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Genetic and Epigenetic Alterations of the Wnt/β-catenin Signaling Pathway in Cancer of the Ampulla of Vater

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Background : Carcinoma of the ampulla of Vater is rare and its pathogenesis is unclear. The role of epigenetic changes in the *APC* or *CDH1*, in the Wnt pathway, has not been reported in ampullary carcinomas. **Methods :** We performed immunohistochemistry on 73 sporadic ampullary carcinomas to identify Wnt-related molecules (APC, β-catenin, E-cadherin, c-erbB2, cyclin D1) and examined mutations in the *CTNNB1*, loss of heterozygosity of 5q21, and the methylation status of the CpG island of *APC* and *CDH1*. **Results :** Thirteen tumors (17.8%) showed abnormal nuclear localization of β-catenin; this was more prominent in the intestinal type than in the pancreaticobiliary type (p=0.01). The loss of APC correlated with the loss of β-catenin or c-erb B2 (p<0.01). The prognosis was worse in the group with APC loss than when APC was maintained (p<0.05). There was no mutation identified in *CTNNB1*. Six (24%) out of 25 informative cases had 5q21 allelic loss. CpG island methylation in *APC* and *CDH1* was detected in 33 (45.2%) and 29 (31.5%) cases, respectively. **Conclusions :** The absence of mutations in *CTNNB1* and the epigenetic alteration of *APC* and *CDH1*, might be characteristic changes in the Wnt/β-catenin signaling pathway during the carcinogenesis of ampullary carcinomas.

Key Words : Ampulla of Vater; Neoplasms; Wnt; APC; Methylation

Ampullary carcinomas account for approximately 6% of the periampullary tumors. It is known that the relatively rare ampullary cancers have a better prognosis and greater respectability potential than adenocarcinoma of the head of the pancreas.[1] There is a transition from the epithelium of the pancreaticobiliary tract to the intestinal epithelium of the duodenal mucosa within the ampulla of Vater. Therefore, epithelial neoplasms of the ampulla can resemble tumors of the duodenal mucosa or the pancreaticobiliary tract. The two major different types, the intestinal and pancreaticobiliary types, have their own histological characteristics. Molecular biological studies have demonstrated variable results in the carcinogenesis of these two histological types of ampullary and periampullary carcinomas.

Studies of the molecular carcinogenesis in ampullary tumors have evaluated p53, K-ras, epidermal growth factor receptor (EGFR), APC, β-catenin, cyclin D1, and microsatellite instability.[2-5] In addition to these numerous molecular markers, Wnts are secreted glycoproteins that regulate cellular growth, motility, and differentiation during embryonic development. Wnts act in a paracrine fashion by activating diverse signaling cascades inside of target cells. The function of this pathway has been explained for embryonic development and tumorigenesis.[8] APC can interact independently with β-catenin and form a complex with axin, conductin, glycogen synthase kinase (GSK) 3β, and diversin. APC has a key function in stabilizing β-catenin; as a result, the proteasome degradation of β-catenin can occur. Cyclin D1, the product of the bcl-1 (PRAD, CCND-1) gene was recently identified as a key transcriptional target of the Wnt pathway.[6] β-catenin is involved in another cellular process; binding of β-catenin to the intracellular domain of the adhesion is important for protein cadherin, which is essential for proper cell adhesion.[7] The role of c-erb B2 (tyrosine kinase receptor family) in cadherin containing intercellular adhesion confers the phosphorylation of β-catenin and this is considered to be a critical

step in modulating the tumor cell motility and metastasis.[8] Such interaction suggests a nuclear localization of the β-catenin molecule.

The correlation between β-catenin gene mutations and accumulation of its protein products is controversial. The abnormal nuclear expression of β-catenin is also related to the mutation and hypermethylation of the APC gene.[9] Deletions of APC in colorectal tumors can stabilize β-catenin and activate the T-cell factor/Lymphoid enhancer factor (TCF/LEF).[10] Some studies have reported different results for the frequency or effects of APC gene silencing associated with mutation or the loss of heterozygosity (LOH) on chromosome 5q (*APC* locus) in ampullary carcinomas.[11,12] However, there are no reports on epigenetic changes of *APC* and the E-cadherin gene (*CDH1*) in sporadic or familial ampullary tumors.

