P16 and *MGMT* hypermethylation predicts surgical outcomes in curative resected mid/distal bile duct cancer

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ABSTRACT

P16 and *MGMT* hypermethylation predicts surgical outcomes in curative resected mid/distal bile duct cancer.

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Extrahepatic bile duct cancer is a primary malignancy arising from epithelium of extrahepatic biliary tract. In patients with extrahepatic bile duct carcinoma, the 5-year survival rate was 38.3%; a very poor prognosis has been reported. In these patients, the important prognostic factors include the TNM stage, cell differentiation and histologic type. Nevertheless, we often encounter a substantial number of patients whose prognosis is not consistent with the TNM stage. Therefore, other prognostic criteria are mandatory than the TNM staging system which has been widely used at present. We aimed to evaluate the potential role of DNA promoter methylation of gene involved in a variety of celluar function including adhesion (CDH1), cell division (p16) and survival (DAPK), to predict disease free survival (DFS) and overall survival (OS) in curative resected mid/distal bile duct cancer.

Sixty-five mid/distal bile duct carcinoma specimens obtained at Severance

Hospital of Yonsei University College of Medicine from January 2000 to December 2006. Methylation of interest loci was confirmed using pyrosequecning.

The significant methylation frequencies (MtI > 5%) of the 3 gene analyzed were 17% for *P16,* 54% for *DAPK*, 60% for *E-cadherin*. *P16* and *DAPK* MtI status correlated with perineural invasion and tumor depth, respectively. In 65 patients, the 3-year and 5-year overall survival was 54.9% and 48.4%, respectively. In multivariate analysis of overall survival, presence of lymph node metastasis and *P16* methylation status were identified as an independent prognostic factors for overall survival. In patient with unmethylated of *P16,* the 3- and 6-yeat survival rates were 60.8 % and 54.9 %, respectively. In patients with a hypermethylated of *P16,* the 3- and 6-yeat survival rates were 27.3 % and 0.0 %, respectively.

P16 hypermethylation can predict overall survival in curative resected mid/distal bile duct cancer. Classification of mid/distal bile duct cancer by both genetic and epigenetic profiles may improve the capability of predicting prognosis and of applying tailored therapy in mid/distal cancer.

Key words : Bile duct cancer, Surgical outcomes, Hypermethylation, Pyroswquencing, $P16^{InK4a}$

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I. INTRODUCTION

Extrahepatic bile duct cancer is a primary malignancy arising from epithelium of extrahepatic biliary tract. Extrahepatic bile duct (EBD) carcinoma is rare malignant tumors, with a frequency of 1 per 100,000 people per year in the United state,[1] whereas the prevalence of EBD carcinoma in Korea is more frequent than that of the Western countries.[2] In patients with EBD carcinoma, the 5-year survival rate was 38.3%; a very poor prognosis has been reported.[3] In these patients, the important prognostic factors include the TNM stage, cell differentiation and histologic type.[3] Nevertheless, we often encounter a substantial number of patients whose prognosis is not consistent with the TNM stage. Therefore, other prognostic criteria are

mandatory than the TNM staging system which has been widely used at present.

DNA methylation refers to the addition of a methyl group to one of the four bases that constitute the coding sequence of DNA.[4,5] Aberrant methylation in tumor suppressor gene associated with poor prognosis.[6] DNA methylation is used as a clinical biomarker in hematopoietic malignancies and colon cancer,[6,7] however, the potential of DNA methylation as prognostic factors is only now beginning to be explored in bile duct cancer.

We reviewed the results of published studies looking at molecular biomarkers and their prognostic significance in bile duct cancer to select molecular markers. The PubMed was searched using the following keywords in varying combinations in order to identify published works in English language to December 2010: bile duct cancer, prognosis, biomarkers and molecular markers. Individual biomarkers were also included in these searches. Gallbladder cancer, Klatskin tumor, intrahepatic cholangiocarcinoma were excluded from survey.

In this study, we evaluated the potential role of DNA promoter methylation of gene involved in a variety of celluar function including adhesion (CDH1), cell division (p16) and survival (DAPK), to predict disease free survival (DFS) and overall survival (OS) in curative resected mid/distal bile duct cancer.

II. MATERIALS AND METHODS

Patients and clinical assessment

Sixty-five mid/distal bile duct carcinoma specimens obtained at Severance Hospital of Yonsei University College of Medicine from January 2000 to December 2006. This study was approved by the Institutional Review Board of Gangnam Severance Hospital. Clinical data were established from chart review. Follow up included radiographic imaging with histological verification of recurrence. Time to recurrence (TTR) and overall survival (OS) were measured in months from the date of diagnosis to the time of disease progression or death.

