

Genetic and Epigenetic Inactivation of Mitotic Checkpoint Genes *hBUB1* and *hBUBR1* and Their Relationship to Survival¹

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Abstract

Sequence alterations of mitotic checkpoint genes, *hBUB1* and *hBUBR1*, were examined, and their gene transcripts were quantified using on-line, real-time quantitative reverse transcription-PCR in surgically resected human colorectal cancers and their neighboring normal tissues. Our results reveal a new *hBUB1* missense mutation (Ala130Ser) but not any *hBUBR1* coding sequence mutations. *hBUB1* and *hBUBR1* mRNA levels were reduced to <10% of the neighboring normal tissues in 3 of 103 and 3 of 109 carcinomas, respectively, and to <50% in 7 and 7 carcinomas, whereas the overall expression levels were markedly higher in cancers than in normal tissues. Carcinomas with reduced *hBUB1* and/or *hBUBR1* mRNA levels, as well as the colon carcinoma harboring the *hBUB1* mutation, were associated with lymph node metastasis ($P < 0.005$) and shorter relapse-free survival after surgery ($P = 0.006$). Thus, *hBUB1* and *hBUBR1* may contribute to a specific driving force in tumor metastasis and progression as a result of nonmutational, as well as mutational, inactivation.

Introduction

The mitotic checkpoint monitors the proper assembly of the mitotic spindle and blocks the onset of anaphase unless all of the chromosomes are stably attached to a specialized region known as kinetochore (1). Failure of the spindle assembly checkpoint machinery may rapidly result in aneuploidy that is frequently observed in many types of human cancer cells. Aneuploidy has long been speculated to be causally involved in tumorigenesis. However, it remains undetermined whether acquired aneuploidy is a specific driving force in the progression of human cancers, rather than a distracting epiphenomenon and, if so, how aneuploidy facilitates cancer progression (2). Aneuploid colorectal cancer cell lines tend to undergo far more variation in chromosome number than near-diploid lines with microsatellite instability (3). In yeast, several gene alterations can give rise to CI,³ such as those involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure, and mitotic checkpoints (4, 5). The murine homologue of the yeast mitotic checkpoint gene, *BUB* (6), regulates exit from mitosis (7). Ectopic expression of mutant alleles of recently identified human homologues (*hBUB1*) in euploid cells disrupted the mitotic checkpoint control (8, 9), suggesting that aneuploidy is a result of defects in the mitotic checkpoint. However, low mutational frequencies of *hBUB1* in certain human cancers suggest the limited role for its mutational inactivation in the pathogenesis

of CI (8, 10–13). Sequence-based qualitative changes preserved in the genomic DNA of tumors have been amply confirmed as a mechanism for gene inactivation in cancer cells, but a nonmutational pathway resulting in reduced transcript levels may be more frequent (14, 15). DNA modifications, such as methylation of cytosine residues in CpG sequences, *e.g.*, are well documented to correlate with epigenetic gene silencing. To elucidate the frequency and physiological consequences of nonmutational silencing of mitotic checkpoint genes in human colorectal cancers, we have established *hBUB1/hBUBR1* LightCycler RT-PCR³ for accurate quantification of their transcripts and determined the expression levels in surgically resected cancer tissues and their neighboring normal colonic mucosa. We found significant association between their nonmutational inactivation and progression of cancers.

Materials and Methods

Patients and Samples. Surgical resections were performed by K. Y. at the Second Department of Surgery, Tokyo Medical and Dental University Hospital. Informed consent was obtained from each patient. Resected specimens were immediately divided, fixed for examination by pathologists, frozen in liquid nitrogen, and stored at -80°C for future RNA extraction. For quantification of *hBUB1* and *hBUBR1* mRNAs, pairs of colorectal carcinoma and adjacent normal tissues were selected using the following criteria: (a) histologically proven primary colorectal carcinomas and simultaneously resected normal colonic epithelial mucosa; (b) availability of complete clinical information, including treatment, outcome, previous and family history, and laboratory data; and (c) no radiotherapy or chemotherapy before surgery. Pairs (111) of specimens from consecutive patients (62 males and 49 females, mean age 62.7 ± 11.0 years, all were Japanese) met the above criteria and classified to Dukes' clinical staging (A to C).

