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Differential Expression of Glu-Tubulin in Basal-Like Phenotype Carcinoma of the Mammary Gland

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ABSTRACT

High levels of tubulin expression have been described in a variety of human malignant tumors, and glu-tubulin, in which the C-terminal tyrosine of α-tubulin is removed by tubulin carboxypeptidase. Over-expression has been reported in the malignant tumors of the mammary gland and correlated with poor prognosis immunohistochemically. Furthermore, Nielsen *et al*. proposed that the use of a panel of four markers (ER, HER 2, CK 5/6, and EGFR) could accurately identify basal-like phenotype carcinoma (BPC) with widely available standard pathologic tools. In our study, major prognostic factors such as patient age, tumor size, histological grade, axillary lymph node metastasis, vessel invasion, and local recurrence in BPC were not significantly different from non BP carcinoma (NBPC). However, the BPC group showed a higher ratio of distant metastasis than that of the NBPC group. In triple-negative carcinoma (TNC) cases, staining for glu-tubulin was observed in 46 cases (63.8%), which consisted of 42 of the 58 BPC patients (72.4%) and 4 of the 14 NBPC patients (28.6%). A significant association was found between the expression of glu-tubulin and BPC, but not NBPC. It seems that our findings also agree with the observation that BPC exhibits aggressive biological behavior and increases the content of glu-tubulin, which plays a greater role in migration and invasion.

Keywords: Glu-Tubulin; Basal-Like Phenotype Carcinoma; Mammary Gland

1. Introduction

Microtubules, which consist of $\alpha\beta$-tubulin heterodimer, play a role in mitosis, cell motility, intracellular transport, and the maintenance of cell shape [1]. High levels of tubulin expression have been described in a variety of human malignant tumors, including ovary, colon, lung, prostate, and breast carcinomas [2-6]. Furthermore, glu-tubulin, in which the C-terminal tyrosine of α-tubulin is removed by tubulin carboxypeptidase, overexpression has been reported in the malignant tumors of the mammary gland and correlated with poor prognosis immunohisto-chemically [7]. We previously showed that the majority of malignant tumors expressed the tubulin protein relative to benign tumors when breast tumor expression of glu-tubulin was evaluated immunohistochemically [8].

Histopathologic studies of triple-negative carcinoma (TNC) of the breast, which is characterized by a lack of estrogen receptor (ER) and progesterone receptor (PgR)

expression and the absence of HER 2 protein overexpression, indicated that these tumors, which are commonly treated with hormonal therapy and anti-HER 2 antibody (trastuzumab) therapy, are mostly unresponsive and typically have a poor prognosis [9]. Furthermore, Nielsen *et al*. suggested that the use of a panel of four markers such as ER, HER 2, CK 5/6, and epidermal growth factor receptor (EGFR) could accurately identify basal-like tumors with widely available standard pathologic tools [10]. They classified HER 2-positive tumors as an HER 2 subtype, ER-positive tumors as a luminal subtype, tumors with negative ER, negative HER 2, and positive CK 5/6 or positive EGFR as a basal-like phenotype carcinoma (BPC), and tumors that were negative for all these four markers as an undetermined subtype. This panel showed a high sensitivity and specificity for the identification of breast carcinomas with BPC and we also adopted these criteria in our definition of BPC. Several reports have shown that BPC is a poor prognostic factor in breast carcinoma [11-13]. Thus, TNC can be classified into two subtypes: BPC

and non-BPC (NBPC), and analyses should to be performed accordingly. As glutubulin is also present in the stable microtubules of malignant tumors with aggressive behavior, we speculated that glu-tubulin expression levels would differ between BPC and NBPC. In this study, we examined BPC in TNC patients and evaluated the biological behavior and impact of the glu-tubulin marker in immunohistochemical evaluations.

