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Significance of PML and p53 Protein as Molecular Prognostic Markers of Gallbladder Carcinomas

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Molecular markers for cancers are not only useful for cancer detection and prognostic prediction, but may also serve as potential therapeutic targets. In order to identify reliable molecular markers for prognostic prediction in gallbladder carcinoma (GBC), we evaluated the immunohistochemical expression of 15 proteins, namely p53, p27, p16, RB, Smad4, PTEN, FHIT, GSTP1, MGMT, E-cadherin, nm23, CD44, TIMP3, S100A4, and promyelocytic leukemia (PML) in 138 cases of GBC using the tissue microarray method. The prognostic significance was analyzed for each protein. Overexpression of p53 and S100A4, and loss of p27, p16, RB, Smad4, FHIT, E-cadherin and PML expression were associated with poor survival. In particular, PML and p53 showed considerable potential as independent prognostic markers. Patients with normal PML and p53 expression displayed favorable outcomes, compared to those showing abnormal expression of either or both proteins (49% *vs.* 23% in a 5-year survival rate; 60 months *vs.* 11 months in median survival, respectively; P=0.009). Thus, PML and p53 are potential candidates for development as clinically applicable molecular prognostic markers of GBC, and may be effective therapeutic targets for the disease in the future. (Pathology Oncology Research Vol 13, No 4, 326–335)

Key words: gallbladder carcinoma, prognosis, PML protein, p53 protein

Introduction

Gallbladder carcinoma (GBC) is the most common malignancy in the biliary tract, and represents 1% of all cancers.[17] Despite recent advances in radiological and surgical techniques, the long-term survival of GBC patients is poor, with the overall 5-year survival rate ranging from 5% to 13%.[7,16] Compared with other common cancers, identification of prognostic markers of GBC has not been extensively studied, since this disease has a relatively low incidence and is usually diagnosed at later stages.[7,16] Moreover, preoperative clinical or radiological staging, an essential process for the prognostic evaluation of GBC, has been disregarded by the clinician, due to its limitations in accurate classification.[10,23] In view of the limited value of stage in GBC, identification of reliable molecular markers may provide important prognostic information, and facilitate adequate treatment plans and targets for a novel therapeutic approach.

Inadequate information is available on the molecular mechanism of gallbladder carcinogenesis. Previous genetic studies on GBC have mainly focused on analyzing mutations of dominant oncogenes (*K-ras)* or tumor suppressor genes (*TP53* and *FHIT*).[35,37] Recent allotyping studies in GBC demonstrated allelic loss of multiple chromosomal regions (3p12-21, 8p21-23, 9p21-23, and 17p12-13),[5,35,36] and analyses on aberrant methylation profiles in GBC disclosed frequent methylation of $p16^{INK4}$, *p73, APC, hMLH1, 3-OST-2, CDH13 (H-cadherin), CDH1 (E-cadherin), RUNX3, RIZ1* and *HPP1*.[13,32] These results suggest that multiple tumor suppressor genes are involved in GBC pathogenesis. However, their roles in the progression and biological behavior of GBC remain to be elucidated.

A number of molecular markers are related to GBC prognosis, including cell cycle regulators (p27,[14] p21,[24]

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p53,[25] RB,[30] and cyclin D1[15]), adhesion-related molecules (epithelial cell adhesion molecule[34] and β-catenin[6]), tumor-associated proteins (RCAS1,[27] MUC1,[18] S100A4,[26] and c-erbB2[19]), and the DNA repair enzyme, MGMT.[20] However, most of these markers have been identified only in single studies comprising a small number of GBC cases. Thus, their prognostic significance remains to be extensively validated, and a reliable molecular marker for GBC is lacking at present.

A novel molecular marker candidate for human cancer, *promyelocytic leukemia (PML)*, is a tumor suppressor gene implicated in the pathogenesis of leukemia and human cancers.[12,28] PML belongs to a large family of proteins harboring a distinct zinc-finger domain designated the RING finger.[28] The PML protein is typically concentrated in subnuclear structures, known as PML nuclear bodies. In the vast majority of acute promyelocytic leukemia patients, *PML* is fused to the *retinoic acid receptor a (RARα)* gene as a consequence of chromosomal translocation. The PML-RARα protein inhibits RARα transcriptional function, and physically associates with PML, leading to its delocalization from nuclear bodies, and consequently, deregulation of the functions of PML and nuclear bodies.[28] Recent studies suggest that PML and PML nuclear bodies play a role in the regulation of apoptosis, growth, and DNA repair, in addition to tumor suppression and transcription.[12,28] PML protein expression is reduced or abolished in various human malignancies, including carcinomas of the prostate, colon, breast and lung, as well as lymphomas, CNS tumors and germ cell tumors.[12] Furthermore, loss of PML expression is associated with tumor progression in prostate, breast, and CNS cancers.[12] However, the clinicopathological significance of PML expression in GBC is yet to be established.

