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# Molecular characterization of intraductal breast carcinomas

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**Abstract** In situ duct carcinoma (DCIS) is a heterogeneous group of lesions which has recently been subdivided into three types: well-differentiated (type I), intermediately differentiated (type II) and poorly differentiated (type III) DCIS. Fourteen cases of DCIS and 11 of DCIS with minimal invasion were analysed for mRNA levels of *β-actin, EGFR, c-cerbB2, MTS1, k-ras, RB, BRCA1, cyclin E,* and *c-myc* genes. A microdissection technique was used on paraffin-embedded tissue. A statistically significantly higher expression of *cyclin E* oncogene and *MTS1* tumor suppressor gene was seen in type III DCIS than in the other types, while no significant differences in the mRNA expression patterns of the other genes were observed. These data are consistent with the fact that poorly differentiated DCIS is a readily recognizable class of tumours that have a particularly aggressive behaviour and probably unique histogenesis.

**Key words** Breast intraductal carcinoma · EGF receptor · c-erbB2 · Cyclin E · c-myc

## Introduction

In situ ductal carcinoma (DCIS) of the breast is an increasingly important clinical problem, as a consequence of widespread mammagraphic screening. Several classification systems for DCIS are in use. Recently a group of European pathologists has proposed a new classification based on cytonuclear differentiation. According to this, cases are grouped into well-differentiated (type I) and poorly differentiated (type III) cases and cases with an intermediate (type II) differentiation [7]. These types appear to differ in several ways. It has been shown that psammomatous calcifications [7], which precipitate on acidic proteins [5], are seen mostly in type I DCIS, while granular calcifications [6], in which calcium salts deposit on DNA fragments [5], are almost unique to type III DCIS [1, 17]. A high proliferative index [1] and positivity with p53 [1] and c-erbB2 [3] proteins are mostly seen in poorly differentiated DCIS. Oestrogen and progesterone receptors [1] and Bc1-2 protein expression [8] are seen in nearly all cases of well-differentiated DCIS. In contrast, progesterone receptors [1] are present in no fewer than 12% of poorly differentiated DCIS. BC1-2 is expressed in only 46% of poorly differentiated cases, which, however, express Bax protein more frequently [8]. It appears, then, that there are two distinctly different groups (types I and III), while type II is ill defined, embracing cases that share characters of well or poorly differentiated DCIS at different times. In an attempt to achieve better definition of the intermediate (type II) group, we studied a series of 25 cases of DCIS (14 cases) and DCIS with invasion, to test the expression of some of the genes involved in the oncogenesis at mRNA level. The genes analysed in paraffin-embedded tissues from breast biopsies were the oncogenes *EGFr, c-erbB2,* cyclin E (*CE*), *c-myc*, and *k-ras*, the tumour suppressor genes *MTS1* (p16) and BRCA1, and the retinoblastoma (*RB*) gene.

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## Materials and methods

Paraffin blocks (25, from 25 patients) were obtained from the Section of Anatomic and Cytopathology "Marcello Malpighi" of the University of Bologna at Bellaria Hospital. All 25 cases had DCIS as a major component. These were classified according to Holland et al. [7] into 8 well-differentiated cases (Fig. 1), 7 intermediately differentiated cases (Fig. 2) and 10 poorly differentiated cases (Fig. 3). In 11 of the 25 cases there was an invasive component, which was the minor part of the lesion and was graded according to Ellis and Elston as seen in Table 1. Comedo-like necrosis in in

