Expression of ΔNp73 Predicts Poor Prognosis in Lung Cancer

Hidetaka Uramoto,1 Kenji Sugio,1 Tsunehiro Oyama,2 Shoji Nakata,1 Kenji Ono,1 Masaru Morita,1 Keiko Funa,3 and Kosei Yasumoto1
1Second Department of Surgery and 2Department of Environmental Health, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan; and 3Department of Cell Biology, Institute of Anatomy and Cell Biology, Göteborg University, Box 420, SE-405 30 Gothenburg, Sweden

ABSTRACT

Purpose: ΔNp73 is an isoform of the p53 homologue p73, which lacks an NH2-terminal transactivation domain and antagonizes the induction of gene expression by p53/p73. The aim of this study was to detect ΔNp73 expression in lung cancer and to evaluate the relationship between the ΔNp73 expression level and the prognosis of patients with resected lung cancer.

Experimental Design: We used immunohistochemistry to analyze the protein expression of ΔNp73 in paraffin-embedded tumor samples from 132 well-characterized lung cancer patients and compared the expression level of ΔNp73, clinical variables, and survival outcome.

Results: Positive expression of ΔNp73 was detected mainly in the cytoplasm of tumor cells in 77 of 132 patients (58.3%) with lung cancer. The incidence of positive expression of ΔNp73 was 52.2, 50.0, and 70.2% in patients with stage I, II, and III, respectively (P = 0.04). Positive expression of ΔNp73 was associated with gender but not associated with age, histologic type, pathological stage, pathological T status, and pathological N status. Lung cancer patients with positive ΔNp73 expression had a poorer prognosis than those with negative ΔNp73 expression. In addition, multivariate analysis of the clinicopathological characteristics of lung cancer indicated that positive expression of ΔNp73 was a significant independent factor for predicting poor prognosis (P < 0.0001, risk ratio = 3.39).

Conclusions: Expression of ΔNp73 may be a useful marker for predicting poor prognosis of patients who underwent resection of lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in North America, and it became the most common cause of cancer-related deaths among Japanese in 1998 (1, 2). Lung cancer is also an aggressive carcinoma with a poor outcome, and the overall survival rate is about ~11 to 14% (3). The Tumor-Node-Metastasis staging system of lung cancer (4) is widely used as a guide for predicting prognosis. Despite therapeutic advances, the survival rate in recent decades has improved little, and the management of patients is far from satisfactory because of its rapid and extensive metastasis (5, 6).

In non–small-cell lung cancer, even after a curative resection for pathological stage I, ~30% of all patients may experience recurrence and eventually die of the disease (6). This suggests that occult metastases are present at the time of surgical intervention. Therefore, it is important to evaluate the malignant potential of tumor cells for a more precise evaluation of the prognosis of patients with lung cancer. Lung cancer is thought to arise from the accumulation of several genetic changes such as mutations and deletions. Recent advances in molecular biology and genetics have created new diagnostic and therapeutic possibilities for clinical oncology. The potential prognostic implications of several biological and molecular parameters, including oncogenes such as K-ras mutation and c-erbB2 overexpression (7–10) and tumor suppressor genes such as p53 (11, 12), have been reported for patients with lung cancer from our laboratory. Expression of p53 is altered in a high proportion of human neoplasms, and it is mutated in half of the various malignant diseases, including lung cancer.

p73 belongs to a family of proteins defined by the p53 tumor suppressor gene (13, 14). p53 and p73 share significant homology in their structural organization as characterized by an NH2-terminal transactivation domain, a central DNA-binding domain, and a COOH-terminal oligomerization domain (15). In addition, both p53 and p73 can block the cell cycle or induce cell death in response to DNA damage (16, 17). However, despite strong functional homology, data from human tumors and p73-deficient mice argue against a classical Knudson-type tumor suppressor role for the p73 gene. p73-deficient mice lack a spontaneous tumor phenotype, and inactivating mutations in human tumors are extremely rare (18). Moreover, although all normal human tissues studied express very low levels of p73, multiple primary tumor types and tumor cell lines overexpress wild-type p73, including cancers of the breast, lung, esophagus, stomach, colon, bladder, ovary, liver, bile ducts, epidermal lining, myelogenous leukemia, and neuroblastoma (18). To date, most studies identifying p73 overexpression in primary human tumors have examined total levels of p73, with only a few exceptions that specifically measured TA (full-length transactivating isoforms) p73 (19, 20), which possess transactivating
domain. Before the discovery of ΔNp73, scientists examined all expressions of p73 in resected lung cancer and paired normal lung using semiquantitative reverse transcription-PCR (21). In mice, a NH2-terminally truncated ΔNp73 protein has recently been found, which was generated from an alternative promoter in intron 3 and lacking a transactivation domain (22). ΔNp73 acts as a potent transdominant inhibitor of the wild-type p53 and the transactivation-competent TAp73 and confers drug resistance to the wild-type p53-harboring tumor cells (23).

