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Expression of Mina53, a novel c-Myc target gene, is a favorable prognostic marker in early stage lung cancer

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ABSTRACT

Mina53, a novel target gene product of c-Myc, is overexpressed in various malignancies. We previously demonstrated that Mina53 is overexpressed in lung cancer patients from the early clinical stages. In this paper, the association between disease prognosis and Mina53 expression in lung cancer patients is analyzed; we found that overexpression of Mina53 in lung cancer patients is associated with favorable prognosis. Statistical analysis using the Kaplan–Meier method showed that patients with negative staining for Mina53 had significantly shorter survival than patients with positive staining for Mina53, especially in stage I or with squamous cell carcinoma. Because the major cause of death in lung cancer patients after surgery is distant metastasis, the effect on cancer cell invasiveness was analyzed for the mechanisms involved in the association with favorable outcome. Overexpression of Mina53 in H226B, a lung squamous cell carcinoma cell line, inhibited cancer cell invasion. Transfection with *mina53* shRNA increased the number of invading cells. These results suggest that Mina53 immunostaining is a useful prognostic marker – especially in the early stage of lung cancer – and that Mina53 negative patients should be managed particularly carefully after surgery.

1. Introduction

Lung cancer is the leading cause of cancer death among males in Japan [1]. Despite development of a therapeutic strategy, improvements in prognosis remain unsatisfactory. Early diagnosis can lead to better prognosis: 5-year survival is 83.9% at pathologic stage IA but only 66.3% at stage IB, according to a recent report of the Japanese Joint Committee of Lung Cancer Registry [2]. Aspects of cancer progression, such as regional lymph node involvement or distant metastasis, are observed in over two-thirds of patients at diagnosis [3]. Extrathoracic metastases are present in 13% of lung cancer patients with small sized primary lesions (smaller than 3 cm) [4]. Therefore, controlling progression is an important step towards prolonging the survival of lung cancer patients.

Myc-induced nuclear antigen with a molecular mass of 53 kDa (Mina53) is overexpressed in various malignancies, such as colon cancer, lymphoma, esophageal cancer, renal cell carcinoma, and neuroblastoma. Mina53 is detected by immunohistochemistry using anti-Mina53 monoclonal antibody [5–10]. To assess the involvement of Mina53 in lung cancer, we examined its expression in lung cancer tissues. We previously demonstrated that Mina53 is overexpressed in 62% of lung cancer patients from the early clinical stages (manuscript submitted). In addition, the enforced expression of Mina53 in NIH/3T3 cells induces cell transformation, and *mina53* transfected NIH/3T3 clones produce tumors in nude mice, suggesting that Mina53 has oncogenic potential. The differentially expressed genes examined by a cDNA microarray showed that Mina53 regulates several genes related with cell adhesion, metabolism, and cytokine/growth factors such as epidermal growth factor receptor (*EGFR*), interleukin-6 (*IL-6*), and hepatocyte growth factor (*HGF*). These results suggest that Mina53 plays an important role in carcinogenesis associated with the cytokine network.

Generally, patients in the early stage of lung cancer have good prognosis following radical resection: 5-year survival is 83.9% at pathologic stage IA. Nevertheless, approximately 20% of lung cancer patients with stage IA show local recurrence or distant metastasis. Therefore, identifying patients at high risk of recurrence among

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those in the early stage of lung cancer is important for determining adjuvant treatment and appropriate management after surgery. In the present study, we found that downregulation of Mina53 in lung cancer cells induced an invasive phenotype and patients who evidenced high expression levels of Mina53 showed favorable prognosis. These results suggest that Mina53 may useful for risk assessment in early stage lung cancer patients.

2. Materials and methods

2.1. Tissue samples and lung cancer cell lines

Tissue samples were obtained from surgical specimens of 101 lung cancer patients. Study patients underwent treatment at Saga Medical School Hospital with surgery but no chemotherapy or thoracic irradiation. The population was a consecutive series of patients treated between 1998 and 2000. Clinical stage was determined by criteria of the International Union Against Cancer. The study protocol was approved by the Clinical Research Ethics Committee of Saga University and all patients gave informed consent for obtaining surgical specimens. Human lung cancer cell lines H226B, A549, and a mouse fibroblast cell line, NIH/3T3 were purchased from American Type Culture Collection (Manassas, VA). H226B, and A549 cells were cultured in RPMI1640 containing 10% fetal bovine serum at 37 °C in 5% CO_2. NIH/3T3 cells were cultured in DMEM containing 10% fetal bovine serum.

