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Mutation analysis of *p31^{comet}* gene, a negative regulator of Mad2, in human hepatocellular carcinoma

Mi-Yong Yun[1,2], Sang Bum Kim[1], Sunhoo Park[1], Chul Ju Han[1], Young-Hoon Han[1], Sun Hee Yoon[1], Sang Hoon Kim[3], Chang-Min Kim[4], Dong-Wook Choi[1], Myung-Haing Cho[5], Gil-Hong Park[2] and Kee-Ho Lee[1,6]

[1]Laboratory of Radiation Molecular Oncology
Division of Radiation Cancer Biology
Korea Institute of Radiological and Medical Sciences
Seoul 139-706, Korea
[2]Department of Biochemistry
College of Medicine, Korea University
Seoul 136-701, Korea
[3]Department of Biology
Department of Life and Nanopharmaceutical Sciences
Kyung Hee University
Seoul 130-701, Korea
[4]National Cancer Center
Goyang 410-769, Korea
[5]Laboratory of Toxicology
College of Verterinary Medicine
Nano Systems Institute-National Core Research Center
Seoul National University
Seoul 151-742, Korea
[6]Corresponding author: Tel, 82-2-970-1312/1314;
Fax, 82-2-970-2402; E-mail, khlee@kcch.re.kr

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Abbreviations: APC, anaphase-promoting complex; HCC, hepatocellular carcinoma

Abstract

Failure of mitotic checkpoint machinery leads to the chromosomal missegregation and nuclear endoreduplication, thereby driving the emergence of aneuploidy and tetraploidy population. Although abnormal nuclear ploidy and the resulting impairment of mitotic checkpoint function are typical physiological event leading to human hepatocellular carcinoma, any mutational change of mitotic checkpoint regulators has not yet been discovered. Therefore, we investigated the mutation of p31^{comet}, a recently identified mitotic checkpoint regulator, in human hepatocellular carcinoma. Of 51 human hepatocellular carcinoma tissue and 6 cell lines tested, five samples exhibited nucleotide sequence variations dispersed on four sites within the entire coding sequence. Among these sites with sequence substitutions, three were found to be missense mutation accompanied with amino acid change but one was a silent mutation. Of these sequence substitutions, two were present in both tumor and non-tumor liver tissues, suggesting the possibility of polymorphism. The present findings indicate that p31^{comet} does not have an impact on the formation of aneuploidy and tetraploidy found in human hepatocellular carcinoma.

Keywords: carcinoma, hepatocellular; MAD2L1 protein, human; mitosis; mutation

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in worldwide, with a high incidence in developing country (Parkin *et al*., 1999). Hepatitis virus infection, alcohol consumption, and aflatoxin B1 are major factors for hepatocarcinogenesis (Hardell *et al*., 1984; Nishioka *et al*., 1991; Feitelson, 1999; Kops *et al*., 2004). However, mechanism of hepatic carcinogenesis is not well known.

Aneuploidy, a phenotype associated with the loss or gain of chromosome number is a critical feature of many types of human cancers (Mitelman, 1983). The defect of mitotic checkpoint plays a major role in inducing the chromosome instability and aneuploidy (Kops *et al*., 2005). The mutations of mitotic checkpoint such as BubRI and Bub I has been identified in a variety of human cancers (Haruki *et al*., 2001; Saeki *et al*., 2002; Lee, 2003; Baker *et al*., 2004; Hanks *et al*., 2004).

Mad2 is a key regulator of spindle checkpoint, by inducing mitosis arrest through the inhibition of APC complex (Fang *et al*., 1998; Luo *et al*., 2000). However, recent reports studying the mutation or expression of Mad2 showed that its defect was not a key factor in inducing tumorigenesis of various types of human tumors. Mutation of human Mad2 gene is very rare in breast, liver, lung and bladder cancers (Takahashi *et al*., 1999; Gemma *et al*., 2001; Hernando *et al*., 2001).

