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SHORT REPORTS

Correlation of Snail expression with histological grade and lymph node status in breast carcinomas

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Snail is a zinc finger transcription factor that triggers the epithelial-mesenchymal transition (EMT) by directly repressing E-cadherin expression. Snail is required for mesoderm and neural crest formation during embryonic development and has recently been implicated in the EMT associated with tumour progression. In a series of human breast carcinomas, we have analysed the expression of Snail and that of molecules of the E-cadherin/catenin complexes. We have also correlated these data with the pathological features of the tumours. We show that Snail expression inversely correlates with the grade of differentiation of the tumours and that it is expressed in all the infiltrating ductal carcinomas (IDC) presenting lymph node metastases that were analysed. In addition, Snail is expressed in some dedifferentiated tumours with a negative nodal status. Considering that Snail is involved in the induction of the invasive and migratory phenotype in epithelial cells, these results indicate that it is also involved in the progression of breast ductal tumours, where it could additionally serve as a marker of the metastatic potential.
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The transcription factor *Snail* was first described in *Drosophila*, where defects in the invagination of the presumptive mesoderm and of germ band retraction were seen in mutant embryos (Grau *et al*., 1984). Subsequently, Snail homologues were identified in different vertebrates including humans (Hemavathy *et al*., 2000; Manzanares *et al*., 2001). During embryonic development *Snail* family members have been implicated in the triggering of epithelial-mesenchymal transitions (EMT) in the precursors of the mesoderm and the neural crest, promoting their delamination and subsequent migration from the primitive streak and the neural tube, respectively (Nieto *et al*., 1994; Sefton *et al*., 1998; LaBonne and Bronner-Fraser, 2000; Cano *et al*., 2000; Carver *et al*., 2001). In epithelial cells, the induction of EMT by Snail is mediated by the direct transcriptional repression of the cell adhesion molecule E-cadherin. Indeed, Snail has been shown to repress E-cadherin expression and to trigger EMT associated with epithelial tumour progression (Cano *et al*., 2000; Batlle *et al*., 2000), the first step in the metastatic cascade. Very recently, two additional *E-cadherin* repressors, SIP1 and E12/E47, have also been described (Comijn *et al*., 2001; Pérez-Moreno *et al*., 2001). With respect to *Snail*, apart from the inverse correlation between its expression and that of *E-cadherin*, a direct correlation has been observed with the invasive and metastatic properties of mouse and human tumour cell lines derived from tissues such as skin, breast, colon, oral mucosa and melanoma (Batlle *et al*., 2000; Cano *et al*., 2000; Poser *et al*., 2001; Yokoyama *et al*., 2001). Furthermore, *Snail* is expressed at the invasive front of skin tumours induced by chemical carcinogenesis in the mouse (Cano *et al*., 2000).

The first evidence that *Snail* is expressed in primary human tumours was recently presented by Cheng *et al*. (2001) who analysed the different mechanisms of E-cadherin inactivation in breast ductal carcinomas. They identified a correlation between *Snail* expression and a reduction or the lack of E-cadherin expression in a high number of tumours. However, since this study was carried out by RT–PCR, it was not possible to perform a direct analysis of the *Snail*-expressing cells within the tumour. The high degree of cellular heterogeneity found in breast cancers and the resulting variability in gene expression, makes it necessary to analyse Snail and E-cadherin expression at the cellular level to directly address the relationship between the two genes and to assess their association with clinicopathological features. In addition, RT–PCR studies can give rise to false positive results since samples from patients with breast cancer may contain different proportions of tumour cells in relation to stroma, inflammatory cells and normal tissue and both Snail and its close family member Slug are expressed in

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fibroblasts (Cano *et al*., 2000; Batlle *et al*., 2000). This expression is not surprising considering that during embryonic development these genes have been proposed to act as factors implicated in maintaining the mesenchymal phenotype (Ros *et al*., 1997).

In order to assess the expression of *Snail* and *E-cadherin* at the cellular level, and in the absence of specific anti-Snail antibodies, we have carried out non-radioactive *in situ* hybridization in tumour sections. We have combined these data with histological, immunohistological and clinicopathological analyses of tumours obtained from 21 patients aged from 35 to 85 and not subjected to chemo- or radiotherapy. Seventeen of them corresponded to infiltrating ductal carcinomas (IDC) and four infiltrating lobular carcinoma (ILC).

