ORIGINAL ARTICLE

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Sodium Iodide Symporter and Phosphatase and Tensin Homolog Deleted on Chromosome Ten Expression in Cholangiocarcinoma Analysis with Clinicopathological Parameters

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Background/Aims: This study was performed to investigate the correlation of sodium iodide symporter (NIS) expression with the functionality and loss of phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression in human cholangiocarcinoma (CCA). **Methods:** Immunohistochemistry for the expression of NIS and PTEN was performed in 60 biopsy specimens of CCA. The clinicopathological parameters were retrospectively identified from medical records. The expression pattern of NIS and loss of PTEN expression were analyzed in association with the clinicopathological characteristics, including survival. **Results:** Normal biliary trees displayed NIS expression, but hepatocytes did not. NIS expression was divided into two patterns: cytoplasmic and membranous. Fifty-nine cases, all except for one case, displayed NIS expression in tumor cells. Twenty-two cases (33.3%) were mixed pattern, and 39 cases (65.05%) were cytoplasmic pattern; the pure membranous pattern was not noted. There was no association between the NIS expression pattern and clinicopathological parameters, including age, sex, differentiation grade, T stage and tumor, node, metastasis stage ($p>0.05$). The survival rates were similar among various NIS expression patterns. Normal hepatocytes and biliary trees exhibited PTEN expression in the nucleus and cytoplasm. CCA cells displayed nuclear staining. Thirty-six (60.0%) of 60 cases displayed a loss of PTEN expression. The loss of PTEN expression was observed in the advanced T-stage group ($p=0.0036$), but there was no association between the loss of PTEN expression and other clinicopathological parameters ($p>0.05$). No association between the loss of PTEN expression and survival was noted. **Conclusions:** NIS is expressed in most types of human CCA. The expression pattern suggests a role in cancer development. PTEN loss expression is common in the context of human CCA, especially in the advanced T stage. **(Gut Liver 2012;6:374-380)**

Key Words: Cholangiocarcinoma; Sodium iodide symporter; Chromosome ten; Immunohistochemistry

INTRODUCTION

Cholangiocarcinoma (CCA) is a notoriously fatal cancer, because of the late clinical presentation, the hardship to diagnose and the lack of effective therapeutic modalities. Even though CCA is rare, the incidence as well as the mortality rate of intrahepatic CCA increase worldwide.[1,2] A few known risk factors related with chronic inflammatory conditions of biliary trees have been noted. But the pathogenesis of the development and progression of CCA and the cause of recently increasing incidence of intrahepatic CCA are unclear. Currently, efforts to develop the novel therapeutic modalities were implicated through inhibition of targeting molecules in critical pathways of carcinogenesis.[3]

The sodium iodide symporter (NIS) is an intrinsic membrane glycoprotein and plays a key role in thyroid hormone production by efficiently accumulating iodide from circulation into thyroid follicular cells, providing the fundamental background for the ^{131}I radiotherapy and diagnosis of some thyroid diseases. Thyroid follicular cells express the NIS at the basolateral plasma membrane.[4] NIS mRNA and protein expressed in variable non-thyroidal tissues also, including salivary and lacrimal glands, gastric mucosa, kidney and mammary gland, which suggest that

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iodide in these tissues might be also transported by functional NIS protein.[5-7] In breast cancer, NIS induced through cAMP and PI3K signaling pathway.[8] In the other organs where express NIS locating in the cytoplasms of tumor cells.[9] Recently, strong and distinct membranous NIS expression in human CCA has been demonstrated, promising as a crucial target for radioiodine therapy.[10]

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor gene and acts as an intermediate regulator of normal cell growth by the PI3K signaling pathway, negatively. Its mutation, such as deletion or inactivation was found during development and progression of many human cancers.[11-13]

In this study, NIS and PTEN expression with their intracellular localization was investigated in human CCA by using immunohistochemistry. The relationship between the expression of NIS and PTEN, and clinicopathological parameters including survival rate were analyzed to search the role of target molecules in the progression of CCA.

MATERIALS AND METHODS

1. Clinicopathologic materials

This study consisted of 60 cases of CCA sorted out from the archival files of the Department of Pathology at Dong-A University Hospital from 2003 to 2006. The materials were all liver biopsy specimens. Pathological and clinical records were reviewed and definitive or suspicious metastatic adenocarcinomas were excluded. The pathological slides with hematoxylin and eosin staining and immunohistochemistry for differential diagnosis were reviewed to confirm the original diagnosis based on the World Health Organization criteria[14] by two pathologists. No chemotherapy or radiotherapy before diagnosis had been administered in any of these cases. This study was approved by the Institutional Review Board of Dong-A University Hospital.

