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Original Paper

The Immunohistochemical Expression of Desmoplakin and its Role *In Vivo* in the Progression and Metastasis of Breast Cancer

E.L. Davies,[1] J.M.W. Gee,[2] R.A. Cochrane,[1] W.G. Jiang,[1] A.K. Sharma,[2] R.I. Nicholson[2] and R.E. Mansel[1]

[1]University Department of Surgery, Academic Unit, University Hospital of Wales, Heath Park, Cardiff, CF4 4XN; and [2]Tenovus Cancer Research Centre, Heath Park, Cardiff, U.K.

Desmoplakin (DP) is a protein located at the inner plaque of desmosomes where it associates with the desmosomal cadherins to form a cell adhesion complex. Reduced expression of DP has been correlated with the progression of several cancers, but its role in *in vivo* breast cancer is yet to be established. The aim of this present paper was to determine the immunohistochemical (IHC) expression of DP in breast cancer specimens ($n = 75$) in comparison with ductal carcinoma *in situ* (DCIS) ($n = 26$), tumour associated normal ($n = 29$) and normal breast tissue ($n = 7$). DP expression was correlated with that of desmosomal cadherin, Desmoglein 2 (Dsg2) and other clinical and IHC prognostic markers. DP staining occurred at the sub-plasma membrane level. There was no significant correlation between the level of DP (as assessed by the H-score) and that of Dsg. Significantly stronger staining was demonstrated in normal breast tissue and well differentiated tumours compared with more moderately or poorly differentiated tumours ($P = 0.04$). A significant inverse correlation was seen between DP staining and tumour size ($P = 0.01$). In a limited series of 8 cases, primary tumours demonstrated significantly stronger staining than the matched metastatic lymph nodes ($P = 0.046$). Of all the IHC markers examined, only Ki-67 showed a significant inverse relationship with DP staining ($P = 0.01$). In summary, the data suggest that loss of DP may be of potential importance in progression of breast cancer *in vivo* from normal, DCIS, well differentiated through to poorly differentiated, large tumours. In addition, this loss may be associated with metastasis. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: breast, invasion, metastasis, adhesion, desmoplakin, desmoglein
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INTRODUCTION

BREAST CANCER affects approximately 1 in 12 women in the U.K. The most devastating aspect of cancer is the spread from the primary site to distant organs. Despite the ever increasing advances in 'modern' treatment modalities, such as surgical, chemo- and radiotherapy, the presence of metastasis is the major cause of treatment failure for cancer patients [1, 2]. Approximately 30% of patients with newly diagnosed tumours already have clinically detectable metastases at the time of presentation. Furthermore, of those 70% of patients free of clinically detectable metastases, only 50% can be cured with local irradiation. The remainder have clinically occult metastases which manifest at a later date [2]. Local invasion and subsequent distant metastasis is a complex multistep process [3–5]. The initial step in this 'metastatic cascade' is the loss of cell–cell adhesion at the primary site. Reduced expression of several cell adhesion molecules, including E-cadherin [6–10] and their associated desmosomal cadherins including Desmoglein 2 (Dsg2) [11–14] have been implicated in this process. We recently reported a loss of Dsg2 expression *in vivo* in breast cancer in comparison with

Correspondence to E.L. Davies, e-mail: wsrjlg@cf.ac.uk.
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both normal breast tissue and ductal carcinoma *in situ* and an association *in vitro* with decreased cell–cell aggregation and increased invasion and motility [15].

Desmosomes play a critical role in the development and maintenance of epithelial tissue integrity. They are composed of a pair of disc-like intercellular junctions, usually less than 0.5 μm in diameter. Their functions include cell–cell adhesion and maintenance of cell integrity via their attachments to the intermediate filaments [16]. Desmosomes occur in all epithelia, cardiac muscle, the arachnoid and piamater and the follicular dendritic cells of the lymphoid system [17–20].

