Lineage infidelity and expression of melanocytic markers in human breast cancer

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Abstract. In previous analyses, dual expression of melanocytic and epithelial molecular markers have been described as indicators of lineage infidelity for breast cancer cells that lose their epithelial identity. Here we demonstrated that this is a much more frequent phenomenon in human breast carcinomas, usually affecting only a part of the tumor. Accordingly we detected, in 18 out of 100 breast carcinomas, immunohistochemically focally positive cells for the melanocytic marker Melan A. The presence and extent of Melan A expression was statistically significantly associated with a reduction in tumor cell differentiation, but not tumor type, size, lymph node metastasis, hormone receptor status or Her-2-neu expression. Microarrays of a further 159 breast cancers showed, in several samples, variably low expression levels of Melan A (and other melanocytic markers) that are consistent with focal expression in many tumors. One case strongly overexpressed Melan A. The transition from an epithelial to a melanocytic phenotype (lineage infidelity) appears to occur much more frequently than previously assumed and occurs in restricted areas of breast cancer during tumor progression, a possible association with a reduction in tumor cell differentiation.

Introduction

Lineage infidelity, a phenomenon widely known from haematopoietic tumors such as acute leukemia (1), was first observed in chronic myeloid leukemia (2), and is defined as the (co-)expression of markers of unrelated cell lineages. The existance of isolated breast cancer cases with dual expression of melanocytic and epithelial markers *in vivo* and several cases with either focal or extensive melanocytic differentiation (3-6), places this phenomenon in a more widespread manner in breast carcinoma. Recently, lineage infidelity has also been described for ovarian cancer (7) yet it has not systematically been analyzed in all types of tumors.

In the present study, we provide evidence to demonstrate that lineage infidelity occurs much more frequently in breast cancer *in vivo* than previously expected and that it is associated with a reduction in tumor cell differentiation.

Material and methods

Breast tumor tissue samples. A series of 100 consecutive breast cancer tissue samples was taken from the files of the Breast Cancer Registry (Munich-Bogenhausen Academic Clinic). The unselected cases covered the time period between August and December 2006. Basic clinical and histopathological information including tumor type, size, nodal status, degree of tumor cell differentiation, proliferative activity and presence/extent of intraductal carcinoma was available. In particular, tumor cell differentiation was determined according to the guidelines by Elston and Ellis (8).

Immunohistochemistry. Formalin-fixed and paraffin-embedded tissue samples from the 100 breast cancers were immunocytochemically tested for Melan A (clone M2-7C10, Linaris, Germany), estrogen and progesterone receptor (Dako, Hamburg, Germany), Her-2-neu oncoprotein and proliferation antigen Ki-67 (Dako, Hamburg) according to the manufacturer's protocols. As negative controls, parallel slides with non-specific (pre-immune) serum were used. Positive controls consisted of either melanoma tissue samples (for the Melan A staining) or breast cancer samples with known expression of hormone receptors (for the estrogen and the progesterone receptor, respectively) or the Her-2-neu oncoprotein and lymph node tissue for the proliferation rate (Ki-67).

The immunostaining for Melan A was evaluated using a semi-quantitative scoring system similar to that previously

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Abbreviations: DCIS, ductal carcinoma *in situ*; EMP, epithelial membrane protein; MAGE, melanoma antigen gene

Key words: breast cancer, melanocytic phenotype, lineage infidelity, tumor progression

