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p53 Protein Expression in Extrahepatic Bile Duct Cancer

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p53 mutations, a tumor suppressor gene located on chromosome 17p, are the most common genetic alterations found in human cancers. Although the p53 expression or mutation has been investigated in a variety of cancers there have been very few studies in extrahepatic bile duct cancers. In this study, we investigated the immunohistochemical expression of p53 in formalin fixed paraffin embedded archival specimens of 36 extrahepatic bile duct cancers in which p53 expression was found in eighteen(50%) cases. There was no significant difference in age, gender, size of tumor, histologic grade, extent of tumor involvement, lymphnode metastasis and tumor resectability according to p53 immunoreactivity. Comparison of survival duration according to p53 expression showed no significant difference.In conclusion, we reported 50 percent of p53 expression in extrahepatic bile duct cancers by immunohistochemical staining and we found no prognostic significance of p53 expression in clinicopathologic parameters.

Key Words: p53 expression, extrahepatic bile duct cancers

Extrahepatic bile duct cancer is a relatively uncommon disease accounting for 0.01~0.46% in all autopsy cases. Choloestatic hepatitis, inflammatory bowel disease, cystic fibrosis and various infections of the biliary tree are associated with extrahepatic bile duct cancers (Bismuth and Malt, 1979).

Multistep genetic changes are associated with the development of human cancers. These changes include activation of cellular proto-oncogenes and inactivation of tumor suppressor genes (Fearon and Vogelstein, 1990). p53 gene is a tumor suppressor gene located on the short arm of chromosome 17 (Isobe *et al.* 1986). It encoded a 53 kD nuclear phosphoprotein which is capable of binding to DNA and acts as a transcriptional factor. Mutation of the p53 gene frequently occurs in association with neoplastic transformation in human tissues (Nigro *et al.* 1989). This mutation often stabilizes the p53 protein, causing it to accumulate within the nucleus to the extent that its overexpression becomes detectable immunohistochemically (Harris 1990). Mutations of the p53 gene have been found in a wide variety of tumors. The frequency of mutation is especially high in lung (Iggo *et al.*1990), colon (Vogelstein *et al.*1989), and bladder carcinomas (Sidransky *et al.* 1991). However there have been very few reports of p53 overexpression in extrahepatic bile duct cancer to our knowledge, although Teh *et al.* (1994) recently reported a 66% positive rate of extrahepatic bile duct/ampullary carcinoma. Thus, we examined the p53 expression in formalin fixed paraffin embedded surgical specimens of extrahepatic bile duct cancer and evaluated its clinical significance in relation to various clinicopathologic parameters.

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MATERIALS AND METHODS

Materials

Thirty-six specimens of the extrahepatic bile duct cancers were collected from the clinical files of Yonsei University College of Medicine between 1988 January and 1992 December. All of the tissue had been fixed in 10% buffered formalin and embedded in paraffin. Representative blocks were selected for immunohistochemistry and 4 μm sections were made from each paraffin block. As positive controls, formalin fixed, paraffin embedded surgical specimens of colon carcinoma were used and processed as described as above. As negative controls, adjacent residual normal bile duct epithelium of the surgical specimens were used. A review of the clinical records was done and the information of the survival duration was obtained from the clinical records, telephone or direct interviews and mail.

Immunohistochemistry

The sections were immunostained for p53 protein by the biotin-streptavidin amplified (B-SA) method using the monoclonal antibody DO-7 (Dako Corp., Santa Barbara, CA, USA). Four μm sections were deparaffinized and hydrated through graded alcohols. Endogenous peroxidase was blocked by immersing the sections for 30 min in 3% hydrogen peroxide in absolute methanol. Nonspecific binding was blocked by incubating the slides in 5% bovine serum albumin in Tris-buffered saline (TBS) for 30 min. The sections were subsequently washed with TBS and incubated with the mouse monoclonal antibody (DO-7, diluted 1:20) for 2 hours at room temperature. The sections were treated for 30 min with secondary biotinylated anti-immunoglobulins diluted 1:10 at room temperature. Next, the sections were incubated for 30 min with concentrated alkaline phosphatase labeled streptavidin complex (StrAviGen Super Sensitive Immunostaining Kit; BioGenex) at room temperature. Reaction products were developed by application of substrate solutions containing naphthol AS-MX phosphate in Tris-HCL which gave a bright red color. Nuclei were counterstained with hematoxylin. The slides were washed with TBS and coverslipped with an aqueous mounting medium. When analyzing p53 reactivity, we considered as positive only the cases in which there was unequivocal nuclear staining.

