CD151 as a potential molecular marker of cholangiocarcinoma stem cells

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CD151 as a potential molecular marker of cholangiocarcinoma stem cells

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The Doctoral Dissertation submitted to the
Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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December 2016

연세대학교
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December 2016

ACKNOWLEDGEMENTS

I always appreciate Professor Si Young Song for his teaching and guidance. I met him in April 1999, my first year of the resident of internal medicine. Since then, I have been so impressed with his patient care, endoscopic skills and research. Since he has been my great mentor, it is an honor for me to have him as my doctoral dissertation supervisors.

I also met four great supervisors, Professor Seungmin Bang, Yun-Han Lee, Young Nyun Park, and Woo Jin Lee. I appreciate them for their guidance and great advices.

I also owe a sincere gratitude to Su Bin Park. She always helped me about all things related to this study. Without her help, I would not have been able to finish this study.

I am also grateful to Professor Ji Young Park who sent a lot of references that were difficult to find.

Last, but not the least, I would like to express my special thanks to my family for their constant love and support that enables me to finish my doctoral dissertation. Thank you.

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ABSTRACT

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Cancer stem cells are currently hypothesized to be responsible for tumorigenesis, metastasis, recurrence, and chemoresistance in the various types of cancer.

CD151 is a member of the transmembrane 4 superfamily, which enhances cell motility, invasion, and metastasis of cancer cells. In intrahepatic cholangiocarcinoma, overexpression of CD151 associated with c-Met is implicated in invasion and metastasis, which result in poor prognosis. However, there are few studies that focus on CD151 in cholangiocarcinoma, and the role of CD151 in cancer stem cells, including cholangiocarcinoma stem cells, which have hardly been documented.

The aim of this study is therefore to characterize the role of CD151 in cholangiocarcinoma stem cell.

To evaluate cholangiocarcinoma stem cell, the author prepared cancer stem like cells using sphere cultivation (SNU-245, SNU-1196). A cDNA microarray was used to gain up-regulated genes between tumorsphere and biliary carcinoma. The expressions of stemness genes and the proteins related to c-Met and Epithelial Mesenchymal Transition (EMT) were evaluated through RT-PCR and western blot to find if tumorsphere has characteristics of stem cell.

Expression of CD151 was compared between adherent cells of SNU-1196 and tumorsphere. To evaluate the role of CD151 in cholangiocarcinoma stem cells, CD151 knockdown was done by CD151 siRNA in SNU-1196 cells and then sphere formation and cytotoxicity assay were performed. In order to evaluate the mechanism of CD151, c-Met, EMT pathway related molecules, β-catenin, and Oct 4 were evaluated through western blot. Proliferation and metastatic capabilities were also evaluated after transfection of CD151 siRNA in SNU-1196.

As a result, CD151 gene was highly up-regulated in both tumorspheres and biliary carcinoma on cDNA microarray. Cultivated tumorsphere had cancer stem cell-like properties, showing highly expressed Notch, Hedgehog, and Wnt pathway related to stem cells in RT-PCR. Expressions of the proteins that related to c-Met, PI3K, EMT pathway, β-catenin, and Oct 4 were more highly increased and CD151 was more highly expressed in tumorsphere than in adherent cells.

After CD151 knockdown, tumorsphere formation was inhibited and chemoresistance was reduced. The expression of the molecules associated c-Met, PI3K, EMT pathways, β-catenin and Oct4 was reduced. Therefore, the characteristics of proliferation and migration were also reduced after transfection of CD151 siRNA in SNU-1196 cells.

The author could suggest that CD151 was more expressed in cholangiocarcinoma stem-like cells and regulated self-renewal, chemoresistance, proliferation and metastatic capabilities. It might interact with c-Met and activate PI3K, EMT pathway, β-catenin, and Oct4. So, it would be a potential cholangiocarcinoma stem cell related marker and therapeutic target in the future.

Key words: CD151, cholangiocarcinoma stem cell, c-Met

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I. INTRODUCTION

Cholangiocarcinoma is a rare malignant cancer of the bile ducts which has poor prognosis.[1] Although treatment is possible with a surgical approach, most cases are inoperable and cancer recurrence is frequent even after surgery. In addition, there is no effective drug treatment for advanced and recurrent cholangiocarcinoma.[2,3] In modern medicine, these poor clinical outcomes are often attributed to cancer stem cells. This is a concept which has been proposed in the last decade and is known as the 'cancer stem cell hypothesis'.[4]

Cancer stem cells are believed to have self-renewal, proliferation, and multilineage differentiation capabilities. They are hypothesized to be responsible for tumorigenesis, metastasis, recurrence, and chemoresistance and are considered to play an important role in the carcinogenesis of various types of cancer.[5,6] Although many cancer stem cell markers have been reported in various solid tumors, few studies in this field have investigated cholangiocarcinoma stem cells. Furthermore, the role of cholangiocarcinoma stem cells in the pathogenesis of cholangiocarcinoma has not been fully understood. Recently, several reports have demonstrated the existence of cholangiocarcinoma stem cells.[7,8] CD133, CD24, EpCAM, and CD44, along

with other adhesion molecules have also been reported as molecular markers of these stem cells.[9-16] However, these markers are controversial and continue to be the subject of much debate.

CD151 protein is a member of the transmembrane 4 superfamily, also known as the tetraspanin family.[17] It is a cell surface glycoprotein that is known to form complexes with integrins and other transmembrane 4 superfamily proteins. CD151 is highly expressed in epithelial and endothelial cells[18,19] and plays a role in the regulation of cell development, activation, growth, and motility. It also enhances cell motility, invasion, and metastasis of cancer cells, especially in colon cancer and fibrosarcoma cells.[20] In hepatocellular carcinoma, CD151 was associated with invasiveness, and CD151 or a combination of CD151/c-Met were independent prognostic factors.[21] Dysregulation of CD151 has been observed in many cancers, and overexpression of CD151 was indicative of poor prognosis in hepatocellular carcinoma and non-small lung, colon, pancreatic, esophageal, gastric, breast, and endometrial cancer.[22-29] In intrahepatic cholangiocarcinoma, CD151 and c-Met were independent prognostic markers.[30]

CD151 interacted laminin-binding integrins α3β1, α6β1, α6β4 and α7β1 in cells in culture and in vivo.[31] CD151 regulated tumor cell migrations through α3β1 and α6β4 on laminin-5[32] and promoted cancer cell metastasis via integrins α3β1 and α6β1.[33,34] CD151 enhanced c-Met signaling pathway through integrin β4 and contributed to tumor cell proliferation and invasive growth.[35] In pancreatic ductal adenocarcinoma, CD151, c-Met, and integrin α3/α6 were also overexpressed and showed poor prognosis.[24] High expression of CD151 and α6 activated epithelial-mesenchymal transition (EMT) through PI3K/APK pathway and resulted in invasiveness of hepatocellular carcinoma.[36] CD151 also promoted neoangiogenesis and progression of hepatocellular carcinoma by modulating matrix metalloproteinase 9 through PI3K/Akt/GSK-3β/SNAIL pathway.[28] However, the studies mentioned here remain among the few to

investigate CD151 in cholangiocarcinoma, and the role of CD151 in cholangiocarcinoma stem cells, has not yet been elucidated.

