Immunohistochemical Expression of 14-3-3 σ Protein in Intraductal Papillary-mucinous Tumor and Invasive Ductal Carcinoma of the Pancreas

TOSHIYUKI OKADA¹,², NORIHIRO MASUDA¹, YASUHUKI FUKAI¹, TATSUO SHIMURA¹, YASUJI NISHIDA³, YASUO HOSOUCHI⁴, KENJI KASHIWABARA⁴, TAKASHI NAKAJIMA² and HIROYUKI KUWANO¹

Departments of ¹General Surgical Science and ²Tumor Pathology, Gunma University Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi, Gunma, 371-8511; ³The Saiseikai Maebashi Hospital, 564-1, Kamishinden-cho, Maebashi, Gunma, 371-0821; ⁴Division of Clinical Pathology, Gunma University Hospital, 3-39-22, Showa-machi, Maebashi, Gunma, 371-8511, Japan

Abstract. Background: 14-3-3 σ (sigma) has been shown to be overexpressed in pancreatic cancers by a c-DNA microarray technique. However, the expression of 14-3-3 σ in intraductal papillary-mucinous tumor (IPMT) of the pancreas remains unclear. Materials and Methods: To evaluate the biological importance of 14-3-3 σ expression in pancreatic carcinogenesis, immunohistochemistry for 14-3-3 σ, CDX2, MUC1, MUC2, p53, p16 and Ki-67 was carried out on 33 IPMTs and the results were compared with those for 14 invasive ductal carcinomas (IDCs). Results: The frequency of 14-3-3 σ immunoreactivity was 70% and 100% in IPMT and IDC, respectively. The frequency of MUC1 and Ki-67 immunoreactivity was significantly higher in IDC than IPMT. In IPMT, dark columnar cell types prevailed over clear columnar cell types in terms of the frequency of the Ki-67 labeling index. Conclusion: The overexpression of 14-3-3 σ was confirmed in both IDC and IPMT. Therefore, this overexpression might occur in the early stages of pancreatic carcinogenesis. Moreover, IPMT composed of dark columnar cells might be a potentially more advanced form than that made up of clear columnar cells.

Pancreatic cancer is the fifth leading cause of cancer death in Japan (1). The mortality and incidence rates are almost identical, since survival rates for pancreatic cancer are extremely low (2) Histologically, 85 to 90% of all pancreatic tumors are ductal adenocarcinomas derived from the exocrine pancreas, especially from the pancreatic duct. Clinically, this type of cancer usually occurs in elderly individuals aged 60 to 80 and occurs more frequently in men than in women. Epidemiologically, many risk factors have been pointed out, but no significant risk factor for pancreatic carcinoma, other than chronic pancreatitis, has yet been identified (3).

Since the first report of pancreatic duct hyperplasia by Sommers et al., pancreatic carcinogenesis has been considered a multi-step process and the existence of precursor lesions is now widely accepted (4-6). Similarly to cervical intraepithelial neoplasia, the term "pancreatic intraepithelial neoplasia" (PanIN) was proposed, in 1999, to reflect the model of genetic progression for pancreatic duct adenocarcinoma (5, 6). In pancreatic duct epithelium, the earliest genetic changes are K-ras gene mutations and overexpression of HER-2/neu gene, which occur in low-grade PanIN lesions, and loss of p16 gene expression to produce PanIN-2 lesions. High-grade PanIN-3 lesions develop through the inactivation of the tumor suppressor genes, p53, DPC4 and BRCA2 (5-8). These tumor suppressor gene abnormalities in duct epithelial changes were confirmed by analysis of loss of heterozygosity (LOH) at several chromosomal loci (9).

A specific pathological entity, known as intraductal papillary-mucinous tumor (IPMT), was established for exocrine tumors of the pancreas 20 years ago (10-13). IPMT is characterized by intraductal papillary growth, excessive production of mucin and some gastroenteric differentiation of the tumor cell. This type of tumor has been referred to by various names, such as intraductal papillary neoplasm, mucin-hypersecreting tumor, intraductal mucin-hypersecreting neoplasm, etc. (14). Now IPMT is histologically subdivided into three types, adenoma, borderline lesion and carcinoma.
according to cellular and structural atypia (13, 15). Moreover, each IPMT subtype has been shown to correspond with each PanIN lesion according to the immunohistochemical expression of various tumor suppressor genes, especially the p16 and DPC4 genes (15, 16). These findings suggest that some IPMTs might be precursor lesions of invasive ductal carcinoma (IDC) of the pancreas. In fact, the incidence of IDC associated with IPMT is reported to be 10-20% (2).

