Immunohistochemical Analysis of p53 Protein and Proliferating Cell Markers (PCNA and Ki 67) in Very Well Differentiated Adenocarcinoma of the Stomach

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Özet: *MİDENİN ÇOK İYİ DİFFERANSİYE ADENOKARSİNOMLARINDA p53 PROTEİN VE HÜCRE PROLİFERANSYON BELİRLEYİCİLERİNİN (PCNA ve Ki67) İMMÜNHİSTOKİMYASAL ANALİZİ*

Bu çalışmada 14 erken mide karsinomu (9 vaka çok iyi diferansiye tubuler adenokarsinom ve 5 vaka iyi diferansiye tubuler adenokarsinom) ile 3 adenom vakasında p53 protein overekspresyonu immünhistokimyasal olarak incelendi. Total 14 erken mide karsinomun %57,144ünde olmak üzere özellikle de çok iyi diferansiye olanların _66,6'sında pozitif immün reaktivite saptandı. Buna karşılık adenom vakalarının hiçbirinde immün boyanma görülmedi. Bu vakalara ayrıca immünhistokimyasal olarak PCNA ve Kİ67 (Hücre proliferasyon belirleyicileri) uygulanarak proliferasyon aktiviteleri değerlendirildi. İki karsinom grubu arasında PCNA immünreaktivitesi açısından farklılık yoktu ancak çok iyi diferansiye karsinomlardaki PCNA reaktivite oranı adenomdan daha yüksek olarak bulundu. Karsinomların her iki grubundaki Ki67 immünreaktivitesi de adenomdan daha yüksekti.

Sonuçta; rutin histolojik materypalde hücre proliferasyon indeksini belirleyen PCNA ya da Ki67 ile p53 proteinin immünhistokimyasal olarak analizinin tubuler adenokarsinom ve tubuler adenom arasındaki borderline vakaların ayırıcı tanısına yardımcı olabileceği düşünülmüştür.

Anahtar kelimeler: p53, PCNA, Ki67, mide adenomu, mide karsinomu, immünhistokimya.

Summary: *Overexpression of p53 protein was analyzed immunohistochemically in 14 early gastric carcinoma (9 cases of very-well differentiated tubular adenocarcinoma and 5 cases of well differentiated tubular adenocarcinoma) and 3 pure adenoma. Positive immunoreactivity was observed in 57.14% of 14 total early gastric carcinoma, especially in 66.6 % of very-well differentiated types. But no staining for p53 was seen in adenomas Immunohistochemistry using the PCNA and Ki67 was also examined in those cases. No diferences in the immunoreactivity for PCNA among two carcinoma groups, however, percentage of PCNA in very-well diferentiated carcinoma higher than adenoma. Immunoreactivity of Ki67 in both of carcinoma groups higher than adenoma.*

There is considerable evidence that immunohistochemical expression of p53 and PCNA and Ki67 for assesing cellular proliferations in routine histological material can allow us to make diferential diagnosis in borderline cases between tubular adenocarcinoma and tubular adenoma.

Key words: p53, PCNA, Ki67, gastric adenoma, gastric carcinoma, immunochemistry.

The histological differential diagnosis between tubular adenocarcinoma and tubular adenoma ismade by various structural and cellular features. However, differences in diagnosis by different pathologists often occur. This tends to happen especially in very-well differentiated tubular adenocarcinoma between differentiated tubular adenocarcinoma and tubular adenoma. Because interpretation of structural and cellular atypia is subjective.

Activation of oncogenes has been demonstrated in a wide variety of human malignancies. It is now thought that the increased expression of on-

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cogene is a prerequisite for the selective growth advantage of cells containing additional gene copies. It also may be the principal contribution of gene amplification to tumorigenesis. It is very important to examine the multistage nature of tumorigenesis, including the activation of oncogenes and the mutation of tumor suppressor genes of naturally occurring human cancer (1). The p53 gene is localized to chromosome arm 17p13. It acts as a tumor suppressor gene. Analysis of this gene in several malignancies has revealed that mutations of the gene are the commonest genetic abnormality in human cancer (2). In human stomach cancer, abnormalities of p53 have been reported and are considered to play important roles in the diagnosis and biologic behaviour of the tumor (3).