The aim of this study was to characterize the role of CD151 in cholangiocarcinoma stem cell. Considering the lack of study regarding CD151 in cholangiocarcinoma stem cells, the comprehension of the CD151 function is important for understanding cancer mechanism and advancement of current treatment. Finally, the development of a targeted therapy for CD151 or related molecules may improve future survival rates for patients with this disease.

II. MATERIALS AND METHODS

1. cDNA microarray analysis

cDNA microarray was performed to gain up-regulated genes in tumorsphere than in adherent cells. The experiment was performed with cells derived from tumorsphere and adherent cells of biliary carcinoma cell line. RNA was extracted by using RNeasy mini kit (Qiagen, Valencia, CA, USA), and RNA quality was verified by A260/A280 ratio and agarose gel electrophoresis with resolution of distinct 28s and 18s. The microarray procedures were carried out following the manufacturer's protocols. The complementary DNA (cDNA) microarray analysis was performed by Digital Genomics (Seoul, Korea). Using 6 μg of template, double stranded cDNA was prepared, after which amplification and biotin-labeling (IVT labeling kit; Affymetrix, Santa Clara, CA, USA) were performed. The labeled cRNA was fragmented and used in the hybridization reaction to GeneChip@ probe array HG U133 plus 2.0 version high-density oligonucleotide array (Affymetrix, Santa Clara, CA, USA). Microarrays were then washed in a

GeneChip Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA) and scanned using a GeneChip Array Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). Expression data were generated using Affymetrix Expression Console software version 1.1 using MAS5 algorithm normalization. Expression intensity data in CEL file were normalized with the MAS5 algorithm to reduce noise. Probe sets that were not identified as ''present'' by the MAS5 detection call in more than 50% of the samples in at least 1 sample group were removed. Genes regulated more than 1.2 fold were taken into consideration. Each individual chip analysis was performed twice with duplicated independent total RNA samples. Human gallbladder cancer chip data which was already analyzed by cDNA microarray compared with normal human gallbladder tissue in our lab was used. The gallbladder cancer genes were selected when expressed more than two times than normal gallbladder ($p<0.01$) and related to the development. The molecules produced by selected genes should be located in the cell membrane and secretory type. With using all the results, the author analyzed the commonly up-regulated genes in gallbladder cancer, SNU-245, and SNU-1196.

2. Cell culture and sphere formation assay

Human biliary tract cancer cell lines: SNU-245 (common bile duct carcinoma), and SNU-1196 (hilar bile duct carcinoma), were purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea). NIH-3T3 mouse embryonic fibroblast cell line was obtained from the American Type Culture Collection (ATCC, VA, USA). All cells were grown in RPMI 1640 medium (Invitrogen Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, UT, USA), and were kept in a humidified incubator at 5% CO_2 and 37°C.

Single cell suspensions at a concentration of 1000 cells/mL were seeded onto

6-well ultra-low attachment culture plates (Corning Inc., NY, USA) for the sphere formation assay, which was performed as described previously[37]. Sphere formation medium consisting of serum-free medium (SFM) supplemented with 10 ng/mL epidermal growth factor (R&D Systems Inc., MN, USA), 10 ng/mL basic fibroblast growth factor (R&D Systems), 1 × insulin-transferrin-selenium (Invitrogen Gibco), 0.5% bovine serum albumin (BSA; Invitrogen Gibco), and 0.5% FBS was used. As a control, cultured cells that were adherent were seeded onto culture dishes (Nalge Nunc Int, NY, USA) with sphere formation medium.

3. Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR)

The expression of CD151 was checked using RT-PCR. Total RNA was extracted from the cells using an RNeasy mini kit (Qiagen, CA, USA), and complementary single-strand DNA was synthesized using the Superscript II system (Invitrogen) according to manufacturer's protocols. β-actin (*ACTB*) was used as a reference housekeeping gene. The primers used are listed in Table 1. After transfection of the siRNA into SNU-1196 cells, RT-PCR analysis was performed at 30 and 96 h post-transfection.

Table 1. Primer Sequences Used For RT-PCR

Gene	Sense	Antisense
Notch3	ATGGTGGGAACTAAACACAGCT	ATGACCCTGGAGGAAGCACA
Hes1	GTGCTGTCTGGATGCGGAGT	GAACACTCACACTCAAAGCCC
Jagged1	CTCAATTACTGTGGGACTCATCA	GAACACTCACACTCAAAGCCC
Ihh	CCTGAACTCGCTGGCTATCT	AATACACCCAGTCAAAGCCG
Gli1	AGAGTCCAGGGGGTTACATA	AGAGTCCAGGGGGTTACATA
Nanog	ACTGTCTCTCCTCTTCCTTCCT	AGAGTAAAGGCTGGGGTAGGTA
Oct4	GTGGAGGAAGCTGACAACAA	AGCAGCCTCAAAATCCTCTC
PTEN	GGACGAACTGGTGTAATGAT	CAGACCACAAACTGAGGATT
FZD7	CCAACGGCCTGATGTACTTT	GCCATGCCGAAGAAGTAGAG
β-catenin	GTATGAGTGGGAACAGGGATTT	CCTGGTCCTCGTCATTTAGC
C-met	CAATGTGAGATGTCTCCAGC	CCTTGTAGATTGCAGGCAGA
Snail	AAGCTTCCATGGCGCGCTCTTT CCTCGTCAGGAAGCCC	GGATCCTCAGCGGGGACATCCT GAGCAGCCGGACTCTTG
Vimentin	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC
CD90	GACCCGTGAGACAAAGAAGC	ACTGTGACGTTCTGGGAGGA
CEACAM1	ATACCTGCCACGCCAATAAC	TTATGCTGAGGGTGGTGTTG
CD151	CTCCCCGGACATACTCTCTG	GTCAGAGCTCACCTGGCTTC
CD104	TCTACACGGACACCATCTGC	GGGCAGTCCTTCTTCTTGTG
β –actin	GGCATCCTCACCCTGAAGTA	GGGGTGTTGAAGGTCTCAAA

4. Transfection of CD151 siRNA in SNU-1196

In order to evaluate the function of CD151, knockout cells were obtained by the transfection of CD151 small interfering RNA (siRNA) (sc-42829; Santa Cruz Biotechnology, CA, USA) composed of a pool of three target specific siRNAs, and a control siRNA-A (sc-37007; Santa Cruz Biotechnology) at a concentration of 33 nM into the cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen).

5. Western blot analysis.

To further analyze CD151 expression, western blotting was carried out for the cells. Cells were lysed in lysis buffer containing 70 mM β-glycerophosphate (pH 7.2), 0.6 mM sodium orthovanadate, 2 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5% TritonX-100, 0.2 mM phenylmethanesulfonylfluoride (PMSF), and 1× complete protease inhibitor (Roche Applied Science, NJ, USA). Protein (20 μg) was loaded onto and resolved on SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked in 5% (w/v) non-fat dry milk and probed with the following primary antibodies: rabbit anti-Met polyclonal antibody (PAb), rabbit anti-phospho-AKT PAb, rabbit anti-phospho-GSK3 PAb, rabbit anti-SNAIL PAb, rabbit anti-Oct4 PAb, mouse anti-CD151 MAb, rabbit anti-Integrin β4 PAb, mouse anti-N-cadherin Mab (Abcam plc., MA, USA), rabbit anti-Occludin PAb, mouse anti-β-catenin MAb, rabbit anti-AKT PAb, rabbit anti-ERK PAb, rabbit anti-phospho-ERK PAb, and mouse anti-GAPDH MAb (Santa Cruz Biotechnology Inc.). Immunoblots were developed with West Pico Chemiluminescent Substrate (Thermo Scientific, IL, USA). The dilution ratio of all primary antibodies was 1:1000.

