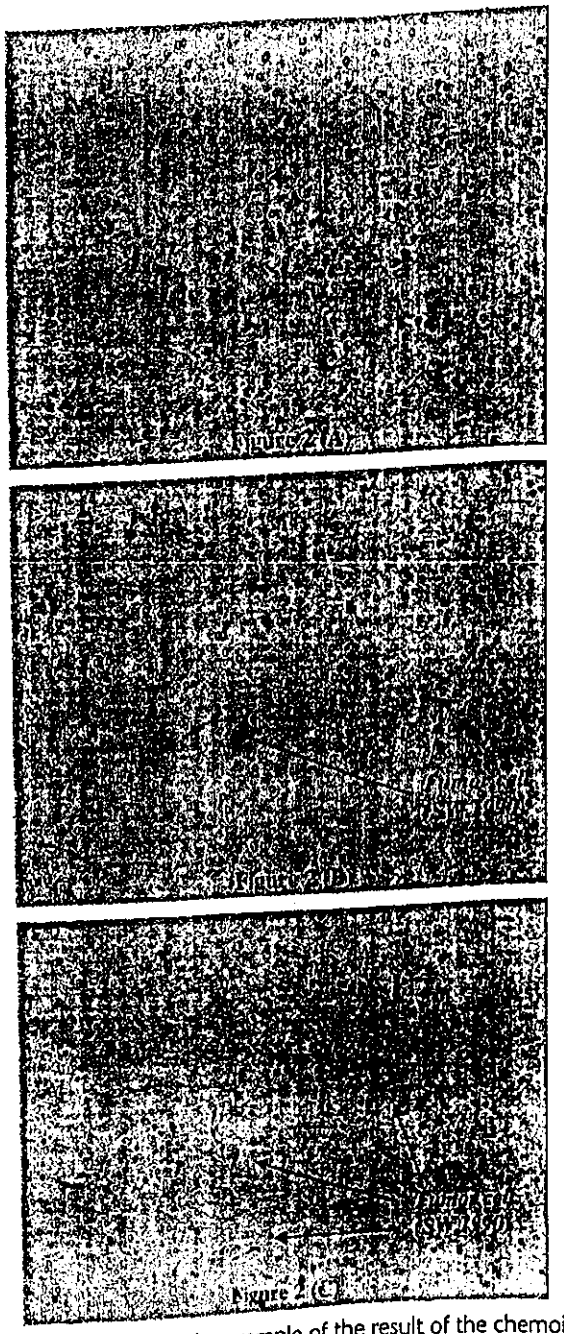

FIGURE 2. Representative example of the result of the chemoinvasion assay using the human pancreatic adenocarcinoma cell line SW.1990. Tumor cells were introduced into the upper chamber either without platelets (tumor cells only) (A), with inactivated platelets (B), or with activated platelets (C). After incubation at 37°C for 24 hours, tumor cells were observed in ×200 view. The number of tumor cells which had infiltrated to the lower side of the filter obviously increased in the presence of platelets.

with three antiplatelet drugs. BxPC-3 was chosen because the invasiveness of BxPC-3 was the most markedly increased in the chemoinvasion assay. The effects of the three antiplatelet drugs were compared for five different concentrations.

Figure 7 shows the results of chemoinvasion assay performed in a similar manner as described above. The number of traversed tumor cells obviously increased when incubated with platelets but without PGI_2 (Fig. 7A). The number of tumor cells decreased under conditions of incubation with PGI_2, but there were no significant differences in cell counts. Under conditions of incubation with EPA, it was obvious that the number of cells decreased depending on the concentration when incubated with activated platelets. There were significant differences in cell counts between concentrations of EPA of 0 μmol/L and 5.0 μmol/L ($P < 0.05$) and between 0 μmol/L and 10.0 μmol/L ($P < 0.01$, Fig. 7B). In the experiment using cilostazol, the number of traversed tumor cells also increased when incubated with platelets but without cilostazol. There were significant differences in cell counts between each of the cilostazol concentrations when incubated with activated platelets. Moreover, in the condition of incubation with inactivated platelets, there were significant differences in cell counts between each concentration of cilostazol except between 0 μg/mL and 1.0 μg/mL (Fig. 7C). It was obvious that the decrease in cell counts by cilostazol depended on its concentration.

ELISA was performed to evaluate whether the expression of pro–MMP-9 decreased when tumor cells were incubated with an antiplatelet drug. Cilostazol was chosen because the inhibition of tumor cell invasion was greatest compared with the other 2 antiplatelet drugs. Figure 8 shows the results of the ELISA. Without cilostazol, the secretion of MMP-9 significantly increased when incubated with platelets compared with incubation without platelets, and there was a significant difference in the secretion of MMP-9 between incubation with inactivated and activated platelets. When incubated with cilostazol, either with inactivated and activated platelets, there were significant differences in the MMP-9 secretion between each concentration of cilostazol, which was concentration dependent.

DISCUSSION

The interaction between tumor cells and platelets and its functions were investigated. It was revealed that the invasiveness of tumor cells was promoted in the presence of platelets and inhibited when incubated with platelets and antiplatelet agents. These changes were paralleled in the secretion levels of MMP-9 from tumor cells.

The invasiveness of tumor cells has been examined using a chemoinvasion assay. This experimental model expresses the traversing of tumor cells through the endothelium into the ECM.[26] In this study, the invasiveness of tumor cells obviously increased in all tumor cell lines when incubated with platelets. Belloc et al[23] recently reported that the invasiveness of tumor cells was promoted by the coculture of tumor cells with activated platelets. In our data, the invasiveness was also increased when incubated with inactivated platelets. Further-

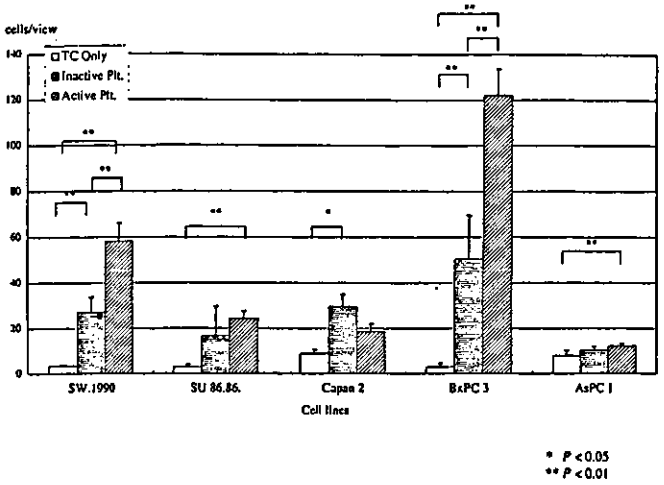

FIGURE 3. Invasiveness of tumor cells analyzed by the chemoinvasion assay. Tumor cells were counted on the lower side of the filter. Results were expressed as the number of cells per microscopic field. Five fields were counted in each of 3 different experiments. In all cell lines, there were significant differences in the number of traversed tumor cells between medium without platelets and with platelets ($*P < 0.05$, $**P < 0.01$).

more, the promotion of tumor cells invasion was greater when incubated with activated platelets than when incubated with inactivated platelets. This result reveals that the presence of platelets can increase the invasiveness characteristics of pancreatic tumor cells, and the activation of platelets may play an important role in promotion of tumor cells invasion.

A gelatin zymography assay was examined to evaluate whether the promotion of tumor cell invasion was accompanied by changes in MMP-9 secretion levels from tumor cells.

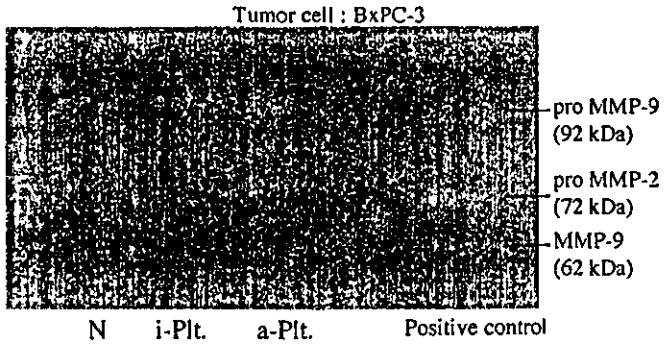

FIGURE 4. Effect of platelets on MMP-9 production by human pancreatic adenocarcinoma cell lines. Examples of the gelatin zymographic analysis: BxPC-3. In this cell line, white broad bands of gelatin lysis against a blue background were detected at 92 kd. The density of the bands on lanes of the sample that had been incubated with platelets (i-Plt., inactivated platelets; a-Plt., activated platelets) obviously increased compared with the band on the no-platelets lane (N, tumor cells only).

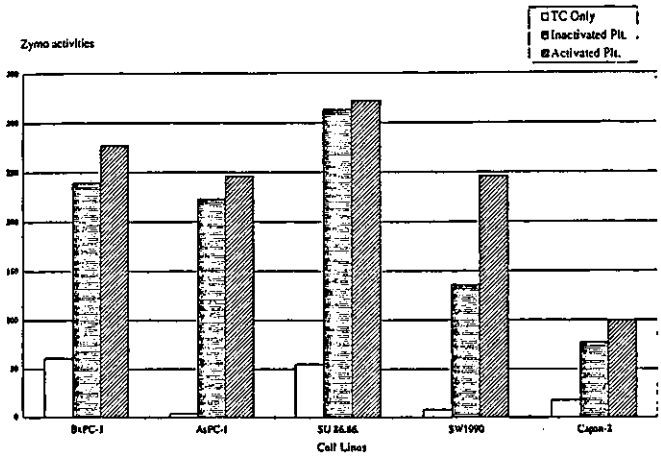

FIGURE 5. MMP-9 activity analyzed using NIH 1.62 image analysis software. In all cell lines, the MMP-9 activity greatly increased when tumor cells were incubated with platelets (i-Plt., inactivated platelets; a-Plt., activated platelets) compared with no-platelets (N, tumor cells only). The activity of samples incubated with activated platelets was greater than in samples incubated with inactivated platelets in all cell lines.

In the assay, it was observed that the expression level of MMP-9 greatly increased when incubated with platelets in all cell lines of human pancreatic cancer. The secretion level of MMP-9 was obviously increased in the medium that was incubated with inactivated platelets compared with without platelets, and it was also increased in the medium incubated with activated platelets compared with inactivated platelets. These data support the concept that the expression of MMP-9 by tumor cells promotes the invasion of tumor cells. Similar results were recently reported by Lindenmeyer et al[24] using human mammary tumor cells. In this study, the secretion level of MMP-9 was increased by platelets, and this increase was caused by platelet aggregation interacting with the tumor cells and not by the soluble factors released during platelet activa-

FIGURE 6. Western blot analysis of MMP-9 secreted from BxPC-3, comparing 3 different media (N, tumor cells only; i-Plt., inactivated platelets; a-Plt., activated platelets). The bands could be detected at 92 kd, meaning the presence of MMP-9. The density of the band on the lane that was incubated with activated platelets (a-Plt.) was obviously greater than the sample with inactivated platelets (i-Plt.). Furthermore, the i-Plt. band was greater compared with the sample without platelets (N).

