

p53, Heat Shock Protein 70 and Topoisomerase II α Expression in Gallbladder Carcinoma

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Background : The present study was designed to investigate the expression of p53, Heat Shock Protein 70 (HSP70), and Topoisomerase (Topo) II α in the preneoplastic lesions and carcinomas of the gallbladder (GB) and to assess the correlation between the expression of these proteins and the clinicopathologic parameters by performing immunohistochemistry.

Methods : The immunohistochemical expressions of p53, HSP70 and Topo II α were evaluated in 38 gallbladder carcinomas and 3 adenomas. Fifteen CISs and 8 dysplasias that were located adjacent to invasive carcinomas were also studied. **Results :** A p53 expression was identified in 22 (57.9%) of the 38 GB carcinomas, in 9 (64.3%) of 14 CISs, and in none of the 8 dysplasias and 3 adenomas. A HSP70 expression was found in 11 (29%) of the 38 carcinomas, in 11 (78.6%) of 14 CISs, and in 4 (57.2%) of 7 dysplasias. A Topo II α expression was present in 36 (94.7%) of the 38 carcinomas, in 13 (92.9%) of 14 CISs, in 7 (100%) of 7 dysplasias and in 3 (100%) of 3 adenomas. p53 overexpression was related to the T stage of the primary tumor, while HSP70 and Topo II α were not related to any of the clinicopathologic parameters. **Conclusion :** p53 may be involved in GB carcinogenesis and in the progression of cancer. p53 may be also helpful for making the differential diagnosis between dysplasia and CIS. A further large study is needed to better elucidate the roles of HSP70 and Topo II α in GB carcinogenesis.

Key Words : Gallbladder; Carcinoma; p53; Heat shock protein 70; DNA topoisomerase II alpha

Gallbladder (GB) carcinoma is the fifth most common type of gastrointestinal carcinoma.¹ Despite the major improvements in diagnostic techniques, it's still not easy to detect GB carcinoma at an early stage; thus, most cases are diagnosed at an advanced stage. Therefore, prevention or detection at an early stage is required to improve the survival rate.

Neoplastic transformation of the biliary epithelium probably requires a number of successive genomic mutations that are similar to the sequence of events proposed for gastrointestinal cancer,² but our knowledge of biliary tract cancer is less extensive. Some studies have reported on the p53 expression in carcinomas of the extrahepatic biliary tract,³ including Klatskin tumors,⁴ and GB carcinomas.⁵ Heat shock proteins (HSP) are constitutively expressed in normal cells, and they play an important role in normal cell metabolism. The mutant p53 protein binds with HSP70, and the p53-HSP70 complex has functional significance for its capacity to transform mutant p53.⁶

Human Topoisomerase (Topo) II functions as a homodimer by cleaving an opening on the DNA duplex, passing a second dup-

lex through the opening and subsequently resealing the break.⁷ The two isoform enzymes are closely related in structure, but they differ in important biochemical and pharmacological properties, including their sensitivity to Topo II-targeting drugs, their cellular localization and their regulation by the cell cycle.⁸ Topo II α is of particular importance because of its association with DNA replication, mitosis and cell proliferation.⁹

Some reports have shown that many clinicopathologic parameters, including age, the histopathologic type and vascular invasion have been associated with the clinical outcome for patients suffering with GB carcinoma,¹⁰ but a true consensus was not reached in these studies. Among these clinicopathologic features, the TNM stage is currently universally accepted as an indisputable prognostic factor. The present study was designed to investigate the possible role of p53, HSP70 and Topo II α in the multistage processes of GB carcinogenesis with using immunohistochemistry, and we wanted to assess their prognostic significance in a series of resected specimens, including dysplasia, carcinoma in situ (CIS), and carcinomas of the GB.

MATERIALS AND METHODS

Materials

Forty-one GB samples were collected. The samples were retrieved from the surgical pathology files of Dong-A University Medical Center between January 2000 and July 2002. The GB carcinomas were reviewed and histologically classified according to the WHO criteria.¹¹ Thirty-eight cases were carcinomas, and the remaining cases were adenomas. Fourteen CISs and 7 dysplasias adjacent to invasive carcinomas were included. The histologic criterion for dysplastic epithelium was that the mucosal cells were composed of cuboidal to columnar cells with various degrees of nuclear atypia. The nuclear changes included pseudostratification, loss of polarity, enlargement and hyperchromasia. In the case of severe nuclear pleomorphism, the presence of large nucleoli and occasional secondary lumen formation without evidence of invasion into the connective tissue stroma was considered as carcinoma in situ.¹²⁻¹⁴

Review of the clinical findings

From the clinical records of each patient, the age, gender, clinical findings, lymph node metastasis and distant metastasis were reviewed, and the stage of the carcinoma was classified according to the AJCC¹⁵ protocol.