6. Proliferation assay

SNU-1196 cells transfected with CD151 siRNA or control siRNA were seeded at 10^3 cells/well in 96-well plates and cultured in a CO_2 incubator at 37℃ for 3 days. After 3 days, 100μl MTT solution (0.5 mg/ml in media) was added per

each well and cells were incubated for 3 hours at 37°C. Next, the solution was discarded and 100μl DMSO was added to the cells. Plates were shaken for 30 minutes and absorptions were measured at 570 nm using Tilter-Tech 96-well multiscanner (Becton Dickinson, Heidelberg, Germany).

7. Cell migration assay

Forty-eight hours after the transfection of CD151 siRNA into SNU-1196, cell migration assays were performed in 24-well transwell plates (8 μm; CoStar, MD, USA), seeded at a concentration of 1×10^4 cells/well in serum free RPMI medium in the upper chambers. The lower chambers were filled with conditioned media derived from NIH3T3 cultures. After 24 h, migrated cells were fixed in 5% glutaraldehyde/PBS and stained with toluidine blue for cell-number scoring and cells were counted using light microscopy. Migrated cells were defined as the cells that had passed into the lower layer, and experiments were performed in triplicate for this assay.

8. Cytotoxicity assay

The cytotoxicity assay of gemcitabine (Eli Lilly Korea, Seoul, Korea) was assessed with a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Inc., St. Louis, MO). SNU-1196 cells transfected with CD151 siRNA or control siRNA were seeded at 10^3 cells/well in 96-well plates and cultured in a CO_2 incubator at 37℃ for 24 hours. The next day, various concentrations of gemcitabine were added and incubated for 72 hours. Treated

cells were incubated in 100μl MTT solution (0.5 mg/ml in media) for 3 hours. The solution was discarded and 100μl DMSO was added to each well. The absorbance of each well was measured at 570 nm using Tilter-Tech 96-well multiscanner (Becton Dickinson, Heidelberg, Germany).

9. Statistical analysis

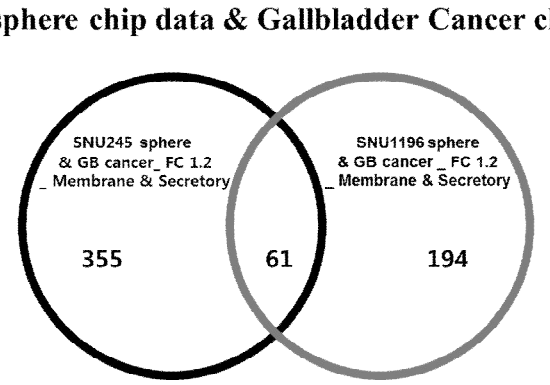
All data were presented as means ± standard deviations. Statistical significance for the results of sphere forming, cytotoxicity, proliferation, and migration assay was determined using independent samples t test and one way ANOVA.

III. RESULTS

1. CD151 gene was up-regulated in both tumorsphere and biliary carcinoma cells

Up-regulated genes were obtained in tumorsphere of both SNU245 and SNU1196 cells comparing with adherent cells by cDNA microarray. Moreover, Gallbladder cancer chip data was also used to find commonly up-regulated genes between tumorsphere and biliary cancer cells. Four hundred sixteen genes were commonly up-regulated between SNU245 tumorsphere and gallbladder cancer cells. Two hundred fifty-five genes were also up-regulated in both SNU1196 tumorsphere and gallbladder cancer cells. Sixty-one genes were highly expressed in all three groups (Figure 1-A). CD151 was confirmed as an up-regulated gene in all three groups (Figure 1-B).

A.



B.

Probe Set ID	SNU245 SP_ FC	SNU1196 SP_ FC	Biliary cancer _FC	Gene Symbol
204306_s_at	1.6	1.4	2.1	CD151

Figure 1. cDNA microarray analysis. A. Upon the microarray data, the genes were selected if the expression level was greater than 1.2-fold ($p<0.05$). Sixty-one genes were highly up-regulated genes in all SNU-245, SNU-1196 tumorsphere, and gallbladder cancer. B. CD151 was confirmed as up-regulated genes in all three groups.

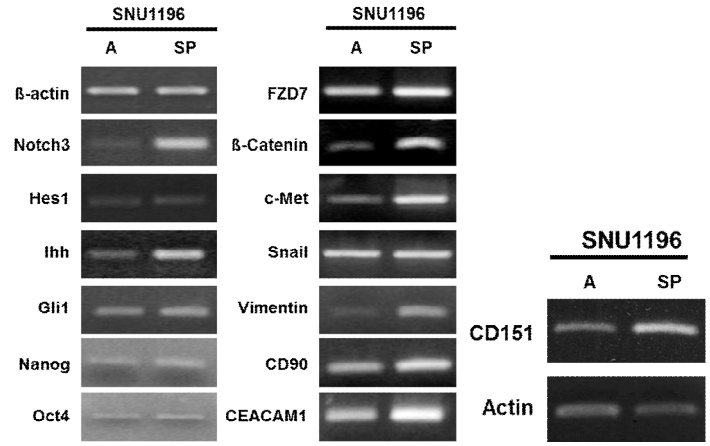
2. The expression of CD151 was higher in tumorsphere than in adherent cells of biliary carcinoma cells

Tumorsphere expressed higher mRNA levels of Notch3, Ihh, Gli1, FZD7, and β-catenin than adherent cells. Upon RT-PCR, the author confirmed that the Notch, Hedgehog, and Wnt signaling were activated in the tumorsphere. c-Met and Vimentin relating to the EMT pathway and CD90 known as liver cancer stem cell marker was also highly expressed in the tumorsphere (Figure 2-A). The

c-Met pathway relating molecules such as c-Met and pAKT were highly expressed in the tumorsphere on western blot. EMT relating molecules such as snail and N-cadherin were also highly expressed in the tumorsphere (Figure 2-B). Based on these results, the author confirmed that the tumorsphere might have cancer stem-like properties.

The expression of CD151 was higher in the tumorsphere than in the adherent cells on both RT-PCR and western blot (Figure 2-A, B).

A.



B.

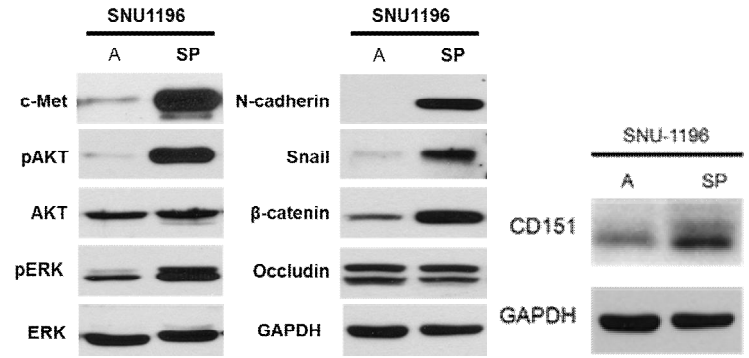
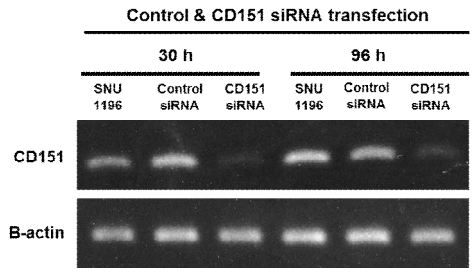


Figure 2. RT-PCR analysis (A) and western blot (B) in the adherent cells and the tumorsphere of the biliary carcinoma cell line, SNU-1196. A. Highly expression of mRNA molecules relating to Notch, Hedgehog, Wnt signaling, and EMT were seen in the tumorsphere. B. The molecules associated with c-Met and EMT pathways were highly expressed in the tumorsphere. RT-PCR and western blot showed that the expression of CD151 was higher in the tumorsphere than in the adherent cells (A; adherent cells, SP; tumorsphere).

