Virchows Arch (1997) 431:317–321

ORIGINAL ARTICLE

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Expression of E-cadherin and its relation to the p53 protein status in human breast carcinomas

Received: 29 May 1997)Accepted: 10 June 1997

Abstract In breast carcinomas the *TP53* gene is altered in 10–30% of cases. Alteration of the gene may lead to a general genomic instability, detected as deletions and/or amplifications at the gene level, and as altered expression at the mRNA and protein level. We have demonstrated a strong association between down-regulation of E-cadherin protein expression and alterations of the p53 protein, detected as *TP53* gene mutation and/or protein accumulation in tumour samples from 210 patients with breast carcinomas (P <0.001). Investigation of allelic imbalance using microsatellite markers located near the E-cadherin locus was also performed. A higher frequency of loss of heterozygosity in the microsatellite marker closest to the E-cadherin locus was observed in samples with down-regulation of E-cadherin protein expression. A higher frequency of down-regulation of the E-cadherin protein expression was found in invasive lobular carcinomas than in invasive ductal carcinomas, although this difference was of borderline significant (P=0.084). Cases in the present series were also immunostained for c-erbB-2 protein overexpression. A significant association between p53 protein accumulation and cerbB-2 protein overexpression was seen (P=0.036). The results of the present study indicate that p53 protein may play a role in regulation of E-cadherin protein expression.

Key words Breast carcinomas · p53 · *TP53* gene · E-cadherin · Immunohistochemistry · Genomic instability

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Introduction

E-cadherin, located on the long arm of chromosome 16, belongs to a group of calcium-dependent transmembrane cell–cell adhesion molecules that bind to one another by homologic interactions and are classified into several subclasses [1]. Cadherins are important for the maintenance of adult tissue architecture, and their function is dependent of the interaction between the cytoplasmic domain and elements of the cellular cytoskeleton [18]. In the adult, almost all epithelial cells express both E-cadherin and P-cadherin. E-Cadherin tends to be distributed uniformly throughout the glandular epithelial layer, whereas P-cadherin is concentrated in the basal layers. The same distribution is found in normal mammary gland. Down-regulation of E-cadherin has been found in variety of human carcinomas, including breast [5].

In experimental systems cadherins decrease tumour invasion [7, 11, 19, 23]. Several groups have studied E-cadherin expression and its prognostic value in different tumour types [8, 12, 14, 16, 17, 20], and down-regulation of E-cadherin expression in breast carcinomas has been found to correlate with differentiation grade. Lindblom et al. [15] found loss of heterozygosity (LOH) at the long arm of the chromosome 16 to be associated with development of distant metastases. Berx et al. [5] have recently demonstrated LOH in the region of chromosome 16 where E-cadherin is located, along with a high frequency of mutations in the E-cadherin gene in lobular breast carcinomas.

In sporadic breast carcinomas several putative tumour suppressor gene loci have been identified through studies of LOH and allelic imbalance. So far, only the *TP53* gene has been found to be mutated in a significant fraction of these tumours [2]. The p53 protein has many functions. It acts as a transcription factor by both transactivating some genes and suppressing transcription of others. The most important action in prevention of tumour formation and progression is probably the “guardian of the genome” function. After DNA damage normal cells stop in G1/S to allow time for repair. Cells with al-

teration in the *TP53* gene do not stop in G1/S. Thus DNA damage will not be repaired and genomic instability will appear, with accumulation of deletions and amplifictions [2, 22]. LOH of several genes, including the gene coding for E-cadherin, may be the result of such instability. Since LOH at the E-cadherin locus is associated with inactivating mutations in the E-cadherin gene [5], reduced protein expression may be the result.

The aim of this study was to evaluate the expression of E-cadherin and its relationship to alterations in the p53 protein, and to evaluate whether p53 protein alterations, and thereby genomic instability leading to high frequency of LOH, leads to reduced expression of E-cadherin in human breast carcinomas. Expression of E-cadherin and p53 alterations were also compared with findings of cerbB-2 expression detected by immunohistochemistry.

