Expression of Aurora-B Kinase and Phosphorylated Histone H3 in Hepatocellular Carcinoma

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Abstract. *Background: Aurora-B, a chromosomal passenger protein forming a complex with INCENP (inner centromere protein) and survivin, regulates stable bipolar spindle-kinetochore attachment in mitosis and chromosome segregation and cytokinesis. It was recently documented that Aurora-B directly phosphorylated histone H3, not only at Ser10, but also at Ser28, which contributed to chromosome number instability and mitotic chromosome condensation. This study aimed at investigating the expression of Aurora-B kinase(Aurora-B) and phosphorylated histone H3 (H3-P) and their roles in hepatocellular carcinogenesis. Materials and Methods: The expressions of Aurora-B and H3-P were examined in hepatocellular carcinoma (HCC) by immunohistochemistry. A hepatoblastoma cell line, HepG2, was targeted and the isolation and characterization of alternative variants of Aurora-B were carried out. The Aurora encoding protein was detected in COS-7 transfected with different Aurora transcripts by Western blot. Finally, the expression of Aurora-B and its variant forms was examined in 17 HCCs by RT-PCR. Results: Immunohistochemically, Aurora-B was observed only in a few cases of HCC, while H3-P expression was more frequently detected in carcinoma foci than in non-carcinoma foci ($p<0.05$). The isolation and characterization of two alternative variant forms of Aurora-B (termed Aurora-B1 and -B2) in the HepG2 cell line were successful. Aurora-B-transfected COS-7 cells expressed two different proteins, one of which was similar to the expression product of Aurora-B1 in size. Aurora-B transcripts were detected in 12 out of 17 (70.5%) HCC cases examined. Aurora-B2 was predominantly detected in 9 (52.9%) cases, while regular Aurora-B and Aurora-B1 were detected in 6 (35.2%) and 7 (41.1%) cases, respectively. Conclusion: Aberrant expression of Aurora-B and H3-P plays a role in hepatocarcinogenesis. Alterative splicing of Aurora-B produces different sizes of proteins in HCC. Temporally altered phosphorylation of histone-H3 in the entire cell cycle may up-regulate the entry of HCC into the cell cycle to enhance their proliferation.*

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Aurora kinase, an oncogenic serine-threonine (S/T) kinase family conserved in yeast cells, nematodes and mammalian cells, is localized to the centrosome, the poles of the bipolar spindle, or midbody (1-4). It plays an essential role in orchestrating mitotic events, including G_2/M transition, centrosome duplication, chromosome condensation, bipolar spindle-kinetochore attachment, chromosome segregation and cytokinesis (5-7). Mammals have three homologs of Aurora kinases, known as Aurora-A, -B and -C, which are expressed in a cell cycle-regulated manner with a peak in G_2/M-phase transition (8-10). They structurally share a conserved catalytic domain in the COOH terminus despite no sequence similarity in the NH_2 terminal. Among them, Aurora-A is involved in spindle assembly and regulates the proper progression of the cell cycle (5, 7, 11). Aurora-C is thought to regulate mitotic chromosome dynamics in mammalian cells. Aurora-B (also called AIM-1 and Stk-5), a chromosomal passenger protein forming a complex with INCENP (inner centromere protein) and survivin, regulates stable bipolar spindle-kinetochore attachment in mitosis and chromosome segregation and cytokinesis.

It was recently documented that Aurora-B directly phosphorylated histone H3, not only at Ser10, but also at

Ser28, which contributed to chromosome number instability and mitotic chromosome condensation (12). Additionally, a significant overexpression of Aurora-B was observed in colorectal tumors, astrocytoma, germ cell tumors and thyroid carcinoma, as well as in human cancer cell lines (12-18). Furthermore, it was observed that the forced expression of Aurora-B produced aneuploid cells with a malignant and aggressive phenotype, suggesting that its aberrant expression was correlated with human tumorigenesis.

Hepatocellular carcinoma (HCC) is a major malignant disease throughout the world, especially in Asian countries. Epidemiological evidence suggested that HCC results from exposure to risk factors, such as hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and prolonged exposure to toxic reagents, such as aflatoxin B1. However, the mechanism of hepato-carcinogenesis remains poorly understood and there is no report regarding Aurora-B expression in HCC. In order to clarify the role of Aurora-B and related molecular aspects in HCC, the expressions of Aurora-B and the phosphorylated histone H3 (H3-P) were immunohistochemically examined in liver sections of HCC patients, HepG2 cell and COS-7 cells transfected with its transcripts. Aurora-B and its alternative variants were analyzed molecularly in a HepG2 line and 17 HCCs.

