Jpn. J. Cancer Res. **91**, 504–509, May 2000

Infrequent Mutation of the *hBUB1* and *hBUBR1* Genes in Human Lung Cancer

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Mitotic checkpoint defects of the cell cycle have been implicated in the development of human cancers. Since *hBUB1* and *hBUBR1*, whose products function in the spindle checkpoint pathway, have been shown to be mutated in a subset of colon cancers with chromosomal instability, we investigated the contribution of these genes to lung cancer development. One hundred and two lung cancer (50 small cell lung cancers and 52 non-small cell lung cancers) and 4 mesothelioma cell line DNAs were analyzed by Southern blot analysis, but no rearrangements or deletions of *hBUB1* and *hBUBR1* were detected. Using single strand conformation polymorphism analysis, we studied all the 25 exons except exon 1 of the *hBUB1* gene in 88 lung cancer DNAs. One lung cancer cell line, NCI-H345, showed a single nucleotide substitution, which resulted in an Arg-to-Gln change at codon 209 (CGA to CAA). Eleven cell line DNAs exhibited a single nucleotide polymorphism in intron 9 of *hBUB1*, all of which were heterozygous. Similar mutation analysis of *hBUBR1* in 47 lung cancer cell line cDNAs revealed a frequent polymorphism at codon 349 (CAA to CGA) leading to a substitution of Gln to Arg but no mutations. Northern blot analyses showed that both *hBUB1* and *hBUBR1* genes were expressed in all of 31 lung cancer cell lines tested with no significant difference in the expression level. Our results suggest that alterations in *hBUB1* and *hBUBR1* rarely contributed to the genetic change of lung cancers.

Key words: Mitotic checkpoint — hBUB1 — hBUBR1 — Lung cancer

Lung cancer cells harbor many structural abnormalities suggestive of allele loss, including non-reciprocal translocations and numerical abnormalities (aneuploidy),[1,2] which have been considered to contribute to the tumor progression through alterations in specific genes located on those chromosomes. Aneuploidy can result from improper allocation of the two chromatids of a chromosome to the two daughter cells during mitosis. Proper assembly of mitotic spindles, especially the stable attachment of chromosomes at the kinetochore, is monitored by the spindle checkpoint machinery, which consists of several proteins including hBUB1, hBUBR1 and hsMAD2.[3–6] Spindle checkpoint disruption appears to be one of the mechanisms leading to aneuploidy in human cancers. First, reduced *hsMAD2* gene expression was implicated in a tumor cell line with chromosomal instability (CIN).[7] Second, mutant alleles of *hBUB1* and *hBUBR1* were detected in four of the 19 colorectal tumor cell lines with CIN.[8,9] Furthermore, two colorectal cancer cell lines with microsatellite instability (MSI) which were transfected with a mutant allele of *hBUB1* showed a disruption of the mitotic checkpoint in a dominant negative fashion.[9] Third, the HTLV-1 retrovirus Tax protein has been shown to inactivate the spindle checkpoint in infected T cells.[10] Thus, we hypothesized that abnormalities in these mitotic checkpoint genes might also occur in lung cancers. We studied genetic changes of the *hBUB1* and *hBUBR1* genes as well as their expression in lung cancer and malignant mesothelioma cell lines. Despite the frequent occurrence of aneuploidy, we found that mutations or downregulation of expression of these genes rarely occur in these thoracic malignancies.

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MATERIALS AND METHODS

Cell lines The tumor cell lines have been described and were deposited at the American Type Culture Collection.[11] The 106 cell lines used in this study included 50 small cell lung cancers (SCLC), 52 non-small cell lung cancers (NSCLC) and 4 mesotheliomas. DNA and RNA were prepared from cell lines by standard techniques.[12] Random-primed, first-strand cDNAs were synthesized from 2 μg of total RNAs using Superscript II according to the manufacturer's instructions (Life Tech. Inc., Rockville, MD).