E-cadherin mRNA was detected in normal epithelial cells (Figure 1c), in all IDCs (17 out of 17, Table 1; Figure 1f,i) and in two of the four ILCs (Table 1; Figure 2c). This is in agreement with several immunohistochemical studies that have reported E-cadherin expression in normal epithelial cells and ductal carcinomas, but low or non-detectable expression in ILCs. Conversely, *Snail* mRNA was not detected in normal breast epithelium (Figure 1b) but it was expressed in a population of stromal fibroblasts (Figure 2e), confirming the caveats inherent to the analysis of *Snail* expression exclusively by RT–PCR. In the tumors, *Snail* was expressed in eight of 17 IDCs (47%; Table 1). Two examples of the IDCs expressing *Snail* are shown in Figure 1d–i, where it can be observed that the areas of *Snail* expression (Figure 1e,h) correlate with a dedifferentiated phenotype (Figure 1d,g) and loss of *E-cadherin* transcripts (Figure 1f,i). This is in keeping with previous results where Snail expression was inversely correlated to *E-cadherin* expression in different mouse and human cell lines (Batlle *et al*., 2000; Cano *et al*., 2000; Poser *et al*., 2001; Yokoyama *et al*., 2001), and shown to act as a direct repressor of *E-cadherin* transcription (Batlle *et al*., 2000; Cano *et al*., 2000). Although not statistically significant, we observed a higher percentage of tumours with reduced *E-cadherin* among the *Snail*-positive cancers (Table 2). As has been shown during embryonic development (Nieto *et al*., 1994; LaBonne and Bronner-Fraser, 2000; Carver *et al*., 2001; Del Barrio and Nieto, 2002) and in chemically-induced tumours in the skin of the mouse (Cano *et al*., 2000), the onset of *Snail* activation represents a local phenomenon associated with EMT and invasiveness. Therefore, previous immunohistological analyses of a relatively wide area of the tumour to assess whether E-cadherin expression is maintained or reduced may be misleading when later compared to the results of *Snail* expression, since a local activation of *Snail* would not account for a deficit in E-cadherin in the tumour. In terms of expression, the inverse relationship between the two can be only properly analysed by simultaneous detection of both proteins once specific anti-Snail antibodies are available, or through the analysis of adjacent sections as we have performed in our *in situ* hybridization studies. In addition, it is worth noting that *E-cadherin* expression in breast cancer *in vivo* seems to be an unstable process subject to temporary down-regulation and re-expression, probably under the control of several regulators (Cheng *et al*., 2001). Indeed, previous observations already addressed the question of the dynamic regulation of E-cadherin expression in tumours induced in nude mice by MDCK-ras cells. These invasive tumours showed *E-cadherin* downregulation and re-expression in their large metastases (Mareel *et al*., 1991). We have also analysed a few cases with a reduced E-cadherin in the absence of *Snail* expression, suggesting that other *E-cadherin* repressors may be present. Good candidates are the recently identified SIP1 and E12/E47 (Comijn *et al*., 2001; Pérez-Moreno *et al*., 2001).

In contrast to IDCs, *Snail* expression was not observed in any of the four ILCs analysed (Table 1, Figure 2b). Immunohistochemical studies of breast cancers have demonstrated that the absence or severe reduction of E-cadherin expression is a characteristic of lobular neoplasms, both *in situ* and infiltrating (Gamallo *et al*., 1993; Vos *et al*., 1997). However, the expression of E-cadherin at both the protein and mRNA level in human breast cancer has not been evaluated. In our sample of ILCs, we observed a good correlation between these two parameters. In two cases the absence of E-cadherin was demonstrated by both *in situ* hybridization and immunohistochemistry whilst in the remaining two cases, both *E-cadherin* mRNA and protein was observed. Interestingly, in one tumour, the protein was found at the cell membrane whereas in the other, it was ectopically located in the cytoplasm (Figure 2f). These different patterns of E-cadherin expression probably represent different mechanisms of E-cadherin inactivation in the different samples. Molecular studies have shown that in lobular cancer, inactivation of the *E-cadherin* gene can occur through allelic loss, gene mutation, and promoter hypermethylation (Berx *et al*., 1996; Droufakou *et al*., 2001). Mutations have been identified throughout the extracellular domain and at exon-intron boundaries, predicting the synthesis of different truncated proteins (Berx *et al*., 1996).

