Biochem. Lett., Vol. 7, PP. 23-41 (2011)

NEW MARKERS FOR BREAST CANCER

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ABSRACT

Background: The ductal carcinoma in situ (DCIS) of the mammary gland represents an early, pre-invasive stage in the development of invasive breast carcinoma. Since DCIS is a curable disease, it would be highly desirable to identify molecular markers that allow early detection. Mice transgenic for the WAP-SV40 early genome region were used as a model for DCIS development. Gene expression profiling was carried out on DCIS-bearing mice and control animals. Additionally, a set of human DCIS and invasive mammary tumors were analyzed in a similar fashion. Enhanced expression of these marker genes in human and murine samples was validated by quantitative RT-PCR. Besides, marker gene expression was also validated by immunohistochemistry of human samples. Furthermore in silico analyses using an online microarray database were performed. Results: In DCIS-mice seven genes were identified that were significantly up-regulated in DCIS: DEPDC1, NUSAP1, EXO1, RRM2, FOXM1, MUC1 and SPP1. A similar up-regulation of homologues of the murine genes was observed in human DCIS samples. Enhanced expression of these genes in DCIS and IDC (invasive ductal carcinoma) was validated by quantitative RT-PCR and immunohistochemistry. Conclusions: By comparing murine markers for the ductal carcinoma in situ (DCIS) of the mammary gland with genes up-regulated in human DCIS-samples we were able to identify a set of genes which might allow early detection of DCIS and invasive carcinomas in the future. The similarities between gene expression in DCIS and invasive carcinomas in our data suggest that the early detection and treatment of DCIS is of

utmost relevance for the survival of patients who are at high risk of developing breast carcinomas.

INTRODUCTION

Early diagnosis and administration of effective treatment is the best strategy to combat cancer [1]. Starting in the early 1980 s, the increasing use of mammography screens has resulted in an increase in diagnosis of the ductal carcinoma in situ (DCIS), especially among women more than 50 years of age [2]. DCIS represents 20-45% of all new cases of mammographically detected breast cancer, and about 10% of all breast carcinomas [3]. Up to 50% of DCIS lesions progress to invasive breast cancer, but there is tremendous variability in the time of progression to invasive disease [4]. Today most DCIS cases are identified as suspicious microcalcifications through mammography. However, the accuracy of mammography in diagnosing DCIS is suboptimal [4]. The main drawback with respect to DCIS is that mammography often underestimates both the pathologic extent of DCIS and the number of tumour foci in patients with multifocal disease [2]. Early detection of DCIS is very important because it is a highly curable disease, with a 10-year cancer-specific survival rate of over 97% [3]. Therefore, biomarkers for DCIS are needed. In many types of carcinomas, biomarkers have enhanced our ability for diagnosis, prognosis, and for therapy prediction. In general, an appropriate biomarker should be useful in defining risks and identifying the early stages of carcinogenesis. Furthermore, biomarkers can be analyzed in a noninvasive and economic way and therefore it is worth investing in the search for more biomarkers. [5]

The use of microarray technologies for gene expression profiling provides insight into the molecular basis of DCIS. Only a few gene expression profiling studies of DCIS have been published to date and most focus on the identification of progression-associated genes by comparison of in situ and invasive disease [6-8]. Gene expression profiling of DCIS is hindered by the limited numbers of samples available. To overcome the latter problem, our study used a transgenic mouse model for DCIS [9]. Mice were transgenic for the WAP-SV40 early genome region, so that expression of the SV40 oncogene is

activated by lactation. The use of these transgenic animals offers the possibility of determining tumor-initiating factors and investigating gene expression at different stages of tumor development. In the present work, we identified molecular markers for the ductal carcinoma in situ. Marker genes identified in the WAP-TNP8 mouse model were further investigated in a small human DCIS cohort. Identification of markers for DCIS and early invasive tumors' is important for early detection and the development of improved therapeutic strategies.

SUBJECTS AND METHODS

Human tissue:

Nineteen freshly frozen human breast tumor samples were obtained from the Robert-Rössle-Biobank at the ECRC (Experimental and Clinical Research Center). Tissue samples were cryopreserved immediately after surgery in liquid nitrogen and stored at -80°C. All participants have given written, informed consent. The study was approved by the local ethics committee (Charité Universitätsmedizin Berlin). The patient cohort consisted of nine DCIS, five invasive ductal carcinoma (IDC) and five healthy control samples obtained from patients with breast reduction surgery. A second panel consisting of human formalin-fixed paraffin-embedded (FFPE) tissue samples was used for immunohistochemical stainings. The panel consisted of 5 healthy, 10 DCIS and 5 IDC. DCIS samples were distinguished according to their grade (5 low grade DCIS/5 high grade DCIS). All samples were reviewed for histological classification according to nuclear grade and classified as low, intermediate, and high nuclear grade; additionally, the TNM Stage and hormone receptor status were determined [10].

