Increased *Sox2* copy number in lung squamous cell carcinomas

HIDEFUMI SASAKI, KEISUKE YOKOTA, YU HIKOSAKA, SATORU MORIYAMA, MOTOKI YANO and YOSHITAKA FUJII

Department of Oncology, Immunology and Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

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Abstract. The transcription factor Sox2 is necessary for foregut morphogenesis. Sox2 is also required for the normal development of the trachea and lung. Recently, *Sox2* amplifications were investigated using large-scale single nucleotide polymorphism arrays in esophageal and lung cancer. We hypothesized that *Sox2* overexpression might be correlated with clinicopathological features of lung cancers. The increased copy number of the *Sox2* gene was analyzed by real-time polymerase chain reaction amplifications in 127 surgically treated non-small cell lung cancer cases from Nagoya City University Hospital, Japan. A total of 87 squamous cell carcinoma (SCC) cases were involved. An increased *Sox2* gene copy number was found in 42 (33.1%) lung cancer patients. Increased *Sox2* copy number status was significantly correlated with gender (females, 9.5% vs. males, 34.1%; p=0.0026), smoking status (never smoker, 4.8% vs. smoker, 32.9%; p=0.0003) and pathological subtypes (squamous cell carcinoma, 44.8% vs. non-squamous cell carcinoma, 7.5%; p<0.0001). However, among the SCCs, the *Sox2* copy number status was not significantly correlated with gender, smoking status, pathological stage or differentiation status. An increased *Sox2* copy number is common within SCC.

Introduction

Lung cancer is a major cause of mortality among malignant diseases due to its high incidence and malignant behavior as well as a lack of major advancements in treatment strategy (1). Lung cancer was the leading indication for respiratory surgery (46.7%) in 2007 in Japan (2), and more than 25,000 patients underwent surgery for lung cancer at Japanese institutions in the same year (2). The clinical behavior of lung cancer is largely associated with its stage. The cure of the disease by surgery is only achieved in cases representing an early stage of lung cancer (3).

Sox2 belongs to a family of evolutionarily conserved transcription factors containing a Sry-related high mobility group (HMG) box (4,5). Sox proteins are recognized as key players in the regulation of embryonic development and determination of cell fate and maintenance (6-9). Later in development, Sox2 is expressed in the endodermal epithelium of the tongue, esophagus, trachea and lung, and plays crucial roles in the differentiation and morphogenesis of these organ systems (5,10-12). Recently, *Sox2* amplifications were investigated using large-scale single nucleotide polymorphism (SNP) arrays in esophageal and lung cancer (13). Sox2 is highly expressed in squamous cell carcinoma (SCC) of the gastrointestinal tract (14). Although Sox2 expression was also investigated in small cell lung cancers (15) and lung SCC (mRNA) (16), the correlation between *Sox2* gene status and lung cancer in Japan has not been previously reported.

To determine the *Sox2* copy number status in Japanese lung carcinoma patients for screening purposes, we investigated the *Sox2* copy number using real-time polymerase chain reaction (real-time PCR) amplifications. The findings were compared against the clinicopathological features of lung cancer.

Patients and methods

Patients. The study group included 127 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School, Japan, between 2001 and 2008. All tumor samples were immediately frozen and stored at -80˚C until assayed. The clinical and pathological characteristics of the 127 lung cancer patients were as follows: 78 cases at stage I, 24 at stage II, and 25 at stages III-IV. The mean age was 66.0 years (range, 29-86). Among the patients, 30 (23.6%) were non-smokers, 94 (74.0%) were male and 87 (68.5%) were diagnosed as having SCC (this study focused mainly on SCC). The samples from these patients had previously been sequenced for EGFR (17-20).

PCR assays for Sox2. Genomic DNA was extracted from lung cancer tissues using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop

Correspondence to: Dr Hidefumi Sasaki, Department of Immunology, Oncology and Surgery, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
E-mail: hisasaki@med.nagoya-cu.ac.jp

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Table I. Clinicopathological data of 127 lung cancer patients.

