Clinical Significance of p16\textsuperscript{INK4A} and p53 Overexpression in Endocrine Tumors of the Gastrointestinal Tract

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Abstract

This study was designed to determine the expression of p16, p53, and CD117 in gastrointestinal tract endocrine tumors. Immunohistochemical studies of p16, p53, and CD117 were performed in 57 gastrointestinal tract endocrine tumors, including 22 poorly differentiated endocrine carcinomas (PDECs) and 35 well-differentiated endocrine tumors (WDETs). Overexpression of p16 and p53 was observed in 16 (73\%) and 10 (45\%) of the PDECs, respectively, whereas only 1 WDET showed overexpression of p53 and none showed overexpression of p16. A total of 18 (82\%) of the PDECs showed overexpression of p16 or p53 proteins. This is closely associated with PDEC (P < .0001). By using overexpression of p16 or p53 as the criteria for PDEC, the sensitivity and specificity are 81.8\% and 97.1\%, respectively, with positive and negative predictive values of 94.7\% and 89.5\%, respectively. CD117 was not detected in any of the 57 gastrointestinal endocrine tumors by immunohistochemical analysis.

Endocrine cells are distributed throughout the body and frequently found in the gastrointestinal tract, pancreas, lung, thyroid, adrenal glands, and many other organs. Although the gastrointestinal tract has the largest population of endocrine cells, endocrine carcinomas of the gastrointestinal tract are rare.\textsuperscript{1} Endocrine tumors in the gastrointestinal tract are classified as well-differentiated endocrine tumor (WDET), including typical carcinoid and atypical carcinoid, and poorly differentiated endocrine carcinoma (PDEC), including small cell carcinoma and large cell carcinoma.\textsuperscript{2} Clinically, WDETs are generally indolent and resistant to chemotherapy, whereas PDECs are more aggressive but may respond transiently to cisplatin-based chemotherapy.\textsuperscript{1,3} There are relatively fewer studies on gastrointestinal tract endocrine tumors compared with pulmonary neuroendocrine tumors. Most staging systems and treatment protocols for gastrointestinal tract endocrine tumors are derived from experience in similar lesions of neuroendocrine tumors in the lung based on the presumption that these diseases are similar in many aspects.\textsuperscript{1}

p16 and p53 are important cell cycle regulators. Genetic alterations involving p16 and p53 genes have been shown to drive the pathogenic development of various types of human cancers, including neuroendocrine tumors of the lung.\textsuperscript{4-13} In pulmonary neuroendocrine tumors, abnormalities in relation to p16 and p53 at DNA, RNA, and protein levels have been reported.\textsuperscript{1,4-10,12,13} Homozygous deletion, loss of heterozygosity (LOH), and hypermethylation of the p16/MTS1 locus, along with changes of clusters of polymorphic microsatellite markers in chromosome 9p21 have been reported in 50\% to 89\% of pulmonary neuroendocrine tumors.\textsuperscript{5,6,8,10,12,13} Aberrant expression of p16 protein has also been reported but with inconsistent results; loss of p16 expression was reported...
in 10% of pulmonary neuroendocrine tumors examined by Brenner et al\textsuperscript{1} and Igarashi et al\textsuperscript{9}, and expression of p16 has also been reported in more than 95% of poorly differentiated neuroendocrine carcinomas.\textsuperscript{4,6,8,13} LOH and mutations of the p53 locus and overexpression of p53 have been frequently observed in pulmonary neuroendocrine tumors.\textsuperscript{1,5,8,11} In addition, 70% to 100% of high-grade pulmonary neuroendocrine carcinoma has been shown to contain p53 mutations and showed the accumulation of p53 protein.\textsuperscript{1,5,7} The c-kit protein (also known as CD117), a type III receptor tyrosine kinase, has been implicated in many human cancers, including pulmonary small cell carcinoma, with the incidence of CD117 expression in poorly differentiated neuroendocrine carcinomas of lung reported in 40% to 89% of cases.\textsuperscript{14-19}

The genetic alterations in the endocrine neoplasms in the gastrointestinal tract remain largely unknown. The expression status of p16 and p53 in endocrine neoplasms in the gastrointestinal tract has rarely been reported. So far, LOH in chromosome 9p21, the locus containing the clusters of CDKN2A/p16\textsuperscript{INK4A}, CDKN2A/p14\textsuperscript{ARF}, and CDKN2B/p15\textsuperscript{INK4B}, has been reported to be a selective target of inactivation in neuroendocrine gastroenteropancreatic tumors.\textsuperscript{20} Alterations of the p16 gene and/or p53 gene overexpression status are observed in gastrointestinal tract small cell carcinoma, PDEC of the stomach and colorectum, and pancreatic endocrine tumors.\textsuperscript{1,5,21-27}