The plaque region of the desmosome is thought to link the intermediate filaments (IFs) to the cell surface. Plaque-associated proteins are located at the inner plaque region and possess the ability to interact both with the IFs and the components of the plaque. The two major plaque-associated proteins are desmoplakin (DP) and plakoglobin (PG). DP I and II are a pair of proteins (molecular weights 250 000 and 215 000 Da, respectively), derived from the same DP gene. DP1 is present in all desmosome-bearing tissues [21], whilst DP2 is more variably expressed, found in lower levels of non-stratified tissues and absent in certain tissue, such as the heart [22].

Several electron microscopical studies of desmosomes have suggested that a reduction in desmosomes is associated with invasive behaviour [23, 24]. These initial studies were followed by immunohistochemical examination of the expression of the different desmosomal components *in vivo*, demonstrating a marked reduction in the expression of both desmoplakin and desmoglein in several carcinomas, including transitional cell carcinomas of the urinary tract [11], squamous cell carcinomas of the oral cavity [13] and adenocarcinoma of the uterine cavity [14]. These studies correlated expression with degree of differentiation and lymph node involvement, demonstrating a DP loss associated with local progression and metastasis. Several studies both *in vitro* [25] and *in vivo* [26] have demonstrated that loss of plakoglobin expression in breast cancer is related to invasive behaviour. In breast cancer, data on the role of DP are limited and inconclusive. To date, only one study has examined DP expression in breast cancer *in vivo* and has surprisingly failed to demonstrate any association with disease progression, although no lymph node data were available to determine its role in metastasis [27]. It is, therefore, apparent that further research is required to establish the role of DP in breast cancer.

Several clinical and immunohistochemical parameters are used as clinical guidelines for assessing the aggressiveness of tumours in terms of potential metastatic ability and cause of death. In the literature, size, grade and lymph node involvement on multivariate analysis are prognostic indicators [28, 29]. Similarly immunohistochemical markers are good indicators of survival and response to certain adjuvant treatments. Patients with oestrogen receptor (ER) positive tumours have prolonged disease-free survival after primary treatment and superior overall survival compared with ER negative patients [29]. Veronese and associates [30] reported that Ki-67 levels predicted 4-year survival independently of ER and lymph node status. High Ki-67 levels have been linked with tumour aggressiveness and disease spread [31]. Finally epithelial growth factor receptor (EGFR) expression has been found to be significantly associated with presence and number of lymph node metastasis [32] and long-term outcome [33].

The aim of this present study was thus to assess the role of desmoplakin I/II in breast cancer progression by comparing the immunohistochemical staining in breast cancer with ductal carcinomas *in situ* (DCIS) and normal breast tissue, and to assess DP's potential role in invasion and metastasis by correlating its expression with a range of clinical (size, grade and lymph node status) and relevant immunohistochemical markers (Dsg 2, Ki-67, EGFR and ER), as well as examining a small available group of matched primary breast cancers with their lymph node metastasis.

PATIENTS AND METHODS

Tissue samples

Seventy-five invasive breast carcinomas (63 ductal, 12 lobular, median size 1.8 cm [range 0.5–7]), 26 DCIS and 29 tumour-associated normal specimens were obtained from the Department of Surgery at the University Hospital of Wales. Seven 'pure' normal breast tissue specimens were also received from reduction mammoplasties. In addition, a small series of eight pairs of primary breast cancer tissue and matched positive metastatic lymph nodes were obtained. The ductal carcinomas were assessed as 15 grade 1, 26 grade 2 and 19 grade 3 tumours (three samples were only available as trucuts and, therefore, not graded). Representative tissue samples from each specimen were snap-frozen in liquid nitrogen, subsequently embedded in OCT compound (Tissue Tek, Raymond Lamb, London, U.K.) and stored at -70°C prior to sectioning and immunohistochemical (IHC) assay.

Immunological reagents

The primary antibodies used were: monoclonal antibody (MAb) to desmoplakin I/II from mouse–mouse hybrid cells (Boehringer Mannheim Biochema, Germany) and mouse MAb to desmoglein 2 (Insight Biotechnology, Middlesex, U.K.). Goat anti-mouse IgG conjugate (Dako Ltd, Bucks, U.K. (Z0420)) was used as the bridging antibody, with mouse peroxidase anti-peroxidase (PAP) (Dako (P0850)) as a tertiary reagent and diaminobenzidine/hydrogen peroxide (DAB) complex (Abbott Laboratories, Chicago, U.S.A.) chromogen.

