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Up-regulation of $p27^{Kip1}$ Correlates Inversely with Anchorage-independent Growth of Human Cancer Cell Lines

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We examined the correlation between anchorage-independent growth and cell cycle-related molecules using 39 human cancer cell lines. They consisted of lung-, colon-, stomach-, breast-, ovarian-, brain-, renal- and melanoma-derived cell lines. Their anchorage-independent growth ability varied, but was not clearly related to the tissue of origin. There was a tendency for the levels of cyclin D1, cyclin E, cyclin A, p27, and p21 to show a tissue-dependent expression pattern. Statistical analysis revealed an inverse correlation of the p27 level with anchorage-independent growth (r=–0.456, P<0.01). Thus, the regulation of p27 is suggested to be linked to the anchorage independence of human cancer cells.

Key words: Human cancer — Anchorage independence — Cell cycle — $p27^{Kip1}$

Cancer cells differ from normal cells in many respects, one being their anchorage independence: cancer cells grow in the absence of substrate attachment, e.g., in soft agar or suspension culture.[1–4] Their growth ability correlates well with their tumorigenicity.[2–4] Many studies have suggested that such a growth advantage is conferred by disruption of the normal cell cycle control.[5–7] In normal cells cultured in suspension, up-regulation of cyclin-dependent kinase (CDK) inhibitors arrests the cell cycle progression by preventing phosphorylation of Rb through inhibition of CDK activity.[8–10] In this study we examined how the cell cycle progression is regulated in anchorage-independent growth (AIG) of human cancer cell lines.

We used the 38 human cancer cell lines in the anticancer drug screening program of the Cancer Chemotherapy Center,[11–13] in addition to human colon cancer DLD-1 cells (in total, 7 lung cancer, 7 colon cancer, 6 stomach cancer, 5 breast cancer, 5 ovarian cancer, 6 brain cancer, 2 renal cancer and 1 melanoma cell lines). We first examined the AIG ability of these cell lines using a plate coated with an antiadhesive polymer, polyHEMA, for the suspension culture and an uncoated plate for the attached culture.[14, 15] Using a poly(2-hydroxyethyl methacrylate) (polyHEMA) plate instead of a soft agar plate allows the cells growing anchorage-independently to be easily harvested and examined together with the attached cells. The morphology of the cells was different between the suspension and the attached cultures; those of DLD-1 and the lung cancer cell lines are shown in Fig. 1 as examples. Most of the cell lines aggregated in the suspension culture, some forming tightly compacted spheroids (e.g., DLD-1, NCI-H522, NCI-H460 and DMS273). The cell growth was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by measuring A_{570} values.[15] The AIG ability was expressed as a ratio, and was calculated by dividing each A_{570} value in the suspension culture by the corresponding A_{570} value in the attached culture. As reported previously, the values for various kinds of untransformed cells, both murine fibroblasts and human epithelial cells, were less than 0.05 and those of oncogene-transformed cells were around 0.4.[15, 16] The values for human tumor cell lines varied, with the average being 0.42 (Fig. 2). Lung and colon cancer cell lines showed relatively high AIG ability, but there was no clear-cut tissue specificity.

We next examined protein expression of molecules related to cell cycle progression by western blotting using specific antibodies.[17] The amount of each molecule was evaluated by an image analyzer and expressed as a relative amount to that in DLD-1 cells as a standard (1.0). The results for DLD-1 and lung cancer cell lines are shown in Fig. 3. The relative amounts of Rb, cyclin D1, cdk4, cyclin E, cdk2, cyclin A, p27, p21 and p16 in the attached and the suspension cultures and the ratio, which was calculated by dividing the amount in the suspension culture by that in the attached culture, are summarized in Table I. There was a moderate tissue dependence of the expression pattern. Lung cancer cell lines expressed high levels of cyclin D1 with low levels of cyclin A; colon