This is the first report on a relationship between ΔNp73 expression and prognosis of patients with lung cancer. This study is a retrospective cohort and designed to detect ΔNp73 expression in lung cancer by using immunohistochemical (IHC) staining and to evaluate the relationship between ΔNp73 expression levels of tumors and the prognosis of the patients.

MATERIALS AND METHODS

Patients and Follow-up. We examined 132 consecutive patients with stage I to III lung cancer who underwent surgical resection between April 1993 and July 1996 at the University of Occupational and Environmental Health, School of Medicine (Kitakyushu, Japan). The inclusion criteria into the study were based on the availability of follow-up data. Clinicopathological data were obtained by retrospective chart review. Tumor stage was classified according to Revisions in the International System for Staging Lung Cancer (4). One hundred thirteen (85.6%), 15 (11.4%), 3 (2.3%), and 1 (0.8%) of 132 patients had received lobectomy, pneumonectomy, partial resection, and segmentectomy, respectively. There were 93 men and 39 women in this series, with a mean age of 66.2 years (range, 40 to 84). The pathological types includes 76 adenocarcinoma, including 7 patients with bronchioloalveolar type, 44 squamous cell carcinoma, 2 adenosquamous cell carcinoma, 3 carcinoïd, 5 large-cell carcinoma, and 2 small-cell carcinoma. Twenty (15.2%), 17 (12.9%), and 3 (2.3%) of 132 patients had received chemotherapy, radiotherapy, and both, respectively. Institutional Review Board-approved informed consent was obtained from all patients or from the patient’s guardian for use of tumor tissue collected at the time of tumor resection.

For the postoperative follow-up, the patients were examined every month within the first year and at ~2- to 4-month intervals thereafter. The evaluations included physical examination, chest roentgen, analysis of blood chemistry, and measurements of classical tumor markers such as carcinoembryonic antigen assay. Chest, abdominal, and brain computed tomographic scans and a bone scintiscan were performed every 6 months until the third year and annually thereafter. If any symptoms or signs of recurrence appeared in these examinations, additional evaluations to locate the site of the recurrent tumors were performed. Survival data were updated in November 2003. A follow-up was available for all patients, ranging from 10 to 3678 days after the primary operation (median follow-up, 50.4 months).

Cell Culture. The MCF-7 and K-562 cell line were maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 60 μg/mL streptomycin in a 5% CO2 atmosphere at 37°C.

Western Blot Analysis. Cytoplasmic proteins were extracted from frozen normal tissues, which were sampled at another segment from tumors and tumor tissues of patients. One hundred μg cytoplasmic proteins were electrophoresed onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal ΔNp73 antiserum raised in rabbits against the exon 3′-peptide MLTVYGDPAR-HLATA (Sigma Genosis, London, United Kingdom). This antibody recognized only ΔNp73 (encoded by ΔNp73 and ΔNp73; ref. 24) without any cross-reactivity with p53 or any TAp73 isomers. A monoclonal anti-actin (Sigma Genosis) was used as a loading control. Detection was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Reverse Transcription-PCR. RNA was extracted essentially as described previously (25) Briefly, total RNA was isolated with the RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer’s protocol and reverse transcribed with cDNA synthesis kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer’s protocol. PCR was carried out by using Taq polymerase (Takara, Tokyo, Japan). Amplification was performed for a predetermined optimal number of cycles. PCR products were separated by electrophoresis on 2% agarose gels, which were stained with ethidium bromide. Sequences of the primers are as follows: ΔN′-p73 sense, 5′-TGGACTTCCGCACTGAAAACG-3′, and antisense, 5′-TGGGACAGGAGCATGGATCTGC-3′; ΔN-p73 sense, 5′-CAACG-GCCCCGATGTCCC-3′, and antisense, 5′-TGTCCTCATGTGCTGTCAGC-3′ (24); and β-actin sense, 5′-GGCACTCAGTGGATGAGCTCCG-3′, and antisense, 5′-GCTGGAAAGGTGGACAGCGA-3′. Positive and negative control was used as K-562 and MCF-7, respectively (26).

