow a disseminated tumour cell to establish a significant metastasis have yet to be identified, p53 expression, contrary to our initial hypothesis, does not appear to be a major factor. Large-scale tumour profiling that permits thousands of genes and proteins to be examined simultaneously may yield clues as to which growth-promoting pathways are consistently altered among recurrent breast cancers [23].

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Conflict of interest statement

The authors of this manuscript have no financial or personal relationships that could bias this work.

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