Virchows Arch (1995) 426:557–562

ORIGINAL ARTICLE

Yasuhiko Kitayama · Haruhiko Sugimura
Masamitsu Tanaka · Shin-ichi Nakamura
Isamu Kino

Expression of p53 and flow cytometric DNA analysis of isolated neoplastic glands of the stomach: an application of the gland isolation method

Received: 19 October 1994 / Accepted: 13 April 1995

Abstract The expression of p53 was studied immunohistochemically in combination with the DNA ploidy pattern by gland isolation in 97 alcohol-fixed gastric lesions. A polyclonal antibody, CM-1, was applied to the paraffin-embedded sections in this study. Overexpression of the p53 protein was found in 73.2% of 41 well or moderately differentiated gastric carcinomas and 52.2% of 23 cases with poor differentiation ($P<0.05$). Immunoreactivity of p53 was also detected in isolated cancerous glands. No p53 immunoreactivity was detected in benign gastric lesions including adenomas, hyperplastic polyps and regions of intestinal metaplasia. In addition, flow cytometric DNA analysis was performed on isolated glandular epithelium adjacent to the portions used for immunostaining. DNA aneuploidy (DA) was detected in 85.7% of the well or moderately differentiated carcinomas and 42.9% of those with poor differentiation($P<0.05$). There was a positive correlation between DA, p53 positivity and the presence of regional lymph node metastasis, but not with other clinicopathological variables. In spite of the limited applicability of this method to poorly differentiated gastric cancer, we found that immunostaining and flow cytometry in combination with the gland isolation method facilitates analysis of gastric carcinogenesis.

Key words Gastric cancer · p53 immunostaining · Gland isolation method · DNA ploidy · Flow cytometry

Introduction

Abnormality of the *p53* gene is the most frequently detected genetic change in common human malignancies [2, 3, 7, 16]. Since antibodies to p53 have been developed, there have been many reports of immunohistochemical studies overexpression of p53 in human cancers [6, 8, 10, 11, 23, 25]. In gastric cancer, p53 positivity in paraffin-embedded materials has been reported at incidences varying from 33% to 57% [14, 18, 24, 26]. Its correlation with various markers such as erbB-2 and PCNA have also been studied [6, 8, 10, 11, 14, 18, 23, 24, 26]. The possible correlation of p53 overexpression with DNA aneuploidy has been discussed in only two reports by Tamura et al. [27] and Kakeji et al. [14]. In the present study, we examined the application of the gland isolation method [1, 4, 5] to the mucosal epithelium of the stomach to measure the DNA content of isolated cancer cells by flow cytometry (FCM) without stromal cell contamination. Simultaneously, we examined the expression of p53 in floating isolated glands to recognize p53 changes at the level of a single gland or several glands, as well as in adjacent alcohol-fixed paraffin-embedded sections, to compare p53 immunoreactivity with the DNA ploidy pattern.

Materials and methods

All specimens were obtained from surgical material at Hamamatsu University Hospital and its affiliated hospitals between 1991 and 1993. The tumours examined were 64 gastric carcinomas and 33 benign lesions (5 adenomas, 8 hyperplastic polyps and 20 regions of intestinal metaplasia). The histological categories of these gastric lesions studied are presented in Table 1.

Tumours were classified histopathologically according to the general rules for gastric cancer study in Japan [12]. Tubular carcinomas were classified histologically into well (tub1) and moderately (tub2) differentiated types according to the degree of glandular formation of the cancer epithelium. Poorly differentiated carcinomas were classified into the solid (por 1) and non-solid (por 2) types. Lymph node metastasis was confirmed by histological study, and the stage and depth of cancer invasion were also examined.

The gland isolation method has been reported by Arai and Kino [1] and Kitayama et al. [15]. Fresh gastric mucosal tissue was washed in calcium-magnesium-free Hanks' (CMFH) fluid at 4° C and cut into pieces of 2–3 mm^3. After incubation for 30 min at 37° C in CMFH with 30 mM ethylenediaminetetracetic acid the sus-

Y. Kitayama · H. Sugimura (✉) · M. Tanaka · I. Kino
First Department of Pathology,
Hamamatsu University School of Medicine, 3600 Handa-cho,
Hamamatsu 431-31, Shizuoka, Japan

S.-I. Nakamura
Department of Pathology, Hamamatsu University Hospital,
Hamamatsu, Shizuoka, Japan

