Increased Expression of Mitotic Checkpoint Genes in Breast Cancer Cells with Chromosomal Instability

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Abstract

Purpose: Most breast cancers have chromosomal instability that seems related to defective mitotic spindle checkpoints. Because the molecular basis of this defect is unknown, we evaluated breast cancer cell lines and tissues for possible defects involving the major mitotic checkpoint genes responsible for maintaining chromosomal stability.

Experimental Design: We analyzed sequences and expression levels (RNA and protein) of eight major spindle checkpoint genes (*MAD1L1, MAD2L1, MAD2L2, BUB1, BUB1B, BUB3, CDC20,* and *TTK*) in a panel of 12 breast cancer cell lines, most with established genetic instability and defective spindle damage checkpoint response. mRNA levels of these genes were also measured in primary tumor samples, and immunohistochemical staining was used to evaluate BUB1B protein levels in a panel of 270 additional cases of breast cancer.

Results: No functionally significant sequence variations were found for any of the eight genes in the breast cancer cell lines with chromosomal instability. More surprisingly, the mRNA and protein levels for these checkpoint genes are significantly higher in the genetically unstable breast cancer cell lines and in high-grade primary breast cancer tissues than in the stable (and checkpoint proficient) MCF-10A and normal mammary epithelial cells, or in normal breast tissues. In fact, overexpression of the BUB1B protein is a marker that recognizes nearly 80% of breast cancers in paraffin-embedded tissues.

Conclusions: Defective mitotic spindle checkpoints in breast cancer are most likely not caused by low expression or mutations of these eight checkpoint genes. High levels of these particular transcripts could represent a cellular compensation for defects in other molecular components of the mitotic spindle damage checkpoint, and increased expression of these genes might be markers of breast cancers with chromosomal instability.

Most breast cancers have significantly aberrant genomic structure, including abnormal numbers of chromosomes. We have previously shown that in breast cancer cell lines, chromosome numbers are both variable and unstable (1), a phenomenon that seems related to defective mitotic spindle checkpoint controls. This chromosomal instability in breast cancer is similar to what has been observed in other cancers, where defective mitotic spindle checkpoint genes have been implicated as a basis of chromosomal instability. For example, mutations in the *BUB1* mitotic checkpoint gene were initially

reported in a small number of colorectal cancers with chromosomal instability (2, 3), Subsequently, mutations of *BUB1* were reported in colon and pancreatic cancers (3, 4), and a truncating mutation of the *MAD2L1* gene was reported for a single breast cancer cell line (4).

Further analyses of the *BUB1, BUB1B,* and *MAD2L1* checkpoint genes, however, have revealed that these mutations are relatively uncommon in cancer. Supporting a possible alternative mechanism for reducing the activity of checkpoint proteins, several studies have provided evidence that low expression levels of key mitotic spindle checkpoint genes could contribute to defective spindle checkpoint controls in cancer. For example, expression of *BUB1* is decreased in many colorectal cancers without mutations (3), and *MAD2L1* is reportedly under-expressed in some breast and ovarian cancers (5). Such reduced expression apparently has functional significance, because deletion of one *MAD2L2* allele results in a defective mitotic checkpoint in both human cancer cells and murine primary embryonic fibroblasts (6) and haploinsufficiency of *BUB1B* in mice results in defective mitotic arrest as well as tumors (7).

Based on such functional studies, mutations or under-expression of mitotic spindle checkpoint genes could still logically be predicted to play a role in causing genomic instability in breast cancer, and the limited analysis of these genes to date certainly does remove these genes from suspicion.