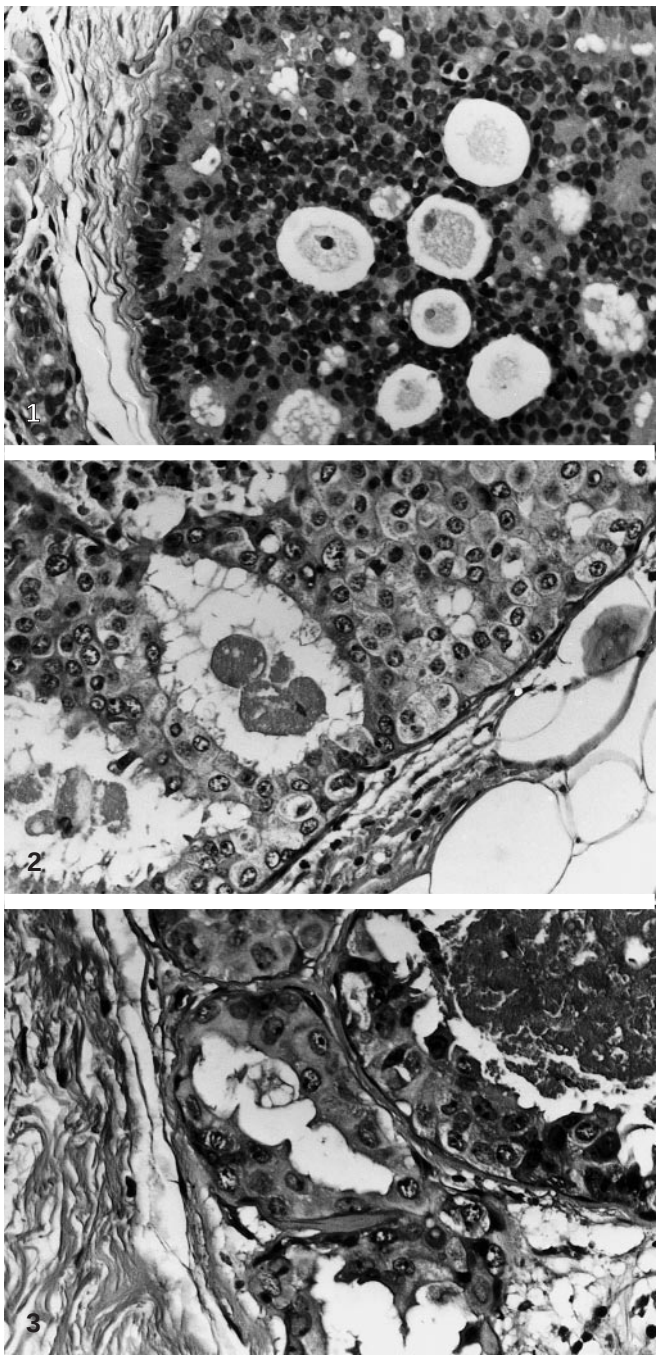

**Fig. 1** Well-differentiated (type I) in situ duct carcinoma. Nuclei are monotonous and cell polarized. H&E, ×175

**Fig. 2** Intermediately differentiated (type II) in situ duct carcinoma. Nuclei are irregular and show an evident nucleolus. A distinct cell polarization is distinctly visible. H&E, ×175

**Fig. 3** Poorly differentiated (type III) in situ duct carcinoma. Nuclei are markedly irregular, and nucleoli are prominent. No cell polarization is seen. H&E, ×200

situ lesions was also taken in account, as it was present in 11 patients (10 cases of type III DCIS and 1 case of type II DCIS).

Ten 6-μm sections were obtained from each formalin-fixed paraffin-embedded block. From these, areas corresponding to the DCIS component were microdissected [11]. Accordingly, areas with the highest nuclear grade were dissected when the case appeared cytologically hetereogeneous. In cases where microinvasion was present, care was taken to dissect cells from areas located away from the invasive part. All specimens were analysed for mRNA level of *β-actin, EGFr, c-erbB2, MTS1* (11 cases), *k-ras, RB, BRCA1, CE*, and *c-myc*.