In addition, information on cell kinetics may be useful adjunct to histologically based tumour classifications in the understanding of tumour behaviour. Various methods are widely used to measure the proliferating cells in gastric cancer as well as in the other cancer (4). However, immunohistochemical techniques are simple and now widely used in routine histopathologic examination. If available, antibodies to cell cycle-related antigens were applicable, this method would be more useful for quantitation of the proliferating cells. A proliferating cell nuclear antigen (PCNA), also called cyclin, is a 36 kd proliferation-associated and the level of synthesis correlates directly with rates of cellular proliferation and DNA synthesis. Elevated levels of PCNA appear in the nucleus during the late G1 and S phases (4,5). Recently, Ki 67-a monoclonal antibody has become available which defines the nuclear antigen present in proliferating cells. A detailed cell cycle analysis showed that Ki67 antigen is expressed through the whole cell cycle and Ki67 antigen is expressed through the whole cell cycle and Ki67 equivalents are available not only in frozen section but also in formalin fixed paraffin embedded tissue (6).

In this study, we examined the immunohistochemical expression of p53 and proliferative activity using PCNA and Ki67, in 14 cases of (9 cases of very well differentiated and 5 cases of well differentiated tubular adenocarcinoma) early gastric carcinoma and 3 cases of pure gastric adenoma and discuss the relationship of the expression of p53 oncogene and PCNA, Ki67 products with differential diagnosis in borderline cases between differentiated tubular adenocarcinoma and tubular adenoma.

MATERIALS AND METHODS

Samples of early gastric carcinoma were available from 14 patients who underwent gastrectomy and pure gastric adenoma were obtained endoscopically from 3 patients at Hamamatsu University Hospital-Japan. The ages of the patients with carcinoma (11 males and 3 females) ranged from 48 to 75 years. All of the early gastric cancers were tubular adenocarcinoma and with tumor invasion confined to the mucosa. Nine cases were diagnosed as very-well differentiated types and 5 cases as well differentiated types. No lymph node metastasis were present in all of the cases. The specimens were fixed in 10% formaldehyde solution and processed routinely for histopathologic examination (H &E).

Immunohistochemical methods: Thin 3 μ slices from paraffin embedded specimens were deparaffinized in a routine manner. They were then boiled in DDW for p53 and PCNA and in 10 mμ citrate buffer for Ki67, for 15 minutes by microwave in order to improve the stainability. After that, they were kept at room temperature for 30 minutes and then in the water for cooling down.

The slides were then immersed in methanol with 0,3 % hydrogen peroxide for 15 minutes to block any endogenous peroxide activitiy. They were washed three times with 0,01 mol/L PBS for 5 minutes each time and treated with 10% normal rabbit sarum for 10 minutes at room temperature. After washing, the slides were incubated with the primary antibody DO7 (Novocastra, UK) -p53 and MIB-1 (Immunotech, France)-Ki67 for 1 hour at room temperature in a moist chamber. The incubation time with the primary antibody PC10 (Novocastra, UK) for PCNA was 30 minutes. The optimal dilution for each of the antibodies was 1:100 for p53 and 1: 50-100 for Ki67 and 1: 150-200 for PCNA, as determined by preliminary studies. They were all diluted with 0,01 mol/L PBS containing 1% bovine serum albumin.

The Histofine method (Nichirei Co Ltd, Tokyo, Japan) was used for immunostaining. After

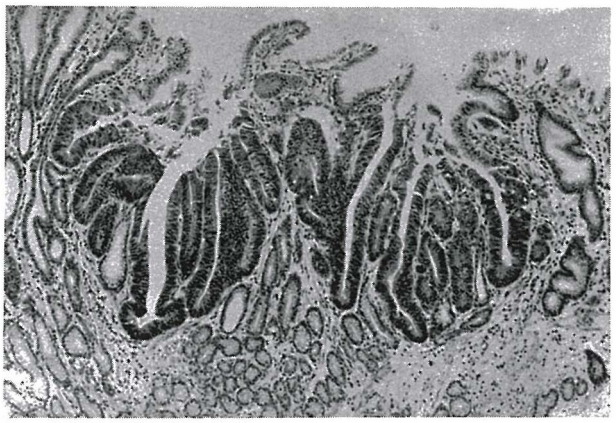

Figure 1a: Very well-differentiated tubular adenocarcinoma; carcinoma limited to the mucosa. HE x 100.

