

# Frequent Loss of Heterozygosity in Region of the *KIP1* Locus in Non-Small Cell Lung Cancer: Evidence for a New Tumor Suppressor Gene on the Short Arm of Chromosome 12<sup>1</sup>

Seisho Takeuchi,<sup>2</sup> Naoki Mori, Michiaki Koike, James Slater, Susan Park, Carl W. Miller, Isao Miyoshi, and H. Phillip Koeffler

Division of Hematology/Oncology, Cedars-Sinai Research Institute, University of California at Los Angeles School of Medicine, Los Angeles, California 90048 [S. T., N. M., M. K., J. S., S. P., C. W. M., H. P. K.], and Department of Internal Medicine, Kochi Medical School, Kochi, Japan [I. M.]

## Abstract

To refine the chromosomal localization of a putative tumor suppressor gene, we analyzed the loss of heterozygosity (LOH) of chromosome 12 in 36 primary non-small cell lung cancer (NSCLC) samples with matched normal DNA using 22 highly informative polymorphic markers. Twelve cases showed LOH at one or more loci on chromosome 12. LOH of chromosome arm 12p was more frequent in large cell carcinoma than squamous cell carcinoma, indicating molecular genetic heterozygosity within the major NSCLC subtypes. We identified the smallest commonly deleted region on chromosome 12p13. This region is flanked by *D12S269* and *D12S308*, including the *KIP1* gene. Mutational analysis of *KIP1* using PCR-single strand conformation polymorphism and Southern Blot analysis showed no homozygous deletions, rearrangements, or point mutations, suggesting that the altered gene in this region is not the *KIP1* gene. These data suggest that a new tumor suppressor gene which is involved in tumorigenesis of NSCLC is in the region of *KIP1*.

## Introduction

Lung cancer is the one of the most common lethal malignancies in the United States. Several alterations of known tumor suppressor genes in lung cancer have been described, including *TP53*, *RBI*, *p15*, and *p16* genes (1-4). However, inactivation of multiple tumor suppressor genes is thought to be critical during tumorigenesis. Cytogenetic studies have shown the involvement of multiple lesions in lung cancer (5, 6). Therefore, additional tumor suppressor genes are probably associated with the development of lung cancer.

The paradigm of alterations of many tumor suppressor genes is a mutation of one allele and loss of the second allele. This reduction to homozygosity can be detected as a LOH<sup>3</sup> of informative markers in the region of the tumor suppressor gene. Thus, LOH analysis is an indirect method to search for a tumor suppressor gene.

Deletions of the short arm of chromosome 12 are observed in 10-20% of patients with NSCLC (7, 8). These findings suggest the presence of a putative tumor suppressor gene on chromosome arm 12p. Molecular analysis of 12p deletions has been very limited in scope.

To define the chromosomal localization of the putative tumor suppressor gene, we have performed fine deletion mapping of chromosome 12 in 36 samples of primary NSCLC with matched normal

DNA using 22 highly polymorphic markers. We identified the smallest commonly deleted region on 12p.

## Materials and Methods

**Samples.** Thirty-six DNAs from primary NSCLC were obtained at the time of surgery after informed consent from patients. These cancers included 16 adenocarcinomas, 12 squamous cell carcinomas, 4 large cell carcinomas, 3 epidermoid carcinomas, and 1 unclassified carcinoma. The corresponding normal DNAs were obtained from the normal lung tissues surrounding the tumors.

