PGP9.5 as a Marker for Invasive Colorectal Cancer

Taiji Yamazaki, Kenji Hibi, Tsunenobu Takase, Ekmel Tezel, Hiroshi Nakayama, Yasushi Kasai, Katsuki Ito, Seiji Akiyama, Tetsuro Nagasaka, and Akimasa Nakao


ABSTRACT

Purpose and Experimental Design: We proved recently that PGP9.5-negative pancreatic cancer patients had significantly better survival rates compared with those who were PGP9.5 positive, and PGP9.5 may be a novel marker for indicating the prognosis of pancreatic cancer patients. In this study, we examined the expression of PGP9.5 in primary colorectal cancers using immunohistochemistry and correlated the result with the clinicopathological features.

Results: Of 74 colorectal cancer specimens examined, 33 cases (46%) showed positive staining with PGP9.5 in most tumor cells, whereas no PGP9.5 expression was detected in adjacent normal epithelium. Subsequently, we correlated PGP9.5 expression in tumors with the clinicopathological features of affected patients and found two significant differences in maximal tumor size and the extent of tumor (P = 0.035 and 0.019, respectively).

Conclusion: This result suggests that PGP9.5 expression is related to tumor progression and may be useful as a marker for invasive colorectal cancer.

INTRODUCTION

There is now good evidence that a series of genetic alterations in both dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of human colorectal cancer. Activation of oncogenes such as the ras gene, and inactivation of tumor suppressor genes such as the APC and p53 genes, have been identified in colorectal cancer (1–3). In addition, we found that several other genes are related to the pathogenesis of colorectal cancer (4, 5). Investigation of genetic changes is important to clarify the tumorigenic pathway of colorectal cancer (6).

PGP9.5 is a ubiquitin hydrolase and widely expressed in neuronal tissues at all stages of neuronal differentiation (7, 8). Ubiquitination of cellular proteins and targeting them for subsequent degradation via ubiquitin-mediated proteolysis is potentially an important mechanism that regulates cell cycle genes (9, 10). In tumors, increased de-ubiquitination of cyclins by PGP9.5 could contribute to the uncontrolled growth of somatic cells (11).

Using the serial analysis of gene expression method, it was found that PGP9.5 was highly expressed in non-small cell lung cancer (12). Further study showed that the expression of PGP9.5 is closely associated with the advanced stages of lung cancer (13). Moreover, we proved recently that PGP9.5-negative pancreatic cancer patients had significantly better survival rates compared with those who were PGP9.5 positive, and that PGP9.5 may be a novel marker for indicating the prognosis of pancreatic cancer patients (14).

These results prompted us to examine the PGP9.5 status in colorectal cancer. In this study, PGP9.5 expression was first confirmed in colorectal cancer cell lines using Western analysis. Next, we examined the expression of PGP9.5 in 74 resected primary colorectal cancers using immunohistochemistry and correlated PGP9.5 expression in tumors with the clinicopathological features of affected patients.

MATERIALS AND METHODS

Tissue Specimens. All cell lines except H460 were kindly provided by the Memorial Sloan-Kettering Cancer Center or purchased from the American Type Culture Collection. A neuroendocrine cell line from lung cancer (H460) was kindly provided by Dr. Takashi Takahashi (Aichi Cancer Center, Nagoya, Japan). Formalin-fixed and paraffin-embedded colorectal cancer samples were obtained from 74 consecutive Japanese patients who had undergone surgical resection in the Second Department of Surgery, Nagoya University Hospital.

Western Analysis. Western analysis was performed as described previously (13). Cells from a T-75 flask were trypsinized and washed with PBS, suspended in 200 μl of radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride, and then incubated on ice for 20 min. Cell lysates (50 μg) were separated on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, United Kingdom). After blocking the nonspecific sites by incubation in PBS + 5% nonfat dry milk, the blot was incubated with polyclonal rabbit antiserum against PGP9.5 (Biogenesis, Sandown, NH) at 1:400 dilution for 1 h at room temperature. After washing, an enhanced chemiluminescence kit (Amersham Pharmacia Biotech UK Ltd.) was used to visualize the antibody binding to PGP9.5 protein. A neuroendocrine cell line, H460, served as a positive control for PGP9.5 protein expression.

Immunohistochemical Analysis. Immunohistochemical analysis was performed as described previously (14). The specimens were fixed with 10% formalin, embedded in paraffin, and cut into 3-μm-thick sections; and the slides were dried at 60°C for 30 min, treated with xylene, and dehydrated in alcohol.
Endogenous peroxidase was blocked with 0.3% H$_2$O$_2$. Microwave treatment was performed for 4 min in Antigen Retrieval Glyca solution (BioGenex Laboratories, San Roman, CA) because it has been shown that the immunoreactivity of PGP9.5 was remarkably enhanced by this method. After blocking with normal goat serum for 20 min, the slides were incubated with polyclonal rabbit antiserum against PGP9.5 (Biogenesis, Sandown, NH) at 1:1000 dilution for 1 h at room temperature. Vectastain ABC kit and DAB Substrate kit (Vector, Burlingame, CA) were used to visualize the antibody binding, and sections were counterstained with hematoxylin. Immunohistochemical staining for PGP9.5 was interpreted by an experienced pathologist (T. N.) without providing any clinical data. In all cases, small nerves served as a positive internal control for PGP9.5 staining. Only cytoplasmic staining above background level was regarded as specific staining. Tumors that had >30% stained cells in five high-power fields were classified as positive staining.