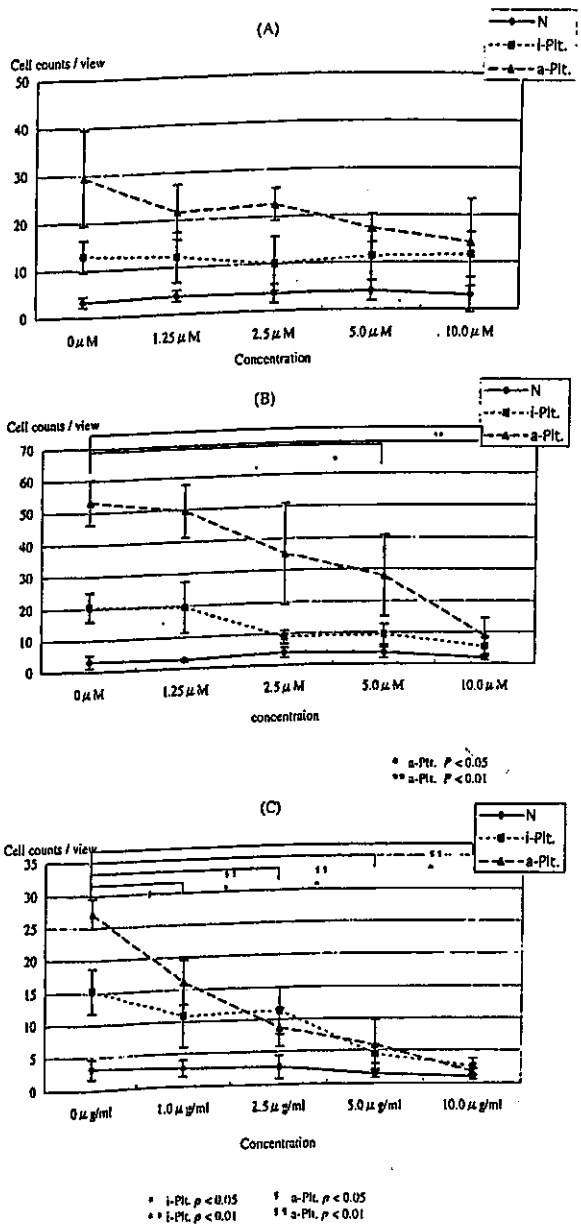

FIGURE 7. Effect of antiplatelet agents on invasiveness of tumor cells analyzed by the chemoinvasion assay. Three different antiplatelet agents were used: PGI_2 (A), EPA (B), and cilostazol (C). Under conditions of incubation with PGI_2, there were no significant differences in the tumor cell counts for each concentration in all medium conditions (N, tumor cell only; i-Plt., with inactivated platelets; a-Plt., with activated platelets). When incubated with EPA, there were significant differences in cell counts among concentrations of EPA with the addition of activated platelets. When incubated with cilostazol, the traversed tumor cells significantly decreased depending on concentrations of cilostazol irrespective of the addition of inactivated or activated platelets.

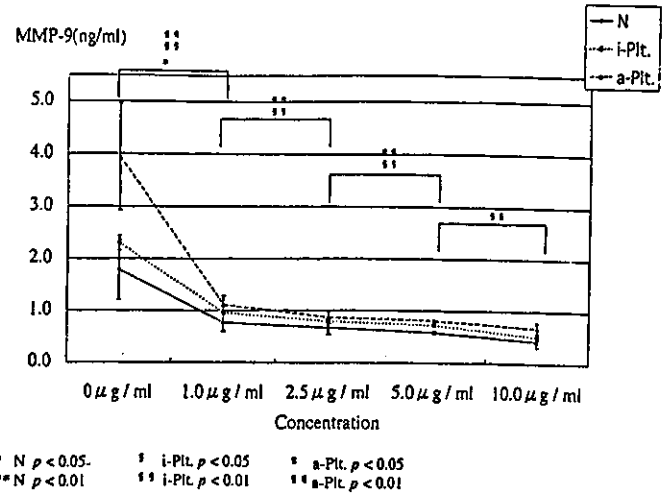

FIGURE 8. Results of the ELISA of incubation with cilostazol. Without the addition of cilostazol, secretion of MMP-9 obviously increased when tumor cells were incubated with inactivated platelets (i-Plt.) or with activated platelets (a-Plt.). With the addition of cilostazol, the secretion levels significantly decreased depending on the concentration of cilostazol in the media incubated with i-Plt. and a-Plt.

tion. It was also reported that synthesis of MMP-9 by monocytes was greatly enhanced in the presence of platelets and that it was tightly regulated and synergistically increased following adhesion to collagen and platelets.[27] Moreover, interruption of intracellular contact between platelets and monocytes dramatically inhibited MMP-9 synthesis. Direct adhesion between platelets and tumor cells as well as between platelets and monocytes may be important in MMP-9 synthesis.

The finding that activated platelets promoted the invasiveness of tumor cells in almost all cell lines suggests that antiplatelet agents may inhibit the invasiveness of tumor cells regardless of the presence of platelets. Furthermore, the inhibition of platelets may lead to a decrease in the hematogenous metastasis of tumor cells and improve the prognosis of patients with malignant cancer. To clarify this hypothesis, the effect of antiplatelet agents on the invasiveness of tumor cells was investigated, and the secretion level of MMP-9 from tumor cells in the presence of platelets was evaluated in parallel.

PGI_2, EPA, and cilostazol were used as antiplatelet agents. PGI_2 is known as a very potent antithrombogenic agent and an inhibitor of platelet aggregation. EPA is a member of the omega-3 fatty acid family. This agent is the competitive inhibitor of the arachidonate cascade. EPA can inhibit generation of thromboxane A_2 (TXA_2) from platelets and inhibit aggregation of platelets. Cilostazol has been widely used in clinical treatment in Japan. Cilostazol is one of the antithrombotic agents acting by selective inhibition of 1 of the 5 phosphodiesterase (PDE) families, called cGMP-inhibited cyclic AMP PDEs.[28]

All antiplatelet agents showed an inhibitory effect on the invasiveness of tumor cells. EPA and cilostazol significantly inhibited the invasion of tumor cells; however, there were no significant differences in tumor cell counts among each concentration of PGI_2. The number of traversed tumor cells was particularly significantly decreased under incubation with cilostazol in all medium conditions regardless of the presence of inactivated or activated platelets.

There have been some reports that the metastasis of tumor cells was suppressed in vivo with PGI_2.[29–31] In those reports, the main reason for distant metastasis was explained as the ability of aggregated platelets surrounding tumor cells to protect them from host immunocytes, and the ability of tumor cells to adhere to the vascular endothelium was inhibited by platelet aggregation. The half-life ($t_{1/2}$) of PGI_2 in aqueous buffer at physiologic pH is very short (approximately 3–5 minutes). Although the antiaggregatory effect of PGI_2 is considerably lengthened ($t_{1/2}$ = 60 minutes) in human plasma because the albumins of several mammalian species significantly prolong the $t_{1/2}$ of PGI_2.[32,33] PGI_2 may soon have been inactivated in vitro, as in our study. Therefore, it is possible that PGI_2 slightly inhibited the invasion of tumor cells.

Inufusa et al[34] reported that cilostazol inhibits metastasis of human lung adenocarcinoma in an animal model. In this report, cilostazol suppressed metastasis of tumor cells by inhibiting the activity of platelet aggregation in a concentration-dependent manner. These results support our data.

ELISA was performed to measure the secretion level of MMP-9 from tumor cells to evaluate the inhibition of invasiveness by cilostazol. The secretion level of MMP-9 from tumor cells was suppressed by cilostazol in a concentration-dependent manner. This suppression was observed under conditions of incubation with both inactivated and activated platelets. There were significant differences in the MMP-9 secretion between 0 μg/mL and 1.0 μg/mL of cilostazol concentration under all 3 conditions of tumor cells only, tumor cell with inactivated platelets, and tumor cells with activated platelets. Inhibition of MMP-9 secretion by cilostazol was directly correlated with the suppression of tumor cell invasion.

There are some reports considering the mechanism of interaction between platelets and tumor cells. Nierodzik et al[35] reported on the role of platelets examined in tumor cell adhesion in vitro and metastasis in vivo. In this report, the adhesion of tumor cells to platelets was inhibited by agents inhibiting platelet integrin IIb-IIIa receptor occupancy as well as IIb-IIIa ligands. Furthermore, it was recently reported that P-selectin, an integral membrane protein receptor, may mediate binding of platelets to tumor cells.[36] These reports suggest that these glycoproteins may play an important role in the mediation of platelet–tumor cell interaction. Many tumor cells have the potential to activate platelets and enhance their aggregation.[8] Tumor cells can express an activator of factor Xa and/or tissue factor-factor VII.[37] Other mechanisms have also been elucidated, such as the activation of platelets by ADP produced by the tumor cells themselves or released by platelets in contact with tumor cells[10] and the production of 12-hydroxyeicosatetraenoic acid (12-HETE), which facilitates the expression of the glycoprotein IIb-IIIa receptor.[38] In our study, the invasiveness of tumor cells and the secretion level of MMP-9 from tumor cells concomitantly increased when incubated not only with inactivated but also with activated platelets. It is suggested that platelets were activated and aggregated by contact with tumor cells in the presence of inactivated platelets, and then the invasiveness and secretion were promoted.

Activation of platelets may promote the invasiveness of tumor cells, and inhibition of platelets may regulate the invasiveness depending on the secretion level of MMP-9 from tumor cells. It is possible that the hematogenous metastasis rate decreased in vivo through the use of antiplatelet agents because not only was the ability of tumor cell protection from host immunocytes and arrest of tumor cells in vessels inhibited, but the secretion level of MMP-9 also decreased, and then the invasiveness was suppressed.

Further in vivo studies are required. Our results suggest the possibility of a novel treatment with antiplatelet agents for patients with malignant tumors.

ACKNOWLEDGMENT

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FAST TRACK

LOSS OF *p16^{INK4a}* EXPRESSION IS ASSOCIATED WITH VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION IN SQUAMOUS CELL CARCINOMA OF THE ESOPHAGUS

Hiroya TAKEUCHI, Soji OZAWA*, Chih-Horng SHIH, Nobutoshi ANDO, Yuko KITAGAWA, Masakazu UEDA and Masaki KITAJIMA

Department of Surgery, School of Medicine, Keio University, Tokyo, Japan

Vascular endothelial growth factor (VEGF) expression has been suggested to correlate with intratumoral microvessel density, tumor advancement and prognosis in esophageal squamous cell carcinoma (ESCC). Previous studies have showed that disruption of cell cycle regulator p16 is related to oncogenesis and tumor progression in ESCC. We hypothesized that VEGF expression in ESCC is reflected by abnormalities in the *p16^{INK4a}* gene. To clarify the regulatory role of *p16^{INK4a}* in VEGF expression *in vitro*, we transferred the *p16^{INK4a}* gene into a *p16^{INK4a}*-deleted ESCC cell line and observed changes in VEGF expression. Furthermore, we immunohistochemically assessed the expression of the cell cycle regulators (p16, p53 and RB) and VEGF in 90 surgically resected specimens of ESCC. Introduction of *p16^{INK4a}* cDNA by the p16 expression vector significantly suppressed cell proliferation in the *p16^{INK4a}*-deleted cell line TE8 ($p < 0.0001$). VEGF secretion by TE8 cells transfected with the *p16^{INK4a}* vector was significantly suppressed as compared to non-transfected TE8 cells ($p < 0.0001$) and TE8 cells transfected with a control vector ($p = 0.0015$). The immunohistochemical studies of ESCC primary tumor specimens showed that loss of p16 expression was significantly correlated with VEGF-positive expression ($p = 0.0004$). The cumulative postoperative survival rate in the group with p16-positive and VEGF-negative expression was significantly higher than in the other groups. Neither p53 nor RB expression had any impact on outcome. Aberrant p53 expression tended to be associated with VEGF expression, but the trend did not reach statistical significance. Our study demonstrated that VEGF expression was correlated with p16 expression in ESCC. Our results suggest that p16 may have a regulatory role in VEGF expression in ESCC.

