High levels of class III β-tubulin expression are associated with aggressive tumor features in breast cancer

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Abstract. Overexpression of class III β-tubulin (TUBB3), a factor that confers dynamic properties to microtubules, is a candidate biomarker for resistance to microtubule-targeting chemotherapeutics in breast and other types of solid cancer. Discrepant results from previous studies, with respect to the association of TUBB3 expression levels with breast cancer phenotype and patient prognosis, prompted the present study to investigate TUBB3 expression in a large cohort of breast cancer cases, with available clinical follow-up data. A preexisting breast cancer prognosis tissue microarray, containing a single 0.6 mm tissue core from each of 2,197 individual patients with breast cancer, was analyzed for TUBB3 expression by immunohistochemistry. The results of the present study revealed that TUBB3 expression was less frequent in lobular breast cancer cases (34%), compared with that of cancer cases of alternative histologies, including breast cancer of no special type (60%; P<0.0001). High TUBB3 positivity was associated with high tumor grade (P<0.0001), negativity for estrogen (P<0.0001) and progesterone receptors (P<0.004), as well as the presence of human epidermal growth factor 2 amplification (P<0.0001) and a triple-negative phenotype (P<0.0001). TUBB3 overexpression was additionally associated with reduced patient survival if all breast cancer cases of any histology were jointly analyzed (P=0.0088); however this link was not evident in the subset of breast cancer cases of no special type, or in a multivariate analysis including the established prognostic factors of tumor stage, grade and nodal stage. In conclusion, the present study demonstrated that TUBB3 overexpression was associated with adverse features of breast cancer, and that TUBB3 may possess a distinct role in lobular breast cancer cases, compared with alternative histological subtypes. The results of the present study do not support a clinically relevant role for TUBB3 as a prognostic marker in breast cancer.

Introduction

Breast cancer is the leading cause of cancer-associated mortality in females worldwide, and >1.5 million females are diagnosed with breast cancer annually (1). Although the majority of breast cancer cases are detected at early stages due to regular screening, gaining an increased understanding of the molecular biology underlying breast cancer may aid further improvements to breast cancer diagnosis and therapy (1).

Microtubules are fibrous cytoskeletal proteins composed of polymers of α- and β-tubulin heterodimers. α- and β-tubulins exist as multiple isotypes, and the individual composition of microtubule fibers varies in specific tissues and among intracellular functions (2). In various compositions, microtubules contribute to a number of cellular mechanisms, including maintenance of cell shape, intracellular transport and chromosome segregation during mitosis and meiosis (2). Class III β-tubulin (βIII-tubulin, TUBB3) is a β-tubulin isotype that has been suggested to possess a significant role in malignant transformation and cancer development (3). TUBB3 expression is typically identified in cells of neuronal origin, where it contributes to the formation of dynamic microtubules, which are essential for neurite formation and maintenance (3). In addition, low levels of TUBB3 expression have been identified in several extra-neuronal normal tissues, including the testis, small intestine and placenta (4). Consequently, although TUBB3 overexpression is frequently observed in brain cancer, variable levels of expression have also been reported in other types of solid tumor, including cancer of the lungs, colon, ovary, kidney, prostate and larynx (4,5). High levels of TUBB3 expression have additionally been linked to poor clinical outcomes in non-small cell lung, gastric, breast and ovarian cancer (6-8), and to a reduced response to

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taxane-based microtubule-targeting anticancer drugs in these cancer types (7,9-11).

Additionally, in breast cancer, several clinical studies have suggested that sensitivity to chemotherapeutic taxane drugs was significantly decreased in breast cancer cases exhibiting increased levels of TUBB3 expression, alone or in combination with other molecular markers (10,12-15). Discrepancies have been reported with respect to associations between TUBB3 expression and breast cancer phenotype. For example, certain studies identified an association between high TUBB3 expression levels and high tumor grade (15,16), advanced tumor stage (16), negative hormone receptor state (16), human epidermal growth factor 2 (HER2) positivity (16,17) and a triple-negative phenotype (17), while alternative studies were unable to confirm these results (10,13,15,17). Similarly, two studies reported an association between TUBB3 over-expression and reduced overall survival rates (16,18), which was not identified in a previous study (12). As these previous studies had analyzed 84-314 cases/study, the present study hypothesized that the analysis of a larger patient cohort may aid the development of an improved understanding of the prognostic value of TUBB3 expression in breast cancer. The present study thus analyzed a large breast cancer prognosis tissue microarray (TMA), containing 2,197 consecutive breast cancer cases, including all histological subtypes and associated molecular data for TUBB3 expression.