Table 1 Incidence of nuclear p53 expression in gastric lesions (detected with polyclonal antibody. CM 1)

Histological subtype[a]	p53 staining				No. positive (%)	
	+++	++	+	−		
Carcinomas						
Tub 1	7	5	6	5	18 (78.3)	*
Tub 2	2	3	7	6	12 (66.7)	
Por 1	1	1	2	3	4 (57.1)	
Por 2	4	1	3	8	8 (50.0)	
Total	14	10	18	22	42 (65.6)	
Non-malignant lesions						
Adenoma				5	0	
Hyperplastic polyp				8	0	
Intestinal metaplasia				20	0	

[a] Histological classification of gastric carcinoma according to the Japanese Research Society for Gastric Cancer (1993). tub 1, well differentiated tubular carcinoma: tub 2, moderately differentiated tubular carcinoma: por 1 and por 2, solid and non-solid types of poorly differentiated adenocarcinoma.
−, +, ++, and +++, indicate increasing order of numbers of positive cells, +++: diffusely positive (>80%) ++: positive (10–80%) +: focally positive (<10%) (* $p<0.05$)

pensions were stirred for 30 min at room temperature in CMFH. Floating epithelial components including isolated glands were selected and promptly fixed in 70% ethanol. In all cases (46 cases), the portion adjacent to the cancer used for glandular isolation was examined histologically for the presence of cancer. In differentiated tumours we were able to verify whether the floating components were neoplastic glands and detected them easily under a stereo-microscope. In the poorly differentiated category, we first identified the floating aggregates when embedded in paraffin and confirmed the presence of cancer and the lack of stromal cell contamination. These materials containing floating neoplastic aggregates had been kept in 70% alcohol at 4° C before further analysis by FCM.

For immunostaining a piece of fresh gastric mucosa obtained just after gastrectomy was cut into pieces of 2–3 mm^3, fixed with 70% ethanol and embedded in paraffin by usual procedures. Rabbit polyclonal antibody CM1 (Novocastra, Newcastle, UK) was used as anti-p53 antibody, and immunostaining was performed by the streptavidin-biotin-peroxidase method according to the company's recommendations (Histofein Kit, Nichirei, Tokyo, Japan). Alcohol-fixed isolated glands were incubated with the first antibody at room temperature for 30 min, washed with phosphate-buffered saline, incubated with the second antibody (biotinylated goat anti-rabbit IgG) for 10 min and the incubated with 0.02% DAB (diaminobenzene tetrahydrochloride, Sigma) for 5 min for staining. Buffer containing Triton-100 (Sigma) was used for each step of rinsing and centrifugation.

Immunostaining of p53 was performed in paraffin sections of the tissue adjacent to the portion from which glands were isolated, and nuclear staining was done by methyl green. Immunoreactivity in paraffin-embedded material was estimated blindly by two independent pathologists. The extent of accumulation was graded as diffusely positive, positive and focally positive (+++, ++, +, respectively), depending on the numbers of p53 immunoreactive cells (+++, >80%; ++, 10–80%; +, <10%). The immunoreactivity of p53 in the isolated glands in each case was compared semi-quantitatively with that in an adjacent paraffin-embedded section.

For flow cytometry single glands isolated from both normal and neoplastic tissues were recovered separately from co-existing stromal cells, and digested with 0.025%–0.0125% pepsin at pH 1.5 (Sigma) at 37° C for 5 min. The suspensions of single nuclei were stained with propidium iodide (Sigma, St. Louis, Mo, USA), treated with RNase (Sigma) at a concentration of 0.25 mg/ml, and passed through 37 μm nylon mesh (Tokyo Screen, Tokyo, Japan). DNA was measured by FCM (Epics Profile, Coulter, Miami, Fla., USA). Normal background epithelium was used as an internal control. Detailed histographic analyses of normal, neoplastic and mixed tissues were carried out as reported previously [19].

The expression of p53 determined by immunostaining of floating glands and the corresponding paraffin-embedded materials were compared with the DNA ploidy pattern of the isolated glands. In two cases of the tub2 subtype of gastric cancer, alcohol-fixed paraffin material were not available.

Results

Immunohistopathology of p53

The results of p53 immunohistochemistry analyses of paraffin sections with antibody CM1 are shown in Table 1. Immunoreactivity for p53 was restricted to the nuclei of gastric cancer cells: no immunoreactivity was detected in the adjacent normal epithelium, stromal cells or cytoplasm of any cells. Representative staining patterns for each subtype of gastric cancer are shown in Figure 1.

