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Comparative Immunohistochemical Studies of p53 and Proliferating Cell Nuclear Antigen Expression and Argyrophilic Nucleolar Organizer Regions in Pancreatic Duct Cell Carcinomas

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Thirty-eight cases of pancreatic duct cell carcinoma were examined for p53 expression and proliferating cell nuclear antigen (PCNA) by enhanced immunohistochemistry, as well as for changes in numbers of argyrophilic nucleolar organizer regions (AgNORs). Fifteen cases (39.5%) showed p53 overexpression, which tended to increase in proportion to the histopathological grading of malignancy. However, tumor stage and lymph node status were not correlated to p53 overexpression. PCNA labeling index (LI) increased with both histologically malignant grading and pathological stage, but was not correlated with lymph node status. The expressions of p53 and PCNA thus did not necessarily reflect the degree of malignant development. In contrast, AgNOR number showed statistically significant correlations with these three indicators of malignancy. A comparative analysis of p53, PCNA LI and AgNOR number showed overexpression of p53 to be correlated to PCNA LI and essentially unrelated to AgNOR number. The present results thus indicate a close relation between p53 and PCNA, while AgNORs appear to be regulated separately from either of them.

Key words: Pancreatic cancer — p53 protein — PCNA — AgNORs — Enhanced immunohistochemistry

Recent advances in molecular biology suggest that deletion and/or point mutation of the p53 gene are closely related to carcinogenesis in various organs, particularly the lungs,[1] breasts[2,3] and colon.[4] However, only a few immunohistochemical studies on p53 protein in PDCs[2] have been conducted owing to difficulties arising from fragility of the antigenicity of p53 protein to formalin-fixation.

A new enhanced immunohistochemical method for the retrieval of antigens in formalin-fixed, paraffin-embedded tissue was developed by Shi *et al.*[5] with the aid of microwave oven heating in the presence of heavy metal solution at over 100°C. They reported immunohistochemical results for 52 polyclonal and monoclonal antibodies, and enhancement effects for 39 of them. Thus, certain antibodies typically unreactive with formalin-fixed tissues can be used with this method to achieve excellent staining.

PCNA, which is implicated in cell proliferation[6] and believed to be regulated by p53,[7] is considered to be a useful indicator for tumor grading because of its increased appearance in various malignant tumors.[8,9] NORs, which are closely related to cell proliferation,[10] also correlate well with the grade of biological malignancy.[11]

In the present study, 38 cases of PDC were investigated for p53 and PCNA expression in combination with change of AgNOR number, especially with respect to cell proliferation. To our knowledge, this is the first comparative analysis of these parameters in PDC.

MATERIALS AND METHODS

Cases Thirty-eight consecutive cases of surgically resected PDC were available from the records of Kitasato University Hospital from April 1986 to April 1992. The patients consisted of 27 males and 11 females with an average age of 59.7 (42 to 73). The tumors were classified and graded according to the General Rules for Cancer of the Pancreas published by the Japan Pancreas Society. Lymph node metastasis was detected in 26 of the 38 cases.

Eight cases of chronic pancreatitis and 10 cases of normal pancreatic tissues surgically removed were prepared for use as negative controls.

All examined tissues were fixed in 10% formalin for 24 to 72 h and routinely embedded in paraffin.

Immunohistochemistry Serial sections from individual cases were cut at 4 μm. Enhanced immunohistochemistry by means of microwave oven heating in the presence of 1% zinc sulfate solution was performed according to the method described by Shi *et al.*[5] Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex method (Vectastain ABC Kit, Vector Labo-

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[2] Abbreviations: PDC, pancreatic duct cell carcinoma; PCNA, proliferating cell nuclear antigen; LI, labeling index; AgNOR, argyrophilic nucleolar organizer region.

ratories, Burlingame, USA). The primary antibodies used were PAb1801 (×50 dilution, Oncogene Science, Manhasset, USA) and CM1 (×1000, Novocastra Laboratories, Newcastle, UK) for mutant and wild-type p53 and PC10 (×100, Novocastra Laboratories) for PCNA.