The aim of this study was to examine the roles of p16 and p53 in gastrointestinal tract endocrine tumors. Because mitosis and necrosis are the basic criteria to differentiate well-differentiated from poorly differentiated gastrointestinal endocrine neoplasms, and p16 and p53 are important cell cycle regulators, we assessed whether p16 and p53 levels would be useful markers for these malignancies. It is important to explore the role of CD117 in gastrointestinal tract endocrine tumors with the hope that targeted therapy may be possible for these tumors. In this study, we explored the involvement of p16, p53, and CD117 in endocrine neoplasms in the gastrointestinal tract by examining their expression levels and assessing clinical significance.

**Materials and Methods**

**Cases**

Cases of primary endocrine tumors in the gastrointestinal tract treated at Taipei Veterans General Hospital, Taipei, Taiwan, from January 1985 to December 2004 were reviewed for the availability of pathologic materials and medical records. The diagnoses of WDETs (including carcinoid and atypical carcinoid) and the PDECs (including small cell carcinoma and large cell endocrine carcinoma) were according to the World Health Organization guidelines on endocrine tumors of the gastrointestinal tract.\textsuperscript{2}

Briefly, a carcinoid is characterized by cells uniform in size with round or oval nuclei, inconspicuous nucleoli, and eosinophilic cytoplasm. The cells are arranged in trabecular anastomosing structures, tubular structures, and solid nests. Mitosis is almost absent, and angioinvasion is infrequent. Typical carcinoids usually have fewer than 2 mitoses per 2 mm\textsuperscript{2} and lack necrosis. Slight nuclear atypia and increased mitoses, 2 to 10 mitoses per 2 mm\textsuperscript{2} and/or foci of necrosis, can be found in atypical carcinoids.

Small cell carcinomas and large cell neuroendocrine carcinomas are considered PDECs. Mitoses are usually greater than 10 per 2 mm\textsuperscript{2}, and necrosis is more commonly found. Small cell carcinomas consist of small cells with scant cytoplasm, ill-defined cell borders, finely granular nuclear chromatin, and absent or inconspicuous nucleoli. Large cell neuroendocrine carcinomas have larger cells with a moderate amount of cytoplasm. Nucleoli are prominent.

Typical carcinoids and small cell carcinomas are more common than atypical carcinoids or large cell neuroendocrine carcinomas in our WDET and PDEC groups, respectively. Neuroendocrine differentiation can be demonstrated by immunohistochemical analysis; the majority of tumor cells were diffusely and strongly positive for chromogranin, synaptophysin, and neuron-specific enolase (NSE), and examples are shown in Image 11. Adenocarcinomas with focal neuroendocrine differentiation were not included in this study.

The medical records and histopathologic sections were reviewed. Clinicopathologic data, including locations of the primary neoplasm, histologic type, staging, metastasis, recurrence, type of treatment, and overall survival were traced. For evaluation of TNM classification, we included barium studies; computed tomography of the chest, abdomen, and brain; ultrasonography of the abdomen; and whole body bone scans. Specimens were obtained through radical surgery, excisional biopsy, or tumor debulking. Small specimens obtained from endoscopic biopsy and needle biopsy were insufficient for this study and were excluded. We found 57 cases for this study and obtained consent from each patient.

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed with antibodies against the following proteins: chromogranin A, synaptophysin, NSE, p16\textsuperscript{INK4A}, p53, and CD117. Immunohistochemical analysis for chromogranin A, synaptophysin, and NSE was done to confirm the diagnosis, for p16 and p53 was performed to study possible pathogenesis, and for CD117, to explore the possibility of c-kit target therapy in gastrointestinal tract endocrine tumors. Clones, antigen retrieval methods, and commercial sources of antibodies are listed in Table II.
Endogenous peroxidase was quenched by hydrogen peroxide, and nonspecific adsorption was reduced by blocking against swine serum. The bound antibodies were detected by the Liquid DAB substrate pack from BioGenex (San Ramon, CA).

Immunopositivity for p16 and p53 was initially scored as follows: 0, undetectable or equivocal individual single cell positivity in fewer than 1% of tumor cells; 1+, heterogeneous positivity in fewer than 30% of tumor cells; 2+, positivity in 30% to 60% of tumor cells; and 3+, diffuse, strong positivity in more than 60% of tumor cells. The cases scored as 3+ were regarded as overexpression, and overexpression was not considered present for cases scored as 0, +1, and +2. All immunohistochemical sections were read by 1 pathologist (A.F.-Y.L.) without knowledge of the diagnosis.

