Osteopontin-Expressing Macrophages in Non-Small Cell Lung Cancer Predict Survival

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Background. **Tumor-associated macrophages (TAMs) are a major component of leukocyte infiltration in the tumor microenvironment. Osteopontin is related to tumor metastasis and proliferation. Osteopontin is expressed not only by tumor cells but also by TAMs. The purpose of the current study was to assess the prognostic significance of osteopontin expressed by TAMs (TOPN) in patients with non-small cell lung cancer.**

Methods. **Tissue microarray was used to detect the expression of TOPN, TAMs, and microvascular density in 159 patients with non-small cell lung cancer undergoing complete pulmonary resection in our hospital between 2003 and 2006. The correlations between TOPN, TAMs, and clinicopathologic data were analyzed with χ^2 tests. Quantitation of TAMs or TOPN and microvascular density analyses was performed using Bonferroni correction and the Student's *t* test. The prognostic value of TOPN was evaluated by univariate Kaplan-Meier survival analysis and multivariate Cox proportional hazard model analysis.**

Results. **In the recurrence and metastasis group, microvascular density was higher than that in the control group (14.4 ± 1.06 versus 8.9 ± 1.02; $p = 0.0002$). In the TOPN-positive group, microvascular density was increased compared with that in the TOPN-negative group (14.3 ± 1.37 versus 10.7 ± 0.91; $p = 0.0273$). Osteopontin expressed by TAMs was an independent predictor for overall survival ($p = 0.017$) and disease-free survival ($p < 0.001$), especially for stage I non-small cell lung cancer. The 6-year overall and disease-free survival rates in TOPN-positive patients were 22.64% and 16.98%, respectively, which were significantly lower than those of TOPN-negative patients (50.00% and 39.62%, respectively).**

Conclusions. **Osteopontin expressed by TAMs is a valuable independent predictor of tumor recurrence and survival in patients with non-small cell lung cancer.**

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Lung cancer is the leading cause of cancer death in the world [1]. Non-small cell lung cancer (NSCLC) comprises approximately 80% of all cases [2]. Although the therapy is more improved, the mortality rate is still high [3]; the overall 5-year survival rate is only 15% [4]. The extremely poor prognosis of NSCLC is largely the result of a high rate of distant metastasis and local recurrence after resection [5]. In the early stage, 40% of patients after complete pulmonary resection and systematic node dissection experience recurrent disease [6]. The current staging system for NSCLC is inadequate for predicting the outcome of treatment, and there is a special need to search for factors that will contribute to identifying the NSCLC patients with a high risk of metastasis and recurrence.

Microvessel density (MVD) is the most recognized indicator to evaluate angiogenesis of solid tumors, and quantitation of intratumor MVD by immunohistochemistry staining for vascular endothelial cell markers, such as CD34, has proven to be a useful prognostic predictor in hepatocellular carcinoma patients [7]. Osteopontin (OPN) is a multifunctional glycoprotein that is expressed in tumor cells of several human carcinomas [8]. Osteopontin has also been shown to be linked to tumor metastasis and proliferation [8–11], and it promotes metastasis of hepatocellular carcinoma and NSCLC cells and invasion by the mitogen-activated protein kinase and nuclear factor kappa B signaling pathway [9]. Macrophages are a major component of leukocyte infiltration in the tumor microenvironment [12]. Tumor-associated macrophages (TAMs) have complex functions in their interactions with cancer cells, which have been associated with worse outcomes in breast cancers [13] and classic Hodgkin lymphoma [14, 15]. Tumor-associated macrophages express a pattern of genes different from those of wound and peritoneal macrophages [16], such as OPN, which is elevated in TAMs [16]. White and colleagues [17] reported OPN-positive macrophages promote the in vitro invasion and metastasis of SK-Hep-1, a human cell line of adenocarcinoma of the liver. Osteopontin-positive macrophages have been identified as a biomarker for recurrence, and OPN is associated with TNM stage and lymphovascular invasion in bulky ampullary cancer [18]. Additionally, we found that OPN was expressed by some macrophages in extracellular matrix

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Abbreviations and Acronyms

DFS = disease-free survival
MVD = microvascular density
NSCLC = non-small cell lung cancer
OPN = osteopontin
OS = overall survival
TAMs = tumor-associated macrophages
TOPN = OPN expressed by TAMs

during out studies on tumor-secreted OPN. However, the role of OPN-positive macrophages remains unclear in NSCLC. In this study, we investigated the prognostic significance of OPN expression in TAMs in NSCLC.

