Infrequent Mutations of the p53 Gene in Pulmonary Carcinoid Tumors

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ABSTRACT

Archival specimens of 25 pulmonary carcinoids including 15 cases of typical carcinoid, 9 atypical carcinoids, and 1 large-cell neuroendocrine carcinoma were analyzed for mutations in exons 5 to 8 of the p53 gene. Mutations were identified in 4 tumors, including 3 out of 15 (20%) typical carcinoids and the single large-cell neuroendocrine carcinoma, but none of the atypical carcinomas showed a mutation. The mutations were acquired during tumor development since they were not present in the corresponding nontumorous tissue. All mutations in the typical carcinoids, a tumor type of epidemiological link to cigarette smoking, were G to A transitions. The level of p53 protein was investigated by immunohistochemistry with the polyclonal antibody CM-1. None of the pulmonary carcinoids investigated showed a positive reaction, despite the presence of missense mutations in two cases. Negative staining of carcinoids with mutations was also observed with the monoclonal antibodies pAb1801 and DO-1. Our data suggest that point mutations of the p53 gene are infrequent in pulmonary carcinoids thus contrasting the findings in other histological types of lung cancer, in particular small-cell lung cancer. Moreover, negative immunostaining for p53 is no indicator for the absence of p53 missense mutations in typical carcinoids.

INTRODUCTION

Pulmonary carcinoids together with SCLC4 are regarded as bronchopulmonary NE neoplasms since they have morphological and functional features in common with their putative progenitors, the pulmonary NE cells (1, 2). SCLC is a highly malignant disease marked by rapid and dissiminated tumor growth in the majority of patients (3). Pulmonary carcinoids, however, show a diverse clinical behavior ranging from dormant tumor nodules to highly malignant cancers. A classification based on histopathological criteria has evolved that defines tumor types that have similar morphological and clinical properties (4, 5); however, auxiliary criteria are still wanted. Investigation of the genetic abnormalities in pulmonary carcinoids may reveal attributes that are helpful for their classification and improve the reliability of prognosis.

The gene that encodes the nuclear phosphoprotein p53 is commonly mutated in various human cancers (6). Somatic mutations of the p53 gene are frequent in SCLC (7–9) and the major histological types of NSCLC (10, 11). The mutational spectrum in these tumors is dominated by G to T transversions and thus distinct from that of most other tumors (8, 11). Because this type of base substitution is induced by some carcinogens in tobacco smoke the unusual mutational pattern may reflect the genotoxic action of this risk factor (12).

Mutations of the p53 gene abrogate the tumor suppressing activity of the wild-type gene that encodes a cell cycle regulatory protein (13, 14). The level of the p53 protein is regulated by rapid protein turnover. The amounts present in normal cells are below the sensitivity of conventional immunohistochemical detection systems (15). However, in many tumor cells p53 has a prolonged half-life and thus accumulates to levels that permit detection by IHC with several antibodies that recognize both wild-type and mutant p53 protein (16, 17). Sequence analysis of the p53 gene in tumors with elevated levels of p53 protein has shown point mutations resulting in amino-acid substitutions at evolutionary conserved sites of the protein (18, 19). Consequently, detection of accumulation of p53 protein by IHC has been proposed as a way of identifying mutations in the p53 gene (18, 19). However, because some types of p53 mutation do not result in accumulation of p53 protein, absence of a positive IHC reaction does not conclusively indicate the presence of a wild-type p53 gene (20).

In order to determine the frequency and type of p53 gene mutations in pulmonary carcinoids, archival samples from 25 patients were analyzed by direct sequencing. Furthermore, accumulation of p53 protein was investigated by IHC to resolve its value as an indicator for p53 gene mutations in this tumor type.