RNA isolation, amplification and microarray analysis

RNA extraction from murine samples was performed using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) [11] with on column DNAse I digestion in accordance with the manufacturer's guide. Human RNA was isolated with RNeasy Lipid Tissue Mini Kit (Qiagen). RNA quality was checked on Agilent 2100 Bioanalyzer

(Agilent Technologies, Böblingen, Germany). For further analysis only samples with a RIN (RNA integrity number) of more than seven were taken. Two-round linear amplification, using 50 ng total RNA, was carried out for the murine samples according to the GeneChip® Two-Cycle Target Labelling protocol (Affymetrix, Santa Clara, CA, USA). In human samples cRNA was amplified from 1 μg of total RNA using the GeneChip® One-Cycle Target Labelling Kit (Affymetrix). Quantities of in vitro transcription and fragmentation products were assessed using the Agilent 2100 Bioanalyzer [12]. Labeled and fragmented cRNA was hybridized for 16 h at 45°C on Affymetrix oligonucleotide Murine Genome 430 2.0 or Human Genome U133 plus 2.0 Arrays. Hybridized arrays were scanned using the Gene-Chip Scanner 3000.

Statistical analysis:

An initial analysis was performed using the Affymetrix Microarray Suite 5.0 (MAS5) software. The percentage of present calls, background noise, the scaling factor, and the ratio of 3' to 5' hybridization for GAPDH and b-actin were used to assess quality of hybridization. Raw image data were converted to CEL files using the Affymetrix GeneChip Operating Software (GCOS).

RESULTS

Identification of murine DCIS markers Gene expression patterns of control samples, of samples taken at different time points after lactation, and of invasive breast tumor's (IDC) from 40 mice (five samples per group) were analyzed. Animals examined one month after activation of the oncogene were excluded from further analysis because of artifacts due to lactation. Histological investigations of all groups were performed. The majority of DCIS arises by month three or later. First a t-test was conducted comparing the control groups (wild type mice + mice before lactation) with mice taken two and three months after lactation. This comparison revealed 230 probe sets which are differentially expressed between control samples and mice in which the development of DCIS had already been induced. A second t-test was conducted in order to compare controls and invasive

mammary tumors. This procedure resulted in a list of 2398 probe sets which were differentially expressed between controls and invasive mammary tumors. To obtain tumor-specific genes that are already up-regulated in DCIS, only genes present in both lists were used for further analysis. A total of 173 probe sets met these criteria and were considered as potential candidate genes for early DCIS detection. These 173 probe sets cover 140 genes. In order to identify a minimal set of genes as final candidates, the distribution of the expression values of the 140 significantly changed candidate genes was investigated. Only genes showing a enhanced expression in the malignant samples were considered. Genes which showed constant up-regulation during DCIS-development and low variance within the groups were chosen as final marker genes. These are: MUC1, SPP1, RRM2, FOXM1, EXO1, NUSAP1 and DEPDC1. Using these seven genes for supervised hierarchical clustering allowed us to separate healthy control samples from all other samples. Again, the tumor samples clustered in the same branch as most of the samples of the late time points (3, 4 and 5 months). To confirm the microarray results, the expression of the seven marker genes was validated by quantitative RT-PCR (Figure 1A). Each group consisted of seven murine samples. Results confirmed very well the findings of the microarray analysis. A comparison of microarray and qRT-PCR box plots showed nearly identical pictures, hence only the RT-PCR results are shown here. With the exception of two cases, the expression of the marker genes was already significantly up-regulated two months after lactation, although in histological investigations almost no DCIS was found. In the case of FOXM1 and DEPDC1 up-regulation in month two was not significant, but that had changed by month three. In most of the genes there was a continuous increase of expression which reached the highest point in the IDC. Analysis of human DCIS samples As a next step we investigated the gene expression of human DCIS samples. To this end we used a set of 19 samples consisting of five healthy controls, five invasive tumors and nine DCIS samples. Expression profiles were recorded by Affymetrix U133 plus 2.0 GeneChips.An unsupervised hierarchical clustering of the human samples shows the healthy samples separated from the DCIS and IDC