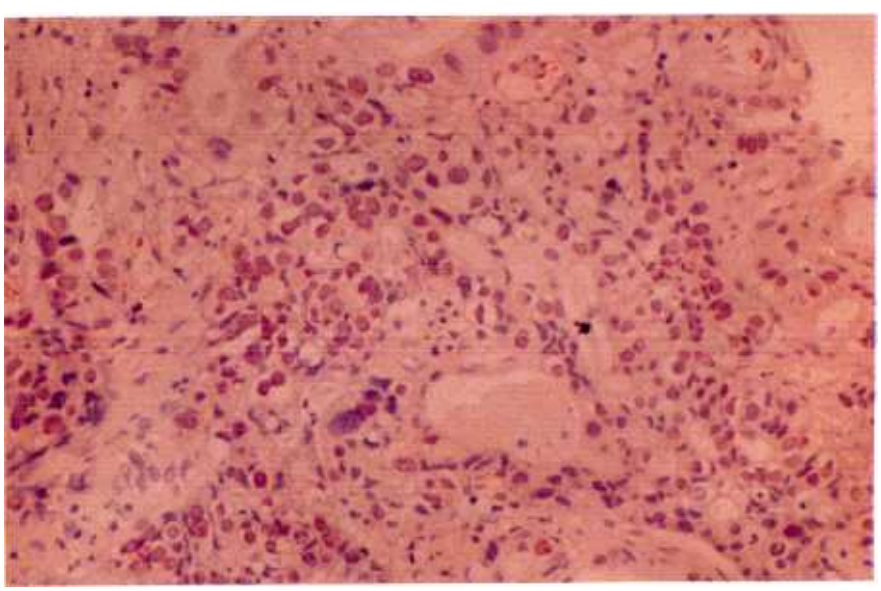

Fig. 1. Positive p53 staining in adenocarcinoma of extrahepatic bile duct cancer(× 100).

Statistical analysis

Either the Chi-square test or the two tailed Fisher's exact test was used to analyze the data. Kaplan- Meier survival curves were constructed from the survival duration and differences were assessed using the log rank test. Probability value of less than 0.05 were regarded as significant.

RESULTS

P53 staining was not present in the non-tumorous cells of the specimens in which p53 immunoreactivity of the cancer cells was demonstrable. In colon cancer cells used as positive controls, positive nuclear staining was demonstrable. Eighteen out of 36 carcinomas (50%) were p53 positive. The immunoreactivity was mainly nuclear with only occasional cytoplasmic staining (Fig. 1).

The tumors were grouped according to the presence or absence of p53-immunoreactivity and they were analyzed according to age, gender, histologic grade, tumor size, and histologic staging for statistical analysis (Table 1). The age and gender distribution of the two groups were not statistically different. There was no increase in the frequency of p53 positivity among the poorly differentiated adenocarcinoma when compared with the better differentiated histologic grades. Although not statistically significant, all the well differentiated types were p53 negative while moderate to severe grade types tended to show more p53 positivity. When the extent of the tumor mass was analyzed on the basis of the criteria of the American Joint Committee on Cancer (AJCC), there was no significant difference in histologic staging according to p53 immunoreactivity. Surgical resectability was analyzed for the correlation with p53 expression and although not statistically significant ($p = 0.08$), extrahepatic bile duct cancers which were surgically resectable, tended to harbor more p53 positive staining (10 of 18 cases; 55.5%) while 5 of 18 cases (27.7%) of the p53 negative group were resectable by surgery. The longitudinal dimension of the tumor mass was evaluated and

Table 1. Relationship between p53 immunoreactivity of tumors and clinicopathological characteristics

	p53 positive (n=18)	p53 negative (n=18)
Sex	12:6	13:5
Mean ages(years)	57.4±13.7	59.3± 9.1
Tmor size(cm)*	2.1± 0.9	3.0± 1.8
Histologic type		
Adenocarcinoma		
well differentiated	0	4
moderately	7	5
pooly	10	9
undifferentiated	1	0
Tumor extent		
T1	3	4
T2	6	5
T3	9	9
T4	0	0
Node metastasis		
N0	12	14
N1	4	3
N2	0	0
N3	2	1
Stage		
I	3	4
II	4	4
III	3	1
IV	8	9
Resectability of Tumor		
Yes	10	5
No	8	13

*$p<0.05$

showed that tumors with negative p53 immunostaining have a larger tumor size than the positive group (2.1 ± 0.9 cm vs. 3.0 ± 1.8 cm , $p < 0.05$). Therefore cancers with the positive p53 group had significantly smaller tumor size than the p53 negative group in our study. Infestation of the bile duct by liver fluke, *Clonorchis sinensis*, confirmed by identification of adult worms from the biliary tract or detection of ovum from the stool exam were found in 4 cases (11%) of which only one case showed p53 immunoreactivity while others disclosed negative immunostaining.

