의학석사 학위논문

Hypoxia enhances tumor-stroma crosstalk that drives progression in hepatocellular carcinoma

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2013년 10월

서울대학교 대학원

의학과 내과학 전공

조 유 리

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지도교수 **윤 정 환**

이 논문을 의학석사 학위논문으로 제출함

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Hypoxia enhances tumor-stroma crosstalk that drives progression in hepatocellular carcinoma

by

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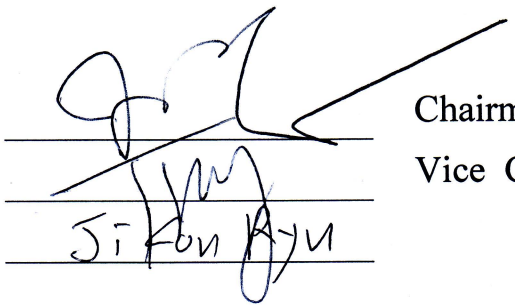

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ABSTRACT

Introduction: The crosstalk between tumor cells and their microenvironment plays a crucial role in the development, progression and metastasis of HCC. Hypoxia, a common feature of advanced hepatocellular carcinoma (HCC), has been known to modulate the development and evolution of the tumor microenvironment. However, the mechanism and functional impact of the tumor-stroma co-evolution in hypoxic HCC remains poorly understood. In this study, we investigated the effects of hypoxia on the tumor-stroma crosstalk in HCC.

Methods: Human HCC cells (Huh-BAT and HepG2) were co-cultured with activated human hepatic stellate cells (HSCs; LX-2) either in a normoxic or hypoxic condition. Cell growth, migration capacity and apoptosis were assessed using the MTS

assay, wound healing assay and DAPI staining. Global gene expression profiling under chronic hypoxia was determined by microarray analysis.

Results: Co-culturing HCC cells with HSCs in hypoxia significantly enhanced proliferation and migration of both cells as compared to normoxic condition by upregulation of PDGF-BB. Co-culturing also decreased bile acid- or TRAIL-induced HCC cell apoptosis. The effects of hypoxic tumor-stroma crosstalk on proliferation and migration were meaningfully inhibited by the PI3K inhibitor compared to cells in a normoxic condition. Unsupervised genome-wide expression profiling showed that 1887 genes were differentially expressed in co-cultured HCC cells with HSCs and 503 genes in HSCs with HCC cells under hypoxia, including genes that encode cell cycle, signaling transduction, cell surface receptors and redox

reaction. Hepatocyte-HSC cross-talk was bidirectional and resulted in the deregulation of functionally relevant gene profiles.

Conclusions: These results indicate that the enhanced bidirectional cross-talk between HCC cells and activated HSCs is an important feature of HCC progression under hypoxia.

Keywords: hepatocellular carcinoma; hepatic stellate cell; microenvironment; hypoxia

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CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

HCC	hepatocellular carcinoma
HSC	hepatic stellate cell
MTS assay	Cell proliferation assay
DAPI	4',6-diamidino-2-phenylindole
BA	bile acid
TRAIL	TNF-related apoptosis-inducing ligand
ELISA	enzyme-linked immunosorbent assay
RT-PCR	reverse transcription-polymerase chain reaction
Akt	protein kinase B (PKB)
PTEN	phosphatase and tensin homolog
PI3K	phosphoinositide 3-kinase
PDGF	platelet-derived growth factor

FGF	fibroblast growth factor
TGF	transforming growth factor
CTGF	connective tissue growth factor

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most fatal cancers with rising incidence around the world[1]. Most of the HCC patients have chronic liver disease or liver cirrhosis which are the main risk factors for the development of HCC[2]. Activation of hepatic stellate cells (HSCs) is known as a central role in the development of hepatic fibrosis. The cross-talk between HCC cells and the surrounding microenvironment is believed to play a pivotal role in modulating the biological behavior of the tumor. During chronic liver damage, HSCs become activated and assume a myofibroblast-like phenotype. They proliferate and migrate towards the area of ongoing tissue remodeling and secrete the extracellular matrix

proteins[3]. Moreover, the stroma of HCC is infiltrated by activated HSCs and myofibroblasts. They are located around the tumor sinusoids, fibrous septa and capsule. The interaction between HCC cells and their microenvironment has been recognized to essentially affect HCC development by triggering cell proliferation and survival as well as the capability to invade the surrounding tissue.

Hypoxia induces survival signals in HCC by activating a variety of cell growth signals. Indeed, hypoxia stimulates angiogenesis by hypoxia-inducible factor-1, vascular endothelial growth factor, basic fibroblast growth factor and angiopoietin-1[4]. Also, hypoxia stimulates HCC cell proliferation by inducing transforming growth factor-ß and platelet derived growth factor[5], facilitates glycolysis

by up-regulating hexokinase-II expression[6], induces immortalization of HCC cells by activating telomerase and histone deacetylase[7] and inhibits cellular apoptosis by inducing the over-expression of inhibitor of apoptosis protein-2 and myeloid cell factor-1[8].

Yet, the molecular framework of crosstalk between tumor cells and their microenvironment in hypoxia and its consequences on carcinogenesis are largely unknown. Especially, the role of activated HSCs in the development and progression of HCC under hypoxia is largely unknown. In this study, we investigated the effects of hypoxia on the tumor-stroma crosstalk in HCC.

