The effect of oxygen-gradient in biochip to the growth of hepatocellular carcinoma

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Directed by Professor Jin Sub Choi

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ABSTRACT

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Introduction: Although cell culture has played a vital role in hepatocellular carcinoma (HCC) research, current culture systems are limited in their ability to reproduce a physiological environment. Hepatocarcinogenesis is a multistep process that causes the gradual arterialization of liver tumors, with the hepatocellular carcinoma (HCC) blood supply being variable according to its stage. We therefore hypothesized that an oxygen-gradient cell culture system might affect the viability of liver cancer cells and their characteristics. We fabricated an oxygen-gradient hydrogel chip with perfusion channels and evaluated such a gradient on both HCC cell lines and patient-derived HCC cells.

Materials and methods: To generate an oxygen-gradient environment within the gelatin hydrogel, two parallel perfusion channels (20 × 20 × 3 mm) were fabricated. Oxygenated media was produced by a catalase reaction with hydrogen peroxide, and the gradient was evaluated by assessing intracellular oxygen concentrations. L929 and Hep3B cell cytotoxicity was assessed using a CellTiter-Glo Luminescent assay kit. For HCC cell lines, Ki67 and MMP9 expressions were used to assess oxygen-gradient effects. Two patient-derived HCC cell samples were also cultured using this oxygen-gradient hydrogel chip system, and their

viability was evaluated using TUNEL analysis, H&E staining, and immunohistochemical staining for CAIX, K19, and CD34.

Result: Both HepG2 and Hep3B cells were viable in the hypoxic and hyperoxic areas of the chip. However, Ki67 expression was significantly increased in Hep3B cells and significantly decreased in HepG2 cells in the hypoxic region of the chip. The expression of MMP-9 was increased in HCC cell lines (i.e., Hep3B and SNU3160 cells) and significantly decreased in HepG2 cells in the hypoxic region of the chip. The patient derived HCC cells with hypoenhancement in MRI at arterial phase was viable in the chip at hypoxic area on H&E staining. Apoptosis was significantly increased in hyperoxic area on TUNEL analysis. Other patient-derived HCC cells with hyperenhancement in MRI at arterial phase were viable in the hyperoxic area of the chip.

Conclusion: The hypoxic conditions of this oxygen-gradient hydrogel chip induced aggressiveness in HCC cell lines. In addition, the chip provided proper oxygenation for culturing patient-derived HCC cells.

Key words: hepatocellular carcinoma, hepatocarcinogenesis, oxygen gradient, biochip, hydrogel

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I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent malignancy and the second most frequent cause of cancer death in Korea.[1] Although liver resection represents the best chance for long-term survival, more than half of these patients suffer from recurrence,[2,3] and no effective adjuvant therapies exist to prevent such recurrences after liver resections.[4] A variety of molecular and genetic pathways are involved in HCC carcinogenesis and progression, so it remains important to develop precision-medicine therapies to cure HCC.[5,6] Although cell culture has played an important role in HCC research, as a model system, it has many deficiencies that limit its utility. One of its deficiencies involves limited oxygen diffusion.[7] In contrast to normal hepatocytes mostly (75%) supplied by the portal vein, most of the HCC blood supply (90–100%) originates from the hepatic artery. Moreover, the multistep hepatocarcinogenesis process causes a gradual arterialization of the blood supply to liver tumors, so the actual blood supply to liver tumors can be variable based on tumor stage. As the HCC grows, the portal vein also supplies blood to these tumors, so tumor growth induces hypoxic conditions around itself that promotes both HCC progression and metastasis.[8] Unfortunately, current cell-culture systems are not able to reproduce a physiological oxygen gradient

around cancer cells.[7] We therefore hypothesized that an oxygen gradient may affect both liver cell viability and other characteristics under cell-culture conditions. First, we developed a perfusable oxygen-gradient hydrogel chip and investigated oxygen-gradient effects on the growth of HCC cell lines (e.g., Hep3B and SNU 3160 cells). We then investigated whether this oxygen-gradient hydrogel chip was suitable for culturing patient-derived primary HCC cells.

II. MATERIALS AND METHODS

1. Fabrication of a hydrogel chip containing perfusable channels

To generate an oxygen-gradient environment within a gelatin hydrogel, two perfusable parallel channels were first fabricated via a specifically designed mold using 3D printing (Fig. 1A and B). This poly lactic acid (PLA) mold (20 × 20 × 3 mm) contained two sets of needles for channels connecting it to a media-perfusion system. Polydimethylsiloxane (PDMS) was then poured onto the PLA mold to fabricate the perfusion chamber and the two needle sets were positioned to produce the two appropriate channels. A 10:1 mixture of 5.5% gelatin:phosphate-buffered saline (PBS) solution and a 10% microbial transglutaminase (mTG) solution (in 5% gelatin) was then poured onto the PDMS mold. Gelatin gelation was completed by incubating the chamber at 37℃ for 30 minutes. The flow-chamber channels were then connected to a perfusion system at a flow rate of 20 μl/min (Fig. 1C and D).

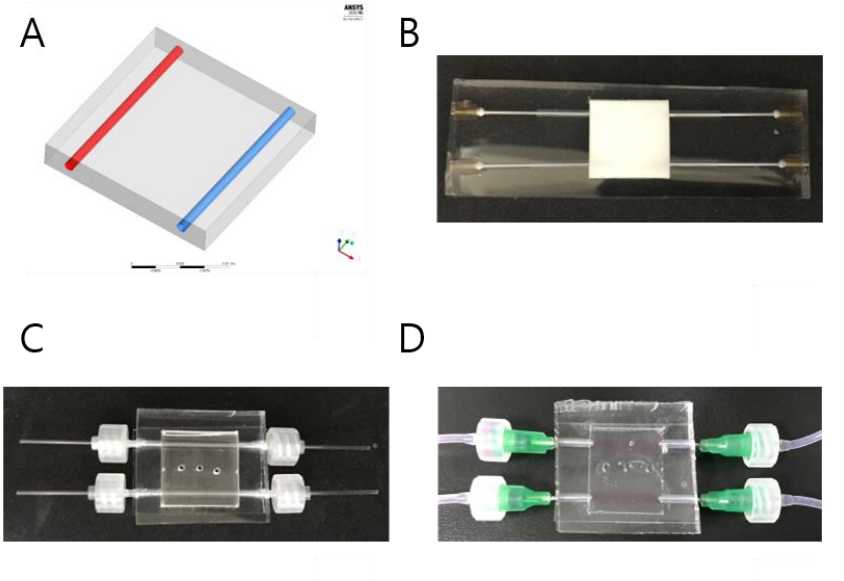

Fig 1. Fabrication of an oxygen-gradient hydrogel chip containing perfusable channels. (A) The gelatin hydrogel chip was designed as a 20 × 20 × 3 mm shape with two 1-mm diameter channels. (B) PLA molding was fabricated using a 3D printer. (C) The gelatin hydrogel was injected into the PDMS bonding mold. (D) Both untreated and oxygenated media could be continuously injected into the two channels, respectively.