DNA extraction from Formalin-fixed Paraffin Embedded (FFPE) Tissue

We extracted DNA from archival FFPE samples following manual microdissection of 5-μm thick H-E stained sections into neoplastic compartments, containing at least 80% tumor cells. The microdissected tissue fragments were transferred into a microcentrifuge tube and incubated in 1.5 ml of xylene for 60 min. After centrifugation at 14,000 rpm for 3 min, the supernatant was removed. This step was repeated once. Subsequently, the tissue samples were washed in 1 ml of 99% ethanol. After centrifugation at 7200 rpm for 3 min the supernatant was discharged. The washing procedure was repeated five times. The samples were air dried at ambient temperature for 30 min. DNA was extracted using QiaAmp DNA Micro kit (Qiagen,

Valencia, CA, USA) according to the manufacturer's instructions. The eluted DNA samples were stored at -20℃

Sodium Bisulfite modification.

Bisulfite modified gDNA was prepared using EZ DNA Methylation-Gold kit (Zymo Research, USA) according to the manufacturer's instructions. The bisulfite reaction was carried out on 1μg gDNA and the reaction volume was adjusted to 20μl with sterile water and 130μl of CT conversion Reagent were added. The sample tubes were placed in a thermal cycler (MJ Research) and performed the following steps : 10min at 98℃, 2hours 30min at 64℃, and stored at 4℃.

The DNA was purified using reagent contained in EZ DNA Methylation-Gold kit (Zymo Research, USA). The converted samples were added into Zymo-Spin IC^{TM} Column containing 600μl of the M-Binding Buffer and mixed by inverting the column several times. The column was centrifuged at full speed for 30sec and discarded the flow-through. The column was washed by adding 200μl of M-Wash Buffer and spined at full speed and then 200μl of M-Desulphonation Buffer was added to the column and let stand at room temperature (20-30℃) for 15-20min. After incubation, the column was centrifuged at full speed for 30sec. The column was washed by adding 200μl of M-Wash Buffer and spined at full speed (repeat this step).

The converted gDNA was eluted by adding 20-50μl of M-Elution Buffer into the column and spin. DNA samples were finally stored at -20℃ until further use.

Pyrosequencing analysis.

We used the bisulfite pyrosequenicng method for methylation analyses of three genes, p16, CDH1, DAPK1, Each primer was designed using PSQ assay design program (Biotage, USA). The primer sequence was listed in the Table 1.

PCR reaction was carried out in a volume of 20μl with 50ng or more converted gDNA, PCR premixture (Bioneer, Korea), 2μl of 10pmole/μl Primer-S, and 2μl of 10pmole/μl biotinylated-Primer-As. The amplification was carried out according to the general guidelines suggested by Pyrosequencing: denaturating at 95℃ for 5min, followed by 45 cycles at 95℃ for 30sec, at each optimal temperature for 30sec, at 72℃ for 30sec and a final extension at 72℃ for 5min. The PCR reaction (4μl) was confirmed by electrophoresis in a 2% Agarose gel and visualized by ethidium bromide staining.

ssDNA template was prepared from 16μl biotinylated PCR product using streptavidin Sepharose® HP beads (Amersham Biosciences, Sweden) following the PSQ 96 sample preparation guide using multichannel pipets.

Fifteen picomoles of the respective sequencing primer sere added for analysis. Sequencing was performed on a PyroMark ID system with the Pyro Gold reagents kit (Biotage, USA) according to the manufacturer's instruction without further optimization. The methylation percentage was calculated by the average of the degree of methylation at 3 to 7 CpG sites formulated in pyrosequencing.

Table 1 PCR primer sequence and sequences for the 3 cancer related genes

Gene	PCR primer sequence	Size (bp)	Sequencing primer
DAPK1[15]	F: 5'- GTTGTAGTAGGTTGGAGAGAGATTGT -3' R: 5'biotin- ACACATACCCACCCAACTTT -3'	110	5'- AGAGAGATTGTTTTTAGTGA -3'
E-Cadherin[15]	F: 5'- GGTTGTGGTAGGTAGGTGAAT -3' R: 5'biotin- AACTTCCCCAAACTCACAAATACTTTAC -3'	135	5'- GTAGGTGAATTTTTAGTTAATTAG -3'
P16^{InK4a}[17]	F: 5' – AGGGGTTGGTTGGTTATTAG -3' R: 5' biotin- CTACCTACTCTCCCCCTCTC -3'	76	5'- GGTTGGTTATTAGAGGGT -3'

