Expression analysis of Hedgehog signaling components in human gallbladder cancer

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<ABSTRACT>

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Background: Although gall bladder (GB) cancer is not a common neoplasm, it shows significant geographic variation in incidence. GB cancer is often diagnosed in advanced stage, obviating curative resection. In advanced stage GB cancers are not amenable to radiotherapy or chemotherapy. It is necessary, therefore, to elucidate molecular mechanism leading to cancer in order to develop effective treatment methods.

Hedgehog (Hh) protein is an essential molecule for normal development of the gastrointestinal tract and the maintenance of the stem cell population, and disruption of Hh signaling is linked to various gastrointestinal tumors. Bmi-1, which is a member of polycomb gene, has recently been identified to play a role in the regulation of stem cell self-renewal and function as a downstream target of the Hh pathway. Deregulation of these processes during carcinogenesis may result in derangement in a stem cell compartment, a key event in carcinogenesis. Strategies aimed at inhibiting these pathways represent a rational therapeutic approach to target cancer stem cells.

AIMS & METHODS: We performed Hh immunohistochemical staining (IHC)

and reverse transcription-polymerase chain reaction (RT-PCR) to investigate the role of Hh signaling and Bmi-1 in human GB cancers. We also analyzed the change of expressions according to tumor stage and degree of differentiation. The GB cancer samples were obtained from patients who underwent radical surgery for GB cancer at the Severance hospital from January 2000 through June 2006. Immunohistochemical staining was carried out using formalin-fixed paraffin section and RT-PCR using fresh frozen tissue for the Hh signaling components (Shh, Ptc-2, Gli-1) and Bmi-1.

RESULTS: Among the total of 59 patients, 19(32.2%) were male and 40(67.8%) were female. Mean age was 61(range: 28-80) years. On RT-PCR, the Ptc-2, Gli-1, and Bmi-1 mRNA levels were increased in human gallbladder cancers compared to non-tumorous tissue. On immunohistochemical staining, 77.0 % (47 of 59), 80.4% (49 of 59), 77.0% (47 of 59), and 71.2% (42 of 59) of the GB cancers were positive for Shh, Ptc-2, Gli-1, and Bmi-1, respectively. The expressions of Shh, Bmi-1, Ptc-2, and Gli-1 (Shh $p = 0.002$; Bmi-1 and Ptc-2 $p<0.001$; Gli-1 $p = 0.001$) was, however, inversely correlated with tumor stage, showing more robust expression in tumors with earlier stage.

There was no difference in the levels of expression and the degrees of histologic differentiation of tumors. (Shh $p = 0.108$; Bmi-1 $p = 0.689$; Ptc-2 $p = 0.804$; Gli-1 $p = 0.613$). Strong expression of Bmi-1 gene was associated with longer survival (shh $p = 0.276$; Bmi-1 $p = 0.025$; Ptc-2 $p = 0.626$; Gli-1 $p = 0.574$).

CONCLUSION: The results suggest that the Hh signaling pathway and Bmi-1 may play a role in the initiation of GB carcinogenesis. As cancers progress, they become less dependent on the Hh signaling and Bmi-1.

Key words: Hedgehog signaling pathway, Bmi-1, Gallbladder cancer

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I. INTRODUCTION

Stem cells are characterized by their ability to self-renew as well as generate differentiated cells within each organ. There is increasing evidence that these cells or their immediate progeny may be targets for transformation. Early events in carcinogenesis may involve deregulation of stem cell self-renewal that leads to a clonal expansion of initiated stem cells.[1, 2] A number of developmental signaling pathways, such as Wnt, Notch, and hedgehog (Hh), have independently been implicated in tumor progression of multiple organs, including the blood, brain, skin, colon and pancreas.[3-6] Furthermore, evidence that these pathways influence one another during tumor progression is accumulating.[7-10]

Hh protein is an essential molecule for gastrointestinal tract development as well as maintenance of stem cell compartment, and there is a link between disruption of the Hh signaling pathway and some gastrointestinal tumorigenesis. Among the three Hh ligands (Shh, Ihh, Dhh) in mammals, it has been reported

that Shh shows the broadest expression patterns, and it is also involved in gastrointestinal tumorigenesis. Although the understanding of Hh signaling has progressed during the last few years, further research is necessary to clarify the complex nature of this signaling pathway. The response to the Hh signal is controlled by two transmembrane proteins, the tumor-suppressor Patched (Ptc) and the proto-oncogene Smoothened (Smo).[8] Smo is a member of the seven transmembrane–receptor family,[20] and its activity is suppressed by the twelve–span transmembrane Ptc. Upon binding to Hh ligands, Ptc releases this inhibition, which leads to Smo activation of a transcriptional response.[8] Expression of Hh ligand and mutational inactivation of Ptc both have the effect of constitutive activation of Smoothened (Smo), a G-protein coupled receptor family protein regulated by Ptc. Downstream targets of the pathway in vertebrate include transcription factor Gli-1, which is related to development of basal cell carcinomas and medulloblastomas.[12]

It has recently been shown that Bmi-1, a polycomb gene, plays a role in the regulation of hematopoietic and neural stem cell self-renewal. Bmi-1 may function as a downstream target of the Hh pathway. Deregulations of these pathways during carcinogenesis may lead to stem cell expansion, a key event in carcinogenesis. These results suggest that strategies aimed at inhibiting these pathways represent a rationale therapeutic approach to target cancer stem cells.