On the other hand, several papers reported an interesting result that argues against the above view of the nonessential role of Mad2 in tu-

morigenesis. Michel and his colleagues observed that near complete depletion of Mad2 protein level by RNA interference induces mitotic failure and apoptosis in tumor and primary human somatic cells (Michel *et al*., 2004). Inhibition of spindle checkpoint signaling by ablating Mad2 results in being invariably lethal as a consequence of massive chromosome loss (Kops *et al*., 2004). These previous reports suggest that Mad2 depletion may play a major role in the suppression of tumorigenesis in many types of human tumors. In the present work, we analyzed the mutation and expression of p31^{comet}, a binding partner for Mad2 (Habu *et al*., 2002; Xia *et al*., 2004; Mapelli *et al*., 2006), in primary human hepatocellular carcinoma tissues and cell lines.

Materials and Methods

Patients and tissue samples

Surgical specimens were obtained from 51 patients who underwent surgical treatment for HCC at Korea Institute of Radiological and Medical Sciences (KIRAMS). Informed consent was obtained from patients for the use of their operated specimens for research purpose. All tissues were surgically resected, immediately frozen and stored in liquid nitrogen until analyzed. Both tumor and adjacent non-tumor liver tissues were collected.

Cell culture and RNA preparation

Four different HCC cell lines, SNU354, SNU368, SNU387 and SNU475, were obtained from Korean Cell Line Bank (http://cellbank.snu.ac.kr/) (Park *et al*., 1995). HepG2, Hep3B, SK-HEP-1 and PLC/PRF/5 cell lines were purchased from ATCC (ATCC, Manassas, VA). These cell lines including Chang liver and Huh7 were cultured in DMEM supplemented with 10% FBS. Total RNA was extracted from the cultured cells and primary tissues using a RNA Isolation kit (QIAGEN) according to the protocol recommended by the manufacturer.

PCR-SSCP and automatic sequencing analysis

Each mRNA sample was examined for the presence of a mutation in the *p31^{comet}* gene by PCR-SSCP analysis. In brief, for reverse transcription, RNA mixture including 1 μg extracted total RNA and RNasin (Promega) was heated at 65°C for 10 min, and then cooled on ice. Reverse transcription reaction was carried out in 20 μl of reverse transcription mixture containing 4 μl of reverse transcription buffer (Gibco BRL), 10 mM of dNTPs, 10 pmol Oligo (dT)15 primer, 10 mM DTT, 200 U M-MulV RTase reverse transcriptase (Gibco BRL) and 5 μl RNA mixture. After incubation at 37°C for 60 min, PCR using reverse-transcribed cDNAs was performed by using 4 sets of oligonucleotide primers for *p31^{comet}* in the presence of [α-^{32}P]dCTP. Oligonucleotide primers used for PCR amplification are shown in Table 1. Thirty-five cycles were used for each primers and consisted of 45 s denaturation at 95°C, 45 s annealing at 56°C, 1 min polymerization at 72°C, and final 10 min extension at 72°C. RT-PCR products were electrophoretically separated on 6% non-denaturing polyacrylamide gels in the presence of 5% glycerol at room temperature, and drying the gels, the gels were exposed to X-ray films at -80°C overnight. The RT-PCR products of primary HCC specimens, and cell lines were subjected to automatic sequencing analysis.

Table 1. Oligonucleotide primers used for PCR amplification of the *p31^{comet}*.

Primer	Sequence	Products size
S1	cccgcgagacctttattcta	450
A1	cctctgccctatgaacagct	
S2	atcatgtatcaacgccagca	432
A2	cttccgagccatattcatgg	
S3	ctgagcacagcagcttgttt	432
A3	aatcacccatctggtttggag	
T-S	tattctaaccgcaaggagta	870
T-A	gcttcttaatcctaaaaacacaatgg	

Semi-quantitative RT-PCR

The RT procedure for semi-quantitation was conducted with the same method as described in RT-PCR-SSCP and automatic sequencing analysis. In brief, PCR was carried out for each gene using S1 and A1 primers (Table 1) for a 450-bp fragment of human *p31^{comet}* and 5'-atcatgtttgagaccttcaacacccc-3' (sense primer) and 5'-catctcttgctcgaagtccagggcga-3'(antisense primer) for a 317-bp fragment of human β-actin. To quantitate the expression level of *p31^{comet}*, β-actin was used as an internal control. The β-actin oligonucleotide primer was added to the same reaction tube and the PCR products were harvested at every two cycle intervals from cycle 28 to cycle 40. Each amplified sample was subjected to electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide.