Recent advances in molecular biology have enabled exploration of the global gene expression pattern in IDC of the pancreas and detected the overexpression of 14-3-3 ß due to hypomethylation of the gene in a majority of cases (17, 18). In the present study, we aimed to clarify the expression pattern of 14-3-3 ß in IPMT as well as in IDC and the biological significance of 14-3-3 ß expression in pancreatic carcinogenesis.

**Materials and Methods**

**Tumor tissues.** A total of 33 IPMTs (24 adenomas, IPMAs, including borderline lesions and nine carcinomas, IPMCs) and 14 IDCs were collected from the pathological files of the Department of General Surgical Science, Gunma University Graduate School of Medicine and the Saiseikai Maebashi Hospital, Japan (Table I). All the tumor materials were obtained at surgery, fixed with 10% formalin and embedded in paraffin for routine pathological examination. Clinical information was obtained from the medical records in each case. From paraffin blocks containing a representative tumor, 5-µm-thick paraffin sections were prepared and stained with hematoxylin and eosin, then used for histochemical re-evaluation by two pathologists.

**Immunohistochemical analysis.** For immunohistochemical analysis, the paraffin sections were dewaxed with xylene and rehydrated through a series of graded alcohols. In order to reduce the endogenous peroxidase activity, the sections were immersed in an absolute methanol solution containing 0.3% H2O2 for 30 min at room temperature. Antigen retrieval was performed with some modifications (Table II). Non-specific antibody binding was reduced with 5% blocking serum in phosphate-buffered saline. The sections were incubated with each primary antibody at 4°C overnight (Table II). They were subsequently incubated with biotinylated secondary antibody for 30 min at room temperature, followed by an avidin-biotin-peroxidase complex solution, according to the manufacturer’s instructions (VECTASTAIN, Vector Laboratories, Burlingame, CA, USA). The peroxidase was visualized with 0.02% 3-3'-diaminobenzidine tetrahydrochloride containing 0.005% H2O2 in 0.01 M tris-phosphate buffer, pH 7.4. Finally, the sections were counterstained lightly with hematoxylin.

<table>
<thead>
<tr>
<th>Name</th>
<th>Clone</th>
<th>Dilution</th>
<th>Treatment*</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-3-3 ß</td>
<td>Polyclonal</td>
<td>1:100</td>
<td>none</td>
<td>Immuno-Biological Laboratories, Gunma, Japan.</td>
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<tr>
<td>p16</td>
<td>JC8</td>
<td>1:100</td>
<td>A</td>
<td>NeoMarkers, Fremont, CA, USA</td>
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<tr>
<td>p53</td>
<td>DO7</td>
<td>1:80</td>
<td>B</td>
<td>Novocastra, Newcastle, UK</td>
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<tr>
<td>MUC-1</td>
<td>Ma69</td>
<td>1:50</td>
<td>C</td>
<td>Novocastra, Newcastle, UK</td>
</tr>
<tr>
<td>MUC-2</td>
<td>Cep58</td>
<td>1:200</td>
<td>C</td>
<td>Novocastra, Newcastle, UK</td>
</tr>
<tr>
<td>CDX2</td>
<td>CDX-2-88</td>
<td>1:50</td>
<td>C</td>
<td>BioGenex, San Ramon, CA, USA</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-1</td>
<td>1:40</td>
<td>C</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

| Tumor cell types of intraductal papillary-mucinous tumors. |
|-----------------|-----------------|-----------------|-----------------|
| Dark columnar cell | Clear columnar cell | Compact cell |
| IPMA (24 cases) | 10 (42) | 14 (58) | 0 (0) |
| IPMC (9 cases) | 5 (56) | 2 (22) | 2 (22) |

**Results**

**Clinical and pathological data.** As shown in Table I, the 33 IPMTs were subdivided histologically into 24 IPMAs and nine IPMCs, of which three included micro-invasive growth. Both IPMT and IDC frequently developed in the head and body of the pancreas. Of the IPMTs, 14 tumors were histologically closely related to the main pancreatic duct,
but 19 tumors had no clear connection to the main pancreatic duct (Table I). Each IPMC or IPMA tended to occur in the main pancreatic duct or the secondary duct, respectively \((p<0.05)\). Histopathologically, the tumor cells of IPMA were classified into three types: dark and clear columnar cell types and a compact cell type, according to Yonezawa \(et al\). (14, 19). As the majority of the IPMAs showed a mixture of dark and clear columnar cells, the classification depended on the predominant cell type (Table III). In IPMA, the clear columnar cells far exceeded the dark columnar cells in number, however, in IPMC, the dark columnar cell type predominated \((p<0.05)\).

**Immunohistochemical results.** The immunohistochemical results are summarized in Table IV. The frequency of MUC1 immunoreactivity was high in IDC compared to IPMT \((p<0.05)\). The frequency of MUC2 immunoreactivity in IPMA, IPMC and IDC was 21%, 33% and 14%, respectively. In all p53-positive tumors, almost all the tumor cell nuclei were diffusely stained for p53. Many IPMAs and IPMCs retained diffuse immunolabeling of p16 in both the nucleus and cytoplasm, however, the immunoreactivity decreased in half of the cases of IDC.

