Expression of tropomyosins in lung cancer - a potential role in carcinogenesis and its utility in a histopathological diagnosis

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Summary. We herein analyzed the relationships between tropomyosin protein expression levels and clinicopathological factors in order to determine the significance of tropomyosins in lung cancers. Although neoplastic cells expressed different isoforms of tropomyosin, overall expression levels were lower than those in bronchial and alveolar epithelial cells. In adenocarcinomas, tropomyosin levels were markedly reduced in poorly differentiated or solid subtype carcinomas, suggesting that a loss in the expression of tropomyosins is involved in the progression of lung adenocarcinomas. The potential utility of the immunohistochemical expression of tropomyosins for a histopathological diagnosis was also investigated. The sensitivity and specificity of a loss in the expression of tropomyosins were 100% and 50%, respectively, which were superior to those for the strong expression of p53 (sensitivity 100% and specificity 44%), a conventional biomarker. An immunohistochemical examination of tropomyosins may assist in the histopathological detection of lung cancer cells in small biopsy specimens.

Key words: Tropomyosin, Lung cancer, Carcinogenesis, Biomarker

Introduction

Lung cancer is one of the most common causes of cancer-related death in the developed world (Hoffman et al., 2002, Spira and Ettinger, 2004). A deeper understanding of the pathological basis of lung cancer is important for the development of novel therapeutic strategies.

We previously identified important molecules involved in lung carcinogenesis through a comprehensive search for downstream targets of KRAS, the most common oncogene (Okudela et al., 2009b, 2013, 2014). Some of the downstream targets of the KRAS oncogene were found to be essential not only in KRAS-mediated carcinogenesis, but also in EGFR- as well as other unknown potential oncogene-mediated lung carcinogenesis (Okudela et al., 2009a,b, 2013, 2014). Thus, investigations into the downstream targets of the KRAS oncogene are considered to be a useful strategy for elucidating the common important molecular bases of lung cancers. Among the downstream targets identified in our recent study (Okudela et al., 2014), we herein focused on tropomyosins.

Tropomyosins are known to promote the contraction of muscle cells, but are also expressed in non-muscle cells, including epithelial cells (O’Neill et al., 2008; Wang and Coluccio, 2010; Choi et al., 2012). Tropomyosins constitute a family of cytoskeletal proteins consisting of many unique variants proper to cell types (O’Neill et al., 2008; Choi et al., 2012), and participate in various essential cellular processes such as polarization, motility, and mitosis (O’Neill et al., 2008;
Tropomyosin expression in lung cancer

Wang and Coluccio, 2010; Choi et al., 2012). Alterations to tropomyosins (gene mutations and disordered expression) have been associated with different pathological conditions such as muscle cell dysfunction and neoplastic transformation (O’Neill et al., 2008; Schmid et al., 2009; Reinersman et al., 2010). Tropomyosin levels were previously reported to be reduced in some types of human cancers, including esophageal cancer (Jazii et al., 2006), colon cancer (Mlakar et al., 2009), urinary bladder cancer (Pawlak et al., 2004), and breast cancer (Raval et al., 2003). However, their expression has not been examined in lung cancers.

We herein investigated the expression of tropomyosin proteins in lung cancer cell lines and surgically resected primary lung cancers, analyzed the relationships between tropomyosin expression levels and clinicopathological factors in order to determine the significance of its expression in lung cancers, and also verified its potential utility in a histopathological diagnosis.

Materials and methods

Cell lines and culture

An immortalized human airway epithelial cell line (16HBE14o, Simian virus 40 (SV40)-transformed human bronchial epithelial cells) described by Cozens et al. (1994) was kindly provided by Grunert DC (California Pacific Medical Center Research Institute). A subclone of 16HBE14o cells, described as NHBE-T in this study, was used. An immortalized airway epithelial cell line (HPL1D, SV40-transformed human small airway epithelial cells) was established by Masuda et al. (1997). Human lung cancer cell lines (A549, H358, H2087, H820, H1819, H441, and H1299) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The human lung cancer cell line LC2/ad was purchased from the Riken Cell Bank (Tsukuba, Japan). The human lung cancer cell lines PC3, PC9, and HARA were from Immuno-Biological Laboratories Co. (Gunma, Japan). The human lung cancer cell lines TKB4, TKB5, TKB6, TKB7, TKB9, TKB14, and TKB20 were obtained from Dr. Hiroshi Kamma via Dr. Takuya Yazawa (Kyorin University School of Medicine) (Yazawa et al., 1999).