samples. The DCIS samples showed a comparative expression profile similar to that of the invasive breast carcinomas (Data not shown). The human data were analyzed in the same fashion as the murine samples. However, we focused on the markers found already in the murine analysis. Statistical analysis revealed a strong up regulation of the seven previously identified marker genes in human DCIS as well. This led us to conclude that the marker genes can be used as early detection markers also for human DCIS. Hierarchical clustering using these seven genes showed that DCIS and invasive carcinomas were clearly separated from healthy samples. Within the malignant branch DCIS and invasive carcinomas could not be distinguished. Microarray results for the seven candidate genes described above were validated by quantitative PCR. Expression differences were highly significant between healthy controls and DCIS samples (Figure 1B). The most important reported functions of each of the seven marker genes are depicted. In order to further investigate the expression of these candidate genes at the cellular level in vivo, we performed immunohistochemical analyses in a panel of healthy human mammary gland tissue samples, DCIS and invasive breast tumors. To do so we used another set of formalin-fixed paraffin-embedded human tissue samples. For each protein multiple immunohistochemical staining were performed (five samples per group). For EXO1 no specific antibody was found. Immunoreaction of the marker genes in healthy tissues was negative or very weak. However, immunoreaction in DCIS and IDC samples in the majority of cases was very intense. The expression of the protein was indicated by pink staining (exemplarily see arrowhead). Positive staining was predominantly visible within the Lumina of the ducts, predominantly epithelial cells showed a positive signal (See arrows for examples). A positive staining was already visible in the low grade DCIS samples. The staining pattern was cytoplasmatic for SPP1, RRM2, FOXM1, DEPDC1 and NUSAP1. Membranous as well as cytoplasmatic staining was visible for MUC1.

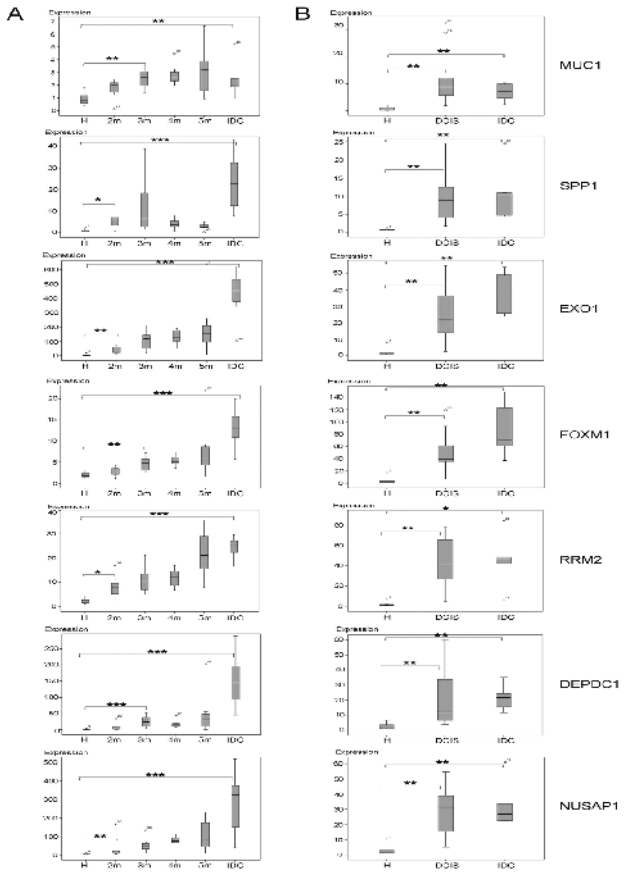

Figure 1 Validation of the marker gene expression by RT-PCR. Relative expression is shown in Box - Whisker - Plots. Gray columns show a 50% range of the data surrounding the median; black lines within each column mark the median; circles mark outliers. Significance was calculated with the Mann-Whitney-U test ($P <= 0.05$*, $P <= 0.01$**, $P <= 0.001$ three stars). A: Panel of the murine samples. Controls are transgenic mice before lactation (H). Months are calculated from the start of lactation (2 m = 2 months; 3 m = 3 months; 4 m = 4 months; 5 m = 5 months; IDC = invasive ductal carcinoma). Each group contains 7 samples. B: Panel of human samples. Controls are healthy tissues from reduction plastics (H).

