HIF-1α regulates A2B adenosine receptor expression in liver cancer cells

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Abstract. Liver cancer exhibits the fourth most common cause of cancer-associated mortality worldwide. Due to the rapid growth, solid tumors undergo severe hypoxia and produce high levels of extracellular adenosine to maintain homeostasis. A previous study indicated that the hypoxic condition in liver cancer increased hepatic adenosine, which is known to facilitate cancer survival and proliferation. Extracellular adenosine has been revealed to regulate pathological and physiological processes in cells and tissues. However, its pathophysiological role in liver cancer remains undetermined. Emerging evidence has indicated that the adenosine A2B receptor promotes the progression of liver cancer. Therefore, it was hypothesized that HIF-1α is a transcriptional regulator of A2B in human liver cancer. The current study determined A2B expression of a number of liver cancer cell lines and performed functional studies of HIF-1α as a master transcriptional regulator of hepatic A2B signaling during hypoxic conditions. The current study aimed to identify the promoter region of A2B, which has a hypoxia response element, by performing luciferase assays. The present study demonstrated that reduced HIF-1α expression is associated with low expression of A2B, and HIF-1α overexpression is associated with A2B induction. Furthermore, the siRNA-mediated downregulation of A2B inhibited the growth and proliferation of HepG2, which is a liver cancer cell line. The relationship between HIF-1α and A2B expression was also identified in human liver cancer specimens. In conclusion, the current study indicated that A2B is induced by the HIF-1α transcriptional regulator during hypoxia, and it may be a potential pharmacologic and therapeutic target for the treatment of patients with liver cancer.

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Introduction

Liver cancer is the fourth leading cause of cancer mortality worldwide (1,2). Despite significant progress over the past decades, the clinical outcomes of patients with liver cancer remain poor and an in-depth understanding of the molecular pathogenesis of liver cancer could facilitate the development of novel diagnostic and therapeutic techniques.

Adenosine is an endogenous nucleoside that controls many physiological processes through interactions with adenosine receptors, such as A1, A2A, A2B, and A3 (3). Adenosine A2 receptors have two segmented isoforms; high-affinity A2A receptor which is highly concentrated in the striatum, and relatively low-affinity A2B receptors which present throughout the brain (4). Generally, adenosines can be found eluted into the

extracellular matrix of ordinary solid organs under low-oxygen conditions (5). However, in cancerous organs, the signaling pathway generated between adenosine and its receptor has been shown to coordinate adenosine accumulation and the increased levels of adenosine in the extracellular fluid of solid tumors stimulate cancer cell proliferation and tumor angiogenesis (6). The relationship between the tumor progression and adenosine A2B receptor expression has been investigated in many types of tumors including bladder (7), breast (8), colon (9), and prostate (10) cancers. While some researchers have shown that A2B is highly expressed in cancerous tissues in patients with HCC (11), the regulatory pathways and the function of A2B in liver cancer remain undiscovered.

The adenosine A2B receptor is reported to be transcriptionally induced by tumor necrosis factor-alpha (TNF-α) (12), interferon-gamma (IFN-γ) (13), and hypoxia-inducible factor-1α (HIF-1α) (14,15). HIF-1α is an oxygen-regulated subunit of HIF-1, a heterodimeric transcription factor consisting of α- and β-subunits. Intra-tumoral hypoxia is the major cause of increased HIF-1 activity in human HCC (16), and HIF-1α protein stabilization in cancer cells leading to the upregulation of multiple HIF-1 target genes that are required for HCC tumor growth, as demonstrated by both genetic and pharmacologic loss-of-function studies (17). HIF-1α mediated expression of adenosine A2B receptor activations in hypoxia has reported in endothelial cells (15), acute lung injury (18), and breast cancer (19). In addition to this, many researchers suggested that hypoxia is one factor for inducing A2B expressions, however, direct HIF-1α mediated A2B increment during tumor progression, especially in liver cancer has not been investigated yet. Therefore, we designed experiments to verify whether transcriptional regulation pathways control the endogenous hepatic adenosine signaling during a low-oxygen condition of liver cancer cells. Furthermore, we also investigated the potential HIF-1α binding regions in the A2B promoter genes. We believe our study will contribute to the development of new therapeutic targets to treat liver cancers.

Materials and methods

Cell culture and hypoxic conditions. We purchased human liver cancer cell lines, including HepG2 (KCLB no. 88065; passage 21), the hepatoblastoma cell line (20), and the hepatocellular carcinoma (HCC) cell lines SK-Hep1 (KCLB no. 30052; passage 49) and SNU-449 (KCLB no. 00449; passage 28) from the Korean Cell Line Bank (KCLB; Seoul, Republic of Korea). HepG2 and SK-Hep1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) high-glucose (Hyclone; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; Mediatech) and 100 U/ml penicillin/streptomycin (HyClone). The HCC SNU-449 cells were maintained in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. We cultured all the cells in a standard humidified incubator at 37°C in an atmosphere of 5% CO_2. To induce hypoxic conditions, we placed cells in a hypoxia incubator (MCO-18M; Sanyo) filled with a mixture of 5% CO_2, 94.5% N_2, and 0.5% O_2 gas. Authentication of the cell lines was done using short tandem repeat (STR) profiling by

the Korean Cell Line Bank (Seoul National University College of Medicine, Seoul, Republic of Korea; http://cellbank.snu.ac.kr/) with proper STR references. The cell lines were tested for mycoplasma contamination before use and were negative for mycoplasma.

Establishment of A2B luciferase constructs. We used a pGL3 basic luciferase vector as a control plasmid and performed luciferase assays with previously designed constructs (15). The full-length construct (1095 bp) had a hypoxia-response element (HRE). The other truncated construct (477 bp) lacked an HRE. Additionally, we used a mutant form of the 1095 bp plasmid with a modified sequence (A*CGTG* was altered to A*ATCG*).