Immunohistochemical staining for desmoplakin

Cryosections of each tumour, as well as DCIS, TAN, normal breast tissue and metastatic LN material, were cut at 5 μm thickness. Slides were fixed by immersing in -20°C acetone for 10 min followed by air-drying for 30 min. Samples were stored at -70°C until required. Prior to assay, structure and grade was confirmed by haematoxylin and eosin staining. For immunostaining, test sections were transferred to phosphate buffered saline (PBS) (0.01 M, pH 7.2–7.4). Non-specific binding was blocked by incubating the slides in 1:10 Normal Goat Serum (in PBS) for 10 min. Thereafter, the test slides were incubated with a 1:10 dilution of DP MAb in PBS for 1 h in a humidity chamber at room temperature. After washing with PBS, the goat anti-mouse IgG conjugate (1:25 dilution in PBS) was applied for 30 min. PAP (1/250 in PBS) for 30 min and DAB/H_2O_2 for 10 min were then applied with intervening PBS washes. The sections were finally washed in distilled water and counterstained with 0.5% (aq.) methyl green. The sections were then dehydrated in graded alcohol concentrations and mounted with DPX mountant. Control sections were included where the primary antibody was replaced with PBS, while MCF7 breast cancer

cell pellets and smooth muscle sections [34] were used as positive and negative controls, respectively.

Immunohistochemical staining for desmoglein 2

Breast cancer cryostat frozen sections were fixed by immediately immersing in -20°C methanol for 5 min followed by -20°C acetone for a further 5 min and finally air-dried. Samples were stored at -70°C until required. The sections were then transferred to 0.01 M PBS. Prior to immunostaining, the sections were tritonised using 0.2% Triton in sodium citrate buffer for 3 min at room temperature. The slides were then washed three times in PBS. Non-specific binding was blocked by incubating the slides in 20% Normal Human Serum (in PBS) for 10 min. Thereafter, the test slides were incubated with a 1:10 dilution of Dsg2 MAb in PBS. After washing with PBS, the goat anti-mouse IgG conjugate (1:50 dilution in PBS) was applied for 1 h. The remaining steps were as described above. Control sections were prepared by replacing the primary antibody with PBS and using MCF7 cells and smooth muscle as positive and negative controls, respectively. In addition, for further quality control purposes, standardised DP positive breast tumour sections were included both for comparison within and between assays.

Immunohistochemical prognostic factors

ER, Ki67 and EGFR immunostaining was routinely performed for each specimen as previously described [35–37].

Assessment of staining

Desmoplakin, Dsg2, ER, Ki-67 and EGFR immunostaining was assessed in breast tissue and if appropriate, lymph node (LN) metastatic sections by two investigators using dual viewing attachment to an Olympus BH-2 light microscope at an ocular magnification of $\times 40$. Control slides were checked for non-specific binding before assessing staining intensity and percentage positivity of the tumour epithelial cells in the test sections. These data were used to construct a staining index (H-score: [38]) for each marker as follows:

$$\begin{aligned}\text{H-score} = {} & ([\%\ \text{cells staining weakly}] \times 1) + \\ & ([\%\ \text{cells staining moderately}] \times 2) + \\ & ([\%\ \text{cells staining strongly}] \times 3)\end{aligned}$$

For the purpose of categorical analysis, the cut-off H-score value for DP and Dsg2 immunopositivity was ≥ 120 and ≥ 40, respectively (i.e median values). The cut-off for ER and EGFR was > 0, whilst Ki-67 was $\geq 30\%$ cells. These latter cut-off points have previously been established to discriminate endocrine sensitivity and insensitivity in breast tumours [39, 40].

Histological assessment

The tumour size was measured on a glass slide, while the grade and tumour type (i.e. ductal or lobular) was determined by the Bloom and Richardson [41] method. In addition, LN status was also documented when available.

