Detection of some mutations in some Mitotic Checkpoint Genes and their Association to Tissue Abnormalities in patients with Breast Cancer

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Abstract

Background: Breast cancer is a multifactorial disease, and several genetic and non-genetic factors contribute to its malignancy. The checkpoint genes Bub1, Bub1B, and MAD2L1 have been shown to be relatively uncommon mutations in cancer. This study aimed to screen for some mutations in some of the mitotic checkpoint genes, including MAD1L1, BUB1, and BUB1B. **Materials and Methods**: Thirty breast cancer biopsy specimens or formalin-fixed, paraffin-embedded (FFPE) tissue were isolated. Then, the patient information of the breast cancer patient was recorded. Histological examination and then genomic DNA extraction were performed for all samples. In the next step, DNA purity and concentration were estimated. For MAD1L1, BUB1 and BUB1B genes, initial design was performed and polymerase chain reaction (PCR) and then gel electrophoresis were performed for PCR products. Electrophoresis bands were cleaned and DNA was sequenced and finally statistical analysis was performed. **Results:** Histological examination showed differences in shape, orientation, and echo pattern between the invasive cancers and DCIS. An irregular shape (72% vs. 35%), a not parallel orientation (42% vs. 9%), and a hypoechoic or complex echo pattern (92% vs. 6%) were more frequent in invasive cancers when compared with the DCIS cases. The results of matching the sequence obtained according to Sanger method with Chromas version 2.6.6 software showed there were multiple mutations in study genes. **Conclusion**: In the present study, after histological examining and PCR product sequencing of all samples, it was determined that all the samples studied had altered genotypes in terms and this alteration associated to histological abnormalities **Keywords**: Breast Cancer, Mitotic Checkpoint Genes, PCR

Introduction:

The latest WHO estimates show that breast cancer is the most common malignancy worldwide in 154 of 185 countries and is the leading cause of cancer-related deaths in more than 100 countries. Globally, this cancer is the most common cancer among women, accounting for 25% of all recorded cancers in women (1,2). In Iraq, breast cancer ranks first among the top ten malignant neoplasms in society, including 19.5% of all cases and 34.3% of women's cancers cases. During 2016, 897 women died from this disease, which is the first cause of cancer deaths among Iraqi women (23.6%) and the second overall cause among men and women (12.1%) after bronchogenic cancer (3). The exact cause of breast cancer has not been identified so far and this disease is considered a multifactorial disease (4). Apart from genetic predisposition, many other factors such as demographic characteristics, clinical characteristics, fertility and environment can affect the incidence of breast cancer in women. Increased risk is associated with advanced age, positive family history, socioeconomic status, diet, endogenous or exogenous hormones, unusual breast diseases, benign tumors, oncogenic viruses, and exposure to carcinogens (5).

Early detection and diagnosis of this disease can be very effective in its treatment. Classification of women based on breast cancer risk factors can be effective in improving risk-free methods and designing targeted breast cancer screening programs (6,7). Breast tumors usually start from ductal proliferation and turn into benign tumors or even metastatic carcinomas after continuous stimulation by various carcinogenic agents (8). There are two hypothetical theories for the initiation and progression of breast cancer: the theory of cancer stem cells and the random theory (9). Cancer stem cell theory states that all tumor subtypes are derived from the same stem cells. Acquired genetic and epigenetic mutations in stem cells or progenitor cells lead to different tumor phenotypes. Random mutations can gradually accumulate in each breast cell (13). BUB1 gene is coding for protein kinase that binds to kinetochore and is involved in the regulation of cyclin-B levels. The gene coding for this protein is located on (2q13) with a length of 40,580 bp and contains 25 exons and 14 transcripts, and the highest expression of this gene has been observed in testicular tissue and lymph nodes. BUB1B gene also coding for protein kinase that plays a key role in SAC activity.

The location of the gene encoding this protein is located on (15q15.1), it has 23 exons and span along 60,128 bp, this gene is most expressed in testicular tissue, lymph nodes, and bone

marrow (21). MAD1L1 gene is a checkpoint component of the mitotic spindle and prevents the onset of anaphase until all chromosomes are properly positioned at the metaphase plate and located in 7p22.3, it has 21 exons, and a length of 8379 bp. MAD1L1 functions as a homodimer and interacts with MAD2L1. MAD1L1 may also play a role in cell cycle control and tumor suppression. This gene is expressed in testis, spleen and 25 other tissues (21). This study was aimed to screening of some mutations in some mitotic varicella genes (MAD1L1, BUB1 and BUB1B) and determining the effect of mutation in the pathological tissue.