Data Analysis and Statistics

Pyrosequencing presents methylation as a continous value. The Methylation index (MtI) at each gene promoter, and for each sample, was calculated as the average value of $^{m}C/(^{m}C+C)$ for all examined CpG in the gene. The methylation status of CpG was analyzed as categorical variable (negative, MtI < 5%; positive, MtI>5%). All clinicopathologic variables, except age and tumor size, were used as categorical variables. Differences in continuous variables between 2 groups were evaluated by the Student *t* test, and differences in categorical variable were evaluated by the chi-square test. Kaplan-Meier method was used to calculate and display disease free survival curve, and the log-rank test was performed to determine differences among all groups. The Cox proportional hazards regression method was used to determine independent prognostic factors. $P < 0.05$ was considered statistically significant.

III. RESULTS

Cliniopathologic characteristics

We studied 65 patients selected based on sample availability. Mean age was 59.3 (± 8.8 years) and consisted of 45 men and 20 women. Of the 65 patients, 9 underwent segmental resection of bile duct, 16 underwent pancreaticoduodenectomy and 40 underwent pylorus preserving pancreaticoduodenectomy. All patients underwent dissection of the lymph nodes in the hepatoduodenal ligament, common hepatic artery, and celiac axis. All patients were not treated with postoperative adjuvant chemotherapy.

Gene specific Methylation Analysis

Pyrosequencing data was successfully generated in 100% of tested samples for *P16*, 100% for *DAPK*, 95% for *E-cadherin*. We consider low level methylation (0-5%) to represent background 'noise' with questionable significance. The significant methylation frequencies (MtI > 5%) of the 3 gene analyzed were 17% for *P16,* 54% for *DAPK*, 60% for *E-cadherin*.

Analysis of Clinicopathologic features and Epigenetic information

The clinicopathogic and molecular features of the 2 group are summarized in Table 2.

Table 2. Clinicopatholgoc characteristics according to Methylation index for the three gene studies

	P16			*DAPK*			*E-cadherin*		
	Unmethylated	methylated	*p-value*	Unmethylated	methylated	*p-value*	Unmethylated	methylated	*p-value*
Age (mean ± SD)	59.2 ± 8.4	59.8 ± 10.7	*0.475*	58.3 ± 9.3	60.2 ± 8.3	*0.350*	57.7 ± 9.8	60.1 ± 8.1	*0.126*
Sex									
Male	36	9	*0.321*	22	23	*0.507*	17	25	*0.425*
Female	18	2		8	12		6	14	
OP name									
Segmental	7	2	*0.418*	4	5	*0.962*	3	3	*0.109*
PD/PPPD	47	9		26	30		36	36	
Tumor gross									
Nodular	4	2	*0.260*	2	4	*0.508*	3	2	*0.269*
Iinfiltrating	50	9		28	31		20	37	
Tumor size (mean ± SD)	1.5 ± 0.5	1.5 ± 0.9	*0.187*	1.7 ± 0.7	1.3 ± 0.5	*0.229*	1.4 ± 0.6	1.5 ± 0.6	*0.744*
Histologic grade									
Well diff	11	3	*0.612*	8	6	*0.352*	2	10	*0.103*
Mod/poor diff	43	8		22	29		21	29	

Depth of invasion									
T1/T2	24	7	*0.300*	10	21	*0.057*	11	19	*0.317*
T3/T4	29	4		19	14		12	19	
Lymph node metasis			*0.702*			*0.786*			*0.355*
Negative	31	7		17	21		12	25	
Positive	23	4		13	14		11	14	
Perineural invasion			*0.005*			*0.399*			*0.189*
No	30	1		16	15		14	17	
Yes	24	10		14	20		9	22	

P16 and *DAPK* MtI status correlated with perineural invasion and tumor depth, respectively. But, patient age and sex, as well as tumor size, histologic grade and lymph node metastasis were not correlated with gene promoter methylation status.

Disease free survival (DFS) and Overall Survival and Epigenetic Alterations

The association of gene methylation with DFS (disease free survival) is evaluated using the Kaplan-Meier method and log-rank test. Table 3 shows unvariate analysis that included known clinicopathologic characteristics and other potential molecular biomarkers. In univariate analysis, depth of invasion, infiltrating formation tumor status had statistically significant factors as poor prognostic factors for DFS. (Table 3).

