Research Article

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Identification of Early Molecular Markers for Breast Cancer

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Abstract

Background: *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%. The biomarkers and immunehistochemical markers (Osteopontin OPN) SPP1, MUC1 (Epithelial Mucin 1), RRM2 (Ribonucleotidereductase), FOXM1 (Forkhead box M1) DEPDC1 (DEP (Disheveled, EGL-10, and Pleckstrin) domain-containing protein), Nucleolar spindle-associated protein (NUSAP1), EXO1 (Exonuclease 1), are expressed strongly right from DCIS and thereby with early diagnosis of this preinvasive condition with progression to invasive carcinoma, improves the prognosis of breast cancer.*

Aim: *To study the markers (Osteopontin OPN) SPP1, MUC1 (Epithelial Mucin 1), RRM2 (Ribonucleotidereductase), FOXM1 (Forkhead box M1) DEPDC1 (DEP (Disheveled, EGL-10, and Pleckstrin) domain-containing protein), Nucleolar spindle-associated protein (NUSAP1), EXO1 (Exonuclease 1 by immunohistochemistry for early detection and prognostication of breast Cancers.*

Methods: *This is a prospective case control study in 50 females with mammographically suspected and FNAC and biopsy proven Ductal Carcinoma in situ (25 cases) and infiltrating ductal carcinoma (25 cases) with 50 healthy control samples. The paraffin blocks of the tissue sections were stained with hematoxylin and eosin evaluated by light microscopy and immunohistochemistry performed with DEPDC1, NUSAP1, EXO1, RRM2, FOXM1, MUC1, SPP1 (Osteopontin).*

Observations: *In DCIS and invasive ductal carcinoma the immunohistochemical marker expression of DEPDC1, NUSAP1, EXO1, RRM2, FOXM1, MUC1 and SPP1are increased as compared to healthy controls where there is absence of any kind of staining for these IHC markers except for some exceptional case where there is weak positive staining.*

Conclusions: *These IHC markers aid in early detection of DCIS and invasive carcinomas as these markers are not expressed in healthy control tissue. The similarities between antigen expression as evidenced by immunohistochemistry in DCIS and invasive carcinomas in our data suggest that the early detection and treatment of DCIS progressing to invasive carcinoma by detection of biomarkers by immunohistochemistry is of utmost relevance for the survival of patients who are at high risk of developing breast carcinoma.*

Keywords: DCIS, EXO1, RRM 2, Invasive Ductal Carcinoma.

Introduction

Ductal Carcinoma in Situ (DCIS) represents 20-45% of all new cases of mammographically detected breast cancer, and about 10% of all breast carcinomas. Up to 50% of DCIS lesions progress simultaneously or coexist with invasive breast cancer, but there is variability in the time of progression to invasive disease [1-2]. There are few biomarkers which can help as predictive marker in DCIS and cancer progression. Osteopontin OPN (SPP1) is a phosphorylated glycoprotein found in all body fluids, extracellular matrix components, and proteinaceous matrix of mineralized tissues. This protein is found to be overexpressed in tumor cells and may be a useful predictor of patient outcome in breast cancer, and OPN may play a functional role in tumour progression and aggressiveness [3-4]. Epithelial mucin1 (MUC1) is an accepted serum tumor marker and cellular tumor antigen [5]. MUC1 protein expression is particularly high in tumors, where it undergoes changes in glycosylation and distribution. Ribonucleotidereductase (RR), RRM2, is overexpressed in breast carcinoma [5]. RR is responsible for the de novo conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates that are essential for DNA synthesis and repair. 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. RRM2 is significantly up-regulated on the RNA as well as on the protein level. FOXM1 expression is specifically elevated in breast carcinomas. DEP

(Disheveled, EGL-10, and containing DEP domain regulate a broad range of cellular functions including a large number of signaling proteins. Nucleolar spindle-associated protein (NUSAP1) was identified in 2003 as a novel 55-kD vertebrate protein with selective expression in proliferating cells, mRNA and protein levels of NUSP1 peak at the transition of G2 to mitosis and abruptly decline after cell division. 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. NUSAP1 expression was significantly increased in DCIS and IDC in our study and is therefore a promising new tumour marker.

Aims and Objectives

Detection of immunohistochemical markers Osteopontin (OPN) SPP1, MUC1 (Epithelial Mucin 1), RRM2 (Ribonucleotidereductase), FOXM1 (Forkhead box M1), DEPDC1 (DEP (Disheveled, EGL-10, and Pleckstrin) domain-containing protein), Nucleolar spindle-associated protein (NUSAP1), EXO1 (Exonuclease1), for early detection and prognostication of breast Cancers.

