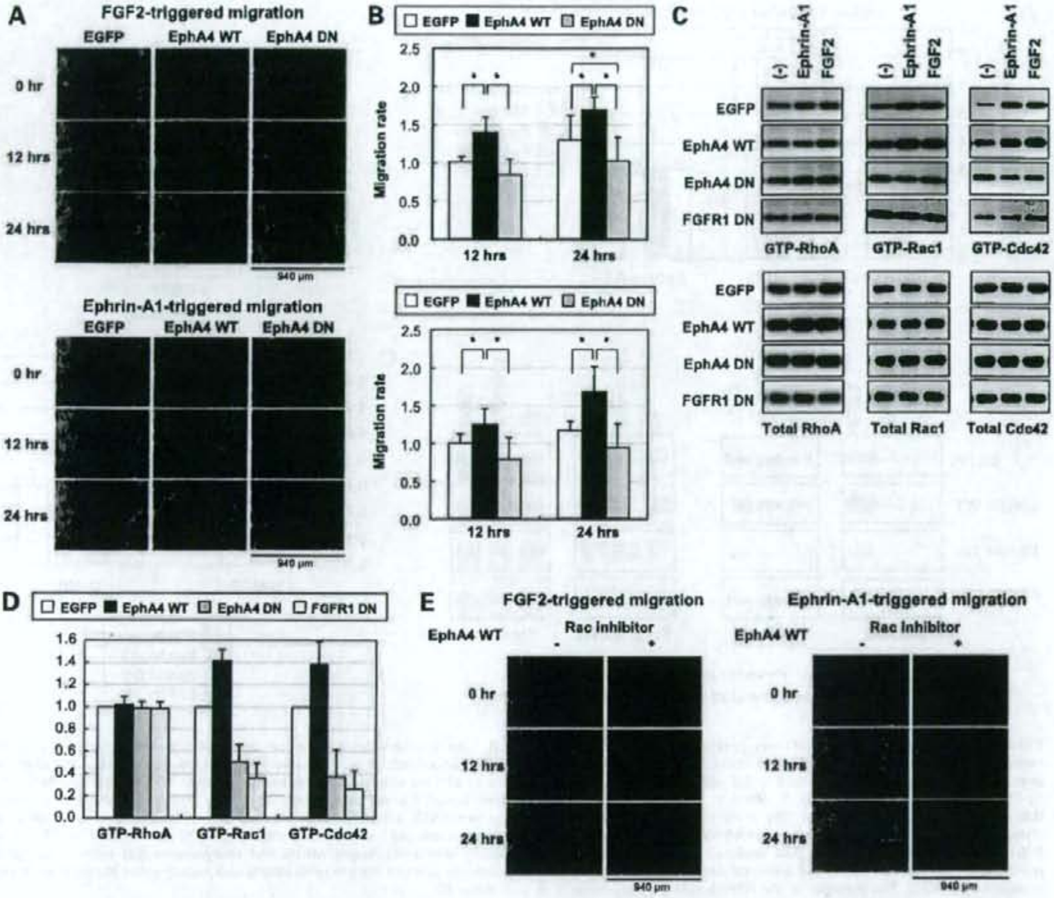

Figure 4. Effect of EphA4 WT on migration in the U251 cells. **A,** evaluation of cell migration by scratch wound assay. Serum-starved cells were cultured in the presence of 20 ng/mL FGF2 (*top*) or 0.5 µg/mL ephrin-A1 (*bottom*). The degree of migration was checked by monitoring EGFP. Pictures were shown at 12 and 24 h after ligand stimulation. **B,** extent of cell migration was quantified and shown graphically (*right*). The areas occupied by the cells were calculated with NIH image software. The ratio of the increased area after 12 and 24 h to that at 0 h was then calculated to quantitate the extent of migration. *Bars,* SD. *, $P < 0.05$ versus the EGFP cells. **C,** effects on activation of Rho GTPases. *Top,* active RhoA, Rac1, and Cdc42 of each cell stimulated by 20 ng/mL FGF2 for 15 min or 0.5 µg/mL ephrin-A1 for 30 min. The active Rho GTPases were pulled down as described in Materials and Methods and checked by immunoblotting using the indicated antibodies. *Bottom,* protein expression of three Rho GTPases using whole protein lysate. **D,** relative densitometric units of the GTP-RhoA, Rac1, and Cdc42 bands in each cell stimulated by FGF2. The densities of the EGFP bands are set arbitrarily at 1.0. *Bars,* SD. **E,** effect of Rac inhibitor on ligand-triggered migration in the transfected U251 cells of EphA4 WT. Cell migration was evaluated by scratch wound assay. Serum-starved cells were cultured in the presence of 20 ng/mL FGF2 (*left*) or 0.5 µg/mL ephrin-A1 (*right*) plus 50 µmol/L Rac inhibitor. The degree of migration was checked by monitoring EGFP. Pictures were shown at 0, 12, and 24 h after stimulation.

sought to examine how the activity of Rho GTPases would be affected by EphA4. Rho GTPase activities were evaluated by immunoblotting following a pull-down assay. FGF2 stimulation activated Rac1/Cdc42 as shown in Fig. 4B. EphA4 WT promoted activation of Rac1/Cdc42 stimulated by FGF2, whereas EphA4 DN, as well as FGFR1 DN, inhibited their activation, suggesting EphA4 promotes FGF2-mediated activation of Rac1/ Cdc42. Similarly, Rac1/ Cdc42 activities stimulated by ephrin-A1 were also promoted by EphA4 WT but were inhibited by EphA4 DN, suggesting EphA4 promotes ephrin-A1-mediated activation. Importantly, the activation was inhibited by FGFR1 DN. As shown in Fig. 3A, ephrin-A1 did not induce FRS2 phosphorylation; therefore, it was suggested that the effect of EphA4 WT on ephrin-A1-triggered activation of Rac1/Cdc42 was, at least in part, through FGFR-mediated signaling not involving FRS2. Consistent with these results, cotreatment of Rac inhibitor with FGF2 or ephrin-A1 drastically inhibited the wound closure in the scratch wound assay (Fig. 4E), showing that Rac inhibition blocked FGF2- or ephrin-A1-triggered glioma cell migration. On the other hand, neither ephrin-A1 nor FGF2 stimulation induced any RhoA activity in EGFP-expressing mock control cells and the activity was not modulated by EphA4 WT, EphA4 DN, or FGFR1 DN. These results suggest that EphA4 enhances FGF2-induced activation of Rac1/Cdc42 and promotes glioma cell migration.

EphA4-FGFR1 Heteroreceptor Complex Enhances FGF2-Triggered Phosphorylation of FGFR1

Based on the results that EphA4 promoted FGF2-mediated cell proliferation and migration as we have shown thus far, we hypothesized a functional relationship between EphA4 and FGFR1, which would affect FGFR1 activity. First, we checked their physiologic interaction by the ectopic expression of EphA4 WT and/or FGFR1 WT retrovirally using the U251 cells. FGFR1 was not detected to be coimmunoprecipitated with EphA4 in EGFP-expressing control cells (Fig. 5A). When stimulated by Ephrin-A1, coimmunoprecipitation of FGFR1 was detectable in EphA4 WT- or FGFR1-expressing U251 cells. This complex formation was most evident in EphA4- and FGFR1-coexpressing cells, meaning that activated EphA4 prefers to forming a receptor complex with FGFR1 in the U251 cells.

To clarify the functional effect of EphA4 on FGFR1 activation, we next examined the tyrosine phosphorylation level of FGFR1 in the U251 cells (Fig. 5B). Without FGF2 stimulation, neither EphA4 WT, EphA4 DN, nor FGFR1 DN affected the phosphorylation level of intrinsic FGFR1. FGFR1 was naturally phosphorylated by FGF2 stimulation in EGFP-expressing cells. This phosphorylation was further enhanced by EphA4 WT. Contrarily, EphA4 DN or FGFR1 DN inhibited FGFR1 phosphorylation by FGF2, revealing that EphA4 strengthened FGF2-mediated phosphorylation of FGFR1. Under this experimental condition, EphA4 was weakly phosphorylated by exogenous addition of FGF2 (Supplementary Fig. S3).[6] These results showed that EphA4 enhanced FGF2-triggered activation of FGFR1 through a EphA4-FGFR1 heteroreceptor complex, which possibly explained the reason that EphA4 promoted downstream signaling of FGFR and contributed to glioma cell proliferation and migration. On the other hand, ephrin-A1 stimulation did not lead to enhanced phosphorylation of FGFR1 in this experimental condition, although EphA4 was clearly phosphorylated by ephrin-A1 (Supplementary Fig. S2).[6] This might, in part, correlate to the result that FRS2 was phosphorylated by FGF2 not by ephrin-A1 (Fig. 3B).

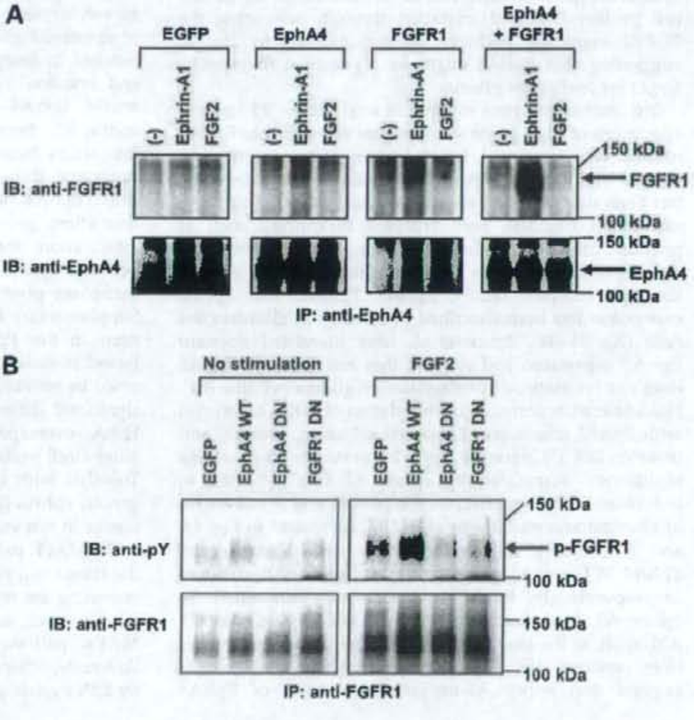

Figure 5. EphA4-FGFR1 interaction. **A,** EphA4 forms a protein complex with FGFR1. The U251 cells coexpressing EphA4 and FGFR1 were used in addition to those expressing EGFP, EphA4, and FGFR1. *Top,* EphA4 protein was immunoprecipitated from the cell lysate treated with or without 0.5 μg/mL ephrin-A1 for 30 min (ephrin-A1) or 20 ng/mL FGF2 for 15 min (FGF2) and separated by SDS-PAGE followed by immunoblotting using an anti-FGFR1 antibody. *Bottom,* reprobing with an anti-EphA4 antibody. **B,** enhanced phosphorylation of FGFR1 by EphA4. Each cell was treated with or without 40 ng/mL FGF2 for 15 min (FGF2) and the tyrosine phosphorylation level of FGFR1 was examined by probing using an anti-phosphotyrosine antibody (*top*). The total levels of precipitated FGFR1 were detected by reprobing with an anti-FGFR1 antibody (*bottom*).

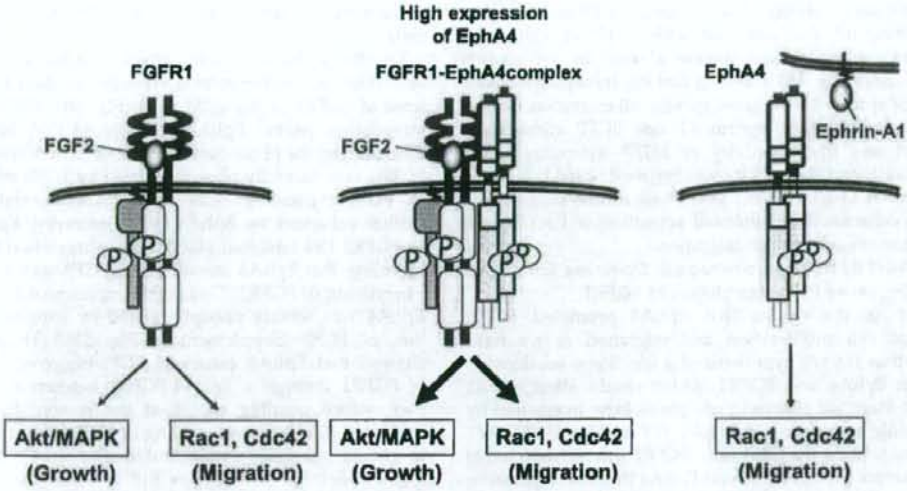

Figure 6. A diagram of the proposed mechanism of enhanced FGFR signaling by EphA4 overexpression.

