Overexpression and Amplification of Aurora-A in Hepatocellular Carcinoma

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ABSTRACT

Purpose: Aurora-A/STK15/BTAK, a centrosome-associated serine/threonine kinase, has been shown to induce chromosomal instability, leading to aneuploidy and cell transformation. The purpose of this study was to investigate the expression and amplification of Aurora-A in hepatocellular carcinoma (HCC).

Experimental Design: Aurora-A mRNA levels were measured in 224 HCCs and 199 paired nontumorous liver tissues by reverse transcription-PCR. Aurora-A mRNA and protein levels of 8 were also measured by reverse transcription-PCR and Western blot hybridization in 8 liver cancer cell lines. Amplification of Aurora-A was determined by Southern blot hybridization in 99 cases.

Results: Aurora-A was overexpressed in 137 of 224 (61%) HCCs and all 8 of the cell lines. Overexpression of Aurora-A was associated with high-grade (grade II-IV), and high-stage (stage IIIB-IV) tumors, p53 mutation, infrequent β-catenin mutation, and poor outcome. Aurora-A overexpression and p53 mutation acted synergistically toward poor prognosis. Amplification of Aurora-A was detected only in 3 HCCs.

Conclusion: The results show that Aurora-A is overexpressed frequently in HCC, and correlated with high grade and high stage, indicating that overexpression of Aurora-A plays a role in the development and progression of HCC.

INTRODUCTION

Carcinogenesis is a multistep process that results from the accumulation of various genetic abnormalities (1). Loss of genomic stability is believed to be one of the driving forces to the development of a tumor and its progression (2). In most cancers, the instability is observed at the chromosome level, resulting in losses and gains of whole chromosomes or large portions thereof. Hence, a variety of chromosomal aberrations, such as abnormal ploidy, are common in cancer cells (3, 4). The centrosomes are thought to play a crucial role in the maintenance of genomic stability by organizing bipolar spindle during mitosis and by ensuring equal segregation of replicated chromosomes into daughter cells (5). Centrosome defects, such as changes in centrosome numbers, organization, and behavior, have been found in some breast cancers and solid tumors in general (6–8). By increasing the incidence of multipolar spindles and related spindle abnormalities, these defects cause chromosome misaggregation and aneuploidy, which are important for progression of malignancy (6–8).

Drosophila aurora and yeast Ipl1 are serine/threonine kinases that are required for centrosome maturation and chromosome aggregation (9–10). Mutations in aurora prevent centrosome separation, leading to the formation of monopolar spindles (10). Homologues of aurora kinase have been identified in Caenorhabditis elegans, Xenopus, mouse, and human (11). On the basis of the similarities in protein sequences, members of the aurora family are grouped into three classes (A, B, and C; Ref. 12). Of the three mammalian aurora homologues, Aurora-A (also called BTAK, STK6, STK15, and Aik1) has attracted intense interest after the discovery that Aurora-A is mapped to chromosome 20q13.2, a region amplified commonly in epithelial cancers (13–16). Amplification of Aurora-A was detected in ~12% of primary breast cancers, as well as in breast, ovarian, colon, prostate, and neuroblastoma cancer cell lines (17). Besides, Aurora-A is overexpressed frequently at mRNA and protein levels in primary cancers without Aurora-A amplification (18–21). Overexpression of Aurora-A transforms NIH3T3 fibroblasts, and induces centrosome abnormality and aneuploidy in near diploid breast cancer cell line (17), indicating that Aurora-A is oncogenic.

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors in southern China, Taiwan, southeastern Asia, and sub-Saharan Africa. HCC is closely associated with hepatitis B and hepatitis C infections, cirrhosis of any etiology, and aflatoxin B1 exposure (22), but the molecular mechanism for the tumorigenesis of HCC is still poorly understood. Gain of chromosome 20q, where Aurora-A is located, was observed frequently in HCC (23–25). To determine the involvement of Aurora-A in HCC, we analyzed the overexpression and amplification of Aurora-A in HCC. Aurora-A mRNA levels were then correlated with various clinicopathological and molecular features.