Table 3 Univariate prognostic factors of Disease Free Survival and overall survival

	DFS			Overall Survival		
	1 yr	3 yr	*p-value*	3 yr	5 yr	*p-value*
Age						
≤ 60 yr	68.9	48.5	*0.923*	54.5	45.5	*0.766*
> 60yr	75.9	41.8		51.7	48.0	
Sex						
M	69.1	42.6	*0.888*	46.5	46.5	*0.843*
F	78.9	51.0		73.7	45.1	
OP name						
Segmental	55.6	33.3	*0.183*	44.4	44.4	*0.944*
PD/PPPD	75.0	41.6		56.6	49.1	
Tumor gross						
Nodular	72.9	44.4	*0.043*	57.1	53.6	*0.003*
Infiltrating	66.7	0.0		33.3	0.0	
Tumor size						
≤ 15 mm	75.0	31.3	*0.407*	56.3	37.5	*0.303*
> 15mm	68.2	48.7		51.7	51.7	
Histologic grade						
Well diff	92.9	51.1	*0.167*	57.1	57.1	*0.703*
Mod/poor diff	66.2	43.2		54.2	45.9	
Depth of invasion						
T1/T2	83.5	54.1	*0.034*	64.5	54.8	*0.565*
T3/T4	61.3	27.5		45.2	41.9	

Lymph node metastais						
Negative	77.8	54.2	*0.110*	66.7	66.7	*0.001*
Positive	63.9	32.1		38.5	23.1	
Perineural invasion						
No	69.0	51.1	*0.559*	55.2	51.7	*0.545*
Yes	75.2	39.4		54.6	45.5	
P16						
Unmethylation	74.5	44.7	*0.107*	60.8	54.9	*0.032*
Methylation	60.5	20.2		27.3	18.2	
DAPK						
Unmethylation	71.7	37.2	*0.607*	48.3	44.8	*0.757*
Methylation	72.7	50.6		60.7	51.6	
E-cadherin						
Unmethylation	63.6	39.7	*0.522*	54.5	45.4	*0.826*
Methylation	78.0	53.3		56.8	51.4	

In multivariate analysis, depth of invasion and infiltrating formation tumor status were identified as an independent prognostic factors for disease free survival. (Table 4).

Table 4. Mutivariate Cox Regression analysis of prognostic factors of Disease Free Survival

Variables	*p-value*	Odd ratio	Confidence interval (95%)	
			Lower	Upper
Infiltrating formation tumor	*0.029*	2.980	1.119	7.938
Deep invasion of Tumor	*0.025*	2.167	1.100	4.268

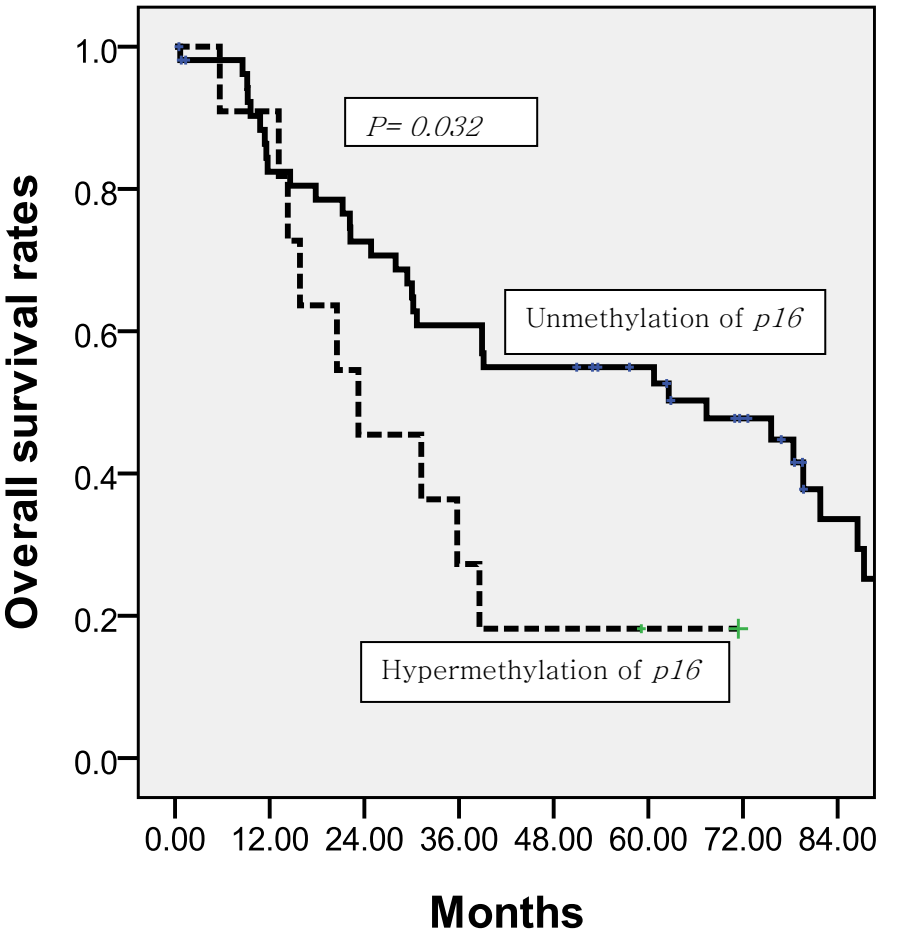
In 65 patients, the 3-year and 5-year overall survival was 54.9% and 48.4%, respectively. In univariate analysis, infiltrating formation tumor, presence of lymph node metastasis, and *P16* methylation status had significantly factors as poor prognostic factors. (Table 3) In multivariate analysis, presence of lymph node metastasis and *P16* methylation status were identified as an independent prognostic factors for overall survival. (Table 5)

