The role of Aurora A in hypoxia-inducible factor 1α-promoting malignant phenotypes of hepatocellular carcinoma

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Abbreviations: HCC, hepatocellular carcinoma; HIF-1α, hypoxia-inducible factor 1α; shRNA, short hairpin RNA; HRE, hypoxia-responsive element; pAk, phosphorylated Akt; ChIP, chromatin immunoprecipitation

Overexpression of both hypoxia-inducible factor 1α (HIF-1α) and Aurora A has been found in hepatocellular carcinoma (HCC). However, whether HIF-1α and Aurora A synergistically promote malignant phenotypes of HCC cells is unknown. The purpose of this study was to investigate the roles and functional correlation of HIF-1α and Aurora A in HCC progression. Immunohistochemistry was performed to detect HIF-1α and Aurora A protein expression in 55 primary HCC and corresponding non-tumor tissues and their clinical significance. Gene knockout technology using short hairpin RNA (shRNA) was used to knockdown expression of HIF-1α or Aurora A and analyze their effects on malignant phenotypes of HCC cells. The transcriptional regulation of Aurora A by HIF-1α and the possible downstream molecular signaling pathways were also determined. Results showed that hypoxia could induce the increased expression of HIF-1α and Aurora A in HCC cells. Also, shRNA-mediated HIF-1α downregulation could lead to the decreased Aurora A expression and inhibition of growth or invasion in HCC cells. Moreover, HIF-1α could transcriptionally regulate Aurora A expression by binding to hypoxia-responsive elements in the Aurora A promoter and recruiting the coactivator-p300/ CBP. Additionally, shRNA-mediated Aurora A knockdown could mimic the effects of HIF-1α downregulation on phenotypes of HCC cells, and overexpression of Aurora A could partially rescue the phenotypical changes of HCC cells induced by HIF-1α downregulation. Further research indicated that activation of Akt and p38-MAPK signaling pathways mediated the downstream effects of HIF-1α and Aurora A in HCC cells under hypoxic condition. Taken together, our findings indicated that Aurora A might be a key regulator of HIF-1α-promoting malignant phenotypes of HCC by activation of Akt and p38-MAPK signaling pathways.

Introduction

Hepatocellular carcinoma is one of the most prevalent fatal malignancies around the world, especially in Asia and Africa.1 In China, HCC is the second leading cancer killer since the 1990s, and HBV infection is highly endemic.2 The high mortality rate is due to its detection at late stage with limited therapeutic options. HCC carcinogenesis is a complicated and partially understood process that is determined by environmental and genetic factors.3 Therefore, a better understanding of the molecular mechanisms governing hepatocarcinogenesis and HCC progression will be helpful to find better prognostic markers that accurately represent biological characteristics of tumors and screen for early-stage disease and design new therapeutic strategies for this lethal disease.

Increasing evidence suggests that intratumor hypoxia is a common characteristic of solid tumors and an important microenvironmental factor of malignant phenotype development.4 Like other solid tumors, HCC have large hypoxic regions, and hypoxia is an important microenvironmental factor in prompting HCC progression and metastasis.5 It has been reported that hypoxia adaptation of tumor cells is regulated by hypoxia-inducible factor 1 (HIF-1).6 HIF-1, a heterodimer, consists of 2 basic helix-loop-helix proteins: α subunit (HIF-1α)
and β subunit (HIF-1β), which have entirely different expression patterns or functions in tumor aggressiveness of different types of human cancers. HIF-1α has been thought to help cells resist hypoxic stress and apoptosis and found to overexpress in a variety of human malignancies, leading to the increased capacity of metastasis and chemoresistance of tumor cells. Consistent hypoxia could lead to resistance to apoptosis as well as treatments, but the molecular mechanisms are not fully understood. Some angiogenesis-related genes such as vascular growth factors (VEGF) and urokinase-type plasminogen activator receptor (uPAR) as well as a few apoptosis regulators such as the anti-apoptotic Mcl-1, Bcl-xL and BIRC5/survivin have been identified as target genes of HIF-1α. Aurora A (STK15) is an important member of Aurora kinase family, which is a novel family of serine/threonine kinases promoting mitotic spindle assembly by regulating centrosome duplication and separation. Previously, we have reported that high Aurora A mRNA level was significantly correlated with tumor stage, more frequent lymph node or hematogenous metastasis, and poor prognosis of HCC patients. We showed that downregulation of Aurora A could lead to growth inhibition and apoptosis induction in HCC cells not only in vitro, but also in vivo. However, its effects on phenotypes of human HCC cells under hypoxic condition have not yet been sufficiently studied. Additionally, the molecular mechanisms leading to Aurora A overexpression in HCC remain undefined. Because Aurora A gene transcription can be regulated by hypoxia and HIF-1α, we hypothesized that Aurora A might play critical roles in HIF-1α-promoting malignant phenotypes of HCC.

To test this hypothesis, we first detected HIF-1α and Aurora A expression in a series of HCC and paired non-tumor tissue samples. Results showed that HIF-1α was correlated with high expression of Aurora A in those tissue samples. To further investigate the roles of HIF-1α in Aurora A overexpression in HCC, we stably transfected shRNA vector targeting HIF-1α into HCC cell lines (HepG2 and BEL-7405) and found that HIF-1α knockdown could significantly inhibit Aurora A expression with consequent suppression in growth, migration, or invasion of HCC cells. It was also found that Aurora A was transcriptionally regulated by HIF-1α, and DNA vector-mediated Aurora A overexpression could partially rescue phenotypical changes of HCC cells induced by HIF-1α knockdown under hypoxic conditions. Further, the roles of activated Akt and p38-MAPK signaling pathways in the downstream effects of HIF-1α and Aurora A in HCC cells under hypoxic condition were investigated. These data indicate that Aurora A might be a key regulator of HIF-1α-promoting malignant phenotypes of HCC under hypoxic condition by activating Akt and p38–MAPK signaling pathways.

**Results**

Expression of HIF-1α and Aurora A in HCC cells or tissues was regulated by hypoxia

First, semi-quantitative RT-PCR and western blotting assays were performed to determine the expression of HIF-1α mRNA and protein in 2 HCC cells (HepG2 and BEL-7405), 3 HCC and paired non-tumor tissues, respectively. As shown in Figure 1A, the level of HIF-1α mRNA or protein in HCC cells or tissues was significantly higher than that in paired non-tumor tissues. Next, the expression of Aurora A mRNA and protein was determined in 2 HCC cells (HepG2 and BEL-7405) and a primary hepatocyte cell line (HH), above 3 paired tissues (Fig. 1B). Results showed that the expression of Aurora A mRNA and protein in 2 HCC cells was significantly higher than that in primary hepatocyte cell line HH. Also, the expression of Aurora A mRNA or protein in HCC tissues was significantly higher than that in paired non-tumor tissues. Thus, the trend of HIF-1α expression was consistent with that of Aurora A expression in HCC cells or tissues. To further determine the effect of hypoxia on expression of HIF-1α and Aurora A gene, HepG2 or BEL-7405 cells were maintained in culture under either normoxic or hypoxic conditions, and total mRNA or protein of HIF-1α and Aurora A were measured by semi-quantitative RT-PCR (Fig. 1C) and western blotting (Fig. 1D) assays. By 30 min of hypoxic exposure, Aurora A mRNA and protein expression increased markedly followed by a gradual decrease over time, but remained elevated compared with normoxic control at 6 h. These results indicated that the expression of HIF-1α and Aurora A was upregulated in HCC cells or tissue samples, and the increased expression of Aurora A protein could be induced by hypoxia.