The inability to detect *Snail* expression in ILCs fits well with previous observations *in vitro* where tumour cell lines with a constitutively inactive *E-cadherin* gene were shown not to express detectable levels of Snail. One example is the bladder transitional-cell carcinoma T24 cell line (Batlle *et al*., 2000; Cano *et al*., 2000) in which *E-cadherin* expression is down-regulated as a result of promoter hypermethylation. This suggests that in the absence of a functionally active *E-cadherin* gene, *Snail* expression tends to be repressed. However, this does not seem to be the case in IDCs, since promoter hypermethylation and *Snail* expression can be observed in the same tumour, although it is not known whether the two mechanisms are operating in the same cells (Cheng *et al*., 2001).

We have also analysed the relationships between *Snail* expression and that of P-cadherin, β-catenin and

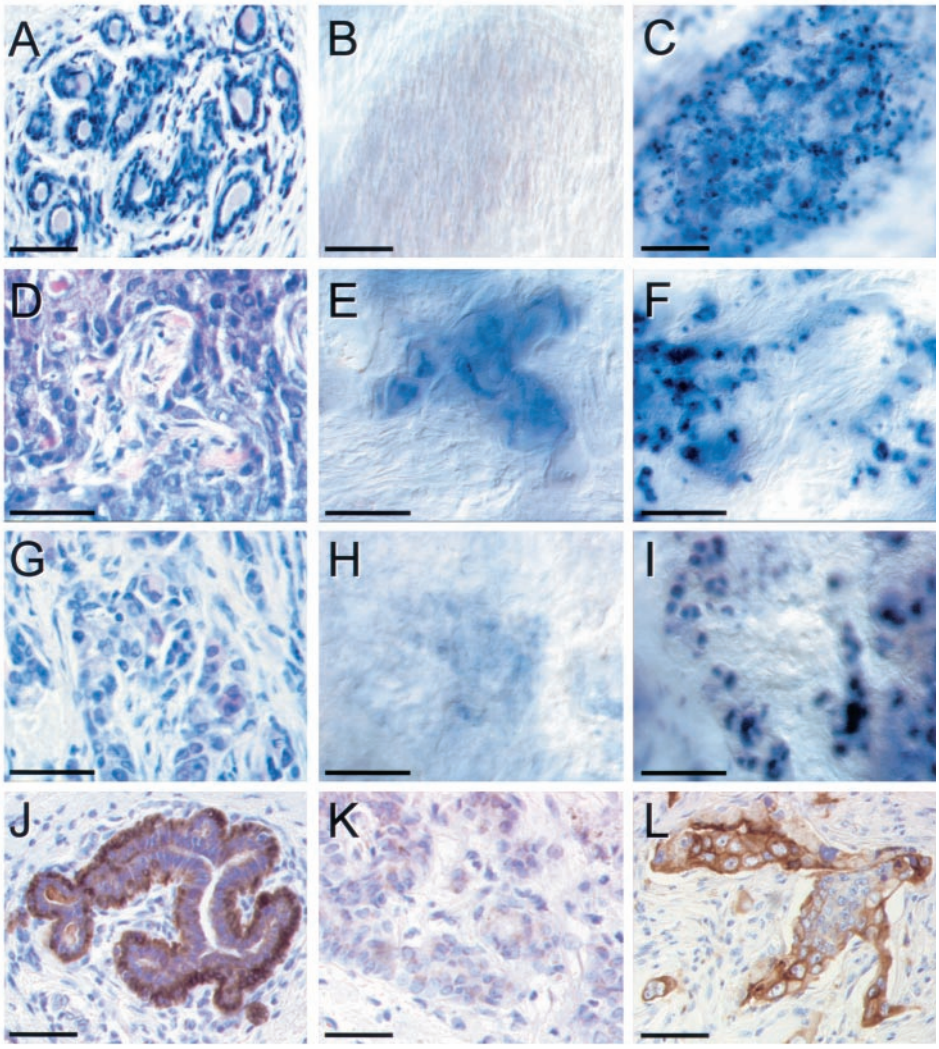

Figure 1 *In situ* hybridization for Snail (**b**, **e**, **h**) and E-cadherin (**c**, **f**, **i**) and immunohistochemical (IHC) analyses for P-cadherin (**j**–**l**) in normal breast tissue (**a**–**c**, **j**) and invasive ductal carcinomas (**d**–**i**, **k**, **l**). (**a**, **d** and **g**) show haematoxylin-eosin staining of the different tissues. Snail transcripts are detected in the undifferentiated IDCs but are absent from the normal epithelial tissue. An inverse correlation between *E-cadherin* and *Snail* expression is observed. Note the specific expression of P-cadherin in the myoepithelial cells of the normal tissue (**j**) and its wide expression in the de-differentiated IDC shown in (**l**). A moderately differentiated P-cadherin-negative tumour is shown in (**k**). For *in situ* hybridization, tumours were gelatin-embedded and 70 μm vibratome sections were processed as described in Nieto *et al*. (1996). Serial adjacent sections were used for *in situ* hybridization for *Snail* and *E-cadherin*, and for haematoxylin-eosin staining after paraffin-embedding and sectioning (8 μm). The *E-cadherin* and *Snail* probes corresponded to the sequences 3205 to 3735 and 695 to 1297 from the translation initiation codon, respectively. P-cadherin IHC was carried out as described in the footnote of Table 1. Calibration bars: 50 μm