DISCUSSION

The identification of gene expression signatures or molecular markers in DCIS is hindered by difficulties in obtaining sufficient numbers of frozen DCIS-samples from the hospital. Thus, we first approached the problem using a mouse model. We choose the WAP-TNP8 mouse model of Schulze-Garg et al. [9] because it is a well described model for DCIS and exhibits long latency in developing invasive tumors. This animal model has been used for detection of different tumor growth kinetics by flat-panel volume computed tomography [13], for the analysis of cell type-specific expression of Casein kinase 1 epsilon (CK1e) [14] and for a molecular imaging study of extra-domain-b fibronectin (EDB-FN) targeting neoangiogenesis by near-infrared fluorescence [15]. In our study, we used this model for determining tumor-initiating factors and investigating gene expression profiles at different stages of tumor development. Gene profiling was confirmed within two panels of human DCIS samples. A panel of fresh frozen human samples was used for another gene expression profiling analysis in order to verify whether the expression of the marker genes identified in the murine samples agrees with that found in the human samples. A second panel of human FFPE samples, including high but also low grade DCIS, was used for a validation of the expression of the candidate genes on the protein level. In this study, we identified seven marker genes which are overexpressed in DCIS and invasive carcinomas and allowed us to distinguish between healthy and DCIS samples. Our marker genes include MUC1, SPP1, RRM2, FOXM1, EXO1, NUSAP1 and DEPDC1. Some of these markers are already known to be related to DCIS; others are completely novel for DCIS and even for breast cancer. In the future, such molecular markers may allow an early detection of DCIS. Epithelial mucin 1 (MUC1) is an accepted serum tumor marker and cellular tumor antigen [16]. According to immunohistological studies MUC1 protein expression is particular high in tumors, where it undergoes changes in glycosylation and distribution [17]. However a low level of expression of MUC1 is also found in healthy, undifferentiated (non-lactating) breast tissue [18]. The correlation

between MUC1 expression and the clinical outcome of the patients is still under debate. While some in-vitro studies showed that MUC1 overexpression promotes cellular invasion [19,20] investigations of MUC1 expression of breast carcinomas have shown a better outcome for patients overexpressing MUC1 [21]. MUC1 was found to be commonly up-regulated in both DCIS and IDC [7]. Our results also confirmed earlier findings showing that MUC1 is also up-regulated on the protein level in DCIS [22]. Similarly, overexpression of Osteopontin (SPP1) has been found in a variety of cancers, including breast, lung, colorectal, stomach, ovarian cancers and melanoma [5, 23]. SPP1 is a phosphorylated glycoprotein secreted by several cell types, including those involved in bone turnover and cells of the immune system [5,24]. SPP1 has been associated with breast cancer progression, invasion and metastasis [24-29] and is present in elevated levels in the blood and plasma of some patients with metastatic cancers [5]. We have found SPP1 to be significantly up-regulated in DCIS. Previously, Reinholz et al. investigated the expression of SPP1 in normal, non-invasive, invasive and metastatic human breast cancer specimens by RT-PCR [30]. They showed that the mRNA level of SPP1 increased in non-invasive, invasive and metastatic breast tumour tissue compared to normal breast tissue. We found an increase in staining intensity for SPP1 in DCIS samples compared to healthy controls, which confirms a study by Oyama et al., who detected positive staining of SPP1 using immunohistochemistry on paraffin-embedded tissues in most cases of low-grade cribiform and high-grade comedotype ductal carcinoma in situ [31]. RRM2, a ribonucleotid reductase (RR), was shown to be overexpressed in human breast carcinoma tissue (DCIS) [32]. RR is responsible for the de novo conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates that are essential for DNA synthesis and repair [33,34]. RR consists of two subunits, M1 (RRM1) and M2 (RRM2). It is known that alterations in RR levels can have significant effects on the biological properties of cells, including tumor promotion and tumor progression. In our findings, RRM2 was significantly up-regulated on the RNA as well as on the protein level. Likewise, the transcription factor forkhead box M1 (FOXM1) was found to be differentially expressed in most solid tumors [35]. FOXM1