Materials and Methods

Pathology. Surgically resected liver specimens from 36 patients with HCC were collected in our hospital from 2000 to 2004. Among them, 31 were moderately- to poorly-differentiated HCC (12 females and 19 males: 65 years±8 years) and 5 with well-differentiated HCC and adenomatous hyperplasia (2 females and 3 males: 58 years±13 years). All patients also suffered from hepatitis B and/or C viral infection. Nineteen cases had a history of liver cirrhosis and the others showed chronic hepatitis. "Normal livers" (2 females and 3 males: 67 years±7 years) came from patients with only mild hepatic dysfunction with no significantly histological change as a negative. All cases were re-evaluated histopathologically and independently by two investigators based on clinical and laboratory data.

Cell culture and transfection. The hepatoblastoma (HepG2) and COS-7 cell lines were obtained from RIKEN cell bank (Japan) and were maintained in MEM (Sigma, USA) containing non-essential amino acid (NEAA) (Invitrogen Corporation, USA) and 10% heat-inactivated fetal bovine serum (FBS, JRH Bioscience, USA) and were grown at 37˚C in an atmosphere of 5% CO_2. To synchronize cells in the G_0-phase, the HepG2 cells were cultured to 70-80% confluence. They were then washed twice with phosphate-buffered saline (PBS) and starved for 24-48 h in culture media containing 0.1% FBS. COS-7 cells were transiently transfected using LipofectAMINE PLUS (Invitrogen) with a total of 1.0 μg of Aurora expression plasmids. The cells were then cultured in medium supplemented with 10% fetal calf serum for 48 h.

Specimen and samples. Tissue specimens were fixed in 10% neutralized formalin, paraffin-embedded, incised into 4-μm consecutive sections and mounted on poly-lysine-coated slides. For the detection of Aurora-B kinase protein, COS-7 cells transfected or not were harvested, homogenized in PBS solution and centrifuged to remove the cell debris. For preparation of histone protein, HepG2 cells were lysed in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 1.5 mM PMSF). Hydrochloric acid was added to a final concentration of 0.2 N to extract nucleus pellets. The acid-soluble proteins were precipitated with 20% trichloroacetic acid and were finally resuspended in water. Total RNA was extracted from a HepG2 cell line and 17 frozen or recent paraffin-embedded tissues of HCC patients using TRIzol reagent (Invitrogen) and a Paraffin Block RNA Isolation Kit (Ambion, USA) according to the manufacturer's protocol. The concentrations of protein or mRNA were quantified using a UV-spectrophotometer.

Immunohistochemistry. Briefly, after deparaffinization, incubation in methanolic H_2O_2, and microwave treatment, sections were incubated with normal goat serum (Sigma Chemical, St. Louis, MO, USA) to prevent non-specific binding. These sections were then incubated with mouse anti-H3-P monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA, 1:1000), mouse anti-Aurora-B kinase monoclonal antibody (Transduction Laboratories, Lexington, KY, USA, 1:20) overnight at 4˚C. After washing slides with Tris-buffered saline (TBS) (pH 7.6), Envision-peroxidase (DAKO, Glostrup, Denmark) was applied to incubate according to its manufacturer's recommendation. For negative control, sections were processed as above, but treated with TBS or mouse serum instead of primary antibodies.

Double immunostaining. To clarify when H3-P would be induced in the cell cycle, a double immunostaining method was performed with antibodies against H3-P and Ki-67. After applying the mouse anti-Ki-67 antibody (DAKO, Glostrup, Denmark, 1:200), Envision-alkaline phosphatase (AP) was applied and colored by Fast Red (Vector Laboratories, Burlingame, CA, USA). Before another staining, the slides were soaked in boiled water for 15 min to avoid double recognition from the first antibody. We preliminarily confirmed the negative staining with peroxidase-conjugated secondary antibodies against mouse IgG. The anti H3-P antibody was secondarily applied. After TBS washing, FITC-conjugated anti-mouse IgG antibody (DAKO, 1:40) was processed for the green color. Covered with Slow Fade (Molecular Probes, Eugene, OR, USA), the sections were observed using an LSM 510 confocal laser scan microscope (Carl Zeiss, Oberkochen, Germany).

RT-PCR. The RNA was converted to cDNA using RAV-2 RTase and a random primer. For Aurora-B subcoloning, complementary DNA (cDNA) of the HepG2 cell line was reverse transcribed by RT-PCR using RAV-2 RTase (Takara, Japan) and antisense primer (5' CAGGAATTCTCAGGCGACAGATTGAAGG 3'). For the Aurora-B products from HepG2, complementary DNA was amplified by Taq polymerase with a sense primer (5' CTCGCTAGCGATGGCCCAGAAGGAGAACTCCTACC 3') and antisense primer (5' CAGGAATTCTCAGGCGACAGATT GAAGG 3'). To examine the splice variants of Aurora-B in the HCC, we amplified them with beta-actin as control using primer sets and PCR conditions described in Table I.