Materials and Methods

50 women reporting with DCIS and suspected breast Cancer at Command Hospital Kolkata in 2014-2016 were enrolled in the study. The patients were divided into 2 groups based upon the clinical suspicion and presenting complaints and examination. Group I-patients with DCIS n =25 patients, Group II-patients with biopsy proven infiltrating ductal carcinoma n=25. Group III Normal controls with no suspected malignancy n=50. The patients had a median age of 54 years (range 18–70 years).The mean follow-up time was 24 months (range 7–18 months). Thin paraffin sections (2-4 μm) were stained with haematoxylin and eosin according to standard procedures and histomorphologically evaluated by light microscopy. A semi-quantitative scoring system was used for the evaluation of the immunohistochemical staining and is explained as- = no immunoreactive cells, + = 1-5 immunoreactive cells, ++ = 5-10 immunoreactive cells, +++ = 10-100 immunoreactive cells, ++++ = >100 immunoreactive cells. Average number of cells per high power field is given, 5 high power fields were evaluated. Immunoreaction of the marker genes in healthy tissues was negative or positive in 4 cases out of 50 with IHC SPP1, MUC1, RRM2, EXO1, positive in 2 cases with IHC FOXM1, NUSAP1 and in 6 cases with DEPDC1. However, immunoreaction in DCIS and IDC samples with IHC marker for was very intense and was +++ (10-100 immunoreactive cells) or ++++ = (>100 immunoreactive cells) in most of the cases except few as enumerated in the table below. The expression of the protein was indicated by brown staining. Positive staining was predominantly visible within the lumina of the ducts, predominantly epithelial cells showed a positive staining. The staining pattern was nuclear and cytoplasmic for SPP1, RRM2, FOXM1, DEPDC1 and NUSAP1 and membranous as well as cytoplasmic staining was visible for MUC1.All p-values are two-sided and 0.05 was used a significance level with a 95 percent confidence interval (95% CI). All analyses were performed using SPSS Statistics Version 22. For the sake of readability, the prefix "log" is not used in the text or graphs when referring to these markers. Chisquare test used to estimate significant associations in non-parametric statistics. Statistical significance level was set at $p < 0.05$.

Observations & Results

The cases of suspected malignancies and the normal controls were notified in a proforma noting all relevant details. The following details were noted-Age, parity, menstrual history, personal history, past history, family history, occupational and drug history. Analysis of the immunophenotype of the ductal carcinoma in situ and invasivecarcinoma of the breast was performed on 50 patients with a complete set of IHC markers SPP1(Osteopontin), RRM2, FOXM1, DEPDC1, NUSAP1 and MUC1.However, immunoreaction in DCIS and IDC samples in the majority of cases was very intense. Positive staining was predominantly visible within the lumina of the ducts, predominantly epithelial cells showed a positive staining. The staining pattern was nuclear and /or cytoplasmic for SPP1, RRM2, FOXM1, DEPDC1, MUC1 and NUSAP1. Membranous/ and cytoplasmic staining was visible for EXO1.

Table 1: Staining pattern of the immunohistochemical analysis of number of cases in healthy controls n=50 and 12 cases of low grade DCIS, 13 High grade DCIS and 25 invasive carcinomas. Samples using a semi-quantitative scoring system in DCIS and IDC.

IHC	Healthy control n=50	Low grade ductal carcinoma in situ(12 cases)	High grade ductal carcinoma in situ(13 cases)	Invasive ductal carcinoma (25 cases)
(Osteopontin OPN)(SPP),	Negative 46 Positive +4	Positive +++ 11 Negative - 1	Positive+++ 12 Negative 1	Positive +++ 24 Negative 1
MUC1(Epithelial Mucin 1),	Negative 46 Pos +4	Positive +++ 11 Negative - 1	Positive+++ 12 Negative 1	Positive +++ 25 Negative 0
RRM2(Ribonucleotidereductase),	Negative 46 Pos +4	Positive +++ 11 Negative - 1	Positive+++ 12 Negative 1	Positive +++ 24 Negative 1
FOXM1(Forkhead box M1)	Negative 48 Pos +2	Positive +++ 11 Negative - 1	Positive+++ 12 Negative 1	Positive +++ 25 Negative 0
DEPDC1(DEP(Disheveled, EGL-10, and Pleckstrin) domain-containing protein),	Negative 44 Pos +6	Positive +++ 11 Negative - 1	Positive+++ 12 Negative 1	Positive +++ 24 Negative 1
Nucleolar spindle-associated protein (NUSAP1) ,	Negative 48 Pos +2	Positive +++ 11 Negative - 1	Positive+++ 12 Negative 1	Positive +++ 25 Negative 2
EXO1 (Exonuclease 1)	Negative 46 Pos 4	Positive +++ 11 Negative - 1	Positive+++ 12 Negative 1	Positive +++ 24 Negative 1

Table 2: Statistical correlation (chi square value and p value) in DCIS and IDC.