Usually, immunoreactivity for 14-3-3 \(\sigma\) is not observed in the parenchyma of the pancreas. However, the pancreatic duct epithelia showed a degree of immunoreactivity varying from negative to strongly positive (Figure 1). Strong 14-3-3 \(\sigma\) immunoreactivity was observed in all IDCs and about 70% of IPMTs were immunohistochemically positive for 14-3-3 \(\sigma\). Immunoreactivity for 14-3-3 \(\sigma\) was present in the cytoplasm and/or nucleus and the staining pattern was heterogeneous, depending on the area in the IPMT (Figure 2). In IDC, strong and diffuse immunoreactivity for 14-3-3 \(\sigma\) was usually observed and the immunoreactivity was stronger than that in IPMT (Figure 3). Regarding the immunohistochemistry for 14-3-3 \(\sigma\), a significant difference was observed between IPMT and IDC \((p<0.05)\). The mean labeling index of Ki-67 increased from IPMA to IDC through IPMC \((p<0.05)\).

In IPMA, the tumor cell types were immunohistochemically analyzed to clarify the biological difference (Table V). Although the frequency was low, immunoreactivity for MUC1 was observed only in IPMA with dark columnar cells. No immunohistochemical difference was detected in CDX2, MUC2, or p16. Regarding immunohistochemistry for 14-3-3 \(\sigma\), the frequency of IPMA with clear columnar cells and dark columnar cells was 64% and 80%, respectively. Based on the Ki-67 labeling index, the frequency of IPMA with dark columnar cells was double that of IPMA with clear columnar cells \((p<0.05)\).

**Discussion**

The 14-3-3 protein comprises a family of highly conserved acidic proteins expressed in all eukaryotic cells (20). There are at least seven different mammalian isoforms of 14-3-3, 25-30 kDa in molecular weight. Of the seven, 14-3-3 sigma (\(\sigma\)) was identified as an epithelial-specific marker, as HME1 in 1992 and stratifin in 1993, which are most abundant in stratified squamous keratinizing epithelium in human tissues (21, 22). Functionally, 14-3-3 \(\sigma\) is associated with control of the G2/M checkpoint in the cell cycle (23).

<table>
<thead>
<tr>
<th>Table IV. Immunohistochemical results in this study.</th>
<th>CDX2</th>
<th>MUC 1*</th>
<th>MUC 2</th>
<th>p53</th>
<th>p16</th>
<th>14-3-3 (\sigma)*</th>
<th>Ki-67*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMA (24 cases)**</td>
<td>3 (13)</td>
<td>2 (8.3)</td>
<td>5 (21)</td>
<td>1 (4.2)</td>
<td>4 (17)</td>
<td>17 (71)</td>
<td>18.1±16.2</td>
</tr>
<tr>
<td>IPMC (9 cases)</td>
<td>2 (22)</td>
<td>1 (11)</td>
<td>3 (33)</td>
<td>1 (11)</td>
<td>2 (22)</td>
<td>6 (67)</td>
<td>33.0±21.1</td>
</tr>
<tr>
<td>IDC (14 cases)</td>
<td>4 (29)</td>
<td>9 (64)</td>
<td>2 (14)</td>
<td>4 (29)</td>
<td>7 (50)</td>
<td>14 (100)</td>
<td>47±8.5</td>
</tr>
</tbody>
</table>

*p<0.005.
**IPMA, intraductal papillary-mucinous adenoma; IPMC, intraductal papillary-mucinous carcinoma; IDC, invasive ductal carcinoma. The percentage of positive tumors are included in parentheses.

<table>
<thead>
<tr>
<th>Table V. Immunohistochemical results in intraductal papillary-mucinous adenoma according to cell types.</th>
<th>CDX2</th>
<th>MUC 1</th>
<th>MUC 2</th>
<th>p53</th>
<th>p16</th>
<th>14-3-3 (\sigma)</th>
<th>Ki-67*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMA-C (14 cases)**</td>
<td>1 (7.1)</td>
<td>0 (0)</td>
<td>2 (14)</td>
<td>1 (7.1)</td>
<td>1 (7.1)</td>
<td>9 (64)</td>
<td>11.6±5.6</td>
</tr>
<tr>
<td>IPMA-D (10 cases)</td>
<td>2 (20)</td>
<td>2 (20)</td>
<td>3 (30)</td>
<td>0 (0)</td>
<td>3 (30)</td>
<td>8 (80)</td>
<td>27.2±21.7</td>
</tr>
</tbody>
</table>

*p<0.05.
**IPMA-C, intraductal papillary-mucinous adenoma with clear columnar cells; IPMA-D, intraductal papillary-mucinous adenoma with dark columnar cells. The percentage of positive tumors are included in parentheses.
of 14-3-3 σ is induced downstream of the activation of p53 and results in a G2/M arrest due to inhibition of the Cdc2-cyclin B1 complex after the relocation of Cdc2 outside the nucleus (24). Recent studies have shown that BRCA-1 is a selective co-activator for 14-3-3 σ, requiring the presence of wild-type p53 and estrogen-responsive RING finger protein (Efp) (25, 26). These findings indicate the 14-3-3 σ gene to be a major G2/M checkpoint control gene.