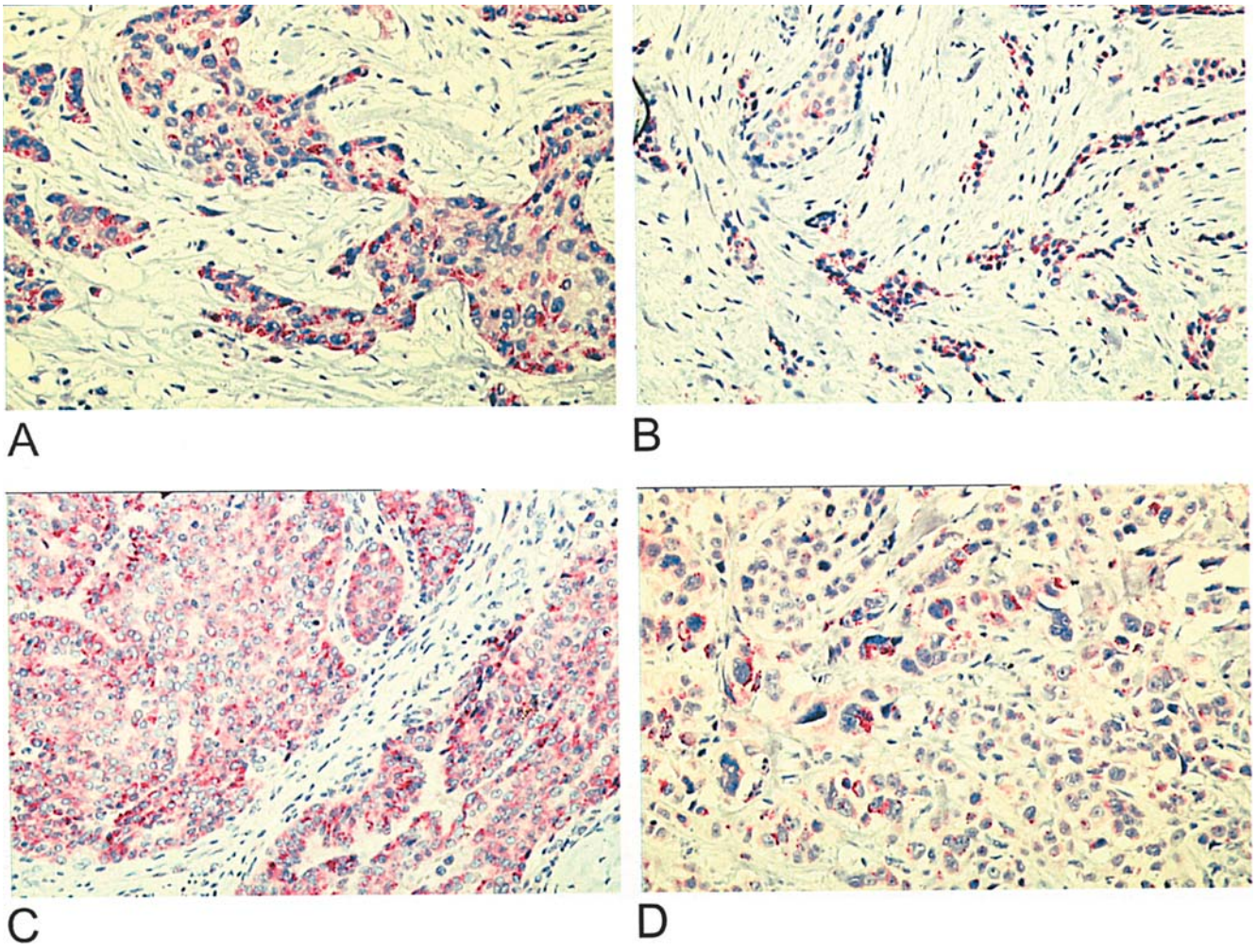

Figure 1. Melan A expression in breast cancer. Immunohistochemical analysis of Melan A expression in positively labeled breast cancer tissue samples revealed an unambiguous and specific cytoplasmatic staining ranging from extensively positively stained tumor areas (examples shown A-C), to focal positive cells (D). Original magnification x400.

applied for the analysis of the hormone receptor status. Briefly, the extent of positively labeled cells was ranked into 5 grades, i.e. 0, 0%; 1, 1-10%, 2, 11-50%; 3, 51-90% and 4, ≥90%; furthermore, the staining intensity was graded into 4 steps with 0=no staining, 1, low; 2, moderate and 3, strong staining. The result was presented as a product of the two assessments.

All other immunostainings were evaluated according to established routine protocols.

Microarrays. Microarray expression analyses of human breast cancers were performed on the dataset GSE1456 obtained from Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). The data set consists of 159 breast cancers from patients who received surgery at Karolinska Hospital from 1994 to 1996 (9). Expression sets were calculated from raw microarray data using the GCRMA algorithms implemented in Bioconductor (10). Agilent GeneSpring 7.1 software was used for the visualization of expression data. Samples were clustered according to the expression of different marker gene groups to enhance visual interpretation by hierarchical clustering with average linkage.

Statistical analysis. The immunohistochemical data were statistically analyzed using Spearman and Chi-square correlation analyses with respect to any significant correlation between the expression of Melan A and clinical/histopathological data in the breast cancer tissue samples.

