In breast carcinoma dysadherin expression is correlated with invasiveness but not with E-cadherin

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Recent advances into molecular pathology of breast cancer have refined diagnostic accuracy and classification systems of the most common malignant neoplasm of women, rendering personalised therapy more possible. Today, there is a plethora of molecular genetic data that indicate differences in pathogenesis between the various types of breast carcinomas and thus support their categorisation, to the patient benefit. The demonstration of lack of E-cadherin expression in lobular neoplasms has had a sound impact with practical applications (Mastracci et al, 2005) In about half of lobular carcinomas, loss of E-cadherin involves genetic changes, that is loss of heterozygosity (LOH) at 16q22.1, while in the other half epigenetic events are involved (Knudsen and Wheelock, 2005; Mastracci et al, 2005). Ductal carcinomas, on the other hand, express E-cadherin, albeit in reduced levels and/or in abnormal cellular locations (Knudsen and Wheelock, 2005). Reduction/loss of E-cadherin has been associated with the development and progression of many epithelial neoplasms. Aberrant E-cadherin expression (heterogeneous, cytoplasmic, or absent) has been detected immunohistochemically in several cancers, including head and neck carcinoma, gastric adenocarcinoma, lobular breast carcinoma, lung cancer, colorectal carcinoma, prostate adenocarcinoma, pancreatic, and bladder cancer (Becker et al, 1994; Hirohashi, 1998; Chang et al, 2002; Charalabopoulos et al, 2002, 2004; Hirohashi and Kanaï, 2003; Mastracci et al, 2005; Massarelli et al, 2005). In the vast majority of these neoplasms such expression has been associated with poor differentiation, increased metastatic potential and poor prognosis. In breast the scenario is more complicated, since lobular carcinoma, that typically does not express E-cadherin has a more favourable outcome than ductal carcinoma, which in general expresses E-cadherin. Furthermore, there are contradictory data on the possible association between E-cadherin expression and high-grade tumours with increased metastatic potential (Oka et al, 1993; Charpin et al, 1997; Heimann et al, 2000; Gillett et al, 2001; Parker et al, 2001; Elzagheid et al, 2002; Gupta et al, 2003; Knudsen and Wheelock, 2005; Rakha et al, 2005). It is clear that there are pieces missing from the puzzle of adhesion molecules and breast carcinoma.

Recently, the cloning and characterisation of dysadherin (FXYD5), a cell membrane glycoprotein that has an anti-cell–cell adhesion function and downregulates E-cadherin in a post-transcriptional manner has been reported, by members of our research team. This novel cancer-associated protein has been detected in head and neck, tongue, oesophageal, gastric, colorectal,
testicular, pancreatic, thyroid, and cervical carcinomas as well as in malignant melanoma and has been associated with tumour aggressiveness (Ino et al, 2002; Tsuji et al, 2002; Aoki et al, 2003; Sato et al, 2003; Shimamura et al, 2003, 2004; Nakaniishi et al, 2004; Shimada et al., 2004a,b; Wu et al, 2004; Batistatou et al, 2005; Nishizawa et al, 2005; Batistatou et al, 2006; Kyzas et al, 2006). A recent in vitro study has demonstrated that dysadherin has prometastatic effects that are independent of E-cadherin expression (Nam et al, 2006). The aim of the present study was to investigate further the expression of dysadherin in breast carcinoma, with particular emphasis to the acquisition of a lobular or a ductal phenotype, in combination with E-cadherin expression.

MATERIALS AND METHODS

One hundred formalin-fixed, paraffin-embedded archival tissue blocks of breast carcinomas were included in the current study and represented an equal number of female patients (mean age 54.5 years, range 35 – 79). The material consisted of 70 invasive ductal carcinomas, no special type, NST (10 Grade 1, 45 Grade 2 and 15 Grade 3, graded using the modified Bloom and Richardson method), in 30 of which an adjacent in situ ductal carcinoma was identified, and 30 invasive lobular carcinomas, in 15 of which an adjacent in situ lobular carcinoma was identified.