Material and Methods

Patients

All NSCLC patients undergoing complete pulmonary resection and systematic lymph node dissection at the Cancer Institute and Hospital of Tianjin Medical University (China) between 2003 and 2006 were considered eligible for retrospective analysis of clinical prognostic factors. Patients who were included in the study met the following criteria: (1) a distinctive NSCLC diagnosis based on World Health Organization criteria; (2) a computed tomography or magnetic resonance imaging scan for brain, chest, and abdomen, and emission computed tomography scan for bone preoperatively to make sure that there was no metastasis; (3) no prior anticancer treatment; (4) adjuvant chemotherapy or radiotherapy, alone or in combination after surgery, for stage II and IIIa patients; (5) complete pulmonary resection and systematic lymph node dissection; and (6) complete clinicopathologic and follow-up data. Ethical approval for human subjects was obtained from the research ethics committee of Tianjin Cancer Institute and Hospital, and informed consent was obtained from each patient. Patients who died within 1 month after surgery were excluded from the study. Tumor staging was based on the most recent International Association for the Study of Lung Cancer TNM classification system [3]. All patients were followed up until December 31, 2012. Patients who were still alive after the last follow-up were censored in the study.

Tissue Microarray and Immunohistochemistry

For tissue microarray construction, 2 experienced pathologists reviewed hematoxylin and eosin–stained sections from each paraffin-embedded, formalin-fixed block. Typical tumor regions were morphologically identified and marked on hematoxylin and eosin–stained slides. Two cores were taken from the marked areas of each block by using tissue cylinders with a diameter of 1.5 mm. To validate the concordance between tissue microarrays and whole tumor sections, we further detected OPN, CD34, and CD68 expression for 30 cases randomly chosen from the 159 patients in comparison with whole tumor sections.

The immunohistochemical examination of OPN and TAMs was performed with monoclonal anti-rabbit OPN antibody at a 1:200 dilution (rabbit, ab8448, Abcam, Cambridge, MA) and CD68 antibody at a 1:100 dilution (clone ED1; Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemistry was performed using a two-step immunoperoxidase technique [19]. After deparaffinization and heat-induced antigen retrieval, tissues were incubated with primary antibodies overnight at 4°C. After half an hour of incubation with secondary antibody, the sections were developed in 3,3′-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Negative control slides with the primary antibodies omitted were included in all assays.

Two investigators who were unaware of the clinical data independently evaluated CD68 staining for TAMs under a light microscope at a magnification of ×400. The numbers of CD68-positive TAMs [20] and of CD34-positive MVD were counted per unit area under a magnification of high-power field (×400) in five fields [21].

Immunofluorescence Staining for CD68 and Osteopontin

Tissue sections were deparaffinized and hydrated, and heat-induced antigen was retrieved using 0.1 mol/L citric acid (pH 6.0) for 20 minutes. Sections were washed with phosphate-buffered saline solution, blocked with 3% bovine serum albumin in phosphate-buffered saline solution, and then incubated with a mouse monoclonal anti-CD68 antibody (1:100) overnight at 4°C. After extensive washing, the sections were incubated with AlexaFluor 555 anti-mouse immunoglobulin G (1:150; BD Biosciences Pharmingen, San Diego, CA) for 90 minutes at room temperature. The sections were washed extensively and incubated with a rabbit monoclonal anti-OPN antibody (1:100) overnight at 4°C. After extensive washing, the sections were further incubated with AlexaFluor 488 anti-rabbit immunoglobulin G (1:150; BD Biosciences Pharmingen) for 90 minutes at room temperature. The sections were washed extensively and incubated with 4′,6-diamidino-2-phenylindole (Santa Cruz Biotechnology, SC-300415, 1 μg/mL). The sections were visualized using a confocal laser scanning microscope (Olympus BX51; Olympus, Tokyo, Japan).

Statistical Analysis

The association between variables was analyzed by χ^2 testing analysis. Quantitation of TAMs or TOPN and MVD analyses was performed using Bonferroni correction and the Student's *t* test. Overall survival (OS) was defined as the interval between the date of surgery and date of death or last follow-up. Disease-free survival (DFS) was defined as the duration between the date of surgery and the date of first recurrence or last follow-up. Disease-free survival and OS were analyzed using the Kaplan-Meier method and compared using the log-rank test, and multivariate analyses were tested by Cox proportional hazard model. A probability value of less than 0.05 was considered significant. The data were analyzed using SPSS (version 18.0; IBM Corp, Armonk, NY) statistical programs for all analyses.