The methodology of quantitation of microvessel density and prognostic value of neovascularization associated with long-term survival in Japanese patients with breast cancer

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Summary
The present study updates results on methodology of quantitation of tumor neovascularization and those on the prognostic value of microvessel density (MVD) in breast cancer tissue previously published in the World J. Surg. 21: 49–56, 1997. The follow-up period of observation of the series was extended to 20 years, and new biological indicators (i.e., proliferating cell nuclear antigen (PCNA), c-erbB-2, and p53) were included in the analysis. There were 109 patients with primary breast cancer, from 1971 to 1979, followed up for a median of 14 years (range, 1–20). A representative median longitudinal section of each breast tumor was immunohistochemically stained with factor VIII-related antigen and analyzed. The three methods of identifying MVD were: (1) average microvessel count (AMC)/mm², (2) central microvessel count (CMC)/mm², and (3) highest microvessel count (HMC)/mm². Thirty-one patients (28.4%) died of breast cancer. There was a relationship between MVD and peritumor blood vessel invasion (AMC: p = 0.0114, CMC: p = 0.0319, and HMC: p = 0.0009). However, there was no relationship between MVD and other factors. Univariate analysis showed that node status (p < 0.0001), histological grade (p < 0.0001), clinical tumor size (T) (p = 0.0002), PCNA (p = 0.0033), p53 (p = 0.0043), mitotic grade (p = 0.0092), AMC (p = 0.0214), and peritumor lymphatic vessel invasion (p = 0.0467) were significantly predictive of overall survival. HMC was borderline significant (p = 0.0702), while CMC and c-erbB-2 were not significant. Multivariate analysis showed that T (p = 0.0005), node status (p = 0.0053), and AMC (p = 0.0485) were independent factors, but neither CMC nor HMC was independent. AMC, a significant independent prognostic factor, might be a better method than the others for evaluating angiogenesis, but further and larger studies are warranted.

Introduction
Axillary lymph-node status has been the most important prognostic factor for breast cancer, associated with reduced overall survival (OS), and has been clinically used as a measure of dissemination of the disease. However, recent studies have suggested that tumor angiogenesis in invasive breast carcinoma is an independent and highly significant prognostic factor, including both node-negative and node-positive patients [1–4]. Weidner et al. reported that they used a method in which they selected the most vascular area in the tumor and counted the microvessels within a × 200 microscopic field. The vessel density indicated representative angiogenesis of the tumor and the microvessel count in the area of the most intense neovascularization is an independent and significant prognostic indicator for early-stage breast cancer [2]. On the other hand, other authors reported that angiogenesis did not predict recurrence in patients with primary breast cancer [5–8]. The studies varied in length of time of patient follow-up, antibody used to detect endothelial cells, method of counting microvessels, and the patients’ race. Thus, the significance of angiogenesis remains controversial.

The purpose of the present study is to update the authors’ previous study published in 1997 [9] by (1) adding a new method to the two previous methods of
quantitation of microvessel density and determining which method is the best and most objective approach to identify angiogenesis and to more accurately predict OS of Japanese patients with breast cancer, who differ in biological behavior from their Caucasian counterparts [10, 11], (2) extending the follow-up period of observation of the series to 20 years, and (3) examining the relationship between microvessel density (MVD) and 3 new biological markers (proliferating cell nuclear antigen (PCNA), c-erbB-2, and p53) in predicting OS rates associated with long-term survival in Japanese patients.

Patients and methods

Patients

Data for this study were analyzed from 109 selected breast cancer patients who underwent mastectomy between 1971 and 1979 at the Tokyo Women’s Medical College Hospital for whom there was sufficient clinical and pathologic material to determine all the biological markers. The distribution of clinicopathologic data for this patient population is listed in Table 1. It shows the main characteristics of the 109 assessable patients. There were 21 patients (19.3%) treated with extended radical mastectomy, 82 (75.2%) with radical mastectomy, and 6 (5.5%) with modified radical mastectomy. Forty-one patients (37.6%) were treated with chemotherapy (Mitomycin C was given intravenously after mastectomy, total dosage ranged from 32–40 mg), 46 patients (42.2%) with radiation (total dosage of 45–55 Gy administered to the axillary, the supraclavicular, and parasternal regions), 15 (13.8%) with both chemotherapy and radiation, and 7 patients (6.4%) were not given any adjuvant treatment.

Pathological studies

The original histologic sections of biopsy and mastectomy specimens were reviewed. Paraffin-embedded tissue samples of 4.5 μm thick sections stained with hematoxylin and eosin (H.E.) were histopathologically assessed. Conventional clinicopathologic features were observed and recorded, including age, menopausal state, the clinical tumor size (tumor size), node status, histological classification, histological grade, mitotic grade, peritumor lymphatic vessel invasion (LVI), and tumor necrosis. The tumor size was determined based on the TNM classification, the histological grade was decided based on the Bloom–Richardson grade [12], and LVI was determined based on H.E. staining. The mitotic grade was divided into three grades based on the number of mitoses in 10 high power fields (×400): Grade I showed no mitoses, Grade II 1–4 mitoses, and Grade III 5 or more mitoses. Sections of the breast tumor that were stained with H.E. were used to select the maximal area of all the cut surfaces of the tumor that included the invasive components.

Immunocytochemical determinations

PCNA

The 4.5 μm thickness sections were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. The sections were then rinsed in distilled water. The endogenous peroxidase activity was blocked with 0.3% of hydrogen peroxide for 20 min. The sections were washed in 0.05 M Tris-buffered saline, and treated with a protein blocking agent (Japan Tanner/Lipshaw Corporation, USA) before staining to reduce nonspecific antibody binding. Immunostaining was performed using a mouse monoclonal anti-human-PCNA antibody (PC10, Novocastra Laboratories, UK) diluted at 1:100 for 24 h at 4°C. Antibody binding was visualized by the streptavidin-biotin-immunoperoxidase method (OMNITAGS kit; Japan Tanner/Lipshaw Corporation, USA), followed by 0.05% 3,3′-diaminobenzidine tetrahydrochloride development. Sections were counterstained with Meyer’s hematoxylin and mounted. A normal human tonsil served as a positive control. As an internal negative control, each tumor was incubated with nonimmune mouse immunoglobulin G (IgG) instead of the primary antibody, followed by the procedure described above.

The growth fraction by PCNA staining was evaluated by counting 500 consecutive cells in a site with a relatively high degree of staining in the proliferating part of a tumor on one slide per tumor at a ×400 magnification, and an index of positive cells to total number of cells (Labeling Index (LI)) was made. LI was evaluated and scored as (−) (< mean of LI) and (+) (≥ mean of LI).

p53

After routine deparaffinization, the sections were pre-treated by microwaving at 500 W in 10 mM citrate buffer (pH 6) as an antigen retrieval. The slides were placed in citrate buffer and microwaved at 500 W for 5 min. After cooling by running them through cold water, they were then slowly rinsed in distilled water. The immunostaining of p53 was performed using a rabbit polyclonal anti-p53 antibody (CM1) (Novocastra Laboratories, UK) diluted at 1:100 for 24 h at 4°C. The