3. CD151 knockdown was confirmed in the biliary carcinoma cell line, SNU-1196, after transfection of CD151 siRNA

To elucidate the role of CD151 in cholangiocarcinoma stem cell, CD151 knockdown was carried out by CD151 siRNA transfection. After transfection of the siRNA into SNU-1196 cells, RT-PCR analysis showed CD151 mRNA to be decreased at 30 and 96 h post-transfection. Furthermore, using western blotting, the expression of CD151 was inhibited at 48 and 72 h post-transfection (Figure 3).

A.



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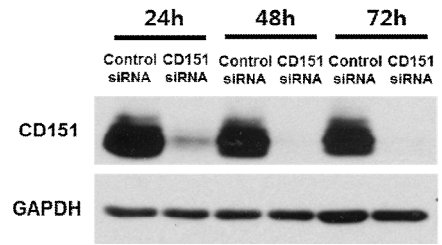


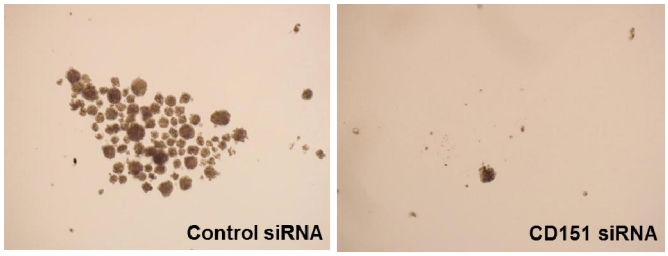
Figure 3. CD151 expression after transfection of CD151 siRNA in SNU-1196. A. RT-PCR results obtained at various post-transfection time points. The expression of CD151 mRNA was decreased at 30 and 96 h. B. Western blot of the SNU-1196 cells lysate at various post-transfection time points. The expression of CD151 was decreased at 24 h and was strongly inhibited at 48 and 72 h.

4. CD151 promoted tumorsphere formation and chemoresistance in SNU-1196

Sphere formation and cytotoxicity assay were done to evaluate the role of CD151 in the cancer stem-like cells.

Sphere formation assays were performed after transfection of control and CD151 siRNA into the SNU-1196 cell line to characterize the effects of CD151 on cancer stem-like cells. On microscopy, tumorsphere formation was still observed after transfection of control siRNA (Figure 4-A, control siRNA), but was completely inhibited after transfection of CD151 siRNA (Figure 4-A, CD151 siRNA). On counting, the number of tumorsphere was significantly decreased after CD151 knockdown (Figure 4-B). Upon these finding, the author could suspect that CD151 might affect the self-renewal property of cancer stem-like cells.

A.



B.

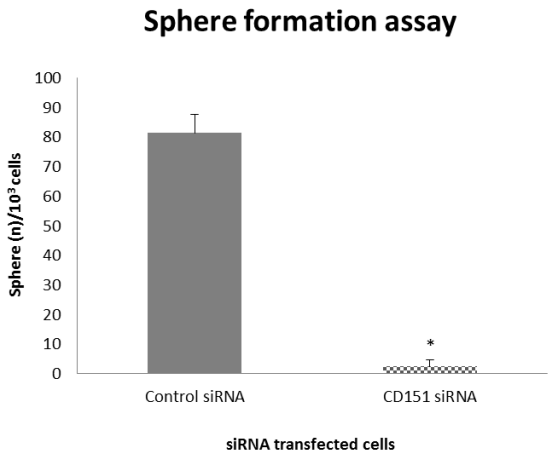


Figure 4. Sphere formation assay of SNU-1196 after transfection of control and CD151 siRNA (OLYMPUS DP71 microscope × 5 magnifications). (B) The number of tumorsphere was markedly decreased after CD151 knockdown (*$p<0.05$).

Cytotoxicity assay showed that the chemoresistance to gemcitabine was decreased after transfection of CD151 siRNA (Figure 5) into SNU-1196. As the dose of gemcitabine was increased, the number of the survived cells was more decreased in CD151 knockdown SNU-1196 cells than in the control group ($p<0.05$). CD151 might also regulate the chemoresistance in the cancer stem like cells.

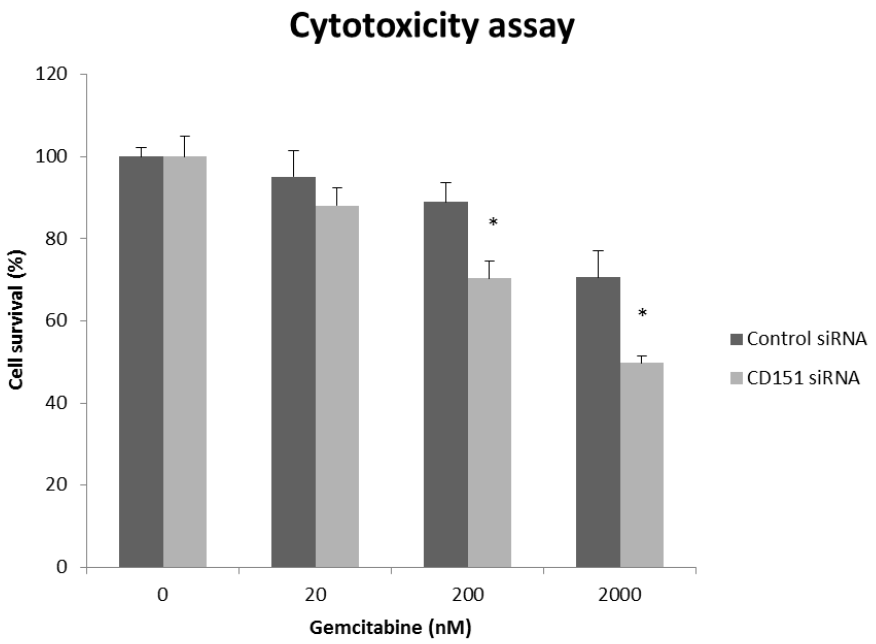


Figure 5. Cytotoxicity assay of SNU-1196 after transfection of control and CD151 siRNA. As the dose of gemcitabine was more increased, the number of survived cells was more decreased after transfection of CD151 siRNA into SNU-1196 than in the control group (*$p<0.05$). Chemoresistance was more reduced in the CD151 knockdown SNU-1196 cells.