HIF-1α and Aurora A expression patterns in tissue samples from HCC patients

To explore the HIF-1α and Aurora A expression patterns, we used 55 HCC tissue samples (median age, 41; range, 20–73 y). By immunohistochemical analysis, we showed that the positive immunostaining for HIF-1α protein was mainly located in the cytoplasm and/or the nucleus of tumor cells in HCC tissues, but the negative immunostaining for HIF-1α protein was shown in corresponding non-tumor tissues (Fig. 2A). The rate of positive immunostaining for HIF-1α protein in HCC tissues (63.2%) was significantly higher than that in non-tumor tissues (8.8%). In addition, the positive immunostaining for Aurora A protein was mainly located in the cytoplasm of tumor cells in HCC tissues, and the negative immunostaining for Aurora A protein was shown in corresponding non-tumor tissues (Fig. 2B). The rate of positive immunostaining for Aurora A protein in HCC tissues (54.6%) was higher than that in corresponding non-tumor tissues (18.5%). Further, we also showed that the expression of HIF-1α in HCC tissues was positively correlated with Aurora A expression (r = 6.161, P = 0.013; Fig. 2C).

Correlation of HIF-1α and Aurora A expression with clinical outcome of patients

Then, we analyzed the correlation of HIF-1α and Aurora A expression with clinicopathologic factors of HCC patients including age, gender, serum HBsAg, tumor size, fibrosis, tumor stage, and lymph node metastasis. As shown in Table 1, positive HIF-1α expression was significantly correlated with advanced tumor stage and lymph node metastasis (P = 0.002 and 0.018, respectively), but not with other clinicopathologic factors. Likewise, we also showed that positive Aurora A expression was
correlated with advanced tumor stage and lymph node metastasis \((P = 0.002\) and \(0.004\), respectively). Survival analysis by the Kaplan-Meier method indicated that positive HIF-1α expression tended to be associated with a poor prognosis of HCC patients. The mean 5-y survival rate of HCC patients with positive or negative HIF-1α was 38.8% or 55.5%, respectively \((P = 0.0047; \text{Fig. 3A})\). It was also found that positive Aurora A expression tended to be associated with a poor prognosis of HCC patients. The mean 5-y survival rate of HCC patients with positive or negative Aurora A was 38.8% or 55.5%, respectively \((P = 0.0047; \text{Fig. 3A})\). It was also found that positive Aurora A expression was correlated with poor prognosis of patients. The mean 5-y survival rate of HCC patients with positive or negative Aurora A was 38.8% or 55.5%, respectively \((P = 0.0047; \text{Fig. 3A})\). It was also found that positive Aurora A expression was correlated with poor prognosis of patients. The mean 5-y survival rate of HCC patients with positive or negative Aurora A was 38.8% or 55.5%, respectively \((P = 0.0047; \text{Fig. 3A})\).

To further determine the prognostic value of HIF-1α expression combined with Aurora A status, we divided all the patients into the following groups: HIF-1α-positive expression and Aurora A-positive group; HIF-1α-negative expression and Aurora A-positive; HIF-1α-negative expression and Aurora A-negative group. Kaplan–Meier survival curves showed that the HIF-1α (+) expression and Aurora A (+) group had the poorest prognosis, with a 5-y overall survival rate of 27.3%, which was significantly lower than that of other groups \((P < 0.05\) for all groups). From these results, it was concluded that both HIF-1α and Aurora A might play critical roles in HCC progression.

pSil/s2 targeting HIF-1α could significantly downregulate the expression of HIF-1α and its target genes in HCC cells under hypoxic condition

DNA sequencing results indicated that 3 different shRNA expression vectors targeting HIF-1α and the negative control (NC) were successfully constructed. Those vectors were named pSil/s1, pSil/s2, and pSil/NC, and those vectors were stably transfected into HepG2 and BEL-7405 cells under hypoxia. Then, semi-quantitative RT-PCR and western blot assays were performed to detect the expression of HIF-1α and HIF-2α mRNA and protein in stably transfected HCC cell lines (Fig. 4A). Compared with mock cells, the levels of HIF-1α mRNA or protein in HepG2 and BEL-7405 cells stably transfected with pSil/s2 were significantly reduced \((P < 0.05)\). However, the levels of HIF-1α mRNA and protein in HCC cells stably transfected with pSil/s1 or pSil/NC showed no significant difference \((P > 0.05)\). Also, we found that pSil/s2 could have no effects on expression of HIF-2α mRNA and protein in HCC cells. Thus, pSil/s2 could effectively and specifically silence endogenous HIF-1α expression in HCC cells. Previous studies have shown that VEGF, MMP2, GLUT-1, and BNIP3 are target genes regulated by HIF-1α in various human cancer cells. Here, the expression of those target genes was examined.

**Figure 1.** Hypoxia induces the increased expression of HIF-1α and Aurora A protein in HCC cells. (A) RT-PCR and western blot assays were performed to detect the expression of HIF-1α mRNA and protein in 2 HCC cell lines (HepG2 and BEL-7405), 3 HCC and corresponding non-tumor tissues. (B) RT-PCR and western blot assays were performed to detect the expression of Aurora A mRNA and protein in 2 HCC cell lines (HepG2 and BEL-7405) and a primary hepatocyte cell line (HH), 3 HCC and corresponding non-tumor tissues. (C) HepG2 and BEL-7405 cells were exposed to hypoxia for 0, 0.5, 2, 4, 6, or 8 h. Total RNA was extracted and subjected to semi-quantitative RT-PCR assay to determine the expression of HIF-1α and Aurora A mRNA. (D) HepG2 and BEL-7405 cells were exposed to hypoxia for 0, 0.5, 2, 4, 6, or 8 h. Total protein was prepared and subjected to western blot assay to determine the expression of HIF-1α and Aurora A protein. β-actin was used as an internal control. All experiments were performed in triplicate. *\(P < 0.01\) and **\(P < 0.05\) vs. cells under normoxia. T, tumor tissues; N, non-tumor tissues; Norma, normoxia.**
genes in HIF-1α-downregulated HCC cells under hypoxia was also determined, and results indicated that the expression of those target genes was significantly reduced in HCC cells stably transfected with pSil/s2 (Fig. 4B).

