Intraductal papillary mucinous neoplasms (IPMN) are pancreatic exocrine tumors composed of dilated main or branch ducts lined by mucin-producing atypical epithelium, which usually proliferate in a papillary fashion (Fig. 1; refs. 1, 2). Based on their architectural and nuclear atypia, the WHO has divided IPMNs into three groups: intraductal papillary mucinous adenoma (IPMA), borderline IPMN (IPMB), and intraductal papillary mucinous carcinoma (IPMC; ref. 1). Borderline lesions and carcinoma are accompanied by less atypical lesions in the vicinity, and transition from adenoma to adenocarcinoma is recognized within lesions. They are considered to exhibit biological changes associated with the progression of adenoma to carcinoma accompanied by several molecular abnormalities. ATM-Chk2-p53 DNA damage checkpoint activation, which is involved in prevention of the progression of several tumors, was analyzed to evaluate the role of the DNA damage checkpoint in the progression of IPMNs.

Purpose: Intraductal papillary mucinous neoplasms (IPMN) are known to show a transition from adenoma to carcinoma accompanied by several molecular abnormalities. ATM-Chk2-p53 DNA damage checkpoint activation, which is involved in prevention of the progression of several tumors, was analyzed to evaluate the role of the DNA damage checkpoint in the progression of IPMNs.

Experimental Design: One hundred and twenty-eight IPMNs were classified into four groups (intraductal papillary mucinous adenoma, borderline IPMN, noninvasive intraductal papillary mucinous carcinoma, and invasive intraductal papillary mucinous carcinoma) and stained immunohistochemically using antibody for Thr68-phosphorylated Chk2. Expression of ATM, Chk2, and p21WAF1 and accumulation of p53 were also analyzed.

Results: Chk2 phosphorylation was shown in all adenomas and showed a significant decreasing trend with the progression of atypia ($P < 0.0001$ by the Cochran-Armitage test for trend). Expression of p21WAF1 also exhibited a decreasing tendency ($P < 0.0001$), reflecting DNA damage checkpoint inactivation. p53 accumulation was mostly detected in malignant IPMNs. It was suggested that the DNA damage checkpoint provides a selective pressure for p53 mutation.

Conclusion: Our findings indicate that DNA damage checkpoint activation occurs in the early stage of IPMNs and prevents their progression. It is suggested that disturbance of the DNA damage checkpoint pathway due to Chk2 inactivation or p53 mutation contributes to the carcinogenesis of IPMNs.

Abstract

The Role of the DNA Damage Checkpoint Pathway in Intraductal Papillary Mucinous Neoplasms of the Pancreas

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Intraductal papillary mucinous neoplasms (IPMN) are pancreatic exocrine tumors composed of dilated main or branch ducts lined by mucin-producing atypical epithelium, which usually proliferates in a papillary fashion (Fig. 1; refs. 1, 2). Based on their architectural and nuclear atypia, the WHO has divided IPMNs into three groups: intraductal papillary mucinous adenoma (IPMA), borderline IPMN (IPMB), and intraductal papillary mucinous carcinoma (IPMC; ref. 1). Borderline lesions and carcinoma are accompanied by less atypical lesions in the vicinity, and transition from adenoma to adenocarcinoma is recognized within lesions. They are considered to exhibit a pattern of progression similar to that seen in colorectal adenocarcinoma (i.e., the adenoma-carcinoma sequence (3, 4)). Some IPMNs are restricted to epithelium, whereas others are associated with invasive carcinoma. Sohn et al. (5) investigated 136 patients with IPMNs, including 52 with invasive carcinoma, and concluded that associated invasive carcinoma is the strongest prognostic factor.

Various biological changes associated with the progression of IPMNs have been reported. Biankin et al. (6) reported that the frequency of loss of p16INK4A and Smad4, cyclin D1 overexpression, and p53 accumulation are greater in IPMC and IPMC with invasive carcinoma. House et al. (7) investigated that IPMNs with invasion tend to have multiple methylated genes, which are related to cell cycle control (p16, p73, and APC), DNA repair (MGMT and hMLH1), and cell adhesion (E-cadherin).

