

Genetic Alteration in Carcinoid Tumors of the Lung

Kenji Sugio, MD,^{1,2} Toshihiro Osaki, MD,¹ Tsunehiro Oyama, MD,¹
Mitsuhiro Takenoyama, MD,¹ Takeshi Hanagiri, MD,¹ Masaru Morita, MD,¹
Koji Yamazaki, MD,² Akira Nagashima, MD,³ Hisashi Nakahashi, MD,⁴
Yoshihiko Maehara MD,² and Kosei Yasumoto, MD¹

Surgically resected specimens of 13 carcinoid tumors of the lung including nine typical carcinoids and four atypical carcinoids, and eight salivary gland type carcinomas (six mucoepidermoid carcinomas and two adenoid cystic carcinomas) were analyzed regarding *p53* expression, loss of heterozygosity (LOH) in chromosome 3p, 9p, and *K-ras* mutation. The overexpression of *p53* was identified in four atypical carcinoid tumors, one mucoepidermoid carcinoma, and one adenoid cystic carcinoma, however, none of typical carcinoids showed *p53* immunoreactivity. LOH in 3p14 was demonstrated in three of seven informative cases in all tumors. LOH in 9p was demonstrated in two of five informative cases in all tumors. Two of three cases with LOH at 3p14 had a poor prognosis, one of which also had LOH at 9p. No mutation of the *K-ras* gene was observed in any of these tumors. These data thus indicate that *p53* overexpression might distinguish atypical carcinoid tumors from typical tumors and might therefore be useful as an adjunct modality in the differential diagnosis of pulmonary carcinoid tumors. The presence of LOH at 3p14 or 9p may thus help to identify lung cancer patients with a poor prognosis. (Ann Thorac Cardiovasc Surg 2003; 9: 149–54)

Key words: lung cancer, carcinoid, *p53*, 3p, 9p

Introduction

Pulmonary carcinoid tumors account for 0.5 to 1% of all tumors of bronchial origin. These tumors together with small cell lung cancer (SCLC) are regarded as bronchopulmonary neuroendocrine tumors.¹⁾ While SCLC is a highly malignant disease marked by rapid and disseminated tumor growth in the majority of patients, pulmonary carcinoids show a relatively benign course and also

demonstrate various malignant potentials, depending on their differentiation, namely, “typical” and “atypical”.²⁻⁴⁾ The differences in the biological behavior between these two pathological types have been analyzed by some immunohistochemical studies,⁵⁻⁷⁾ and by some gene analyses.^{8,9)}

The *p53* protein regulates the cell cycle negatively through the transactivation of the p21/WAF1 gene,¹⁰⁾ and induces apoptosis through the transactivation of the *Bax* gene.¹¹⁾ The *p53* gene is one of the most frequently mutated tumor-suppressor genes in human tumors.¹²⁾ The mutations of the *p53* gene usually prolong the half life of the protein and results in accumulation. The accumulated protein is readily detectable in the nuclei by immunohistochemistry (IHC). In lung cancer, *p53* mutations are found in more than 50% of non-small cell lung cancer (NSCLC) and in about 90% of SCLC.¹³⁾ In addition, the overexpression of *p53* was also detected in about 50 to 60% of NSCLC.^{14,15)}

A loss of heterozygosity (LOH) in chromosome 3p and 9p is frequently detected in lung cancer.^{16,17)} Although the

From ¹Second Department of Surgery, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, ²Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, ³Department of Chest Surgery, Kitakyushu Municipal Medical Center, Kitakyushu, and ⁴Department of Respiratory Surgery, Matsuyama Red Cross Hospital, Matsuyama, Japan

Received September 5, 2002; accepted for publication December 13, 2002.

Address reprint requests to Kenji Sugio, MD: Second Department of Surgery, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan.

Table 1. The sequence of the primer for the PCR reaction to detect LOH

Gene	PCR step	Primer orientation	Primer sequence
<i>p53</i>	1st	Sense	5'-ACTGCCACTCCTTGCCCCATTC
		Anti-sense	5'-AGGGATACTATTCAGCCCCGAGGTG
	2nd	Sense	5'-GCCACTCCTTGCCCCATTC
		Anti-sense	5'-ATACTATTCAGCCCCGAGGTG
3p14 (D3S1228)	1st & 2nd	Sense	5'-TCCTTAACTCTTTCTCTGTGAGTTG
		Anti-sense	5'-TCTAGGAAAGGGATTAGGAAGGA
3p25 (D3S1038)	1st & 2nd	Sense	5'-TCCAGTAAGAGGCTTCCTAG
		Anti-sense	5'-AAAGGGGTTTCAGGAAACCTG
9p (IFNA)	1st	Sense	5'-TGCGCGTTAAGTTAATTGGTT
		Anti-sense	5'-GTAAGGTGGAAACCCCACT
	2nd	Sense	5'-GCGTTAAGTTAATTGGTTTG
		Anti-sense	5'-AGGTGGAAACCCCACTGGA