On the other hand, Gallbladder cancer, which is frequently diagnosed at later stage, is an unfavorable tumor showing aggressive biologic nature, especially in advanced stage. Advanced gallbladder cancers are not amenable to chemotherapy or radiotherapy. The overall five-year survival rates vary from 0

to 12% in most reported series.[14] At present, only surgical resection of all apparent tumor is associated with improved five-year survival.[15-17] Therefore, urgent efforts are needed for identification of cancer-specific cellular targets that might form the basis for innovative therapeutic approaches.

Recent studies have shown that the Hh pathway plays important roles in cell differentiation, tissue patterning, and embryonic development.[11-13] The role of the Hh pathway in human gallbladder cancers, however, is still unclear, and there is little information available about the expression of Hh signaling and Bmi-1 in a series of human gallbladder cancer.

The purpose of this study, therefore, is to investigate the expression of Hh pathway signaling proteins in gallbladder cancers. We elucidated the role of Hh signaling and the polycomb gene Bmi-1 in the formation of gallbladder cancer.

In this study, we tried to reveal the correlations between the expression of the Hh signaling pathway and Bmi-1 and the various clinicopathologic factors, such as age, lymph node metastasis, pathology, and tumor stage of gallbladder cancer.

II. MATERIALS AND METHODS

We performed RT-PCR and immunohistochemical staining to evaluate the differences according to tumor stage and differentiation of adenocarcinoma, and also to investigate the role of the Hh signaling pathway including sonic hedgehog (Shh), Patched-2 (Ptc-2), Gli-1, and Bmi-1, a downstream transcription factor, in human gallbladder cancer.

1. Subjects

The human gallbladder cancer samples were obtained from patients who underwent routine surgery for gallbladder cancer at the Department of Surgery, Severance hospital from January 2000 to June 2006. Surgically resected specimens from patients with gallbladder cancer were examined by immunohistochemical staining and RT-PCR for aberrant expression of Hh signaling components (Shh, Ptc-2, Gli-1) and Bmi-1.

2. Methods

A. Immunohistochemical Staining for Shh, Bmi-1, Ptc2, and Gli1

Immunohistochemical staining was performed to study altered protein expression in 59 human gallbladder cancer tissues. Each antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), commercially available rabbit monoclonal antibodies agonist Shh (1:100), Bmi-1 (1:100), Ptc-2 (1:100), Gli-1 (1:100), were used as primary antibodies.

The 59 gallbladder cancer tissue were frozen and stored in liquid nitrogen

until further use. For the immunohistochemical study, some of these tissue specimens were fixed in 10% neutralized buffered formalin solution for 24 hours. Each patient's clinical status was classified according to the pathological stage of the tumor size, lymph node, and metastasis (pTNM) classification system.[18]

In short, a paraffin section of the gallbladder cancer tissue from a patient was deparaffinized with xylene and rehydrated in PBS.

Antigen retrieval was processed by submerging in citrate buffer (pH 6.0) and micro-waving. The sections were then treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with 1% BSA to block the non-specific binding. The primary monoclonal anti Shh, Ptc-2, Gli-1, and Bmi-1 antibody (1:100, Santa Cruz, CA, USA) were incubated overnight at 4 ℃. After washing, the tissue section was immersed in brown 3.3'-diaminobenzidine hydrochloride (DAB) staining or AEC (3-amino-9ethyl carbazole) as a substrate; then it was counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted by crystal mount. The degree of immunostaining of the formalin-fixed, paraffin embedded sections was evaluated by two independent observers; moderate to strong cytoplasmic as well as nuclear staining was considered a positive reaction. The distribution of Shh, Bmi-1, Ptc-2, and Gli-1 was scored on a semi-quantitative scale as follows: negative (<10% of the cells were positive), weakly positive (small cell clusters, but 10-50% of the cells were positive) and strongly positive (> 50% of the cells were positive).

B. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 4 samples out of 59 gallbladder carcinoma and non-tumorous tissues of the patients using AGPC method.[19] The pathologic tumor stage of extracted four samples, by AJCC 6th edition, were as follows: stage II_B ($T_2N_1M_0$) in two samples; stage I_A($T_1N_0M_0$) and I_B ($T_2N_0M_0$) in the other two samples, respectively. Extracted RNA, which was reverse-transcribed with a cDNA, was synthesized with 5 μg of total RNA and oligo (dT) primer in 50 μl of a solution containing reverse transcriptase. The reverse-transcribed samples were used as templates for amplification of Shh, Bmi-1, Ptc-2, Gli-1, and G3APDH fragment, which was used as an internal quantitative control. The PCR primer sequences of Bmi-1 were as follows: 5'-AGCAGAAATG CATCGAACAA-3' and anti-sense primer 5'-CCTAACCAGATGATGAAG TTGCT-GA-3'.Shh:5'-AATGCCTTGGCCATCTCTGT-3',Gli-1:5'-GGAAGT CCTATTCACGCCTTGA-3', Ptc-2: 5'-CT GCC GAGA ACGCTTCCCA-3'. As for G3APDH, the primer sequences were as follows: sense primer 5'-CCCCTG-GCCAAGGTCATCCAT GACAACTTT-3' and antisense primer 5'-GGCCATGAGGTCCACCAC-CC TGTTGCTGTA-3'. The PCR reactions were performed following the cycling parameters on a Minicycler ™ PCR system (MJ Research, Inc.). Shh and Gli-1: 10 min at 94 ℃ followed by 35 cycles of 1 min at 94 ℃, 1 min at 56 ℃, 1 min at 72 ℃, and a final cycle at 72 ℃ for 10 min.; Ptc-2: 10 min at 94 ℃ followed by 35 cycles of 1 min at 94 ℃, 1 min at 53 ℃, 1 min at 72 ℃, and a final cycle at 72 ℃ for 10 min.; Bmi-1: 10 min at 94 ℃ followed by 30 cycles of 1 min at 94 ℃, 1 min at 53 ℃,

and 1 min at 72 ℃, and a final cycle at 72 ℃ for 10 min. Quantitation of the PCR products were scanned and performed using a Quantity One Program (Bio-Rad, Hercules, CA). When expressions of the Shh, Pic-2, Gli-1, and Bmi-1 mRNA in tumor were seen 2-3 times more than in normal gallbladder tissue, it was defined as increase in the expressions.

3. Statistical analysis

The relationship between the results of the immunohistochemical study and the clinicopathologic parameters was determined using the SPSS ver 13.0 (SPSS Inc, Chicago, IL, USA). Univariate and multivariate analyses were done using the proc logistic module. A P value < 0.05 was considered statistically significant.

III. RESULTS

1. Clinical Characteristics and Laboratory findings in enrolled patients

Among the total 59 patients, 19(32.2%) were male and 40(67.8%) were female. Median age was 62 (range: 28-80) years. 54 patients (91.5%) had the risk factors for gallbladder cancer. Of these patients, 24(40.7%) patients were female and of old age (>60 years). 12(20.3%) and 3(5.1%) patients had the medical history of gallstone and gallbladder polyp, respectively. Also, 3(5.1%) patients had the history of gallbladder infection such as salmonella typhi. One patient had been diagnosed as AUPBD (Anomallous Union of Pancreaticobilliary Duct). At admission, 40(67.8%) patients had some presenting symptoms and signs. Of these, most common symptom was right upper quadrant pain (55.9%). On the other hand, 32.2% did not have any specific symptoms or signs associated with gallbladder cancer. The median total bilirubin level of the enrolled patients was 0.5 mg/dL, and CA 19-9 level was 14.9 U/mL. The clinical characteristics of the 59 patients are summarized in Table 1.

Table 1. Clinical characteristics and laboratory findings of patients

	N= 59
Age(mean ± SD)	61.00 ± 9.76
Male : Female	19 : 40
Presenting Sx & Sign	
RUQ pain	55.9% (33/59)
No symptom	32.2% (19/59)
Jaundice	6.8% (4/59)
Wt loss & general weakness	5.1% (3/59)
Laboratory findings (median[range])	
Total bilirubin (mg/dL)	0.5 (0.1-9.5)
Alk (IU/L)	84.0 (31 - 410)
r-GT (IU/L)	39.0 (10-618)
CA 19-9 (U/mL)	14.9 (0-3710)
Number of risk factors	
Multiple risk factors	47.5% (28/59)
Single risk factors	44.1% (26/59)
None	8.5% (5/59)
Type of risk factors	
Old age (>60) and female	40.7% (24/59)
GB stone	20.3% (12/59)
GB polyp	5.1% (3/59)
GB infection(salmonella typhi)	5.1% (3/59)
AUPBD	1.7% (1/59)