Results

Immunohistochemical expression of Melan A in breast cancer tissues. Immunohistochemical analysis of Melan A expression in 100 consecutive and non-selected breast cancer tissue samples revealed unambiguous and specific cytoplasmatic staining in 18 cases (Fig. 1) ranging from a few groups of positive cells to extensively positively stained tumor areas. Melan A was not present in normal breast tissue, stromal cells or endothelia. Classification of the 100 cases according to the 3-step differentiation score (8), resulted in 11, 69 and 20 patients in G=1, G=2 and G=3, respectively.

We correlated the presence and extent of Melan A staining with patient age, tumor type, and tumor size, degree of tumor cell differentiation, estrogen and progesterone expression levels, Her-2-neu oncoprotein expression and

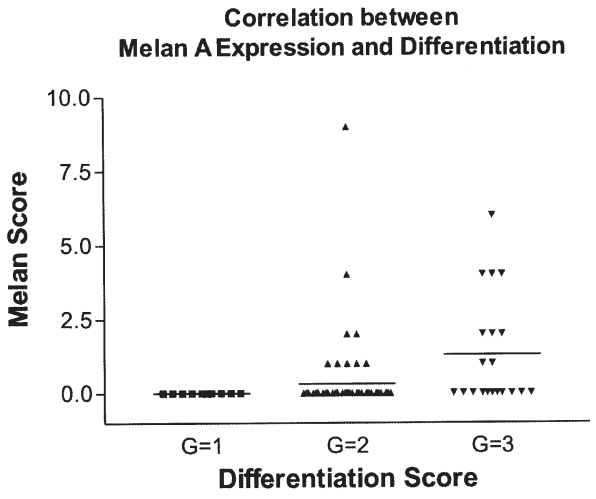

Figure 2. Correlation between Melan A expression and tumor differentiation. Melan A expression was seen significantly more frequently in tumors of poor differentiation (Spearman p=0.01). None of the cases in G=1 (n=11) expressed Melan A, whereas 9 cases in G=2 (n=69) and 9 cases in G=3 (n=20) were Melan A positive. The extent of Melan A staining (score) for most of the cases was highest in G=3.

tumor cell proliferation rate (Ki-67). Presence and extent of Melan A staining was significantly correlated with the degree of tumor cell differentiation (Spearman p=0.01). None of the well-differentiated carcinomas (G=1) expressed Melan A, whereas 9 out of 69 cases in G=2 (moderately differentiated carcinomas) and 9 out of 20 cases in G=3 (poorly differentiated carcinomas) were Melan A positive (Fig. 2). The majority of the cases in G=3 had a much higher staining intensity for Melan A. Accordingly, Melan A expression was observed significantly more frequently in tumors of poor differentiation.

Melan A expression also significantly correlated with the tumor type [occuring more frequently, but not exclusively in invasive ductal carcinomas (IDC); (Chi-square test 0.005)] and particularly in IDC with enhanced intraductal tumor proliferation (IDC with increased DCIS) (Spearman p=0.03). Other parameters tested; in particular estrogen and progesterone receptor expression, Her-2/neu expression, tumor size, patient age or cell proliferation rate (Ki-67), were not significantly associated with Melan A expression.

Microarray analysis of breast cancer tissue samples and breast cancer cell lines. Since our analyses had demonstrated a notable number of human breast cancer samples containing Melan-A positive cells, we analyzed a breast cancer microarray dataset containing 159 breast cancers for the expression of genes encoding various melanocytic and mammary markers: melanoma associated antigens (MAGE) and commonly used melanoma markers such as Melan-A, GP100/HB145 and tyrosinase A, S100 proteins and breast cancer markers (Fig. 3A-C). Fig. 3 shows that the expression of most of the genes analyzed is highly variable.

While a part of the tumors expressed no or low amounts of mammaglobins, the other part presented very high expression levels (Fig. 3C). Several breast cancers revealed lack of keratin 18 or 7 or other epithelial markers. Melanoma markers were expressed to variable extents by many of the breast cancer types. We identified one tumor that expressed extremely high levels of Melan-A together with melanoma specific markers including GP100/HB145, tyrosinase A and several MAGEs. This tumor (GSM107166) was positive for epithelial markers such as keratin 18 and EMP1, 2 and 3, but lacked the expression of keratin 7 and the mammaglobin genes. It was estrogen receptor negative, classified differentiation grade 2, and belonged to the basal subtype. The patient relapsed and succumbed to breast cancer. A number of other tumors with comparatively low keratin 18 expression levels (Fig. 3C) were negative for melanoma markers (data not shown).