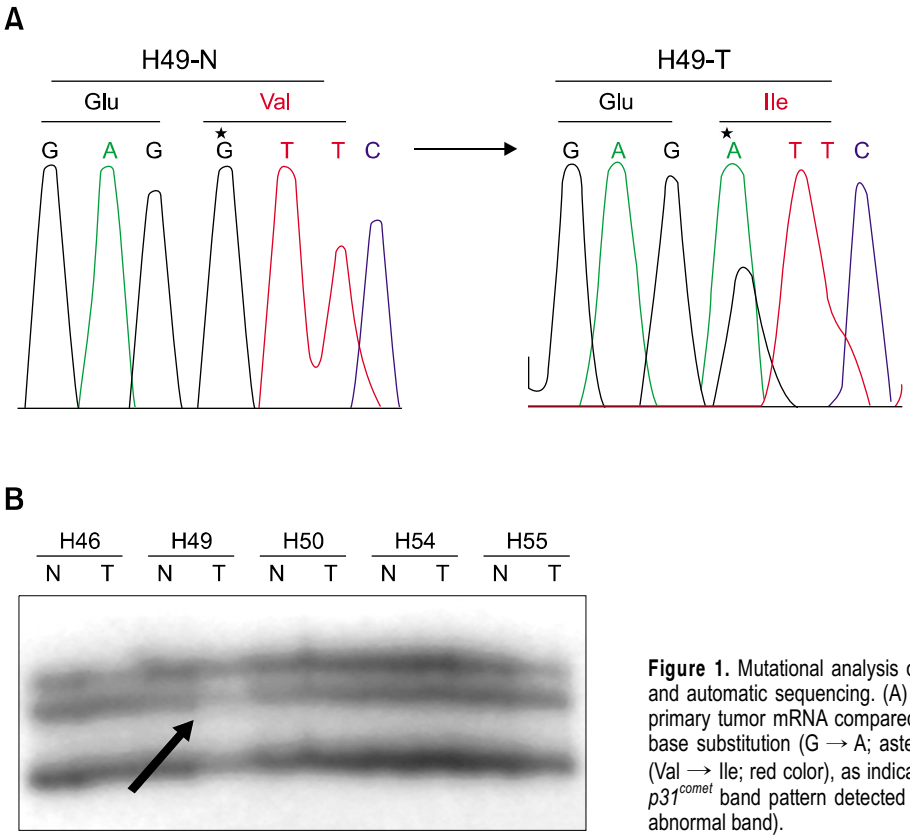
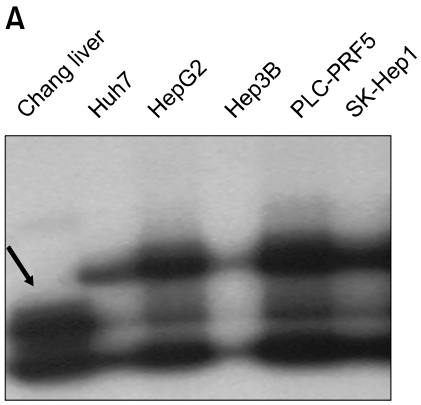


Figure 1. Mutational analysis of the human *p31*^{comet} gene by PCR-SSCP and automatic sequencing. (A) Automatic sequencing of PCR products of primary tumor mRNA compared to those of non-tumor mRNA. The single base substitution (G → A; asterisk) produces the amino acid substitution (Val → Ile; red color), as indicated in the forward sequence. (B) Abnormal *p31*^{comet} band pattern detected by PCR-SSCP in H49 (an arrow indicates abnormal band).