Here, we analyze the prognostic significance of 15 molecular markers in GBC, namely p53, p27, p16, RB, Smad4, phosphatase and tensin homolog (PTEN), fragile histidine triad (FHIT), glutathione S-transferase P1 (GSTP1), O^6-methylguanine-DNA methyltransferase (MGMT), E-cadherin, nm23, CD44, tissue inhibitor metalloproteinase 3 (TIMP3), S100A4 and PML. Our results show that PML and p53 have independent prognostic significance in GBC, and their combined expression profile may be a useful molecular marker for GBC.

Materials and Methods

Patients and samples

We examined a total of 138 GBC samples surgically resected at the Seoul National University Hospital and the National Medical Center (Seoul, Korea) between 1991 and 1999. Clinicopathologic data, including age, gender, tumor size, histological tumor type, growth pattern, angiolymphatic or perineural invasion, and stage were obtained by reviewing the relevant medical charts and pathological records, and examining H&E-stained glass slides from each case. The patient group comprised 81 females and 57 males with a mean age of 61.7 years (range, 36-87 years). The mean tumor diameter was 3.9 cm (range, 1-18 cm). All carcinomas were classified according to World Health Organization (WHO) criteria,[3] and included 115 adenocarcinomas (51 well differentiated, 51 moderately differentiated and 13 poorly differentiated), 5 papillary adenocarcinomas, 2 squamous cell, 6 adenosquamous, 3 small cell, 5 undifferentiated, and 2 mucinous carcinomas. For statistical analysis, the histologic type was arbitrarily divided into two groups, specifically, papillotubular (well- to moderately differentiated adenocarcinomas and papillary adenocarcinomas) and non-papillotubular (the remaining histologic types). Growth pattern was classified as polypoid or infiltrative, based on the presence or absence of predominantly nodular or papillary lesions. Carcinomas were staged according to the criteria of the AJCC cancer staging manual.[10] The mean follow-up period was 34 months (range, 2-132 months). The cases lost to follow-up and those ending in death from causes other than gallbladder cancer were regarded as censored data during the analysis of survival rates.

Immunohistochemistry

All tissues were routinely fixed in 10% buffered formalin, and embedded in paraffin blocks. Core tissue biopsies 2 mm in diameter were obtained from individual paraffin-embedded tissues (donor blocks), and arranged in new recipient paraffin blocks (tissue array block) using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). Since a single sample from each tumor is sufficient for distinguishing protein expression or molecular alterations related to clinical outcome,[33] we analyzed one tissue core from the most cellular and representative portion in each case. The number of tumors tested ranged from 119 to 136 due to the lack of cores on some slides or insufficient tumor cell numbers in some cores.

Sections (4 μM) were subjected to immunostaining using the avidin-biotin peroxidase complex (ABC) method. The primary antibodies are summarized in *Table 1*. Antigen retrieval was performed using citrate buffer solution (Antigen Unmasking Solution, Vector Laboratories, Burlingame, CA, USA) in an 800 W microwave oven for 15 min, a pressure cooker for 6 min, or an autoclave for 15 min. DAB (3,3'-diaminobenzidine) was used as the chromogen. Non-immune serum or PBS was used in place of primary antibodies as controls. Positive immunostaining was identified as unequivocal brown staining in the nucleus, cytoplasm or cell membrane, regardless of intensity. The staining results were estimated semi-quantitatively by two pathologists (HJC and WHK), based on the percentage