A2B reporter assay. To confirm the transcriptional activity of the adenosine A2B receptor, we used HepG2 cells as an easily transfectable cellular model. First, to investigate the A2B promoter region, we used HepG2 cells to measure the reporter gene activity. We co-transfected cells with 2 µg of A2B-Luc promoter-reporter and 0.02 µg Renilla reporter vector for 24 h using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Next, we exposed the cells to hypoxic conditions for up to 12 h. To prepare whole protein lysates, we used 1x passive lysis buffer (cat no. E1910; Promega). To perform the luciferase assay, we followed the protocol of the Dual-Luciferase Reporter Assay (Promega).

RNA interference. Small interfering RNAs (siRNA) specific to either HIF-1α (HIF-1α-siRNA), A2B (A2B-siRNA), or scrambled sequences (scr-siRNA) were prepared by Bioneer Corporation. We used 0.5 µg of siRNA for transfections using Lipofectamine® 2000 reagent (Invitrogen). The sequences of siRNAs were: Scrambled-siRNA (scr-siRNA), sense, 5'-CCU ACGCCACCAAUUUCGU (dTdT)-3', antisense, 5'-ACGAAA UUGGUGGCGUAGG (dTdT)-3'; HIF-1α-siRNA, sense, 5'-GUGGUUGGAUCUAACACUA (dTdT)-3', antisense, 5'-UAGUGUUAGAUCCAACCAC (dTdT)-3'; A2B-siRNA, sense, 5'-GAGACUUCCGCUACACUUU (dTdT)-3', antisense, 5'-AAAGUGUAGCGGAAGUCUC (dTdT)-3'.

HIF-1α plasmid overexpression. We seeded HepG2 cells into 6-well plates at a density of $1.0x10^6$ cells per well and incubated them for 24 h at 37°C. The cells were transfected with a HIF-1-pcDNA3.1-expressing plasmid using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). We confirmed HIF-1α overexpression by western blot. The negative control plasmid pcDNA3.1(+) and the HIF-1-pcDNA3.1-expressing plasmids were purchased from Addgene. We treated cells with range from 3 to 300 nM of echinomycin (cat no. 5520; Tocris Bioscience), a specific inhibitor of HIF-1 DNA binding activity, to achieve chemical interference of HIF activity.

BrdU assay. Exponentially growing HepG2 cells were digested, centrifuged, collected, and inoculated into 96-well culture plates at a density of $5.0x10^3$ cells/well. After culturing for 24 h in DMEM containing 10% FBS, the cells were transfected with scrambled-siRNA (scr-siRNA) and A2B-siRNA. HepG2 cell proliferation activity was analyzed using

Table I. Clinical characteristic of five patients with HCC in the Asan Medical Center.

Patients	P1	P2	P3	P4	P5
Grading	Grade III	Grade III	Grade III	Grade III	Grade III
Gender	F	M	M	M	F
Age (years)	38	54	62	65	68
Risk Factors	HBV	HBV	HBV	HBV	HBV
AST (U/l)	174	82	133	191	190
ALT (U/l)	128	72	87	147	137
Albumin (g/dl)	3.5	3.6	3.3	3.0	2.8
Tumor size (cm)	3.7x3.3x3.3	2.4x1.5x1.0	4.5x4.0x3.3	11.6x10.8x9.6	14.2x10.8x7.5

P, patient; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

BrdU cell proliferation assay kits (cat no. 2750; Millipore), 24, 48, 72, and 96 h after siRNA transfection. The BrdU reagent was added to the culture wells at each time point, and the plates were incubated at 37˚C for an additional 4 h. The cells were then fixed, and the BrdU levels were detected colorimetrically following the manufacturer's instructions. To plot the growth curve, a microplate reader (Sunrise TECAN, Labx, Canada) was used to measure absorbance at 450 nm. Experiments were repeated four times.

Cell growth and viability test. Cell growth and viability were determined by performing a 3-(4,5-dimethythi-azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 12 well cell culture plates (NEST Biotechnology). HepG2 cells were seeded at a density of 5.0×10^4 per well for 24 h and cultured in DMEM containing 10% FBS. The cells were then transfected with scr-siRNA or A2B-siRNA. After incubation for 24, 48, and 72 h, 20 μl of MTT solution (5 mg/ml in phosphate buffer saline) was added to each well, and the plates were incubated in the dark for 4 h at 37˚C. Cell growth and viability were expressed as the percentage of absorbance of MTT-treated cells relative to that of untreated cells at a wavelength of 540 nm. Measurements for all treatment groups were taken four times.

Patient tissue samples. We collected five individual human HCC specimens from patients with HCC, who visited the Asan Medical Center (Division of Liver Transplantation and Hepatobiliary Surgery; Seoul, Republic of Korea) between 2014 and 2015 (Table I). None of the patients received any form of treatment before surgical procedures. We separately collected small pieces of the tumor and adjacent normal tissues and snap-froze them into liquid nitrogen. Frozen samples were stored at -80˚C until use. The Institutional Review Board of Asan Medical Center reviewed and approved the collection and use of patient specimens (approval no. 2016-0582). All patients who provided tissue samples agreed to donate their specimens and provided written informed consents.

RNA extraction and RT-PCR. We isolated total RNA from human livers and liver cancer cells using QIAzol reagent (Qiagen). We homogenized the frozen tissues and liver cancer cell suspensions in QIAzol reagent. The homogenates

Table II. Sequences of Primers used in the study.