Results

Mutation of *p31*^{comet} gene in human hepatocellular carcinoma

From 1.28 kb mRNA sequence data of human *p31*^{comet} (Habu *et al*., 2002), we designed 4 primer sets to cover and overlap the entire coding region for the sequence analysis in human hepatocellular carcinoma (Table 1). For cross-checking sequence abnormality and allelic variation, two analytical methods, PCR-SSCP and automatic nucleotide sequence were adopted for the present study. Of 51 human hepatocellular carcinoma specimens, we identified that four cases (7.8%) harbored sequence variations within the coding region of *p31*^{comet} (Figure 1A). At nucleotide 19 near N-terminal region, a G to A transversion was accompanied with amino acid change from valine to isoleucine (sample #; H49). PCR-SSCP analysis further supports that this substitution occurred only on one allele (Figure 1A). The second sequence variation at nucleotide sequence 226, located at 31 bp apart from Mad2 binding site (Habu *et al*., 2002), was a C to G transversion changing an amino acid from leucine to valine (sample #; H9). The third substitution was a G to T transversion located at nucleotide 602, changing an amino acid from serine to isoleucine. This type of substitution was shown in two patient specimens (sample #; H53 and H61). Of these mutations, the sequence variation at nucleotide 19 was found to be present only in tumor specimens but not in counterpart non-tumor liver tissues (Figure 1A and 1B). However, the other two sequence variations at nucleotide 226 and 602 were observed in both tumor and non-tumor liver tissues, suggesting the possibility of polymorphism on *p31*^{comet} coding region. In an analysis using liver cancer cell lines, Chang liver cell line exhibited the aberrant band shift (Figure 2A), and a single nucleotide substitution without amino acid change at nucleotide position 720 (Figure 2B), indicating the silent mutation. The types of sequence variations of *p31*^{comet} found in hepatocellular carcinoma tissues and cell lines was summarized (Table 2), three with amino acid substitution and one without amino acid change. The previous reports that Mad2 mutation frequency is extremely low (Takahashi *et al*., 1999; Gemma *et al*., 2001; Hernando *et al*., 2001), together with our present findings illustrate a notion



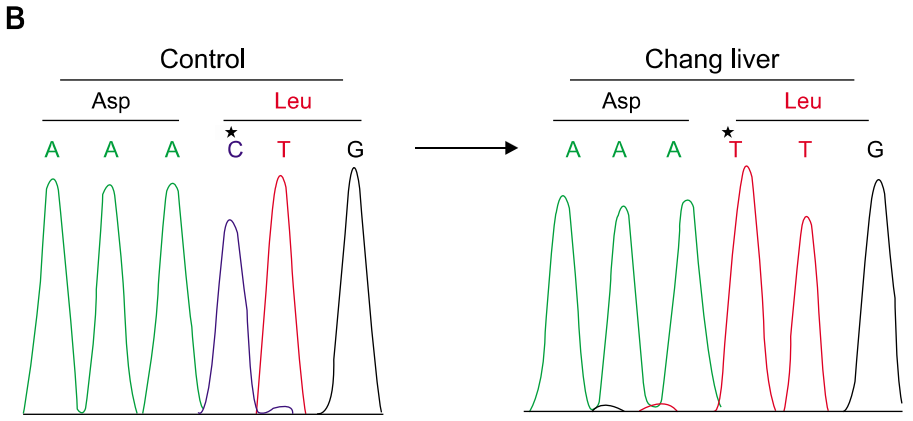


Figure 2. Mutational analysis of the human *p31*^{comet} gene by PCR-SSCP and automatic sequencing in hepatocellular carcinoma cell lines. (A) Abnormal *p31*^{comet} band pattern detected by PCR-SSCP in Chang liver cell (an arrow indicates abnormal band). (B) Automatic sequencing of PCR products of Chang liver cell's mRNA compared to those of primary non-tumor mRNA. The single base substitution (C → T; asterisk) produces no substitution of the amino acid.

Table 2. Identification of mutation in the *p31*^{comet} gene.