MATERIALS AND METHODS

Tissue Samples. The tissue samples used in this study were from patients who were admitted to the Department of Thoracic Surgery at the Technical University of Munich during 1981 to 1989. Tumor tissue was excised at the time of fiberoptic biopsy or operation, fixed in formalin, and embedded in paraffin wax. Paraffin blocks were drawn from the files of the Department of Pathology and reclassified histologically (4, 5). Of a total of 25 cases, 15 were typical carcinoids, 9 were atypical carcinoids, and 1 case was a large-cell NE carcinoma. Tumor size was defined as the maximum diameter of the tumor, N and M staging was performed according to standard criteria for NSCLC (3). To assess the sensitivity of our IHC system, 15 cases of SCLC drawn from the same archive and analyzed in a previous study (9) were included in the IHC analysis.

Sequence Analysis. Exons 5, 7, and 8 of the p53 gene were investigated by direct sequencing as described previously (9). In addition, exon 6 was analyzed in this study. In brief, tumor tissue was scratched off from a 5-μm section under microscopic control, dewaxed, resuspended in sterile water, and heat denatured. DNA for direct sequencing was synthesized by two successive rounds of PCR using nested primers. The sequences of the primers for PCR of exon 6 were: left external, GGTTGCCCAGGGTCGGG; right external, CACTTACCCTCCTCCC; left internal, AGGCCTCTGATFCCTCACTG; right internal, CTCCCAGAGACCCCAGTTGC (all 5' to 3'). Mutations were confirmed by additional separate PCR and direct sequencing. Whenever feasible, an additional confirmation of mutant sequences was performed by restriction enzyme digestion of the mutation bearing PCR product as previously described (9).

Immunohistochemistry. The polyclonal anti-p53 antibody CM-1 (Medac, Hamburg, Germany) was used for immunohistochemical detection of p53 protein in formalin-fixed, paraffin-embedded tissue sections (17, 21). For proteolytic pretreatment, tissue was incubated with Pronase E (Merck, Darmstadt, Germany) 0.1% (w/v) in TBS buffer (145 mM NaCl-20 mM Tris, pH 7.6) at 37°C for 2 min. To reduce nonspecific background staining, sections were incubated with normal goat serum (Dako, Hamburg, Germany) 1:5 in TBS following a second blocking step with 1% bovine serum albumin (Sigma, Deisenhofen, Germany). Sections were incubated with primary antibody diluted 1:300 in TBS for 90 min at room temperature. Preimmune rabbit serum was used as a negative control. Localization of the primary antibody was achieved by subsequent incubation with a biotinylated goat-anti-rabbit anti-
were used (22, 23). To improve antigenicity, deparaffinized sections were conjugated to alkaline phosphatase (Dako). Alkaline phosphatase activity was visualized using the manufacturer’s protocol. Between all following steps, slides were washed 3 times with TBS for 5 min. Sections were incubated for 2 h with primary antibody diluted 1:100 in TBS containing 1% bovine serum albumin pretreated with antigen retrieval solution (BioGenex, San Ramon, CA) following the manufacturer’s protocol. Between all following steps, slides were washed 3 times with TBS for 5 min. Sections were incubated for 2 h with primary antibody diluted 1:100 in TBS containing 1% bovine serum albumin (Sigma) at room temperature. Slides were then incubated with secondary antibody (Dako) diluted 1:30 in TBS containing 20% nonimmune serum for 30 min followed by incubation with APAAP (alkaline phosphatase anti-alkaline phosphatase) complex (Dako) diluted 1:100 in TBS for 30 min. Alkaline phosphatase activity was visualized with new-fuchsine (Chroma, rainbow) as a chromogen. Omission of the first and second antibody resulted in negative staining.

## RESULTS

**p53 Point Mutations in Pulmonary Carcinoids.** The sequence of exons 5 to 8 of the p53 gene including their flanking intron regions was determined in archival tissue samples of 25 pulmonary carcinoids. Mutations were identified in 3 of 15 typical carcinoids, in none of the 9 atypical carcinoids and in 1 of 1 large-cell NE carcinoma (Table 1). For example, a G to A transition at the second base of codon 229 was identified in a typical carcinoid (case 9; Fig. 1A). This mutation was confirmed by restriction analysis with Rsal (Fig. 1B). In both the sequence and restriction analysis a faint wild-type sequence pattern was detectable at the site of mutation which was most likely due to the normal DNA contributed by the fibrovascular stroma present in this tumor. No wild-type sequence was detectable in all other tumors with a mutation thus indicating the loss of the wild-type allele. Restriction analysis of exon 7 PCR products generated from nontumorous tissue of this patient showed only the wild-type pattern (case 9; Fig. 1B). Therefore, the mutation was a somatic event that occurred during tumor development. Nontumorous tissue was also available from all other patients that showed a mutation in the tumor and the somatic nature of these alterations was confirmed by sequence analysis in each case.