Table 1. Primers for real-time PCR

Gene	Reference sequence	Forward primer	Reverse primer
BUB1	NM_004336	GAGTGATATCTTCAGCTTGTG (633-653)	AACAACCTGCTCAACATCAAC (734-754)
BUB1B		ACGTTATTAGAAAGAGCTGTAG (432-453)	CATATCCAAAGGCTCATTGC (529-548)
BUB3	NM_004725	ATGCGGCTCAAGTACCAGC (215-233)	GATCAGTGTTCAAATCATGC (313-332)
MAD2L1	NM_002358	GCGCGTGCTTTTGTTTGTG (47-65)	AGTAAAGGTTTCAGATGGATAT (185-206)
MAD1L1	NM_003550	TACCAGATCGACATCACCAC (2091-2110)	TCTCCAGTAGCTGCATCTTG (2194-2213)
MAD2L2	NM_006341	TGCATCTCATCCTCTACGTG (191-210)	TCCTGGATATACTGATTCAGC (288-308)
CDC20	NM_001255	CCTCTGGTCTCCCCATTAC (1325-1343)	ATGTGTGACCTTTGAGTTCAG (1420-1440)
TTK	NM_003318	AATGCTGGAAATTGCCCTGC (608-627)	GAACCGGAAAATGATTCTTGG (707-727)
ACTB	NM_001101	AATCTGGCACCACACCTTCTA (325-345)	ATAGCACAGCCTGGATAGCAA (474-494)

In this study, we comprehensively evaluated eight major mitotic spindle checkpoint genes in 12 breast cancer cell lines that have been previously characterized for chromosomal instability and mitotic spindle defects and in primary tumor samples of high-grade breast cancers. Specifically, we sequenced transcripts of the *MAD1L1*, *MAD2L2*, *MAD2L2*, *BUB1*, *BUB1B*, *BUB3*, *CDC20*, and *TTK* genes and measured mRNA levels by real-time PCR and protein levels by immunoblot. Notably, the *MAD1L1*, *MAD2L2*, *BUB3*, *TTK*, and *CDC20* checkpoint genes have not, to our knowledge, been previously evaluated in breast cancer (other than measurements of transcripts of these genes in microarray analyses). We found no sequence variations of any of these genes that would be suggestive of a functionally significant somatic mutation. Surprisingly, we found increased rather than decreased levels of checkpoint gene transcripts in the breast cancer samples compared with those in primary breast epithelial cells. Extending these findings, we tested a large series of breast tissues for expression of a checkpoint gene protein as a potential marker for breast cancer.

Materials and Methods

Breast cancer cell lines, culture conditions, and primary breast cancers. The 12 breast cancer cell lines (MDA-MB-231, HCC1806, T47D, HCC1937, HCC38, MCF7, HCC1143, BT549, SKBR3, BT-20, Hs578T, and DU4475) and the nontumorigenic breast cancer epithelial cell line MCF10A were obtained from the American Type Culture Collection (Manassas, VA) and cultured in recommended medium at 37°C 5% CO_2. Immortalized mammary epithelial cells (ME16C) were provided by Dr. Jerry Shay (University of Texas, Dallas, TX; ref. 8) and maintained in MEGM (Clonetics, San Diego, CA). Cultures of primary mammary epithelial cells were established and maintained in MEGM using previously described methods (9). Cultures of the various cells were harvested at 70% to 80% confluency for experiments to measure gene expression.

In compliance and approval from the institutional review board for use of human subjects, snap-frozen samples of primary breast cancer were obtained from surgical pathology specimens and used for measurements of gene mRNA levels. Frozen sections of the tissues were examined for each case to assure that at least 70% of the cells in each sample analyzed were cancer cells. Immunohistochemistry studies were done on tissue microarrays, which used small core biopsy samples of routinely prepared paraffin tissue blocks. All samples used for research were in excess of tissue required for routine diagnosis, and patient identifiers were not linked to specimens.

Sequence analysis of checkpoint gene transcripts. At 80% confluency, cells were harvested, and RNA was isolated using RNAeasy Mini kit (Qiagen, Valencia, CA), according to manufacturer's protocol. cDNA synthesis was done using Oligo(dT)$_{18}$ (Invitrogen, Carlsbad, CA,) and Superscript II RNase H reverse transcriptase (Invitrogen), following the manufacturers' recommendations. PCR amplifications were done using Platinum Taq DNA Polymerase (Invitrogen) with the following conditions: 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension step of 72°C for 5 minutes. Reverse transcription-PCR products were purified using a QIAquick PCR purification kit (Qiagen), and automated fluorescent sequencing was done using appropriate primers (sequences available upon request) on ABI Prism model 3700 automated sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed using National Center for Biotechnology Information Blast databases and software. Each sequence was verified twice, using primers in both the forward and reverse directions. Heterozygous as well as homozygous sequence variants were detected by this method.