Immunohistochemical studies

Immunohistochemical staining was performed by using the DAKO EnVision Kit (Dako, Denmark). Immunoperoxidase studies were performed on sections prepared from the formalin-fixed and paraffin-embedded specimens that were dewaxed and rehydrated in a graded series of alcohol solutions. The endogenous peroxidase was inactivated by dipping the sections into 3% aqueous hydrogen peroxide for 10 min. Antigen retrieval was performed via microwave treatment in 10 mmol/L citrate buffer, pH 6.0, for 10 min. Diluted primary antibodies for anti-Human p53 protein (DO7, 1:100, Dako, Denmark), anti-Human HSP70 (1:2,000, Dako, Denmark), and anti-Human Topo II α (clone Ki-S1, 1:200, Dako, Denmark) were treated at room temperature for 1 h. After the primary incubation, the sections were incubated with the secondary antibody. The sections were lightly counterstained with Mayer's hematoxylin.

To evaluate the expression of p53, the accumulation of nuclear p53 in more than 10% of the neoplastic cells was considered

positive. The staining intensity of HSP70 was evaluated semi-quantitatively and then graded arbitrarily on a 4-point scale: 0, baseline staining (the expression intensity of normal epithelial cells); 1, light but definitely positive staining; 2, moderate staining; 3, intense staining. The HSP70 level in the tissue specimens having a score of 0 was considered to be the basal level of expression. Tissues having a score of 1 or greater were considered to represent an increased expression of HSP70. Semi-quantitative scores were used for Topo II α staining according to the percentage of positively stained cells (0, no positive tumor cells; 1, <30% positive tumor cells; 2, 30% to 60% positive tumor cells; 3, >60% positive tumor cells).

Statistical analysis

Statistical analysis was performed using SPSS 10.0K for Microsoft Windows. The relationships between each of the measured protein expressions and the patients' variables were determined by Pearson's χ^2 test, Kendal's tau-b test, Spearman's correlation test and linear by linear association. *p* values less than 0.05 were considered to be significant.

RESULTS

Clinical and histopathologic findings of carcinomas

There was a slight predominance of females (F:M=2.4:1) in the study group. The age at the time of diagnosis varied from 38 to 77 years (average: 60.4 years). The distribution of GB carcinomas according to the TNM stage demonstrated that stage I disease accounted for 6 (18.4%) of the carcinomas, stage II accounted for 15 (26.8%) of the carcinomas, stage III accounted for 10 (26.3%) of the carcinomas, stage IVA accounted for 3 (7.9%), and stage IVB accounted for 4 (10.5%) of the carcinomas; whereas the distribution according to the T stage showed that T1 accounted for 6 (15.8%) of the carcinomas, T2 accounted for 18 (47.4%) of the carcinomas, T3 accounted for 10 (26.3%), and T4 accounted for 4 (10.5%) of the carcinomas. Nodal metastasis and distant metastasis were observed in 15.8% and 7.9% of the 38 carcinomas, respectively. Five (13.2%) of the 38 carcinomas and all the adenomas were accompanied by the presence of gallstones. The most prevalent site for carcinomas was the fundus (63.2%), followed by the body (28.9%) and the neck (7.9%).

The histological types of the 38 carcinomas are summarized in Table 1. Twenty-nine of the 38 carcinomas were conventional