Gene	Primer sequence
Adora1	Forward 5'-TGCACTGGCCTGTTCTGTAG-3'
	Reverse 5'-CTGCCTCTCCCACGTACAAT-3'
Adora2a	Forward 5'-GGAGTTTGCCCCTTCCTAAG-3'
	Reverse 5'-CTGCTTCCTCAGAACCCAAG-3'
Adora2b	Forward 5'-ATCTCCAGGTATCTTCTC-3'
	Reverse 5'-GTTGGCATAATCCACACAG-3'
Adora3	Forward 5'-CCTGGGCATCACAATCCACT-3'
	Reverse 5'-ACCCTCTTGTATCTGACGGTA-3'
Gapdh	Forward 5'-GAGTCAACGGATTTGGTCGT-3'
	Reverse 5'-TTGATTTTGGAGGGATCTCG-3'

were mixed thoroughly after adding chloroform and were centrifuged at 16,000 x g for 15 min. The aqueous phase was removed, and the RNA was precipitated with isopropyl alcohol. RNA was pelleted, washed with 70% ethanol, dried, and eluted using DEPC-treated water. We used a spectro-photometer Nanodrop 2000 (Nanodrop) to quantify the final concentration.

We quantified transcription of relevant genes using real-time reverse transcription-polymerase chain reaction (RT-PCR) in a CFX Connect Real-Time PCR Detection System (Bio-Rad) with 5x HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne), according to the manufacturer's instructions. In brief, the samples were first denatured at 95˚C for 15 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C-60˚C for 15 sec, and elonga-tion at 72˚C for 20 sec. The data are expressed as the fold changes in the treatment groups relative to the control level and were normalized to *GAPDH* levels using the delta-delta Ct methods (21). Table II lists the sense and antisense primer sequences.

Protein isolation and western blotting. We extracted protein samples from ~2.0×10^6 cells or 70 mg of frozen liver tissues. The samples were homogenized in lysis buffer (RIPA; Biosesang), quantified with BCA protein assay kit (Thermo
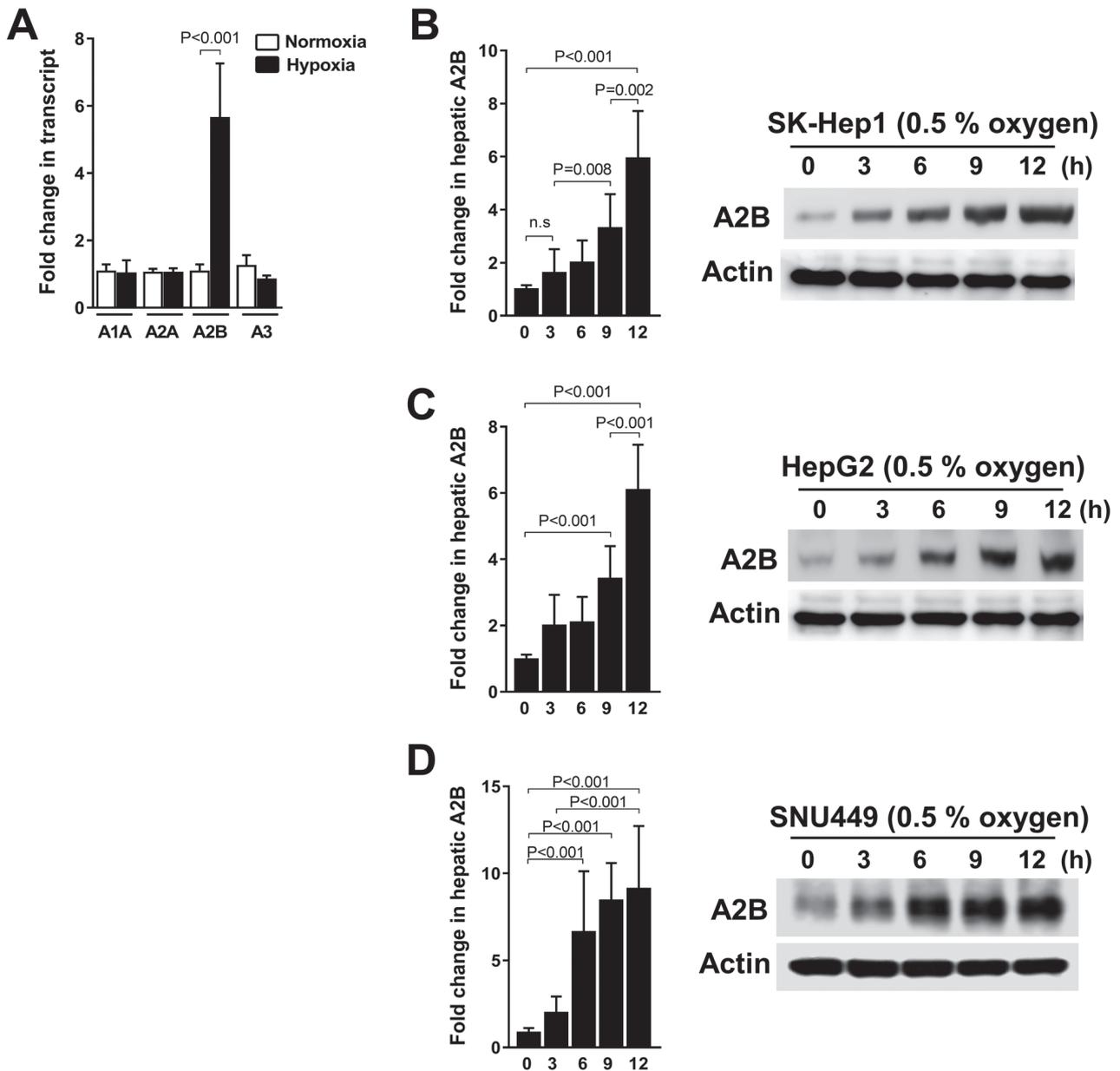
Figure 1. Adenosine receptor isoform expression profiles in liver cancer cell lines and A2B induction following hypoxic growth of representative liver cancer cell lines. (A) Transcript expression profiles of adenosine receptor isoforms, including A1, A2A, A2B and A3, in liver cancer cell lines (SK-Hep1, HepG2 and SNU-449). (B) Transcriptional and protein expressions of A2B in liver cancer cell lines (B) SK-HEP1, (C) HepG2 the hepatoblastoma cell line and (D) SNU-449 grown under hypoxic (0.5% O₂) or normal conditions. Transcriptional expression patterns and levels were detected using reverse transcription-quantitative PCR. The results were analyzed relative to GAPDH and according to fold changes relative to the control. For western blot analysis, actin was used as a loading control. Data are presented as the mean ± standard deviation.