Statistical analysis

Statistical analysis of the data was performed using SPSS for Windows software. Spearmans correlation and two-tailed non-parametric analyses using the Mann–Whitney U Test, as well as a Chi-squared analysis (with Fisher's exact test for small numbers) was applied. Analysis on matched breast cancer and LN metastasis material was performed using Wilcoxon Matched Signed Ranks Test. Values were considered statistically significant when $P < 0.05$, and trends were noted where sample numbers were small.

RESULTS

Distribution of DP staining and its relationship to progression

The luminal epithelial cells of the lobules and ductules of 'pure' normal breast tissue generally show strong, brown immunostaining with the DP MAb, median H-score = 140 (range 45–180, Figure 1a). The immunostaining was discretely localised to the sub-plasma membrane area. Tumour-associated normal tissue demonstrated a comparably high positive staining pattern, median H-score = 125 (range 55–220). The associated myoepithelium in all normal breast structures also stained positively, although no staining was seen in the surrounding stromal tissue. DP staining was also strong in the epithelium of DCIS ($n = 26$) median H-score = 120 (range 70–200, Figure 1b), with no significant difference in level of staining when statistically compared with both normal ($n = 7$) and tumour-associated normal ($n = 29$) breast tissue.

Within cancers, however, staining was more heterogeneous between samples with diminished staining not infrequent. DP staining was often strong in the epithelium of well-differentiated tumours (Grade 1, $n = 15$), median H-score = 130 (range 5–190, Figure 1c), with no significant differences in staining between these tumours and normal breast structures and DCIS. In contrast, however, DP staining was statistically significantly diminished ($P = 0.04$) in both moderately ($n = 26$) and poorly differentiated ($n = 19$) tumours (grades 2 and 3), median H-score = 112.5 (range 1–200, Figure 1d and e) compared with normal breast tissue. There was a non-significant trend for more poorly differentiated tumours to have reduced levels of DP compared with their well-differentiated counterparts ($P = 0.17$).

Quality control of immunostaining was maintained throughout the study by inclusion of positive control breast tumour and MCF7 cell to pellet sections both within DP assays and between assays. Thus, there was a very low intra-assay (7.8%) and inter-assay (8.5%) coefficient of variation recorded. Moreover, non-specific staining was very rare in patient material when PBS control and PBS test sections were compared, whilst smooth muscle negative control sections never stained positively for DP in this assay.

Relationship between DP staining and clinical prognostic markers

There were no significant differences ($P = 0.29$) between tumour epithelial DP staining in ductal ($n = 63$, median H-score = 120, range 1–200) and lobular carcinomas ($n = 12$, median H-score = 132.5, range 14–195). However, an inverse trend was observed between DP staining and the size of the primary tumour ($P = 0.079$). Whilst this trend was observed at all size cut-offs, it reached statistical significance using a 3-cm cut-off ($P = 0.01$), with larger tumours having lower DP levels (i.e. median H-score tumours < 3 cm = 120 [range 5–200], $n = 52$; ≥ 3 cm = 75 [range 1–70, $n = 10$]; no size documented, $n = 13$).

No correlation was shown between DP expression and lymph node status ($P = 0.24$). However, when the expression of DP was compared in the small sample series comprising eight primary breast cancers with matched LN metastasis material, there was a significant reduction ($P = 0.046$) in

immunostaining within the tumour epithelial cells in the lymph node. Thus, the breast tumours generated a median H-score of 115 (range 5–180), whilst matched metastatic LN material had a lower median H-score of 62.5 (range = 5–140). Indeed, only one of the 8 cases had DP staining in the lymph node metastases comparable with that in the primary breast tumour sample.