**Immunohistochemical Detection of p53 Protein.** In order to evaluate the sensitivity of our detection system, IHC was first performed on 15 formalin-fixed, paraffin-embedded specimens of SCLC with known mutational status in exons 5 to 8 of the p53 gene (9). These tumors had been routinely processed and stored under identical conditions as the pulmonary carcinoids investigated in this study. Staining was evaluated without knowledge of the mutational status of each case. Five cases of SCLC with mutations that resulted in amino acid substitutions in the p53 protein (missense mutations) showed intense to moderate nuclear staining in the majority of neoplastic cells (Fig. 2A). A positive reaction was also detectable in one SCLC that had no mutation in exons 5 to 8 (Fig. 2B). Five cases of SCLC without p53 mutation showed faint staining in less than 10% of the tumors cells or no staining at all. Two SCLC with somatic mutations that resulted in no change of the p53 primary structure (neutral mutations) and two with mutations that created premature translation stop signals (non-sense mutations) also showed no immunoreaction. All nonneoplastic tissue adjacent to tumors was negative.

In a second round 13 cases of typical carcinoid, 8 cases of atypical carcinoid, and 1 large-cell NE carcinoma were investigated with the monoclonal anti-p53 antibody CM-1. Three cases of SCLC from the first series were used as positive controls. All pulmonary carcinoids including two cases of typical carcinoid with missense mutations and one large-cell NE carcinoma with a nonsense mutation showed no immunostaining whereas the controls showed positive staining (Table 1). Identical results were obtained in repeated analyses. Finally, IHC was performed using the monoclonal antibodies pAb1801 and DO-1. All three positive controls showed intense nuclear staining with both negative staining.

### Table 1 Summary of the pulmonary carcinoid tumor cases studied

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^a TC, typical carcinoid; AC, atypical carcinoid; LC-NEC, large-cell neuroendocrine carcinoma.

^b Result of CM-1 immunostaining = - no staining; ND, not done.

^c Maximum tumor diameter; NA, not available.

^d Confirmed by restriction enzyme digestion with Rsal.

^e Alive with multiple metastases.

^f Died, not from tumor.

^g Unpublished data.
DISCUSSION

Archival specimens of 25 pulmonary carcinoids were examined for alterations of the p53 gene. Mutations were detected in 4 samples, including 3 out of 15 typical carcinoids (20%) and the single case of large-cell NE carcinoma. All mutations were acquired during tumor development since they were not present in the corresponding nontumorous tissue. The missense mutations resulted in the substitution of evolutionary conserved amino acids within and outside the domains of continuous interspecies homology (24).

The clinical features of the pulmonary carcinoids investigated comprised the full spectrum of this disease ranging from minute lesions to metastasizing disease with fatal outcome but there was no overt relation between the presence of a mutation and clinico-pathological stage or survival (Table 1). Although the typical carcinoids with a mutation were rather large (>3 cm) as compared with reported size ranges (4, 5), tumor size was not significantly related to the mutational status (Wilcoxon rank sum test). Different p53 mutants exhibit different biological activities (25, 26) and may even be functionally silent (27). A strong indication of the functional significance of the mutations observed in the typical carcinoids is given by the concomitant loss of the wild-type p53 gene in the tumors. However, we cannot exclude that these mutations have only a minor effect on tumor growth and hence show no overt relation to clinical behavior. In the large-cell NE carcinoma normal function of p53 was lost due to a non-sense mutation that resulted in truncation of structural motifs essential for nuclear localization (28). No mutation was identified in the atypical carcinoids. However, assuming that the mutation frequency in this tumor type of intermediate malignancy is as low as in typical carcinoids, analysis of a higher number of samples may be necessary.