Therefore, we evaluated the potential role, clinical and pathological significance of genetic and epigenetic alterations Wnt signaling pathway genes and their association with ampullary carcinogenesis.

MATERIALS AND METHODS

Patients and tissue specimens

Formalin-fixed and paraffin-embedded tissues collected between 1994 and 2003 were retrieved from the archives of the department of pathology at the Korea University Medical Center. Seventy-three patients who had undergone pancreaticoduodenectomy due to a sporadic ampullary or periampullary carcinoma were selected for inclusion in the study. None of the cases had a history of familial adenomatous polyposis. The tumors were classified as (intra) ampullary, periampullary, and mixed type, according to their location. Every case had their main tumor burden in the ampullary area, not in the duodenum or pancreas. The institutional review board of Korea University Medical Center approved this study.

Immunohistochemistry

Three paired cores from the neoplastic lesions and adjacent normal mucosa of the prepared paraffin blocks were selected and tissue microarray blocks were constructed. Immunohistochemical staining using diaminobenzidine as the chromogen was performed with an automated staining system (Autostainer Plus, Dako, Denmark). The antibodies used were β-catenin (1:200, mouse monoclonal; Transduction Laboratories, KY, USA), APC (1:200, rabbit; Santa Cruz Biotechnology, CA, USA), cyclin D1 (1:50, mouse monoclonal; Santa Cruz Biotechnology), E-cadherin (1:100, mouse monoclonal; Zymed, CA, USA), and c-erb B-2 (1:50, rabbit; Zymed). Biotinylated sheep anti-mouse or anti-rabbit IgG (Dako) was used as a secondary antibody.

All sections were scored depending on the extent and intensity of staining from the three selected cores of the tissue array as previously described.[13] Briefly, the intensity of staining was graded according to a 4-tiered scale of 0 to 3 (with 3 as the most intense staining). The extent of positive immunoreactivity was graded according to the percentage of stained cells in the region of interest: 0 points for 0%, 1 point for <20%, 2 points for 20-50%, and 3 points for >50%. An overall score was obtained by the sum of the intensity and the extent of the positive staining. Cases with a final score of more than three were defined as positive. Abnormal nuclear localization of β-catenin was also examined. Two independent, experienced pathologists graded all sections without knowledge of the patients' clinical status.

DNA extraction

Manual microdissection for DNA extraction on H&E stained slides was performed from the formalin fixed, paraffin-embedded specimens using a 27 1/2-gauge injection needle under a low-power (X4) objective view. Tumor areas with more than 90% cellularity were chosen for excision. Paired normal tissue was also obtained and analyzed to exclude germ line mutations. DNA was extracted by method as described previously.[14]

β-catenin gene (*CTNNB1*) mutation analysis-direct sequencing

The DNAs from each sample were amplified by PCR using a known oligonucleotide primer pair: forward, 5′-ATGGAACCAGACAGAAAAGC-3′: reverse, 5′-GCTACTTGTTCTGAGTGAAG-3′. A 200-bp fragment of exon 3 of *CTNNB1* encompassing the region for GSK-3β phosphorylation was then amplified. PCR reactions were performed under standard conditions in a 50 μL volume containing 2 μL of genomic DNA, 10 mmol/L dNTP mix, 2.25 U AmpliTaq Gold (PE Applied Biosystems, CA, USA), 0.125 U PFU DNA polymerase (Stratagene, CA, USA), and 20 pmol of forward and reverse primers. The PCR conditions consisted of an initial denaturation at 95℃ for 10 min, then 40 cycles at 94℃ for 1 min, 58℃ for 1 min,

and 72℃ for 2 min, and a final extension step at 72℃ for 10 min. The PCR products were purified using QIA Quick PCR Purification Kit (QIAGEN, Hilden, Germany), and cycle sequencing reactions were performed using a BigDye™ Terminator V3.0 Ready Reaction Cycle Sequencing Kit (PE Applied Biosystems). Finally, the cycle sequencing products were electrophoresed and analyzed on an ABI PRISM 3100 DNA Sequencer (PE Applied Biosystems). All the sequences were verified in both the sense and anti-sense directions.