The correlation of survival with the presence of p53 expression was examined. Kaplan Meier

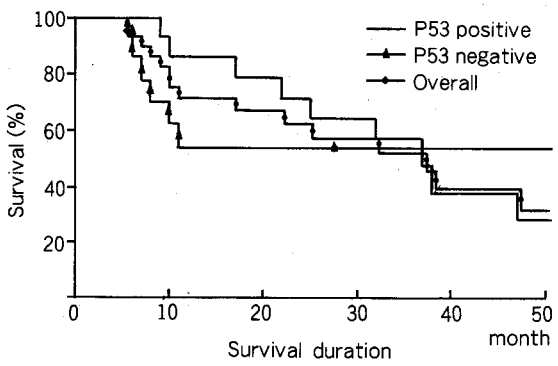

Fig. 2. Kaplan-Meier survival curve for 29 patients according to p53 mutation status of tumors.

survival plots for 29 patients, whose survival duration was obtained, were generated for having tumors with and without p3 expression. There was no statistically significant difference between those two groups (Fig. 2).

DISCUSSION

Previous studies of p53 immunohistochemical staining on a variety of human cancers have shown that mutated p53 protein is expressed widely in a variety of human malignancies (Nigro *et al.* 1989). More than 50% of human malignancies derived from the epithelial, mesenchymal, hematopoietic, and lymphoid tissues as well as the central nervous system, have been shown to have an altered p53 gene (Chang *et al.* 1993). Most p53 gene alterations are the missense mutations giving rise to an altered protein which are detected by immunohistochemical methods. It has been suggested that p53 mutation is an essential occurence in the multistep development in human carcinogenesis.

Extrahepatic bile duct cancer is one of the rare malignancies and although there have been few reports on the incidences and clinical significance of p53 immunoexpression on intrahepatic cholangiocarcinoma (Choi *et al.* 1993; Terada *et al.* 1994), and gall bladder carcinoma (Kamel *et al.* 1993), only two studies dealing with the incidence of p53 immunoreactivity on extrahepatic bile duct cancer (Kim *et al.* 1993; Teh *et al.* 1994) have been published thus far. Even the last study mentioned, included extrahepatic bile duct and ampullary cancer together, which allows for the possibility that there might be a chance for error in the different subtypes of tumor manifestations. Teh *et al* (1994) reported that ninety-two percent of the gallbladder cancer stained for p53 protein compared with only 66% of the extrahepatic bile duct/ampullary cancers. Because the incidence and pattern of staining are different, they proposed that these are different tumors with different etiologies and pathogenesis. The present study revealed that p53 was expressed in 50 percent of extrahepatic bile duct cancers. The incidence was lower than previously reported by Teh *et al.* (1994). Since it is difficult to differentiate the true origins of ampullary cancers and no statistically significant difference between ampullary and extrahepatic cancers has been established, this may reflect different tumors with different manifestations. As Terada *et al* (1994) pointed out, immunohistochemical demonstration of p53 protein in formalin-fixed paraffin embedded tissue might be varied according to adequate tissue preparation such as microwave oven heating and protease pretreatment, and several factors must be taken into account in evaluating the clinical significance of p53 positivity.

The expression of p53 protein has been shown to correlate with worsening tumor grades in some malignancies (Soini *et al.* 1992) but not in others, such as colorectal carcinomas (Starzynska *et al.* 1992) and gastric carcinomas (Martin *et al.* 1992). In gallbladder cancer an association between p53 expression and higher tumor grades have been reported by several authors (Kamel *et al.* 1993) as well as negative correlation in extrahepatic bile duct cancers (Teh *et al.* 1994). In our study, no significant difference was observed in histologic grades between p53 positive and negative groups although all four cases of well differentiated types were p53 negative. Because of the small numbers involved we failed to verify that it might suggest that just as in gall bladder cancers, there may exist positive correlation between p53 expression and tumor grades. However the clinical relevance between positive p53 expression and tumor grades should

be evaluated with further studies. Extent of tumor involvement analyzed by TNM staging did not show any significant difference according to p53 expression. Hamelin *et al* (1994) in investigating the relationship between prognosis and p53 mutation in colorectal cancers, reported that there was no relationship between p53 expression and histologic staging, but rather that p53 mutation showed as the only independent prognostic factor besides histologic grading. We could not find any significant association between p53 expression and histologic staging. Interestingly, although not statistically significant, extrahepatic bile duct cancers which were surgically resectable, tend to harbor more p53 positive staging. Moreover, cancers with the positive p53 group had significantly smaller tumor size than the p53 negative group in our study which might suggest that the presence of p53 overexpression could be one of the prognostic factors of resectability of extrahepatic bile duct cancers.

A consistent picture is now emerging correlating the loss of normal p53 function with shortened survival in patients with carcinoma of the breast or lung: the presence of a p53 mutation is an independent unfavorable prognostic factor (Thor *et al.* 1992; Quinlan *et al.* 1992). Although this association may not hold for all types of cancer, recent data suggest that this may be true for gastric cancer (Martin *et al.* 1992). Our data showed that there was no difference in survival duration for patients having tumors with and without p53 expression. However, due to the small number of cases for the evaluation of survival this may not hold true when a larger number of cases are evaluated. We were able to reach no conclusive results, except that further investigation, with expanded cases and better tissue preparation, including ones evaluating the p53 genetic sequence for specific mutations would be warranted in verifying the role of p53 mutation in carcinogenesis of extrahepatic bile duct cancers in the future.

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