Table 5. Mutivariate Cox Regression analysis of prognostic factors of Overall Survival

Variables	*p-value*	Odd ratio	Confidence interval (95%)	
			Lower	Upper
Presence of lymph node	*0.004*	2.789	1.398	5.566
Hypermethylation of *P16*	*0.025*	2.485	1.120	5.516

In patient with unmethylated of *P16,* the 3- and 5-year survival rates were 60.8 % and 54.9 %, respectively. In patients with a hypermethylated of *P16,* the 3- and 5-year survival rates were 27.3 % and 0.0 %, respectively (Fig 1)

Fig 1. Survival rates according to hypermethlyation of *P16*



IV. DISCUSSION

Extrahepatic bile duct cancer is classified as proximal, middle, or distal bile duct cancer according to the anatomical location of the tumor in the biliary tree. As a result of differences in anatomy of the bile duct and consideration of local factors that relate to respectability, extrahepatic bile duct cancers have been divided into perihilar and distal bile duct cancers and separated in perihilar and distal group and separate staging classification according to 7th edition of TNM stage. And gallbladder carcinoma is also considered in some series to represent a sub-type of bile duct cancer. But we excluded proximal bile duct cancer and gallbladder cancer in this study.

Pyrosequencing is a sequencing-by-synthesis method that allows accurate quantitative assessment of DNA methylation at CpG sites with high resolution. Pyrosequencing is more efficient and less expensive than combined bisulfite restriction analysis and is more reliable and accurate than a primer extension approach.[8,9] In this study, we have first used pyrosequencing to determine the methylation profile of mid/distal bile duct cancer in a quantitative manner and could successfully analyze nearly 100% for pyrosequencing reaction.

Tumorigenesis is a multistep process in which defects in various cancer genes accumulate. We reviewed the results of published studies looking at molecular biomarkers and their prognostic significance in bile duct cancer to select molecular markers. The PubMed was searched using the following keywords in varying combinations in order to identify published works in

English language to December 2010: bile duct cancer, prognosis, biomarkers and molecular markers. As the results, we selected putative oncogenes that had been previously identified in bile duct cancer.

The percentage of tumors with methylated gene promoters was 54% for *DAPK* and 60% for *E-cadherin*. The high methylation frequency presented for CpG islands at *DAPK* and *E-cadherin* suggests that many signaling pathways could be involved in the development and progression of mid/distal bile duct cancer. However, association of methylated *DAPK* and *E-cadherin* with patient clinicopathological data does not supports their role in tumor progression. There have some reports to date that hypermethylated *DAPK* was associated with poor prognosis in EBD cancer. But, these studies contains with gallbladder cancer and ampulla of vater cancer.[10,11]

Rate of hypermethylation of $P16^{InK4a}$ in bile duct cancer was reported to be 18-83%,[12] while that in this study was 17%, lower than that in previous studies.[10,13] This results may be due to the diversity of criteria on the selection of the subjects in the previous studies.

$P16^{InK4a}$ inactivation has been reported to be an early and critical event in tumor progression in some types of tumors.[14,15] A recent study has shown that the re-expression of *P16* inhibits lymphangiogenesis and lymphatic metastasis of pancreatic cancer in the mouse model.[16] We observed that promoter hypermethylation of the $P16^{InK4a}$ was associated with perineural invasion which was early event in bile duct cancer.[17] This result on $P16^{InK4a}$

hypermethylation suggests that *P16* hypermethylation may represent an early alteration preceding the phenotypic alteration in the mid/distal bile duct cancer.

