Jagged1 DNA Copy Number Variation Is Associated with Poor Outcome in Liver Cancer

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Short title; Jagged1 genomic amplification in liver cancer

Kazunori Kawaguchi, Masao Honda, Taro Yamashita, Hikari Okada, Takayoshi Shirasaki, Masashi Nishikawa, Kouki Nio, Kuniaki Arai, Yoshio Sakai, Tatsuya Yamashita, Eishiro Mizukoshi and Shuichi Kaneko

Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

Address correspondence to Shuichi Kaneko M.D., Ph.D.,
Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa, Ishikawa 920-8641, Japan
Phone: +81-76-265-2233, Fax: +81-76-234-4250
Email: skaneko@m-kanazawa.jp

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Abstract

Notch signaling abnormalities are reported to be involved in the acceleration of malignancy in solid tumors and stem cell formation or regeneration in various organs. We analyzed specific genes for DNA copy number variations in liver cancer cells and investigated whether these factors relate to clinical outcome. Chromosome 20p, which includes the ligand for Notch pathways, Jagged1, was found to be amplified in several types of hepatoma cells and its mRNA was upregulated according to α-fetoprotein gene expression levels. Notch inhibition using Jagged1 shRNA and γ-secretase inhibitors produced significant suppression of cell growth in α-fetoprotein-producing cells with suppression of downstream genes. Using *in vivo* hepatoma models, the administration of γ-secretase inhibitors resulted in reduced tumor sizes and effective Notch inhibition with widespread apoptosis and necrosis of viable tumor cells. The γ-secretase inhibitors suppressed cell growth of the EpCAM+ fraction in hepatoma cells, indicating that Notch inhibitors could suppress the stem cell features of liver cancer cells. Even in clinical liver cancer samples, the expression of α-fetoprotein and Jagged1 showed significant correlation, and amplification of the copy number of Jagged1 was associated with Jagged1

mRNA expression and poor survival after liver cancer surgical resection. In conclusion, amplification of Jagged1 contributed to mRNA expression that activates the Jagged1-Notch signaling pathway in liver cancer and led to poor outcome.

Introduction

Globally, liver cancer has one of the poorest prognoses of all cancers [1]. Many liver cancer patients experience recurrence after effective treatments, including surgical resection at stage I, leading to high mortality rates. The etiologies of liver cancer include hepatitis B virus (HBV) and hepatitis C (HCV) infection, any other chronic liver injury such as primary biliary cirrhosis or autoimmune hepatitis, metabolic diseases such as non-alcoholic steatohepatitis or diabetes, and exposure to aflatoxin, a harmful chemical substance. Although the direct oncogenic factor of HCV-related liver cancer has not yet been discovered, HBV is reported to be associated with oncogenic factors of viral infection, including HBV-x genome. Moreover, a recently developed effective antivirus therapy for both HCV and HBV, using direct-acting agents in HCV or nucleos(t)ide analogues in HBV, has led to some patients developing liver cancer without developing advanced liver disease like cirrhosis. However, this oncogenic mechanism has not been resolved completely even in HBV and could become a major problem in the near future. Serological tests are performed to diagnose liver cancer, to determine the appropriate therapies, and to monitor their effects. The most important serological tumor markers are α-fetoprotein (AFP) or PIVKA-II. AFP is also associated with angiogenesis, and tumor cell growth,

invasion, and metastasis. From hierarchical expression analysis using microarray, ephrin family genes have been found to be linked to AFP elevation or angiogenesis [2]. Serum AFP elevation or overexpression in the tissue can be seen in liver cancer samples; however, other proteins have also been discovered. For example, glypican 3 is significantly elevated and EpCAM plays an important role in liver cancer progenitor cells [3-5]. Recent bioinformatics techniques have been applied to find proteins related to abnormal cellular signaling in liver cancer samples. We examined gene expression patterns using RNA samples of liver cancer tissue, including clinical samples, in addition to hepatoma cell lines or hepatoma cells in experimental small animals. At the DNA level, genomic alterations such as single nucleotide polymorphisms, loss of heterogeneity, or copy number amplifications or deletions were found and these resulted in specific changes in liver cancer samples [6, 7]. Southern blotting, FISH (fluorescence *in-situ* hybridization), and CGH (comparative genomic hybridization) have been used in cytogenetic studies. Recent progress in bioinformatics techniques has led to the discovery of specific genomic alterations in solid tumors such as tumors of the breast, colon, and pancreas, and these have been useful for diagnostics and in the selection of therapy. The

same techniques have also resulted in the identification of specific genomic alterations in liver cancer. Altered genomic patterns in liver cancer influence patient mortality. A specific genomic location altered in liver cancer samples was reported to be associated with carcinogenesis or mitosis of the tumor cells. Previously, we reported that genomic alteration patterns affect the expression patterns in hepatoma cell lines and are associated with AFP production [8]. Referring to related genes on microarray, we could estimate the types of genes involved and the candidate cell-cell interaction genes. Since it was important to determine whether a specific gene could be involved with a possible molecular therapeutic target or one of the pathways in a molecular target in clinical samples, we used microarray results to detect genomic abnormalities.