2. Production of oxygenated media

Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin was used as the culture medium for the patient-derived liver cancer tissues as well as for the cell lines (e.g., Hep3B, SNU3160, and HepG2 cells). Oxygenated media was produced through a reaction between catalase (catalase from bovine liver, Sigma-Aldrich, St. Louis, MO) and hydrogen peroxide (H_2O_2). To control the oxygen concentration in the media, 100 μM H_2O_2 was added to 1 μM catalase per 10 ml of the complete media at a rate of 10 μl/hr (Fig. 2).

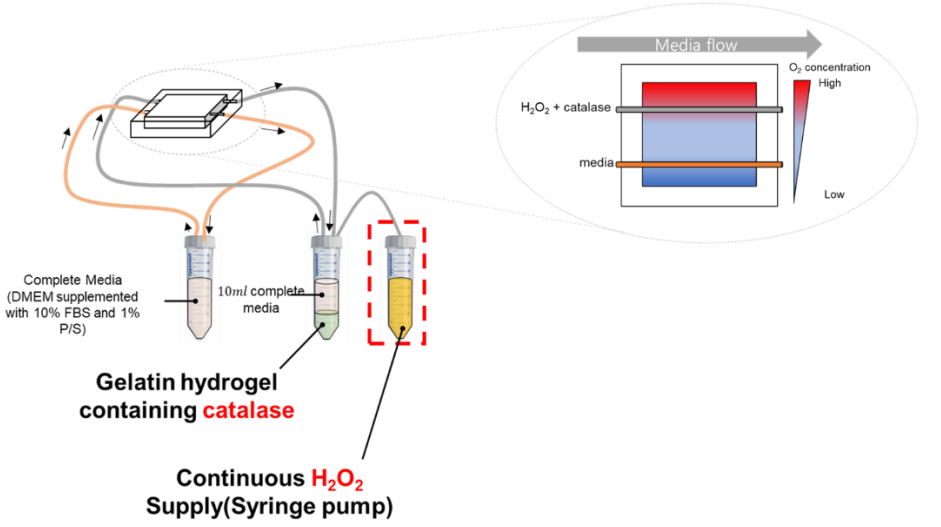

Fig 2. Experimental setup of the oxygen-gradient hydrogel chip. DMEM with 10% FBS and 1% penicillin and streptomycin was used for the complete media. H_2O_2 was continuously infused at a rate of 20 μl/min into the gelatin hydrogel containing catalase to form an oxygen gradient in the chip.

3. Gelatin degradation test

Long term exposure to catalase may result in degradation of the gelatin hydrogel. We therefore performed a degradation test by varying the concentration of catalase with the H_2O_2. 10% gelatin hydrogel was cross linked by mTG in a 10:1 ratio at 37°C for 30 minutes. Then, 0, 1, 5, and 10 μM catalase concentrations were tested with the gelatin for 3 days. The gelatin was degraded by the third day using 5 μM catalase, so we fixed the catalase concentration at 1 μM (Fig. 3).

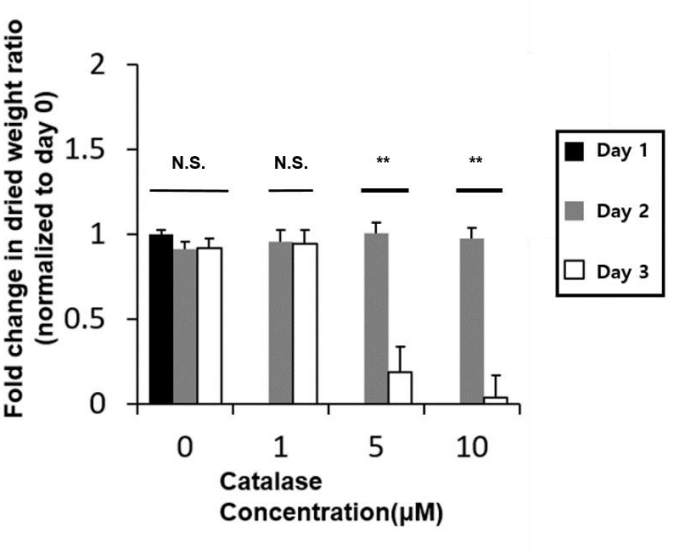

Fig 3. The gelatin degradation test using catalase. The gelatin hydrogel was degraded by the third day using 5 μM (and above) catalase. ** $p < 0.01$, N.S., not significant.

4. Cell cytotoxicity assay

Mouse fibroblasts (L929 cells) and hepatocellular carcinoma cells (Hep3B cells) were cultured in DMEM containing 10% FBS and 1% penicillin. The cells were seeded at a density of 2 × 104 cells/well in a final volume of 100 μl in 96-well plates. Twenty-four hours after plating, the cells were treated with H_2O_2 at each concentration for 3 days in an atmosphere of 5% CO2 at 37℃. Cell viability was estimated using the CellTiter-Glo Luminescent Cell Viability Assay kit (G7570, Promega) according to the manufacturer's protocol.

5. Cell culture

HepG2 (hepatoblastoma) cells, SNU3160 (hepatocellular carcinoma) cells, and Hep3B (hepatocellular carcinoma) cells were maintained in an atmosphere of 5% CO2 at 37℃ in DMEM supplemented with 10% FBS, 1% penicillin, and

1% streptomycin. Cells (2 × 105 cells) were embedded in the oxygen-gradient chip and their densities determined after being incubated for 4 days with oxygenated media.

6. Patient-derived HCC tissues embedded in the oxygen-gradient biochip

Fresh tumor tissue samples were harvested from surgically resected specimens (peripheral areas only; gross findings by the surgeon determined the central necrotic areas). These tumor tissues were rinsed with DMEM containing 10% FBS and 1% penicillin, and then minced into small pieces (average = 3 mm3). These tissues were then embedded into the oxygen-gradient hydrogel biochip with perfusable system and incubated at 37℃ in a humidified atmosphere (95% air and 5% CO2) for 4 days.

7. Staining of live and dead cells

Oxygen-gradient chip cells were incubated with oxygenated media for 4 days. The samples were washed three times with PBS, and then stained with calcein-AM and ethidium-1 (live/dead viability/cytotoxicity kit for mammalian cells) for 1 hr in a 5% CO2 atmosphere at 37℃. The fluorescence signals were detected using a confocal microscope (LSM700; Carl Zeiss, Germany)

8. Ki67 immunofluorescence

Oxygen-gradient chip cells were incubated with oxygenated media for 4 days, and then fixed using 10% neutral-buffered formalin, embedded in paraffin, and sectioned (5 μm). After deparaffinization, the samples were stained using an anti-Ki67 antibody (Abcam, ab15580) at room temperature for 3 hr, followed by an incubation with a fluorescent secondary antibody (Alexa 647) at room

temperature for 1 hr. The fluorescence signals were assessed using a confocal microscope (LSM700).

