Acta Med. Nagasaki 34 : 73–77

Search for Cancer Antigens ; Monoclonal Antibodies and Oncogenes

Hiroshi Shiku, Kuniko Yoshida*, Hideo Tsuchiyama*, Eiichi Nakayama

*Depatments of Oncology and * Pathology, Nagasaki University School of Medicine*

INTRODUCTION

Oncogens originally identified as viral genes with transforming activities in experimental animals are now considered to play important roles in the oncogenesis of human cells [1]. Activation of these genes are often observed in various human tumors by analyzing their DNA and RNA with the corresponding molecular probes [2-7]. A variety of mechanisms result in either the increased production of normal gene products or the production of aberrant gene products. These may include gene amplification, translocation, mutation, and rearrangement. Gene products thus abnormally expressed in a cell may transform that particular cell and eventually lead to the establishment of cancer. Under the physiological condition, however, levels of their transcripts and products are also known to vary depending on the cell growth and differentiation.

To data, about 50 oncogenes have been isolated. Among them ras gene is the first oncogene with transforming activity isolated from human cancer cells. Subsequent analyses strongly suggest that activation of ras gene might participate in oncogenesis of various human cancers.

Two major reasons prompted us to study details of the profiles of expression of ras gene products p21 at the cellular level ; the first, precise information on the cell types expressing ras p21 is essential in investigations of functional roles of ras gene products under physiological conditions and in the state of oncogenic processing. the second, it is to answer the question of whether expression of ras p21 gene products is greater in certain types of human cancer, and if so, whether expression of ras genes is associated with cellular transformation, and whether ras gene products are useful as cancer markers. Monoclonal antibodies that react with the ras gene product p21 are particularly useful for this purpose since they allow us to detect p21 at both tissue and cellular levels.

We report here the production of anti p21 monoclonal antibodies and immunohistochemical analysis of stomach cancer by prepared monoclonal antibodies.

MATERIALS AND METHODS

Preparation of anti ras p21 monoclonal antibodies.

(BALB/c x C57BL/6) F_1 mice were immunized 4 times with v-Ki-ras p21 at 2-week intervals ; the first time subcutaneously with 50 μg of v-Ki-ras p21 and complete Freund adjuvant, the second time subcutaneously with 100 μg and incomplete Freund adjuvant, and the third and fourth times intraperitoneally with 100 μg and 200 μg, respectively. Three days after the last immunization, spleens of immunized mice were removed, and spleen cells were fused with NS − 1 cells as described previously [16]. Culture fluids of hybridomas were assayed for the presence of anti ras p21 antibody by enzyme linked immunosorbent assays and immunofluorescence assays. Limiting dilution was carried out twice or three times to obtain monoclonality.

IMMUNOHISTOCHEMISTRY

Avidin-Biotin complex techniques were employed (Vectastain, ABC Kits, Vector Labs). Sections (4mm) were dewaxed and rehydrated through alcohol. Endogenous peroxidase activity was blocked by incubating sections for 30 min in methanol with 0.3% H_2O_2. After washing sections with phosphate buffered saline (PBS), normal horse serum was added to sections and incubated for 20 min at the room temperature. The sections were then incubated at the room temperature for 30 min with anti ras p21 monoclonal antibodies (10 μg/ml). After washing sections with PBS, biotinylated horse anti mouse immunoglobulin antibodies were added and further incubated at the room temperature for 30 min, then excess antibodies were washed away with PBS. Sections were incubated with Peroxidase labeled Avidin-Biotin complexes for 60 min. The substrate was developed by an incubation with diaminobenzidine (0.5 mg/ml) in 0.05M Tris hydrochloric acid buffer (pH7.2) with 0.01% H_2O_2 for 5 min. The sections were washed and counterstained with the hematoxylin staining.

RESULTS

1. Incidence of p21 expression in stomach cancer.

We prepared 16 clones of anti ras p21 monoclonal antibodies (RASK-1 to 16). Their specificities were confirmed by enzyme linked immunosorbent assays and immunoblotting assays with ras p21 produced by E. coli and also with cellular lysates. Using one of these monoclonal antibodies, RASK-3, which reacts with p21 of all ras gene family, expression of ras genes in stomach cancer were analyzed by means of immunohistochemical stainings.

Cells at the cancerous area are generally strongly positive. In each individual cell, cytoplasma is diffusely stained. Morphologically normal epithelial cells are negative. Parietal cells and intestinal metaplasia are, however, often positive.

A summary of analysis in 80 cases of stomach cancer is shown in Table 1. Expression of p21 in cancer cells and normal epithelial cells on the same slides was evaluated. Cases were classified into 4 groups based on the proportion of p21 positive cells among the corresponding cell population. In 35 cases, more than 80% of cancer cells express p21 and in 28 cases, 50 to 80% of cancer cells express p21. Together, in 63 cases more than 50% of cancer cells express p21. On the otherhand, p21 positive morphologically normal epithelial cells are consistently less than 50%. In 75 cases, less than 5% of normal epithelial cells express p21. These figures clearly indicate that expression of p21 is more dominant in cancer cells than in normal epithelial cells. Based on this data, we defined "positive" when more than 50% of cells express p21, "partial positive" when 5% to 50% of cells express p21, and "negative" when less than 5% of cells express p21.