Real-time quantitative PCR measure expression level of mitotic checkpoint genes. Total RNA was extracted as above. The primers for SYBR Green real-time quantitative PCR were designed specific for each checkpoint gene and for *ACTB* (*β-actin*) as an endogenous control (Table 1). Quantification was based on standard curves constructed by serial dilutions of RNA. The assays were repeated in their entirety for each measurement.

Multiplex RCR was done in 96-well plates using 50 ng RNA per assay and Taqman reverse transcription reagents and SYBR Green PCR master mix (Applied Biosystems). PCR conditions were 30 minutes at 48°C and 10 minutes at 95 °C followed with 40 cycles at 95°C for 30 seconds and 60°C for 1 minute. Data were collected using the ABI PRISM 7900HT SDS analytic thermal cycler (Applied Biosystems). Each sample was tested in triplicate, and dissociation curves of each sample were used to check the specificity of amplification. For verification of dissociation curve results, PCR reactions were examined by 2% agarose gel electrophoresis. All experiments with intra-assay or inter-assay coefficient of variation of >10% were repeated. The relative expression level of each gene was normalized to the level present in MCF10A cells, using the level of *ACTB* as a reference standard.

Cell sorting. To isolate G_0-G_1 and G_2-M populations of cells, cultures were harvested at about 80% confluency and resuspended in prewarmed RPMI 1640 containing 2% fetal bovine calf serum, at a density of 10^6/mL. Hoechst 33342 (Sigma, Milwaukee, WI) was then added at a concentration of 5 μg/mL, and the suspension was incubated at 37°C for 90 minutes in a shaker bath. Cells were pelleted, resuspended in fresh cold medium, and sorted using FACSStar Plus (Becton Dickinson, Mountain View, CA).

Immunoblot analysis of proteins. Cells were harvested and lysed in buffer containing 50 mmol/L Tris-HCl (pH 7.7), 150 mmol/L NaCl, 0.5% NP40, 1 mmol/L DTT, 10% glycerol, and 0.5 μmol/L protease inhibitor cocktail (Sigma, St. Louis, MO). Samples of the lysates (50 μg) were then separated using SDS-PAGE electrophoresis and transferred to Bio-Rad (Hercules, CA) membranes. Specific antisera used to

probe the membranes included goat polyclonal anti-MAD2L2, rabbit polyclonal anti-CDC20 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-BUB1B, monoclonal anti-BUB3 (BD Biosciences, San Diego, CA), and rabbit polyclonal anti-β-actin (Santa Cruz Biotechnology). Subsequently, membranes were hybridized to horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology) and visualized with enhanced chemiluminescence (Amersham, Piscataway, NJ).

Immunochemical staining. Breast cancer tissues from a total of 270 patient samples, represented on tissue microarrays, were analyzed using a 1:100 dilution of the same monoclonal anti-BUB1B antibody described above. Immunohistochemical staining of paraffin-embedded pellets of the cell lines corresponded to the expression seen on immunoblots (data not shown), confirming specificity of the antibody for tissue analysis. Slides were pretreated with steam vapors of Target Retrieval solution (DakoCytomation, Carpinteria, CA) according to manufacturer's instructions, and immunochemistry was developed using the conventional streptavidin-avidin-biotin complex kit from DakoCytomation. Staining was scored on the following scale: 0, no staining; 1+, minimal staining; 2+, moderate to strong staining in at least 20% of cells; 3+, strong staining in at least 50% of cells.

Results

Transcripts of mitotic spindle checkpoint genes lack mutations. To begin our analysis, we sequenced transcripts of the eight major checkpoint genes in the 12 breast cancer cell lines. A total of nine sequence variations, all previously reported as polymorphisms, were found in the transcripts from the cell lines (Table 2). No sequence variation that would likely result in a functional change in the protein (e.g., change of amino acid charge) was found. Because these breast cancer cell lines with defective mitotic spindle checkpoints do not have mutations of these common checkpoint genes, we conclude that such mutations are not a prevalent cause of chromosomal instability in breast cancer.

