Tubulin Detyrosination Is a Frequent Occurrence in Breast Cancers of Poor Prognosis

Agnes Mialhe, Laurence Lafanechère, Isabelle Treilleux, Nadine Peloux, Charles Dumontet, Alain Brémont, Meng-Hong Panh, Raoul Payan, Jürgen Wehland, Robert-Louis Margolis, and Didier Job


ABSTRACT

Tubulin, the dimeric subunit of microtubules, is a major cell protein that is centrally involved in cell division. Tubulin is subject to specific enzymatic posttranslational modifications including cyclic tyrosine removal and addition at the COOH terminus of the α-subunit. Tubulin is normally extensively tyrosinated in cycling cells. However, we have previously shown that detyrosinated tubulin accumulates in cancer cells during tumor progression in nude mice. Tubulin detyrosination, resulting from suppression of tubulin tyrosine ligase and the resulting unbalanced activity of tubulin-carboxypeptidases, apparently represents a strong selective advantage for cancer cells. We have now analyzed the occurrence and significance of tubulin detyrosination in human breast tumors. We studied a total of 134 breast cancer tumors from patients with or without known complications over a follow-up period of 31 ± 10 months. The mean age of the patients at the time of diagnosis was 57 years. For each patient, detailed data concerning the histology and extension of the tumor were available. Tumor cells containing detyrosinated tubulin were visualized by immunohistochemical staining of paraffin-embedded tissue sections.

Cancer cells with detyrosinated tubulin were observed in 53% of the tumors and were predominant in 19.4% of the tumors. Tubulin detyrosination correlated to a high degree of significance (P < 0.001) with a high Scarff-Bloom-Richardson (SBR) grade, a known marker of tumor aggressiveness. Among SBR grade 1 tumors, 3.8% were strongly positive for tubulin detyrosination compared with 65.4% of the SBR grade 3 tumors. The SBR component showing the strongest correlation with tubulin detyrosination was the mitotic score. In the entire patient population, neither the SBR grade nor the detyrosination index had significant prognostic value (P = 0.11, P = 0.27, respectively), whereas a combined index was significantly correlated with the clinical outcome (P = 0.02). A preliminary subgroup analysis indicated that tubulin detyrosination may define high- and low-risk groups in breast cancer tumors with an SBR grade of 2. Our study shows that tubulin detyrosination is a frequent occurrence in breast cancer, easy to detect, and linked to tumor aggressiveness.

INTRODUCTION

Microtubules are major cytoskeletal structures, centrally involved in the control and mechanics of cell division, being the principal components of the mitotic spindle in eukaryotic cells (1). The building block of microtubules is the β-tubulin heterodimer. Tubulin is subject to specific posttranslational modifications including a cycle of tyrosine removal and addition at the COOH terminus of the α subunit (2, 3). This cycle involves two enzymes, TTL (4) and an ill-defined tubulin carboxypeptidase (5, 6), and generates two major forms of tubulin: tyrosinated tubulin (Tyr-tubulin) and Glu-tubulin. A third tubulin species (Δ2-tubulin) arises by removal of the COOH-terminal Glu residue from the α chain of Glu-tubulin (7, 8). Tyr-tubulin is the dominant tubulin species in cycling cells (9, 10). Glu-tubulin is abundant in neurons but can be present in stable microtubules of other cell types (10–14). Δ2-tubulin normally has high neuronal specificity (7, 8, 13, 15). However, we have previously observed an abnormal accumulation of Glu-tubulin and Δ2-tubulin in cancer cells of both fibroblastic and epithelial origin, during tumor growth in nude mice (15). This accumulation is attributable to TTL suppression and apparently represents a strong selective advantage for cancer cells (15). Here, we have tested whether accumulation of Glu- and Δ2-tubulin also occurred in human tumor cells and whether tubulin detyrosination was related to tumor severity.