Discussion

Our study revealed that highly expressed EphA4 in malignant gliomas functions as an accelerator of glioma cell proliferation and migration through promoting the FGFR1 signaling pathway as summarized in Fig. 6, suggesting that EphA4 might be a potential therapeutic target for malignant glioma.

Our microarray gene expression analysis for 32 surgical specimens of high-grade astrocytomas showed that EphA4 mRNA was expressed 4-fold higher in tumors than in normal brain tissue. Elevated expression of Eph members has been described in various human cancers (18). EphA4 expression has also been reported in cancers such as prostate, colon, and melanoma, although it has never been described in association with malignant gliomas. Among the Eph receptor family, EphA2, EphA5, and EphB2 expression has been described previously in glioblastoma cells (20, 34–36). Bruce et al. have identified aberrant EphA5 expression and showed that activation of EphA5 does not promote cell proliferation in glioma cell line (34). Nakada et al. reported phosphorylation of R-Ras associated with EphB2 effects on glioma cell adhesion, growth, and invasion (20, 35). Recently, EphA2 elevated in glioblastoma multiforme stimulated by ephrin-A1 was reported to induce an inhibitory effect on the growth and invasiveness of glioblastoma multiforme cells (36). As shown in Fig. 4A and *B* (*bottom*), the promotion of cell migration by EphA4 WT was observed under ephrin-A1 stimulation. Correspondingly, Rac1/Cdc42 activities stimulated by ephrin-A1 were promoted by EphA4 WT (Fig. 4C and *D*). Although, to the best of our knowledge, EphA4 has never been reported about glioma cell motility, these results suggest that ephrin-A1-triggered activation of EphA4 enhances Rac1/Cdc42 activation and promotes glioma cell migration (Fig. 6).

Recently, Nakada et al. revealed that ephrin-B3 promotes glioma invasion using U251 that is a high expressor cell line of ephrin-B3 and that knockdown of ephrin-B3 in U251 resulted in morphologic change and decreased migration and invasion induced by EphB2 (37). Although, in this article, EphA4 was not discussed in association with ephrin-B3, there are some previous reports about the interaction between ephrin-B3 and EphA4 (38, 39). In summary, these studies provide the evidence that EphrinB3/EphA4 has an important role in neuronal circuit formation, growth, and development. Based on this information, there is a possibility that the interaction between ephrin-B3 and EphA4 plays some role in the malignant phenotype of cancer. However, as shown in Supplementary Fig. S2A,[6] EphA4 was not phosphorylated even in the EphA4-overexpressing U251 cells without ligand stimulation, although ectopically expressing EphA4 could be activated by endogenous ephrin-B3. There was no significant difference between the mock control and the EphA4-overexpressing cells without ligand stimulation in glioma cell proliferation and migration (Figs. 3A and 4A). Together with these data, the interaction between endogenous ephrin-B3 and EphA4 did not appear to affect the results in our experimental condition.

The MAPK pathway is well known to play a major role in the tumor cell proliferation (40). There are several reports regarding the regulation of this pathway by Eph signaling (6). However, some studies report negative regulation of MAPK pathway by Eph receptor activation (41–43). Reversely, others show positive regulation of the pathway by Eph signaling, although the precise mechanism has not been shown (44–46). In addition, Yokote et al. reported a positive-regulating mechanism of the MAPK pathway by EphA4 through direct interaction with the FGFR receptor and its activation (32). Our report also showed that FGFR1 signaling was enhanced by EphA4 through their interaction followed by an increased MAPK pathway in the U251 glioma cells. Corresponding to the increased MAPK, proliferation of glioma cells was also promoted by EphA4. It was important that this enhancement was dependent on FRS2 phosphorylation, which was suggested by the findings that either FGFR1 DN or EphA4 DN inhibited FRS2-MAPK signaling enhanced by EphA4. In addition, it may be considered that the glioma cells have little or less negatively regulating molecules for the MAPK pathway such as Ras-GAP but dominantly possess a positively regulating mechanism such as the enhancement of FGFR signaling by EphA4. From our results that ephrin-A1 stimulation alone did not affect the MAPK pathway, it is considered that the strength of these two negative and positive regulation may be different among the cell types, which may explain the discrepancy in the previous results regarding the regulation of the MAPK pathway by Eph receptors.

The FGFR-mediated phosphatidylinositol 3-kinase/Akt signaling pathway was also enhanced by EphA4, which was suggested by the findings that phospho-Akt was increased by EphA4 WT but inhibited by EphA4 DN (Fig. 3C). However, the Akt pathway was found to be activated by ephrin-A1 stimulation itself and to be inhibited by EphA4 DN. In addition, FGFR1 DN had no effect on the Akt pathway mediated by ephrin-A1 stimulation. This means that EphA4 has its own Akt activating pathway, except for the enhancement of FGFR-mediated Akt pathway in the U251 cells as reported that Akt pathway was mediated by Eph receptors (44).

Rho family GTPases are signaling molecules influencing cell motility by cytoskeletal reorganizing (46). Eph signaling modulates the balance of Rho versus Rac and Cdc42 activities (3, 6). As shown in Fig. 4, neither ephrin-A1 nor FGF2 changed the Rho activity of the U251 cells. The reason might be that the U251 glioma cells expressed little ephexin, a guanine nucleotide exchanging factor for Rho, which was identified as a direct signaling molecule relayed from EphA4 (data not shown; ref. 47). On the contrary, Rac1 and Cdc42 were both activated by FGF2 or ephrin-A1 stimulation and EphA4 further enhanced these activities. Interestingly, both Rac1 and Cdc42 activations were found to be inhibited by EphA4 DN and FGFR1 DN. This means that FGFR1 and EphA4 are both necessary to activate Rac1 and Cdc42 by either FGF2 or ephrin-A1 and a close functional association is suggested between their receptors. These results were consistent with the promoted migration by EphA4 shown in Fig. 4A and could explain why EphA4 enhanced the migration in the U251 cells. The contribution of other types of guanine nucleotide exchanging factors for Rac1 or Cdc42 might be important for the enhanced migration, but the precise mechanism remains to be elucidated.

As shown in Fig. 5, we clearly showed that EphA4 formed a complex with FGFR1 and FGFR1 activity was enhanced by EphA4. These results supported the reason why EphA4 enhanced FGFR-mediated MAPK, Akt pathway, and Rac1/Cdc42 activities. These findings were supported by the report showing the interaction between EphA4 and FGFR (32). This report also showed the direct association between two molecules and how they assembled to relay their signal. It is noteworthy that different types of receptor protein tyrosine kinases such as EphA4 and FGFR1 are able to interact with each other and enhance their functions. This means cross-talk between the receptors may exist at the plasma membrane and could produce a more diverse signaling mechanism in living cells. In the glioma cells, the EphA4-FGFR1 interaction is considered to promote proliferation and migration by acting as a enhancer for the malignant phenotype.

Long-term survival of patients with malignant gliomas has improved very little despite aggressive multimodality treatments including surgery, radiotherapy, and cytotoxic chemotherapy (48). One limitation of the conventional treatment is that these therapeutic strategies are not properly based on the malignant glioma-specific biological properties. Therefore, it is important to know the molecular-based characteristics (bioinformatics) of malignant gliomas to achieve a therapeutic breakthrough. Recently, the molecular targeting approach has been developed in treating many types of cancer (49). To realize the molecular targeting therapy in malignant gliomas, one strategy is to identify key molecules using the microarray technique (50). From this view, we analyzed gene expression in malignant gliomas using a DNA microarray and found high expression of EphA4 in malignant gliomas. We then clarified the functional significance of the overexpression of EphA4 using a human glioma cell line, U251 cells. Our collective results provide the typical strategy to analyze functionally the molecule that has been identified from the gene expression study. We expect EphA4-FGFR1 signaling may become a candidate as a potential target in therapeutic intervention for malignant gliomas and cell-based screening for an EphA4-specific inhibitor is ongoing based on our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Epidermal growth factor receptor lacking C-terminal autophosphorylation sites retains signal transduction and high sensitivity to epidermal growth factor receptor tyrosine kinase inhibitor

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Constitutively active mutations of epidermal growth factor receptor (EGFR) (delE746_A750) activate downstream signals, such as ERK and Akt, through the phosphorylation of tyrosine residues in the C-terminal region of EGFR. These pathways are thought to be important for cellular sensitivity to EGFR tyrosine kinase inhibitors (TKI). To examine the correlation between phosphorylation of the tyrosine residues in the C-terminal region of EGFR and cellular sensitivity to EGFR TKI, we used wild-type (wt) EGFR, as well as the following constructs: delE746_A750 EGFR; delE746_A750 EGFR with substitution of seven tyrosine residues to phenylalanine in the C-terminal region; and delE746_A750 EGFR with a C-terminal truncation at amino acid 980. These constructs were transfected stably into HEK293 cells and designated HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr, respectively. The HEK293/D cells were found to be 100-fold more sensitive to EGFR TKI (AG1478) than HEK293/Wt. Surprisingly, the HEK293/D7F and HEK293/D-Tr cells, transfected with EGFR lacking the C-terminal autophosphorylation sites, retained high sensitivity to EGFR TKI. In these three high-sensitivity cells, the ERK pathway was activated without ligand stimulation, which was inhibited by EGFR TKI. In addition, although EGFR in the HEK293/D7F and HEK293/D-Tr cells lacked significant tyrosine residues for EGFR signal transduction, phosphorylation of Src homology and collagen homology (Shc) was spontaneously activated in these cells. Our results indicate that tyrosine residues in the C-terminal region of EGFR are not required for cellular sensitivity to EGFR TKI, and that an as-yet-unknown signaling pathway of EGFR may exist that is independent of the C-terminal region of EGFR. (*Cancer Sci* 2009)

Epidermal growth factor receptor (EGFR), also termed HER1/ErbB-1, is overexpressed and activated in many cancers.(1–3) Small-molecule inhibitors of EGFR tyrosine kinase and antibodies have been shown to exhibit antitumor activity in several tumors.(4–6) Somatic mutations of EGFR tyrosine kinase in non-small cell lung cancer have been shown to be associated with hyperresponsiveness to gefitinib, a selective EGFR tyrosine kinase inhibitor (TKI).(7,8) Many investigators have subsequently reported that EGFR mutations are strong determinants of the tumor response to EGFR TKI.(9,10) Approximately 90% of non-small cell lung cancer-associated EGFR mutations in two reports consisted of two major EGFR mutations, namely, delE746_A750 in exon 19 and L858R in exon 21.(11) We previously reported hypersensitivity to EGFR TKI of a PC-9 cell line with delE746_A750 in exon 19, one of the commonly encountered mutations mentioned above, and this deletion mutant of EGFR was constitutively active and activated the ERK and Akt pathway.(12–16) Binding of the receptor with its ligand leads to homodimerization and heterodimerization of the receptor tyrosine kinase.(17,18) Thus, EGFR is a ligand-activated tyrosine kinase that ultimately delivers cellular growth signals.