MATERIALS AND METHODS

Tissue Samples. From January 1983 to December 1997, 1033 surgically resected primary and 188 recurrent HCCs were pathologically assessed at the National Taiwan University Hos-
pital. The tissue samples were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored in deep freezer. Of these, 224 patients who already had DNA or RNA samples taken from resected primary HCCs were selected for this study. The definition of unifocal HCC was based on combined analyses of pathological features, hepatitis B virus DNA integration pattern, α-fetoprotein mRNA expression, and mutation patterns of β-catenin and p53 genes, as described previously (26–28). The 224 patients included 179 males and 45 females with a mean age of 55.26 years (range, 14–88 years). All of the patients had adequate liver function reserve at the time of surgery, and all of the tumors were surgically resectable. Cirrhosis was present in 83 patients (37%), including 22 patients with incomplete septal (early) cirrhosis, whereas 141 patients (63%) had no cirrhosis due to patient selection bias for surgery. None of these patients had received transhepatic arterial embolization or chemotherapy before surgery.

Histological Study and Tumor-Node-Metastasis Staging. Tumor grade was divided into three groups, well-differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III and IV). The resected HCC was staged as stages I, II, IIIA, IIIB, and IV, as described previously (26, 29). The staging was based on the International Union Against Cancer criteria, with slight modification. Stage I HCC included tumors that were ≤2 cm and showed no evidence of liver and vascular invasion. These tumors were either encapsulated with no liver invasion (stage IA) or unencapsulated with liver invasion (stage IB). Stage II HCCs included tumors that were ≤2 cm for which vascular invasion was limited to small vessels in the tumor capsule, as well as encapsulated tumors >2 cm with no evidence of liver or vascular invasion. Stage IIIA HCCs included invasive tumors >2 cm with invasion of small vessels in the tumor capsule and/or satellites near the tumor, but no portal vein invasion. Stage IIIB HCCs included tumors with invasion of the portal vein branch near the tumor, but not of the distant portal vein in the liver parenchyma. Stage IV included tumors with involvement of major portal vein branches, satellites extending deeply into the surrounding liver, tumor rupture, or invasion of the surrounding organs. Seven, 93, 37, 28, and 59 tumor specimens were classified as stage I, II, IIIA, IIIB, and IV HCC, respectively.

The intrahepatic tumor recurrence was based on imaging diagnosis with ultrasonography and/or computed tomography, supplemented with elevated serum α-fetoprotein. Among the 224 patients studied, 205 were eligible for the evaluation of early intrahepatic tumor recurrence (≤1 year). Nineteen patients who died within 1 year after resection and had no information or were negative for intrahepatic tumor recurrence were excluded from the evaluation of early recurrence.

Reverse Transcription-PCR (RT-PCR). RT-PCR was used to determine the expression of Aurora-A in 224 samples of HCCs and 199 corresponding nontumorous liver parenchyma, as is described elsewhere (29). S26 ribosomal protein mRNA, a housekeeping gene, was used as the internal control (30). Briefly, 2 μl reverse transcription product, 1.25 units Pro Taq polymerase (Protech Technology Enterprise, Taipei, Taiwan), Pro Taq buffer, and 200 μM each dATP, dCTP, dGTP, and dTTP were mixed with primer pairs for Aurora-A and S26 in a total volume of 30 μl. PCR was performed in an automatic DNA thermal cycler 480 (Perkin-Elmer Corp.), with initial heating at 94°C for 2 min, followed by the Touch Down PCR program, 22 cycles of 94°C for 30 s, annealing for 1 min (the annealing temperature is reduced by 1°C per 2 cycles from 65°C to 55°C), 72°C for 1 min, and final 72°C for 10 min. Aurora-A CDNA was amplified using Aurora-A-F (AATTGCAAGTTTTTTGGTGTT) and Aurora-A-R (AAACTCTGATGACATGTTCTGTCG) oligonucleotides. The primers for S26 are S26F (CCGGTCCTC-CAAGATGACAAAG) and S26R (GTTGCTTTTGGCGG-GCTTCA). PCR was stopped at the exponential phase of the amplified genes, 29 cycles for Aurora-A and 23 cycles for S26. The products were electrophoresed on a 2% agarose gel. The concentrations of the PCR fragments were determined with the IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA). The Aurora-A mRNA levels were determined by the ratio of signal intensity of Aurora-A to that of S26 measured by 1D Image Analysis software (Kodak Digital Science, Rochester, NY) and scored as high (ratio >1.0) or low (ratio <1.0). The Aurora-A mRNA level of nontumorous liver rarely exceeded a ratio of 1.0.