**Analysis of LOH Using Microsatellite Markers.** The LOH analysis was performed by PCR-amplification of microsatellite sequences. The genetic map of chromosome 12 was compiled mainly from the Généthon microsatellite map and GDB (9-12). Some markers have been assigned to the same location in a 0 cM cluster. Primers were obtained from Research Genetics (Huntsville, AL) or synthesized by the Cedars-Sinai Medical Center Molecular Biology Core and listed in Fig. 1. Each PCR reaction contained 10-100 ng of DNA, 10 pmol of each of the primers, 2 nmol each of the four deoxyribonucleotide triphosphates (Pharmacia, Stockholm, Sweden), 0.5 units of Taq DNA polymerase (Boehringer-Mannheim, Indianapolis, IN), and 2  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dCTP (ICN, Irvine, CA) in 20  $\mu$ l of the specified buffer with 1.5 mM MgCl<sub>2</sub>. Samples were amplified by 30-35 cycles of denaturing for 40 s at 94°C, annealing for 30 s at 55°C, and extending for 1 min at 72°C in a programmable thermal controller (MJ Research, Inc., Water Town, MA). After amplification, PCR samples were diluted 5-fold in loading buffer containing 20 mM EDTA, 96% formamide, and 0.05% of both bromophenol blue and xylene cyanol. The products were heated to 95°C for 5 min, and 1.5  $\mu$ l of each sample was electrophoresed through a 5-6% polyacrylamide gel containing 8.3 M urea for 3-4 h at 85 W. Subsequently, the gels were dried and subjected to autoradiography using Kodak XAR film at -80°C. LOH was inferred when a visible reduction in the ratio of allele radiographic signal intensities occurred in the tumor sample relative to that in the corresponding normal sample.

**Analysis of LOH Using a Polymorphism in Exon 1b of the *KIP1* Gene.** The *KIP1* gene has been mapped to 12p (13-17). As we reported previously, exon 1b of the *KIP1* gene contains a polymorphism; at codon 109, guanine is substituted for thymine (GTC→GGC), resulting in an amino acid substitution of glycine for valine (Val→Gly). By analyzing this polymorphism using PCR-SSCP, we examined LOH of the *KIP1* gene. PCR-SSCP was performed as described previously (18).

**Statistical Analysis.** Fischer's Exact test was performed for statistical evaluation of data. Probability values were two tailed, with  $P \leq 0.05$  regarded as statistically significant.

## Results

We examined thirty-six NSCLC samples for LOH of chromosome 12 using 22 highly informative polymorphic markers. Fig. 2 displays a representative autoradiograph showing LOH. The patterns of LOH are shown in Fig. 3.

Twelve samples showed LOH at least at one locus on chromosome 12. Of these 12 cases, three of them (nos. 75, 121, and 135) showed LOH at all informative loci on chromosome 12. This is consistent with

Received 11/2/95; accepted 12/28/95.

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.

<sup>1</sup> Supported in part by NIH Grants DK42792 and CA42710, the Concern Foundation, the Parker Hughes Trust, and the Louis Shushan Fund.

<sup>2</sup> To whom requests for reprints should be addressed, at Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Boulevard, B208, Los Angeles, CA 90048. Phone: (310) 855-4609; Fax: (310) 659-9741.

<sup>3</sup> The abbreviations used are: LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer; PCR-SSCP, PCR-single-strand conformation polymorphism.

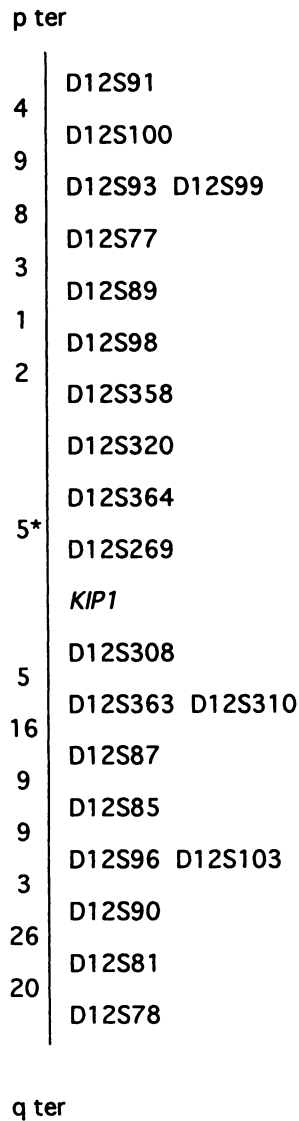


Fig. 1. A genetic map of chromosome 12. The genetic map of chromosome 12 was compiled mainly from the Génethon microsatellite map and GDB. Some markers have been assigned to the same location in a 0-cM cluster. Genetic distances are shown in centimorgans. \*, the distance between *D12S358* and *D12S3083* is 5 cM.

loss of one whole chromosome 12 in these three cases. Two cases (nos. 73 and 139) showed terminal deletions of 12p. Six cases (nos. 79, 101, 113, 117, 127, and 143) showed interstitial deletions of chromosome arm 12p. A total of 11 samples showed LOH at least at one locus on chromosome arm 12p. In addition, one case (no. 97) showed terminal deletions of 12q.