The mutational spectrum of NSCLC and SCLC is marked by a prevalence of G to T transversions that are ascribed to the genotoxic action of tobacco smoke, which is a major risk factor for these tumors (12). The development of carcinoids, however, shows no epidemiological link to cigarette smoking (29). Therefore it is interesting that all mutations in the typical carcinoids were G:C to A:T transitions, which is the most frequent type of base substitution in many tumor types (6, 30). The limited data available on the epidemiology of large-cell NE carcinomas suggest a positive relation to smoking history (4, 5). Interestingly, the large-cell NE carcinoma investigated here showed a G to T transversion. Further investigations on a larger series of patients are required to define the relation between the mutational spectrum and etiological important carcinogens in pulmonary carcinoids.

To evaluate the sensitivity of our IHC analysis, a series of formalin-fixed, paraffin-embedded samples of SCLC with known mutational status were investigated. Accumulation of p53 protein was detected in all SCLCs with p53 missense mutations whereas tumors with nonsense mutations showed no immunoreaction. Interestingly, one immunopositive SCLC contained only wild-type sequence in exons 5 to 8. We cannot exclude that accumulation of p53 protein in this tumor results from a mutation outside the region investigated by direct sequencing. However, the presence of elevated protein levels in tumors that did not present a detectable genetic alteration was also reported by others (31–33). Furthermore, accumulation of normal p53 was identified in an inherited cancer susceptibility syndrome indicating the presence of mechanisms of p53 stabilization other than missense point mutation (34).

None of the pulmonary carcinoids investigated showed accumulation of p53 to levels detectable by IHC with the polyclonal antibody CM-1. The large-cell NE carcinoma had a non-sense p53 mutation, so negative staining in this case is in accordance with the findings in
Fig. 2. Immunohistochemical detection of p53 protein in tissue sections of SCLC. A, SCLC with a missense p53 mutation (Arg175 to His175) presenting diffuse nuclear staining in the majority of neoplastic cells. B, SCLC with no mutation in exons 5 to 8 showing positive nuclear staining. For both A and B the primary antibody was CM-1. × 400.

SCLC and other tumors (20). Two cases of typical carcinoid with negative staining, however, had missense point mutations. An identical mutation at codon 216 (GTG to ATG, valine to methionine) was identified in an epithelial ovarian cancer and this tumor showed a high level expression of the p53 protein by IHC with the monoclonal antibody pAb1801 (35). Therefore, IHC was also performed using pAb1801 and, in addition, DO-1. Again, both carcinoids with missense mutations showed no staining with either antibody thus indicating that negative staining is not due to epitope differences between the antibodies used. The observed negative staining may be due to the lack of mRNA expression of the mutant p53. However, since we could not obtain suitable tissues for the analysis of RNA, control of expression of mutant p53 at the transcriptional level was prohibited. We cannot exclude that negative staining in these cases is due to overfixation or delayed fixation which allowed degradation of the p53 protein. However, considering that IHC was positive in all 5 cases of SCLC with missense mutations that were drawn from the same archive the rate of failure seems to be low. Therefore, these data suggest that the mutational change of the p53 protein primary structure is not the sole determinant of its turnover rate. Transfection experiments in cell lines have indicated that the specific cellular environment also affects the stability of p53 (25, 36). Because p53 expression shows a positive relation to proliferative activity (20, 37–40) the undetectable levels of mutant protein in the typical carcinoids might be due to the slow growth rate of these tumors (41). Negative immunostaining with different anti-p53 antibodies in carcinoids was also reported by several investigators (19, 42, 43). Our results suggest, therefore, that unlike the situation in many, and especially highly proliferating, tumors, negative staining for p53 in typical carcinoids is no indicator for the absence of p53 mutations in this tumor type. Consequently, the validity of IHC as a screening method for detecting p53 gene mutations should be confirmed for each tumor type under investigation.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Arne Luz and Dr. Mike Atkinson for helpful discussions, Inge Bruemmer for excellent technical assistance, Dr. Kay Friedrichs for support, and Dr. Bernhard Horsthemke for critical reading.

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