5. CD151 interacted with c-Met, and activated PI3K and EMT pathway, and promoted β-catenin and Oct4 in SNU-1196

Previous results showed that CD151 was expressed in the cancer stem like cells and regulated the stemness and chemoresistance. In order to gain a more detailed insight into the effects of CD151 in cancer stem like cells, various

proteins were examined for their expression levels in the SNU-1196 cells by western blot at 72 h post-transfection of CD151 siRNA, based on the result of western blot in the tumorsphere. The expression of c-Met, pAKT, and pGSK3 were decreased after CD151 knockdown. Both the c-Met signaling pathway and the PIK3 pathway were affected. The expression of β-catenin and Oct4 was also decreased after CD151 knockdown. EMT pathway-associated molecules such as Snail and N-cadherin were reduced after CD151 siRNA transfection into SNU-1196 (Figure 6).

Upon these results, the author could postulate that CD151 interacted with c-Met and activated the PI3K pathway which promoted the EMT pathway and increased intra-nuclear β-catenin. Although the author does not understand the exact mechanism, increased β-catenin induced the Oct4. These molecules might be responsible for stemness, infiltration, and metastasis of cancer stem cells (Figure 6, center box).

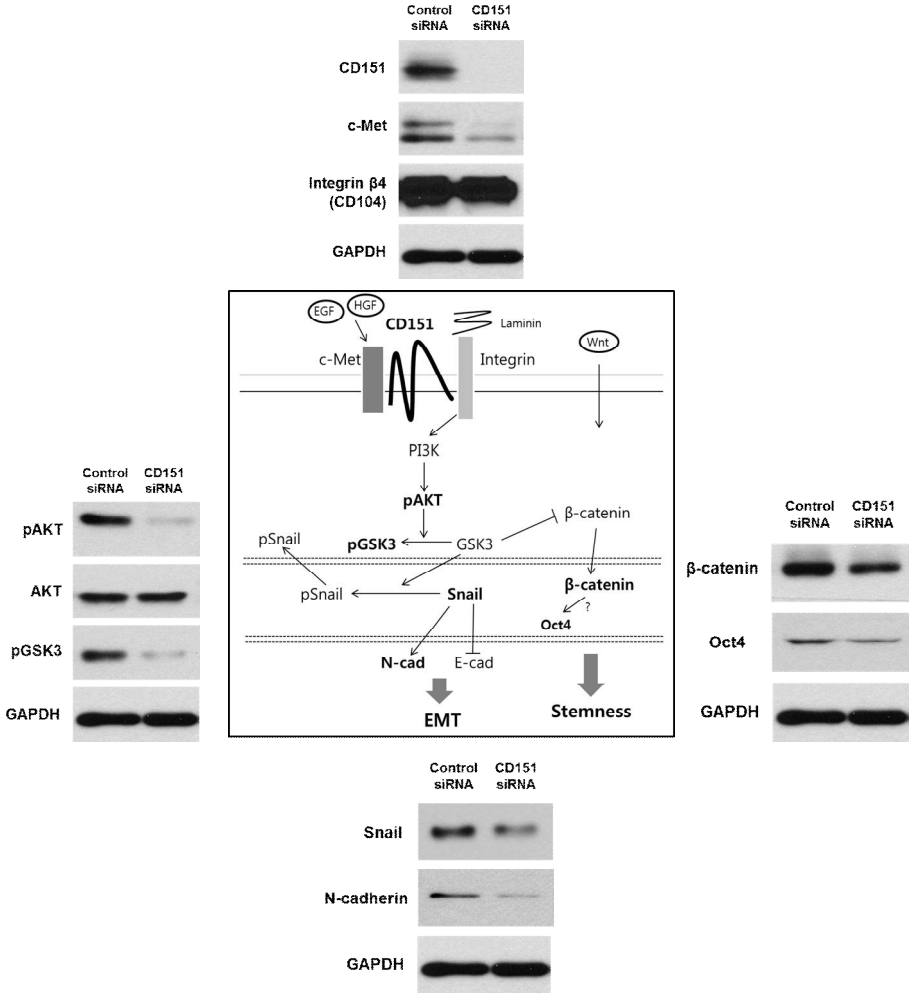


Figure 6. Summary of postulated pathway associated with CD151 and western blot assay in SNU-1196 after transfection of control and CD151 siRNA. CD151 interacts with c-Met and activate subsequent pathway; PI3K pathway. Activated PI3K pathway induced the activation of EMT pathway and promoted β-catenin and Oct4 (solid lines; cell membrane, dot lines; nuclear membrane).

6. CD151 promoted proliferation and migration of SNU-1196.

Because cancer stem cells also have proliferation and metastasis capabilities, the author evaluated proliferation and migration assay after CD151 knockdown in SNU-1196 cells.

Cellular proliferation was significantly decreased after transfection of CD151 siRNA, compared with SNU-1196 cells and transfection of control siRNA. After transfection of control siRNA, cellular proliferation was reduced by 6% than SNU-1196 cells, but by 35% in CD151 knockdown cells (Figure 7, $p<0.05$).

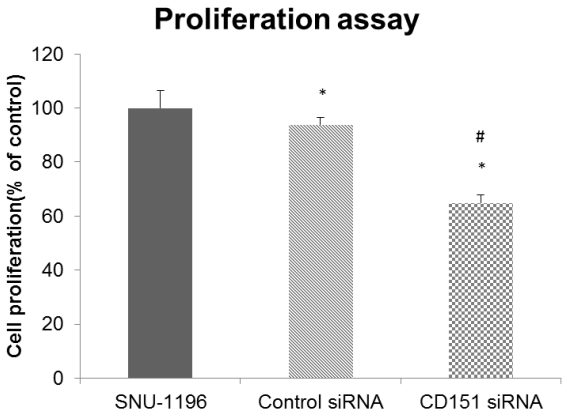


Figure 7. Proliferation assay of SNU-1196 after transfection of control and CD151 siRNA. Cell proliferation was reduced by 6% and 35% after transfection of control siRNA and CD151 siRNA respectively, compared with SNU-1196. Significance was tested by one way ANOVA test with * representing comparison with SNU-1196 and # with control siRNA with p value of <0.05.

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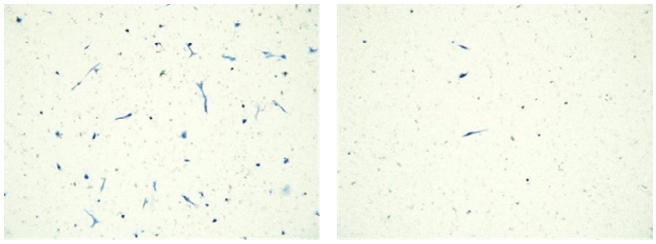
Migration assays were carried out 48 hours after transfection. The number of cells that migrated were markedly decreased after CD151 knockdown when counted under the microscope (Figure 7-A), and there was a statistically significant difference in the number of migrated cells between the control and CD151 knockdown cells (53.0±1.0 vs. 10.7±2.3 respectively, $p<0.05$, Figure 7-B).