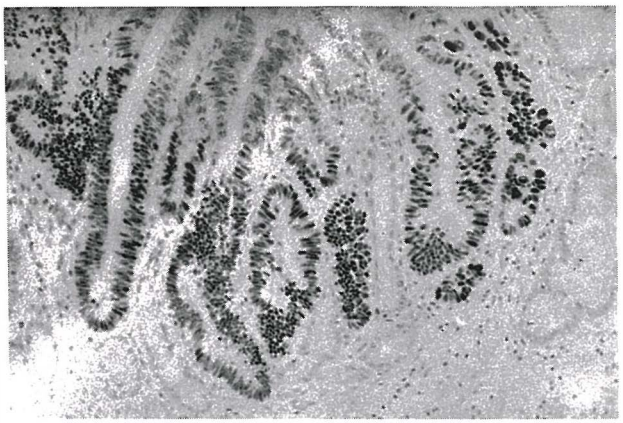

Figure 1b: Immunohistochemical staining of p53 in same case (very well diferentiated tubular adenocarcinoma). Most cells demonstrate nuclear p53 expression. x 200.

washing in 0,01 mol/L PBS, the slides were incubated 10 minutes at room temperature with biotinylated antimouse immunoglobulin. The slides were washed with 0.01 mol/L PBS for three times and then incubated at room temperature with peroxidase-conjugated streptavidin. After incubation, they were washed with PBS. A final wash was followed by immersion for about 10 minutes in a solution containing 20 mg/dl 3-3' - diaminobenzidine (DAB) and 10 ml/dl 30% - hydrogen peroxide in 0,05 % Tris -HCI buffered at pH 7.6. The slides were counterstained with Hematoxylin and dehydrated in alcohol, cleared in xylene and mounted in DPX.

Immunohistochemical analysis of case, using p53, Ki67 and PCNA was based on the percentage and the intensity of positive staining. A semiquantative immunoreactivity grading system was devised in which the whole of a section was assessed at al lower power (x100). Tumors were allocated a low grade of immunoreactivity if the observer assessed less than 50% tumor cell nuclei to be positive.

In an immunoreativitiy-positive samples, intensity of staining evaluated such as two grade: (+) and (++).

RESULTS

Using the monoclonal antibody, p53 protein was detected in cell nuclei in 8 (57.14%) of 14 total early gastric carcinoma. Especially, positive immunoreactivity was observed in 66.6% of very-well diferentiated types (Figure 1a,1b) but none of the 3 pure adenomas (Table I) (Figure 2). No immunoreactivity of p53 was observed in epithelialcells of non-carcinomatous normal gastric

Table I: Immunohistochemical Detection of p53 .

Diagnosis	No of Cases	P53 immunore activity	Immunoreactivity grade		Intensity	
			Low	High	+	++
Very-well * difg. tubular adenocarcinoma	9	6(66,6%)	2 33.3%	4 66,6%	1 16,6%	5 83,3%
Well dif.* tubular adenocarcinoma	5	2(40,0%)	1 50%	1 50%	1 50%	1 50%
Adenoma	3	0(100%)				

* Adenocarcinoma limited to the mucosa - early gastric carcinoma.

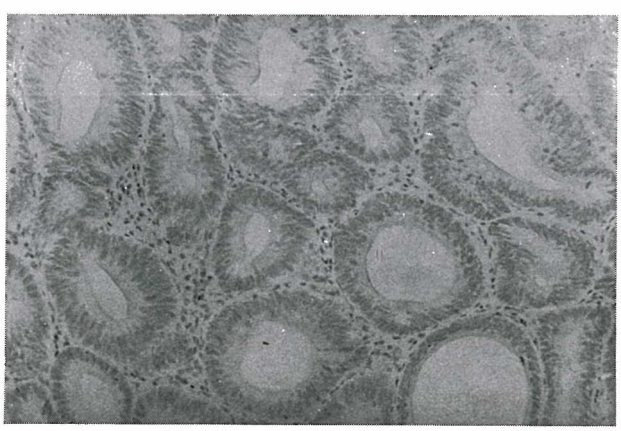

Figure 2: p53 negative cells in gastric tubular adenoma. x 200.