MATERIALS AND METHODS

Cell line and culture

Human HCC cell lines and hepatic stellate cell line were used in this study: Huh-BAT, a well-differentiated HCC cell line[9]; HepG2, a poorly differentiated HCC cell line[10]; and LX-2, an activated human HSC line[11]. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) or in RPMI-1640 supplemented with 10% fetal bovine serum, 100,000 U/L penicillin, and 100 mg/L streptomycin. Cells were incubated either under standard culture conditions (20% O_2 and 5% CO_2, at 37℃) or under hypoxic culture conditions (1% O_2 and 5% CO_2, at 37℃). To avoid confounding factors related to serum induced signals, cells were serum starved overnight for all the experiments.

Cell proliferation analysis

Cell growth was measured colorimetrically using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI), which is based on the cellular conversion of 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium salt (MTS) into soluble formazan by dehydrogenase enzymes produced only in metabolically active, proliferating cells. Following each treatment, 20 uL of CellTiter 96® AQueous One Solution reagent was added into each well of a 96-well plate. After 1 hour of incubation, the absorbance for each

well at 490 nm wavelength was recorded using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

Migration assays

Migration was assessed applying time-lapse scratch assays (wound-healing-assay). Here, HCC cells were seeded in high density into 6-well plates. After adherence, cells were washed with serum-free DMEM and either in conditioned medium from activated HSC or control medium. Subsequently, the cell layer was scratched by a pipette tip in a definite array, and the migration into this array was documented and measured after 24 and 48 hours. Each analysis was performed in triplicate and repeated three times with consistent results.

Apoptosis analysis

Apoptosis was assessed by examining apoptosis-associated nuclear changes (i.e. chromatic condensation and nuclear fragmentation) using DNA binding dye, 40,6-diamidino-2-phenylindole dihydrochloride (DAPI) and fluorescence microscopy (Carl Zeiss, Jena, Germany).

Quantification of various growth factors

Expressions of various growth factors were quantified in nuclear extracts with the ELISA-based kit and quantitative real-time PCR analysis (RT-PCR). Real-time PCR analysis was performed according to the manufacturer's instructions (SYBR Green PCR Master Mix; Applied Biosystems) using the iCycler thermal 7300 Real-Time PCR System (Applied

Biosystems).

cDNA microarray analysis

In order to find the global gene expression profiling under chronic hypoxia, microarray analyses were performed. Total RNA from Huh-BAT cells and LX-2 cells cultured under standard and hypoxic culture conditions were extracted and purified using the Illumina Total Prep RNA Amplification Kit (Illumina, Inc., San Diego, CA). Following fragmentation, 0.75 ug of cRNA were hybridized to the Illumina HumanHT-12 Expression Beadchip according to the protocols provided by the manufacturer. Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner. Array data export processing

and analysis was performed using Illumina GenomeStudio version 2011.1 (Gene Expression Module version 1.9.0)

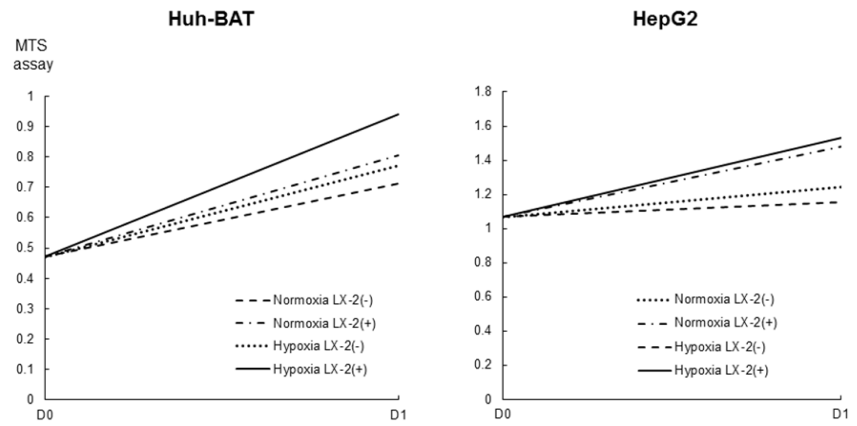
Data analysis

All experimental results represent at least three independent experiments using cells from a minimum of three separate isolations and are expressed as means ± standard deviation. Comparisons between groups used a Mann-Whitney U test. All analyses used SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant.

RESULTS

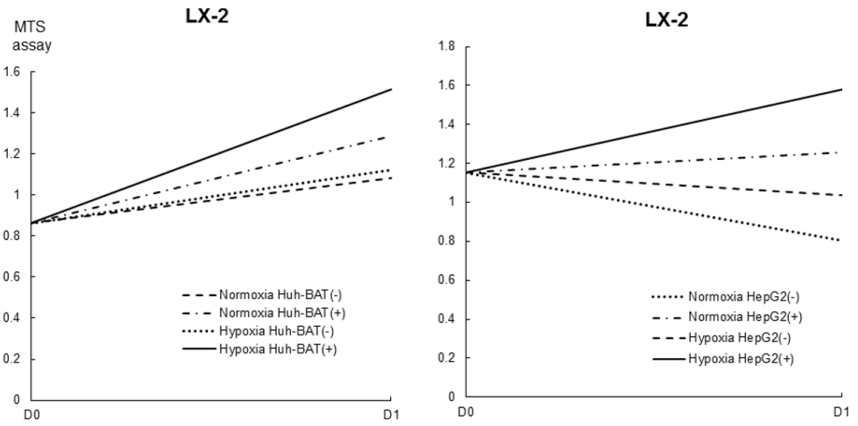
Enhanced cell proliferation and migration by co-culturing in hypoxia

We tried to find whether co-culturing HCC cells with HSCs enhances cell proliferation and migration. As shown in Figure 1, HCC cells showed the increased proliferation when they were cultured with HSCs (Fig. 1A.) and HSCs showed the increased proliferation when they were cultured with HCC cells (Fig. 1B.) by MTS assay.