stimulates proliferation and cell cycle progression by promoting entry into both S-phase and mitosis. In addition, it plays a role in the proper execution of mitosis. FOXM1 is implicated in the tumourigenesis of more than 20 types of human tumors and contributes to both tumor initiation and progression [36]. FOXM1 is broadly expressed in breast epithelial cell lines and seems to be significantly increased in transformed breast epithelial cell lines. Consistently, FOXM1 expression is specifically elevated in breast carcinomas [37]. Using immunohistochemistry, Bektas et al. analysed FOXM1 expression in human invasive breast carcinomas and normal breast tissues on a tissue microarray [38]. In contrast to what could be expected from GO-analysis, they found a strong cytoplasmatic expression of the transcription factor FOXM1, resulting most likely from its strong overexpression. Additionally, using RT-PCR, FOXM1 was found to be overexpressed in breast cancer in comparison to normal breast tissue both on the RNA and protein level. Furthermore, FOXM1 was found to be overexpressed during progression from DCIS to invasive breast cancer [7]. Our findings confirm these results. FOXM1 was significantly overexpressed already on the DCIS level and was even higher expressed in IDC. In contrast, overexpression of EXO1, NUSAP1 and DEPDC1 in IDC and DCIS had not yet been described. We found these genes significantly up-regulated in DCIS as well as in IDC. EXO1 (exonuclease 1) has been implicated in a multitude of eukaryotic DNA metabolic pathways that include DNA repair, recombination, replication, and telomere integrity. This makes EXO1 a logical target for mutation during oncogenesis [39]. However, Rassmussen et al. have shown high expression levels of human EXO1 transcripts in liver cancer cell lines and in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue [40]. This is a first hint that EXO1 is up-regulated in tumors. Nucleolar spindle-associated protein (NUSAP1) was identified in 2003 as a novel 55-kD vertebrate protein with selective expression in proliferating cells [41]. mRNA and protein levels of NUSP1 peak at the transition of G2 to mitosis and abruptly decline after cell division. Interestingly, NUSAP1 was found to be up-regulated in melanoma cells by gene expression profiling of a series of melanoma cell lines

[42]. Proteins such as NUSAP that show little or no expression in G1 and G0 may be reliable histochemical markers for proliferation and might therefore be useful for cancer prognosis [41]. NUSAP1 expression was significantly increased in DCIS and IDC in our study and is therefore a promising new tumor marker. DEPDC1 (DEP domain containing 1) is also a newly detected gene. Kanehira et al. identified DEPDC1 as a novel gene that is highly overexpressed in bladder cancer samples, but not expressed in any human organs (heart, liver, kidney, and lung) except the testis [43]. Our findings show that DEPDC1 is significantly up-regulated in DCIS and IDC. Preliminary results from a study of the functional relevance of DEPDC1 show that it seems to be an important gene for proliferation as well as for migration and invasion (C.S. manuscript in progress). We found that the seven putative marker genes are strongly up-regulated in mice and in human DCIS samples. This reveals that the mouse model we used reflects human breast cancer development. Previously, Klein et al. [44] compared the expression profile of 24 human breast tumours and six WAP-SVT/t mice breast tumors. They found 597 genes which are overexpressed in breast cancer in mice [44]. Their list also contains DEPDC1, NUSAP1, MUC1, EXO1, and RRM2. Some of our marker genes have been described previously in human breast cancer. In a 22-gene signature investigated by Martin et al. [45], FOXM1 and RRM2 were included. This signature accurately predicts breast cancer outcome [45]. Additionally, Ma et al. developed a gene expression index for tumor grade in breast cancer patients which included RRM2 [6]. This is further evidence that the candidate genes we identified are important in tumour development. Candidate genes were further validated using Oncoming http://www.oncomine.org, a database for online cancer gene expression analysis. In the data set of Richardson et al. which compared normal breast tissue with IDC, six of our seven marker genes are significantly up-regulated in IDC [46]. Additionally, also using Oncoming to search for the tumor grade and the prognostic impact, we found that all the marker genes except MUC1 were significant for prognosis in the calculation of this database. Using a p-value of 0.001 these genes are up-regulated in multiple expression analyses in patients with a poor prognosis. This is an indication that

our panel of marker genes could also be useful as a prognostic tool. Looking at the tumor grade, all the genes except MUC1 and SPP1 were significantly up-regulated in samples with a high tumor grade in Oncomine. Thus, the marker genes might indicate a high grade of malignancy. One explanation for this could be that in the analysis of the human samples, we used predominantly samples with a high tumor grade. On the other hand, in the case of the murine samples, the specimens we investigated were from a very early time point, where no DCIS (or few) were pathologically found. In accordance with recent gene expression studies, our data support the hypothesis that critical molecular events which have a profound influence on development, progression and outcome of human breast cancer occur at an early stage. Despite significant morphologic differences between the different stages, expression profiles of early lesions are highly similar to the more advanced, invasive lesions [47]. This has been demonstrated also on the protein level [48]. Sorlie et al. claimed that extensive studies of DCIS and other pervasive stages of tumors will enhance this hypothesis and substantiate the value of gene expression-based classification in the prognosis of breast cancer at an early stage [49]. Furthermore Ma et al. [50] showed that the tumor microenvironment of invasive breast tumors also participates in tumourigenesis even before tumor cells invade into stroma. This is a further hint that changes during breast cancer development occur at a very early time point and that also the tumor microenvironment plays an important role in the transition from pervasive to invasive growth. We took a step in this direction by showing on the RNA level as well as on the protein level that the marker genes we found are already significantly up-regulated on the level of DCIS and likewise later on the IDC level.

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