Jpn. J. Cancer Res. 83, 1299–1303, December 1992

Absence of Activating Mutations in the Transmembrane Domain of the c-*erb*B-2 Protooncogene in Human Lung Cancer

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The rat *neu* gene is known to be activated by a point mutation in its predicted transmembrane domain. Overexpression of the human homologue of *neu*, the c-*erb*B-2 gene, in human lung cancer has been reported, and a similar activating point mutation has been suggested. Therefore, we tested for possible aberrations of the c-*erb*B-2 gene in the region of the transmembrane domain in surgical specimens of human primary lung cancer from 190 patients, and also examined 24 metastases and 26 specimens of noncancerous portions of the lung of the same patients. Single-strand conformation polymorphism analysis of polymerase chain reaction products revealed no point mutations in the target domain in any of these specimens.

Key words: c-*erb*B-2 protooncogene — Human lung cancer — Single-strand conformation polymorphism analysis

Recent evidence indicates that acumulation of genetic changes is required for the genesis of human cancers. The human c-*erb*B-2 protooncogene is one of the target genes for a genetic change. The gene encodes a receptor-type tyrosine kinase, $p185^{erbB-2}$, related to, but distinct from the epidermal growth factor receptor.[1-3] Amplification of the gene and overexpression of the gene product have been found to be related to the clinical prognosis of primary breast cancer.[4-9] In human lung cancer also, overexpression of the c-*erb*B-2 gene and its correlation with a poor clinical outcome have been reported.[10-12] However, we found previously that amplification of the c-*erb*B-2 gene was rare in lung cancer (1 of 51 tumors).[13]

The rat homologue of the human c-*erb*B-2 protooncogene, named *neu*, is frequently activated in chemically induced neuro- and glioblastomas by a single base substitution, an T to A transversion resulting in replacement of a valine residue by a glutamic acid residue at position 664 in the predicted transmembrane domain.[14, 15] This activated *neu* oncoprotein shows increased tyrosine kinase activity and increases the phosphotyrosine content in transformed cells.[16, 17] The activating point mutation facilitates homodimerization of $p185^{neu}$, which leads to receptor activation and increased enzymatic function.[18] The activation of the tyrosine kinase is linked to the transforming ability of the $p185^{neu}$ oncoprotein.[19]

From these results, we anticipated that in tumor cells without c-*erb*B-2 amplification an activating mutation might facilitate c-*erb*B-2-mediated signaling and contribute to their malignancy. Such an activating point mutation is likely to occur in the nucleotide sequence of the transmembrane domain as in the case of the *neu* oncogene. In this study we examined DNAs from human primary lung cancers for possible activation by a point mutation in the transmembrane domain of the c-*erb*B-2 gene. For this purpose we used single-strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR) products.[20, 21] Our results indicate that point mutations in the transmembrane domain of the c-*erb*B-2 protooncogene are rarely, if ever, involved in human lung cancers.

MATERIALS AND METHODS

Surgical specimens and DNA preparation Specimens of lung cancers and normal portions of lung tissue were obtained from the National Cancer Center Hospital, Tokyo. Histological identification of these cancers was performed in the Pathology Division of the National Cancer Center Research Institute, Tokyo. High-molecular-weight DNA was prepared as described previously[13] according to the method of Blin and Stafford.[22]

PCR-SSCP analysis Oligonucleotide primers were synthesized by the phosphoroamidite method with a 394 DNA/RNA synthesizer and purified using Oligonucleotide Purification Cartridges (Applied Biosystems). The names, nucleotide sequences and nucleotide positions in the c-*erb*B-2 cDNA sequence[2] of the primers synthesized were PL1, AGCCAGCCCTCTGACGTCCA, 2115–2134; PR1, GATCTTCTGCTGCCGTCGCT, 2201–2220; and PR2, TGTACTTCCGGATCTTCTGC, 2211–2230. The lengths of the DNA fragments amplified with PL1 and PR1 (F1) and with PL1 and PR2 (F2) were 106 bp and 116 bp, respectively. The 5′-ends of the primers were labeled by the polynucleotide kinase reaction with [γ-^{32}P]ATP as described previously.[23]

DNA samples (0.1 μg) were subjected to the PCR under the conditions described previously.[24] Thirty cycles of the reaction at 94, 60 and 72°C for 0.5, 0.5 and 1 min, respectively, were performed in a DNA Thermal Cycler (Perkin-Elmer Cetus). The product was diluted 100 times with 98% formamide, 20 m*M* EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol, and heated at 80°C for 2 min, and 1 μl samples of the diluted reaction mixture were promptly applied to two 6% polyacrylamide gels (acrylamide: N,N-bisacrylamide, 49:1), one containing 5% glycerol, and the other without glycerol. Electrophoresis in the gels with and without glycerol was performed at 35 W for 130 min and 80 min, respectively, keeping the temperature of the gels at 25°C with a water cooling system. The gels were then dried on filter paper and exposed to X-ray film at −80°C for 2 to 5 h with an intensifying screen.