The profiles of p53 immunoreactivity in isolated glands were consistent with those of paraffin-embedded sections. Positive staining was observed in 42 of 64 cases of gastric carcinoma (65.6%): 73.2% in well or moderately differentiated cases (n=41) and 52.2% in poorly differentiated cases (n=23). There was a significant correlation of the histological type with p53 immunoreactivity, but not with the numbers of positive cells (+++, ++, +) (Tables 1, 3). There was also a correlation between p53 immunoreactivity and the histological classification according to the general rules of the Japanese Research Society for Gastric Cancer, which was agreed upon in all cases by two pathologists.

A correlation was found between p53 positivity and lymph node metastasis (Table 3). Of seven lymph node negative and p53 positive cases, four were focally positive, two were positive and only one showed diffuse positive p53 staining. There was no correlation between immunoreactivity for p53 and the clinical stage. Cases of stage IV cancer showed p53 overexpression less frequently than those of stages II and III (Table 3), although the number of cases examined was small.

Adenomas, hyperplastic polyps and mucosa with intestinal metaplasia were all negative for p53.

DNA ploidy pattern of isolated glands and comparison with p53 immunoreactivity

As shown in Figure 2, the gland isolation method enabled us to observe the characteristic features of neoplastic glands under stereo-microscopy, as reported previously [15]. In the five samples of poorly differentiated carcinoma in which DNA analysis could not be done, stromal fibrosis was too severe to allow a sufficient epithelial component to be harvested by this isolation method. Table 2 shows the DNA ploidy pattern obtained with FCM