Two very informative samples (nos. 117 and 143) showed LOH at *KIP1* but retained both alleles at the distal neighboring locus *D12S269*. One of these samples (no. 117) maintained heterozygosity at the proximal neighboring locus *D12S308* (Fig. 3). All the other four samples with interstitial deletions (nos. 79, 101, 113, and 127) contained *KIP1* in the deleted regions. Taken together, a tumor suppressor gene probably exists between the smallest commonly deleted region flanked by *D12S269* and *D12S308*.

LOH frequency of 12p was compared among adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. LOH of 12p was found in 3 of 4 (75%) large cell carcinomas, 7 of 16 (44%) adenocarcinomas, and 1 of 12 (8%) squamous cell carcinomas. LOH of 12p was significantly more frequent in large cell carcinoma than in squamous cell carcinoma ( $P = 0.027$ ).

## Discussion

In this study, we analyzed the LOH of chromosome 12 in 36 primary NSCLC samples with matched normal DNA using 22 highly informative polymorphic markers. All of our samples were obtained at the time of initial surgery for the primary lung cancer; therefore, these results reflect the genetic changes that are important in development, rather than in metastatic spread, of NSCLC.

Twelve samples showed LOH at least at one locus on chromosome 12. This frequency is higher than that reported by previous allelotyping analysis, which is 10–20% (7, 8).

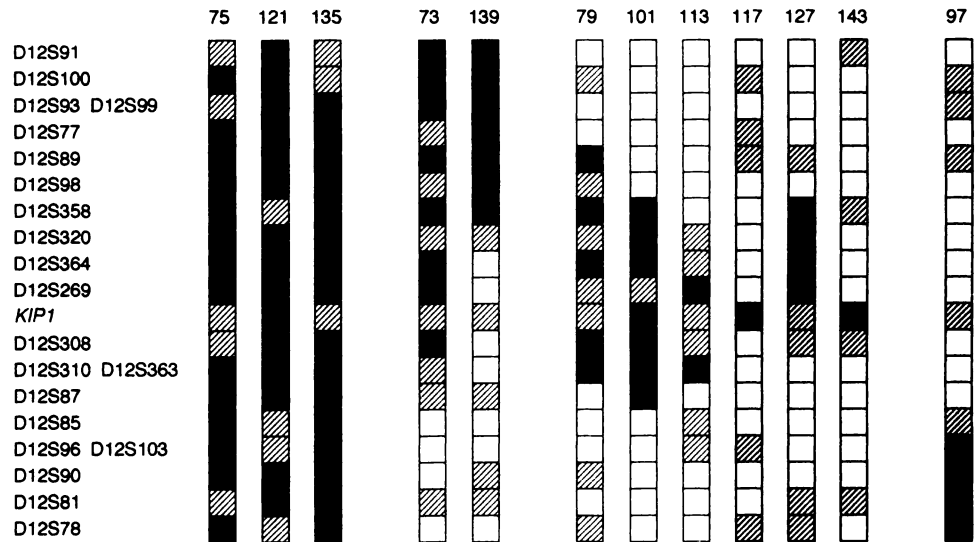
We found that LOH of 12p was significantly more frequent in large cell carcinomas than in squamous cell carcinomas. LOH on 17q was found more frequently in adenocarcinomas than squamous cell carcinomas (19). However, for most of the other chromosome arms, LOH was more frequently reported in squamous cell carcinoma than in adenocarcinoma (8). These observations indicate molecular genetic heterozygosity within the major NSCLC subtypes.

We identified the smallest commonly deleted region on chromosome 12p13. This region is flanked by *D12S269* and *D12S308*. This smallest commonly deleted region on 12p contains the cyclin-dependent kinase inhibitor gene *KIP1*. This gene was considered to be a good



Fig. 2. Representative autoradiograph showing LOH at the *KIP1* locus. Sample no. 83 retained heterozygosity at *D12S269*, *KIP1* locus, and *D12S308*. Sample no. 117 shows LOH only at the *KIP1* locus, with apparent retention of heterozygosity at *D12S269* and *D12S308*. Sample no. 121 shows LOH at *D12S269*, *KIP1* locus, and *D12S308*. T, tumor DNA; N, normal DNA.