2. Immunohistochemistry

Immunohistochemical study for NIS was performed on formalin-fixed, paraffin-embedded, 4 μm-thick tissue sections, using DAKO EnVision kit (DakoCytomation, Glostrup, Denmark). The primary antibody was a mouse monoclonal antibody directed against hNIS (Neomarkers, Fremont, CA, USA) and PTEN (DBS, Pleasanton, CA, USA) used in a 1:150 and 1:50 dilution with nonspecific reaction reducing solution, respectively. Deparaffinization of all the sections was performed through a series of xylene baths, and rehydration was performed with graded alcohol solutions. To enhance immunoreactivity, microwave antigen retrieval was performed at 750 W for 30 minutes in citrate buffer (pH 6.0). After blocking endogenous peroxidase activity with 5% hydrogen peroxidase for 10 minutes, for NIS, protein blocking solution was treated for 30 minutes and primary antibody incubation was performed at 4°C, overnight. For PTEN, primary antibody was incubated for 1 hour at room temperature. Secondary antibody was applied for 30 minutes at room temperature. After washing the tissue samples in Tris-buffered saline for 10 minutes, 3, 3'-diaminobenzidine tetrachloride was used as a chromogen, and Mayer's hematoxylin counterstain was applied. Thyroid tissue from Graves' disease and tonsil were used as a positive control of NIS and PTEN, each.

3. Interpretation of immunohistochemistry

Immunoreactivity of NIS expression was evaluated by the presence of cytoplasmic or membranous staining. The staining intensity was divided into negative, weak and strong. Positivity was defined when 10% or more of tumor cells were stained in cytoplasm or cell membrane.[9]

Immnunoreactivity of PTEN expression was defined by the presence of nuclear staining in tumor cells. Staining grading was classified by the extent and the intensity, applying 'quickscore' method proposed by Detre *et al*.[14] Briefly, the proportion of tumor cells with nulcear staining was categorized into 1 to 6 scores (1, 0% to 4%; 2, 5% to 19%; 3, 20% to 39%; 4, 40% to 59%; 5, 60% to 79%; 6, 80% to 100%). The average intensity, corresponding to negative, weak, moderate and strong staining was assigned a score from 0 to 3, each. I used an additive quickscore by addition of extent score and intensity score, and defined the 3 or more of additive score as positivity.

4. Statistical analysis

Statistical analyses were performed using MedCalc for Windows version 9.3.9 (MedCalc software, Mariakerke, Belgium). Data were analysed by the chi-square test and chi-square test for trend. Survival was analysed by Kaplan-Meir curve and the difference was analysed by log-rank test. The p-values less than 0.05 were considered as statistically significant.

RESULTS

1. Clinicopathological characteristics

The ages of the 60 patients ranged from 33 to 78 years (median age, 60 years), and there were 46 men (76.7%) and 14 women (23.3%). All cases were intrahepatic CCA. Histologically, they consisted of 6 well differentiated (10.0%), 29 moderately differentiated (48.3%), and 25 poorly differentiated (41.7%) CCA. T stage of CCA showed T1, 13 cases (21.7%), T2, 5 cases (8.3%), T3, 38 cases (63.3%) and T4, 4 cases (6.7%). According to tumor, node, metastasis (TNM) stage, stage I was 7 cases (16.7%), stage II, 4 cases (6.6%), stage III, 37 cases (61.7%) and stage IV, 12 cases (20.0%).

2. NIS expression in CCA

In non-neoplastic liver (data not shown), medium to large bile ducts showed basolateral and luminal membranous expression and bile ductules showed cytoplasmic expression. Reactively

proliferating bile ductules showed stronger cytoplasmic expression. Hepatocytes did not show NIS expression.