We found several tumors expressing GP100/HB145 and tyrosinase and a larger group expressing one of the two markers in addition to MAGEs. A subgroup of GP100/HB145 positive tumors synthesized very high levels of the MAGEs A6, A2, A12 and A3 (Fig. 3B).

S100 proteins are preferentially but not exclusively synthesized by melanomas and among the 159 breast cancers we did not find even a single case without the expression of at least one of the isoforms (Fig. 3A).

Discussion

Lineage infidelity, the (co-)expression of markers of unrelated cell lineages, has been described for several cancers (7,11) yet it has not been systematically analyzed. The transition of regional phenotypes is frequent in ovarian epithelial cancers where it appears to be regulated by Hox genes (7). Previous case studies and/or small series of breast cancer cases provided evidence that melanocytic differentiation may occur in epithelial carcinomas of the mammary gland (3-6). However, it has been repeatedly assumed that a dual expression pattern of epithelial and melanocytic differentiation markers comes from a secondary 'invasion' of melanocytes into the tumor (3,6). While such a variety of cell types may occur in carcinomas arising in the nipple where the mammary gland and the epidermis join, this is very difficult to fathom in carcinomas occurring in the interior parts of the gland or in metastases.

In previous studies the expression of melanocytic markers by breast cancers was associated with either focal or extensive melanocytic differentiation (3-6). Additionally, since the first described case of 'pigmented breast carcinoma' by Azzopardi and Eusebi (12), a further 16 cases with 'dermal breast cancer metastases with melanocyte colonization' (13,14) have been described, some of which may also be categorized as breast carcinoma with partial melanocytic differentiation. As a consequence, the results of lineage infidelity are seen in occasional cases of manifest breast cancer.

We felt that this aspect of breast cancer required further attention since lineage infidelity is likely to influence the outcome. We therefore investigated the frequency of melanocytic differentiation in breast cancer cases and the potential association with pathohistological and prognostic parameters. This was accomplished in a dual approach: A series of 100 breast cancer tissue samples was analyzed immunohistochemically and an independent second series covered the microarray data from 159 mammary carcinomas. These series revealed several cases with unambiguously