Plasmid construction and sequencing. Aurora-B cDNAs were amplified by RT-PCR, purified from agarose gel digested and

Table I. *Oligonucleotide primers and optimum conditions for the amplification of Aurora-B and the beta-actin gene.*

Genes	Primers	Product size (bp)	Reaction conditions
Aurora-B	Forward 5' gtgtacttggctcgggagaa 3 Reverse 5' tcctcctccggtcataaaaa 3''	178	95˚C for 9 min, 48 cycles at 94˚C for 60 sec, 62˚C for 60 sec, and 62˚C for 10 min
Aurora-B1	Forward 5' gtcaccccatctgcacttgt 3' Reverse 5' ctcaggccaaaggcataaaa 3'	180	95˚C for 9 min, 48 cycles at 94˚C for 60 sec, 64˚C for 60 sec and 64˚C for 10 min
Aurora-B2	Forward 5' atcttaaccaggcggcactt 3' Reverse 5' actcctccatgattgcaggt 3'	216	95˚C for 9 min, 48 cycles at 94˚C for 60 sec, 65˚C for 60 sec, and 65˚C for 10 min
beta-actin	Forward 5' tcctgtggcatcgacgaaact 3' Reverse 5' gaagcatttgcggtggacgat 3'	315	95˚C for 9 min, 48 cycles at 94˚C for 60 sec, 65˚C for 60 sec, and 65˚C for 10 min

ligated into the NheI and EcoR I sites of the Ptarget expression vector (Promega). The positive clones were screened by enzyme digestion and colony-PCR. Positive plasmid DNA, purified using a Plasmid Midi kit (Qiagen) was subsequently sequenced to confirm the Aurora-B sequence using DSQ-1000L DNA Sequencer (Shimadzu, Japan). The sequences were analyzed using BLAST, Clustal W and DNASIS sequence analysis programs (Hitachi software, Japan).

Western blot. The proteins were mixed with 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 7% glycerol and 0.01% bromophenol blue and were boiled for 5 min. Fifteen micrograms of target proteins were separated by SDS-PAGE and electrolotted to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). Following blocking non-specific protein binding sites using 5% milk in TBST (tris-buffered saline containing 0.2% Tween-20), the membranes were incubated with antibodies against Aurora-B kinase (1:1000) or H3-P (1:5000). After removal of unbound primary antibodies by washing with TBST, the membrane was incubated in anti-mouse horse-radish peroxidase-conjugated secondary antibody (eBioscience, 1:5000). Immunoreactive bands were visualized by X-ray films using enhanced chemiluminescence (ECL; Amersham, UK) according to the manufacturer's instructions.

Evaluation of immunohistochemistry. The immunoreactivity to Aurora-B and H3-P was localized in the cell nucleus. One hundred cells from 3 representative fields of each section were randomly selected and counted by two independent observers to calculate the proportion of positive cells. According to the positive rates, Aurora-B kinase expression was classified into 3 categories of less than 1% (<1%), 1%-10% and more than 10% (>10%). As for H3-P, the positive rates were considered as its expression index.

Statistical analysis. Mann-Whitney *U*-test was performed to compare the H3-P index of different groups. $P<0.05$ was considered as statistically significant. SPSS 10.0 software for windows was employed to analyze all data.

Results

Aurora-B kinase expression in HCC. Aurora-B kinase was diffusely expressed in cancer cell nuclei, while expression was not detected in normal hepatocytes, normal or proliferating epithelial cells of bile duct. Aurora-B expression was not found in non-carcinoma foci, but in 5 out of 35 moderately- to poorly-differentiated HCC, among which 3 cases belonged to the 1% to 10% labeling index group of Aurora-B and the 2 cases to the more than 10% labeling index group. In these five cases, Aurora-B expression was not only restricted to cells showing mitotic figures, but also appeared in non-mitotic cells (Figure 1, A and B). Aurora-B expression was also detected in asynchronous HepG2 cells (Figure 3A).

To confirm Aurora-B expression in HCC, the catalytic portion of Aurora-B cDNA was isolated and subcloned from HepG2 cells and three different clones, including normal Aurora-B and two alternative variants (termed Aurora-B1 and -B2) were obtained. Aurora-B1, an Aurora-B transcript, retained 46 bp of intron 5 and loses 96 bp of exon 5, while Aurora-B2 encoded the entire sequence of missing exon 6, a novel variant (Figure 2, GenBank accession number: ABO11450). Two protein bands were observed from the cell homogenate of Aurora-B-transfected COS-7 cells: one corresponded to Aurora-B protein (41 kDa), while the other band was lower than 41 kDa, similar to Aurora-B1.