Key words: *transfection; immunohistochemistry; p53; RB; prognosis*

Angiogenesis is essentially required for tumor growth and progression.[1] It increases the supply of nutrients and oxygen to the tumor cells.[2] High intratumoral microvessel density has been documented to be associated with aggressive tumor proliferation, systemic metastasis and prognosis in a variety of cancers.[3–6]

Vascular endothelial growth factor (VEGF) is one of the principal angiogenic factors.[7,8] VEGF specifically induces the proliferation and migration of endothelial cells. VEGF also increases vascular permeability, which may allow cancer cells to enter the blood circulation. VEGF secretion has been demonstrated in various carcinomas[9] and VEGF expression has been reported to correlate with intratumoral microvessel density, tumor advancement, and prognosis in several solid carcinomas.[10–12]

Previous studies have shown that disruption of cell cycle regulators is related to oncogenesis and tumor progression. Two cell cycle regulators, p53 and p16, play important roles in the transition from G1 to S phase of the cell cycle. They induce cell cycle arrest *via* independent pathways, the p53-p21-RB pathway and the p16-cyclin D1-RB pathway, respectively, and induce apoptosis cooperatively.[13] The *p53* and *p16^{INK4a}* genes have been identified as tumor suppressor genes in various tumors.[14–17] Maesawa *et al.*[18] reported homozygous deletion of *p16^{INK4a}* in 5 (16%) of 31 patients with primary ESCCs, with *p16^{INK4a} de novo* methylation in 6 patients (19%) and a *p16^{INK4a}* mutation in one patient (3.2%). In another study, loss of heterozygosity (LOH) on chromosome 9p21-22, on which *p16^{INK4a}* is located, was found in 18 (36%) of 50 primary ESCC tumors.[19] *p53* mutations in exon 4–10 were identified in 16 (44.4%) of 36 primary ESCC tumors,[20] and 30 of 55 (55%) ESCC tumors were found to exhibit LOH on chromosome 13q21, on which the *RB* gene is located.[21]

Esophageal squamous cell carcinoma (ESCC) has one of the highest malignant potentials of any tumor. The postoperative 5-year survival rate of ESCC patients is low, ranging from 24–45%.[22] Direct invasion to adjacent organs, lymph node metastasis and distant organ metastasis have been valued as useful indicators for predicting outcome in ESCC, and several molecular markers have been evaluated as the important prognostic factors of ESCC.[23–26] We reported previously that loss of p16 expression and VEGF expression were correlated with poor outcome in patients with ESCC.[27,28] The association between p53 status and outcome in ESCC, however, remains controversial.[29,30] The relation between the dysfunctions of these cell cycle regulators and VEGF expression in ESCC is largely unknown and no clear clinical studies on p16 status and VEGF expression in ESCC or other carcinomas have ever been conducted. Harada *et al.*[31] reported that *p16^{INK4a}* gene transfer into *p16^{INK4a}*-deleted glioma cells significantly reduced VEGF expression and secretion rather than *p53* gene transfer, and they also reported that exogenous p16 expression suppressed neovascularization *in vivo*. We therefore hypothesized that VEGF expression in ESCC is reflected by the abnormalities in the *p16^{INK4a}* gene.

To clarify the regulatory role of *p16^{INK4a}* in VEGF expression *in vitro*, we observed changes in VEGF expression by *p16^{INK4a}* gene transfer into a *p16^{INK4a}* -deleted ESCC cell line. Furthermore, we immunohistochemically assessed expression of cell cycle regulators p16, p53, RB and VEGF in ESCCs. We also investigated whether p16 in comparison with p53 status is correlated with VEGF expression in ESCC.

MATERIAL AND METHODS

Cell lines and plasmid constructions

Human ESCC cell lines TE 1 and TE8 were obtained from Dr. Nishihira (Tohoku University, School of Medicine, Sendai, Japan). The *p16^{INK4a}* gene has been reported to be the wild-type in TE1 cells, whereas homozygous deletions of the *p16^{INK4a}* gene have resulted in loss of p16 expression in TE8 cells.[27,32,33] A wild-type *p16^{INK4a}* expression vector, pCDKN2WT, was kindly provided by Dr. H-J Su Huang.[34] Wild-type *p16^{INK4a}* cDNA synthesized by the

*Correspondence to: Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81-3-3355-4707. E-mail: ozawa@sc.itc.keio.ac.jp

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reverse transcriptase-polymerase chain reaction was cloned into the *Bam*HI/*Xho*I sites of mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA).

Transfection

TE1 and TE8 cells were transfected with plasmid pCDKN2WT or pcDNA3 (no insertion of p16 cDNA) by the lipofection method (Lipofectamine Reagent, Life Technologies, Inc.). Cells (2×10^5) were plated on a 35-mm culture dish and incubated in 1 ml of serum-free DMEM with 5 μl of Lipofectamine and 2 μg of the expression vectors. After 12 hr, the medium was changed to 2 ml of serum-free DMEM. The supernatant was collected 48 hr after the medium change and used for ELISA assay. Cells were split and exposed to G418 at 800 μg/ml (Geneticin; Gibco BRL-Life Technologies) with 10% FBS DMEM for 7 days. The total number of G418-resistant pCDKN2WT-transfected cells was compared to the G418-resistant pcDNA3-transfected cells by using a hemocytometer to count cells that excluded trypan blue.

Western blot analysis

Western blot analysis to detect p16 protein was carried out as described previously.[27] Cell lines were lysed, and approximately equal amounts of total cellular protein were run on 10–20% SDS-polyacrylamide gradient gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). p16 protein was detected using monoclonal mouse antihuman p16 antibody PMG175-405 (Pharmingen, San Diego, CA), followed by reaction with enhanced chemiluminescence reagents (Amersham). Finally, the blot was exposed to autoradiography film.

ELISA

A human VEGF immunoassay kit (Quantikine, Minneapolis, MN) was used for the quantitative determination of VEGF concentrations in cell culture supernatants. A murine monoclonal antibody specific for VEGF was pre-coated onto a microplate. Standards (recombinant human VEGF 165) and samples were applied into the wells, followed by a horseradish-peroxidase-conjugated polyclonal antibody specific for VEGF. The intensity of color that developed in proportion to the amount of VEGF due to stabilized hydrogen peroxide and tetramethylbenzidine was measured. A standard curve was drawn by plotting the logs of standard VEGF concentrations, and it was used to determine the VEGF values of the samples.

Patients

The surgical specimens of 90 patients (median age, 61 years; range = 40–83; 78 men and 12 women) with squamous cell carcinoma of the thoracic esophagus, who had undergone R0 (curative) resection of the esophagus at Keio University Hospital (Tokyo, Japan) between 1991 and 1996, were examined after signing an informed consent form. Pathological examination of the specimen was carried out according to the Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus of the Japanese Society for Esophageal Disease[35] and the TNM classification.[36] The stages and clinicopathological background factors are summarized in Table I. Patients were followed in the outpatient clinic, and diagnostic examinations, consisting of chest X-ray, computed tomography, and ultrasonography, were carried out every 6 months to detect recurrence. The maximum follow-up period was 72 months, and the median observation period was 27 months.

Immunohistochemistry for p16, p53, RB and VEGF

Four micrometer sections from 10% formalin-fixed and paraffin-embedded tissue were freshly cut and deparaffinized in xylene for protein detection by immunohistochemistry. Slides that contained the most invasive lesion of the tumor were selected and subjected to an antigen retrieval step, in Target Unmasking Fluid (Pharmingen) for 10 min at 90°C for p16, p53 and RB, and in 10 μg/ml pepsin in 0.01 N HCl buffer (pH 2.5) for 30 min at room

TABLE I – ASSOCIATION BETWEEN EACH FACTORS AND CLINICOPATHOLOGICAL CHARACTERISTICS[1]

Characteristics	Patients	p16-negative cases	*p*	p53-positive cases	*p*	pRB-negative cases	*p*	VEGF-positive cases	*p*
Total	90 (100)	49 (100)		47 (100)		16 (100)		32 (100)	
Clinical factors									
Age(yrs)									
< 60	38 (42)	21 (43)		19 (40)		9 (56)		11 (34)	
≥ 60	52 (58)	28 (57)	0.89	28 (60)	0.72	7 (44)	0.33	21 (66)	0.26
Gender									
Male	78 (87)	44 (90)		41 (87)		13 (81)		28 (88)	
Female	12 (13)	5 (10)	0.52	6 (13)	0.99	3 (19)	0.77	4 (13)	0.99
Location[2]									
Ut	14 (16)	7 (14)		7 (15)		3 (19)		4 (13)	
Mt	49 (54)	26 (53)		21 (45)		10 (63)		15 (47)	
Lt	27 (30)	16 (33)	0.82	19 (40)	0.07	3 (19)	0.55	13 (41)	0.26
Pathological factors									
pT factor[3]									
1	28 (31)	13 (27)		14 (30)		4 (25)		7 (22)	
2	10 (11)	4 (8)		5 (11)		2 (13)		4 (13)	
3	52 (58)	32 (65)	0.27	28 (60)	0.94	10 (63)	0.84	21 (66)	0.37
pN factor[4]									
0	30 (33)	12 (24)		14 (30)		8 (50)		8 (25)	
1	60 (67)	37 (76)	0.05	33 (70)	0.46	8 (50)	0.21	24 (75)	0.46
Histological stage									
I	13 (14)	4 (8)		5 (11)		3 (19)		3 (9)	
IIA	17 (19)	8 (16)		9 (19)		5 (31)		5 (16)	
IIB	23 (26)	12 (24)		13 (28)		2 (13)		7 (22)	
III	37 (41)	25 (51)	0.12	20 (43)	0.75	6 (38)	0.36	17 (53)	0.37
Histological type									
Well diferentiated	18 (20)	11 (22)		9 (19)		3 (19)		9 (28)	
Moderately differentiated	69 (77)	36 (73)		36 (77)		13 (81)		22 (69)	
Poorly differentiated	3 (3)	2 (4)	0.73	2 (4)	0.87	0 (0)	0.70	1 (3)	0.36

[1]Values are *n* (%).–[2]According to the Guideines for Clinical and Pathological Studies on Carcinoma of the Esophagus of the Japanese Society for Esophageal Diseases. Ut, upper thoracic esophagus; Mt, middle thoracic esophagus; Lt, lower thoracic esophagus.–[3]pT1, tumor invades lamina propria or submucosa; pT2, tumor invades muscularis propria; pT3, tumor invades adventitia.–[4]pN0, no regional lymph node metastasis; pN1, regional lymph node metastasis.

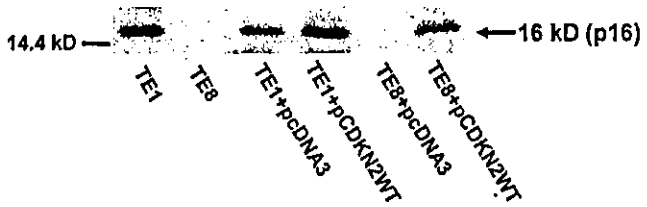

FIGURE 1 – Western blot analysis of ESCC cell lines, TE1 and TE8. p16 expression was detected both in TE1 and TE8 after the transfection of pCDKN2WT.