PCR and single strand conformation polymorphism (SSCP) analyses For SSCP analysis, we developed 30 primer sets from the *hBUB1* genomic sequence[13] and 18 primer sets from the *hBUBR1* cDNA sequence (Genbank

accession #AF046079). Detailed information on PCR primers and conditions are available upon request to the authors. The PCR product was diluted with formamide-dye solution and electrophoresed on MDE gels (FMC Bioproducts, Rockland, ME) containing 10% or no glycerol for *hBUB1* and 10% glycerol for *hBUBR1*. Aberrant bands were reamplified with the same primer set and the purified PCR products were sequenced using an Applied Biosystems model 377 DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT) with a PCR primer and a BigDye terminator Cycle sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA).

Southern and northern blot analyses For Southern blot analysis, 5 μg of high-molecular-weight DNA from each cell line was digested with *Eco*RI or *Hin*dIII, electrophoresed in 0.7% agarose gel, and transferred to Hybond N+ (Amersham, Arlington Heights, IL). Hybridization and washing were performed using standard techniques.[12] For northern blot analysis, 10 μg of total RNA from each cell line was electrophoresed in formaldehyde-1.0% agarose gel and transferred to Hybond N+. Hybridization and washing were performed using standard techniques.[12] The hybridized membranes of northern blot were exposed to scientific imaging films (Kodak, New York, NY) or BAS-IP (Fuji Photo Film Co., Ltd., Tokyo), which were analyzed by a BAS 2000 Image analyzer (Fuji) to quantify the intensity of signals. The DNA probes used were a 3.5 kb insert of a cDNA construct (pBI-GFP) of the *hBUB1* gene[9] and a 1.1 kb RT-PCR product of the *hBUBR1* gene (Genbank accession #AF046079, from nt 437 to nt 1513) amplified from an Epstein-Barr (EB) virus-transformed B lymphoblastoid (BL) line cDNA. A fragment of the *β-actin* gene was synthesized by RT-PCR using a primer set of β-actin-s (5′-CAAGAGATGGCCACGGCTGCT-3′) and β-actin-as (5′-TCCTTCTGCATCCTGTCGGCA-3′).

RESULTS

Searching for mutations in the *hBUB1* and *hBUBR1* genes To test lung cancer and mesothelioma cell lines for gross structural changes, we performed Southern blot analysis. Genomic DNAs extracted from 102 lung cancer and 4 mesothelioma cell lines were digested with *Eco*RI or *Hin*dIII restriction enzymes, transferred to membranes, and hybridized with a full-length cDNA probe of the *hBUB1* gene or a 1.1 kb RT-PCR product of the *hBUBR1*