Correlation between DP staining, Dsg2 and IHC markers

There was no significant correlation between the desmosomal cadherin Dsg2 and DP immunostaining ($P = 0.38$). In addition, there was no marked correlation between DP expression and either ER or EGFR (i.e. $P = 0.2$ and $P = 0.17$, respectively). However, there was a trend for highly proliferative, Ki-67 positive tumours ($n = 5$) to have reduced levels of DP (median H-score = 110) compared with Ki-67 negative tumours ($n = 65$, 130, median H-score = $P = 0.07$). This relationship reached statistical significance within the ductal carcinoma subgroup ($P = 0.01$).

DISCUSSION

The results showed that epithelial cell DP expression is found in the sub-plasma membrane area of positive cells, and is reduced in breast cancers in relation to both normal breast tissue, tumour-associated normal breast tissue and DCIS. This phenomenon is particularly prominent for grade 2/3, moderately/poorly differentiated tumours. It is thus possible that loss of DP may be important in the progression from

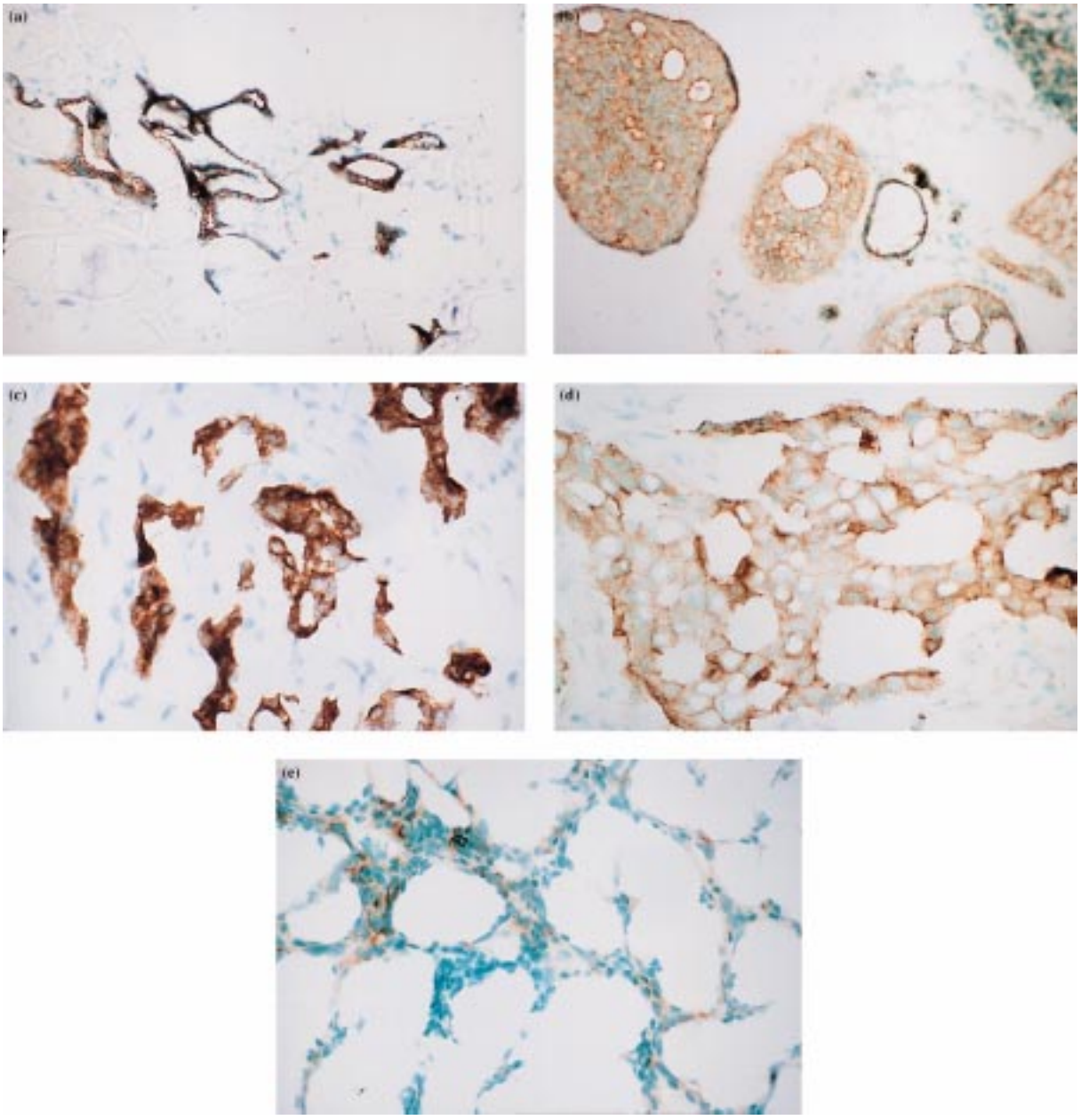

Figure 1. (a) Strong positive DP staining in 'pure' normal breast tissue (×20 magnification); (b) moderate positive staining in ductal carcinoma, *in-situ* (×20 magnification); (c) strongly DP positive staining well differentiated tumour (grade 1) (×40 magnification); (d) moderately positive DP staining moderately differentiated tumour (grade 2) (×40); and (e) weakly positive staining poorly differentiated tumour (grade 3) (×40).

normal phenotype to breast cancer. This has been suggested in similar studies examining DP expression in other tumour types, e.g. uterine adenocarcinoma and oral squamous cell carcinomas [13, 14]. Importantly, weaker staining occurred in high grade, larger sized and highly proliferative tumours, phenotypic features which have been linked to tumour aggressiveness in terms of metastatic capacity and cause of death [28–33]. Moreover a limited matched sample analysis ($n = 8$) revealed that weaker DP staining occurred once tumour cells had left the primary tumour and metastasised to the lymph node. In addition, similar relationships have been demonstrated both with progression and metastatic spread for other desmosomal components e.g. E cadherin in breast cancer [6–10].

