Haploinsufficiency of RAD51B Causes Centrosome Fragmentation and Aneuploidy in Human Cells

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Abstract
The Rad51-like proteins, Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3, have been shown to form two distinct complexes and seem to assist Rad51 in the early stages of homologous recombination. Although these proteins share sequence similarity with Rad51, they do not show functional redundancy. Among them, Rad51B is unique in that the gene maps to the human chromosome 14q23-24, the region frequently involved in balanced chromosome translocations in benign tumors particularly in uterine leiomyomas. Despite accumulating descriptive evidence of altered Rad51B function in these tumors, the biological significance of this aberration is still unknown. To assess the significance of reduced Rad51B function, we deleted the gene in the human colon cancer cell line HCT116 by gene targeting. Here, we show that haploinsufficiency of RAD51B causes mild hypersensitivity to DNA-damaging agents, a mild reduction in sister chromatid exchange, impaired Rad51 focus formation, and an increase in chromosome aberrations. Remarkably, haploinsufficiency of RAD51B leads to centrosome fragmentation and aneuploidy. In addition, an ~50% reduction in RAD51B mRNA levels by RNA interference also leads to centrosome fragmentation in the human fibrosarcoma cell line HT1080. These findings suggest that the proper biallelic expression of RAD51B is required for the maintenance of chromosome integrity in human cells. (Cancer Res 2006; 66(12): 6018-24)

Introduction
The centrosome is the primary microtubule-organizing center in vertebrate cells and forms the poles of the mitotic spindles that facilitate chromosome segregation (1). A direct link between centrosome abnormalities and chromosome instability has been suggested by the significant correlation between centrosome amplification and aneuploidy in human cancers (2, 3). Inactivation of tumor suppressor genes or amplification of oncogenes also induces centrosome amplification and aneuploidy in mammals (4–7). Such correlations have led to the hypothesis that centrosome amplification plays a causal role in chromosome instability. DNA double-strand breaks (DSB) are repaired either by nonhomologous end joining or homologous recombination (8, 9). Rad51 promotes homologous DNA pairing and strand exchange, thereby playing a central role in the early stages of homologous recombination (10). Defects in homologous recombination repair have been shown to cause centrosome abnormalities. An increase in the number of centrosomes has been observed in rodent and chicken cells deficient in BRCA1, BRCA2, Mre11, XRCC2, XRCC3, Rad51, or Rad51D (6, 11–15). It should be noted that the increases in centrosome numbers in cells deficient in BRCA1, BRCA2, Mre11, or Rad51 have resulted from centrosome amplification, whereas such increases in cells deficient in other proteins have resulted from centrosome fragmentation. Consequently, the frequency of aneuploidy has been found to be increased in rodent cells deficient in BRCA1, BRCA2, XRCC2, or XRCC3. It has been reported that BRCA1 is localized at the centrosome (16). In addition, ataxia-telangiectasia mutated (ATM) is involved in centrosome amplification in Rad51-deficient DT40 cells (14). Despite these observations, the primary causes for these aberrations have not been fully characterized.

Rad51B (Rad51L1), a member of the Rad51 paralogue family, plays a role in homologous recombination in concert with Rad51 and other Rad51 paralogues by directly associating with Rad51C (17–19). Additionally, biochemical evidence that Rad51B binds to the Holliday junction has suggested that the protein may play a role in the late phase of homologous recombination (20). Consistent with this finding, Rad51C or Rad51C-associated proteins have been proposed to be components of Holliday junction resolvase (21), although there is not yet any direct evidence that Rad51B is involved in the resolution of recombination intermediates. Cellular functions of Rad51B have been investigated in chicken DT40 cells. Hypersensitivity to DNA-damaging agents, decreases in sister chromatid exchange (SCE) and gene targeting, impaired damage-dependent Rad51 focus formation, and an increase in chromosome aberrations have been observed in RAD51B−/− DT40 cells (22). Although Chinese hamster ovary (CHO) cells have been used for the functional analysis of XRCC2, XRCC3, and Rad51C, CHO cells deficient in Rad51B have not been available thus far. RAD51B−/− mice die in the early embryonic stages, suggesting that Rad51B plays a role in development (23).