chromosome 3p regions are the most frequent sites for genetic alterations in SCLC and NSCLC,^{16,18)} the extent of such abnormalities in carcinoid tumors remains to be investigated. A *ras* gene mutation is found in 10 to 30% of adenocarcinoma and about 80% of these mutations occur at codon 12 of the *K-ras* gene, which is also reported to be a poor prognostic factor.¹⁹⁻²¹⁾ This mutation, however, has never previously been detected in SCLC.

In this study, we evaluated the genetic abnormalities in carcinoid tumors and also tried to clarify the differences between typical and atypical carcinoids.

Materials and Methods

Tissue samples

The tissue samples which were surgically resected between 1982 and 1995, were excised at the time of operation, fixed in buffered formalin, and embedded in paraffin wax. They included nine typical carcinoids and four atypical carcinoids, and eight salivary gland type carcinomas (six mucoepidermoid carcinomas and two adenoid cystic carcinomas) that are considered to show a low-grade malignant behavior, were also analyzed. Informed consent to participate in the study was obtained from all patients before the operation.

Immunohistochemical detection of *p53* protein

IHC for *p53* protein was performed. Briefly, the sections were immersed in citrate buffer and irradiated 5 times for 5 minutes each in a domestic microwave oven for antigen retrieval. They were then stained with mouse mono-

clonal antibody DO-1 (Oncogene Science Inc., Cambridge, MA, USA) against the *p53* protein using a Labeled Strept Avidin Biotin (LSAB) kit (DAKO Japan Co., Ltd., Kyoto, Japan). The sections were subsequently examined for nuclear staining under a light microscope.

Microdissection of the materials from stained slides and DNA extraction

We utilized the microdissection technique extensively to collect cells under direct microscopic observation from hematoxylin and eosin stained slides of the paraffin-embedded materials. The materials were digested in 50 μ l of buffer consisting of 20 mM Tris (pH 8.0), 1 mM EDTA, 0.5% Tween 20, and 200 mg/ml proteinase K for 24-36 h at 42°C, and then were incubated for 15 min at 95°C to inactivate the proteinase K. We used 5 μ l of the digested samples for each PCR reaction as a template DNA. The DNAs from 10 of 13 carcinoids, three of six mucoepidermoid carcinomas and two adenoid cystic carcinomas were available for analyses to detect the LOH and mutation, however, no others were suitable for analysis due to degradation.

Microsatellite analysis at *p53*, 3p and 9p

The primers used for analyses of LOH in the microsatellite markers at *p53*, 3p and 9p are described in Table 1. The first PCR reaction was performed in 50 μ l reaction mixtures containing 20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each deoxyribonucleoside triphosphate (dATP, dGTP, dCTP, dTTP), 0.2 μ M of outer primer pairs, 1.5 units of Taq DNA polymerase, and 5 μ l

Table 2. Immunoreactivity and genetic alteration of the cases with carcinoid tumor, mucoepidermoid carcinoma and adenoid cystic carcinoma

	<i>p53</i> IHC	3p14 LOH	3p25 LOH	9p LOH	<i>K-ras</i> mutation
Carcinoid tumor	4/13	1/4	0/4	1/4	0/10
Typical	0/9	0/2	0/1	0/1	0/4
Atypical	4/4	1/2	0/3	1/3	0/6
Mucoepidermoid carcinoma	1/6	1/1	0/2	0/0	0/3
Adenoid cystic carcinoma	1/2	1/2		1/1	0/2
Total	6/18	3/7	0/6	2/5	0/15

IHC, immunohistochemistry; LOH, loss of heterozygosity.

of template DNA. The first PCR product was diluted 1:100 in dH₂O, and 1 μ l of the dilution was used as a template for the second PCR reaction using the same primer as the first PCR (3p) or nested primer (*p53*, 9p). The reaction was performed using 0.5 μ l of [α -³²P] CTP in a volume of 10 μ l reaction, and 30 cycles were used. Following the PCR, the labeled reaction products were denatured and then electrophoresed in 6% polyacrylamide gels containing 7 M urea for 2-3 h at 60 W. After electrophoresis, the gels were dried and exposed to X-ray film at room temperature for 12-24 h.