Recently, the DNA damage checkpoint pathway, including the ATM-Chk2-p53 pathway, was reported to be involved in tumorigenesis. The ATM-Chk2 pathway was originally shown to be activated in response to double-strand DNA breaks caused by ionizing radiation (8, 9). Responding to DNA damage, ATM phosphorylates Chk2 at Thr68, and activated Chk2 phosphorylates downstream proteins, such as p53, activation of which leads to cell cycle arrest and DNA repair or apoptosis (Fig. 2). Bartkova et al. (10) showed that Chk2 phosphorylation occurred in colon adenoma and the early stage of urinary bladder cancer and decreased in advanced carcinoma. They thus suggested that the ATM-Chk2 DNA damage signaling pathway was activated and delayed or prevented tumor progression in the early stage of tumorigenesis. Gorgoulis et al. (11) reported the activation of the DNA damage checkpoint in precancerous lesions of lung and skin as...
indicated by phosphorylation of Chk2 and H2AX. It is proposed that frequent inactivation of p53 in carcinomas supports DNA damage checkpoint activation in precursor lesions because activated checkpoint might confer a selective pressure for p53 dysfunction (11, 12). Indeed, aberrations of checkpoint-related genes, such as ATM, Chk2, and p53, have been reported in the literature and their role as tumor suppressors has been widely accepted (13, 14). However, the role of the DNA damage checkpoint pathway in pancreatic neoplasms has not yet been investigated.

The existence of IPMNs with the adenoma-carcinoma sequence prompted us to investigate the status of the DNA damage checkpoint pathway in a large series of IPMNs. Activation of the pathway was evaluated by immunohistochemistry using antibody for Thr68-phosphorylated Chk2. We also investigated expression of ATM, Chk2, and p21WAF1, which is known as a downstream effector of the ATM-Chk2-p53 pathway, and accumulation of p53. The purpose of this study was to clarify the involvement of the DNA damage checkpoint pathway in the tumorigenesis and progression of IPMNs.

Materials and Methods

Tissue samples. One hundred and twenty-eight cases of IPMN were collected from the files of the Department of Anatomic Pathology of Kyushu University. All samples were obtained by surgery between July 1986 and January 2006. The original H&E slides for each case were reviewed by three pathologists (Y.M., T.I., and M.T.) independently, and the IPMNs were classified into four groups (IPMA, IPMB, noninvasive IPMC, or invasive IPMC) according to the WHO criteria (1). Because of the heterogeneity of the atypia in IPMN, we determined which lesion had the highest degree of architectural and cellular atypia in each case and selected it as a representative section. Normal ductal epithelia in these pancreata, which were available in 119 cases, were also evaluated.

Immunohistochemistry. Serial 3-μm sections were prepared from the selected paraffin blocks, deparaffinized in xylene, and rehydrated in ethanol. Endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 30 min. Antigen retrieval was achieved by microwave in citrate buffer at pH 6.0. A Histofine SAB-PO kit (Nichirei) was used for immunohistochemical labeling. Each section was exposed to 10% nonimmunized rabbit serum (Chk2, ATM, p53, and p21WAF1) or goat serum (phospho-Chk2) for 10 min to block nonspecific binding of the antibodies and then incubated with primary antibodies at 4°C overnight. We used the following antibodies obtained from commercial suppliers: rabbit monoclonal anti-phospho-Chk2 (Thr68) antibody (80F5, 1:200; Cell Signaling), mouse monoclonal anti-Chk2 antibody (DCS-270, 1:200; MBL), mouse monoclonal anti-ATM antibody (ATX08, 1:50; Novus Biologicals), mouse monoclonal anti-p53 (PAb1801, 1:100; Oncogene), and mouse monoclonal anti-p21WAF1 (EA10, 1:200; Calbiochem). The sections were conjugated with biotinylated anti-rabbit (phospho-Chk2) or anti-mouse (Chk2, ATM, p53, and p21WAF1) immunoglobulin solution for 20 min followed by 20-min incubation with peroxidase-labeled streptavidin. For visualization of the reaction products, 3,3′-diaminobenzidine was used as a chromogen, and then nuclear counterstaining with hematoxylin was done.

Evaluation. Immunohistochemical staining was assessed by the three pathologists independently. To assess the expression of Chk2 and phospho-Chk2, we used the scoring system described by Eymin et al. (15). Scores were calculated by multiplying the percentage of positive cells (0-100) by the intensity score (0-3); when the score was ≥10, the tumor was considered positive for the antibody. Based on published reports, the criteria for staining with other antibodies were as follows: ≥5% nuclear and cytoplasmic staining was considered positive for ATM.
and ≥10% nuclear staining was considered positive for p21WAF1 (17) or p53 accumulation (6).