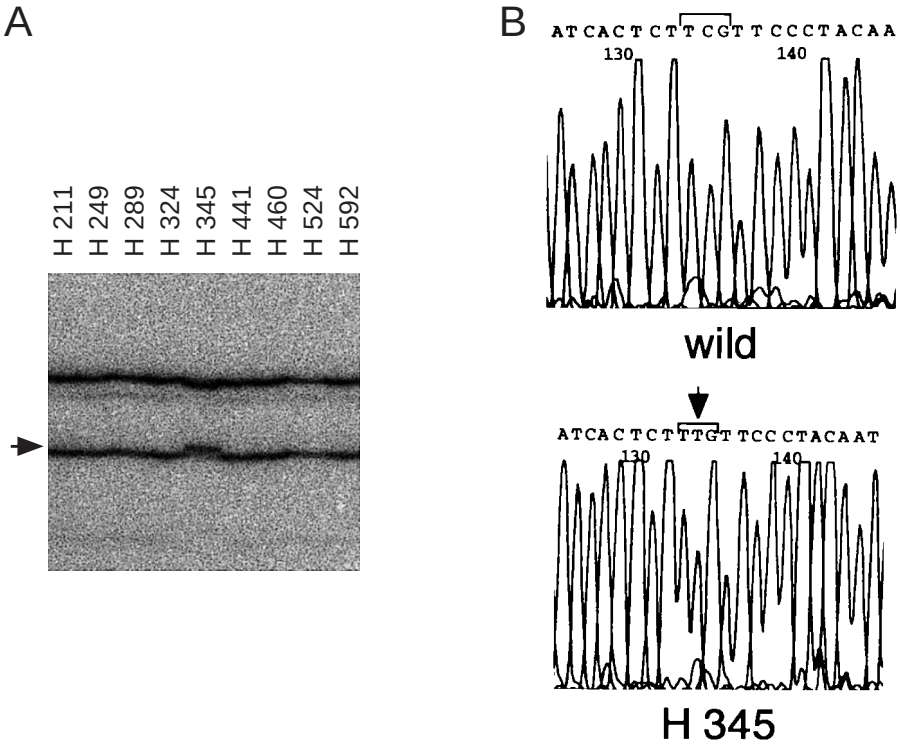

Fig. 1. Identification of a nucleotide change of the *hBUB1* gene in NCI-H345 cell line. A. SSCP analysis of the fragment amplified with primers of hBUB1s6880 (5′-AGTTTCCTTTAAGTGCTC-3′) and hBUB1as7081 (5′-TTGTATTTCTGGGCTCTC-3′). B. Sequence analysis of the aberrant SSCP band with the anti-sense primer. An arrow indicates the nucleotide change and brackets indicate the anti-sense strand sequence of codon 209.

gene. All the cell line DNAs showed multiple bands for these probes with no rearrangements or deletions (data not shown).

Next, we performed SSCP analysis to test whether lung cancer cell lines had point mutations or small deletions of the *hBUB1* and *hBUBR1* genes. We analyzed all 25 exons of the *BUB1* gene except exon 1, using 30 primer sets for 88 lung cancer cell line DNAs (46 SCLCs and 42 NSCLCs). In one SCLC cell line, NCI-H345, an aberrant band was detected homozygously using a primer set covering exon 8 of the *hBUB1* gene (Fig. 1A). Sequence analysis of this band revealed a single nucleotide substitution at codon 209 (CGA to CAA), which results in an amino acid change from Arg to Gln (Fig. 1B). We could not determine whether this change was a somatic mutation or a rare polymorphism due to nonavailability of constitutional DNA from this patient. Eleven of the 88 cell lines tested for *hBUB1* showed another aberrant SSCP band (data not shown). Sequence analysis revealed that this mobility change was due to one single nucleotide substitution in intron 9, which is a T-to-C change 8 bp upstream of exon 10. All the 11 cell lines (5 SCLCs and 6 NSCLCs) were heterozygous for this polymorphism. To determine if this intronic polymorphism causes alternative splicing, we performed RT-PCR analysis using a primer set located in exons 8 and 10. However, these cell lines did not express any messages of aberrant size (data not shown).

Since the genomic structure was not available, we performed SSCP analysis of the *hBUBR1* gene using cDNAs from 47 lung cancer cell lines (24 SCLCs and 23 NSCLCs). We detected a frequent single nucleotide change at codon 349 (CAA to CGA), which results in an amino acid change of Gln to Arg (data not shown). There were 24 cell lines expressing a Gln form, 14 cell lines expressing an Arg form, and 9 cell lines with both forms. NIH-H1304 cell line cDNA exhibited an aberrant SSCP band in the coding region (Fig. 2A). However, sequence analysis of this band showed a single nucleotide substitution at codon 952 (GAC to GAT) without an amino acid change (Fig. 2B).

Expression of *hBUB1* and *hBUBR1* To test whether these genes were downregulated without genetic changes,