Materials and methods

Breast cancer TMA. The breast cancer TMA utilized in the present study has been previously described in detail (19). Briefly, 2,197 formalin-fixed (buffered neutral aqueous 4% solution), paraffin-embedded tumors were assembled in a TMA format. A custom-made semiautomatic robotic precision instrument was used to punch out one tissue cylinder (diameter, 0.6 mm) for each case, from representative tumor areas of each patient tissue block. The histological grade of each sample was determined according to a modified scoring system devised by Elston and Ellis [Bloom-Richardson-Elston (BRE) score] (20). Several examples of molecular data utilized for the present study were available from previous studies, including data obtained via immunohistochemistry (IHC) for estrogen receptor (ER), progesterone receptor (PR) and Ki67 (19,21) expression, as well as amplification data obtained via fluorescence *in situ* hybridization (FISH) for HER2. The use of these tissues for FISH and protein expression analysis was approved by the ethics committee of the University of Hamburg (Hamburg, Germany). The median patient age was 62 years (range, 26-101 years) and median follow-up time was 68 months (range, 1-176 months). Clinicopathological parameters of the assayed cancer specimens are described in Table I.

IHC. Freshly cut TMA sections were subjected to immuno-histochemical analysis. Primary rabbit monoclonal antibody specific for TUBB3 (dilution, 1:150; cat no. ab68193; Abcam, Cambridge, MA, USA) was added to the sections, slides were deparaffinized and subsequently exposed to heat-induced antigen retrieval for 5 min in an autoclave (Systec 2540 EL;

Systec GmbH, Linden, Germany) at 121°C in pH 7.8 Tris-EDTA-citrate buffer (Sigma-Aldrich, St. Louis, MO, USA). Following incubation (at 37°C for 60 min), bound antibody was visualized using the EnVision™ kit (Dako, Glostrup, Denmark). Internal staining in nerves and axons on the TMA slide served as a positive control, as previously described (22). The staining intensity and the percentage of positively stained tumor cells were recorded for each tissue spot. Staining intensity was determined by visual inspection of each TMA spot under a microscope (magnification, x200; Axioskop 40; Carl Zeiss, Inc., Oberkochen, Germany). Staining intensity scores were assigned as follows: No visible staining, 0; faint staining, 1+; medium staining, +2; and strong staining, 3+. In addition, the fraction of positively stained cells (5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100%) was estimated in each spot by visual inspection. A final score was calculated from these two parameters according to the following criteria, as previously described (23): Negative score, staining intensity of 0; weak score, staining intensity of 1+ in ≤70% of tumor cells, or 2+ in ≤30% of tumor cells; moderate score, staining intensity of 1+ in >70% of tumor cells, 2+ in 31-70% of tumor cells, or 3+ in ≤30% of tumor cells; and high score, staining intensity of 2+ in >70% of tumor cells, or 3+ in >30% of tumor cells (Fig. 1).

Statistical analysis. Statistical calculations were performed using JMP 9 software (SAS Institute Inc., Cary, NC, USA). Contingency tables and χ^2 test were performed to identify associations between tumor phenotype and molecular parameters. Survival curves were calculated according to Kaplan-Meier. The Log-Rank test was applied to identify any significant survival differences between groups. Cox proportional hazards regression analysis was performed to investigate statistical independence and significance between pathological, molecular and clinical features. P<0.05 was considered to indicate a statistically significant difference.