Despite similar relationships to progression, there was no significant correlation between DP staining and the desmosomal cadherin (Dsg2) staining. This may be explained by the fact that DP binds not only Dsg2, but also Dsg1 and 3, Dsc1–3. In addition, the different desmoglein isoforms show different patterns of tissue distribution. Recent evidence has indicated that the desmosomal cadherin Dsg2, is expressed in all desmosome-bearing tissues described [42], whereas Dsg1 and 3 have a more limited tissue distribution. Desmocollin (Dsc) 1–3 also show a complexed pattern of tissue distribution with Dsc 2 demonstrating a broader tissue distribution than Dsc 1 and 3 [43, 44].

Interestingly, although we showed a trend for high-grade tumours to have a reduced level of DP expression, there were occasionally grade 1 tumours which also had very low levels, and conversely grade 2/3 tumours which expressed high levels. This is borne out by the study by Dervan and associates [27] who failed to demonstrate a relationship with DP expression and breast cancer progression or grade. In addition, although we observed a significant reduction in DP expression in metastatic lymph nodes compared with the primary tumours, there was one case where DP expression in the lymph node metastasis was comparable to the primary. Thus, we propose that although loss of desmosomal protein expression may aid progression and metastasis, it is not an essential or absolute parameter, and additional factors including loss of other cell adhesion molecules may also be important, such as cadherin [6–10]. Another explanation might be that desmosomes and their associated proteins in DP-positive, grade 3 or LN-positive invasive carcinomas may be expressed but are in some way functionally impaired, compared with both normal breast tissue and their more well differentiated tumour counterparts [16]. This is an important area, which needs further evaluation. Key parameters for example may include phosphorylation status [39], as seen for plakoglobin [25], the necessary complexes with desmosomal glycoproteins, desmoglein and plakoglobin and somatic mutations as seen for E-cadherin [46–48].

In conclusion, loss of desmoplakin in breast cancer is likely to be important in the progression and metastasis of breast cancer *in vivo*, and larger confirmatory studies are needed. However, it is notable that delineation of a causative or merely phenotypic role for DP in these phenomena and moreover in overall patient survival remains uncertain. In this light, further investigations are required to assess the functionality of the DP expressed, whilst gene transfer studies examining the effect of introduction or elimination of DP in a panel of breast cancer cell lines would also be highly relevant. Future studies in this direction are planned in our department.

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