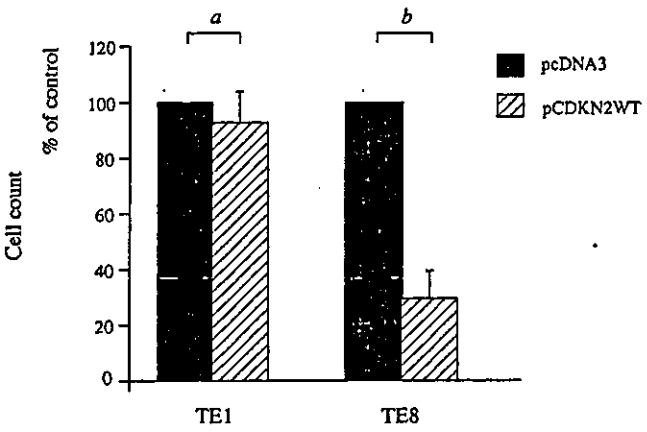

FIGURE 2 – Effects of *p16* gene introduction in ESCC cell growth. Viable cell counts were determined after G418 selection. The results are shown as a percentage of the vector control (pcDNA3) group setting to 100%. Values are the means ± SD of 4 independent experiments. (*a*) $p = 0.23$; (*b*) $p < 0.0001$.

temperature for VEGF. The primary antibodies were a monoclonal mouse antihuman p16 antibody (PMG175-405 [dilution 1:100], Pharmingen), a monoclonal mouse antihuman p53 antibody (DO-7 [dilution 1:100], DAKO, Glostrup, Denmark), a monoclonal mouse antihuman RB antibody (PMG3-245 [dilution 1:1000], Pharmingen), and a monoclonal mouse antihuman VEGF antibody ([dilution 1:50], IBL Inc., Gunma, Japan). The sections were reacted with PMG175-405 at room temperature for 1 hr for p16 staining, DO-7 at 4°C overnight for p53 staining, PMG3-245 at 4°C overnight for RB staining, and VEGF antibody at room temperature for 1 hr. Negative control slides were treated with nonspecific mouse IgG1 under equivalent conditions. The DAKO labeled streptavidin-biotin kit provided the secondary reagents. Slides were developed with diaminobenzaminidine and counterstained with Mayer's hematoxylin.

We utilized the criteria described previously to assess the immunohistochemical results.[27,28,30,37–39] Staining was evaluated independently by 2 observers without knowledge of the clinicopathological factors. Five fields within the most invasive area of the tumor were selected at random and the stained cells were counted. p16 staining was considered positive if >80% of the tumor cells exhibited nuclear staining.[27] If <80% of the tumor cells exhibited nuclear staining, the specimen was considered to be negative. We previously defined 80% as the cut-off value for p16-positive staining, because about 80% of cells in normal epithelium show nuclear staining, and highly keratinized cells, such as cancer pearls in ESCC, do not exhibit nuclear staining even in p16-positive ESCC.[27] A similar cut-off value for p16 was determined independently by another group.[30,40] p53 and RB staining was evaluated as positive if >10% of the tumor cells exhibited nuclear staining and negative if <10% of the cells exhibited nuclear staining.[21] VEGF staining was considered positive if >80% of cancer cells exhibited

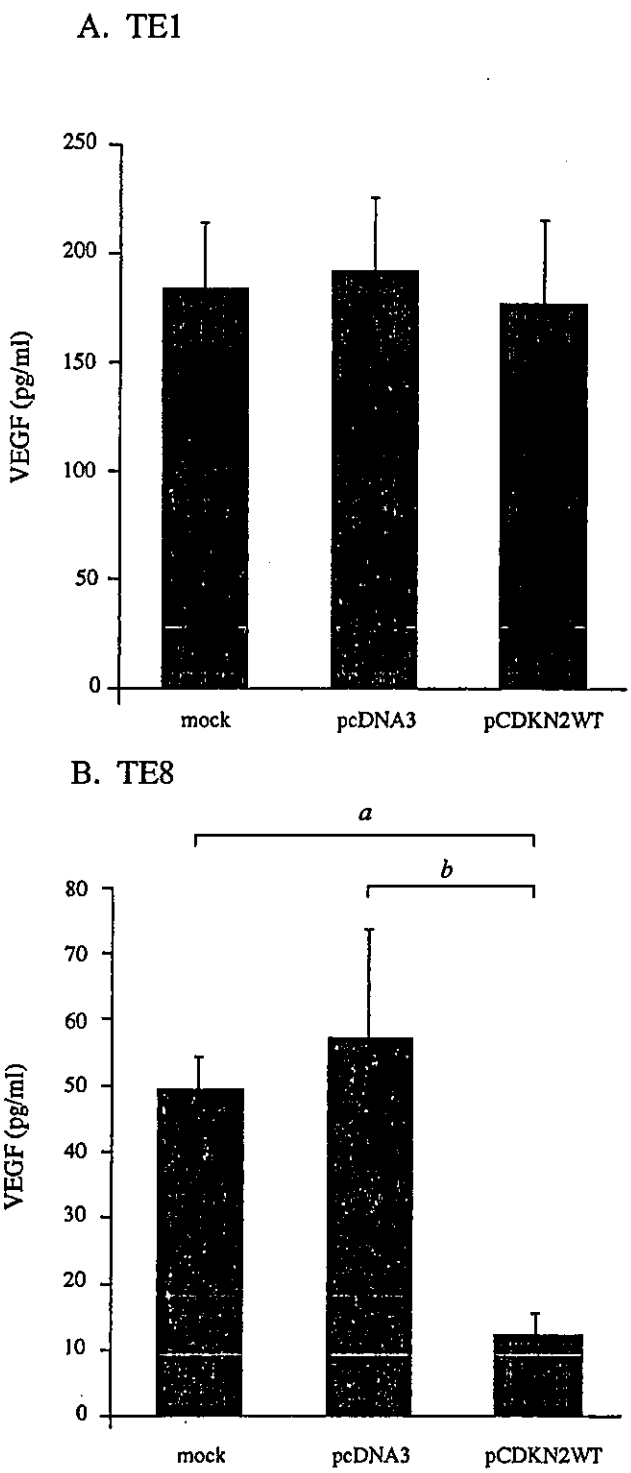

FIGURE 3 – Effect of p16 expression on supernatant VEGF protein levels. The supernatant VEGF protein was analyzed using ELISA assay. Values are the means ± SD of 4 independent experiments. (*a*) TE1, $p < 0.0001$; (*b*) TE8; $p = 0.0015$.

cytoplasmic positivity.[28] If cytoplasmic staining was manifested in <80% of the tumor cells, the specimen was judged to be negative. The reliability of the anti-p16 antibody PMG175-405 and the anti-RB antibody PMG3-245 was previously confirmed by Western blot analysis in studies of ESCC cell lines (data not shown).[27]

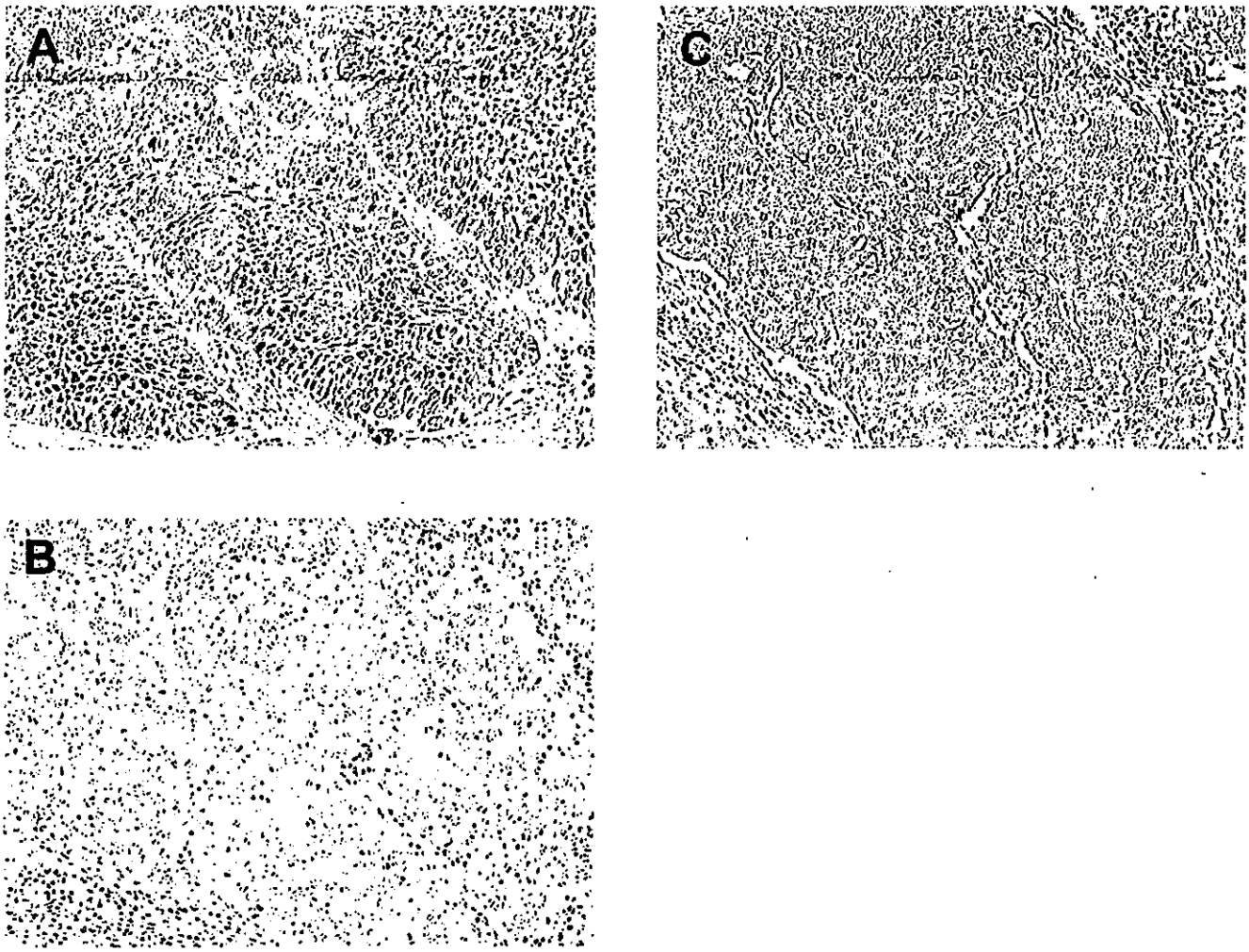

FIGURE 4 – Immunohistochemical staining for p16 and VEGF in paraffin-embedded specimens of primary ESCC. (*a*) Positive staining for p16; staining was detected in the nucleus. (*b*) Negative staining for p16. (*c*) Same specimen as (*b*), a serially cut section. Positive staining for VEGF in the cytoplasm of tumor cells. Magnification = ×200.

Parabasal cells of normal esophageal epithelium and inflammatory cells were used as a positive control for p16 staining.[27] Normal epithelium was used as a positive control for RB staining.[21,39]

Statistical analysis

Patients groups were compared using the χ^2 test and Mann-Whitney *U*-test. The cumulative survival rates for the patients groups were calculated by the Kaplan-Meier method and compared by using the Mantel-Cox log-rank test. A multiple regression model was used for multivariate analysis of variables associated with VEGF expression. All statistical analyses were carried out using the StatView-J 4.02 (Abacus Concepts Inc., Berkeley, CA), and all *P*-values that were two-sided at a value of <0.05 were considered to be statistically significant.

RESULTS

p16 gene transfer and exogenous p16 expression in ESCC cell lines

The p16 protein was expressed only in TE1 cells before transfection, and it was not detected in TE8 cells by Western blot analysis. The 2 ESCC cell lines (TE1 and TE8) were transfected with plasmid pCDKN2WT or pcDNA3 (p16-null vector). After the transfection with pCDKN2WT, p16 expression was detected in both cell lines by Western blot analysis (Fig. 1). The introduction of pCDKN2WT significantly reduced the cell count by 29% of pcDNA3 transfection after G418 selection in the TE8 cells ($p < 0.0001$; Fig. 2). There was, however, no significant difference in cell count between TE1 cells transfected with pCDKN2WT and pcDNA3 (Fig. 2).