A



B

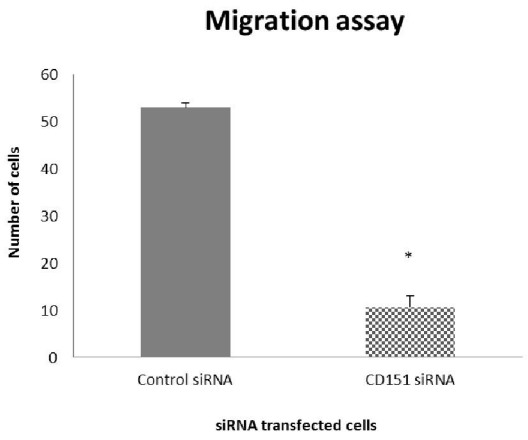


Figure 8. Migration assay of SNU-1196 after transfection of control and CD151 siRNA. A. The migrated cells were markedly decreased after CD151 knockdown. B. The number of migrated cells, defined as cells that had relocated into the lower compartment of the transwell plates. Control siRNA; 53.0±1.0 migrated cells, CD151 siRNA cells; 10.7±2.3 migrated cells, *$p<0.05$.

IV. DISCUSSION

The author used two biliary carcinoma cell lines, SNU-245 and SNU-1196 in this study. As the culture of the SNU-1196 cell line was relatively easy and it has been shown to have superior tumorsphere forming abilities and demonstrated greater stemness characteristics than the other cell lines, the author chose it for further analysis. Additionally, SNU-1196 holds mutant variants of a range of genes such as p53 and p16 and it was originally derived from Klatskin tumor of the hepatic duct and was indicative of poor prognosis.

For proving that tumorsphere has cancer stem cells properties, the author showed expression of 'stemness gene' was higher in tumorspheres than in adherent cells. Furthermore, the colony formation assay and *in vivo* tumorigenecity experiments were also performed (Data not shown). As briefly summarized, the results showed that the number of colonies was 80.3±2.1 and 149.7±4.9 in adherent cells and tumorsphere of SNU-1196 respectively ($p<0.05$). 10^3 cells from both adherent cells and tumorspheres of SNU-1196 were selected and implanted individually on the flank of nude mouse. After 12 weeks from the transplantation, mice were sacrificed to access tumor volumes. The size was 88 mm^3 and 1,440 mm^3 respectively ($p<0.05$). Based on these results, the author confirmed that the tumorsphere of SNU-1196 cells displayed a greater colony-forming efficiency, higher proliferative output, and greater tumorigenic capacity in vivo when compared with the adherent cells.

cDNA microarray was performed in order to find the commonly up-regulated genes between tumorsphere and biliary cancer. Sixty-one genes were activated in SNU-245, SNU-1196 tumorsphere, and gallbladder cancer; CD151, Transgelin 2 (TAGLN), aldehyde dehydrogenase 3 family, memberA1 (ALDH3A1), fibroblast growth factor receptor 4 (FGFR4), chemokine receptor 1 (CCR1), ATP-binding cassette, sub-family, member 1 (ABCA1),

v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (ERBB2), aldehyde dehydrogenase 4 family, and memberA1 (ALDH4A1), etc. The genes were carefully selected considering the molecules were membranous, secretory and associated with cell's development. Among them, the author gave attention to CD151 because some recent studies show that it was associated with motility, invasion, and metastasis in various carcinomas including cholangiocarcinoma, but there were hardly any studies about the role in cholangiocarcinoma stem cells.

At first, the expression of CD151 was performed also in cancer tissues (Data not shown). The stain was definitively seen on the membrane of cells in cholangiocarcinoma tumor tissue. The expression of CD151 was also seen in both adherent and tumorsphere cells. However, CD151 expression was higher in tumorsphere cells than in adherent cells. Because we proved that tumorsphere had cancer stem-like properties by RT PCR of stemness gene, the colony formation assay and *in vivo* tumorigenecity experiments, tumorsphere formation and cytotoxicy assay were investigated after CD151 knockdown to evaluate the effect of CD151 on cancer stem-like cells. Since tumorsphere was inhibited and showed reduced chemoresistance after CD 151 knockdown, the author assumed that CD151 knockdown SNU-1196 cells showed decreased cancer stem cell properties and CD151 might regulate cancer stem-like cells.

In order to reveal how CD151 controls cancer stem-like cells, the author took notice of c-Met and EMT pathways because both related proteins were more highly expressed in tumorspheres than in adherent cells (Figure 2-B). Infiltration and metastasis of cancer stem cells were also known to associate with c-Met and EMT pathway.

CD151 was already known to make complex with integrin families and c-Met. For evaluation of immune complex with CD151, immunoprecipitation test was done. After immunoprecipitation was achieved by anti-CD151

antibody, integrin β4 and c-Met were expressed on the western blot (Data not shown).

In cancer cells, activation of c-Met results in cell growth, differentiation, infiltration, and apoptosis via various signal pathways. RAS-MAPK, PI3K-AKT, and STAT activity have been associated with c-Met activation.[38,39] The Wnt/β-catenin pathway was also activated by interactions with HGF/c-Met complex.[40] Overexpression of c-Met has been demonstrated to be related to poor prognosis in various cancers.[39] Interestingly, a c-Met has been exhibited to be a pancreas stem cell marker in normal mouse tissue[41] and has also been presented as a pancreatic cancer stem cell marker.[42] As overexpression of c-Met and abnormal activation of c-Met/HGF has been seen in the benign proliferation of biliary epithelial cell and bile duct cancer, it was suspected as cause.[43-45] Overexpression of c-Met is also linked to expression of EGFR and associated with poor prognosis in biliary cancer.[46]

In this study, expression of c-Met, pAKT, and pGSK3 was decreased after CD151 knockdown. EMT related molecules were also affected and the mesenchymal marker, expression of SNAIL and N-cadherin, was decreased after CD151 knockdown. Interestingly, the expression of molecules related to the RAS/MAPK pathway was not changed after transfection of CD151 siRNA (Data not shown). The author could guess that CD151 regulates cholangiocarcinoma stem-like cells by c-Met and PI3K-AKT pathway. Expression of β-catenin and embryonic stem cell marker, Oct4 was also decreased after CD151 knockdown. A β-catenin and Oct4 were already known to be associated with cancer stem cells. As one study suggested that Wnt/β-catenin regulated lung cancer stem cell through Oct4 in lung cancer A549 cells,[47] the author could presume that CD151 might control cholangiocarcinoma stem-like cells through β-catenin and Oct4. As another, a different study showed that Oct4 controlled colorectal cancer metastasis through EMT pathway,[48] the result of EMT related molecules and Oct4 in this

study was notable. Although the author could not explain the exact function and interaction of these molecules, the author could guess CD151 regulate cholangiocarcinoma stem-like cells through c-Met, PI3K, EMT pathway, β-catenin and Oct4.

For the evaluation of the effect of CD151 on the proliferation and metastasis of cholangiocarcinoma, proliferation and migration assay were performed after CD151 knockdown. As expected, proliferation and migration was significantly decreased after transfection of CD151 siRNA into SNU-1196. Interestingly, one study shows that CD151 did not affect the proliferation of prostate cancer cells and promoted migration and invasion only.[49] However, the results of this study showed that CD151 might regulate the proliferation and metastasis of cholangiocarcinoma.

In summary, CD151 was highly expressed in cancer stem-like cells of cholangiocarcinoma and the cancer stem cell properties such as self-renewal and chemoresistance were weakened when CD151 was knocked down. The related pathway and molecules between CD151 and cholangiocarcinoma stem-like cells might be c-Met, PI3K, EMT pathway, β-catenin and Oct4, and CD151 might control the proliferation and metastasis of cholangiocarcinoma.