An interesting feature of Rad51B in cancer genetics is that the gene maps to the chromosome break point in some benign tumors that harbor balanced chromosome translocations involving 14q23-24 (24). The involvement of Rad51B in benign tumors was first found in uterine leiomyomas harboring a balanced chromosome translocation between chromosomes 12 and 14 with the high mobility group protein HMGA2 (HMGC) as the partner (25, 26). Chimeric transcripts encoding either RAD51L1/HMGA2 or HMGA2/RAD51L1 have been found in some uterine leiomyomas (25, 27, 28). In pseudo-Meigs’ syndrome, which is characterized by uterine leiomyomas, ascites, and pleural effusion, a combination of the HMGA2/RAD51L1 fusion and a loss of the second RAD51L1 allele were observed (29). In addition, RAD51B is involved in other types

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References
of balanced chromosome translocations in pulmonary chondroid hamartomas (30) and thymomas (31). These studies indicate that at least one allele of \textit{RAD51B} is altered in some benign tumors, but the key pathologic event is still unclear. For this reason, the elucidation of the significance of \textit{RAD51B} haploinsufficiency has been awaited.

To investigate the putative role of Rad51B in human cells, we sequentially knocked out the gene by gene targeting in the human colon cancer cell line HCT116. Here, we show that haploinsufficiency of \textit{RAD51B} leads to a defect in homologous recombination repair as well as to centrosome fragmentation and increased aneuploidy. A reduction in \textit{RAD51B} levels by RNA interference (RNAi) also leads to centrosome fragmentation in the human fibrosarcoma cell line HT1080. Thus, a loss of the proper biallelic expression of \textit{RAD51B} leads to chromosome instability by preventing centrosome integrity in human tumor cells.

**Materials and Methods**

Targeted inactivation of the \textit{RAD51B} gene. Targeting vectors were designed to insert promoterless drug resistance genes in exon 2 in the frame. A 2.2-kb 5′ homology arm was amplified from the isogenic DNA of HCT116 cells using the primers 5′-TAAGGCGAAATGGCAGA-3′ and 5′-TATCGATGTTTCTTGCTACCCAT-3′. A 1.6-kb 3′ homology arm was amplified using the primers 5′-ATAGCTAAAGAGCTGTGTGACCG-3′ and 5′-TACTAGTATGAGCGCTACACTTG-3′. Both arms were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) by the TA cloning method. The 3′ arm was cut out with SpeI digestion and subcloned into the vector containing the 5′ arm. Neomycin, hygromycin, and blasticidin resistance genes were inserted into the \textit{ClaI} site of the vector containing homology arms. Gene targeting in HCT116 was done as described previously (32).

Ectopic expression of the \textit{RAD51B} cDNA. The human \textit{RAD51B} cDNA was amplified from cDNA derived from normal human cells using the primers 5′-CGCGGGGAAACTGTGTAAA-3′ and 5′-GGCAAGATGAA-CAGGTTTGC-3′. The cDNA was cloned into pCR2.1, and the sequence was confirmed. The expression vector was designed to insert the \textit{RAD51B} cDNA under the control of the murine sarcoma virus enhancer and the mouse mammary tumor virus promoter. The transfected cells were selected in the presence of 900 μg/mL Zeocin (Invitrogen).

Growth rate and sensitivity to DNA-damaging agents. To measure growth rate, the cells were plated at a density of 10^4 per 60-mm dish and cultured. The cells were counted on the days indicated. To measure sensitivity to DNA damage, the cells were irradiated with a 60Co source or treated with mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan) in suspension for 10 minutes and plated at a density of 2 × 10^3 per 60-mm dish. After 7 days of culturing, the colonies of wild-type (WT) cells were counted. Because knockout and complemented cells grew more slowly than WT cells, we factored growth rate into the counting of colonies; the colonies of these cells were further cultured and counted after 9 to 10 days.

SCE and gene targeting. The frequency of SCEs was measured essentially as described previously (32). Wild-type cells were cultured in 16 μmol/L 5-bromodeoxyuridine for 32 hours. Because the mutant showed slow growth, \textit{RAD51B}^{+/−/−} and the cDNA-expressing cells were cultured in the agent for 40 and 36 hours, respectively. To examine MMC-induced SCE, the cells were incubated in the presence of 0.8 μg/mL MMC for 8 hours. The gene-targeting frequency was examined using \textit{RAD54B-hyg} and \textit{RAD51C-par} vectors (32).
Antibodies. We used commercially available antibodies to Rad51 from Oncogene Research (San Diego, CA) and to γ-tubulin and β-tubulin from Sigma (St. Louis, MO).