Fig. 3. Summary of LOH analysis of chromosome 12 in NSCLC. Twelve samples that showed LOH at one or more loci are presented. For the markers that are assigned to the same location by linkage analysis, the result obtained from each marker was combined. The status of each chromosome locus is indicated by shading as LOH (black), retention of heterozygosity (white), and not informative (cross-hatched). Patient nos. are listed at the top of each column.



candidate for a tumor suppressor gene because it acts as a negative regulator of cellular proliferation (13, 14). We previously examined the mutational status of the *KIP1* gene by PCR-SSCP and Southern Blot analysis using most of the samples used in this study (18). No detectable point mutations or homozygous deletions were found. These findings suggest that the *KIP1* gene is unlikely to be the target tumor suppressor gene of NSCLC. Taken together, another tumor suppressor gene that is important in tumorigenesis of NSCLC is probably close to the *KIP1* gene.

We found recently that the same region as identified in the present study was frequently deleted in acute lymphoblastic leukemia of childhood (20). The LOH of the same region both in NSCLC and in acute lymphoblastic leukemia of childhood suggest that either a critical tumor suppressor gene exists in this region with a broad range of activity or more than one tumor suppressor gene is present in this region.

#### Acknowledgments

We are grateful to Alfred Reiter (Department of Pediatrics IV, Hannover Medical School, Hannover, Germany) for performing statistical evaluation of data. We also thank Gail Jao for excellent secretarial help.

#### Note Added in Proof

Recently, a new chromosome 12 map was published [(K. Krauler *et al.*, Nature (Lond.), 377 (Suppl 3): 321, 1995)], in which *KIP1/p27* was mapped between *D12S98* and *D12S358*. Nevertheless, *KIP1/p27* continues to be in the frequently deleted region in most cases with LOH of chromosome 12.

#### References

- Chiba, I., Takahashi, T., Nau, M., D'Amico, D., Curiel, D., Mitsudomi, T., Buchhagen, D., Carbone, D., Piantadosi, S., Koga, H., Reissman, P., Slamon, D., Holmes, E. C., and Minna, J. D. Mutations in the *p53* gene are frequent in primary, resected non-small cell lung cancer. *Oncogene*, 5: 1603-1611, 1990.
- Harbour, J. W., Lai, S.-L., Whang-Peng, J., Gazdar, A. F., Minna, J. D., and Kaye, F. J. Abnormalities in structures and expression of the human retinoblastoma gene in SCLC. *Science* (Washington DC), 241: 353-357, 1988.
- de Vos, S., Miller, C. W., Takeuchi, S., Gombart, A. F., Cho, S. K., and Koeffler, H. P. Alterations of CDKN2 (p16) in non-small cell lung cancer. *Genes Chromosomes & Cancer*, 14: 164-170, 1995.
- Okamoto, A., Hussain, S. P., Hagiwara, K., Spillare, E. A., Rusin, M. R., Demetrick, D. J., Serrano, M., Hannon, G. J., Shiseki, M., Zariwala, M., Xiong, Y., Beach, D. H., Yokota, J., and Harris, C. C. Mutations in the *p16<sup>INK4</sup>/MTS1/CDKN2*, *p15<sup>INK4B</sup>/MTS2*, and *p18* genes in primary and metastatic lung cancer. *Cancer Res.*, 55: 1448-1451, 1995.