9. Measurement of the intracellular oxygen gradient

Cells cultured in the oxygen-gradient chip were incubated with oxygenated media for 4 days. The media was then replaced with fresh complete media containing Image-iT Green Hypoxia Reagent (final concentration of 1 μM) and incubated for 1 hr at 37℃ in an incubator. The fluorescence signals were assessed using a confocal microscope (LSM700).

10. Histological analysis

Human HCC liver tissue samples (surgically resected specimens; IRB approval: 4-2016-0728) were minced and then embedded in the oxygen-gradient hydrogel chip for 4 days. The samples were then fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned (5 μm). Liver samples from the oxygen-gradient chip were also subjected to hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis.

Four μm-thick tissue sections were processed for immunohistochemical (IHC) staining using an automated system (Ventana BenchMark XT, Ventana Medical Systems, Tucson, AZ) according to the manufacturer's recommendations. The slides were dried at 60℃ for 1 hr and then deparaffinized using EZ Prep (Ventana Medical Systems) at 75℃ for 4 min. Cell conditioning was performed using CC1 solution (Ventana Medical Systems) at 100 ℃ for 8 min. Anti-CD23, -CAIV, and -K19 antibodies were used (Table 1). Antibody incubations were at 37℃ for 32 min, and immunoreactivities were detected using an OptiView DAB IHC Detection Kit (Ventana Medical Systems). Counterstaining (Hematoxylin I; Ventana Medical Systems) was processed for 4 min at room

temperature.

Table 1. Antibodies for immunohistochemical staining

Antibody	Source	Clone	Dilution	Antigen retrieval
CD34	DAKO (Glostrup, Denmark)	QBEnd-10	1:50	Automated immunostainer
CAIX (pAb)	Novus Biological (Littleton, CO, USA)		1:1000	Automated immunostainer
K19	DAKO (Glostrup, Denmark)	RCK108	1:100	Automated immunostainer

Abbreviations: CD34, cluster of differentiation of 34; CAIX, Carbonic Anhydrase IX; K19, Keratin 19; pAb, polyclonal antibody.

11. TUNEL analysis.

To analyze apoptosis in the human HCC liver samples after embedding in the oxygen-gradient hydrogel chip, the Click-iT Plus TUNEL assay kit (Promega, Madison, WI, USA) was used according to the manufacturer's instructions. The fluorescence signals were assessed by confocal microscopy (LSM700). Apoptotic cell frequency in the liver sections was quantified according to the percentage of TUNEL-positive cells in five randomly chosen microscopic fields per specimen.

12. Quantitative RT-PCR analysis

Total RNA was prepared from the oxygen-gradient biochip samples using TRIzol® reagent (MRC, TR 118). RNA (1 μg) was then subjected to reverse transcription using a cDNA synthesis kit (TaKaRa, RR036A-1). The resulting cDNA was then subjected to quantitative PCR analysis using SYBR® Green (ABI, 467659) and human-specific primer pairs. The primer-pair sequences for human cDNA were as follows:

MMP-9- Forward: 5'-CCTGGGCAGATTCCAAACCT-3',

Reverse: 5'-GCAAGTCTTCCGAGTAGTTTTGGAT-3'.

Rn18S- Forward: 5'- CGGCTACCACATCCAAGGAA-3',

Reverse: 5'-GCTGGAATTACCGCGGCT-3'.

The Rn18S ribosomal RNA was used as an internal control.

13. Statistical analysis

Data were analyzed using two-tailed Student's t-tests for comparisons between two groups or using a one-way analysis of variance (ANOVA) with Tukey's honest significant difference post-hoc tests for multiple comparisons. GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA) was used for all the tests to determine statistical significance. Differences with P values less than 0.05 were considered significant.

III. RESULTS

1. H_2O_2 cytotoxicity and the oxygen gradient in the hydrogel chip

Mouse fibroblasts (L929) and hepatocellular carcinoma cells (Hep3B) were treated with different concentrations of H_2O_2 to evaluate cytotoxicity. At 3 days, 100 μM H_2O_2 (and less) did not affect cell viability in L929 and Hep3B cells (Fig. 4).

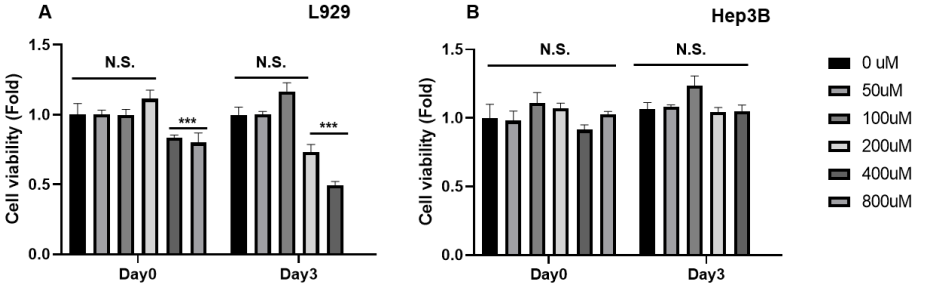

Fig 4. Optimization of the H_2O_2 concentrations in fibroblast and HCC. Mouse fibroblasts (L929 cells) (A) and HCC cells (Hep3B) (B) were treated with the indicated concentrations of H_2O_2 for 3 days. Data are mean ± SD from three independent experiments. ** p<0.01, *** p<0.001

Oxygenated complete media was created by reacting H_2O_2 with 1 μM catalase. To further verify whether an oxygen gradient was formed intracellularly, the oxygen concentration was determined using an intracellular oxygen-concentration kit, where green fluorescence indicated low intracellular oxygen levels (hypoxic conditions) in HCC cells. The results showed a separation of hyperoxic and hypoxic regions in the oxygen-gradient hydrogel chip (Fig. 5).