V. CONCLUSION

This study demonstrates that CD151 was more expressed in cholangiocarcinoma stem-like cells and regulated self-renewal, chemoresistance, proliferation and metastatic capabilities. It interacted with c-Met, PI3K/AKT, EMT pathway, β-catenin, and Oct4, and resulted in having cancer stem cell-like properties. Therefore, CD151 should be considered as a potential molecular marker of cholangiocarcinoma stem cells. It may be expected to be a therapeutic target of cholangiocarcinoma stem cells in the future. Further studies towards this end are required.

REFERENCES

1. Steiner PE, Higginson J. Cholangiolocellular carcinoma of the liver. Cancer 1959;12:753-9.

2. Morise Z, Sugioka A, Hoshimoto S, Kato T, Tanahashi Y, Ikeda M, et al. Patient with advanced intrahepatic cholangiocarcinoma with long-term survival successfully treated with a combination of surgery and chemotherapy. J Hepatobiliary Pancreat Surg 2008;15:545-8.

3. Igami T, Nishio H, Ebata T, Yokoyama Y, Sugawara G, Nimura Y, et al. Surgical treatment of hilar cholangiocarcinoma in the "new era": the Nagoya University experience. J Hepatobiliary Pancreat Sci 2010;17:449-54.

4. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730-7.

5. Alison MR, Lim SM, Nicholson LJ. Cancer stem cells: problems for therapy? J Pathol 2011;223:148-62.

6. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-11.

7. Sell S, Dunsford H. Evidence for the stem cell origin of hepatocellular carcinoma and cholangiocarcinoma. Am J Pathol 1989;134:1347-63.

8. Chiba T, Zheng YW, Kita K, Yokosuka O, Saisho H, Onodera M, et al. Enhanced self-renewal capability in hepatic stem/progenitor cells drives cancer initiation. Gastroenterology 2007;133:937-50.

9. Fan L, He F, Liu H, Zhu J, Liu Y, Yin Z, et al. CD133: a potential indicator for differentiation and prognosis of human cholangiocarcinoma. BMC cancer 2011;11:320.

10. Wang M, Xiao J, Jiang J, Qin R. CD133 and ALDH may be the molecular markers of cholangiocarcinoma stem cells. Int J Cancer 2011;128:1996-7.

11. Shimada M, Sugimoto K, Iwahashi S, Utsunomiya T, Morine Y, Imura S, et

al. CD133 expression is a potential prognostic indicator in intrahepatic cholangiocarcinoma. J Gastroenterol 2010;45:896-902.

12. Leelawat K, Thongtawee T, Narong S, Subwongcharoen S, Treepongkaruna S. Strong expression of CD133 is associated with increased cholangiocarcinoma progression. World J Gastroenterol 2011;17:1192-8.

13. Agrawal S, Kuvshinoff BW, Khoury T, Yu J, Javle MM, LeVea C, et al. CD24 expression is an independent prognostic marker in cholangiocarcinoma. J Gastrointest Surg 2007;11:445-51.

14. Su MC, Hsu C, Kao HL, Jeng YM. CD24 expression is a prognostic factor in intrahepatic cholangiocarcinoma. Cancer Lett 2006;235:34-9..

15. Keeratichamroen S, Leelawat K, Thongtawee T, Narong S, Aegem U, Tujinda S, et al. Expression of CD24 in cholangiocarcinoma cells is associated with disease progression and reduced patient survival. Int J Oncol 2011;39:873-81.

16. Boer CJ, Krieken JH, Janssen-van Rhijn CM, Litvinov SV. Expression of Ep-CAM in normal, regenerating, metaplastic, and neoplastic liver. J Pathol 1999;188:201-6.

17. Maecker H, Todd S, Levy S. The tetraspanin superfamily: molecular facilitators. FASEB J 1997;11:428-42.

18. Sincock PM, Mayrhofer G, Ashman LK. Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: comparison with CD9, CD63, and alpha5beta1 integrin. J Histochem Cytochem 1997;45:515-25.

19. Zöller M. Tetraspanins: push and pull in suppressing and promoting metastasis. Nat Rev Cancer 2008;9:40-55.

20. Kohno M, Hasegawa H, Miyake M, Yamamoto T, Fujita S. CD151 enhances cell motility and metastasis of cancer cells in the presence of focal adhesion kinase. Int J Cancer 2002;97:336-43.

21. Ke AW, Shi GM, Zhou J, Wu FZ, Ding ZB, Hu MY, et al. Role of

overexpression of CD151 and/or c-Met in predicting prognosis of hepatocellular carcinoma. Hepatology 2009;49:491-503.

22. Tokuhara T, Hasegawa H, Hattori N, Ishida H, Taki T, Tachibana S, et al. Clinical significance of CD151 gene expression in non-small cell lung cancer. Clin Cancer Res 2001;7:4109-14.

23. Hashida H, Takabayashi A, Tokuhara T, Hattori N, Taki T, Hasegawa H, et al. Clinical significance of transmembrane 4 superfamily in colon cancer. Br J Cancer 2003;89:158-67.

24. Zhu GH, Huang C, Qiu ZJ, Liu J, Zhang ZH, Zhao N, et al. Expression and prognostic significance of CD151, c-Met, and integrin alpha3/alpha6 in pancreatic ductal adenocarcinoma. Dig Dis Sci 2011;56:1090-8.

25. Suzuki S. Prognostic significance of CD151 expression in esophageal squamous cell carcinoma with aggressive cell proliferation and invasiveness. Ann Surg Oncol 2011;18:888-93.

26. Kwon M, Park S, Choi J, Oh E, Kim Y, Park Y, et al. Clinical significance of CD151 overexpression in subtypes of invasive breast cancer. Br J Cancer 2012;106:923-30.

27. Voss M, Gordon N, Maloney S, Ganesan R, Ludeman L, McCarthy K, et al. Tetraspanin CD151 is a novel prognostic marker in poor outcome endometrial cancer. Br J Cancer 2011;104:1611-18.

28. Shi GM, Ke AW, Zhou J, Wang XY, Xu Y, Ding ZB, et al. CD151 modulates expression of matrix metalloproteinase 9 and promotes neoangiogenesis and progression of hepatocellular carcinoma. Hepatology 2010;52:183-96.

29. Yang YM, Zhang ZW, Liu QM, Sun YF, Yu JR, Xu WX. Overexpression of CD151 predicts prognosis in patients with resected gastric cancer. PLoS One 2013;8:e58990.

30. Huang XY, Ke AW, Shi GM, Ding ZB, Devbhandari RP, Gu FM, et al. Overexpression of CD151 as an adverse marker for intrahepatic

cholangiocarcinoma patients. Cancer 2010;116:5440-51.

31. Sterk LM, Geuijen CA, Berg JG, Claessen N, Weening JJ, Sonnenberg A. Association of the tetraspanin CD151 with the laminin-binding integrins α 3β1, α6β1, α6β4 and α7β1 in cells in culture and in vivo. J Cell Sci 2002;115:1161-73.

32. Winterwood NE, Varzavand A, Meland MN, Ashman LK, Stipp CS. A critical role for tetraspanin CD151 in α3β1 and α6β4 integrin–dependent tumor cell functions on laminin-5. Mol Biol Cell 2006;17:2707-21.

33. Fei Y, Wang J, Liu W, Zuo H, Qin J, Wang D, et al. CD151 promotes cancer cell metastasis via integrins α3β1 and α6β1 in vitro. Mol Med Rep 2012;6:1226-30.