**$P < 0.01$

Fig. 1A. Increased proliferation of HCC cells when they were cultured with HSCs



**$P < 0.01$

Fig. 1B. Increased proliferation of HSCs when they were cultured with HCC cells

This activation was more enhanced in hypoxic cells than in normoxic cells. Also, a stronger migration of HCC cells in hypoxia was observed compared with in normoxic condition ($P < 0.01$; Figure 2).

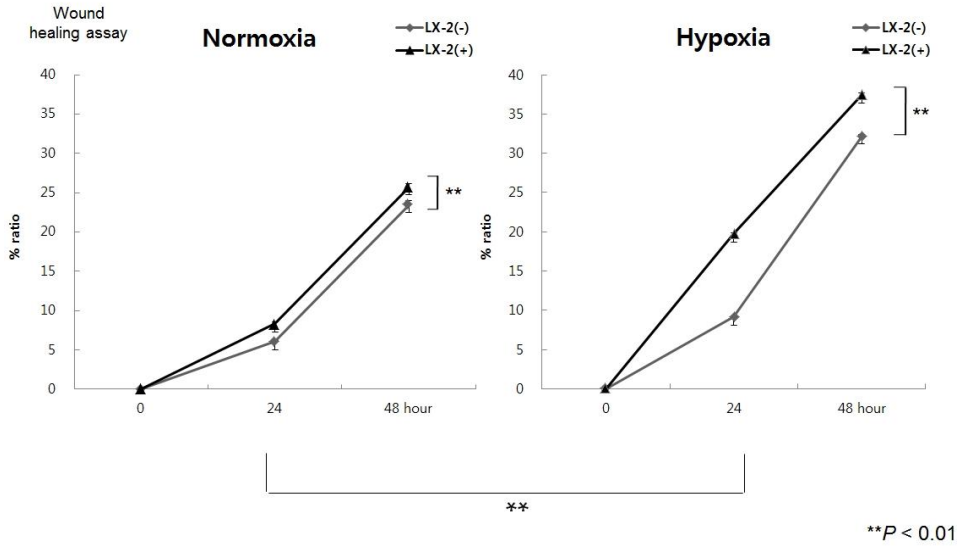


Fig. 2. Stronger migration of HCC cells in hypoxia

To investigate the molecular pathways supporting this enhanced cell proliferation in hypoxia by co-culturing, we explored the expression of phosphorylated-Akt (*p*' -Akt). As shown in Figure 3A, *p*' -Akt expression level was more increased in hypoxia. We found that *p*' -Akt (a

downstream kinase of PI3K) was activated in HCC cells when they were co-cultured with HSCs in hypoxic condition. We then assessed if the PI3K/Akt signal is responsible for HCC cell proliferation. When LY294002 (a PI3K inhibitor) was administered in co-cultured cells, HCC cell proliferation significantly decreased by 40.3% in hypoxic condition compared with single cultured HCC cells. This effect was more prominent in hypoxic condition than in normoxic condition (Fig. 3B.).

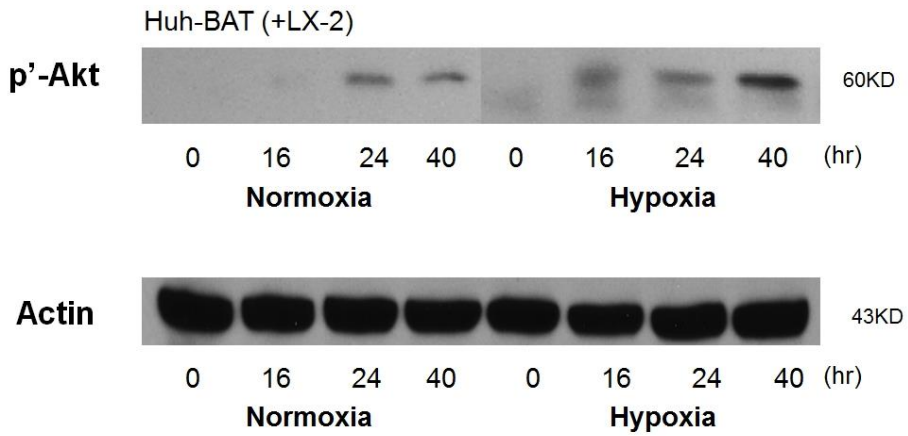


Fig. 3A. Increased *p'* -Akt expression level in hypoxia

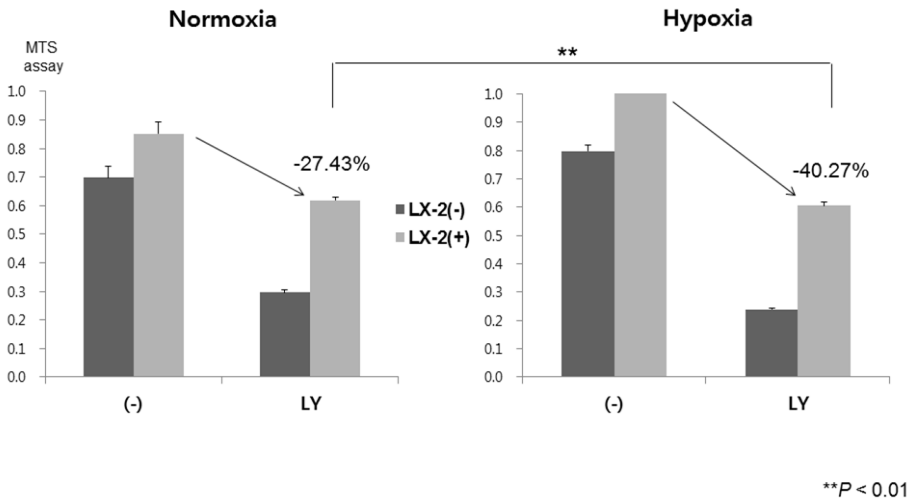


Fig. 3B. Decreased HCC cell proliferation in hypoxic condition compared with single cultured HCC cells, more than in normoxic condition