Increased mRNA and protein levels of mitotic spindle checkpoint genes in breast cancer. Previous studies have implicated decreased expression rather than mutations of checkpoint genes as a cause of defective mitotic spindle damage checkpoints in
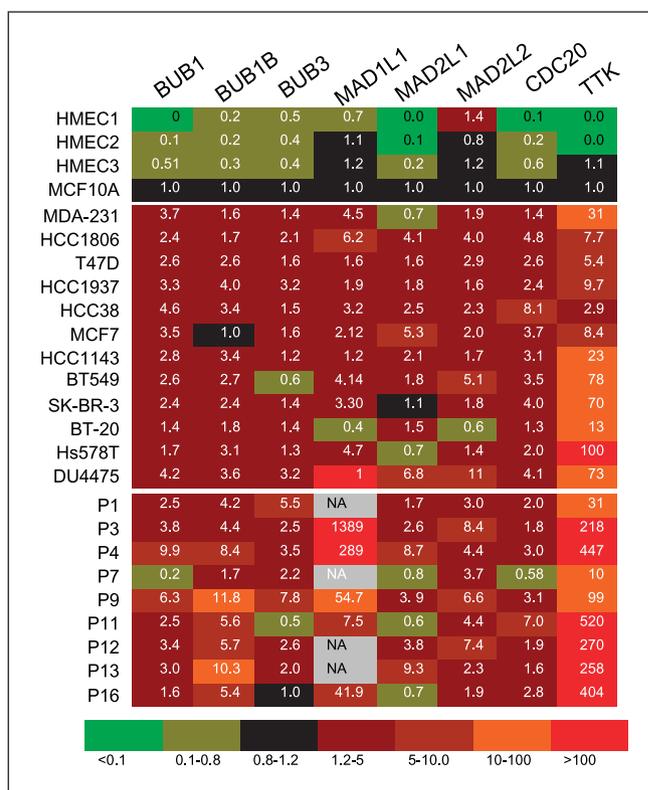
	BUB1	BUB1B	BUB3	MAD1L1	MAD2L1	MAD2L2	CDC20	TTK
HMEC1	0	0.2	0.5	0.7	0.0	1.4	0.1	0.0
HMEC2	0.1	0.2	0.4	1.1	0.1	0.8	0.2	0.0
HMEC3	0.51	0.3	0.4	1.2	0.2	1.2	0.6	1.1
MCF10A	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MDA-231	3.7	1.6	1.4	4.5	0.7	1.9	1.4	31
HCC1806	2.4	1.7	2.1	6.2	4.1	4.0	4.8	7.7
T47D	2.6	2.6	1.6	1.6	1.6	2.9	2.6	5.4
HCC1937	3.3	4.0	3.2	1.9	1.8	1.6	2.4	9.7
HCC38	4.6	3.4	1.5	3.2	2.5	2.3	8.1	2.9
MCF7	3.5	1.0	1.6	2.12	5.3	2.0	3.7	8.4
HCC1143	2.8	3.4	1.2	1.2	2.1	1.7	3.1	23
BT549	2.6	2.7	0.6	4.14	1.8	5.1	3.5	78
SK-BR-3	2.4	2.4	1.4	3.30	1.1	1.8	4.0	70
BT-20	1.4	1.8	1.4	0.4	1.5	0.6	1.3	13
Hs578T	1.7	3.1	1.3	4.7	0.7	1.4	2.0	100
DU4475	4.2	3.6	3.2	1	6.8	11	4.1	73
P1	2.5	4.2	5.5	NA	1.7	3.0	2.0	31
P3	3.8	4.4	2.5	1389	2.6	8.4	1.8	218
P4	9.9	8.4	3.5	289	8.7	4.4	3.0	447
P7	0.2	1.7	2.2	NA	0.8	3.7	0.58	10
P9	6.3	11.8	7.8	54.7	3.9	6.6	3.1	99
P11	2.5	5.6	0.5	7.5	0.6	4.4	7.0	520
P12	3.4	5.7	2.6	NA	3.8	7.4	1.9	270
P13	3.0	10.3	2.0	NA	9.3	2.3	1.6	258
P16	1.6	5.4	1.0	41.9	0.7	1.9	2.8	404