Fifty-nine cases of 60 CCA showed NIS expression in cancer cells. NIS was expressed in cytoplasm or membrane of tumor cells. In cytoplasmic pattern, staining was thick granular (Fig. 1A) or fine granular (Fig. 1B). In membranous pattern, luminal

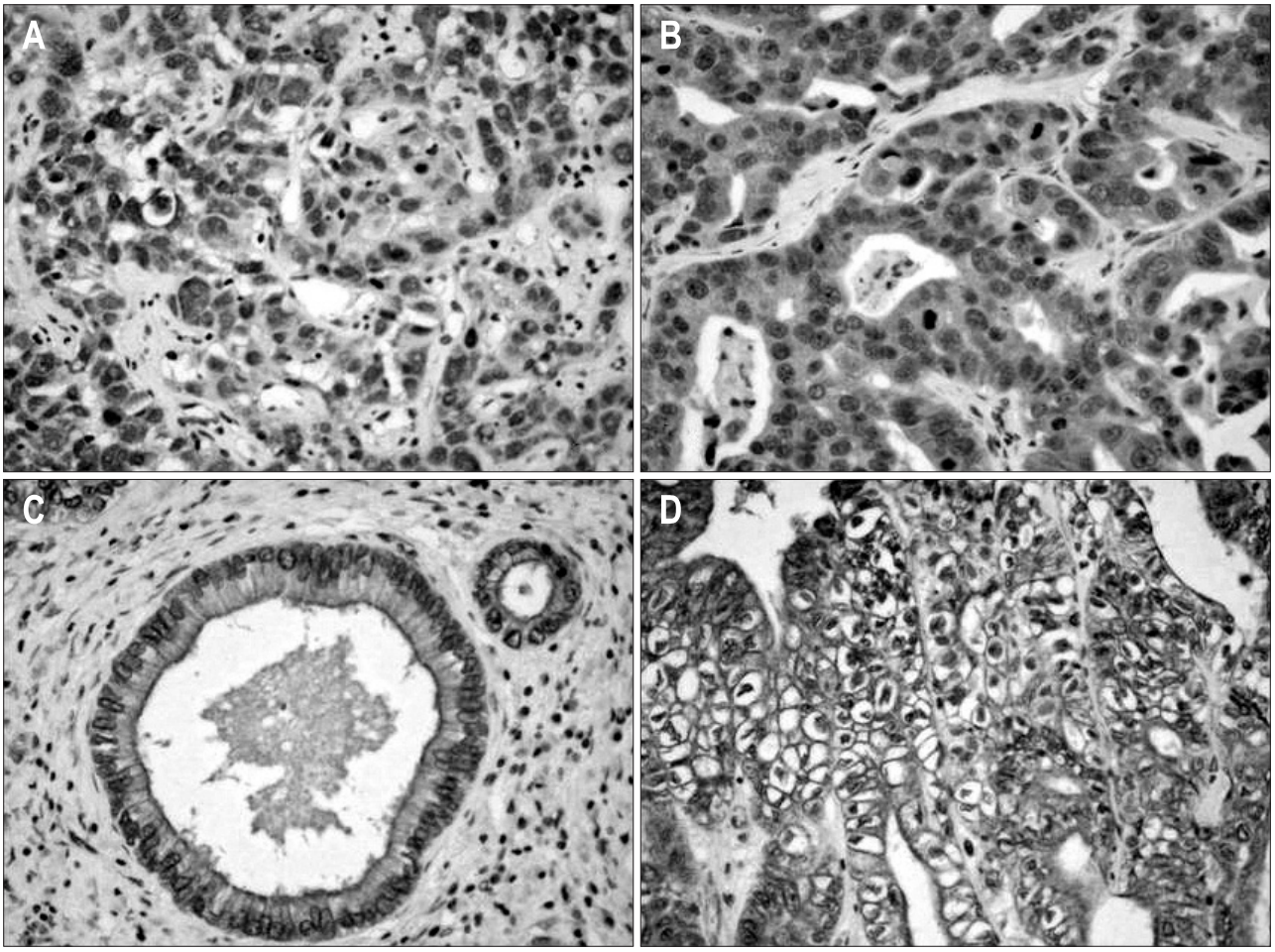

Fig. 1. hNIS expression in cholangiocarcinomas (CCA) (anti-hNIS, IHC, ×400). (A) Poorly differentiated CCA cells exhibit granular cytoplasmic staining. (B) Moderate differentiated CCA cells display homogenous cytoplasmic staining. (C) Well-differentiated CCA cells exhibit luminal surface and lateral membranous staining. (D) Moderately differentiated CCA cells exhibit membranous staining.
NIS, sodium iodide symporter.