Materials and Methods

Hepatoma and non-hepatoma cell lines

For genomic and gene expression analysis, we selected seven hepatoma cell lines: Huh7, HepG2, Hep3B, Huh6, and PLC/PRF/5, all of which produce AFP, and two cell lines, HLE and SKHep1, which do not. Human cervical cancer cell

line HeLa and bile duct cancer cell line KMBC, neither of which produce AFP, were also analyzed as non-hepatoma cells. THLE-5b cells, a normal hepatocyte immortalized by using SV40, was selected for normal gene expression control for real-time detection polymerase chain reaction (RTD-PCR) gene expression analysis [9].

Clinical liver samples (frozen liver cancer samples)

Genetic analysis of frozen tumor samples from 38 liver cancer patients was conducted using RTD-PCR. Most samples were collected during surgery. The patients included 17 who had HBV-related liver cancer and 18 who had HCV-related liver cancer; the remainder had other etiologies. We observed AFP expression in hepatoma tissue samples and it was upregulated in around 37% of samples (6 HBV, 6 HCV, 2 non-B and non-C). Surgery was performed on patients diagnosed from October 2000 to September 2006 at Kanazawa University Hospital. All patients gave informed consent for their tissues to be used for genomic analysis. By isolating genomic DNA from eight patients with elevated AFP expression (one sample was from an autopsy liver), we observed genomic copy number changes using RTD-PCR by comparing the samples with

non-cancerous tissues.

Clinical liver samples

Formalin-fixed paraffin-embedded (FFPE) samples from 115 liver cancer patients were selected and genomic DNA was isolated by checking the tumor area under a microscope using similar H-E stained slices and scraping off tumor cells from samples on the glass slide. Genomic DNA isolation was performed using Min-elute FFPE kit (Qiagen, Venlo, Netherlands). Surgery had been performed in patients diagnosed from 2004 to 2010 who gave consent for the genetic analysis of tissues. Of these samples, 31 were HBV-related liver cancer, 54 were HCV-related, 1 was both HBV and HCV infected, and 29 were non-HBV and non-HCV.

BAC array hybridization and comparison with in-house cDNA chip

Seven hepatoma cell lines and two non-hepatoma cell lines were used for BAC microarray CGH. Manufactured microarray slides contained 1440 BAC clones, which covered most major chromosome regions (Macrogen, Seoul, South Korea). Genomic DNA (0.5 μg) from tumor cells was labeled by Cy5-dUTP (GE

Healthcare Life Sci., Uppsala, Sweden) and the same amount of control genomic DNA from peripheral blood mononuclear cells (PBMCs) of healthy volunteers was labeled by Cy3-dUTP (GE) and hybridized onto slides at 37 °C overnight. The microarray technique was similar to the conventional method. A microarray scanner was used to calculate the intensity of each dye, and we analyzed the BAC clone spots in each chromosomal region using MAC Viewer™ (Macrogen) and viewed the log ratio chart. We compared the expression profiles from the results of the cDNA in-house microarray (Kanazawa chip Ver 2.1, 9600 spots) and analyzed the same cell line samples [8]. These BAC array CGH datasets have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/); GEO Series accession number GSE77224.

Real-time detection PCR of Notch-related genes

RTD-PCR was performed using cDNA from clinical samples or cell lines to measure the expression of Jagged1 and AFP. We also checked the expression levels of other Notch-related genes, Notch1 and HES1. We measured the genomic copy number variation (CNV) of Jagged1 using RTD-PCR for genomic DNA samples. We used master mix reagent for RTD-PCR (Life Technologies,

Carlsbad, CA) and performed amplification using ABI7900 (Life Technologies). For CNV analysis, we used two different areas of the Jagged1 probe, and CNV calculation was performed using CopyCaller^{TM} software (Life Technologies).

Cell growth assay after Jagged1 shRNA silencing

We measured cell line growth after Jagged1 expression inhibition using shRNA vector (MISSION shRNA, Sigma-Aldrich St. Louis, MO). Cell growth for up to 120 h in Huh7, HepG2, HLE, and SKHep1 cells was compared using Jagged1 and a non-targeting negative control after infection by these lentivirus transfected vectors. After puromycin selection, we compared cell growth using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Notch inhibition by Notch inhibitors (γ-secretase inhibitors)

To disrupt Notch signaling in hepatoma cells, we used two Notch inhibitors, L-685,485 and DAPT ((3,5-Difluorophenylacetyl)-Ala-Phg-OBu^{t}) (Peptide Institute, Inc., Minou, Japan). These drugs, γ-secretase inhibitors (GSI), are administered to prevent amyloid beta accumulation and have been used in clinical trials in Alzheimer patients. In addition to Alzheimer's research, many

trials are ongoing for solid malignant tumors, such as breast cancers, lung cancer, and colorectal cancers [10, 11]. L-685,458 strongly inhibits amyloid beta, thus it is widely used in basic research, while DAPT has similar inhibitory effects but needs a greater concentration to inhibit signal peptide peptidase (SPP) [12, 13]. We observed cell proliferation in four hepatoma cell lines (Huh7, HepG2, HLE, and SKHep1) after administrating 10 μM of these drugs. We measured data from five wells for each sample and calculated absorption to measure cellular growth. We changed the medium at 24, 48, 72, 96, and 120 h and checked cell growth at 0, 72, 120, and 168 h (Huh7 and SKHep1), or 0, 48, 96, and 168 h (HepG2 and HLE). The reason for varying the measurement times was that cellular growth differed depending on cell lines and we could not estimate the confluent state for these cell sets. Moreover, we checked the expression of the downstream gene, HES1, in Huh7 and HLE by RTD-PCR.