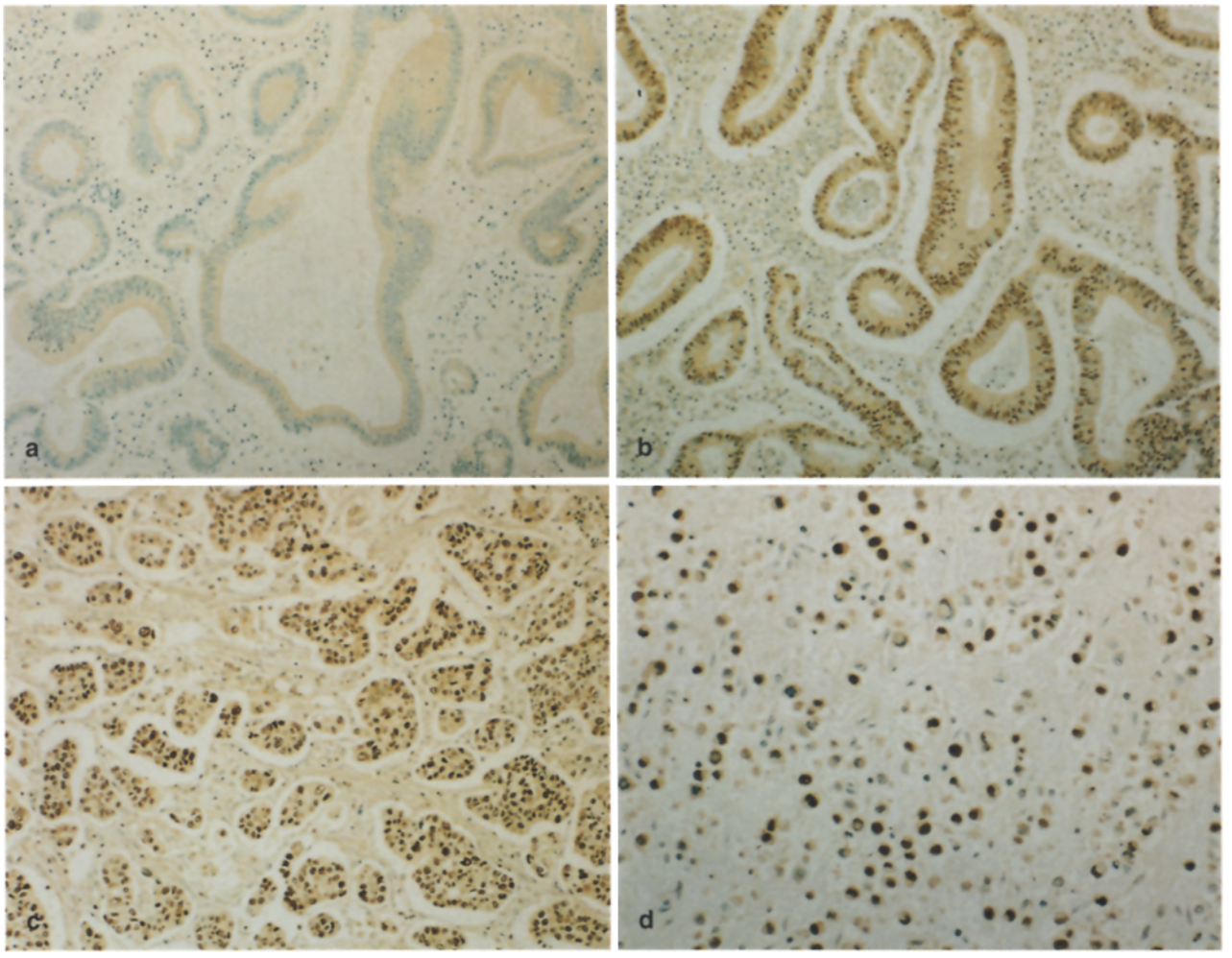

Fig. 1a–d Immunohistochemical staining of p53 in paraffin sections (polyclonal antibody, CM-1). **a** Gastric adenoma showing negative staining in the tumour nuclei, ×40. **b** Positive p53 nuclear staining in a well differentiated tubular carcinoma (tub 1), ×50. **c** p53 positive nuclear staining in non-solid type tumour (por 2), ×100. **d** Signet ring cells, ×200. Stromal cells show no staining

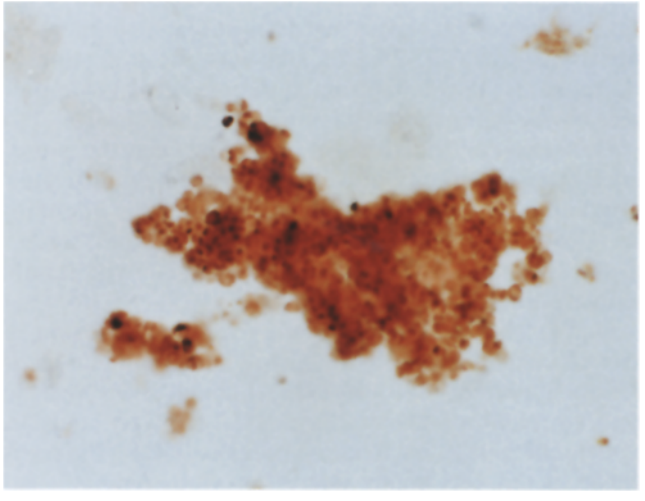

Fig. 3 p53 immunohistochemical staining of isolated epithelial sheets in which p53 positive cells are diffusely present, ×100

from isolated single epithelial glands in relation to histological type. We detected DNA aneuploidy (DA) in 37 of 46 gastric carcinomas (80.4%) by this procedure. The ratio of aneuploidy was found not to differ in different types (Table 2). However, combination of tub 1 and tub 2 types as cancers with well or moderate differentiation and por 1 and por 2 types as those with poor glandular differentiation showed that 86.1% of the former and 60.0% of the latter had DA (not significant). All 20 benign lesions examined were found to have diploid patterns.

We demonstrated p53 immunoreactivity in floating isolated glands, as shown in Figure 3. Immunoreactivity for p53 was found in two of nine diploid cases (22.2%), which all showed focal positive staining in contrast to 29 of 37 aneuploid cases (78.4%; Table 3).

Discussion

Although there are many reports on the prevalence or distribution of p53 immunoreactivity in gastric cancers [14, 18, 24, 26], there have been few studies on ethanol-fixed material, which are likely to give different results since p53 immunoreactivity is greatly influenced by the