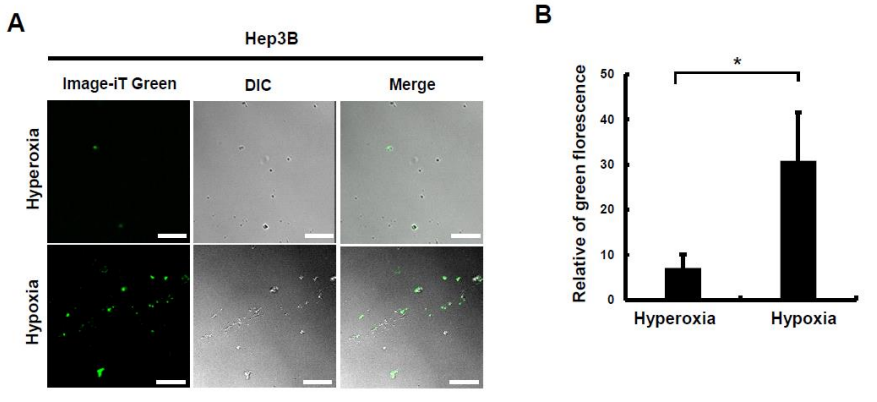

Fig 5. Construction of an oxygen-concentration gradient in a hydrogel chip containing perfusable channels. (A) Detection of intracellular oxygen

concentrations by hypoxia reagents. Green fluorescence indicates a low oxygen concentration. Scale bars represent 20 μm. (B) Quantification of green fluorescence. Data are mean ± SD from 6 or 7 cells per group. *p<0.05

2. HepG2 and Hep3B cell viability in the oxygen-gradient hydrogel chip

The application of live/dead staining was used to evaluate cell viability, and Hep3B and HepG2 cells were used (Hep3B representing HCC, and HepG2 representing hepatoblastoma). These cell lines remained viable in the oxygen-gradient hydrogel chip for 4 days according to this assay (Fig. 6).

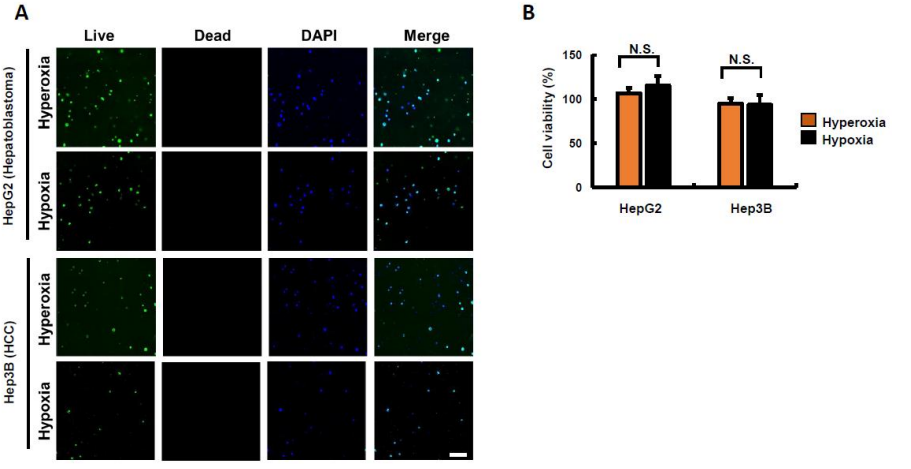

Fig 6. Hepatoblastoma (HepG2) and HCC (Hep3B) cells cultured in the perfused oxygen-gradient chip without cytotoxicity. (A) Cytotoxicity was assessed using a live/dead staining kit. The fluorescence signal for live cells was green, and red for dead cells. Nuclei were stained with DAPI, and representative single optical sections and overlays (merge) are shown. Scale bars, 100 μm. (B) Quantification of cell viability. Data are mean ± SD from three independent experiments. N.S., not significant.

3. Effect of an oxygen gradient on the expression of Ki67 in cell lines

Ki67 expression reflected cell proliferation. HepG2 (hepatoblastoma) and Hep3B (HCC) cells were incubated in the oxygen-gradient hydrogel chip for 4 days. Ki67 expression was significantly increased in Hep3B (HCC) cells in the hypoxic region. However, opposite results were seen in HepG2 (heptaoblastoma) cells (Figs. 7 and 8).

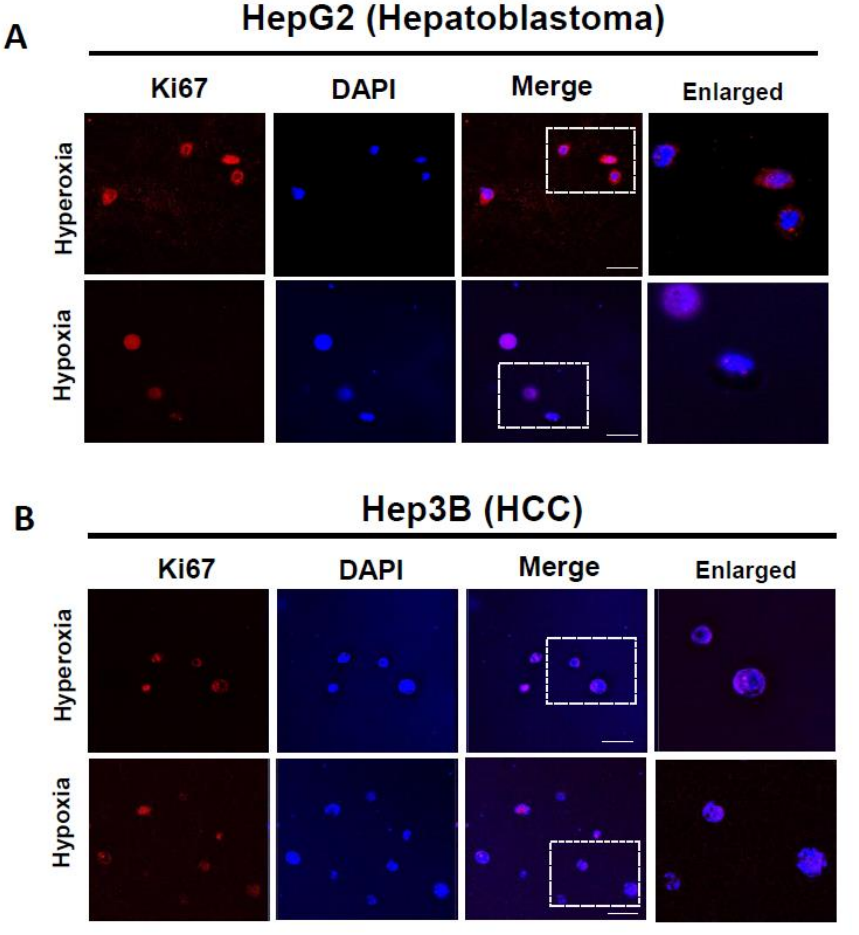

Fig 7. Ki67 expression in hepatoblastoma (HepG2) and HCC (Hep3B) cells within the oxygen-gradient hydrogel chip. Confocal microscopy assessment of Ki67 expression. Ki67 expression (red) in HepG2 (A) and Hep3B (B) cells

under hypoxic or hyperoxic conditions. Nuclei were stained with DAPI, and representative single optical sections and overlays (merge) are shown. Enlarged images were two times magnified image of white dotted box. Scale bars represent 20 μm.