Total RNA was extracted from paraffin-embedded tissues as previously described [13, 14]. For every gene, three oligonucleotides were synthesized, two of which were mRNA sense and one, antisense. For all the sequences studied the first sense and the antisense oligonucleotide were in two successive exons of the gene and they were used for amplification of the sequence. The second sense oligonucleotide spanned the included intron and was used as a probe for the amplified product. The segments of mRNA studied were very short, between 75 and 100 bases. The oligonucleotide sequences are reported in Table 2.

After spectrophotometric quantitation, all mRNA specimens were diluted to the same concentration, so that the same quantity of target RNA was used for each sample (400 ng of total RNA). The RNAs were treated with 2.5 U of AMV reverse transcriptase (Promega) in 10 μl final volume containing Tris-HCl pH 8.3 (42°

**Table 1** DCIS with microinvasion (*DCIS* duct carcinoma in situ, *DCI* duct carcinoma invasive, *N.V.* not evaluable, very small DCI, *TUBUL.* tubular carcinoma)

| Case | DCIS type | DCI grade |
|---|---|---|
| 1 | 2 | 2 |
| 2 | 2 | 1 |
| 3 | 3 | 2 |
| 4 | 3 | 2 |
| 5 | 3 | N.V. |
| 6 | 3 | 3 |
| 7 | 2 | N.V. |
| 8 | 3 | 3 |
| 9 | 3 | 3 |
| 10 | 1 | TUBUL. |
| 11 | 3 | 2 |

**Table 2** Oligonucleotide sequences

| Name | Type | Sequence |
|---|---|---|
| β-Actin 1 | Sense | aaggccaaccgcgagaagatga |
| β-Actin 2 | Probe | cccagatcatgtttgagaccttcaacaccc |
| β-Actin 3 | Antisense | tggatagcaacgtacatggctg |
| *MTS1-1* | Sense | atgtgccacggtacctgc |
| *MTS1-2* | Probe | agaggcagtaaccatgcccgcatagat |
| *MTS1-3* | Antisense | ttcaatcggggatgtctga |
| *EGFr1* | Sense | ggctctggaggaaaagaaag |
| *EGFr2* | Probe | tttgccaaggcacgagtaaca |
| *EGFr3* | Antisense | tcaaaagtgcccaactgcgt |
| *e-erbB2* | Sense | tgtctgaattctcccgca |
| *c-erbB2* | Probe | agcgctttgtggtcatccaga |
| *c-erbB2* | Antisense | aaggtgctgtccaagggactg |
| *CE1* | Sense | gtgctcacccggcccggt |
| *CE2* | Probe | acccgggtccacagggatgcgaagga |
| *CE3* | Antisense | gccgccgtcctccttcat |
| *BRCA1* | Sense | ctgtctggagttgatcaagg |
| *BRCA1* | Probe | gcaaattttgcatgctgaaacttctc |
| *BRCA1* | Antisense | aggccctttcttctggtt |
| *c-myc1* | Sense | ttctctgaaaggctctcc |
| *c-myc2* | Probe | cgctggatttttttcgggatgcccctcaa |
| *c-myc3* | Antisense | tcgaggtcatagttcctg |
| *k-ras1* | Sense | gagggctttctttgtgta |
| *k-ras2* | Probe | gaagatattcaccattatagagaacaa |
| *k-ras3* | Antisense | cttcagagtccttaactctt |
| *RB1* | Sense | ctcacctcccatgttgctca |
| *RB2* | Probe | aagaaccatataaaacagctgttataccc |
| *RB3* | Antisense | ctatccgtgcactcctgttc |

C) 50 mM, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 1 mM dNTPs and 15 pmol of downstream antisense primer. Reaction was proceeded at 42° C for 60 min. The amplification was performed by adding 40 µl of master mix containing 10 mM of Tris-HCl pH 8.3, 55 mM of KCl, 15 pmol of upstream sense primer and 1.2 U of AmpliTaq Polimerase (Perkin-Elmer). After a denaturation step of 3 min at 95° C, 5 cycles of 95° C/1 min, 55° C/1 min, 72° C/1 min and 35 cycles of 95° C/30 s, 55° C/30 s, 72° C/30 s were performed in a Tween block apparatus with heated cover system (Ericomp).