For the determination of p21 and Bax levels in Aurora-A-overexpressed cells, total RNA was extracted from Aurora-A or empty vector-transfected cells and analyzed for p21 and Bax expression by RT-PCR. The primers used were p21-F (ggcgcctggcatctcact), p21-R (ctctctatcaacgccgtag), Bax-F (tctcatcagcagaggg), and Bax-R (ccagatgggtaggtcgccg). PCR was performed using Touch Down program and stopped at the exponential phase of the amplified genes, 28 cycles for both genes.

Cell Culture. All of the cell lines used in this study were maintained in DMEM plus 10% FCS supplemented with penicillin and streptomycin.

Western Blot Hybridization. To determine the expression levels of Aurora-A in liver cancer cell lines, ~5 × 10⁶ cells were taken up in 50 μl of loading buffer and boiled for 5 min. After a 30-s vortexing step to shear the genomic DNA, 30 μl of this extract was electrophoresed through SDS-10% polyacrylamide gels. After transfer to a polyvinylidene difluoride membrane, the proteins were detected with anti-Aurora-A antibody (1:1000; Cell Signaling, Beverly, MA), followed by horseradish peroxidase-conjugated secondary antibody and chemiluminescence reagents (Amersham, Piscataway, NJ). The same membrane was reprobed with anti-β-actin (1:2000; Sigma, St. Louis, MO) as a control for equivalent protein loading.

Immunofluorescence Staining. Cells on 18-mm coverslips were fixed in 4% paraformaldehyde for 15 min. Fixed cells were blocked in PBS/5% FCS/0.1% Triton X-100 and subsequently incubated for 2 h in primary antibodies (Aurora-A, 1:250; γ-tubulin, 1:200; Sigma). Cells were washed repeatedly in PBS/0.1% Triton X-100 and incubated for 45 min with a secondary antibody diluted 1:500 in same antibody buffer. Nuclei were counterstained with 0.5 μg/ml 4’,6-diamidino-2-phenylindole for 10 min and washed in PBS.

Cloning and Transfection. The full-length Aurora-A was kindly provided by Dr. Tang K. Tang (Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan). Aurora-A-pCMV-Tag2B was constructed by cloning into the BamHI-XhoI site of pCMV-Tag2B. Using LipofectAMINE (Invitro) according to the manufacturer’s protocol, HEK 293 cells were...
transiently transfected with Aurora-A-pCMV-Tag2B or empty vector.

**Southern Hybridization.** Genomic DNA samples (5–15 μg) of HCC and nontumorous liver tissues were isolated using the phenol-chloroform method, digested with BgII, HindIII, or EcoRI (Life Technologies, Inc., Gaithersburg, MD), subjected to electrophoresis on a 0.8% agarose, and transferred to Zeta-probe nylon membrane (Bio-Rad Labs, Richmond, CA), as described (31). To avoid cross-hybridization of Aurora-A pseudogene, an Aurora-A intron 2 probe was used. The membrane was hybridized with 32P-labeled probe, washed, and autoradiographed at −70°C with X-Omat AR film (Eastman Kodak Co., Rochester, NY). The membranes were rehybridized with a β-globin probe as a control.

**Analysis of p53 and β-Catenin Mutations.** Mutations of the p53 tumor suppressor gene and β-catenin gene were detected by direct sequencing of exon 2 to exon 11 of the p53 tumor suppressor gene and exon 3 of β-catenin as described previously (26, 32). Cases with incomplete study were excluded from statistical analysis.

**Follow-Up Observation.** During the follow-up period up to 175 months, 178 patients (79%) had been followed for >10 years or until death. At the end of follow-up, 60 patients remained alive, 14 of whom had survived for >10 years.

**Statistical Analysis.** The analyses were performed using the Statistica for Windows software (Statsoft, Inc., Chicago, IL). Two-tailed χ² was used for univariate analysis. The cumulative survival after tumor removal was calculated with the log-rank test. P < 0.05 were considered statistically significant.