Factors		*Sox2* gene status		p-value
		Amplified Patients (n=42)	Normal Patients (n=85)	
Mean age (years)	66.0±10.2	67.1±8.7	65.5±10.8	0.4134
Stage				
I		26 (61.9%)	52 (61.2%)	0.9999
II-IV		16 (38.1%)	33 (38.8%)	
Lymph node metastasis				
N0		29 (55.8%)	56 (65.9%)	0.2779
N(+)		23 (44.2%)	29 (34.1%)	
Smoking status				
Never smoker		2 (4.8%)	28 (32.9%)	0.0003
Smoker		40 (95.2%)	57 (67.1%)	
Differentiation				
Well-differentiated		6 (15.4%)	21 (31.3%)	0.1046
Moderate/poor		33 (64.6%)	46 (68.7%)	
Pathological subtype				
Squamous		39 (92.9%)	48 (56.5%)	
Non-squamous		3 (7.1%)	37 (43.5%)	<0.0001
Age				
≤65		18 (42.9%)	39 (45.9%)	0.8501
>65		24 (57.1%)	46 (54.1%)	
Gender				
Male		38 (90.5%)	56 (65.9%)	0.0026
Female		4 (9.5%)	29 (34.1%)	

N0, lymph node metastasis negative; N(+), lymph node metastasis positive; Squamous, squamous cell carcinoma.

Technologies, Inc., Rockland, DE, USA) and adjusted to a concentration of 2.5 ng/ml.

We then used 5 μl of each DNA for the PCR assays. The *Sox2* copy number was analyzed by quantitative real-time PCR and performed on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR-Green kit (Qiagen, Inc., Valencia, CA, USA) (21,22). The PCR run was performed in triplicate for each patient. The *Sox2* primers used for amplification were 5'-CAAAGAAAAA CGAGGGAAAT-3' and 5'-ATGGGATTGGTGTTCTCTTT-3'. Total DNA content was estimated by assaying *LINE-1* elements for each sample using the primers 5'-AAAGCCGCTCAA CTACATGG-3' and 5'-TGCTTTGAATGCGTCCCAGAG-3'.

The cycling conditions were as follows: initial denaturation at 95˚C for 15 min, followed by 40 cycles at 94˚C for 15 sec, 56˚C for 30 sec, and 72˚C for 34 sec.

Immunohistochemistry. Sox2 and p63 protein expression were evaluated by immunohistochemistry (IHC) using an anti-Sox2 antibody (rabbit polyclonal; Thermo Scientific, Rockford, IL, USA) and a p63 ready-to-use antibody (mouse, clone 4A4; Dako, Carpinteria, CA, USA). We used a standard protocol for the immunostaining of the samples. Sections (4 μm) were cut from paraffin tissue blocks from the non-small cell lung cancer tumors. The slides were treated with xylene then dehydrated in alcohol. For epitope retrieval, specimens were exposed to 10 mM citrate buffer (pH 6.0) and heated to approximately 10 min in a microwave. Endogenous peroxidase activity was blocked with H_2O_2 in methanol. Sections were incubated with blocking solution (10% Block Ace) and then reacted with anti-Sox2 (x250) or p63 antibody overnight at 4˚C. After washing out the excess antibody with phosphate-buffered saline (PBS), samples were incubated with a peroxidase-conjugated anti-mouse antibody (Mouse HRP EnVision™+; Dako) for 45 min. After washing out the excess antibody with PBS, 3,3'-diaminobenzidine (DAB) substrate (10 min) was used to visualize the antibody binding, and the sections were counterstained with hematoxylin. Sox2 and p63 staining was evaluated under a light microscope at x400 magnification. Tumor nuclear staining intensity was graded on a scale of 1-4. The percentage of positive tumor nuclei was evaluated and a proportion score was attributed (1, <5%; 2, 5-25%; 3, 25-50% and 4, >50%), as previously described (23,24).