IHC Staining. A 3-μm section was obtained from each of the 132 formalin-fixed, paraffin-embedded samples of primary lesions. All specimens were stained with H&E for histopathologic diagnosis. IHC staining was performed by a streptavidin-biotin-peroxidase complex method (27). Sections were briefly immersed in citrate buffer [0.01 mol/L citric acid (pH 6.0)] and incubated for two 5-minute intervals at 100°C in a microwave oven for antigen retrieval. They were then incubated with the ΔNp73 antibody diluted at 1:1000 overnight in a cold room by using a Labeled Streptavidin Biotin kit (CA930 13, DAKO LSAB kit: Dako Corp., Carpinteria, CA). Antibody was diluted in PBS containing 2% BSA.

IHC Evaluation. All slides were evaluated for immunostaining by two observers (H. Uramoto and K. Sugio) using a blind protocol (observers had no information on the clinical outcome or other clinicopathologic data). Cells were judged positive for ΔNp73 when the cytoplasm or both the nuclei and cytoplasm were stained. To evaluate the correlation with clinicopathological characteristics, ΔNp73 expression scores were divided into two groups: positive or negative. Negative controls were processed by immunostaining with a preimmune serum and by exclusion of the primary antibody.

Statistical Analysis. Statistical significance was evaluated using the Pearson’s χ2 test. Survival curves were plotted according to the Kaplan-Meier method (28), and differences between the curves were analyzed by a log-rank test (29). The
Cox proportional hazards model was applied to the multivariate survival analysis (30). The results of the Cox proportional hazards model did not change when the follow-up was within 5 years. The statistical difference was considered significant if the $P$ was $<0.05$. Data were analyzed with the use of Abacus Concepts, Survival Tools for StatView (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Western Blot Analysis. To confirm specificity of the anti-ΔNp73 antibody, Western blot analysis was performed with samples extracted from frozen tumors and the corresponding normal tissues from eight patients. Fig. 1 shows representative data of the analyses. ΔNp73 expression was found in the tumor tissue but not in the normal tissue of case B, confirming the IHC staining of the same tumor material. In contrast, no ΔNp73 expression was detected in neither tumor nor normal tissue of case A showing negative ΔNp73 staining.

Reverse Transcription-PCR. Reverse transcription-PCR analysis of selected samples of human tumor tissue, the corresponding normal tissues, and two control cell lines (26) showed that Δ’Np73 and ΔNp73 were abundantly expressed in the tumor tissue but not in the normal tissue of case B (Fig. 2), confirming the IHC staining of the same material (Fig. 3).

IHC Detection of ΔNp73 Expression in Lung Cancer. In all 132 specimens, 77 (58.3%) were stained positive for ΔNp73, mainly in the cytoplasm of tumor cells, and in 6 (4.5%) cases, where positive expression of ΔNp73 was found both in the nuclei and cytoplasm. However, no reactivity was found in the surrounding normal stromal cells. Typical appearances of positive ΔNp73 staining are shown in Fig. 3A (adenocarcinoma) and Fig. 3B (squamous cell carcinoma), respectively. Fig. 3C (squamous cell carcinoma) shows negative staining of the tumor cells. The relationship between ΔNp73 expression and various clinicopathologic characteristics of the patients is summarized in Table 1. The incidence of positive expression of ΔNp73 was 52.2, 50.0, and 70.2% in patients with stage I, II, and III, respectively ($P = 0.04$). No significant difference was observed, except in gender and pathological stage, between the ΔNp73 expression and the age at operation, histologic type, pathologic stage, pathological T status, or pathological N status.

