Tumor Neoangiogenesis by CD31 and CD105 Expression Evaluation in Breast Carcinoma Tissue Microarrays

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ABSTRACT

Purpose: The aim of the study was to evaluate CD31 and CD105 immunohistochemical expressions in tissue microarrays from 360 breast carcinomas.

Study design: Computerized (ACIS/Chromavision) assisted image analysis was performed to compare immunoreactions in tissue microarrays with those in current paraffin and frozen sections. We also aimed to determine the CD105 and CD31 prognostic significance and relevance in routine practice by correlating results of immunodetections with patients’ (n = 360) outcome (14.3-year follow-up).

Results: The results show (a) that in tissue microarrays, the CD31 and CD105 expression quantified by image analysis device did not correlate with the measurements assessed on routine paraffin sections; (b) that CD105 expression is endowed of a prognostic significance in paraffin sections in terms of overall survival (P < 0.01), whereas in contrast, CD31 on paraffin sections did not correlate with patients overall survival; (c) that semiquantitative analysis of CD105 expression correlated with the image analysis measurements in frozen sections (p = 0.671, P < 0.01) and paraffin (p = 0.824, P < 0.01) sections. However, paraffin sections were less immunostained than frozen ones.

Conclusions: It is concluded (a) that CD105 may be suitable in paraffin sections to evaluated neoangiogenesis; and (b) that tissue microarrays are not suitable substrates for neoangiogenesis evaluation as a prognostic indicator in breast carcinomas, in contrast to current tissue sections.

INTRODUCTION

Angiogenesis in tumor can be evidenced by immunocytochemical labeling of the endothelial cells that line the basal membrane along vessel walls. In routine pathology practice, the labeling of vessels is sometimes useful for diagnosing emboli in malignant tumors. Neoangiogenesis in malignancies reflects the capacity of tumors to produce blood-borne metastases (1–5). Thus neoangiogenesis evaluated by quantification of tumor vessels can be used as a prognostic marker (6–11). In previous studies, we have shown along with other authors (reviewed in Refs. 10 and 11) that CD31 and CD105 expressions in breast carcinomas (8, 12, 13) were endowed with prognostic significance, particularly when immunocytochemical procedures were performed on frozen sections that are ideal tissue substrates for immunodetection. But frozen samples are not easy to handle in routine practice (smaller tumors detected by screening program). Also, semiquantitative evaluation of immunohistochemical labeling is easy to perform and cost-effective, but computerized systems of image analysis, developed to quantify positive immunoprecipitates within tissue sections, are more reproducible and therefore more acceptable for clinical and pathological use (10–18). The purpose of the present retrospective study was (a) to compare the immunohistochemical expressions of CD31 and CD105 in frozen sections with those in paraffin sections and on tissue microarrays from 360 breast carcinomas, evaluated by semiquantitative analysis and by a computerized system of image analysis including a specific software to count vessels; and (b) to correlate the results with patients’ outcome (14.3-year follow-up). More specifically, our goal was to evaluate whether CD105 immunocytochemical expression labeling of the activated endothelial cells lining the neovessels retained a prognostic significance on paraffin sections and more specifically in tissue microarrays, observed on frozen sections (12, 13) when evaluated semiquantitatively or by image analysis and compared with patients’ long-term follow-up (14.3 years).

MATERIALS AND METHODS

Patients. The study included 360 patients ranging in age from 26 to 81 year old (mean ± SD = 54 ± 11.6 years) with breast carcinoma who underwent surgery from January 1986 to December 1991. They did not receive chemotherapy or hormone therapy before surgery. Axillary node excision combined with wide local excision with margin clearance of mastectomy was realized in the Department of Oncologic Gynecology (Hôpital de La Conception). All specimens were examined by the same group of three senior pathologists experienced in breast carcinoma diagnosis and screening (C. C., L. A., J-P. D.).

The follow-up period ranged from 8 to 15 years (median 14.3 years). The records for 2001 showed that 184 patients relapsed, of whom 79 died (median survival, 97 months), and 176 were disease-free. Overall survival was calculated as the period from surgery until date of death. Metastasis-free and
recurrence-free survival were calculated as the period from surgery until the date of first metastasis and recurrence.

The mean tumor size was 22.4 ± 10.4 mm; 24% of tumors were 10 mm or smaller; 42% were between 10 and 20 mm; 19% were more than 20 mm but not more than 30 mm; and 15% were larger than 30 mm.

Microscopic examination of surgical specimens was performed on paraffin-embedded sections stained with hematoxylin, eosin, and safranin.