Results

Technical issues with interpretation of the TMA. A total of 1,652 (75.2%) tumor samples were interpretable in the TMA analysis. Reasons for non-interpretable cases (545 spots; 24.8%) included a lack of tissue samples or an absence of unequivocal cancer tissue in the TMA spot.

Enhanced TUBB3 expression is significantly associated with high-grade breast cancer cases, ER-and PR-negative tumors and the presence of HER2 amplification. TUBB3 immunostaining was localized to the cytoplasm of the cells. Representative images of positive and negative TUBB3 immunostaining are exhibited in Fig. 1. In total, positive immunostaining was detected in 55.7% of the 1,652 interpretable breast cancer cases, including 1.9% of tumors exhibiting weak, 14.5% exhibiting moderate and 39.3% exhibiting high levels of immunostaining, according to the aforementioned predefined criteria (23). TUBB3 expression was significantly less frequent in lobular breast cancer cases (34%) when compared with other types of breast cancer, including the largest group of breast cancer cases of no special type (60%; P<0.0001). Increased

Table I. Composition of breast cancer prognosis TMA and associations between tumor phenotype and TUBB3 immunohisto-chemistry.

Clinical feature	On TMA, n	Analyzable, n	TUBB3 immunohistochemistry				P-value
			Negative, %	Weak, %	Moderate, %	Strong, %	
All cancers	2197	1652	44.3	1.9	14.5	39.3	
Histology							
No special type	1531	1211	40.3	2.2	14.7	42.8	
Lobular carcinoma	311	179	65.9	0.0	17.9	16.2	<0.0001[b]
Cribriform carcinoma	64	52	53.8	1.9	11.5	32.7	
Medullary carcinoma	57	52	34.6	3.8	15.4	46.2	
Tubular carcinoma	56	29	62.1	0.0	6.9	31.0	
Papillary carcinoma	30	23	56.5	4.3	8.7	30.4	
Mucinous carcinoma	69	48	41.7	0.0	10.4	47.9	
Other rare types[a]	79	58	50.0	0.0	10.3	39.7	
Tumor stage							0.5921
1	804	559	44.4	2.3	14.1	39.2	
2	1015	803	43.6	2.0	15.3	39.1	
3	124	94	51.1	0.0	11.7	37.2	
4	242	189	44.4	1.1	13.2	41.3	
BRE grade							<0.0001
1	539	383	55.4	1.3	9.7	33.7	
2	839	589	48.9	1.9	15.8	33.4	
3	646	542	31.9	2.8	16.1	49.3	
Nodal stage							0.1549
0	936	695	47.3	1.7	13.1	37.8	
1	783	590	41.7	1.5	16.3	40.5	
2	121	102	41.2	4.9	13.7	40.2	
ER status							
Negative	474	398	31.9	2.8	16.8	48.5	<0.0001
Positive	1544	1178	48.5	1.6	13.7	36.2	
PR status							
Negative	1265	984	40.3	1.7	14.8	43.1	0.0039
Positive	661	523	48.8	2.5	14.5	34.2	
HER2 FISH							
No amplification	1349	1051	45.4	2.1	13.8	38.7	<0.0001
Amplification	282	246	29.3	2.0	19.1	49.6	

[a]including adenoid-cystic carcinoma, apocrine carcinoma, atypical medullary carcinoma, carcinosarcoma, clear cell carcinoma, histio-cytic carcinoma, lipid rich carcinoma, metaplastic carcinoma, neuroendocrine carcinoma, signet ring carcinoma and small cell carcinoma; [b]comparison between lobular breast carcinoma and breast cancer of no special type. TMA, tumor microarray; TUBB3, class III β-tubulin; BRE, Bloom-Richardson-Elston; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor 2; FISH, fluores-cence *in situ* hybridization; n.s., not significant.

levels of TUBB3 expression were significantly associated with high-grade breast cancer cases (P<0.0001), ER-nega-tive (P<0.0001) and PR-negative (P=0.0039) tumors and the presence of HER2 amplification (P<0.0001), although were not associated with tumor stage (P=0.5921) or presence of nodal metastasis (P=0.1549). All results are summarized in Table I. For all subsequent analyses, the small fraction of breast cancer cases that exhibited weak expression (n=31; 1.9%) was combined with the subset of breast cancer cases

that exhibited moderate expression (n=239; 14.5%), into a single 'low expression' group.