Cell Lines. Human hepatoma cell line HepG2 and human endometrial cancer cell line HHUA were purchased from the American Type Culture Collection (Manassas, VA), human leukemia cell line HL60 (JCRB0085) and human colon cancer cell lines DLD-1 (JCRB9094) and LoVo (JCRB9083) were purchased from Human Research Resources Bank (Osaka, Japan), and human leukemia cell line K562 was purchased from Dainippon Pharmaceutical (Osaka, Japan). All cell lines were grown in monolayers in appropriate media: RPMI 1640 with 20% FCS³ for HL60, RPMI 1640 with 10% FCS for DLD-1 and K562, DMEM for HepG2, Ham's F-12 with 15% FCS for HHUA, and Ham's F-12 with 20% FCS for LoVo.

Sequence Analysis for *hBUB1*. Total RNA was extracted from 5 mg of tissue or 10^7 cultured cells by the acid guanidinium thiocyanate-phenol-chloroform extraction using Isogen (WAKO Pure Chemicals, Inc., Osaka, Japan) and DNase I (Takara, Shiga, Japan). cDNA was synthesized with random primer (Life Technologies, Inc., San Diego, CA), and the PCR amplifications were carried out at 35 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 20 s). The following primer sets were used for amplification of *hBUB1* (GenBank; AF046078): 220–1010 nt: forward 5'-AGATAAGAA-GAAATACCACAATGACCCAAG-3' and reverse 5'-TTACTCTGGGAC-CTTTCCTGGGAAGCG-3'; 959–1614 nt: forward 5'-GTGGAGACATCCATGAGGATCT-3' and reverse 5'-AGAAGAGATGATCTTATTGACTCCCAAG-3'. Part of the amplified products was electrophoresed in agarose gel and purified with high pure PCR product purification kit (Roche Molecular Biochemicals, Mannheim, Germany) and directly sequenced using

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³ The abbreviations used are: CI, chromosomal instability; BUB, budding uninhibited by benzimidazole; RT-PCR, reverse transcription PCR; FCS, fetal calf serum; SSCP, single-strand conformational polymorphism.

the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) with ABI 3100 Genetic Analyzer (Applied Biosystems). The sequence was finally compared with the wild-type *hBUB1* sequence. We confirmed all mutations by repeated experiments using DNAs extracted from the tumors and corresponding tissues.

PCR-SSCP Analysis for *hBUBR1*. Tumor cDNAs were subjected to PCR-SSCP analysis to search for mutations of *hBUBR1* mRNA. We used the following four sets of primers for amplification of *hBUBR1* (GenBank; AF046079): 13–972 nt: forward 5'-CAGGACGAGGACCTGAGC-3' and reverse 5'-TGCTTGCAGCTCATTCTCT-3', 912–1571 nt: forward 5'-TACAGTCCAGCCATGGATAG-3' and reverse 5'-AGTGAAGTTTCTCTGGCACA-3', 2063–2773 nt: forward 5'-AAGACAGTCGTGAAGCCA-3' and reverse 5'-CCACTATCTTCAAGCTTGATT-3', 2725–3305 nt: forward 5'-CAGGATCCCTATGATTGTAAC-3' and reverse 5'-GGTAGTG-CATCTAAATGTGTC-3'. PCR was carried out at 35 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 20 s). Each PCR product was digested with *TthHB* 8 I (13–972 nt: 228, 159, 237, and 336 bp), *EcoRI* (912–1571 nt: 356 and 304 bp), *HhaI* (2063–2773 nt: 332 and 379 bp), and *HinI* (2725–3305 nt: 315 and 266 bp), respectively, and digested products were electrophoresed, stained, and visualized with SYBR Green II (FMC BioProducts, Rockland, ME).