Tyr-1068, Tyr-1148, and Tyr-1173 in the C-terminal region are the major autophosphorylation sites in human EGFR. These C-terminal phosphorylation sites of EGFR interact with adaptor proteins.(19,20) Phosphorylation of the C-terminal autophosphorylation sites of EGFR, triggered by epidermal growth factor (EGF), in turn trigger an intracellular signal cascade involving proteins such as ERK, Akt, Janus kinase, and signal transducer and activator of transcription.(15,21,22) Src homology and collagen homology (Shc) is a molecular adaptor protein that binds phosphorylated tyrosines within activated EGFR, and is itself phosphorylated on tyrosine residues upon stimulation of EGFR. The phosphorylated CH1 site of Shc then engages the binding site for the SH2 domain of growth factor receptor-bound protein (Grb) 2. The SH3 domain of Grb2 directly interacts with the guanyl nucleotide exchange factor son of sevenless homolog (Sos).(23,24) Sos catalyzes the conversion of GDP to GTP on Ras, resulting in Ras activation. Activated GTP-Ras recruits Raf kinase to the plasma membrane, resulting in Raf activation and phosphorylation of its downstream target ERK kinase.(25,26)

Phosphorylation of tyrosine residues at the C-terminal region of EGFR is believed to be important in cell signaling triggered by wild-type EGFR.(27,28) However, the role of this region in an active mutant of EGFR (delE746_A750) has yet to be elucidated in detail. To clarify the biological functions of the tyrosine residues at the C-terminal region of EGFR, we constructed several mutants with C-terminal-truncated or substitution of tyrosine residues to phenylalanine in the C-terminal region. We showed that EGFR lacking C-terminal autophosphorylation sites still generated signals, with retention of cellular hypersensitivity to EGFR TKI.

Materials and Methods

Expression constructs. The method used to obtain full-length cDNA of wild-type *EGFR* has been described previously.(12) Wild-type *EGFR* cDNA and 15 bp-deletion *EGFR* (delE746_A750) were introduced into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) with a myc-tag at its C-terminus. The *EGFR* cDNA with substitution of seven tyrosine residues to phenylalanine in the C-terminal region was amplified by mutagenesis; the QuikChangeα

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Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used for the polymerase chain reaction and a primer set was synthesized (Supporting Information Table S1). The cDNA of the C-terminal-truncated *EGFR* with 15-bp deletion (*EGFR-D-Tr*) was amplified using the following primer set: forward, CCT CCT CTT GCT GCT GGT GGT G; reverse, GAA CAAGCT TGA CAA GGT AGC GCT GGG GGT CTC. After the polymerase chain reaction products were cut with *Cla*I and *Hind*III, they were ligated to the *Cla*I and *Hind*III sites of the pcDNA3.1 expression vector containing EGFR-D cDNA. The cDNA of wild-type *EGFR* with the C-terminal truncation at amino acid 980 (*EGFR-Wt-Tr*) was made from the *Cla*I and *Xho*I fragments of the pcDNA3.1 expression vector containing wild-type EGFR and the *Cla*I and *Xho*I fragments of the pcDNA3.1 expression vector containing *EGFR-D-Tr*.

Epidermal growth factor receptor cDNA with the myc-tag in pcDNA3.1 was cut and introduced into a pQCLIN retroviral vector (BD Biosciences Clontech, San Diego, CA, USA) together with enhanced green fluorescent protein (EGFP) followed by the internal ribosome entry sequence, to monitor the expression of the inserts indirectly. A pVSV-G vector (Clontech, Palo Alto, CA, USA) for constitution of the viral envelope, pGP vector (Takara, Yotsukaichi, Japan), and the pQCXIX constructs were cotransfected into HEK293 cells using FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). Briefly, 80% confluent cells cultured in a 10-cm dish were transfected with 2 µg pVSV-G vector plus 6 µg pQCXIX vector. Forty-eight hours after the transfection, the culture medium was collected and the viral particles were concentrated by centrifugation at 15 000*g* for 3 h at 4°C. The viral pellet was then resuspended in fresh Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA). The titer of the viral vector was calculated by counting the EGFP-positive cells that were infected in serial dilutions of a virus-containing medium and then determining the multiplicity of infection. HER2 and HER3 introduced retrovirally into HEK293 cells were used as positive controls in western blotting.

Cell culture and transfection. The human embryonic kidney HEK293 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Sigma) in a humidified atmosphere of 5% CO_2 at 37°C. The HEK293 cells were transfected with the viral vectors.

***In vitro* growth-inhibition assay.** The growth-inhibitory effects of AG1478 (Biomol International, Plymouth Meeting, PA, USA) in HEK293/Wt, HEK293/Wt-Tr, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were examined using a 3, 4, 5-dimethyl-2H-tetrazolium bromide (MTT) assay as described previously.(29)

Immunoprecipitation. The culture cells were washed twice with ice-cold phosphate-buffered saline (PBS) (–), and lysed with a lysis buffer containing 20 mM Tris-HCl (pH 7.0), 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM Na pyrophosphate, 50 mM NaF, 1 mM Na orthovanadate, 1% TritonX-100, and the Complete Mini protease inhibitor mix (Roche Diagnostics). The lysates were cleared by centrifugation at 15 000 *g* for 10 min and the protein concentrations of the supernatants were measured using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA).

The cell lysates (500 µg) were immunoprecipitated by overnight incubation with 3 µg anti-EGFR antibody, anti-HER3 antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-HER2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-c-Myc (Roche Diagnostics), followed by further incubation with protein-G agarose (Santa Cruz Biotechnology) for 1 h. Bound proteins were washed three times with lysis buffer and eluted in Laemmli sample buffer containing 2-mercaptoethanol. The eluted proteins were subjected to 2–15% gradient sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immmunoblotted as described above.

Immunoblotting. Whole-cell lysates and the immunoprecipitates were separated using 2–15% gradient SDS-PAGE and blotted on to a polyvinylidene fluoride membrane. The membrane was probed with anti-EGFR, anti-HER3 (Upstate Biotechnology), anti-phospho(Tyr845)-EGFR, anti-phospho(Tyr1068)-EGFR, anti-phospho(Tyr1173)-EGFR, anti-HER2, anti-phospho-tyrosine, anti-p44/42 mitogen-activated protein (MAP) kinase, anti-phospho-p44/42 MAP kinase, anti-Shc, anti-phospho-Shc (Cell Signaling, Beverly, MA), anti-Sos (Santa Cruz), anti-Grb2 (BD Biosciences, San Jose, CA), and anti-c-Myc (Roche Diagnostics) antibodies by incubation for 2 h at room temperature and then with horseradish peroxidase-conjugated anti-rabbit IgG antibody or anti-mouse IgG antibody for 1 h at room temperature. Finally, the proteins were visualized with an enhanced chemiluminescence western blotting detection system (GE Healthcare, Piscataway, NJ, USA).

Chemical crosslinking assay. After treatment or no treatment with EGF (R&D Systems, Minneapolis, MN, USA) the chemical crosslinking assay was carried out in intact cells as described previously.(13) The transfected cells were washed with ice-cold PBS (+) and incubated for 30 min at room temperature in PBS (+) containing 2 mM crosslinker bis(sulfosuccinimidyl)suberate (Pierce Biotechnology). The reaction was terminated with 20 mM Tris (pH 7.5) for 15 min at room temperature. The cells were washed with PBS (+), and 15 µg protein was resolved by 2–15% gradient SDS-PAGE and then immunoblotted with anti-EGFR and anti-phospho-EGFR antibodies.

Results

Epidermal growth factor receptor lacking C-terminal autophosphorylation sites (EGFR-D-Tr and EGFR-D7F) retains signal transduction. To examine the role of the tyrosine residues in the C-terminal region of EGFR in signal transduction, we constructed vectors containing wild-type EGFR, a deletion mutant (delE746_A750 EGFR) with C-terminal truncation, or a mutant with substitution of seven tyrosine residues in the C-terminal region (Fig. 1a), and transfected these vectors into HEK293 cells with rather low expression levels of endogenous EGFR. The expression of exogenous EGFR in the transfectants was confirmed by immunoblotting with anti-EGFR antibodies (Fig. 1b).

In order to examine the signal transduction of EGFR in the transfectants, we analyzed the phosphorylation status of EGFR and its downstream molecules. Phosphorylation of EGFR at the Y845 and Y1173 tyrosine residues was detected in HEK293/Wt and HEK293/D cells cultivated in medium containing 10% fetal bovine serum (Fig. 2a). Enhanced phosphorylation of the Y1068 tyrosine residue was observed specifically in the HEK293/D cells, suggesting that Y1068 is constitutively active in delE746_A750 EGFR. This phenomenon is consistent with our previous reports.(29,30) On the other hand, no significant phosphorylation of Y845, Y1068, or Y1173 was observed in the HEK293/D7F and HEK293/D-Tr cells. ERK and Akt are major downstream pathways of EGFR. We examined the phosphorylation of ERK and Akt in the transfectants. Increased phosphorylation of ERK was observed in the HEK293/D7F, HEK293/D-Tr, and HEK293/D cells, even though HEK293/D7F and HEK293/D-Tr cells were transfected with EGFR lacking the C-terminal autophosphorylation sites.

We also examined ligand-dependent signals in these cells under the 1% serum starve medium (Fig. 2b). Ligand-stimulated phosphorylation of EGFR was observed in the HEK293/Wt cells transfected with wild-type EGFR. Constitutive phosphorylation of EGFR and a further increase in the EGFR phosphorylation response to EGF were observed in the HEK293/D cells. On the other hand, no significant phosphorylation in response to EGF binding was observed in the HEK293/D7F and HEK293/D-Tr cells. Downstream of EGFR, increased phosphorylation of ERK and Akt was observed in response to EGF in the HEK293/D7F

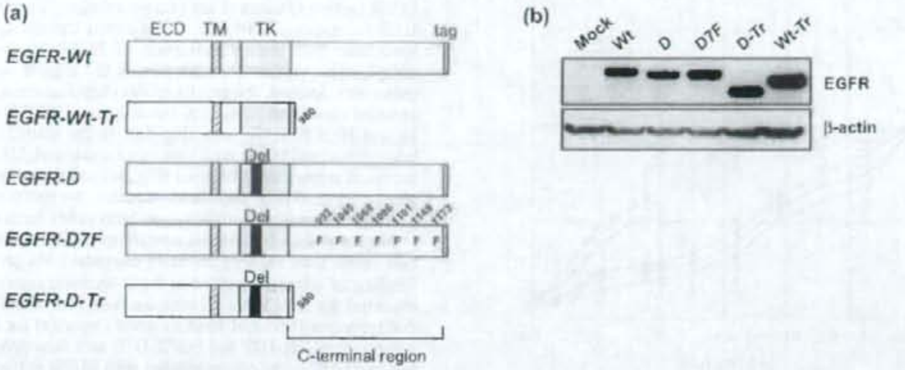

Fig. 1. Epidermal growth factor receptor (EGFR) constructs and their expression. (a) Structures of the various EGFR mutants. EGFR-Wt, wild-type human EGFR; EGFR-Wt-Tr, wild-type kinase domain of EGFR with C-terminal truncation at amino acid 980; EGFR-D, EGFR with a 15-bp deletion from the tyrosine kinase domain (delE746_A750); EGFR-D7F, 15-bp deletion of EGFR (delE746_A750) and substitution of seven tyrosine residues to phenylalanine (Y992F, Y1068F, Y1045F, Y1068F, Y1086F, Y1148F, Y1173F); and EGFR-D-Tr, 15-bp deletion of EGFR (delE746_A750) with C-terminal truncation at amino acid 980. EGFR-Wt, EGFR-D, EGFR-D7F, and EGFR-D-Tr contained a myc-tag. EGFR-Wt-Tr contained a flag-tag. ECD, extracellular domain; TK, tyrosine kinase; TM, transmembrane. (b) Stable transfectants were lysed and cell lysates containing equal amounts of protein were immunoblotted with anti-EGFR antibody recognizing the extracellular domain of EGFR. A band with a molecular weight of ~170 kDa was detected in the HEK293/Wt, HEK293/D, and HEK293/D7F cells, and a band of lower molecular weight was detected in the HEK293/D-Tr and HEK293/Wt-Tr cells. Mock, HEK293/Mock; Wt, HEK293/Wt; Wt-Tr, HEK293/Wt-Tr; D, HEK293/D; D7F, HEK293/D7F; and D-Tr, HEK293/D-Tr.