Table 1. Summary of clinical data and Immunohistochemical stain

Characteristics	No. of Patients (%)	p53 (%)		Heat shock protein 70 (%)			Topoisomerase II α (%)			
		-	+	0	1	2	0	1	2	3
Stage		*								
I	6 (18.4)	3 (50.0)	3 (50.0)	4 (66.7)	2 (33.3)	0 (0.0)	0 (0.0)	5 (83.3)	1 (16.7)	0 (0.0)
II	15 (26.8)	7 (46.7)	8 (53.3)	11 (73.3)	4 (26.7)	0 (0.0)	1 (6.7)	7 (46.7)	4 (26.7)	3 (20.0)
III	10 (26.3)	4 (40.0)	6 (60.0)	8 (80.0)	1 (10.0)	1 (10.0)	1 (10.0)	4 (40.0)	4 (40.0)	1 (10.0)
IVA	3 (7.9)	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	3 (100)	0 (0.0)	0 (0.0)
IVB	4 (10.5)	1 (25.0)	3 (75.0)	2 (50.0)	1 (25.0)	1 (25.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)
T Stage		†								
T1	6 (15.8)	4 (66.7)	2 (33.3)	4 (66.7)	2 (33.3)	0 (0.0)	0 (0.0)	5 (83.3)	1 (16.7)	0 (0.0)
T2	18 (47.4)	9 (50.0)	9 (50.0)	13 (72.2)	5 (27.8)	0 (0.0)	2 (11.1)	8 (44.4)	5 (27.8)	3 (16.7)
T3	10 (26.3)	3 (30.0)	7 (70.0)	8 (80.2)	1 (10.0)	1 (10.0)	0 (0.0)	5 (50.0)	4 (40.0)	1 (10.0)
T4	4 (10.5)	1 (25.0)	3 (75.0)	2 (50.0)	1 (25.0)	1 (25.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
Nodal metastasis										
Negative	32 (84.2)	14 (43.7)	18 (56.3)	21 (65.6)	9 (28.1)	2 (6.3)	1 (3.1)	20 (62.5)	8 (25.0)	3 (9.4)
Positive	6 (15.8)	3 (50.0)	3 (50.0)	6 (100)	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)	3 (50.0)	1 (16.7)
Distant metastasis										
Negative	35 (92.1)	16 (45.7)	19 (54.3)	25 (71.4)	8 (22.9)	2 (5.7)	2 (6.3)	19 (59.4)	8 (25.0)	3 (9.4)
Positive	3 (7.9)	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	2 (66.7)	1 (33.3)	0 (0.0)
Histologic Type by WHO										
Papillary adenocarcinoma	4 (10.5)	1 (25.0)	3 (75.0)	2 (50.0)	0 (0.0)	2 (50.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)
Adenocarcinoma										
Well differentiated	13 (34.2)	5 (38.5)	8 (61.5)	10 (76.9)	3 (23.1)	0 (0.0)	0 (0.0)	11 (84.6)	1 (7.7)	1 (7.7)
Moderately differentiated	9 (23.7)	5 (54.6)	4 (44.4)	8 (88.9)	1 (11.1)	0 (0.0)	1 (11.1)	2 (22.2)	3 (33.3)	3 (33.3)
Poorly differentiated	7 (18.4)	3 (42.9)	4 (57.1)	3 (42.9)	4 (57.1)	0 (0.0)	0 (0.0)	4 (57.1)	3 (42.9)	0 (0.0)
Mucinous carcinoma	1 (2.6)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)
Signet ring cell carcinoma	1 (2.6)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)
Undifferentiated carcinoma	1 (2.6)	0 (0.0)	1 (100)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)
Mixed adeno. & neuro. [‡]	1 (2.6)	0 (0.0)	1 (100)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)
Mixed adeno. & carcino. [§]	1 (2.6)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)

*p=0.015 by linear by linear association; †p=0.020 by Kendall's tau_b test; ‡Mixed adenocarcinoma and neuroendocrine carcinoma; §Mixed adenocarcinoma and carcinoid tumor.

adenocarcinomas, and the remaining 9 cases consisted of 4 cases of papillary adenocarcinoma, and 1 case each of mucinous carcinoma, an undifferentiated carcinoma (pleomorphic type), a mixed adenocarcinoma, a neuroendocrine carcinoma and a mixed adenocarcinoma-carcinoid tumor signet ring carcinoma. All 3 adenomas were of the tubular type. One adenoma showed foci of well-differentiated adenocarcinoma and this was categorized as an adenoma because the mechanisms of carcinogenesis between the adenoma-carcinoma model and the dysplasia-CIS-carcinoma model are known to be different. Among the 38 carcinomas, 14 cases of CIS and 7 cases of dysplasia were present around the main tumor.