Immunostaining. The cells were cultured on glass slides, fixed in ice-cold methanol for 10 minutes, washed in PBS, and blocked in fetal bovine serum for 15 minutes. Rad51 focus formation was examined as described previously (33). The cells were either non-treated or irradiated with 8 Gy and stained at 2.5 hours after irradiation with anti-Rad51 antibody. The cells were also treated with 0.8 μg/mL MMC for 1 hour and stained at 2 hours after treatment. Centrosomes were detected by anti-γ-tubulin antibody. Microtubules were detected by anti-β-tubulin antibody. The cells were counterstained with 4',6-diamidino-2-phenylindole.

Fluorescence in situ hybridization analysis. Chromosome-specific centromere probes were obtained from Vysis (Downers Grove, IL). Hybridization was done according to the manufacturer’s protocol.

Small interfering RNA transfection. To knock down Rad51B in stably transformed cells, a DNA fragment flanked by the BamHI and HindIII sites containing the sense target sequence (5'-AGCACAAGGTTGCTGATG-3'), the hairpin loop sequence (5'-TTCAAGAGA-3'), and the antisense target sequence was synthesized and inserted into pBSi-hU6 Neo (Takara, Otsu, Japan). Similarly, the control sequence (5'-TACCAGCTAACAACATCA-3') was used to construct the vector used for a negative control. Transfected cells were selected in the presence of 400 μg/mL G418.

Real-time reverse transcription-PCR. Total RNA was extracted from cytoplasm (34). Total RNA (500 ng) was reverse transcribed in a total of 20 μL reaction mixture. Real-time PCR was carried out with the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) in a 20-μL reaction volume containing 1 μL cDNA using SYBR Green (Qiagen, Valencia, CA) for the detection of PCR products. The PCR primers were as follows: RAD51B, 5'-CAGTGTGAAATCACCCTGTA-3' and 5'-CTTGATGTTGATGACAATGAGGTG-3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GCCCGTCAAGGCTGAGAAC-3' and 5'-ATGGTGGTAGAAGGCACCTGAT-3'. The expression level of the RAD51B gene was evaluated as the ratio of its mRNA to that of GAPDH mRNA.

Results

Rad51B is required for gene targeting. The RAD51B gene was sequentially knocked out by gene targeting in the HCT116 cell line (Fig. 1A). Southern blot analysis revealed that this cell line harbors four RAD51B alleles (Fig. 1B). Rad51B-null cells were not generated because the targeting frequencies in the triple knockout cells were extremely low. The effect of the triple knockout on gene targeting was examined at two independent loci (32). The frequency at the RAD54B locus was 6.1% (25 of 407) in WT cells, whereas it was 1.4% (2 of 141) in RAD51B+/−/−/− cells. Similarly, the frequencies at the RAD51C locus were 0.3% (12 of 3,985) in WT cells and 0% (0 of 1,436) in RAD51B+/−/−/− cells. These differences were statistically significant (P < 0.05, Fisher’s exact test). In addition, the frequencies in RAD51B+/−/−/− cells were 5.6% (4 of 72) at the RAD54B locus and 0.33% (4 of 1,216) at the RAD51C locus, indicating a difference in gene-targeting frequency between RAD51B+/−/−/− and RAD51B+/−/−/− cells. This finding suggests that Rad51B is required for gene targeting in human cells; thus, the present results are in accord with those of a previous report using chicken DT40 cells (22). Northern blot analysis confirmed that the expression levels correled well with the targeting events; the level of Rad51B in RAD51B+/−/−/− cells was approximately one fourth that of the WT cells (Fig. 1C). The level of Rad51B by ectopic expression was almost identical to that in WT cells. We then investigated Rad51B function using the RAD51B+/−/−/− cell line.