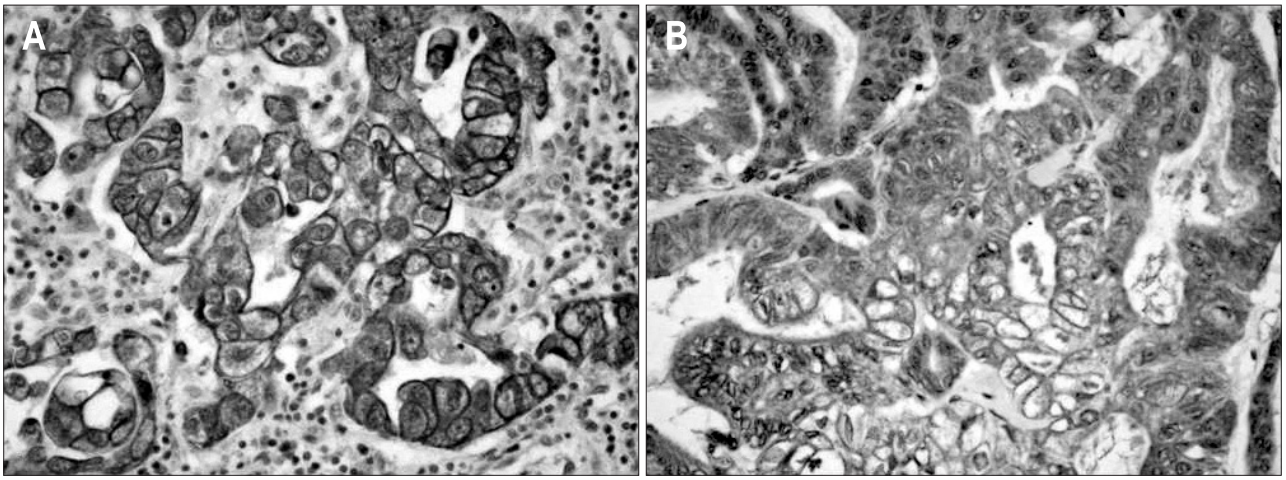

Fig. 2. hNIS expression in cholangiocarcinoma (CCA) (anti-hNIS, IHC, ×400). Moderately differentiated CCA cells display concurrent cytoplasmic and membranous staining (A) and cytoplasmic or membranous staining (B).
NIS, sodium iodide symporter.

and basolateral membrane (Fig. 1C) or whole cell membrane (Fig. 1D) were stained. The cases representing concurrent cytoplasmic and membranous staining in same cancer cells (Fig. 2A) or mixed cytoplasmic and membranous staining (Fig. 2B) in a cancer were categorized as cytoplasmic and membranous pattern. Twenty cases (33.3%) showed cytoplasmic and membranous pattern, and 39 cases (65.0%) showed cytoplasmic pattern. Thirty-seven cases (61.7%) showed strong staining intensity and 22 cases (36.7%) showed weak intensity.

3. PTEN expression in CCA

In non-neoplastic liver, hepatocytes showed strong nuclear and weak cytoplasmic staining and biliary epithelial cells showed nuclear staining, as well (data not shown). Thirty-six cases (60.0%) of CCA showed loss of PTEN expression (Fig. 3) and 24 cases (40.0%) showed positive PTEN expression (Fig. 4).

4. Relation of hNIS expression pattern and loss of PTEN expression with clinicopathological parameters

There were no significant associations between NIS expression pattern and clinicopathological parameters, such as sex, age, histologic differentiation, T stage and TNM stage (Table 1).

The loss of PTEN expression related with tendency of advanced T stage (p=0.0036). Otherwise, there were little relationships between PTEN expression and clinicopathological parameters, such as age, sex, histologic differentiation, and TNM stage (Table 1).

5. Relation of NIS expression pattern and loss of PTEN expression with survival

Follow-up data of all 60 cases were evaluated and 6 cases (10.0%) were alive. Ten cases were lost. There were no relation between survival and NIS expression pattern (p=0.8473, log-rank test) and loss of PTEN expression (p=0.5292, log-rank test (Fig. 5). CCA, showing coincidental membranous NIS expression and loss of PTEN expression were 14 cases (23.3%). This functional group versus others with clinicopathological parameters (Table 2) and survival (Fig. 5) showed no associated relationship between them (p=0.8533, log-rank test).