Expression of p53

A p53 immunohistochemical expression was noted in the nuclei of the tumor cells (Fig. 1). Twenty-two (57.9%) of the 38 carcinomas and 9 (64.3%) of the 14 CIS cases demonstrated a p53 expression. None of the cases of dysplasia showed a posi-

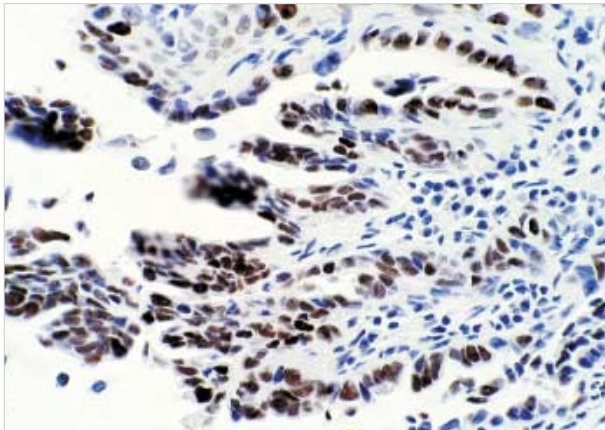
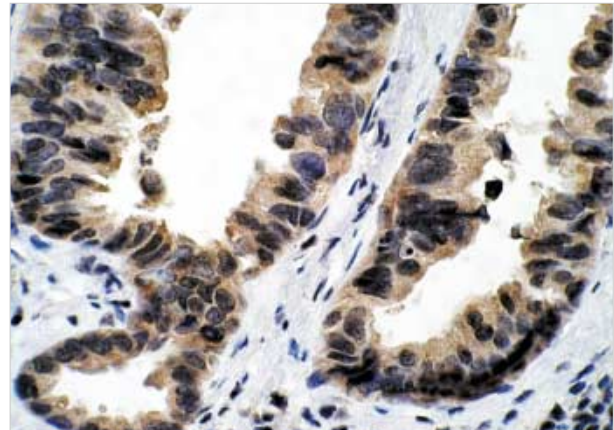
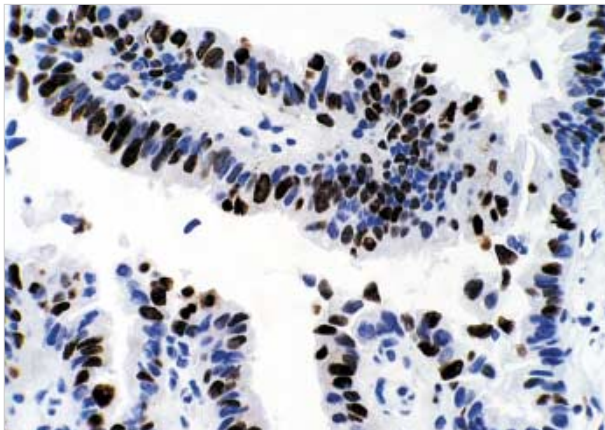
tive p53 expression. An increased frequency of p53 expression was significantly correlated with the T stage of the primary tumor (p=0.02) and the TNM stage (p=0.015) (Table 1). However, no significant correlation was noted for nodal metastasis, distant metastasis, cholelithiasis, the histological type or grade and the p53 expression. One case among the 3 adenomas had a positive p53 expression.

Expression of HSP70

HSP70 showed dark brown cytoplasmic staining (Fig. 2). Eleven (29%) of the 38 carcinomas exhibited a positive HSP70 expression. The CIS cases exhibited a positive expression in 11 (78.7%) of 14 specimens, and 4 (57.20%) of the 7 dysplasia specimens exhibited a positive reaction. Most of the cases were weakly positive. The frequency of an HSP70 expression frequency was decreased when comparing the premalignant lesions (dysplasia and CIS) to carcinomas, but this decrease was not significant (Table 2). No significant correlation between the HSP70

Table 2. Immunohistochemical reactions in the adenoma, dysplasia, carcinoma in situ and carcinoma of the gallbladder

	No.	p53		Heat shock protein 70			Topoisomerase II α (%)			
		Negative	Positive	0	1	2	0	1	2	3
Adenoma	3	2 (66.7)	1 (33.3)	3 (100)	0 (0.0)	0 (0.0)	0 (0.0)	3 (100)	0 (0.0)	0 (0.0)
Dysplasia	7	7 (100)	0 (0.0)	3 (42.9)	3 (42.9)	1 (14.3)	0 (0.0)	6 (85.7)	1 (14.3)	0 (0.0)
Carcinoma in situ	14	5 (35.7)	9 (64.3)	3 (21.3)	7 (50.0)	4 (28.6)	1 (7.1)	8 (57.1)	4 (28.6)	1 (7.1)
Carcinoma	38	16 (42.1)	22 (57.9)	27 (71.1)	9 (23.7)	2 (5.3)	2 (5.3)	21 (55.3)	11 (28.9)	4 (10.5)