Western blotting

Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, and transferred onto PVDF membranes (Amersham, Piscataway, NJ). The membranes were incubated with nonfat dry milk in Tris-buffered saline containing Tween-20 (TBS-T) to block non-immunospecific protein binding, and then with a primary antibody against tropomyosins (this antibody binds to different unspecified isoforms derived from any of the four TPM genes (TPM1, NG_007557.1; TPM2, NG_011620; TPM3, NG_008621; TPM4, NG_015841) (FL-284; Santa Cruz, Santa Cruz, CA)) or β-actin (Sigma, St. Louis, MO). After washing with TBS-T, the membranes were incubated with animal-matched HRP-conjugated secondary antibodies (Amersham). Immunoreactivity was visualized with an enhanced chemiluminescence system (Amersham).

Primary lung cancer

A total of 196 tumors (167 adenocarcinomas, 20 squamous cell carcinomas, 4 large cell carcinomas, and 5 small cell carcinomas) were removed by radical surgical resection at the Kanagawa Prefectural Cardiovascular and Respiratory Center (Yokohama, Japan). Twenty biopsy specimens were also from the Kanagawa Prefectural Cardiovascular and Respiratory Center. This study was approved by the Ethics Committees of Yokohama City University and Kanagawa Prefectural Cardiovascular Respiratory Center Hospital. Informed consent for research use of the resected materials was obtained from all subjects. Histological types and disease stages were determined according to the International TNM Classification System (seventh edition of the UICC) (Travis et al., 2004).

Immunohistochemistry

Tumor tissues and culture cells were fixed with buffered 10% formaldehyde solution and embedded into paraffin wax. Sections from the largest tumor sections and cell pellets were deparaffinized, rehydrated, and incubated with 3% hydrogen peroxide, followed by 5% goat serum to block endogenous peroxidase activities and non-immunospecific protein binding. Sections were boiled in citrate buffer (0.01 M, pH 6.0) for 15 minutes to retrieve masked epitopes and then incubated with the primary antibody against tropomyosins (FL-284; Santa Cruz), Ki-67 (MIB1; DAKO, Ely, UK), or p53 (DO7; DAKO). Immunoreactivity was visualized using an Envision detection system (DAKO), and nuclei were counterstained with hematoxylin. Tropomyosin expression levels were classified as negative (level 0), faint (level 1), modest (level 2), and strong (level 3). The faint level was defined as weaker than that in bronchiolar and alveolar epithelial cells, but not negative. The modest level was defined as an equivalent level to that in the bronchioles and alveoli. The strong level was defined as an unequivocally stronger level. The score of immunohistochemical expression was determined as an average level (if 30%, 10%, 50%, and 10% of neoplastic cells in tumor sections covering the largest diameters were the negative, faint, modest, and strong levels, respectively, the average level was calculated as “1.4 = 0.3×0 + 0.1×1 + 0.5×2 + 0.1×3”)
calculated as the proportion of positive nuclei cells by counting 500–1000 cancer cells. The MIB1 labeling indices of <10% and ≥10% were classified as low and high levels because this classification criteria has excellent prognostic value to evaluate malignant potential (risk of recurrence) (Woo et al., 2009).

Search for KRAS and EGFR gene mutations

The tumorous part was dissected from formalin-fixed, paraffin-embedded tissue sections. DNA was purified using the conventional phenol/chloroform extraction method (Okudela et al., 2009a). Mutations in the KRAS oncogene (exons 2 and 3) and EGFR oncogene (exons 18, 19, 20, and 21) were analyzed by direct sequencing, according to a method described elsewhere (Okudela et al., 2009a).