Chromosome 5q21 allelic losses

Allelic losses on chromosome 5q21 were evaluated by microsatellite assays using the microsatellite marker D5S346, which is located 30-70 kb downstream from the APC gene. The assays were performed by fluorescent-labeled PCR amplification using a fluorescent dye-labeled forward primer and and unlabeled reverse primer. Primer sequences were as follows: forward, 5′-ACTCACTCTAGTGATAAATCGGG-3′: reverse, 5′-AGCAGATAAGACAGTATTACTAGTT-3′. The forward primer was end-labeled with 6-FAM (PE Applied Biosystems). The PCR reactions were performed in 15 μL reaction volumes that contained 40 ng of genomic DNA, 9 μL of ABI Prism True Allele PCR Premix (PE Applied Biosystems), 5 pmol of 6-FAM-labeled forward primer, and 10 pmol of unlabeled reverse primer. The cycling conditions were as follows: denaturation at 95℃ for 6 min, and then 45 cycles at 94℃ for 45 s, 55℃ for 45 s, and 72℃ for 1 min; this was followed by a final extension at 72℃ for 1 min. The resulting PCR products were diluted with 30 μL of H_2O, and a 1.0 μL aliquot of each diluted fluorescent-labeled PCR product was combined with 12 μL of formamide and 0.5 μL of GeneScan 400HD (ROX) size standard (PE Applied Biosystems). The samples were then capillary electrophoresed on an ABI 3100 automated DNA sequencer and analyzed using GeneScan Analysis software version 3.7 (PE Applied Biosystems). Allelic loss was considered present when there was at least a 50% reduction in the height of a heterozygous peak in the microsatellite marker compared to normal control DNA.

Bisulfite modification of DNA and methylation specific PCR (MSP)

The method of determination of the status of CpG island methylation of *APC* and *CDH1* was as previously described.[15] DNA was denatured with 2 mol/L NaOH at 37℃ for 10 min, followed by incubation with 3 mol/L sodium bisulfite (PH 5.0) at 50℃ for 16 h in the dark. After treatment, DNA was purified using the DNA purification kit (Promega, Madison, WI, USA) as recommended by the manufacturer, incubated with 3 mol/L NaOH at room temperature for 20 min, and then precipitated with ethanol. The methylation status of each gene was determined using 2 μL of modified DNA as a template for the PCR using primers specific for methylated and unmethylated alleles. The primer sequences used have all been previously reported and can be found in the report referenced after each gene.[16,17] PCR was performed for 35 cycles at 95℃ denaturing, 55.5℃ annealing (53.5℃ for *CDH1*), and 72℃ extension with a final extension step of 5 min. Seven μL of PCR products were electrophoresed on 5% acrylamide gels and visualized by ethidium bromide staining. Commercially manufactured methylated and unmethylated DNAs (CpGenome Universal Methylated and Unmethylated DNA, Chemicon, USA) were used as a control. Samples showing signals approximately equivalent to that of the marker (7 ng/μL) were scored as methylated.

Statistical analysis

The data was analyzed with Statistical Package Service Solution software (SPSS 10.1 for Windows, SPSS Inc., IL, USA). Statistical analyses were performed with the chi square test or Fisher's exact test. Correlations between the immunoprofile or mutation and the clinical outcome were calculated using the Kaplan-Meier method and analyzed by log rank tests. Statistical significance was defined as p values <0.05.