Immunohistochemistry

We performed immunostaining on formalin-fixed, paraffin-embedded tissue sections using the EnVision System (DAKO Corp., Netherlands), and the monoclonal antibodies: NCC-M53 against dysadherin and E-cadherin (CM170B, Biocare Medical, CA, USA). Briefly, 4-μm-thick tissue sections were deparaffinised in xylene; rehydrated through graded concentrations of alcohol and heated in a microwave oven for two cycles of 15 min each at 300 W, in citrate buffer, for antigen retrieval. Endogenous peroxidase activity was blocked with H2O2 solution in methanol (0.01 M), followed by incubation (30 min at room temperature and overnight at 4°C respectively). Then the slides were washed for 10 min with PBS and were visualised with the EnVision system using diaminobezidine tetrahydrochloride as a chromogen. Finally, all sections were counterstained with haematoxilin. Positive staining of endothelial cells and lymphocytes was used as an internal positive control for dysadherin. As an internal positive control for E-cadherin, positive staining of non-neoplastic ducts and acini, as well as in endothelial cells of vessels and lymphocytes, as described previously (Batistatou et al, 2005, 2006). Dysadherin immunostaining was observed in the membranes of the neoplastic cells and it was heterogeneous throughout the neoplasm (Figure 1C). In particular, preferential expression in diffuse than in compact infiltrative areas was detected. Overall, ‘positive dysadherin expression’ was found in six out of 10 (60%) Grade 1, 34 out of 45 (75.5%) Grade 2 and all 15 (100%) Grade 3 neoplasms (Table 1, Figure 1B). Interestingly, in the adjacent in situ ductal carcinoma a small proportion of neoplastic cells (<10%) exhibited membranous immunostaining for dysadherin (Figure 1D). Dysadherin expression was not correlated with E-cadherin expression in IDC (P>0.05).

Lobular carcinoma

None of the 30 infiltrating lobular carcinomas showed preserved E-cadherin expression (Table 1, Figure 2A). The vast majority

| Histologic type | Preserved E-cadherin expression | ‘Positive’ dysadherin expression |
|-----------------|---------------------------------|-------------------------------|
| Invasive ductal carcinoma |                               |                               |
| Grade 1 (10)    | 10 (100 %)                      | 6 (60%)                       |
| Grade 2 (45)    | 37 (82.2%)                      | 34 (75.5%)                    |
| Grade 3 (15)    | 6 (40%)                         | 15 (100%)                     |
| Total (70)      | 53 (75.7%)                      | 55 (78.6%)                    |
| Invasive lobular carcinoma |                       |                               |
| Total (30)      | 0 (0%)                          | 30 (100%)                     |

immunostaining was considered as aberrant expression and was not included in the immunopositive cases.

Statistical analysis

Analyses were conducted in SPSS software version 11.0 (SPSS, Inc, Chicago, IL, USA). For comparisons between antibodies’ expression with clinicopathological variables we used the χ2 test. The level of statistical significance was P<0.05.

RESULTS

Ductal carcinoma

Membranous E-cadherin expression was detected in epithelial cells of non-neoplastic ducts and acini and this served as internal positive control. In neoplastic cells there was some variation in distribution, depending on the grade and the pattern of stroma infiltration. Specifically, all 10 (100%) Grade 1, 37 out of 45 (82.2%) Grade 2 and six out of 15 (40%) Grade 3 neoplasms showed preserved E-cadherin expression (Table 1, Figure 1A). In immunopositive Grade 2 and Grade 3 tumours the expression of E-cadherin was more heterogeneous, with variations in intensity and distribution of positive cells. Thus, cells in clusters or in tubular structures exhibited higher percentage and more intense membranous staining than individual cells infiltrating the stroma. In the periphery of the invasive ductal carcinoma an intraductal component was observed in several cases. In this in situ ductal component the expression of E-cadherin was similar to the non-neoplastic epithelial cells, homogeneous and stronger than the adjacent invasive component (Figure 1B).