<0.1	0.1-0.8	0.8-1.2	1.2-5	5-10.0	10-100	>100

Fig. 1. Relative expression levels of checkpoint genes in nonmalignant breast cells (*top*), breast cancer cell lines (*middle*), and primary tumor samples (*bottom*). Expression for each gene was normalized to level of β-actin in each cell line, and expression was then compared with levels in MCF10A cells. Scale for relative expression is shown in bar below. NA, not amplified in given sample.

breast cancer. To determine whether decreased expression of checkpoint genes could be responsible for defective checkpoints seen in most of these cell lines, we quantitatively measured transcripts of the eight major mitotic checkpoint genes in the 12 breast cancer cell lines, in "control" breast

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Table 2. Sequence variations in checkpoint gene transcripts from breast cancer cell lines

Genes	Accession no.	Nucleotide position	Sequence variants	SNP cluster ID	Frequencies of sequence variants
BUB1	AF046078		No variants noted		
BUB1B	AF046079	324	AAA(Lys)-AAG(Lys)	rs1801389	2/12-11/12
		1088	CAA(Gln)-CGA(Arg)	rs1801376	6/12-2/12-5/12 hetero
		1206	GCA(Ala)-GCG(Ala)	rs1047130	3/12-10/12
BUB3	AF053304		No variants noted		
MAD1L1	AF083811	663	CAC(His)-CAT(His)	rs11557345	11/12-2/12
		1673	CGC(Arg-R)-CAC(His)	rs1801368	10/12-3/12
		1698	GCG(Ala)-GCA(Ala)	reported	11/12-2/12
		1833	GCC(Ala)-GCT(Ala)	reported	12/12-1/12
MAD2L1	NM_002358	503	CCA(Pro)-CCG(Pro)	rs1801614	12/12-1/12
MAD2L2	NM_006341		No variants noted		
CDC20	NM_001255	542	TAT(Tyr)-TAC(Tyr)	rs1801338	11/12-2/12
TTK	NM_003318		No variants noted		

NOTE: For each gene with sequence variations found (column 4), the projected amino acid changes are listed. Frequencies of the respective sequence variants are also tabulated (column 6). Note that all of the variations have been previously reported as polymorphisms.
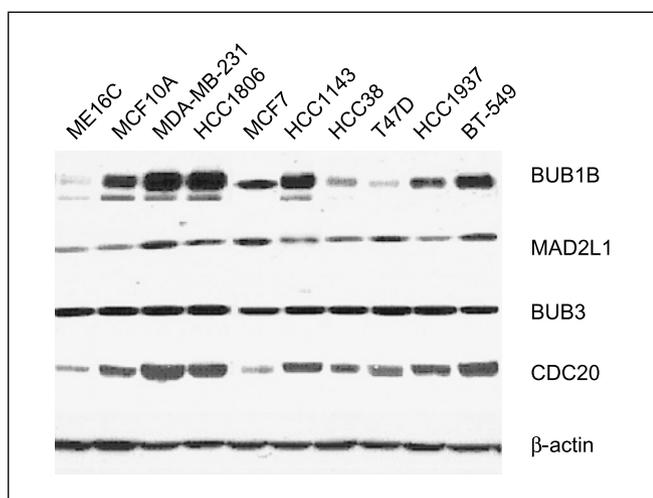Abbreviation: SNP, single nucleotide polymorphism.

Fig. 2. Immunoblot of spindle checkpoint gene protein levels in breast cancer cell lines and the nonmalignant MCF10A and ME16C cells. β-Actin levels as control (*bottom*).

epithelial cell lines (MCF10A and primary mammary epithelial cells) with intact mitotic spindle damage response, and in tissue samples from high-grade breast cancers.