Materials and methods

Material for this study was obtained from 210 primary breast carcinoma patients admitted to Ullevål Hospital during the period from 1987 to 1994. The mean age of the patients at diagnosis was 64.5 years (range 28–91 years). Lymph node dissection was performed in 186 patients: 108 of the 186 (58.0%) patients were lymph node negative and 78 (42.0%), lymph node positive at the time of surgery.

Twenty-eight of the tumours were classified as invasive lobular carcinomas, 160 as invasive ductal carcinomas and 20 as belonging to other types. Two of the tumours were unclassified. Sixty-five of the tumours were classed as grade 3 (31%), 126 (61%) as grade 2 and 17 (8%) as grade 1 tumours. Grading of the tumours was based on the recommendations from Elston and Ellis [9]. Clinical stage was based on the TNM classification of 1988 [3]. All samples included in this study were judged after histological evaluation to contain more than 20% tumour tissue. Mean observation time was 2.8 years (range 1.5–4.4 years). Sixty-four patients (30%) had recurrences or distant metastases (to liver, lungs, skeleton, CNS) during the follow-up period. Thirty-six patients had died of disseminated breast cancer, and 20 of other causes.

Fresh tumour tissue obtained at surgery was frozen in liquid nitrogen and stored at −70 °C. Frozen sections were cut and immunostained for E-cadherin protein, applying the avidin–biotin–peroxidase complex (ABC) method [13]. Briefly, frozen sections were air-dried and fixed in cold acetone at 4 °C for 10 min. After preincubation with mouse serum for 20 min at room temperature, the sections were incubated with a monoclonal antibody (428B from Euro Diagnostica) for 16–22 h at 4 °C. The sections were then incubated with the biotin-labelled secondary antibody for 30 min and washed in PBS, followed by incubation with ABC for 60 min. The peroxidase reaction was carried out by using diaminobenzidine as chromogen. All series included positive and negative controls. Only cells with distinct membrane staining were scored as E-cadherin protein positive. The proportion of immunopositive cells was estimated semiquantiatively: + means 5–10%, ++ 10–50%, and +++ more than 50% positive cells.

Antibodies NCL CB11 from Nova Castra laboratories were used to detect cerbB-2 protein. The same staining procedure was applied as for E-cadherin protein. Only cells with distinct membrane staining were scored as positive. The proportion of immunopositive cells was estimated semiquantitatively: + 1–10%, ++ 10–50%, and +++ more than 50% positive cells.

p53 alterations were detected as mutations in the *TP53* gene and/or p53 protein accumulation. These data have been reported previously [6]. In 38 of the tumours a p53 protein alteration was detected either by protein accumulation and/or as a mutation: 31 tumours showed protein accumulation and 26 tumours had a mutation [6].

In the investigation of LOH blood samples were available for only 14 of the 38 patients with reduced/absent E-cadherin protein expression in the tumour tissue. These 14 samples, together with 36 samples with strong immunoreactivity for E-cadherin in the primary tumour, were analysed for LOH using the following microsatellite markers: D16S186, D16S265, D16S398, D16S496 and D16S515, all mapping to 16q22.1 where the E-cadherin gene is located. These microsatellite markers were selected on the basis of information obtained from the Genomic Data Base. The microsatellite marker D16S496 maps closest to the E-cadherin gene. Microsatellite genotyping was performed in microtitre plates by PCR, using 50–100 ng DNA in a 10-µl reaction volume containing 200 µM dATP, dGTP and dTTP, 2.5 µM dCTP, 0.7 µCi (a-32P)-dCTP, 20 µM (3 pmol) of each primer and 0.3 U Taq DNA polymerase and 1×PCR buffer II (Perkin Elmer). The oligonucleotides were purchased from Research Genetics.

