Search for Mutations and Examination of Allelic Expression Imbalance of the p73 Gene at 1p36.33 in Human Lung Cancers

Shuji Nomoto, Nobuhiro Haruki, Masashi Kondo, Hiroyuki Konishi, Takao Takahashi, Toshitada Takahashi, and Takashi Takahashi

Laboratories of Ultrastructure Research [S. N., N. H., M. K., H. K., Takao T., Takas. T.J and Immunology [To. T], Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan

Abstract

We examined 61 lung cancer cases to determine whether alterations of p73, a novel monoallelically expressed p53-like molecule, may be involved in the pathogenesis of lung cancer. Allelic loss at the p73 locus at 1p36.33 was observed in 42% (11 of 26 informative cases), and squamous cell carcinoma tended to carry this lesion most frequently. Somatic mutations in the p73 gene itself, however, were not detected, despite our extensive search. We found interindividual differences in the allelic expression of p73 in normal lung, as well as intertissue variance, even within the same individual, but preferential loss of the expressed allele appeared to be an unlikely mechanism for p73 inactivation. This study, consequently, suggests the presence of an as yet unidentified tumor suppressor gene or genes within the subtelomeric region of 1p, warranting further studies aimed at its isolation.

Introduction

Allelic losses are hallmarks of chromosomal regions harboring tumor suppressor genes. Cytogenetic and molecular genetic studies have demonstrated frequent chromosomal deletions in lung cancer on 3p, 5q, 8p, 9p, 11p, 13q, 17p, 18q, and 22q, suggesting the presence of tumor suppressor genes in the affected chromosomal regions (1). Indeed, we and others have reported genetic alterations of candidate tumor suppressor genes in lung cancer, which include p16 on 9p, Rb on 13q, and p53 on 17p, as well as Smad2 and Smad4/DPC4 on 18q (2–8). Among these genetic lesions, inactivation of p53 appears to be the most frequent, suggesting an important role of this gene in the pathogenesis of lung cancer.

Recently, Kaghad et al. (9) reported a novel gene encoding a protein, which they termed p73, with remarkable sequence similarity to the DNA-binding, transactivation, and oligomerization domains of p53. It was shown that p73 could activate transcription of p53-responsive genes and inhibit cell growth in a p53-like manner, by inducing apoptosis (10). Interestingly, the p73 gene was mapped to the 1p36.33 region, which is known to be frequently deleted in lung cancer, as well as intertissue variance, even within the same individual, but preferential loss of the expressed allele appeared to be an unlikely mechanism for p73 inactivation. This study, consequently, suggests the presence of an as yet unidentified tumor suppressor gene or genes within the subtelomeric region of 1p, warranting further studies aimed at its isolation.

Materials and Methods

Tissue Specimens. Tumor samples, along with uninvolved lung tissue where available, were collected from 61 patients diagnosed histologically as having lung cancers (4 cases of SCLC, 26 cases of adenocarcinoma, 28 cases of squamous cell carcinoma, and 3 cases of large cell carcinoma). All tissues were quickly frozen in liquid nitrogen and stored at —80°C until analyzed. In addition, normal lung specimens obtained from three patients undergoing thoracic surgery due to noncancerous diseases, as well as the peripheral blood cells of their parents, were also examined.

Examination of Allelic Loss of the p73 Locus by PCR-SSCP Analysis. PCR-SSCP analysis was used to distinguish two distinct alleles, representing tightly linked polymorphisms in exon 2 (9). The primer pair used was: GS1 (sense; 5'-GAGGCCCACTTGGCTGCC) and GAS1 (antisense; 5'-AGAGGTCGCTCAAAAGCTTG). PCR amplification was carried out using genomic DNAs in the presence of [32P]dCTP, followed by electrophoretic separation on 6% nondenaturing polyacrylamide gels with 5% glycerol at room temperature. The PCR amplification consisted of 35 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s) after the initial denaturation step (94°C for 5 min). Tumor specimens were scored as having allelic loss when the decrease in signal intensity seen in the densitometric tracing was >50%.

Southern Blot Analysis. Southern blot analysis was carried out by using EcoRI-digested DNAs. A cDNA probe, which covered the entire open reading frame of the p73 gene, was prepared by PCR amplification using the following three sets of oligonucleotide primer pairs: S1 (sense; 5'-GAGCCCACTTGCCCTGCC) and GASI (antisense; 5'-CGTGGGCCGCTGGTTGGA); S2 (sense; 5'-GAGCCCACTTGCCCTGCC) and GAS2 (antisense; 5'-GAGCCCACTTGCCCTGCC); and S3 (sense; 5'-GGCCCACTTGCCCTGCC) and GASS (antisense; 5'-GGCCCACTTGCCCTGCC). The PCR amplification consisted of 35 cycles (94°C for 1 min, 60°C for 45 s, and 72°C for 1 min) after the initial denaturation step (94°C for 5 min).