Nucleotide position	Substitution		Sample ID
	Nucleotide	Amino acid	
19	GTT→ATT	Val→Ile	H49
226	CTT→GTT	Leu→Val	H9
602	AGC→ATC	Ser→Ile	H53, H61
721	CTG→TTG	No change	Chang liver cell

that a circuit of p31^{comet} and Mad2 may not contribute to the formation of aneuploidy and tetraploidy found in human hepatocellular carcinoma.

Transcriptional level of p31^{comet} in human hepatocellular carcinoma

Expression of human p31^{comet} gene varies during cell cycle progression in which the level transiently increases at mitosis phase (Habu *et al.*, 2002). Due to the relatively low frequency of *p31*^{comet} mutation, we attempted to examine the expressional level in hepatocellular carcinoma. In an expression analysis of 51 human hepatocellular carcinoma tissues by quantitative RT-PCR, the majority of them including samples with p31^{comet} mutation did not exhibit differences of p31^{comet} transcriptional level between tumor and non-tumor liver tissues (Figure 3A and 3B). The absence of the expression variation was further confirmed in 8 human liver cancer cell lines (Figure 3C). The present expression analysis suggests that p31^{comet} transcriptional level, itself, may not contribute to the formation of aneuploidy or teraploidy in human hepatocellular carcinoma.

Discussion

Disruption of genes participating in the regulation of mitotic checkpoint can lead to abnormal chromosome segregation, and then bear the aneuploid cells. Mad2 is one of the major components in the regulation of mitotic checkpoint. Dysfunction of Mad2 in tumor has been reported in a few paper, however its role in cancer development is unclear. Saeki and Tamura reported that about 65% of