2. The expression of Shh signaling and Bmi-1 in human gallbladder cancer by RT-PCR and Immunohistochemical staining

Expressed patterns and degrees of Shh, Ptc-2, Gli-1, and Bmi-1 in human gallbladder carcinomas were examined through immunohistochemical analysis and RT-PCR. The relative levels of expression of Shh, Ptc-2, Gli-1, and Bmi-1 in 4 gallbladder cancer tissues were compared with those of non-tumorous tissues by RT-PCR. The expression levels were determined as a ratio between Shh, Ptc-2, Gli-1, and Bmi-1 and the reference gene (GAPDH) to correct for variation in the amounts of mRNA. The Ptc-2, Gli-1, and Bmi-1 mRNA levels

were increased in 3 specimens (these specimens' pathologic stage were stag I_A, I_B and II_B by AJCC 6^{th} edition) out of the examined 4 human gallbladder cancers, and Shh in all of the 4 examined human gallbladder cancers, when compared with those of corresponding normal mucosal tissue (Fig. 1).

In the immunohistochemical study, 77.0% (47 of 59), 77.0% (47 of 59), 78.7 % (48 of 59), and 71.2% (42 of 59) of the gallbladder cancers were positive for Shh, Ptc-2, Gli-1, and Bmi-1, respectively. The expressions of Shh, Bmi-1, Ptc-2, and Gli-1 in human gallbladder cancer by immunohistochemical staining were tabulated in Table 2.

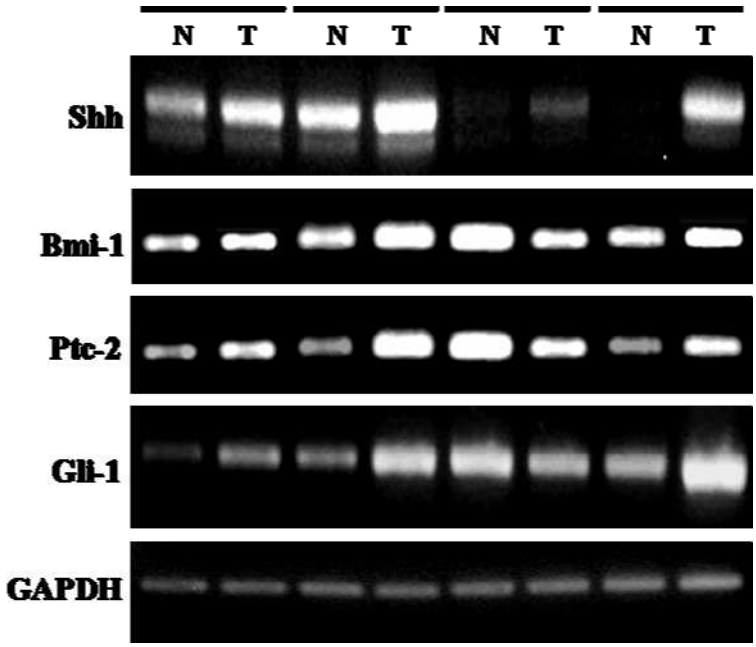

Figure 1. Representative expressions of Shh, Ptc-2, Gli-1, and Bmi-1 in human gallbladder cancer by RT-PCR. GAPDH served as internal control. T represents tumorous part, and N nontumorous part of the neoplasm. Pathologic stages of the four extracted tumors were IA (T1N0M0), IB (T2N0M0), IIB (T2N1M0) and IIB (T2N1M0) from left to right.

Table 2. Expressions of Shh signaling and Bmi-1 in human gallbladder cancers detected by immunohistochemistry

	Shh			Bmi-1			Ptc-2			Gli-1		
	++	+	-	++	+	-	++	+	-	++	+	-
T-stage												
T1 (n=12)	8	3	1	10	2	0	9	2	1	11	1	0
T2 (n=22)	11	6	5	11	8	3	12	8	2	14	5	3
T3 (n=19)	10	6	3	5	8	6	8	5	6	8	5	6
T4 (n= 6)	2	1	3	1	0	5	2	1	3	3	1	2
N-stage												
- (n=33)	14	12	7	18	9	6	15	10	8	19	7	7
+ (n=26)	12	7	7	14	8	4	16	6	4	17	5	4
Overall stage												
I-A (n=12)	8	3	1	10	2	0	9	2	1	11	1	0
I-B (n=12)	7	5	4	4	6	2	5	7	4	8	4	4
II-A (n=7)	1	1	0	1	3	3	1	1	0	0	2	0
II-B (n=18)	9	6	4	10	4	4	11	4	4	10	4	5
III (n= 4)	1	1	2	0	0	4	0	1	3	1	1	2
IV (n= 6)	5	0	1	2	3	1	5	1	0	6	0	0

Stage by AJCC 6th edition

3. Correlation between clinical parameters and expression of Hh signaling molecules and Bmi-1 level

There were no significant relationships between histopathological differentiation and expression level of Hh components (Shh p = 0.108; Bmi-1 p=0.689; Ptc-2 p=0.804; Gli-1 p= 0.613). The stronger degree of staining had a tendency to longer survival time. But there was no statistically significant difference in survival time according to the degree of immunohistochemical staining, except Bmi-1 in univariate analysis (Shh p = 0.276; Bmi-1 p = 0.025; Ptc-2 p = 0.626; Gli-1 p = 0.574) (Table 3, Fig 2). In multivariate analysis, however, the expression degree of immunohistochemical staining of Bmi-1 also did not show statistically significant correlation with survival time (Table 4).