Statistical analysis. Clinicopathologic characteristics were compared among the four groups using the χ² test, Fisher’s exact test, or ANOVA with Bonferroni correction. The Cochran-Armitage test was used to determine trends of immunohistochemical staining. All analyses were done using Statistical Analysis System for Windows, release 8.2 (SAS Institute, Inc.).

Results

Clinicopathologic data. The 128 IPMNs analyzed in this study comprised 46 IPMAs, 30 IPMBs, 25 noninvasive IPMCs, and 27 invasive IPMCs. The clinicopathologic features of the patients are summarized in Table 1. The neoplasms showed a tendency to arise in the pancreas head in elderly men. Larger cysts, mural nodules, and main duct type were significantly correlated with malignancy. These findings are consistent with previous reports (5, 18). This group can therefore be considered as an average population of IPMNs.

Chk2 phosphorylation in IPMNs. All of the IPMAs showed nuclear staining for phospho-Chk2, whereas normal ductal epithelia in 31 of 119 (26%) cases were positive for phospho-Chk2, indicating that the DNA damage checkpoint was fully activated in adenoma. With progression of atypia, the rate of phospho-Chk2 staining decreased: 46 of 46 (100%) of IPMAs, 29 of 30 (96.7%) of IPMBs, 21 of 25 (84.0%) of noninvasive

Table 1. Clinicopathologic features

<table>
<thead>
<tr>
<th></th>
<th>IPMA (n = 46)</th>
<th>IPMB (n = 30)</th>
<th>IPMC (n = 64)</th>
<th>Total (n = 71)</th>
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<td>Age (y)</td>
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<tr>
<td>Mean ± SD</td>
<td>64.3 ± 8.5</td>
<td>67.3 ± 7.4</td>
<td>68.3 ± 6.4</td>
<td>65.5 ± 9.1</td>
<td>66.0 ± 8.1</td>
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<td>20</td>
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<tr>
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<td>Size (mm)</td>
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<td></td>
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<tr>
<td>Median (25%, 75%)</td>
<td>25.5 (15.0, 35.0)</td>
<td>26.5 (20.0, 40.0)</td>
<td>36.0 (30.0, 50.0)</td>
<td>33.5 (28.0, 47.0)</td>
<td>30.0 (20.0, 40.0)</td>
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<tr>
<td>Mural nodule</td>
<td></td>
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<td>19</td>
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<td>39</td>
<td>17</td>
<td>10</td>
<td>8</td>
<td>74</td>
</tr>
</tbody>
</table>

*Statistically significant.
Fig. 3. A to D, IPMB. A, H&E stain. B, positive nuclear stain for phospho-Chk2. C, positive nuclear stain for Chk2. D, positive nuclear stain for ATM. E, noninvasive IPMC showing positive stain for phospho-Chk2. F, invasive IPMC showing negative stain for phospho-Chk2. G, IPMB showing positive nuclear stain for p21WAF1. H, noninvasive IPMC showing positive nuclear stain for p53.
IPMCs, and 16 of 27 (59.9%) of invasive IPMCs (Figs. 3A, B, E, and F and 4A). The Cochran-Armitage test showed a significant decreasing trend between adenoma and invasive carcinoma ($P < 0.0001$). Less atypical lesions around phospho-Chk2-negative areas showed phospho-Chk2 staining. This suggests that activation of Chk2 is gradually disrupted in the transition to malignancy.

**ATM and Chk2 protein expression.** To investigate the cause of the loss of phospho-Chk2 staining, immunohistochemistry for Chk2 and ATM expression was done. Chk2 showed nuclear staining, whereas ATM showed nuclear and/or cytoplasmic staining. Chk2 expression was preserved in all tumors except one noninvasive IPMC (Figs. 3A and C and 4B), and ATM expression was preserved in all except one case of invasive IPMC (Figs. 3A and D and 4C). Both cases were negative for phospho-Chk2 staining and exhibited positive staining in surrounding less atypical lesions. Normal ductal epithelia in all cases were positive for both antibodies (Fig. 4B and C).