Statistical analysis

Differences in the mean values of the immunohistochemical scores for tropomyosins among the groups classified based on various clinico-pathological factors were analyzed by a one-way ANOVA. Recurrence curves were plotted using the Kaplan-Meier method, and the absolute risk of recurrence at five years was estimated from these curves. Differences in the disease-free survival (DFS) span and rate were analyzed using the log-rank test. P values less than 0.05 were considered significant.

Results

Expression of tropomyosins in lung cancer cell lines

The Western blotting analysis revealed many bands of various densities (Fig. 1A). The densitometric levels of these bands in some lung cancer cell lines were similar to or higher than those in the non-cancerous immortalized airway epithelial cell lines, but were markedly lower in other cancer cell lines (Fig. 1B). Immunocytochemical expression was faint in some cell lines, which showed lower levels of whole tropomyosin proteins in the Western blot analysis (Fig. 1B,C). Although the antibody used was not specific enough to discriminate the different subtypes of tropomyosins, the levels evaluated by the Western blot analysis generally appeared to be associated with immunohistochemical signal intensity.

No correlation was detected between tropomyosin levels and driver oncogene mutations (Fig. 1B).

Expression of tropomyosins in primary lung cancers

Airway epithelial cells in the bronchioles and alveoli strongly expressed tropomyosin proteins in the cytoplasm (Fig. 2A). They also occasionally expressed tropomyosin proteins in the nucleus (Fig. 2A). Tropomyosins are known to be cytoplasmic proteins. A nuclear signal may be an artifact owing to the oozing of abundant amounts of cytoplasmic tropomyosin proteins (or other unknown causes during the process of tissue section preparation). Thus, cytoplasmic signals were evaluated. The levels of tropomyosins expressed in neoplastic cells varied, even in individual tumors, with some expressing tropomyosins faintly or at an undetectable level (Fig. 2A). Expression levels were scored as described in the

<table>
<thead>
<tr>
<th>Expression score</th>
<th>ADC (167)</th>
<th>0.739±0.507</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Grade</td>
<td>ADC (167)</td>
<td>0.739±0.507</td>
</tr>
<tr>
<td>WEL (105)</td>
<td>0.816±0.530</td>
<td></td>
</tr>
<tr>
<td>MOD (47)</td>
<td>0.670±0.402</td>
<td></td>
</tr>
<tr>
<td>POR (15)</td>
<td>0.413±0.504</td>
<td></td>
</tr>
<tr>
<td>*Subtype</td>
<td>ADC (167)</td>
<td>0.739±0.507</td>
</tr>
<tr>
<td>BAC (90)</td>
<td>0.861±0.513</td>
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</tr>
<tr>
<td>ACI (36)</td>
<td>0.632±0.479</td>
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</tr>
<tr>
<td>PAP (18)</td>
<td>0.572±0.416</td>
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</tr>
<tr>
<td>SOL (14)</td>
<td>0.450±0.508</td>
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<tr>
<td>MUC (9)</td>
<td>0.722±0.424</td>
<td></td>
</tr>
<tr>
<td>SOC (20)</td>
<td>0.325±0.438</td>
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</tr>
<tr>
<td>Grade</td>
<td>ADC (167)</td>
<td>0.739±0.507</td>
</tr>
<tr>
<td>WEL (3)</td>
<td>0.13±0.058</td>
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</tr>
<tr>
<td>MOD (8)</td>
<td>0.28±0.500</td>
<td></td>
</tr>
<tr>
<td>POR (9)</td>
<td>0.42±0.458</td>
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<tr>
<td>LCC (4)</td>
<td>0.025±0.050</td>
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<tr>
<td>SCC (5)</td>
<td>0.300±0.283</td>
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</tr>
<tr>
<td>Vascular involve/ADC (167)</td>
<td>ADC (167)</td>
<td>0.739±0.507</td>
</tr>
<tr>
<td>Present (14)</td>
<td>0.543±0.372</td>
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</tr>
<tr>
<td>Absent (143)</td>
<td>0.757±0.515</td>
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<tr>
<td>Lymphatic canal involve/ADC (167)</td>
<td>ADC (167)</td>
<td>0.739±0.507</td>
</tr>
<tr>
<td>Present (19)</td>
<td>0.700±0.514</td>
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</tr>
<tr>
<td>Absent (148)</td>
<td>0.744±0.460</td>
<td></td>
</tr>
<tr>
<td>*MIB1 labeling index/ADC (167)</td>
<td>ADC (167)</td>
<td>0.739±0.507</td>
</tr>
<tr>
<td>Low level (&lt;10%)</td>
<td>(95)</td>
<td>0.85±0.428</td>
</tr>
<tr>
<td>High level (≥10%)</td>
<td>(72)</td>
<td>0.58±0.532</td>
</tr>
<tr>
<td>Oncogenic mutation/ADC (152)</td>
<td>ADC (167)</td>
<td>0.739±0.507</td>
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<tr>
<td>KRAS (11)</td>
<td>0.71±0.681</td>
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<tr>
<td>EGFR (49)</td>
<td>0.79±0.583</td>
<td></td>
</tr>
<tr>
<td>NONE (92)</td>
<td>0.72±0.412</td>
<td></td>
</tr>
</tbody>
</table>