The expressions of all Aurora-B variant forms were observed with variable intensities in HCC (Figure 3). As summarized in Table II, expression of one or more Aurora-B mRNA alternative variants was detected in 12 out of 17 cases (70.5%) in HCC samples. The expression of regular Aurora-B and Aurora-B1 was detected in 6 (35.2%) and 7

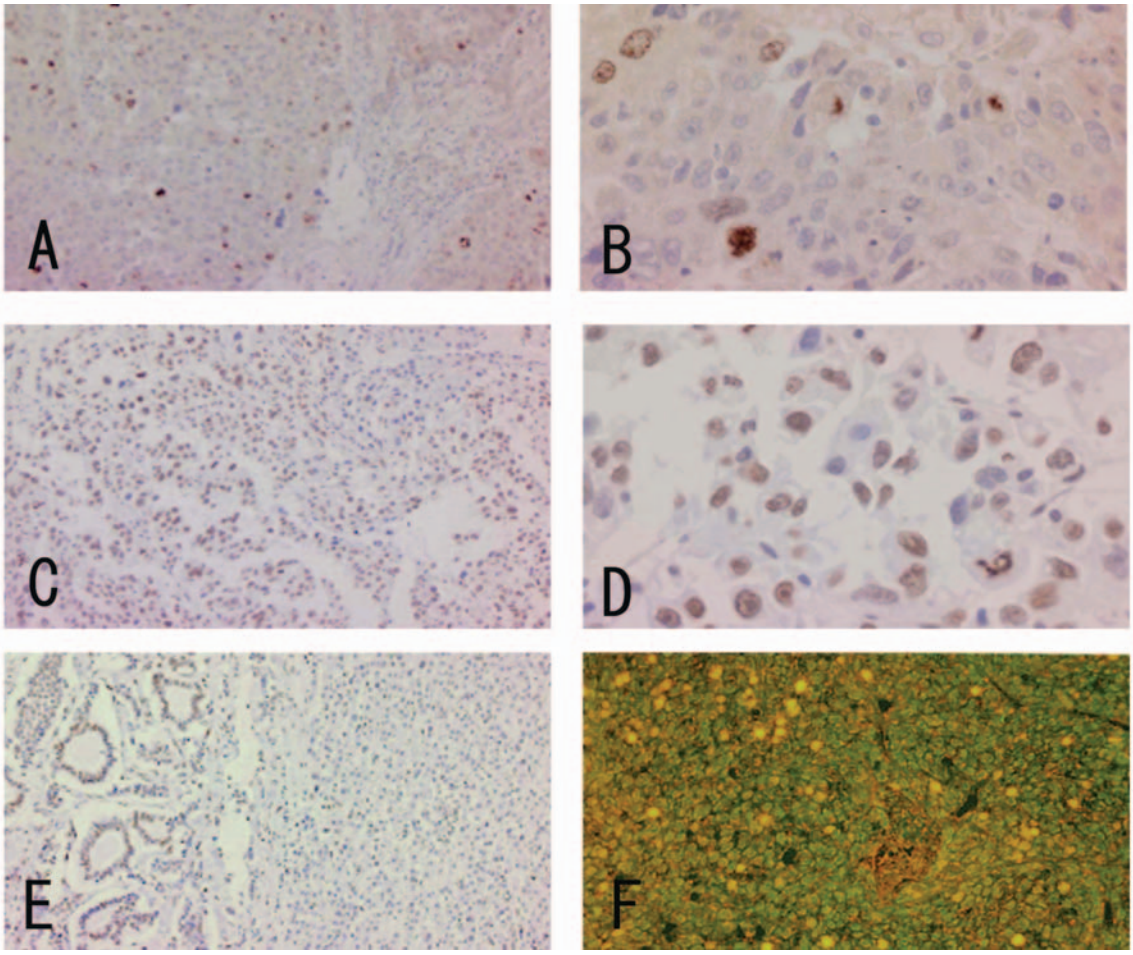

Figure 1. *Immunohistochemical staining of Aurora-B (A,B) , H3-P (C-E) and a double immunostaining of Ki-67 (MIB-1) and H3-P (F) in human liver specimens. A) Lower magnification of moderately-differentiated HCC showing a thick trabecular structure. The expression of Aurora-B kinase was scattered throughout cancerous areas. Positive staining was mainly restricted to mitotic cells and was sporadically expressed in the nucleus of cells without mitosis. B) Higher magnification of the same specimen of (A) (A: x100, B: x400). C) Low magnification of moderately-differentiated HCC showed a thin trabecular structure. H3-P overexpression was observed diffusely in the cancer area. D) Higher magnification of the same specimen of (A). Frequent H3-P expression was observed, not only in cells with mitosis but also in those without. E) Moderately-differentiated HCC showing a pseudoglandular structure was noted on the left side and the background liver combined with chronic hepatitis C was shown on the right side. The labeling index of H3-P in the HCC area was obviously higher than the background liver (C: x100, D: x400, E: x200). F) Double immunostaining of Ki-67 (MIB-1) and H3-P showing a thick trabecular structure in moderately-differentiated HCC. Ki-67 expression was labeled with Fast Red (red) and H3-P was recognized by FITC (green). Cells co-expressing Ki-67 and H3-P were yellow, mainly belonging to the G_1- or S-phase or the G_2- or M-phase. Ki-67 (–)/H3-P(+) cells were green and were mainly in the G_0-phase.*

(41.1%) cases, respectively, while the Aurora-B2 form was predominantly detected in 9 (52.9%) cases. Co-expression of regular Aurora-B or alternative variants (Aurora-B1 and/or Aurora-B2) was detected in almost all positive cases of regular forms. Similar to the tissue sample results, all three Aurora-B transcript forms were found in HepG2 cells (Figure 4).