Two multiplex PCR reactions were performed combining the following microsatellite markers: D16S186 and 398, D16S265 and 515. The microsatellite marker F16S496 was amplified separately. PCR reactions were performed in an MJ Research PTC-100 PCR machine. The PCR conditions were as follows: 94 °C for 5 min, followed by 27 cycles of 94 °C for 30 s, 55 °C for 1 min 15 s, 72 °C for 15 s and ending at 72 °C for 2 min. PCR products were separated on 6% denaturating polyacrylamide gels (1:19bis:acrylamide), containing 1×TBE and 7.7 M urea, for 1.5–2 h at 100 W constant in 0.5×TBE buffer. The gels were transferred to filter paper, dired, and exposed to Fuji Medical X-ray films, RX. Several exposures were performed for each gel (between 1 h and 4 days). The autoradiograms were scored visually by three independent observers. The intensities of the alleles in tumour samples from heterozygotes were compared with those observed in the matched blood samples. Total or partial loss of one allele in the tumour DNA was scored LOH. Cases with LOH were analysed at least twice for verification.

Statistical analysis was performed by the Chi-square test with Yates correction and Fisher exact when appropriate (total number <50).

Results

Tissue samples from 210 tumours were examined for E-cadherin protein immunoreactivity. A strong membrane staining in more than 50% of the cells (+++) was seen in 127 (60.5%) of the cases (Fig. 1A), in 10–50% (++) of the cells in 45 (21.4%) cases and in 5–10% of the cells (+) in 8 cases (3.8%). Thirty tumours (14.3%) did not show any E-cadherin immunoreactivity. Normal breast epithelial cells were strongly immunostained.

Invasive lobular carcinomas had more often down-regulation of the E-cadherin protein than invasive ductal carcinomas (Table 1), although this difference was of only borderline significance (P=0.084). Nine (32.1%) of the tumours of invasive lobular type showed reduced or absent E-cadherin expression, as opposed to 26 (16.3%) of the invasive ductal carcinomas. In both histological groups down-regulation of the E-cadherin protein occurred more frequently in tumours with p53 alterations than in tumours without p53 alterations.

No association was seen between reduced E-cadherin and tumour grade. When E-cadherin protein expression and presence of lymph node metastases were compared, no association was seen.

Successful staining for the cerbB-2 protein was achieved in 200 tumours. Of these, 22 (11.0%) showed staining in more than 50% of the cells (Fig. 1B). In 10

tumours (5.0%) staining was seen in 10–50% of the cells. Three tumours (1.5%) showed staining in few cells, and in 165 (82.5%) no staining was seen.

Of the 50 paired samples analysed LOH was detected in one or more of the microsatellite markers employed in 35 (70.0) of the samples. The marker closest to the E-cadherin gene, D16S496, showed LOH in 10 of the 35 informative tumours (28.6%). Seven of these were found among the 14 samples without detectable E-cadherin protein expression in the tumour tissue. This is significantly more frequent than LOH in tumours with normal E-cadherin expression (P=0.053). The other microsatellite markers (D16S186, D16S265, D16S398 and D16S515) did not show significantly higher LOH in E-cadherin-negative than in E-cadherin-positive samples, although there was tendency to higher LOH in E-cadherin negative samples (Table 2). LOH was detected only in one sample for microsatellite marker D16S398. Examples of tumours with LOH at the D16S496, D16S185 and D16S515 are shown in Fig. 2.

Markedly reduced or absent expression of the E-cadherin protein was significantly associated with alterations in the *TP53* gene scoed as mutations alone or as accumulation and/or gene mutations (P <0.001, Table 3). In cases with a normal p53 gene, 86.3% of the samples showed strong (++ or +++) membrane staining. Only 13.7% showed weak or no staining (0 or +). In samples with mutation in the *TP53* gene only 50% showed strong membrane staining (++ or +++).

When E-cadherin protein expression and cerbB-2 overexpression were compared no association was seen. Table 4 shows the relationship between p53 alterations and cerbB-2 protein expression. No significant association was found between mutations in the *TP53* gene and cerbB-2 protein expression. Comparison of p53 protein accumulation and cerbB-2 expression revealed a significant association (P=0.036). When *TP53* gene mutation and p53 protein accumulation were compared no significant association was observed.