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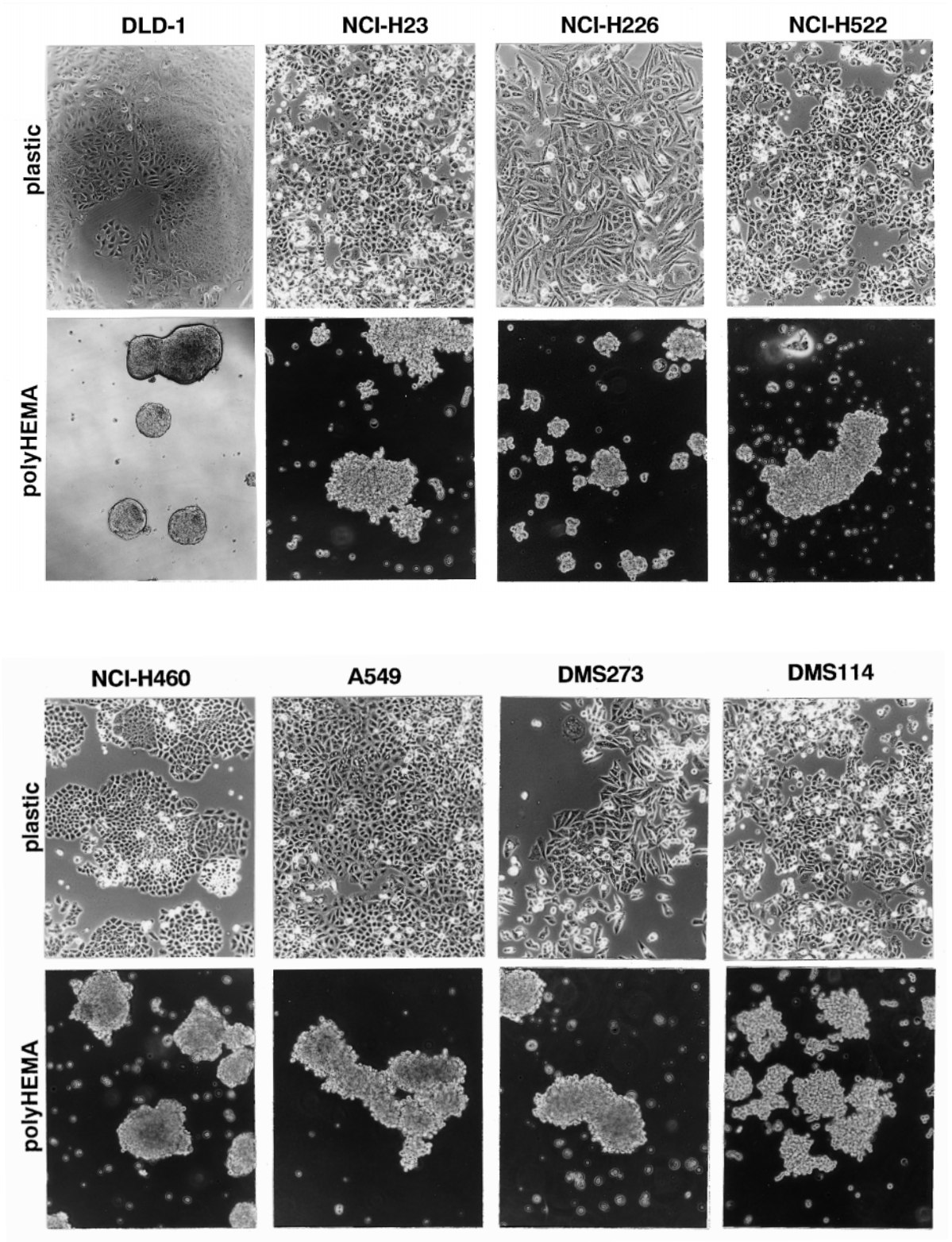

Fig. 1. Morphology of DLD-1 and lung cancer cell lines. Cells were cultured for 4 days in 10 cm dishes coated with polyHEMA (polyHEMA) or uncoated (plastic).