TUBB3 expression is associated with patient survival. Follow-up data were available for 1,650 breast cancer cases with interpretable TUBB3 results, including 1,209 cases of no special type. If all breast cancer cases were jointly analyzed, a statistically significant association existed between low and high TUBB3 expression and shortened raw survival (P=0.0088;

Table II. Multivariate analysis of overall survival, including tumor stage, BRE grade, nodal stage and TUBB3 expression.

Clinicopathological parameter	Hazard ratio	95% Confidence interval	P-value
Tumor stage			0.0039
pT2 vs. pT1	1.3	1.0-1.7	
pT3 vs. pT2	0.9	0.6-1.3	
pT4 vs. pT3	1.6	1.1-2.5	
BRE grade			<0.0001
G2 vs. G1	1.3	0.9-1.7	
G3 vs. G2	2.1	1.7-2.6	
Nodal stage			<0.0001
pN1 vs. pN0	2.3	1.8-3.0	
pN2 vs. pN1	2.7	2.1-3.6	
TUBB3			0.0806
Low vs. negative	1.4	1.0-1.8	
High vs. low	0.8	0.6-1.1	

BRE, Bloom-Richardson-Elston; TUBB3, class III β-tubulin; n.s., not significant.
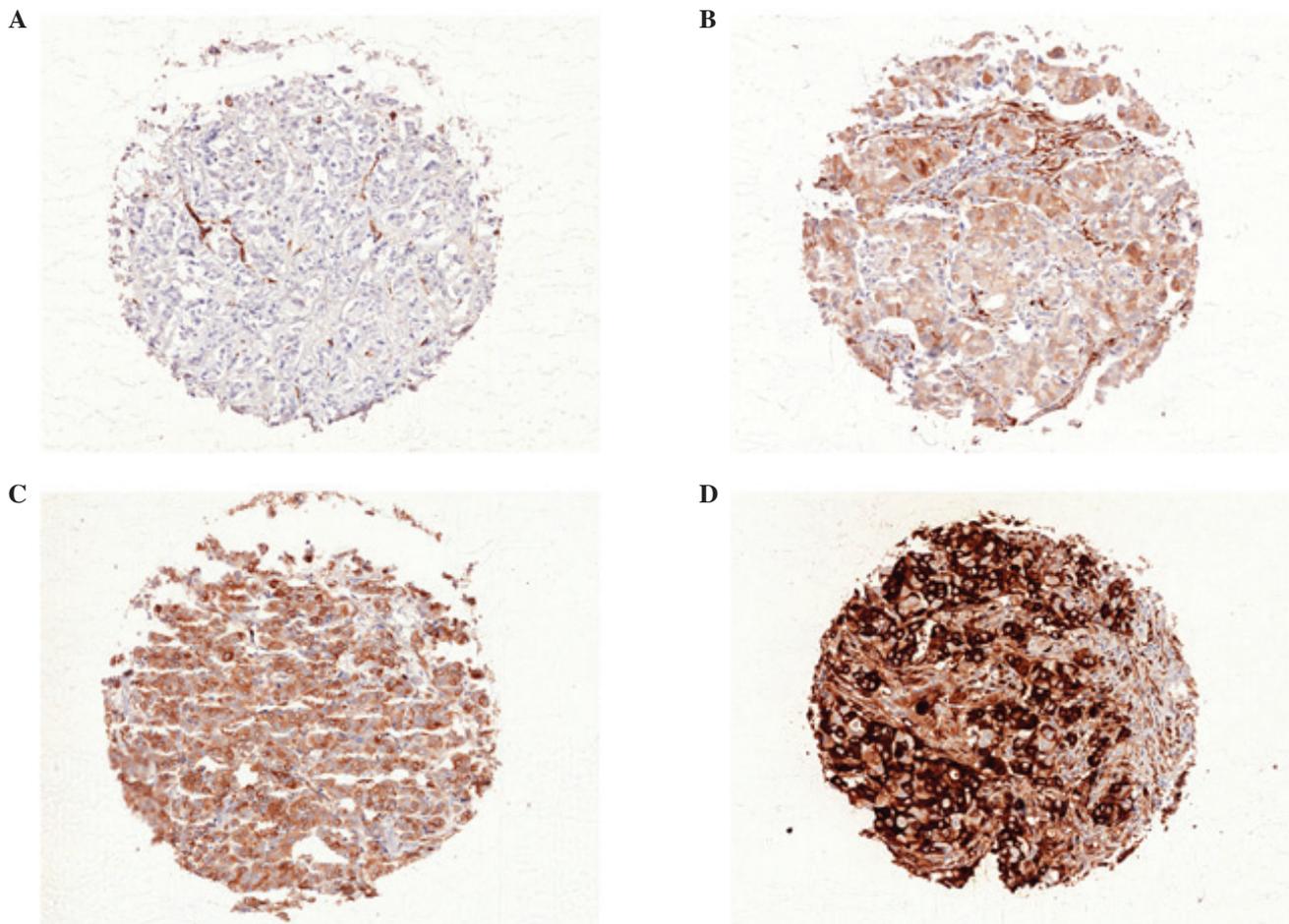
Figure 1. Representative examples of TUBB3 immunostaining analysis results in breast cancer tissue samples. (A) Negative (0), (B) weak (1+), (C) moderate (2+) and (D) strong TUBB3 (3+) expression. TUBB3, class III β-tubulin.