*Significant in a one-way ANOVA analysis; *histology (ADC versus LCC, P=0.0056; ADC versus SQC, P=0.0006); *ADC grade (WEL versus MOD, P=0.0065; MOD versus POR, P=0.0475); *ADC subtype (BAC versus ACI, P=0.0227; BAC versus PAP, P=0.0268; BAC versus SOL, P=0.0062); *MIB1 labeling index (P=0.0006); ADC, adenocarcinoma; SQC, squamous cell carcinoma; LCC, large cell carcinoma; SCC, small cell carcinoma; WEL, well differentiated; MOD, moderately differentiated; POR, poorly differentiated carcinomas; BAC, bronchioalveolar carcinoma; ACI, acinar adenocarcinoma; PAP, papillary adenocarcinoma; SOL, solid adenocarcinoma; MUC, mucinous adenocarcinoma; NONE, specimens without KRAS or EGFR mutations.
Materials and methods section. The expression scores of all the tumors examined are shown (Fig. 2B). Overall, neoplastic cells appeared to express these proteins at a lower level than those in bronchial and alveolar epithelial cells, and some tumors exhibited no immunohistochemical expression.

Relationships between tropomyosin levels and pathological factors

Among the histological types examined, squamous cell carcinomas and large cell carcinomas expressed slightly lower levels of tropomyosins than adenocarcinomas (Table 1). Among the adenocarcinomas, these levels appeared to be lower in poorly differentiated carcinomas and in solid subtypes. Tropomyosin expression levels were also lower in adenocarcinomas with high growth activity (a high MIB1 labeling index) (Table 1). These levels were not associated with other factors, including lymphatic canal invasion, vessel invasion, or driver oncogene mutations (Table 1).

Relationship between tropomyosin levels and DFS

A total of 132 patients with adenocarcinomas at pathologic stage I were available for the DFS analysis. Immunohistochemical scores of <0.75% and ≥0.75% were classified as low and high based on a receiver operating characteristic curve (area under the curve 0.720, 95% confidential interval 0.582-0.858). Seventy-five patients were low expressers while 57 were high expressers. The incidence of recurrence was higher in low expressers (17.3% (13/75) versus 3.5% (2/57)). A significant difference was observed in the five-year DFS rate (log-rank test, P=0.0082) (Fig. 3).

The utility of tropomyosin expression in histopathological diagnoses

Neoplastic cells in most of the tumors examined in the present study appeared to express tropomyosin proteins at a lower level than those in bronchial and alveolar epithelial cells (Fig. 2B), whereas some tumors

Table 2. Immunohistochemical expression of tropomyosins in lung lesions difficult to define by biopsy examinations.