MATERIALS AND METHODS

Patient Population. Entered in this study were tumor samples from 134 breast cancer patients treated at the Center Léon Bérard, between January 1996 and December 1998. The end point for clinical follow-up was December 31, 1999. All of the tumors were primary breast tumors. The study included tumors from all of the patients who had locoregional recurrence, metastasis, or death during the period of follow-up. Among these tumors, tumors <40 mm in size (n = 34) were paired with tumors of the same size from patients of the same age with no event. Tumors ≥40 mm in size (n = 18) could not be paired because of difficulties in finding subjects of the same age in the relatively small-sized population of patients with similar tumors and no event (n = 48). All of these 48 patients were included in the study. The median follow-up (from diagnosis of the tumor to December 1999) was 32.2 months (range, 12.2–48.2 months). All of the patients were seen on a regular basis, every 6 months. The principal patient characteristics are described in Table 1.

Tumor samples were collected at the Department of Pathology of the Center Léon Bérard. Tumors were pure histological variants of invasive breast carcinomas comprising 100 ductal, 15 lobular, and 19 other variants (including mucinous, tubular, and metaplastic). The mean largest diameter of collected tumors was 37.4 mm (range, 2–100 mm; Table 1). The histopathological type and grade of the carcinomas were evaluated according to WHO criteria and SBR grading, respectively (16). The SBR grade includes three histological parameters: tubular differentiation, nuclear pleomorphism, and mitotic count.

Immunohistochemistry of Paraffin-embedded Tissue. The cellular content in Glu-tubulin and in Δ2-tubulin was evaluated by immunohistochemical analysis, using specific antibodies. Tumor samples were Bouin-fixed within 2 h after surgical removal. Sections of 4 μm in thickness from paraffin-embedded blocks were deparaffinized in xylene and rehydrated in a decreasing ethanol series (100 to 50%). At this stage, tissue sections assigned to Δ2-tubulin immunohistochemistry were treated for antigen retrieval. Antigen retrieval involved treatment in a sodium citrate solution [10 mM (pH 6.0)] in a 700-W microwave oven, three times for 5 min each. The sections were left

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2 A. M. and L. L. contributed equally to this work.

3 To whom requests for reprints should be addressed, at Laboratoire du Cytosquelette, Inserm U356, DBS/CS, CEA/Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France. E-mail: djob@cea.fr.

4 The abbreviations used are: TTL, tubulin tyrosine ligase; Glu-tubulin, detyrosinated tubulin; Δ2-tubulin, α tubulin lacking both tyrosine and glutamic acid from its COOH terminus; SBR, Scarff-Bloom-Richardson; NS, not significant.
Table 1  Patient characteristics

<table>
<thead>
<tr>
<th>No.</th>
<th>134</th>
</tr>
</thead>
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<tr>
<td>Age (yr)</td>
<td>55.5</td>
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<tr>
<td>Median</td>
<td>25.8-85.8</td>
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<tr>
<td>Range</td>
<td>20-40 mm</td>
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<tr>
<td>Tumor size</td>
<td>≥40 mm</td>
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<tr>
<td>Node involvement</td>
<td>N0</td>
</tr>
<tr>
<td>Metastasis</td>
<td>0</td>
</tr>
<tr>
<td>Receptor status</td>
<td>ER+PgR+</td>
</tr>
<tr>
<td>SBR Grade</td>
<td>1</td>
</tr>
<tr>
<td>Glu-tubulin grade</td>
<td>1, negative</td>
</tr>
<tr>
<td>Clinical evolutiona</td>
<td>No complication</td>
</tr>
<tr>
<td>Metastasis</td>
<td>32 (23.9%)</td>
</tr>
<tr>
<td>Death related to cancer</td>
<td>14 (10.4%)</td>
</tr>
<tr>
<td>Death not related to cancer</td>
<td>5 (3.7%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0.7%)</td>
</tr>
</tbody>
</table>

* ER, estrogen receptor; PgR, progesterone receptor.
* a For patients with complications, the most severe complication is taken into account.
* b Node involvement and metastatic expansion were rated according to the TNM classification of the International Union against Cancer.