Fig. 2A), which was less marked (statistically insignificant) when the analysis was restricted to the subset of breast cancers of no special type (P=0.1583; Fig. 2B). For all breast cancer cases, this association was not observed following multivariate analysis including the established prognosticators of tumor stage stage (P=0.0039), BRE grade (P<0.0001) and nodal
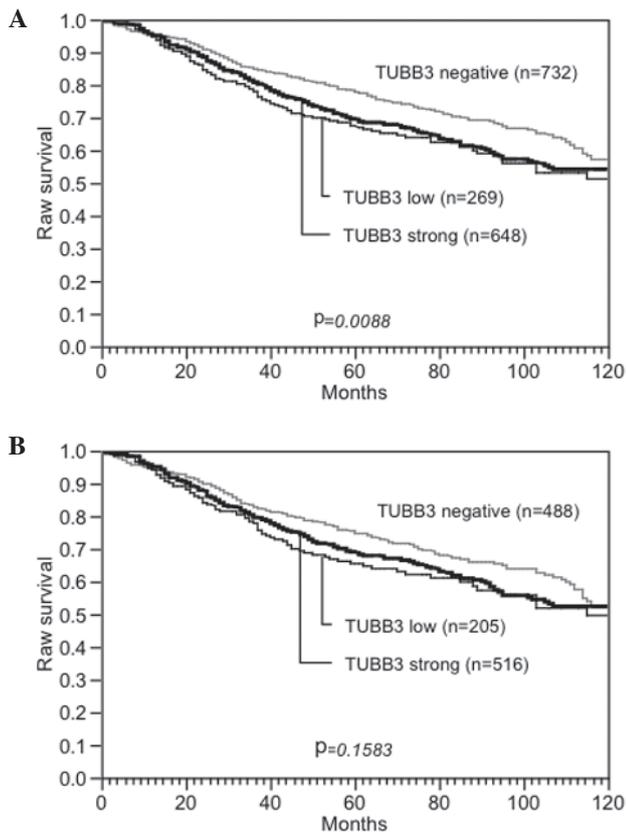
Figure 2. Prognostic impact of TUBB3 immunohistochemical analysis results on patient survival in (A) all arrayed types of breast cancer and (B) the subset of breast cancer of no special type. TUBB3, class III β-tubulin.
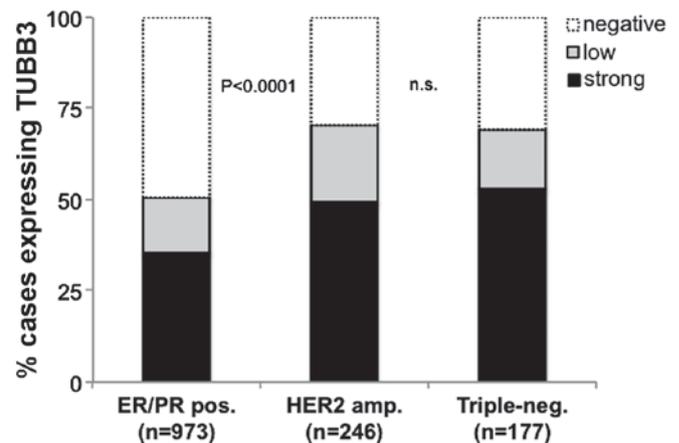