Direct DNA sequencing DNA fragments showing a mobility shift on PCR-SSCP analysis were eluted from polyacrylamide gel as described previously,[25] and were amplified by 35 cycles of the PCR under the same conditions as described above. The reaction mixture was diluted and deionized in a Centricon 30 microconcentrator (Amicon). Nucleotide sequences were determined using a dsDNA Cycle Sequencing System (Gibco BRL). In addition to the primers described above, the following primers approximately 10 bp internal to the fragment ends were used for sequencing: PL3, TCTGACGTCCATCATCTCTG; PL4, TCTGACGTCCATCGTCTCTG; and PR3, TGCCGTCGCTTGATGAGGAT. Sequencing products were analyzed in 8% polyacrylamide gel containing 7 *M* urea.

RESULTS

PCR-SSCP analysis of the c-*erb*B-2 gene in human lung cancers We analyzed the sequence encoding the transmembrane domain of the c-*erb*B-2 gene in DNAs from 190 surgical specimens of primary human lung cancers and 24 metastases together with those of 26 normal portions of the lung of the same patients by PCR-SSCP analysis. As the complete nucleotide sequence of the human c-*erb*B-2 gene has not yet been determined, we deduced the possible sequence of the exon encoding the transmembrane domain from the known nucleotide sequence of the cDNA[2,3] and tried to amplify the corresponding region using two sets of primers. As indicated in Fig. 1, both sets of primers provided amplified DNA fragments spanning the whole region of the putative transmembrane domain, and these fragments were of the nucleotide lengths expected from the nucleotide sequence of the cDNA,[2] indicating that the regions amplified were in one exon.

Representative results of SSCP analysis of PCR products are shown in Fig. 2. Lanes 1 and 2 contain DNA from normal human liver tissue as controls. In all, 6 bands, named B1 to B6, were detected. Normal DNA (lane 1) as well as the DNAs from some lung cancers (lanes 8 and 10) showed the same additional bands, suggesting the presence of a polymorphic nucleotide substitution. The slower (B1 and B2) and faster (B3 to B6) moving bands correspond to the two separated complementary strands. The DNAs in the faster moving multiple bands B3 and B4 were confirmed to have exactly the same nucleotide sequence, suggesting that these bands are due to the presence of two different conformations. The DNAs in bands B5 and B6 also had the same nucleotide sequence. LuC154C (lane 8) gave only bands B2, B5 and B6. This result indicated that the tumor was homozygous for the polymorphism. This result also indicated that single-stranded DNA in band B2 and that in bands B5 and B6 are complementary, while the single-stranded DNA in band B1 is complementary to that in bands B3 and B4.

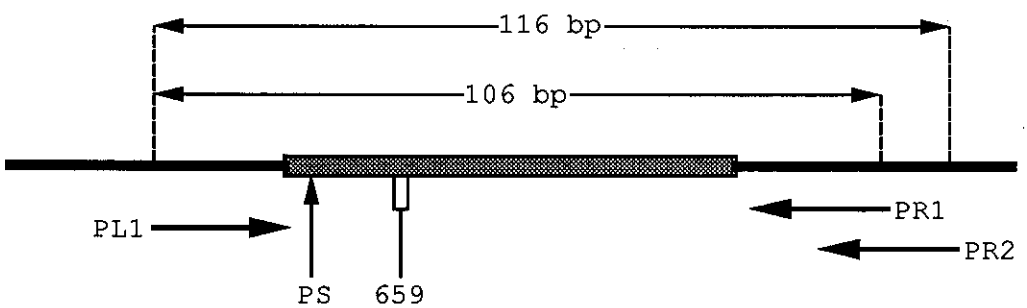

Fig. 1. PCR-SSCP analysis of the c-*erb*B-2 gene. The region encoding the transmembrane domain is indicated by a shaded box. PL1, PR1 and PR2 indicate synthetic oligonucleotide primers (20 mers) for the PCR. PS indicates a polymorphic site at codon 655. The codon of the human c-*erb*B-2 gene equivalent to that activated by a point mutation in the rat *neu* gene is the indicated codon 659.

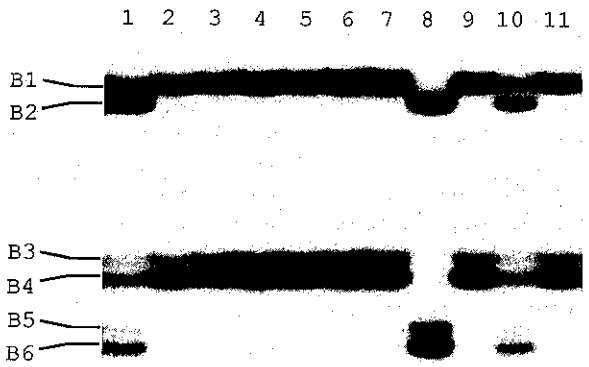

Fig. 2. PCR-SSCP analysis of the transmembrane domain of the c-*erb*B-2 gene. Genomic DNAs from lung cancer and noncancerous tissue were analyzed. Primers PL1 and PR2 were used for the PCR. Electrophoresis was performed in 6% polyacrylamide gel at 35 W at 25°C. Sources of amplified DNA fragments: lanes 1 and 2, normal liver DNA LiC18N and LiC21N, respectively; lane 3 to 11, cancerous lung tissues of LuC148C, LuC149C, LuC151C, LuC152C, LuC153C, LuC154C, LuC156C, LuC157C and LuC158C.