Table 1. **Antibodies used for immunohistochemical studies**

Antibody	*Biological activity*	*Source*	*Dilution*	*Antigen retrieval*	*Expression*	
					Normal	*Cancer*
p53	Cell cycle, apoptosis DNA repair	DAKO (Carpinteria, CA)	1:100	MW	Negative	Nucleus
p27	Cell cycle	Calbiochem (San Diego, CA)	1:200	PC	Nucleus	Loss
p16	Cell cycle	Pharmingen (San Diego, CA)	1:50	AC	Nucleus	Loss
RB	Cell cycle	Pharmingen (San Diego, CA)	1:50	MW	Nucleus	Loss
Smad4	TGF-β signal	Santa Cruz (Santa Cruz, CA)	1:50	MW	Nucleus	Loss
PTEN	Insulin receptor signal	AG Scientific (San Diego, CA)	1:50	MW	Cytoplasm	Loss
FHIT	Nucleotide metabolism	Zymed (South San Francisco, CA)	1:250	MW	Cytoplasm	Loss
GSTP1	Detoxification	Gift from Dr. SC Park, Seoul Nat'l Univ., Seoul, Korea	1:1000	MW	Cytoplasm	Loss
MGMT	DNA repair	Chemicon (Temecula, CA)	1:50	MW	Nucleus	Loss
E-cadherin	Adhesion	Transduction (Lexington, NY)	1:200	MW	Membrane	Loss
nm23	Metastasis	Novocastra (Newcastle, UK)	1:250	Not done	Cytoplasm	Loss
CD44	Adhesion	Novocastra (Newcastle, UK)	1:40	MW	Membrane	Loss
TIMP3	Apoptosis	Chemicon (Temecula, CA)	1:50	MW	Cytoplasm	Loss
S100A4	Metastasis	DAKO (Carpinteria, CA)	1:500	MW	Negative	Cytoplasm
PML	Cell cycle, apoptosis DNA repair	MBL (Woburn, MA)	1:200	MW	Nucleus	Loss

MW, microwave; PC, pressure cooker; AC, autoclave

of positive cells: 0, <10%; 1, 10-24%; 2, 25-49%; and 3, 50%. For statistical analysis, the cutoff values for high expression were 10% or more for RB, PTEN, FHIT, GSTP1, MGMT, E-cadherin and PML, 26% or more for Smad4, nm23, CD44, TIMP3 and S100A4, and 50% or more for p53, p27 and p16.

Statistical analysis

Survival curves were estimated using the Kaplan-Meier method, and the log-rank test was used to compute differences between the curves. Multivariate analysis using the Cox proportional hazards regression model was performed to assess the prognostic values of protein expression. Correlation coefficients between protein expression and clinicopathologic findings were estimated using the Pearson correlation method. A P value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS-PC software (SPSS, Chicago, IL, USA).

Results

Kaplan-Meier survival analysis of clinicopathologic parameters

The overall 5-year survival rates according to stage were 92, 69, 22 and 8% for stages I, II, III and IV, respectively. The median survival duration in each stage was >75 months, >72 months, 11 months, and 7 months, respectively. The results of the Kaplan-Meier survival analysis and log-rank tests for the clinicopathologic parameters in GBC are summarized in *Table 2*. Among the clinicopathologic parameters, infiltrative growth type (P=0.008), nonpapillotubular histologic type (P=0.005), presence of lymphatic, venous or perineural invasion (P<0.0001), advanced stage (stage III + stage IV; P<0.0001), and positive resection margin (P=0.005) were significantly related to poor survival.

Kaplan-Meier survival analysis according to protein expression, and correlation between protein expression and clinicopathologic findings

Protein expression analysis revealed the following rates of high expression in GBC samples: 38% for p53; 36% for p27; 74% for p16; 88% for RB; 51% for Smad4; 12% for PTEN; 39% for FHIT; 89% for GSTP1; 69% for MGMT; 73% for E-cadherin; 68% for nm23; 17% for CD44; 91% for TIMP3; 11% for S100A4; and 78% for PML (*Fig. 1, Table 3*). Of the 15 proteins studied, overexpression of p53 (P=0.022) and S100A4 (P=0.025), and loss of expression of p27 (P=0.015), p16 (P=0.024), RB (P=0.011), Smad4 (P=0.023), FHIT (P=0.001), E-cadherin (P=0.009) and PML (P=0.014) were associated with reduced 5-year survival rates and shorter median survival duration (*Table 3*).

Data on the correlation between protein expression status and clinicopathologic findings are summarized in *Table 4*. Most proteins, with the exception of p53, RB, GSTP1

and CD44, were correlated with stage. Loss of p27 or FHIT expression was significantly associated with multiple clinicopathologic parameters, including lymphatic and perineural invasion, pT, pN and stage, whereas the expression status of p53 and CD44 was not correlated with any of the parameters tested (*Table 4*).

Multivariate analysis of clinicopathologic parameters and protein expression

Multivariate survival analysis using a forward selection procedure of the Cox proportional hazard model for all the clinicopathologic parameters and protein expression revealed that advanced stage (hazard ratio [HR], 3.694; 95% confidence interval [CI], 1.704-8.007; P=0.001), perineural invasion (HR, 2.855; 95% CI, 1.501-5.541; P=0.001), p53 overexpression (HR, 2.745; 95% CI, 1.496-5.308; P=0.001) and loss of PML expression (HR, 0.288; 95% CI, 0.151-0.546; P<0.0001) were independent predictors of poor prognosis.