<table>
<thead>
<tr>
<th>Case</th>
<th>Tropomyosin</th>
<th>p53</th>
<th>Tentative diagnosis on biopsy specimens</th>
<th>Final diagnosis on resected specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>Adenocarcinoma, suspected</td>
<td>Adenocarcinoma, lepidic non-mucinous subtype</td>
</tr>
<tr>
<td>2</td>
<td>+/-</td>
<td>-</td>
<td>Adenocarcinoma, suspected</td>
<td>Adenocarcinoma, lepidic non-mucinous subtype</td>
</tr>
<tr>
<td>3</td>
<td>+/-</td>
<td>-</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, lepidic mucinous subtype</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, lepidic subtype</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+/-</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, lepidic subtype</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+/-</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, mixed with the micropapillary subtype</td>
</tr>
<tr>
<td>7</td>
<td>+/-</td>
<td>+/-</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, mixed with the micropapillary subtype</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, solid subtype</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>+</td>
<td>Poorly differentiated carcinoma, suspected</td>
<td>Adenocarcinoma, solid subtype</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>-</td>
<td>Poorly differentiated carcinoma, suspected</td>
<td>Adenocarcinoma, solid subtype</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>Poorly differentiated carcinoma, suspected</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>12</td>
<td>+/-</td>
<td>+/-</td>
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<td>Squamous cell carcinoma</td>
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<td>13</td>
<td>-</td>
<td>-</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>+</td>
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<td>Large cell carcinoma</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>+/-</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Large cell carcinoma (neuroendocrine)</td>
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<td>16</td>
<td>+</td>
<td>+/-</td>
<td>Adenocarcinoma, possible</td>
<td>Collapse fibrosis with entrapped alveolar epithelia</td>
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<tr>
<td>17</td>
<td>+</td>
<td>-</td>
<td>Adenocarcinoma, possible</td>
<td>Collapse fibrosis with entrapped alveolar epithelia</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>+/-</td>
<td>Adenocarcinoma, possible</td>
<td>Pneumonia with reactive epithelial cell hyperplasia</td>
</tr>
<tr>
<td>19</td>
<td>+</td>
<td>-</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Granulomas (histiocytes and fibroblastic cells)</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>+/-</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Granulomas (histiocytes and fibroblastic cells)</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>+/-</td>
<td>Adenocarcinoma, possible</td>
<td>Pneumonia with reactive epithelial cell hyperplasia</td>
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<td>-</td>
<td>+</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, lepidic subtype</td>
</tr>
<tr>
<td>24</td>
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<td>Adenocarcinoma, papillary subtype</td>
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<tr>
<td>25</td>
<td>+</td>
<td>-</td>
<td>Adenocarcinoma, possible</td>
<td>Pneumonia with reactive epithelial cell hyperplasia</td>
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<tr>
<td>26</td>
<td>+</td>
<td>-</td>
<td>Adenocarcinoma, possible</td>
<td>Pneumonia with reactive epithelial cell hyperplasia</td>
</tr>
<tr>
<td>27</td>
<td>-</td>
<td>-</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Adenocarcinoma, solid subtype</td>
</tr>
<tr>
<td>28</td>
<td>-</td>
<td>+</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>29</td>
<td>+</td>
<td>-</td>
<td>Adenocarcinoma, possible</td>
<td>Adenocarcinoma, lepidic subtype</td>
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<tr>
<td>30</td>
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<td>+</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Squamous cell carcinoma</td>
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<tr>
<td>31</td>
<td>+</td>
<td>+/-</td>
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<td>-</td>
<td>Adenocarcinoma, possible</td>
<td>Pneumonia with reactive epithelial cell hyperplasia</td>
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<tr>
<td>34</td>
<td>+</td>
<td>+</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>35</td>
<td>+</td>
<td>+/-</td>
<td>Poorly differentiated carcinoma, possible</td>
<td>Granulomas (histiocytes and fibroblastic cells)</td>
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</tbody>
</table>