Assessment of VEGF secretion by ELISA showed no significant changes after transfection with pcDNA3, compared to the non-transfected cells in the TE8 cells (Fig. 3). VEGF secretion was suppressed significantly in the TE8 cells transfected with pCDKN2WT, however, compared to non-transfected cells ($p < 0.0001$) and pcDNA3-transfected cells ($p = 0.0015$). No significant difference in VEGF secretion was found between the pCDKN2WT-transfected TE1 cells and pcDNA3-transfected TE1 cells (Fig. 3).

p16, p53, RB and VEGF expression in primary human esophageal carcinomas

Associations between clinicopathological background factors and immunohistochemical results are summarized in Table I. Of the 90 thoracic ESCC specimens, 41 (46%) were positive for p16 and 49 (54%) were negative (Fig. 4*a*,*b*). The p16-negative (loss of p16 expression) cases seemed to be associated with pN factor, but the association did not reach statistical significance ($p = 0.052$). Forty-seven (52%) of the 90 cases were evaluated as p53-positive, and 43 cases (48%) as p53-negative. Sixteen cases (18%) were

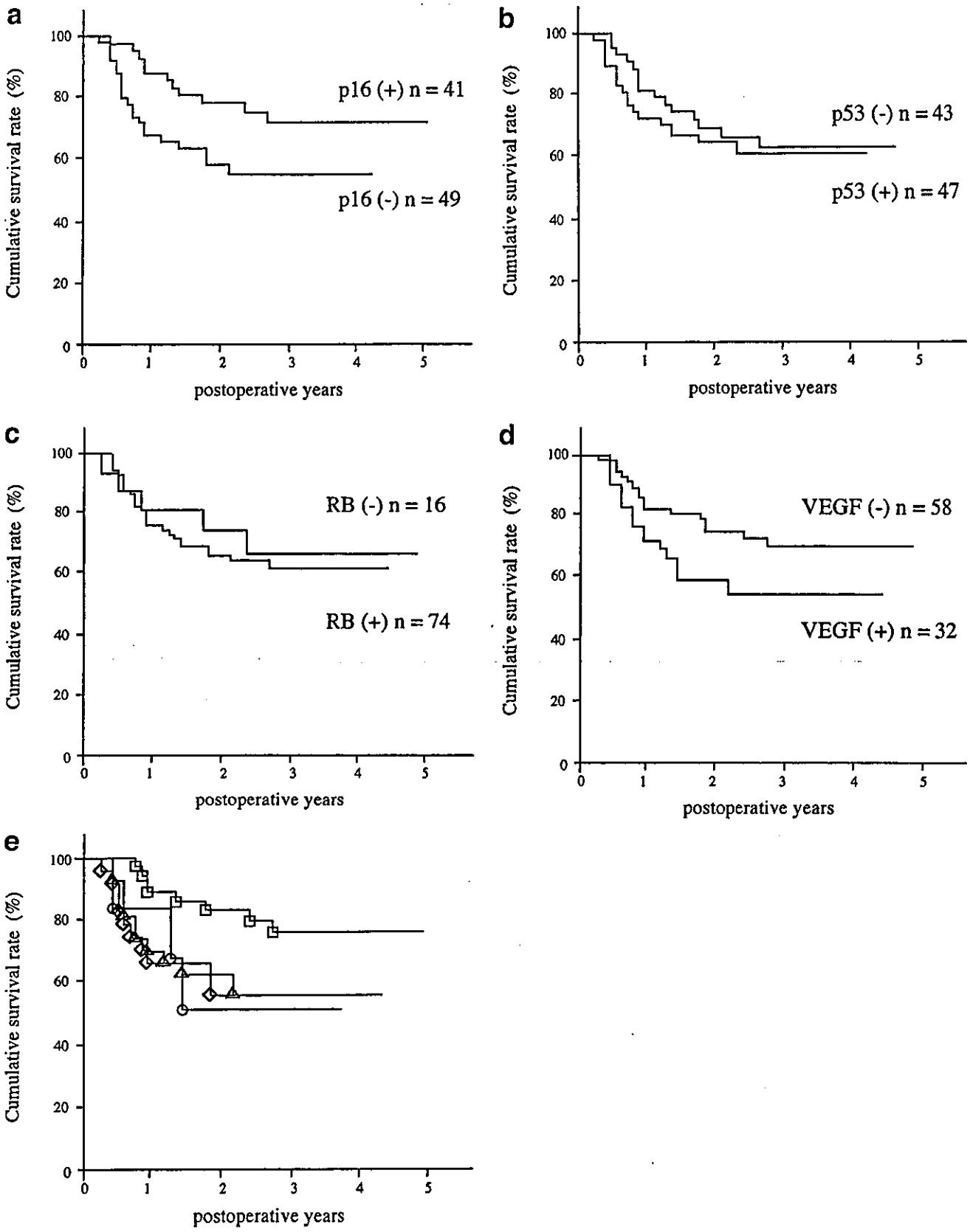

FIGURE 5 – Cumulative survival curves after curative surgery. (*a*) Patients with and without p16 expression, $p = 0.02$. (*b*) Patients with and without p53 expression, $p = 0.51$. (*c*) Patients with and without RB expression, $p = 0.57$. (*d*) Patients with and without VEGF expression, $p = 0.04$. (*e*) Patients with p16 or VEGF expression after curative surgery. ○, p16+/VEGF+ ($n = 6$); □, p16+/VEGF− ($n = 35$); △, p16−/VEGF+ ($n = 26$); ◇, p16−/VEGF− ($n = 23$).

TABLE II – RELATIONSHIP BETWEEN p16 AND VEGF EXPRESSION

	VEGF (%)		Total[1]
	Positive	Negative	
p16-positive	6 (19)	35 (60)	41
p16-negative	26 (81)	23 (40)	49
Total	32 (100)	58 (100)	90

[1] $p = 0.0004$.

TABLE III – RELATIONSHIP BETWEEN p53 AND VEGF EXPRESSION

	VEGF (%)		Total[1]
	Positive	Negative	
p53-positive	20 (63)	27 (47)	47
p53-negative	12 (38)	31 (53)	43
Total	32 (100)	58 (100)	90

[1] $p = 0.147$.

TABLE IV – RELATIONSHIP BETWEEN p16/p53 AND VEGF EXPRESSION[1]

	VEGF (%)		Total
	Positive	Negative	
p16(+)/p53(−)	3 (11)	23 (88)	26 (100)
p16(−)/p53(−)	9 (53)	8 (47)	17 (100)[2]
p16(+)/p53(+)	3 (20)	12 (80)	15 (100)
p16(−)/p53(+)	17 (53)	15 (47)	32 (100)[3]
Total	32 (36)	58 (64)	90 (100)

[1](+), positive; (−), negative.–[2]$p = 0.005$.–[3]$p = 0.056$.

RB-negative, and 74 cases (82%) were RB-positive. Neither p53 nor RB expressions was significantly correlated with any of the clinicopathological background factors. VEGF-positive staining was found in 32 (36%) of the 90 cases, and 58 cases (64%) were VEGF-negative (Fig. 4*c*). No clinicopathological background factors were associated with VEGF expression.

The cumulative postoperative survival rate for patients without p16 expression was significantly lower than that for patients with p16 expression (Mantel-Cox log-rank test, $p = 0.02$; Fig. 5*a*), whereas there was no prognostic impact of p53 ($p = 0.51$; Fig. 5*b*) or RB ($p = 0.57$; Fig. 5*c*) expression. The postoperative survival rate of the patients with VEGF expression was also significantly lower than that of the patients without VEGF expression ($p = 0.04$; Fig. 5*d*).

Only 6 cases (19%) of the 32 VEGF-positive cases were positive for p16 and 26 (81%) were negative. By contrast, 35 (60%) of the 58 VEGF-negative cases were positive for p16 and 23 (40%) were negative (Table II). Loss of p16 expression was significantly correlated with VEGF expression ($p = 0.0004$). The cumulative postoperative survival rate in the group with p16-positive and VEGF-negative was significantly higher than in the other groups ($p < 0.05$; Fig. 5*e*), except for the p16-positive and VEGF-positive group ($p = 0.12$). The p53-positive cases tended to be associated with VEGF-positive expression, but the trend was not statistically significant ($p = 0.147$; Table III).

We also investigated whether p53 status affected the association between p16 and VEGF expression. Among the p53-negative patients ($n = 43$), loss of p16 expression was significantly associated with VEGF-positive expression ($p = 0.005$; Table IV). The association was weakened in the p53-positive cases ($p = 0.056$; Table IV). Multivariate regression analysis showed loss of p16 expression to be an independent variable linked to VEGF expression ($p = 0.003$; Table V).

DISCUSSION

In our current study VEGF expression in primary human esophageal carcinomas was correlated with p16 expression rather than with p53 expression. VEGF secretion by $p16^{INK4a}$-deleted ESCC cells was significantly suppressed by the introduction of $p16^{INK4a}$ cDNA *in vitro*, whereas introduction of $p16^{INK4a}$ cDNA produced no significant change in the wild-type $p16^{INK4a}$ cell line.

TABLE V – REGRESSION COEFFICIENTS OF VARIABLES ASSOCIATED WITH VEGF EXPRESSION UPON MULTIVARIATE REGRESSION ANALYSIS

Variable	Coefficient	SE	*p*
Age	0.005	0.005	0.36
Gender	0.046	0.139	0.74
pT	0.034	0.054	0.53
pN	−0.047	0.114	0.68
Metastatic lymph nodes (*n*)	0.015	0.009	0.10
p16	−0.312	0.103	0.003
p53	−0.031	0.101	0.76
RB	0.218	0.134	0.11

Previous studies have reported that VEGF expression in ESCC is correlated not only with high intratumoral microvessel density, but with lymph node metastasis and poor postoperative survival.[20,28] These findings suggest that VEGF expression allows aggressive tumor proliferation and development of metastasis as a result of angiogenesis and increased vascular permeability. In our study, no correlations were found between VEGF expression and clinicopathological factors and the patients with VEGF expression had significantly worse postoperative survival than the patients without VEGF expression. These results were consistent with reports by other investigators.[41]

Some studies have demonstrated a correlation between aberrant p16 expression and poor prognosis in ESCC.[27,30,40] It remains unclear, however, why aberrant p16 expression in ESCC is associated with a poor prognosis. The association may be due to more invasive and progressive proliferation by cancer cells with $p16^{INK4a}$ alterations as a result of the disruption of cell cycle regulation than by cancer cells with wild-type $p16^{INK4a}$.[42] Moreover, the immunohistochemistry findings demonstrated that an incidence of VEGF expression without p16 expression was significantly higher than with p16 expression. No other investigations have reported the relation between p16 status and VEGF expression, however, our results suggest that $p16^{INK4a}$ may regulate VEGF expression, which allows tumor neovascularization and the development of metastasis, in ESCC.

p53 status was not correlated with any clinicopathological factors or postoperative survival in this study. Other investigations have reported that an incidence of aberrant p53 expression detected by immunohistochemistry is approximately 50% in ESCC and there was no relationship between p53 status and postoperative survival.[29,30] Our findings are consistent with the results of these studies. p53 has been identified as an important factor in VEGF expression.[43] VEGF expression was suppressed by the introduction of *p53* cDNA into *p53*-mutated cells,[44,45] and a correlation between p53 status and VEGF expression has been reported in some solid carcinomas.[46–48] Uchida *et al.*[20] have reported that VEGF expression was correlated significantly with p53 mutation in 36 ESCC tumors, whereas our results did not show a statistically significant association between VEGF expression and p53 expression. Another immunohistochemical study also found no correlation between VEGF and p53 expression in ESCC.[49] The discrepancy may be attributable to the difference between the polymerase chain reaction single-strand conformation polymorphism used to detect *p53* mutations and the immunohistochemistry used to detect aberrant p53 expression. We also need to keep in mind the presence of other factors (*e.g.*, hypoxia) that can induce VEGF expression.