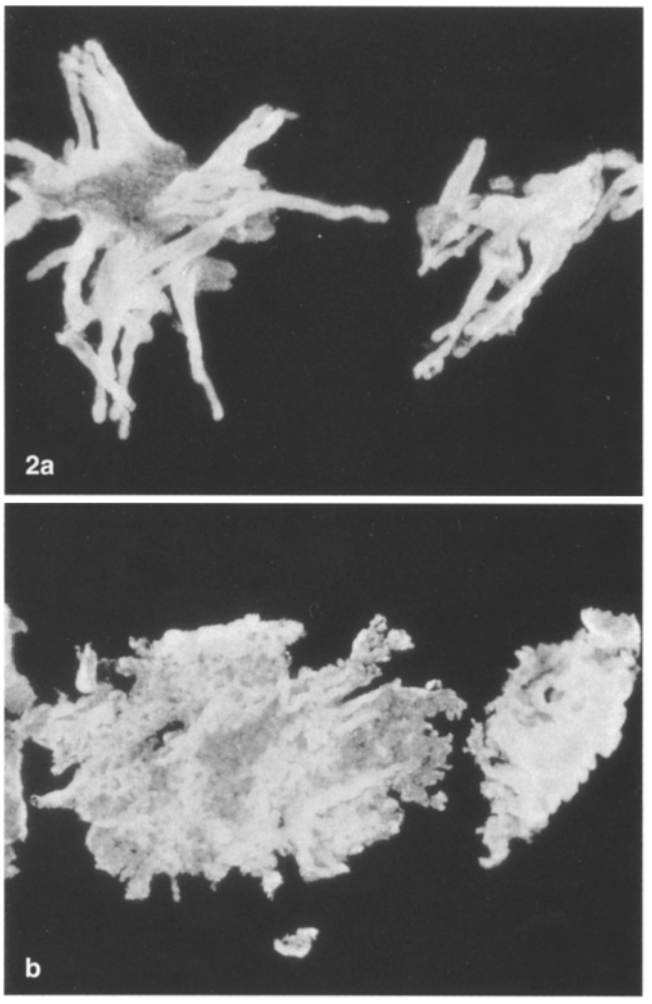

Fig. 2 Stereo-microscopic photograph of isolated normal fundic glands (**a**) and neoplastic glands (**b**) without stromal cell contamination, ×25

Table 2 Flow cytometric DNA analysis of gastric lesions

Histology	Diploidy	Aneuploidy (%)		Total
Carcinomas				
Tub 1	2	14 (87.5)	NS	16
Tub 2	3	17 (85.0)		20
Por 1	2	4 (66.7)		6
Por 2	2	2 (50.0)		4
Total	9	37 (80.4)		46
Non-malignant lesions				
Adenoma	4	0		4
Hyperplastic polyp	8	0		8
Intestinal metaplasia	8	0		8

conditions of fixation or heat treatment [9]. Recently, Martin et al. [18] and Starzynska et al. [26] examined CM-1 immunostaining in formalin-fixed gastric cancer tissue and reported immunoreactive p53 staining in gastric carcinoma at rates of 57% and 47.3%, respectively.

Table 3 p53 Immunohistochemistory on paraffin section and clinico-pathological variables of gastric carcinomas

		No. examined	No. positive (%)	P value
Sex:	male	39	26 (66.7)	NS
	female	25	16 (64.0)	
Age:	<60	17	12 (70.6)	NS
	>60	47	30 (63.8)	
Type:	well or moderately differentiated	41	30 (73.2)	<0.05
	poorly differentiated	23	12 (52.2)	
Stage:	I	12	6 (50.0)	NS
	II	23	18 (78.3)	
	III	19	14 (73.7)	
	IV	10	4 (40.0)	
Lymph-node metastasis				
	positive	50	35 (70.0)	<0.05
	negative	14	7 (50.0)	
DNA ploidy:	diploid	9	2 (22.2)	<0.05
	aneuploid	37	29 (78.4)	

The difference from our results, which showed a higher prevalence of p53 (65.6%), was probably due to differences in methods of fixation [6]. Since our samples were minced into pieces of 2–3 mm^3 promptly after surgery or biopsy, fixed in 70% ethanol and embedded as rapidly as possible, p53 antigenicity might have been better preserved. For a comparison of possible differences due to fixation conditions, adjacent samples fixed in formalin were also stained in the same manner, and found to show less immunoreactivity than in the ethanol-fixed material (data not shown).

The results in Table 1 showing restricted localization of p53 to nuclei and lack of immunoreactivity in benign gastric lesions are consistent with most previous reports, although there is one report cytoplasmic staining or positivity in flat adenomas and dysplastic gastric mucosa [13, 17].

Clinicopathological analysis revealed no correlation of the extent of immunoreactivity of p53 with age or the stage of cancer. However, our data, unlike most previous reports [21, 22, 25, 28, 29] and with the exception of Martin et al. [18], demonstrated the use of p53 as a possible biological marker for lymph node metastasis.