Quantification of mRNAs Using On-line, Real-time, and Quantitative RT-PCR Method. For quantification of *hBUB1* and *hBUBR1* mRNA, we used the LightCycler (Roche Molecular Biochemicals) PCR protocol, in which fluorescence emission attributable to binding of SYBR Green I dye to amplified products could be detected and measured essentially as described (16–18). Total RNA was extracted from colorectal cancer and normal tissues using RNAzol B (Tel-Test, Inc., Friendswood, TX) and cDNA synthesized with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to manufacturers' instructions. Each amplification reaction (DNA Master SYBR Green I; Roche Diagnostics) contained 50 nM template cDNA, 0.5 μ M primer DNA, and 4 mM MgCl₂. TaqStart antibody (Clontech Laboratories, Inc., Palo Alto, CA) was used to prevent generation of nonspecific amplification products. Experiments performed with duplicates were repeated at least twice for two primer sets for each sample (*hBUB1*: primer 1, forward 5'-AGAGCCCAGGAGACTTC ACA-3', reverse 5'-GTGCCTGGGTACTGTTT-3', primer 2, forward 5'-CCTTTGGAG-AACGCTCTGTCAA-3', reverse 5'-TGTGAAGTCTCTGGGCTCT-3'; *hBUBR1*: primer 1, forward 5'-CTCGTGGCAATACAGCTTCA-3', reverse 5'-CTGGTCAATAGCTCGGCTTC-3', primer 2, forward 5'-AGCCAGAA-CAGAGGACTCCA-3', reverse 5'-CAGGCTTCTGGTGCTTAGG-3'), and the mean values are shown. After the completion of each extension step (72°C), the fluorescence of each sample was measured at 82°C to exclude any possible nonspecific reactions. After the amplification was finished, the products were subjected to a temperature gradient from 65°C to 95°C at 0.2°C/s with continuous fluorescence monitoring to produce a melting profile of the products. The fluorescence data were quantitatively analyzed using 2-fold serially diluted normal control colonic mucosa samples included in each run to produce a standard curve. For verification of the melting curve results, the PCR reactions were examined by 1.5% agarose gel electrophoresis. All experiments with intraassay or interassay coefficient of variation >10% were retested.

Statistical Analyses. Associations between high, normal, and low expression groups and the following clinical parameters were compared using the χ^2 test. These include Dukes' stages (A–C), tumor size at surgery, histology (classified into well-differentiated, moderately differentiated, or poorly differentiated), presence or absence of familial history of colorectal and other cancers, and duplicate cancer. Relapse-free survival was determined as the interval between surgery and detection of the first relapse. Relapse-free survival attributable to *hBUB1/hBUBR1* expression levels were plotted with the use of Kaplan-Meier estimates and compared with the use of Log-rank test with one or two degrees of freedom. The median duration of follow-up was calculated according to the method of Korn. Differences between two populations were judged significant at confidence levels > 95% ($P < 0.05$). The statistical analyses were performed with StatView-J, ver 4.5 (Abacus Concepts, Berkeley, CA) or ver 5.0 (SAS Institute, Inc., Cary, NC).

Results

We first assessed the potential for *hBUB1* and *hBUBR1* mutations in 31 surgically resected human cancers (22 colorectal carcinomas, 7

hepatocellular carcinomas, and 2 clear cell renal carcinomas), as well as six human cancer cell lines (LoVo, HHUA, DLD-1, HepG2, HL60, and K562). Both genes were expressed in all of the above tissues and cell lines, and one major band of the expected size was observed for RT-PCR products. Coding regions of *hBUB1* were sequenced from PCR-amplified tumor cDNA, and a missense mutation (GenBank AF046078; nt 437 GCT to TCT transition) replacing Ala to Ser at codon 130 was identified in an ascending colorectal carcinoma of a 51-year-old female. The mutation was somatic and not found in the adjacent normal epithelial tissue (Fig. 1). Sequence alterations were not detected in the remaining carcinomas or six cancer cell lines. Furthermore, PCR-SSCP analysis did not detect any *hBUBR1* mutations in any cancer tissues or cell lines. These results reveal a new *hBUB1* mutation, confirm the low frequency mutation of *hBUB1*, and further demonstrate that *hBUBR1* mutation is also very rare in human cancers.

We next quantified transcript levels of *hBUB1* and *hBUBR1* using a LightCycler-based, on-line, real-time, and quantitative RT-PCR method in 111 human colorectal carcinomas and adjacent normal colonic mucosa (Fig. 2). The *hBUB1* and *hBUBR1* genes were expressed in all normal colonic mucosa but inactivated (mRNA levels < 10% of the neighboring normal tissues) in 3 (2.9%) of 103 and 3 (2.8%) of 109 carcinomas, respectively (9 and 2 failed in correct quantification); 2 carcinomas showed inactivation of both genes. *hBUB1* and *hBUBR1* mRNA were reduced to <50% of normal colonic mucosa in seven (6.8%) and seven (6.4%) carcinomas, respectively, whereas the overall expression levels were markedly higher in cancers than in normal tissues (*hBUB1* 8.68 \pm 1.11-fold; *hBUBR1* 6.85 \pm 1.20-fold, mean \pm SE). The variations of *hBUB1* and *hBUBR1* mRNA in normal colonic mucosa as expressed in SE of mean copy number were 9.0 and 19.1%, respectively.