DISCUSSION

CCA is the cancer originated from intrahepatic or extrahepatic biliary tree. Curative surgical resection is the only hope for

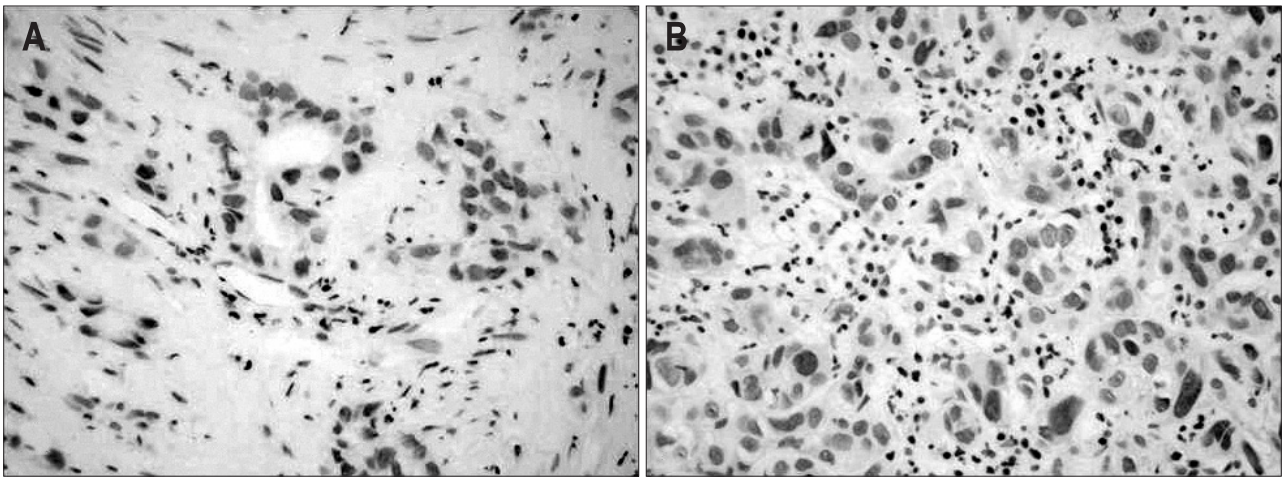

Fig. 3. Loss of phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression in cholangiocarcinoma (CCA) (anti-PTEN, IHC, ×400). Moderately differentiated CCA cells (A) and poorly differentiated CCA cells display negative staining (B).

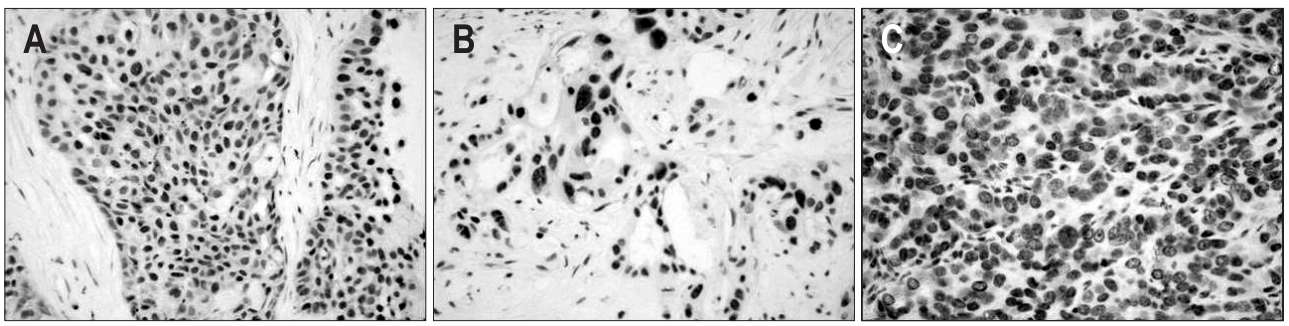

Fig. 4. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression in cholangiocarcinoma (CCA) (anti-PTEN, IHC, ×400). Moderately differentiated CCA cells display distinct nuclear staining in a focal and weak pattern (A), focal and strong pattern (B), and diffuse and strong pattern (C).

Table 1. Relationship between the Expression Pattern of NIS and PTEN with Clinicopathological Parameters in Cholangiocarcinomas

		NIS			PTEN		
		Cyto and memb pattern	Cyto pattern	p-value*	Negative staining	Positive staining	p-value*
Total		20	39		36	24	
Gender				0.2259			0.5419
Male	45 (76.3)	13	32		31	15	
Female	14 (23.3)	7	7		5	9	
Age				0.3018			0.3306
<60	27 (45.8)	11	16		17	10	
≥60	32 (54.2)	9	23		19	14	
Differentiation				0.8154			0.4605
Well	6 (10.2)	2	4		5	1	
Moderate	29 (49.2)	12	17		17	12	
Poor	24 (40.6)	8	16		14	11	
T stage				0.3053			0.0036†/0.2200
T1	13 (22.0)	6	7		5	8	
T2	5 (8.5)	1	4		2	3	
T3	37 (62.7)	13	24		29	9	
T4	4 (6.8)	0	4		0	4	
TNM stage				0.6664			0.2605
I	7 (11.9)	3	4		3	4	
II	4 (6.8)	1	3		2	2	
III	36 (61.0)	15	21		26	11	
IV	12 (20.3)	3	9		5	7	