**Statistical Analysis**

The association between immunohistochemical staining and histologic type was estimated by using the Pearson $\chi^2$ test and Fisher exact test, depending on the number of samples. Computation was achieved by using the Stata software program (Stata, College Station, TX). The significance level chosen was a $P$ value less than .05, and all tests were 2-sided.

**Results**

Tissue samples from 57 patients with gastrointestinal tract endocrine neoplasms were used in this study. Among them, 22 cases were gastrointestinal tract PDECs, including...
6 cases in the esophagus, 9 gastric lesions, 1 small intestinal lesion, and 6 colorectal lesions. In addition, tissue samples of WDETs were available from 35 patients, including 11 atypical carcinoid lesions of the colon with lymph node and/or liver metastasis, 23 cases of colon carcinoid, and 1 gastric solitary carcinoid without evidence of metastasis or recurrence after at least 5 years follow-up. Clinical information for the 22 PDECs is summarized in **Table 2**.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sex/Age (y)</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Survival (mo)</th>
<th>p16</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>M/59</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>32</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>M/45</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>37</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>M/74</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>17</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>M/64</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>16</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M/82</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M/72</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

| Stomach           | M/69        | 4 | 0 | 1 | 4             | 3   | 0   |
|                   | M/77        | 2 | 1 | 0 | 91            | 3   | 3   |
|                   | M/70        | 2 | 1 | 0 | 65            | 3   | 3   |
|                   | M/68        | 3 | 1 | 1 | 7             | 1   | 0   |
|                   | M/85        | 3 | 1 | 0 | 6             | 3   | 3   |
|                   | M/66        | 3 | 1 | 0 | 16            | 3   | 2   |
|                   | M/87        | 3 | 1 | 0 | 5             | 3   | 3   |
|                   | M/88        | 4 | 1 | 0 | 25            | 3   | 0   |

| Small intestine   | M/84        | 3 | 0 | 0 | 9             | 3   | 3   |
|                   | F/32        | 3 | 2 | 1 | 0             | 0   | 1   |
|                   | F/57        | 3 | 1 | 1 | 4             | 3   | 2   |
|                   | F/35        | 3 | 3 | 0 | 6             | 3   | 2   |
|                   | M/66        | 3 | 3 | 1 | 35            | 3   | 3   |
|                   | F/61        | 3 | 2 | 1 | 7             | 3   | 3   |
|                   | M/79        | 3 | 1 | 1 | 34            | 0   | 1   |

* Immunohistochemical scoring was as follows: 0, undetectable or positivity in fewer than 1% of tumor cells; 1+, heterogeneous positivity in fewer than 30% of tumor cells; 2+, positivity in 30%-60% of tumor cells; 3+, diffuse and strong positivity in more than 60% of tumor cells.

† No follow-up information; survival not determined.

**Immunohistochemical Analysis for p16 and p53**

Immunohistochemical analysis for p16 and p53 was performed in tissue samples from 22 PDECs and 35 WDETs. Of 22 PDECs, 16 (73%) showed diffuse, strong (3+) p16 positivity, whereas none of the WDETs was scored as p16 overexpression. Overexpression of p53, as demonstrated by the diffuse, strong 3+ signals, was observed in 10 (45%) of the PDECs and 1 (3%) of the WDETs. Representative images of the immunohistochemical stains for p16 and p53 are shown in **Image 2** and **Image 3**, respectively. The profiles of patients and immunohistochemical results for p16 and p53 are summarized in Table 2 and **Table 3**. Overexpression of p16 or p53 was noted in 18 PDECs and 1 WDET. The difference in the frequency of p16 and p53 overexpression in these 2 groups was statistically significant ($P < .0001$) (Table 3).

Our finding that overexpression of p16 and p53 is significantly more frequent in gastrointestinal tract PDECs suggests...
the usefulness of p16 and p53 levels as diagnostic markers for this malignancy. When applying overexpression of p16 or p53 as a criterion for PDEC, the sensitivity and specificity of detection are 81.8% and 97.1%, respectively, and the positive and negative predictive values are 94.7% and 89.5%, respectively.

**Immunohistochemical Analysis for CD117**

All 57 cases of the gastrointestinal neuroendocrine neoplasms were negative for CD117. Gastrointestinal endocrine neoplasms did not immunohistochemically express detectable CD117 in our cases.