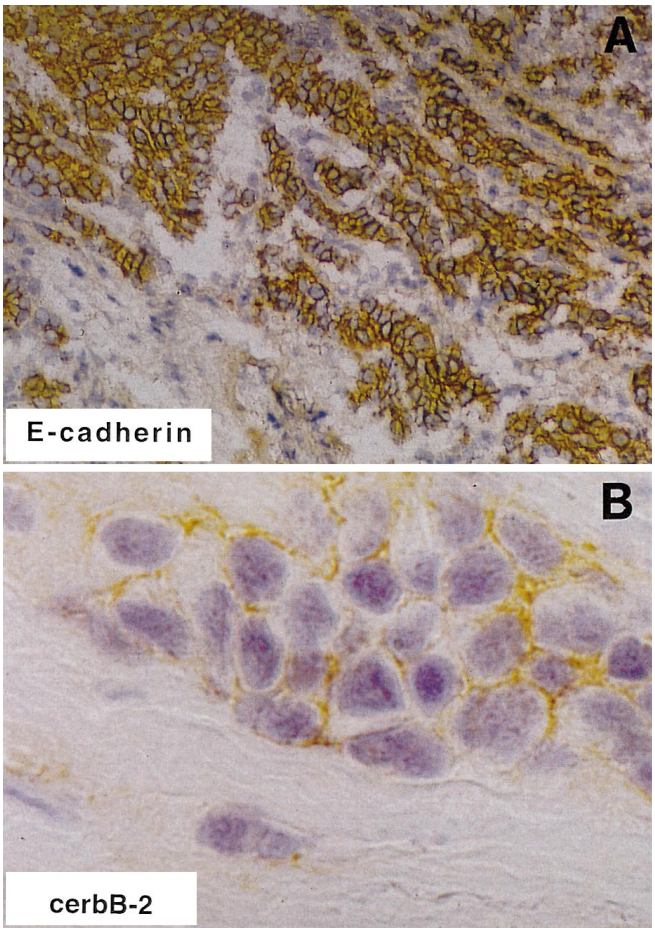

Fig. 1 **A** Tumour cells showing strong immunoreactivity to E-cadherin protein. **B** Tumour cells are stained with antibodies to the cerbB-2 protein. The tumour cells showed strong staining at the cell membrane

Table 1 Expression of E-cadherin protein in different types of breast carcinomas

	E-cadherin expression		
	0/+	++/+++	
Invasive ductal	26(16.3%)	134(83.7%)	
Invasive lobular	9(32.1%)	19(67.9%)	P=0.084

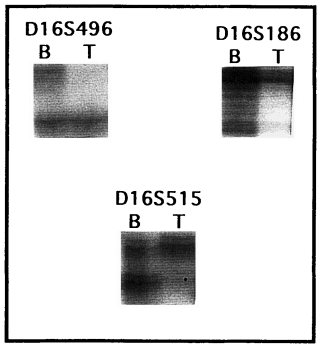

Fig. 2 Examples of microsatellite analyses of breast carcinomas demonstrating loss of heterozygosity. Blood DNA samples are denoted "*B*" and tumour DNA samples, "*T*"

Table 2 Frequency of LOH of different microsatellite markers in samples with reduced/absent E-cadherin protein expression compared to samples with strong protein expression of E-cadherin

		D16S496 LOH		D16S186, 265, 515 LOH	
		Positive	Negative	Positive	Negative
E-cadherin protein expression	0/+	7(50.0%)	7(50.0%)	8(72.7%)	3(27.3%)
	++/+++	3(14.3%)	18(85.7%)	24(66.7%)	12(33.3%)
		P=0.053		Not significant	

Table 3 Relationship between p53 protein and E-cadherin protein expression (*N.S.* not significant)