Table II: Immunohistochemical Detection of PCNA.

Diagnosis	No of Cases	Immunoreactivity grade		Intensity	
		Low	High	+	++
Very-well * difg. tubular adenocarcinoma	9	2 22,2%	7 77,7%	7 77,7%	2 22,2%
Well dif.* tubular adenocarcinoma	5	1 20,0 %	4 80%	1 20%	4 80%
Adenoma	3	3 100%	ND	3 100%	ND

* Adenocarcinoma limited to the mucosa - early gastric carcinoma ND: none detected

and intestinal metaplastic glands including the areas adjacent to the carcinoma. Interstitial cells were negative for p53. No diference of staining intensitiy was observed between very-well differentiated and well differentiated groups for p53.

Immunohistochemical analysis for PCNA and Ki67 have demonstrated that both of them presents a strong staining in the nuclei of tumor cells and germinal center cells of lymph follicles All of the examined adjacent mucosa to the tumor showed positive nuclear staining for both of them in the middle zone of normal gastric glands whilst in areas of intestinal metaplasia, it was located in the lower two-thirds of gland.

The PCNA immunoreactivity are detailed in Table II. High percentage of PCNA positive cells were observed very-well differentiated carcinomas (Figure 3) and percentage of PCNA immunoreactivity were not significantly different in both of carcinoma groups, although well differentiated tumors were more frequently high level of staining intensity than very-well differentiated tumors. However, PCNA immunoreactivity of adenoma was lower than very-well differentiated carcinomas and no significant differentation of staining intensitiy was observed between very-well differentiated carcinoma and adenoma.

The Ki67 staining pattern summarized in Table III. The rate of Ki67 positive cells in carcinoma cases (very-well and well differentiated groups) were higher than adenoma (Figure 4). The differences in the intensity of Ki67 among the carcinoma groups were small and not significant.

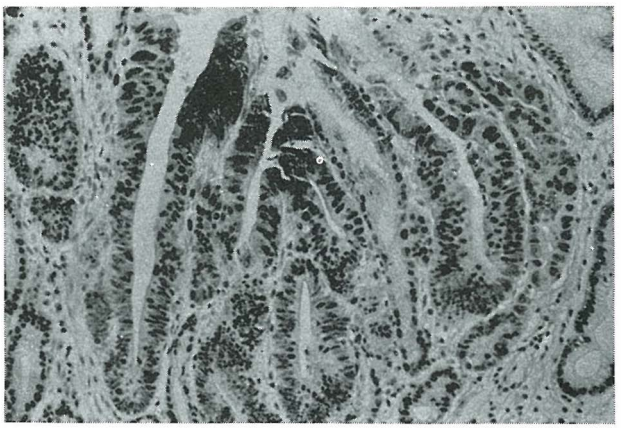

Figure 3: Immunohistochemical staining of PCNA in the case of Fig 1. High percentage of positive nuclei throughout the glands. x 200.

DISCUSSION

Abnormalities in p53 have been demonstrated in a wide variety of Common human malignancies. Overexpression of the p53 protein as determined by immunohistochemistry has been demonstrated to be closely correlated with the presence of DNA mutations in the p53 gene in different types of cancer, such as primary lung cancer (7), breast, colon and ovarian carcinomas (3). Thus, simple immunohistologic methods can provide strong evidence of such p53 DNA mutation (7). In human gastric cancer p53 mutations have been detected in 9 of 24 cases by nucleic acid-based studies (9). Martin et al. (3) reported immunohistochemical analysis of p53 in human gastric cancer and demonstrated that 57% of cases were positive for p53. Recently, Kakeji et al (10). demonstrated that immunoreactivity of p53 were found in 54% of gastric cancer. In this study, 57,14% of gastric carcinomas were positive for p53. Our results are consistent with previous results. Results from another group showed positivity in 27% of 48 well differentiated early gastric carcinamas (11). The discrepancy of our findings on the rate of p53 positivity compared with those of the study of Tohdo et al (11) may be due to the different methods and/or the small case number of our study. It is reported that no immunoreactivity for p53 was in the pure adenomas, but positive immunoreactivity was observed in 10,5% of the carcinomas arising in adenomas (17). In our results, 3 pure adenoma cases were negative for p53 and however, positive immunoreactivity was observed in 66.6

Table III: Immunohistochemical Detection of Ki67.