Unexpectedly, we found almost uniformly increased mRNA transcript levels of these checkpoint genes (*MAD1L1*, *MAD2L1*, *MAD2L2*, *BUB1*, *BUB1B*, *BUB3*, *CDC20*, and *TTK*) in the breast cancer cell lines relative to levels in MCF10A and primary mammary cells. The results are summarized in Fig. 1, where relative expression levels for all samples are normalized to those measured in the MCF10A cell line. The relative increases in expression range from up to 11-fold for the *MAD2L2* gene and up to 520 fold for the *TTK* gene. Notably, the levels of the checkpoint genes in the breast cancer cell lines were never found to be below the range of expression measured in the normal breast epithelial cells.

We also measure transcript levels of the eight checkpoint genes in nine different tissue samples of poorly differentiated (Nottingham grade 3) invasive breast cancers. As shown in Fig. 1 (*bottom*), the levels of these transcripts in the cancer tissues mirrored our findings in the breast cancer cell lines, with almost uniformly increased expression of checkpoint genes in cancer compared with normal breast.

To confirm overexpression of these genes at the protein level, protein levels of four genes (*BUB1B*, *MAD2L1*, *BUB3*, and *CDC20*) were measured semiquantitatively by immunoblot using commercially available antisera. For these experiments, protein levels in the cancer cells were compared with those in MCF-10A and immortalized, nontumorigenic mammary epithelial cells (ME16C). As shown in Fig. 2, these four checkpoint proteins are generally present in significantly higher levels in the cancer cell lines than in MCF10A or ME16C cells. Although BUB1B protein levels are somewhat lower in several of the cancer cell lines than in the MCF10A cells, all breast cancer cell lines have significantly higher levels of BUB1B than the ME16C cells (Fig. 2) and cultured primary mammary epithelial cells (data not shown). Thus, checkpoint genes seem to be generally expressed at high levels in breast cancer at both the transcriptional and translational level.

Cell cycle variation does not explain high levels of checkpoint gene expression in breast cancer cells. Because previous investigations found checkpoint genes (e.g., *CDC20*, and mouse *BUB1B*) to be expressed in a cell cycle–dependent manner (10, 11), we considered the possibility that high levels of checkpoint gene expression in cancer cells simply reflect a high rate of proliferation. Although the rate of proliferation of the cancer cells does not differ significantly from that of MCF-10A cells or early-passage primary mammary cells, we compared expression levels for G_0-G_1 and G_2-M fractions of flow cytometry sorted MCF-7, MDA-231, and primary mammary cells (Fig. 3). As shown in Fig. 3, the difference in expression between G_2-M cells and G_0-G_1 cells is less than the difference in gene expression across unsorted (and unsynchronized) cells. Thus, cell cycle–dependent variation in expression is insufficient to completely explain the high levels of checkpoint gene transcripts observed in breast cancer cells compared with nonmalignant breast cells.

Increased expression of BUB1B characterizes high-grade breast cancer. We tested the commercially available antisera to checkpoint proteins and found that antiserum to the BUB1B protein (but not other antisera) stains formalin-fixed, paraffin-embedded tissues in a manner consistent with immunoblot results (data not shown). Therefore, we used this antiserum to evaluate expression of BUB1B in a panel of 270 primary breast cancer samples represented on tissue microarrays. Most (77%) of the breast cancers in this set of tumors stained positive (2+ to 3+) for BUB1B, whereas none of the 18 normal breast ductal tissues showed significant staining for this antigen. Representative staining is shown in Fig. 4. The staining in breast cancer for BUB1B is cytoplasmic, similar to the pattern reported for colorectal cancer cells (12). By contrast, for normal colonic epithelium, BUB1B staining is seen with the expected nuclear localization (12), whereas no staining was seen in normal breast epithelium with the conditions used in this study. Some breast cancers show heterogeneous staining for BUB1B (e.g., Fig. 4B).