Our data confirmed a difference between the prevalence of p53 positivity in well and moderately differentiated, and poorly differentiated cancers. As this might reflect aetiological heterogeneity of these subtypes, studies on the spectra of *p53* mutations in each subtype should be interesting.

Our DNA ploidy data benefit from the exclusion of stromal contamination by the gland isolation method, as already reported [1, 4, 5, 19]. Although there was no clear correlation between histological subtypes and DA (not significant) as we reported previously [15], the prevalence of DA was higher than in previous reports. Cancers with well or moderately differented grades tended to

show high frequencies of DA. This method enabled us to detect a small aneuploid peak or near diploid aneuploidy which cannot be detected by conventional FCM DNA analysis. Substantial numbers of cases of isolated neoplastic glands show diploid DNA pattern. Interestingly, p53 abnormality was found more frequently not only in cancer cells with aneuploidy, which is consistent with the report of Tamura et al. [27], but also in two of nine diploid cases. It has been thought that p53 is expressed from the later G1 to S phase of the cell cycle [14, 30] and that the higher frequencies of p53 positivity in aneuploid cells reflect abnormal proliferation of tumour cells having aneuploid DNA. It is possible that we detected p53 immunoreactivity in the diploid cancer cases because our immunohistochemical procedure could detect small amounts of probably mutant p53 and revealed the extent of heterogeneities in terms of p53 accumulation in the population of tumour cells. This would imply that a genetic change such as a *p53* point mutation occurs before the following large scale chromosomal aberration in the later stage of gastric cancer [20]. However, the number of cases examined was small and the glands analysed here may have been heterogeneous in DNA ploidy. Alternatively it might be that immunoreactive p53 does not necessarily indicate mutation, as proposed by Fisher et al. [6]. DNA sequencing of further cases will be needed to clarify the role of immunoreactive p53 in diploid cancerous glands.

Gland isolated followed by immunostaining or flow cytometry is better than processing from paraffin-embedded materials, not only because an isolated gland has no stromal cell contamination, but also because it provides better material for future DNA extraction. Since 5 unsuccesful cases out of a total of 15 were all poorly differentiated subtypes, this procedure has limitations. However, we believe the combination of p53 analysis and DNA aneuploidy with the gland isolation method will facilitate the analysis of gastric carcinogenesis at the single or several gland level, and provide ideal specimens for genomic analysis.

Acknowledgements We are grateful to Mr. Igarashi and Mrs. Goto for their excellent technical assistance. This work was supported in part by the Ministry of Education, Culture and Science, the Ministry of Health and Welfare and the Smoking Research Foundation.