In addition to the enhanced immunohistochemical staining, conventional immunohistochemical staining was conducted in all cases for confirmation.

Breast cancer tissue for which the presence of p53 point mutation had been proved was employed as a positive control. Non-immune swine serum or phosphate-buffered saline were used as a control for specificity of mouse or rabbit primary antibody binding. PCNA LI values were calculated after counting PCNA-positive nuclei per 1000 nuclei in randomly selected high-power fields of PDC (Olympus, BH-2, ×40 objective, ×10 ocular). Percentages of p53-positive cells were evaluated by the same procedure.

AgNOR staining This staining was conducted according to a modified version of the method described by Ploton *et al.*[12] AgNOR dots per nucleus were counted per 100 cells in viable cancerous lesions of each case at high power, and the average numbers were calculated.

Statistical analysis For statistical evaluation of the data obtained, chi-square and Wilcoxon-Mann-Whitney tests were conducted using a Fisher's software package.[13] Standard deviations (SD) were routinely calculated.

RESULTS

Expression of p53 Fifteen out of 38 cases (39.5%) with PDC showed positive reactions for both PAb1801 and CM1. The detection rate for the p53 protein by PAb1801 and CM1 was the same, though their binding ability differed. PAb1801 showed 7 cases of strongly positive versus 8 of weakly positive, and CM1 demonstrated 10 cases of strongly positive versus 5 of weakly positive. A similar tendency was noted for cases of more than 50% positive cells (Table I). In addition, the immunohistochemical staining with CM1 was more strictly limited to the nuclei as compared to PAb1801 (Fig. 1a, b). Neither chronic pancreatitis cases nor normal pancreas tissue showed p53 protein positivity.

With regard to the correlation between p53 expression and histologically malignant grading, lymph node involvement and pathological stage assessed according to the General Rules for Cancer of the Pancreas, statistically significant differences were noted between the papil-

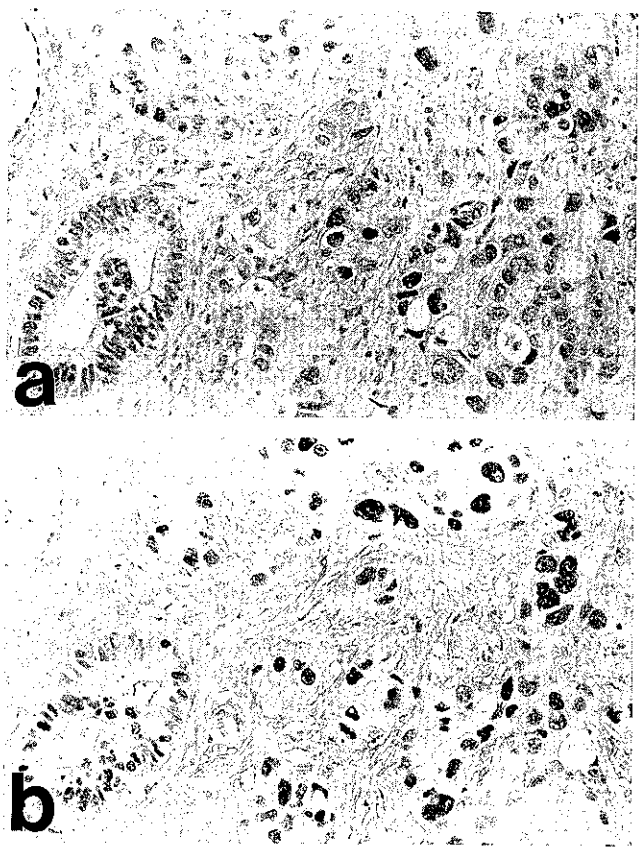

Fig. 1. Positive staining for the p53 protein in PDC. a) PAb1801, ×100. b) CM1, ×100. The positive reaction is more strictly limited to the nuclei of cancer cells with CM1, which also stains a higher proportion of cells than PAb1801.