RESULTS

Analysis of Glu-tubulin in Cancer Cells. Tissue sections stained with Glu-tubulin antibody were examined for presence of cells containing Glu-tubulin as described above. In normal areas of breast tissue sections, Glu-tubulin staining was detected in the cytoplasm of fibroblasts (Fig. 1A) and of endothelial cells. The nuclei of myoepithelial cells were occasionally stained. This nuclear labeling may result from a cross-reaction with a nuclear protein in this cell type because tubulin is normally undetectable in cell nuclei. Most importantly, normal epithelial cells were never stained (Fig. 1A). In contrast, Glu-tubulin was detected in transformed epithelial cells (Fig. 1C and D). Stromal cells within the tumor were also stained. For the scoring of tumors, slides were microscopically evaluated by three observers (A. M., L. L., I. T.) without knowledge of either the prior histological results or the clinical outcome. The percentage of positively stained transformed epithelial cells in the infiltrating component were estimated by each investigator. Tumors were scored according to the percentage of positive cancer cells: grade 1, negative for Glu-tubulin staining (Fig. 1B); grade 2, positive, with <50% of cancer cells positive for Glu tubulin (Fig. 1C); and grade 3, strongly positive with ≥50% of cancer cells positive for Glu-tubulin (Fig. 1D). When the scores determined by the investigators differed significantly, a consensus scoring was decided by joint examination. Nerve fibers known to exhibit high levels of Glu-and Δ2-tubulin (13) were used as internal positive controls. Negative controls were obtained by omission of the primary antibody. To test the robustness of the Glu-tubulin scoring, a sample of 34 slides was scored again by an independent observer (M. H. P.), and the scoring was compared with the consensus scoring. Scores were identical in 28 cases, showing a good reproducibility of the scoring (Kendall concordance coefficient, 0.79). With this scoring, >50% of the tumors were positive for Glu-tubulin staining (Table 1). Thus, tubulin detyrosination is a common occurrence in breast cancer cells.

Other sections from the same tumor blocks as those used for Glu-tubulin scoring were stained with Δ2-tubulin antibody. These tumor sections were analyzed without knowledge of the Glu-tubulin score. Staining of normal cell types was almost identical to the staining obtained with Glu-tubulin antibody, except that the nuclear staining of myoepithelial cells was almost constant, whereas fibroblasts were only exceptionally stained (Fig. 1E). Normal epithelial cells were never stained (Fig. 1E). In contrast, Δ2-tubulin could be detected in cancer cells (Fig. 1F). The scoring was done as described for Glu-tubulin. Over 20% of the tumors scored positive for Δ2-tubulin staining (Table 1). Ninety % (28 of 31) of the Δ2-tubulin-positive tumors were also Glu-tubulin positive. There was a strong overall correlation between positivity for Δ2-tubulin and positivity for Glu-tubulin (P < 0.00001).

Relationship between Tubulin Detyrosination and Markers of Tumor Severity. Tubulin detyrosination could occur at random among breast tumors or could be related to tumor severity, as assessed by clinical and cytological markers. To test which of these possibilities was correct we analyzed the relationship between the tumor detyrosination score and known markers of tumor severity. Major prognostic factors in breast tumors include patient age, tumor size and axillary lymph node involvement (17). Cytological markers include the steroid receptor status and the so-called SBR grade. In our population, detyrosination was unrelated to age, tumor size, axillary node involvement and receptor status (Table 2). In contrast, there was a highly significant association between positivity for Glu-tubulin and high SBR grade (Table 2). The SBR grade has three components, scoring tubular differentiation, nuclear pleiomorphism, and the proportion of mitotic cells, respectively (16). Nuclear pleiomorphism was not significantly related to detyrosination (Table 2). Interestingly, detyrosination was strongly and specifically correlated with the mitotic score. In strongly detyrosinated tumors, the mitotic score was high (Table 2). The score of tubular differentiation also differed among detyrosination classes, but this was probably the result of statistical fluctuations: the score was lower in the grade 2 Glu-tubulin-positive tumors than in the two other groups (Table 2) and did not differ significantly between Glu-tubulin-negative tumors (grade 1) and strongly positive (grade 3) tumors (P = 0.32, NS). In contrast, the
SBR grade and the mitotic score both differed significantly between the two extreme classes of negative and strongly positive ($P < 0.001$ and $P < 0.01$, respectively).