NIS, sodium iodine symporter; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Cyto, cytoplasmic; Memb, membranous; TNM, tumor, node, metastasis.
*Chi-square test; †Chi-square test for trend.

cure. Conventional chemotherapy and radiotherapy have been ineffective in patients with inoperable CCA. Even though the patient received curative surgical resection, the recurrence rate is high. Because the mortality of CCA is high and its incidence is increasing, new therapeutic modalities and diagnostic methods are required. Several molecular targeting strategies against CCA have been developed and progressed to preclinical trials.[3] Although the pathogenesis of CCA development and progression remains unclear, several molecular alterations in relation with proliferation, growth, anti-apoptosis and invasiveness have been reported.[2]

Recently, human CCA has been reported to express NIS in tumor cells.[10] NIS expression in normal and cancer cells is interesting because it might be a target molecule for new diagnostic method or therapeutic modality. NIS expression already has been reported to predict ^{131}I uptake, which may lead to effective radioiodide therapy. One of the novel study showed prostate cancer cells expressed exogenous NIS after adenoviral gene transfer and it retained for a long time, thus suggested that potential therapeutically effective ^{131}I therapy is applicable in NIS-expressing extra-thyroidal cells, even in the absence of I- organification.[15] In normal liver, as previous reports mentioned NIS in intrahepatic biliary trees,[10] weak but constant NIS expression in cholangiocytes was observed and those findings are consistent with biliary NIS acting as a transporter. NIS expressed on the plasma membrane of bile ducts, and in the cytoplasm of bile ductules, but NIS expression was significantly stronger in proliferating bile ductules than in normal bile ducts. Such NIS up-regulation in proliferating bile ductular cells was also reported in breast tissue.[9]

In our study, the remarkable NIS expression in almost all of intrahepatic CCA except 1 was demonstrated. Most of the CCA cells (65%) expressed NIS in the cytoplasms. Twenty cases (33.3%) expressed NIS in the cell membrane in addition to cytoplasm. By literatural review, only one study was reported worldwide which showed immnunohistochemical results for NIS expression of human CCA by Liu *et al*.[10] They showed NIS accumulation in the cytoplasm in 11 of the total 20 patients, the plasma membrane in 2 patients, and in both compartments in 7 patients respectively. In this study, the NIS expressed in larger numbers of patients and represented similar results although exclusive membranous staining was not observed. Both studies