There was no correlation between the expression degree of immunohistochemical staining and clinicopathological parameters, such as age, gender, tumor marker, and clinical lab finding (Total bilirubin, GOT/GPT, Alk, r-GT) either in univariate and multivariate analysis. But multivariate analysis using data from the whole parameters showed that the expression degree of immunohistochemical staining of the Shh signaling pathway molecules and tumor stage were significant covariates. Stronger stained cases were more frequently seen in cancer with earlier tumor stage (Shh $p = 0.002$; Bmi-1 and Ptc-2 $p < 0.001$; Gli-1 $p = 0.001$)(Fig 3). These findings suggested that the Shh signaling pathway and Bmi-1 might play a role in the initiation and development of human gallbladder cancer, and Bmi-1 especially was correlated with human gallbladder cancer prognosis and survival rate.

Table 3.Survival rate according to the degree of Bmi-1 immunohistochemical staining in gallbladder cancer tissue

Bmi-1 expression degree	Number of patients	Survival rate (%)			P value
		1yr	3yr	5yr	
0	14	61.5 %	23.1 %	7.7 %	0.025
1+	18	75.1 %	53.8 %	40.3 %	
2+	27	84.7 %	67.8 %	67.8 %	

Table 4. Results of multivariate logistic regression analysis with survival time

Categories	*p*-value	Odds ratio	95% confidence limits
Stage	0.036	0.684	-364.8-4.90
T-stage	0.052	0.735	-48.3 – 753.6
N-stage	0.247	0.402	-449.0-1189.0
Pathology	0.270	0.319	-142.5-475.0
Postoperative Therapy	0.339	0.186	-118.1-328.8
Shh	0.305	0.240	-423.0-138.7
Bmi-1	0.053	0.622	-5.50-740.7
Ptc-2	0.233	0.390	-619.1-158.8
Gli-1	0.218	0.452	-156.7-635.8

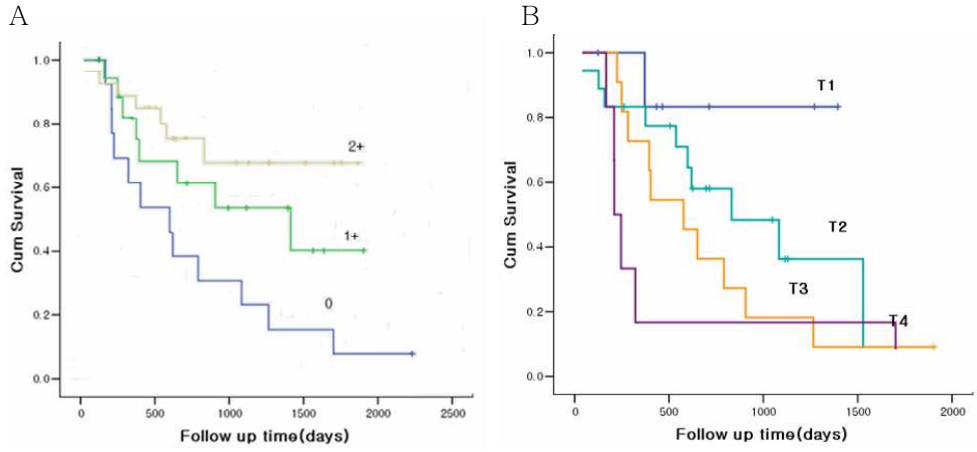

Figure 2. Survival rates according to the expression degree of Bmi-1 (A) and T-stage (B) in gallbladder cancer tissue