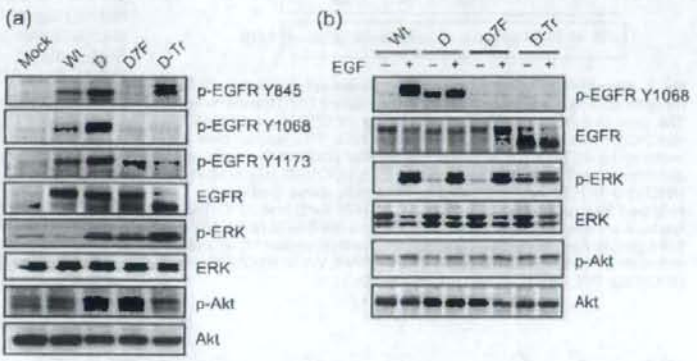

Fig. 2. Epidermal growth factor receptor (EGFR) lacking C-terminal autophosphorylation sites retains EGFR signal transduction. (a) The HEK293/Mock, HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were lysed, and the cell lysates were immunoblotted with anti-phospho-EGFR (p-EGFR Y845, Y1068, Y1173), anti-EGFR (recognizing the extracellular domain), anti-phospho-ERK, anti-ERK, anti-phospho-Akt, and anti-Akt antibodies. (b) The HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were incubated in 1% serum starve medium for 12 h, followed by treatment with 10 ng/mL epidermal growth factor for 10 min at 37°C. The cell lysates were immunoblotted. Mock, HEK293/Mock; Wt, HEK293/Wt; D, HEK293/D; D7F, HEK293/D7F; and D-Tr, HEK293/D-Tr.

and HEK293/D-Tr cells, as well as the HEK293/Wt and HEK293/D cells. These results indicate that EGFR lacking the C-terminal autophosphorylation sites (EGFR-D-Tr and EGFR-D7F) retained signal transduction ability.

Transfectants with EGFR lacking C-terminal autophosphorylation sites retain their hypersensitivity to EGFR TKI. EGF stimulation increased the growth of HEK293/Wt cells significantly but did not affect their sensitivity to AG1478 (data not shown). To examine the role of the C-terminal region of EGFR in cellular sensitivity to EGFR TKI, the sensitivity of these transfectants was examined by growth-inhibition assay (Fig. 3a). HEK293/Wt and HEK293/Wt-Tr cells with normal EGFR in relation to the kinase domain were relatively resistant to EGFR TKI, with IC_{50} values of 3.0 ± 0.97 and 8.1 ± 0.99 µM. On the other hand, HEK293/D (0.028 ± 0.018 µM), HEK293/D7F (0.047 ± 0.030 µM), and HEK293/D-Tr (0.017 ± 0.017 µM) cells were ~100 times more sensitive to AG1478 compared to HER293/Wt cells (Fig. 3a), suggesting that the cells transfected with EGFR lacking C-terminal phosphorylation sites retained hypersensitivity to EGFR TKI. There were no differences in the proliferation rates of these cell lines under the absence of drug exposure (data not shown).

To elucidate the effect of EGFR TKI on the EGFR-triggered signal cascade, the phosphorylation status of EGFR and ERK was examined in the transfectants treated with AG1478 under the 1% serum starve medium (Fig. 3b). AG1478 at a concentration of 20 nM inhibited the phosphorylation of EGFR in HEK293/D cells, but not in the other cell lines. The increased phosphorylation of ERK observed in the HEK293/D, HEK293/D7F, and HEK293/D-Tr cells was inhibited by AG1478 at 20 nM. These results suggest that signal transduction from C-terminal-truncated EGFR to downstream molecules allows sensitivity to EGFR TKI to be retained, just like the deletion mutant of EGFR (delE746_A750).

Endogenous HER families are not involved in the dimerization of EGFR-D-Tr and EGFR-D7F. We hypothesized that the signals from EGFR lacking the C-terminal autophosphorylation sites were transduced through heterodimerization with endogenous EGFR, HER2, or HER3. No significant endogenous EGFR expression or its phosphorylation was observed in the HEK293/Mock cells (Fig. 4a). Very low levels of intrinsic HER2 or HER3 expression were detected in the HEK293 cells, and the expression levels seemed not to be involved in significant drug sensitivity nor increased signal transduction (Fig. 4b,c). Therefore, it is not likely that heterodimerization of

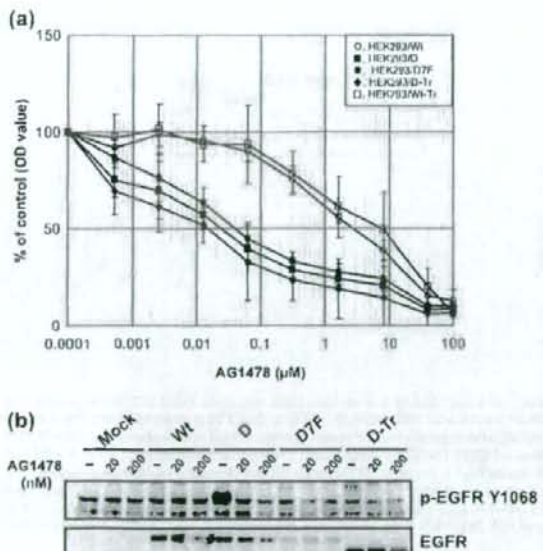

Fig. 3. Sensitivity of cell growth and downstream epidermal growth factor receptor (EGFR) signaling to AG1478 in the mutant EGFR transfectants. (a) The growth-inhibitory effect of AG1478 in HEK293/Wt, HEK293/Wt-Tr, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells. The seeded cells were exposed to AG1478 for 72 h and the cellular proliferative activity was determined by MTT assay. (b) The HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were incubated in 1% serum starve medium for 12 h, followed by exposure to 20 or 200 nM AG1478 for 3 h at 37°C. The cell lysates were immunoblotted with anti-phospho-EGFR (p-EGFR Y1068), anti-EGFR (recognizing the extracellular domain), anti-phospho-ERK, or anti-ERK antibodies. Mock, HEK293/Mock; Wt, HEK293/Wt; Wt-Tr, HEK293/Wt-Tr; D, HEK293/D; D7F, HEK293/D7F; D-Tr, HEK293/D-Tr.

EGFR lacking C-terminal autophosphorylation sites with endogenous HER receptors contributes to the signal transduction. It is thus speculated that homodimerization of EGFR lacking C-terminal autophosphorylation sites transduces the signals to downstream molecules. Indeed, the results of the chemical crosslinking assay revealed clear homodimerized bands in the HEK293/Wt, HEK293/D, and HEK293/D7F cells (Fig. 5a). In the HEK293/D-Tr cells, homodimerized bands with lower molecular weights (indicated by the black arrow) were detected (Fig. 5a) and these dimers were not phosphorylated (Fig. 5b). Taken together, we speculate that EGFR lacking C-terminal autophosphorylation sites form homodimers.

Despite a lack of C-terminal autophosphorylation sites, transfected cells retain their capacity for EGFR-dependent Shc phosphorylation. Binding of adaptor proteins to the C-terminal region of EGFR is essential for EGFR signal transduction. It is widely recognized that tyrosines 1068 and 1086 are most important for Sos and Grb2 activation; EGFR-D7F and EGFR-D-Tr lack these tyrosine residues. Sos and Grb2 were coprecipitated with EGFR in the HEK293/Wt and HEK293/D cells, but not in the HEK293/D7F or HEK293/D-Tr cells (Fig. 6a). The bands were confirmed by reblotting of the membranes used for immunoblotting (data not shown). Another adaptor protein, Shc, also binds to the C-terminal region of EGFR, and phosphorylation of Shc activates the ERK pathway. An increase in phosphorylated p46 and p52 Shc was observed in the HEK293/D, HEK293/D7F, and HEK293/D-Tr cells compared with the HEK293/Mock and HEK/Wt cells (Fig. 6b). The phosphorylation of Shc observed in the HEK293/D, HEK293/D7F, and HEK293/D-Tr cells was completely inhibited by 20 nM AG1478 (Fig. 6c). These results suggest that EGFR lacking C-terminal autophosphorylation sites activates Shc in a C-terminal-independent manner, and that Shc-mediated signals may be involved in the hypersensitivity to EGFR TKI of HEK293 cells expressing EGFR lacking C-terminal autophosphorylation sites.

Discussion

In the present study, we investigated the relationship between phosphorylation of tyrosine residues in the C-terminal region of EGFR and cellular sensitivity to EGFR TKI. Increased phosphorylation of Shc and ERK was observed in HEK293/D7F and HEK293/D-Tr cells, which expressed EGFR lacking autophosphorylation sites in the C-terminal region. Previous reports have demonstrated

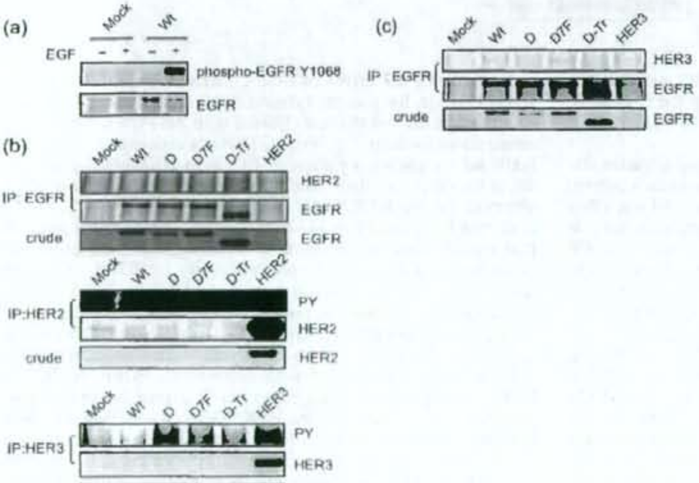

Fig. 4. Heterodimerization of mutant epidermal growth factor receptor (EGFR) with endogenous receptors of the HER family in mutant EGFR transfectants. Expression of endogenous EGFR and response to epidermal growth factor stimulation. HEK293/Mock, HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were incubated in 1% serum starve medium for 12 h followed by the addition of 10 ng/mL epidermal growth factor for 10 min at 37°C. (a) The whole-cell lysates of HEK293/Mock and HEK293/Wt cells containing equal amounts of protein were immunoblotted with anti-phospho-EGFR (p-EGFR Y1068) and anti-EGFR (recognized extracellular domain). (b,c) The lysates were immunoprecipitated with anti-EGFR, anti-HER2, or anti-HER3 antibodies, and immunoblotted with anti-EGFR, anti-HER2, anti-HER3, or anti-phosphotyrosine antibodies to detect the dimerization and phosphorylation of EGFR and endogenous HER2 or HER3. HER2, HER2-introduced HEK293 cells as a positive control; HER3, HER3-introduced HEK293 cells as a positive control.

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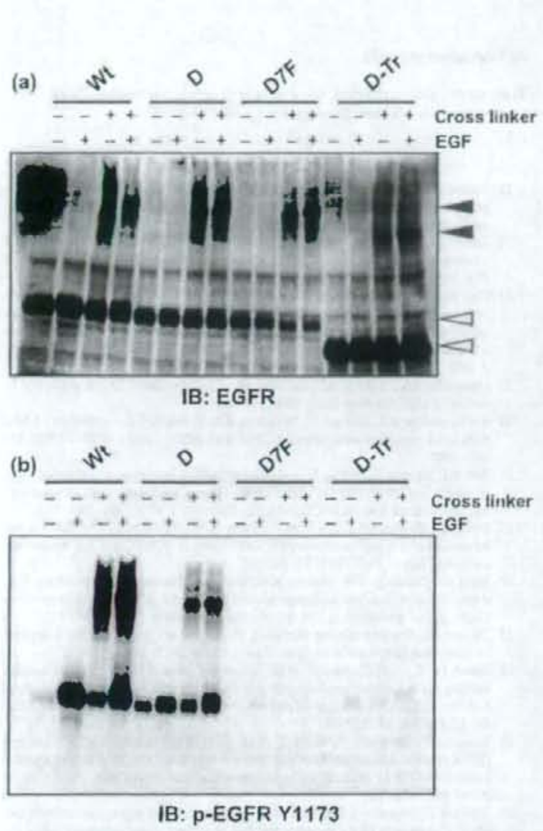

Fig. 5. The cells under 1% serum starved medium were allowed to react with 2 mM of the chemical crosslinking regent BS_3 before the crosslinking reaction was quenched. The cell lysates were immunoblotted with (a) anti-epidermal growth factor receptor (EGFR) (recognizing the extracellular domain) and (b) anti-phosphoEGFR (p-EGFR Y1173) antibodies to detect the dimerization and phosphorylation of wild-type and mutant EGFR. Black arrow, EGFR dimer; open arrow, EGFR monomer. Mock, HEK293/Mock; Wt, HEK293/Wt; D, HEK 293/D; D7F, HEK293/D7F; D-Tr, HEK293/D-Tr. EGF, epidermal growth factor.

that cells expressing EGFR lacking C-terminal autophospholylation sites retain EGF-induced mitogenic and transforming activity.[27,28] Our data and these previous reports suggest that there exist other EGFR signaling pathways besides those mediated by the C-terminal tyrosine residues. In addition, these signaling pathways are operative in the active EGFR mutant (delE746_A750) as well as wild-type EGFR.[28] The results of our growth-inhibition assay demonstrated the hypersensitivity of HEK293/D7F and HEK293/D-Tr cells to EGFR TKI, and phosphorylation of ERK and Shc in these cells was also inhibited. These results suggest that this EGFR signaling pathway contributes to tumor cell growth.