+, strong level of expression; +/-, faint level; -, no detectable expression.
Protein lysates from lung cancer cell lines were subjected to a Western blot analysis for tropomyosin (TPM) and β-actin (ACTB) (A). The signal intensities of the six major bands (*a*-f) were evaluated by NIH imaging. Tropomyosin levels were normalized to those of ACTB. Normalized levels are shown (B). The band "b" was faintly observed in some cell lines (H820, HARA, and PC10); however, the level of b was too low to be visualized in the plot. The status of the immunocytochemical expression of tropomyosin (IE: S, strong expression; F, faint expression) and major driver oncogene mutations (KRAS and EGFR: +, mutated (PC3 and PC9, EGFR exon 19 deletion mutations); A549, H358, H441, KRAS exon 2 point mutations; -, not mutated) are shown (B). Representative photographs of the results of immunocytochemistry are shown (C). LC2AD cells strongly expressed the tropomyosin protein (right panel), while H2087 cells showed faint expression (right panel).
Fig. 2. The expression of tropomyosin proteins in tumors and non-tumorous epithelia was immunohistochemically examined. Representative photographs from the bronchioles, alveoli, and different subtypes of adenocarcinomas (ADC) (bronchioloalveolar carcinoma (BAC), acinar (ACI), papillary (PAP), and solid (SOL), mucinous (MUC) subtypes), squamous cell carcinomas (SQC), large cell carcinomas (LCC), and small cell carcinomas (SQC), are shown (A). Expression levels were classified into negative, faint, modest, and strong. The faint level was defined as weaker than that in bronchiolar and alveolar epithelial cells, but not negative (ADC/ACI, ADC/PAP, and SCC). The modest level was defined as a level equivalent to that in the bronchioles and alveoli (ADC/MUC, and SCC). The strong level was defined as an unequivocally stronger level (ADC/BAC, some of the neoplastic cells in this panel were judged to be strong). Negative expression is shown in the panel of ADC/SOL. The immunohistochemical scores of all the tumors examined are shown (B). The dashed line indicates the level of non-tumorous epithelial cells (NC).
exhibited no immunohistochemical expression (Fig. 2B), suggesting the potential utility of the immunohistochemical expression of tropomyosins. Among the series of biopsy specimens in our faculty (the Kanagawa Prefectural Cardiovascular and Respiratory Center), cases suspected of being malignant, but that were difficult to define due to the small number of cells or very slight morphological changes were selected (Table 2). Tropomyosin expression was immunohistochemically examined and some representative results are shown (Fig. 4). The sensitivity and specificity of the immunohistochemical expression of tropomyosins as a biological marker to detect neoplastic cells in these difficult cases were analyzed, and were also compared to those of p53, which is a marker that is commonly used to detect cancer cells (Kitamura et al., 1996; Liu and Gelmann, 2002) (Table 3). All specimens with tropomyosin-negative atypical cells had malignancies (Table 2). The sensitivity of the tropomyosin examination was excellent (Table 3), whereas its specificity was not satisfactory, but was still superior to that of the p53 examination (Table 3). The combination of tropomyosin and p53 examinations slightly improved specificity (Table 3).

**Discussion**

The present study focused on tropomyosins and investigated the significance of their expression in lung cancers. This is the first study to demonstrate the aberrant expression of tropomyosins in lung cancers. Overall, tropomyosin protein levels were lower in lung cancer cells than in non-cancerous cells, and were absent in some tumors. Tropomyosin protein levels differed among the histological types; squamous cell carcinomas and large cell carcinomas expressed slightly lower levels of tropomyosins than adenocarcinomas. These levels may be the potential cause of the histological features of lung cancer cells. Therefore, elucidating the molecular mechanisms altering the expression of tropomyosin is attracting increasing interest. Moreover, among the adenocarcinomas examined, tropomyosin protein levels were lower in high-grade tumors, such as poorly differentiated carcinomas, solid subtypes, or tumors exhibiting strong proliferating activity (high MIB1 labeling index) (Table 1). Furthermore, adenocarcinoma patients with lower tropomyosin levels had poorer outcomes (Fig. 3). These results suggest that the down-regulated expression of tropomyosins is involved in the progression of lung adenocarcinomas. Although tropomyosins are downstream targets of oncogenic KRAS, their down-regulation was detected not only in KRAS-mutated tumors, but also EGFR-mutated tumors and tumors without these mutations (Table 1). The two major driver oncogenes of KRAS and EGFR, and also the other minor drivers, are known to transmit oncogenic signals via a common pathway (Marks et al., 2008; Schmid et al., 2009; Reinersman et al., 2010). The down-regulated expression of tropomyosins may represent an essential event in this common oncogenic pathway.