Effect of HIF-1α expression on growth, colony formation, and apoptosis of HCC cells under hypoxic condition

Previous studies have shown that HIF-1α plays a critical role in cancer progression.19,20 However, whether the overexpression of HIF-1α affects the growth of HCC cells is still unclear. In this study, the results of MTT assay indicated that shRNA-mediated HIF-1α knockdown could significantly reduce the growth rate of HCC cells in a time-dependent manner (Fig. 5A), and the highest inhibitory rate was 58.02 or 55.36% for HepG2 or BEL-7405 cell line, respectively, on day 5 (P < 0.01). Then, the effect of HIF-1α downregulation on colony formation capacity of HCC cells was determined. Compared with that in pSil/NC-transfected cells, the number of colonies in pSil/s2-transfected HepG2 or BEL-7405 cells was significantly reduced by 53.8% or 56.3%, respectively (P < 0.05; Fig. 5B). Finally, we analyzed the effect of HIF-1α downregulation on apoptosis of HCC cells by flow cytometry (Fig. 5C). Compared with those of pSil/NC-transfected cells, the apoptotic rate of pSil/s2-transfected HepG2 or BEL-7405 cells was significantly increased by 14.31% or 15.78%, respectively (P < 0.05). The expression of cleaved caspase-3 and PARP proteins in pSil/s2-transfected HepG2 or BEL-7405 cells was significantly increased in comparison with that in pSil/NC-transfected cells (P < 0.05; Fig. 5D). Thus, shRNA-mediated HIF-1α knockdown could inhibit growth, reduce colony formation, and enhance apoptosis of HCC cells under hypoxic condition.

Effect of HIF-1α expression on migration and invasion of HCC cells under HCC cells under hypoxic condition

Tumor cell migration and invasion are directly correlated with metastasis, and the overexpression of HIF-1α has been found to play a critical role in tumor invasion and metastasis.21 To determine whether repression of HIF-1α expression inhibits HCC cell migration and invasion, wound healing and transwell cell invasion assays were performed under hypoxic conditions. As shown in Figure 6A, at 24 h, HepG2 or BEL-7405 cells stably transfected with pSil/s2 showed 9.1 or 13.0% wound closure, respectively (P < 0.05), and those cells stably transfected with pSil/NC showed 22.6 or 21.7% wound closure, respectively, suggesting that inhibition of HIF-1α could lead to the decreased migration of HCC cells. Also, the number of pSil/s2-transfected HepG2 or BEL-7405 cells that passed through the filter was significantly less than that of pSil/NC-transfected cells (Fig. 6B). Consistent with the results of the wound-healing assay, shRNA-mediated downregulation of HIF-1α could inhibit the in vitro migration.

Figure 2. Expression patterns of HIF-1α and Aurora A in 55 cases of HCC tissues and corresponding non-tumor tissues. (A) The overall immunoreactivity of HIF-1α was high in HCC tissues compared with non-tumor tissues. Right: percentage of cases with positive HIF-1α in HCC tissues and non-tumor tissues. (B) The overall immunoreactivity of HIF-1α was high in HCC tissues compared with non-tumor tissues. Right: percentage of cases with positive HIF-1α in HCC tissues and non-tumor tissues. Original magnification, ×100; insets ×200. (C) Tumors with HIF-1α expression were positively correlated with Aurora A expression. R values and P values from Fisher exact test. Bars, 50 μm.
invasion of HCC cells. These data indicated that HIF-1α could promote migration and invasion of HCC cells under hypoxic conditions.

HIF-1α was involved in hypoxic induction of Aurora A expression in HCC cells

To explore whether HIF-1α is involved in the hypoxic induction of Aurora A expression, the effect of HIF-1α downregulation on Aurora A expression in HCC cells was determined under hypoxic conditions. As shown in Figure 7A, pSil/s2 targeting HIF-1α could significantly inhibit the expression of hypoxic induction of Aurora A mRNA in both stably transfected HepG2 and BEL-7405 cells (P < 0.05). However, pSil/NC had no effect on expression of hypoxic induction of Aurora A mRNA. The same results were observed at the protein level of Aurora A gene (P < 0.05; Fig. 7B). These data clearly indicated that HIF-1α was involved in hypoxic induction of Aurora A in HCC cells.

Hypoxia activates Aurora A transcription by increasing the recruitment of HIF-1α to potential HREs on Aurora A promoter

The above experiments provided important clues to correlation between HIF-1α and Aurora A. Sequence analysis using the UCSC genome browser revealed that 3 short HRE (hypoxia-responsive element) consensus motifs (HRE1: −236 ~−240; HRE2: −319 ~−323; HRE3: −332 ~−336) were identified within the −1000 bp region preceding the transcriptional start site of Aurora A gene (Fig. 8A), which is also reported in other researches. Then, we investigate whether HIF-1α can regulate the activity of Aurora A promoter under hypoxic condition. First, we performed a CHIP assay in HepG2 cells to identify whether HIF-1α binds to the Aurora A promoter under hypoxia. The chromatin was immunoprecipitated with anti-IgG (negative control) and anti-HIF-1α antibodies, and the DNA precipitated in the complexes was subjected to PCR amplification with primers flanking the region containing 3 HREs. As shown in Figure 8B, an enrichment of the Aurora A promoter was detected using anti-HIF-1α antibody in HepG2 cells under hypoxic (P < 0.01) but not under normoxic conditions (P > 0.05), but no obvious signals were observed using a negative control antibody or no antibody under both conditions. Moreover, it was shown that hypoxia could significantly increase the activity of Aurora A promoter in HCC cells (P < 0.01; Fig. 8C). Also, when pSil/s2 (or pSil/NC) and luciferase reporter vector harboring wild-type Aurora A promoter (pGL3-Auro-pro) were co-transfected into HepG2 cells under hypoxia, we found that shRNA-mediated HIF-1α knockdown could significantly reduce the activity of Aurora A promoter in HCC cells (P < 0.05; Fig. 8D). Finally, whether HREs were essential for the transactivation of Aurora A gene induced by hypoxia was investigated. Then, luciferase reporter vectors harboring wild-type promoter or 3 different mutant HRE sites (mut1, mut2, or mut3) were constructed and transfected into HepG2 cells under hypoxia. Results showed that the luciferase activity of cells transfected with either mutant reporter vectors was significantly lower than that of cells transfected with wild reporter vector (Fig. 8E), showing the essential role of HRE sites involved in the hypoxia-induced transactivation of Aurora A gene in HCC cells.