Fisher Scientific, Inc.), and immunoblotted with anti-adenosine A2B receptor antibody (1:1,000 dilution; ab40002, Abcam) followed by rabbit anti-goat IgG HRP (1:20,000 dilution; A5420, Sigma-Aldrich); or anti-HIF-1α antibody (1:1,000 dilution; cat no. 610959, BD Biosciences) followed by goat anti-mouse IgG HRP (1;5,000; sc-2005, Santa Cruz Biotechnology). We also probed the nitrocellulose membranes with actin-peroxidase conjugate (1:20,000 dilution; A3854, Sigma-Aldrich). The protein signals were detected using an enhanced chemiluminescence solution (SuperSignal™ West Femto; Thermo Fisher Scientific, Inc.), and images were obtained using the ImageQuant LAS 4000 system (GE Healthcare Biosciences).

Statistical analysis. We performed all statistical analyses using the GraphPad Prism 6.0 software (GraphPad Software). All other data are presented as the mean ± SD. For western blot analyses, we repeated each experiment three times. We used a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxia triggered A2B induction is time-dependent in liver cancer cells. In this study, we investigated whether A2B is upregulated under hypoxic conditions or not. Thus, we grew

three liver cancer cell lines, SK-Hep1, HepG2, and SNU449, under hypoxic conditions. We consistently detected the significant expression of only A2B mRNA levels compared to the expression of other receptor genes (A1, A2A, and A3) under low-oxygen conditions (Fig. 1A). As shown, the A2B mRNA level and protein expressions were expressed in a time-dependent manner during hypoxia in SK-Hep1 cells (Fig. 1B). Additionally, A2B is upregulated under hypoxic conditions in HepG2 (Fig. 1C) and SNU449 cells (Fig. 1D). These data reveal a selective induction of A2B during hypoxic conditions.

HIF-1α directly regulated the expression of A2B by binding to the HRE in the A2B promoter region. To verify if A2B is transcriptionally induced by HIF-1α, we first searched for the human A2B promoter region, which has an HRE, containing -642 to -647 bp, at the 5'-CACGTGG-3' sequence and its typical HIF-1α ancillary HAS site (5'-CGGGGAG-3') at -546 to -541 (Fig. 2A). To investigate the function of the HIF-1α binding site, we used two modified constructs, a full-length promoter construct (1095 bp) and a truncated construct (477 bp) of HIF-1α (Fig. 2B). As shown in the results, the full-length HRE construct-transfected cells had significantly higher activity than the truncated construct. Next, to define whether the full-length construct was acted upon by HIF-1α, we performed luciferase assays with site-directed mutagenesis constructs. The mutant form of this construct had an HRE A*ATC*G change from A*CGT*G (Fig. 2C). The mutant construct (1095 Mut) exhibited low luciferase assay activity levels, as hypothesized. These results confirmed the A2B promoter region has an HRE site in it.

HIF-1α upregulates A2B expression during hypoxia. To confirm the HRE function within the promoter region of A2B, we performed experiments with HIF-1α siRNA and HIF-1α pcDNA plasmids. We transfected HIF-1α siRNA for 12 h (Fig. 3A) and then exposed the cells to hypoxia for different durations of time (Fig. 3B). HIF-1α siRNA-transfected cells displayed low A2B expression levels while HIF-1α is being absent. In contrast, the cells overexpressing HIF-1α exhibited high A2B expression levels (Fig. 3C) and hypoxia time dependency (Fig. 3D). Moreover, we treated cells with echinomycin (a cell-permeable inhibitor of HIF-1α-mediated gene transcription) and observed a dose-dependent decrease in the A2B mRNA and protein expression levels during low-oxygen conditions (Fig. 3E and F). Our results suggest HIF-1α is a transcriptional regulator of A2B.

