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Geminin expression in small lung adenocarcinomas: Implication of prognostic significance

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ABSTRACT

Geminin is an important molecule which plays a role in cell cycle regulation, and this has been considered to be a useful biomarker of cell proliferation. The purpose of this study was to evaluate the pathological and prognostic significance of geminin expression in small lung adenocarcinoma (AC). We performed Western blot analysis of five human lung AC cell lines and immunohistochemistry on 100 surgically resected specimens of lung AC with a diameter less than 3 cm. We counted the number of positively stained tumor cells, and calculated the labeling indices (LIs). Geminin proteins were variably detected in all five cell lines examined on Western blotting. The mean LIs for geminin, Ki-67, and MCM7 were 7.5%, 12.3%, and 18.5%, respectively. The geminin LIs were associated with some clinicopathological profiles including gender, histological grade, subtypes, N-status, p-factor, and tumor stage. A significantly worse prognosis was noted in the higher geminin LIs group than in the lower group ($p < 0.01$). Multivariate Cox regression analysis also confirmed that geminin LIs was an independent prognostic marker in stage IA lung AC patients. These results suggest that geminin is overexpressed in small lung ACs, and geminin LIs might be a useful prognostic indicator in patients with lung AC.

1. Introduction

Lung cancer is currently the leading cause of cancer death in Japan and worldwide [1]. Although the prognosis of patients mainly depends on the tumor stage, the long-term survival rate of stage IA non-small cell lung cancer (NSCLC) is still poor, even when resected completely [2–4]. Among NSCLC, the incidence of lung adenocarcinoma (AC) has recently increased due to the availability of computed tomography (CT) screening and the accuracy enhancement of high-resolution CT (HRCT), especially among women and non-smokers [5–7]. In spite of various studies into the prognostic biomarkers of lung AC [8–12], these have not significantly contributed to improving the prognosis. It is hoped that useful clinical biomarkers predicting the prognosis more precisely will be identified and utilized to improve patients' prognosis with lung AC. Among a number of cancer biomarkers, we have investigated the proteins associated with the cell cycle and evaluated their clinical implications.

Eukaryotic cell division is regulated through multiple convergent pathways to restrict genome duplication to once and only once each time a cell divides [13]. In the cell cycle, the minichromosome maintenance complex (MCM2-7) plays an important role for the facilitation of the DNA replication, and MCM2 and MCM7 have been shown to be reliable proliferating biomarkers of small lung AC [10,12]. Geminin, which is expressed during S, G2, and M phases, is also a protein which plays a crucial role for the regulation of cell cycle. After the beginning of DNA replication in early S phase, geminin inhibits recruitment of the MCM complex onto chromosomes and prevents a second cycle of replication [14–16]. Some immunoblotting and immunohistochemical studies have demonstrated that geminin is specifically expressed in proliferating lymphocytes, male germ cells and various epithelial cells including those in the skin, uterine cervix, and colon [17–19]. As for malignant neoplasms, several reports revealed that geminin was overexpressed in various human cancer cells, and its expression was increased with tumor grade [20–24], leading to poor prognosis [25–27]. Therefore, it appears that geminin might reflect the proliferative activities of malignant tumors and could be a useful prognostic biomarker. Moreover, geminin might become a new biomarker such as Ki-67 or MCM complex from which the values have been established as useful biomarkers, especially in lung cancer [10,12,28].

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There have been no studies which have evaluated the pathobiological significance of geminin in human lung AC. In this study, we examined the expression of geminin in human lung AC with a diameter less than 3 cm, and assessed its prognostic implication in comparison with the expression of Ki-67 and MCM7.

2. Materials and methods

2.1. Cell culture and Western blot analysis

Expression of geminin was examined in five human lung AC cell lines: A549 and PC14 (purchased from Riken Cell Bank, Tsukuba, Japan), LK87 and LCSC#1 (provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan), and Ma-1 (provided by Dr. T. Hirashima, Osaka Prefectural Habikino Hospital, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2 in air. The subconfluent cells were rinsed in phosphate-buffered saline (PBS) and solubilized in lysis buffer [150 mM NaCl, 20 mM Tris–HCl (pH 7.4), 0.1% sodium dodecyl sulfate, and 1% Triton X-100, containing a mixture of proteinase inhibitors (5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml trypsin inhibitor)] for 60 min on ice. Lysates were centrifuged at 12,000 rpm for 5 min. The protein concentrations were determined by Bradford protein assay (Bio-Rad Lab, Richmond, CA, USA) using bovine serum albumin as the standard. Equal amounts (30 μg) of the proteins were resolved by electrophoresis on the 12% sodium dodecyl sulfate-polyacrylamide gel, and then electro-transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Waltham, MA, USA). After blocking the nonspecific binding with 10% skim milk in PBS for 1 h at room temperature, the blotted membrane was incubated with rabbit anti-geminin antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-β-actin antibody (1:2000; Sigma, St. Louis, MO). The blots were developed with peroxidase-labeled secondary antibodies. After extensive washing, specific bands were detected using an enhanced chemiluminescence system (ECL detection system, Amersham Biosciences, UK).