In human carcinogenesis, loss of the tumor suppressor function of 14-3-3 σ can be explained by the inactivation of the gene by hypermethylation at its locus, or the proteolysis of the protein by Efp, which is concerned with increased cell proliferation, leading to tumorigenesis (26). The down-regulation of 14-3-3 σ expression is obvious in cancers of the breast (27, 28), colon (29), stomach, lung (30), liver (31), oral cavity (32) and vulva (33). Extensive studies of breast cancer have revealed that loss of 14-3-3 σ expression occurs early in carcinogenesis (34). In pancreatic cancers, by contrast, 14-3-3 σ was shown to be overexpressed due to hypomethylation of the gene (17, 18). Our present immunohistochemical study clearly demonstrated strong overexpression of 14-3-3 σ protein in IDC of the pancreas and the IPMT that followed. In non-neoplastic duct epithelia, heterogeneous 14-3-3 σ expression was observed by immunohistochemistry. Our previous immunohistochemical study showed that 14-3-3 σ-positive cells were generally observed only at periductal or periglandular locations in the normal pancreas (30).

IPMT is a precursor lesion of pancreatic adenocarcinoma, since the overall incidence of IDC associated with IPMT is 10-20% (2). Furthermore, IPMT shows similar genetic alterations in IDC and PanIN, although the frequency and stage of neoplastic progression at which these alterations occur differ from those in PanIN. Biankin et al. compared IPMN with a PanIN progression model for pancreatic cancer from the viewpoint of genetic alterations, and suggested that mutation of the Ki-ras gene and the overexpression of HER-2/neu are closely associated with low-grade PanIN and IPMA, while abnormalities of p16, p53, DPC4 and BRCA2 appear in high-grade PanIN and IPMC (16). In our immunohistochemical study, abnormalities of p16 and p53 were commonly found in IPMC and IDC, though the frequencies differed. Moreover, 14-3-3 σ overexpression was immunohistochemically confirmed in pancreatic IDC, as well as in the majority of IPMT cases, the latter finding having never been
reported before. Therefore, in pancreatic carcinogenesis, overexpression of 14-3-3σ might be an early event with similar timing to the occurrence of Ki-ras gene mutation or HER-2/neu overexpression (16). The biological significance of 14-3-3σ expression in pancreatic carcinogenesis is now believed to be its anti-apoptotic effect, achieved by inhibiting the pro-apoptotic proteins bad and bax (18).

Clinicopathologically, IPMT is now a well-accepted entity (2, 13). Clinically, IPMT predominantly involving the main pancreatic duct is associated with an invasive adenocarcinoma such as colloid carcinoma and conventional tubular adenocarcinoma (35). Therefore, it is reasonable that IPMC arises mostly in the main pancreatic duct, as shown in Table I. According to the immunohistochemistry for MUC proteins, MUC1 and MUC2 are characteristically expressed in IDC and IPMT, respectively (14, 15, 19, 36). In addition to these MUC proteins, MUC5AC was also expressed in IPMT (14). As shown in Table IV, our IPMT and IDC cases showed a similar tendency to the previous immunohistochemical results for MUC1 and MUC2 expression (33-37). Recently, aberrant expression of the CDX2 homeobox gene was shown in IPMT, but not in IDC. However, our results contradict previous results.

Yonezawa et al. cytologically classified IPMT into two main types, dark and clear columnar cells, and revealed that IPMT composed of dark and clear cells showed MUC2-positive and -negative expression, respectively (14). Although our study could not confirm the difference in MUC2 expression between IPMT with clear or dark columnar cells, IPMA tended to be mainly composed of clear cells and IPMC of dark columnar cells. As shown in Table V, the mean labeling index of Ki-67 was significantly higher in IPMA with dark columnar cells than that with clear columnar cells. No difference in the expression of 14-3-3σ was observed between IPMA with dark or clear columnar cells. Our previous study of 14-3-3σ in colon cancers has already shown that the Ki-67 labeling index does not necessarily correlate with 14-3-3σ expression (29). These findings suggest that IPMA composed of dark columnar cells might be a potentially more advanced form than that composed of clear columnar cells.

References


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