Figure 3. Association between TUBB3 immunohistochemical analysis results and subsets of breast cancer according to ER/PR state (ER and/or PR positive, HER2 negative), presence of HER2 amplification (HER2 positive irrespective of ER/PR state) and total lack of ER/PR/HER2 positivity (triple-negative). TUBB3, class III β-tubulin; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor 2; pos, positive; amp, amplified; neg, negative; n.s., not significant.

stage (P<0.0001), in addition to the TUBB3 immunostaining results (P=0.0806; Table II).

TUBB3 expression is significantly associated with triple negative breast cancer. ER, PR and HER2 data were combined to identify a triple-negative phenotype in 177 (12.7%) cases, an ER- and/or PR-positive phenotype in 973 (69.7%) cases and a HER2-positive (presence of HER2 amplification) phenotype in 246 cases (17.6%) of 1,396 breast cancer cases with interpretable data for TUBB3 expression. TUBB3 expression was significantly associated with subsets of breast cancer exhibiting HER2 amplification or a triple-negative phenotype: 70% each of triple-negative tumors and HER2-amplified breast cancer cases were classified as TUBB3-positive, compared with only 51% of ER/PR-positive specimens (Fig. 3).

High TUBB3 expression is associated with cell proliferation. Immunohistochemical data regarding Ki67 expression were available for 1,276 breast cancer cases possessing interpretable TUBB3 results. A high proliferation index (Ki67 labeling index; i.e., the % of Ki67-positive cancer cells per high power field) was associated with high TUBB3 immunostaining if all types of breast cancer were jointly analyzed (P<0.0001); however, subset analysis revealed that this association was primarily driven by BRE grade, as there was no statistically significant increase in cell proliferation rates within the subgroups of breast cancer cases of identical BRE grade (Fig. 4).

Discussion

The results of the present study demonstrated that TUBB3 is frequently expressed in breast cancer cases, and is associated with adverse prognostic features, including a triple-negative hormone receptor status and the presence of HER2 amplification.

Positive TUBB3 staining was detected in 55.7% of the 1,652 breast cancer samples that were successfully analyzed in the present study. A total of 40% of cancer cases evaluated exhibited high immunostaining according to the predefined criteria. These data were concurrent with the results of earlier IHC studies investigating TUBB3 expression, which had analyzed between 46 and 1,205 cases of breast cancer (10,13,15,16,18). Despite variable thresholds for TUBB3 expression levels, these previous studies reported TUBB3 positivity in 34-62% of samples. In the present study, utilization of a TMA enabled the analysis of a markedly larger cohort of cancer cases, enabling evaluation of the potential biological and clinical roles of TUBB3 expression. Although the analysis was limited to a single tumor specimen of 0.6 mm in diameter per patient, the breast cancer prognosis TMA utilized in the present study has previously been proven to be effective in identifying associations between biomarkers and clinicopathological parameters of breast cancer (19,21,24-26).