RESULTS

Clinical and pathological findings

There were more male patients than female (43:30) and their mean age was 60 (range: 40-80). The distribution of clinical and pathological staging for the patients was as follows; stage I: 7 cases, stage II: 36 cases, stage III: 16 cases, and stage IV: 14 cases. The tumors were divided into three gross subtypes according to their location; intra-ampullary, periampullary and mixed. There were 58 cases of intra-ampullary lesions and 15 cases of periampullary lesions (including 9 cases of the mixed type). There were 53 predominant intestinal type adenocarcinomas and only 20 pancreaticobiliary type tumors. For the histological grades, 28 cases were well differentiated, 34 cases were moderately differentiated and 11 cases were poorly differentiated

(Table 1).

Immunohistochemistry

Normal duodenal epithelium and many of the ampullary tumors showed strong membranous staining for β-catenin. Loss of membrane staining and abnormal nuclear localization of β-catenin were observed in 26 (35.6%) and 13 cases (17.8%), respectively (Table 1). The poorly differentiated tumors showed a loss of membranous expression of β-catenin and E-cadherin in most cases (p=0.002) (Fig. 1). As the E-cadherin membranous expression disappeared, the catenin staining also decreased (p<0.001). Nuclear β-catenin accumulation was more prominent in the intestinal type tumors than in the pancreaticobiliary type tumors (p=0.017) (Table 1). The APC protein was normally expressed in the crypts of normal colonic and duodenal mucosa. The antibody used in our study was targeted to the last 20 amino acids of the C-terminus of the human APC. APC was lost in 74% of cases and its loss was most prominent in patients with stage 4 disease (Fig. 1) (p=0.014). The cases that

Table 1. Clinicopathological characteristics of the patients and the results of immunohistochemistry

Clinicopathologic information	No. of cases (%)	Immunohistochemical staining (%)					
		β-catenin		APC loss	Cyclin D1	Cadherin loss	c-erb B2
		Loss	Nuclear				
Sex							
Male	43 (54.8)	14 (19.2)	9 (12.3)	31 (42.5)	32 (43.8)	10 (13.7)	3 (4.1)
Female	30 (45.2)	12 (16.4)	4 (5.5)	23 (31.5)	19 (26.0)	7 (9.6)	2 (2.7)
Histologic type							
Intestinal	53 (72.6)	19 (14.3)	12 (16.4)*	39 (53.4)	34 (46.6)	12 (16.4)	4 (5.5)
Pancreaticobiliary	20 (27.4)	7 (9.6)	1 (1.4)	15 (20.5)	17 (23.3)	5 (6.8)	1 (1.4)
Histologic grade							
WD	28 (38.4)	4 (5.5)	6 (8.2)	18 (24.7)	21 (28.8)	2 (2.7)	2 (2.7)
MD	34 (46.6)	14 (19.2)	6 (8.2)	26 (35.6)	23 (31.5)	8 (11.0)	3 (4.1)
PD	11 (15.0)	8 (11.0)†	1 (1.4)	10 (13.7)	7 (9.6)	7 (9.6)†	0 (0)
Location							
Ampullary	58 (79.5)	23 (31.5)	11 (15.1)	44 (60.3)	42 (57.5)	13 (17.8)	5 (6.8)
Periampullary	15 (20.5)	3 (4.1)	2 (2.7)	10 (13.7)	9 (12.3)	4 (5.5)	0 (0)
LN metastasis							
No	58	20 (27.4)	12 (16.4)	45 (61.6)	42 (57.5)	15 (20.5)	5 (6.8)
Yes	15	6 (8.2)	1 (1.4)	9 (12.3)	9 (12.3)	2 (2.7)	0 (0)
Clinical stage							
Stage I	7 (9.6)	0 (0)	1 (1.4)	2 (2.7)	5 (6.8)	0 (0)	1 (1.4)
Stage II	36 (49.3)	12 (16.4)	9 (12.3)	28 (38.4)	26 (35.6)	10 (13.7)	4 (5.5)
Stage III	16 (21.9)	7 (9.6)	1 (1.4)	11 (15.1)	10 (13.7)	3 (4.1)	0 (0)
Stage IV	14 (19.1)	7 (9.6)	2 (2.7)	13 (17.8)†	10 (13.7)	4 (5.5)	0 (0)
Total	73 (100)	26 (35.6)	13 (17.8)	54 (74.0)	51 (69.9)	17 (23.3)	5 (6.8)

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; LN, lymph node.
Fisher's exact test *, p<0.05; †, p<0.01.