Decreased bile acid- or TRAIL-induced HCC cell apoptosis

To determine whether co-culturing in hypoxia participates in decreasing HCC cell apoptosis, we evaluated bile acid (BA)- or TRAIL-induced HCC cell apoptosis by DAPI staining. Both bile acid- and TRAIL-

induced HCC cell apoptosis, when co-cultured with HSCs, significantly decreased compared to single cultured HCC cell in hypoxic condition (Fig. 4A.). Moreover, BA induced enhanced expressions of caspase-9, 7, and 8 under hypoxic condition were attenuated by co-culturing with HSCs (Fig. 4B.).

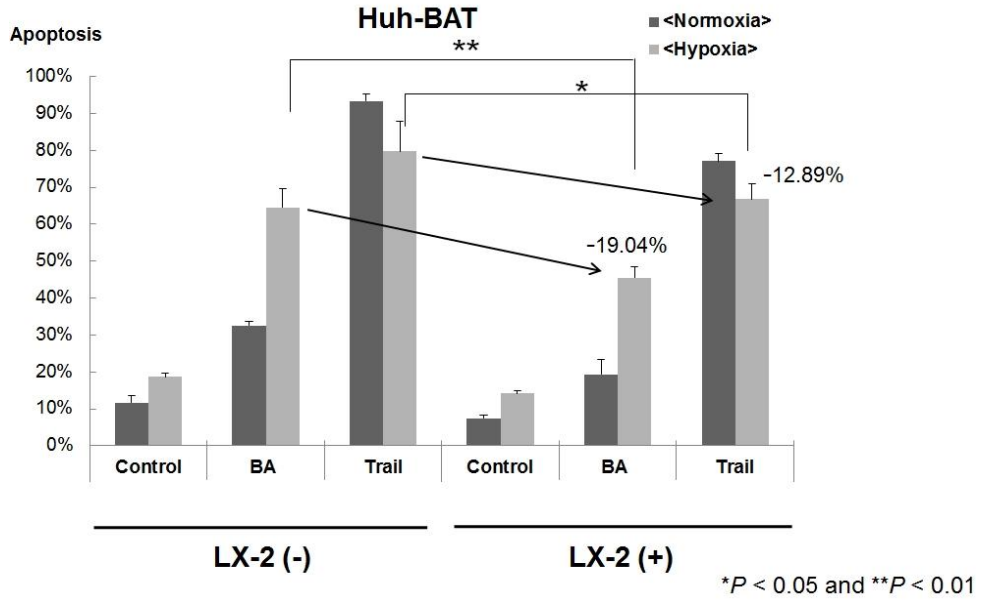


Fig. 4A. Bile acid- and TRAIL-induced HCC cell apoptosis was decreased when co-cultured with HSCs in hypoxic condition.

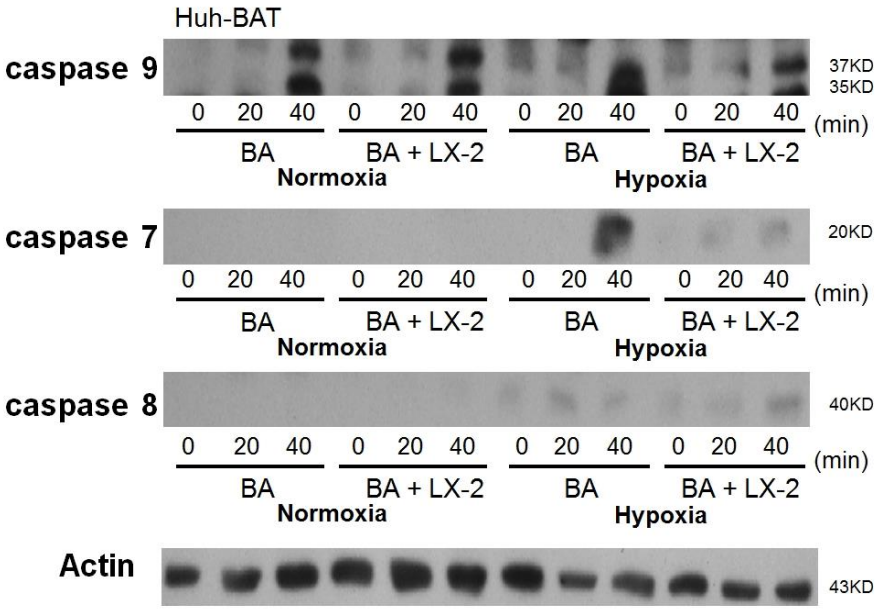


Fig. 4B. Bile acid induced caspase-9, 7, and 8 under hypoxic condition were attenuated by co-culturing with HSCs.

Growth factors from co-cultured cells

To explore the most abundant growth factor from co-cultured cells, we investigated the expressions of several growth factors by using quantitative RT-PCR. As shown in Figure 5A, platelet-derived growth factor (PDGF)-B mRNA was most abundantly up-regulated in LX-2 cells

which were co-cultured with Huh-BAT cells in hypoxic condition.