Materials and methods:

DNA Extraction: Tissue samples were taken from 30 breast cancer patients and the DNA extraction was carried out by Quick-DNA™ FFPE Kit depending on the manufacturing procedure.

Polymerase Chain Reaction: In order to amplify the desired fragment for each SNP, PCR reaction was performed on the extracted DNA of all samples. For this purpose, the primer sequences were first selected. Then, the specific primers of each SNP were blasted on the NCBI site, and thus the sequence of the primers was determined (Table 4-2).

Table (1): Specific primers for BUB1, BUB1B and MAD1L1 genes

Gene	Sequence (5′→3′ direction)	Cg%	Tm (°C)	Product Size(bp)
BUB1	GCCTGGCTTTGTTTGTGTTT	55%	58	192
	GCCTGGCTTTGTTTGTGTTT	57%	58	
BUB1B	TGAGGCCACAGTGTCTGTTC	60%	62	174
	CTGAGGCAGCAATCTGTGAG	58%	62	
MAD1L1	ATGCCTGCTCTCCTCACTGT	62%	60	242
	GCTTCTTTCCCCAATTAGCC	60%	62	

Maxime PCR PreMix kit (i-Taq) 20µlrxn: Is a product that contains a mixture of the following components: i-Taq DNA Polymerase, dNTP mix, reaction buffer. The first reason is that it has all the components of PCR, so we can perform PCR by just adding template DNA, primer set and D.W.

PCR Reaction and steps: The materials required for PCR reaction to BUB1, BUB1B and MAD1L1 genes was carried out by mixing:5 µl Taq PCR PreMix, 10 picomols (1µl) Forward and Reverse primer, DNA templet 2 µl and 16 µl D.W. The PCR steps are shown in Tables (2).

Table (2): PCR Reaction Temperature cycle for BUB1, BUB1B and MAD1L1 genes

Steps	Temperature	Time	No. Of cycles
Initial denaturation	95 C°	5 min	1 cycle
Denaturation	95C°	45sec	35cycles
Annealing of BUB1	58 C°	45sec	
Annealing of BUB1B	60 C°		
Annealing of MAD1L1	62 C°		
Extension	72C°	45 sec	
Final extension	72C°	5 min	1 cycle
Holding	4 C°	10 min	1 cycle

Gel electrophoresis: Electrophoresis was used to confirm the target size. PCR products were separated on 2% agarose gel electrophoresis and observed by exposure to ultraviolet light (302 nm) after staining with ethidium bromide.

Gene Sequencing: was performed by Macrogen Korea, Homology search using Basic Local Alignment Search Tool (BLAST) program available at National Center for Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm. nih.gov) is available and the BioEdit program

was performed. PCR products were purified and sequenced using BigDye Terminator v3.1 Cycle Sequencing kit on ABI 3130 Genetic Analyzer.

Sequences Analysis: The analysis of nucleotide databases using NCBI's Basic Local Alignment Search Tool Bio ID program for sample identification and submitted to GenBank (ID). Sample sequences were obtained from the NCBI nucleotide database (www.ncbi.nlm.gov/nucleotide) and included in a multiple alignment using the Bio ID program.

Histological Examination: The breast specimens were formalin-fixed, paraffin-embedded tissue blocks subsequently stained with hematoxylin and eosin. Histological tumor types were divided into invasive cancers and ductal carci-noma in situ (DCIS). Invasive cancer was graded as grade 1 (well differentiated), grade 2 (moderately differentiated), or grade 3 (poorly differentiated) according to the Scarff-Bloom-Richardson System (1957). DCIS cases were classified as group 1 (nonhigh grade DCIS without comedo-type necrosis), group 2(nonhigh grade DCIS with comedo-type necrosis),or group 3 (high grade DCIS with or without comedo-type necrosis) according to the Van Nuys Classification (1995)

Statistical Analysis: In order to perform statistical analysis, first all the data were entered in an excel file. Then, using spss software version 22, the variables were compared based on the chi-square test, and P value less than 0.05 was considered significant.