2.2. Patients and surgical specimens

This study enrolled a total of 100 consecutive patients with lung AC who underwent curative resection at Tottori University Hospital between January 1997 and December 2005. All the 100 tumors were diagnosed as lung AC with a diameter less than 3 cm (pT1). Routinely, neutral buffered formalin (pH 7.4)-fixed and paraffin-embedded tumor tissue samples were sectioned in 3 μm serial slices. The sections were stained by hematoxylin with eosin (HE) and elastic-van-Gieson (EvG) stain. The patients included 51 males and 49 females, with a mean age of 68.1 ± 9.8 (S.D.) years old (range: 26–85 years). Histological specimens had been reviewed by a qualified pathologist at diagnosis and assessed for histological subtype and tumor grade according to the World Health Organization (WHO) criteria [1]. These included 25 bronchioloalveolar carcinomas (BACs), and 75 non-BACs consisting of 7 acinar, 19 papillary, 8 solid AC with mucin, and 41 AC with mixed subtypes. Among 25 BACs, there were 2 cases of mucinous BAC and 2 cases of minimally invasive adenocarcinoma (MIA) which had small invasive foci (less than 5 mm) in the tumor. BAC was noted in 17 cases (17/25: 68%) of 49 female patients and in 8 cases (8/25: 32%) of 51 male patients, the frequency being significantly higher in the female patients ($p = 0.03$). Well, moderate, and poorly differentiated ACs were 46, 43, and 11 cases, respectively. Tumor stage of the disease at pathological diagnosis was determined according to the UICC guidelines of the TNM classification of malignant tumor [29]. The pathological stage of lung cancer was IA in 83 patients, IB in 4, IIA in 3, IIB in 1, IIIA in 8, IIIB in 1, respectively. Pleural involvement (p-factor) was classified as p0, p1, p2, and p3; p0 included tumor with no pleural involvement or reaching the visceral pleura but not extending beyond its elastic pleural layer; p1 included tumor reaching visceral pleural elastic layer but not exposed on the pleural surface; p2 included tumor exposed on the pleural surface; and p3 included tumor invading parietal pleura or chest wall. Selected operative procedures were lobectomy in 77 patients, segmentectomy in 8, and wedge resection in 15, respectively. We classified the tumor size as follows: less than 20 mm or exactly 20 mm (≦20 mm) and more than 20 mm (>20 mm, ≦30 mm). The median follow-up period was 61.4 months (range: 2–141 months). The study protocol was approved by the institutional review board.

2.3. Immunohistochemistry

Tissue sections were de-waxed in xylene, rehydrated though a graded series of ethanol solution, rinsed in distilled water for 5 min, and then immersed in 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min to block endogenous peroxidase. For antigen retrieval, sections were microwaved in 0.01 mol/L of sodium citrate buffered saline (pH 6.0) for 20 min at 95 °C, using a microwave processor model MI-77 (Azumaya, Tokyo, Japan). After being rinsed in PBS for 5 min, the slides were pre-blocked with a solution of 2% FBS at room temperature for 20 min, and incubated at 4 °C overnight with the antibodies. The following monoclonal antibodies were used for immunohistochemistry; rabbit anti-geminin antibody (1:100 dilution; Santa Cruz Biotechnology), mouse anti-MCM7 antibody (1:100 dilution; Santa Cruz Biotechnology), mouse Ki-67 antibody (1:50 dilution; MIB-1, Dako, Glostrup, Denmark), and mouse anti-P53 antibody (1:50 dilution; DO7, Dako, Glostrup, Denmark). A subsequent reaction was initiated by the streptavidin-biotin-peroxidase complex technique (SAB method) using a Histofine SAB-PO (M) immunohistochemical staining kit (Nichirei, Tokyo, Japan). The immunoreactions were visualized with 0.2 mg/mL 3,3′-diaminobenzidine and 20 μL/dL hydrogen peroxide in 0.05 M Tris–HCl buffer (pH 7.6). Finally, the slides were counterstained with 0.1% hematoxyline and then dehydrated and mounted.

2.4. Evaluation of immunohistochemical findings

To evaluate geminin, Ki-67, MCM7, and P53 expression, positively stained tumor cell nuclei were counted. Counts were performed in high-magnification fields using the FLOVEL Image Filling System FlvFs (FLOVEL Inc., Tachikawa, Japan). Both positive and negative cells within the fields were counted and any stromal or inflammatory cells were excluded. The percentage of positive cells was determined by the three authors (T.H., K.S., and H.I.) independently. For geminin, Ki-67, and MCM7, at least 1000 tumor cells were counted in areas showing a high frequency of cells with these positive nuclei, and these labeling indices (LIs) were calculated using the following formula: LI = number of positive cells/total number of cells × 100. Geminin, Ki-67, MCM7, and P53 LIs were categorized as high if these LIs were equal to or more than the mean LIs (geminin = 7.5%, Ki-67 = 12.3%, MCM7 = 18.5%, and P53 = 16.4%, respectively). To confirm the specificity of the immunostaining results, sections immunoreacted without the primary antibodies were used as negative controls.