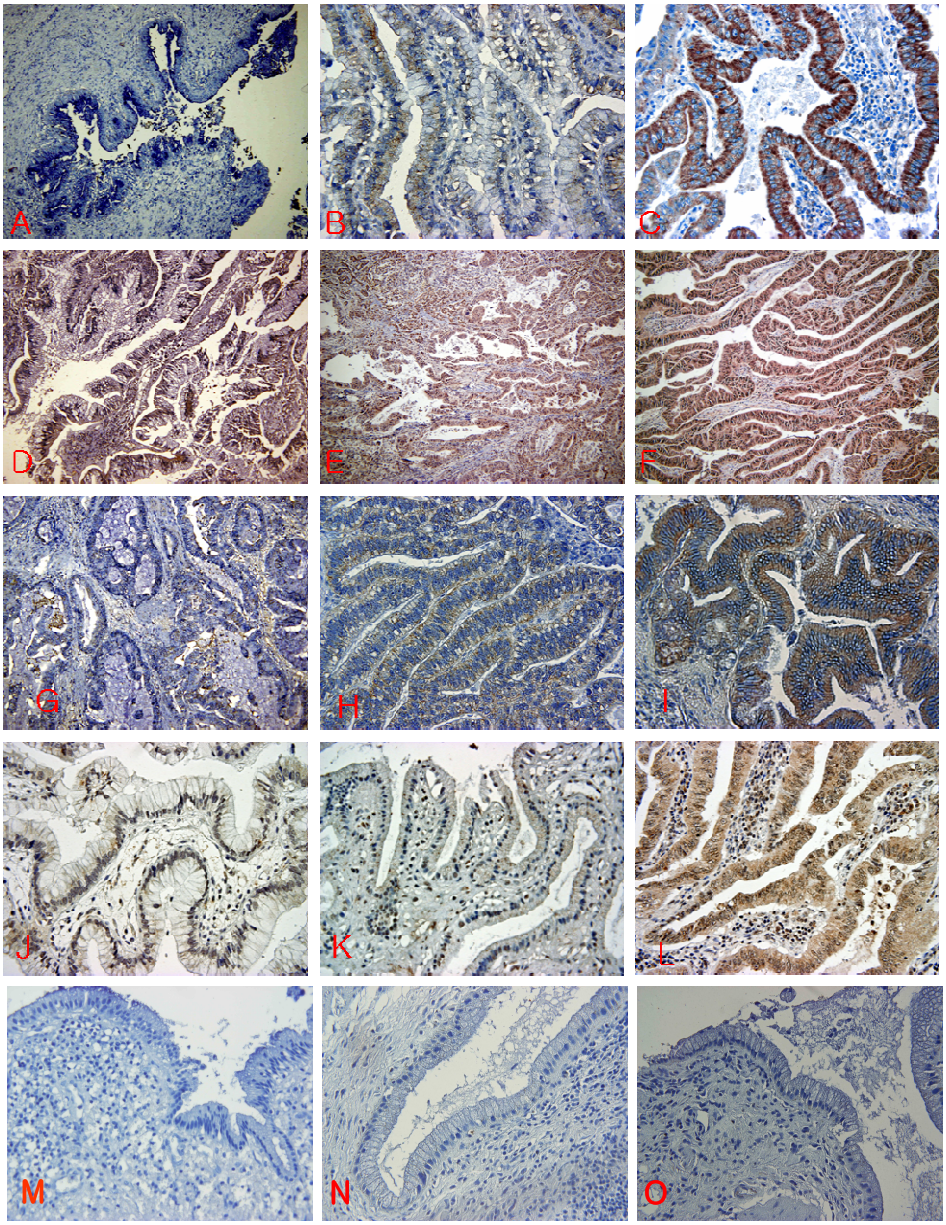

Figure 3. Immunohistochemical study of Shh signaling and Bmi-1 on human gallbladder cancers. A, No expression of shh in human gallbladder cancer (original magnification x100). B, Weak expression of Shh in human gallbladder cancer (original magnification x400). C, Strong expression of Shh in human

gallbladder cancer (original magnification x400). D, No expression of Bmi-1 in human gallbladder cancer (original magnification x 100). E, Weak expression of Bmi-1 in human gallbladder cancer (original magnification x100). F, Strong expression of Bmi-1 in human gallbladder cancer (original magnification x100). G, No expression of Ptc-2 in human gallbladder cancer (original magnification x400). H, Weak expression of Ptc-2 in human gallbladder cancer (original magnification x400). I, Strong expression of Ptc-2 in human gallbladder cancer (original magnification x400). J, No expression of Gli-1 in human gallbladder cancer (original magnification x400). K, Weak expression of Gli-1 in human gallbladder cancer (original magnification x400). L, Strong expression of Gli-1 in human gallbladder cancer (original magnification x400). M, No expression of Shh in human non-tumorous gallbladder tissue (original magnification x400). N, No expression of Bmi-1 in human non-tumorous gallbladder tissue (original magnification x400). O, No expression of Ptc-2 in human non-tumorous gallbladder tissue (original magnification x400).