**p21$^{WAF1}$ expression and p53 accumulation.** To investigate downstream of Chk2, we studied p21$^{WAF1}$ expression and p53 accumulation. Normal epithelia in 7 of 119 (6%) cases were positive for p21$^{WAF1}$. Forty-one IPMAs (89%), 21 IPMBs (70%), 16 noninvasive IPMCs (64%), and 11 invasive IPMCs (41%) exhibited p21$^{WAF1}$ expression (Figs. 3G and 4D). p21$^{WAF1}$ expression showed a significant decreasing trend between adenoma and invasive carcinoma ($P < 0.0001$). Accumulation of p53 was seen in no normal epithelia (0%), one IPMA (2%), no IPMB (0%), five noninvasive IPMCs (20%), and eight invasive IPMCs (30%) and was shown to have an increasing tendency (Figs. 3H and 4E; $P < 0.0001$).

**Discussion**

In this study, we showed that Chk2 was phosphorylated in most of the IPMNs, including all cases of adenoma. Although Chk2 was originally reported to be phosphorylated in response to DNA double-strand breaks, recent studies have shown Chk2 phosphorylation in tumors of the lung, urinary bladder, colon, breast, and skin (10, 11, 15, 19). Chk2 phosphorylation has even been detected in preneoplastic lesions of the lung and skin (11). These findings suggest that the DNA damage checkpoint pathway is activated in the early stage of neoplasia, and our present data were consistent with this. However, the mechanism of Chk2 phosphorylation in neoplasms remains to be elucidated. Bartkova et al. (10) proposed that oncogene-induced DNA damage and telomere dysfunction activate the DNA damage pathway, and Gorgoulis et al. (11) mentioned that DNA replication stress due to aberrant stimulation of cell proliferation induces DNA damage. It has been reported that activation of oncogenes, such as $KRAS$ mutation or c-erbB-2 overexpression, occurs in the early stage of IPMN (20–22). Recently, mutated $HRAS$ was reported to activate the ATM-Chk2 pathway in both human fibroblast cultures and mouse keratinocyte tumor models (23, 24). Mutated KRAS might have similar effects on the DNA damage checkpoint activation in IPMNs because KRAS has a high degree of sequential analogy with HRAS and mutated forms of HRAS and KRAS have similar abilities to cause morphologic and growth transformation in cultured cells (25, 26). Unexpectedly, even normal ductal epithelia showed positive staining for phospho-Chk2 in some cases, but the proportion of these cases was much lower. It is

**Fig. 4.** Results of immunohistochemical analysis. A, phospho-Chk2 staining showed a significant decreasing trend ($P < 0.0001$, Cochran-Armitage test). B, one phospho-Chk2–negative noninvasive IPMC showed Chk2-negative staining. C, one phospho-Chk2–negative invasive IPMC with invasion showed ATM-negative staining. D, p21$^{WAF1}$ expression showed a significant decreasing trend ($P < 0.0001$, Cochran-Armitage test). E, p53 accumulation showed a significant increasing trend ($P < 0.0001$, Cochran-Armitage test).

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Research.
therefore reasonable to conclude that the DNA damage checkpoint is activated in neoplasms. Although some reports have documented that Chk2 was not phosphorylated in normal tissue (e.g., lung, urinary bladder, and breast), others have documented that Chk2 was phosphorylated in normal bone marrow, testicular, and esophageal tissue (10, 11, 19, 27). Unlike lymphocytes in bone marrow and stem cells in testes, which undergo physiologic DNA double-strand breaks, the process of Chk2 activation in the normal esophageal epithelium remains unclear. There is also no evidence supporting physiologic DNA damage in pancreatic ductal epithelium.

Considering the multifocal lesions and the frequent recurrence after margin negative resection of IPMNs, some of the morphologically normal ductal epithelium in the vicinity of the IPMN may already have activated oncogenes and hence show activation of the DNA damage response (5, 28).