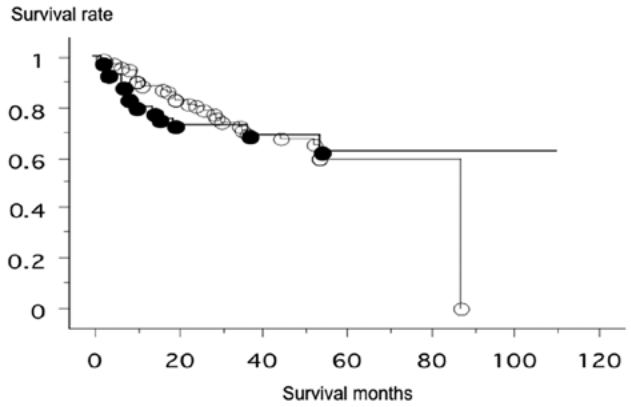

Figure 1. The prognosis of patients with an increased *Sox2* gene copy number (●, n=42, 13 were deceased; mean survival, 40.8 months) and patients with a normal *Sox2* copy number (○, n=85, 27 were deceased; mean survival, 62.8 months) was not significantly different (log-rank test, p=0.9299).

Statistical analysis. Statistical analyses were performed using the Mann-Whitney U-test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using the Spearman's test and the χ^2 test. The overall survival of lung cancer patients was evaluated by the Kaplan-Meier method, and differences were determined by the log-rank test. All analyses were performed using the StatView software package (Abacus Concepts Inc., Berkeley, CA, USA), and $p<0.05$ was considered significant.

Results

Sox2 gene status in Japanese lung cancer patients. Using the primer sets for the *Sox2* gene, 42 of the 127 lung cancer patients were found to have more than 4 copies of the *Sox2* gene. The clinicopathological background of the patients is shown in Table I. The *Sox2* gene copy status was significantly correlated with gender (male, 40.4% vs. female, 12.1%; p=0.0026), tobacco smoking (non-smokers, 6.6% vs. smokers, 41.2%; p=0.0003) and pathological subtype (squamous cell carcinoma, 44.8% vs. non-squamous cell carcinoma, 7.5%; p<0.0001), but not with pathological stage (stage I vs. II-IV, p=0.3878) or age (≤65 vs. >65; p=0.8052). The increased *Sox2* gene copy number was observed in 2/13 (15.4%) of the adenosquamous cell carcinomas and 1/26 (3.8%) of the adenocarcinomas. There was a tendency towards a higher degree of differentiation within the normal *Sox2* copy number group when compared with the increased *Sox2* copy number group (p=0.1046). The overall survival of the 127 lung cancer patients from Nagoya City University, who were followed-up until December 31 2009, was studied in reference to the *Sox2* gene status. The survival rate of patients with an increased *Sox2* gene copy number (n=42, 13 were deceased) and patients with a normal *Sox2* copy number (n=85, 27 were deceased) was not significantly different (log-rank test, p=0.9299; Fig. 1).

Immunohistochemistry. The immunohistochemical evaluation was performed according to the scoring system described in Materials and methods. IHC was performed for 72 patients, since the tissue blocks were not available for the other patients. The p63-positive (≥4) ratio (Fig. 2) was more closely correlated with squamous histology (adenosquamous + SCC, 95.2% vs. adenocarcinoma, 60%; p=0.0004) than the Sox2-positive ratio (adenosquamous + SCC, 85.4% vs. adenocarcinoma, 45.5%; p=0.001) (Fig. 3). The p63 IHC was correlated with an increased *Sox2* copy number (p=0.0226). The Sox2-positive IHC cases had a tendency towards an increased *Sox2* copy number (p=0.1035). However, the p63 IHC status (p=0.8188) and Sox2 IHC status (p=0.3394) did not correlate with the prognosis of lung cancers.

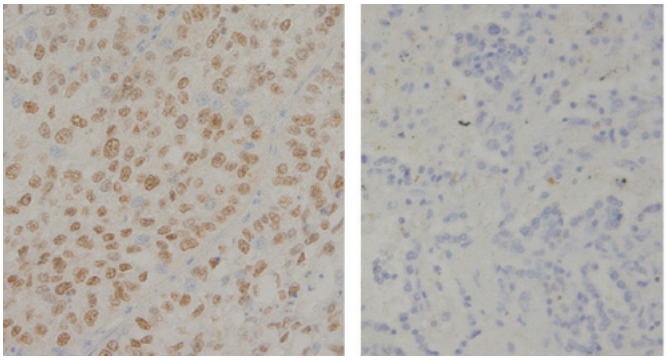

Figure 2. Immunohistochemistry for p63. Left, p63-positive (≥4) section. Right, p63-negative section.