2. Materials and Methods

Materials

The patients included in this study were 554 patients with primary breast carcinoma who were treated at the Department of Pathology, Dokkyo Medical University Koshigaya Hospital between 2005 and 2011 for which paraffin blocks were available. For each case, all available hematoxylin and eosin—stained sections were reviewed to confirm the diagnosis of mammary disease by three observers who had no knowledge of either prior histological results or clinical outcomes and selected a representative block for immunostaining [14]. The sources, dilutions, and cutoff values of antibodies are listed in **Table 1**. Specimens were judged to be positive for ER or PgR when at least 1% of the tumor cells stained positively. HER2 was judged on the basis of a staining score range of 0 - 3 by the Hercep test, with a score of 0 or 1 rated as negative and a score of 2 or 3 as positive. Based on these results, 72 of the 554 patients (12.9%) were judged to have triple negative carcinoma (TNC).

3. Immunohistochemical Staining for Basal Marker (CK 5/6), Epidermal Growth Factor Receptor, and Glu-Tubulin

CK 5/6 and EGFR were carried out to classify the 72 TNC cases as BPC or NBPC. Cases were considered CK 5/6 and EGFR positive when at least 10% of the tumor cells showed membrane staining. TNC was judged to be BPC when the specimen was found to be positive for either CK 5/6 or EGFR. TNC found to be negative for

Table 1. Sources, dilutions, and cutoff values of the antibodies used in this study.

Antibody	Clone	Manufacturer	Dilution	Cutoff values
ER	SP1	Dako Cytomation	Prediluted	$\geqq 10\%$ (positive)
PgR	1E2	Dako Cytomation	Prediluted	$\geqq 10\%$ (positive)
HER 2	4B5	Dako Cytomation	Prediluted (Hercep test)	0.1 + (negative)
CK 5/6	D 5/16 B4	Nichirei Biosciences	Prediluted	$\geqq 10\%$ (positive)
EGFR	31G7	Nichirei Biosciences	Prediluted	$\geqq 10\%$ (positive)
glu-tubulin	polyclonal	Cosmo Bio	1:500	$\geqq 10\%$ (positive)

these markers was diagnosed as NBPC. Based on these markers, 58 of the 72 TNC patients were judged to be BPC and 14 were NBPC. Glu-tubulin was evaluated by immunohistochemical analysis using a specific antibody [15-18]. Five-micrometer-thick unstained sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series (100% - 50%). After antigen retrieval with L.A.B. solution (Polyscience, Inc., Warrington, PA) for 20 min at 60°C, endogenous peroxidase activity was quenched by exposure to 5% H_2O_2 overnight at room temperature. Sections were then permeabilized with phosphate-buffered saline containing 0.5% Triton X-100 for 6 min. After washing twice with phosphate-buffered saline containing 0.3% Triton X-100 for 10 min, unspecific antibody binding was blocked with 1% goat serum for an hour. Sections were then incubated for 2 days at 4°C with a rabbit polyclonal anti-glu-tubulin antibody (Cosmo Bio, Tokyo, Japan) at 1:500 dilutions. After washing, sections were further processed with a Histofine SAB-PO(R) kit (Nichirei Corp., Tokyo, Japan) following the manufacturer's instructions. A color reaction was developed with 3, 3'-diaminobenzidine. After counterstaining with hematoxylin, sections were dehydrated, mounted with mount-quick (Daido Sangyo Co. Ltd., Tokyo, Japan), and analyzed using an Olympus BX 51 microscope. Nerve fibers known to express high levels of glu-tubulin were used as positive controls and were included in each staining run. Cases were considered glutubulin-positive if cytoplasmic and/or membranous staining was observed in more than 10% of the cancer cells.

4. Comparison of Clinicopathological Findings for BPC and NBPC

Patients were classified as BPC or NBPC, and these two disease groups were compared in relation to the following clinicopathological parameters: age, tumor diameter, histological grade, lymph node status, vessel invasion status, local recurrence, distant metastasis, overall survival in months, and glu-tubulin overexpression.

