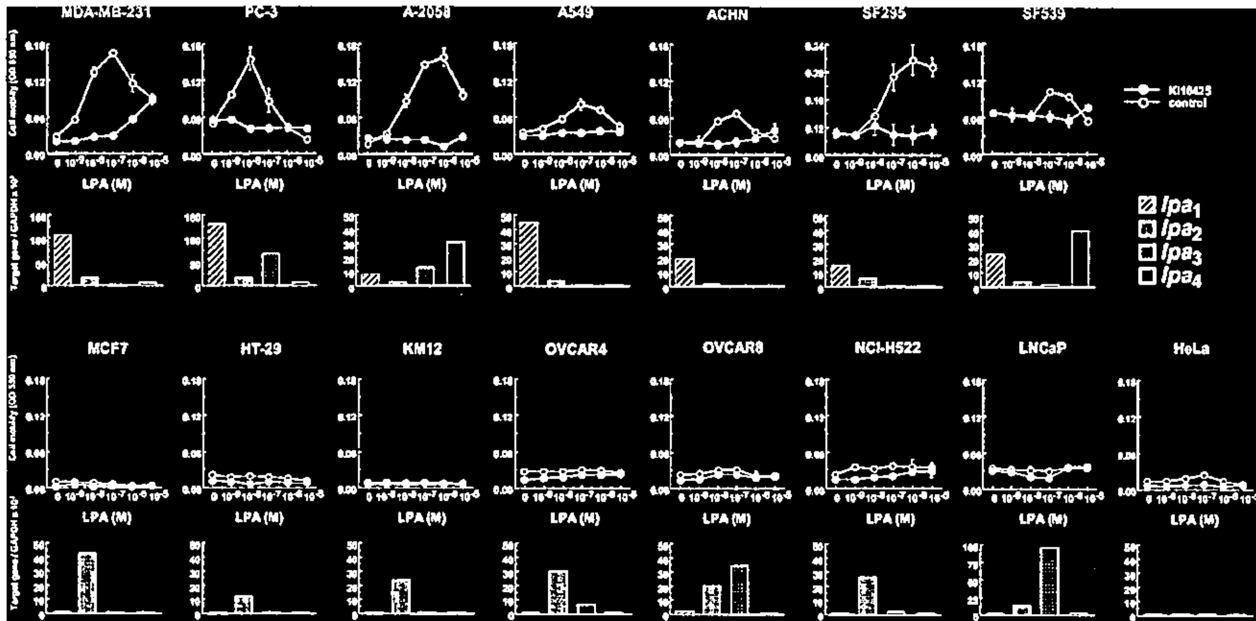

FIG. 4. **LPA_1 is involved in LPA-induced cell motility in multiple carcinoma cell lines.** In each cell line, the *upper panel* shows migratory response to LPA either in the absence (*open circles*) or presence (filled circles) of an LPA_1-selective antagonist, Ki16425 (1 M), evaluated by the Boyden chamber assay. The *lower panel* shows expression of each LPA receptor mRNA (lpa_1, lpa_2, lpa_3, and lpa_4) measured using quantitative real-time RT-PCR. The LPA-induced migratory responses appear to be parallel with the expression of LPA_1.

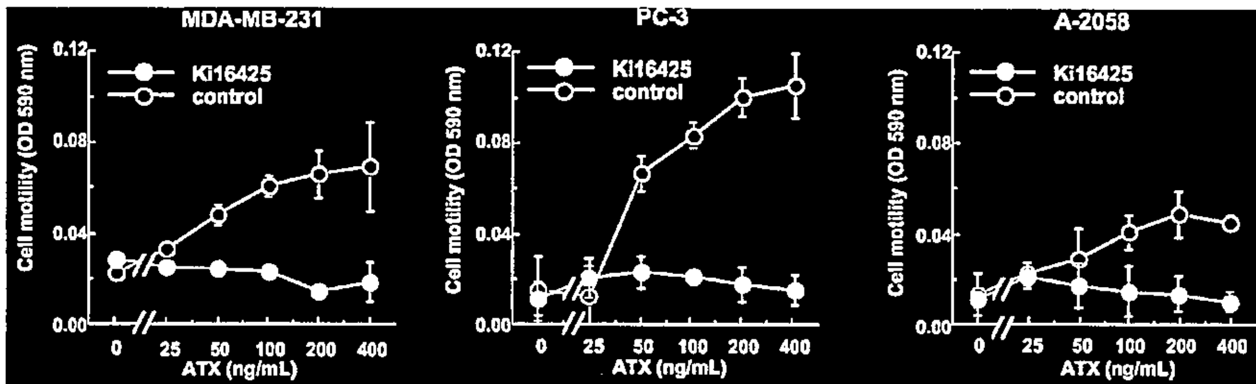

FIG. 5. **Ki16425 inhibits ATX-induced cell motility of carcinoma cell-expressing LPA_1.** ATX stimulates cell motility of three LPA_1-expressing carcinoma cell lines (MDA-MB-231, PC-3, and A-2058), and the motility is suppressed by an LPA_1-selective antagonist, Ki16425.

reduced in lpa_1 / MSF cells and markedly reduced in lpa_1 / - lpa_2 / MSF cells (Fig. 8). These results indicate that LPA_1 has a major role in both Rac1 and RhoA activation induced by LPA stimulation (Fig. 9). The Rac1 activation is predominantly dependent on LPA_1, whereas RhoA activation is less dependent on LPA_1. LPA_2 does contribute to the activation of RhoA in the absence of LPA_1 expression. In addition, RhoA can be activated to some extent (Fig. 8) in the absence of LPA_1 and LPA_2, indicating the presence of other LPA receptors and/or indirect mechanisms of RhoA activation (Fig. 9).

DISCUSSION

LPA is a multifunctional signaling molecule with diverse activities, including stimulation of cell motility. Recent identification of lysophospholipase D, an LPA-producing enzyme, as ATX, a cell-motility stimulating factor of cancer cells (10, 11), indicated that the activity is one of the intrinsic functions of LPA. In this study we showed that among the four LPA receptors identified so far, LPA_1 has a crucial role in LPA-induced cell migration of fibroblast cells (Fig. 2) and multiple cancer cells (Fig. 4), based on the observation that inactivation of LPA_1 either by gene-targeting technique or by a receptor-selective antagonist resulted in loss of migratory response. In addition, we observed that the migratory response induced by ATX again disappeared in these cells (Figs. 3 and 5). These results clearly show that ATX exerts its function through LPA production and the following LPA_1 activation. Both LPA_1 and ATX are highly expressed in brain (14, 29). Recently it was reported that oligodendrocytes express LPA_1 and ATX during myelination (30, 31). We also found that LPA_1 and ATX are highly enriched within certain regions of developing mouse brain, such as olfactory bulb.[2] The colocalization of the two genes suggests that they also function co-operatively in physiological condition.

The migratory responses were found to be PTX-sensitive (Fig. 1A). By contrast, in a previous report LPA was found to stimulate cell motility of other cell types, such as lymphoma

[2] M. Fukaya, M. Watanabe, J. Aoki, and H. Arai, unpublished result.

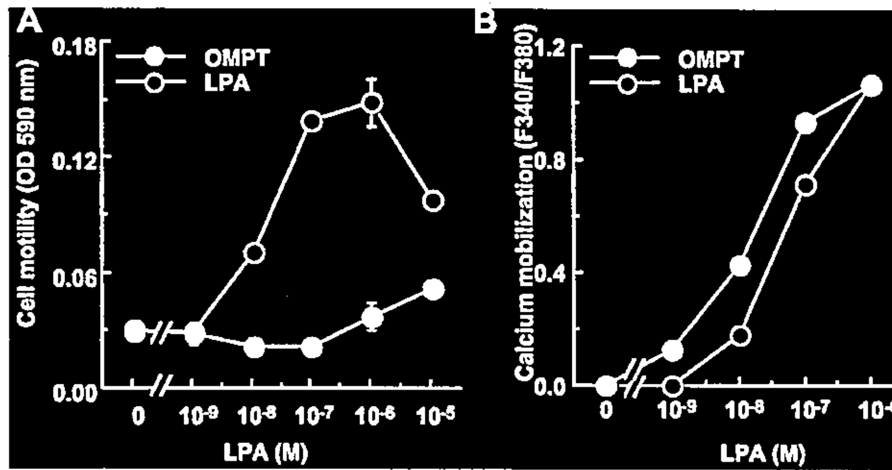

FIG. 6. LPA_3 is not involved in LPA-induced cell motility. *A*, OMPT did not stimulate cell motility of LPA_3-expressing cell, A-2058. *B*, OMPT induced an intracellular calcium mobilization of the cells more efficiently than LPA.

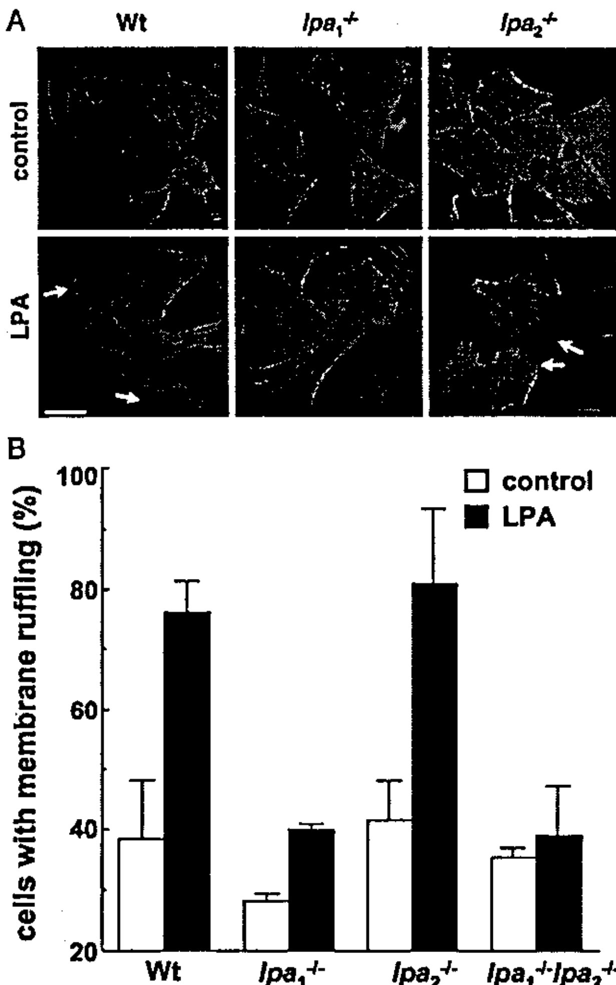

FIG. 7. LPA induces lamellipodia formation through LPA_1. *A*, fluorescence microscopy of BODIPY FL phallacidin-stained MSF cells from wild-type, lpa_1 $'$, and lpa_2 $'$ mice before (*upper panels*) or after (*lower panels*) LPA stimulation (1 M, 3 h). The lamellipodia formation (*arrows*) was observed in wild-type and lpa_2 $'$ MSF cells but rarely observed in lpa_1 $'$ MSF cells. *Bar*, 20 m. *B*, percentage of wild-type, lpa_1 $'$, lpa_2 $'$, and lpa_1 $'$ lpa_2 $'$ MSF cells with lamellipodia after stimulation with 1 M LPA.