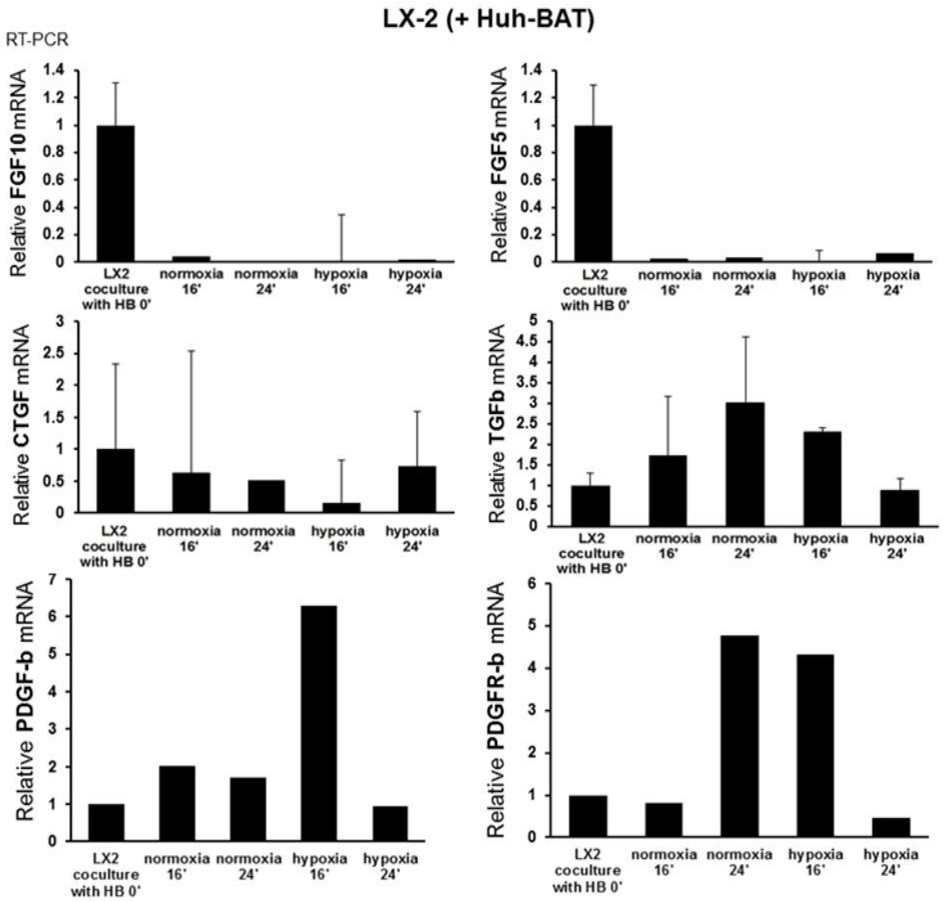


Fig. 5A. Platelet-derived growth factor (PDGF)-B mRNA was most abundantly up-regulated in LX-2 cells which were co-cultured with Huh-BAT cells in hypoxia.

As shown in Figure 5B, the expression of PDGF-B mRNA in Huh-BAT cells was also enhanced when co-cultured with LX-2 cells in hypoxic condition.

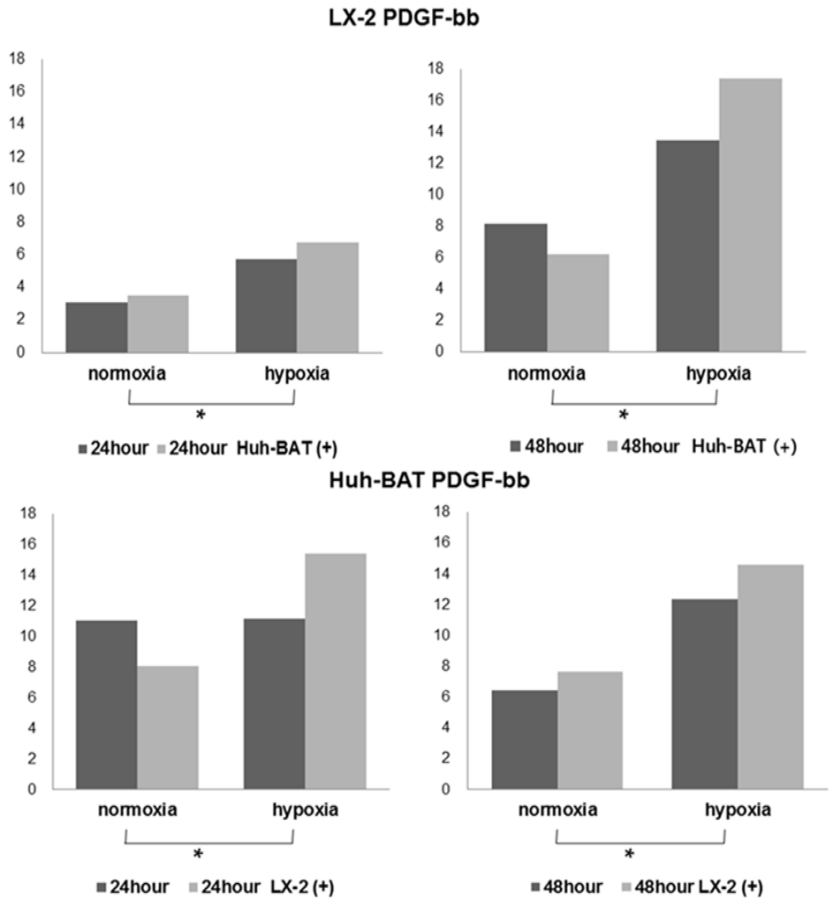
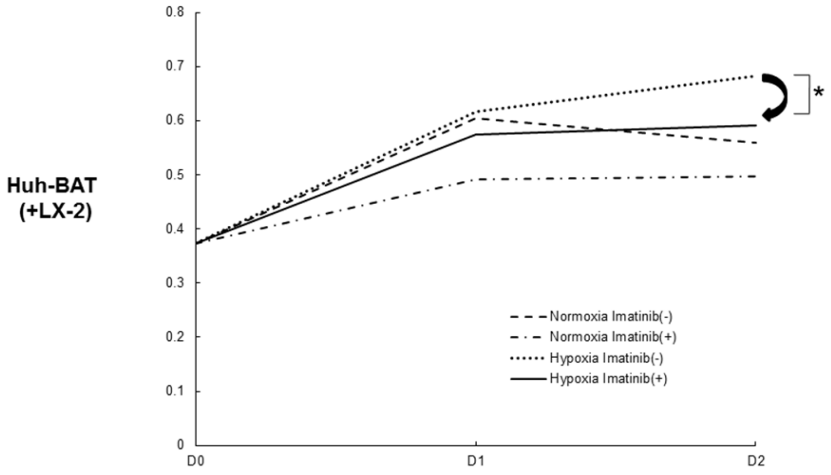