Previous researches have shown that HIF-1α activates transcription through recruitment of the coactivator-p300/ CBP. Then, we performed additional CHIP assays to investigate whether hypoxia enhances p300 recruitment to the HREs region of Aurora A promoter (~500 --100 bp), and results showed that...
p300 could recruit to the Aurora A promoter after only 30 min of hypoxia. The recruitment of p300 to the Aurora A promoter was time limited, as after 4 h of hypoxia, p300 recruitment decreased (Fig. 9). However, under normoxic conditions, no PCR products were detected following immunoprecipitation with p300 antibody (Fig. 9). The specificity of the approach was demonstrated by the absence of PCR products when IP was performed using control IgG, or after IP with p300 antibody and PCR using primers corresponding to an upstream region of the Aurora A promoter that does not contain an HRE (−1000−−600 bp). These results suggest that HIF-1α regulates Aurora A expression by binding and recruiting p300 to the region of Aurora A promoter, including HREs.

shRNA vector targeting Aurora A could mimic the phenotypical changes of HCC cells induced by pSil/s2 targeting HIF-1α under hypoxic condition

To determine the effects of Aurora A expression on malignant phenotypes of HCC cells under hypoxia, shRNA expression vector targeting Aurora A (pSil/shAuro) was constructed and stably transected into HepG2 and BEL-7405 cells. Under hypoxic condition, pSil/shAuro could significantly induce the decreased expression of Aurora A mRNA and protein but had no effects on the expression of HIF-1α mRNA and protein in HepG2 or BEL-7405 cells (Fig. 10A). It was observed that downregulation of Aurora A could significantly lead to the decreased capacity of growth and colony formation in HCC cells under hypoxic condition (Fig. 10B and C). Compared with that of pSil/NC-transfected cells, the apoptotic rate of pSil/shAuro-transfected HepG2 and BEL-7405 cells could also be increased by approximately 13.4% and 11.2%, respectively (P < 0.05; Fig. 10D). Next, we analyzed the effect of Aurora A downregulation on migration and invasion of HCC cells, and showed that shRNA-mediated Aurora A knockdown could inhibit migration and invasion of HCC cells (P < 0.05; Fig. 10E and F). Taken together, Aurora A knockdown could mimic the effects of HIF-1α downregulation on malignant phenotypes of HCC cells under hypoxic conditions.

Overexpression of Aurora A could partially rescue phenotypical changes of HCC cells induced by pSil/s2 targeting HIF-1α

To further investigate the roles of hypoxia-induced Aurora A upregulation in malignant phenotypes of HCC cells under hypoxia, pMD18/Auror vector was transiently transfected into BEL-7405 cells stably transfected with pSil/s2. Forty-eight hours after transfection, qRT-PCR and western blotting assays were performed to detect the expression of Aurora A (Fig. 11A). Results showed that pMD18/Auror could rescue the decreased expression of Aurora A both mRNA and protein in pSil/s2-transfected HCC cells (P < 0.05). MTT and colony-formation assays indicated that overexpression of Aurora A could partially rescue the growth inhibition of HCC cells induced by pSil/s2 targeting HIF-1α (Fig. 11B and C). Also, overexpression of Aurora A could partially rescue the increased apoptosis of HCC cells induced by pSil/s2 targeting HIF-1α (Fig. 11D). Furthermore, overexpression of Aurora A could partially counteract inhibition of migration and invasion in HCC cells induced by pSil/s2 targeting HIF-1α (Fig. 11E and F). Therefore, overexpression of Aurora A could partially rescue phenotypical changes of HCC cells induced by HIF-1α downregulation, further suggesting that Aurora A might be a key regulator of HIF-1α-promoting malignant phenotypes of HCC cells.

**Figure 4.** pSil/s2 targeting HIF-1α effectively and specifically reduces HIF-1α expression and its target genes in HCC cells under hypoxia. (A) Semi-quantitative RT-PCR and western blot assay was performed to detect the expression of HIF-1α and HIF-2α mRNA and protein. Compared with mock or pSil/NC-transfected cells, pSil/s2 but not pSil/s1 could significantly induce the decreased levels of HIF-1α mRNA and protein expression in HepG2 or BEL-7405 cells. However, both pSil/s2 and pSil/s1 could have no effects on expression of HIF-2α mRNA and protein in HCC cells. (B) Semi-quantitative RT-PCR and western blot assays were performed to detect the expression of HIF-1α target genes VEGF, MMP-2, GLUT1, and BNIP3 in HepG2 or Hep-3B cells in comparison to pSil/NC-transfected cells. All experiments were performed in triplicate. *P < 0.05 vs. pSil/NC-transfected cells.
Activation of Akt and p38-MAPK signaling pathways mediated the downstream effects of HIF-1α and Aurora A in HCC cells under hypoxic condition

It has been reported that Akt and p38-MAPK signaling pathways can be activated by hypoxia in various cells, and the activation of both 2 signaling pathways was monitored by the phosphorylated Akt (pAkt) and p38 (p-p38). The effect of hypoxia on expression of pAkt and p-p38 proteins was determined. As shown in Figure 12A, hypoxia treatment could lead to the induction of pAkt and p-p38 in BEL-7405 cells, whereas the expression of total Akt and p38 proteins showed no obvious changes, implying that hypoxia could lead to the activation of PI3K/Akt and p38-MAPK signaling pathways. Whether activation of Akt and p38-MAPK signaling pathways mediated the downstream effects of HIF-1α and Aurora A in HCC cells is further determined (Fig. 12B). Under the hypoxic condition, we found that both pSil/s2 targeting HIF-1α and pSil/shAuro could significantly reduce the expression of pAkt and p-p38 proteins ($P < 0.05$). More importantly, pMD18/Auror could partially rescue the decreased expression of pAkt and p-p38 proteins. Then, we further analyzed the effects of the Akt-specific inhibitor (MK-2206) and p38-MAPK-specific inhibitor (SB-202190) on growth and apoptosis of BEL-7405 cells under hypoxic condition (Fig. 12C). Cells treated with MK-2206 (3.0 μmol/L) or SB-202190 (50 μg/ml) or combined treatment had significantly reduced proliferation compared with control cells. Analysis of apoptosis indicated that MK-2206 or SB-202190 or combined treatment could induce the increased apoptosis and expression of cleaved caspase-3 or PARP proteins compared with control cells. At the same time, we analyzed the effects of the Akt-specific inhibitor (MK-2206) and p38-MAPK-specific inhibitor (SB-202190) on migration and invasion of BEL-7405 cells under hypoxic conditions (Fig. 12D). Results showed that MK-2206 or SB-202190 or combined treatment could significantly reduce the capacity of migration and invasion in HCC cells. These data indicate that inhibition of Akt and p38-MAPK signaling can mimic the downstream effects of HIF-1α and Aurora A downregulation in HCC cells under hypoxic conditions.

Table 1. Correlation of HIF-1α and Aurora A expression with clinicopathological factors of HCC patients

| Clinicopathological factors | Positive HIF-1α | Negative HIF-1α | $P$ value | Positive Aurora A | Negative Aurora A | $P$ value |
|----------------------------|----------------|----------------|-----------|-------------------|-------------------|-----------|
| Age (n = 30) | 0.609 | | |< 55 | 20 | 15 | 0.672 |
| ≥ 55 | 10 | 10 | 23 | 12 | | |
| Gender | | | | Male | 21 | 13 | 0.171 |
| Female | 9 | 12 | | 25 | 9 | 0.052 |
| Serum HBsAg | | | | Negative | 11 | 9 | 0.959 |
| Positive | 19 | 16 | | 13 | 7 | 0.874 |
| Tumor size | | | | < 5 cm | 14 | 11 | 0.843 |
| ≥ 5 cm | 16 | 14 | | 16 | 9 | 0.959 |
| Fibrosis | | | | F1/F2 | 20 | 12 | 0.162 |
| F3/F4 | 10 | 13 | | 22 | 10 | 0.352 |
| Tumor stage | | | | I-II | 6 | 15 | 0.002* |
| IIA-IV | 24 | 10 | | 8 | 13 | 0.002* |
| Lymph node metastasis | | | | No | 12 | 18 | 0.018* |
| Yes | 18 | 7 | | 14 | 16 | 0.004* |