plakoglobin. Normal breast epithelial cells express E-cadherin, β-catenin and plakoglobin at the cell membrane whereas P-cadherin is expressed by myoepithelial cells (Figure 1j). All IDCs expressed E-cadherin, β-catenin and plakoglobin at the cell membrane, but expression was reduced in nine (53%), 11 (65%), and 13 (76%) of the tumours respectively (Table 1). P-cadherin expression was detected in six (35%) IDCs. Examples of negative and positive tumours are shown in Figure 1k and l, respectively. In poorly differentiated IDCs, a reduction in E-cadherin expression is frequently associated with anomalous P-cadherin expression, suggesting the presence of common regulatory mechanisms (Palacios *et al*., 1995; Gamallo *et al*., 2001). However, in this study no correlation between P-cadherin and *Snail* expression was observed ($P=0.29$). Although this could again be the result of local *Snail* activation that cannot be compared with the general levels of P-cadherin expression, this seems an unlikely explanation since we have previously reported the absence of a correlation between *Snail* and *P-cadherin* expression in different murine cell lines (Cano *et al*., 2000). Moreover, we did not find any relationship between *Snail* expression and the expression of β-catenin ($P=0.63$), plakoglobin ($P=0.62$), oestrogen receptors ($P=0.29$) or progesterone receptors ($P=0.37$). Recently, it has been proposed that Snail levels may be

Table 1 Pathological, *in situ* hybridization and immunohistochemical results of the complete series

Case	*Type*	*Grade*	*LNM*	*Snail*	*E-CD*	*E-CD (ISH)*	*P-CD (IHC)*	*β-catenin*	*Plakoglobin*	*ER*	*PR*
1	IDC	1	0	–	+	+	–	+	+	+	+
2	IDC	1	0	–	+	↓	–	↓	↓	+	–
3	IDC	1	0	–	+	+	–	+	+	+	+
4	IDC	1	0	–	+	+	+	+	↓	+	+
5	IDC	2	0	+	+	↓	–	↓	↓	+	–
6	IDC	2	0	–	+	+	+	+	+	–	–
7	IDC	2	1	+	+	+	–	↓	↓	+	+
8	IDC	2	0	–	+	↓	–	↓	↓	+	+
9	IDC	2	0	–	+	+	–	↓	↓	+	+
10	IDC	2	0	+	+	↓	–	↓	↓	+	+
11	IDC	2	3	+	+	+	–	+	+	+	+
12	IDC	3	0	+	+	↓	+	↓	↓	–	–
13	IDC	3	0	–	+	↓	–	↓	↓	+	+
14	IDC	3	6	+	+	↓	–	↓	↓	+	+
15	IDC	3	1	+	+	+	+	+	↓	–	–
16	IDC	3	1	+	+	↓	+	↓	↓	–	–
17	IDC	3	0	+	+	↓	+	↓	↓	–	–
18	ILC	–	4	–	+	↓*	+	↓	↓	+	–
19	ILC	–	0	–	+	↓	+	↓	↓	+	+
20	ILC	–	0	–	–	–	–	↓	↓	+	–
21	ILC	–	0	–	–	–	–	↓	↓	+	+