H3-P expression in HCC. H3-P was strongly expressed in the nuclei of HCC and some of proliferating bile ductules, but was not expressed in normal hepatocytes or normal bile ducts. In HCC, H3-P was frequently observed in nuclei showing mitotic figures, but also in round nuclei of HCC cells (Figure 1C, D). As shown in Table III and Figure 1E, the H3-P indices of liver cirrhosis and chronic hepatitis/ normal liver were significantly lower than those of well-differentiated and moderately/poorly-differentiated carcinomas ($p<0.05$).

After double staining, only some H3-P-positive cells were also positive for Ki-67, while most of the H3-P-positive cells were negative for Ki-67 (Figure 1F). From Western blotting analysis, cultured HepG2 cells were found to express H3-P not only in the G_2/M-phase but also in the G_0-phase (Figure 4B).

Table II. *Results of Aurora-B and alternative variant transcript expressions in frozen and paraffin-embedded tissue of HCC patients.*

Sample no.	Expression of Aurora-B form		
	Regular	–B1	–B2
1	–	–	–
2	–	–	–
3	+	+	+
4	+	+	–
5	–	+	–
6	–	+	–
7	–	+	+
8	–	–	–
9	–	–	–
10	–	–	–
11	–	–	+
12	–	+	+
13	–	–	+
14	+	–	+
15	+	–	+
16	+	+	+
17	+	–	+

Aurora-B transcript was examined from 11 frozen tissue samples (Nos. 1-11) and 6 paraffin-embedded samples (Nos. 12-17) of HCC patients; + positive; – negative.

Table III. *H3-P labeling indices among several groups of HCC.*

Groups	N	H3-P labeling index (mean±SD)
mod-poor HCC	31	51.2±17.2
well HCC	5	41.8±5.1
LC	19	12.5±5.6*
CH/ Nor	17	4.7±2.2*

SD, standard deviation; mod-poor HCC, moderately- to poorly-differentiated HCC group; well HCC/AH, well differentiated HCC and adenomatous hyperplasia group; LC, the regenerative nodules of liver cirrhosis group; CH/ Nor, chronic hepatitis and normal liver group. *Compared with well- or mod-poor HCC ($p<0.05$).

Discussion

Aurora-B, a chromosomal passenger protein, is important for the process of cell cycle regulation. The impaired regulation of Aurora-B expression in human cells shows evidence of multinuclearity and ploidy. In addition, the overexpression of Aurora-B was recently reported in various human cancers (13-19).

We immunohistochemically observed the expression of Aurora-B kinase in human liver specimens of HCC for the first time. Our results indicate that Aurora-B kinase was expressed in few moderately- to poorly-differentiated HCCs with frequent mitosis. Western blot revealed Aurora-B expression in the HepG2 cell line, as well. To clarify Aurora-B protein expression, we subcloned its regular form and two variants and transfected them into COS-7 cells to produce different encoding proteins. Interestingly, the Aurora-B transfectant encoded two different proteins, one of which was similar to the aurora-B1 in size, suggesting that there might be different translation start sites in the regular Aurora-B mRNA. The findings provide novel insight into the involvement of the Aurora-B variant and its encoding proteins in hepatocarginogenesis.

Based on the sequencing of different Aurora-B, Aurora-B mRNA expression was examined, including those of its two variant forms, by RT-PCR. We found that Aurora-B and its alternative transcripts were exhibited in most HCC examined (70.5%) in this study. The Aurora-B2 form was dominantly expressed (52.9%). Interestingly, all positive regular Aurora-B cases were co-exhibited with one or both variant forms, while some positive variant forms were expressed without regular Aurora-B in 6 out of 17 HCCs (35.2%). To our knowledge, this is the first report to show the splice variants of Aurora-B transcript in HCC. Sorrentino *et al.* (17) reported that Aurora-B overexpression in thyroid carcinoma and its cell lines, as well as the block of Aurora B expression induced, either by RNA interference or by using an inhibitor of Aurora kinase activity, significantly reduced the growth of thyroid anaplastic carcinoma cells. These findings indicated that Aurora-B expression might play some role in hepatocarcinogenesis. Until now, the different positive rates of Aurora-B protein and mRNA have been controversial. Taken together with all data, we can infer that the low positive rate of Aurora-B protein expression in the paraffin-embedded samples of liver might be due to the poor efficacy of the primary antibody, antigen mask or low translation of its mRNA. Additionally, molecular conformation and pathophysiological function of protein encoded by Aurora need to be further investigated.