A comparison of immunostaining results with the phenotype of the assayed types of cancer revealed that increased TUBB3 expression levels were associated with certain features of aggressive breast cancer, including advanced tumor grade, loss of ER/PR expression, HER2 amplification and triple-negative phenotypes; however, TUBB3 expression levels exhibited no significant associations with tumor stage and metastatic growth. These results appear to disprove a major role for TUBB3 in tumor progression or metastasis. The results of the present study are supported by those of previous studies, which reported increased levels of
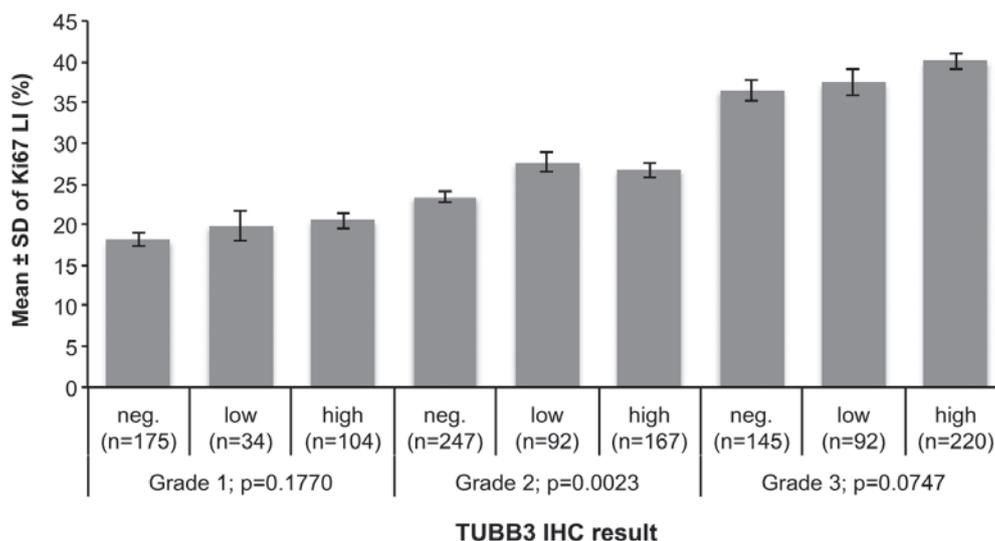
Figure 4. Association between TUBB3 IHC analysis results and cell proliferation measured by Ki67 IHC in subsets of breast tumors with various Bloom-Richardson-Elston grades. SD, standard deviation; LI, labeling index; TUBB3, class III β-tubulin; neg, negative; IHC, immunohistochemistry.

TUBB3 expression in grade 3 tumors, compared with those of grade 1 and 2 tumors (15,16), in hormone receptor-negative cancer compared with hormone receptor-positive cancer (16), in HER2 amplified cancer (16,17) and in triple-negative cancer (17). The fact that none of these previous studies identified all of these associations is potentially due to the comparatively small sample sets used, ranging between 84 and 314 tumors. The fact that all associations were able to be clearly visualized in the present study, which analyzed >1,600 breast cancer cases, emphasizes the importance of analyzing as large as possible a tumor set for candidate biomarker validation.

Notably, the subtype of lobular cancer demonstrated significantly lower TUBB3 expression compared with that of other breast cancer types. Given that loss of E-cadherin function is the primary characteristic feature of lobular breast cancer (27), it is possible that TUBB3 expression does not provide a selective advantage in an E-cadherin-negative molecular background, or that E-cadherin signaling may be involved in regulating TUBB3 expression. E-cadherin maintains epithelial integrity by binding to the cytoplasmic α- and β-catenin proteins, which link cadherin to the actin cytoskeleton (28). Loss of E-cadherin induces epithelial mesenchymal transition, a significant event in the development of cancer (27). It may be hypothesized that loss of E-cadherin function in lobular breast cancer cases results in such marked changes to the cytoskeleton, that the increase in microtubule dynamics induced by TUBB3 overexpression may exert little or no additional effects. However, a recent study identified that drug-induced microtubule disassembly *in vitro* resulted in aberrant expression of E-cadherin (29), supporting a direct molecular link between tubulin turnover and E-cadherin expression. To the best of our knowledge, only one study, including 44 breast cancer cases of no special type and 40 lobular breast cancer cases (13), had previously investigated the variations in TUBB3 expression between these histological subtypes, however this study identified no significant differences, in contrast to the results of the present study.