Fig. 5B. Enhanced expression of PDGF-B mRNA in Huh-BAT cell, when co-cultured with LX-2 cell in hypoxic condition

To determine whether PDGF-B mRNA accelerates the evolution of tumor-stroma crosstalk, we administered imatinib in co-cultured cells. Imatinib is a competitive tyrosin-kinase inhibitor and suppresses PDGF by inhibiting its receptor (PDGF-Rβ). When imatinib was administered in Huh-BAT cells which were co-cultured with LX-2 cells, the proliferation of HCC cells was significantly decreased in hypoxic condition (Fig. 5C.).



*P < 0.05 and **P < 0.01

Fig. 5C. Decreased proliferation of HCC cells in hypoxic condition, when imatinib was administered in Huh-BAT cells which were co-cultured with LX-2 cells

cDNA microarray analysis

Unsupervised genome-wide expression profiling showed that 1887 genes were differentially expressed in co-cultured HCC cells with HSCs and 503 genes in HSCs with HCC cells under hypoxia, including genes that encode cell

cycle, signaling transduction, cell surface receptors and redox reaction (Fig. 6A-C.). Hepatocyte-HSC cross-talking was bidirectional and resulted in the deregulation of functionally relevant gene profiles.

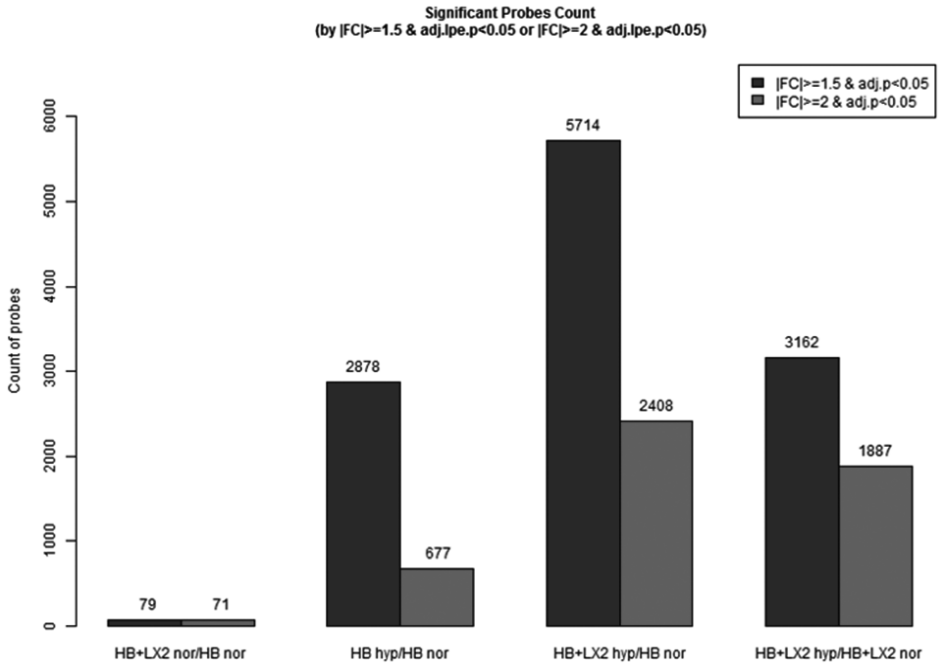


Fig. 6A. Count of significant probes in HCC cells which were co-cultured with HSCs