Silencing of A2B in liver cancer cell suppressed cell growth and proliferation. As increased expression of adenosine A2B was observed in liver cancer cells, we tested whether the direct knock-down of A2B expression could affect cell growth and proliferation in liver cancer cells. We transfected HepG2 (the hepatoblastoma cell line) cells with A2B-siRNA and maintained them for up to 96 h. The A2B-siRNA effectively inhibited A2B expressions compared to scr-siRNA (Fig. 4A). Treatment with A2B-siRNA for 24 to 96 h significantly (P<0.001) decreased cell proliferation, as revealed by the BrdU assay (Fig. 4B). Similarly, the MTT assay also showed that cell growth was greatly decreased when cells were treated with
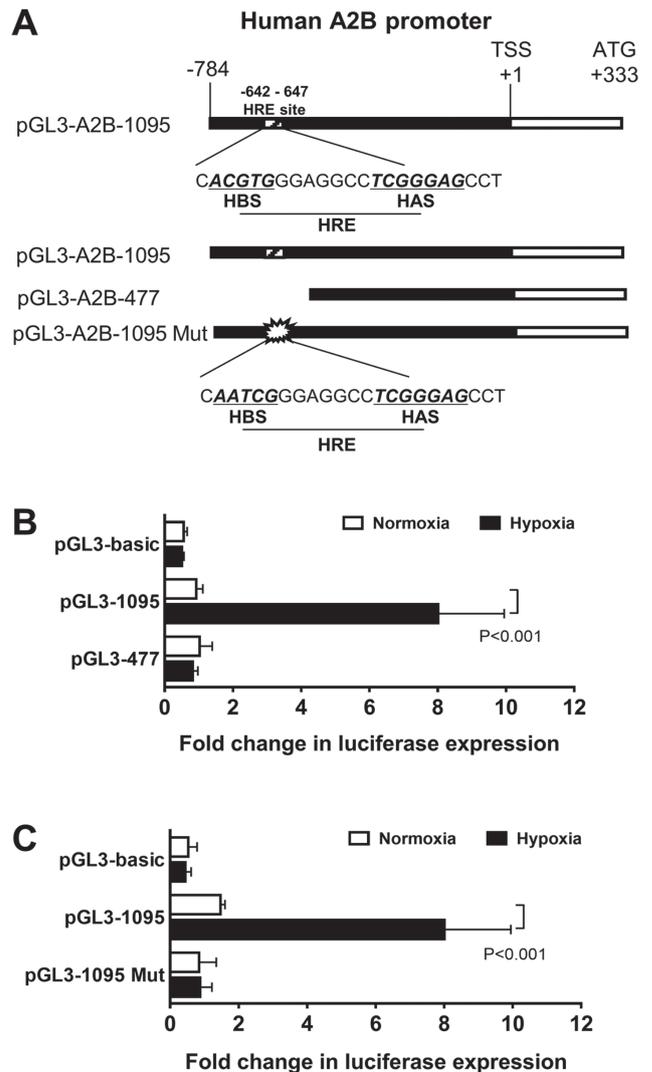

Figure 2. Luciferase reporter assays were performed to analyze A2B transcriptional activity under hypoxic conditions. (A) Map of luciferase reporter constructs indicating the position of HRE-related A2B promoter (-642 to -647). Relative positions of the HBS and HAS. (B) HepG2 cells were transfected with a full-length construct pGL3-1095 or a truncation construct pGL3-477 plasmid. The empty pGL3-basic vector was transfected as a control vector. All transfected cells were grown under 0.5% O_2 hypoxia conditions for 12 h (P<0.05; n=5). *Renilla* expression was used to normalize all luciferase expressions. (C) For site-directed mutagenesis, cells were transfected with a pGL3-1095 Mut construct and cultured under hypoxic conditions for 12 h. The luciferase assay activity was measured and normalized with *Renilla* (P<0.05; n=4). Data are expressed as the mean ± standard deviation. A2B, adenosine A2B receptor; HBS, HIF binding site; HAS, HIF ancillary site; Mut, mutant.

A2B-siRNA (Fig. 4C). Additionally, the relative cell viabilities were significantly (P<0.001) lowered after transfection of cells with A2B-siRNA (Fig. 4D). As A2B deficient liver cancer cells show consistent decrease in cell growth and proliferation, we could conclude that A2B takes an important role in liver cancer cell proliferation.