LNM: Lymph node metastases; E-CD: E-cadherin; ISH: *in situ* hybridization; IHC: Immunohistochemistry; P-CD: P-cadherin; ER: oestrogen receptor; PR: progesterone receptor. IDC: infiltrating ductal carcinoma. ILC: infiltrating lobular carcinoma; +: postive; –: negative; ↓: Reduced; *case 18 showed cytoplasmic E-cadherin expression, see Figure 2f. All immunostainings were performed on paraffin-embedded tissue sections, using a step of heat-induced antigen retrieval prior to exposure to the primary antibody. Mouse anti-human E-cadherin (clone 4A2c7, Zymed, San Francisco, CA, USA), P-cadherin, β-catenin, and plakoglobin monoclonal antibodies (Transduction Laboratories, Lexington KY, USA) were used at a dilution of 1:200, 1:250, 1:1000, and 1:1000 respectively. Membrane expression of E-cadherin, β-catenin, and plakoglobin was semiquantitatively estimated as maintained (+) or reduced (↓) using a composite score obtained by adding the values of the immunoreaction intensity and the relative abundance of the immunoreactive cells, as previously reported in Gamallo *et al*. (1993) and Palacios *et al*. (1995). A tumour was considered to be P-cadherin (P-CD)-positive when at least 5% of the cells were labelled by P-cadherin antibodies

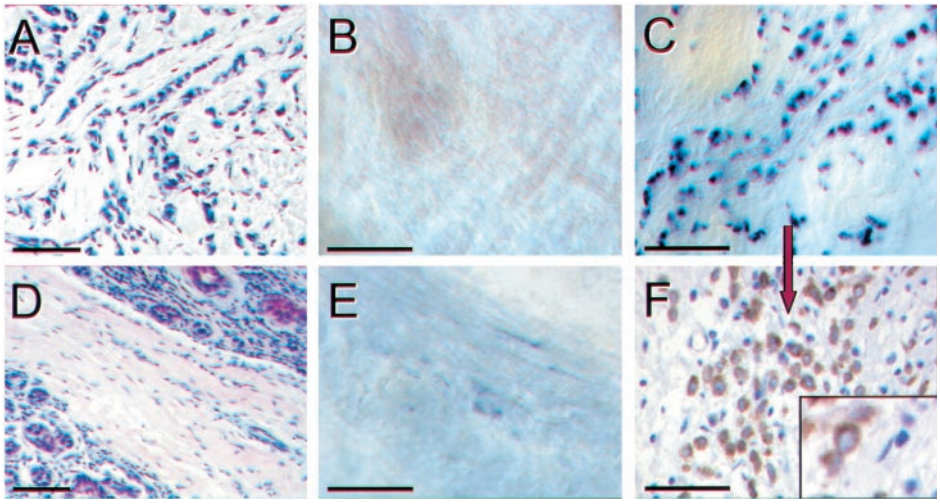

Figure 2 *Snail* and *E-cadherin* expression in invasive lobular carcinomas (**a**–**c**, **f**) and in tumour stroma (**d**, **e**). (**a** and **d**) show haematoxylin-eosin stainings. *Snail* transcripts are absent from the lobular carcinoma which expresses E-cadherin at the mRNA level (**c**) and shows cytoplasmic expression of the protein (see the inset in **f**). *Snail* expression is also detected in scarce fibroblastic cells present in the stromal regions (**e**). Calibration bars: 50 μm

regulated by integrin linked kinase (ILK) in colon cancer cell lines, where ILK seems to activate *Snail* and to regulate Tcf-mediated transcription upon the nuclear translocation of β-catenin (Tan *et al*., 2001). Although we can not exclude a role of ILK in the control of Snail expression in breast cancer, we have not detected β-catenin nuclear accumulation in any of our tumours, including those that expressed *Snail*.