Table 2. Correlation of immunoprofiles; APC, β-catenin, E-cadherin, and c-erb B2

Markers	β-catenin (%)				c-erb B2 (%)		Total (%)
	P	N	m	nc	P	N	
E-cadherin							
P	46 (63.0)	10 (14.0)	48 (65.8)	8 (11.0)	3 (4.1)	53 (72.6)	56 (76.7)
N	1 (1.4)	16 (21.9)†	12 (16.4)	5 (6.8)	2 (2.7)	15 (20.5)	17 (23.3)
APC							
P	16 (21.9)	3 (4.1)	16 (21.9)	3 (4.1)	4 (5.5)	15 (20.5)	19 (26.0)
N	31 (42.5)	23 (31.5)*	44 (60.3)	10 (14.0)	1 (1.4)	53 (6.8)†	54 (74.0)
Total	47 (64.4)	26 (35.6)	61 (83.6)	13 (17.8)	5 (6.8)	68 (93.2)	73 (100)

P, positive; N, negative; m, membranous; nc, nucleocytoplasmic.
Fisher's exact test *, p<0.05; †, p<0.01.

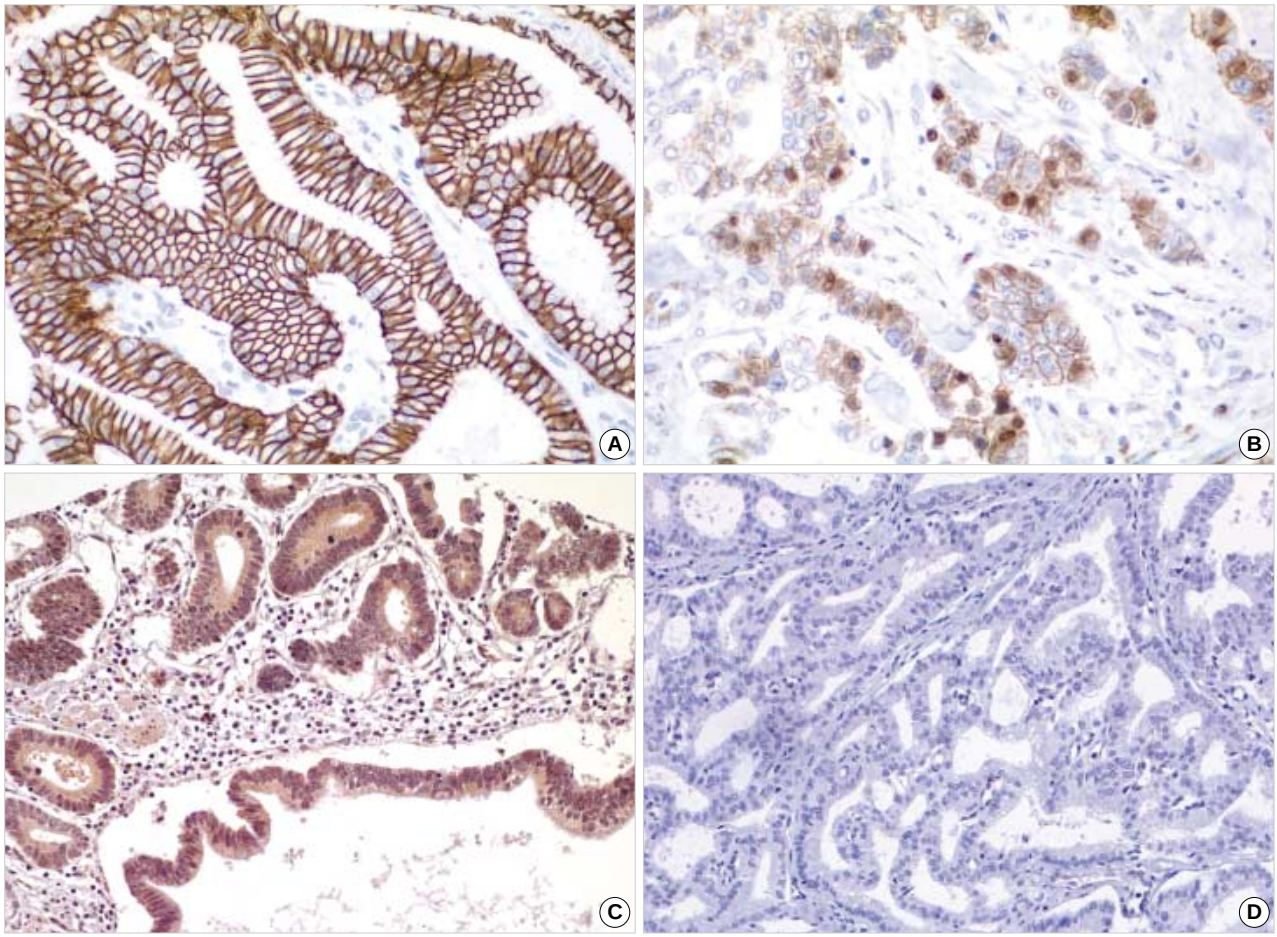

Fig. 1. Various patterns of immunoreactivity. Normal strong membranous expression of β-catenin in tumor glands (A) and abnormal nuclear localization of β-catenin in some tumors (B). Positive expression of APC in tumor glands (C) and negative reactivity for APC in the other ampullary carcinoma (D).

had loss of APC were positively correlated with cases that were negative for β-catenin or c-erb B2 (p=0.04 and p=0.004) (Table 2). There were only five cases with c-erb B2 positive staining. The overexpression of an assumed transcriptional target, cyclin-D1 (69.9%), tended to increase in cases with nuclear accumulation of β-catenin. However, this difference was not statistically significant (p=0.07).

Genetic and epigenetic changes in Wnt pathway genes

None of the 73 cases available for study showed a mutation in exon 3 of *CTNNB1* by direct sequencing. Twenty-five cases were informative (heterozygosity, 34.2%) and there were 5q21 allelic losses identified in six cases (24%) (Fig. 2). All six cases had intra-ampullary carcinoma and five of the cases had stage II disease. There were no other statistically significant correlations with the other results of the immunohistochemistry or with the clinical and pathological information.

Thirty-three (45.2%) cases had *APC* methylation and 29 (31.5%) cases had *CDH1* methylation (Fig. 3). The normal tissue, of these cases, also revealed a methylated pattern; but at a much lower frequency (11.0% and 8.2%, respectively for each gene). The pancreaticobiliary subtype tumors had more frequent CpG island methylation of *CDH1* than the intestinal type tumors (p=0.03).

Analysis of clinical outcome

The 5-year survival rate for our cases was 8.22%; the mean survival was 25 months and the median was 20 months. Patients older than 50 years had a worse prognosis than their younger counterparts did (p=0.006). Patients with clinically higher stages (stage 3 and 4) had a worse prognosis than those with lower stage disease (stages 1 and 2) (p=0.023). Well differenti-