Cell separation of EpCAM+ and EpCAM- Huh7 cells

Since Huh7 cells include the heterogenic EpCAM+ stem cell feature and this stem cell population is associated with the malignant characteristics of cells, we separated EpCAM+ and EpCAM- fractions using FACS cell sorter (FACS Aria III,

BD, Franklin Lakes, NJ). Cells were spread onto culture dishes after sorting and incubated at 37 °C in 5% CO_2. After several passages, we administrated 10 μM of L-685,458 and DAPT. Relative cellular growth rates were analyzed using Cell Counting Kit-8.

GSI treatment for NOD-SCID mouse hepatoma models

Seven-week-old NOD-SCID mice (NOD.CB17-Prkdc<SCID>/J, male) (Charles River Laboratories, Wilmington, MA) were given subcutaneous injections of 1 x 10^5 of Huh7 cells. Percutaneous administration of 5 mg/kg doses of L-685,458 and 20 mg/kg doses of DAPT dissolved in DMSO, or DMSO alone (control) was started. To compare tumor size, we injected these drugs three times per week, sacrificed the mice after 2 weeks, and measured the hepatomas. To assess survival, we injected drugs twice a week and observed the mice for up to 6 weeks after the injections. Thereafter, we sacrificed the remaining live mice, and applied immunofluorescence. Several specific polyclonal antibodies were added to paraffin-embedded tumor samples before staining with DAB and hematoxylin. We calculated the CK19 stained areas in the cells and compared the control and GSIs-treated mouse tumor samples using Image J software (NIH).

Results

20p genomic amplification of hepatoma cell lines using array-based CGH

We analyzed the genomic amplification and deletion of hepatoma cells using BAC microarray CGH. Five of the cell types produce AFP (Huh7, Hep3B, Huh6, PLC/PRF/5, HepG2) while two do not (SKHep1 and HLE). The genomic DNA of these cells was previously analyzed using cDNA microarray CGH, and it was found that AFP production is associated with a different genomic cluster of cells [8]. A similar result was observed in cDNA and BAC array CGH but some results were different. This was because the target DNA on the array slide was different and exons but not introns were present, in contrast to genomic DNA, thus the intron sequence was skipped. Moreover, the length of the cDNA was shorter than the BAC clone. The genomic DNA was amplified sequentially in some chromosome locations and deleted in others. The 20p position was particularly amplified compared with other chromosome locations, although we found other genomic alterations at other locations (Figure 1A). Since we have already reported that AFP-positive and -negative hepatoma cells can differentiate between genomic amplification and expression, we mixed the data of each spot

from the BAC array and calculated the average AFP-positive and -negative characteristics (Figure 1B). The copy number differed at other chromosomal locations based on AFP-producing status—these differences were strong, especially in the 20p location.

Amplification and overexpression of Jagged1 gene in AFP-positive hepatoma cells

We selected genes in each of the BAC clones to focus on 20p in the BAC microarray (Table 1). Although not all spots in the BAC array expressed genes, several were found during the analysis. From 9800 in-house genes of cDNA microarray, several were located in the 20p area (underlined genes) and we compared the average expression ratio in AFP-positive and -negative cell lines for some genes. We found that the Jagged1 gene showed a distinct pattern (Table 1, Figure 1A). The underlined genes in Table 1 are listed on the in-house 9800 microarray. The expression of other genes was not upregulated in AFP-positive cells. Using cDNA microarray, we previously showed that AFP-positive and -negative cells had differential gene expression and genomic alterations [5]. We focused on the Jagged1 gene and analyzed its role in

hepatoma cells or clinical samples. Using the RTD-PCR method, Jagged1 genes showed distinct upregulation in AFP-positive cell lines and downregulation in AFP-negative cell lines (Figure 1C). Furthermore, the data showed that Jagged1 gene was not upregulated in non-hematoma cell lines such as HeLa. We analyzed alterations in the Jagged1 copy number using RTD-PCR and found that most of the cell lines showed similar expression, except HLE cells (Figure 1D).

Cell growth suppression in AFP-producing hepatoma cells after Jagged1 shRNA silencing

Strong suppression of cell growth in Huh7 and HepG2 cell lines was found after shRNA silencing of Jagged1 gene (Figure 2). However, AFP-negative cells, HLE and SKHep1, showed no growth suppression. We also performed Jagged1 siRNA knock-down and evaluated cell growth; however, no significant suppression was observed, even in Huh7 and HepG2 cells (data not shown). RTD-PCR confirmed that downstream HES1 gene expression was suppressed in Huh7 for Jagged1 siRNA (Supplement Figure 1). The reason for this result may be because the induction of Jagged1 knockdown by siRNA was not efficient

and successful transfection might only have occurred in a small number of cells.