5. Statistical Methods

All immunostains were initially reviewed and scored by 2 pathologists. A χ^2 test was used to compute the statistical association and a P value of 0.05 was considered significant. The Kaplan-Meier method was used to calculate the overall survival in months and the survival rates. The log-rank test was used to test for significance, and a P-value of <0.05 was considered to indicate significance. Statistical analysis was carried out using Excel 2012 software.

6. Results

The histological and clinical characteristics of the BPC

and NBPC cases identified in this study are summarized in **Table 2**. The ages of the 58 BPC cases, ranged from 28 to 87 years (mean, 57 years). The mean tumor size was 2.7 cm at the maximum diameter. The ages of the14 NBPC cases, ranged from 44 to 84 years (mean, 59 years). The mean tumor size was 2.4 cm at the maximum diameter. Major prognostic factors such as patient age, tumor size, histological grade, axillary lymph node metastasis, and vessel invasion were not significantly different between BPC and NBPC cases [14]. Local recurrence was found in 16 TNC patients, which consisted of 14 of the 58 BPC patients (24.1%) and two of the NBPC patients (14.3%). The incidence of local recurrence was higher in the BPC group than in the NBPC group, but this difference was not significant. In addition, the BPC group showed a significantly high ratio of visceral metastases, such as to the brain and lungs (**Table 2**). Complete follow-up data were available in 66 of the 72 patients diagnosed with TNCs. Of these patients, 11 died of the disease after the initial diagnosis of a primary lesion and 55 patients were alive 5 years after the initial diagnosis. These patients consisted of 9 patients from the BPC group and two patients from the NBPC group, but the difference was not significant.

In TNC cases, staining for glu-tubulin was observed in 46 cases (63.8%) (**Table 3**), consisting of 42 of the 58 BPC patients (72.4%) and 4 of the 14 NBPC patients (28.6%). The staining pattern of breast carcinoma was predominantly characterized by the cytoplasmic staining of epithelial cells (**Figure 1**). In most tumors, some areas also showed cell-cell boundary staining. Only 26 cases showed no significant staining. A significant association
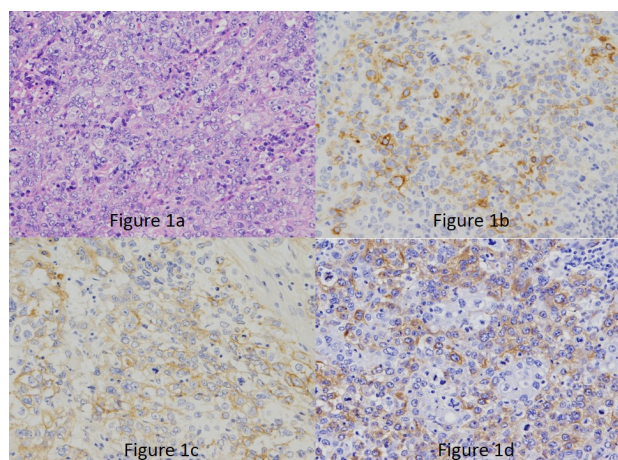

Figure 1. (a): Basal-like phenotype carcinoma (BPC) showing a high-grade invasive ductal carcinoma not otherwise specified, composed of sheets of marked pleomorphism (H&E 200×); (b): Cytokeratin 5/6 (200×); (c): EGFR (200×); (d): carcinoma with strong and diffuse cytoplasmic reactivity of tumor cells to the glu-tubulin antibody scored as positive (200×).

Table 2. The relationship between histological carcinoma characteristics.