Tumors were classified as ductal carcinomas (67%), lobular carcinomas (19%), and carcinomas of other types including tubular, mucinous, medullary, papillary, apocrine, and mixed (14%). Tumors were scored as grade 1 (23%), grade 2 (52%), and grade 3 (25%). Tumor grading, initially using Scarff, Bloom, and Richardson scores, was reevaluated according to Elston and Ellis (19, 20).

A mean of 14.4 (SD ± 4.1) lymph nodes was found in axillary node excision, and 172 patients (55.3%) were node negative.

**Tissue Sections and Tissue Microarrays.** Fresh tissue fragments were sampled by pathologists (C. C., L. A., J.-P. D.) immediately after intraoperative diagnosis. The fragment size varied according to the tumor size (average size, 5 mm long, 4 mm wide, and 3 mm thick). Fragments were obtained from dense tumor areas that lacked grossly visible adipose tissue. They were then dipped promptly in liquid nitrogen and stored at −80°C (embedded in current tissue embedding material to avoid dehydration during storage) in the laboratory tumor library. Immunodetection studies were performed on 5-μm-thick frozen sections (Leica CM 3050 cryostat; Leica Microsystems, Rueil Malmaison, France).

Tissue microarrays were performed using an Alphelys (78370 Plaisir, France) device as described previously (21, 22), providing microsections of 600 μm in diameter. Three punches were assessed (three tissue microarray blocks) in appropriate tumor areas selected on H&E paraffin standard sections.

**Immunostaining Procedure and Quantification of CD31 and CD105 Immunostained Vessels.** Paraffin sections were performed after fixation in formalin (10% buffered formalin), Automated immunoperoxidase procedures were performed using monoclonal (5.6 E) mouse antihuman CD31/PECAM (10, 11) and monoclonal (8E11; Ref. 23) antihuman CD105 (Novocastra; Tebu, Le Perray en Yvelines, France), and the Ventana Benchmark device with Ventana kits (Ventana, Tucson, AZ).

Measurements of the immunostaining were assessed in the most vascularized areas (so-called hotspots; Refs. 24 and 25) using a ×40 objective (0.5 mm field diameter) with a Zeiss Axiosplan microscope (Carl Zeiss International, Gottingen, Germany). The mean value of the vessel count in the four fields was retained as the final value in frozen and paraffin sections (whereas in tissue microarrays the total section was screened) for semiquantitative analysis (C. C., J.-P. D., L. A.) as described previously (12, 13). Densitometry was performed using an ACIS I device (Microm, Francheville, France).

**Statistical Analysis.** The Kaplan-Meier method was used to analyze disease-free and overall survival rates. The difference between curves was evaluated with the Mantel Cox test (or log rank test) for observations regarding censored survival or events. All computations were done with NCSS 2000 statistical software (Ness, Kaysville, UT). CD31- and CD105-stained vessels were stratified and correlated with major events during the course of the disease (distant metastases or local recurrence) and with the overall survival to define immunohistochemical thresholds of prognostic significance. The optimal CD31 and CD105 cutoff points endowed with prognostic significance were determined after statistical validation (26).

The correlation between measurements of CD31 and CD105 in frozen, paraffin, and tissue microarray sections was assessed by computation of Spearman’s correlation coefficient, available in NCSS 2000 software.

**RESULTS**

**CD31 and CD105 Expressions on Frozen Sections.**

The semiquantitative analysis showed that the mean number of vessels stained with anti-CD105 was 11%, and with CD31, 23%. As previously shown (12, 13), the expression of CD105 correlated with overall survival or metastases-free status of node-negative patients (P ≤ 0.01; cutoff, n = 15) in contrast to that of CD31 (cutoff, n = 30; Fig. 1; Fig. 2; Fig. 3).

Densitometry by image analysis showed similar results with a significant correlation (ρ = 0.671, ρ = 0.71, P < 0.01) between automated and semiquantitative analysis.

**CD31 and CD105 Expression on Paraffin Sections.**

The semiquantitative analysis showed that CD31 and CD105 expressions were reduced on paraffin sections compared with frozen sections (Fig. 4; Fig. 5; Fig. 6; Fig. 7).

CD105 but not CD31 expression significantly correlated with patients overall and metastasis-free survival (P < 0.01; cutoff, n = 5). Node-negative patients with CD105 expression in fewer than five stained vessels survived (83 of 99) and were metastasis free (79 of 99) more than those with greater than 5 CD105-stained vessels (50 of 73 alive; 26 of 73 with metastasis), whatever the tumor size, type, or grade (Cox proportional hazard regression model).

Densitometry by image analysis showed similar results with a significant correlation (ρ = 0.824, ρ = 0.781, P < 0.01)

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**Fig 1** Kaplan Meier survivorship plot of overall and metastasis-free survival in frozen sections. A greater risk of death is observed in patients (n = 360) within tumor in which CD105 immunoeexpression is greater.
between automated and semiquantitative analysis and between results from automated image analysis in both frozen and paraffin sections ($p = 0.615$, $p = 0.659$, $P < 0.01$).