The amplification fragments were tested by dot blot hybridization using as probe the internal oligonucleotide, labelled with $^{32}$P-ATP (Amersham) using T4-Polinucleotide Kinase (BioLabs). Hybridization was performed in SSC 6X, 0.25% powdered milk at 50° C for all the probes but *MTS1*, *k-ras* and *c-myc*, which were hybridized at 55° C. The single spots were cut from the membranes and counted in a β-counter (Backman).

For the relative quantitation of any mRNA the linearity conditions between the log of the target RNA and the log of the amplification products were analysed as reported elsewhere [15]. For all the sequences studied, 400 ng of total RNA with 40 cycles of amplification were in the range of linearity condition. The results were standardized for the level of RNA degradation for each sample on the β-actin quantity (only for β-actin were the linearity conditions reached, with 10 ng of total RNA for each sample).

A competitive quantitative analysis is not available for RNA in paraffin-embedded tissues, because of the different level of degradation among different tissue samples. Negative controls with parallel extraction and RT-PCR out of paraffin block without tissues were carried out.

For statistical analysis Student's test was performed.

## Results

Correlations were sought between levels of expression of the tested genes and the three types of DCIS. The variation among different groups was especially evident for *MTS1* and *CE*, but also for the *EGFr* and *RB* genes. The expression of *CE* was high only for type III DCIS ($P<0.001$; Fig. 4). The tumour suppressor *MTS1* was highly expressed only in type III DCIS, and the level of expression was higher in all the type III cases than in the other two types ($P<0.01$; Fig. 5). The *EGFr* and *RB* genes were on average more highly expressed in type III than in types I and II, but without a clear-cut difference (Figs. 6, 7). For *EGFr* the difference was at the limit of statistical significance ($P<0.05$), and for *RB* the difference was not significant.

Variation of expression was not found for the oncogenes *k-ras*, *c-myc* and *c-erbB2* or for the tumour suppressor gene *BRCA1*. The level of expression of *k-ras* was low in all groups, but in 2 cases of type III DCIS the level of expression was 5 times that in the other cases.

The relationship between the tested genes and the presence of intraductal necrosis in tumours was also studied. A positive correlation with necrosis was statistically not significant for the *RB* gene (Fig. 8), but was more evident for the *CE* gene ($P<0.001$; Fig. 9).

We also checked for correlations between gene expression and associated stromal invasion. On average, the *RB* and *c-erbB2* genes were expressed more strongly, in the cases associated with stromal invasion, but this correlation was not statistically significant (Figs. 10, 11). Stromal invasion was also related to low *EGFr* gene expression (Fig. 12), but this difference was not statistically significant.