Influence of ΔNp73 Expression on Survival. The overall 5-year survival rate for the patients with positive ΔNp73 expression and negative ΔNp73 expression was 32.1 and 71.4%, respectively ($P < 0.0001$; Fig. 4). Among the patients with stage I ($n = 67$), the 5-year survival rate for the patients with positive and negative ΔNp73 expression was 56.6 and 74.5%, respectively ($P = 0.067$; Fig. 5A). Of the patients with stage II ($n = 18$), the 5-year survival rate for the patients with positive and negative ΔNp73 expression was 22.2 and 88.9%, respectively ($P = 0.001$; Fig. 5B). The 5-year survival rate for stage III patients ($n = 47$) with positive and negative ΔNp73 expression was 9.1 and 52.2%, respectively ($P = 0.0006$; Fig. 5C). Five variables (gender, pathological stage, pathological T status, pathological N status, and expression of ΔNp73) were found significantly to affect the survival of all patients by univariate analysis (Table 2). Furthermore, a multivariate analysis demonstrated that three variables (pathological T status, pathological N status, and expression of ΔNp73) were independently associated with the survival of all patients (Table 3). Given the present data, the estimated statistical power is $>90\%$ with the condition of 0.05 $\alpha$ error. The detectable relative risk is estimated 2.3 with 90% of statistical power and 2.0 with 80% of statistical power. Expression level of ΔNp73 did not affect the survival of patients at stage IV significantly (data not shown). Therefore, ΔNp73 might affect extension and spreading of local tumors to the regional lymph nodes (lymphatic locoregional metastasis) rather than hematogenous systemic metastasis. Positive expression of ΔNp73 was associated with an increased risk of death by a
factor of 3.39 as seen by multivariate analysis ($P < 0.0001$). Among the patients with adenocarcinoma, the 5-year survival rate in the patients with positive and negative ΔNp73 expression was 37.0 and 72.7%, respectively ($P < 0.01$; data not shown). The 5-year survival rates of positive and negative ΔNp73 squamous cell carcinoma were 26.1 and 64.2%, respectively ($P < 0.01$; data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All cases</th>
<th>Gender</th>
<th>Age (y)</th>
<th>Histological type</th>
<th>Pathologic stage</th>
<th>pTNM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>132</td>
<td>Male</td>
<td>&lt;65</td>
<td>Adenocarcinoma</td>
<td>I</td>
<td>T1</td>
</tr>
<tr>
<td>Positive</td>
<td>77</td>
<td>93</td>
<td>46</td>
<td>Squamous cell carcinoma</td>
<td>II</td>
<td>T2</td>
</tr>
<tr>
<td>Negative</td>
<td>55</td>
<td>39</td>
<td>86</td>
<td>Adenosquamous cell carcinoma</td>
<td>III</td>
<td>T3</td>
</tr>
<tr>
<td>P</td>
<td>0.03</td>
<td></td>
<td>0.50</td>
<td>Carcinoid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>58.3</td>
<td>64.5</td>
<td>54.3</td>
<td>Large-cell carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>21</td>
<td>Small-cell carcinoma</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### DISCUSSION

The p53 tumor suppressor gene belongs to a family that includes the two recently identified homologues, p63 and p73. p73 mutations are rare: they are present in 0.5% of tumors, compared with 50% of those that have p53 mutations. Therefore, p73 etiology is more complex than that of classical tumor suppressor genes (16). Cisplatin is one of the most potent antitumor agents known and displays a clinical activity against lung cancers. The response to cisplatin is influenced not only by p53 but also by the status of a network containing p53 and p73 (15). Most human tumors show a deregulation of the E2F family of transcription factors through the loss of cyclin-dependent kinase inhibitor INK4, overexpression of cyclin D, or loss of RB. E2F1 directly regulates p73 by binding to E2F-binding sites within the TAp73 promoter. c-Myc and E1A also signal to p73. We previously reported that p73 interacts with c-Myc (31) and CTF2 (32) to regulate Y-box–binding protein 1 and HMG1 expression, respectively, which are up-regulated in cisplatin-resistant cell lines.

One of the complications in assessing the role of p73 is the presence of ΔNp73, which might be a putative oncogene, by efficiently counteracting the transactivation of function, apoptosis, and growth suppression mediated by wild-type p53 and TAp73. The ΔNp73 isoform is not expressed in normal tissues.
but is overexpressed in breast cancer cell lines (33), ovarian cancer (34), vulval cancer (35), and neuroblastoma (36). Thus far, there is only one study on the prognostic value of Np73 expression (37). Casciano et al. (37) reported that it is strongly associated with reduced survival (hazard ratio = 7.93; \( P < 0.001 \)) and progression-free survival (hazard ratio = 5.3; \( P < 0.001 \)) and that Np73 expression plays a role in predicting a poorer outcome independently of age, primary tumor site, stage, and MYCN amplification.