Remarkably, staining for BUB1B is particularly prevalent in grade 2 (86%) and grade 3 (94%) ductal breast cancers, with many fewer cases of grade 1 ductal cancer (18%) and no cases of mucinous breast cancer staining above background for this antigen (Table 3). Not unexpectedly (because of the high prevalence of BUB1B staining), expression was not found to be
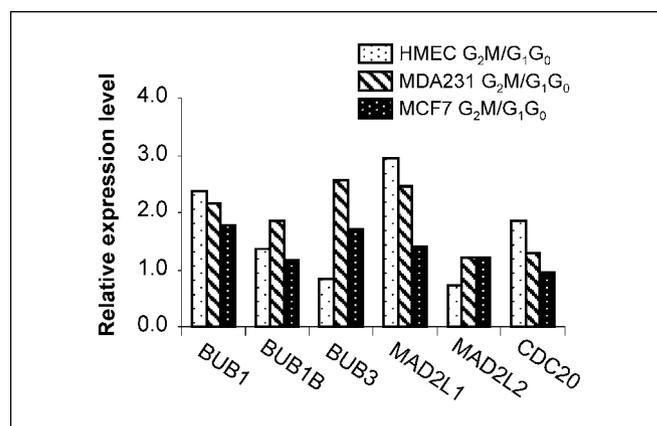

Fig. 3. Expression levels of spindle checkpoint genes in sorted fractions of primary breast epithelial cells (HMEC) and breast cancer cells. Ratios of levels in G_0-G_1 fraction to levels in G_2-M fraction.
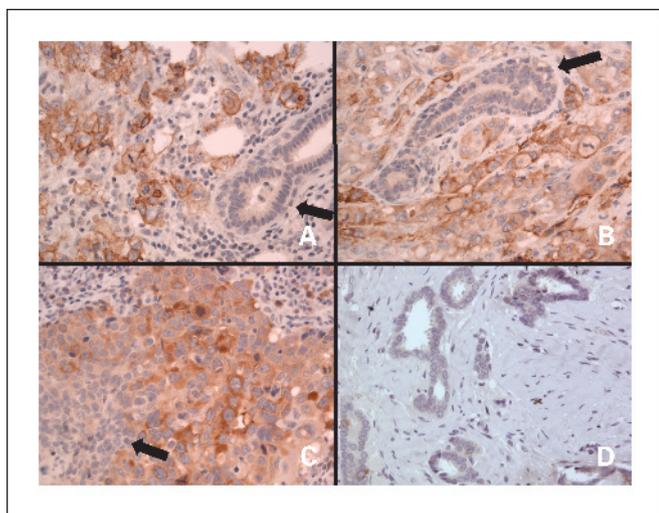
Fig. 4. Immunohistochemical staining for BUB1B in breast cancer tissues. *A-C* from grade 3 ductal cancers; *D,* from a grade 1 ductal cancer. *A* and *B,* arrows designate nonmalignant breast ducts that do not stain significantly for BUB1B. *C,* arrow designates area of tumor with minimal staining (heterogeneous expression). *A-C,* expression was interpreted as 3+; *D,* expression was interpreted as 0. Photographs were taken at 400× magnification.

a significant independent variable predictive of survival among the patients with cancers represented on the arrays (data not shown).

Discussion

Checkpoint genes, which delay mitosis when chromosomes are imperfectly aligned, have drawn considerable attention for their possible role in the phenomenon of genomic instability in neoplasia. This attention was fueled by early reports showing mutations in the *BUB1* gene in some cases of colorectal cancer and low expression of the *MAD2L1* gene in one breast cancer cell line. Interestingly, even a hemizygous mutation of *BUB1* seems to be functionally significant, and haploinsufficiency of checkpoint genes, such as *BUB1B*, in genetically engineered mice was sufficient to result in slippage of cells from mitotic arrest and enhancement of tumor development. Thus, whereas checkpoint genes do not have characteristics of classic tumor suppressor genes, it would seem that even partial reduction of function for one of these genes could have profound effects on mitotic stability and maintenance of genomic integrity.

Based on such observations, the role of mitotic spindle damage checkpoint genes in the development of chromosomal instability, mutations, or decreased expression of these checkpoint genes in breast cancer might be expected. However, with one exception (5), previous studies on the checkpoint genes in breast cancer failed to find possible mutations or loss of gene expression for the checkpoint genes examined (4, 13–17). Nevertheless, the fragmented nature of these previous studies, which did not include any analysis of *MAD1L1, MAD2L2, BUB3, TTK,* or *CDC20* genes, does not by any means exclude this class of genes from being involved in the pathogenesis of genomic instability in breast cancer. By conducting a more comprehensive evaluation, we can now be more confident in concluding that neither mutations nor underexpression of these checkpoint genes are responsible for the defective checkpoints that are so prevalent in this type of cancer.