IV. DISCUSSION

Gallbladder cancer is the most common type of bile duct cancer and the sixth most common form of digestive-tract malignancy. Though it is relatively uncommon, the incidence of gallbladder cancer shows significant geographic variation. It is one of the most lethal malignancies, especially when diagnosed at advanced stage. Moreover, it is typically resistant to chemotherapy or radiotherapy.[35] Five year survival rates for gallbladder cancer vary from 0 to 12% in most reported series.[14] Since the symptoms are seldom suggestive of cancer and imaging techniques sometimes can not depict the lesions, gallbladder cancers are often overlooked before surgery. At present, the only procedure associated with improved 5-year survival is surgical excision of all apparent malignancy.[15-17] Due to its relative rarity, in-depth investigation of molecular pathogenesis relevant to gallbladder carcinogenesis has seldom been carried out. To improve outcomes in patients with gallbladder cancer and to develop an effective agent such as cancer-specific targeted agent, it is necessary to understand better underlying mechanisms of carcinogenesis at molecular level.

The cancer stem cell hypothesis have recently come into spotlight, regarding carcinogenesis. A number of genes involved in developmental program, such as Wnt, Notch, and hedgehog, have been found to play a role in regulating the self-renewal of stem cells. There have been ample evidence suggesting that, of these developmental signaling pathways, the Hh signaling pathway in particular is involved in tumor growth and differentiation. There are three known Hh ligands

(Shh, Ihh, Dhh) in mammals, and among these, Shh is mostly widely studied to show the broadest expression pattern, and to be most closely linked to some gastrointestinal tumorigenesis. Deregulation of the Hh pathway has been proposed to be a component in stem cell activation in cancers, and therefore represents an attractive target for cancer therapy.[21]

It has recently been reported that Bmi-1, which belongs to polycomb group genes, plays an important role in the regulation of self-renewal of hematopoietic[22] and neuronal stem cells.[23] Bmi-1 has been identified as a cell cycle-regulatory factor and is known as a negative regulator of cyclin-dependent kinase p16INK4a and p14ARF.[24, 25] The INK4a/ARF locus is a critical downstream target of the Bmi-1 transcriptional repressor activity involved in the cell cycle regulation of a number of malignant tumors.[26-29] It has been determined that activation of the Hh signaling increases Bmi-1 expression. In normal development, Hh and the downstream transcription factor Bmi-1 play an important role in regulating stem cell self-renewal. Deregulation of these processes during carcinogenesis may result in stem cell expansion, a key event in carcinogenesis. Besides, activation of the Hh signaling pathway as well as Bmi-1 has been shown to result in the generation of carcinomas in vitro or transgenic models.[30, 31] Inhibitors of the Hh signaling, such as cyclopamine and related compounds, have been shown to have antitumor activity with minimal systemic toxicity in mouse tumor models.[33,34] These results suggest that strategies aimed at inhibiting these pathways represent a rational therapeutic approach to target cancer stem cells.

It has recently been reported the roles of pathway include cell differentiation,

tissue patterning and embryonic development.[11-13] In terms of gastrointestinal tumorigenesis, although the Hh signaling pathway seems to play an important role in the early phase of carcinogenesis,[36] there is little available information about its role in the pathogenesis of gallbladder cancer. It is intriguing to explore whether the Hh signaling is also involved in gallbladder cancer. More importantly, these studies may reveal requirements of key molecules for tumor survival and thus could lead to novel therapies for treatment of gallbladder cancer patients.

In this study, we systemically investigated the expression of the Shh signaling and their downstream cell cycle-regulator genes in gallbladder cancer. We found that the mRNA expression levels of the Shh signaling pathway and Bmi-1 were significantly higher in gallbladder cancer tissue than paired adjacent normal gallbladder tissue by RT-PCR. Immunohistochemical staining analysis showed that the increase in the expression degrees of immunohistochemical staining of the Shh signaling pathway molecules and Bmi-1 were significantly correlated with early stage gallbladder cancer. In RT-PCR analysis, however, mRNA expression levels of Bmi-1, Ptc-2, and Gli-1 in human gallbladder cancer tissue were lower than paired non-tumorous tissue in only one tissue sample. This result could probably be explained by the fact that this sample's tumor stage was relatively advanced (stage IIb by AJCC 6th edition). In other words, because expressions of these signaling pathway molecules gradually lessen as cancer progresses, the level of expression even lower than non-tumorous tissue could be seen in cancer tissue sample with advanced tumor stage.

These results coincide with other studies' results mentioned above[11-13,36] that the Shh signaling pathway may have a more critical role in the genesis and initiation of cancer mainly in early stage, and as cancer progresses, the roles gradually disappear. This study provides the first report that the Shh signaling pathway molecules and Bmi-1 are differentially expressed in human gallbladder cancers according to tumor stage.

On the other hand, other parameters, such as several clinicopathologic findings and histological dedifferentiation, were not correlated with the expression degree of immunohistochemical staining of the Shh signaling pathway molecules in human gallbladder cancer. Further investigations are needed, however, to determine the clinicopathologic importance of the Shh signaling pathways and Bmi-1 in human gallbladder cancer.