Except for a well-differentiated ascending colon carcinoma classified as Dukes' stage A (patient 33, Fig. 2A), all carcinomas with *hBUB1* and/or *hBUBR1* mRNA levels <50% of normal tissues, as well as the ascending colon carcinoma harboring the Ala130Ser

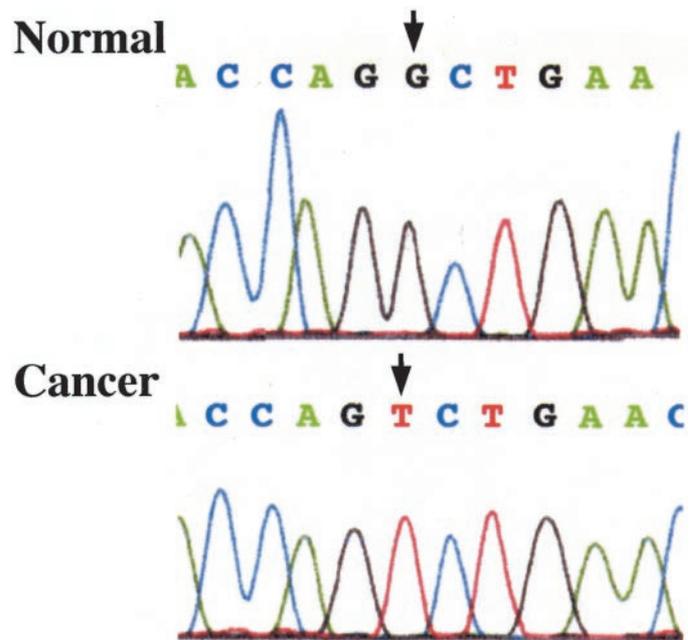


Fig. 1. Nucleotide sequence analysis of transcripts. RT-PCR of *hBUB1* exon from an ascending colon carcinoma (bottom panel) in 51-year-old female and simultaneously resected adjacent normal colonic epithelial cells (top panel) revealed a somatic missense mutation. Arrow, the position of GCT to TCT transition (nt. 437) replacing Ala to Ser at codon 130.