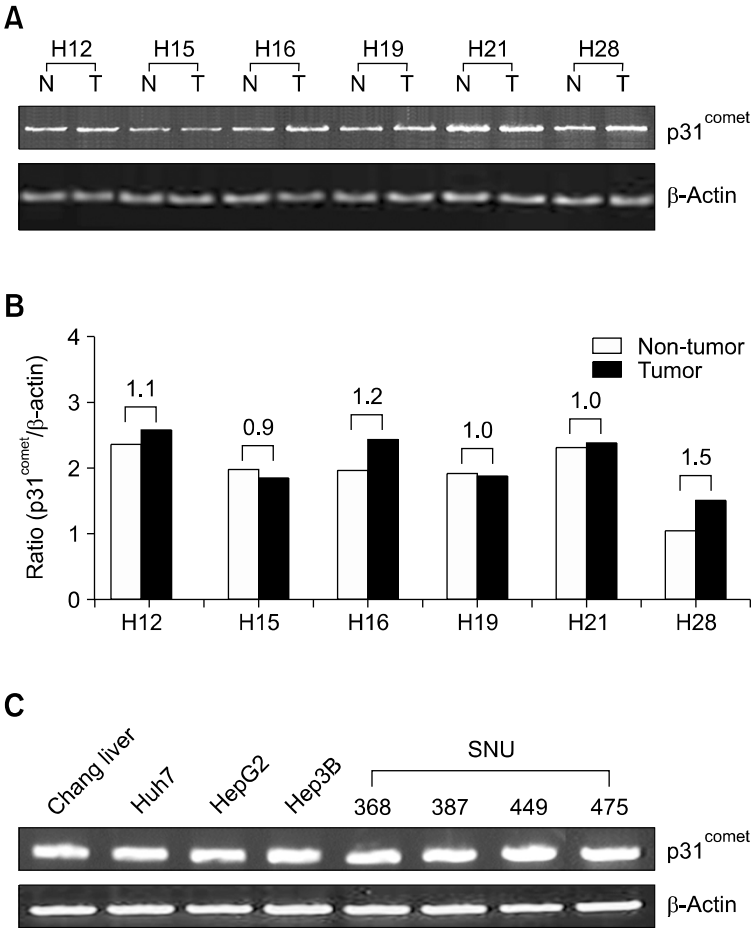


Figure 3. Semi-quantitative RT-PCR of human *p31*^{comet} in hepatocellular carcinoma primary tissues and cell liens. Transcriptional levels of *p31*^{comet} in each primary tissue (A) and HCC cell line (C) were measured by quantitative RT-PCR. β-actin gene expression was used on an internal control. Similar results were obtained from three different experiments. (B) To determine the levels of p31^{comet}, the images of p31^{comet} and β-actin band intensities were scanned and the relative p31^{comet} mRNA levels (ratios of the values of p31^{comet} mRNA/β-actin mRNA) of HCC tissues were compared with those of non-tumor liver tissues. The cut off value of the relative ratio for counting differential expression between HCC and corresponding non-tumor liver tissues was 2-folds, according to the previous quantitative analysis (Kim *et al.,* 2004). Ratios of tumor versus non-tumor are shown on top of the graph.

aneuploid HCC cell lines exhibited an impaired spindle checkpoint, but they could not find any mutation in mitotic checkpoint genes (Saeki *et al*., 2002). These data suggest that mutations of other mitotic checkpoint regulator may contribute to induce tumorigenesis of liver. In the present study, we analyzed the mutation and expression of *p31*^{comet}, a mitotic checkpoint regulator counteracting Mad2 function. We performed mutation analysis of the *p31*^{comet} gene in 6 human hepatocellular carcinoma cell lines and 51 primary tumor tissues. Four different nucleotide substitutions were detected on the coding region of *p31*^{comet} (Table 2). Of these four mutations, three were defined as a somatic missense mutation, a substitution accompanying with amino acid change and one was a silent mutation without amino acid change. Since two types of the somatic mutations were found in both tumor and non-tumor liver tissues, we can not exclude the possibility whether these mutations might be a polymorphism.

p31^{comet} has a Mad2 binding region from 55 to 64 amino acids and is detected as a 34 kDa protein on SDS-PAGE gel (Habu *et al*., 2002). Transient depletion of p31^{comet} by antisense oligonucleotide induces a minor apoptosis following a delay in the onset of anaphase in HeLa cells treated with nocodazole, a spindle poison agent (Habu *et al*., 2002). Similar to the depletion of p31^{comet}, overexpression of p31^{comet} was found to induce strongly cell death of HCC cell lines without the treatment of nocodazole (unpublished data), indicating that the abnormal expression of p31^{comet} may drive the cells to the pathway for apoptosis induction. However, the mutation frequency of p31^{comet} in HCC tumor specimens was low and the transcriptional level was shown to be similar to their corresponding non-tumor liver tissues. In conclusion, as predicted from the results describing low level of mutation rate and expression variation of the *p31*^{comet} gene, p31^{comet} may not play a role in the pathogenesis of human liver cancer. We need further study for the analysis of *p31*^{comet} mutation and malfunction in other primary tumor

tissues.

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References

Baker DJ, Jeganathan KB, Cameron JD, Thompson M, Juneja S, Kopecka A, Kumar R, Jenkins RB, de Groen PC, Roche P, van Deursen JM. BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. Nat Genet 2004;36:744-9

Fang G, Yu H, Kirschner MW. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev 1998;12:1871-83

Feitelson MA. Hepatitis B virus in hepatocarcinogenesis. J Cell Physiol 1999;181:188-202

Gemma A, Hosoya Y, Seike M, Uematsu K, Kurimoto F, Hibino S, Yoshimura A, Shibuya M, Kudoh S, Emi M. Genomic structure of the human MAD2 gene and mutation analysis in human lung and breast cancers. Lung Cancer 2001;32:289-95

Habu T, Kim SH, Weinstein J, Matsumoto T. Identification of a MAD2-binding protein, CMT2, and its role in mitosis. EMBO J 2002;21:6419-28

Hanks S, Coleman K, Reid S, Plaja A, Firth H, Fitzpatrick D, Kidd A, Mehes K, Nash R, Robin N, Shannon N, Tolmie J, Swansbury J, Irrthum A, Douglas J, Rahman N. Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B. Nat Genet 2004;36:1159-61