In our present study, the introduction of $p16^{INK4a}$ cDNA *in vitro* suppressed not only cell proliferation, but the VEGF secretion in the $p16^{INK4a}$-deleted cell line whereas there was no significant suppression in the wild $p16^{INK4a}$ cell line. These results are consistent with those of other studies[31] and may support the clinical significance of $p16^{INK4a}$ for VEGF expression in the current im-

munohistochemical studies *in vivo*. Our immunohistochemical findings showed that VEGF expression may also be regulated by p16 via a p53-independent pathway. The molecular mechanism by which exogenous p16 expression causes downregulation of VEGF secretion remains unclear. G1 arrest due to exogenous p16 expression may suppress the transcription of various factors related to VEGF synthesis. D'Amico *et al.*[50] reported recently that $p16^{INK4a}$ has a function that inhibits cyclin D1 promoter activity in mammary gland tumorigenesis. Moreover cyclin D1 protein levels were increased in $INK4a/Arf^{+/-}$ mice, which showed reduced levels of p16 protein.[50] In our previous study, p16 expression in ESCC tumors was inversely correlated with cyclin D1 overexpression.[27] Shintani *et al.*[51] reported that cyclin D1 overexpression induced VEGF expression in ESCC cells. Taken together, these findings suggest that p16 may have a regulatory role in VEGF expression in ESCC via the p16-cyclin D1 pathway. Harada *et al.*,[31] however, have speculated that p16 may not only have a cell cycle regulating function, but another unknown function associated with VEGF synthesis and secretion. Another study has indicated that demethylation of the $p16^{INK4a}$ gene results in downregulation of VEGF expression in lung cancer cell lines[52] and a more recent study suggested that endogenous p16 inhibits colon tumor progression and tumor angiogenesis related to VEGF level.[53] These evidences seem to corroborate our own findings. Our results make it possible to explain the correlation of aberrant p16 expression with malignant potential of ESCC. Although further studies will be required to explore the mechanism between $p16^{INK4a}$ function and VEGF expression, restoration therapy of wild-type $p16^{INK4a}$ gene may be useful in addition to the selective modalities for inhibition of angiogenesis via VEGF suppression in patients with p16-negative ESCC.

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COMPARISON OF K-RAS POINT MUTATION DISTRIBUTIONS IN INTRADUCTAL PAPILLARY-MUCINOUS TUMORS AND DUCTAL ADENOCARCINOMA OF THE PANCREAS

Minoru KITAGO[1], Masakazu UEDA[1],* Koichi AIURA[1], Keiichi SUZUKI[1], Sojun HOSHIMOTO[1], Shin TAKAHASHI[1], Makio MUKAI[2] and Masaki KITAJIMA[1]

[1]*Department of Surgery, School of Medicine, Keio University, Tokyo, Japan*
[2]*Department of Diagnostic Pathology, School of Medicine, Keio University, Tokyo, Japan*

Intraductal papillary-mucinous tumors (IPMT) consist of cells with varying histologic degrees of severity and exhibit multiple tumor loci; however, whether or not these lesions exhibit the same genetic changes has not been clarified. To investigate this point, we analyzed K-ras mutations in multiple IPMT lesions from each patient enrolled in our study and compared our findings to those for patients with ductal adenocarcinoma of the pancreas (DC). Twenty IPMT specimens and 7 DC specimens were resected, microdissected and analyzed for the presence of K-ras mutations. The mutated genes were then sequenced using a genetic analyzer. K-ras mutations were observed in 80% of IPMT and 100% of DC patients. More than 2 types of K-ras mutation were observed in the main tumors of 43.8% of IPMT and 0% of DC patients. K-ras mutations in peritumoral and separated lesions were observed in 66.7% and 62.5% of IPMT patients, respectively. At least one identical mutation between the main tumor and the peritumoral or separated lesions was recognized in all of the IPMT patients with those lesions. Different mutations from those in the main tumor were observed in 40% of IPMT patients with separated lesions. The survival curve of IPMT-carcinoma patients with more than 2 types of K-ras mutation in the main tumor was better than that with one type of K-ras mutation. IPMT patients exhibit a remarkably genetic heterogeneity in main tumor and have good prognosis.

Key words: *intraductal papillary-mucinous tumors; postoperative survival rate; microdissection; non-RI SSCP; heterogeneity*

Intraductal papillary-mucinous tumor (IPMT) of the pancreas is a relatively new tumor classification that has been the topic of an increasing number of reports. IPMT is associated with the massive dilatation of the pancreatic duct and its branches by the copious production of mucin. IPMT was first described by Ohhashi *et al.*[1] in 1982 as a "mucous-secreting pancreatic cancer." This entity has since been reported not only in Japan,[2–4] but also in Europe and the United States[5–8] under a variety of names. The term IPMT has been adopted by the World Health Organization's International Histological Classification of Tumors.[9] IPMT can be further classified as a benign adenoma, a moderate dysplasia (borderline) or a carcinoma (noninvasive or invasive), but reliably distinguishing one type from another is difficult to perform preoperatively, and all IPMTs are suspected of having malignant potential.[6–8] Compared to ductal adenocarcinoma (DC) of the pancreas, IPMT clearly has a more favorable natural history characterized by a much longer survival period and a much higher cure rate.[5–8]

Inasmuch as various oncogenes are now known to be involved in the pathogenesis of cancer and of pancreatic carcinomas in particular, the lower incidence of malignancy and the reduced aggressiveness of IPMT compared to DC may be the result of a different spectrum of genetic changes. K-ras mutation, a type of mutation that is frequently detected in pancreatic carcinomas,[10–16] may be an important event in the neoplastic process of IPMT.[12,17,18] K-ras mutations have been recently shown to occur at a relatively early stage of multistep carcinogenesis in pancreas lesions. These mutations have also been used as a clonal marker in myelodysplastic syndromes.[19]

IPMT, like DC, arises from the epithelial lining of pancreatic ducts. Histologically, however, IPMT lesions differ from DC lesions by exhibiting varying degrees of hyperplasia and neoplasia at different tumor loci.[5] Whether the histologic variation observed in IPMT lesions corresponds with a variation in genotype has not been clarified. To investigate this point, DNA must be selectively extracted from the lesions in question and analyzed. Using microdissection, DNA samples are not contaminated with DNA from normal tissue or other histologically different tissues. Few studies have analyzed K-ras mutations in several different IPMT lesions (IPMT has a large number of foci than DC) from the same individual. We obtained specimens using a microdissection method and analyzed the distribution of K-ras mutations in a large number of different IPMT lesions. We then compared our results to those obtained for DC lesions. In addition, we also compared the clinical features of IPMT and DC patients. Our observations provide useful information on the role of K-ras mutations in tumorigenesis.

MATERIAL AND METHODS

Patients

Twenty surgically resected IPMT specimens and 7 DC specimens were obtained from 18 men and 9 women, ranging in age from 52–80 years (mean, 65 years). All patients were seen at Keio University Hospital (Tokyo, Japan) between 1988 and 1999. All IPMT lesions were classified according to the World Health Organization's International Histological Classification of Tumors.[9] Using the criteria outlined by this classification system, the IPMT lesions were divided into IPMT-adenomas (benign adenoma and borderline moderate dysplasia) and IPMT-carcinomas (malignant). The IPMT-carcinoma group included both *in situ* and infiltrating carcinomas. Follow-up examinations were scheduled for all patients: 3 clinical stagings were scheduled during the first 2 years after diagnosis, 2 stagings were scheduled between the third and fifth years, and one staging was scheduled at the beginning of the sixth year. Paraclinical investigations consisted of computed tomography and ultrasonography examination and a serologic test for tumor markers (CEA, CA19-9). The mean observation period

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*Correspondence to: Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81-3-3355-4707. E-mail: tiger@i-berry.ne.jp

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was 45 months (range, 2–118 months). All patients were followed to death and living status.

Materials

The resected specimens were immediately fixed in 10% buffered formalin. After 1 or 2 weeks, the fixed specimens were serially sectioned (5 mm) and embedded in paraffin using routine methods. The paraffin sections were then stained with hematoxylin and eosin. After the slides from each individual patient were reviewed, the main tumor, peritumoral lesions and separated lesions were designated for microdissection. Pathologically distinct lesions located at least 1 cm away from the main tumor were defined as separated lesions; lesions within the vicinity of the main tumor and that did not satisfy the requirements of separated lesions were defined as peritumoral lesions.

DNA extraction

DNA was extracted from the paraffin sections according to a previously described procedure with minor modifications.[20–22] Serial sections (4- or 10-μm thick) were made from the paraffin-embedded tissue blocks and placed on glass slides. The 4-μm sections were stained with hematoxylin and eosin, but the 10-μm sections were stained with hematoxylin and eosin after microdissection. Using comparative microscopic observations of the hematoxylin and eosin-stained sections for orientation, tiny fractions of the epithelial lesions (10 to 1,000 cells) were dissected from the 10-μm section using a micromanipulator (Zeiss, Oberkochen, Germany). These microdissection samples were transferred to 5 μl of proteinase K digestion buffer (20 μg of proteinase K/mL in 10 mM Tris/HCl and 1 mM EDTA [pH 8]) in PCR vials followed by inactivation of the proteinase K. Digestion was performed for 10 min at 55°C to demask the DNA followed by the inactivation of proteinase K (15 min, 96°C).

Enriched PCR by Bst-N1 digestion

The DNA was amplified by PCR according to a previously described method with minor modifications.[21,23] Amplifications were performed using a thermal cycler (Perkin-Elmer Corp., Branchburg, NJ) and a kit (Takara Corp, Tokyo, Japan) according to the following protocol: 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 30 sec followed by an additional 7 min at 72°C. The following synthetic oligonucleotides were used as primers: primer A, 5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′; primer B, 5′-TCAAAGAA TGGTCCTGGACC-3′; primer C, 5′-TAATATGTCGACTAAAACAAGATTTACCTC-3′. The underlined bases represent mismatches from the normal K-ras DNA sequence. The first PCR was performed using primers A and B, generating a 157-bp fragment containing 2 Bst-N1 restriction sites in the wild-type K-ras allele; if a mutation was present at the first or 2nd position in codon 12, the fragment only contained one Bst-N1 site. Next, 10 μl of the PCR product was digested with 20 units of Bst-N1 (New England Biolabs, Beverly, MA) in a 25-μl mixture containing 10 mM of DTT and 5 μg of 0.1% BSA. The reaction was continued for 16 hr at 60°C. After enzyme inactivation at 96°C, 5 μl of the product was reamplified using primers A and C for 40 cycles of the protocol used for the first PCR, generating a 135-bp fragment. For each amplification, DNA from Panc1 diluted with normal DNA at a ratio of 1:250 and DNA from MKN1 carrying the wild type K-ras sequence were amplified as positive and negative controls.

Non-RI SSCP analysis of K-ras gene mutations

After confirming the reliability of the 2-step PCR by electrophoresis through an 8.0% acrylamide gel, producing a 135-bp fragment, the K-ras gene mutations were detected using a non-RI single-strand conformation polymorphism (SSCP) analysis, as described previously with minor modifications.[21,24] After denaturation at 85°C for 10 min, 2 μl of the PCR product was mixed with 10 μl of a loading solution containing 90% deionized formamide, 20 mM EDTA and 0.05% bromphenol blue and xylene cyanol. The loading solution (10 μl) was then applied to a 15% polyacrylamide gel (Atto Corp., Tokyo, Japan). Electrophoresis was performed at a constant voltage of 200 V produced using an ECPS 3000/150 power supply (Pharmacia LKB Biotechnology, Tokyo, Japan) in a continuous buffer system consisting of 25 mM Tris and 192 mM glycine. During the electrophoresis, the buffer temperature was adjusted to 18°C using a cooling pipe with continuously circulating, cool (14–16°C) water. The running time was 4 hr. The gel was then stained with silver using a kit purchased from Daiichi Pure Chemical Company, Ltd. (Tokyo, Japan) according to the manufacturer's instructions.