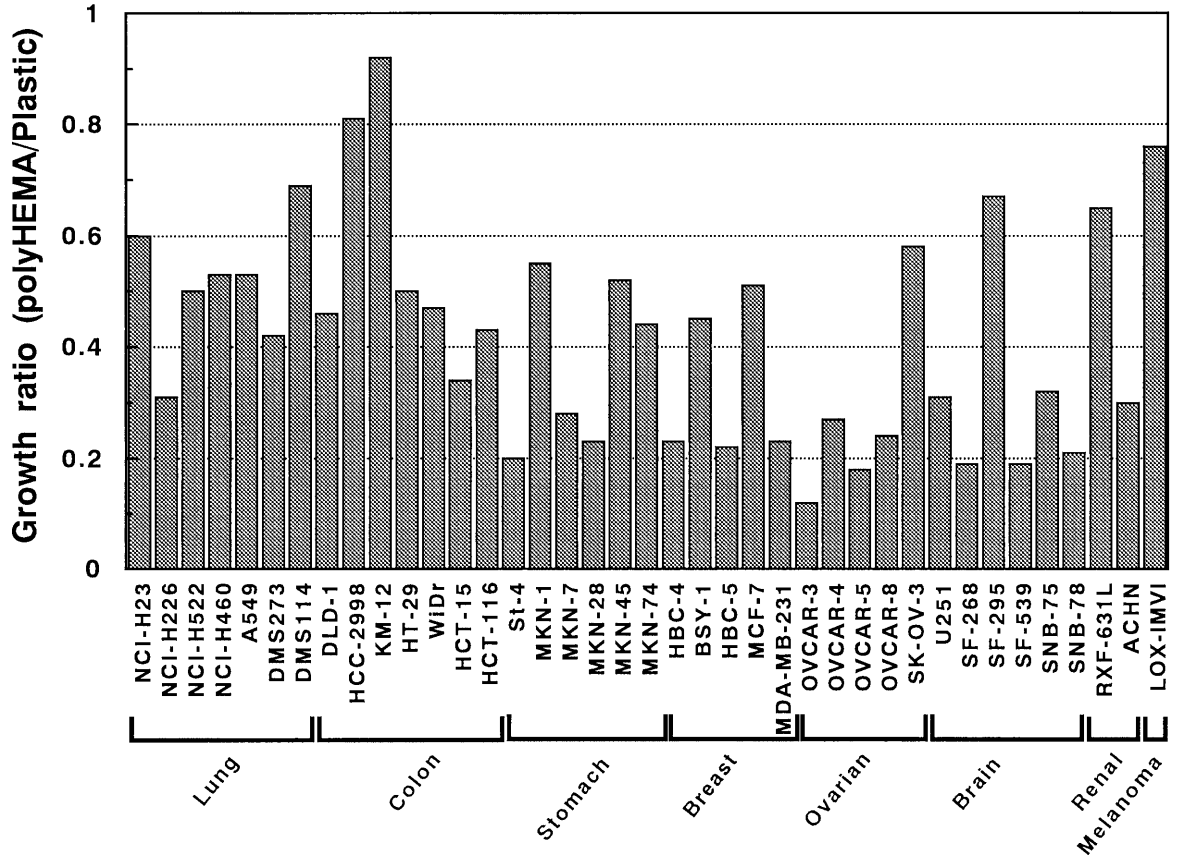

Fig. 2. Anchorage-independent growth of human cancer cell lines. Cells were inoculated in RPMI1640 supplemented with 5% fetal bovine serum into 96-well plates coated or uncoated with polyHEMA. After culture for 4 days at 37°C with 5% CO_2, cell growth was assessed using MTT.[15] The growth ratio was calculated by dividing the A_{570} value in a polyHEMA-coated plate by that in an uncoated plate. Cell plating numbers (cells/well) were as follows: 1250, DMS273; 2500, NCI-H460, BSY-1; 3500, HBC-5; 3750, HCT-116, OVCAR-3, OVCAR-4; 4000, HBC-4; 5000, DLD-1, NCI-H226, A549, St-4, MKN-1, MKN-7, MKN-28, MKN-74, OVCAR-8, U251, SF-295, SF-539; 6250, NCC-2998, HT-29, MKN-45, MCF-7, OVCAR-5; 7500, NCI-H23, WiDr, HCT-15, SK-OV-3, SF-268, SNB-78, RXF-631L, ACHN, LOX-IMVI; 8750, NCI-H522; 1000, DMS114, KM-12, MDA-MB-231, SNB-75. Cell plating number was determined on the basis of the growth rate of each cell line.