Prognostic significance of the combined PML and p53 expression profile

To estimate the prognostic significance of the combined PML and p53 expression profile, we reclassified cases into 4 groups, specifically, PML (normal)/p53 (normal), PML (normal)/p53 (overexpression), PML (loss)/p53 (normal), and PML (loss)/p53 (overexpression). The results of the Kaplan-Meier survival analysis are shown in *Fig. 2*. The 5-year survival rate was 49% in patients displaying normal expression of PML and p53, whereas those with abnormal expression of PML and/or p53 had a 5-year survival rate of 23% (P=0.009). The median survival duration periods in these groups were 60 months *vs.* 11 months, respectively. Multivariate analysis revealed that the prognostic significance of the PML/p53 expression profile was independent (HR, 2.132; 95% CI, 1.128-3.734; P=0.008) once calculations were adjusted for pT, pN, pM, stage, and lymphatic, venous or perineural invasion.

***Table 2.* Kaplan-Meier survival analysis according to clinicopathologic parameters of gallbladder carcinomas**

Clinicopathologic findings (total case no.)			*5-year survival rate (%)*		*Median survival (months)*	*P value*
			KM estimate	*SE*		
Age	≤ 61 years	(n=63)	38	6	24	NS
	> 61 years	(n=75)	33	6	11	
Gender	Female	(n=81)	37	6	15	NS
	Male	(n=57)	36	6	22	
Size[a]	< 4 cm	(n=71)	42	6	22	NS
	≥ 4 cm	(n=53)	31	7	12	
Growth pattern	Polypoid	(n=71)	47	6	46	P=0.008
	Infiltrative	(n=67)	25	5	10	
Histology	Papillotubular	(n=107)	40	5	25	P=0.005
	Non-papillotubular	(n=31)	23	8	8	
Lymphatic invasion[b]	(-)	(n=53)	60	7	>75	P<0.0001
	(+)	(n=80)	19	5	10	
Venous invasion[c]	(-)	(n=73)	55	6	75	P<0.0001
	(+)	(n=56)	13	5	8	
Perineural invasion[d]	(-)	(n=66)	59	6	>75	P<0.0001
	(+)	(n=63)	13	4	9	
pT stage	T1 + T2	(n=67)	59	6	>75	P<0.0001
	T3 + T4	(n=71)	15	4	9	
pN stage	N0	(n=81)	54	6	75	P<0.0001
	N1 + N2	(n=57)	11	4	8	
pM stage	M0	(n=119)	40	5	24	P<0.0001
	M1	(n=19)	11	7	4	
Stage	I + II	(n=46)	77	7	>75	P<0.0001
	III + IV	(n=92)	16	4	9	
Residual disease	R0	(n=107)	42	5	28	P=0.001
	R1 + R2	(n=31)	15	7	8	

[a]Size was unknown in 14 cases; [b]lymphatic invasion could not be checked in 5 cases; [c]venous invasion could not be checked in 9 cases; [d]perineural invasion could not be checked in 9 cases; KM estimate, Kaplan-Meier estimate; SE, standard error; NS, statistically not significant