DNA sequencing

DNA sequencing was performed in the samples that did not exhibit the normal pattern of electrophoretic mobility (n = 154). Sequencing reactions were performed using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and the products were analyzed using an ABI Prism 310 Genetic Analyzer.

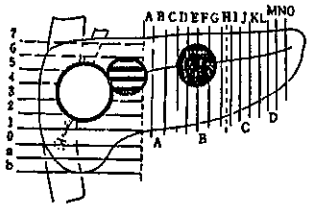
Statistical analysis

The mean values were compared using a Student's t-test. Statistical comparisons between groups were made using a chi-square test or a Fisher exact test. Postoperative survival rates were calculated using the Kaplan-Meier method, and the differences between curves were measured using the log-rank test. Statistical significance was defined as $p < 0.05$.

RESULTS

A total of 266 duct lesions from 20 patients with IPMT and 88 duct lesions from 7 patients with DC were identified. Ductal lesions were classified by a pathologist according to the criteria of the World Health Organization's International Histological Classification of Tumours.[9] There were 168 lesions (mean 8.4 lesions/case) from IPMT patients (n = 20 cases) and 55 lesions (mean 7.8 lesions/case) from DC patients (n = 7 cases) found in the main tumor. There were 26 lesions from IPMT patients (n = 7 cases) and 16 lesions from DC patients (n = 5 cases) designated as peritumoral lesions. There were 43 lesions from IPMT patients (n = 10 cases) and 6 lesions from DC patients (n = 2 cases) designated as separated lesions (Fig. 1, Table I).

At least one K-ras mutation was detected in the main tumors of 80.0% (16/20) of IPMT and 100% of DC patients, as shown in Table II (not a significant difference). At least one K-ras mutation was detected in 83.3% (5/6) of the IPMT-carcinoma and in 78.6% (11/14) of the IPMT-adenoma patients.



	Main tumor ○	Peritumoral lesion ⊖	Separated lesion ●
IPMT	168 (n=20)	26 (n=7)	43 (n=10)
DC	55 (n=7)	16 (n=5)	6 (n=2)
	223 (n=27)	42 (n=12)	49 (n=12)

FIGURE 1 – Designation of main tumor ○, peritumoral lesions (=) and separated lesions ● for microdissection. Pathologically distinct lesions located at least 1 cm away from main tumor were defined as separated lesions; lesions within the vicinity of main tumor and that did not satisfy the requirements of separated lesions were defined as peritumoral lesions.

TABLE I – GENOTYPES OF MICRO-DISSECTED LESIONS ACCORDING TO TUMOR SITE

Patient number	Main tumor PH, BeAd, BoAd, Ca	Peritumoral lesion FH, PH	Separated lesion FH, PH	n	Total	Sequences
IPMT-carcinoma						
Case 1	6/10 (GAT, codon 13)		0/5[1]		6/15	GAT/codon 13
Case 2	0/8[1]				0/8[1]	wild
Case 3	5/33 (AGT3, GAT2)	0/4[1]		0/1[1]	5/38	AGT/GAT
Case 4	4/10 (GAT)		3/8 (GTT2, GAT1)	1/6 (GTT)	8/24	GAT/GTT
Case 5	6/8 (GTT)			0/1[1]	6/9	GTT
Case 6	2/6 (GAT1, GTT1)				2/6	GAT/GTT
	23/75	0/4	3/13	1/8	27/100	5/6 cases (83.3%)
IPMT-adenoma						
Case 7	0/2*				0/2[1]	Wild
Case 8	1/10 (GTT)		1/3 (GTT)		2/13	GTT
Case 9	8/16 (GAT7, CGT1)		0/6[1]	0/9[1]	8/31	GAT/CGT
Case 10	1/9 (GCT)			0/2[1]	1/11	GCT
Case 11	0/2*	0/4[1]	0/2[1]		0/8[1]	Wild
Case 12	3/3 (GTT2, AGT1)	2/4 (AGT)	3/10 (AGT3)	0/1[1]	8/18	AGT/GTT
Case 13	2/3 (GAT)	1/1 (GAT)	2/4 (GAT)	1/1 (GAT)	6/9	GAT
Case 14	0/3*		0/1[1]		0/4[1]	Wild
Case 15	1/10 (TGT)	0/1[1]			1/11	TGT
Case 16	3/7 (codon 13 2,GTT2)	1/6 (codon 13)	0/1[1]	0/5[1]	4/19	GTT/codon 13
Case 17	6/8 (GTT3, GAT3)		3/3 (GAT2, CGT1)	0/1[1]	9/12	GTT/GAT/CGT
Case 18	4/8 (GAT)	1/6 (GAT)		0/1[1]	5/15	GAT
Case 19	7/7 (GTT)			0/1[1]	7/8	GTT
Case 20	1/5 (GAT)				1/5	GAT
	37/93	5/22	9/30	1/21	52/166	11/14 cases (78.6%)
	60/168	5/26	12/43	2/29	79/266	16/20 cases (80%)
	Ca	FH, PH	FH, PH	n	Total	Sequences
DC						
Case 21	2/7 (GTT)	0/2[1]			2/9	GTT
Case 22	5/8 (GTT)	2/2 (GTT)			7/10	GTT
Case 23	2/2 (GAT)	2/7 (AGT1, GTT1)		0/1[1]	4/10	AGT/GAT/GTT
Case 24	1/2 (GAT)				1/2	GAT
Case 25	6/6 (GTT)	2/2 (AGT)	1/2 (AGT)	2/5 (AGT)	11/15	GTT/AGT
Case 26	2/11 (GAT)				2/11	GAT
Case 27	8/19 (GAT)	1/3 (GAT)	0/4[1]	1/5 (GAT)	10/31	GAT
	26/55 (47.3%)	7/16	1/6	3/11	37/88 (42.0%)	7/7 cases (100%)

[1]Zero mutations.–N, normal epithelium; FH, flat hyperplasia; PH, papillary hyperplasia; BeAd, benign adenoma; BoAd, borderline adenoma; Ca, carcinoma; wild, GGT.

TABLE II – NUMBER AND VARIETY OF K-RAS MUTATIONS FOUND IN MAIN TUMORS OF IPMT AND DC PATIENTS

	≥1 K-ras (%)	≥2 K-ras (%)
IPMT	16/20 (80%)	7/16 (43.8%)[1]
IPMT-carcinoma	5/6 (83.3%)	3/5 (60%)[2]
IPMT-adenoma	11/14 (78.6%)	4/11 (36.4%)
DC	7/7 (100%)	0/7 (0%)[1,2]

[1]IPMT *vs.* DC $p < 0.05$.–[2]IPMT – carcinoma *vs.* DC $p < 0.05$.

More than 2 kinds of K-ras mutation were detected in the main tumors of 43.8% (7/16) of IPMT patients with multiple K-ras mutations in their main tumor and in 0% (0/7) of the DC patients (IPMT *vs.* DC, $p < 0.05$). More than 2 kinds of K-ras mutation were detected in the main tumors of 60% (3/5) of IPMT-carcinoma patients with multiple K-ras mutations in their main tumor and in 36.4% (4/11) of the IPMT-adenoma patients (IPMT-carcinoma *vs.* DC, $p < 0.05$).

The following transitions were observed in the main tumors of DC patients: GGT to GAT (4/7 cases, 57.1%) and GGT to GTT (3/7 cases, 42.9%). In the IPMT patients, the following single K-ras mutations were found: GGT to GAT (4/9 cases, 44.4%), GGT to GTT (3/9 cases, 33.3%), GGT to TGT (1/9 cases, 11.1%) and GGT to GCT (1/9 cases, 11.1%). In IPMT patients with multiple K-ras mutations, the following transitions were detected: GGT to GAT (5/7 cases, 71.4%), GGT to GTT (4/7 cases, 57.1%), GGT to AGT (2/7 cases, 28.6%) and GGT to CGT (2/7 cases, 28.6%).

K-ras mutations in peritumoral lesions were observed in 66.7% (4/6) of IPMT patients who had one or more K-ras mutations in their main tumor and in 80% (4/5) of DC patients with K-ras mutations in main tumor. Identical mutation patterns between main tumor and peritumoral lesions were observed in 100% (4/4) of IPMT patients with K-ras mutations in both main tumor and peritumoral lesions and in 50% (2/4) of DC patients.

K-ras mutations in separated lesions were observed in 62.5% (5/8) of IPMT patients who had one or more K-ras mutations in main tumor and in 50% (1/2) of DC patients with K-ras mutations in main tumor. Identical mutation patterns between main tumor and separated lesions in at least one lesion were observed in 100% (5/5) of IPMT patients with K-ras mutations in both main tumor and separated lesions and in 0% (0/1) of the DC patient. Different mutation patterns between main tumor and separated lesions were observed in 40% (2/5) of IPMT patients with K-ras mutations in both main tumor and separated lesions and in 100% (1/1) of the DC patient.

The number of lesions with a K-ras mutation in patients with different stages of neoplasia showed the following: normal epithelium 6.9% (2/29), hyperplasia (flat and papillary hyperplasia) 29.8% (28/94), adenoma (benign and borderline adenoma) 31.4% (37/118), IPMT-carcinoma 48% (12/25) and DC-carcinoma 47.3% (26/55). The number of lesions with a K-ras mutation increases as the histologic grade becomes more severe.

The clinicopathologic characteristics of the patients are shown in Table III. The average age of the 6 patients with IPMT-carcinoma was 72 years (range, 66–80 years), while that of the 14

TABLE III - CLINICOPATHOLOGICAL CHARACTERISTICS OF PATIENTS ACCORDING TO HISTOLOGICAL FINDINGS

Clinicopathological characteristics	IPMT		DC	
	IPMT-adenoma (n = 14)	IPMT-carcinoma (n = 6)	(n = 7)	
Age [year (range)]	63.6 ± 6.8 (52–73)[1]	71.8 ± 5.1 (66–80)[1]	63.2 ± 9.3 (52–78)[1]	IPMT-Ad *vs.* DC p = 0.9212 IPMT-Ad *vs.* IPMT-Ca p = 0.0173 IPMT-Ca *vs.* DC p = 0.0710
Sex (M:F)	12:2	3:3	3:4	
Size of main tumor (cm)	3.2 ± 1.7	4.3 ± 2.7	4.5 ± 1.9	n.s
Stage of main tumor				
I	[2]	3		
II	[2]	1		
III	[2]	2	3	
IVA	[2]		4	
IVB	[2]			

[1]Results are expressed as the mean ± SE.–[2]Not present.

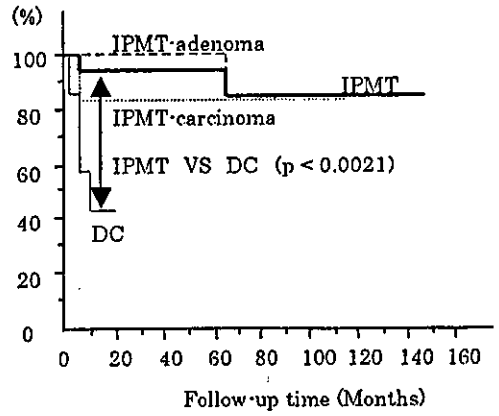


FIGURE 2 – Survival rates for IPMT (n = 20) (IPMT-carcinoma [n = 6], IPMT-adenoma [n = 14]) and DC (n = 7) patients (p = 0.0021).