We demonstrated the hypersensitivity of the transfectants (HEK293/D7F and HEK293/D-Tr cells) to AG1478. We previously reported the hypersensitivity of transfectants carrying mutant EGFR to AG1478 as well as gefitinib, ZD6474, and erlotinib.[8,15,31] Therefore, it can be easily speculated that the HEK293/D7F and HEK293/D-Tr cells would also be hypersensitive to the clinically available EGFR TKI and AG1478.

Somatic EGFR mutation in lung cancer has been reported, and over 20 types of mutations have been reported.[10] The L858R point mutation in exon 21 of EGFR is a major point mutation (such as in delE746_A750) that contributes to EGFR TKI hypersensitivity.[32] Interestingly, we constructed cells that overexpressed EGFR-Wt-Tr and EGFR-D-Tr, and a mutant truncated form of EGFR similar to EGFR-Wt-Tr was previously found in patients with glioblastoma.[33] The mutant was truncated at amino acid 958 of EGFR and the frequency was relatively high in 7 of 48 patients. Therefore, it would be of interest to determine in future studies whether this C-terminal-truncated form of delE746_A750 EGFR, similar to EGFR-D-Tr, might be identifiable in human materials in the clinical setting.

We attempted to clarify the signaling pathway from the C-terminal region of EGFR. We observed the phosphorylation of ERK and Shc in HEK293/D7F and HEK293/D-Tr cells, and these phosphorylations were inhibited by exposure to AG1478. These phosphorylations were not observed in HEK293/Mock, HEK293/Wt, or HEK293/Wt-Tr cells. Our results suggest that the constitutively active mutant EGFR lacking C-terminal autophosphorylation sites is sufficient for activation of the downstream pathway. However, it remains unknown how signals are transduced from EGFR without a C-terminal region to Shc, as no direct binding of Grb2 or Shc with EGFR lacking the C-terminal region was detected in the HEK293/D7F and HEK293/D-Tr cells (Fig. 6a). We attempted to identify the mediator molecules binding to EGFR-D-Tr and EGFR-D7F by mass analysis of immunoprecipitates; however, no clear mediator molecules were identified. As a possible indirect mechanism, Sasaoka *et al.* postulated that ErbB2–Shc signals from EGFR lacking C-terminal autophosphorylation sites.[34] However, we consider this unlikely from the results of our experiments because no significant expression of Erb2 was detected in the HER293 cells.

The results of the crosslinking assay demonstrated that a complex of lower molecular weight was present in the HEK293/D-Tr cells compared with the HER293/Wt cells, indicating that truncated EGFR forms homodimers in the HEK293/D-Tr cells. Thus, it can be speculated that homodimerized truncated EGFR directly transduces signals downstream.

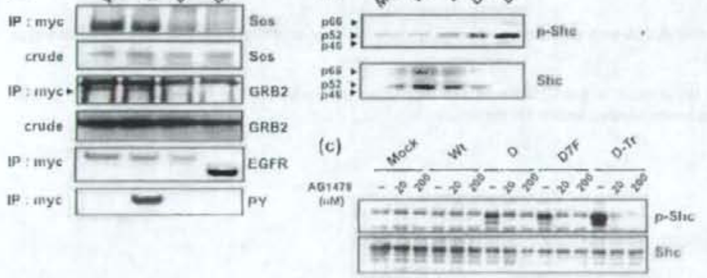

Fig. 6. Interaction between mutant epidermal growth factor receptor (EGFR) and adaptor proteins. The cells were cultured under normal conditions. (a) The lysates of HEK293/Wt, HEK293/D, HEK293/D7F, and HEK293/D-Tr cells were immunoprecipitated with anti-myc tag antibody; the precipitates were immunoblotted with anti-son of sevenless homolog (Sos) and anti-growth factor receptor-bound protein (Grb) 2 antibodies. (b) Whole-cell lysates containing equal amounts of protein were immunoblotted with anti-phospho-Src homology and collagen homology (Shc) and anti-Shc antibodies. (c) The cells incubated in 1% serum starve medium for 12 h were treated with 20 or 200 nM AG1478 for 3 h and then the lysates were immunoblotted with anti-phospho-Shc or anti-Shc antibodies. Mock, HEK293/Mock; Wt, HEK293/Wt; D, HEK293/D; D7F, HEK293/D7F; D-Tr, HEK293/D-Tr. PY, anti-phospho-tyrosine.

In conclusion, our results indicate that an as-yet-unknown signaling pathway of EGFR exists that is independent of the C-terminal region of EGFR, and these regions are not required for cellular sensitivity to EGFR TKI.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer set for epidermal growth factor receptor cDNA with substitution of seven tyrosine residues to phenylalanine in the C-terminal region

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Establishment and molecular profiling of a novel human pancreatic cancer panel for 5-FU

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Ten novel human pancreatic carcinoma cell lines (Sui65 through Sui74) were established from a transplantable pancreatic carcinoma cell line. All the cell lines resembled the original clinical carcinoma in terms of the morphological and biological features, presenting with genetic alterations such as point mutations of *K-ras* and *p53*, attenuation or lack of *SMAD4* and *p16* and other relevant cellular characteristics. Using this panel, we evaluated the effects of 5-FU in suppressing the proliferation of pancreatic carcinoma cells. When tested *in vitro*, although Sui72 was highly susceptible to 5-FU, the other cell lines were found to be resistant to the drug. When Sui72 and Sui70 were implanted subcutaneously in SCID mice followed by treatment with 5-FU, the drug was found to be effective against Sui72 but not Sui70, consistent with the results *in vitro*. In order to identify the molecular determinant for high sensitivity of Sui72 to 5-FU, we examined the mRNA expression levels of the metabolic enzymes of 5-FU. Decreased expression of DPYD was observed in Sui72 as compared with other cell lines (0.1 *versus* 0.6 ± 0.5, 0.1-fold).

It is believed that the novel cell lines established in the present study will be useful for analyzing the pattern of progression of pancreatic cancer and for evaluating the efficacy of anticancer agents. (*Cancer Sci* 2008; 99: 1859–1864)

Pancreatic cancer is an intractable cancer with a prognosis poorer than that of any other cancer of the gastrointestinal tract. In Japan, the 5-year survival rate of patients with pancreatic cancer is 5.5%, which is extremely low as compared with that of patients with colorectal cancer (64.6%), gastric cancer (58.8%) or even hepatic cancer (17.1%).(1,2) The number of patients with this cancer has been increasing significantly in recent years, and the mean life expectancy of patients with this cancer is only about 1.5 years. Pancreatic cancer, often characterized by pain, jaundice and digestive dysfunction, causes much pain and stress to the patient. These characteristics make this cancer an important open issue in medicine and healthcare. Currently, no valid clinical means are available for the prevention, diagnosis or treatment of this cancer. Treatment of pancreatic cancer with local therapy alone, that is surgical resection and radiotherapy, has limitations, and chemotherapy needs to be considered.(2) In the past, various adjuvant chemotherapy regimes, primarily based on 5-FU, have been attempted;(3–5) however, no effective therapy has as yet been established for pancreatic cancer.

In recent years, close attention has been paid to the development of cancer treatment methods based on information about the molecular mechanism of onset and progression of cancer.(2,6,7) Thus, exploration of molecular markers and target molecules for treatment is expected to play a key role in cancer management. For this kind of preclinical research, in particular, for evaluation of the efficacy of molecule-targeted drugs, the development of a panel of cultured cells derived from clinical cancer specimens is indispensable. To date, a number of cell lines derived from human pancreatic cancer have been established(8–15) and have contributed greatly to advancing cancer research(15,16) in terms of analysis of biological features and evaluation of the efficacy of anticancer agents. However, after multiple passages, some of these cell lines lose their original features (e.g. histopathological characteristics of the tumor formed after implantation, etc.).

The present study was undertaken to establish 10 novel cell lines derived from human pancreatic carcinoma that would reliably reflect the clinical features of pancreatic carcinoma. We investigated the proliferative characteristics and genetic alterations in these cell lines, then, using the panel of cell lines, we evaluated the efficacy of conventional 5-FU-based chemotherapy against pancreatic cancer and attempted to elucidate the determinants of sensitivity of these pancreatic cancer cell lines to 5-FU-based anticancer agents.

Materials and Methods

Establishment of the cell lines. All the cell lines were established *in vitro* from xenotransplantable tumors (taco series) originating from primary or metastatic human pancreatic cancer (Table 1) (T. Oda and A. Ochiai, unpublished data). The cell lines were established by s.c. back transplantation of the primary tumor for the 8th transplant generation, then tumors were removed for *in vitro* cultivation. Fresh tumor specimens obtained under sterile conditions were washed five times in Roswell Park Memorial Institute medium (RPMI)-1640 containing streptomycin (500 μg/mL) and penicillin (500 IU/mL). The tumor tissue specimens were trimmed of fat and necrotic materials and minced with a scalpel. The tissue pieces were transferred together with Dulbecco's Modified Eagle Medium (DMEM) + Ham's F12 + 5% fetal bovine serum (FBS), at 10–15 fragments per dish, to 60-mm culture dishes.(17) The dishes were left undisturbed for 24 h at 37°C in a 5% CO_2/95% air atmosphere. The medium was composed of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (1:1) supplemented with 5% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 IU/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (Immuno-Biological Laboratories [IBL], Fujioka, Japan). After 48 h, the medium was replaced by RPMI-1640 medium (IBL, Fujioka, Japan) supplemented with 10% FBS, 100 IU/mL penicillin G sodium and 100 mg/mL streptomycin sulfate. The dishes containing the tissue fragments were observed weekly under an inverted phase microscope. The dishes were initially trypsinized (0.05% trypsin and 0.02% ethylenediametetraacetic acid [EDTA]) to selectively remove overgrowing fibroblasts. In addition, we also attempted to remove the fibroblasts mechanically and transfer only the tumor cells. Half of the medium was changed every 4–6 days. The cultures were first split after 3–8 months of cultivation, and the

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Abbreviations: i.p., intraperitoneal; s.c., subcutaneous; TPA, tissue polypeptide antigen; 5-FU, 5-fluorouracil; SCID, severe combined immunodeficiency.

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Table 1. Establishment of ten human pancreatic cancer cell lines from the xenotransplantable tumors

Cell line	Source	Origin		Histology
	xenograft tumor	Age/sex	Source (TNM)	
Sui65	taco-1	63/F	Peritoneum (metastatic focus)	Tubular adenocarcinoma c/w meta
Sui66	taco-2	74/M	Pancreas	Tubular adenocarcinoma
Sui67	taco-4	73/F	Pancreas	Tubular adenocarcinoma
Sui68	taco-5	53/M	Pancreas	Adenocarcinoma
Sui69	taco-6	54/M	Pancreas	Tubular adenocarcinoma
Sui70	taco-7	53/M	Pancreas	Adenocarcinoma
Sui71	taco-12	65/M	Liver (metastatic focus)	Adenocarcinoma c/w meta
Sui72	taco-13	76/F	Pancreas	Tubular adenocarcinoma
Sui73	taco-15	65/F	Pancreas	Tubular adenocarcinoma
Sui74	taco-16	72/F	Pancreas	Adenocarcinoma

cells were passaged thereafter at a 1:10 or 1:20 ratio.(18) They were then judged, established and designated (Table 1). All of the cell lines were routinely tested for *Mycoplasma* using a PCR *Mycoplasma* Detection kit (Takara, Kyoto, Japan), and no contamination was detected. This study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all of the patients from whom the tumor specimens were obtained. The study protocol was approved by the institutional review board of the National Cancer Center (approved number: 17–43).