**Tubulin Detyrosination and Clinical Outcome.** A total of 46 patients had complications related to breast cancer (recurrence and metastasis, death) within the period of observation. The patient selection process (see “Materials and Methods”) equalized major prognostic factors such as age and tumor size among patients with or without complications. Therefore, in the studied population, neither age nor tumor size was related to the clinical outcome ($P = 0.67$ and $P = 0.32$, respectively). The relationship between the clinical outcome and both the detyrosination and SBR classes is shown in Table 3. Neither the detyrosination index nor the SBR index had significant prognostic value in our study ($P = 0.27$ and $P = 0.11$, respectively), although in both cases, a trend toward accumulation of patients with complications in the highest-grade class was observed. Could the detyrosination score be used to improve the prognostic value of the SBR grade? It is the clinician’s experience that the SBR classification is nonambiguous for the extreme classes (1 and 3) but that the class 2 tumors (which are the most numerous) pose a problem, some of them being almost class 1, and others almost class 3. We tried to use the detyrosination score to reclassify the SBR2 tumors: SBR2 tumors were considered as SBR3 if they were strongly positive for Glu-tubulin (grade 3). This defined a corrected SBR index, designated SBRc. The SBRc correlated significantly with the occurrence of complications ($P = 0.02$, Table 3). The apparently improved prognostic value of SBRc compared with SBR was explained by subgroup analysis that showed that among SBR grade 2 patients, cancer complications occurred in 5 of 8 patients with a detyrosination index of 3 and in only 15 of 58 patients with lower detyrosination grades ($P < 0.05$, Fisher’s exact test).

**DISCUSSION**

The present study shows that tubulin detyrosination is a specific and common occurrence in human breast primary tumor cells. Glu-tubulin was absent in the epithelial cells of normal breast tissue but was observed in over 50% of carcinomatous tumor cells. $\Delta 2$-tubulin accumulation was also frequent, although of somewhat more restricted occurrence than Glu-tubulin accumulation. In cultured cycling cells, Glu-tubulin levels can vary to some extent as a function of the cell cycle (10, 18, 19), whereas $\Delta 2$-tubulin has only been observed after TTL suppression (15). Therefore, in principle, $\Delta 2$-tubulin accumulation is a more specific marker of TTL suppression than Glu-tubulin accumulation. However, in the present study, Glu- and $\Delta 2$-tubulin signals were very strongly correlated, which indicated that both $\Delta 2$-tubulin accumulation and Glu-tubulin accumulation resulted...
from TTL suppression. In cultured cells with suppressed TTL activity, Glu-tubulin is much more abundant than Δ2-tubulin (8, 15). Therefore, Glu-tubulin is an easier marker of TTL loss to detect than is Δ2-tubulin. This probably accounts for the excess number of Glu-tubulin-positive tumors compared with the number of Δ2-tubulin-positive tumors observed in this study. We have found Glu-tubulin scoring of tumors to be easy and reproducible among observers. This scoring should, therefore, be easy to perform in routine clinical practice and offers a unique possibility to detect the loss of a putative tumor suppressor gene (TTL) by the accumulation of an abnormal variant of a major cell protein (Glu-tubulin).

Our study shows that tubulin detyrosination does not occur at random among breast tumors. Instead, tubulin detyrosination is more frequent in tumors with a high SBR grade, a known marker of tumor severity (16). The detyrosination grade was apparently unrelated to the differentiation status of the tumor, as assessed by the tumor histomorphology and steroid receptor status, whereas it strongly correlated with the mitotic score. These results agree with previous studies, which indicated that the tyrosination cycle is not a differentiation marker (2, 3, 20) and that its inhibition in cancer cells somehow favors tumor growth (15).

Is tubulin detyrosination a clinically useful marker of tumor prognosis? Our preliminary data indicate that tubulin detyrosination may define high- and low-risk groups in breast cancer patients, which represent the majority of breast tumor patients, with an SBR grade of 2. A simple combination of the SBR and of the detyrosination grades apparently yielded an index with improved prognostic value. These results are encouraging but are based on the analysis of groups of small size and obviously need to be confirmed by studies of much larger patient populations. We believe that the ease of the Glu-tubulin scoring and its potential usefulness justify its evaluation in such studies. The ubiquity of the tubulin tyrosination cycle (2, 3, 20) suggests that TTL elimination may occur in several types of cancers. In a preliminary limited survey of colon and lung cancers, we have, indeed, observed Glu-tubulin and Δ2-tubulin positive tumors. Therefore, assaying tubulin detyrosination may be of clinical interest in epithelial cancers in general.

**REFERENCES**

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