*The $P$ value was determined using the Mann–Whitney U test, the chi-square test and Fisher exact probability test.
Figure 5. pSil/s2-mediated knockdown of HIF-1α significantly inhibits growth, reduces colony formation capacity and increases apoptosis of HCC cells under hypoxia. (A) MTT analysis of growth in pSil/NC or pSil/s2-transfected HepG2 and BEL-7405 cells. Compared with pSil/NC-transfected cells, the growth of pSil/s2-transfected HCC cells was significantly inhibited. (B) The results of colony-formation assay. Compared with that in pSil/NC-transfected cells, the number of colony formation in pSil/s2-transfected HCC cells was significantly reduced. (C) Flow cytometric analysis of apoptosis in pSil/NC or pSil/s2-transfected HepG2 and BEL-7405 cells. (D) Western blotting analysis of cleaved caspase-3 or PARP protein expression in pSil/NC or pSil/s2-transfected HepG2 and BEL-7405 cells. All experiments were performed in triplicate, *P < 0.05 and **P < 0.01 vs. pSil/NC-transfected cells.
Discussion

To date, the molecular basis of HCC pathogenesis is not fully defined, and this has hindered the development of novel cancer therapeutics. Thus, identification of novel molecular mechanisms involved in HCC carcinogenesis and development is needed. Aurora A is an oncogenic serine/threonine kinase, which can cause cell transformation and centrosome amplification when overexpressed.22 In our previous studies, it was shown that overexpression of Aurora A in HCC possessed both clinicopathological and prognostic importance, and RNA interference-mediated Aurora A downregulation could lead to growth inhibition of HCC cells. However, the molecular mechanisms of Aurora A overexpression in HCC are still unknown, and understanding the regulatory mechanism of Aurora A is a key step in developing new strategies for cancer diagnosis and treatment. Under normal physiological conditions, as well as diseases such as cirrhosis and cancer, hypoxia helps to regulate expression of many human genes.23 In this regard, HIF-1α has been identified as a key regulator of the hypoxic responses, activating various tumor-associated genes, including VEGF, MMP-2, GLUT1, and BNIP3, which have been reported to be involved in such biological processes as energy metabolism, cell survival, and angiogenesis.24-26 The overexpression of HIF-1α has been found to promote and maintain a more malignant phenotype in several human cancers.27,28 Although others have shown that HIF-1α could transcriptionally activate the expression of Aurora A gene,18 the correlation between the 2 proteins in HCC and the roles of Aurora A upregulation in HIF-1α-promoting malignant phenotypes of HCC remain unclear. Thus, in this study, we focused on the roles of HIF-1α-mediated Aurora A upregulation in HCC oncogenesis.

First, we determined the expression of HIF-1α and Aurora A in HCC cells or tissues at both mRNA and protein levels, and showed that the expression levels of both HIF-1α and Aurora A in HCC cells or tissues were significantly higher than that in paired non-tumor tissues, suggesting that the trend of HIF-1α expression might be consistent with that of Aurora A expression.

Figure 6. shRNA-mediated knockdown of HIF-1α significantly inhibits migration and invasion of HCC cells under hypoxia. (A) The capability of cell migration was determined using a wound-healing assay. A confluent monolayer of pSil/NC or pSil/s2-transfected HepG2 or BEL-7405 cells was wounded. Photographs were taken immediately (0 h) and at 24 h after wounding; quantification of wound closure was done. The data present the mean distance of cell migration to the wound area at 24 h after wounding in 3 independent wound sites per group. (B) The capability of cell invasion was determined using a transwell invasion assay. The pSil/NC or pSil/s2-transfected HepG2 or BEL-7405 cells were subject to transwell assays. After 24 h, invasion cells were counted after staining with crystal violet. All experiments were performed in triplicate. *P < 0.05 vs. pSil/NC-transfected cells.
in HCC. Then, we investigated the hypoxic regulation of Aurora A gene in HCC cells, and found that Aurora A was significantly induced by hypoxia, and this upregulation was directly mediated by HIF-1α. By immunostaining of HIF-1α and Aurora A protein in HCC tissues, we found that HIF-1α protein was aberrantly expressed in either cytoplasm or nucleus in HCC cells, while Aurora A protein was mainly located in the cytoplasm. Meanwhile, the positive rates of both HIF-1α and Aurora A in HCC tissues were significantly higher than those in non-tumor tissues. Furthermore, it was found that the expression of HIF-1α protein in HCC tissues was positively associated with expression of Aurora A protein in accordance with other published results on nasopharyngeal carcinoma. Results of clinicopathological significance showed that both HIF-1α and Aurora A expression were significantly correlated with advanced tumor stage and lymph node metastasis. Upregulation of HIF-1α has been observed in a variety of human malignancies and is thought to be associated with poor survival, which was in agreement with our results that HCC patients with positive HIF-1α had a poor survival. In the same research group, we also found that HCC patients with positive Aurora A immunostaining had a poor survival, which was consistent with our previous studies demonstrating that HCC patients with high Aurora A mRNA expression had a poorer survival. From these data, it was concluded that HIF-1α might play a physiologic role in regulating Aurora A expression during HCC progression. To the best of our knowledge, this is the first study on functional correlation between HIF-1α and Aurora A in HCC to be reported.

Hypoxia exerts a biologically inducible stress and a strong selective pressure in a tumor, and HIF-1α is the major transcription factor that is specifically activated during hypoxia. A hypoxia-mediated increase in HIF-1α plays critical roles in tumorigenesis and progression of many cancers through HIF-1α-dependent gene activation. In the present study, we showed that shRNA-mediated HIF-1α knockdown could significantly reduce the capacity of growth and colony formation of HCC cells while increasing cell apoptosis. Liu and his colleagues showed that antisense hypoxia-inducible factor 1α gene therapy could enhance the therapeutic efficacy of doxorubicin to combat HCC. Also, Yang et al. reported that HIF-1α downregulation by small interfering RNA inhibited proliferation, induced apoptosis, and enhanced radiosensitivity in chemical hypoxic human hepatoma.