Table 1. Expression of ras p21 in 80 cases of stomach cancer determined by the ABC method

	% of positive cells			
	>80	50-80	5-50	<5
Cancerous Parts	35 (44%)	28 (35%)	5 (6%)	12 (15%)
Non-cancerous Parts	0	0	5 (6%)	75 (94%)

2. Incidence of p21 expression in non-cancerous stomach.

Expression of p21 was also examined in 53 cases of noncancerous stomach and results are summarized in Table 2. In 7 of 8 cases of atypical hyperplasia, more than 50% of the epithelial cells were positive, and in 2 of 15 cases of hyperplastic polyp, 5 to 50% of the cells were

Table 2. Expression of p21 in formalin fixed tissues of 53 cases of non-cancerous stomach determined by the ABC method

Tissue	No of Cases	Positive	Partially Positive	Negative
Atypical Hyperplasia(ATP)	8	7(88%)	0	1(13%)
Hyperplastic Polyp	15	0	2(13%)	13(87%)
Ulcer	13	2(15%)	1(8%)	10(77%)
Gastritis and others	17	0	3(18%)	14(82%)

p21-positive. In 3 cases of gastric ulcer, epithelial cells expressed p21. These 3 cases of gastri gc ulcer showed dominant regenerative patterns of the epithelium with healing of the ulcer and the same regenerating epithelial cells were p21 positive.

3. Correlation of p21 expression in stomach cancer with histological types.

Correlation of p21 expression in cancer with their histological types was examined (Table 3). In the tubular types of adenocarcinoma, 90% of well differentiated cancers, 79% of moderately differentiated cancers, and 41% of poorly differentiated cancers were positive in p21 expression, indicating some correlation of p21 expression with histological types of cancer.

Table 3. Expression of ras p21 in stomach cancer: Correlation with histological types of cancer

Histological Type	Cases tested	Cases positive	%positive
Papillary	9	8	89
Tubular			
Well diff.	10	9	90
Moderat. diff..	24	19	79
Poorly diff.	27	11	41
Mucinous	4	4	100
Signet ring	6	0	0
Total	80	51	64

Discussion

In most cases stomach cancer cells were strongly p21-positive. In many cases, a variety of normal cells, such as parietal, smooth muscle, and ganglion cells, were also found to express ras p21. However, normal epithelial cell, chief cells, and mucous cells of the stomach were mostly p21-negative. Thor et al. reported the similar findings and suggested that many of the cell types with enhanced ras p21 expression have been associated with ion exchange functions and/or produce hormone products which effect ion exchange mechanisms in effector organ to maintain homeostasis within the normal physiologic range[8]. Chesa et al. also reported from immnohistochemical analysis with anti ras p21 monoclonal antibodies that in many cell lineages, well differentiated cells usually express p21 more than poorly differentiated cells[10]. P21 is known to have GTP binding capacity and thus is related to cellular regulation of adenyl cyclase like other members of the G protein family. In highly-differentiated cells with specified functions, p21 may be dominantly expressed because it participated in critical cellular functions.

A crucial question is whether dominance of p21 expression in cancer cells is a consequence of cellular transformation. In stomach cancer, expression of p21 was apparently higher in cancer cells than in normal epithelial cells. This tendency was marked in moderately to well differentiated cancers.

Moderately to well differentiated types of stomach cancer have been considered to originate from epithelial cells of the stomach. There is also circumstantial evidence that these types of cancer may appear via intestinal metaplasia. Interestingly, our analyses showed that p21 expression is marked in moderately to well differentiated cancer, intestinal metaplasia, and atypical hyperplasia, but not in normal epithelial cells and hyperplastic polyps. These results may indicate that expression of p21 in epithelial cells of the stomach has increased as a consequence of cellular changes to premalignant status such as intestinal metaplasia and atypical hyperplasia. Alternatively, all these p21-positive gastric epithelial cells share morphological and other cellular characteristics with p21-positive epithelial cells of the intestine. From the pattern of p21 distribution observed in the present study we may illustrate pathways of cellular changes as shown in Fig. 1. If this is the case, moderately to well differentiated cancer, atypical hyperplasia, and intestinal metaplasia start to express p21 as a consequence their metaplastic changes to intestinal epithelial cells. Expression of p21 by these differentiated cancers is therefore preexistent to cytological transformation. This interpretation may support the view of Chesa et at. that p21 is related to cellular differentiation rather than to the maintenance of transformed

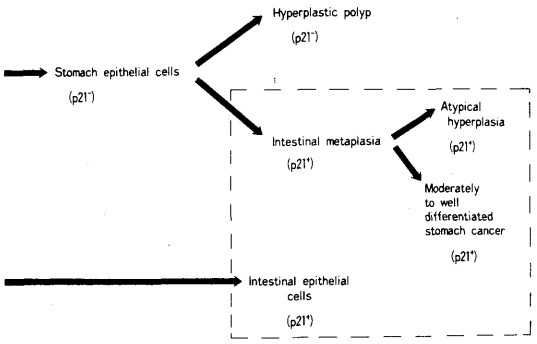

Fig. 1

phenotypes[9].