cancer cell lines expressed low levels of cyclins D1 and E; stomach cancer cell lines showed high cyclin A with low p27; breast cancer cell lines showed high p27; brain cancer cell lines had high cyclin A with low cyclin E; and renal cancer cell lines had high cyclin D1 with low cyclin E. Not all cancer cell lines expressed every tested molecule, however. For example, the expression of Rb was not detected in OVCAR-8, SF-539 or SNB-75, but p16 was detected in those three lines. The expression of p27 was detected in all cell lines, but p16 and p21 were detected in only some of them. p21 was expressed well in stomach and breast cancer cell lines. Furthermore, the expression of almost all tested molecules differed between the attached and the suspension cultures. Among them, p27 was always up-regulated in the suspension culture of most cell lines, though the degree of up-regulation varied.

We searched for correlations between the levels of tested molecules and AIG abilities, but found only one significant correlation. As shown in Fig. 4, the p27 ratio (suspension culture/attached culture) correlated inversely with the AIG ability (r=–0.456, P<0.01). This suggests that up-regulation of p27 in the suspension culture suppresses AIG of human cancer cells. Our recent data indicated that p27 is greatly up-regulated in untransformed fibroblasts cultured in suspension and inhibits the AIG, while the reduced level of p27 in Ras-transformed cells is strongly associated with the AIG.[17] Although the precise mechanism of p27 regulation is unclear, the cells with