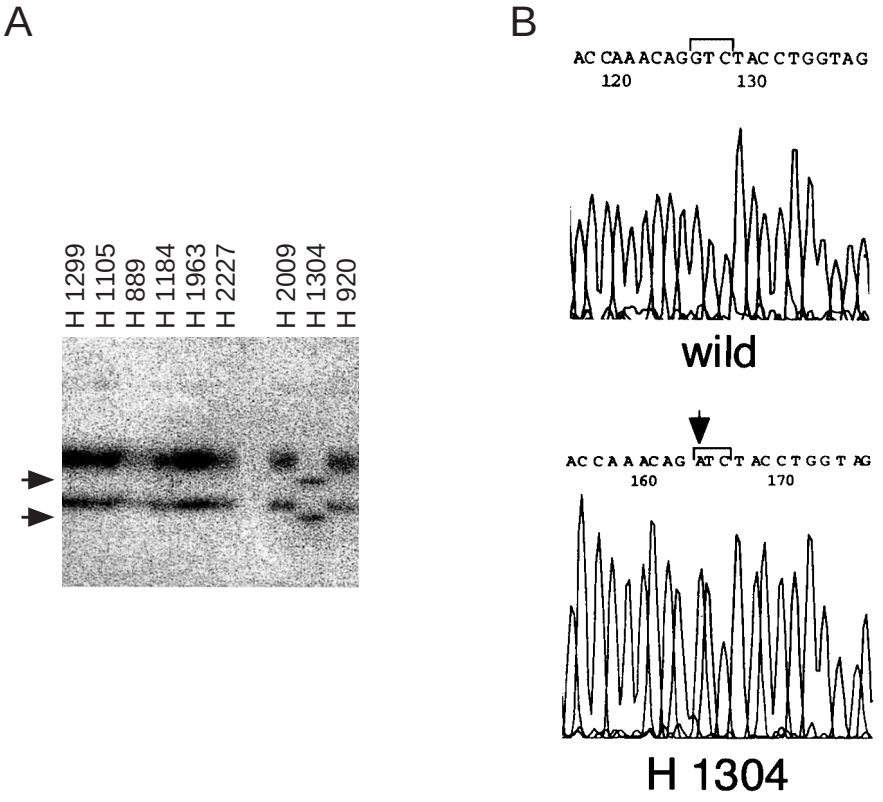

Fig. 2. Identification of a nucleotide change of the *hBUBR1* gene in NCI-H1304 cell line. A. SSCP analysis of the fragment amplified with primers of hBUBR17s2809 (5′-GATGTTTTTACCCTCAGC-3′) and hBUBR17as3027 (5′-AGAATCCGCACAAAGAAT-3′). B. Sequence analysis of the aberrant SSCP band with the anti-sense primer. An arrow indicates the nucleotide change and brackets indicate the anti-sense strand sequence of codon 952.

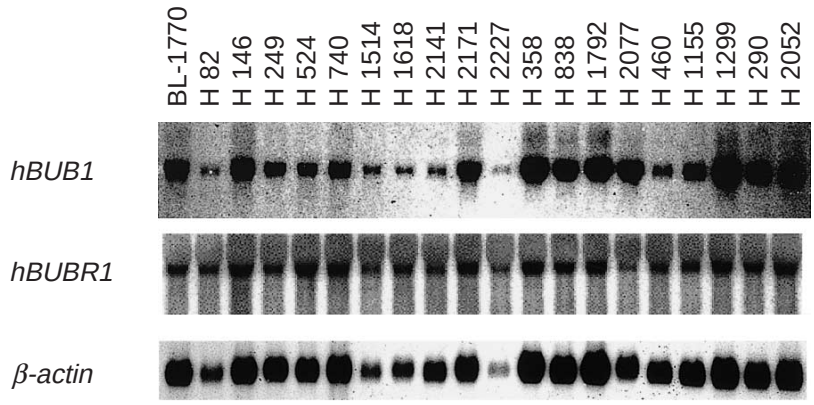

Fig. 3. Representative northern blot analysis of *hBUB1*, *hBUBR1*, and *β-actin*. The histologic types were an Epstein-Barr (EB) virus-transformed B cell line: BL-1770; SCLC: NCI-H82, H146, H249, H524, H740, H1514, H1618, H2141, H2171, and H2227; NSCLC: NCI-H358, H838, H1792, H2077, H460, H1155, and H1299; and mesothelioma: NCI-H290 and H2052.

we performed northern blot analysis. Thirty-one tumor cell lines (13 SCLCs, 14 NSCLCs, 2 unclassified lung cancers and 2 mesotheliomas) and one BL-line as a control were studied for the expression of *hBUB1* and *hBUBR1*, and the signal intensity of each sample was compared with that of β-actin as an internal control (Fig. 3). All of the expected mRNA-sized transcripts were expressed in the cell lines tested. The difference in signal intensity between the highest and lowest gene expression levels was approximately 4-fold for *hBUB1*, and 4-fold for *hBUBR1*.