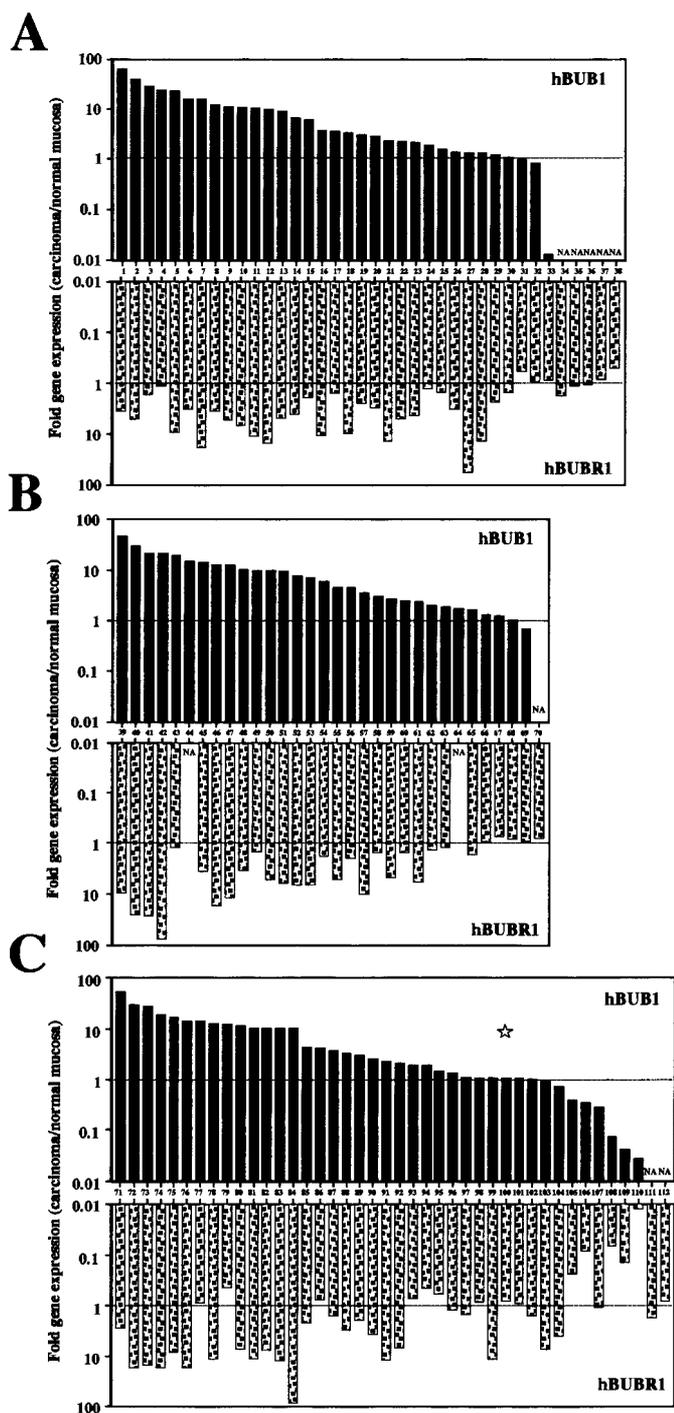


Fig. 2. Quantification of *hBUB1* (top panel) and *hBUBR1* (bottom panel) mRNA using LightCycler RT-PCR in human colorectal carcinoma. Serial dilution of control samples was used to generate a standard curve, and the relative mRNA values from simultaneously resected cancer and normal mucosal tissues from each patient were shown. Carcinomas were classified as Dukes' stage A (A), B (B), or C (C). NA, accurate quantification not available. ☆, the Ala130Ser missense mutation.

hBUB1 mutation, were classified as Dukes' stage C (Fig. 2C). The entire population distributed from early to late stages (Fig. 2, *hBUB1*, Dukes' stages: 33 at A, 31 at B, and 39 at C; *hBUBR1*, 38 at A, 30 at B, and 41 at C). These results reveal a statistically significant association of *hBUB1* or *hBUBR1* gene suppression with advanced stage cancer with lymph node metastasis (Dukes' stage C, *hBUB1*, $\chi^2 = 7.139$, $P < 0.05$; *hBUBR1*, $\chi^2 = 12.104$, $P < 0.005$). Neither *hBUB1* or *hBUBR1* suppression was significantly associated with any

of the other clinical manifestations: familial history of colorectal and other cancers, duplicate cancer, tumor histology, or tumor size.

After a median follow-up of 22 months, 15 of the 111 patients relapsed; the distributions of first relapsed events were 5 local or regional recurrences, 6 metastases, and 4 both. Classification of the patients into the following three categories created remarkable statistical differences on the basis of the regression analyses: (a) high expression groups: mRNA levels $>200\%$ of control tissues (*hBUB1*, 70 patients; *hBUBR1*, 59 patients); (b) normal expression group: mRNA levels between 50 and 200% (*hBUB1*, 25 patients; *hBUBR1*, 43 patients); and (c) low expression group: mRNA levels $<50\%$ (*hBUB1*, 8 patients, including Ala130Ser mutation; *hBUBR1*, 7 patients). The estimated median relapse-free survival from the date of surgery for both *hBUB1* and *hBUBR1* genes were 21, 31, and 13 months for high, normal, and low expression group, respectively (Fig. 3). There was statistically significant differences in relapse-free survival among the three groups (*hBUB1*, $\chi^2 = 6.496$, $P < 0.05$; *hBUBR1*, $\chi^2 = 22.399$, $P < 0.0001$). Patients in the groups with both high and low expression groups had shorter relapse-free survival compared with corresponding normal groups (*hBUB1*, high versus normal $P < 0.05$, low versus normal $P = 0.35$; *hBUBR1*, high versus normal $P < 0.0001$, low versus normal $P = 0.006$).