Results and discussion:

Obtained from DNA extraction After performing PCR, we examined the resulting fragments on the gel. The primary results are given in Table (1-3). In this table, the sign + and - means getting or not getting the result. After DNA extraction, the extracted samples were electrophoresed on a 2% agarose gel and observed with a gel dock device. The results of the extracted samples on 2% agarose gel are shown in figures (1,2,3)

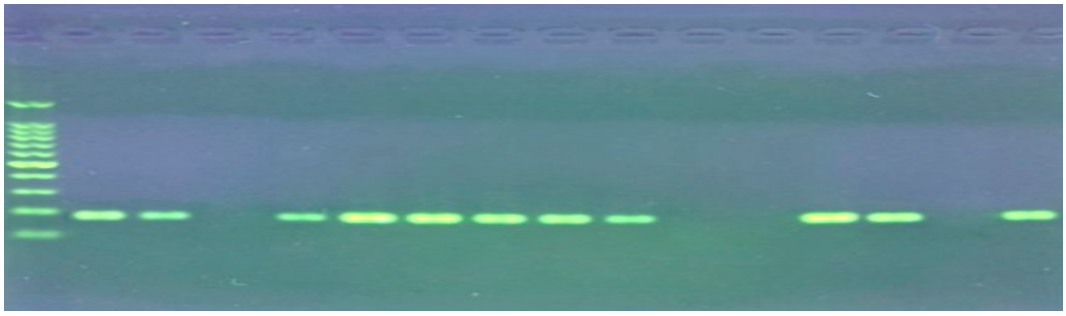

Figure (1): BUB1 gene PCR product. The band size is 192 bp. The product was electrophoresed on 2% agarose with a voltage of 25 volts/cm.

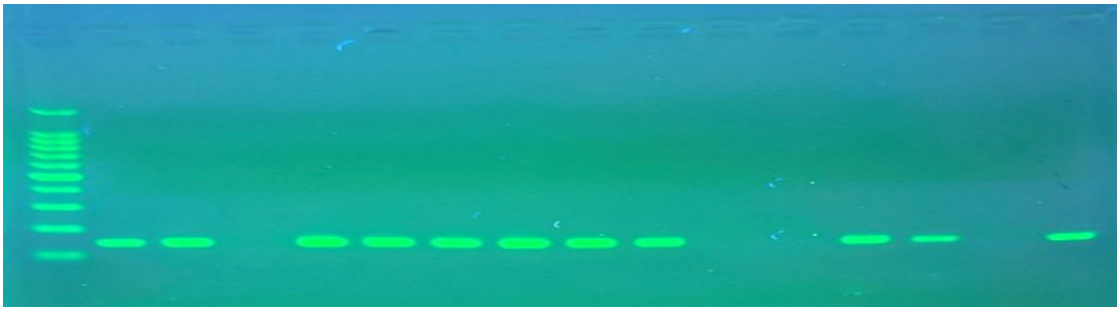

Figure (2): PCR product of BUB1B gene. The band size is 174 bp. The product was electrophoresed on 2% agarose with a voltage of 5 volts/cm2.

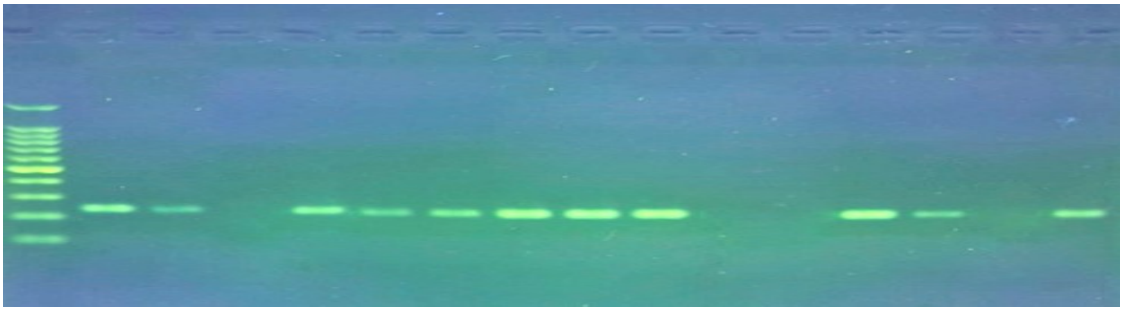

Figure (3): MAD1L1 gene PCR product. The band size is 242 bp. The product was electrophoresed on 2% agarose with a voltage of 5 volts/cm2.