Results

In 31 lung cancer specimens for the presence of allelic loss at the p73 locus and for neuroblastoma is paternally imprinted and maternally expressed, based on the finding of significant maternal bias with regard to origins of discrete 1p36 deletions in neuroblastoma cases.

Here, we investigated the potential involvement of p73 alterations in the pathogenesis of lung cancer by examining 61 lung cancer specimens for the presence of allelic loss at the p73 locus and for mutations and allelic expression imbalance of the p73 gene, with reference to the p53 gene status in each of lung cancer cases.

Received 12/23/97; accepted 2/17/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, Japan, as well as by a Grant-in-Aid for the Second Term Comprehensive Ten-Year Strategy for Cancer Control and by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

2 To whom requests for reprints should be addressed, at Laboratory of Ultrastructure Research, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan.

The abbreviations used are: SCLC, small cell lung cancer; SSCP, single-strand conformation polymorphism; RT-PCR, reverse transcription-PCR.
Identification of frequent allelic loss at the p73 locus, especially in squamous cell carcinomas, led us to investigate whether the p73 gene itself is altered as a target for the frequent allelic loss at Ip36.33, where p73 resides. Forty-four lung cancer specimens with known p53 status were chosen for this purpose and were further examined. Southern blot analysis was performed first to detect gross genomic alterations but failed to identify any such abnormalities (data not shown). RT-PCR-SSCP analysis was then performed to search for subtle mutations in the p73 gene. Distinct mobility shifts were observed with the aid of S5-AS5 and S7-AS7 primer pairs (Fig. 2), as well as the S8-AS8 PCR primer set, in both lung cancer specimens and the corresponding normal lung tissues. Subsequent sequence analysis revealed that these mobility shifts were due to silent nucleotide substitutions at codon 336 (GCC to GCT), codon 349 (CAT to CAC), codon 557 (GGC to GCA), and codon 610 (GGG to GGA), indicating that they probably reflect sequence polymorphisms among individuals. We noted that these sequence variations were concordant, suggesting that they probably reflect sequence polymorphisms among individuals.

RT-PCR products of lung cancer specimens showing distinct PCR-SSCP patterns were cloned into the EcoRV site of pBluescript SKIII(-) (Stratagene) after polishing, and the resulting plasmid DNAs prepared from pooled clones were sequenced. RT-PCR products of the corresponding normal lung RNAs were also subjected to PCR-SSCP and sequencing analysis.

Results and Discussion

Sixty-one lung cancer cases were examined for the presence of allelic loss at the p73 locus by PCR-SSCP analysis, as a result of which two distinct alleles representing tightly linked polymorphisms in exon 2 could be distinguished. Among the 26 informative cases, allelic loss at the p73 locus was observed in 11 (42.3%; 1 of 2 SCLCs, 3 of 13 adenocarcinomas, 6 of 10 squamous cell carcinomas, and 1 of 1 large cell carcinoma; Table 1 and Fig. 1). Allelic loss at the p73 locus was observed more frequently in squamous cell carcinoma (60%, 6 of 10 informative cases) than it was in adenocarcinoma (23%, 3 of 13 informative cases).

Identification of frequent allelic loss at the p73 locus, especially in squamous cell carcinomas, led us to investigate whether the p73 gene itself is altered as a target for the frequent allelic loss at Ip36.33, where p73 resides. Forty-four lung cancer specimens with known p53 status were chosen for this purpose and were further examined. Southern blot analysis was performed first to detect gross genomic alterations but failed to identify any such abnormalities (data not shown). RT-PCR-SSCP analysis was then performed to search for subtle mutations in the p73 gene. Distinct mobility shifts were observed with the aid of S5-AS5 and S7-AS7 primer pairs (Fig. 2), as well as the S8-AS8 PCR primer set, in both lung cancer specimens and the corresponding normal lung tissues. Subsequent sequence analysis revealed that these mobility shifts were due to silent nucleotide substitutions at codon 336 (GCC to GCT), codon 349 (CAT to CAC), codon 557 (GGC to GCA), and codon 610 (GGG to GGA), indicating that they probably reflect sequence polymorphisms among individuals. We noted that these sequence variations were concordant, suggesting that they probably reflect sequence polymorphisms among individuals. We noted that these sequence variations were concordant, suggesting that they probably reflect sequence polymorphisms among individuals.
mutations in the p73 gene itself are rare in lung cancer, if present at all. Thus, the conventional two-hit theory did not appear to be applicable as a major inactivation mechanism leading to loss of function of this p53-like molecule in lung cancer.