HIF-1α levels are associated with A2B transcript and protein levels in human liver cancer specimens. Solid tumors, including liver cancer, usually experience hypoxic conditions due to the fast growth (22). To confirm the relationship between adenosine A2B receptor expression
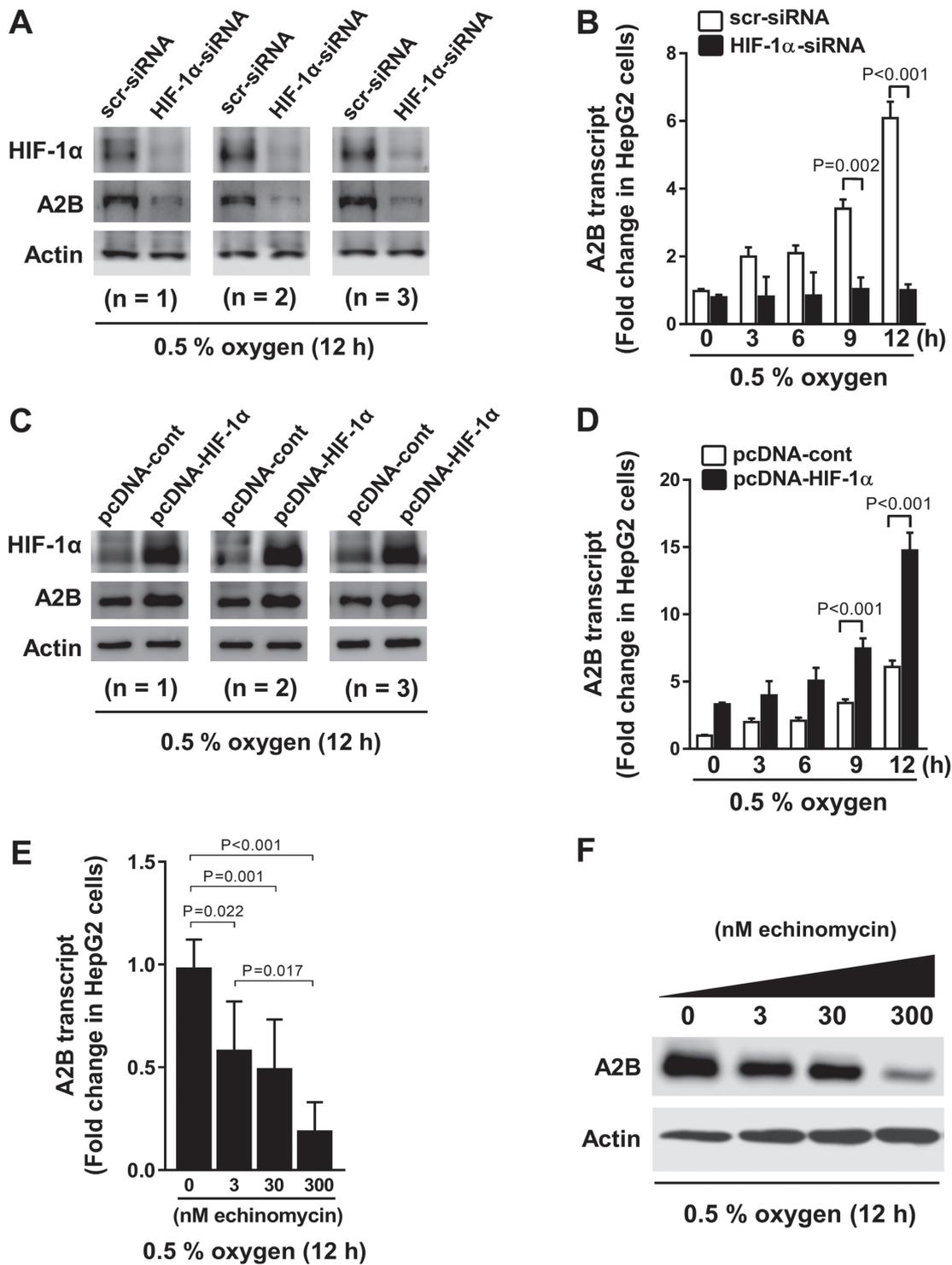
Figure 3. A2B expression profiles in liver cancer cell lines with gain- or loss- of HIF-1α function under hypoxic conditions. Western blot analyses of the expression of (A) HIF-1α and A2B and A2B transcript levels under hypoxic (0.5% oxygen) conditions for (B) up to 12 h in HepG2 cell lines. Cells were transfected with either scrambled-siRNA or HIF-1α-siRNA. Western blot analyses of (C) HIF-1α and A2B expression and A2B transcript levels under hypoxic (0.5% oxygen) conditions for (D) up to 12 h in HepG2 cell lines. Cells were transfected with either empty vector pcDNA3.1 (pcDNA-cont) or HIF-1α-pcDNA3.1 (pcDNA-HIF-1α) plasmid. (E) Cells were treated with different doses of echinomycin, a HIF-1α chemical inhibitor, for (F) up to 12 h. The expression of A2B transcript and protein levels were analyzed using RT-qPCR and western blot analysis, respectively. Data are presented as the mean ± standard deviation (n=4). Actin was used as a loading control housekeeping gene for use in western blot analysis. *GAPDH* was used as the housekeeping gene for RT-qPCR. A2B, adenosine A2B receptor; HIF-1α, hypoxia inducible factor-1α; siRNA, small-interfering RNA; RT-q, reverse transcription-quantitative.

and human liver cancer, we tested A2B induction from both tumor tissues and the adjacent normal tissues came from identical individuals diagnosed as a liver cancer. As expected, HIF-1α protein expression was high in the five

human liver cancer specimens compared to its expression in normal tissues. A2B protein expression levels were also increased, suggesting that HIF-1α correlates with A2B in tumors, according to the fold changes of HIF-1α and A2B
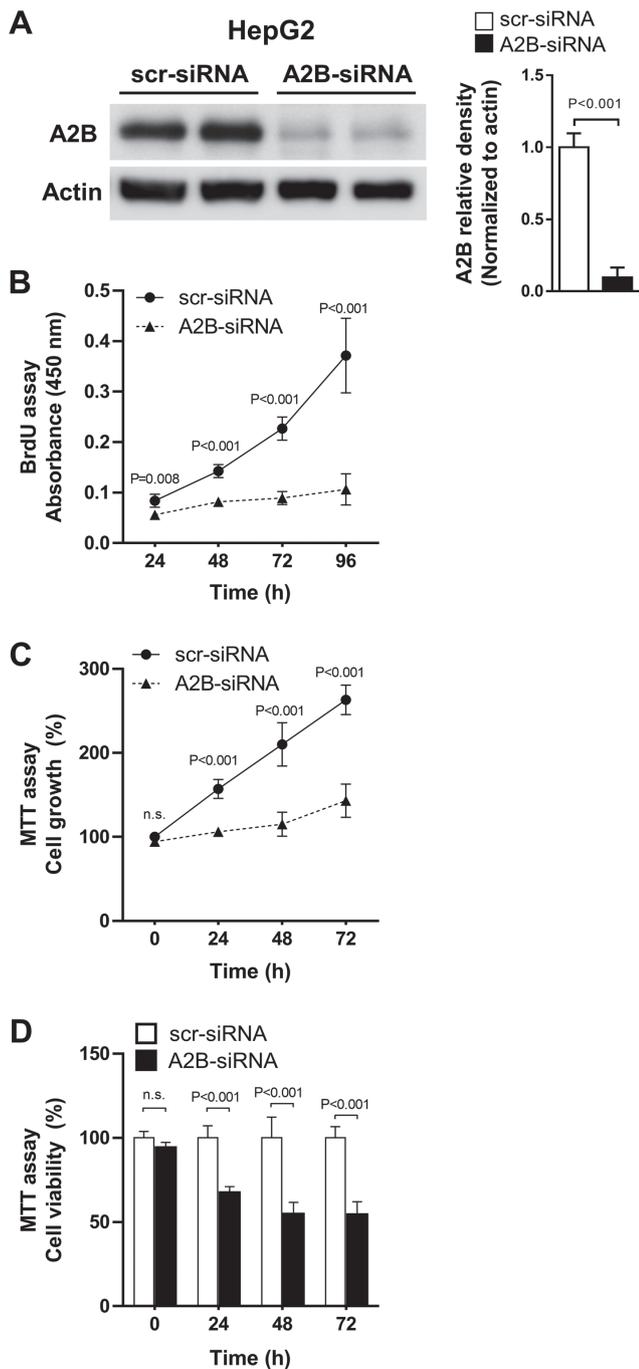
Figure 4. siRNA-mediated A2B silencing inhibits HepG2 cell proliferation. (A) HepG2 cells were transfected with either scr-siRNA or A2B-siRNA. Transfection efficiency of A2B-siRNA was measured using western blot analysis. Actin was used as a loading control. (B) Cell proliferation was determined by measuring BrdU incorporation for up to 96 h (n=4). (C) Relative cell growth rates were examined by the MTT assay. (D) Cell viability of cells at each time-point was measured by MTT assay. Data are presented as the mean ± standard deviation (n=4). Cells transfected with scr-siRNA was used as control and P<0.05 was considered to indicate a statistically significant result. siRNA, small-interfering; A2B, adenosine A2B receptor; scr, scrambled.