In our study, Chk2 showed a decreasing trend of phosphorylation with the progression of IPMNs. In addition, p21WAF1 expression decreased and p53 accumulation increased in the transition of IPMN from benign tumor to malignancy. Aberrations of the DNA damage checkpoint pathway are frequently recognized in malignant IPMNs, suggesting that checkpoint pathway disruption contributes to the IPMN progression. Bartkova et al. (10) reported a decrease of Chk2 phosphorylation in advanced urinary bladder carcinoma. Gorgoulis et al. (11) found that some lung cancers and melanomas showed no phospho-Chk2 staining, whereas their precursor lesions did. Our data indicate that, in some cases, loss of phospho-Chk2 staining could be attributed to ATM or Chk2 protein depletion. However, the cause of Chk2 inactivation in the other cases is uncertain. Loss of p53-binding protein 1 expression might prevent Chk2 phosphorylation, as concomitant defects of p53-binding protein 1 expression and Chk2 phosphorylation have been reported in lung cancer and melanoma (11, 19). Alternatively, it is possible that phosphatases, such as Wip1, are involved in Chk2 dephosphorylation (29).

In the present study, expression of the cyclin-dependent kinase inhibitor p21WAF1 exhibited a gradual decline parallel to phospho-Chk2 staining. Down-regulation of p21WAF1 according to tumor progression has been reported in gastric and colonic neoplasms, and expression of p21WAF1 is regulated by Chk2 via both p53-dependent and p53-independent pathways (30–33). The reduction of p21WAF1 expression observed in this study is thought to reflect inactivation of the DNA damage checkpoint pathway in IPMNs. Biankin et al. (34) reported an increasing trend of p21WAF1 in intraepithelial neoplasia associated with invasive ductal carcinoma of the pancreas, but the genetic differences between invasive ductal carcinoma and IPMNs might account for this discordance.

p53 abnormalities in IPMNs are documented in the literature (6, 20, 35). Although p53 accumulation is less common in IPMNs than in pancreatic invasive ductal carcinoma, some IPMNs show it (36). Similar to our data, the frequency of p53 accumulation increased in malignant IPMNs. Halazonetis (12) proposed that frequent p53 inactivation in human cancer cells is due to DNA damage checkpoint activation. Without DNA damage checkpoint pathway activation, there would be no selective pressure for p53 dysfunction. In other words, p53 mutation in malignant IPMNs supports activation of the DNA damage checkpoint in IPMNs.

It has been shown that Chk2 activation leads to cellular senescence (32, 33, 37). Senescence is a state of permanent cell cycle arrest accompanied by specific morphologic alterations and has been reported to be induced by certain oncogenes, such as Ras (38, 39). Recently, several authors have shown that senescence occurs in premalignant tumor cells and plays a role in limiting carcinogenesis, and they have suggested that attenuation of senescence due to deficiency of its effectors

Fig. 5. Schema of hypothesis for the progression of IPMNs. In premalignant IPMNs, DNA damage checkpoint activation inhibits oncogene-induced cell proliferation and tumor growth. Activated oncogenes and disruption of the checkpoint pathway due to dysfunction of checkpoint-related molecules or phosphatase overexpression result in continuous cell proliferation, which leads to explosive tumor growth and acquisition of invasiveness and metastatic abilities.
results in progression to malignancy (40–42). It has been reported that the DNA damage checkpoint pathway is involved in the induction of oncogene-induced senescence and that inactivation of the DNA damage checkpoint due to suppression of mediator proteins, such as ATM, terminates senescence (23, 24). Our data showed that both Chk2 activation and expression of p21 \( ^{WAF1} \), which is reported to be a senescence effector protein, reached a peak in adenoma lesions and they decreased gradually in the progression to invasive malignancy (33). It is therefore suggested that premalignant IPMNs may undergo senescence and that disruption of senescence might lead to malignancy.

We hypothesize a model for the progression of IPMNs as shown in Fig. 5. In premalignant IPMN, the activated DNA damage checkpoint inhibits oncogene-induced cell proliferation. Inactivation of the DNA damage checkpoint leads to continuous proliferation of tumor cells. Sustained replication increases the occurrence of molecular abnormalities, and shortage of nutrients due to the increased cell population might provide selective pressure for molecular abnormalities that enable cells to invade or metastasize (43). In other words, disruption of the DNA damage checkpoint results in acquisition of the characteristics of malignancy. Considering the good prognosis of benign and borderline lesions, development of methods to maintain the DNA damage checkpoint would enable us to avoid the use of invasive treat. Detailed exploration of the molecular abnormalities leading to DNA damage checkpoint inactivation, such as dysfunction of p53-binding protein 1 and overexpression of Wip1, will contribute to progress in the treatment of IPMNs.

References


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