Growth and sensitivity to DNA-damaging agents of rad51b-mutant cells. The rad51b-mutant cells grew at a slightly slower rate than the WT cells, with a doubling time of 21 hours versus 16 hours (Fig. 2A). We next examined the sensitivity of rad51b-mutant cells to DNA damage by measuring their ability to form colonies following exposure to DNA-damaging agents. The rad51b-mutant exhibited mild hypersensitivity to γ-irradiation (2-fold; Fig. 2B). The mutant cells also exhibited mild hypersensitivity to the DNA interstrand cross-linking agent MMC (2.5-fold; Fig. 2C). The expression of the transfected cDNA partially complemented this phenotype. Thus, the present findings suggest that Rad51B plays a role in DNA DSB repair.
Rad51B is involved in SCE. Lower levels of SCE have been found in cells deficient in homologous recombination repair (35). In the present study, the frequencies of spontaneous SCEs were 4.3 ± 1.8 (mean ± SD) in WT cells (n = 131) and 3.4 ± 1.5 in rad51b-mutant cells (n = 116; Fig. 3A). Despite a small frequency reduction in the mutant cells, this difference was statistically significant (P < 0.001, Mann-Whitney U test). The frequencies of MMC-induced SCEs were 8.3 ± 3.9 in WT cells (n = 98) and 6.7 ± 3.6 in mutant cells (n = 120). This difference was also statistically significant (P < 0.001). The expression of the RAD51B cDNA increased the frequencies of SCEs to the WT levels. These findings suggest that Rad51B is involved in homologous recombination by using sister chromatids.

RAD51B haploinsufficiency impairs Rad51 focus formation.

DNA damage induces the formation of Rad51 foci in the nucleus (33). A decrease in Rad51 focus formation has been observed in cells deficient in genes that are involved in the early stages of homologous recombination in concert with Rad51 (36–38). We therefore examined damage-dependent focus formation of Rad51 in the rad51b-mutant cells (Fig. 3B). Because many cells contained one to five foci even in the absence of DNA damage, a cell containing more than five foci was scored as positive. The percentage of positive cells after irradiation was 73.7 ± 5.1% (mean ± SD) among WT cells, 64.7 ± 4.7% among RAD51B+/−/− cells, and 50.7 ± 5.9% among RAD51B+/-/+ cells (Fig. 3C). The expression of RAD51B in RAD51B+/-/+ cells increased the percentage to 73 ± 4.6%. Similarly, the percentage of positive cells after MMC treatment was 64.7 ± 1.4% among WT cells, 46 ± 8.1% among RAD51B+/−/− cells, and 37.3 ± 1.4% among RAD51B+/-/+ cells (Fig. 3C). The expression of RAD51B in RAD51B+/-/+ cells increased the percentage to 60.7 ± 2%. These findings suggest that Rad51B is required for the recruitment of Rad51 to damaged sites in the nucleus.

Rad51B is required for chromosome stability. Because a defect in homologous recombination has been shown to promote chromosome aberrations (39), we did chromosome analysis by preparing metaphase spreads in the presence of colcemid. Spontaneous chromatid-type and chromosome-type aberrations, including gaps, breaks, and exchanges, were significantly (8-fold) increased in rad51b-mutant cells (Fig. 3D). This chromosome damage was reduced by the expression of the RAD51B cDNA. These findings suggest that Rad51B is required for the maintenance of chromosome stability in human cells.

**RAD51B haploinsufficiency leads to centrosome fragmentation.** We next examined centrosome aberrations by immunostaining γ-tubulin because centrosome fragmentation has been observed in other mutant cells deficient in XRCC2, XRCC3, or Rad51D (13, 15). In the rad51b mutant, cells with multiple centrosomes were more frequently observed (Fig. 4A and B). The presence of smaller centrosome-like structures indicated that these numerous abnormalities were caused by centrosome fragmentation rather than by centrosome amplification. The frequency of aberrant numbers of interface centrosomes (more than two) was 5% in WT cells, whereas it was 11.5% in RAD51B+/-/+ cells and

![Figure 3](https://example.com/figure3.jpg)