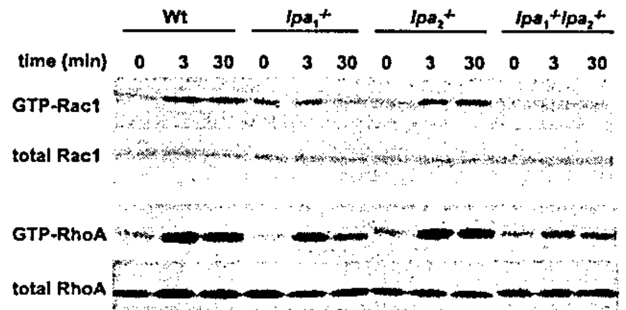

FIG. 8. LPA-induced activation of Racl and RhoA in MSF cells. MSF cells were serum-starved for 24 h and stimulated with 1 M LPA. Activated RhoA and Racl were isolated using GST-PAK (*Racl, upper panel*) or GST-Rhotekin (*RhoA, lower panel*) coupled to glutathione-Sepharose beads. Racl and RhoA bound to the beads were detected by Western blotting using specific antibodies.

cells, in a PTX-insensitive manner (6). Because lymphocytes express LPA_2 predominantly with no detectable expression of LPA_1 (data not shown), it is possible that LPA_2 is involved in LPA-induced migratory response of non-carcinoma neoplasms, such as lymphoma cells. Splenocytes and thymocytes isolated from wild-type, lpa_1 $'$, lpa_2 $'$, and lpa_1 $'$ lpa_2 $'$ mice did not show a migratory response to LPA in our system (data not shown). In addition, in the absence of selective agonists or antagonists for LPA_2, we could not test the migratory effect of LPA_2 on lymphoma cell migration. Further study is necessary to show the role of LPA_2 in migratory response of lymphoma cells.

We previously showed that LPA_1 and LPA_2 had redundant functions in mediating multiple endogenous LPA responses, including phospholipase C activation, Ca^2 mobilization, cell proliferation, and stress fiber formation in mouse embryonic fibroblasts (20). In this study, we demonstrated that LPA-induced lamellipodia formation was severely affected in lpa_1 $'$ MSF cells (Fig. 7, *A* and *B*). In addition, we showed that LPA activates Racl in an LPA_1-dependent manner, whereas it activates RhoA either in LPA_1-, LPA_2-dependent or LPA_1-, LPA_2-independent pathway (Fig. 8). Thus, LPA_1 is able to activate both Racl and RhoA regardless of LPA_2 expression (Fig. 9). Because the activation of both RhoA and Racl is essential for LPA-stimulated cell migration (12), the lack of Racl activation in lpa_1 $'$ MSF cells explains why the cells could not migrate in response to LPA. Many reports have

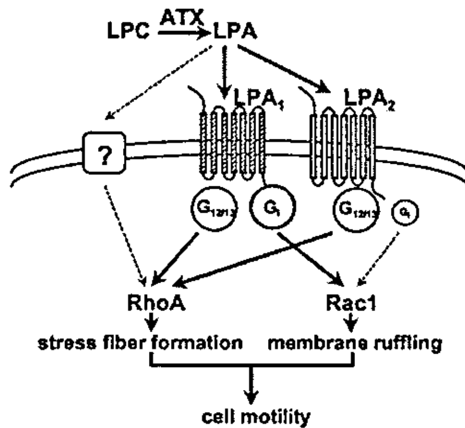

FIG. 9. **Model for LPA- or ATX-induced cell motility.** ATX activates Rac1 and RhoA through G_i and $G_{12/13}$, respectively, by producing LPA. The activation of Rac1 and RhoA results in membrane ruffling and stress fiber formation, which finally lead to activation of cell motility. The Rac1 activation is predominantly dependent on LPA_1, whereas RhoA activation is induced by either LPA_1 or LPA_2 activation. LPA_1- and LPA_2-independent pathways also contribute partially to RhoA activation.

shown that $G_{i/o}$ and $G_{12/13}$ regulate the activation of Rac1 and RhoA, respectively (12, 32). Thus, our results again suggest that LPA_1 couples with both $G_{i/o}$ and $G_{12/13}$, whereas LPA_2 mainly couples with $G_{12/13}$ as we indicated previously using cells transfected with each LPA receptor (Fig. 9) (20, 33). van Leeuwen *et al.* (12) recently showed that LPA_1, when overexpressed in B103 neuroblastoma cells, mediates LPA-induced cell migration through concomitant activation of Rac1. However, the role of other LPA receptors in cell motility remained to be solved, which we did in this study (Figs. 2 and 8). We showed that multiple carcinoma cells utilize the same mechanism for their LPA-induced cell motility (Fig. 4). It is well accepted that cell motility is closely linked to the metastatic and invasive potential of cancer cells. In addition, the activating pathways of both Rac1 and RhoA were also implicated in tumor invasion and metastasis (34, 35). Furthermore, there is accumulating evidence that elevated expression of ATX is observed in various cancer tissues (36, 37) and that the expression is closely linked to the invasive and metastatic potency of cancer cells (38). We therefore propose that both ATX and LPA_1 are the potential targets for cancer therapy.

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Csk defines the ability of integrin-mediated cell adhesion and migration in human colon cancer cells: implication for a potential role in cancer metastasis

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Progression of human colon cancer is often associated with elevated expression and activity of the Src family tyrosine kinase (SFK). SFK is ordinarily in equilibrium between inactive and primed states by a balance of negative regulatory kinase Csk and its counteracting tyrosine phosphatase(s), both of which act on the regulatory C-terminal tyrosine of SFK. To evaluate the contribution of the regulatory system of SFK in cancer progression, we here modulated the equilibrium status of SFK by introducing wild-type or dominant-negative Csk in human epithelial colon cancer cells, HCT15 and HT29. Overexpression of wild-type Csk induced decreased SFK activation, increased cell–cell contacts mediated by E-cadherin, decreased the number of focal contacts and decreased cell adhesion/migration and *in vitro* invasiveness. Conversely, expression of a dominant-negative Csk resulted in elevated SFK activation, enhanced phosphorylation of FAK and paxilllin, enhanced cell scattering, an increased number of focal contacts, dramatic rearrangement of actin cytoskeleton and increased cell adhesion/migration and *in vitro* invasiveness. In these scattered cells, however, localization, expression and phosphorylation of either E-cadherin or β-catenin were not significantly affected, suggesting that the E-cadherin-mediated cell–cell contact is indirectly regulated by SFK. Furthermore, all these events occurred absolutely dependent on integrin-mediated cell adhesion. These findings demonstrate that Csk defines the ability of integrin-SFK-mediated cell adhesion signaling that influences the metastatic potential of cancer cells.
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Keywords: Src; Csk; colon cancer; integrin

Introduction

The Src family tyrosine kinase (SFK) has been shown to play an important role in proliferation, differentiation, adhesion and migration of a variety of animal cells. These diverse functions are due to the pivotal role of SFK as membrane-attached molecular switches that link a variety of extracellular cues to crucial intracellular signaling pathways (Thomas and Brugge, 1997). The kinase activity of c-Src is known to be highly elevated in the majority of human colon cancers, and there is an increasing trend during further disease progression, with the highest values in metastasis disease (Talamonti *et al.*, 1993). In addition, some types of adenomas and ulcerative colitis, which possess a high risk of developing malignant disease, also course with increased c-Src activity (Cartwright *et al.*, 1994). This increased c-Src activity does not appear to result solely from an increase in the amount of the c-Src protein, suggesting that its specific activity is enhanced in some cases (Bolen *et al.*, 1987; Cartwright *et al.*, 1989). A subset of colon cancers has a nonsense mutation in the C-terminal regulatory domain of c-Src. This mutation has been demonstrated to be activating, tumorigenic and to promote metastasis (Irby *et al.*, 1999). However, since the frequency of this mutation appears to be very low, it seems unlikely to play a significant role in the progression of most colon cancers (Daigo *et al.*, 1999; Nilbert and Fernebro, 2000; Wang *et al.*, 2000; Laghi *et al.*, 2001). On the other hand, some cases of colon cancer have shown an upregulation of other members of SFK, such as Yes and Lck, suggesting that SFK upregulation in cancer cells might be a more common event (Veillette *et al.*, 1987; Boardman and Karnes, 1995).