Results obtained from trench sequencing:

PCR products confirmed by gel electrophoresis were sequenced by the Sanger method with Chromas version 2.6.6 software, which are shown in Figure (3-4).

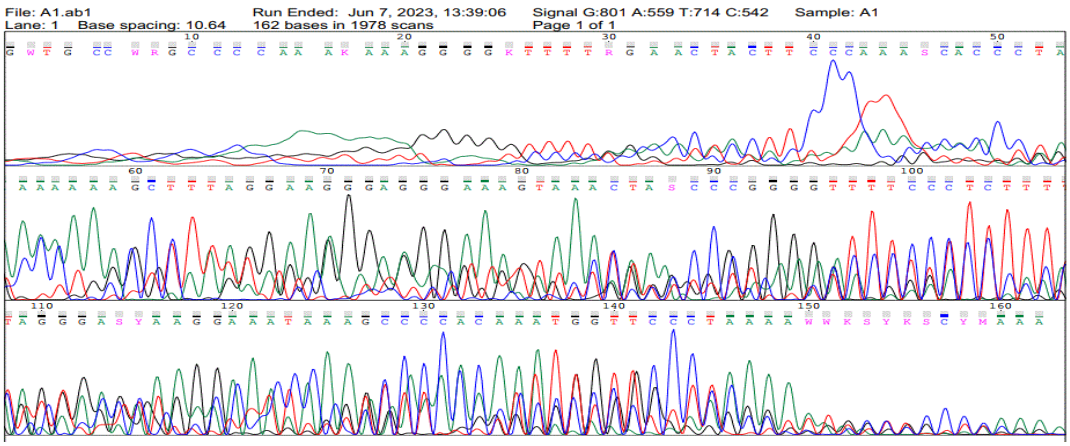

Figure (4): Diagram resulting from sequencing with Chromas software

Table (4): The results of matching the sequence according to Chromas version 2.6.6 software

Gene	W.T	Mutant	Site	Name of mutation	a.a change	Type of Mutation
BUB1	CCA	CTT	2641	Sub:CA>TT	Q > L	Substitution
	ATAA	----	2730	Del: ATAA	---	Frameshift
	TCCGT	-----	3030	Del:TCCGT	---	Frameshift
BUB1B	CAT	CGG	2778	Sub:AT>GG	H>R	Substitution
	AAA	TAA	2962	Sub:A>T)	N>stop	Substitution
	TCT	TAT	3001	Sub:C>A	S > R	Substitution
MAD1L1	CCA	CTT	2641	Sub:CA>TT	Q > L	Substitution
	TAT	TTT	2823	Sub:A>T	R>P	Substitution
	C--	CAA	3069	Ins:AA	---	Frameshift
	CTA	CAA	3078	Sub:T>A	L> Q	Substitution

After examining the sequence of all the samples, it was found that all the examined samples had altered genotypes in terms of the variants in these regions and in their correspondence with the reference sequence, genetic changes were observed in the variants in question and the upstream and downstream regions that were sequenced.

Histological Examination Results:

Biological markers correlated with the histological grade in invasive cancers. ER negativity, PR negativity, and HER-2/neu positivity were more frequent in grade 3 invasive cancers than in grade2/grade 1 invasive cancers (pB0.0001). There was no significant difference between invasive cancers and DCIS for the presence of the biological markers (p0.05). Results of the univariate and multivariate regression models comparing the ultrasound findings of the 30 breast cancers. Differences were seen in shape, orientation, and echo pattern between the invasive cancers and DCIS. An irregular shape (72% vs. 35%), a not parallel orientation (42% vs. 9%), and a hypoechoic or complex echo pattern (92% vs. 6%) were more frequent in invasive cancers when compared with the DCIS cases.