References

1. Arai T, Kino I (1989) Morphometrical and cell kinetics studies of normal human colorectal mucosa. Acta Pathol Jpn 39: 725–730
2. Banks L, Matlashewski G, Crawford L (1986) Isolation of human p53 specific monoclonal antibodies and their use in the studies of human p53 expression. Eur J Biochem 159:529–534
3. Bartec J, Barcova J, Vojtesk B, Staskova Z (1990) Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. Oncogene 6:1699–1703
4. Bjerknes M, Cheng H (1981) Method for the isolation of intact epithelium from the mouse intestine. Anat Rec 199:565–574
5. Cheng H, Bjerknes M, Amar J (1984) Method for determination of epithelial kinetic parameters of human colonic epithelium isolated from surgical and biopsy specimens. Gastroenterology 86:78–85
6. Fisher CJ, Gillet CE, Vojtesk B, Millis RR (1994) Problem with p53 immunohistochemical staining: the effect of fixation and variation in the method of evaluation. Br J Cancer 69: 26–31
7. Harris CC, Holstein M (1993) Clinical implications of the p53 tumor-suppressor gene. N Engl J Med 329:1318–1327
8. Hiyoshi H, Matsuno Y, Kato H, Shimosato Y, Hirohashi S (1992) Clinicopathological significance of nuclear accumulation of tumor suppressor gene p53 product in primary lung cancer. Jpn J Cancer Res 83:101–106
9. Igarashi H, Sugimura H, Maruyama K, Kitayama Y, Ohta I, Suzuki M, Tanaka M, Dobashi Y, Kino I (1994) Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. APMIS 102:295–307
10. Iwaya K, Tsuda H, Hiraide H, Tamaki K, Tamakuma S, Fukutomi T, Mukai K, Hirohashi S (1990) Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. Jpn J Cancer Res 85:835–840
11. Janet M, Bruner JH, Saya CH (1993) p53 protein immunostaining in routinely processed paraffin-embedded sections. Mod Pathol 6:189–193
12. Japanese Research Society for Gastric Cancer (1993) The general rules for gastric cancer study, 12th edn. Kanehara, Tokyo, pp 42–75
13. Joypaul VB, Newman LE, Hopwood D, Grant A, Quershi S, Lane DP, Cuschieri A (1993) Expression of p53 protein in normal, dysplastic, and malignant gastric mucosa: an immunohistochemical study. J Pathol 170:279–283
14. Kakeji Y, Korenaga D, Tsujitani S, Baba H, Anai H, Maehara Y, Sugimachi K (1993) Gastric cancer with p53 overexpression has high potential for metastasis to lymph nodes. Br J Cancer 67:589–593
15. Kitayama Y, Nakamura S, Sugimura H, Kino I (1995) Cytophotometric and flow cytometric DNA content of isolated glands in gastric neoplasia. Gut 36:516–521
16. Lane DP, Benchimol S (1980) p53 oncogene or anti-oncogene. Genes Dev 4:1–8
17. Lauwers GY, Wahl SJ, Melamed J, Rojas-Corona R (1993) p53 expression in precancerous gastric lesions: an immunohistochemical study of PAb 1801 monoclonal antibody on adenomatous and hyperplastic gastric polyps. Am J Gastroenterol 88:1916–1920
18. Martin HM, Filipe MI, Morris RW, Lane DP, Silvestre F (1992) p53 expression and prognosis in gastric carcinoma. Int J Cancer 50:859–862
19. Nakamura S, Goto J, Kitayama Y, Kino I (1994) Application of the crypt-isolation technique to flow cytometry analysis of DNA content in colorectal neoplasms. Gastroenterology 106:100–107
20. Ochi H, Douglas OH, Sandberg AA (1986) Cytogenetic studies in primary gastric cancer. Cancer Genet Cytogenet 22:295–307
21. Renault B, Broek M, Fodde R, Wijnen J, Pellegata SM, Amadori D, Khan MP, Ranzani NG (1993) Base transitions are the most frequent genetic changes at p53 in gastric cancer. Cancer Res 53:2614–2617
22. Sano T, Tsujino T, Yoshida K, Nakayama H, Haruma K, Ito H, Nakamura U, Kajiyama G, Tahara E (1991) Frequent loss of heterozygosity on chromosomes 1q, 5q, and 17p in human gastric carcinomas. Cancer Res 51:2926–2931
23. Sasano H, Miyazaki S, GooukonU, Nishihira T, Sawai T, Nagura H (1992) Expression of p53 in human esophageal carcinoma: an immunohistochemical study with correlation to proliferating nuclear antigen expression. Hum Pathol 23:1238–1243
24. Sasano H, Date F, Imatani A, Asaki S, Nagura H (1993) Double immunostaining for c-erbB-2 and p53 in human stomach cancer cells. Hum Pathol 24:584–589

25. Scott N, Sagar P, Stewart J, Blair GE, Dixon MF, Quirke P (1991) p53 in colorectal cancer: clinicopathological correlation and prognostic significance. Br J Cancer 63:317–319
26. Starzynska T, Bromey M, Ghosh A, Stern PL (1992) Prognostic significance of p53 overexpression in gastric and colorectal carcinoma. Br J Cancer 66:558–562
27. Tamura G, Kihara T, Nomura K, Terada M, Sugimura T, Hirohashi S (1991) Detection of frequent p53 gene mutation in primary gastric cancer by cell sorting and polymerase chain reaction single-strand conformation polymorphism analysis. Cancer Res 51:3056–3058
28. Uchino S, Noguchi M, Ochiai A, Saito T, Kobayashi M, Hirohashi S (1993) p53 mutation in gastric and colorectal cancer. Int J Cancer 54:759–764
29. Yamada Y, Yoshida T, Hayashi K, Sekiya T, Yokota J, Hirohashi S, Nakatani K, Nakano H, Sugimura T, Terada M (1993) p53 gene mutation in gastric cancer metastasis and gastric cancer cell lines derived from metastasis. Cancer Res 51:5800–5805
30. Yonemura Y, Fushida S, Tsugawa K, Ninomiya I, Fonseca L, Yamaguchi A, Miyazaki I, Urano T, Shiku H (1993) Correlation of p53 expression and proliferative activity in gastric cancer. Anal Cell Pathol 5:277–288