The C-terminal Src kinase (Csk) is a cytoplasmic tyrosine kinase that serves as an essential and a common negative regulator of SFK through specific phosphorylation at the C-terminal regulatory site of SFK (Tyr-529 in human c-Src) (Okada and Nakagawa, 1989; Nada *et al.*, 1991). The phosphorylated C-terminal tyrosine binds intramolecularly to the SH2 domain of SFK, thereby generating an inactive conformation (Okada *et al.*, 1991; Sabe *et al.*, 1992a). The importance of Csk as a negative regulator for SFK and the absence of functional redundancy have been demonstrated by the observation that Csk knockout mice, which are lethal at prenatal stage, exhibited constitutive activation of multiple SFK members (Imamoto and Soriano, 1993; Nada *et al.*, 1993). It is

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now believed that the activity of SFK is determined by an equilibrium between an inactive (phosphorylated) and a primed (dephosphorylated) state regulated by a balance of Csk and its counteracting tyrosine phosphatase(s). Upon cell stimulation, SFKs at a primed state become functionally activated to relay signals into the cells. Thus, perturbation of the equilibrium status of SFK, potentially induced by up- or downregulation of Csk and/or a tyrosine phosphatase(s) during development and/or cancer progression, may greatly affect the sensitivity of the cells to extracellular cues. In relation to cancer progression, it has been reported that c-Src-induced transformation of cells bearing v-Crk was suppressed by Csk overexpression (Sabe *et al.*, 1992b). In addition, downregulation of Src kinase activity by adenovirus-mediated csk gene transfer has been shown to abrogate the metastatic phenotype of colon cancer cells (Nakagawa *et al.*, 2000). We previously reported a reduction of Csk protein in about 60% of human colon cancer cases in which there was an elevation of Src activity, indicating that reduction of Csk may be one of the causes of the elevated specific activity of c-Src (Rengifo-Cam *et al.*, 2001). These findings suggest that Csk could be involved in the regulation of some cancer progression, through controlling the activity status of multiple SFKs. However, the precise function of Csk in human colon cancer cells has not been yet established. To address this issue, we examined the effects of modulation of Csk function on the phenotypes of human colon cancer cells. We here show that the equilibrium status of SFK controlled by Csk could control the integrin-mediated cell adhesion and migration implicated in the metastatic potential of human colon cancer cells.

Results and discussion

Expression and activity of SFK in human epithelial colon cancer cells

We first analysed protein expressions of Csk and SFK and the activity status of SFK in human colon cancer cell lines (Dan *et al.*, 2002), HCC2998, HCT15 and HT29 (Figure 1a). Consistent with previous observations (Rosen *et al.*, 1986; Rengifo-Cam *et al.*, 2001), HCT15 and HT29 cells showed substantial elevation of c-Src expression and a reduced expression of Csk compared with HCC2998 cells, a highly differentiated adenocarcinoma cell line (Kobayashi *et al.*, 1999). It was also shown that all these cells express multiple members of SFK including Yes, Fyn, Lyn and Lck, although there were no dramatic changes in their expressions among them. The activity status of SFK was then assessed with anti-Src pY418 antibody that can recognize autophosphorylated tyrosines of SFKs (SFK pY418), since SFK autophosphorylation is directly associated with its function (Sabe *et al.*, 1992a, b). HCC2998 cells demonstrated only faint signals for SFK autophosphorylation, while HCT15 and HT29 cells showed substantial phosphorylation on SFKs potentially corresponding to c-Src and c-Yes. In contrast, phosphorylations at the negative regulatory sites, Y529,

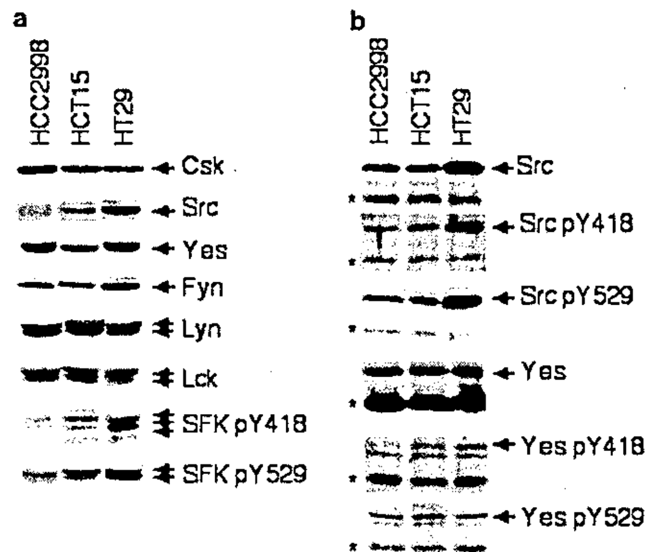


Figure 1 Expression and phosphorylation status of SFK in human colon cancer cells. (**a**) Total cell lysates from HCC2998, HCT15 and HT29 cells were obtained using ODG buffer, and equal amounts (20 μg protein) were analysed by Western blotting using specific antibodies against the indicated proteins. Anti-Src pY418 and pY529 detected multiple members of phosphorylated SFKs indicated by arrows. (**b**) c-Src and c-Yes proteins were immunoprecipitated using total lysate (500 μg protein) from the cancer cells, and probed with anti-Src, anti-Yes, anti-Src pY418 and anti-Src pY529 antibodies. Asterisks indicate the bands of immunoglobulin heavy chain

were steadily detected in all cell types with the highest levels in HT29 cells. To verify the phosphorylation levels of individual SFK members, c-Src and c-Yes proteins were immunoprecipitated and subjected to Western blotting (Figure 1b). In HT29 cells, c-Src was substantially phosphorylated at both Y418 and Y529, revealing that some c-Src are at primed state, while others are downregulated by Csk. In the case of c-Yes, phosphorylation at Y418 was the most evident in HT29 cells, while Y529 appeared to be less phosphorylated in the same cell line. These observations demonstrate that not only c-Src but also some other SFKs including c-Yes are basally primed in advanced cancer cells such as HT29, but only slightly activated in highly differentiated HCC2998 cells. This raises the possibility that perturbation of the balance between SFK and Csk, potentially achieved by elevated expression of SFK, downregulation of Csk or both, might influence the activity status of SFK in cancer cells. In order to address the role of such regulatory system of SFK in cancer progression, we here modulated the SFK–Csk balance by introducing Csk into HCT15 and HT29 cells, and observed its effects on the activity status of SFK and the phenotypic features of cancer cells.

Modulation of SFK by Csk influences cell morphology, cell–cell interaction, cell motility and in vitro *invasiveness in colon cancer cells*

We introduced wild-type Csk (Csk K+) or kinase-negative mutant of Csk (Csk K) in HCT15 and HT29

cells using an adenovirus expression system (Takayama *et al.*, 1999). Adenovirus infection readily induced protein expression in almost 100% of the cells, and did not affect the viability or growth rate of the cells (data not shown). The expression of Csk K+ suppressed the autophosphorylation of SFK (pY418) while that of Csk K enhanced it (Figure 2a), demonstrating that Csk K+ suppressed the activity of SFK and Csk K acted dominant negatively in inducing the activation of SFK. The effect of Csk overexpression on the morphologies of HCT15 and HT29 cells were observed by phase contrast microscopy (Figure 2b). Overexpression of Csk K+ in either cell types induced a dramatic change in cell morphology; the majority of cells formed compact cell aggregates (Figure 2bB and G). On the other hand, cells expressing Csk K (Csk K cells) appeared scattered and exhibited a mesenchymal-like phenotype, although HT29

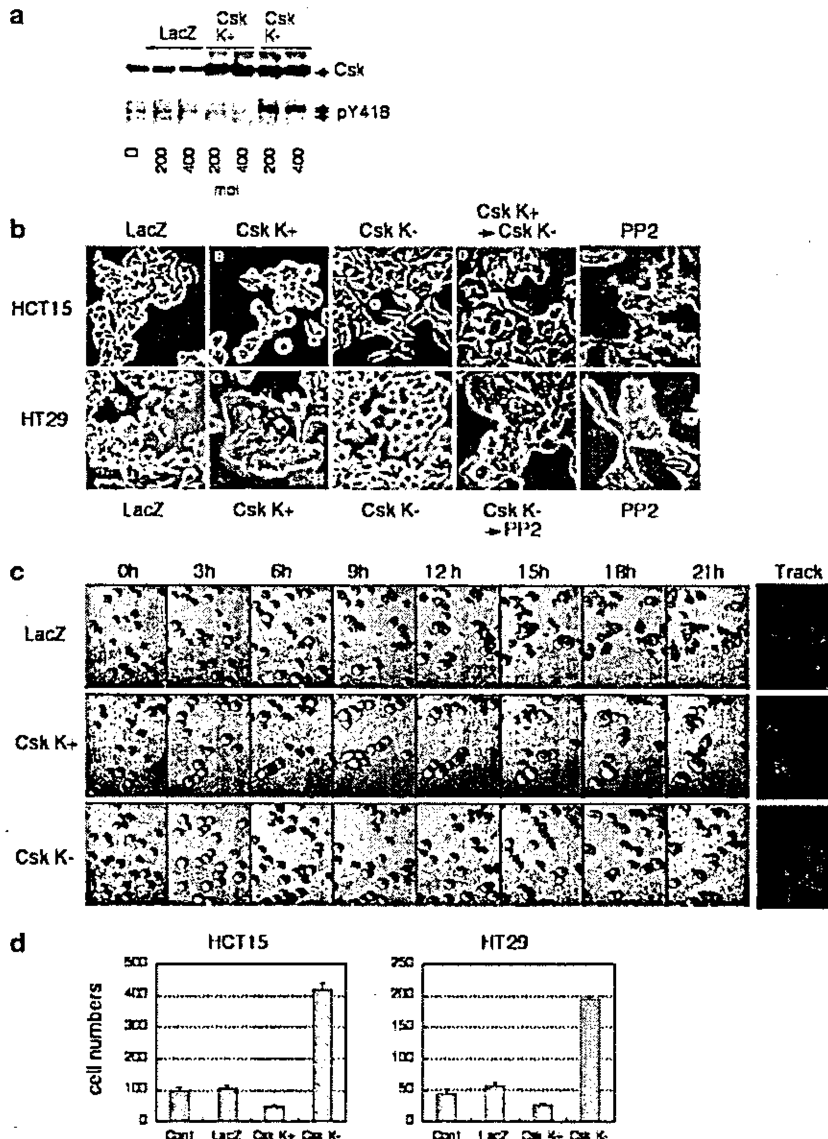


Figure 2 Effect of adenovirus-mediated overexpression of Csk on cell morphology, cell–cell interaction, cell motility and *in vitro* invasiveness in colon cancer cells. (**a**) Expression of Csk protein induced by adenovirus-mediated gene transfer. HCT15 cells were infected with indicated m.o.i. of adenoviruses carrying LacZ, wild-type Csk (Csk K+) and kinase-negative mutant Csk (Csk K), and the total cell lysates were subjected to Western blotting with anti-Csk (upper panel) and anti-Src pY418 (lower panel) antibodies. Location of Csk and phosphorylated SFKs are indicated by arrows. (**b**) Phase-contrast pictures were taken from HCT15 and HT29 cells after 48 h treatment with LacZ (A and F), Csk K+ (B and G) or Csk K (C and H), respectively. HCT15 cells treated with Csk K+ were subjected to secondary treatment with Csk K (D). HCT15 cells were incubated with 10 μM PP2 for 24 h (E). HT29 cells treated with (I) or without (J) Csk K were incubated with 10 μM PP2 for 24 h. (**c**) HT29 cells infected with indicated viruses for 24 h were replated, and their cell movements was monitored for 21 h by time-laps video microscopy. Images at 3 h intervals are shown. In each cell type, five cells were arbitrarily selected and tracked to assess the ability of cell motility using Move-tr/2D software (right panels). (**d**) *In vitro* invasiveness of HCT15 and HT29 cells without virus infection (control) or those infected with virus carrying LacZ, Csk K+ and Csk K . Cells (1.0 10^5) were seeded into matrigel invasion chambers. After 48 h, membranes were detached and cells were stained and counted under light microscopy. Results are expressed as mean values of three experiments