Effect of Notch signal inhibition of hepatoma cells by γ-secretase inhibitors

Two types of γ-secretase inhibitors, L-685,458 and DAPT, were used for the cellular reaction of Notch pathway inhibition among hepatoma cell lines. L-685,458 and DAPT diluted with DMSO were added to cells and cell proliferation was compared with the control (DMSO alone). Inhibition occurred in Huh7 and HepG2, which are AFP-producing cell lines, whereas there was no significant inhibition in non-AFP-producing cell lines, HLE and SKHep1 (Figure 3A). We calculated IC50 values for these cells and the values of L-685,458 treated groups were smaller than those treated with DAPT and were smaller in Huh7 cells than in other cells (Table 2). Notch-related gene expression after 120 h showed that HES1 expression was suppressed using the two types of GSI in both Huh7 and HLE (Figure 3B). However, other Notch signaling-related genes, Jagged1, Notch1, and Notch2, were not altered apart from Notch1 expression after DAPT treatment in HLE, suggesting that only downstream genes are affected by GSIs.

Effectiveness of γ-secretase inhibitors in EpCAM+ cancer stem cells

Notch signaling plays a role in the functions of stem cells and EpCAM+ is strongly associated with cancer stem cells in hepatomas. Cell growth was compared after GSI treatment in EpCAM+ and EpCAM- Huh7 cell fractions after separation using a cell sorter. We observed significant cell growth suppression in EpCAM+ cells with DAPT, and L-685,458 suppressed both EpCAM+ and EpCAM- features, suggesting that GSI have anti-tumor effects, even in hepatoma cells with the EpCAM+ cancer stem cell feature (Figure 3C).

Anti-tumor effect of Notch inhibitors in mouse hepatoma models

Slower tumor formation was observed after 2-week administration of Notch inhibitors, L-685,458 and DAPT (Figure 4A). The effect was more pronounced for DAPT (p=0.04) and after a longer period of observation, there was a tendency for earlier death in control cases (Figure 4B). The dynamic tumor status in GSI cases after longer administration showed tumor necrosis and apoptosis (Figure 4C). We found that EpCAM staining was weak in GSIs groups except in necrotic areas, suggesting GSIs had inhibited EpCAM production

(Figure 4C). Moreover, CK19 staining was significantly reduced in GSI cases (p<0.001), which indicated that the malignant characteristics of the hepatoma cells were controlled (Figure 4D). Brighter hematoxylin staining in GSI-treated cases indicated that HES1 staining was diminished in the nucleus (Figure 4C). Jagged1 and Notch1 were stained by immunofluorescence in control tumor samples; however, these stains were not changed in the L-685,458 or DAPT treatment groups. This result showed that ligands and receptors were not affected by γ-secretase inhibitors (Supplement Figure 2A). This is reasonable data; we checked RTD-PCR data of HES1 and found a decrease after γ-secretase inhibitor treatment (Supplement Figure 2B).

Association between amplification of Jagged1 genome in liver cancer tissues and poor prognosis

Genomic DNA in FFPE samples from surgically resected liver cancer cases was analyzed and the cases were followed for up to 5 years after surgery. High Jagged1 CNV cases (≥1.5 fold) and low CNV cases (<1.5 fold) were divided and the clinical characteristics compared. Advanced TMN classification and positive vascular invasion were associated with high Jagged1 CNV (Table 3). Multivariate analysis showed that high Jagged1 CNV (≥1.5 fold) in cases of liver

cancer recurrence after surgical resection was associated with survival (Table 4). Copy number alterations were analyzed using two different areas of the Jagged1 genome locus and the proximal copy number multiple of 0.5 was calculated. CNV analysis of the Jagged1 locus in 110 liver cancer samples revealed that high CNV cases (≥1.5 fold, 65 cases) had significantly lower survival rates (p=0.019) compared with low CNV cases (<1.5 fold, 45 cases) (Figure 5A). We also classified three groups (CNV≥2.0-fold, 28 cases; 2.0≥CNV>1.5, 37 cases; CNV<1.5-fold, 45 cases), and higher CNV groups showed poorer survival rates compared with lower CNV cases (p=0.014) (Figure 5B). AFP elevation status was analyzed since Jagged1 amplification is observed especially in AFP-producing hepatoma cells *in vitro*. We classified the two groups according to the presence (≥100 ng/mL) or absence of AFP elevation. There was significantly higher Jagged1 CNV in AFP-elevated cases (Figure 5C).

The expression of AFP and Jagged1 genes among frozen resected liver cancer tissues were compared using RTD-PCR. AFP and Jagged1 expression showed significant correlation after the selection of AFP-upregulated samples (p<0.05). Moreover, there was a strong correlation between AFP expression and Jagged1 genome amplification (p<0.01) (Figure 5D, 5E). Jagged1 expression and

Jagged1 genome amplification also correlated (p<0.05) (Fig. 5F), suggesting that the AFP value was associated with Jagged1 expression and genome amplification in clinical samples. We also confirmed a correlation between Jagged1 and Notch1 expression by RTD-PCR using these samples. We assessed Notch1 and downstream HES1 genes and found a strong correlation, which suggested that Notch signaling was upregulated in AFP-positive clinical tissues (Supplement Figure 3AB).