We have performed a statistical analysis to compare the expression of *Snail* with the differentiation grade of IDCs. Our data support that a relationship exists between Snail expression and IDC tumour differentiation. In 47% of these tumours *Snail* was detected. Most of the grade 3 tumours and more than a half of grade 2 tumours expressed *Snail*, but it was not found in any of the grade 1 IDCs (Table 2). Indeed, in grade

1 tumours that presented well differentiated cribiform *in situ* components, they were also negative for *Snail* expression (Figure 3a–c). In grade 3 IDCs that expressed *Snail*, the *in situ* component also expressed *Snail* when it was poorly differentiated as seen in the comedo-type one shown in Figure 3d–i. The expression of *Snail* in this type of poorly differentiated *in situ* components is in agreement with previous analysis (Van Diest, 1999) indicating that they lead to dedifferentiated infiltrating ductal carcinomas, whereas well differentiated *in situ* tumours progress to well differentiated, tubular, and cribiform infiltrating carcinomas. A relationship between *Snail* expression and cell differentiation has also been observed both *in vitro* and during early embryonic development (Sefton *et al*., 1998; Cano *et al*., 2000). Well differentiated cell lines derived from human breast (MCF7) and colon (HT29P and LoVo) carcinomas do not express *Snail* mRNA, whereas it is expressed in dedifferentiated cell lines from breast (MB435S) and melanoma (A375P) (Cano *et al*., 2000; see also Batlle *et al*., 2000).

Table 2 Relationships between Snail expression and pathological and immunohistochemical features in infiltrating ductal carcinomas

	Snail-negative	*Snail-positive*	P
Grade			
1	4 (100%)	0	
2	3 (43%)	4 (57%)	
3	1 (17%)	5 (83%)	0.034
Metastases			
Negative	8 (67%)	4 (33%)	
Positive	0	5 (100%)	0.029
E-cadherin			
Preserved	5 (62%)	3 (38%)	
Reduced	3 (33%)	6 (67%)	0.347

Fischer's exact test was used to determine the statistical significance of the relationships between *Snail* expression and the pathological and immunohistochemical variables. The SPSS software package for Windows (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis

In addition to the relationship between *Snail* expression and differentiation, we have found that, in IDCs, *Snail* expression correlates with the presence of lymph node metastases. It was expressed in all the IDCs with lymph node metastases and all the *Snail*-negative tumours were also node negative (Table 2). This is in agreement with previous results where ectopic Snail expression in MDCK epithelial cells not only induced a profound alteration in cell morphology (spindle cells expressing higher levels of mesenchymal markers such as vimentin and fibronectin), but also induced the acquisition of tumorigenic and invasive properties (Cano *et al*., 2000). Tumour progression toward an invasive state which will eventually lead to the formation of metastases depends on, at least in part, the active movement of neoplastic cells across the extracellular matrix. Migration of individual cells or small groups of cells, as observed in IDCs, requires loss of cell-cell contact and the acquisition of migratory properties. In terms of cellular behaviour, this is reminiscent of the process of EMT. Very probably, both neoplastic and physiological invasion of tissues by