Detection of point mutation of *K-ras* gene using PCR-based designed RFLP

To detect *ras* mutations, we utilized a modification of the designed RFLP method using nested PCR reactions to detect point mutations in codons 12 and 13 of the *K-ras* gene, as described previously.^{22,23} Briefly, mismatched primers were used to introduce a new restriction site (*Bst* NI for codon 12, *Bgl* I for codon 13) into the PCR product derived from the wild-type allele. Wild type alleles were digested and were found to yield a smaller product than the mutant forms which were digestive resistant. Both bands could be recognized after electrophoresis.

Results

Immunohistochemical detection of *p53* protein

Anti-*p53* immunostaining reactions showed the protein to be restricted to the nucleus in the positive control sections. The histological classification of the tumors examined and the immunostaining obtained with the anti-*p53* DO-1 antibody are summarized in Table 2. In carcinoid tumors, none of the typical carcinoids lacked any immunoreactive cells, whereas all four cases of atypical carci-

noid showed nuclear *p53* immunoreactivity. In four of the atypical carcinoids with positive *p53*, three cases were *p53* positive in almost all tumor cells whereas one case showed scattered positive cells in the tumor. One of six mucoepidermoid carcinomas, and one of two adenoid cystic carcinomas showed positive *p53* immunoreactivity.

LOH analysis at *p53*

The LOH of the *p53* gene was analyzed using a microsatellite marker within the gene. Only one case of atypical carcinoid was informative for the LOH analyses and showed LOH. Other three cases with atypical carcinoids were not informative. No LOH was determined in one informative case with typical carcinoids. No LOH was determined in one informative case with mucoepidermoid carcinoma, and other cases were not informative.

LOH analysis at 3p and 9p

We tested our samples for LOH at the 3p14, 3p25 loci using microsatellite markers (Fig. 1). In 3p, three of seven informative cases showed LOH in 3p14 locus, whereas none of the six informative cases showed LOH in 3p25 locus. Two of the three cases with LOH at the 3p14 locus showed a *p53* overexpression. In 9p, LOH of the INFA locus was detected in two of five cases, one was a carcinoid tumor and one was a case of adenoid cystic carcinoma.

PCR-based designed RFLP analysis for *K-ras* mutations

We screened for a mutation of codons 12 and 13 in the *K-ras* gene in the DNA from 10 cases with carcinoid tumors, three cases with mucoepidermoid carcinomas and



Fig. 1. LOH determined by a microsatellite analysis at the 3p14 (D3S1228) locus. LOH was determined in cancer tissue in patients 4, 5, and 8. Lanes 1, 2, carcinoid tumor (typical); 3, 4, carcinoid tumor (atypical); 5, 6, 7, mucoepidermoid carcinoma; 8, 9, adenoid cystic carcinoma. N, normal tissue; T, cancer tissue.

two cases with adenoid cystic carcinomas. As a result, no mutation of codons 12 and 13 was detected in any of the samples examined.

Prognosis of cases with carcinoid tumors

All cases with carcinoid tumors were followed more than five years after surgery. All cases with typical carcinoid tumors are alive, however, in cases with atypical carcinoid tumors, three of four cases were dead within three years after surgery. Only one case with atypical carcinoid tumor is alive without recurrent disease, which had *p53* overexpression but had no other abnormal genetic alterations.

Discussion

IHC for *p53* expression has now become an established method for the assessment of the *p53* status. In this study, we used anti-*p53*-antibody DO-1 and a microwave for antigen retrieval, under the same conditions as previously reported.¹⁵ The immunoreactivity of the *p53* protein by IHC usually indicated a point mutation. However, the presence of elevated protein levels in the tumors that had no detectable gene mutations has been reported by others.^{15,24} The accumulation of wild-type *p53* protein indicates the presence of a mechanism of *p53* stabilization other than a missense point mutation, which may be

caused by such DNA damage as ionizing radiation or chemotherapeutic agents which showed a physiological response to allow for DNA repair.^{25,26}