Few prior studies have investigated bile duct cancer methylation status and prognosis. We found that $P16^{InK4a}$ promoter hypermethylation was associated with a poor prognosis, thus indicating that it could be an important biomarker in patients with mid/distal bile duct cancer. Some reports have demonstrated that $P16^{InK4a}$ promoter methylation is associtated with prognostic biomarkers in many cancers.[18-20] It remains unclear how *P16* methylation affects prognosis in this studies. Therefore, further studies will be needed.

There some limitations in this study. First, the frequency of hypermethylation of *P16* is relative small. Thus some subgroup analyses are based on small number of patients, which are relected in the wide confidence intervals. These results should be interpreted carefully. Second, our proportion of our study participants are asian people with mid/distal bile duct cancer, and thus our results are not generalized to other race.

Nonetheless, we have some advantage include a homogenous and large volume of patients diagnosed with mid/distal bile duct cancer. This approach allows for greater generalizability and higher sensitivity, since multiple factors were taken into consideration in examining the associations with survival. Moreover, there are few epigenetic studies that have reported on the prognostic value of gene promoter methylation status in bile duct cancer.

V. CONCLUSION

In conclusions, this candidate-base study describes the hypermethylation profile of three oncogenes in mid/distal bile duct cancer to elucidate the clinical implication of aberrant DNA hypermethylation. $P16^{InK4a}$ hypermethylation can predict overall survival in curative resected mid/distal bile duct cancer. Further study is warranted to validate the use of high frequency methylated genes as potential biomarkers for prognosis in mid/distal bile duct cancer. Classification of mid/distal bile duct cancer by both genetic and epigenetic profiles may improve the capability of predicting prognosis and of applying tailored therapy in mid/distal cancer. Therefore, further research with large number of patients is needed to investigate the pathophysiology and possible role of DNA hypermethylation in patients with mid/distal bile duct cancer.

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ABSTRACT(IN KOREAN)

P16 and *MGMT* hypermethylation predicts surgical outcomes in curative resected mid/distal bile duct cancer.

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박 준 성

연구 목적 : 우리나라에서 담도암은 소화기 암중 위암, 간암, 대장암 다음으로 흔한 암이며, 담도암의 예후는 매우 불량한 것으로 보고 되며, 예후에 관련된 인자로서 진단 당시의 병기, 암세포의 분화도 및 조직유형이 중요하다. 그럼에도 불구하고, 진단 당시의 병기와는 다른 환자들의 예후를 드물지 않게 경험하여, 현재 많이 이용되는 TNM분류법 만이 아닌 추가적인 예후 인자의 필요성이 제기된다. 따라서 본 연구는 암의 후성적 변화인 프로모터 CpG island 과메틸화 여부가 담도암에서 환자의 예후를 예측할 수 있는 진단적 표지자로서의 임상적 유용성에 대해 분석해 보고자 한다.

대상 및 방법: 2000년 1월부터 2006년 12월까지 담도의 원발성 악성 종양으로 연세대학교 의과대학 세브란스 병원에 입원하여 근치 수술을 받은 환자 중 조직의 보관상태가 양호하고 의무 기록과

수술 후 생존 여부의 추적이 가능한 65명을 대상으로 하였다. 각 유전자의 CpG island의 과메틸화를 pyroswquencing으로 정량화하여 기존의 예후 인자와의 비교하였다.

결과: 전체 65명 대상 환자에서 CpG island의 과메틸화는 P16에서는 17%, DAPK에서는 54%, E-cadherin에서는 60%이었다., P16의 과메틸화는 신경절 침윤, DAPK의 과메틸화 정도는 종양의 침윤 정도와 통계학적 연관성이 있었다. P16의 과메틸화 정도에 따른 5년 생존율은 과메틸화군이 0%, 비메틸화군이 54.9%로 통계학적으로 유의한 차이가 있었으며, 다변량 분석에서도 중요한 예후인자로 판명되었다.

결론:, P16의 과메틸화를 보인 환자들이 더욱 나쁜 예후를 보여 P16의 메틸화 여부가 담도의 원발성 악성 종양에서 예후 인자로서의 기능은 할 수 있다고 여겨진다.

핵심되는 말 : 담도암, CpG island, 과메티화, 생존률, *P16*