Table I. p53 Protein Antibody Immunoreactivity in PDC

Pretreatment	PAb1801 (n=38)				CM1 (n=38)			
	−	+	++	>50% positive cells	−	+	++	>50% positive cells
Microwave (+)	23	7	8	4	23	5	10	7
Microwave (−)	36	1	1	0	34	2	2	1

Microwave, treated by microwave-oven heating.
−, negative; +, weakly positive; ++, strongly positive.

Table II. Relationship between Pathologic Features and p53 Expression, PCNA LI or AgNOR Number

	No. of cases	p53 positive cases (%)		P value	PCNA positivity (mean±SD%)		P value	AgNOR number		P value
Normal pancreas	10	0		*	4.16±0.92		*	1.43±0.36		*
Chronic pancreatitis	8	0			4.66±1.27			1.29±0.30		
Pancreatic cancer	38	15 (39.5)		*	26.60±12.67		*	2.51±0.59		*
Stage I	2	0	2/11	NS	11.80±5.80	16.27±5.60	*	1.61±0.11	2.04±0.34	**
II	9	2 (22.2)			17.27±5.37			2.14±0.29		
III	15	6 (40.0)	13/27		23.87±7.79	30.82±12.37		2.37±0.38	2.70±0.57	
IV	12	7 (58.3)			39.50±11.66			3.12±0.49		
Histologic grade of malignancy										
papillary	2	0	4/19	**	16.05±3.47	18.62±8.21	*	2.16±0.38	2.24±0.47	**
well	17	4 (23.5)			18.92±8.61			2.25±0.48		
moderately	12	7 (58.3)	11/19		29.10±7.55	34.59±11.32		2.49±0.39	2.78±0.59	
poorly	7	4 (57.1)			44.00±10.79			3.28±0.55		
Lymph node involvement										
positive	26	10 (38.5)		NS	29.27±13.06		NS	2.69±0.56		**
negative	12	5 (41.7)			20.83±9.98			2.12±0.46		

*, $P<0.0005$; **, $P<0.05$; NS, not significant.

lary and well differentiated carcinoma groups and the moderately and poorly differentiated carcinoma groups. However, no significant variation was found for pathological stage or lymph node involvement (Table II).

PCNA LI PCNA LI values were significantly different between cancerous lesions and non-cancerous tissues (Table II), depending also on histologic grade and stage. For lymph node involvement, no meaningful relation was obtained. Fig. 2a, b illustrates PCNA features in cancer and normal tissue. The microwave oven heating method dramatically improved PCNA expression.

AgNOR number Statistically significant differences were apparent between cancers and non-cancers, and also for stage, grade and lymph node involvement (Table II). Fig. 3a, b illustrates AgNOR staining in PDC and chronic pancreatitis.

Correlation among p53 overexpression, PCNA LI and AgNOR number Values for PCNA LI between the positive and negative p53 expression groups were statistically significantly different. However, no significant difference between p53 expression and AgNOR number could be found (Table III).

Analysis of the relation between p53 expression and PCNA LI In the p53-positive group, 13/15 were distributed in the PCNA LI range higher than 25%. In contrast, in the p53-negative group, 18/23 were distributed in the range below 25% (Table IV).

DISCUSSION

Most previous studies using conventional immunohistochemical procedures to detect p53 expression were performed on cryostat sections, since p53 protein is very labile and its antigenicity deteriorates during routine processing for formalin-fixed and paraffin-embedded sections.[14–16] The enhanced immunohistochemical method applied in the present study was nevertheless successful at detecting p53 protein since in a pilot study on breast cancer using the same antibodies for p53, the results were similar to those using fresh frozen tissue (data not shown). The mechanisms underlying microwave oven recovery of antigen are not clear, since this treatment has no effect on alcohol-fixed paraffin sections. Possibly, cross-linking of proteins caused by formaldehyde may be altered by microwave heating. Some studies have used heavy metal salts with formalin for tissue fixation.[17] Re-fixation by heavy metal salt solution may improve the immunoreactivity of antigens.[18]