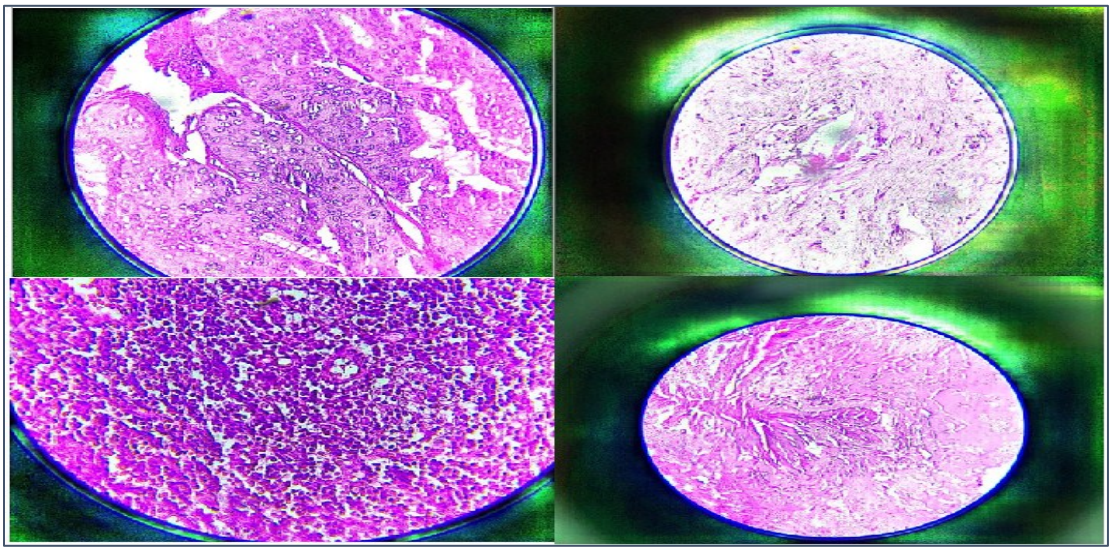

Figure (5): Images of breast Cancer tissues in patients

Statistical Review of patients Information:

The total number of patients was 30, the youngest of whom was 20 years old and the oldest was 68 years old. The average age of the patients was 45.6 years. Out of this number, 7 people (23.33%) were men, the rest (76.66%) were women. The distribution of patients in age groups is given in Table (5).

Table (5): Age range of patients

Age	Number	Percentage
20-30	3	10%
31-40	9	30.0%
41-50	7	23.33%
51-60	7	23.33%
61-70	4	13.33%

According to table (6) and based on the results of Chi-square statistical test, there is a significant difference between age in both male and female groups.

Table (6): Chi-square test results between male and female groups

Variable	X^2	Df	P value
Age	20/417	4	0/0000
Number	30		

Discussion:

Cancer is one of the most common diseases worldwide and the second cause of death after cardiovascular diseases. Breast cancer is the most common type of cancer among Iraqi women, because it accounted for the highest percentage of malignant tumors in women until 2018 (34). Breast cancer involves the patient, family and society and wastes many financial and spiritual resources. Although the primary cause of breast carcinoma is unclear, many risk factors have been documented. It lists "smoking, alcohol consumption, and diet" as factors that can vary and depend

on lifestyle. On the other hand, other factors have been documented "age, race, gender and family history". In addition, hormones play an important role in some forms of breast carcinogenesis. It can be said here: "Aging, a history of breast cancer in the family, specific changes in the breasts, genetic changes, history of pregnancy and menopause, physical inactivity, alcohol consumption, diet and nutrition, race and radiation therapy to the chest are risk factors for breast tumorigenesis. (35). Several studies have shown that low expression levels of key mitotic spindle checkpoint genes can help control defective spindle checkpoints in cancer. For example, according to reports, MAD2L1 is less expressed in some breast and ovarian cancers (36). Apparently, such reduced expression has functional significance, because deletion of the MAD2L2 allele leads to a defective mitotic checkpoint in human cancer cells and early embryonic fibroblasts (37) and haploid insufficiency of BUB1B in mice leads to defective mitotic arrest as well as tumors. It becomes (38).

In this study, 30 breast cancer patients were sampled. The average age of these patients was 45.6 years, the youngest was 20 years old and the oldest was 68 years old. Of these, 7 were men and 23 were women. The results of SPSS analysis showed that there is a significant relationship between the age of disease and gender (P=0.000). This work showed 30 affected patients were sampled to investigate the relationship between mitosis checkpoint gene mutations and breast cancer tissue abnormalities. DNA was extracted from the tissues and then PCR. Sequencing was done by Sanger method. In the next step, we compared the obtained sequence with NCBI to see if the sequence we obtained is the same with the sequence available in NCBI.