Discussion

Genomic alterations such as genome copy number gain or loss in cancerous cells can be analyzed using microarray based CGH [6, 14]. Amplified or deleted regions are found in various types of cancers but these specific abnormal regions differ in different organs [15]. Some reports show that regions of genomic alterations in cancer are associated with expression abnormalities [16, 17]. We previously reported a significant association between altered genomic copy number and gene expression patterns in hepatoma cells, and using hierarchical clustering analysis by microarray found that the factor involved was AFP-producing status [8]. Chromosome 20p locations in hepatoma cells

apparently differed in terms of genomic copy number, and AFP-producing hepatoma cells differed from non-AFP-producing cells. A previous report showed genomic alterations in liver cancer using CGH analyses and frequent gains were observed in 1q, 6p, 8q, and 17q, whereas losses were observed in 4q, 8p, 13q, 16q, and 17p. In addition to these gained locations, 20p11 was also present in some liver cancer tissue [18]. Moreover, five BAC clones for this experiment were found listed in BAC array CGH, but only Jagged1 gene was a candidate because the different expression patterns were associated with the AFP-producing status of hepatoma cells using microarray.

Notch signaling pathway is widespread and essential for cell-cell interactions or intracellular transduction by Notch intracellular domain (NICD) [19]. Notch ligands and receptors perform major roles in this pathway and abnormalities such as point mutations are reported to be the cause of Alagille syndrome, a systemic disorder in humans [20, 21]. In the oncology field, Notch signaling abnormalities are related to carcinogenesis or tumor vessel abnormalities [22, 23]. Notch signaling-targeted therapy has been developed in the field of neural diseases such as Alzheimer's disease. One of the Notch inhibitors, γ-secretase inhibitor, restricts cleavage of the Notch receptor in the cell membrane and disturbs signal

transduction by NICD. Clinical trials were underway but reports of gastrointestinal toxicity halted further study [24]. This drug is useful for inhibiting aggregation of amyloid protein in Alzheimer's disease [25], while its effectiveness has been reported in the oncology field in targeted molecular therapy [26]. For example, hematopoietic tumor therapy is highly effective and various clinical studies in other solid tumors are ongoing [27-29].

Some genomic alteration studies have already been performed in liver cancer samples and many reports using array CGH method or other new bioinformatics techniques have been published [30]. Genome amplification of cancer cells usually shows that the check point system for DNA repair was destroyed leading to gene amplification, especially in malignant cells, including those of liver cancer. However, it is important to consider that liver cancer includes several histological types and genomic alteration patterns differ by type; variations occur in differentiation, stem cell features, AFP production, and so forth. The etiologies also differ since advanced chronic liver injury can be caused by HBV, HCV, or other factors. Some reports suggest that genomic abnormalities in liver cancer cause poor survival and candidate genes have been reported; however, they are not associated with the development of any clinical drugs [31]. Our method

selected genes by microarray analysis but we concluded that these candidate genes would be important for future effective liver cancer therapy. The relationship between genomic and expression alterations can explain their oncological significance. We investigated the importance of AFP-producing status in hepatoma cells using genome microarray analysis of expression. We focused on Jagged1 gene because it was both amplified and overexpressed in AFP-positive hepatoma cells. In another report, Jagged1 expression was associated with HBV-x genes [32]; however, we included HCV-infected clinical liver cancer cells. Mutations in Jagged1 gene are reported to cause abnormalities of the bile duct cells and Alagille syndrome in the gastroenterology field. However, an association with cancer has not been investigated. By conducting Notch-related basic research, Notch ligand and receptor expression abnormalities have been linked to several types of cancers. Jagged1 gene expression abnormalities in liver cancer have already been reported, although copy number changes have not been discussed [33]. Yet, recent cancer research has revealed that copy number changes in specific genes are associated with carcinogenesis or the aggressiveness of the cancer and correlate with patient survival [34, 35]. In the hepatological field, some reports have mentioned links

between genomic alterations, severity of liver cancer, and survival [36, 37]. Our study revealed a correlation between AFP elevation and Jagged1 genomic copy number changes. Based on array CGH data and PCR-based CNV assay, Jagged1 CNV was not reduced in HLE cells even though these are not AFP-producing cells. This means Notch signaling, including Jagged1 underexpression, is applicable to non-AFP producing cells, but the decrease in Jagged1 CNV does not fully correlate in these cells. This data indicated that HLE cells are an exception but Notch transduction suppression was significant. Furthermore, the data showed that CNV value was not elevated, which indicated that Jagged1 CNV did not change in these cells and underexpression of Jagged1 was not directly associated with a decrease in Jagged1 CNV. We analyzed the genomic alterations of Jagged1 genes and AFP elevation using surgically resected samples. AFP elevation and overexpression of Jagged1 gene were calculated and related to liver cancer cases with elevated AFP. Using surgical samples, RTD-PCR showed that AFP expression and Jagged1 genomic copy number changes also correlated. We concluded that the genomic copy numbers of Jagged1 gene were associated with AFP expression. Notch-related gene expression analysis after Jagged1 knockdown indicated that Notch signal

transduction affected HES1 gene by modulation of the Notch ligand.