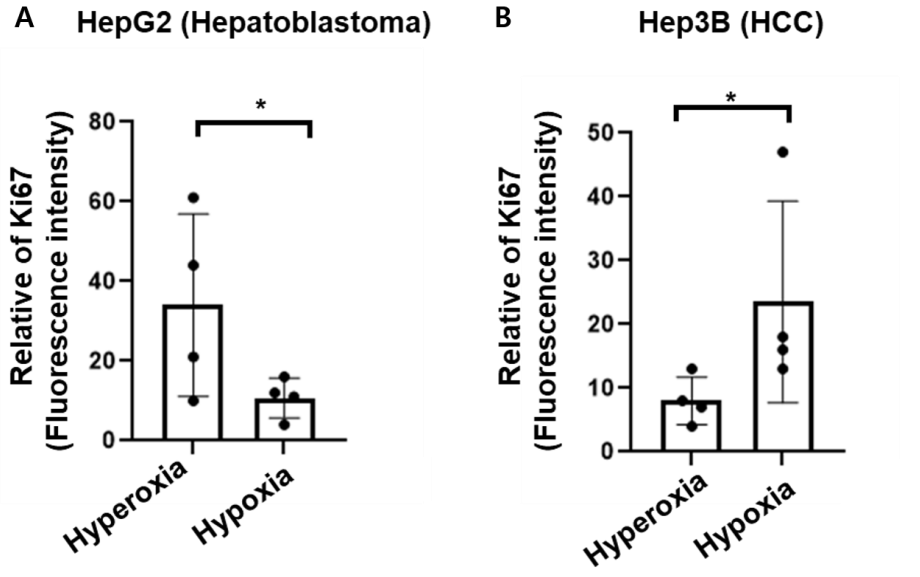

Fig 8. Quantification of Ki67 expression in hepatoblastoma (HepG2) and HCC (Hep3B) cells using the oxygenated and perfusable system. The relative expressions of Ki67 in HepG2 (A) and Hep3B (B) cells in response to either hypoxia or hyperoxia. * $p<0.05$

4. Effect of an oxygen gradient on the expression of matrix metalloproteinase 9 (MMP-9) in cell lines

Extracellular matrix metalloproteases are known to be crucial factors for tumor invasiveness. MMP-9 has specifically been reported to be predictive for both tumor recurrence and patient survival in those with HCC-related liver

resections.[9] Accordingly, Hep3B, SNU3160, and Hep3B cells were incubated in the oxygen-gradient hydrogel chip for 4 days and evaluated for the expression of MMP-9. In the HCC cell lines Hep3B and SNU3160, MMP-9 mRNA was increased in hypoxic regions of the hydrogel chip. However, opposite results were seen in HepG2 (heptaoblastoma) cells (Fig. 9).

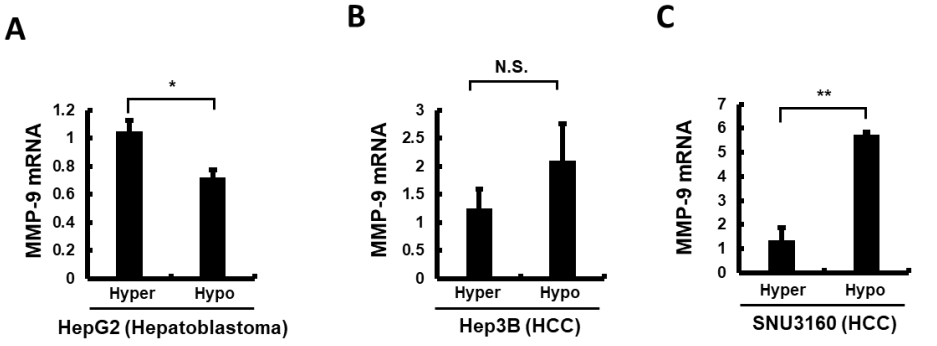

Fig 9. The expression of MMP-9 in hepatoblastoma (HepG2) and HCC (Hep3B, SNU3160) cells using the oxygenated and perfusable system. Total mRNA was isolated from cells incubated in the oxygenated/perfused system for 4 days and qRT-PCR was used to assess MMP-9 expression. (A) The expression of MMP-9 mRNA in HepG2 cells was significantly increased in hyperoxic areas of the oxygen-gradient hydrogel chip. The expression of MMP-9 was increased in both Hep3B (B) and SNU3160 (C) cells in the hypoxic regions of the oxygenated/perfusable system. *$p<0.05$, **$p<0.001$

5. Effect of an oxygen gradient on the viability of patient-derived HCC samples using an oxygen-gradient hydrogel chip

Cancer tissues harvested from two HCC patients were embedded into the oxygen-gradient hydrogel chip.9 The arterial enhancement patterns for these two tumor samples were opposite each other according to preoperative magnetic resonance imaging (MRI) assessment. The hypoenhanced

arterial-phase tumor sample was viable in the hypoxic region of the oxygen-gradient hydrogel chip (Fig. 10 A–D), and apoptotic cells were replaced in hyperoxic region based on H&E staining (Fig. 10E).

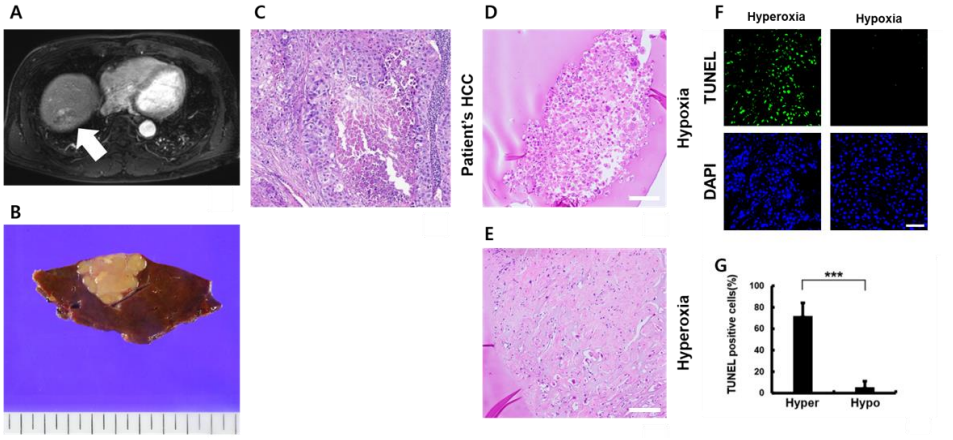

Fig 10. Effect of an oxygen gradient on patient-derived liver cancer samples (hypoenhanced MRI arterial phase). (A) Hypoenhanced HCC (white arrow) in the right superior liver was surgically resected. (B) The tumor was 3.2 cm, considered to be nodular with perinodular gross extensions. (C) The patient's HCC sample showed a macrotrabecular pattern. (D) Observed HCC appearance in the hypoxic area (H&E, 10×). (E) Replacement of the apoptotic lesion in the hyperoxic area (H&E, 10×). (F) Abundant TUNEL-positive cells were detected in the hyperoxic area. (G) TUNEL-positive cells were significantly increased under hyperoxic conditions. Scale bars represent 200 μm. *** $p<0.001$

In addition, TUNEL-positive cells were significantly increased under the hyperoxic conditions (Fig. 10F and G). Based on IHC staining, the primary HCC cells were positive for CAIX (30%), K19 (10%), and for CD24. The HCC cells cultivated under hypoxic conditions were positive for both CAIX and CD34. However, there were no CAIX-, K19-, or CD34-positive cells in the hyperoxic regions (Fig. 11).