densitometry results (Fig. 5A-C). Collectively, HIF-1α, as a master transcription factor, can bind to the A2B promoter region and induce expression of A2B, thereby promoting tumor growth and survival. Moreover, we also confirmed the higher level of A2B gene expression in the HCC specimens by qRT-PCR. By contrast, we found no associations between

the expression levels of mRNAs of A1, A2A, or A3 between normal and HCC specimens (Fig. 5D).

Discussion

In this study, we defined the various expression patterns of adenosine receptor isoforms from three different liver cancer cell lines and human liver cancer specimens. We found that the A2B expression patterns were persistently higher than those of A1, A2A, and A3 receptor subtypes in liver cancer cell lines when maintained under low-oxygen conditions. Those patterns were also observed in human liver cancers. Under the hypothesis that hypoxia-mediated HIF-1α activation leads to liver cancer cell proliferation by activating adenosine A2B receptors, we followed transcription factor binding assays utilizing A2B promoter constructs and constructs with site-directed mutagenesis and have identified HIF-1α as the key regulator of A2B-induction during hypoxic conditions. We also investigated loss- and gain-of-function using HIF-1α siRNA and overexpressing vectors. We found that the expression levels of A2B were modulated HIF-1α-dependently. In addition to this, echinomycin treatment dose-dependently inhibited A2B expressions under hypoxia, which indicate transcriptional induction of A2B by HIF-1α. We also showed that active deprivation of A2B expression by siRNA suppresses cell growth and proliferation of the hepatoblastoma cell line, HepG2. Those results indicate the activation of hepatic A2B signals during low oxygen condition is related to liver cancer cell proliferation. Consistent with our in vitro results, human liver cancer specimens showed elevated levels of HIF-1α along with A2B in both transcript and protein levels. These data implicate HIF-1α in the regulation of adenosine-elicited cancer growth in liver.

We have previously studied the HIF-1α stabilization during hypoxic conditions in liver cell lines (23) and identified the protective role of HIF-1α stabilization mediated by hypoxia-induced inhibition of succinate dehydrogenase, concomitant increases in glycolytic capacity, and improved tricarboxylic acid (TCA) cycle function in alveolar epithelium (24). Also the pharmacologic studies with HIF activator or inhibitor treatment implicated HIF-1α-stabilization increases in liver cancer proliferation (25).

On the other hand, studies have shown the key roles of adenosine in HCC cell proliferation (26). Adenosine also upregulates endothelial cell proliferation by associating with A2B in porcine and rat arterial endothelial cells (27). In retinal endothelial cells, activated A2B can initiate neovascularization through increased angiogenic growth factor expression (28). In contrast, adenosine inhibits the growth of cardiac fibroblasts and aortic and vascular smooth muscle cells through the activation of A2B, in addition to increasing the proliferation of peripheral micro-vessels, but exerts the opposite effect on cardiac tissue and capillaries (29). Thus, A2B can promote cancer proliferation and expansion by initiating neovascularization around cancer masses (30).