Discussion

Several lines of evidence now indicate that defects of mitotic checkpoint are important causes of CI (8). Our mutational analyses of *hBUB1* and *hBUBR1* genes in 31 surgically resected cancers and six human cancer cell lines revealed only one somatic missense *hBUB1* mutation but none of *hBUBR1* mutation. Ala at codon 130 is not conserved in the *BUB1* genes of other organisms, and it remains unknown whether mutations in this region may alter function. Irrespective of the potential of the previously unreported Ala130Ser substitution to suppress *hBUB1* function and induce subsequent CI, our data support recent reports suggesting low mutational frequency of *hBUB1* gene (10–13) and further demonstrate that mutation of *hBUBR1* gene is also very rare in sporadic human cancers.

Alternative, nonmutational mechanisms for gene inactivation during the development of cancer include an epigenetic process that is marked by DNA modifications, such as methylation of cytosine residues in CpG sequences (15). Aberrant promoter methylation and the associated loss of gene expression, e.g., may be more frequent than the mutational inactivation in some tumor suppressor genes (14, 15). However, it remains challenging to demonstrate in any given tumor the frequency and magnitude of reduced expression levels of specific genes that are mechanistically involved in the pathogenesis of cancer. Because the ability to monitor real-time amplification of DNA provides the means to accurately estimate the starting copy number of any DNA template, we have used LightCycler-based RT-PCR to assess the expression levels of *hBUB1* and *hBUBR1* genes in 111 human colorectal cancers by comparing their mRNA levels with those of simultaneously resected normal colonic mucosa tissues. Our quantification results demonstrated that somatic nonmutational silencing of *hBUB1* or *hBUBR1* genes occurs in a fraction of colorectal carcinomas mostly with advanced clinical stage. We have also quantified the expression levels of other genes, such as *GAPDH*, *c-myc*, *integrins*, four G-protein coupled receptors, *preproendothelin-1*, and *endothelin converting enzyme* in all carcinomas showing down-regulated *hBUB1* or *hBUBR1* levels, but *hBUB1* or *hBUBR1* down-regulation was not associated with silencing of other genes (data not shown). Taken together, the results suggest that nonmutational silencing of *hBUB1* and *hBUBR1* genes may be more predominant than their mutational inactivation, thereby contributing to the development of aneuploidy in

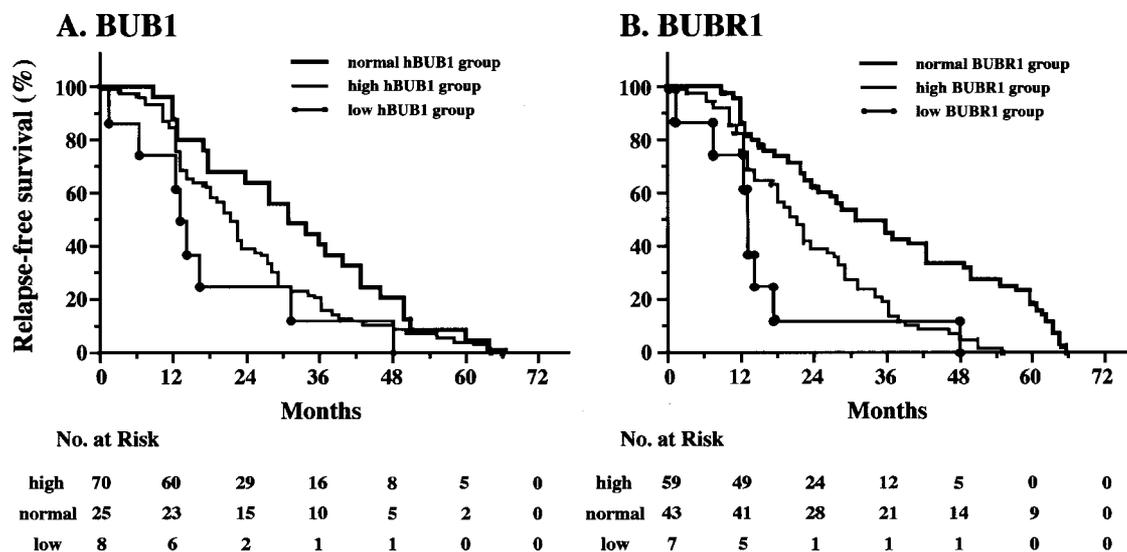


Fig. 3. Probability of relapse-free survival from the date of surgery among the patients in the three groups with differential gene expression levels of *hBUB1* and *hBUBR1*. Patient 100 (Fig. 2C) with Ala130Ser *hBUB1* mutation was classified to low expression group. The median treatment-free intervals for the groups with high, normal, and low expression groups were 21, 31, and 13 months, respectively, for both *hBUB1* and *hBUBR1* genes. The differences between the curves were significant (*hBUB1*, $P < 0.05$; *hBUBR1*, $P < 0.0001$).