cells still exhibited round shape (C and H). When the aggregated HCT15 cells expressing Csk K+ were subsequently infected with virus carrying Csk K , the cells became scattered again, demonstrating that the morphological changes can be regulated reversibly (D). As reported previously (Nam *et al.*, 2002), treatment with PP2, a specific inhibitor of SFK, induces cell aggregation in both cell types (E and J). Cell aggregation could also be induced by the PP2 treatment in HT29 cells expressing Csk K (I). The morphology of PP2-treated cells is somewhat similar to that of Csk K+ cells, supporting the idea that morphological changes induced by Csk expression are mediated by the modulation of SFK activity.

To further characterize Csk-expressing cancer cells, the infected HT29 cells were replated onto collagen-coated dishes and their behaviors were observed for 21 h under time-laps video microscopy. The images at 3 h intervals and the tracks of five cells in each cell type are shown in Figure 2c. Overexpression of Csk K+ appeared to attenuate cell motility, and also the cells tended to associate with each other resulting in the formation of compact cell aggregates. In contrast, the Csk K cells actively migrated without forming the cell aggregates. We then assessed the *in vitro* invasive potential of the infected HCT15 and HT29 cells by matrigel assay (Figure 2d). The Csk K cells efficiently invaded matrigel, whereas the invasiveness of Csk K+ cells was rather suppressed. This supports the previous observation showing that adenovirus-mediated csk gene transfer can abrogate the metastatic phenotype of colon cancer cells *in vivo* (Nakagawa *et al.*, 2000). Taken together, these observations suggest that the activity status of SFK regulated by Csk might influence the ability of cell migration implicated in the metastatic potential of human colon cancer (Avizienyte *et al.*, 2002).

Modulation of SFK by Csk influences integrin-SFK-mediated cell adhesion signaling

We then addressed the molecular mechanisms that determine the phenotypes of the infected HT29 cells. When cells were plated onto culture dishes coated with collagen I, evident changes in morphology were observed again; overexpression of Csk K+ induced formation of large cell aggregates, while that of Csk K facilitated the cell scattering and destabilized cell–cell contacts (Figure 3aA–C). However, when cells were plated onto laminin-coated dishes, the morphological change became more moderate and large cell aggregates were stably formed even in Csk K cells (Figure 3aD–F). These findings suggest that the effects of Csk are dependent on cell adhesion mediated by specific integrins that can respond to collagen I. To confirm this, cell adhesion assay on collagen I was performed. As shown in Figure 3b, cell adhesion was apparently suppressed by the expression of Csk K+, while it was greatly enhanced by that of Csk K . The changes in tyrosine phosphorylation of cellular proteins during cell adhesion process were then examined by Western blotting (Figure 3c). In suspended cells, SFK is phosphorylated dominantly at Y529 in Csk K+ cells, while in Csk K cells, it is phosphorylated at Y418. This can be attributed to a change in the equilibrium of SFK to an inactive state in Csk K+ cells and to a primed state in Csk K cells. Upon cell adhesion, the primed SFK in Csk K cells became functionally activated resulting in a dramatic increase in tyrosine phosphorylation of several SFK targets including FAK and paxillin, which play crucial roles in the formation of focal contacts. Phosphorylation of FAK Y397 was also induced, reflecting the activation of FAK implicated in the regulation of cell migration and invasion (Hauck *et al.*, 2002). In contrast, inactive state of SFK in Csk K+ cells cannot be activated through integrin signaling, and consequently there is no significant increase in the phosphorylation of cell adhesion-related proteins. The importance of cell adhesion mediated by specific integrin was further supported by the observation that tyrosine phosphorylation of cell adhesion proteins was not induced even in Csk K cells when plated onto laminin or poly- D-lysine-coated dishes (data not shown). These results show that Csk is critical to define the sensitivity of the cells to the integrin-SFK-mediated cell adhesion signaling.

Modulation of SFK by Csk influences focal contact formation and actin cytoskeletal organization

We next investigated the cellular localizations of tyrosine phosphorylated proteins (pY), vinculin, as markers of focal contact, and actin fiber (F-actin) by confocal microscopy (Figure 4a). Csk K+ cells showed reduced numbers of staining patches of pY and vinculin that were localized only at the edge of large cell aggregates, suggesting that focal contacts are formed at places that can directly interact with the substrate (Figure 4a and c, B). Consistent with this, staining of F-actin was evident only at the edge of cell aggregates as well (Figure 4a and c, E). In contrast, in Csk K cells, the number of focal contacts was greatly increased, and they appeared surrounding the individual cells (Figure 4a and c, C). F-actin was also enriched around the focal contacts of each cell (Figure 4a and c, F). At the middle surface of Csk K+ cells, F-actin appeared highly concentrated at cell–cell contacts probably for the support of E-cadherin/β-catenin complex (Figure b, E), whereas in Csk K cells, such actin support was substantially reduced (Figure b, F). These observations indicate that expression of Csk K+ suppresses the formation of focal contacts while that of Csk K enhances it through the increased phosphorylation of cell adhesion machinery, and that there is a drastic rearrangement of actin cytoskeletal organization accompanied by the formation of focal contacts.

Modulation of SFK by Csk does not affect the E-cadherin/β-catenin complex

We here addressed the mechanism by which cell–cell contact is stabilized in Csk K+ cells, while it is

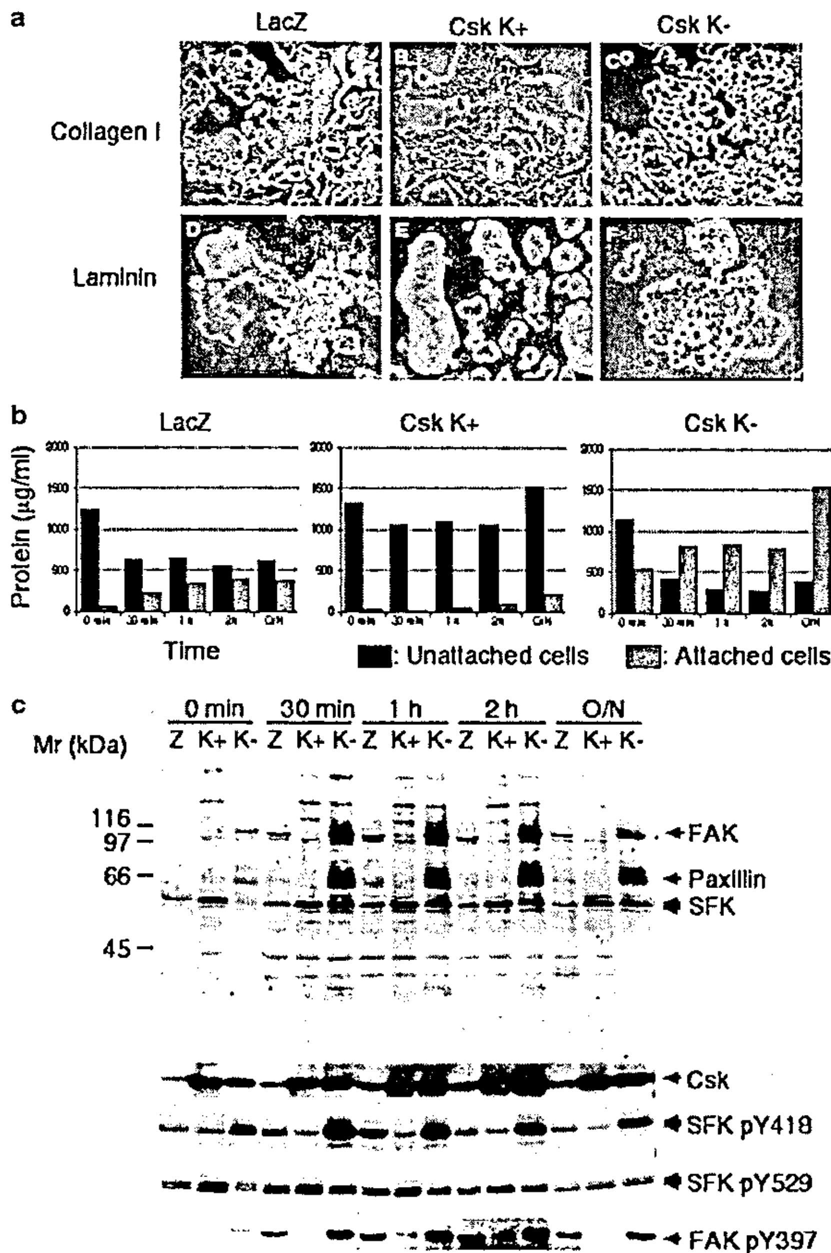
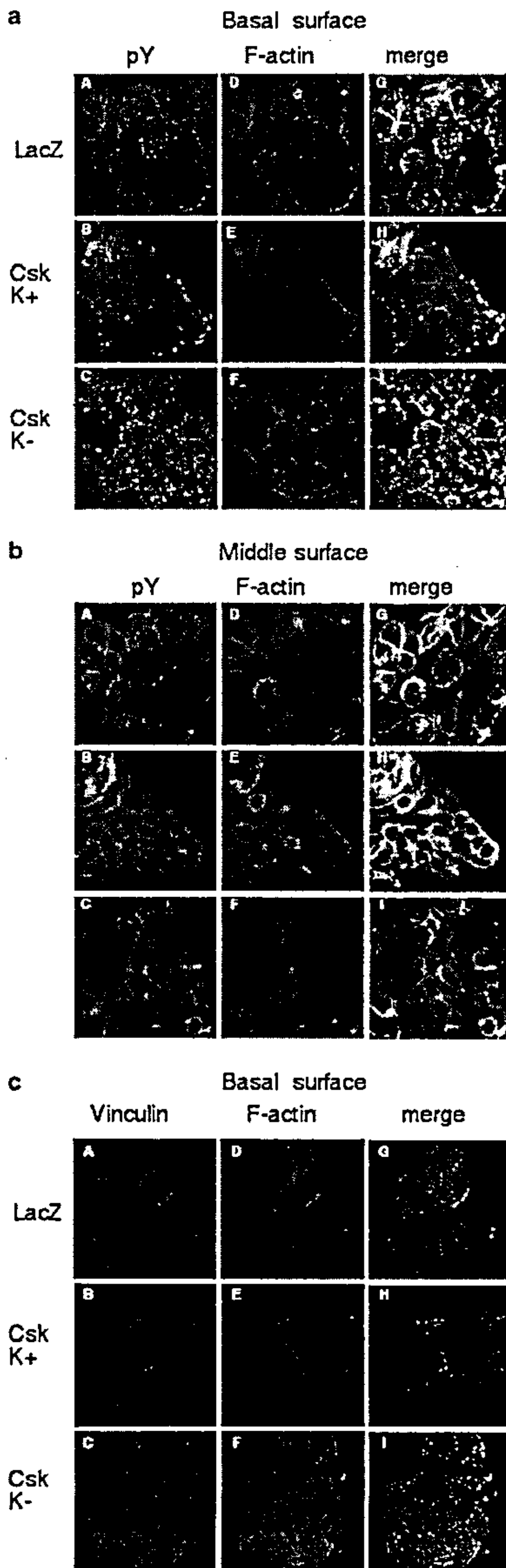