**Discussion**

In this study, we examined the expression of p16, p53, and CD117 in 22 PDECs of the gastrointestinal tract (6 in the esophagus, 9 in the stomach, 1 in the small intestine, and 6 in the colon) and 35 WDETs (34 in the colon and 1 in the stomach) by immunohistochemical analysis. Our results showed that overexpression of p16 and/or p53 is a frequent event in gastrointestinal tract PDECs, whereas WDETs seldom display overexpressed patterns of p16 or p53 (P < .0001); therefore, p16 and p53 levels may be regarded as supplementary tests in the diagnosis of this malignancy, with a sensitivity of 81.8%
and a specificity of 97.1%. Such information is very useful for the differential diagnosis of low-grade vs high-grade endocrine neoplasms in the gastrointestinal tract when the histopathologic diagnosis, which carries therapeutic and prognostic significance, is sometimes difficult owing to overlapping features.

In most cases, the distinction between WDETs and PDECs is straightforward and mainly based on morphologic features and mitotic activities, but it is difficult in some cases. In 1 of our WDETs representing an infiltrating rectal mass, 6 × 4 × 4 cm, in the mucosa to serosa in a 45-year-old man, the tumor cells microscopically had features of carcinoid with slight cellular pleomorphism [Image 4A] and were diffusely and strongly positive for chromogranin A, synaptophysin, and NSE, but lymphatic tumor emboli and perineural infiltration were noted [Image 4B] and [Image 4C]. The MIB-1 proliferating index was 4.5% [Image 4D]. There was no p16 or p53 overexpression. The diagnosis of this case as PDEC (large cell endocrine carcinoma) or WDET (atypical carcinoid) was difficult; after extensive discussion, well-differentiated endocrine carcinoma, atypical carcinoid, was chosen as the diagnosis. With extended follow-up of the patient, we will know the clinical course.

The only patient with WDET with overexpressed p53 in this study had lymph node and liver metastasis and died 1...
month after operation. Review of the histopathologic sections revealed insufficient mitotic count or necrosis to make a diagnosis of PDEC. Although p53 mutation or overexpression is uncommon in gastrointestinal carcinoids, it was suggested that overexpression of p53 may confer a more malignant phenotype. It would be of interest to expand the current study until more cases are available to investigate whether p16 and p53 may have a significant role in predicting clinical course and differential diagnosis in these difficult cases.

We also compared the status of p16 and p53 with clinicopathologic data, including sex, age, and TNM status. Our results showed that p16 and p53 levels were not significantly associated with clinical and tumor-specific factors (data not shown). The prognostic role of p16 and p53 was not evaluated in the 22 PDEC cases because of the differences in the locations of the primary lesions, staging, and recurrences. Moreover, the patients with PDEC were admitted from 1985 to 2005 and had been through various therapeutic regimens, including different drugs and dosages for chemotherapy and radiation therapy that had evolved during the past 2 decades. The mean ± SD survival of the 22 patients with gastrointestinal tract PDEC was 20.3 ± 23.1 months and for patients with WDET with metastasis was 71.8 ± 51.8 months. The average follow-up period for patients with WDET without metastasis was 136.8 months (range, 84-250 months), and there was no disease-related mortality during follow-up. In patients with WDET, p16 and p53 status are not useful for predicting the possibility of metastasis because most WDETs, with or without metastasis, do not show significant differences in p16 and p53 protein expression levels.

Genetic alterations in gastrointestinal tract endocrine neoplasms are largely unknown; the control of cell proliferation, such as the p16/cyclin D1/Rb pathway, may be involved. The gene cluster in 9p21, containing CDKN2A/p16INK4A, CDKN2A/p14ARF, and CDKN2B/p15INK4B, has been reported to be a selective target of inactivation in small cell carcinoma of the gastrointestinal tract, gastrinoma, insulinoma, nonfunctional pancreatic neuroendocrine tumor, and carcinoid. It was demonstrated that genetic or epigenetic alterations in the 9p21 region led to the inactivation of the p16/MTS1 gene in 22% to 87.5% of gastrinomas and nonfunctioning pancreatic neuroendocrine tumors. Alteration of p16 expression in PDECs of the colon and stomach were reported. The proper regulation of the p16/cyclin D1/Rb pathway is of central importance for the control of G1 to S transition in cell cycle progression.