DISCUSSION

Almost all lung cancers harbor many cytogenetic abnormalities, including aneupoidy,[14–16] and 20–30% of them show abnormal sizes of microsatellite repeats.[17] This suggests that lung cancer may be genetically unstable at two distinct levels; the former with CIN and the latter with MSI. However, in lung cancer, mismatch repair gene mutations have not been demonstrated and MSI is relatively infrequent.[17] Thus, the genetic changes in lung cancer may not be due to sub-optimal DNA repair but could result from near normal mutation rates which nonetheless become significant due to multiple rounds of cellular division that accompany the waves of clonal expansion through which advanced cancers evolve.[18] A recent report has demonstrated an impaired mitotic checkpoint in 44% of lung cancer cell lines by showing failure of mitotic arrest after nocodazole treatment, which normally inhibits spindle-kinetochore interaction through microtubule disruption.[19] This result would be consistent with the notion of CIN occurring in lung cancer, although other supporting evidence is needed, such as fluorescence *in situ* hybridization to show the higher frequency of losses or gains of chromosomes during mitosis. To elucidate the possible mechanisms underlying the CIN phenotype in lung cancer, we performed a systematic examination of the *hBUB1* and *hBUBR1* genes in thoracic malignancies; both of these have been implicated as target genes of spindle checkpoint disruption in other types of human cancers.

Our results revealed that the NCI-H345 cell line harbored a single nucleotide substitution in the *hBUB1* gene, which resulted in an amino acid change from Arg to Gln. However, we could not determine whether this substitution was a somatic mutation or simple polymorphism, because the DNA derived from the patient's normal tissue was not available. Since this cell line did not show a wild-type band on SSCP, it was strongly suggested that the allele loss of the wild-type *hBUB1* gene locus occurred in this cell line, which is thought to be a classical route of inactivation of a tumor suppressor gene. Although it remains possible that mutations of the *hBUB1* gene may contribute to the development of a subset of lung cancers, the amino acid residue corresponding to codon 209 (Arg) in the *hBUB1* gene is Gln in the mouse *BUB1* gene. Thus, it may be possible that the amino acid change of NCI-H345 is a silent mutation in terms of molecular function. On the other hand, a single nucleotide substitution at codon 349 (CAA to CGA) resulting in Gln-to-Arg substitution in the *hBUBR1* gene was identical to that reported in normal tissues from Japanese individuals.[20] Our results confirmed that these changes were also detected in BL-lines, indicating that this alteration represents a polymorphism.

All cell lines expressed mRNA messages of the *hBUB1* and *hBUBR1* genes with a maximum 4-fold difference in levels. It is not yet clear whether this relatively slight downregulation of these genes contributes to carcinogenesis. However, a previous report has shown that a breast

cancer cell line that is sensitive to taxol and nocodazole, had reduced *MAD2* expression and failed to arrest in mitosis after nocodazole treatment, suggesting that the reduced expression could be sufficient to cause disturbance of the spindle checkpoint.[7] Therefore, it will be important to test the functional significance of high and low levels of gene expression.

The underlying mechanisms of CIN phenotype have been sought in lung cancer as well as other types of tumors by others. It has been reported that one adenosquamous cell lung cancer line had a single nucleotide substitution of the *hBUB1* gene without an amino acid change in a study of 25 lung cancers (8 SCLCs and 17 NSCLCs).[21] RT-PCR-SSCP analysis of the *hsMAD2* gene has shown that only a silent polymorphism at codon 143 was detected in 46 lung cancer cDNAs (21 cell lines and 25 primary cases).[19] In addition, a recent study of 32 sporadic digestive tract cancers has shown a missense mutation of the *hBUB1* gene, but no change of the *hsMAD2* gene.[22] Thus these mitotic checkpoint genes seem to be rarely mutated in human malignancies, although other cell cycle machinery, such as cyclin E, GADD45 and c-myc, may be involved in chromosome instability.[23–25] In conclusion, our results are consistent with the previous reports, indicating that further investigations are needed to search for alterations in other components of the spindle checkpoint machinery.

ACKNOWLEDGMENTS

The authors thank Dr. C. Lengauer for sending the cDNA constructs of *hBUB1* and the genomic sequence information of the *hBUB1* gene before publication and Dr. K. M. Fong for helpful comments on the manuscript. This work was supported by SPORE grant CA70907.

(Received December 3, 1999/Revised February 17, 2000/ Accepted February 24, 2000)

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