The serine 10 motif of histone H3, selectively phosphorylated in the G_2/M-phase of the cell cycle, is regulated by Aurora-B kinase and is considered to play several important roles, *e.g.,* in chromosome segregation (20-22). However, the mechanism of histone-H3 phosphorylation and the role of cell kinetics remain unclear. Our immunostaining analysis showed a high H3-P index in the HCC, compared with non-cancerous lesions. This revealed that the increased phosphorylation of histone-H3 might have effects on the malignant transformation of hepatocytes and may be considered as a molecular marker reflecting hepatocarcinogenesis. Although some cancer cells co-expressed H3-P and Aurora-B kinase in the nuclei, most of them showed histone H3 phosphorylation without aberrant

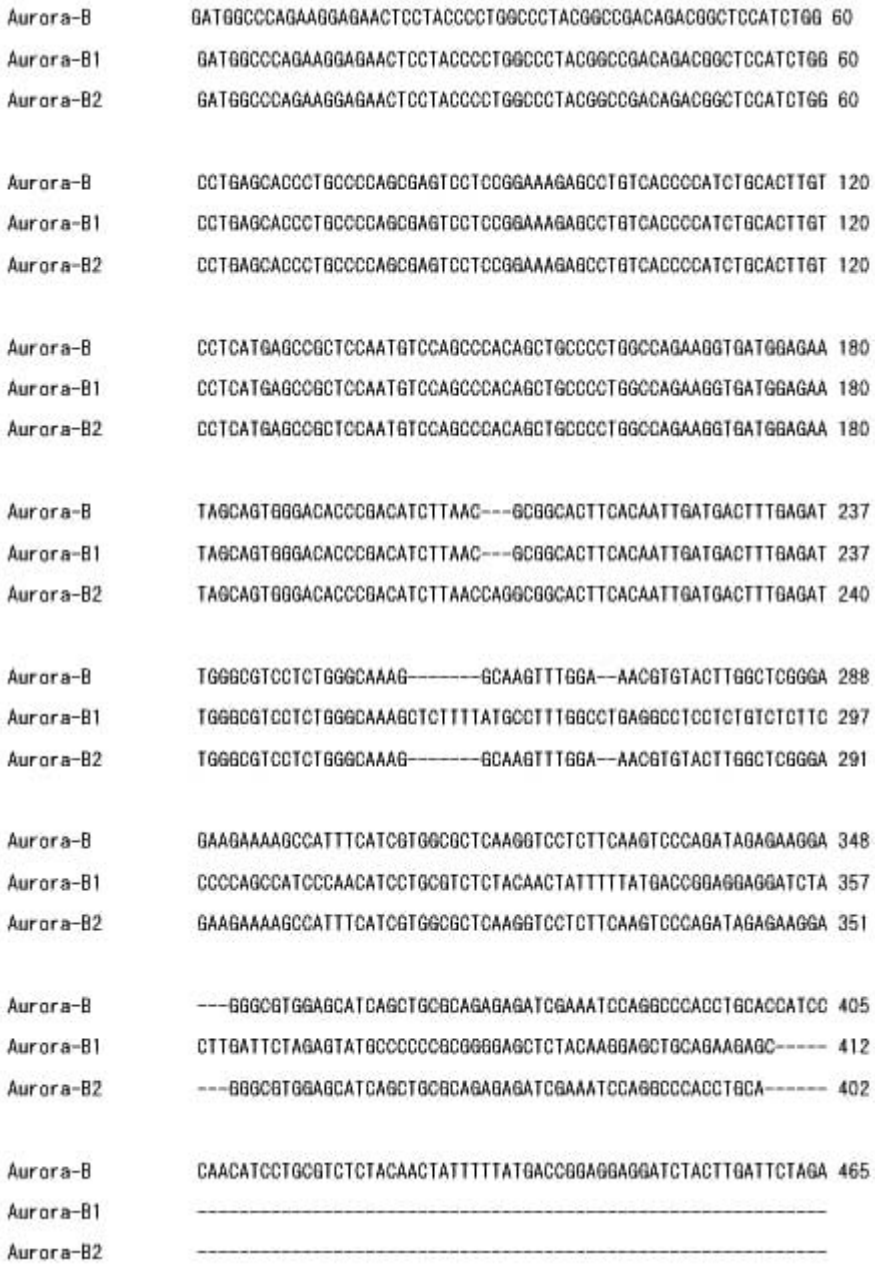

Figure 2. *Nucleotide alignment of regular Aurora-B and two alternative variant forms. Dots represent deleted sequences. Schematic representation of Aurora-B genes. Sequence analysis of cDNA encoding Aurora-B identified three different transcripts. The Aurora-B1 transcript lost 96 bp of exon 5 and retained 46 bp of intron 5. Aurora-B2 encoded the entire part of the missing exon 6. Arrows indicate PCR primers.*

Aurora-B kinase expression immunohistochemically. One possibility for this is that other kinases, such as Aurora-A, MSK1, MSK2, Pak1 and IKK-alpha can phosphorylate the histone H3, as well (23-26). Another possibility is that Aurora-B kinase expression might not be immunochemically detected by our antibody.