Animal experimentation. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation, and the experiments were conducted in accordance with the Guideline for Animal Experiments of the National Cancer Center. Female SCID mice (C.B.17/Icr Jcl-scid) were purchased from CLEA Japan (Tokyo, Japan), and maintained under specific-pathogen-free conditions. Six- to 8-week-old mice were used for this experiment. The mice were housed in filter-protected cages and reared on sterile water. The ambient light was controlled to provide regular 12-h light : 12-h dark cycles.

Western blot analysis. *SMAD4* and *p16* expression was examined in all the cell lines using Western blotting.(19) An osteosarcoma (SaOs2) and a human embryonic kidney (293) cell line were used as positive controls for *p16* and *SMAD4*, respectively. Monoclonal mouse antihuman antibodies to *DPC4/Smad 4* (cloneB8, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and to *p16* (clone G175-405, PharMingen, San Diego, CA, USA) were used.

Therapeutic studies with 5-fluorouracil (5-FU). S.C. implantation of 5×10^5 cultured cells suspended in 0.1 mL RPMI-1640 medium was conducted in 6-week-old female SCID mice. To evaluate the antitumor activity, 3 days after the tumor cell implantation, the mice were divided into three groups of six mice each according to the tumor volume on Day 0. The experimental mice were divided into a control group that received vehicle alone (saline), and experimental groups that received i.p. inoculation of different doses of the drug (50 and 100 mg/kg/head). On Days 3, 10 and 17, tumor-bearing mice received an i.p. injection of 5-FU. 5-FU was purchased from Sigma (St. Louis, MO, USA) and dissolved in saline before being injected. Tumor growth was measured weekly, in terms of the tumor diameter, with calipers. At appropriate intervals, or when moribund, the mice were sacrificed and the tissues were examined macroscopically for metastasis in various organs and then processed for histological examination, as described previously.(20)

Direct sequencing. Samples from the cell lines were analyzed for the presence of mutations in exon 1 of the K-*ras* (Kirsten rat sarcoma-2 viral oncogene homolog) gene and exons 5–8 of the p53 gene by direct sequencing of the PCR-amplified DNA fragments. PCR and direct sequencing were performed as described previously(21) using minor modifications.

Real-time reverse-transcription polymerase chain reaction. RNA derived from each cell line was converted to complementary DNA using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA, USA). Real-time reverse transcription–polymerase chain reaction (RT-PCR) were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7900HT Fast Real-time PCR system (Applied Biosystems). The PCR conditions were as follows: one cycle of denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Obtained data was normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPD) expression. The following primers were used: DHFR-FW: 5′-GCA AAT AAA GTA GAC ATG GTC TGG A-3′; DHFR-RW: 5′-AGT TTA AGA TGG CCT GGG TGA-3′; FPGS-FW: 5′-TCT GCC CTA ACC TGA CAG AGG TG-3′; FPGS-RW: 5′-TCG TCC AGG TGG TTC CAG TG-3′; TP-FW: 5′-GAG GCA CCT TGG ATA AGC TGG A-3′; TP-RW: 5′-GCT GTC ACA TCT CTG GCT GCA TA-3′; UPP1-FW: 5′-GTA CTA TGC CCG GTG CTC CAA C-3′; UPP1-RW: 5′-CTC TGC CTT GAA GCA GGA ATC CA-3′; PRPS1-FW: 5′-AAC GCA TGC TTT GAG GCA GTA GTA G-3′; PRPS1-RW: 5′-CTG ATG GCT TCT GCA AGG ATC ATA-3′; DPYD-FW: 5′-CAA CGT AGA GCA AGT TGT GGC TAT G-3′; DPYD-RW: 5′-AGT CGA CAA TAG GGC AAA CAC TGA-3′; TYMS-FW: 5′-ATC ATC ATG TGC GCT TGG AAT C-3′; TYMS-RW: 5′-TGT TCA CCA CAT AGA ACT GGC AGA G-3′; OPRT-FW: 5′-CTG GCT CCC GAG TAA GCA TGA-3′; OPRT-RW: 5′-CTG CTG AGA TTA TGC CAC GAC CTA-3′; TK1-FW: 5′-ATT CTC GGG CCG ATG TTC TC-3′; TK1-RW: 5′-GCG AGT GTC TTT GGC ATA CTT GA-3′; MTHFR-FW: 5′-GGA CAC TAC CTC ACC TGC CAG TAT C-3′; MTHFR-RW: 5′-CCA GAA GCA GTT AGT TCT GAC ACC A-3′; NP-FW: 5′-CAA CCT ACC TGG TTT CAG TGG TCA-3′; NP-RW: 5′-CCG GTC GTA GGC ATC AGA CA-3′; UCK2-FW: 5′-TTC GTC AAG CCT GCC TTT GAG-3′; UCK2-RW: 5′-TGG ATG TGC TGC ACG ATG AG-3′; GAPD-FW: 5′-GCA CCG TCA AGG CTG AGA AC-3′; GAPD-RW: 5′-ATG GTG GTG AAG ACG CCA GT-3′.

Results

Establishment and characterization of pancreatic cancer cell lines. Ten cell lines derived from human pancreatic cancers (Sui65, Sui66, Sui67, Sui68, Sui69, Sui70, Sui71, Sui72, Sui73 and Sui74) were newly established in the present study (Table 1). All the cell lines formed mono-layered sheets with clustering on confluence (Fig. 1 upper column). These cell lines exhibited the typical morphologic features of epithelial cells, characterized by sheets of polygonal cells in a pavement-like arrangement. The Sui65, 67, 68, 70 and 71 cell lines were found to secrete carbohydrate antigen19-9 (CA19-9), the concentration of which in the culture supernatants varied from 69 to 450 units/mL (Table 2). Production of TPA was also detected from all of the cell lines. The doubling times of the cell lines varied from approximately 20.8 to 55.2 h in the RPMI-1640 medium

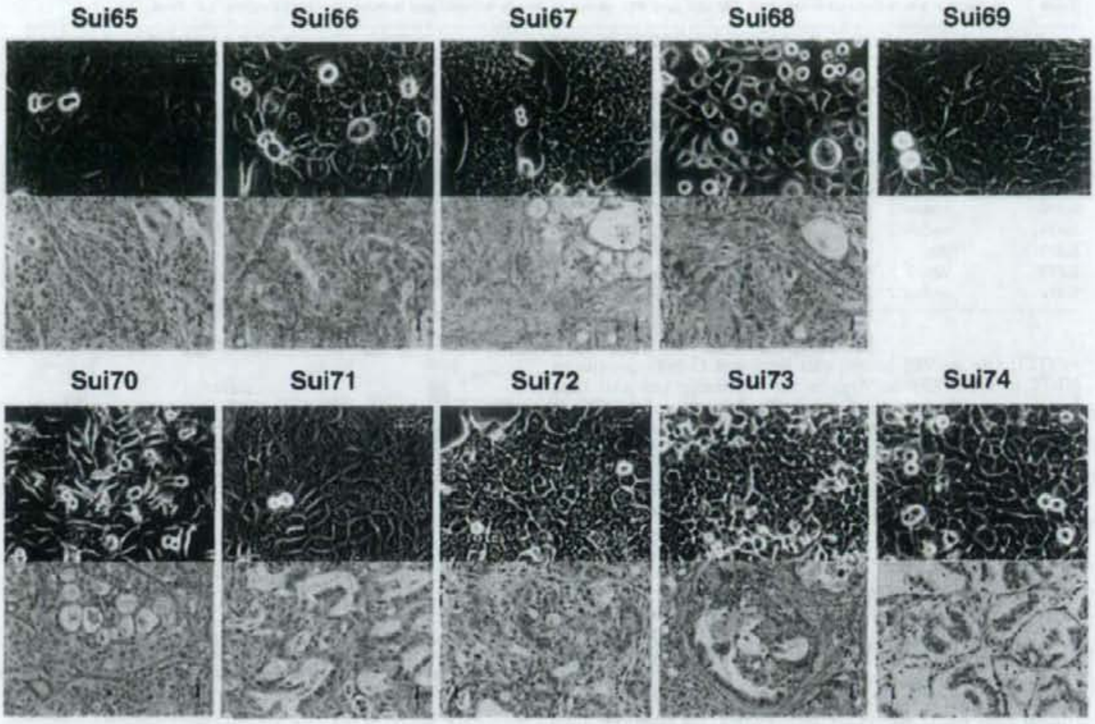

Fig. 1. Phase-contrast micrographs of the 10 established cell lines in this study (Sui65-Sui74) at the 25–30th passage (upper column). Original magnification, ×200. Histological section of a tumor established by s.c. injection of one of the cell lines into a SCID mouse (lower column). HE Stain, ×400.

Table 2. Biological characterization of newly established human pancreatic cancer cell lines

Cell line	Histological typing[†]		Growth[‡]				Tumor marker[§]			
	Original	Xenografts	Pattern	in CDM	in Agar	DT (h)	CEA ng/mL	CA19-9 u/mL	TPA u/L	CA125 u/mL
Sui65	Tub.ad.	Mod.tub.ad.	M	+	+	48.6	<0.5	69	2600	32.2
Sui66	Tub.ad.	Mod./well.	M	+	+	41.2	1.2	<6	1800	2.2
Sui67	Tub.ad.	Poor.tub.ad.	M	+	+	35.1	2.6	420	1600	1.5
Sui68	Ad.	Poor./mod.	M	+	+	43.3	5.1	450	980	3.2
Sui69	Tub.ad.	(–)	M	–	–	55.2	0.6	<6	2200	<1.0
Sui70	Ad.	Mod.tub.ad.	M	+	+	20.8	<0.5	290	14 000	7.5
Sui71	Ad.	Well.tub.ad.	M	+	+	24.1	<0.5	170	7100	9.3
Sui72	Tub.ad.	Mod.tub.ad. Sarcoma	M	+	+	27.8	31.6	<6	4400	<1.0
Sui73	Tub.ad.	Well./mod.	M	+	+	23.5	<0.5	<6	2400	1.5
Sui74	Ad.	Mucinos ca.	M	+	+	47.5	<0.5	<6	1800	13.5

[†] The tumorigenicity of the cell lines were tested by s.c. injection of 5×10^5 cultured cells suspended in 0.1 mL RPMI-1640 medium into mice. Histological typing of the pancreatic cancer was conducted in accordance with the 'General Rules for the Study of Pancreatic Cancer (1993)', as tub (tubular adenocarcinoma), or Muci. ca. (Mucinous carcinoma).

[‡] M, monolayer; CDM, composed of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) medium supplemented with 0.05% bovine serum albumin (BSA); +, positive; –, negative; DT, doubling time.
The doubling time of each line was determined as described previously.(28)

[§] Secretion of CEA, CA19-9, TPA and CA125 was tested by radioimmunoassay and immunoradioassay at SRL Laboratories (Tokyo, Japan).

supplemented with 10% FBS. When injected s.c., all of the cultured cell lines established from human pancreatic cancers, except for Sui69, survived and showed tumorigenicity (Fig. 1, lower column). These biological properties are summarized in Table 2. All the tumorigenic cell lines were also found to be strictly anchorage independent (60–90% efficiency).