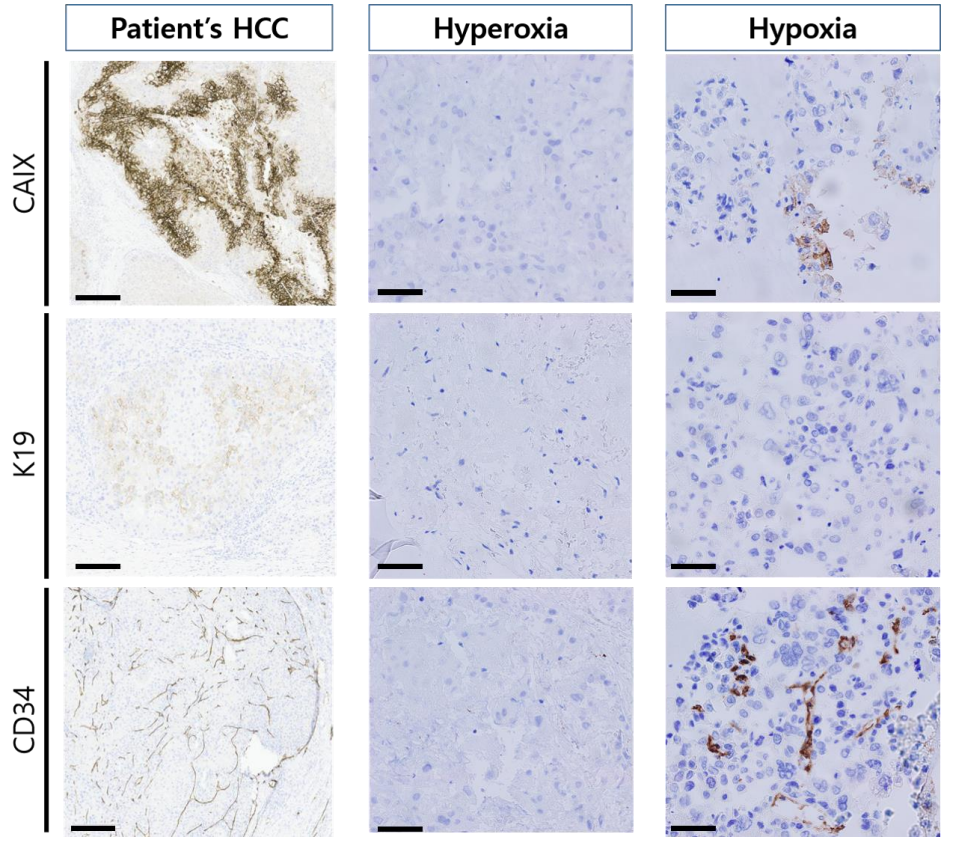

Fig 11. Immunohistochemical staining of patient-derived liver cancer samples (hypoenhanced MRI arterial phase). The patient HCC sample had focal CAIX-positive cells (30%), K19-positive cells (10%), and was positive for CD34 (10×, Scale bars represents 200 μm). There were focal CAIX- and CD34-positive cells which were cultivated under hypoxic biochip conditions. However, there were no IHC-positive cells after cultivation under hyperoxic chip conditions (20×, Scale bars represents 100 μm).

In contrast, the hyperenhanced arterial-phase tumor sample remained viable in the hyperoxic region and showed apoptosis in hypoxic region (Fig. 12 A–E). For this sample, TUNEL-positive cells were also significantly increased under hypoxic conditions (Fig. 12F and G).

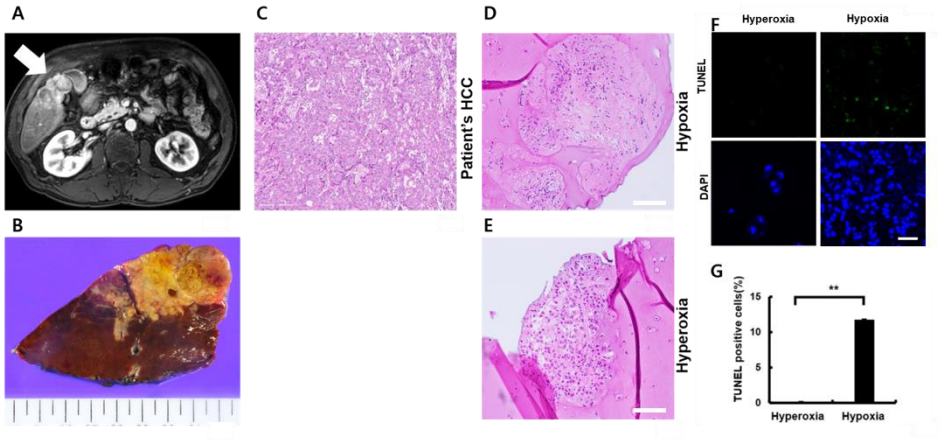

Fig 12. Effect of an oxygen gradient on patient-derived liver cancer samples (hyperenhanced MRI arterial phase). (A) Hyperenhanced HCC (white arrow) in the right superior liver was surgically resected. (B) The tumor was 6.3 cm, and considered to be grossly infiltrated. (C) The patient HCC sample was 30% of the macrotrabecular type, with a compact with fibrous stroma and lymphocytic infiltration. (D) The HCC observed from the hyperoxic area (H&E, 10×). (E) Replacement of the apoptotic lesion in the hypoxic area (H&E, 10×). (F) Abundant TUNEL-positive cells were observed in the hypoxic area. (G) TUNEL-positive cells were significantly increased under hypoxic conditions. Scale bars represent 200 μm .**$p<0.01$

Based on IHC staining, the primary HCC cells were focally positive for CAIX (10%), K19 (5%), and for CD34. The HCC cells cultivated under hyperoxic conditions were positive for both K19 and CD34. The cells cultivated under hypoxic conditions were only weakly positive for both K19 and CD34. However, no CAIX-positive cells were observed under either hyperoxic or hypoxic conditions (Fig. 13).