In the present study, TUBB3 expression was significantly associated with shortened survival when all 1,649 cancer cases were jointly analyzed. In a multivariate analysis including established prognosticators, the hazard ratio for overall survival was increased by expression of TUBB3, however, this was not dependent on the expression level in multivariate analysis. These results suggest that TUBB3 may not be an optimal prognostic marker for routine application in breast cancer diagnosis. This may be explained by the fact that TUBB3 overexpression was linked to certain adverse prognostic features, including HER2 amplification and a triple-negative phenotype, however not to other significant prognostic factors, including tumor stage and metastatic growth. For example, the fraction of TUBB3-positive cases was identical in early (pT1) and late (pT4) stage cancer, or in nodal-negative (pN0) and nodal-positive (pN+) tumors, and was not unequivocally linked to tumor cell proliferation in the present study. The results of the present study support those of a previous study, which reported that TUBB3 messenger RNA expression was associated with reduced survival, although the authors did not identify a significant association when TUBB3 expression was determined by TUBB3 IHC analysis (16). Furthermore, in concordance with the results of the present study, Horak *et al* (17) reported an association between TUBB3 overexpression and HER2-enriched and basal-like cancer types, as well as a triple-negative phenotype. Increased levels of TUBB3 have additionally been linked to adverse phenotypes and poor prognosis in various other types of solid cancer, including colon (3), prostate (5), lung (9,30), ovarian (31,32) and neurological cancer (33). The adverse effects of TUBB3 may be linked to its significant role in rendering microtubules dynamic. High microtubule plasticity is required for cellular processes that also have significant roles in cancer cells, including cell motility, mitotic spindle formation and cell division. Microtubules containing α/TUBB3-heterodimers are more dynamic than those assembled from other isotypes, for example α/β class II, which results in the generation of less flexible microtubules (34,35). It is thought that the TUBB3 isotype is responsible for generating the highly dynamic microtubules

required for neurite formation and motility in neuronal tissues (33).

Several studies have suggested that TUBB3 may be a clinically relevant biomarker for the prediction of responses to drugs targeting microtubules, including vinca alkaloids, taxanes and epothilone analogues in breast (10,13-15), lung, ovarian, prostate, breast, stomach and pancreatic tumors (36). These drugs constitute a significant class of chemotherapeutic agent, which impair microtubule assembly and activity and inhibit cell division via inhibition of the mitotic spindle and induction of apoptosis, in solid tumors and hematological malignancies (37,38). It has thus been suggested that the increase in microtubule dynamics conferred by TUBB3 may provide resistance to microtubule-targeting drugs (39). However, the two largest studies of TUBB3 and breast cancer, including 314 specimens analyzed by the Hellenic Cooperative Oncology Group and >1,200 specimens from the BCIRG001 trial, were unable to identify an association between TUBB3 expression levels and the benefit of inclusion of Paclitaxel into the treatment regimen (16,18). It is possible that the discrepant conclusions arising from the present and previous studies may be associated with the study size and/or composition of the cancer specimens, with respect to histological subtypes and alterations in clinically relevant molecular pathways, including ER or HER2 signaling. The results of the present study contribute to the ongoing discussion, indicating that it may be possible that lobular breast cancer, which demonstrated the lowest levels of TUBB3 expression amongst all histological subtypes included in the present study, may benefit more from tubulin-targeting agents, compared with alternative histological subtypes of cancer, provided that TUBB3 expression levels posses predictive value for responses to therapy.

In conclusion, the present study emphasized the significant role for TUBB3 in breast cancer, based on its association with prognostic adverse molecular subtypes, including HER2 positive and triple-negative tumors. In addition, the results of the present study demonstrated variations in TUBB3 expression levels between the two most frequent histological subtypes of breast cancer, lobular cancer and tumors of no special type. However, the comparatively low prognostic power of TUBB3 measurement by IHC appears to disprove a potential role for this factor as a clinically relevant prognostic biomarker in breast cancer.

Acknowledgements

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