Analysis of p21 expression in cancer by the immunohistostaining were reported in colon cancer, mammary cancer, and prostate cancer [11-15]. In general, cancer cells were found to express p21 more dominantly than begign or normal cells. There are, however, reports on colon cancers in which investigators observed no significant difference of p21 expression between cancer cells and normal cells.

Further analysis is no doubt needed for evaluating ras genes as tumor marker. These may include, in addition to expansion of numbers and types of tissues, use of monoclonal antibodies specific for each of Ki-, N-, and Ha-ras gene products, and those specific for point mutation sites of peptides.

REFERENCES

1) Bishop, J. M. Viral Oncogenes Cell 42 : 23-38, 1985.
2) Ellis, R. W., DeFeo, D., Shin, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowy, D. R., and Scolnick, E. M. The src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature 292 : 506-511, 1981.
3) Der, C. J., Krontiris, T. G., Cooper, G. M. Trandforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc. Natl. Acad. Sci. USA. 79 : 3637-3640, 1982.
4) Shimizu, K., Goldfarb, M., Suard, Y., Perucho, M., Li, Y., Kamata, T., Feramisco, J., Stavnezer, E., Fogh, J., and Wigler, M. H. Three human transforming genes are related to the viral ras oncogenes. Proc. Natl. Acad. Sci. USA. 80 : 2112-2116, 1983.
5) Lane, M., Sainten, A., Cooper, G. M. Activation of related trandforming genes in mouse and human mammary carcinomas. Proc. Natl. Acad. Sci. USA. 78 : 5185-5189, 1981.
6) Murray, M. J., Shilo, B. Z., Shin, C., Cowing, D., Hsu, H. W., and Weinberg, R. A. Three different human tumor cell lines contain different oncogenes. Cell 25 : 355-361, 1981.
7) Peruchi, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J., and Wigler, M. Human-tumor-derived cell lines contain common and different transforming genes. Cell 27 : 467-476, 1981.
8) Thor. A., Ohuchi, N., Hand, P. H., Callahan, R., Weeks, M. O., Theillet, C., Lidereau, R., Escot, C., Page, D. L., Vilasi, V., and Schlom, J. Biology of disease ras gene alterations and enhanced levels of ras p21 expression in a spectrum of benign and malignant human mammary tissues. Lab. Invest., 55 : 603-615, 1986.
9) Chesa, P. G., Rettig, W. J., Melamed, M. R., Old, L. J., and Niman, H. L. Expression of $p21^{ras}$ in normal and malignant human tissues : Lack of association with proliferation and malignancy. Proc. Natl. Acad. Sci. USA, 84 : 3234-3238, 1987.
10) Hand, P. H., Thor, A., Wunderlich, D., Murano, R., Caruso, A., and Schlom, J. Monoclonal antibodies of predefined specificity detect activated ras gene expression in human mammary and colon carcinomas. Proc. Natl. Acad. Sci. USA. 81 : 5227-5231, 1984.
11) Thor, A., Hand, P. H., Wunderlich, D., Caruso, A., Murano, R., and Schlom, J. Monoclonal antobodies difine differential ras gene expression in malignant and benign colonic diseases. Nature 311 : 562-564, 1984.
12) Kerr, I. B., Lee, F. D., Quintanilla, M. and Balmain, A. Immunocytochemical demonstration of p21 ras family oncogene product in normal musoca and in premalignant and malignant tumours of the colorectum. Br. J. Cancer 52 : 695-700, 1985.
13) Williams, A. R. W., Piris, J., Spandidos, D. A., and Wyllie, A. H. Immunohistochemical detection of the ras oncogene p21 product in an experimental tumor and in human colorectal neoplasms. Br. J. Cancer 52 : 687-693,

1985.
14) Ohuchi, N., Thor, A., Page, D. L., Hand, P. H., Halter, S. A., and Schlom, J. Expression of the 21000 molecular weight ras protein in a spectrum of benign and malignant human mammary tissues. Cancer Res. 46 : 2511-2519, 1986.
15) Viola, M. V., Fromowitz, F., Oraves, S., Deb, S., Finkel, G., Lundy J., Hand, P., Thor, A., and Schlom, J. Expression of ras oncogene p21 in prostate cancer. N. Engl. J. Med. 314 : 133-137, 1986.