**Fig. 1.** p53 Expression in GB Carcinoma. Well differentiated adenocarcinoma of GB showed dark brown nuclear staining.**Fig. 2.** HSP70 Expression in GB carcinoma. Well differentiated adenocarcinoma showed moderate (+2) cytoplasmic staining in brown color.**Fig. 3.** Topo II α Expression in GB Carcinoma. Well differentiated adenocarcinoma showed dark brown nuclear staining in moderate (+2) distribution.

expression and the clinicopathologic parameters was noted (Table 1). All the adenomas demonstrated no expression.

Expression of Topo II α

Topo II α showed dark brown nuclear staining (Fig. 3). The expression of Topo II α was identified in 36 (94.7%) of the 38 carcinomas, in the 3 (92.9%) of 14 CISs, and in 7 (100%) of the

7 dysplasias (Table 2). No significant correlation between the Topo II α expression and the clinicopathologic parameters was noted (Table 1). All the adenomas showed a positive expression.

Co-expression of protein and the clinicopathologic findings

The p53 expression was significantly correlated with the HSP70 expression (correlation coefficient: 0.291) ($p=0.043$) (Table 3) and the Topo II α expression (correlation coefficient: 0.318) ($p=0.023$) (Table 4). The coexpression of 2 or 3 of the proteins was not correlated to the TNM stage or any other clinicopathologic parameters.

DISCUSSION

The clinical outcome of GB carcinoma has improved very little during the past couple of decades. The frequency of GB cancer increases with age, with peaking during the 7th decade of life. The median age at diagnosis is 70.3 years for GB carcinoma based on the data from the California Tumor Registry.¹⁶ Chung *et al.*¹⁷ have reported that the female to male ratio was

Table 3. Correlation of p53 and heat shock protein 70 in gallbladder carcinoma

	Grading of positivity	HSP70 (%)			Total
		0	1	2	
p53	Negative	14 (51.9)	3 (33.3)	0 (0.0)	17
	Positive	13 (48.1)	6 (66.7)	2 (100.0)	21
	Total	27	9	2	38

p=0.043 by linear by linear association.
correlation coefficient=0.291.

1.7:1 and mean age was 64 years in a study of 132 primary GB carcinomas. In our study, there was a predominance of female subjects (F:M=2.4:1), and the mean age at the time of diagnosis was 60.4 years. The incidence rate of GB carcinoma varies in different parts of the world and it also varies among different ethnic groups in the same country. Among the risk factors, gallstones are the main risk factor for GB cancer.¹⁸ Gallstones are found in at least 70% of GB cancer in Western countries,¹⁸ whereas in Korea only 30% of GB carcinomas are associated with gallstones.¹⁹ In our study, only 5 (13.2%) of the 38 GB carcinomas had gallstones, but all the cases of adenoma exhibited gallstones.

More than 90% of GB carcinomas are adenocarcinomas. Upon gross examination, approximately 10% to 37% of GB carcinomas cannot be identified with complete certainty, and their macroscopic findings are similar to those of chronic cholecystitis, but in a previous report, most of the cases were weakly positive.²⁰ About 60% of carcinomas originate in the fundus of the GB, 30% in the body and 10% in the neck.¹⁴ Most GB adenocarcinomas are well-to-moderately differentiated. In our study, 29 cases (76.3%) of the 38 GB carcinomas were conventional adenocarcinomas. Well to moderately differentiated adenocarcinomas were observed in 21 (55%) of the 38 cases. Tumor in 24 of the 38 cases (58.5%) that we examined originated in the fundus.

TP53 gene abnormalities are well-known to frequently occur in GB carcinomas. Although the frequency of p53 immunohistochemical staining in GB carcinomas has widely varied, two thirds of the studies showed a frequency greater than 50%.²¹ In addition, deletions at the TP53 locus were reported in GB carcinomas,²² which indicates that inactivation of the TP53 locus plays an important role in GB carcinogenesis. The frequency of p53-positive immunoreaction in the GB carcinomas in our study was 55.3%, which corresponds to previous studies, and the frequency of a p53 expression was increased as the TNM and T stage increased. Our data suggested that p53 might play an important role in GB carcinogenesis, as has been previously reported.