Genetic alterations in the established pancreatic cancer cell lines. The results are summarized in Table 3. *K-ras* mutations were observed in eight of the 10 cell lines (80%). Activating mutations were detected in the 2nd base of codon 12 of *k-ras* in eight cell lines. The mutations were G-to-A transitions (GGT to GAT, Gly to Asp) in five cell lines, G-to-T transversions (GGT

Table 3. Molecular alterations of k-ras, p53, SMAD4 and P16 genes in newly established human pancreatic cancer cell lines

Cell line	Gene mutation				Gene expression	
	k-ras		p53		SMAD4/DPC4	p16
Sui65	codon12	GGT(Gly) → GAT(Asp)	codon248	CGG(Arg) → CAG(Gln)	–	–
Sui66	codon12	GGT(Gly) → GAT(Asp)	codon133	ATG(Met) → AAG(Lys)	+	–
Sui67	codon12	GGT(Gly) → GAT(Asp)	codon248	CGG(Arg) → TGG(Trp)	–	+
Sui68	codon12	GGT(Gly) → GAT(Asp)	codon245	GGC(Gly) → AGC(Ser)	+	–
Sui69	codon12	GGT(Gly) → GTT(Val)	codon175	CGC(Arg) → CAC(His)	–	–
Sui70	codon12	GGT(Gly) → GTT(Val)	codon175	CGC(Arg) → CAC(His)	–	–
Sui71	codon12	GGT(Gly) → GAT(Asp)	codon253	ACC(Thr) → CCC(Pro)	–	+
Sui72	Wt		codon135	TGC(Cys) → TAC(Tyr)	+	–
Sui73	Wt		Wt		+	–
Sui74	codon12	GGT(Gly) → CGT(Arg)	Wt	–	+	

to GTT, Gly to Val) in two cell lines and G-to-G transitions (GGT to GGT, Gly to Arg) in the remaining one cell line. Inactivating mutations of *p53* were found in eight of the 10 cell lines (80%), and included missense mutations.

Next, *SMAD4/DPC4* gene expression was checked by Western blot analysis. Marked expression of this gene was observed in Sui66 and Sui73, while the expression was less marked in Sui68 and Sui72; expression was altogether absent in the remaining six cell lines. Expression of the *P16* product was noted in Sui67, Sui71 and Sui74, but the levels were quite low or absent in the other cell lines (Table 3).

Therapeutic studies with 5-FU. Using this panel of human pancreatic cancer-derived cell lines, we evaluated the effect of 5-FU in suppressing the proliferation of each cell line *in vitro*. Sui69 was excluded from the analysis, since its proliferation rate was quite slow. The other nine cell lines were subjected to the MTT assay and the results are shown in Fig. 2. Sui72 was about 10-fold more susceptible to the drug, whereas the other cell lines were resistant to the drug.

Based on this result, we conducted a study *in vivo*, in which the highly drug-susceptible Sui72 and the resistant Sui70 were implanted subcutaneously in SCID mice. The 5-FU levels tested were 50 and 100 mg/kg/head. As shown in Fig. 3, tumor formation was markedly suppressed in all the mice implanted with Sui72. This suppressive effect was dose dependent, suggesting the effectiveness of 5-FU against this cell line. On the other hand, 5-FU did not inhibit the formation of tumor following implantation of Sui70, reflecting the findings *in vitro*.

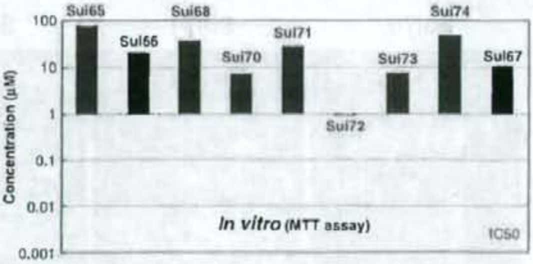

Fig. 2. Inhibition of growth of various human pancreatic cancer cell lines by 5-fluorouracil (5-FU) *in vitro*. The cell-growth inhibitory effects of 5-FU were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay as described elsewhere.[21]

We attempted to identify the cause of the hypersensitivity of Sui72-5-FU by RT-PCR (Table 4). The mRNA expression levels of genes associated with the metabolisms of 5-FU, i.e. DHFR, EPGS, ECGF (TP), UPP1, PRPS1, TYMS (TS), UMPS (OPRT), TK1, MTHFR, NP (PNP) and UCK2 (UMPK) were analyzed in Sui72

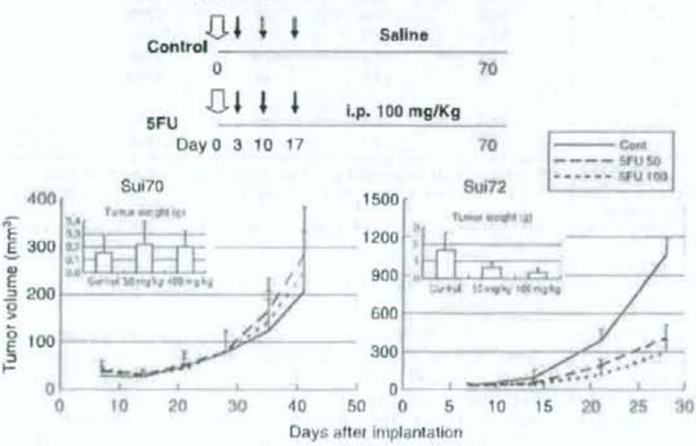

Fig. 3. Effect of 5-fluorouracil (5-FU) on Sui70 and Sui72 tumor growth in the SCID mouse. Six mice from each group were sacrificed when moribund, or on Day 28 or 40. The tumor mass was measured at predetermined time intervals in two dimensions with calipers, and the tumor volume was calculated according to the equation $(l \times w^2)/2$ [l = length, w = width].[18] Pancreatic carcinoma was confirmed by histopathology.

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Table 4. mRNA expression levels of genes related with 5-FU metabolism in a high sensitive Sui72 cells and in other human pancreatic cancer cell lines

Genes	Expression[†] The others[‡]	Expression[†] Sui72	Fold
DHFR	32.7 ± 37.8	33.1	1.0
FPGS	10.3 ± 18.3	n.d.	n.d.
TP	3.1 ± 4.6	0.1	0.0
UPP1	3.8 ± 5.0	1.6	0.4
PRPS1	9.1 ± 5.2	3.9	0.4
DPYD	0.6 ± 0.5	0.1	0.1
TYMS(TS)	49.9 ± 33.7	35.6	0.7
UMPS(OPRT)	7.2 ± 4.3	6.2	0.8
TK1	46.6 ± 46.3	28.4	0.6
MTHFR	4.4 ± 7.8	9.6	2.2
NP(PNP)	15.7 ± 8.0	23.3	1.5
UCK2(UMPK)	15.8 ± 9.7	29.7	1.9

n.d.: Not detectable by reverse transcription–polymerase chain reaction.

[†] Ratio of target gene/GAPD × 10-3 (fold).

[‡] The average ± SD of other cell lines except for Sui72.

as compared with other cell lines. Remarked decreased expression of DPYD was observed in Sui72 compared with other cell lines (0.6:/–0.5). These results suggest that lower expression of DPYD is the molecular determinant of high sensitivity to 5-FU in Sui72 cells.

Discussion

A number of cultured cell lines have been established from human pancreatic cancers(8–14) and have contributed greatly to advancing cancer research by allowing biological characterization (analysis of the features of proliferation, progression, etc.) of this cancer and being useful as a preclinical research tool in the evaluation of anticancer agents.(15,16) However, after multiple passages, some of these cell lines lose their initial properties (e.g. the histopathological features of the tumors formed following their implantation). There seems to be a universal necessity for enriching the research resources through establishment of new cancer cell lines which would reliably reflect the clinical features of this cancer. Pancreatic cancer is usually characterized by stromal cell infiltration, therefore it is relatively difficult to establish pancreatic cancer cell lines.(16) Bearing this in mind, we first attempted to establish pancreatic cell lines from the tumors formed in SCID mice following implantation of primary or metastatic pancreatic cancer tissue. In this way, we established 10 cell lines of the Sui series. Half of the 10 cell lines were positive for the tumor marker CA19-9, while all were positive for TPA. The histopathological profile of most of the 10 cell lines resembled that of the original tumor. These findings indicate that the cell lines of this Sui series are pancreatic cancer cell lines reliably reflecting the clinical features of pancreatic cancer. However, one of these cell lines (Sui69) did not form a tumor in the SCID mice, even though it was transplantable. This Sui69 cell line exhibited very slow proliferative activity. We are currently studying this cell line in NOD-SCID or NOG mice.

Pancreatic cancer cells often exhibit genetic alternations. Point mutation of the oncogene *K-ras*, loss of heterozygosity (LOH; 9p, 17p, 18q, 1q, etc.), point mutation of tumor suppresser genes at these chromosomal locations (*p16*, *p53*, *DPC4/AMAD4*, etc.), methylation of the promoter region, etc., have been reported in pancreatic cancer.(22–25) Of the 10 cell lines established in this study, *K-ras* point mutation(26,27) and *p53* mutation(28) were noted in eight cell lines, and the genetic alterations found resembled those seen in the clinical materials. In Sui73, both *K-ras* and *p53* were wild type. The *SMAD4/DPC4* gene is known to show a high frequency of alterations (50%), including mutation (20%) and deletion (30%).(29,30) Marked expression of the *SMDA4/DPC4* gene was observed in two cell lines, and less marked expression in two cell lines; expression was altogether absent in the remaining six cell lines. Analysis of expression of the *P16/CDKN2A/INK4A* product in the 10 established cell lines revealed its expression in three cell lines, but the expression was quite low or altogether absent in remaining seven cell lines. It has been suggested that in pancreatic cancer free of *P16* mutations or deletions, expression of this gene is absent because of abnormal methylation of the gene expression–adjusting region and that most pancreatic cancers show malfunctioning of *P16*.(30,31) These changes are seen commonly in many cases of pancreatic cancer and seem to determine the proliferative potential and tumorigenicity of pancreatic cancer cells.

When treating pancreatic cancer, surgical resection, one or a various combination of surgical resection, systemic chemotherapy and radiotherapy is selected depending on the stage of the cancer.(2,25,32) Conventionally, various regimens of adjuvant chemotherapy, primarily involving 5-FU, have been attempted,(3–5) but no valid means of treating pancreatic cancer have yet been established. We attempted to evaluate the efficacy of 5-FU (a drug used as a standard therapy for this cancer in the past) against the cell lines established by us in this study. When tested *in vitro*, Sui72 was susceptible to the drug, whereas the remaining eight lines (Sui70, etc.) were found to be resistant to the drug. This finding was endorsed by the results of the *in vivo* study. We then explored the molecular determinant of sensitivity of the Sui72 cell line to 5-FU. The results of the analysis suggest that the decreased expression of DPYD may be involved in the mechanism of cellular sensitivity to 5-FU. In addition, decreased expression of TYMS was also observed in Sui72 as compared with other cell lines. This observation might be consistent with high sensitivity to 5-FU of Sui72 cells.

Throughout this study, it was shown that the new cell lines of the Sui series are useful for research on pancreatic cancer (e.g. evaluation of pancreatic cancer cell proliferation and progression, evaluation of the efficacy of anticancer agents, and so on).

New anticancer agents (e.g. TS-1, which reinforces the efficacy of 5-FU),(33–35) and gemcitabine hydrochloride (GEM) have recently become available for clinical use,(36–38) with the expectation of extending the survival period of patients with pancreatic cancer. To date, however, no chemotherapeutic agents more efficacious than GEM for pancreatic cancer have been developed. Clinical studies have therefore been carried out, focusing on developing treatment regimens containing GEM in combination with some other drugs. If GEM were combined with TS-1 or other molecule-targeted drugs, further extension of the survival period of pancreatic cancer patients may be expected. In the near future, we propose to carry out preclinical studies to evaluate the efficacy of various anticancer agents in SCID mice implanted with the new cell lines derived from human pancreatic cancer and to identify the genes, etc. which determine the susceptibility of pancreatic cancer cells to anticancer agents. It also seems to be essential to develop a model of orthotopic implantation, with the goal of establishing a drug evaluation system more relevant to the clinical setting.(25)

In conclusion, we established 10 cell lines derived from human pancreatic cancers that were found to possess biological characteristics and genetic alterations unique to pancreatic cancer. These new cell lines are expected to be highly useful for analyzing the pattern of pancreatic cancer progression and evaluating the efficacy of anticancer agents.