Dysadherin expression was detected in myoepithelial cells of ducts and acini, but not in non-neoplastic epithelial cells, as well as in endothelial cells of vessels and lymphocytes, as described previously (Batistatou et al, 2005, 2006). Dysadherin immunostaining was observed in the membranes of the neoplastic cells and it was heterogeneous throughout the neoplasm (Figure 1C). In particular, preferential expression in diffuse than in compact infiltrative areas was detected. Overall, ‘positive dysadherin expression’ was found in six out of 10 (60%) Grade 1, 34 out of 45 (75.5%) Grade 2 and all 15 (100%) Grade 3 neoplasms (Table 1, Figure 1B). Interestingly, in the adjacent in situ ductal carcinoma a small proportion of neoplastic cells (<10%) exhibited membranous immunostaining for dysadherin (Figure 1D). Dysadherin expression was not correlated with E-cadherin expression in IDC (P>0.05).

Lobular carcinoma

None of the 30 infiltrating lobular carcinomas showed preserved E-cadherin expression (Table 1, Figure 2A). The vast majority
was completely negative, while only in two of them, <20% of neoplastic cells showed weak membranous and cytoplasmic immunopositivity. Interestingly, the adjacent in situ lobular carcinoma was completely negative, as well (Figure 2B).

All the 30 infiltrating lobular carcinomas exhibited ‘positive dysadherin expression’ (Figure 2C). In this in situ lobular component the expression of dysadherin was limited to a small proportion (<10%) of neoplastic cells (Figure 2D).

**DISCUSSION**

Two of the most important characteristics of neoplastic cells are their abilities to grow locally and to metastasise. For both of these processes tumour cells must initially dissociate from each other, either singly or in small nests and invade the surrounding stroma. Today it is generally accepted that at least for carcinomas, adhesion molecules, in particular E-cadherin,
play a pivotal role in this process by being downregulated (Hirohashi, 1998; Charalabopoulos et al., 2002; Hirohashi and Kanai, 2003). In general, there is an association between aberrant E-cadherin expression, tumour dedifferentiation and poor clinical outcome.

Regarding breast cancer, E-cadherin expression varies depending on the histological subtype. Thus, in ductal carcinoma E-cadherin is expressed, albeit in reduced levels and aberrant cellular locations. Although E-cadherin correlates inversely with the grade of the tumour, reduced E-cadherin expression is not adequate to predict clinical outcome and there are contradictory studies on the association between E-cadherin and survival (Knudsen and Wheelock, 2005). Moreover, it has been reported that in other breast cancers with known poor prognosis, such as inflammatory breast cancer, there is overexpression of E-cadherin (Knudsen and Wheelock, 2005). An interesting concept is that possibly the reduction of E-cadherin expression in breast carcinomas, other than lobular, is transient, due to epigenetic modifications. Several mechanisms for reversible reduction of E-cadherin expression in human neoplasms have been reported (Hirohashi, 1998; Charalabopoulos et al., 2002; Hirohashi and Kanai, 2003). Among them, recently, a novel cell membrane glycoprotein named 'dysadherin' (from the Greek prothema dys-, which means difficulty, or aberration, or reversibility) has been found to downregulate E-cadherin in a post-transcriptional manner and reduce cell–cell adhesiveness in vitro studies and in animal models. Dysadherin is a member of the FXYD family (FXYD5 or Related to Ion Channel). It is located at chromosome 19 and has a single transmembrane domain. It interacts with and modulates the properties of the Na⁺, K⁺ ATPase (Ino et al., 2002; Lubarski et al., 2005). In human tissues increased dysadherin expression has been correlated with the development of metastasis and poor prognosis in gastric, pancreatic, colorectal, oesophageal, thyroid, tongue and cervical carcinomas, as well as in malignant melanoma (Aoki et al., 2003; Sato et al., 2003; Shimamura et al., 2003, 2004; Nakaniishi et al., 2004; Shimada et al., 2004a, b; Wu et al., 2004; Batistatou et al., 2005, 2006; Nishizawa et al., 2005; Kyzas et al., 2006). Furthermore, in a small pilot series of breast cancer patients, dysadherin expression was correlated with poor prognosis (Ino et al., 2002). In most of these neoplasms, as well as in testicular tumours and lymph node metastases of colorectal adenocarcinoma, increased dysadherin expression was correlated with reduced E-cadherin expression (Aoki et al., 2003; Sato et al., 2003; Shimamura et al., 2003, 2004; Wu et al., 2004; Batistatou et al., 2005, 2006). In invasive ductal carcinoma, as reported in this study, there is an increase in dysadherin expression, which is not related to E-cadherin expression. This lack of association has also been reported in pancreatic, primary colorectal and gastric carcinomas (Shimamura et al., 2003; Shimada et al., 2004b; Batistatou et al., 2005). Furthermore, in the in situ ductal carcinoma dysadherin was not expressed. On the basis of these data we would like to propose that in ductal carcinomas, dysadherin can promote invasion independently of the E-cadherin expression.