IHC	Healthy controls n=50	Low grade ductal carcinoma in situ (12 cases),High grade ductal carcinoma in situ(13 cases) Invasive ductal carcinoma (25 cases)Total 50 cases	Statistical analysis		P Value	Chi square value, Positive predictive value PPV, Negative predictive value. NPV
(Osteopontin OPN) (SPP),	Negative46 Positive +4	Positive +++ 47 Negative 3	Alpha df P-value Test Statistic Critical Value	0.05 1 5.62 E-13 51.977136 3.84145882	<0.5	Chi square 51 PPV 95% NPV 88%
MUC1(Epithelial Mucin 1),	Negative 46 Pos +4	Positive +++ 48 Negative 2	Alpha df P-value Test Statistic Critical Value	0.05 1 2.52E-14 58.08 3.84145882	<.05	Chi square value 58 PPV 96% NPV 92%
RRM2 (Ribonucle-otidereductase)	Negative 46 Pos +4	Positive +++ 47 Negative 3	Alpha df P-value Test Statistic Critical Value	0.05 1 1.61E-13 54.4250392 3.84145882	<0.5	Chi square 54 PPV 95% NPV 88%
FOXM1(Forkhead box M1)	Negative 48 Pos +2	Positive +++ 48 Negative 2	Alpha df P-value Test Statistic Critical Value	0.05 1 2.97E-15 62.2841444 3.84145882	<0.5	Chi square 62 PPV 97% NPV 92%
DEPDC1	Negative 44 Pos +6	Positive +++ 24 Negative 1	Alpha df P-value Test Statistic Critical Value	0.05 1 1.23E-12 50.43 3.84145882	<0.5	Chi square value 50 PPV 94% NPV 88%
(NUSAP1) ,	Negative 48 Pos +2	Positive +++ 48 Negative 2	Alpha df P-value Test Statistic Critical Value	0.05 1 2.97E-15 62.2841444 3.84145882	<0.5	Chi square 62 PPV97 NPV 92
EXO1 (Exonuclease 1)	Negative 46 Pos 4	Positive +++ 47 Negative 3	Alpha df P-value Test Statistic Critical Value	0.05 1 1.61E-13 54.4250392 3.84145882	<0.5	Chi square 54 PPV95 NPV 88

Discussion

In our study we found strong immunohistochemical expression (nuclear and or cytoplasmic positivity) of DEPDC1 in ductal carcinoma in situ and invasive carcinoma. Man Yicun and Kanehira M et al studied expression of the DEPDC1 gene in five breast cancer cell lines and indicated a relationship between DEPDC1 and aggressiveness of breast cancer cells [4,5]. It has been established with this study that DEPDC1 expression is associated with different breast cancer cell lines, we observed that high expression of DEPDC1 was associated with a more malignant behavior with metastases in breast cancer. Epithelial mucin 1(MUC1) is an accepted serum tumor marker and cellular tumor antigen [6-9]. According to immunohistological studies. B Van Der Vegt studied the expression pattern of MUC1 and found its expression related to tumour characteristics and clinical outcome of invasive ductal breast carcinoma [10].This has been demonstrated also on the protein level. Increased expression of osteopontin in patients with triple-negative breast cancer [11]. In this study, MUC1 was found to be up-regulated in both grades of DCIS and IDC in our study.Wang X1 and Reinholz et al studied increased expression of osteopontin in patients with triple-negative breast cancer and other workers and studied the correlation of osteopontin expression with breast cancer progression and metastases and found significant correlation as we have found in our study with osteopontin strong cytoplasmic expression in ductal carcinoma in situ and invasive ductal carcinoma [12-22]. In our study RRM2 levels were significantly correlated with ductal carcinoma in situ and invasive breast cancers ($p < 0.001$). Zhang et al found that RRM2 was significantly related to aggressiveness and associated with poor outcome in breast cancers especially in ER-negative breast cancer [23]. In our findings, RRM2 was significantly upregulated as expressed by immunohistochemistry in DCIS and IDC [24]. Using immunohistochemistry, Bektas et al. analysed FOXM1 expression in human invasive breast carcinomas [25]. They found a strong cytoplasmic expression of the transcription factor FOXM1, resulting most likely from its strong overexpression.

Rassmussen et al have shown high expression levels of human EXO1 transcripts in cancer cell lines, but not in the corresponding non-neoplastic tissue showing that EXO1 is up-regulated in tumors [26]. Nucleoli spindle-associated protein (NUSAP1) was identified in 2003 as a novel 55-kD vertebrate protein with selective expression in proliferating cells with 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. 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. NUSAP1 expression was significantly increased in DCIS and IDC in our study and is therefore a promising new tumor marker.

Conclusions

We found seven immunohistochemical markers DEPDC1, NUSAP1, EXO1, RRM2, FOXM1, MUC1 and SPP1 which are strongly expressed at a very early stage of premalignancy and preneoplasia that is ductal carcinoma in situ(DCIS) and is a preinvasive condition of infiltrating ductal carcinoma which is invasive breast carcinoma. Using a p-value of 0.001 these markers corresponding to genes are upregulated in multiple expression analyses in patients of DCIS which progress to invasive carcinoma whether low or high grade being not the criteria. In DCIS and invasive ductal carcinoma the immunohistochemical marker expression of DEPDC1, NUSAP1, EXO1, RRM2, FOXM1, MUC1 and SPP1 are increased. The IHC markers aid in early detection of DCIS and progression to invasive carcinomas and these are not expressed in healthy controls. The similarities between antigen expression as evidenced by immunohistochemistry in DCIS (whether low or high grade 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. With the aid of these immunohistochemical marker which are not very expensive the progressive disease from DCIS to invasive carcinoma is diagnosed as these are mostly negative in healthy controls with significant p value ($p<0.05$).

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