**CD105 and CD31 Expressions on Tissue Microarray Sections.** The semiquantitative analysis showed that CD31 and CD105 expressions in tissue microarrays were reduced compared with frozen and paraffin sections (Fig. 8; Fig. 9; Fig. 10). In addition, CD105 and CD31 did not significantly correlate with patients' outcome in tissue microarrays.

Densitometry by image analysis showed different results from those in paraffin sections, with no significant correlation between measurements on tissue microarrays and current paraffin sections.

In brief, the results show that (a) with both methods of measurement (semiquantitative or by image analysis), CD31 and CD105 expressions are correlated in frozen and paraffin sections; but (b) CD31 and CD105 expressions in current tissue sections, frozen or paraffin, do not correlate with those assessed in tissue microarrays.

**DISCUSSION**

The optimal conditions for antigen detection by immunohistochemical procedures are usually assessed on frozen sections, in which antigens are not damaged by fixation and heating during paraffin embedding. But frozen sections are less easy to handle than paraffin sections currently used in routine practice of pathology laboratories. And screening programs enable detection of significantly smaller tumors, measuring most often less than 20 mm in diameter. Therefore, sampling of breast tumors for storage in the tissue library of frozen tumors can now be assessed in our institution in only 30% of palpable tumors, because priority is given to optimal microscopic diagnosis, on which the patients' monitoring is essentially based after surgery, explaining the development of tissue microarrays for research purposes (reviewed in Refs. 21 and 22). This method is cost-effective, because about 600 different tumor fragments (and much more) may be tested on a single glass slide with the same antibody. This sophisticated and cost-effective method has proved to provide an excellent substrate for evaluating many
immunohistochemical markers in tumors essentially targeting epithelial carcinomatous cells.

However, the validation of this technique requires some pretests comparing, when possible, the expression of markers both in current “large” tissue sections and in tissue microarrays. Our results show that using standardized analysis (automated immunohistochemistry/Ventana Benchmark and densitometry by image analysis/ACIS I Microm), the vessel counts in tissue microarrays did not correlate with those in current “large” paraffin sections. And this likely results from the fact in that in current tissue microarray, the stromal areas are not large enough to appropriately evaluate tumor angiogenesis. Indeed, tissue microarray construction is usually assessed after a preselection, by microscope examination, of areas in the paraffin block that are the most representative of tumors and include carcinomatous rather than stromal areas, which are heterogeneous in breast carcinomas. Our results suggest that for a particular study design targeting the stromal surfaces especially to evaluate the immunostaining along vessels within the stromal compartment of carcinomas, the use of tissue microarrays does not appear to be appropriate. However, specific construction selecting stroma of carcinomas may be relevant.3 The quantification by image anal-

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analysis of immunoprecipitates developed mainly for research purposes (13–17) is expensive and time consuming. In the study, to obtain more reproducible measurements correlated with the patients’ follow-up, we used an automated procedures for microscopic images capture (ACIS hardware/software), which is significantly less time consuming for CD105 and CD31 immunostaining evaluation in paraffin or frozen tissue sections and tissue microarrays, in comparison with semiquantitative evaluation of vessels labeling. Although densitometry by image analysis is basically more accurate, our results using quantitative (ACIS) and semiquantitative (observers, C. C., J-P. D., L. A.) were significantly \((P < 0.01)\) correlated \((\rho = 0.671, \rho = 0.71\) for CD105 and \(\rho = 0.824, \rho = 0.659\) for CD31, respectively; Spearman coefficient), suggesting that semiquantitative analysis may be sufficient for current practice in institutions unable to use image analyzers. Moreover, the correlations of the vessels labeling with the patients’ outcome using both methods were similar, showing that greater CD105 immunoperoxidase correlated with a poorer survival. Our results show that anti-CD105, which labels activated endothelial cells (and thus neoangiogenesis), is an better indicator of poorer survival than CD31 in routine tissue sections or than CD34, as shown by others (8), when either semiquantitative (12) or automated image analyses were performed.

In conclusion, although tissue microarrays are very relevant for evaluating immunocytochemical markers within tumor cells, they are not suitable for evaluating neoangiogenesis in the stromal component of tumor and likewise should also not be suitable for evaluating other stromal markers, except if specific construction including only tumor stromal compartment can be performed. In addition, CD105 immunohistochemical expression is a prognostic indicator more sensitive than pan-endothelial markers and can be used on paraffin sections and semiquantitatively quantified, although frozen sections and computerized assisted image analysis are more accurate and more standardized but less cost-effective.

REFERENCES

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