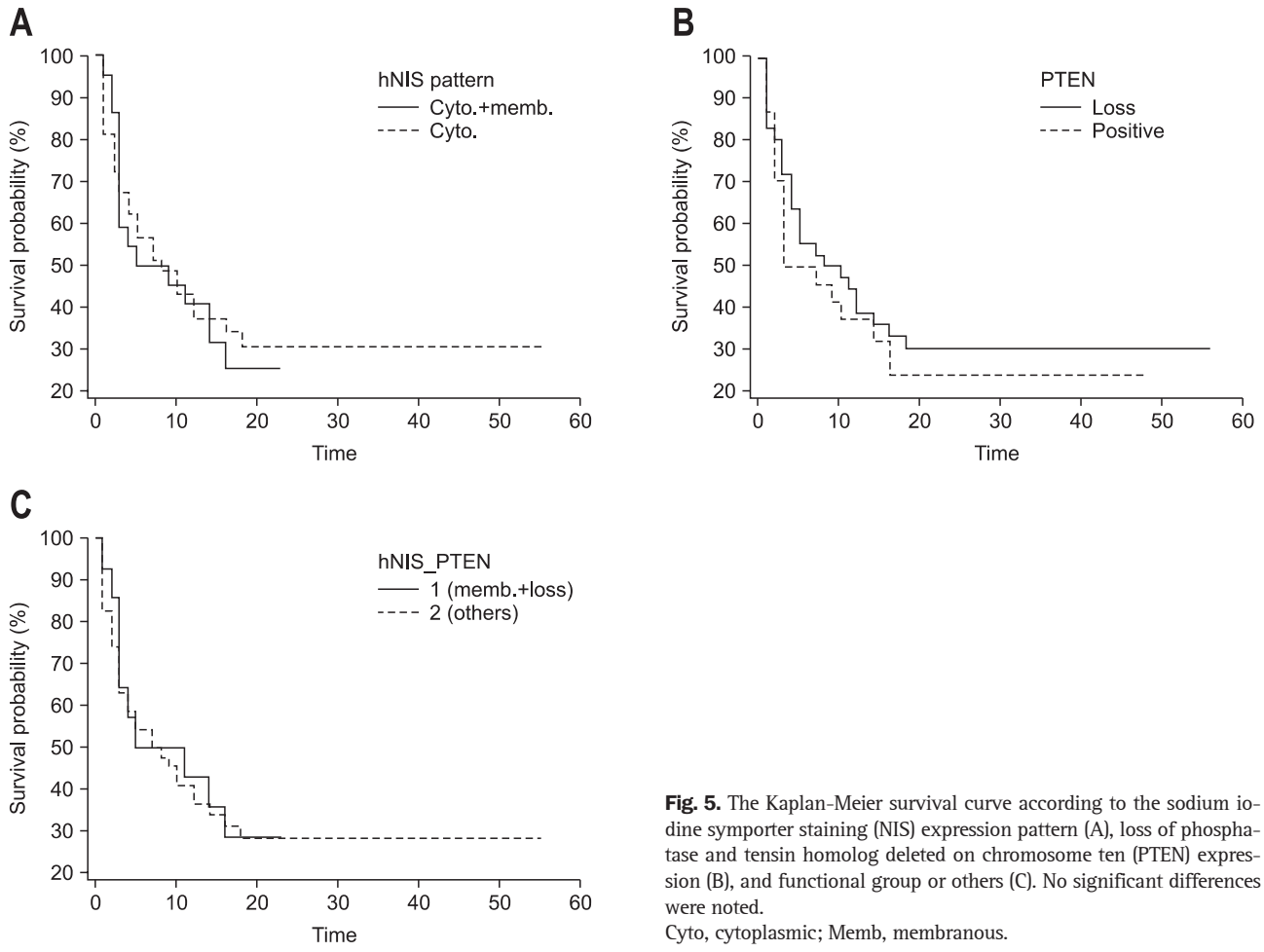

Fig. 5. The Kaplan-Meier survival curve according to the sodium iodine symporter staining (NIS) expression pattern (A), loss of phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression (B), and functional group or others (C). No significant differences were noted.
Cyto, cytoplasmic; Memb, membranous.

revealed that a significant proportion of human CCA expressed NIS at the cell and thus NIS might be some function in cancer development especially in membranous expression patterns. The results of these studies suggest that ^{131}I radiotherapy may have a therapeutic option for CCA, as was for breast cancer. But, NIS expression did not affect clinical results, it suggest that NIS affect only in cancer development not in cancer progression and cancer differentiation.

In breast cancers, up-regulation of mammary NIS glycoprotein is induced through the cAMP and PI3-K signaling.[8] Activation of PI3K alone is sufficient to increase NIS expression and radioiodide uptake in breast cancer cells, and cAMP activation increases NIS promotor activity and NIS mRNA but is not sufficient to increase radioiodide uptake. PI3K signaling pathway involved in the regulation of NIS expression seems to have different roles in thyroid cells or extra-thyroidal cells, even in normal or cancer cells. PTEN is a tumor suppressor gene and has been known to regulate normal cell growth and suppress tumor cell growth by antagonizing tyrosine kinases in the PI3K signaling pathway. Its mutation, deletion or inactivation were found during development and progression of many human cancers.[12,13] The involvement of PTEN loss in development and progression of human CCA, especially intrahepatic, has been rarely studied. Even limited biopsied intrahepatic CCA, 60.0% of 60 biopsied CCA showed loss of PTEN expression, and the loss of PTEN was observed in advanced T stage group, in this study. Chung *et al*.[16] reported that the decreased PTEN expression in CCA was observed in the patients with increasing depth of invasion, advanced T classification, and progressed stage grouping, and the presence of invasion of the pancreas and duodenum. Those CCA were not intrahepatic but all extrahepatic cases. With the status of PTEN expression in CCA, further evaluation of the relation between NIS expression and PTEN status was pursued. Concurrent membranous NIS expression and loss of PTEN expression were occurred in 23.3%. Although they are not related with any clinicopathological impacts, it is supposed these cases can be as functional group on the basis of PI3K signaling pathway in NIS regulation of cancer cells. Because this study was done in small numbers of cases and not evenly stratified in each stage, further studies with larger number of cases including surgically removed specimens and molecular level experiments should be followed.