**Figure 7.** Hypoxic induction of Aurora A is dependent on HIF-1α in HCC cells. (A) Semi-quantitative RT-PCR and western blot assays were performed to detect the expression of HIF-1α or Aurora A mRNA and protein in mock HepG2 cells or HepG2 cells stably transfected with pSil/NC or pSil/s2 under normoxia or hypoxia for 24 h. (B) Semi-quantitative RT-PCR and western blot assays were performed to detect the expression of HIF-1α or Aurora A mRNA and protein in mock BEL-7405 cells or BEL-7405 cells stably transfected with pSil/NC or pSil/s2 under normoxia or hypoxia for 24 h. β-actin was used as an internal control. All experiments were performed in triplicate. *P < 0.05 and **P < 0.01.
SMMC-7721 cells, suggesting that inhibition of HIF-1α gene could lead to sensitization of HCC cells to therapeutic agents. The similar effects have been found in other human tumors, such as prostate carcinoma, oral squamous cell carcinoma, glioma, pancreatic cancer, and so on. In this study, we found that silencing of HIF-1α could significantly inhibit migration and invasion of HCC cells. Similarly, a study by Chio and colleagues showed that the knockdown of HIF-1α by adenovirus-mediated shRNA inhibited angiogenesis and tumor growth in HCC cell lines. These data suggest that high expression of HIF-1α not only

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**Figure 8.** Hypoxia increases the transcription of Aurora A by enhancing the recruitment of HIF-1α to HREs in Aurora A promoter in HCC cell line (HepG2). (A) Three potential HRE sites (site 1, site 2, and site 3, starting at position −236 ~−240, −319 ~−323, and −332 ~−336 of human Aurora A promoter, respectively) were identified. (B) Chromatin immunoprecipitation analysis. HIF-1α binds to the Aurora A promoter in vivo. HepG2 cells were cultured under normoxia or hypoxia for 24 h. Purified DNA was analyzed by PCR using primers as described in "Materials and Methods". A sample representing 10% of the chromatin used as a starting material for the immunoprecipitation is also shown (input). The amplified PCR fragments were analyzed on 1.5% agarose gel. The densities of the acquired bands were quantified by densitometric analysis using the Labworks Image Acquisition and normalized to Input included in each experiment. (C) Dual luciferase assay. HepG2 cells were transfected with pGL3-Auro-pro or pGL3-Basic under normoxic or hypoxic conditions for 24 h. (D) Dual luciferase assay. HepG2 cells were co-transfected with pGL3-Auro-pro and pSil/NC or pSil/s2 under normoxic or hypoxic conditions for 24 h. (E) Dual luciferase assay. HepG2 cells were transfected with wild-type or HRE mutant Aurora A promoter constructs under hypoxic conditions for 24 h. All experiments were performed in triplicate. NS, not significant. *P < 0.05 and **P < 0.01.
represents a potential prognostic molecular marker, but also contributes to HCC cell proliferation, apoptosis, and invasion. At the same time, we found that hypoxia could induce an increased expression of Aurora A gene at both transcriptional and translational levels. Tumor hypoxia is an important regulator for the expression of many genes that are involved in malignant transformation and tumor development, and thereby the transcription factor HIF-1α has been reported to be a key regulatory factor for many tumor-associated genes. Then, we investigated whether HIF-1α was involved in the transcriptional activation of Aurora A by hypoxia. A CHIP assay in HepG2 cells showed that an enrichment of the Aurora A promoter could be detected under hypoxia but not under normoxia. To further define the binding sites of HIF-1α in the Aurora A promoter that are contributing to increased expression under hypoxic conditions, we performed luciferase based reporter assays using 1.0 kb gene sequence immediately upstream of the translation start site, and observed that hypoxia could induce increased activity of Aurora A promoter-reporter construct. Also, silencing of HIF-1α could significantly increase the activity of Aurora A promoter-reporter construct in HepG2 cells under hypoxia. Three potential short HRE consensus motifs in the Aurora A promoter were identified by using the UCSC genome browser. Then, mutagenesis experiments and subsequent reporter gene assays indicated that the activity of 3 promoter-reporter constructs with mutated HRE was significantly reduced compared with wild promoter-reporter construct, and of the various mutated promoter-reporter constructs tested the mutation of the site we designated as HRE-2, located within the −320 bp region, appeared to have the largest impact on a decrease of reporter activity under hypoxic mimic conditions. This is consistent with a similar high basal reporter activity reported by Klein and colleagues.18 More importantly, our results are consistent with the notion that, with hypoxia, HIF-1α recruits p300 to the HRE of the Aurora A promoter. Of course, except for p300, whether other coactivators are involved in the HIF-1α-induced transcriptional regulation of Aurora A expression still needs to be further elucidated. However, the functional correlation between HIF-1α and Aurora A in HCC is unclear. The data further suggested exploration of the roles of Aurora A overexpression in HIF-1α-promoting malignant phenotypes of HCC cells. Functional analyses indicated that shRNA-mediated Aurora A knockdown could mimic the effects of HIF-1α downregulation on malignant phenotypes of HCC cells, and overexpression of Aurora A could rescue those HIF-1α downregulation-induced phenotypical changes of HCC cells, including not only growth but also invasion in an in vitro model. To our knowledge, this is the first study about the important roles of Aurora A in HIF-1α-promoting migration and invasion of tumor cells. Activation of various growth factor-activated signaling pathways plays critical roles in malignant transformation and tumor development.43 Among various growth factor-activated signaling pathways, the PI3K/Akt and MAPK signaling pathways have been shown to be the important growth factor-activated pathways in the tumorogenesis of many types of cancers, including HCC.44,45

Previously, it has been reported that overexpression of Aurora A could induce the activation of PI3K/Akt and MAPK signaling pathways in human cancers.46,47 However, whether activation of both signaling pathways mediated the downstream effects of HIF-1α and Aurora A is unclear. In this study, it was found that hypoxia could induce the increased expression of phosphorylated AKT (pAkt) or p38 (p-p38) proteins. Also, shRNA-mediated downregulation of either HIF-1α or Aurora A could significantly reduce the expression of pAkt and p-p38 proteins, while upregulation of Aurora A could rescue the increased expression of pAkt and p-p38 proteins induced by siSil/s2 targeting HIF-1α. Meanwhile, the Akt- or p38-MAPK-specific inhibitor could inhibit growth, enhance apoptosis, and reduce migration and invasion in HCC cell. These experimental data suggest that activation of Akt and p38-MAPK signaling pathways may mediate the downstream effects of HIF-1α and Aurora A in HCC cells.

Taken together, our findings indicate that HIF-1α is overexpressed and positively correlated with Aurora A expression levels in HCC, and can promote malignant phenotypes of HCC cells through activation of Akt and p38-MAPK signaling pathways by transcriptionally regulating Aurora A expression. This study has several limitations. First, since the number of patients in the present study is small, further study of a larger case population is necessary to confirm the clinical significance of HIF-1α and Aurora A expression in HCC. Second, in the present study, we have only focused on in vitro cell models, further research on the roles of Aurora A in HIF-1α-promoting tumorigenesis and metastasis of tumor cells in an in vivo model might strengthen the significance of our study.

Materials and Methods

Ethics statement
The study was approved by the research ethics committee of Jinling Hospital and the Jiangsu Province Medical Association.
Tissues used in the study were obtained with informed written consent from each patient.

**Cell culture**

Two human hepatoma cell lines (HepG2 and BEL-7405) and a primary hepatocyte cell line (HH) were cultured in growth medium (DMEM; GIBICO) supplemented with 10% fetal bovine serum (Gibco Ltd) and 1% penicillin-streptomycin (Sigma). All cell lines were maintained at 37 °C with 5% CO₂. Hypoxic culture conditions were achieved with a multigas incubator containing a gas mixture composed of 94% N₂, 5% CO₂, and 1% O₂.