Histone H3 modification appears in the late G_2-phase, peaks in the metaphase and then decreases in anaphase and telophase. In contrast, phosphorylated histone H3 was involved in transcripted gene regulation during interphase (12, 20). To explore when H3-P would be regulated in the cell cycle, a double immunostaining method was performed

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Aurora-B        GTATGCCCCCCGCGGGGAGCTCTACAAGGAGCTGCAGAAGAGCTGCACATTTGACGAGCA 525
Aurora-B1       ------------------------------------------TGCACATTTGACGAGCA 429
Aurora-B2       ------------------------------------------------------------

Aurora-B        GCGAACAGCCACGATCATGGAGGAGTTGGCAGATGCTCTAATGTACTGCCATGGGAAGAA 585
Aurora-B1       GCGAACAGCCACGATCATGGAGGAGTTGGCAGATGCTCTAATGTACTGCCATGGGAAGAA 489
Aurora-B2       ------------ATCATGGAGGAGTTGGCAGATGCTCTAATGTACTGCCATGGGAAGAA 449

Aurora-B        GGTGATTCACAGAGACATAAAGCCAGAAAATCTGCTCTTAGGGCTCAAGGGAGAGCTGAA 645
Aurora-B1       GGTGATTCACAGAGACATAAAGCCAGAAAATCTGCTCTTAGGGCTCAAGGGAGAGCTGAA 549
Aurora-B2       GGTGATTCACAGAGACATAAAGCCAGAAAATCTGCTCTTAGGGCTCAAGGGAGAGCTGAA 509

Aurora-B        GATTGCTGACTTCGGCTGGTCTGTGCATGCGCCCTCCCTGAGGAGGAAGACAATGTGTGG 705
Aurora-B1       GATTGCTGACTTCGGCTGGTCTGTGCATGCGCCCTCCCTGAGGAGGAAGACAATGTGTGG 609
Aurora-B2       GATTGCTGACTTCGGCTGGTCTGTGCATGCGCCCTCCCTGAGGAGGAAGACAATGTGTGG 569

Aurora-B        CACCCTGGACTACCTGCCCCCAGAGATGATTGAGGGGCGCATGCACAATGAGAAGGTGGA 765
Aurora-B1       CACCCTGGACTACCTGCCCCCAGAGATGATTGAGGGGCGCATGCACAATGAGAAGGTGGA 669
Aurora-B2       CACCCTGGACTACCTGCCCCCAGAGATGATTGAGGGGCGCATGCACAATGAGAAGGTGGA 629

Aurora-B        TCTGTGGTGCATTGGAGTGCTTTGCTATGAGCTGCTGGTGGGGAACCCACCCTTTGAGAG 825
Aurora-B1       TCTGTGGTGCATTGGAGTGCTTTGCTATGAGCTGCTGGTGGGGAACCCACCCTTTGAGAG 729
Aurora-B2       TCTGTGGTGCATTGGAGTGCTTTGCTATGAGCTGCTGGTGGGGAACCCACCCTTTGAGAG 689

Aurora-B        TGCATCACACAACGAGACCTATCGCCGCATCGTCAAGGTGGACCTAAAGTTCCCCGCTTC 885
Aurora-B1       TGCATCACACAACGAGACCTATCGCCGCATCGTCAAGGTGGACCTAAAGTTCCCCGCTTC 789
Aurora-B2       TGCATCACACAACGAGACCTATCGCCGCATCGTCAAGGTGGACCTAAAGTTCCCCGCTTC 749

Aurora-B        CGTGCCCATGGGAGCCCAGGACCTCATCTCCAAACTGCTCAGGCATAACCCCTCGGAACG 945
Aurora-B1       CGTGCCCATGGGAGCCCAGGACCTCATCTCCAAACTGCTCAGGCATAACCCCTCGGAACG 849
Aurora-B2       CGTGCCCATGGGAGCCCAGGACCTCATCTCCAAACTGCTCAGGCATAACCCCTCGGAACG 809

Aurora-B        GCTGCCCCTGGCCCAGGTCTCAGCCCACCCTTGGGTCCGGGCCAACTCTCGGAGGGTGCT 1005
Aurora-B1       GCTGCCCCTGGCCCAGGTCTCAGCCCACCCTTGGGTCCGGGCCAACTCTCGGAGGGTGCT 909
Aurora-B2       GCTGCCCCTGGCCCAGGTCTCAGCCCACCCTTGGGTCCGGGCCAACTCTCGGAGGGTGCT 869

Aurora-B        GCCTCCCTCTGCCCTTCAATCTGTCGCCTGA 1036
Aurora-B1       GCCTCCCTCTGCCCTTCAATCTGTCGCCTGA 940
Aurora-B2       CCTCCCTCTGCCCTTCAATCTGTCGCCTGA  900
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Figure 2. *continued*