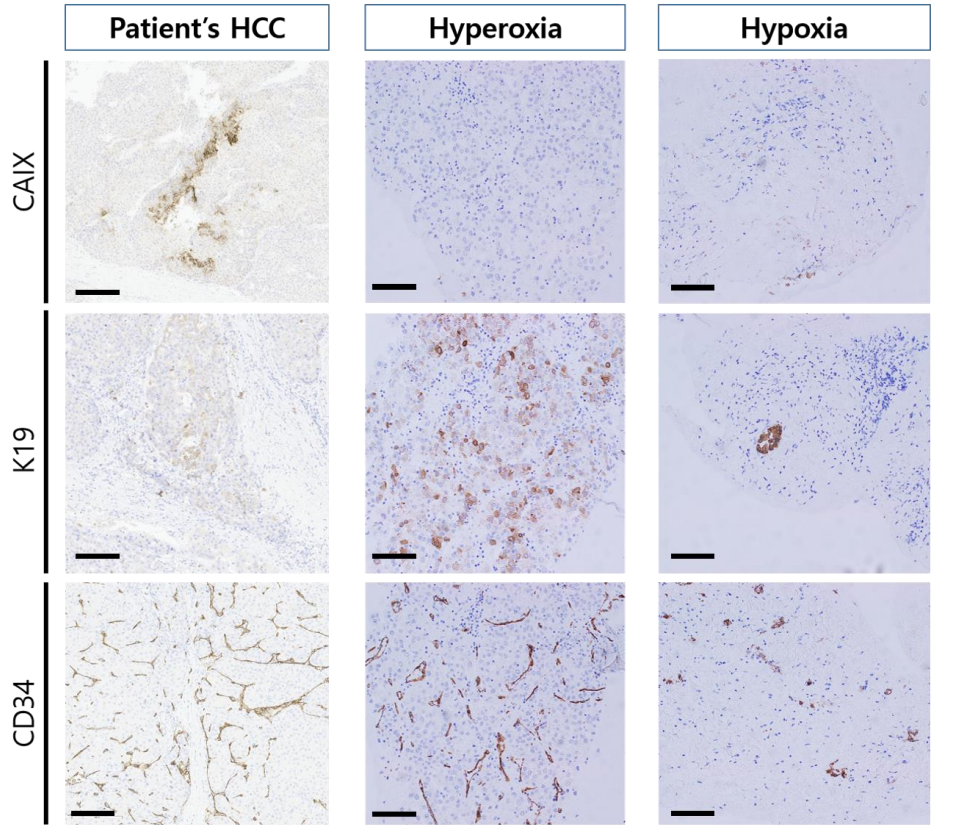

Fig 13. Immunohistochemical staining of patient-derived liver cancer samples (hyperenhanced MRI arterial phase). Patient HCC samples had focal CAIX positivity (10%), K19 (5%), and were positive for CD34 (10×, Scale bars represents 200 μm). Positive cells for both K19 and CD34 were seen in the hyperoxic regions of the biochip. Focal cells positive for both K19 and CD 34 were seen in the hypoxic regions of the oxygen-gradient chip. However, CAIX-positive cells were seen in both the hyperoxic and hypoxic regions (10×, Scale bars represents 200 μm).

IV. DISCUSSION

Although, current treatments of HCC mostly focused on loco-regional therapy such as liver resection, radiofrequency ablation, transarterial chemoembolization, et al. Unfortunately, relapse of the tumor is very frequent even after the effective loco-regional therapy of HCC. Therefore, tailored therapy for the patient after local treatment is mandatory to cure the disease. Effective culture system reflecting real tumor environment is fundamental tool for developing new generation drugs and predictive biomarkers.

Here, we describe the development of an oxygen-gradient hydrogel chip and an investigation into the effect of an oxygen gradient on HCC cell lines and cells surgically resected from the livers of patients with HCC. According to previous studies, the chances of establishing cell lines from fresh tumor samples were extremely low. Even with current technological advances for cell cultures, the success rate has been reported to be less than 20% for fresh patient-derived HCC tissues,[10] and the success rate for establishing 3D HCC organoids from patient samples was only 26%.[11] HCC cell lines normally have a high oxygen consumption rate (200–400 attomoles/cell/sec), but cells on the bottom of culture dishes seeded at high density can have pericellular oxygen concentrations near zero within four hours.[7] Given that an HCC intra-tumoral arterial blood supply provides hyperoxic blood, and the portal vein supplies the tumor with relatively hypoxic blood, overall blood oxygenation is considered to be variable during the multistep hepatocarcinogenesis process.[12] Therefore, equally variable oxygen concentrations may be required for HCC culture conditions.

The oxygen-gradient hydrogel chip described here provides hypoxic as well as hyperoxic media conditions. Using fluorescence imaging of intracellular oxygen gradients, significantly hypoxic and hyperoxic regions could be distinguished (Fig. 5). Although the oxygen gradient established by the chip did not affect

viability for HepG2 and Hep3B cells (Fig. 6), it did affect Ki67 and MMP-9 expression levels (Figs. 8 and 9). In contrast to the hepatoblastoma cell line (HepG2), Ki67 and MMP-9 expressions were increased in the Hep3B and SNU3160 HCC cell lines. Several studies have reported that Ki67 expression acts as a marker for cellular proliferation, and a marker for prognosis prediction in patients with HCC.[13-15] MMP-9 has also been shown to be a crucial marker for potential HCC invasiveness, with MMP-9 expression reported to be a predictive marker for both HCC tumor recurrence and patient survival after surgical tumor resections.[9] These findings suggest that hypoxia may induce HCC aggressiveness.

In contrast to the HCC cell lines, the patient-derived HCC samples were observed to be viable under both hyperoxic and hypoxic conditions. Hypoenhanced (MRI arterial phase) patient-derived HCC cells remained viable within the hypoxic region of the oxygen-gradient hydrogel chip, and a partial increase in apoptotic cells was observed in hyperoxia region of the chip using both H&E staining and TUNEL assessments (Fig. 10). However, the hyperenhanced (MRI arterial phase) patient-derived HCC samples demonstrated the opposite result (Fig. 12). As hyperenhancement represents a mainly arterial blood supply (hyperoxic), and hypoenhancement represents a mainly portal vein supply (hypoxic), these findings suggest that the present oxygen-gradient hydrogel biochip can provide the proper oxygen conditions for culturing different primary liver cancer cells (Fig. 14).