Lobular breast carcinomas, typically exhibit loss of E-cadherin expression, but they tend to have a more favourable clinical outcome than the more common ductal carcinomas. This loss is an early event affecting not only lobular carcinoma in situ but even atypical lobular neoplasia (Mastracci et al., 2005). This silencing of E-cadherin is attributed to genetic as well as epigenetic events (Knudsen and Wheelock, 2005; Mastracci et al., 2005). In approximately 50% of lobular carcinomas loss of E-cadherin involves LOH at the chromosomal region of 16q, which includes the E-cadherin gene CDH1 locus and mutations in the remaining allele (Kanai et al., 1994; Vos et al., 1997; Huiping et al., 1999; Knudsen and Wheelock, 2005; Mastracci et al., 2005). This LOH definitely accompanies mutations in cases of invasive lobular carcinoma, however, the classic pattern of LOH coupled with inactivating mutations in lobular carcinoma in situ has not been confirmed. In the other 50% loss of E-cadherin is attributed to epigenetic events, with hypermethylation of the E-cadherin promoter region at CpG islands being one of the most important ones and possibly occurring very early, even at the stage of atypical lobular hyperplasia (Sarrio et al., 2004; Shibata et al., 2004; Knudsen and Wheelock, 2005; Mastracci et al., 2005).

In this study we have confirmed the loss of E-cadherin expression, in situ and invasive breast carcinoma. On the basis of the rare expression of dysadherin in lobular carcinoma in situ we can conclude that dysadherin is not responsible for E-cadherin downregulation in lobular carcinoma. An interesting finding from our study is the difference in dysadherin expression between in situ and invasive lobular carcinoma. In breast carcinoma the progression from in situ to invasive disease is not clearly defined and the specific events that mark the transition to an invasive tumour are under intense investigation. Lack of E-cadherin expression cannot be associated with an invasive phenotype, since it is also evident in the in situ component. On the other hand, we have shown that dysadherin is specifically and constantly expressed in invasive lobular carcinomas. On the basis of this finding we propose that dysadherin is a possible causative player in the process of acquiring an invasive phenotype, as well as a possible marker for invasiveness. It has also been proposed that loss of E-cadherin expression is responsible for the distinct pattern of invasion observed in lobular neoplasms (Knudsen and Wheelock, 2005; Mastracci et al., 2005). We would like to add that another major contributor to this characteristic invasion pattern, with single cells arranged in cords is the expression of dysadherin. The latter possibly acts, either alone or in conjunction with loss of E-cadherin, by allowing cells to dissociate from each other. Studies on the function of dysadherin are available by experimental data, where dysadherin appears to play an important role in neoplastic cell invasion and metastasis (Ino et al., 2002; Nam et al., 2006). The exact molecular mechanisms of these effects have not been elucidated yet. Recently, it has been shown that, besides downregulating E-cadherin, dysadherin can promote invasion at least in breast cancer cells in vitro, through an E-cadherin-independent mechanism. This mechanism involves enhanced signaling through the NF-κB pathway, which leads to increased production of the tumour-promoting (C-C motif) ligand 2 (CCL2) (Nam et al., 2006).

In conclusion, in this study we have investigated the role of specific adhesion/dysadhesion molecules in the development of breast carcinoma. We have selected ductal carcinoma which is by far the most common type, and lobular carcinoma which has a distinctive microscopic appearance. We have shown similarities and differences between these two types. Interestingly, in ductal as well as in lobular carcinoma, dysadherin was expressed only in the invasive and not in the in situ component, and this expression was independent of E-cadherin. Thus, dysadherin may play an important role in breast cancer progression by promoting invasion and, particularly in lobular carcinomas, it might also be used as a marker of invasion.

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