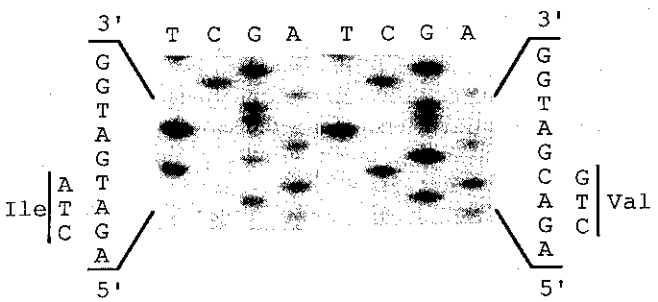

Fig. 3. Nucleotide sequence of the DNA fragments showing different mobilities on SSCP analysis. Single-stranded DNAs in bands B1 (left panel) and B2 (right panel) obtained from LuC140C were eluted from the gel, amplified and subjected to nucleotide sequencing analysis.

Analyses of the DNAs from 24 metastases and 26 normal portions of the lung gave the same results as those for DNAs from the primary lung cancers of the respective patients.

Direct DNA sequencing For determination of the changes of the nucleotide sequence causing the observed mobility shifts of single-stranded DNA fragments, the single-stranded DNA fragments in bands B1 to B6 of the two controls and of some lung cancers were eluted from the gel and amplified by the PCR. The resulting DNA fragments were then subjected to the cycle sequencing procedure. As shown in Fig. 3, two different patterns of sequencing ladders were obtained. Each of them represents one of the two sequences carrying a known polymorphic nucleotide substitution in amino acid codon 655 reported by Papewalis *et al.*[26] The DNA fragment giving bands B1, B3 and B4 contains the ATC codon, while that giving B2, B5 and B6 carries the GTC codon. All mobility shifts detected in this analysis were due to this polymorphic nucleotide substitution. We could not detect any mutation in the transmembrane domain of the c-*erb*B-2 protooncogene in 190 primary human lung cancers or 24 metastases.

DISCUSSION

Overexpression of p $185^{erbB\text{-}2}$ has been observed in human lung cancer as well as in human breast cancer.[5-12] Amplification of the c-*erb*B-2 gene has been demonstrated in breast cancer, but seems to be infrequent in lung cancer.[8, 13] Instead of amplification, a mutation at critical positions in the coding region might activate the c-*erb*B-2 gene, because such a mutation has been found in the transmembrane domain of the analogous rat *neu* oncogene, where a single point mutation specifies a substitution of glutamic acid (GAG) for valine (GTG) at codon 664.[14, 15] The nucleotide sequence at the equivalent codon 659 of the human c-*erb*B-2 gene is GTT (valine) and would require a double mutation to be converted to glutamic acid (GAA or GAG). If aberrations of the c-*erb*B-2 gene are involved in the genesis of lung cancers, the region of the gene encoding the transmembrane domain seems a likely position for such mutations.

We analyzed the transmembrane domain in DNAs from 190 surgical specimens of primary human lung cancers and 24 metastases by the PCR-SSCP method. This method has been used successfully for detection of various DNA aberrations, including point mutations,[21, 27-29] and is especially useful for analyzing surgical tumor specimens containing a significant proportion of normal cells,[25] in which it is sometimes very difficult to detect point mutations by conventional methods.

We detected mobility shifts in DNA fragments, amplified from the transmembrane domain of the c-*erb*B-2 gene, in 42 tumors of 190 patients. However, analyses of normal DNAs by the PCR-SSCP method and determination of nucleotide sequences of both normal and tumor DNAs revealed that these mobility shifts were due to a known polymorphic nucleotide substitution in codon 655.[2, 3, 26] We did not observe any mobility shift due to mutational change of the nucleotide sequence in the region analyzed. Although PCR-SSCP analysis is efficient, it may not detect all possible nucleotide substitutions and, therefore, we cannot eliminate false-negative cases.[30] However, as we did not detect any mutations even by determination of nucleotide sequences is some

tumor DNAs showing no mobility shift on SSCP analysis, our results strongly suggest that point mutations in the transmembrane domain of the c-*erb*B-2 gene are rarely, if ever, involved in the genesis of human lung cancers.

From analysis of 58 individuals, Papewalis *et al.* reported that the ratio of alleles carrying ATC and GTC at codon 655 was 0.68:0.32.[26] Our analysis of 380 alleles of 190 patients revealed that the ratio of the frequencies of the ATC allele and the GTC allele was 0.88:0.12. This difference in relative frequencies might reflect a difference in genetic diversity between Japanese and Caucasians.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan, a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, a grant from the Special Coordination Fund of the Science and Technology Agency of Japan, a grant from the Princess Takamatsu Cancer Research Fund, and a grant from the Naito Foundation. Richard Sachse is a Foreign Research Fellow of the Foundation for Promotion of Cancer Research, Tokyo.

(Received June 29, 1992/Accepted September 10, 1992)

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