In hepatoma cells, γ-secretase inhibitors can be used both *in vitro* and *in vivo*. The results showed that, compared with the control, these drugs significantly inhibited growth in AFP-producing hepatoma cells. However, growth was strongly inhibited using DAPT but not L-685,458 in KMBC cell lines (data not shown). This result suggested that any bile duct cell-related cause could be efficiently suppressed by the administration of Notch inhibitors. We used two types of Notch inhibitor L-685,458 and DAPT. The former is a more efficient SPP (signal peptide peptidase) inhibitor than DAPT, and SPP is essential for HCV replication [12]. This inhibitor does not directly inhibit Jagged1 because it is a ligand in the cells and in cell interactions, but from our results, there appears to be some association, which will be investigated in further *in vivo* experiments. Recent clinical oncology research showed that the chemical was more effective in combination with currently used drugs, such as sorafenib, for advanced stages of liver cancer than as a monotherapy [38-40]. It may provide effective data for *in vitro* or *in vivo* models. Since Notch signaling exists even in non-AFP-producing cells, it can suppress downstream HES1 genes via GSI in, for example, HLE cells. Since GSI catalyzes the membrane protein that affects

Notch1 cleavage, it may not reduce Jagged1, Notch2, or even Notch1. Hence, our data implies that the expression of upstream Jagged1 expression and other receptors are not affected. Furthermore, the reason why Notch1 was significantly suppressed by DAPT only in HLE might be because it was suppressed in both Huh7 and HLE cells, and showed Notch1 consumption by not restricting Jagged1-Notch1 signaling, especially in HLE. We demonstrated an anti-tumor effect in AFP-producing hepatoma cells and that GSI caused cell growth suppression even in EpCAM+ Huh7 cells. This data suggests that GSIs could be aimed towards the more malignant features of cells as promising new targeted molecular therapy. Liver progenitor cells and cancer stem cells are sensitive to GSI treatment based on our experiments. Previous studies show that hepatoma cell lines and liver cancer tissues are heterogeneous and cancer stem cells expressing EpCAM can be separated [3, 41]. Our experiments showed that anti-tumor effects occurred in the EpCAM-positive fraction *in vitro*, although it is difficult to observe precise reactions in clinical tissues. DAPT administration resulted in anti-tumor effects only in the EpCAM-positive cell fraction. Since these cells are particularly malignant, we would expect good prognoses following GSI administration.

We used NOD-SCID mouse models implanted with human hepatoma cells and performed GSI treatment. Tumor formation was suppressed and survival was extended by treatment, which indicates that *in vivo* Notch suppression causes effective anti-tumor effects in hepatomas. Although metastasis was not observed, earlier death due to tumor enlargement and more dominant viable tumor cells occurred in control cases. GSI was dissolved in DMSO, which was also used in control cases. Although DMSO shows cytotoxicity in animal models, the amount (50 μL/animal) used in this study did not affect survival rates [42].

Jagged1 CNV analysis of surgically resected liver cancer patients showed that amplification was associated with poor survival, and copy number gain resulted in poor prognosis. Genomic copy number alterations are associated with survival and this result indicated that these changes were associated with malignant characteristics and influenced patient survival. This region may exist in other regions of the liver cancer genome. Statistical analysis showed that high AFP levels are associated with high Jagged1 CNV. Moreover, we found significantly shorter OS in higher Jagged1 CNV cases that were dependent on CNV values. A comparison of CNV ≥1.5 and <1.5 groups, showed that AFP values were unchanged; even if CNV was high at surgical resection AFP is not since it is an

earlier stage. However, even if AFP is not elevated and Jagged1 CNV is high, the clinical course is worse, earlier recurrences of liver cancer occur and AFP rapidly increases at the end stage. However, when AFP is high at surgical resection, many cases show high Jagged1 CNV; conversely when AFP is low, although some patients have higher CNV, in many cases it is lower and the clinical course is quite good (Figure 5C).

We explained that Jagged1 genomic amplification and overexpression is related to Notch signal enhancement and AFP positivity and the malignant characteristics of liver cancer cells were related to these abnormalities. Though the mechanism of AFP production and Notch enhancement were not directly explained, Notch signal transduction was associated with the activation of cancer stem cells, including those in liver cancer, and we found that the EpCAM-positive fraction in hepatoma cells are sensitive to Notch signal inhibition. Advanced and end-stage liver cancer clinical cases show much higher AFP levels and most show Notch activation including Jagged1 and Notch1 overexpression. Notch pathways are related to angiogenesis, and AFP-upregulated cases show higher levels of angiogenic factors, such as the Ephrin family reported previously by our group, thus it is reasonable to expect

Notch signaling activation in AFP-upregulated cases[2]. Epidemiological studies show that liver cancer has a poor prognosis; therefore, it is important to focus on molecules that are well-known liver tumor markers, such as AFP.

Our results showed that the Jagged1 genomic region and Notch activity are associated with the malignant characteristics of liver cancer and can predict the outcome of liver cancer therapy.