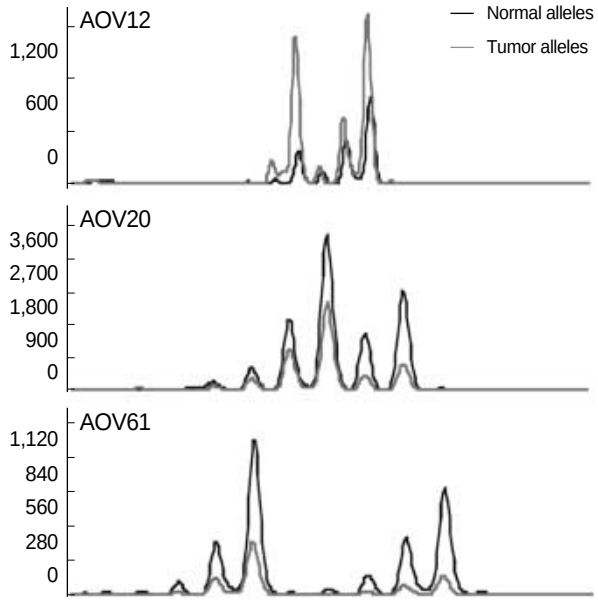

Fig. 2. Representative three cases of biallelic *APC* inactivation in sporadic ampullary carcinomas. The ratio of smaller alleles to the larger alleles in normal tissue are changed (the relative ratio of A to C; 2.31, 2.12, and 1.89, respectively).

ated tumors and intestinal type tumors had a more favorable prognosis than did the poorly differentiated or pancreaticobiliary types (p=0.024 and 0.005, respectively). The loss of APC was correlated with a worse prognosis (p=0.042) (Fig. 4). The presence of LOH of *APC* and the status of the CpG island methylation was not correlated with survival.

DISCUSSION

Because of its unique anatomical location and biochemical characteristics, the ampulla of Vater displays different biological features than the other gastrointestinal and pancreaticobiliary tracts. The malignant tumors that develop in this region have a variable biological behavior based on their histological and anatomical classification. Ampulla of Vater cancer is not common; these lesions require pancreaticoduodenectomy in most cases for a cure. There is controversy with regard to the pathogenesis of this cancer because of the anatomy and function of this area.[18,19] These tumors may occur sporadically, or they may be associated with familial adenomatous polyposis (FAP) and Gardner's syndrome.

The Wnt/β-catenin signaling pathway has been associated with the development of many types of malignant tumors, especially gastrointestinal malignancies including colon cancer.[6] However, pancreatic cancers have no or a much lower frequency of *CTNNB1* and *APC* mutations.[20,21] Additionally, in other solid tumors such as a hepatoblastoma, the abnormal nuclear accumulation of beta-catenin is found without any additional somatic mutation in *APC*.[22] Achille *et al*. and Imai *et al*. found 17% (only one case out of 18 in MCR) and 47%, respectively, of *APC* mutations in sporadic ampullary cancers.[11] Kawakami *et al*. reported a 19% nuclear accumulation of β-catenin and one case with a mutation; Yamazaki *et al*. found 30% and 13% with nuclear accumulation and mutations of the β-catenin gene in ampullary carcinomas.[23,24]

Fig. 3. The status of methylation in CpG islands of *APC* and *CDH1* by methylation specific PCR. lane 1, marker; lane 2-11, tumor; lane 12-15, normal; lane 16, methylated DNA; 17, unmethylated DNA; 18 and 19, DW.

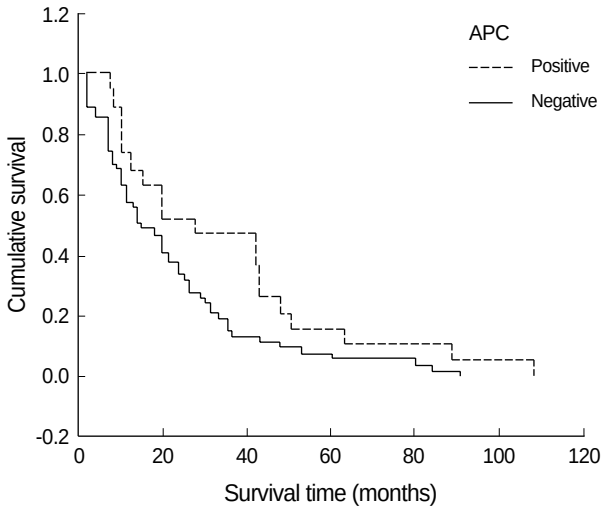

Fig. 4. The survival curve according to APC immunohistochemical staining in ampullary carcinomas. Cases with APC loss shows worse prognosis than the other group (p=0.042).