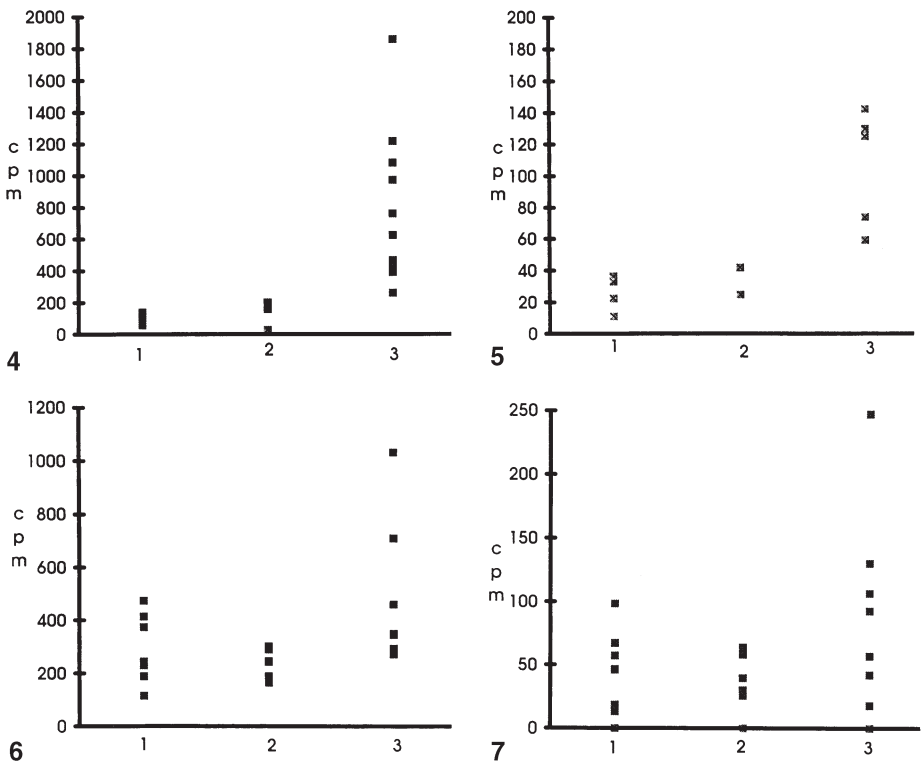


**Fig. 4** Relative standardized quantitation of cyclin E mRNA in different types of DCIS (*1, 2, 3*)

**Fig. 5** Relative standardized quantitation of MTS1 mRNA in different type of DCIS (*1, 2, 3*)

**Fig. 6** Relative standardized quantitation of EGFr mRNA in different type of DCIS (*1, 2, 3*)

**Fig. 7** Relative standardized quantitation of RB mRNA in different type of DCIS (*1, 2, 3*)

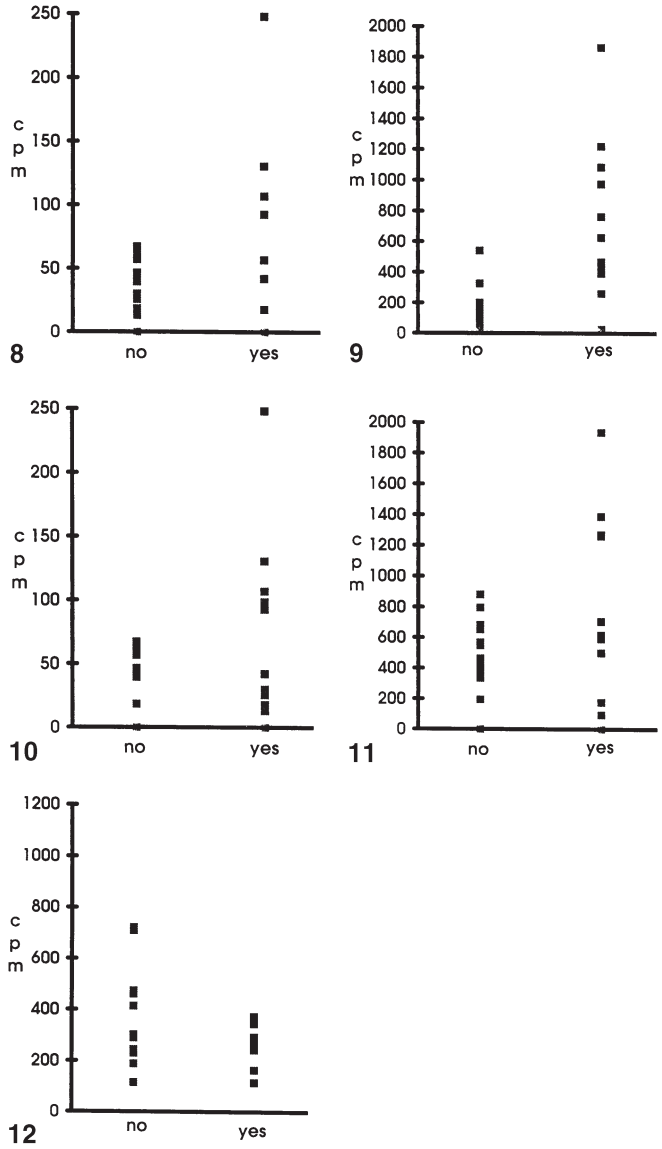


**Fig. 8** Relative standardized quantitation of RB mRNA in breast in situ duct carcinomas with (*yes*) or without (*no*) intraductal necrosis