	TNC (72)	BP (58)	NBP (14)	P value
Age				
mean	58	57	59	
≦ 50	23	19	4	
>50	49	39	10	0.9858
Tumor size				
mean	2.6	2.7	2.4	
≦ 2	33	28	5	
>2	39	30	9	0.3972
Histological grade				
I	2	1	1	
II	6	4	2	0.3432
III	64	53	11	
Lymph node status				
Negative	38	30	8	
Positive	34	28	6	0.7155
Vessel invasion				
Negative	39	32	7	
Positive	33	26	7	0.7274
Local recurrence				
Negative	56	44	12	
Positive	16	14	2	0.6616
Distant metastasis				
Brain	3	3	0	
Lung	4	4	0	
Liver	1	1	0	0.0405
Bone	3	3	0	
Other lymph node group	3	3	0	

Table 3. Relationship between glu-tubulin expression and intrinsic subtypes.

		BP	NBP	P value
Glu-tubulin	negative	16	10	0.0006
	positive	42	4	

BP: Basal-like Phenotype NBP: Non-Basal-like Phenotype.

was found between the expression of glu-tubulin and BPC, but not NBPC.

7. Discussion

Recent studies using cDNA microarray technology and unsupervised cluster analysis have provided new insights into the classification of invasive breast cancers. These studies have resulted in the identification of several breast cancer subgroups that differ in their gene expression signatures and clinical course [19-22]. The majority of BPs lack ER, PgR, and HER 2 expression, and several

reports have adopted a TNC definition for BPCs, an approach that is convenient in routine practice. However, it should be noted that an overlap between TNC and BPC exists and gene expression cannot be applied to clinically available formalin fixed, paraffin-embedded tissues. This method is relatively expensive and has not been established in routine management. On the basis of the above reason, a convenient method to identify these BPCs in pathological specimens would involve certain markers for immunohistochemical analysis. Several immunohistochemical analysis surrogates have been proposed for the routine identification of BPC. Nielsen *et al.* proposed an immunohistochemical analysis surrogate based on four markers (ER, HER 2, CK 5/6, and EGFR) and we arranged and adopted these criteria for the definition of BPC [10]. As a result, in our study, among the 72 cases of TNCs, 58 cases completely overexpressed CK 5/6 or EGFR and had a possible classification of BPC, which was true for as the majority of TNCs based on the simple analysis of ER, PR, and HER 2 [23]. Major prognostic factors such as patient age, tumor size, axillary lymph node metastasis, vessel invasion, and local recurrence were not significant. Furthermore, there were no significant differences in the histological grade corresponding to tubule formation, nuclear pleomorphism, and mitotic counts. However, for our present patient series, distant metastasis was clearly significant in BPC patients. This result indicates that BPC has a different biological behavior from NBPC with no clear morphologic features.

A preliminary limited survey of previous reports indicated the differential expression of tubulin in several types of carcinomas [2-7]. Moreover, in human breast cancers, the accumulation of glu-tubulin has been correlated with poor prognosis using an immunohistochemical approach [7]. In this report, we evaluated the glu-tubulin expression of a large number of breast cancer cases to examine the hypothesis that glu-tubulin expression may be significantly different between BPC and NBPC. Numerous studies have investigated microtubules in cell migration, and a form of stabilized tubulin was shown to accumulate in the microtubules of migrating cells [24-26]. Stable microtubules such as glu-tubulin increase cell migration because they act as a directed membrane and are involved in cell-organ transportation of the cell, regulating adhesion and contraction [27,28]. In previous reports, BPC demonstrated a specific pattern of distant metastasis with a high ratio of visceral metastases, such as to the brain and lung [29,30]. The mechanism leading to this result is still unclear. However, BPC has a higher ratio of distant metastasis than NBPC and it seems that those findings are compatible with the observation of increases in the content of glu-tubulin, which plays a role in migration and invasion.

In summary, we reported glu-tubulin expression in a series of invasive breast carcinomas of distinct subclasses such as BPC and NBPC based on immunohistochemical staining. Considering the fact that biological behavior and glu-tubulin expression is obviously different between BPC and NBPC, may need to be established different pathological and clinical strategies for TNC patients.

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