Although previous studies that have focused specifically on mitotic checkpoint genes in breast cancer have not found increased expression of these genes, it is notable that some microarray-based gene expression profile studies of breast cancer have found elevated levels of selected genes in subsets of breast cancer. For example, levels of *BUB1* mRNA were reported to be high in breast cancers with extremely poor outcome (18), and *MAD2L1* levels were reportedly relatively elevated in basal-like breast cancers that respond to chemotherapy (19). Although the transcript levels of many checkpoint genes are below the level required for effective measurement by microarray analysis, these results complement our findings in suggesting that elevated transcript levels of checkpoint genes are common in breast cancers, including subtypes of breast cancer with aggressive clinical biology.

When evaluating this gene expression data of cell culture and tissue samples, it is important to recognize that mitotic checkpoint genes are normally expressed in a cell cycle–dependent manner (10, 11). To verify that breast cancer cells are not deficient in checkpoint proteins at the time they are likely needed most, we measured transcripts in G_2-M cells isolated by flow cytometry. These measurements confirmed that the high expression of checkpoint genes in cancer cells persists through mitosis. Furthermore, our immunohistochemical staining for BUB1B protein showed expression in mitotic cells as well as nondividing cells. Thus, it seems unlikely that cell cycle–specific deficiencies in checkpoint protein levels are responsible for loss of the checkpoint controls in breast cancer.

What, then, is the significance of overexpression of checkpoint genes in breast cancer? One possible explanation of this observation would be that high expression of these proteins represents a partial compensation for other defects in the mitotic spindle checkpoint. Indeed, total loss of checkpoint gene function actually seems catastrophic, even for cancer cells (20); thus, it would seem reasonable that cells would have such compensatory mechanisms to prevent total chaos at mitosis. Alternatively, it could be speculated that overexpression of checkpoint genes actually contributes to defective checkpoints and genomic instability. The possibility of this apparent paradox is supported by observations that overexpression of wild-type *BUB1* or *MAD3* gene leads to chromosome instability in yeast (21).

Table 3. Expression of BUB1B by immunohistochemistry in breast cancer tissues

Histologic type	No. tested	No. (%) positive
Grade 1 ductal	40	7 (18%)
Grade 2 ductal	76	65 (86%)
Grade 3 ductal	137	129 (94%)
Mucinous	6	0
Lobular	11	7 (64%)

NOTE: Cases with 0 or 1+ staining (similar to normal breast tissue) are classified as negative, and cases with 2+ or 3+ staining are classified as positive. The percentages of positive cases for grade 1 ductal and mucinous breast cancers are significantly less ($P \ll 0.001$ by χ^2 statistic) than for grade 2 and 3 ductal breast cancers. Overall, 77% of tumors stained positive for BUB1B.

If overexpression of checkpoint genes contributes to cancer cell survival, or if this overexpression contributes to the malignant phenotype, it is possible that the related pathways could represent potential targets for cancer therapy. A more tangible implication of this overexpression is that increased expression of these genes could be markers of genetic instability in breast cancer. In fact, a single antibody, specific for BUB1B, selectively stained >90% of grade 2 and 3 ductal breast cancers without reacting significantly to normal breast ductal structures. Remarkably, positive staining was seen in relatively few cases of grade 1 ductal breast cancer (18%) and no cases of mucinous breast cancer, which have many fewer genomic alterations than ductal cancers have and thus probably do not have a comparable level of genomic instability (22). Although the sensitivity of this one antibody alone may not be sufficient for application as a diagnostic marker for breast cancer, there is certainly an implication that it could be a part of a panel of markers used to differentiate benign breast cells from malignant cells in some diagnostic situations. Moreover, because the high expression of this protein seems to be relatively selective for high-grade cancers, the potential of this marker for classification of breast cancer should be explored.

Acknowledgments

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