Hempen et al. (2003) sequenced the entire coding regions of the BUB1 and BUB1B genes in pancreatic cancer cell lines and xenografts to determine sequence alterations of the BUB1 and BUB1B genes in pancreatic cancer. Although only polymorphic changes were found in the BUB1B gene, the aneuploid pancreatic cell line Hs766T had two novel missense variants (p.[Y259C; H265N]) in the BUB1 gene. These mutations were on the same allele associated with a wild-type BUB1 allele. This change was not found in other samples, articles, or 110 additional chromosomes from a reference population. Compared to two cell lines with microsatellite instability (MIN), the wild-type pancreatic cell line TP53 Hs766T had a defective mitotic spindle checkpoint, indicating a cell line with chromosomal instability (CIN) (39). The results of this study are consistent with our results.

Langerød et al (2003) selected 20 cases with genomic instability by comparative genomic hybridization (CGH), and without somatic TP53 (p53) mutations, and sequenced the entire coding region of the BUB1 gene. The results showed that genomic instability as copy number changes by CGH in TP53 wild-type breast carcinomas is not caused by somatic mutations in the BUB1 gene (40). The results of this study were not consistent with our study.

Mafalda et al. (2008) determined the mRNA expression levels of major mitotic checkpoint genes that were not inhibited by the benzimidazole family (BUB1, BUBR1, BUB3) and the MAD gene family (MAD1, MAD2L1, MAD2L2) by quantitative PCR in 39 cc and in 36 samples. The normal kidney tissue was analyzed. analyzed these tumors by comparative genomic hybridization (CGH) to assess the relationship between mitotic checkpoint defects and the pattern of chromosomal alterations in this subset of RCC. BUB1, BUBR1, MAD1 and MAD2L1 showed significant differences in tumor tissue compared to the control group (BUB1, BUBR1 and MAD2L1 were overexpressed, while MAD1 was under expressed). Overexpression of BUB1 and BUBR1 was significantly associated with the number of genomic copy number changes ($p<0.001$ for both genes) (41). This study was consistent with the results of our study.

M. de Voer et al. (2013) performed genome-wide analysis of copy number and genetic mutations of 208 patients with familial or early-stage (40 years or younger) colorectal cancer. Heterozygous or haploid insufficiency mutations in BUB1 and BUB3 genes of spindle assembly were identified in 2.9% of them. In addition to colorectal cancer, these patients had various aneuploidies in multiple tissues and variable malformation features. The results showed that mutations in BUB1 and BUB3 cause mosaic aneuploidy and increase the risk of developing colorectal cancer at a young age (42). This study was consistent with the results of our study.

Germline mutations in BUB1 and BUB3 have been reported to increase the risk of developing colorectal cancer (CRC) at a young age, in the presence of variegated aneuploidy and malformation features reminiscent of mosaic aneuploidy syndrome. We performed a mutational analysis of BUB1 and BUB3 in 456 hereditary non-polyposis CRC families and 88 polyposis cases. Four novel or rare germline variants, one splice site and three missense variants, were identified in four families. Neither diverse aneuploidy nor malformation traits were observed in the carriers. Obvious functional effects were observed in the heterozygous form for c.1965-1G>A, but not for c.2296G>A (p.E766K), despite common positive segregation in the family. BUB1 c.2473C>T

(p.P825S) and BUB3 c.77C>T (p.T26I) remained uncharacterized significant variants. As of today, the rarity of gain-of-function mutations identified in familial series and/or early onset does not support the inclusion of BUB1 and BUB3 testing in routine genetic diagnostics of familial CRC.

Conclusion:

Many genetic and environmental factors including demographic characteristics, clinical, reproductive and environmental characteristics, advanced age, positive family history, socioeconomic status, diet, endogenous or exogenous hormones, unusual breast diseases, benign tumors, oncogenic viruses and exposure Carcinogens cause breast cancer.In the present study, after examining the sequence of all the samples, it was found that all the investigated samples had altered genotypes in terms of the variants in these regions, and in their correspondence with the reference sequence, genetic changes were observed in the variants in question and the sequenced upstream and downstream regions. ; Therefore, further study in this field is also recommended to examine the resulting changes in the sequence of genes involved in mitotic checkpoints.

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