		E-cadherin protein expression		
		0/+	++/+++	
TP53 gene mutations	Negative	25(13.7%)	159(86.3%)	
	Positive	13(50%)	13(50.0%)	*P* <0.001
p53 protein accumulation	No accumulation	30(16.8%)	149(83.2%)	
	Accumulation	8(25.8%)	23(74.2%)	N.S.
p53 alterations[a]	*p53* normal	19(11.0%)	153(88.9%)	
	p53 abnormal	19(50.0%)	19(50.0%)	*P* <0.001

[a] Scored as protein accumulation and/or gene mutations

Table 4 Relationship between p53 protein and cerbB-2 protein overexpression

		cerbB-2 Protein overexpression		
		0	+	
TP53 gene mutation	Negative	144(82.8%)	30(17.2%)	
	Positive	21(80.8%)	5(19.2%)	N.S.
p53 protein accumulation	No accumulation	144(85.2%)	25(14.8%)	
	Accumulation	21(67.5%)	10(32.3%)	*P*=0.036
p53 protein alterations[a]	p53 normal	136(84.0%)	26(16.0%)	
	p53 abnormal	29(70.0%)	9(30.0%)	N.S.

[a] Scored as protein accumulation and/or gene mutations

Discussion

The chromosome region 16q22.1 containing the E-cadherin gene is frequently affected by LOH in prostatic and breast carcinomas [4, 15, 21], and somatic mutations in the E-cadherin gene have been described [4, 21]. The strong association between p53 abnormalities and down-regulation of the E-cadherin protein in tumour tissue from patients with breast cancer in this study is a novel finding. The LOH studies showed a significantly higher frequency of allelic imbalance in the S16D496 marker, located closest to the E-cadherin gene, in tumours with down-regulation of E-cadherin expression. This indicates that LOH may have a role in down-regulation of the E-cadherin protein, although relatively few samples from this cohort were available for the LOH investigations. Mutations of the *TP53* gene may result in a high frequency of LOH at the E-cadherin locus as a result of genomic instability, and hence unmask a recessive mutation in the E-cadherin gene in the remaining allele. Both LOH alone and/or with an inactivting mutation on the other allele will cause a reduced or absent protein expression. LOH at other sites as a result of p53 protein alterations has been reported previously in breast carcinomas. Eyofjord et al. [10] found a significant association between p53 protein alterations and allelic loss on chromosome 17.

However, no LOH was detected in three tumours with absent E-cadherin protein. This may indicate that mechanisms other than LOH also play an important part in regulation of the E-cadherin protein expression (at the transcription, translation or post-translation level).

When the present material was subdivided into different histological types, a higher frequency of reduced E-cadherin protein expression were found in invasive lobular carcinomas than in invasive ductal carcinomas. This may be caused by a higher frequency of mutations in the remaining allele of E-cadherin gene in invasive lobular carcinomas than in invasive ductal carcinomas, as reported by Berx et al. [5].

To investigate the role of p53 in amplification, we evaluated the status of cerbB-2 protein expression in the same material. A significant association was seen between p53 protein accumulation and cerbB-2 protein immunoreactivity. A stronger and more widely distributed cerbB-2 protein immunoreactivity was seen in grade 3 tumours than in grade 1 and 2 tumours, although this difference was not significant. The same distribution was seen on comparison of p53 protein accumulation and the grade of the tumours [6]. Overexpression of the cerbB-2 protein seems to follow p53 protein accumulation more than *TP53* gene mutations.

Our results indicate that the p53 protein has a role in E-cadherin protein expression in breast carcinomas. LOH, as a result of p53 alterations and genomic instability, is probably one important mechanism. However, down-regulation of E-cadherin protein expression also occurs in tumours without detectable p53 alteration and LOH in the E-cadherin locus, indicating that other mechanisms than LOH also regulate E-cadherin protein expression.

Acknowledgements This study was supported by The Norwegian Cancer Society. Excellent technical assistance from Ms. Ellen Hellesylt is gratefully acknowledged.

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