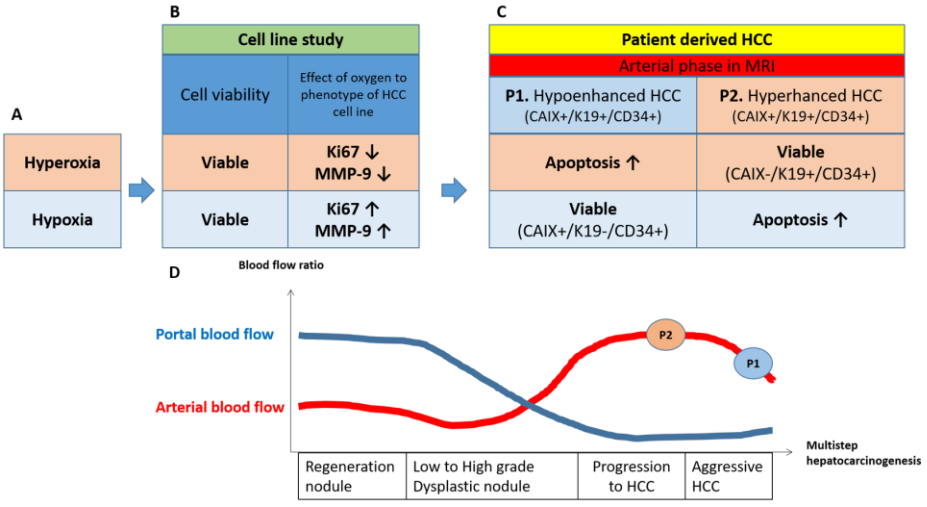

Fig 14. Highlight of the result. (A) The oxygen-gradient hydrogel chip produced hyperoxic and hypoxic area. (B) Although, HCC cell lines were viable in both hyperoxic and hypoxic area in the chip, oxygen condition affected the expression of Ki67 and MMP-9 which were associated with aggressiveness of HCC. (C) HCC of the patient 1 (P1) which was hypoenhanced in the arterial phase of MRI were viable in hypoxic area in the chip. However, HCC of the patient 2 (P2) which was heyperenhaced in the arterial phase of MRI were viable in hyperoxic area in the chip. (D) Arterial enhancement pattern of P1 was accordant to aggressive HCC in multistep hepatocarcinogeneis while that of P2 matched with typical HCC.

We found that oxygen concentration was a crucial factor for these primary cell cultures and for determining HCC cell-line characteristics. As oxygen conditions for the patient-derived HCC cells are likely to be different for each patient, it is interesting that several previous studies only suggested uniform oxygen conditions, such as either normoxia or hyperoxia.[7,16-18] Although previous studies have suggested the establishment of metabolic zones through chip-based oxygen gradients, only normal hepatocyte culture conditions were described, and the reported conditions were considerably more complex

compared to our oxygen-gradient hydrogel chip.[7,19]

Despite our development of an effective culture system using an oxygen-gradient hydrogel chip, the mechanisms that regulate the growth of HCC cells by oxygen concentration should be evaluated further. HCC oxygen responses involve key mechanisms. First, hypoxia-inducible factor-1 (HIF1) has been shown to act as a transcription factor that is activated under hypoxic conditions to overcome low oxygen levels,[20,21] promoting the expression of hypoxia-responsive genes during HCC tumorigenesis and cancer progression. In addition, high HIF1 expression has been reported to promote both HCC cell proliferation and survival, and was associated with poor HCC prognosis. Second, the molecular pathway linking nuclear factor E2-related factor 2 (Nrf2) and Kelch-like ECH associated protein 1 (Keap1) has been shown to be essential for redox homeostasis functions.[22,23] Nrf2 is known to be a master transcription factor responsible for the elimination of radical oxygen species (ROS), and Keap1 has been shown to act as an Nrf2 suppressor. Furthermore, this Nrf2/Keap1 pathway is known to be activated by SQSTM1/p62, a general ROS-response target gene, without Keap1 oxidation, and this composite p62/Keap1/Nrf2 pathway has been shown to be upregulated or dysregulated in patients with HCC. However, oxygen's role in these mechanisms is not fully understood for HCC patients. Therefore, our present system may be used to manipulate and control the oxygen gradient; a useful tool for investigating oxygen's functional mechanisms in HCC.

V. CONCLUSION

The hypoxic conditions of an oxygen-gradient hydrogel chip induced aggressiveness in HCC cell lines and the chip provided the proper oxygen concentration for culturing patient-derived HCC samples. Further studies and development of the current system to high throughput screening are needed for personalized medicine of HCC.

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ABSTRACT(IN KOREAN)

바이오칩 내 산소농도 구배에 따른 간암세포의 성장에 대한 영향

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한 대 훈

배경: 간암 세포 배양은 간암 연구에 있어 매우 중요하지만, 현재의 간암세포 배양 방식은 간암세포의 생리학적인 환경을 재현하는 데에 한계가 있다. 간암세포의 발생과정은 다단계로 구성되어 있으며, 간암이 진행됨에 따라 간암세포로의 동맥형성이 증가되었다가 감소하는 양상을 보이게 된다. 따라서, 간암세포를 배양할 때에 산소 농도가 간암의 성장 및 특성에 영향을 줄 것이라고 가정하였고, 이에 산소가 투과할 수 있는 통로를 포함한 산소농도 구배 하이드로겔 바이오칩을 제작하여 산소농도가 간암 세포주 및 환자 유래 간암세포의 성장에 어떤 영향을 주는지 연구하였다.

방법: 산소농도 구배를 형성하기 위하여 두개의 산소 투과 통로를 포함하여 20 x 20 x 3mm 크기의 하이드로겔 칩을 제작하였다. 촉매를 함유한 배양액에 과산화수소를 순환 접촉하여 조성한 고산소 농도의 배양액과 일반 배양액이 바이오칩 내 산소구배를 발생할 수 있도록 하였다. L929, Hep3B 간암세포주를 이용하여 바이오칩 내부의 세포 독성을 CellTiter-Glo Luminescent assay kit 을 이용하여 평가하였고, Ki67,

MMP-9의 발현 여부를 검사하여 산소농도 구배가 간암세포주에 미치는 영향을 분석하였다. 또한, 실제 환자에서 유래한 간암 수술조직을 산소농도 구배 바이오칩에서 배양을 한 후 TUNEL 분석 및 CAIX, K19, CD34 면역항암화학 염색을 시행하였다.

결과: HepG2 및 Hep3B 세포주 모두 바이오 칩 내부의 저산소 및 고농도 산소 영역에서 생존하고 있었지만, 간모세포종 세포주인 HepG2 와 달리 간암세포주인 Hep3B에서는 저산소 농도에서 Ki67의 발현이 증가되어 있었다. 또한, 저산소 영역에서는 Hep3B 및 SNU 3160 의 간암세포주의 MMP-9 발현이 증가 되어 있었다. MRI 의 동맥조영기에서 조영이 감소된 양상을 보이는 환자의 간절제술 후 획득한 간암조직을 바이오칩에 배양하하였을 때에는 저산소 농도에서 간암조직이 생존하고 있었지만, 고농도 산소 영역에서는 세포사멸이 증가하였고, MRI의 동맥조영기에서 조영이 증가된 양상을 보이는 환자의 간암조직은 고농도 산소 영역에서 생존하고 있었으며, 저농도 산소 영역에서는 사포사멸이 증가되었다.

결론: 저농도의 산소는 간암 세포주의 공격성을 증가시키는 것으로 보인다. 또한, 산소농도 구배 바이오칩은 환자 유래 간암조직의 배양에 적절한 산소농도를 공급해준다.

핵심되는 말: 간세포암, 간암발생기전, 산소농도, 바이오칩, 하이드로겔