Table I. Expression of Cell Cycle-related Molecules in Human Cancer Cell Lines

	Cell line	Rb			cycD1			cdk4			cycE			cdk2			cycA			p27			p21			p16		
		P	H	H/P	P	H	H/P	P	H	H/P	P	H	H/P	P	H	H/P	P	H	H/P	P	H	H/P	P	H	H/P	P	H	H/P
Lun	NCI-H23	+	+		2+	2+		+	+		+	+		+	+		+	+		+	+		+	+		−	−	
	NCI-H226	+	+		2+	2+		+	+		±	±	↑	+	±	↓	−	−		±	+	↑	+	+		−	−	
	NCI-H522	±	±		±	±	↓	+	+		+	+		+	+		−	−		±	±	↑	−	−		+	+	
	NCI-H460	+	+		+	2+	↑	±	±		±	±		±	+		−	−		±	+	↑	−	−		−	−	
	A549	±	+	↑	+	+	↓	+	+		+	+		+	±	↓	−	−		+	+		−	−		−	−	
	DMS273	+	+		+	+	↑	2+	2+		2+	+		+	+		+	+		±	+	↑	+	+		+	+	
	DMS114	2+	2+		2+	2+		2+	2+		±	+	↑	+	+		−	−		2+	2+		−	−		+	+	
Col	DLD-1	+	+		+	+		+	+		+	+		+	+		+	+		+	2+	↑	−	−		−	−	
	HCC-2998	±	±		±	±	↑	2+	2+		+	+		+	+		−	−		±	±		−	−		+	+	
	KM-12	2+	2+		±	±		+	+		±	±		+	+		+	+		+	+		−	−		−	−	
	HT-29	+	+		±	±		±	+	↑	±	±		±	+	↑	2+	2+		±	2+	↑	−	−		−	−	
	WiDr	+	+		±	±		+	+		+	±		±	±		2+	2+		±	+	↑	−	−		−	−	
	HCT-15	+	+		±	±		+	+		±	±	↓	±	±	↓	±	±		+	2+	↑	−	−		−	−	
	HCT-16	+	+		+	+		+	+		+	+		2+	+		−	−		+	2+	↑	+	+		−	−	
Sto	St-4	±	+	↑	+	+	↑	±	±	↑	+	+		±	±		±	±		±	2+	↑	−	+	↑	−	−	
	MKN-1	+	+		+	+	↓	+	+		2+	2+		+	+		2+	+	↓	±	±		±	±		+	+	
	MKN-7	+	±	↓	±	±		±	±	↓	±	±		+	+		2+	±	↓	±	+		−	−		−	−	
	MKN-28	+	+		+	2+	↑	±	+		±	±	↑	+	+		2+	±	↓	±	+	↑	±	+	↑	−	−	
	MKN-45	+	+		+	+		+	+		+	+		+	+		−	−		±	±		+	+		−	−	
	MKN-74	+	+		±	+	↑	±	+	↑	±	+	↑	±	+	↑	+	+	↓	±	+	↑	±	+	↑	−	−	
Bre	HBC-4	+	+	↓	+	+		±	±		+	±	↓	±	±	↓	−	−		±	+	↑	+	±	↓	−	−	
	BSY-1	±	±	↓	+	+		+	+		2+	2+		+	+		2+	2+		±	+	↑	±	±		+	+	
	HBC-5	+	+	↓	+	2+	↑	±	+	↑	2+	2+	↑	+	+		+	±	↓	2+	2+	↑	±	+	↑	±	+	↑
	MCF-7	+	+		+	+		+	+		+	2+		+	+		−	−		2+	2+		+	+		−	−	
	MDA-MB-231	+	+		2+	+	↓	+	+		±	±		+	±	↓	2+	2+	↓	2+	2+		+	+		−	−	
Ova	OVCAR-3	+	+		±	±	↑	2+	2+		2+	2+	↑	+	+		2+	2+		±	2+	↑	−	−		±	+	↑
	OVCAR-4	+	+		±	±		±	+	↑	+	2+	↑	+	2+		+	+		±	2+	↑	−	−		−	±	↑
	OVCAR-5	+	±	↓	+	2+		±	±		±	+		+	±	↓	−	−		+	2+	↑	−	−		−	−	
	OVCAR-8	−	−		+	+		+	2+		+	2+	↑	+	+	↑	+	+		2+	2+		−	−		±	+	↑
	SK-OV-3	+	+		±	+	↑	±	±	↑	±	±		±	±		−	−		±	+	↑	−	−		−	−	
Bra	U251	+	+	↑	+	+		±	+	↑	±	±		+	+	↑	2+	+	↓	+	2+		−	−		−	−	
	SF-268	+	+		+	+		+	+		±	±	↑	+	+		2+	2+		+	+		−	−		−	−	
	SF-295	+	+		+	+		±	±	↑	±	±	↑	+	+		2+	2+		+	2+		−	−		−	−	
	SF-539	−	−		±	±	↑	+	+		±	+	↑	+	+		±	±		±	+	↑	−	±	↑	+	+	
	SNB-75	−	−		±	±		+	+		+	2+	↑	+	+		+	+		+	+		−	−		+	+	
	SNB-78	±	±		±	±	↑	±	+	↑	±	±	↑	±	+	↑	−	−		±	+	↑	−	−		−	−	
Ren	RXF-631L	±	+		2+	2+		+	+		±	±		+	+		−	−		+	+		+	+		−	−	
	ACHN	+	+		+	2+		+	+		±	±		+	2+		±	±		2+	2+		+	+		−	−	
Mel	LOX-IMVI	+	+		2+	2+		+	+		±	±		+	+		±	±		+	+		+	+		−	−	

Each expression level was expressed as a relative amount to that in DLD-1 cells in attached culture as a standard (1.0) and classified as number of times the mean values. 2+, >mean×2; +, ~mean; ±, <mean×0.5; –, below the limit of detection. The expression ratio (H/P) was calculated by dividing each amount in a polyHEMA-coated plate by that in an uncoated plate and classified as 2-fold increase (↑) or decrease (↓).