- Whang-Peng, J., Knutsen, T., Gazdar, A., Steinberg, S. M., Oie, H., Linnoila, I., Mulshine, J., Nau, M., and Minna, J. D. Nonrandom structural and numerical chromosome changes in non-small-cell lung cancer. *Genes Chromosomes & Cancer*, 3: 168-188, 1991.
- Johansson, M., Jin, Y., Mandahl, N., Hambræus, G., Johansson, L., Mitelman, F., and Heim, S. Cytogenetic analysis of short-term cultured squamous cell carcinoma of the lung. *Cancer Genet. Cytogenet.*, 81: 46-55, 1995.
- Tsuchiya, E., Nakamura, Y., Weng, S.-Y., Nakagawa, K., Tsuchiya, S., Sugano, H., and Kitagawa, T. Allelotype of non-small cell lung carcinoma: comparison between loss of heterozygosity in squamous cell carcinoma and adenocarcinoma. *Cancer Res.*, 52: 2478-2481, 1992.
- Sato, S., Nakamura, Y., and Tsuchiya, E. Difference of allelotype between squamous cell carcinoma and adenocarcinoma of the lung. *Cancer Res.*, 54: 5652-5655, 1994.
- Weissenbach, J., Gyapay, G., Dib, C., Vignal, A., Morissette, J., Millasseau, P., Vaysseix, G., and Lathrop, M. A second-generation linkage map of the human genome. *Nature* (Lond.), 359: 794-801, 1992.
- Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. The 1993-94 Génethon human genetic linkage map. *Nat. Genet.*, 7: 246-339, 1994.
- Pearson, P. L., Matheson, N. W., Flescher, D. C., and Robbins, R. J. The GDB human genome data base anno 1992. *Nucleic Acids Res.*, 20 (Suppl.): 2201-2204, 1992.
- Stegmaier, K., Pendse, S., Barker, G. F., Bray-Ward, P., Ward, D. C., Montgomery, K. T., Krauter, K. S., Reynolds, C., Sklar, J., Donnelly, M., Bohlander, S. K., Rowley, J. D., Sallan, S. E., Gilliland, D. G., and Golub, T. R. Frequent loss of heterozygosity at the *TEL* gene locus in acute lymphoblastic leukemia of childhood. *Blood*, 86: 38-44, 1995.
- Polyak, K., Lee, M.-H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massagué, J. Cloning of *p27<sup>KIP1</sup>*, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*, 78: 59-66, 1994.
- Toyoshima, H., and Hunter, T. p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. *Cell*, 78: 67-74, 1994.
- Bullrich, F., Maclachlan, T. K., Sang, N., Druck, T., Veronese, M. L., Allen, S. L., Chiorazzi, N., Koff, A., Heubner, K., Croce, C. M., and Giordano, A. Chromosomal mapping of member of the *cdc2* family of protein kinases, *cdk3*, *cdk6*, *P1SSLRE*, and *P1TALRE*, and a cdk inhibitor, *p27<sup>KIP1</sup>*, to regions involved in human cancer. *Cancer Res.*, 55: 1199-1205, 1995.
- Pietenpol, J. A., Bohlander, S. K., Sato, Y., Papadopoulos, N., Lin, B., Friedman, C., Trask, B. J., Roberts, J. M., Kinzler, K. W., Rowley, J. D., and Vogelstein, B. Assignment of the human *p27<sup>KIP1</sup>* gene to 12p13 and its analysis in leukemia. *Cancer Res.*, 55: 1206-1210, 1995.
- Ponce-Castañeda, M. V., Lee, M.-H., Latres, E., Polyak, K., Lacombe, L., Montgomery, K., Mathew, S., Krauter, K., Sheinfeld, J., Massagué, J., and Cordon-Cardo, C. *p27<sup>KIP1</sup>*: chromosomal mapping to 12p12-12p13.1 and absence of mutations in human tumors. *Cancer Res.*, 55: 1211-1214, 1995.
- Kawamata, N., Morosetti, R., Miller, C. W., Park, D., Spirin, K. S., Nakamaki, T., Takeuchi, S., Hatta, Y., Simpson, J., Wilczynski, S., Lee, Y. Y., Bartram, C. R., and Koeffler, H. P. Molecular analysis of the cyclin-dependent kinase inhibitor *p27<sup>KIP1</sup>* gene in human malignancies. *Cancer Res.*, 55: 2266-2269, 1995.
- Fong, K. M., Kida, Y., Zimmerman, P. V., Ikenaga, M., and Smith, P. J. Loss of heterozygosity frequently affects chromosome 17q in non-small cell lung cancer. *Cancer Res.*, 55: 4268-4272, 1995.
- Takeuchi, S., Bartram, C. R., Miller, C. W., Reiter, A., Seriu, T., Zimmermann, M., Schrappe, M., Mori, N., Slater, J., Miyoshi, I., and Koeffler, H. P. Acute lymphoblastic leukemia of childhood: identification of two distinct regions of deletion on the short arm of chromosome 12 in the region of *TEL* and *KIP1*. *Blood*, in press, 1996.