We showed that A2B is induced under hypoxic conditions. In general, solid cancers, such as breast, lung, colon, and prostate cancers undergo similar low-oxygen growth conditions (31); moreover, to maintain homeostasis under low-oxygen supply
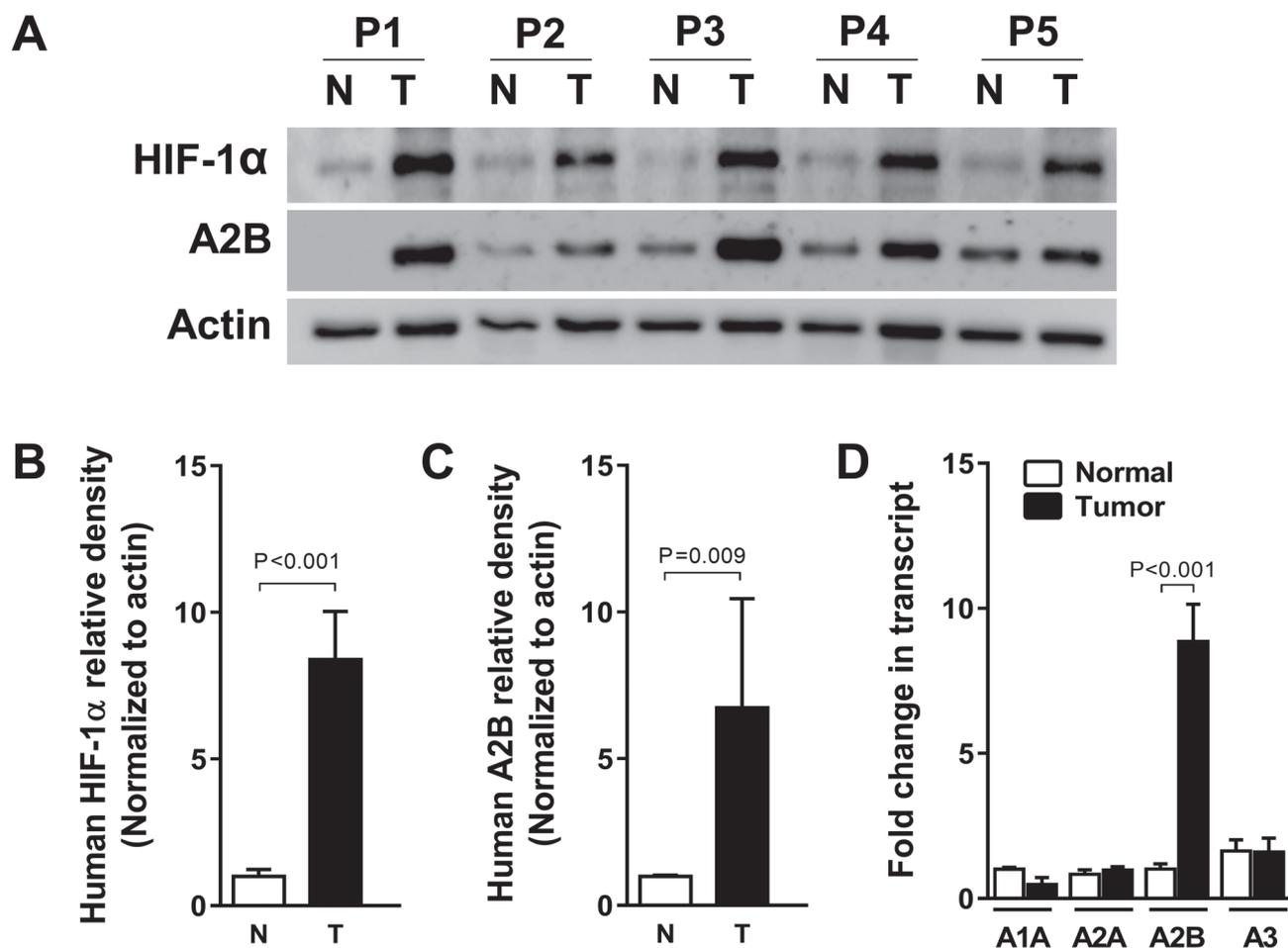
Figure 5. Protein levels of A2B and HIF-1α measured in human HCC specimens and in non-cancerous tissue specimens, and mRNA levels of A2B isoforms. (A) HIF-1α and A2B protein expression were detected using western blot analysis. Specimens from five patients (P1-P5) who underwent HCC hepatectomy and non-cancerous tissue specimens around the tumors. Actin was used as a loading control. Relative expression of (B) HIF-1α and (C) A2B by densitometric quantification of western blots. (D) mRNA levels of A2B and other adenosine isoform receptors from five patients. The expression levels are presented relative to those of the control housekeeping gene GAPDH. The results are presented as the mean ± standard deviation; $P < 0.05$; Student's t-test. A2B, adenosine A2B receptor; HIF-1α, hypoxia inducible factor-1α; HCC, hepatocellular carcinoma; T, HCC hepatectomy; N, non-cancerous tissue specimens.

conditions, specific genes need to be systematically activated and expressed or inactivated.

It was previously reported that bleomycin, a chemical irritant which induce HIF-1α, can mediate A2B receptor expression while inducing adenosine accumulation in acute lung injury (32). In addition, HIF-1α mediated expression of A2B in endothelial cells (15), lung injury (18), breast cancer (19), and dendritic cells (33) has been reported. Nevertheless, the hypoxia mediated A2B signaling mechanism promoting liver cancer cell proliferation is yet to be elucidated.

Other researchers have suggested that adenosine receptors are highly controlled and, thus, adenosine responses might be regulated by the surface expression of adenosine receptors. Consistent with our study, Eltzschig *et al* reported that microarray analyses of cDNA derived from endothelial cells subjected to various periods of hypoxia revealed significant changes in the adenosine receptor's profiles, wherein the prominent phenotypic change favored A2B expression with concomitant downregulation of A1 and A2 subtypes (34). In another study, Feoktistov and Biaggioni suggested that hypoxia increases A2B expression levels releasing vascular endothelial growth factor through decreasing adenosine (35).

Human endothelial and smooth muscle cells have also been shown to express A2B (36). Moreover, hypoxia reduces matrix metalloproteinase-9 (MMP-9) production by human monocyte-derived dendritic cells and requires the activation of adenosine A2B via the cAMP/protein kinase (PKA) signaling pathway (37,38). Collectively, these data suggest that A2B increases HIF-1α dependently under hypoxic conditions and might promote liver cancer cell proliferation.

In summary, we investigated the expressions of A2B and HIF-1α in liver cancer cells to find an association suggesting that under hypoxic conditions, HIF-1α induces A2B expression to maintain tumor proliferation. Our results show that the upregulated A2B plays a critical role in liver cancer cell growth, suggesting A2B as a potential target for drug design; thus, an effective A2B inhibitor might be useful for treating liver cancers.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JHK and ET designed the study. JL and JK conducted the experiments. JHK, GWS, YIY, SH, GCP and SGL performed human specimen handling. YHJ, VAK, BJK and NK contributed to the interpretation of the results. GCP and ET were involved in manuscript writing and analyzing the data. SGL, GCP and ET supervised the work. All authors provided critical feedback and conducted the research, analyzed the data and prepared the manuscript.

Ethics approval and consent to participate

The Institutional Review Board (IRB) of Asan Medical Center (Seoul, Republic of Korea) reviewed and approved the collection and use of patient specimens (approval no. 2016-0582). All patients who provided tissue samples agreed to donate their specimens and provided written informed consents.

Patient consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

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