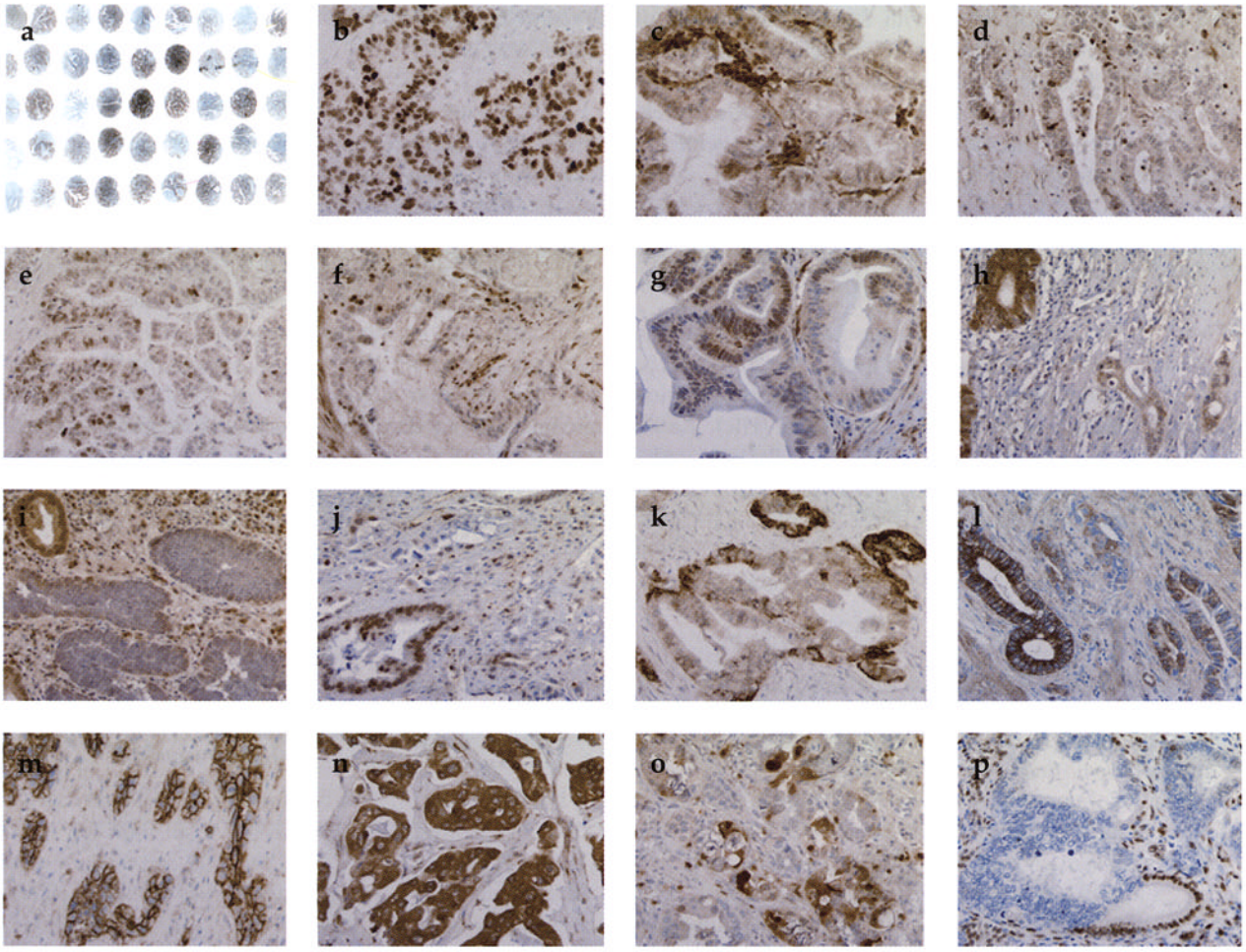


Figure 1. *Immunohistochemical staining using tissue microarray* ***(a).*** *Gallbladder carcinomas displaying positive expression of p53* ***(b);*** *negative expression of p27* ***(c);*** *negative expression of p16* ***(d);*** *negative expression of RB* ***(e);*** *negative expression of Smad4* ***(f);*** *positive expression of PTEN* ***(g);*** *negative expression of FHIT* ***(h);*** *negative expression of GSTP1* ***(i);*** *negative expression of MGMT* ***(j);*** *negative expression of E-cadherin* ***(k);*** *negative expression of nm23* ***(l);*** *positive expression of CD44 (M); positive expression of TIMP3* ***(n);*** *positive expression of S100A4* ***(o);*** *negative expression of PML (P).*

Discussion

Stage is the most important factor in the survival, management and prognosis of GBC patients as well as those with other carcinomas.[10] However, accurate clinical or pathologic classification of the TNM stage in GBC may be limited, as preoperative radiological diagnosis or staging of the disease is difficult,[23] and 10% to 40% of GBC cases are incidentally detected during or after cholecystectomy for benign diseases, including cholelithiasis or cholecystitis.[10,21] Moreover, due to the anatomic features of gallbladder promoting early local spread of cancer, and lack of specific signs and symptoms, GBC is usually diagnosed at the later stages.[23] Thus, identification of reliable molecular prognostic markers is more important in GBC than in other malignancies, since their application for serum, bile or small biopsy tissue samples should provide important prognostic information.

We screened for the prognostic significance of 15 molecular markers in GBC using tissue microarray, which provides a rapid, standardized and cost-effective method for high-throughput clinicopathologic studies.[33] This technique permits comprehensive analysis of the protein expression profiles and identification of prognostic subclasses in a large number of samples,[33] but has potential limitations associated with the acquisition of information from only a tiny area in each tumor. However, tissue heterogeneity does not negatively influence the predictive power of microarray results,[33] and previous analyses of single readable cores in tissue microarray matched the staining patterns of whole sections with a concordance rate of >90%.[29] Moreover, the relatively large number of cases included in a tissue microarray study compensates for the false negative results created by a small-sized tumor tissue chip.[33]