We analyzed the *p53* gene expression by IHC analyses in carcinoid tumors and salivary gland type tumors of the lung, that are considered to show a low-grade malignant behavior. Pulmonary neuroendocrine tumors include a spectrum of histological variants ranging from relatively indolent typical carcinoids to highly malignant small cell carcinomas. The behavior of atypical carcinoid tumors is intermediate between that of a typical carcinoid tumor and small cell carcinoma. In carcinoid tumors, no immunoreactivity was detected in any of 21 cases including 13 typical and 8 atypical carcinoids by Lohmann et al.,²⁷ and in only one of 18 carcinoids by Wang et al.⁵ However, Roncalli et al. reclassified the atypical carcinoids into three groups,²⁸ and *p53* immunoreactivity was thus strictly confined to the cases belonging to the more aggressive subsets, that is, 10 of 16 cases showed *p53* overexpression, however, no immunoreactivity was observed in the less aggressive atypical carcinoids and typical carcinoids.²⁹ We were able to confirm the previous reported results in which *p53* immunoreactivity was frequently detected in atypical carcinoids, but not in typical carcinoids.²⁹ Although the number of our atypical carcinoid cases was small in this study, we demonstrated that all atypical carcinoid tumors showed an abnormal *p53*

expression. These cases were also characterized by a cell proliferation analysis using PCNA and AgNORs, which showed a significantly high proliferation (in preparation). These data suggest that *p53* overexpression might thus be related to a deregulated cell proliferation, and might thus be a useful marker for evaluating atypical carcinoid tumors from typical carcinoid tumors in the lung.

LOH at multiple loci on the short arm of chromosome 3 is the most frequent genetic lesion in lung cancer.¹⁶⁾ Recently, the FHIT gene was identified at 3p14.2, in which 80% of SCLC and 40% of NSCLC showed abnormalities in RNA transcripts.^{30,31)} In addition, the LOH at 3p loci was also reported to be associated with poor prognosis in lung cancer.¹⁸⁾ Deletions in the short arm of chromosome 9 have also been observed in lung carcinomas.¹⁷⁾ In our preliminary study, the frequency of LOH was higher in 3p14 than in 3p25, and two of the three cases with LOH at 3p14 died of the disease; one was mucoepidermoid carcinoma and other was adenoid cystic carcinoma. One of these patients with LOH at 3p14 also demonstrated LOH at 9p. These data suggest that the putative tumor suppressor gene in 3p14 might thus play a critical role in carcinogenesis and may also help to identify these lung cancer patients with a poor prognosis.

A *ras* gene mutation is found in 10 to 30% of adenocarcinoma,¹⁹⁻²¹⁾ however, it has never previously been detected in both carcinoid tumor and SCLC. No *ras* gene mutation was found in the tumors examined in this study, that suggested that *ras* mutation does not play a critical role in carcinogenesis in these tumors.

In conclusion, these data indicate that *p53* overexpression might thus distinguish atypical carcinoid tumors from typical tumors and might therefore be a useful adjunct modality for making a differential diagnosis of pulmonary carcinoid tumor.

Acknowledgment

We thank Dr. Brian T. Quinn for critical comments. This work was supported in part by a Grand-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), and a Research Grant for Promotion of Occupational Health from VOEH, Japan.

References

1. Travis WD, Linnoila RI, Tsokos MG, et al. Neuroendocrine tumors of the lung with proposed criteria for large-cell neuroendocrine carcinoma. *Am J Surg Pathol*

- 1991; **15**: 529–53.
2. Arrigoni MG, Woolner LB, Bernatz PE. Atypical carcinoid tumors of the lung. *J Thorac Cardiovasc Surg* 1972; **64**: 413–21.
3. Warren WH, Gould VE, Faber LP, Kittle CF, Memoli VA. Neuroendocrine neoplasms of the bronchopulmonary tract. A classification of the spectrum of carcinoid to small cell carcinoma and intervening variants. *J Thorac Cardiovasc Surg* 1985; **89**: 819–25.
4. McCaughan BC, Martini N, Bains MS. Bronchial carcinoids. Review of 124 cases. *J Thorac Cardiovasc Surg* 1985; **89**: 8–17.
5. Wang DG, Johnston CF, Anderson N, Sloan JM, Buchanan KD. Overexpression of the tumour suppressor gene *p53* is not implicated in neuroendocrine tumour carcinogenesis. *J Pathol* 1995; **175**: 397–401.
6. Costes V, Marty-Ane C, Picot MC, et al. Typical and atypical bronchopulmonary carcinoid tumors: a clinicopathologic and KI-67-labeling study. *Hum Pathol* 1995; **26**: 740–5.
7. Brambilla E, Negoescu A, Gazzeri S, et al. Apoptosis-related factors *p53*, *Bcl2*, and *Bax* in neuroendocrine lung tumors. *Am J Pathol* 1996; **149**: 1941–52.
8. Lai S, Brauch H, Knutsen T, et al. Molecular genetic characterization of neuroendocrine lung cancer cell lines. *Anticancer Res* 1995; **15**: 225–32.
9. Przygodzki RM, Finkelstein SD, Langer JC, et al. Analysis of *p53*, *K-ras-2*, and *C-raf-1* in pulmonary neuroendocrine tumors. Correlation with histological subtype and clinical outcome. *Am J Pathol* 1996; **148**: 1531–41.
10. el-Deiry WS, Tokino T, Velculescu VE, et al. *WAF1*, a potential mediator of *p53* tumor suppression. *Cell* 1993; **75**: 817–25.
11. Oltvai ZN, Milliman CL, Korsmeyer SJ. *Bcl-2* heterodimerizes in vivo with a conserved homolog, *Bax*, that accelerates programmed cell death. *Cell* 1993; **74**: 609–19.
12. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994; **54**: 4855–78.
13. D'Amico D, Carbone D, Mitsudomi T, et al. High frequency of somatically acquired *p53* mutations in small-cell lung cancer cell lines and tumors. *Oncogene* 1992; **7**: 339–46.
14. Carbone DP, Mitsudomi T, Chiba I, et al. *p53* immunostaining positivity is associated with reduced survival and is imperfectly correlated with gene mutations in resected non-small cell lung cancer. A preliminary report of LCSG 871. *Chest* 1994; **106** (Suppl): 377S–81S.
15. Mitsudomi T, Oyama T, Nishida K, et al. *p53* nuclear immunostaining and gene mutations in non-small-cell lung cancer and their effects on patient survival. *Ann Oncol* 1995; **6** (Suppl): S9–13.
16. Hibi K, Takahashi T, Yamakawa K, et al. Three dis-