human colorectal cancers. Therefore, it is too early to dismiss the roles of cell cycle checkpoint genes for the pathogenesis of CI simply because of the low mutational rates of *hBUB1* and *hBUBR1* in human cancers (10, 11, 19).

Our data have linked *hBUB1/hBUBR1* gene down-regulation/mutation to metastasis of colorectal cancers. The *hBUB1* and *hBUBR1* mRNA levels were reduced to <10% of the neighboring normal tissues in three (2.9%) and three (2.8%) carcinomas, respectively, and all carcinomas with inactivation of one or both genes showed advanced clinical stage with lymph node metastasis (Dukes' stage C). *hBUB1* and *hBUBR1* mRNAs between 10 and 50% of normal colonic mucosa were observed in four (6.8%) and four (6.4%) carcinomas, respectively, all of whom except patient 33 again showed Dukes' stage C. An ascending colon carcinoma harboring Ala130Ser mutation (patient 100, Fig. 2C) also showed Dukes' stage C. Down-regulation/mutation of *hBUB1* or *hBUBR1* was not significantly associated with other clinical manifestations, such as tumor histology or tumor size. Therefore, association of *hBUB1/hBUBR1* down-regulation/mutation with Dukes' stage C in the present study indicates their role in colorectal cancer metastasis, rather than tumor size enlargement. Kaplan-Meier analyses demonstrated significantly shorter relapse-free survivals of low expression groups than their corresponding normal expression groups. The statistical differences were stronger in *hBUBR1* comparisons than *hBUB1*. These findings further lend credence to the notion that *hBUB1/hBUBR1* down-regulation/mutation is involved in colorectal cancer metastasis.

Our data also demonstrated that both *hBUB1* and *hBUBR1* genes were markedly up-regulated in most colorectal cancers, but their mechanism and significance remain unknown. Patients of high *hBUB1* and *hBUBR1* expression groups had significantly shorter relapse-free survival than those of corresponding normal expression groups. However, unlike the case with low expression groups, which showed significant association with lymph node metastasis and shorter relapse-free survival than normal expression group, the increased expression levels of *hBUB1* and *hBUBR1* were not associated with advanced clinical stage. These results suggest that deregulated

hBUB1 and *hBUBR1* expression may simply be one of many epiphenomena associated with carcinomas showing faster progression.

Inactivations of other spindle/kinetochore checkpoint genes might also contribute to the pathogenesis of CI, because in yeast, BUB1 may work in concert with *MAD1-3*, *BUB3*, and *MPS1* in MAD/BUB checkpoint, which delays the exit from mitosis unless all chromosomes have attached to the mitotic spindle (20, 21). *BUB2* and *BFA1/BFR4* in BUB2 checkpoint monitors the location of the anaphase spindle to ensure the location of one nucleus to each progeny cell (20, 21). However, the above cell cycle checkpoint genes other than *hBUB1/hBUBR1* still largely remain functionally characterized in mammalian cells. On the other hand, mutations in *hBUB1* have demonstrated a dominant negative effect by disrupting the mitotic checkpoint, thereby suggesting that acquired aneuploidy is a specific driving force in tumor progression rather than an epiphenomenon (8). Therefore, it will be important to determine whether inactivation of cell cycle checkpoint genes other than *hBUB1* and *hBUBR1* may also be causally involved in the pathogenesis of CI.

In conclusion, the mitotic checkpoint genes *hBUB1* and *hBUBR1*, which were overexpressed in most colorectal cancers, may contribute to acquired aneuploidy more frequently than currently considered as a result of epigenetic inactivation. Their reduced expression may lead to increased cancer metastasis and subsequent relapse.

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