**Statistical Analysis.** The $\chi^2$ test and Student’s $t$ test were used to examine the association between PGP9.5 expression and clinicopathological features. Survival rates by the Kaplan-Meier method and statistical significance were examined using the log-rank test.

**RESULTS**

We first examined the expression of PGP9.5 in colorectal cancer cell lines by Western analysis. We found that 1 of 14 colorectal cancer cell lines showed PGP9.5 expression (Fig. 1). Although the expression rate of PGP9.5 was not high, this result confirmed that PGP9.5 has a role in some colorectal cancers as well as lung and pancreatic cancers (13, 14).

We next examined PGP9.5 expression in primary colorectal cancers using immunohistochemistry. In normal colorectal tissues, we found that PGP9.5 staining was restricted to nerves that served as a positive internal control. The positive staining of neurons is consistent with the fact that PGP9.5 was reported as a neuron-specific peptide widely expressed in neuronal tissue (Ref. 7; Fig. 2A). In colorectal cancer samples, PGP9.5 staining was mainly observed in the cell cytoplasm. Of 74 colorectal cancer specimens examined, 33 cases (46%) showed positive staining with PGP9.5 in most tumor cells, whereas no PGP9.5 expression was detected in adjacent normal epithelium (Fig. 2B).

To seek the role of PGP9.5 expression in colorectal cancer, we examined the correlation of PGP9.5 expression with the clinicopathological features. There was no significant difference in the distribution of patients with positive or negative PGP9.5 expression in terms of sex, age, tumor histology, location, distant metastasis, lymph node metastasis, Tumor-Node-Meta-

**DISCUSSION**

Colorectal cancer is one of the most aggressive cancers, and it occurs at a high incidence in most countries (15). To eliminate this fatal cancer from patients, we perform surgical operations and subsequent chemotherapy and radiotherapy. For
this purpose, it is important to seek genetic alteration as a new parameter for estimation of the malignancy of the cancer.

To date, little is known about the role of PGP9.5 in cancer. PGP9.5 belongs to the ubiquitin COOH-terminal hydrolase family. Current accumulating data suggest that these enzymes play an important role in the cellular proteolytic pathway that regulates many cellular processes including cell cycle and death (7, 11). Ubiquitin COOH-terminal hydrolases are Mr 25,000 enzymes involved in the translational processing of pro-ubiquitin gene products as well as in the release of ubiquitin from tagged proteins, i.e., de-ubiquitination by which the degradation of cyclins decreases, possibly contributing to the overexpression of these molecules in tumors (10, 16, 17). In our preliminary data, we found that the expression of some cell cycle genes, such as cyclin G2, was induced by PGP9.5 expression using cDNA microarray technology.

In lung cancer, PGP9.5 expression was found to be independent of neuronal differentiation and strongly associated with the pathological stage of the cancer (13). It was proved for the first time that increased expression of PGP9.5 is associated with cancer development. In pancreatic cancer, a significant negative correlation was reported between overexpression of PGP9.5 and postoperative survival (14). Multivariate analysis also suggested PGP9.5, along with tumor stage and extrapancreatic plexus invasion, as strong predictors of the outcome. In this study, we investigated the correlation of PGP9.5 expression with the clinicopathological features of colorectal cancer patients. Although PGP9.5 expression was not associated with pathological stage or survival, colorectal cancer with PGP9.5 expression showed significant invasiveness, i.e., PGP9.5-positive staining was correlated with maximal tumor size and the extent of tumor. This result further supported the concept that PGP9.5 status might have an important role for tumor progression.

This study provides solid evidence for further studies on the molecular mechanism of PGP9.5 expression in colorectal cancer and also confirms that PGP9.5 may be a novel marker for various human cancers. Work in progress on the downstream target of de-ubiquitination by PGP9.5 will elucidate a part of the tumorigenic pathway of colorectal cancer.

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<table>
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<tr>
<th>Clinicopathological feature</th>
<th>No. of cases</th>
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| 22–92 years                | 74          | 67.2 ± 11.2 | 61.4 ± 15.3 | 0.74*
| Sex                        |             |        |        |
| Male                       | 44          | 23     | 21     | 0.11*
| Female                     | 30          | 10     | 20     |        |
| Maximal tumor size 15–100 mm| 74          | 52.9 ± 17.2 | 43.9 ± 18.1 | 0.035*
| Extent of tumor ≤ mt<      | 16          | 3      | 13     | 0.019*
|                             | 58          | 30     | 28     |        |
| Histology                  |             |        |        |
| Well                       | 5           | 2      | 3      | 0.17* |
| Mod                        | 65          | 34     | 31     |        |
| Other                      | 4           | 0      | 4      |        |
| Lymph node metastasis      |             |        |        |
| +                          | 37          | 17     | 20     | 0.82* |
| −                          | 37          | 16     | 21     |        |
| Liver metastasis           |             |        |        |
| +                          | 16          | 8      | 8      | 0.62* |
| −                          | 58          | 25     | 33     |        |
| TNM stage                  |             |        |        |
| I, II                      | 31          | 14     | 17     | 0.93* |
| III, IV                    | 43          | 19     | 24     |        |
| Vital status               |             |        |        |
| Alive                      | 29          | 17     | 12     | 0.72* |
| Dead                       | 24          | 12     | 12     |        |
| Total                      | 74          | 33     | 41     |        |

* Mean ± SD.
* Student’s t test.
* χ² test.
* mt, muscular tunic; Well, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma.


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