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Evaluation of the Safety and Compliance of 3-Week Cycles of Vinorelbine on Days 1 and 8 and Cisplatin on Day 1 as Adjuvant Chemotherapy in Japanese Patients with Completely Resected Pathological Stage IB to IIIA Non-small Cell Lung Cancer: A Retrospective Study

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Objective: With regard to adjuvant chemotherapy for non-small-cell lung cancer, the usefulness of combined chemotherapy with cisplatin (CDDP) and vinorelbine (VNR) has been reported. However, poor compliance has been reported with VNR administered weekly by the conventional method for 16 consecutive weeks, and there is no report on the safety and compliance of adjuvant chemotherapy with CDDP and VNR in Japanese patients.

Methods: The subjects were 25 non-small-cell lung cancer patients who received CDDP and VNR as adjuvant chemotherapy at the Shizuoka Cancer Center between April 2005 and April 2008. The treatment schedule included combined treatment, with CDDP at 80 mg/m^2 administered on Day 1 and VNR at 25 mg/m^2 administered on Days 1 and 8. The treatment was repeated every 3 weeks, and each 3-week treatment schedule was designated as one cycle. A total of four cycles were administered.

Results: The main adverse events were Grade 3 or more severe neutropenia (76%), anemia (12%), anorexia (12%) and nausea (12%). Thus, the adverse events were mostly mild. There were no treatment-related deaths. The rate of completion of the four cycles was 92%. The mean dose of CDDP and VNR was 312 and 195 mg/m^2, respectively. The mean dose administered of either drug was 97.5% of the scheduled dose.

Conclusion: This study was retrospective and had some limitations, for example, non-hematological toxicity would be evaluated milder. However, it was considered that adjuvant chemotherapy with CDDP administered on Day 1 and VNR administered on Days 1 and 8 every 3 weeks was safe, and that the rate of completion of the four cycles was also satisfactory in Japanese patients.

Key words: adjuvant chemotherapy – non-small-cell lung cancer – cisplatin – vinorelbine – Japanese patients – safety – compliance

INTRODUCTION

The radical treatment for clinical stages IA–IIB and part of IIIA non-small cell lung cancer (NSCLC) is a surgical resection. However, 40–70% of patients with pathological stage IB to IIIA NSCLC who are treated by surgical resection develop recurrence and show a fatal outcome (1). Recurrence in these cases occurs even when all the lesions that are detectable by diagnostic imaging are totally resected; therefore, they have been considered to be caused by micrometastatic lesions already present at the time of resection. Based on the recognition of such lesions as the possible cause of recurrence, there have been some attempts to administer postoperative adjuvant chemotherapy to selected patients of NSCLC with the aim of reducing the mortality associated with postoperative recurrence.

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Before 2000, most clinical studies on postoperative adjuvant chemotherapy have failed to demonstrate its usefulness for survival (2–5). In these studies, the chemotherapeutic regimen included drugs that existed before the development of third-generation drugs, and the number of patients included was small. These factors seem to have had an influence on the failure of the treatments reported from the studies. In 1995, a meta-analysis of many previous clinical studies on postoperative adjuvant chemotherapy was performed, which suggested the usefulness of this strategy, because an improvement in the 5-year survival rate in the postoperative adjuvant chemotherapy group by 5% was observed as compared with that in the course observation group ($P = 0.08$) (6). Since the publication of this report, many studies have been conducted to investigate the usefulness of postoperative adjuvant chemotherapy, as follows: International Lung Cancer Trial (IALT) based on cisplatin (CDDP) (7); JBR 10 trial by the combined use of CDDP and vinorelbine (VNR); Adjuvant Navelbine International Trialist Association (ANITA) trial by the combined use of CDDP and VNR (8,9). The JBR 10 trial showed that CV (CDDP+VNR) improved the 5-year survival rate by 15% in patients with pathological stage IB to IIB NSCLC. The ANITA trial showed that CV improved the 5-year survival rate by 8.6% in patients with pathological stage IB to IIIA NSCLC. On the other hand, the Cancer and Leukemia Group B (CALGB) 9633 trial showed that combined therapy with carboplatin and paclitaxel improved the 4-year survival rate by 12% in patients with pathological stage IB NSCLC (10). A longer-term follow-up study, however, revealed no statistically significant improvement in the survival rate (11).

Based on the results of these studies, CDDP-based regimens, particularly CV, have been recommended as postoperative adjuvant chemotherapy for NSCLC (12). In the JBR 10 trial and ANITA trial, VNR was administered weekly for 16 weeks, and the median dose of VNR could be administered in only 52% of the patients. Gebbia et al. (13), who conducted a Phase III study of combined use of CDDP at 100 mg/m^2 administered on Day 1 plus VNR at 25 mg/m^2 administered on Days 1, 8 and 15 every 4 weeks and combined use of CDDP at 80 mg/m^2 administered on Day 1 plus VNR at 30 mg/m^2 administered on Days 1 and 8 every 3 weeks for Stage IIIB/IV NSCLC reported the absence of any significant difference in the survival period or the efficacy rate between the 3- and 4-week cycles of CV described above, and that the 3-week cycle showed excellent safety. It has also been confirmed that the combined use of CDDP at 80 mg/m^2 administered on Day 1 plus VNR at 25 mg/m^2 administered on Days 1 and 8 every 3 weeks is effective for Stage IIIB/IV NSCLC in Japanese patients (14). However, there is no report on the safety and compliance of postoperative adjuvant chemotherapy with CV administered in this schedule in Japanese patients.

Under these circumstances, we retrospectively assessed the safety and compliance of postoperative adjuvant chemotherapy with CDDP at 80 mg/m^2 administered on Day 1 plus VNR at 25 mg/m^2 administered on Days 1 and 8 every 3 weeks in Japanese patients.

PATIENTS AND METHODS

There were 26 patients with histopathologically or cytologically diagnosed NSCLC between April 2005 and April 2008 at the Shizuoka Cancer Center, who underwent surgical complete resection and then received postoperative adjuvant chemotherapy with the CV regimen. Based on the inclusion criteria [(i) age $\geq$18 years, (ii) pathological stage IB to IIIA and (iii) performance status (PS), 0–2] and exclusion criteria [(i) double cancer, (ii) serious complications such as active infectious diseases, serious heart diseases, poorly controlled hypertension diabetes mellitus and radiologically distinct interstitial pneumonia and (iii) prior chemotherapy] for the study, 25 patients were included as subjects for the present study and one patient was excluded because of prior neo-adjuvant chemotherapy. CDDP at 80 mg/m^2 was administered on Day 1 and VNR at 25 mg/m^2 was administered on Days 1 and 8. The combined use of these drugs was repeated every 3 weeks, and each 3-week treatment schedule was designated as one cycle. As a rule, patients were in the hospital from Day 1 to 3 of chemotherapy and thereafter they were discharged and administered VNR on Day 8 in outpatient setting. A total of four cycles were administered. In regard to the antiemetic therapy, granisetron plus dexamethasone were used according to the ASCO guidelines. Granulocyte colony-stimulating factor was used when patients with febrile neutropenia or Grade 4 neutropenia were judged as requiring its administration by the physician-in-charge. Dose reduction of the anticancer drugs was based on the judgment of the respective physicians-in-charge, but, as a rule, when Grade 3 or more severe non-hematotoxicity or Grade 4 hematotoxicity appeared, the doses of CDDP and VNR in the subsequent cycles were reduced to 60 and 20 mg/m^2, respectively. If further dose reduction was required, the treatment was discontinued. Complete blood count and biochemistry were usually examined at least once per week.

These patients were examined for the patient background characteristics, adverse events, treatment compliance and relapse-free survival. Adverse events were evaluated until 4 weeks after the completion of chemotherapy according to the Common Terminology Criteria for Adverse Events (CTCAE) Version, 3.0. Relapse-free survival was defined as time from the first administration of adjuvant chemotherapy to relapse or death, and calculated using Kaplan–Meier method.

RESULTS

Table 1 shows the patient characteristics. The number of male patients, 18 (72%), was larger than that of the female patients, and the median age of the patients was 62 years. The number of patients with Stage IIIA was the largest, that is, 10 (40%). In relation to the histological type of the lesions, ~2/3 of all the patients had adenocarcinoma. Most of the patients had undergone lobectomy as the surgical procedure, and most had a PS of 0. The interval from the operation to the start of this treatment ranged from 29 to 79 days, with a median of 41 days.

All the four cycles could be completed in 23 of the 25 patients (92%). The treatment was discontinued in two of the 25 patients (8%): it was discontinued after one cycle because of prolonged myelosuppression in one patient, and the Day 8 dose in the fourth cycle was omitted because of Grade 3 neutropenia and the study period ended without its administration because of prolonged myelosuppression in the other patient. There was no patient in whom the treatment was discontinued because of treatment rejection by the patient, recurrence or death. The mean cumulative dose of CDDP and VNR was 312 (97.5% of the scheduled dose) and 195 mg/m^2 (97.5% of the scheduled dose), respectively.

Table 2 shows the adverse events. The Grade 3 or 4 adverse events observed in the patients included neutropenia in 76%, leukopenia in 20%, anemia in 12%, anorexia in 12% and nausea in 12% of the patients. The dose needed to be reduced because of the development of adverse events in five patients. The reasons for dose reduction were Grade 3 elevation of AST and ALT in first cycle in one patient, Grade 3 fatigue and Grade 4 neutropenia in second cycle in another patient, Grade 4 neutropenia and Grade 1 elevation of the serum creatinine in first cycle in another patient, Grade 3 febrile neutropenia and Grade 1 elevation of the serum creatinine in first cycle in another patient, Grade 1 elevation of the serum creatinine in third cycle in the other patient. There was no patient who died within 30 days of the treatment, or there were no treatment-related deaths.

Table 1. Patient characteristics ($n = 25$)

Gender [No. (%)]	
Male	18 (72%)
Female	7 (28%)
Age (year)	
Median	62
Range	39–74
Stage [No. (%)]	
IB	1 (4%)
IIA	8 (32%)
IIB	6 (24%)
IIIA	10 (40%)
Histology [No. (%)]	
Ad.	16 (64%)
Sq.	4 (16%)
Large	3 (12%)
Others	2 (8%)
Operation [No. (%)]	
Lobectomy	22 (88%)
Pneumonectomy	3 (12%)
PS	
0	19 (76%)
1	6 (24%)

Ad, adenocarcinoma; Sq, squamous cell carcinoma; PS, performance status.

Table 2. Adverse events ($n = 25$)

	Gr1	Gr2	Gr3	Gr4	Gr3–4
Leukopenia	4	11	5	0	20%
Neutropenia	0	2	10	9	76%
Anemia	10	11	3	0	12%
Thrombocytopenia	3	1	0	0	0
Fatigue	15	4	1	0	4%
Anorexia	10	11	3	0	12%
Nausea	12	6	3	0	12%
Vomiting	8	3	1	0	4%
Constipation	7	[illegible]	0	0	0
Hiccough	0	8	0	0	0
Alopecia	6	1	0	0	0
AST	2	0	1	0	4%
ALT	7	0	1	0	4%
Creatinine	8	0	0	0	0
Febrile neutropenia	0	0	1	0	4%
Infection	0	1	0	0	0
Phlebitis	0	5	0	0	0

Gr, grade; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

The Kaplan–Meier curve of relapse-free survival is shown in Figure 1. There were seven events and all of them were relapse. Median relapse-free survival had not been reached. Estimated 1 year relapse-free survival was 67.3%. One patient died because of disease progression after relapse of lung cancer.

DISCUSSION

The major trials on postoperative adjuvant therapy using the combination regimen of CDDP and VNR include the JBR 10 trial and the ANITA trial. In the present study, CDDP at 80 mg/m^2 was administered on Day 1 and VNR at 25 mg/m^2 was administered on Days 1 and 8 every 3 weeks, and each 3-week treatment schedule was designated as one cycle. In the JBR 10 trial, CDDP at 50 mg/m^2 was administered on Days 1 and 8 and VNR at 30 mg/m^2 was administered on Days 1, 8, 15 and 22 every 4 weeks, and each 4-week treatment schedule was designated as one cycle. In this trial, however, the dose of VNR was reduced to 25 mg/m^2 because