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Figure legends

Figure 1

In vitro genomic copy number and expression analysis in hepatoma or

non-hepatoma cells. (A) Genomic copy number changes of chromosome 20 in each cell line using BAC microarray. Copy number changes in each chromosome area were calculated using commercial software (MAC viewer, Macrogen Inc., South Korea). Human hepatoma cell lines (Huh7, HepG2, Huh6, Hep3B, PLC/PRF/5, HLE, and SKHep1) and human non-hepatoma cell lines (KMBC and HeLa) were analyzed by comparing with healthy human volunteer PBMCs as control. Red dotted lines with arrows indicate Jagged1 gene copy number changes in 20p12.2. Values above zero show copy number gains and those below indicate copy number losses. Average log2 (Cy3/Cy5) signal ratios of BAC clones were calculated for each sample and a threshold of 0.25 log2 ratio units was used for defining copy number gains and losses. (B) Mean copy number changes between hepatoma (yellow) and non-hepatoma (green) cell lines and AFP positive (yellow) and negative cells (green). (C) RTD-PCR analysis of Jagged1 gene in cell lines. These values are log based Jagged1/β-actin expression ratios. Huh7, Hep3B, HepG2, PLC/PRF/5, and Huh6 cells are AFP-producing, whereas SKHep1, HLE and HeLa are not. (D) RTD-PCR analysis of Jagged1 genome in cell lines. These values were calculated by comparing changes with genomic changes in healthy PBMC.

Figure 2

Jagged1 inhibition of hepatoma cells impacts on tumor growth *in vitro*. Cell growth after Jagged1 shRNA transfection in Huh7, HepG2, HLE, and SKHep1 cells. Huh7 and HepG2 are AFP-producing cells and HLE and SKHep1 are non-producing cells. In Jagged1 shRNA transfected cells (black), cellular growth was compared with control shRNA transfected cells (red). *** $P<0.001$.

Figure 3

Notch inhibitor impact on AFP-producing and EpCAM+ hepatoma cells *in vitro*. (A) Relative cellular growth curve of hepatoma cell lines after administration of γ-secretase inhibitors L-685,458 and DAPT. We administered 10 μM of each reagent dissolved in DMSO at 0 h, 72 h, 120 h, and 168 h and blank reagents that included only DMSO (control). *** $P<0.001$; Control versus L-685,458 and DAPT. (B) Relative expression of HES1, Jagged1, Notch1, and Notch2 after L-685,458 and DAPT treatment in Huh7 and HLE cells. Total RNA was isolated after 120 h administration. *** $P<0.001$. (C) Relative cellular growth of L-685,458 or DAPT-treated Huh7 cells after separation of EpCAM+ and EpCAM- fractions

using FACS. We compared 120 h of cellular growth in each with the DMSO control. * *P*<0.05.

Figure 4

Anti-tumor effect of γ-secretase inhibitors in NOD-SCID mouse models implanted with Huh7 cells. (A) Tumor size comparison between L-685,458, DAPT, and negative control after 2 weeks of treatment. Tumor diameters of each of the five tumor samples were analyzed. * *P*<0.05. (B) Cumulative survival rate compared between GSI (L-685,458 and DAPT) and control cases. Survival rates of each of the five samples were compared and calculated using the log-rank t test. ** *P*<0.01. (C) Immunostaining using anti-caspase 3, CK19, HES1 and EpCAM antibodies for control and L-685,458-treated tissues. Immunostaining was performed using DAB, and cellular nuclei were stained by hematoxylin. Scale bars = 100μm. (D) Quantification of CK19-positive cells between control and GSIs. Calculations were performed for each of five CK19 immunostained slides. *** *P*<0.001.

Figure 5

Amplified Jagged1 liver cancer patients have poor survival. We analyzed Jagged1 CNV in liver cancer tissue in FFPE and frozen tissue to compare genomic alterations and expression using RTD-PCR. (A) Overall survival data after surgical resection of liver cancer was divided into two groups (CNV<1.5 and ≥1.5 (CNV: copy number variations)). The *p*-value was calculated using log-rank t test. We followed patients for up to 60 months after surgery. * *P*<0.05. (B) Overall survival data divided into three groups (CNV<1.5, CNV=1.5 and CNV≥2). * *P*<0.05. (C) Comparison of CNV values in two groups of high (≥100 ng/mL) and low (<100 ng/mL) AFP using the Mann-Whitney U test. We used the day of surgery as the approximate date that the serum AFP values were obtained. ** *P*<0.01. (D) Relationship between Jagged1 and AFP expression by RTD-PCR using frozen liver cancer tissues. We analyzed only AFP upregulated liver cancer tissues. (E) Relationship between Jagged1 genomic alterations and AFP expression. (F) Relationship between Jagged1 genomic alterations and expression.

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Table 1 BAC clones of chromosome No. 20 on microarray slides and representative genes