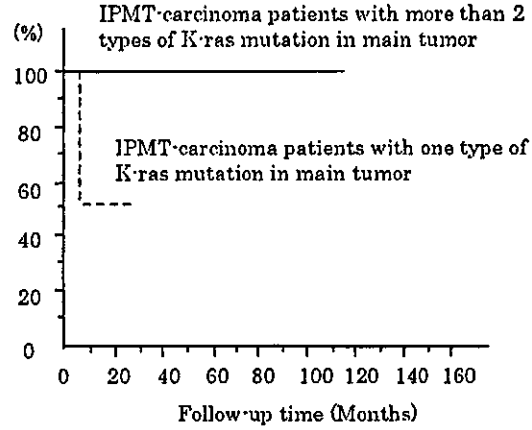


FIGURE 3 – Survival rates for IPMT-carcinoma with more than 2 types of K-ras mutation in primary tumor (n = 3) and one type of K-ras mutation in primary tumor (n = 2).

patients with IPMT-adenoma was 64 years (range, 52–73 years) and that of the 7 patients with DC was 63 years (range, 52–78 years). The IPMT-carcinoma group was older than the DC group and significantly older than the IPMT-adenoma group ($p < 0.05$).

The 5-year survival rate after resection for the IPMT group was 94.7% (IPMT-carcinoma group, 83.3%; IPMT-adenoma, 100%), which was significantly better than that of the DC group (p = 0.0021; Fig. 2). Survival rate of IPMT-carcinoma patients with one type K-ras mutation in primary tumor was better than that with more than 2 types K-ras mutation in primary tumor (Fig. 3).

DISCUSSION

The progression of human pancreatic carcinoma is strongly associated with the presence of K-ras mutations, with mutations found in 70–100% of lesions.[10–16] Thus, K-ras mutation may be an important event in the neoplastic process. Recently, K-ras mutations have been shown to occur at a relatively early stage of carcinogenesis in pancreas.[7,16,22] In our study, at least one K-ras mutation was found in 80% of IPMT patients and 100% of DC patients (no significant difference). The reported percentage of IPMT patients with a K-ras mutation ranges from 0–86%[5,17,18,25–28] but was 81 and 86% in the two most recent studies.[17,18] The most probable suggested reason for this large discrepancy is that the constituent cells of IPMTs are often heterogeneous; in the more recent studies, multiple specimens were examined in each subject.[17] Our results show that the frequency at which K-ras mutations were observed was not different between IPMT and DC patients.

In our present study, only one kind of mutation was observed in the main tumor of each DC subject, whereas more than 2 types of K-ras mutation were observed in 43.8% of IPMT patients ($p < 0.05$). In particular, more than 2 types of K-ras mutation were observed in 60% of IPMT-carcinoma patients; this incidence was higher than that of IPMT-adenoma patients, but the difference was not significant. These results support the heterogeneous nature of IPMT cells and suggest that the degree of heterogeneity may progress as the tumor advances. Lyons *et al.*[19] concluded that ras mutations in myelodysplastic syndromes were heterogeneous and suggested that they may occur at an early stage of carcinogenesis. Lyons *et al.* further suggested that ras mutations might be useful as a clonal marker for studying myeloid malignancies. Similarly, the presence of different K-ras mutations in the same pancreatic carcinoma increases the likelihood that the tumor arose from multiple foci.[15] Previous studies have reported multiple kinds of K-ras mutations in 3–13% of pancreatic carcinomas.[12,15,16,29] Motojima *et al.*[15] reported that the detection of different mutations in the same tumor suggests that pancreatic carcinomas may be multicentric. While Fujii *et al.*[30] reported that the allelic heterogeneity of IPMT might, in part, be due to the slow growth rate of these neoplasms, we believe that the increasing heterogeneity is intrinsic to the carcinogenesis of pancreatic cancer.

The types of K-ras mutations found in the main tumors of the IPMT patients in our study were not particularly different from those in the DC patients. Our analyses showed that all the mutations occurred at codon 12 with the exception of 2 cases where mutations occurred at codon 13 (GGC to GGG). GGT to GAT and GGT to GTT transitions at codon 12 were common in both IPMT and DC patients. These results are compatible with those of previous reports.[15,22]

To study the genetic and clonal associations between the main tumor and other pancreatic ductal lesions, we investigated the presence and type of K-ras mutations in main tumor, peritumoral lesions and separated lesions. K-ras mutations were frequently observed not only in main tumor, but also in the peritumoral and separated lesions of both IPMT and DC patients. Without K-ras mutation in main tumor of IPMT patients, the mutation was not observed in peritumoral or separated lesions. In each IPMT patient with one or more peritumoral lesions, at least one identical mutation was observed in both the main tumor and the peritumoral lesions. In DC patients with peritumoral lesions, the same mutation in both locations was observed only 50% of the time. With regard to separated lesions, at least one identical mutation was observed in both the main tumor and the separated lesion in all of the IPMT patients with one or more separated lesions. However, different mutations were also observed in the separated lesions of 40%(2/5) of the IPMT patients with separated lesions. Moskaluk *et al.*[31] reported that K-ras mutations often occur within pancreatic ducts and pancreatic ducts in the parenchyma surrounding K-ras positive tumors may be vulnerable to K-ras mutations. However, the number of lesions with a K-ras mutation increased as the degree of abnormality increased from normal epithelium to hyperplasia, to adenoma and to carcinoma in IPMT patients. These results support the hyperplasia–adenoma–carcinoma sequence in the progression of IPMT. Z'graggen *et al.*[18] reported the same results and concluded that K-ras mutation was an important event in carcinogenesis for most IPMT lesions. Our results also support this conclusion, even though K-ras mutations occur at a relatively early stage in the carcinogenesis of the pancreatic lesions and are liable to occur within the pancreatic ducts of the parenchyma surrounding a K-ras positive tumor.

Clinically, the average ages of the IPMT-adenoma, IPMT-carcinoma and DC patients were 64, 72 and 63 years, respectively. Patients with IPMT-carcinoma tended to be older than patients with DC and were significantly older than patients with IPMT-adenoma. IPMT has mainly been reported in elderly men between the ages of 60 and 70 years. This observation suggests that IPMT may slowly evolve from adenoma to carcinoma according to a multistage process. Patients with IPMT have been previously reported to have a good clinical prognosis after resection because of the slow progression and low malignancy rate associated with this tumor.[6–8] In our study, the 5-year survival rate for IPMT patients who underwent a resection was 94.7% (IPMT-carcinoma, 83.3%; IPMT-adenoma, 100%). This survival rate is significantly better than that for DC. Azar *et al.*[7] reported that the overall actuarial 3-year survival rate of IPMT patients was 79%. Thus, patients with IPMT have a favorable long-term survival rate after adequate resection, unlike patients with DC.

In follow-up examinations for patients who have undergone operations, it is useful to distinguish whether or not the tumor is slow growing. A genetic heterogeneous primary tumor, which has more than 2 types of K-ras mutation in main tumor, may indicate a slow-growing tumor. In fact, the survival rate of IPMT-carcinoma patients with a genetic heterogeneous primary tumor was better than that of IPMT-carcinoma patients with a genetic homogenous primary tumor, which has one type K-ras mutation in main tumor.

In conclusion, IPMTs not only exhibit a clinically characteristic slow growth rate, but their main tumor is remarkably genetic heterogeneity. IPMT is thought to occur according to a hyperplasia – adenoma – carcinoma sequence, because of at least one identical mutation between the main tumor and the peritumoral lesion and the stepwise increase of K-ras mutations from normal epithelium to hyperplasia, to adenoma and to carcinoma. K-ras mutation appears to be an important event in IPMT carcinogenesis and occurs at a relative early stage, as in DC carcinogenesis.

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Original Paper

Platelets Counts Closely Correlate with the Disease-Free Survival Interval of Pancreatic Cancer Patients

Keiichi Suzuki[1], Koichi Aiura[1], Minoru Kitagou[1], Sojun Hoshimoto[1], Shin Takahashi[2] Masakazu Ueda[1], Masaki Kitajima[1]

[1]Department of Surgery, Keio University School of Medicine, Tokyo, and [2]Nippon Kokan Hospital Kanagawa, Japan

Corresponding Author: Dr. Koichi Aiura, Department of Surgery, Keio University School of Medicine 35, Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan

Tel: +81 3 3353 1211, Fax: +81 3 3355 4707, E-mail: aiurako@sc.itc.keio.ac.jp

ABSTRACT

Background/Aims: We investigated the relationship between preoperative platelets count and the outcome of pancreatic cancer patients.

Methodology: We retrospectively reviewed the clinical records of 105 patients with invasive ductal carcinoma of the pancreas who underwent radical resection at our institution over a 20-year period. The patients were divided into two groups based on their preoperative platelets counts: a group with thrombocytosis (≥40x10⁴/mL) and a group with non-elevated platelets counts (<40x10⁴/mL). Histopathological findings, survival rates, recurrence patterns, and disease-free survival intervals were compared between the two groups.

Results: The outcome of the group with thrombocytosis was significantly poorer than that of the group with non-elevated platelets counts (p=0.043). The mean disease-free interval of patients with thrombocytosis was 4.9 months as opposed to 46.5 months for those with non-elevated platelets counts (p=0.006). Patients were classified into four groups according to changes in platelets counts after surgery. Consistently, the outcome of the group with both pre- and postoperative non-elevated platelets counts was the best compared to groups with post- and/or postoperative thrombocytosis.

Conclusions: Our clinical data suggest that high platelets counts in the preoperative and postoperative period may be associated with a poor outcome and shortening of postoperative disease-free survival interval.

KEY WORDS: Liver metastases; Prognostic factor; Thrombocytosis; Radical resection

ABBREVIATIONS: Pancreatoduodenectomy (PD); Pylorus-preserving Pancreatoduodenectomy (PpPD); Distal Pancreatectomy (DP); Splenectomy (SP); Total Pancreatectomy (TP); Intraoperative Radiotherapy (IOR); Interleukin-6 (IL-6); Macrophage Colony-Stimulating Factor (M-CSF); Vascular Endothelial Growth Factor (VEGF); Platelets-Derived Growth Factor (PDGF); basic Fibroblast Growth Factor (bFGF)

INTRODUCTION

Cancer of the pancreas is the fourth most common malignancy-induced cause of death in the United States (1). It is a discouraging disease with a poor prognosis, most likely reflecting the more invasive character of frequent metastases even in the early stage. Even after radical resection, the overall median survival time of patients with pancreatic adenocarcinoma is only 18 to 20 months; their overall 5-year survival rate is approximately 10% (2). Although the mechanisms of metastases have been studied by many investigators to improve the survival of pancreatic cancer patients, they involve many processes and factors, making it necessary to investigate both the characteristics of the tumor cells and the interactions between the tumor cells and host cells.

Promotion of metastases by platelets has been noted in cancer patients, and the distant metastases rate of tumor cells has been reported to be decreased in a laboratory animal model of thrombocytopenia (3). Moreover, the distant metastases rate was found to increase 10-150 times when platelets were activated by thrombin *in vivo* (4), and the metastases rate was decreased by both anti-platelets antibody (5) and prostacyclin (6), which inhibits platelets functions. It has recently been reported that the prognosis is poorer in lungs and uterine cervix cancer patients whose preoperative platelets count is increased (7-10). The above findings suggest that thrombocytosis may also affect the prognosis of patients with digestive organ cancers, including pancreatic cancer.

Pancreatic cancer has a high potential for hematogenous metastases, especially to the liver (11), and there have been several reports of a potential hazard for intraoperative hematogenous tumor cell dissemination (12-14). Recent studies have reported a risk of shedding cancer cells into a portal vein while manipulating pancreatic head lesions during pancreatoduodenectomy or distal pancreatectomy to treat pancreatic cancer (15), and platelets appear to facilitate the formation of liver metastases before and after surgery for pancreatic cancer. It is important to study the preoperative and postoperative relationships between platelets and tumor cells in light of the formation of metastases.

In this study, we investigated the relationship between both pre- and postoperative platelets counts and the disease-free survival of pancreatic cancer patients, we also attempted to elucidate the role of platelets in this disease.

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