Our hypothesis was that deregulated Np73 can bestow oncogenic activity upon the p73 gene by functionally inactivating the suppressor action of p53 and TAp73 (23) in lung cancer cells. If so, the detection of Np73-positive cells may help us to identify the patients at high risk for recurrence. In the current study, we investigated the associations between Np73 expression and various clinicopathologic characteristics of patients with lung cancer. We provide clinical evidence that ΔNp73 is frequently overexpressed in lung cancer specimens. We showed that tumor-specific up-regulation of ΔNp73 occurs at the protein level in primary tumors. Moreover, univariate and multivariate analysis demonstrated that among the clinicopathologic T and N factors, positive expression of ΔNp73 was a significant independent factor for predicting poor prognosis. Thus, ΔNp73 expression level may be a marker of malignant potential of lung cancer.

Recently, Zaika et al. (23) reported that ΔNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors but not in normal tissues. They also showed that ΔNp73 can build a complex with wild-type p53 as demonstrated by coimmunoprecipitation from cultured cells and primary tumors. More recently, Frasca et al. (38) reported that normal thyrocites do not express p73, whereas most malignancies of thyroid are positive for p73 expression and that the loss of p73 biological activity in neoplastic thyroid cells is partly explained by its interaction with transcriptionally inactive variants of p73 (ΔNp73) and mutant p53. Our findings agree with these studies. Furthermore, we found that the ΔNp73 gene expression in lung cancer patients may be independently associated with shorter survival. In our studies, there is no significant relationships between ΔNp73 expression and p53 alteration (data not shown).

We previously reported the usefulness of biomarkers such as p53 (11, 12), vascular endothelial growth factor (39), YB-1 (40, 41), CK (42–44), 8-hydroxydeoxyguanosine (45), c-erbB-2 (10), 3p (46), k-ras (7–9), Fas (27), and telomerase activity (47) to determine accurate staging of diseases and selection of candidates for adjuvant therapy. Notably, this requires proper interpretation shown in interplay the gene profile of individual tumors. On the other hand, understanding how groups of lung cancer cell genes are co-coordinately expressed in response to...
physiologic, immunologic, and microenvironmental stimuli is also another important goal. Thus, a better understanding of gene expression of tumor may find molecular targets for effective therapy.

In conclusion, positive expression of ΔNp73 may be a useful marker in predicting poor prognosis. To address these issues, it may be necessary to use such a promising molecular marker as ΔNp73 for stratification in the setting of prospective randomized clinical trials for patients with lung cancer. By assessing the ΔNp73 expression, it may be possible to select patients who might benefit the most from adjuvant chemotherapy and to provide benefits for patients using combination gene knockdown methods such as small interfering RNA for ΔNp73 combined with traditional treatments.

ACKNOWLEDGMENTS

We thank Yoshihisa Fujino and Ayako Yamasaki for statistical advice and technical assistance, respectively.

REFERENCES


Table 3 Multivariate analyses of various prognostic factors

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Unfavorable</th>
<th>Favorable</th>
<th>Risk ratio</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
<td>1.02</td>
<td>0.56–1.85</td>
<td>0.94</td>
</tr>
<tr>
<td>T status</td>
<td>Pathologic T3–4</td>
<td>Pathologic T1–2</td>
<td>2.79</td>
<td>1.64–4.72</td>
<td>0.0001</td>
</tr>
<tr>
<td>N status</td>
<td>Pathologic N1–3</td>
<td>Pathologic N0</td>
<td>2.06</td>
<td>1.25–3.38</td>
<td>0.004</td>
</tr>
<tr>
<td>ΔNp73 expression</td>
<td>Positive</td>
<td>Negative</td>
<td>3.39</td>
<td>1.94–5.88</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>


Expression of ΔNp73 Predicts Poor Prognosis in Lung Cancer

Hidetaka Uramoto, Kenji Sugio, Tsunehiro Oyama, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/20/6905

Cited articles
This article cites 46 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/20/6905.full#ref-list-1

Citing articles
This article has been cited by 21 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/10/20/6905.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.