Out of the 15 molecular markers screened in this study, 11 were correlated with advanced tumor stage, while 9 (p53, S100A4, p27, p16, RB, Smad4, FHIT, E-cadherin and PML) were associated with survival of GBC patients. These findings, along with previous results from other genetic studies,[1,5,13,32,35-37] suggest that multiple tumor suppressor and tumor-related genes are involved in the progression and carcinogenesis of GBC. Our results are consistent with earlier reports on the prognostic significance of S100A4 and p27 in GBC.[14,26] S100A4 is a calcium-binding protein tandemly arranged with other *S100* genes at chromosome 1q12.[26] The protein induces metastasis and regulates the motility or invasiveness of cancer cells, and its expression is associated with poor survival in GBC patients.[26] p27 is a member of the INK family of cyclin-dependent kinase inhibitors that blocks G1 to S phase transition in the cell cycle.[14] Decreased expression of p27 is significantly associated with shorter survival in patients with GBC.[14] *FHIT* is a candidate tumor suppressor gene involved in the carcinogenesis of GBC,[37] but its prognostic significance is yet to be evaluated in GBC. In the present study, loss of FHIT expression was significantly correlated with non-papillotubular histologic type, lymphatic or perineural invasion, increased depth of tumor invasion (pT3 + pT4), lymph node metastasis, advanced stage (stage III + stage IV), and poor survival. Although its prognostic significance was not independent of stage, differences in the median survival between groups displaying loss of FHIT and normal FHIT expression (10 months vs. 72 months, respectively; P=0.001) were comparable to those between stage I + stage II and stage III + stage IV (>75 months vs. 9 months, respectively; P<0.0001).

Among the markers analyzed, abnormal expression of p53 and PML, as well as stage and perineural invasion were independently related to poor prognosis of GBC. Inactivation of the p53 tumor suppressor gene is the most

***Table 3.* Kaplan-Meier survival analysis according to protein expression in gallbladder carcinomas**

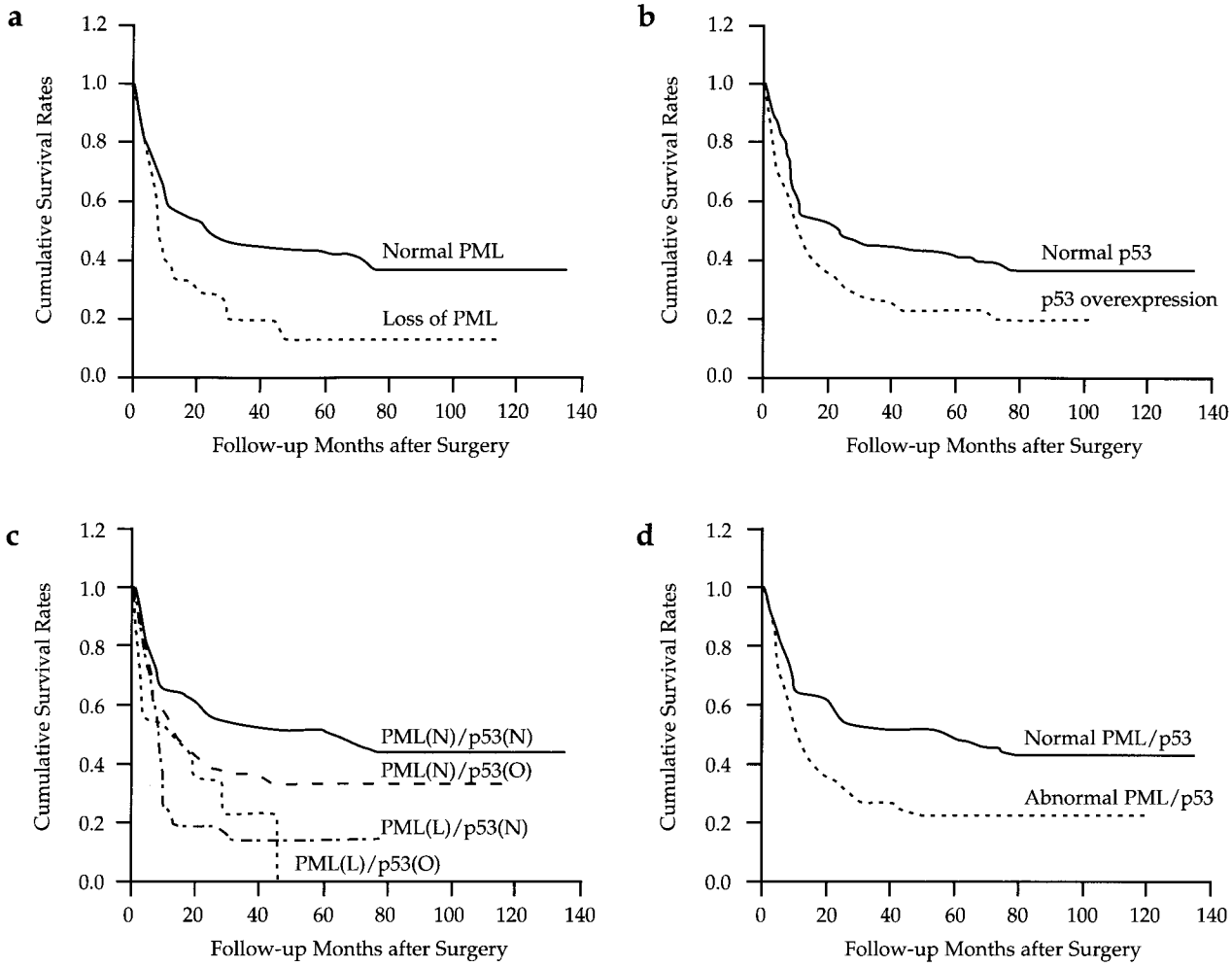
Protein expression		*5-year survival rate (%)*			*Median survival (months)*	*P value*
Markers		*(Case no.)*	*KM estimate*	*SE*		
p53	low	(n=84)	42	5	24	P=0.022
	high	(n=52)	24	6	11	
p27	low	(n=83)	28	5	10	P=0.015
	high	(n=46)	47	8	43	
p16	low	(n=34)	19	7	10	P=0.024
	high	(n=96)	42	5	25	
RB	low	(n=16)	14	9	6	P=0.011
	high	(n=115)	39	4	21	
Smad4	low	(n=65)	26	5	11	P=0.023
	high	(n=69)	43	6	39	
PTEN	low	(n=106)	35	5	14	NS
	high	(n=15)	53	13	>25	
FHIT	low	(n=73)	25	5	10	P=0.001
	high	(n=47)	56	8	72	
GSTP1	low	(n=13)	38	13	21	NS
	high	(n=109)	38	5	17	
MGMT	low	(n=37)	22	7	10	P=0.07
	high	(n=82)	44	6	24	
E-cadherin	low	(n=36)	22	7	8	P=0.009
	high	(n=96)	46	5	22	
nm23	low	(n=41)	43	8	28	NS
	high	(n=87)	32	5	12	
CD44	low	(n=104)	33	5	14	NS
	high	(n=22)	45	12	60	
TIMP3	low	(n=11)	36	15	10	NS
	high	(n=116)	36	5	17	
S100A4	low	(n=107)	40	5	21	P=0.025
	high	(n=13)	15	10	7	
PML	low	(n=28)	13	8	9	P=0.014
	high	(n=101)	42	5	24	