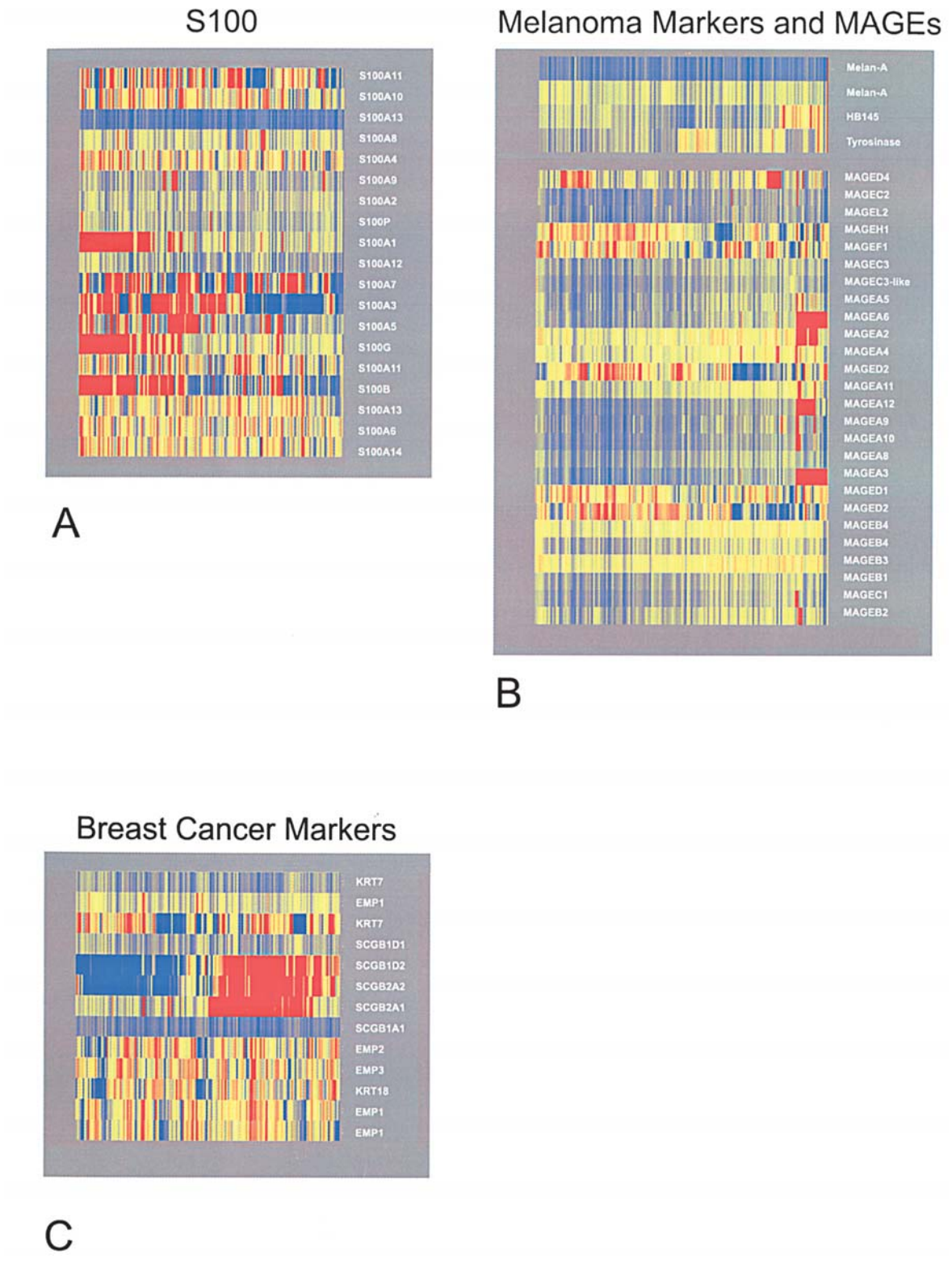

Figure 3. Microarray analysis of Melan A expression in breast tumors and breast cancer cell lines. Gene expression data of melanoma (S100, MAGEs, Melan A, GP100/HB145 and tyrosinas A) and breast cancer (mammaglobins, keratin 7 and 18 and epithelial membrane proteins) markers of 159 breast cancers (GEO dataset GSE1456) were extracted from Affymetrix HGU133A GeneChip data. Samples (columns) were clustered according to the expression levels of the genes in each group for better visualization. For several genes two or three probe sets were present on the chip. Expression levels are indicated by color (blue, low; yellow, intermediate and red, high expression). (A) S100 protein encoding genes. (B) Melanoma markers and melanoma associated antigens: Melan A, tyrosinase A, GP100 (HB145) and melanoma associated antigens (MAGEs). (C) Breast cancer markers: keratin 7 (KRT7); keratin 18, (KRT18); epithelial membrane proteins (EMP1, 2 and 3) and mammaglobins (secretoglobulins, SCGBs).

positive results for Melan A and other melanocytic markers. Eighteen of the 100 cases revealed focal to widespread Melan A-positive staining. This was statistically associated with poor tumor cell differentiation suggesting that lineage infidelity is a phenomenon of the loss of control of cellular differentiation. There was no statistical correlation between Melan A

expression and any other prognostic parameter suggesting that other tumor parameters; such as tumor size or expression of hormone receptors, are not correlated with lineage infidelity.

The analysis of 159 breast cancer samples by microarray for the presence of the melanocyte markers Melan A, melanoma-associated antigens, and S100-protein isoforms as well as epithelial markers such as diverse cytokeratins, epithelial membrane antigens and various mammaglobin isoforms, principally confirmed the immunohistochemical data. A single tumor showed strong overexpression of Melan A. This case was not exceptional in respect neither to clinical nor histopathological data. The other tumors showed variably low levels of Melan A expression. These tumors contained a small subpopulation of cells with a relatively high expression level of Melan A. As a consequence of the heterogeneity of the cell population within the tumor, the Melan A expression level of the tumor as a whole cannot adequately reflect the expression level of single cells or small cell groups.

The association between Melan A expression and differentiation grading suggests that lineage infidelity is closely linked to a reduction in cellular differentiation. Furthermore, we provided circumstantial evidence that this phenomenon occurs much more frequently than previously assumed. In consequence, the immunohistochemical identification of focal positivity for Melan A in otherwise (clinically) clear-cut breast carcinoma should not be interpreted as melanoma metastases. In summary, the identification of Melan A might prove an important marker for reduction in tumor cell differentiation.

Acknowledgements

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