This study showed that a significant proportion of CCA expressed NIS in the plasma membrane and two third of CCA lost PTEN expression. Although the loss of PTEN related with advanced T stage group, the functional group, showing concurrent

Table 2. Relationship of the Functional Group with the Clinicopathological Parameters in Cholangiocarcinomas

		Functional group*	Others	p-value†
Total		14	46	
Gender				0.3408
Male	46 (76.7)	10	36	
Female	14 (23.3)	4	10	
Age				0.5319
<60	27 (45.0)	7	20	
≥60	33 (55.0)	7	26	
Differentiation				0.7417
Well	6 (10.0)	1	5	
Moderate	29 (48.3)	8	21	
Poor	25 (41.7)	5	20	
T stage				0.4365
T1	13 (21.7)	2	11	
T2	5 (8.3)	0	5	
T3	38 (63.3)	12	26	
T4	4 (6.7)	0	4	
TNM stage				0.4158
I	7 (11.7)	1	6	
II	4 (6.6)	0	4	
III	36 (61.7)	11	25	
IV	12 (20.0)	2	10	

TNM, tumor, node, metastasis.
*The functional group comprises cases exhibiting cytoplasmic and membranous sodium iodine symporter staining and negative phosphatase and tensin homolog deleted on chromosome ten staining; †Chi-squared test.

membranous NIS expression and loss of PTEN was not associated with clinicopathlogical parameters and survival. In conclusion, these data suggest that NIS might be a candidate target molecule for ^{131}I radioiodine therapy in CCA. The significance of PTEN loss in the development of CCA should be further studied.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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REFERENCES

1. Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD. Cholangiocarcinoma. Lancet 2005;366:1303-1314.
2. Lazaridis KN, Gores GJ. Cholangiocarcinoma. Gastroenterology 2005;128:1655-1667.
3. Sirica AE. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. Hepatology 2005;41:5-15.
4. Dai G, Levy O, Carrasco N. Cloning and characterization of the thyroid iodide transporter. Nature 1996;379:458-460.
5. Spitzweg C, Joba W, Eisenmenger W, Heufelder AE. Analysis of human sodium iodide symporter gene expression in extrathyroidal tissues and cloning of its complementary deoxyribonucleic acids from salivary gland, mammary gland, and gastric mucosa. J Clin Endocrinol Metab 1998;83:1746-1751.
6. Spitzweg C, Joba W, Schriever K, Goellner JR, Morris JC, Heufelder AE. Analysis of human sodium iodide symporter immunoreactivity in human exocrine glands. J Clin Endocrinol Metab 1999;84:4178-4184.
7. Spitzweg C, Dutton CM, Castro MR, et al. Expression of the sodium iodide symporter in human kidney. Kidney Int 2001;59:1013-1023.
8. Knostman KA, Cho JY, Ryu KY, et al. Signaling through 3',5'-cyclic adenosine monophosphate and phosphoinositide-3 kinase induces sodium/iodide symporter expression in breast cancer. J Clin Endocrinol Metab 2004;89:5196-5203.
9. Wapnir IL, van de Rijn M, Nowels K, et al. Immunohistochemical profile of the sodium/iodide symporter in thyroid, breast, and other carcinomas using high density tissue microarrays and conventional sections. J Clin Endocrinol Metab 2003;88:1880-1888.
10. Liu B, Hervé J, Bioulac-Sage P, et al. Sodium iodide symporter is expressed at the preneoplastic stages of liver carcinogenesis and in human cholangiocarcinoma. Gastroenterology 2007;132:1495-1503.
11. Alvaro D, Barbaro B, Franchitto A, et al. Estrogens and insulin-like growth factor 1 modulate neoplastic cell growth in human cholangiocarcinoma. Am J Pathol 2006;169:877-888.
12. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943-1947.
13. Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997;15:356-362.
14. Detre S, Saclani Jotti G, Dowsett M. A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. J Clin Pathol 1995;48:876-878.
15. Spitzweg C, O'Connor MK, Bergert ER, Tindall DJ, Young CY, Morris JC. Treatment of prostate cancer by radioiodine therapy after tissue-specific expression of the sodium iodide symporter. Cancer Res 2000;60:6526-6530.
16. Chung JY, Hong SM, Choi BY, Cho H, Yu E, Hewitt SM. The expression of phospho-AKT, phospho-mTOR, and PTEN in extrahepatic cholangiocarcinoma. Clin Cancer Res 2009;15:660-667.