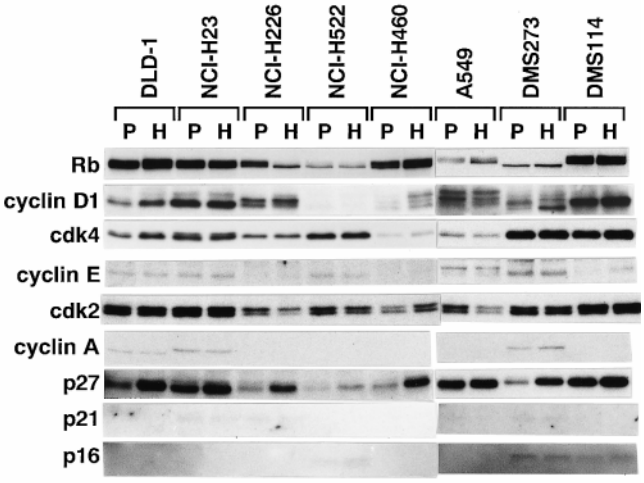

Fig. 3. Expression of cell cycle-related molecules in DLD-1 and lung cancer cell lines. Cells were cultured for 4 days in 10 cm dishes coated (H) or uncoated (P) with polyHEMA. Subconfluent cells were lysed in 20 m*M* HEPES (pH 7.5), 150 m*M* NaCl, 1% Triton X-100, 10% glycerol, 1 m*M* EDTA, 50 m*M* NaF, 50 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4 and 25 μg/ml each of antipain, leupeptin and pepstatin. Equal amounts of protein extracts were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Antibodies used were: anti-Rb (sc-15), cyclin D1 (sc-92), cdk4 (sc-260), cdk2 (sc-163), cyclin A (sc-239), p27 (sc-528), p21 (sc-397), p16 (sc-468), all from Santa Cruz Biotechnology; anti-cyclin E (06–134) was from Upstate Biotechnology.

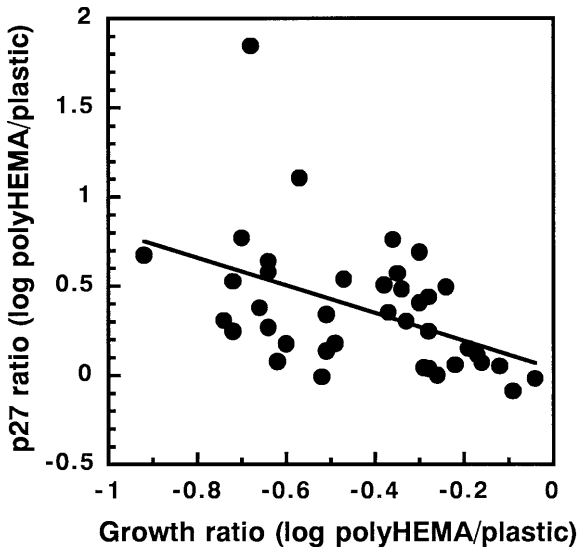

Fig. 4. Inverse correlation between p27 up-regulation and anchorage-independent growth. Statistical analysis was done between p27 ratio (polyHEMA/plastic) and anchorage-independent growth ability expressed as growth ratio (polyHEMA/plastic) as shown in Fig. 1. $r=-0.456$, $P<0.01$.

little or no difference between the attached and the suspension cultures had possibly lost their normal growth control machinery for preventing AIG. Thus the regulation of p27 may be linked to anchorage independence and therefore to tumorigenicity of human cancer cells. Although the derived tumor histology and the phenotype of the cell lines used here have been reported,[11–13] detailed clinical information such as staging is unavailable, making a direct comparison of our data with clinical data impossible. It was recently reported, however, that a lower level of p27 expression in breast, colorectal, and gastric carcinoma is associated with a poorer prognosis (survival) of patients.[18–22] Therefore, a reduced level of p27 in suspension cultures could be an indicator of poor prognosis in the clinical field. Analysis of the levels of p27 and other tested molecules showed that the level of cyclin D1 is correlated with that of p27 in attached cultures ($r=0.568$, $P<0.001$; data not shown), although no correlation was seen in suspension cultures ($r=0.299$; data not shown). This finding suggests that the up-regulation of p27 exceeds that of cyclin D1 in suspension culture. Thus, the balance between the levels of the two molecules might be a critical factor for AIG.

During the preparation of this manuscript, a research group at NCI, NIH, USA reported a new approach to the identification of new anticancer drugs using a database of more than 100 target molecules in 60 human cancer cell lines.[23] Although we have discussed only cell cycle-related molecules in this paper, we have also been investigating other target molecules such as receptors and oncogenes. These current results should be helpful in studies aimed at finding new anticancer drugs and in studies on drug action.

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