Figure 3 Effect of Csk overexpression on cell adhesion signaling in HT29 cells. (a) Phase-contrast pictures of HT29 cells infected with adenovirus carrying LacZ (A and D), Csk K+ (B and E) or Csk K (C and F). Cells were seeded onto plastic slides coated with collagen I (A, B and C) or plastic slides coated with laminin (D, E and F). (b) Cell adhesion assay on collagen I. HT29 cells infected with adenovirus carrying LacZ, Csk K+ or Csk K were once detached and replated onto collagen I-coated dishes. After indicated periods, adherent and nonadherent cells were separately collected and the amounts of cellular proteins were determined. Here '0 min' means the minimum period taken between replating and cell harvesting. Protein amounts of nonadherent and adherent cells are indicated by black and gray bars, respectively. (c) Changes in tyrosine phosphorylation in response to cell adhesion. After replating the infected HT29 cells onto collagen I-coated dishes for indicated periods, total cell lysates were obtained, and total tyrosine phosphorylation level (upper panel) and expression of Csk and tyrosine phosphorylation of Src Y418, Src Y529, FAK Y397 (lower panels) were analysed by Western blotting. Z; cells expressing LacZ, K+; Csk K+ cells, K ; Csk K cells

destabilized in Csk K cells. It is known that E-cadherin plays a major role in cell–cell contact of epithelial cells (Hyafil *et al.*, 1980). Indeed, an anti-E-cadherin antibody could completely inhibit the formation of compact cell aggregates in Csk K+ cells (Figure 5a), suggesting that SFK activity under the control of Csk is involved in the regulation of E-cadherin-mediated cell–cell contacts. Previous reports have suggested a role for activated form of Src in dissociating the E-cadherin/β-catenin complex (Irby and Yeatman, 2002). Thus, we investigated the localization of E-cadherin and its partner β-catenin in HT29 cells expressing Csk K+ or Csk K . However, there was no apparent difference in the staining patterns of either E-cadherin or β-catenin in any type of cells (Figure 5b). Both E-cadherin and β-catenin were predominantly localized at the membrane



even in the scattered cells. Tyrosine phosphorylation of E-cadherin and β-catenin by the activated form of Src has previously been proposed to trigger ubiquitin-mediated degradation (Aberle *et al.*, 1997) or endocytosis of the E-cadherin/β-catenin complex (Fujita *et al.*, 2002). To test the possible effect of up- or down-regulation of Csk on this mechanism, we determined the protein amounts of the E-cadherin/β-catenin complex and other potential Src targets, α-catenin and $p120^{CTN}$, and the tyrosine phosphorylation of E-cadherin/β-catenin complex. As shown in Figure 5c, there was no significant change in the amounts of any protein tested in either Csk K+ or Csk K cells. In addition, only a subtle increase in tyrosine phosphorylation of either E-cadherin or β-catenin was detected even in Csk K cells. These findings demonstrate that there is no apparent change in E-cadherin/β-catenin related cell–cell contact machinery and that the activity of endogenous SFK is not sufficient for the phosphorylation-mediated endocytosis of the E-cadherin/β-catenin complex. This raises the possibility that cell–cell contact mediated by E-cadherin/β-catenin is deregulated by SFK through an indirect pathway. Supporting this possibility, we observed that there was a dramatic mobilization of F-actin from cell–cell contacts to focal contacts by modulating SFK function (Figure 4a). Thus, it seems likely that rearrangement of actin cytoskeleton induced by SFK activation causes a loss of actin-support for the E-cadherin/β-catenin complex, thereby destabilizing clustering of the complex that allows high-affinity interaction.

Csk could control metastatic potential of cancer cells through regulating integrin-SFK-mediated cell adhesion signaling

In summary, we here showed that upregulation of Csk in human epithelial cancer cells suppresses the activation of SFK upon cell adhesion, thereby allowing cell–cell contacts mediated by E-cadherin leading to epithelial-like phenotype, and conversely that downregulation of Csk greatly enhances integrin-mediated activation of SFK, which accompanies increased phosphorylation of cell adhesion proteins, cell scattering with mesenchymal-like phenotype, increased number of focal contacts, reorganization of cytoskeletal support, enhanced cell adhesion and migration and *in vitro* invasiveness. These observations suggest that Csk plays a key role in the regulation of the activity status of SFK that influences epithelial–mesenchymal transition (EMT) and the metastatic potential of epithelial cancer cells. Src has been implicated in the regulation of EMT (Frame, 2002), and

Figure 4 Effect of Csk overexpression on the formation of focal contacts and arrangements of actin cytoskeleton. HT 29 cells were infected with adenovirus as described above, and stained with 4G10 (a and b) or anti-vinculin antibody (c) followed by detection with FITC-conjugated anti-mouse IgG. F-actin was stained by rhodamine-phalloidine. Immunofluorescence confocal microscopy pictures were taken at the cell basement in contact with the substratum (basal surface) or 2 μm above the basal surface (middle surface)

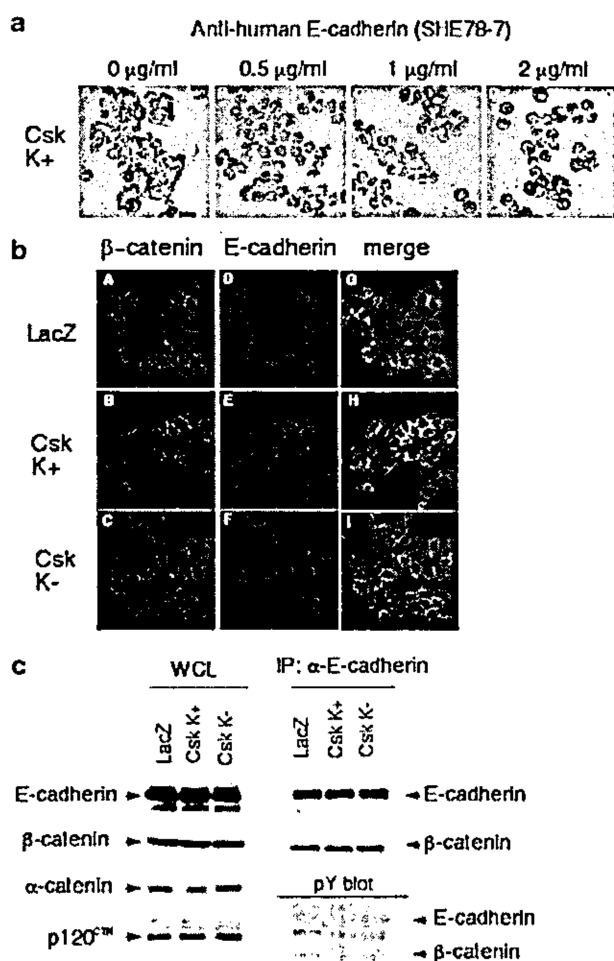


Figure 5 Effect of Csk overexpression on localization, expression and phosphorylation of E-cadherin/β-catenin complex. (a) Inhibition of cell–cell contacts by anti-E-cadherin antibody. HT29 cells were infected with adenovirus carrying Csk K+ in the presence of indicated concentrations of anti-E-cadherin antibody (SHE78-7). After 48 h, cells were observed under phase-contrast microscopy. (b) HT29 cells infected with indicated adenoviruses were stained with anti-β-catenin and anti-E-cadherin antibodies followed by Cy3-conjugated and FITC-conjugated secondary antibodies, respectively. Pictures were taken at the middle surface (2 μm above the basal surface). (c) Total cell lysates from HT29 cells, treated as described above, were subjected to Western blotting using antibodies against E-cadherin, β-catenin, α-catenin and p120^{CTN} (left panels). For analysis of tyrosine phosphorylation of E-cadherin/β-catenin complex, the complex was immunoprecipitated with anti-E-cadherin antibody and then probed with anti-E-cadherin, anti-β-catenin or 4G10 (right panels)