Diagnosis	No of Cases	Immunoreactivity grade		Intensity	
		Low	High	+	++
Very-well * difg. tubular adenocarcinoma	9	ND	9 100%	2 22,2%	7 77,7%
Well dif.* tubular adenocarcinoma	5	ND	5 100%	ND	5 100%
Adenoma	3	3 100%	ND	1 33,3%	2 66,6%

* Adenocarcinoma limited to the mucosa - early gastric carcinoma ND: none detected

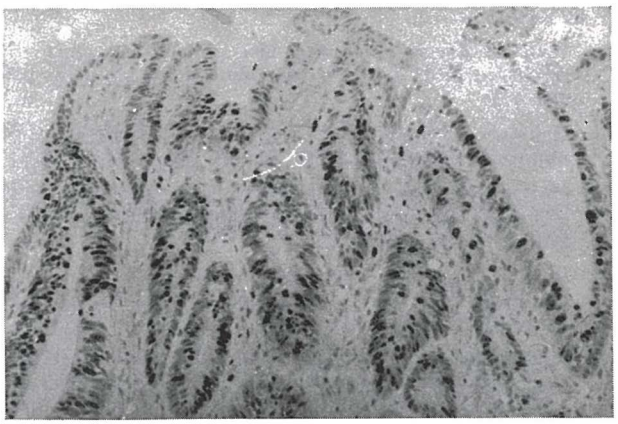

Figure 4: Immunohistochemical staining for Ki67: High percentage and intensity of Ki67 positivity in an other very well differentiated tubular adenocarcinoma case. x 220.

% of very well differentiated carcinomas. In all studies, p53 immunoreactivity was observed only in the nuclei of carcinoma cells, but not in those of adjacent nonpathologic gastric mucosa (11,12,15,17) such in this study. In addition, all findings that is consistent with a strong association of p53 immunoreactivity with malignant phenotype.

The results suggest that mutation of the p53 gene has an important role to play at an early stage. Additionally, we showed that applicability of immunohistochemical detection of p53 protein in routinely formalin fixed material. Immunoreactivity of p53 can allow as to make differential diagnosis in borderline cases between tubular adenocarcinoma and tubular adenoma.

In fact, recent immunohistochemical studies of PCNA have shown that PCNA immunoreactivity was a marker of cell proliferation in fixed histologic material (4). It would be expected that tumors with higher mitotic rates and higher degrees of atypia might show greater degrees of PCNA positivity (5). There are reports that PCNA may be a good indicator of prognosis in gastrointestinal lymphoma (13), hemangiopericytomas (14), and gastric carcinoma (15,16). In this study, the proliferating cell nuclear antigen positivity rates of the two carcinoma groups were not different, although well differentiated tumors were more staining intensity than very well differentiated tumors. Filipe et al (17). demonstrated that in the normal gastric glands, PCNA staining was largely confined to the middle zone of the gland whilst in areas of high grade dysplasia, to expended proliferative cell population throughout the glands and the PCNA positivity rates of dysplastic lesions were observed higher than normal mucosa. In this study, PCNA positivity of adenoma were lower than very well differentiated tumors. Our findings are consistent with previous results.

The Ki67 antibody enables the immunohistochemical detection of larger numbers of cycling cells in formalin fixed material (6). Monoclonal antibody Ki67 for the detection of growth fraction in tumors has previously been reported in malignant lymphoma (18) and breast tumors (19). There have been reports about the proliferating activity of gastric cancer measured by the use of Ki67 monoclonal antibody. High proliferative activity of Ki67 was observed in gastric cancer cells and there was no correlation between the Ki67 rates and the histologic grade (20,21). İn this study, Ki67 positivity rates of the two carcinoma groups were not different, but Ki67 positivity rates of very well differentiated carcinomas were higher than adenoma. There is considerable evidence that those immunohistochemical methods for assessing cellular proliferation in histological material provides useful information about biological behaviour and prognostic importance. Additionally, may be of some help in routine practice in discriminating between normal, borderline and carcinoma in the stomach.

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