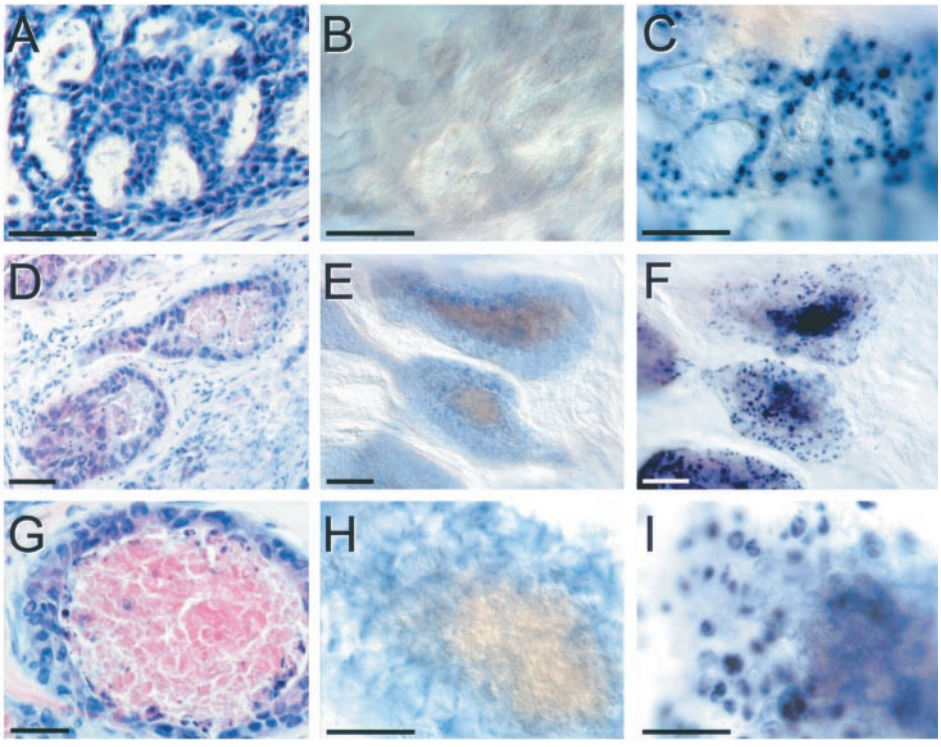

Figure 3 *Snail* and *E-cadherin* transcripts in *in situ* components of two IDCs. (**a**–**c**) shows a well differentiated *in situ* component depicting high levels of *E-cadherin* expression and absence of *Snail* transcripts. (**d**–**i**) show a dedifferentiated comedo-type *in situ* component. Note high levels of *Snail* expression (**h**) in areas with disorganized *E-cadherin* expression (**i**). Calibration bars: 50 μm

migrating cells relies upon identical molecular mechanisms (Kohn and Liotta, 1995; Locascio and Nieto, 2001). The Snail family of transcription factors are implicated in triggering EMT in embryonic development and a similar role can be proposed for Snail in IDC tumour progression as previously suggested in mouse skin chemically-induced carcinomas (Cano *et al*., 2000). In this context, it would be interesting to analyse whether other E-cadherin repressors, such as E12/E47 and SIP1 (Comijn *et al*., 2001; Pérez-Moreno *et al*., 2001) can play a role in maintaining the invasive phenotype.

Interestingly, *Snail* is expressed in IDCs with a poor grade of differentiation, but that do not present lymph node metastases (Table 2). Considering the role of *Snail* in inducing EMT and invasive properties in epithelial cells, it is tempting to speculate that Snail could be a prognostic marker of malignancy in IDCs. The identification of potentially metastatic tumours is a long-standing goal for oncologists and would be extremely useful in the design of more specific therapies. The loss of E-cadherin expression is considered as a poor prognostic sign and it has been correlated with the transition from adenoma to carcinoma (Perl *et al*., 1998). Since Snail is a direct repressor of *E-cadherin* transcription, the correlation we have found is very likely to be meaningful. It is worth noting that although we have not analysed *Snail* expression in metastases, we would expect it to be down-regulated during cell re-attachment, as occurs during embryonic development once *Snail*-expressing migratory cells have reached their destination (Nieto *et al*., 1994; Sefton *et al*., 1998). This would also be in agreement with the unexpected finding of E-cadherin re-expression in axillary lymph node metastases (Bukholm *et al*., 2000) and in metastases generated in some experimental systems (Mareel *et al*., 1991). This reversibility in gene expression favours mechanisms of transcriptional regulation and reinforces the idea that epigenetic mechanisms rather than irreversible genetic loss may confer upon breast cancer cells a selective advantage for progression (Cheng *et al*., 2001).

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