There are only scattered p16+ cells, usually fewer than 1%, demonstrated in the normal mucosa of the esophagus and intestine, which were scored as 0 and served as the internal control (Image 2A). Cervical squamous cell carcinomas with p16 overexpression scored as 3 served as positive controls for p16 immunohistochemical studies. A high level of p16 expression has been demonstrated to be associated with functional inactivation of Rb in several types of tumors, including non–small cell lung carcinoma and neuroendocrine tumors of the lung. The Rb gene is a prototypical tumor suppressor gene encoding a nuclear phosphoprotein that acts as a cell cycle regulator for G1 to S transition. Our results showed that overexpression of p16 was observed in 73% of PDEC cases (16/22) and none of 35 WDETs, suggesting that dysfunction of the p16/cyclin D1/Rb pathway through altered expression of p16 is a major event leading to the pathogenic development of PDEC, but not WDET, and warrants further investigation of the functionality of the p16/cyclin D1/Rb pathway in these tumors.

The molecular mechanism leading to p16 overexpression is not clear in endocrine tumors in the gastrointestinal tract. Overexpression of p16 has been shown to be associated with human papillomavirus infection in cancers of the cervix and tonsil. In malignant melanoma, coamplification of CDK4 and MDM2 was found, which may substitute for loss of p16INK4A and p14ARF function in a subset of melanomas.

Overexpression of p53 is more frequently observed in gastrointestinal tract PDECs than in well-differentiated tumors. Overexpression of p53 is observed in more than 50% of human cancers, including small cell carcinoma of the lung, with an incidence of 42% to 100%. Expression of p53 was also demonstrated in well-differentiated neuroendocrine neoplasms of the lung, but at a much lower incidence, 0% and 20% for typical carcinoid and atypical carcinoid, respectively. Induction of small cell lung cancer by somatic inactivation of TP53 and Rb1 in a conditional mouse model was reported. Overexpression of p53 protein was observed in PDECs of the stomach and colon, whereas carcinoid tumors seldom display p53 accumulation or gene mutation. In this study, we confirmed a similar observation in gastrointestinal tract endocrine neoplasms in that 45% of poorly differentiated and well-differentiated endocrine tumors.

| Table 3 |

Immunohistochemical Profiles of p16INK4A and p53 in Poorly Differentiated and Well-Differentiated Endocrine Neoplasms in Various Locations of the Gastrointestinal Tract

<table>
<thead>
<tr>
<th>Antigen/</th>
<th>No. of Cases</th>
<th>No. (%) of Cases With ( P^1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>p16</td>
<td>Overexpression</td>
</tr>
<tr>
<td>PDEC</td>
<td>22</td>
<td>16 (73)</td>
</tr>
<tr>
<td>WDET</td>
<td>35</td>
<td>0 (0)</td>
</tr>
<tr>
<td>WDET</td>
<td>35</td>
<td>1 (3)</td>
</tr>
<tr>
<td>PDEC</td>
<td>22</td>
<td>16 (73)</td>
</tr>
</tbody>
</table>

PDEC, poorly differentiated endocrine carcinoma; WDET, well-differentiated endocrine tumor.

* Overexpression was defined when immunohistochemical analysis showed strong positive 3+ signals (diffuse and strong positivity in more than 60% of tumor cells).
† Pearson \( \chi^2 \) test.
differentiated gastrointestinal tract endocrine carcinomas (10/22) and 3% of WDETs (1/35) expressed p53. Overexpression of p53 may indicate that the p53/BAX apoptotic pathway is disrupted, and, hence, tumor cells are resistant to various death stimuli, ie, irradiation, drugs, and cytotoxins. This may cause a more aggressive clinical course and a more malignant phenotype.

In pulmonary poorly differentiated neuroendocrine carcinomas, the incidence of CD117 expression is high, ranging from 40% to 89%, whereas in well-differentiated neuroendocrine neoplasms of lung, the CD117 expression rate is low, ranging from 0% to 7%. Akintola-Ogunremi et al reported that CD117 was detected in 15 of 66 primary colorectal neuroendocrine carcinomas but in none of 19 colorectal carcinoid lesions. In our study, there was no CD117 expression in 34 colorectal WDETs and 6 colorectal PDECs, and there was no CD117 expression in 17 endocrine neoplasms in other parts of the gastrointestinal tract either. Our results are similar to those of Akintola-Ogunremi et al in that CD117 was negative in colorectal WDETs. We also consider it possible to find some CD117+ colorectal PDECs if more colorectal PDECs become available. We therefore conclude that CD117 expression in colorectal endocrine tumors is low and the incidence is probably lower than that of pulmonary neuroendocrine neoplasms. It is still debatable whether CD117 expression is related to prognosis in pulmonary neuroendocrine carcinomas. The absence of CD117 expression in the 57 cases, including well and poorly differentiated
endocrine tumors in the gastrointestinal tract, suggests that these tumors are less likely to respond to the currently available tyrosine kinase inhibitors against c-kit.

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References