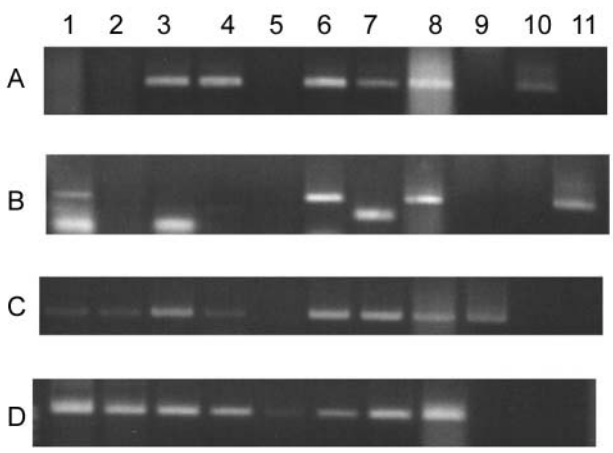

Figure 3. *RT-PCR analysis of Aurora-B transcripts in HCC. Total RNA was isolated from frozen and paraffin-embedded tissue of HCC patients (lanes 1-7) and the HepG2 cell line (lane 8). RT-PCR was conducted using primer pairs specific for the regular form (A), and the Aurora-B1 (B) and Aurora-B2 (C) forms. Each clone of regular Aurora-B (lane 10), Aurora-B1 (lane 11) and Aurora-B2 (lane 9) was amplified as positive and negative controls beta-actin amplification was used as an internal control (D).*

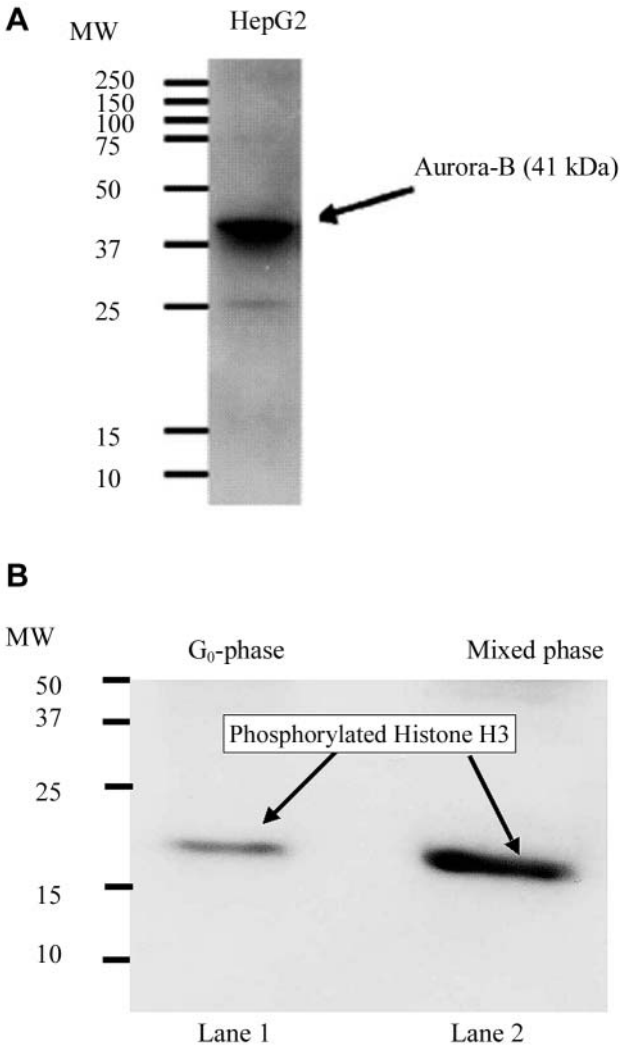

Figure 4. *A) Western blotting analysis of Aurora-B kinase in asynchronous HepG2 cells. Arrow indicates Aurora B (41 kDa). B) Western blotting analysis of phosphorylated Histone H3 expression in acid-soluble proteins extracted from the HepG2 cell line in the G_0-phase (lane 1) and mixed phase (lane 2). Arrows indicate phosphorylated Histone H3 (17 kDa).*

with antibodies against H3-P and Ki-67 (MIB-1), a well-known proliferating marker expressed in the G_1-, S-, G_2- and M-phases of the cell cycle. Ki-67-negative cells are regarded as belonging mainly to the G_0-phase, thus, it was found that most cancer cells with H3-P expression did not belong to the G_2/M-phase of the cell cycle, as supported by a higher expression of phosphorylated histone H3 in HepG2 cells synchronized into G_0-phase. These observations suggest that H3-P temporally changed in the HCC cells, possibly up-regulating their proliferation.

In conclusion, an abnormal expression of Aurora-B and H3-P was found to be involved in hepatocarcinogenesis. Alterative splicing of Aurora-B encoded its different sizes of protein in the HCC. Temporally altered phosphorylation of histone H3 in the entire cell cycle might force the entry of hepatocellular carcinoma cells into the cell cycle and promote their proliferation. Further experiments are necessary to elucidate the structural and functional properties of this alternative splicing in HCC.

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