Hardell L, Bengtsson NO, Jonsson U, Eriksson S, Larsson LG. Aetiological aspects on primary liver cancer with special regard to alcohol, organic solvents and acute intermittent porphyria--an epidemiological investigation. Br J Cancer 1984;50:389-97

Haruki N, Saito H, Harano T, Nomoto S, Takahashi T, Osada H, Fujii Y, Takahashi T. Molecular analysis of the mitotic checkpoint genes BUB1, BUBR1 and BUB3 in human lung cancers. Cancer Lett 2001;162:201-5

Hernando E, Orlow I, Liberal V, Nohales G, Benezra R, Cordon-Cardo C. Molecular analyses of the mitotic checkpoint components hsMAD2, hBUB1 and hBUB3 in human cancer. Int J Cancer 2001;95:223-7

Kim BY, Lee JG, Park S, Ahn JY, Ju YJ, Chung JH, Han CJ, Jeong SH, Yeom YI, Kim S, Lee YS, Kim CM, Eom EM, Lee DH, Choi KY, Cho MH, Suh KS, Choi DW, Lee KH. Feature genes of hepatitis B virus-positive hepatocellular carcinoma, established by its molecular discrimination approach using prediction analysis of microarray. Biochim Biophys Acta 2004;1739:50-61

Kops GJ, Foltz DR, Cleveland DW. Lethality to human cancer cells through massive chromosome loss by inhibition of the mitotic checkpoint. Proc Natl Acad Sci USA 2004;101: 8699-704

Kops GJ, Weaver BA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. Nat Rev Cancer 2005;5:773-85

Lee H. Impaired phosphorylation and mis-localization of Bub1 and BubR1 are responsible for the defective mitotic checkpoint function in Brca2-mutant thymic lymphomas. Exp Mol Med 2003;35:448-53

Luo X, Fang G, Coldiron M, Lin Y, Yu H, Kirschner MW, Wagner G. Structure of the Mad2 spindle assembly checkpoint protein and its interaction with Cdc20. Nat Struct Biol 2000;7:224-9

Mapelli M, Filipp FV, Rancati G, Massimiliano L, Nezi L, Stier G, Hagan RS, Confalonieri S, Piatti S, Sattler M, Musacchio A. Determinants of conformational dimerization of Mad2 and its inhibition by p31comet. EMBO J 2006;25:1273-84

Michel L, Diaz-Rodriguez E, Narayan G, Hernando E, Murty VV, Benezra R. Complete loss of the tumor suppressor MAD2 causes premature cyclin B degradation and mitotic failure in human somatic cells. Proc Natl Acad Sci USA 2004;101:4459-64

Mitelman F. Catalogue of chromosome aberrations in cancer. Cytogenet Cell Genet 1983;36:1-515

Nishioka K, Watanabe J, Furuta S, Tanaka E, Iino S, Suzuki H, Tsuji T, Yano M, Kuo G, Choo QL. A high prevalence of antibody to the hepatitis C virus in patients with hepatocellular carcinoma in Japan. Cancer 1991;67:429-33

Park JG, Lee JH, Kang MS, Park KJ, Jeon YM, Lee HJ, Kwon HS, Park HS, Yeo KS, Lee KU. Characterization of cell lines established from human hepatocellular carcinoma. Int J Cancer 1995;62:276-82

Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. Int J Cancer 1999; 80:827-41

Saeki A, Tamura S, Ito N, Kiso S, Matsuda Y, Yabuuchi I, Kawata S, Matsuzawa Y. Frequent impairment of the spindle assembly checkpoint in hepatocellular carcinoma. Cancer 2002;94:2047-54

Takahashi T, Haruki N, Nomoto S, Masuda A, Saji S, Osada H, Takahashi T. Identification of frequent impairment of the mitotic checkpoint and molecular analysis of the mitotic checkpoint genes, hsMAD2 and p55CDC, in human lung cancers. Oncogene 1999;18:4295-300

Xia G, Luo X, Habu T, Rizo J, Matsumoto T, Yu H. Conformation-specific binding of p31 (comet) antagonizes the function of Mad2 in the spindle checkpoint. EMBO J 2004;23:3133-43