The down-regulated expression of tropomyosins through the ras-mediated oncogenic pathway in mouse fibroblastic cells was initially reported by Cooper et al. in 1985, and was suggested to be essential to oncogene-mediated transformation through a disruption in cytoskeletal organization (Raval et al., 2003; Stehn et al., 2006; Helfman et al., 2008; O’Neill et al., 2008; Choi et al., 2012). Previous studies demonstrated that tropomyosin levels were markedly reduced in highly invasive malignant cell lines (Bhattacharya et al., 1988; Takenaga et al., 1988), and the restoration of tropomyosins suppressed invasive activity (Mahadev et al., 2002; Bharadwaj et al., 2005). A marked reduction in or the loss of the expression of tropomyosins has also been reported in human malignancies (Bharadwaj and Prasad, 2002; Raval et al., 2003; Jazii et al., 2006; Stehn et al., 2006), in some of which the involvement of DNA hypermethylation was demonstrated (Bharadwaj and

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**Table 3. The utility of tropomyosin expression in a histopathological diagnosis.**

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<td>Specificity</td>
<td>50% [12/24]</td>
<td>44% [12/27]</td>
<td>60% [12/20]</td>
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P, positive (strong level); N, negative (faint level, no detectable expression)

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**Fig. 3. Relationships between tropomyosin levels and disease-free survival in stage I lung adenocarcinomas (132 cases) were analyzed by the log-rank test (P=0.0082). Kaplan-Meier survival curves are shown (five-year disease-free survival rates were 17.3% [13/75] and 3.5% [2/57] in the low and high expressers, respectively).**
Prasad, 2002). These findings are consistent with our results, and support the down-regulated expression of tropomyosins promoting the progression of carcinogenesis. However, only a few studies have so far examined the involvement of tropomyosins in human malignancies (Stehn et al., 2006; Helfman et al., 2008; O’Neill et al., 2008; Choi et al., 2012). Thus, further investigations on the potential role of and mechanisms underlying the down-regulated expression of tropomyosins in human malignancies, including lung cancers, are warranted.

On the other hand, the results of the present study suggest that the immunohistochemical expression of tropomyosins has the potential to become a useful biological marker for the histopathological diagnosis of lung adenocarcinomas in small biopsy specimens. However, its specificity was not satisfactory, and, thus, it may be difficult to distinguish bronchioloalveolar carcinoma cells from reactive pneumocyte hyperplasia or poorly differentiated carcinoma cells from a reactive swelling of mesenchymal cells. Some modifications, such as a combination with other biomarkers, are required for practical use.

In summary, the down-regulated expression of tropomyosins was identified as a common event in lung cancer cells, especially in adenocarcinomas with highly malignant activity. Furthermore, an immunohistochemical examination of tropomyosins may have potential utility in the histopathological detection of lung cancer cells in biopsy specimens.

Fig. 4. Biopsied tissues containing a small number of neoplastic cells with slight morphological changes were immunohistochemically examined for the expression of tropomyosin proteins. Representative photographs of hematoxylin and eosin staining (left panels) and immunohistochemistry (right panels) of two different specimens are shown. The immunohistochemical examination clearly defined neoplastic cells (negative) from non-tumor epithelial cells (positive).
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