## RESULTS

### Expression of Aurora-A in HCC, Liver, and Liver Cancer Cell Lines.

We used RT-PCR for large-scale analysis of Aurora-A mRNA expression in HCC. Among 224 unifocal primary HCCs, Aurora-A was overexpressed in 137 tumors (61%; Fig. 1). Of these 224 HCCs, RNA samples of nontumorous liver parenchyma were available for 199 patients. In the nontumorous liver, Aurora-A mRNA was overexpressed in 10 cases (5%). Aurora-A was overexpressed in all 8 of the liver cancer cell lines at the mRNA and protein levels (Fig. 2). The protein expression level of Aurora-A in the liver cancer cell lines was parallel with the mRNA expression level, indicating that the expression of Aurora-A is regulated at transcription level, and mRNA level can represent the protein expression level.

To determine the subcellular localization of Aurora-A in HCC, immunofluorescent staining was performed on Hep3B cell lines. Aurora-A colocalized with γ-tubulin at centrosome at interface and at centrosome, and mitotic spindle at metaphase (Fig. 3), as was reported in other cells (11).

### Clinicopathologic Correlation of Aurora-A Expression in HCC.

To elucidate the role of Aurora-A in HCC, we correlated the Aurora-A expression with clinicopathologic features of HCC. As shown in Table 1, Aurora-A overexpression in HCC did not correlate with age, gender, and chronic hepatitis B virus infection, but was associated with serum α-fetoprotein elevation (P = 0.031).

Histologically, HCCs with Aurora-A overexpression were more frequently grade II-IV than those without Aurora-A overexpression (P < 0.0001). Aurora-A overexpression was associated with portal vein tumor invasion (stage IIIB and IV HCC; P = 0.025), regardless of tumor size.

HCCs with Aurora-A overexpression was associated with a significantly lower 10-year survival rate than HCCs without Aurora-A overexpression (P < 0.007; Table 1).

### Correlation and Synergy between Aurora-A Expression, and p53 and β-Catenin Mutations.

p53 and β-catenin are the two most frequently mutated genes in HCC (26, 32). To identify the potential interplay between these oncogenic events, we analyzed the relation of mutations of these two genes and Aurora-A overexpression. In this study, p53 mutation was found in 88 (49%) of 178 HCCs examined. Overexpression of Aurora-A correlated with p53 mutation (P = 0.04; Table 1). Moreover, HCCs with Aurora-A overexpression and p53 mutation had the worst survival than those with p53 mutation or Aurora-A overexpression alone (Fig. 5).

Mutations of exon 3 of β-catenin were identified in 32 (15%) of 214 HCCs examined. In contrast to the association of Aurora-A overexpression with p53 mutation, Aurora-A was more frequently overexpressed in HCCs without β-catenin mutation (P = 0.024).

### Aurora-A Overexpression and p53 Target Genes.

Because Aurora-A overexpression was closely correlated with p53
mutation and Aurora-A was known to interact directly with p53 (33), we then analyzed the effects of Aurora-A on p53 target genes. RT-PCR analysis of Aurora-A-overexpressed HEK 293 cells demonstrated that Aurora-A did not significantly alter the transcriptional level of p21 and Bax (Fig. 6).

Amplification of Aurora-A in HCC. Southern blot analysis demonstrated amplification of Aurora-A only in 3 of 99 tumors (3%; Fig. 7). Overexpression of Aurora-A was detected in all of these tumors.

DISCUSSION

Genetic instability, by generating mutations in oncogenes and tumor-suppressor genes, provide cancer cells with a selective growth advantage, thereby leading to clonal outgrowth, and, hence, is a major driving force for malignant transformation and tumor progression (34–36). Two forms of genetic instability have been described in cancers, chromosomal instability and microsatellite instability (37). Microsatellite instability involves the inactivation of DNA mismatch repair genes and is present in only a minor subset of tumors. Chromosomal instability is much more frequent in cancer, but its mechanism is poorly understood. Inactivation of mitotic spindle checkpoint genes, such as BUB and MAD2, have been implicated in aneuploidy formation, but BUB mutation and MAD2 down-regulation can only be detected in a small fraction of aneuploidy cancer (38, 39). Amplification and overexpression of Aurora-A are more frequent in human cancer (17–21, 40, 41). By causing centrosome abnormalities and chromosomal instability, overexpression of Aurora-A can promote tumor formation and progression (17).