We analyzed 73 cases of ampullary carcinoma by focusing on the expression of Wnt pathway related molecules. There were 17.8% of the cases with abnormal nuclear localization of β-catenin. However, we could not identify any mutations of the β-catenin gene. Achille *et al.* reported a 37% (versus 24% in present study) LOH at the *APC* locus in sporadic ampullary

tumors.[25] The cases with LOH at 5q21 might have been due to other alterations in the *APC* (i.e., nondisjunction, gene conversion, or mitotic recombination) gene other than a mutation. The abnormal nuclear location of β-catenin in our cases (17.8%) was thought to be due to other mechanisms such as a mutation, LOH or hypermethylation in the APC gene rather than alteration of the β-catenin gene. We found CpG island methylation of the *APC* and *CDH1* promoter region in 33 (45.2%) and 29 (31.5%) cases, respectively. In addition, the incidence of methylation of *CDH1* was higher in the pancreaticobiliary type compared to the intestinal type of tumors. Therefore, these results suggest that epigenetic transcriptional silencing, of these two genes, is associated with tumorigenesis and Wnt pathway activation in the development of ampullary cancers.

Our findings of APC loss in 74% of the cases studied is similar to a previous study on colon cancer showing 83% loss using the same antibody.[26] Altered APC gene products due to a mutation (generally a truncating mutation), might have the potential to exert dominant negative effects on the wild type APC gene products.[27] In the present study, APC loss was more prominent in cases with a loss of membranous staining intensity of β-catenin. Furthermore, the Kaplan-Meier analysis revealed that patients with reduced APC expression lived for a shorter period as compared to those patients who maintained cytoplasm expression. Therefore, APC as a gatekeeper gene product may be a candidate prognostic marker for ampullary carcinomas.

Moore *et al*. reported that pancreatic ductal adenocarcinomas and their terminal excretory structure (the ampulla of Vater) have common molecular features when they analyzed K-ras, p53, p16, and DPC4/Smad4.[28] The absence of a mutation in *CTNNB1* in this study is consistent with the above findings. A specific and well known subtype, pancreaticobiliary type tumors, have shown a tendency to infiltrate the surrounding tissues more frequently; these tumors have a much higher rate of K-ras mutations.[4] Our twenty pancreaticobiliary type tumors also showed E-cadherin loss and a worse prognosis compared to cases with an intestinal type tumor. Abnormal nuclear accumulation of β-catenin was more frequent in the intestinal type tumors than in the pancreaticobiliary type tumors. Our 5-year survival rates were lower (8.22%) than in other reports (30%). This is because the ratio of patients with stage IV disease was relatively high (19% in our study vs 1% in other studies).[29]

The relatively high incidence of cyclin D1 expression in this study could be due to various other signaling activities that contributed to the activation of cyclin D1. The Wnt/β-catenin signaling pathway is composed of a network of a variety of complicated molecules, with the major function of tumorigenesis and maintenance of homeostasis by controlling inappropriate levels of β-catenin. The pancreatic carcinomas and ampulla of Vater cancers might have different molecular biological characteristics than colorectal carcinomas. Recently, Kim *et al*. reported that the methylation profile and genetic alterations of duodenal carcinomas were distinct from biliary and ampullary carcinomas.[30]

In conclusion, the result of this study showed that the promoter CpG island methylation of *APC* and *CDH1* might be a contributing factor in the carcinogenesis of ampullary carcinomas. The lack of a β-catenin gene mutation in our cases suggests that the activating mechanism of the Wnt/β-catenin signaling pathway, in the carcinogenesis of ampulla of Vater cancers, might be different based on the characteristic differences in molecular and clinical behavior of the two major subtypes (intestinal vs pancreaticobiliary) of ampullary carcinoma.

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