KM estimate, Kaplan-Meier estimate; SE, standard error; NS, statistically not significant

***Table 4.* Correlation between protein expression and clinicopathologic parameters** (*correlation coefficients*)

Markers	*Size*[a]	*Growth pattern*[b]	*Histology*[c]	*Lymphatic invasion*[d]	*Venous invasion*[e]	*Perineural invasion*[f]	*pT stage*[g]	*pN stage*[h]	*pM stage*[i]	*Stage*[j]
p53	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
p27	NS	NS	NS	*P*=0.022 (-0.203)	*P*=0.013 (-0.225)	*P*=0.022 (-0.207)	*P*=0.003 (-0.256)	*P*=0.037 (-0.182)	NS	*P*<0.001 (-0.348)
p16	NS	NS	*P*=0.012 (-0.221)	NS	NS	*P*=0.008 (-0.239)	*P*=0.004 (-0.253)	NS	*P*=0.023 (-0.200)	*P*<0.0001 (-0.308)
RB	NS	NS	NS	NS	*P*=0.043 (0.183)	NS	NS	NS	NS	NS
Smad4	NS	NS	NS	*P*=0.011 (-0.223)	NS	NS	*P*<0.0001 (-0.341)	NS	NS	*P*<0.0001 (-0.394)
PTEN	NS	NS	NS	NS	NS	*P*=0.003 (-0.279)	*P*=0.036 (-0.191)	NS	NS	*P*=0.010 (-0.233)
FHIT	*P=0.019* (-0.228)	NS	*P*<0.0001 (-0.321)	*P*=0.013 (-0.230)	NS	*P*=0.015 (-0.227)	*P*<0.0001 (-0.327)	*P=0.009* (-0.238)	NS	*P*<0.0001 (-0.370)
GSTP1	NS	NS	*P*=0.011 (-0.231)	NS	NS	NS	NS	NS	NS	NS
MGMT	NS	NS	*P*=0.016 (-0.220)	NS	NS	NS	NS	*P*=0.002 (-0.287)	NS	*P*=0.006 (-0.250)
E-cadherin	NS	NS	*P*<0.0001 (-0.455)	NS	NS	NS	NS	NS	*P*=0.005 (-0.246)	*P*=0.05 (-0.171)
nm23	NS	NS	*P*=0.005 (0.245)	*P*<0.0001 (0.342)	NS	NS	NS	NS	NS	*P*=0.044 (-0.179)
CD44	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
TIMP3	NS	NS	NS	NS	NS	NS	NS	NS	NS	*P*=0.015 (-0.215)
S100A4	NS	NS	NS	NS	*P*=0.015 (0.230)	NS	NS	NS	*P*=0.026 (0.203)	*P*=0.029 (0.200)
PML	NS	NS	NS	*P*<0.0001 (-0.318)	NS	NS	NS	NS	*P*=0.018 (-0.207)	*P*=0.003 (-0.255)