The antibodies for p53 protein in this study were PAb1801, a monoclonal antibody directed to p53 amino acid residues 32 to 79 out of 393 amino acids[19] and CM1, a polyclonal antibody directed to a broader sequence.[20] Both react with wild-type and mutant-type p53 protein.[21] Mutant-type p53 protein is more stable, with a longer half-life, making possible its detection by immunohistochemistry,[1] whereas this cannot be done for wild-type p53 protein due to its short half-life.[15, 22, 23] Barton *et al.*[24] demonstrated immunodetectable p53 protein (CM1) in 23% of PDC and suggested that p53 activation is an important event in human PDC tumorigenesis. They considered that the CM1 antibody could detect a high proportion of cases of overexpression of mutant p53 in archival pathological material. In this study, the figure of about 40% of the cases showing p53

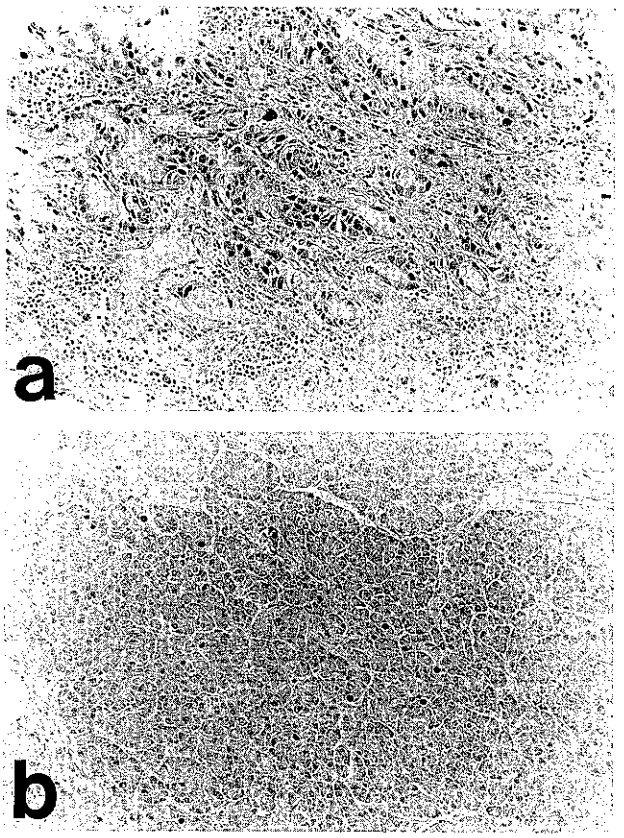

Fig. 2. Anti-PCNA immunohistochemical staining. a) Most nuclei of PDC cells are strongly stained. ×40. b) Normal pancreas has a low frequency of positive nuclei, with rather weaker staining. ×40.