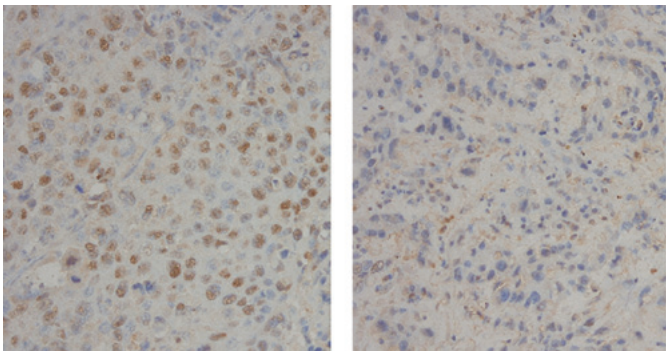

Figure 3. Immunohistochemistry for Sox2. Left, Sox2-positive (≥4) section. Right, Sox2-negative section.

Discussion

In this analysis, we found an increased *Sox2* gene copy number in 33% of the Japanese lung cancer patients. The *Sox2* gene statuses were mainly correlated with squamous histology.

The HMG domain is a DNA-binding motif that was originally discovered in abundant nonhistone components of chromatin. The proteins containing this motif are considered as architectural components in the assembly of nucleoprotein complexes, and they regulate transcription by interacting with the minor groove of the DNA helix and modulating DNA structure by bending the DNA helix (25). At least 20 *Sox* genes have so far been identified in mammals and more in

other vertebrates (26-28), and they have been implicated in the regulation of a variety of developmental processes. Sox2 has a recognized role in lung branching morphogenesis and differentiation in embryonic development. Mice engineered to overexpress Sox2 in the developing lung epithelium have a marked reduction in the number of airways (5). Sox2 is modulated in concert during the course of tracheal and esophageal development (12).

Sox2 is highly expressed in SCC of the gastrointestinal tract (14). Sox2 was expressed in 81% of esophageal SCC cases and 91% of anal canal SCC cases, compared with 13% and 17% of esophageal and rectal adenocarcinoma cases, respectively (14). In the upper gastrointestinal tract, Sox2 is expressed in the developing foregut endoderm and is thought to play a role in establishing the boundary between the keratinized, squamous-lined esophagus and the glandular hindstomach (12). The high correlation between p63 and Sox2 expression was reported in esophageal cancer and raised the issue of a possible contribution of Sox2 to squamous cell carcinogenesis, perhaps via disturbed differentiation and patterning in the context of other procarcinogenic events.

Copy number increase in the 3q26-qter region is observed in various squamous cell cancers including lung, esophagus, head and neck and cervix (13). Two studies have reported the identification of Sox2 as a novel oncogene in lung and esophageal SCC (13,29). The knockdown experiments demonstrated that Sox2 was necessary for lung squamous cell viability by protecting cells from apoptosis (13,29). The suppression of Sox2 with shRNA constructs reduced proliferation and colony formation in the 3q26.33-amplified cell lines but not in the controls (13). Although Sox2 itself was not transforming, the combination of Sox2 with the FOXE1 or FGFR2 isoforms promoted anchorage-independent growth in cancers (13).

Sholl *et al* demonstrated that Sox2 IHC expression was more common in poorly differentiated adenocarcinoma of the lung (23), and this study also demonstrated that there was a tendency towards a higher degree of differentiation within the normal *Sox2* copy number group compared to the increased *Sox2* copy number group (p=0.1046). However, Hussenet *et al* addressed the relationship between the Sox2 expression level and histological differentiation status in lung SCC and found no significant changes (29). They found that Sox2 is highly expressed in both aggressive and non-aggressive tumors with no statistically significant differences between the two groups (29). This result is consistent with the very high expression levels of Sox2 protein observed in the majority of primary SCC cases (13,23,29).

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