BAC clone ID	Chromosome location	BAC size	Representative genes	Genbank ID	Average expression ratio of AFP+ cells /AFP- cells
BAC40_E05	20p13	110124	LOC343637, RSPO4		
BAC109_E03	20p13	97397	ATRN, GFRA4, ADAM33	NM_139321	0.88
BAC127_K22	20p12.3	91214	C20orf30, PCNA, CDS2	NM_002592	0.98
BAC85_D15	20p12.3	100000			
BAC155_K05	20p12.3	90398	PLCB1		
BAC143_E21	20p12.2	98802	PLCB4	NM_000933	1.08
BAC86_O12	20p12.2	136522	JAG1	NM_000214	2.14
BAC165_M10	20p12.1	88468			
BAC86_O11	20p12.1	153399	MACROD2, CR596518		
BAC81_M07	20p12.1	126920	MACROD2		
BAC81_K08	20p12.1	88837	KIF16B		
BAC29_A11	20p12.1	131740	RPL7AL3, SNRPB2, OTOR	NM_003092	0.4
BAC132_A05	20p12.1	93613			
BAC96_K10	20p12.1	112559	PCSK2, BFSP1	NM_002594	0.99
				NM_001195	0.98
BAC44_G02	20p11.23	110517	DTD1		
BAC75_H16	20p11.23	108696	KIAA1272, RALGA1A2 C20orf74, AK026194		
BAC63_I12	20p11.22	104597	NKX2-2		
BAC110_E03	20p11.21	113928	VSX1, ACSS1		
BAC36_M13	20p11.21	173706	ENTPD6, PYGB, C20orf22,	NM_001247	1.6
			PPIAP2, BC128043, ABHD12,	NM_002862	0.79
			CD39L2		
BAC204_A05	20q11.21	89320	CBFA2T2		
BAC140_D09	20q11.23	125023			
BAC43_F22	20q12	171393	LOC339568		
BAC239_E02	20q12	91366	CR612573, CR593014		
BAC97_K09	20q12	76966	LPIN3, EMILIN3, KIAA1335, CH06		
BAC21_G16	20q12	109908			
BAC52_H17	20q12	114526	PTPRT	NM_133170	0.87
BAC75_I24	20q13.12	86258	ADA, AK090842, CR597563	NM_000022	0.71
			CR616794, WISP2	NM_003881	1.11
BAC12_K18	20q13.13	109372	STAU1		
BAC193_H13	20q13.13	102377			
BAC72_N10	20q13.2	95499			
BAC51_F06	20q13.2	224038			
BAC97_O05	20q13.2	102916			
BAC63_G09	20q13.32	121623	PPP4R1L, RAB22A		
BAC142_K22	20q13.32	81986	PHACTR3, SYCP2		
BAC42_C03	20q13.33	102953	CDH4, AK097866	NM_001794	1.11

Underlined representative genes; 9800 in-house cDNA microarray listed genes.

Table 2　IC50 values of L-685,458 and DAPT in hepatoma cell lines

	IC50 (μM)	
	L-685,458	DAPT
Huh7	12.91±0.55	118.26±40.42
HepG2	12.69±4.60	155.27±7.58
HLE	21.76±0.84	244.86±7.95
SKHep1	12.18±2.90	153.24±14.38

Table 3 Clinical characteristics of liver cancer patients who had undergone surgical resection

Clinical category	CNV ≥ 1.5	CNV < 1.5	*p*-value
No. of patients	n = 68	n = 47	
Sex (M vs. F)	50 vs. 18	39 vs. 8	0.234
Age (years)	63 (38-82)	66 (44-80)	0.054
Viruses (HBV / HCV / non-B non-C)	21 / 33 /15[a]	11 / 22 / 14	0.540
Child Pugh score (5 / 6 / ≥7)	55 / 5 / 6[b]	36 / 7 / 1[c]	0.054
AFP (≥100 ng/mL vs. <100)	27 / 41	11 / 36	0.067
PIVKA-II (≥100 mAU/mL vs. <100)	35 / 31	26 / 19	0.622
Histological grading (mod.-poor. vs. well-mod.)	57 / 11	37 / 7	0.970
Tumor size (mm)	45 (7-230)	35 (10-150)	0.359
Tumor morphology (multi vs. uni)	26 / 42	13 / 34	0.239
Vascular invasion (+ vs. -)	15 vs. 53	1 vs. 46	0.002**
Liver cancer recurrence (+ vs. -)	31 vs. 26	32 vs. 12	0.059

a One patient had a double infection of HBV and HCV.

b The score for two patients could not be calculated.

c The score for three patients could not be calculated.

** $P<0.01$.

Table 4 Cox regression analysis of clinical and genomic variables associated with survival in liver cancer

Variable	Univariate	Multivariate	
	p-value	*p*-value	HR (95% CI)
Sex (M vs. F)	0.824	0.104	2.639 (0.820 - 8.497)
Age (≥60 y.o. vs. <60 y.o.)	0.551	0.612	1.258 (0.519 - 3.050)
Jagged1 CNV (≥1.5 vs. <1.5)	0.002**	0.018*	3.494 (1.243 - 9.816)
AFP (≥100 vs. <100)	0.079	0.187	1.897 (0.733 - 4.909)
PIVKAII (≥100 vs. <100)	0.001**	0.930	0.956 (0.353 - 2.591)
Liver cancer recurrence (Yes vs. No)	0.139	0.001**	9.032 (2.472 - 33.00)
Child-Pugh scores (≥7 vs. <7)	0.024*	0.113	2.704 (0.789 - 9.268)
Histological grading (mod.-poor. vs. well-mod.)	0.221	0.371	2.000 (0.438 - 9.146)
Tumor size (≥30 mm vs <30 mm)	0.003**	0.060	3.073 (0.954 - 9.899)
Vascular invasion (+ vs -)	0.001**	0.553	1.423 (0.443 - 4.574)

* $P<0.05$ ** $P<0.01$