requirement of integrin signaling in such process has also been reported (Avizienyte *et al.*, 2002). However, most of the studies on the function of Src were performed using v-Src or active form of Src having a mutation at the C-terminal regulatory site. A natural Src mutant, Src531 (Irby *et al.*, 1999), has also been used, but this mutation does not seem to occur frequently (Daigo *et al.*, 1999; Nilbert and Fernebro, 2000; Wang *et al.*, 2000; Laghi *et al.*, 2001). These molecules cannot be regulated by Csk and show constitutively high activity. Using these activated forms of Src, it has been proposed that deregulation of cell–cell contacts mediated by E-cadherin is due to SFK-induced phosphorylation of E-cadherin/β-catenin complex leading to endocytosis and degradation of the complex (Aberle *et al.*, 1997; Fujita *et al.*, 2002). In the present study, however, significant phosphorylation and degradation of E-cadherin/β-catenin complex were not observed, suggesting that endogenous SFK activity is not sufficient or too weak to be responsible for such degradation pathway of E-cadherin/β-catenin complex in some cancer cells. Nonetheless, the E-cadherin-mediated cell–cell contacts were destabilized by the activation of SFK. In this study, we proposed the possibility that endogenous SFK dominantly regulates integrin-mediated cell adhesion in any cell type. Thus, it would be possible that deregulation of cell–cell contacts may result from secondary events such as reorganization of actin cytoskeleton, although more detailed analysis for the molecular mechanism underlying these events should be necessary. As already mentioned, other members of SFK, Yes and Lck, are upregulated in some colon cancers (Veillette *et al.*, 1987; Boardman and Karnes, 1995). In HCT15 and HT29 cells used in this study, a variety of SFK members, that is, Src, Yes, Fyn, Lyn and Lck, are expressed and some are indeed activated. In addition, multiple members of SFK, including Src, Fyn, Hck and Fgr, have been shown to be involved in integrin signaling that is critical for the regulation of metastasis (Suen *et al.*, 1999; Cary *et al.*, 2002). Taking these observations together with the fact that SFK has redundant function, it is likely that deregulation of the balance between multiple SFKs and Csk achieved by elevated expression of SFKs, down-regulation of Csk or both could influence the metastatic potential of some cancer cells through primarily regulating integrin-mediated cell adhesion signaling.

We showed here that upregulation of Csk function could attenuate the metastatic potential of human colon cancer cells. Our results may provide an insight for therapy in cancer metastasis. Inhibitors of SFK, such as PP2, are known to have harmful side effects. Instead, Csk might be a more selective target for cancer therapy. Gene transfer of Csk has already been undertaken and it successfully suppressed the metastatic activity of colon cancer cells (Nakagawa *et al.*, 2000). Since the structure of Csk is now available (Ogawa *et al.*, 2002), a specific compound that binds to the SH2 domain to activate Csk may be designed; alternatively, a drug that can activate the expression of Csk may be useful.

Materials and methods

Cell culture and reagents

Colon adenocarcinoma cell lines HCC2998, HCT15 and HT29, and a gastric cancer cell line MKN22 were obtained from the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (Tokyo, Japan). Cells were cultured in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/l glutamine, 50 μg/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37°C and 5%

CO_2 in a humidified atmosphere. Cells were cultured in collagen I, laminin or poly- D-lysine-coated dishes (BD Biocoat, CA, USA) and used for experiments after reaching 80% of confluence. PP2 was purchased from Calbiochem (La Jolla, CA, USA). Stock solutions of this compound were prepared in 100% DMSO (Sigma, St Louis, MO, USA) and stored at 70°C. Coating was performed with 50 μg/ml collagen I on Lab-Tech plastic chamber (Nalge Nunc International, Rochester, NY, USA) (strong coat). Ax1CAT-lacZ, Ax1CATcsk K+ and Ax1CATcsk K adenovirus were obtained as described elsewhere (Takayama et al., 1999).

Western blotting and immunoprecipitation

Cells were washed, and lysed in ODG buffer (50 mM Tris-Hcl, pH 8.0, 1 mM EDTA, 0.25 M NaCl, 20 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P-40, 2% octyl-D-glucoside, 5 mM β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 5% glycerol). Proteins separated by SDS–PAGE were then transferred onto a nitrocellulose membrane (Schleicher & Schuell, NH, USA). Subsequently, the membrane was treated with blocking buffer (Tris-buffered saline containing 0.1% Tween 20 (Tween-TBS)) for 2 h at room temperature. The blocked membrane was probed with primary antibodies and further incubated with a secondary antibody conjugated with horseradish peroxidase. The immunoreactivity was visualized with an enhanced chemiluminescence system (Perkin-Elmer Life Sciences, MA, USA). Primary antibodies used were antiphosphotyrosine (clone 4G10), anti-Yes, anti-FAK (clone 77), anti-E-cadherin (clone 36), anti-$p120^{CTN}$ (clone 98) and anti-α catenin (clone 5) from Transduction Laboratories (KY, USA); anti-v-Src (clone Mab 327) from Calbiochem (San Diego, CA, USA); anti-Csk (clone c-20), anti-Fyn (clone FYN3), anti-Lyn (clone 44), anti-Lck (clone 2102) and anti-β-catenin (clone H-102) from Santa Cruz Laboratories (CA, USA); anti-Src pY418 and pY529, and FAK pY397 from Biosource Laboratories (CA, USA). Horseradish peroxidase-conjugated anti-mouse IgG (Zymed Laboratories Inc., CA, USA) and horseradish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories Inc., CA, USA) were used as secondary antibodies. For immunoprecipitation, precleared lysate (500 μg protein) was incubated with indicated antibodies and Protein G-Sepharose (Amersham Pharmacia, Buckinghamshire, UK) for 2 h at 4°C. The immunoprecipitate was washed with ODG buffer and analysed by Western blotting.

Cell motility and invasion assay

HT29 cells infected with adenovirus for 24 h were replated on 35 mm glass-bottom microwell dishes (IWAKI) coated with 50 μg/ml collagen I. Cell movements were monitored using Olympus IX71 microscope for 24 h. Images were collected with Cool SNAP HQ CCD camera (Roper) at 6 min intervals, digitized and stored as image stacks using MetaMorph 5.0 software (Universal Imaging). Movements of individual cells were analysed using Move-tr/2D software (Library, Tokyo). For *in vitro* invasion assay, cells (1.0 10^5) were seeded into matrigel invasion chambers (BD Biocoat, CA, USA) in serum-free RPMI media (500 μl), with full RPMI media (700 μl) in the well below. Cells were allowed to grow for 48 h, after which the layer of cells in the chamber was carefully scraped off and cells adhering to the membrane beneath the chambers were stained with crystal violet and counted.

Immunofluorescence staining and confocal microscopy

HT29 cells were infected with adenovirus carrying lacZ (control), Csk K+ or Csk K on collagen-coated glass coverslips for 2 days. After fixation with 3.7% formaldehyde in PBS for 10 min, cells were incubated with anti-E cadherin, anti-β-catenin, anti-vinculin or antiphosphotyrosine (4G10) antibody in Tris-buffered saline containing 0.1% Tween 20; and further incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) or Cy3-conjugated anti-rabbit IgG antibody (Chemichon, CA, USA). Rhodamine-phalloidin (Eugene, OR, USA) was used to stain F-actin. The stained cells were observed by immunofluorescence microscopy and/or confocal microscopy using an LSM510 (Zeiss, Germany). For inhibition assay of cell–cell contacts, HT29 cells were infected with adenovirus carrying Csk K+ in the presence of indicated concentrations of anti-human E-cadherin antibody (SHE78-7, TAKARA, Japan) for 48 h.

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基　礎

がん分子標的と創薬スクリーニング

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基　礎

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はじめに

この20年で達成された分子生物学の急速な進歩は，がんの発生・増殖・進展の機序解明に革新的役割を果たした．そして，ヒトゲノム構造解読の完了は，がんの分子基盤解明にいっそう拍車をかけている．このような流れの中で，新規抗がん剤開発の方向は，がん細胞に発現した特定分子機能を標的とする分子標的治療薬に向けられている．

本稿では，がん分子標的治療薬開発の現状とゲノム創薬の流れを概観し，筆者らが抗がん物質評価に用いているcancer cell informaticsの創薬における位置づけについて述べてみたい．

がん分子標的

図1にがん治療の分子標的と考えられるおもなものを示す．がん遺伝子産物，増殖因子とその受容体，シグナル伝達分子，ホルモン受容体，細胞周期関連蛋白質，テロメラーゼ関連分子，アポトーシス関連分子，血管新生関連分子，抗がん剤耐性・感受性因子，転写因子，浸潤転移関連分子などがある．これらには，がん細胞に特異的に発現するもの，正常細胞にもあるが量的にがん細胞に多く発現するもの，あるいは局所でがんの間質をなす宿主側組織で高発現するものなどが含まれる．いずれにせよ，分子標的治療薬は，がんのいわばアキレス腱となる特徴的分子を選択的に狙おうとするものである．従来の抗がん剤の多くは，DNA合成や蛋白質合成など基本的機能を障害し，ためにがん細胞のみならず正常細胞にも広範に毒性が及び，当然の帰結として強い副作用を惹起する．それに対し，分子標的治療薬はがん選択性に基づく，高い有効性と副作用の軽減を狙うものである．

分子標的治療薬の開発

分子標的治療薬の開発は，従来薬のそれとまったく逆の流れで行われる(図2)．すなわち，その開発は，「はじめに標的ありき」で始まり，細胞レベル，次に個体レベルという順で有効性を証明していくことになる．以下にまず，近年から最近にかけ実際に新薬として相次いで承認されたおもな分子標的治療薬を紹介する．