34. Yang W, Li P, Lin J, Zuo H, Zuo P, Zou Y, et al. CD151 promotes proliferation and migration of PC3 cells via the formation of CD151-integrin α3/α6 complex. J Huazhong Univ Sci Technolog Med Sci 2012;32:383-8.

35. Franco M, Muratori C, Corso S, Tenaglia E, Bertotti A, Capparuccia L, et al. The tetraspanin CD151 is required for Met-dependent signaling and tumor cell growth. J Biol Chem 2010;285:38756-64.

36. Ke AW, Shi GM, Zhou J, Huang XY, Shi YH, Ding ZB, et al. CD151 amplifies signaling by integrin α6β1 to PI3K and induces the epithelial–mesenchymal transition in HCC Cells. Gastroenterology 2011;140:1629-41.

37. Jung DE, Wen J, Oh T, Song SY. Differentially expressed microRNAs in pancreatic cancer stem cells. Pancreas 2011;40:1180-87.

38. Boccaccio C, Comoglio PM. Invasive growth: a *MET*-driven genetic programme for cancer and stem cells. Nat Rev Cancer 2006;6:637-45.

39. Eder JP, Wounde GFV, Boerner SA, LoRusso PM. Novel therapeutic inhibitors of c-MET signaling pathway in cancer. Clin Cancer Res 2009;15(7):2207-14.

40. Huang FI, Chen YL, Channg CN, Yuan RH, Jeng YM. Hepatocyte growth

factor activates Wnt pathway by transcriptional activation of LEF1 to facilitate tumor invasion. Carcinogenesis 2012;33(6):1142-1148.

41. Oshima Y, Suzuki A, Kawashimo K, Ishikawa M, Ohkohchi N, Taniguchi H. Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-Met by flow cytometric cell sorting. Gastroenterology 2007;132:720-32. 48

42. Li C, Wu JJ, Hynes M, Dosch J, Sarkar B, Welling TH, et al. c-MET is a marker of pancreatic cancer stem cells and therapeutic target. Gastroenterology 2011;141:2218-27.

43. Varnholt H, Asayama Y, Aishima S, Tagucji K, Sugimachi K, Tsuneyoshi M. c-Met and hepatocyte growth factor expression in combined hepatocellular carcinoma and cholangiocrcinoma. Oncol Rep 2002;9:35-41.

44. Terada T, Nakanuma Y, Sirica AE. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and in hepatolithiasis. Hum Pathol 1998;29:175-80.

45. Sanada Y, Osada S, Tokuyama Y, Tanaka Y, Takahashi T, Yamaguchi K. Critical role of c-Met and Ki67 in progress of biliary carcinoma. Am Surg 2010;76(4):372-9.

46. Miyamoto M, Ojima H, Iwasaki M, Shimizu H, Kokubu A, Hiraoka N, et al. Prognostic significance of overexpression of c-MET oncoprotein in cholangiocarcinoma. Bri J Cancer 2011;105:131-8.

47. Teng Y, Wang X, Wang Y, Ma D. Wnt/β-catenin signaling regulates cancer stem cells in lung cancer A549 cells. Biochem Biophys Res Commun 2010;392:373-9.

48. Dai X, Ge J, Wang X, Qian X, Zhang C, Li X. OCT4 regulates epithelial-mesenchymal transition and its knockdown inhibits colorectal cancer cell migration and invasion. Oncol Rep 2013;29:155-60.

49. Ang J, Fang BL, Ashman LK, Frauman AG. The migration and invasion of human prostate cancer cell lines involves CD151 expression. Oncol Rep 2010;24:1593-97.

ABSTRACT(IN KOREAN)

담도암 줄기 세포의 분자표지자로서 가능성 있는 CD151

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최근 암줄기 세포가 다양한 암에서 종양형성, 전이, 재발, 항암제내성을 일으킨다고 가정하고 있다.

CD151은 transmembrane 4 superfamily의 하나로 세포의 이동뿐 아니라 암세포의 침습이나 전이에 관여한다. 간내 담도암에서 CD151과 c-Met의 과발현이 암의 침습과 전이와 연관이 있어 불량 예후를 보인다는 연구와 같이 담도암에서 CD151의 역할에 대한 연구는 있었으나 담도암 줄기세포에서의 역할에 대한 연구는 현재 없다. 이에 본 연구에서는 CD151의 담도암 줄기세포에서 역할을 대해서 알아보고자 하였다.

담도암 줄기세포에 대한 평가를 위해 본 연구에서는 2개의 담도암 세포주(SNU-245, SNU-1196)를 이용하여 tumorsphere를 배양하여 담도암 줄기 양 세포를 얻었다. cDNA microarray를 이용하여 tumorsphere와 담도암에서 과발현된 유전자를 얻었다. Tumorsphere가 줄기세포의 특성을 갖는 지를 보기 위해 RT-PCR과 western blot을 통하여 줄기세포 관련 mRNA와

c-Met과 EMT 관련 단백질을 확인하였다. CD151의 발현을 SNU-1196 세포주의 부착 세포와 tumorsphere에서 비교하였다. CD151의 담도암 줄기 세포에서의 역할을 확인하기 위하여 SNU-1196을 CD151 siRNA로 처리하여 CD151 발현을 억제한 후에 sphere formation assay, cytotoxicity assay를 시행하였다. 또한 c-Met, EMT pathway 관련 인자 및 β-catenin, Oct4의 발현을 western blot을 통하여 확인하였으며 proliferation assay 및 migration assay를 시행하였다.

결과적으로 cDNA microarray에서 CD151이 tumorsphere와 담도암 모두에서 과발현됨을 알 수 있었다. 배양된 tumor sphere는 RT-PCR에서 줄기세포와 관련된 Notch, Hedgehog, Wnt 관련 인자들이 과발현되고 있고, western blot에서 c-Met, PI3K, EMT 관련 인자, β-catenin, Oct4의 발현이 증가되어 있어서 암줄기 세포의 특성을 가지고 있었다. CD151은 부착 세포보다 tumorsphere에서 발현이 더 증가되어 있었다. CD151의 발현을 억제하는 경우 tumorsphere 형성이나 chemoresistance가 감소하는 것으로 보아 CD151을 억제하는 경우 암줄기 세포의 특성이 사라짐을 볼 수 있었다. CD151을 SNU-1196 세포주에서 억제한 후 c-Met, PI3K, EMT 관련 인자, β-catenin, Oct4 모두 발현이 감소하는 것으로 볼 때 CD151은 이들을 통해서 작용을 함을 생각해 볼 수 있었다. 또한 CD151 억제 시 proliferation과 migration이 모두 감소하는 것을 확인할 수 있었다.

결론적으로 CD151은 담도암줄기 양 세포에서 발현이 증가되어 있었으며 자가재생, 항암제내성, 증식, 전이능력을

조절하였다. c-Met과 상호 작용을 하여 PI3K 및 EMT pathway, β-catenin, Oct4를 활성화시켜 작용을 하는 것으로 생각된다.

따라서, CD151은 담도암 줄기세포와 관련된 인자로 생각되며 지며 향후 진단적 혹은 치료적 표지자로 이용될 수 있는 중요한 인자로 기대해 볼 수 있겠다.

핵심되는 말: CD151, 담도암 줄기세포, c-Met