**Fig. 9** Relative standardized quantitation of cyclin E mRNA in breast in situ duct carcinomas with (*yes*) or without (*no*) intraductal necrosis

**Fig. 10** Relative standardized quantitation of RB mRNA in breast in situ duct carcinomas with (*yes*) or without (*no*) stromal invasion

**Fig. 11** Relative standardized quantitation of c-erbB2 mRNA in breast in situ duct carcinomas with (*yes*) or without (*no*) stromal invasion

**Fig. 12** Relative standardized quantitation of EGFr mRNA in breast in situ duct carcinomas with (*yes*) or without (*no*) stromal invasion

## Discussion

The aim of this study was to characterize the different DCIS types, as defined by Holland et al. [7], from a molecular point of view. Accordingly, we microdissected the tissues from breast biopsies containing DCIS of various types and studied the mRNA levels of several genes.

The *MTS1* gene was more highly expressed in all cases of type III examined than in cases of the better differentiated DCIS. *CE* seemed to characterize the less well differentiated group. In these cases *CE* always had a higher level of expression than in type I and II DCIS; it has been reported for invasive breast cancer that *CE* is overexpressed in high stages and grades of tumours [9]. It seems that the same phenomenon is seen in intraductal carcinomas. In our cases *CE* overexpression was found only in poorly differentiated DCIS. CE was also positively correlated with intraductal necrosis, which was present mostly in type III DCIS and was related to an increased expression of *RB*. This was also associated with stromal invasion, mostly in poorly differentiated tumours. Therefore, it seems that *RB* overexpression in these cases is connected with more actively proliferating DCIS and might represent a late phase of tumour progression in the same lesions.

A surprising result was found for the cell membrane proteins, EGFr and c-erbB2. EGFr appeared to be highly expressed in type III DCIS, but had a low level of expression in those cases in which stromal invasion was present. In the same cases a high expression of *c-erbB2* was also present. This type of inversion between *EGFr* and *c-erbB2* had already been shown at protein level [12]. It is possible that these two oncogenes, when studied in combination, might predict which DCIS is more prone to invade the breast stroma.

The oncogene *k-ras* expression was increased only in 2 poorly differentiated DCIS, and in both cases the expression of *c-erbB2* was also very high. Expression of *k-ras* might be strictly related to the activation of the homonymous signaling pathway [2], and in this respect might be connected to the signal transduction activated by *c-erbB2* overexpression.

No difference was seen in the expression of *BRCA1* and *c-myc*, in keeping with the fact that the level of *c-myc* RNA expression has been reported to be normal in all the breast cancer cell lines studied [18].

Therefore, it seems that type III DCIS differs from the other types of DCIS (I and II) in having statistically significantly higher *EC* and *MTS1* expressions. These data are consistent with the fact that poorly differentiated DCIS are a class of tumours that deserve recognition as having more aggressive behaviour than the other types [4, 10]. Types I and II are linked by similar molecular patterns, which probably indicate a common histogenesis, in contrast to type III DCIS; this is genetically different. Consequently the proposal of three classes of DCIS [7] appears to be consistent with the present data. Type II DCIS, which is genetically related to well-differentiated DCIS, is probably the result of a dedifferentiation phenomenon. In spite of the genetic link, type II DCIS differs from well-differentiated forms, as its biological behaviour is far more aggressive [10]. Therefore, it seems that the subdivision of DCIS into three classes is supported by both genetic and clinical data.

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