1. Trastuzumab(Herceptin®)

Trastuzumabは，HER2の細胞外ドメインに結合するヒト型モノクローナル抗体で，承認

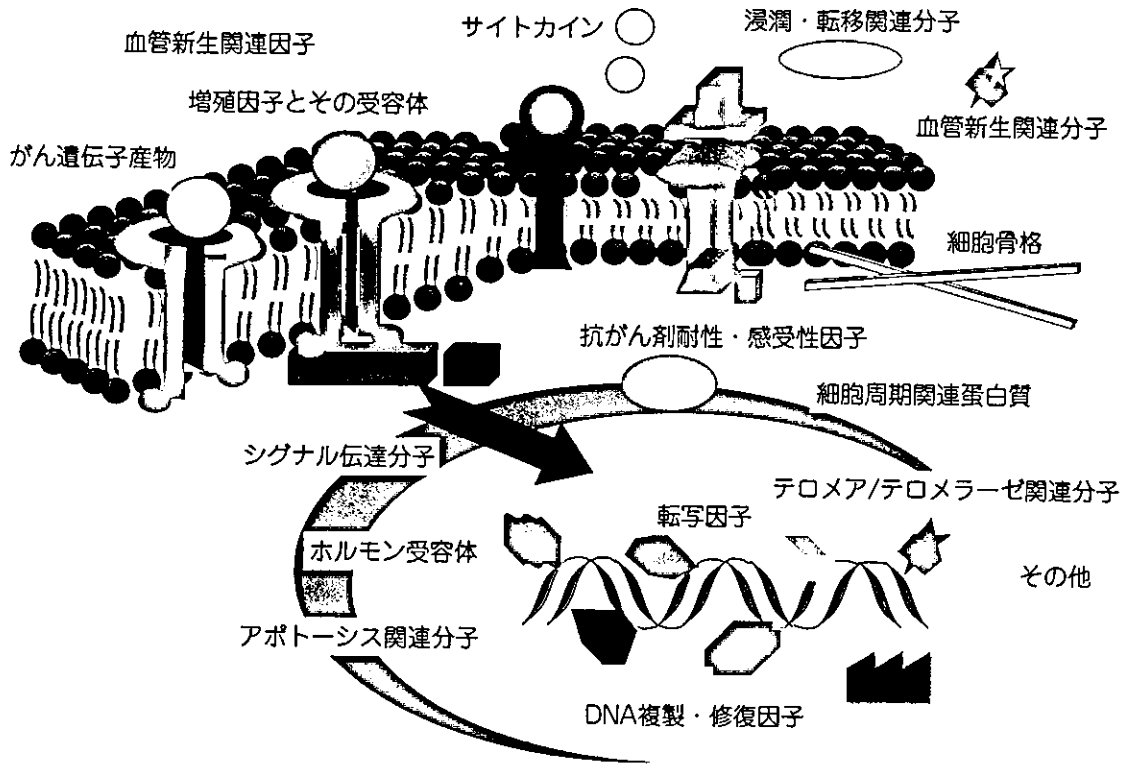

図 1. がん治療の分子標的

された分子標的治療薬の第一号である．HER2 (ErbB2) は，乳がん，卵巣がん，肺がん，胃がんなどの腺がんで過剰発現が知られ，ことに乳がんでは予後因子の一つとして重要である[1,2]．Trastuzumab は，HER2 へのリガンドの結合を阻害してがん増殖を阻害する．HER2 陽性の転移性乳がんの標準的治療として，他剤との併用療法で用いられている．

2. Imatinib (Gleevec®)

Imatinib は，慢性骨髄性白血病 (CML) の治療に画期的成果をもたらした．すなわち，CML の慢性期症例の 90%に，さらにこれまで手の施しようのなかった急性転化期の症例でも約 30%に完全寛解をもたらし，しかも副作用がわずかであった[3]．CML では，染色体相互転座により *bcr/abl* キメラ遺伝子が生じ，その産物 BCR/ABL 蛋白質の持つ強力なチロシンキナーゼ活性が悪性化の要因とされる．Imatinib は，BCR/ABL チロシンキナーゼの ATP 結合部位に特異的な競合阻害薬である．さらに，imatinib は，PDGF 受容体および KIT のチロシンキナーゼ活性を阻害する．KIT は，消化管間質腫瘍 (gastrointestinal stroma tumor：GIST)，セミノーマほか多くのがん種に発現している．最近 imatinib は，これまで外科切除以外に治療法がないとされた GIST に対し高い奏効率を示し[4]，GIST に対しても承認された．

3. Gefitinib (Iressa®)

EGF 受容体 (EGFR) は，多くのがんでの過剰発現が知られている．Gefitinib は，EGFR の ATP 結合部位に競合的に結合し EGFR チロシンキナーゼ活性を阻害する．現在，肺がんの分子標的薬として非常に注目されている．ただし，副作用として間質性肺炎がみられ，その原因解明が待たれる．また，有効性に関しても EGFR チロシンキナーゼの阻害だけでは説明

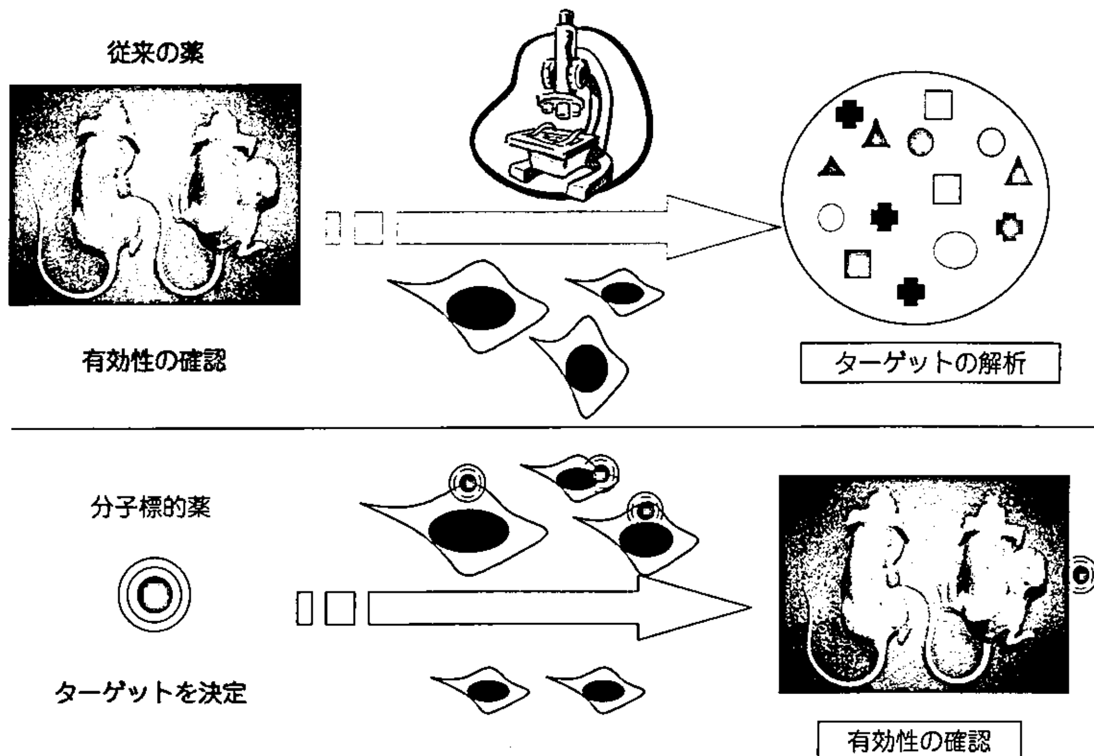

図 2. 分子標的治療薬開発と従来の抗がん剤開発の違い

分子標的治療薬の開発は，従来薬のそれとまったく逆の流れで行われる．すなわち，その開発は，「はじめに標的ありき」で始まり，細胞レベル，次に個体レベルという順で有効性を証明していく．

できない点があり，血管新生阻害作用など[5]，別の局面での作用の解析も進められている．

4. Bortezomib (Velcade®)

Bortezomib は，プロテアソームの阻害薬としては初の分子標的治療薬として 2003 年 5 月に FDA によって，多発性骨髄腫に対して承認された．プロテアソームは，真核細胞内での蛋白質分解の主要な経路を司る酵素複合体である．細胞周期やアポトーシスに対しても，関連蛋白質の分解を通じてそれらの制御に関係していると考えられている[6]．

5. Vivacizumab (Avastin®)

血管新生阻害薬は待望久しかったが，vivacizumab がついにその第一号とし 2004 年 2 月に FDA によって承認された．転移性大腸がんに対する 5Fu との併用治療が認められた．Vivacizumab は，血管新生に重要な VEGF に結合しその作用を中和する抗体として期待される．

6. そのほかの開発中の分子標的治療薬

表 1 に現在臨床開発中の種々の分子標的治療薬の一部をまとめた．Erb B ファミリーのチロシンキナーゼを標的とするものが最も競合している．セリン/スレオニンキナーゼ阻害薬のうち，mTOR や CDK 阻害薬にも興味がもたれる．キナーゼ阻害薬は，今のところ ATP 結合部位への競合阻害をするものしか開発されていない．今後，基質蛋白質を特異的に阻害するようなものが出れば，新しい制御につながるかも知れない[7]．RAS 経路関連では，MEK，RAF キナーゼの阻害薬の有効性がどこまで達成されるか，また，HDAC では，アイソザイム特異性と