**Patients and tissue samples**

A total of 55 primary HCC and corresponding non-tumor tissues were collected from the Department of Pathology, Jinling Hospital, Nanjing University. All specimens were embedded in paraffin. All patients were clearly diagnosed as having hepatocellular carcinoma based on the clinicopathological findings. All tumors were histopathologically confirmed to contain at least 80% malignant cells, and none of the participants received preoperative treatment. The tumor type and the grade of cell differentiation were designated based on the criteria of World Health Organization (WHO), whereas the pathological stage of each tumor was determined by the International Union Against Cancer (UICC) TNM classification. Details of clinical and pathological characteristics of the patients were summarized in Table 1. Informed consent was obtained from all subjects prior to carrying out the study, and this study was approved by the Review Board of Hospital Ethics Committee. The study was performed in...

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**Figure 10.** shRNA-mediated knockdown of Aurora A could significantly inhibit growth, enhance apoptosis and reduce migration or invasion of HCC cells under hypoxia. (A) RT-PCR and western blot assays were performed to detect the mRNA and protein expression of Aurora A or HIF-1α in pSil/NC or pSil/shAuro-transfected HepG2 or BEL-7405 cells under normoxic or hypoxic conditions, respectively. (B) MTT and colony formation analysis of growth in pSil/NC or pSil/shAuro-transfected HepG2 or BEL-7405 cells. (C) Hoechst staining analysis of apoptosis in pSil/NC or pSil/shAuro-transfected HepG2 or BEL-7405 cells according to the previous method. White arrow indicates a cell with condensed nucleus. (D) Analysis of the effect of shRNA-mediated Aurora A knockdown on migration of HepG2 or BEL-7405 cells using a wound-healing assay according to the previous method. (E) Analysis of the effect of shRNA-mediated Aurora A knockdown on invasion of HepG2 or BEL-7405 cells using a transwell invasion assay according to the previous method. All experiments were performed in triplicate. *P < 0.05 or **P < 0.01 vs. pSil/NC-transfected cells.
compliance with the Helsinki Declaration. None of the patients received chemotherapy or radiotherapy prior to surgery.

Construction of plasmid vectors and transfection

Short hairpin RNAs (shRNA) specifically targeting human HIF-1α gene (GenBank No.U22431) and Aurora A gene (GenBank no. NM_003600) were designed to knockdown HIF-1α and Aurora A expression. The shRNA sequences targeting HIF-1α and Aurora A were as follows:

HIF-1α-s1: sense, 5′-GATCCGTAGC-CTCTTTGACAACTTTCAAGAGAGTTTGTCAAAGAGGCTACAGA; HIF-1α-s2: sense, 5′-GA-TCCCTGATGACCAGCAACTTGATTCAAGAGATCAAGTTGCTGGTCATCAGAGA-3′;

Aurora A-shRNA: sense 5′-GATCCATGCCCTGTCTTAACGTGCATTCAAGAGATGACAGTAAGACAGG-GCATAGA-3′.

Negative control (NC) shRNA, sense: 5′-GATCCAAGCTGAAGTACAACCTTCT-TCAAGAGAGAAGGTTGTACTTCAGCTTAGA-3′.

All the above sequences were inserted into the BglII and HindIII enzyme sites of pSilencer4.1-CMVneo vector, respectively. The recombinant plasmids were named pSil/s1, pSil/s2, pSil/shAuro, and pSil/NC, respectively. The recombinant vectors were confirmed by the digestion analysis of restriction endonuclease, and all the constructed plasmids were confirmed by DNA sequencing. The plasmid vector (pMD18/Auro) expressing Aurora A was purchased from Sino Biological Inc. The cell transfection was performed in opti-MEM with the transfection reagent Lipofect AMINE PLUS (Invitrogen) following the manufacturer’s protocols. After 6 h of transfection, the cells were incubated with the fresh medium under either the normoxic or hypoxic (1.0% O2) condition for a further 24 h. The cell lines transfected with knockdown constructs were stably selected with G418 (400 mg/mL) 48 h later after transfection, and individual clones were isolated and maintained in a medium containing G418 (100 mg/mL). The cells were collected for further researches.

Immunohistochemistry

Paraffin-embedded, formalin-fixed tissues were immunostained for Aurora A and HIF-1α protein (Santa Cruz Biotechnology) using standard immunohistochemistry procedures described previously. Immunostained slides were analyzed independently by 2 authors. Slight differences were resolved by simultaneous viewing. Sections were scored semi-quantitatively for the extent of immunoreaction as follows: 0, 0% immunoreactive cells; 1, <5% immunoreactive cells; 2, 5–50% immunoreactive cells; and 3, >50% immunoreactive cells. Also, the intensity of staining was scored semi-quantitatively as 0, negative; 1, weak; 2, intermediate; and 3, strong. The final immunoreactions score was defined as the sum of both

Figure 11. Overexpression of Aurora A could partially rescue phenotypical changes of HCC cells induced by pSil/s2-mediated HIF-1α knockdown under hypoxia. (A) RT-PCR and western blot assays were performed to detect the expression of Aurora A mRNA and protein in BEL-7405 cells stably transfected with pSil/s2 (pSil/NC as control) or co-transfected with pSil/s2 and pMD18/Auro, respectively. (B) MTT analysis of growth in HepG2 cells stably transfected with pSil/s2 (pSil/NC as control) or co-transfected with pSil/s2 and pMD18/Auro. (C) The colony-formation capacity of BEL-7405 cells stably transfected with pSil/s2 (pSil/NC as control) or co-transfected with pSil/s2 and pMD18/Auro was determined using the previous method. (D) Hoechst staining analysis of apoptosis in BEL-7405 cells stably transfected with pSil/s2 (pSil/NC as control) or co-transfected with pSil/s2 and pMD18/Auro according to the previous method. White arrow indicates a cell with condensed nucleus. (E) A wound-healing assay was performed to detect the migration of BEL-7405 cells stably transfected with pSil/s2 (pSil/NC as control) or co-transfected with pSil/s2 and pMD18/Auro according to the previous method. (F) A transwell invasion assay was performed to detect the invasion of BEL-7405 cells stably transfected with pSil/s2 (pSil/NC as control) or co-transfected with pSil/s2 and pMD18/Auro according to the previous method. All experiments were performed in triplicate. *P < 0.05 and **P < 0.01.
parameters (extension and intensity), and samples were grouped as negative (0), weak staining (1–2), moderate staining (3) and strong staining (4–6). For statistical purposes, only moderate and strong final immunoreaction scores were considered to be positive, and the other final scores were considered to be negative.