Figure 3. Rad51B plays a role in the Rad51-dependent recombination repair pathway. A, frequency of SCEs. Columns, mean from at least 98 mitotic cells; bars, SD. B, damage-dependent Rad51 focus formation. The cells were treated with 0.8 μg/mL MMC for 1 hour and stained at 2 hours after treatment. C, decreased levels of damage-dependent Rad51 focus formation. Percentage of cells containing more than five damage-induced Rad51 foci. Columns, mean of three independent experiments; bars, SD. A total of 100 cells were examined for each cell line. D, spontaneous chromosome aberrations in the absence of DNA-damaging agents. Data are the numbers of aberrations per 100 cells. A total of 100 mitotic cells were examined for each cell line.
14% in RAD51B+/+/+ cells (Table 1). The expression of the RAD51B cDNA in the mutant reduced the frequency of such aberrations to 5%. Similarly, in metaphase, the frequency was 6% in WT cells, 13.5% in RAD51B+/+/+ cells, and 25.5% in RAD51B+/+/+ cells. The expression of the RAD51B cDNA in the mutant reduced the frequency to 10.5%. Thus, haploinsufficiency of RAD51B leads to increased centrosome fragmentation.

RAD51B haploinsufficiency leads to increased aneuploidy. Extra centrosome-like structures can unequally distribute chromosomes to daughter cells, which is thought to cause aneuploidy. We therefore examined the frequency of aneuploidy by fluorescence in situ hybridization (FISH) using two independent chromosome-specific centromere probes (Fig. 4C; Table 2). At chromosome 7, the frequencies of aneuploidy represented by one or three signals were 5.4% in WT cells, whereas the corresponding frequency was 9.8% in RAD51B+/+/+ cells and 14% in RAD51B+/+/+ cells. At chromosome 17, the frequencies of aneuploidy were 3.8% in WT cells, whereas the corresponding frequency was 7.4% in RAD51B+/+/+ cells and 10.2% in RAD51B+/+/+ cells. The differences between WT cells and the mutant cells were statistically significant (P < 0.05, Fisher's exact test). Expression of the RAD51B cDNA in the mutant reduced the frequency of aneuploidy to 6.4% at chromosome 7 and 4.2% at chromosome 17 (P < 0.05). Thus, haploinsufficiency of RAD51B leads to increased aneuploidy.

It is possible that RAD51B+/+ cells will acquire more copies of the WT allele and form significant subpopulations due to increased aneuploidy. To investigate this possibility, we examined a difference in RAD51B expression levels between originally isolated cells and cells passaged 25 times by Northern blot analysis and found no difference. Thus, despite the problem of chromosome segregation, the RAD51B+/+ status is stable.

A reduction in RAD51B levels leads to centrosome fragmentation in HT1080 cells. To confirm that a reduction in RAD51B levels leads to centrosome fragmentation in other human cells, we knocked down the gene in HT1080 cells by small interfering RNA (siRNA) transfection. Real-time PCR analysis revealed that the mRNA ratio of RAD51B/GAPDH was 2.5 ± 0.6 × 10−4 (mean ± SD; n = 3) in cells transfected with a control vector, whereas it was 1.2 ± 0.1 × 10−4 in cells transfected with a Rad51B knockdown vector. Thus, an ~50% reduction in RAD51B mRNA levels was achieved by RNAi. The frequency of aberrant numbers of interface centrosomes was 1.5% in cells transfected with the control vector, whereas it was 4% in cells transfected with the RAD51B knockdown vector (Table 1). In metaphase, the frequency was 2.5% in the control cells and 16.5% in RAD51B knockdown cells. Thus, like the haploinsufficiency of RAD51B in HCT116 cells, the 50% reduction in RAD51B mRNA levels in HT1080 cells leads to centrosome fragmentation.

Discussion

In the present study, we have shown that haploinsufficiency of RAD51B leads to centrosome fragmentation and increased aneuploidy in the human cancer cell line HCT116. In addition,
we have confirmed that Rad51B plays a role in homologous recombination repair in concert with Rad51 in human cells, which is consistent with the previous finding in DT40 cells (22). However, the absence of RAD51B haploinsufficiency in knockout mice and DT40 mutants may argue against this interpretation of the findings (22, 23). This apparent discrepancy may be the result of species differences and/or differences between cell types. One finding of the present study, that a 50% reduction in RAD51B mRNA levels by RNAi in HT1080 cells also leads to centrosome fragmentation, suggests that the effects of RAD51B haploinsufficiency may not be specific to HCT116 cells. Furthermore, the present findings are also supported by evidence of haploinsufficiency for XRCC2 in mouse cells (40). XRCC2−/− mice and XRCC2−/− DT40 cells have been shown to exhibit no apparent abnormalities (37, 41), whereas haploinsufficiency for the gene was clearly observed in chromosome aberrations and centrosome fragmentation in XRCC2−/− mouse embryonic fibroblasts. Rad51B forms a complex with Rad51C, Rad51D, and XRCC2 (BCDX2), suggesting a functional similarity between these Rad51 paralogues (42, 43). These subtle effects of haploinsufficiency on genomic instability, even if they are much weaker than the effects of homozygous mutations, are likely to be of considerable importance in carcinogenesis when they confer a growth advantage.