2.2. Immunohistochemical staining

Immunohistochemical analysis with anti-Mina53 antibody was performed as follows. For antigen retrieval, deparaffinized 4 μm-thin tissue sections were microwaved in 0.01 M sodium citrate buffer (pH 6.0). After tissue sections were blocked with 3% hydrogen peroxide in phosphate-buffered saline for 30 min at room temperature, each section was incubated overnight at 4 °C with anti-Mina53 antibody, at a dilution of 1:500, and MIB-1 mouse monoclonal antibody against Ki-67 (DAKO Co., Carpinteria, CA), at 1:100. Anti-Mina53 monoclonal antibody was established using a hybridoma clone, M532, secreting an IgG2a antibody as described previously [5]. A DAKO ENVISSION system (DAKO Corp., Carpinteria, CA) was used for detection. Counter-staining was performed with hematoxylin. Immunohistochemical examination with each antibody was evaluated by the following criteria and scored according to sequential arbitrary cutoffs. Staining of the cancer cell nuclei with anti-Mina53 antibody in whole tissue section, with no remarkable staining of parenchymal cells, was taken as positive and scored as − (0–10% positive cells), ± (10–30%), + (30–60%), or ++ (more than 60%). The Ki-67 labeling index was defined as the percentage of nuclear-stained cells among at least 1000 tumor cells for each section; representative areas in each tissue section were selected and cells were counted in at least four fields in these areas (score −, Ki-67 labeling index from 0 to 10%; score ±, 11 to/25%; score +, 26 to/50%; score ++, more than 50%). All slides were concomitantly scored by three researchers.

2.3. Transient transfection of mina53 expression plasmid and mina53 shRNA

The 1.5-kb HindIII/NotI fragment of *mina53* cDNA was ligated into the NheI/NotI site of pCAGGS mammalian expression vector to produce pCAGGS/*mina53* [4,11]. The cDNA fragment was obtained by PCR (GeneAmp 9600, PerkinElmer) using a cDNA library of HEL cells as described previously [4]. One day after seeding in 10 cm dishes, 5×10^5 cells were transfected with the indicated amount of pCAGGS/*mina53* or pCAGGS using LipofectAMINE (Invitrogen, Corp. CA). After 30 h of incubation in complete medium, western blot analysis was performed to determine the Mina53 expression. The *mina53* shRNA construct was established as follows. To obtain human U6 siRNA vector based on pcDNA3, the human U6 promoter, which contains the cloning sites HindIII and BamHI, was amplified from human genomic DNA with primer pairs 5′-AGATCTGAATTCCCCAGTGGAAAGACGCGCAGGC and 5′-AGATCTA-AGCTTCTCGAGGATCCCGCGTCCTTTCCACAAGATATATAAACCCAAG, then ligated into the BglII site of pcDNA3. To construct the pcDNA3-GFP-hU6 siRNA vector, GFP cDNA fragment obtained from pEGFP-N3 (Clontech Laboratories, Inc. Mountain View, CA) was ligated into the multi-cloning site of human U6 siRNA vector. The partial DNA fragments of human *mina53* DNA were chemically synthesized and cloned into pcDNA3-GFP-hU6siRNA cleaved with HindIII and BamHI to produce pU6shmina53(892)/GFP. The synthesized DNAs for pU6shmina53(892)/GFP were 5′-GATCCCCAGG-TGGAATCCACAACTGTTTTC AAGAGAAACAGTTGTGGATTCCACCT-GTTTTTGGAAG and 5′-AGCTCTTCCAAAAACAGGTGGAATCCACAA-CTGTTTCTCTTGAAAACAGTTGTGGATTCCACCTGGG. Transfection was performed in the same way as with the *mina53* expression plasmid.

2.4. Western blot analysis

Whole cell lysates were prepared from lung cancer cell lines or cancer tissues using lysis buffer containing Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1 μg/ml leupeptin, 10 μg/ml aptrotinin, and 1 mM phenolmethylsulfonyl fluoride. Fifty micrograms of protein were separated using a 10% NuPAGE electrophoresis system (NOVEX, San Diego, CA), transferred to a nitrocellulose membrane (Schleicher & Schell, Inc., Keene, NH), blocked with 5% milk at 4 °C overnight, and then reacted with anti-Mina53 or anti-Actin (Santa Cruz Biotechnology, CA) antibodies. An ECL kit (Amersham Corp., Arlington Heights, IL) was used for detection.

2.5. Analysis of cell proliferation, cell cycle, and apoptosis

Cells were transiently transfected by the indicated amount of pCAGGS/*mina53* or pCAGGS, then counted using trypan blue staining. After 5×10^3 cells were cultured, the number of cells was counted at the indicated point in time. The experiments were undertaken three times. The analysis of cell cycle was performed as follows. At the indicated time following transient transfection of A549 cells with pCAGGS/*mina53* or pCAGGS, both floating and adherent cells were collected by trypsinization and fixed with 2% paraformaldehyde followed by 70% ethanol. Cells were treated with RNase A (0.25 mg/ml) at 37 °C for 30 min and stained with propidium iodine (50 μg/ml). Cellular DNA content was analyzed by flow cytometry and the cell cycle profiles were determined with CELLQuest™ software (Becton Dickinson, NJ). As for analysis of apoptosis, cells were simultaneously stained with Annexin V and propidium iodide (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. Stained cells were conducted to a FACS analysis to determine the number of apoptotic cells.

2.6. In vitro cell invasion assay

The in vitro cell invasion assay was performed using BD BioCoat® Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ). Twenty-four hours after transfection with pCAGGS/*mina53* or pCAGGS, 1.0×10^5 cells were added to each of the upper chambers with serum-free growth medium, whereas the lower wells contained growth medium with 5% FBS. After an additional 22 h of incubation, non-invading cells on the upper side of the chamber membranes were removed. Cells invading the opposite side of the chamber membranes were examined. The invading