The exact sequence of molecular changes that leads to neo-

Table 4. Correlation of p53 and topoisomerase II α in gallbladder carcinoma

	Grading of positivity	Topo II α (%)				Total
		0	1	2	3	
p53	Negative	2 (100)	11 (52.4)	3 (27.3)	1 (25.0)	17
	Positive	0 (0.0)	10 (47.6)	8 (72.7)	3 (75.0)	21
	Total	2	21	11	4	38

p=0.023 by linear by linear association.
correlation coefficient=0.318.

plastic transformation in the GB epithelium remains uncertain. More detailed understanding of the earliest molecular abnormalities in GB carcinogenesis may eventually provide methods for risk assessment and the early detection of GB carcinoma. Many studies have reported that excess accumulation of p53 in GB dysplasia ranges from 0% to 32% and the p53 expression in CIS ranges from 45% to 86%, suggested that TP53 abnormality might be an early event.²³ The presence of deletions at the TP53 locus in the histologically normal epithelium near GB carcinomas, and also the presence of TP53 mutation in precursor lesions, indicates an important role for TP53 in the early phase of GB carcinogenesis.²³ In contrast, Kim *et al.*²⁴ reported, with using direct sequencing methods, that TP53 gene alterations were found only in the advanced stages of GB carcinoma. In our study, a p53 expression was observed in 9 (64.3%) of the 14 CIS cases, but the dysplasia cases demonstrated no expression. The normal or regenerating epithelium of the GB did not exhibit p53 immunoreaction. The overall findings from our study suggest that accumulation of p53 protein might be an important discriminator between dysplasia and CIS as a marker for malignant transformation.

In response to environmental or physiological stress such as heat, ethanol, heavy metals or amino acid analogues, cells show an increased synthesis of intracellular HSPs or stress proteins.²⁵ HSP70 is believed to play multiple important roles in the cell cycle and in the various processes of carcinogenesis.²⁵ The related reports have, so far, primarily focused on *in vitro* or animal studies and little is known about the HSP70 expression in human malignant diseases.⁶ Further, some mutant types of p53 have the ability to bind to HSP70 and to transactivate a number of promoters.²⁶ There has been no previous reported study concerning the HSP70 expression in GB carcinoma prior to this research.

Our results demonstrated an HSP70 expression in 11 (28.9%) of the 38 carcinomas, in 11 (78.7%) of the 14 CISs, and in 4 (57.1%) of the 7 dysplasias. The intensity was generally weak. All the adenomas showed no HSP70 expression. Although the expression of HSP70 might play a role in early events of the dys-

plasia-CIS carcinoma model in GB carcinogenesis, no clinicopathologic correlation with the HSP70 expression was identified in the present study. Lee *et al.*²⁷ reported that the HSP70 scores were related to the stage and p53 expression in gastric carcinoma. In our study, the HSP70 expression was significantly related to the p53 expression, and this relationship suggests a possible role in tumor progression through a pattern of p53-HSP70 complex formation. Furthermore, the structural and functional interaction of these proteins and their *in situ* detection in lesion should be researched in future studies on GB carcinoma.

In addition to its physiological function, Topo II α serves as a target for many anticancer drugs such as etoposide, teniposide and doxorubicin; these chemotherapeutic agents are known as Topo II α inhibitors.⁸ Topo II α analysis has been proposed as a marker for cell proliferation.²⁸ Tuccari *et al.*²⁹ studied the Topo II α expression in breast tumors and they demonstrated a relationship between the clinical aggressiveness of the tumor and the Topo II α expression. In our study, all 7 dysplasias and 3 adenomas we examined and most of the cases of carcinomas (except 2 cases) and CISs (except 1 case) showed a positive immunoreaction for Topo II α , while the normal GB mucosal epithelial cells showed no immunoreaction. Although the Topo II α expression was related to the p53 expression, the Topo II α expression was not related to any of the clinicopathologic parameters. In this research, a heterogeneous expression of Topo II α was observed within a single tumor, as was also reported by Yamazaki *et al.*³⁰ Yamazaki *et al.*³⁰ suggested that the observed heterogeneous expression may be related to the differences in the sensitivity of tumor cells to Topo II α inhibitor and this may have clinical consequences.

In conclusion, p53 may play an important role in GB carcinogenesis and in the progression of carcinoma. In addition, p53 may be a diagnostic discriminator between dysplasia and CIS. Taking into consideration the limitations of our study, in terms of the small sample size, a further large study may be necessary in order to further elucidate the roles of HSP70 and Topo II α in GB carcinogenesis.

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