Although centrosome aberrations have been observed in cells defective in other recombination genes, the molecular mechanisms underlying these aberrations are unclear. Centrosome amplification has been shown to arise from a DNA damage-induced mechanism during the prolonged G2 phase in Rad51-deficient DT40 cells (14). Deletion of ATM reduced, but did not completely abolish, G2-phase centrosome amplification, thus indicating ATM-dependent and ATM-independent mechanisms. However, a recent study using CHO cells argues against this interpretation (44).

Centrosome aberrations found in cells deficient in a member of the Rad51 parologue family seem to be centrosome fragmentations (13, 15). A recent observation has indicated that centrosomes split into fractions containing only one centriole, which leads to the formation of multipolar spindles with extra centrosome-like structures in the presence of incompletely replicated or damaged DNA during mitosis (45). This result is also observed in CHO cells deficient in XRCC3, suggesting that aneuploidy in this cell line arises from extra centrosome-like structures. It is therefore possible that centrosome fragmentation resulting from haploinsufficiency of RAD51B could be explained by centrosome splitting.

Aneuploidy is a hallmark of genetic instability observed in human cancers, although the direct cause remains a matter of debate (46). Extra centrosome-like structures are likely to lead to the assembly of multipolar spindles, which may in turn lead to the unequal distribution of chromosomes to daughter cells. However, supernumerary centrosomes do not always result in multipolarity, as shown by a recent finding that dynein plays a role in the prevention of multipolar spindles by centrosomal clustering (47). Consistent with this finding, multipolar spindles are rarely detected in the rad51b mutant, despite the increase in the incidence of supernumerary centrosomes. It is therefore unlikely that multipolar spindles play a causal role in increased aneuploidy in this mutant. This raises the question of how supernumerary centrosomes lead to aneuploidy. A possible clue may come from the characterization of the practical consequences of centrosome amplification in p53−/− mouse embryonic fibroblasts (48, 49). In cells with two centrosomes at one spindle pole, a small fraction of chromosomes can be bioriented between incompletely separated centrosomes. Although these cells may divide in a bipolar fashion, this abnormal mitosis is likely to lead to the loss or gain of a few chromosomes.

From the viewpoint of tumor biology, it is of great importance that at least one allele of RAD51B is altered in some benign tumors, although the key pathologic alteration is still unclear. It is noteworthy that functional domains of Rad51B, such as Walker A and B motifs, are often lost as a consequence of the chromosome translocation, in contrast to the translocation partner HMGA2, the coding region of which is not rearranged by the translocation (28). It is well established that benign tumors do not usually develop into malignant tumors. However, a small proportion of benign tumors transforms into malignant tumors; <1% of uterine leiomyomas progress into uterine sarcomas. Given that the chromosome translocation involving RAD51B results in the loss of one functional RAD51B allele, the present finding that haploinsufficiency of the gene leads to aneuploidy implies chromosome instability in tumors harboring these translocations. The present study indicates that rad51b-mutant cells grow at a slightly slower rate than WT cells, implying that centrosome fragmentation and aneuploidy are not directly linked to a growth advantage. Aneuploidy is likely to contribute to a growth advantage only when genes that control cell growth are altered by the accumulation of chromosome instabilities. These observations lead to the hypothesis that the haploinsufficiency of RAD51B, even if not directly linked to malignant transformation, may play a role in the early steps of tumor development by inducing chromosome instability. To verify this hypothesis, a detailed analysis of RAD51B in association with pathologic and clinical studies in tumors involving 14q23-24 will be needed.

**Table 2. Distribution of chromosome numbers**

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NOTE: A total of 500 cells were scored for each line.

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