[a]Size: <4 cm vs. ≥4 cm; [b]growth pattern: polypoid vs. infiltrative; [c]histology: papillotubular vs. non-papillotubular; [d]lymphatic invasion: absent vs. present; [e]venous invasion: absent vs. present; [f]perineural invasion: absent vs. present; [g]pT stage: pT1+pT2 vs. pT3+pT4; [h]pN stage: pN0 vs. pN1+pN2; [i]pM stage: pM0 vs. pM1; [j]stage: stage I+II vs. stage III+IV; NS, statistically not significant



PML/p53 expression	*Case No*	*5-yr survival rate (%)*		*Survival duration*	
		*KM estimate**	*SE*	*Mean*	*Median*
Normal PML and p53	58	49	7	69 mo	60 mo
PML(N)/p53(N)	58	49	7	69 mo	60 mo
Abnormal PML and/or p53	65	23	6	37 mo	11 mo
PML(N)/p53(O)	38	32	8	46 mo	13 mo
PML(N)/p53(N)	16	13	8	18 mo	9 mo
PML(N)/p53(O)	11	0	0	19 mo	14 mo

Figure 2. *Kaplan-Meier survival curves showing significantly different survival rates for patients with gallbladder carcinoma, according to PML and p53 expression.* ***(a)*** *PML (P=0.014);* ***(b)*** *p53 (P=0.022);* ***(c)*** *PML/p53 expression profile. PML (normal, N)/p53 (N) vs. PML (N)/p53 (overexpression, O) vs. PML (loss, L)/p53 (N) vs. PML (L)/p53 (O) (P=0.015);* ***(d)*** *Normal expression of PML and p53 vs. abnormal expression of PML and/or p53 (P=0.009).*

common genetic alteration in human cancers,[31] and the prognostic significance of p53 overexpression has been reported in several malignancies, including those of stomach,[22] colon,[11] and endometrium.[4] In GBC, the reported immunohistochemical expression rate of p53 ranges from 39.6% to 70%,[2,8,25] and thus its prognostic significance is a subject of some controversy. These discrepancies may arise from differences in the sources of antibodies, immunohistochemical methods (i.e. antigen retrieval) or variations in the cutoff values used to define positive immunohistochemical expression. The small sample sizes assessed for p53 expression in GBC (20 to 60 cases) may

be another factor affecting proper statistical analysis. The current study represents the largest clinicopathologic analysis of GBC in terms of the p53 expression state.

To our knowledge, this is the first report on the prognostic significance of PML in human GBC. In particular, loss of PML expression is related to poor prognosis and lymphatic invasion, metastasis, and stage of GBC, suggesting that the protein may also be involved in GBC progression. However, despite its prognostic significance, the clinical application of PML as a predictor of GBC may be limited, as loss of protein expression was identified in only 22% of our GBC samples. Accordingly, we examined the use of both PML and p53 expression profiles in the prognostic analysis of GBC. Patients with normal PML and p53 expression showed favorable outcomes, compared to those displaying abnormal expression of PML and/or p53 (49% *vs.* 23% in a 5-year survival rate; 60 months *vs.* 11 months in median survival, respectively; p=0.009). The significance of the combined PML/p53 expression profile in predicting outcome of GBC was independent in multivariate analysis.

Although the molecular mechanism of PML inactivation and its biological activity remain to be clarified, our results form the basis for the development of clinically applicable PML/p53-based molecular screening of GBC cells in bile, and highlight the importance of PML as a target for novel therapeutic agents. Given the identified roles of p53 and PML in the regulation of apoptosis, growth and DNA repair,[9,12,28] these molecular markers may provide additional information related to therapeutic responses for neoadjuvant chemotherapy or radiotherapy in patients with GBC.

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