To study the role of Aurora-A in HCC, we analyzed its expression level in 224 cases, and detected overexpression of Aurora-A in 61% of the cases. Aurora-A overexpression was more frequently associated with higher grade, higher stage, and worse 10-year survival. Pervious studies indicated that most cancers with aneuploidy show a more malignant phenotype than diploid cancers (42, 43). These observations suggest that overexpression of Aurora-A, by causing chromosomal instability, may contribute to a more malignant phenotype of HCC.

Inactivation of p53 results in centrosome hyperamplification, leading to aberrant mitosis and chromosomal instability (44, 45). The p53 protein interacts directly with Aurora-A, and suppresses Aurora-A-induced centrosome amplification and cellular transformation in a transactivation-independent manner (33). Centrosome amplification and aneuploidy induced by Aurora-A was visualized with anti-aurora-A antibody and Rhodamine-conjugated secondary antibody (red). DNA was labeled with 4',6-diamidino-2-phenylindole staining (blue). Aurora-A shows perinuclear dot-like staining in interphase cells and located at mitotic spindle in metaphase cells. B, γ-tubulin, a marker of centrosome, was stained with anti-γ-tubulin antibody and FITC-conjugated secondary antibody. C, merge of A and B. Aurora-A and γ-tubulin colocalized at centrosome and mitotic spindle.
Aurora-A overexpression are exacerbated in a p53−−/− background (46). In this study, we found that Aurora-A overexpression was correlated with p53 mutation in HCC, and tumors with both Aurora-A overexpression and p53 mutation had worse prognosis than p53 mutation alone, but Aurora-A overexpression did not significant affect the expression level of p53 target genes. These observations provide in vivo evidence that Aurora-A and p53 had a synergistic effect contributing toward tumor progression and, hence, poor prognosis.

We also showed that Aurora-A overexpression was negatively associated with β-catenin mutation. In previous study, we showed that β-catenin mutation in HCC was associated with lower grade, lower stage, and better 5-year survival (26). Laurent-Puig et al. (47) classified HCC into two groups. One is characterized by chromosome stability, β-catenin mutation, and chromosome 8p loss. The other is characterized by chromosomal instability, Axin 1, and p53 mutation. The negative association of Aurora-A overexpression with β-catenin mutation and positive association with p53 mutation suggest that Aurora-A overexpression is associated with the latter group and may account for the genomic instability in these tumors.

Aurora-A was detected in only 3% of HCCs examined. Although Aurora-A amplification coincided with overexpression, many more cases showed Aurora-A overexpression without amplification. Thus, the expression of Aurora-A is likely to be regulated not only by gene amplification but also by other mechanisms such as transcriptional activation.

### Table 1

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Fig. 4 Cumulative survival curve for 224 patients with primary unifocal hepatocellular carcinoma (HCC). HCC with Aurora-A mRNA overexpression, designated Aurora-A (+), had a significantly worse 10-year survival rate than HCC without Aurora-A mRNA overexpression, designated Aurora-A (−).

Fig. 5 Cumulative survival curve for 178 patients with primary unifocal hepatocellular carcinoma in relation to increased (+) or normal (−) expression of Aurora-A and the presence (+) or absence of p53 mutation. Hepatocellular carcinoma with Aurora-A overexpression and p53 mutation had the worst survival rate.

Amplification of Aurora-A was detected in only 3% of HCCs examined. Although Aurora-A amplification coincided with overexpression, many more cases showed Aurora-A overexpression without amplification. Thus, the expression of Aurora-A is likely to be regulated not only by gene amplification but also by other mechanisms such as transcriptional activation.
Another possibility is that Aurora-A gene amplification occurred in only a proportion of the tumor cells and is undetectable by Southern blot analysis. Discrepancy between amplification and overexpression rates has also been reported in gastric cancer (39), bladder cancer (21), and pancreatic cancer (40), indicating that transcriptional activation may be a major cause of Aurora-A overexpression in human cancers.

In summary, we have demonstrated that Aurora-A is highly expressed in HCC. Overexpression of Aurora-A may have important pathogenic and prognostic significance in HCC.

REFERENCES

Fig. 7 Southern blot hybridization demonstrated amplification of Aurora-A in 3 tumors (T; tumor; N; nontumorous liver parenchyma). β-Globin served as a control for DNA quantity.
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