Based on our study results, the Shh signaling pathway and Bmi-1 were involved in the tumorigenesis of gallbladder cancer and might play a role in the tumor initiation and development in human gallbladder cancer. As cancer progresses, however, these signaling pathway molecules' role in human gallbladder cancer gradually diminishes.

V. CONCLUSION

Shh, Ptc-2, Gli-1, and Bmi-1 immunohistochemical staining results show stronger expression in the earlier stage of gallbladder carcinomas. This suggests that the stronger expression of Shh, Bmi-1, Ptc-2, and Gli-1 is associated with lower grade malignant potentials. In particular, the strong expression degree of Bmi-1 is correlated with good prognosis or survival rate in human gallbladder cancer.

In conclusion, this study suggests that the Shh signaling pathway components and Bmi-1 are involved in human gallbladder cancer initiation and tumor development. There is a significant correlation between Bmi-1 and good prognosis in human gallbladder cancer.

As cancer progresses, however, the roles of the Shh signaling pathways and the downstream molecules, Bmi-1, in human gallbladder cancer gradually grows weaker.

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< ABSTRACT(IN KOREAN)>

인체 담낭암에서의 Hedgehog 신호전달체계 물질의 발현에 관한 분석

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배경: Hedgehog (Hh) 단백은 소화기암 발생에 필수적인 물질로, Hedgehog 신호 전달 체계의 혼란은 소화기암의 발생과 연관되며, 또한 최근 조혈 및 신경 줄기세포의 자가재생 능력의 조절과 연관되는 것으로 알려져 있는 Bmi-1은 Hedgehog 신호전달체계의 하류 전사 인자로써 작용한다. 이러한 과정들이 적절히 조절되지 못할 경우 암 발생의 중요한 원인이 되며, 이러한 사실들은 종양줄기세포를 표적으로 하는 치료적 접근의 합리적 가능성을 제시한다. 한편, 담낭암은 전세계적으로 드문 암이지만 그 발생률에 있어서 상당한 지역적 편차를 보이며 늦은 진단과 불만족스러운 치료성적으로 인해 예후가 좋지 않은 암으로 알려져 있어 새로운 치료방법의 개발이 절실히 요구되고 있다.

목적 및 방법: Shh, Patched-2, Gli-1 및 Bmi-1을 포함하는 Hedgehog 신호전달체계가 인체 담낭암에서 어떤 역할을 하는지 알아보기 위하여, 2000년 1월부터 2006년 6월까지 신촌 세브란스 병원에서 담낭암으로 수술적

절제를 받은 환자로부터 얻은 담낭암 조직을 사용하여 면역화학염색 및 역전사효소 중합 연쇄반응을 시행하여 암의 분화도와 병기 등에 따른 차이를 평가하였다.

결과: 총 59명의 환자 중에서 19명(32.2%)은 남성이었고 40명(67.8%)은 여성이었으며, 환자의 평균나이는 61(범위: 28-80)세였다. 각각 4쌍의 정상과 암 조직의 역전사효소 중합 연쇄반응 비교에서, 정상조직에 비해 담낭암 조직에서 Shh, Ptc-2, Gli-1 및 Bmi-1의 발현이 상대적으로 증가되어 있었으며 (Shh, 100%(4/4)., Ptc-2, Gli-1, Bmi-1, 75%(3/4)), 면역화학염색에서는 Shh, Ptc-2, Gli-1과 Bmi-1에 대하여 각각 77.0 % (47/59), 80.4% (49/59), 77.0% (47/59) ,71.2% (42/59)의 환자에서 양성반응을 보였다. 낮은 병기일수록 강하게 염색되는 경향을 보였지만 (Shh p = 0.002., Bmi-1 and Ptc-2 $p < 0.001$., Gli-1 $p = 0.001$)., 담낭암의 분화도와 면역염색 정도간에 통계적 유의성은 없었다 (shh p = 0.108., Bmi-1 p=0.689., Ptc-2 p= 0.804., Gli-1 $p = 0.613$). 또한, Shh, Ptc-2, Gli-1 및 Bmi-1 모두 염색강도가 높을수록 더 긴 생존기간을 갖는 경향을 보였지만 Bmi-1만이 통계적으로 의미 있는 연관성을 보였다 (Shh p = 0.276., Bmi-1 $p = 0.025$., Ptc-2 $p = 0.626$., Gli-1 $p = 0.574$).

결론: Hedgehog 신호전달체계와 Bmi-1은 인체 담낭암의 발현에 관여하고, 특히 Bmi-1은 예후와 강한 연관성을 보이지만, 암이 진행됨에 따라 인체 담낭암에서 이들의 역할은 점차 감소한다.

핵심되는 말: Hedgehog 신호전달체계, Bmi-1, 담낭암