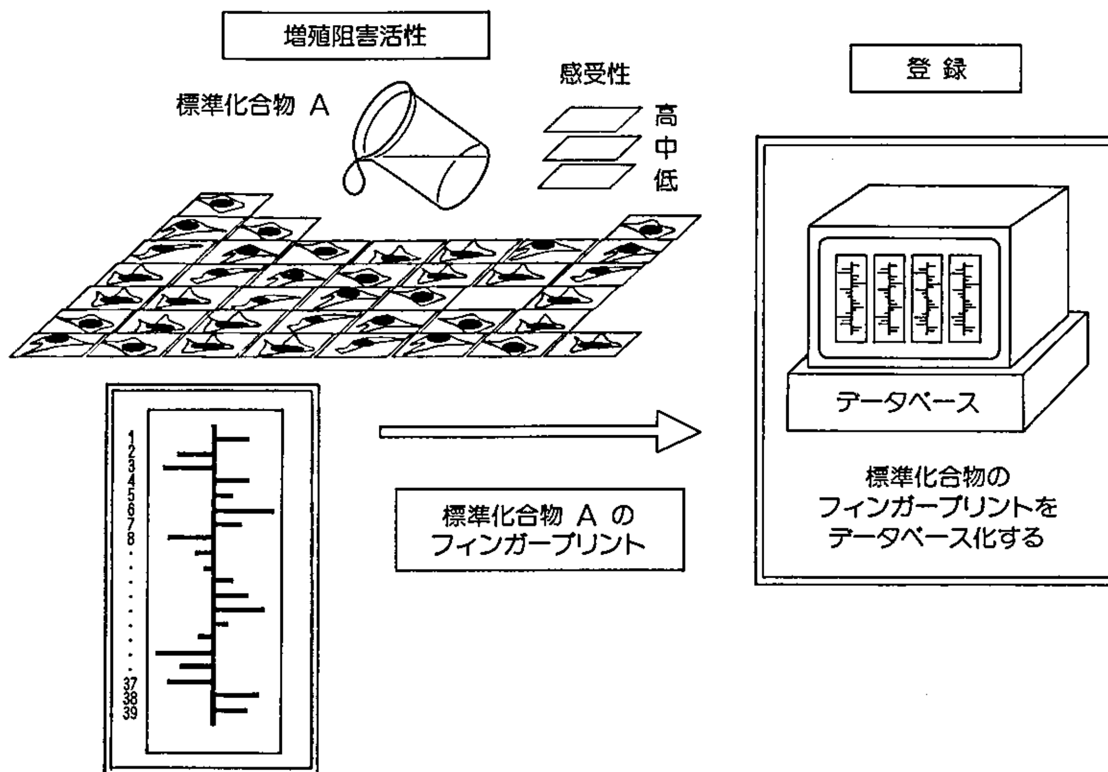

図 4. Cancer cell informatics におけるフィンガープリントデータベースの構築

がん細胞パネルの各がん細胞株は一つの薬剤に対しおのおの異なる感受性を持つ．その結果，一つの薬剤について各がん細胞株の薬剤感受性をパネル全体でみると，その薬剤固有のパターン(フィンガープリント)が得られる．標準化合物のフィンガープリントをデータベース化することにより標準化合物の作用メカニズムがフィンガープリントの形でデータベース化できる．

プラットフォームである．がん細胞株数十系を一組とし(がん細胞パネル)，さまざまな抗がん剤に対するその感受性をデータベース化し，データマイニングを導入することによって，化合物の薬理評価に役立つ辞書的機能を持つユニークなシステムが構築できる(図4〜6)．紙面の都合で詳しくは述べないが，ウェットな実験(感受性試験)とドライな研究手法(データベース構築とデータマイニング)とを連携させたこのシステムは，がん化学療法研究手法としてこれまでにない特徴を持っている．たとえば，抗がん剤研究や抗がん剤探索に応用する場合，化合物の作用メカニズムを予測できるという特徴をもつ．新規作用メカニズムをもつ抗がん剤を探索したい，開発中の抗がん剤と既存の抗がん剤との差別化ができるかどうかを知りたい，あるいは，開発した分子標的治療薬が想定した標的以外への作用を持つかを知りたい時，ことに威力を発揮する．したがって，図3のゲノム創薬のパイプラインの中で cancer cell informatics は，探索から前臨床までを広くサポートするシステムとして役立つものと考える．Cancer cell informatics により，筆者らは新規抗がん物質 MS-247 や thiazinotrienomycin B を見いだすとともに[9,10]，製薬企業の抗がん剤開発の支援も進めている．さらに，がん細胞パネルにおける遺伝子発現をデータベース化し，上述の抗がん剤感受性データと比較解析することにより，抗

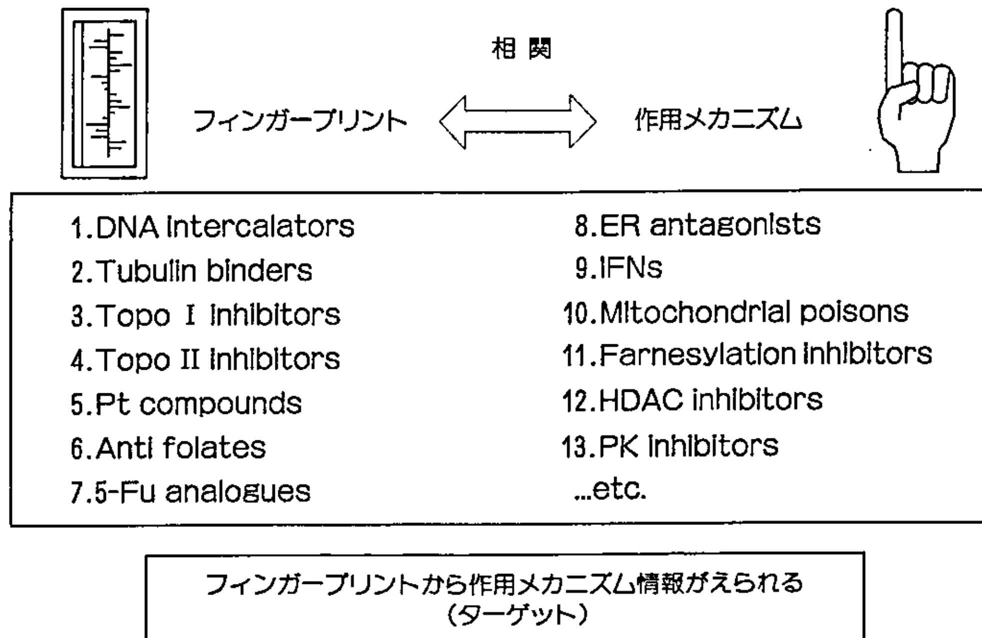

図 5. 化合物フィンガープリントはそのメカニズムを示す

フィンガープリントを指標に(似ているもの同士を集める)，化合物を分類するとできあがった分類は，作用メカニズムによる分類とよく一致する．

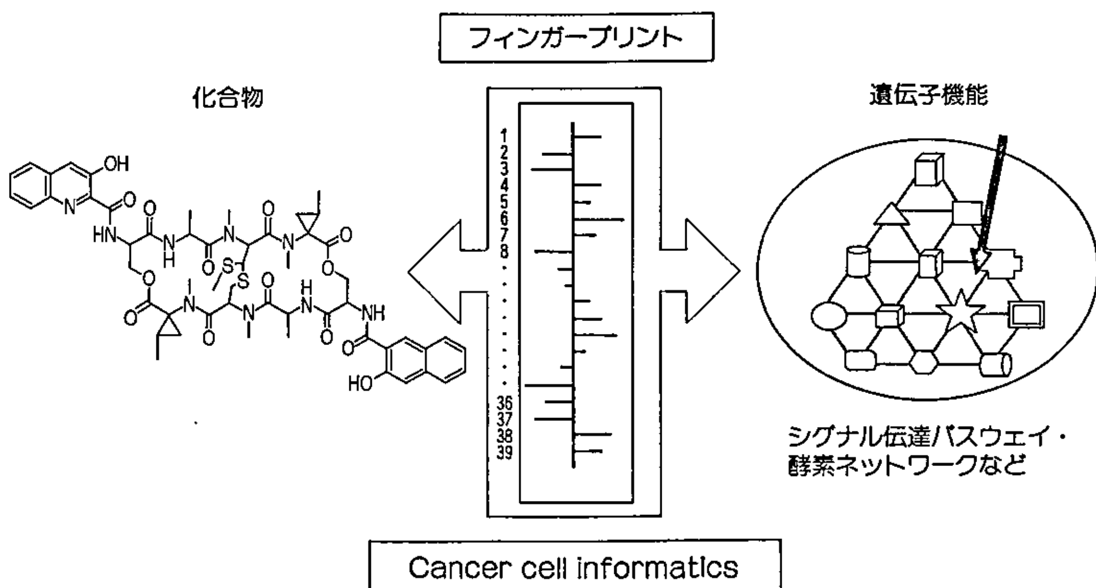

図 6. フィンガープリントは化合物と遺伝子産物機能と関連づける

がん細胞パネルを用いることによって，化合物のフィンガープリント(化合物フィンガープリント)が得られる．一方，がん細胞パネルにおける遺伝子発現を調べることによって，個々の遺伝子の発現レベルについてフィンガープリント(遺伝子発現フィンガープリント)が得られる．両者の相関関係を網羅的に解析することによって，互いの関連が予想される化合物と遺伝子の組合せが抽出できる．

表 2. Cancer cell informaticsの有用性

Cancer cell informaticsの有用性は リード探索・最適化に有用である
1. 新規作用機作を持つリードの探索
2. 弱い活性のリードをもとに，より高い活性の物質をデータベースの中から探索
3. 分子標的薬剤を見直す一他の標的への作用はないのか？
4. 抗がん剤以外の化合物の分類(他の医薬品，毒物など)
5. 遺伝子機能を化合物と対応づける(化学遺伝学に貢献)

がん剤の分子標的予測，抗がん剤感受性に影響する遺伝子候補，あるいは診断・治療のバイオマーカー候補の探索も可能である[11~13]．この系は，オーダーメイド診断・治療への基盤情報を提供するツールとしてポストゲノム研究分野での活用，さらに将来は化学遺伝学分野での活用も期待される(図6，表2)．

おわりに

がんの分子生物学に立脚して生まれた分子標的治療のコンセプトは，最近相次ぐ分子標的治療薬の承認により現実のものとなり，がん化学療法は，新たな展開を迎えた．これらに続く分子標的治療薬の開発競争は激化の様相を示している．問題点としては，分子標的治療薬にも従来の抗がん剤と同様に耐性発現がみられているのでその克服が必要となろう．また，標的が発現しているのみでは有効性につながらない場合もあるので，個々のがんの遺伝子発現プロファイルにおける当該分子標的の位置づけを見極め，標的への打撃がそのがん細胞に与えるダメージを正確に予測できるような感受性診断法の開発も望まれる．今後，有望な標的に対し多様な分子標的治療薬の開発が行われていくものと予想され，それらの併用による真の意味でのオーダーメイド治療の実現を期待したい．

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Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells

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