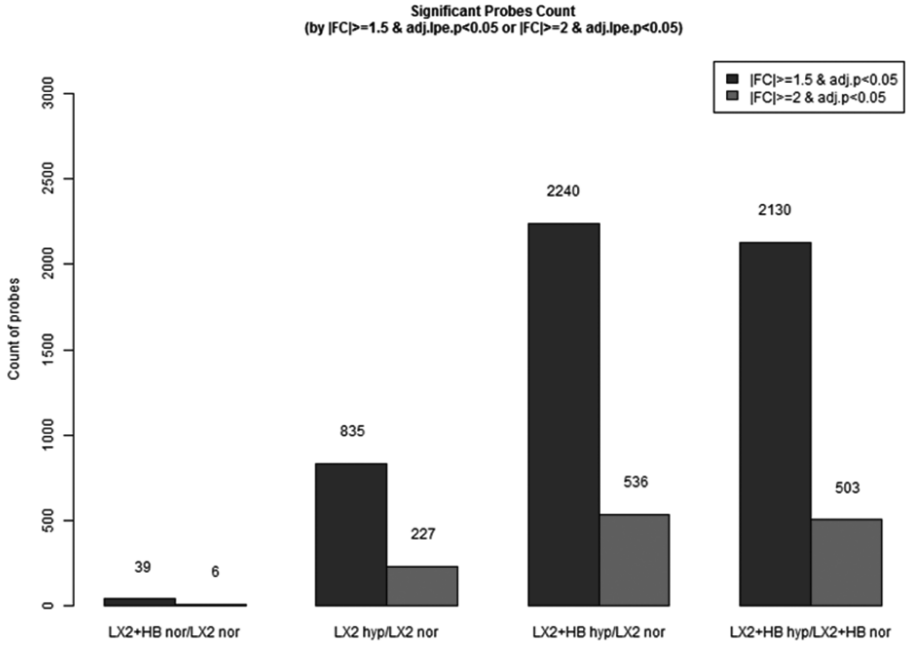


Fig. 6B. Count of significant probes in HSCs which were co-cultured with HCC cells

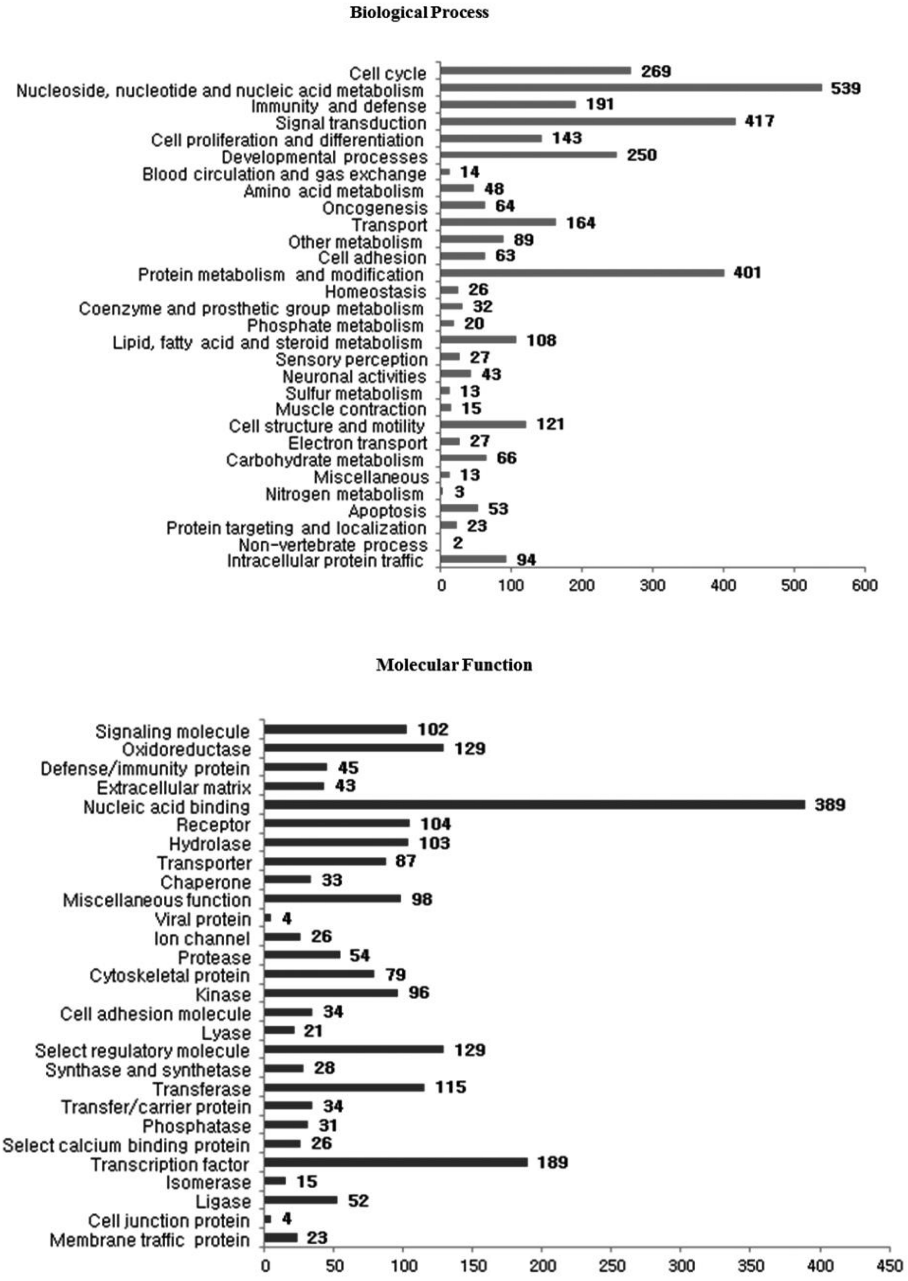


Fig. 6C. Biologic processes and molecular functions of significant probes

DISCUSSION

HCC cells under hypoxic condition usually show more rapid progression than those with an adequate vascular supply[12,13]. HSCs have been reported to play a role in the tumoral progression of HCC, since their presence in the peritumoral tissue is associated with strong vascular invasion and with more aggressive overall pathological characteristics[14]. However, how HSCs modulate the invasive HCC phenotype is still unclear. Therefore, in this study we aimed to analyze the cross-talk of activated HSCs on the tumorigenicity of HCC cells. Our experiments show that activated HSCs induced the proliferation and migration of HCC cells and suppressed

the bile acid- or TRAIL-induced HCC cell apoptosis in hypoxic condition. PI3K inhibitor suppressed HCC cell proliferation which was enhanced by hypoxic tumor-stroma crosstalk, which means PI3K/Akt pathway is involved in the progression of HCC. PI3K/Akt pathway is known as a promising target with respect to its frequent dysregulation in HCC and its central role in regulating cell proliferation, migration, survival and angiogenesis[15,16]. Activated PI3K/Akt pathway by tumor-stroma crosstalk has been shown to affect the migratory activity and proliferation of HCC cells, suggesting that this molecular pathway may be critical in the aggressiveness of HCC in hypoxic condition.