**Semi-quantitative RT-PCR assay**

RNA isolation and semi-quantitative RT-PCR was performed as previously reported. For HIF-1α, the forward primer was 5'-TCGACACAGCCTGGATATGA-3' and the reverse was 5'-CGGCTCGGCGAACAGAAAGTT-3', the amplicon size is 247 base pair (bp). For HIF-2α, the forward primer was 5'-ATCATGCGACTGGCAATCAG-3', and the reverse was 5'-TCCTCATGGTCGCAGGGATG-3', the amplicon size is 260 base pair (bp). For Aurora A, the forward primer was 5'-CTACAATGAGCTGCGTG-3', and the reverse was 5'-GGTCTCTGCTCATCAA-3', the amplicon size is 575 bp. For β-actin, the forward primer was 5'-CTACAATGAGCTGCGTG-3', and the reverse was 5'-GGTCTCTGCTCATCAA-3', the amplicon size is 575 bp. The cycling program was performed as follows: 1 cycle of 94 °C for 3 min; 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 90 s; followed by a final elongation step of 72 °C for 10 min. Then, RT-PCR products were electrophoresed through a 1.5% agarose gel with ethidium bromide. Signals were quantified by densitometric analysis using the Labworks Image Acquisition. Results were normalized against those of β-actin.

**Western blot assay**

The transfected cells were harvested by suspension in lysis buffer (1 mM dithiothreitol, 0.125 mM EDTA, 5% glycerol, 1.0 mM phenylmethylsulfonylfluoride, 1.0 mg/mL leupeptin, 1.0 mg/mL pepstatin, 1.0 mg/mL aprotinin, 1% Triton X-100). All experiments were performed in triplicate. β-actin was used as an internal control. NS, not significant. *P < 0.05 and **P < 0.01.
μ

After 24 h, the medium was replaced with new medium containing repeated at least 3 times.

the wound area was determined.

chosen areas were measured, and the distance of cell migration to and observed under a microscope. A minimum of 5 randomly

medium. Twenty-four hours after wounding, cultures were fixed

suberib, and cells were washed and incubated in a serum-free

culture medium of living cells; changes in nuclear morphology

were detected by fluorescence microscopy using a filter for

colony formation; visible colonies were manually

were electrophoresed on a 12% SDS-polyacryl-a-mide gel and transferred onto PVDF membranes (Sigma Co), and then blocked in PBS containing 5% nonfat milk and 0.2% Tween-20 for 1 h at room temperature. The membrane was incubated overnight at 4 °C with rabbit anti-Aurora A (1:200), rabbit anti-HIF-1α (1:100), and anti-HIF-2α (1:100), rabbit anti-cleaved caspase-3 (1:100) or anti-PARP (1:150), rabbit anti-phosphorylated Akt (pAkt) (1:100), rabbit anti-phosphorylated p38 (p-p38) (1:300), rabbit anti-total Akt (1:200), or anti-total p38 (1:100) proteins in PBS containing 0.1% Tween-20 followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (Sigma) for 1.5 h. Then the membrane was washed several times with PBS containing 0.2% Tween-20. As an internal control, the same membrane was hybridized to anti-actin antibody (1:500) as control for loading equal amount of protein among each sample followed by incubation with horseradish peroxidase-conjugated anti-mouse IgM (Sigma) as above. The bands were visualized by chemiluminescence using a chemiluminescence kit (Invitrogen), and Lab Works Image Acquisition and Analysis Software (UVP) were used to quantify band intensities. Antibodies were purchased from Univ-bio Inc.

Cell proliferation assay

The cells were cultured in 96-well plates and were harvested for a standard tetrazolium bromide (MTT) assay as previously described.17 All assays were performed in Octuplicate and repeated at least 3 times.

Cell cycle

A total of 500 transfected cells were placed in a fresh 6-well plate and maintained in DMEM containing 10% fetal bovine serum. After 24 h, the medium was replaced with new medium containing 48 h (400 μg/ml). After 14 d, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted. Each experiment was performed in triplicate.

Flow cytometric analysis of apoptosis

An annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Oncogene Research Products) was used to detect apoptosis according to the manufacturer’s instructions.

Hoechst staining assay

Cells were cultured on six-well tissue culture plates to confluence, and Hoechst 33342 (Sigma) was added to the culture medium of living cells; changes in nuclear morphology were detected by fluorescence microscopy using a filter for Hoechst 33342 (365 nm). For quantification of Hoechst 33342 staining, the percentages of Hoechst-positive nuclei per optical field (at least 50 fields) were counted. Each experiment was performed in triplicate.

Wound-healing assay

Cell migration was measured using a wound-healing assay. In brief, cells were seeded in 12-well plates and cultured to confluence. Wounds of 1.0 mm width were created with a plastic scriber, and cells were washed and incubated in a serum-free medium. Twenty-four hours after wounding, cultures were fixed and observed under a microscope. A minimum of 5 randomly chosen areas were measured, and the distance of cell migration to the wound area was determined.

Transwell invasion assay

The cells were seeded into inserts at 800 per insert in serum-free medium and then transferred to wells filled with the culture medium containing 10% FBS as a chemoattractant. After 24 h of incubation, non-invading cells on the top of the membrane were removed by scraping. Invaded cells on the bottom of the membrane were fixed, followed by staining with 0.05% crystal violet. The number of invaded cells on the membrane was then counted under a microscope. Each experiment was performed in triplicate.

Dual-luciferase reporter assay

The 5'-flanking sequence (−1000 to +300) of human Aurora A was amplified using genomic DNA isolated from HepG2 cells using the Qiagen genomic DNA isolation kit (Qiagen). The PCR fragment was gel-purified and cloned into the BglII site of pGL3- basic vector (Promega) to obtain pGL3-Auro-pro. To generate HRE mutants of the human Aurora A promoter, a QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) was utilized. The pRL-TK plasmid containing the Renilla luciferase gene driven by the herpes simplex virus thymidine kinase promoter was co-transfected with the constructs, and the luciferase activity was normalized. Dual Luciferase Reporter Assays were performed in a Tristar multimode microplate reader LB 941 (Berthold Technologies). Experiments were performed in triplicate.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as previously reported using a kit from Upstate Biotechnology Inc, according to the supplied protocol. Semi-quantitative RT-PCR assay was performed to generate a 169 bp amplification product by using primers specific for the Aurora A sequence: sense, 5’-GGCGCAGAGTCTGCATTC-3’; reverse, 5’-GAGATAAGTCCAAGGAGGT-3’. A standard curve was prepared using serial dilutions of the pGL3-Auro-Pro promoter construct. The amount of Aurora A promoter that was present in the immunoprecipitation, and input fraction was calculated from the standard curve. The input represents 1.0% of the material used in the immunoprecipitation assay. The immunoprecipitation/input ratio of the vehicle-treated sample was considered as 100%, and the immunoprecipitation/input ratio of the LPS-treated sample was expressed as a percentage of the untreated.

Statistical analyses

The SPSS16.0 program was used for general statistical and survival analysis. Experimental data were expressed as the mean ± s.e.m. Fisher’s exact test was used to compare the expression of HIF-1α and Aurora A with various clinicopathologic factors. The Spearman test was used to analyze the association between HIF-1α and Aurora A. Survival rates were estimated using the Kaplan-Meier method, and statistical analysis was performed using the log-rank test for equality of the survival curves. The differences between groups were analyzed using Student t test when only 2 groups, or assessed by one-way analysis of variance (ANOVA) when more than 2 groups, were compared. All tests performed were 2-sided. Differences were considered statistically significant at P < 0.05.
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No potential conflicts of interest were disclosed.

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