Genomic imprinting has been suggested as playing a role in certain pediatric tumors, such as Wilms' tumor, based on the findings of highly selective loss of maternal alleles in these tumors (17, 18). Although neuroblastomas had previously been reported to exhibit selective maternal loss of 1p36 (11), Kaghad et al. (9) found that the p73 gene was monoallelically expressed in peripheral blood cells of all five of their cases and in a single neuroblastoma line, lending support to the notion that p73 might be a candidate tumor suppressor gene in this childhood tumor. We previously reported the identification of genomic imprinting of a cyclin-dependent kinase inhibitor, p57kip2, at 11p15 and selective loss of the expressed maternal allele in lung cancer specimens, suggesting that p57kip2 may be a target for frequent 11p15 deletions in lung cancer (4). We, therefore, examined whether the p73 gene is also monoallelically expressed in the lung and whether the frequent allelic loss at the p73 locus observed in this study occurs selectively on the expressed allele of p73. Among 61 lung cancer cases examined in this study, 26 proved to be useful for this study because of their heterozygosities for the polymorphism within its 5'-untranslated region. In contrast to the report by Kaghad et al. (9), we observed biallelic expression in the majority (25 of 26) of the normal lungs, although slight variations in certain specimens, such as cases 39 and 42, were identified in this analysis. Although marked allelic expression imbalance was observed only in a single case (Fig. 3, case 48), we had fortunately collected various normal tissues from this patient at necropsy. Examination of the allelic expression status of p73 revealed significant variations among various normal tissues taken from this individual. The p73 gene was preferentially expressed from the A1 allele in lung and liver, whereas expression from the A2 allele was predominant in the stomach. Small intestine, spleen, and kidney exhibited almost equal expression of the p73 alleles. These findings indicated that allelic expression of the p73 gene in the lung varies among individuals and that there is also considerable intertissue variation, even within the same individual.

Marked allelic expression imbalance was also observed in an additional case among three normal lung specimens collected from patients with noncancerous diseases (data not shown). It is of note that the expressed allele in this particular case could be determined to have been paternally derived, in contrast to the findings of Kaghad et al. (9), in which the expressed allele in peripheral blood cells was of maternal origin. Because parental origins of the expressed allele of p73 have been determined only in one case each by us and Kaghad et al. (9), further studies are obviously necessary to determine whether p73 is expressed from a specific parental allele in a tissue-specific manner or whether it is monoallelically expressed regardless of the parental origin. In this regard, it is interesting to note that Mitsuya et al. (19) recently demonstrated the presence of either paternal or biallelic expression of WTI in human fibroblasts and lymphocytes, in contrast to the previous findings concerning its maternal or biallelic expression in human placental villus and brain tissue (20). It is, therefore, possible that a tissue- and individual-specific modifier(s) might be present and might determine the allelic expression pattern, resulting in a variable allele-specific expression pattern of certain genes, such as p73 and WTI.

Here, we have shown that it appears that the p73 gene quite infrequently carries mutations in lung cancer, if they occur at all. Because marked allelic expression imbalance in the lung was observed in a very small fraction of the cases examined, it appears to be unlikely that preferential loss of the expressed allele serves as an important inactivating mechanism. We, thus, conclude that the p73 gene is unlikely to play a major role in the pathogenesis of lung cancer. However, the present demonstration of highly frequent allelic loss at the p73 locus at 1p36.33 suggests that there may be a tumor suppressor gene or genes within this subtelomeric region of chromosome 1p, which may be involved specifically in the development of squamous cell carcinoma. The presence of frequent allelic loss at 1p has also been described in various other tumor types, including neuroblastoma, breast cancer, melanoma, and hepatocellular carcinoma (11–16). Although such frequent involvement of 1p in lung cancer has not yet been reported (21), our use of the subtelomeric marker p73 in this study seemed to have enabled us to identify frequent allelic loss. An obvious next step toward positional cloning of this putative tumor suppressor gene will be to narrow down its potential location, using increasingly available, closely spaced microsatellite markers. Future studies should lead to the isolation and
characterization of the target gene(s), ultimately leading to a better understanding of the molecular pathogenesis of this fatal disease.

References

Search for Mutations and Examination of Allelic Expression Imbalance of the \( p73 \) Gene at 1p36.33 in Human Lung Cancers

Shuji Nomoto, Nobuhiro Haruki, Masashi Kondo, et al.

*Cancer Res* 1998;58:1380-1383.

Updated version

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/58/7/1380

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.