PDGF-BB was enhanced in co-cultured cells and

PDGF-BB accelerated the evolution of tumor-stroma crosstalk. Also, imatinib suppressed the HCC cell proliferation which was co-cultured with hepatic stellate cell in hypoxic condition. The effect of imatinib is partly mediated by the inhibition of PDGF, which is highly expressed in the liver[17]. PDGF is a heat-stable positively charged hydrophilic protein of 30 kDa. It consists of two related peptide chains, PDGF-A and PDGF-B that contain intramolecular disulfide bonds, linking the submits. All possible isoforms (i.e., PDGF-AA, PDGF-BB and PDGF- AB) are biologically active; PDGF-BB being a strong mitogen for vascular smooth muscle cells[18]. PDGF-BB is one of the key events involved in the neoplastic progression of liver. Recently in a

transgenic mouse model, PDGF-B overexpression was shown to be associated with the accelerated hepatic tumor progression[19]. Hypoxia might have activated an autocrine-paracrine anti-apoptotic mechanism that involves up-regulation of PDGF-B and PDGF-ß receptor-dependent activation of the PI3K/Akt signaling pathway to enhance tumor-stroma cross-talk.

The interaction of tumor cells with the microenvironment has been recognized to be central for cancer progression and metastatic colonization[20]. Yet, the molecular framework of the tumor-host crosstalk in the specific tissue context and its consequences on carcinogenesis are largely unknown and rather based on observations from individual cell types. In this study we tackled the issue

on the progression of malignant hepatocytes and its modulation by activated HSCs. We showed the evidence that paracrine feedback mechanisms governed by activated HSCs strongly affected the malignant progression of neoplastic hepatocytes by induction of PDGF-BB and PI3K/Akt pathway. These results further suggest that combining chemotherapy with a PI3K inhibitor or a PDGF receptor inhibitor under hypoxic condition might enhance the efficacy of chemotherapeutic agents against HCC cells which are activated by HSCs.

In conclusion, our current study demonstrates that hypoxia induced the up-regulation of PDGF-BB which accelerated the evolution of tumor-stroma crosstalk partly through PI3K/Akt pathway. And the enhanced bidirectional cross-talk between HCC cells and activated

HSCs is an important feature of HCC progression under hypoxia.

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국문 초록

저산소증이 종양과 간질 사이의 상호작용을 촉진하여 간세포암의 진행에 미치는 영향 및 기전에 관한 연구

서론: 간세포암와 주변의 미세환경과의 상호작용은 암의 발생, 진행 및 전이에 있어 중요한 역할을 한다. 간세포암 진행 과정에서는 저산소증이 흔히 유발되며, 저산소증 조건에서 미세환경이 영향을 받아 암의 진행에 영향을 미친다는 것이 알려져 있다. 하지만 저산소증 조건에서의 간세포암에서 종양과 기질 사이의 상호작용이 어떠한 기전을 통하는지에 대하여는 자세히 알려져 있지 않다. 따라서 이 연구에서는 간세포암에서 저산소증이 종양과 기질 사이의 상호작용에 미치는 영향과 그 기전을 알아보고자 한다.

방법: 간암세포주(Huh-BAT와 HepG2)를 활성화된 간성상세포주(LX-2)와 정상산소조건과 저산소증 조건에서 각각 공동배양을 하였다. 세포 증식, 세포 이동 및 아포토시스는 각각 MTS 분석법, wound healing 분석법 및 다피 염색법을 통하여 측정하였다. 저산소증에서의 범용유전자 발현 자료를 수집하기 위하여 마이크로어레이 자료 분석을 시행하였다.

결과: 저산소증 조건에서 간세포암과 간성상세포를 공동배양하는 것은 정상산소조건에서와 비교하였을 때, PDGF-BB의 발현 증가를 통하여 각각의 세포 증식과 세포 이동을 촉진시켰다. 또한 두 세포의 공동배양은 담즙- 또는 TRAIL 유도 간세포암 아포토시스를 감소시켰다. 저산소증에 의하여 증가한 각 세포의 증식은 PI3K 억제제에 의하여 억제되었다. 범용유전자 발현 자료 수집을 통하여 확인한 결과, 저산소증에서 간성상세포와 공동배양된 간세포암에서는 총 1887개의 유전자가, 간세포암과 공동배양된 간성상세포에서는 총

503개의 유전자가 유의하게 다르게 발현되었다. 이들은 세포 주기, 신호 전달, 세포 표면 수용체 및 산화환원반응에 관여하는 유전자들이었다. 간세포와 간성상세포와의 상호작용은 양방향성이었으며 관련 있는 유전자의 발현을 변화시켰다.

결론: 결론적으로 양방향성의 간세포암과 활성화된 간성상세포와의 상호작용은 전산소증 조건에서 간세포암의 진행에 중요한 역할을 하며, 이에 관여하는 기전들은 추후 간세포암의 치료제 개발에 기여할 수 있을 것이다.

주요어 : 간세포암, 간성상세포, 저산소증, 상호작용

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