- tinct regions involved in 3p deletion in human lung cancer. *Oncogene* 1992; **7**: 445–9.
17. Kishimoto Y, Sugio K, Mitsudomi T, et al. Frequent loss of the short arm of chromosome 9 in resected non-small-cell lung cancers from Japanese patients and its association with squamous cell carcinoma. *J Cancer Res Clin Oncol* 1995; **121**: 291–6.
 18. Horio Y, Takahashi T, Kuroishi T, et al. Prognostic significance of p53 mutations and 3p deletions in primary resected non-small cell lung cancer. *Cancer Res* 1993; **53**: 1–4.
 19. Slebos RJC, Kibelaar RE, Dalesio O, et al. K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* 1990; **323**: 561–5.
 20. Sugio K, Ishida T, Yokoyama H, Inoue T, Sugimachi K, Sasazuki T. ras gene mutations as a prognostic marker in adenocarcinoma of the human lung without lymph node metastasis. *Cancer Res* 1992; **52**: 2903–6.
 21. Fukuyama Y, Mitsudomi T, Sugio K, Ishida T, Akazawa K, Sugimachi K. K-ras and p53 mutations are an independent unfavourable prognostic indicator in patients with non-small-cell lung cancer. *Br J Cancer* 1997; **75**: 1125–30.
 22. Sugio K, Kishimoto Y, Virmani AK, Hung JY, Gazdar AF. K-ras mutations are a relatively late event in the pathogenesis of lung carcinomas. *Cancer Res* 1994; **54**: 5811–5.
 23. Sugio K, Gazdar AF, Albores-Saavedra J, Kokkinakis DM. High yields of K-ras mutations in intraductal papillary mucinous tumors and invasive adenocarcinomas induced by N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine in the pancreas of female Syrian hamsters. *Carcinogenesis* 1996; **17**: 303–9.
 24. Lang FF, Miller DC, Pisharody S, Koslow M, Newcomb EW. High frequency of p53 protein accumulation without p53 gene mutation in human juvenile pilocytic, low grade and anaplastic astrocytomas. *Oncogene* 1994; **9**: 949–54.
 25. Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci U S A* 1992; **89**: 7491–5.
 26. Lane DP. Cancer. p53, guardian of the genome. *Nature* 1992; **358**: 15–6.
 27. Lohmann DR, Fessler B, Putz B, et al. Infrequent mutations of the p53 gene in pulmonary carcinoid tumors. *Cancer Res* 1993; **53**: 5797–801.
 28. Roncalli M, Doglioni C, Springall DR, et al. Abnormal p53 expression in lung neuroendocrine tumors. Diagnostic and prognostic implications. *Diagn Mol Pathol* 1992; **1**: 129–35.
 29. Warren WH, Memoli VA, Gould VE. Well differentiated and small cell neuroendocrine carcinomas of the lung. Two related but distinct clinicopathologic entities. *Virchows Arch* 1988; **55**: 299–310.
 30. Ohta M, Inoue H, Cotticelli MG, et al. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 1996; **84**: 587–97.
 31. Sozzi G, Veronese ML, Negrini M, et al. The FHIT gene 3p14.2 is abnormal in lung cancer. *Cell* 1996; **85**: 17–26.