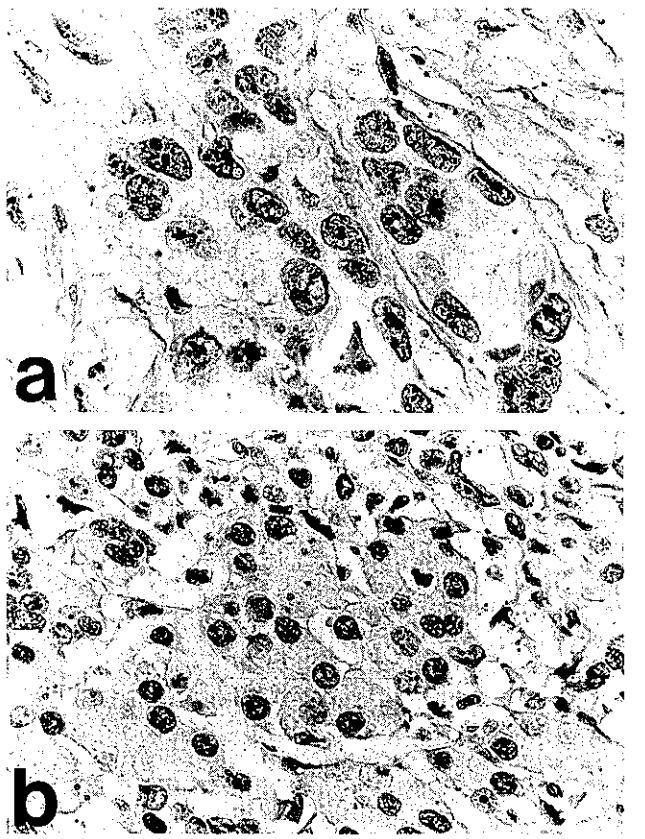

Fig. 3. AgNOR staining. a) A PDC demonstrates bizarre nuclei containing single or several clumped AgNORs with large numbers of minute or dispersed AgNORs. ×160. b) Chronic pancreatitis shows small, round nuclei containing a relatively small number of AgNORs. ×160.

expression in PDC therefore seems reasonable, even using an enhanced immunohistochemical method. Walker *et al.*[15] suggested that detectable p53 protein is related to histologically malignant grading, but not significantly correlated to lymph node status or pathological stage in breast cancers. In colorectal cancers, the incidence of p53 overexpression was high in cases with distant metastasis, but was not affected by pathologic features such as tumor size or depth of invasion.[25] In the present study, the incidence of p53 expression significantly increased in proportion to histologically malignant grading, whereas no differences in pathologic stage and lymph node involvement could be found. Our results are thus quite similar to those for breast cancer. In PDC, we consider that p53 expression might increase in proportion to cancer progression and would not necessarily represent actual biological malignancy. PCNA expression might increase in proportion to malignant grading within limited steps of cancer development, as well as p53 expression.

Table III. Relationship between p53 Detection and PCNA LI or AgNOR Number

	p53 detection		*P* value
	positive (n=15)	negative (n=23)	
PCNA LI (mean±SD)	36.13±11.73	20.39±8.96	<0.0001
AgNOR number	2.68±0.64	2.40±0.54	NS

Visakorpi *et al.*[16] reported high levels of p53 protein immunoreactivity in tumors showing a high PCNA LI (twice that of p53-negative tumors), and there is a good deal of evidence for such a tendency in tumors with histologically high-grade malignancy and DNA aneu-

Table IV. Relationship between p53 Expression and PCNA LI

PCNA LI	p53 detection: positive (%) (n=15)			p53 detection: negative (%) (n=23)		
<10	0	2 (13.4)	*	3 (13.0)	18 (78.2)	*
11–25	2 (13.3)			15 (65.2)		
26–40	8 (53.4)	13 (87.6)		4 (17.4)	5 (21.8)	
>41	5 (33.3)			1 (4.4)		

* $P<0.0005$.

ploidy. However, this p53 expression does not necessarily reflect the actual progress of malignant development. In the present study, positive ratios of p53 and PCNA expression and their distribution were essentially the same, which might lead to the conclusion that expression of mutant-type p53 is closely related to that of PCNA. Recent studies also showed that wild-type p53 protein inhibits cycle progression to the S phase[26] and that inhibition of cell cycle progression to this phase following wild-type p53 gene induction is accompanied by selective down-regulation of PCNA mRNA and protein expression.[7]

No correlation between p53 expression and AgNORs could be found in the present study, while the opposite was the case for p53 and PCNA expression. AgNOR number under normal conditions varies according to cell cycle stage, increasing in the M and early G1 phases and decreasing in late G1, S and G2,[27] in contrast to PCNA. It is still unclear why the AgNOR number increases in malignancy. It seems likely that NORs have functions distinct from those of p53 and PCNA.

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