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Characterization of Endogenous E2F Transcriptional Activities in Hepatocellular, Gastric and Colorectal Cancer Cell Lines

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In many human cancers, genetic alterations in E2F-pRB pathway have been frequently observed. The E2F transcription factors are known to play pivotal roles in the control of the G1/S transition of cell cycle, and their activities are highly elevated in the cells infected with a DNA tumor virus. We investigated the endogenous E2F transcriptional activities of human hepatocellular, gastric and colorectal cancer cells using a synthetic E2F-responsive reporter vector. As the result we could classify the cells into three categories according to the endogenous E2F transcriptional activity, i.e., low level or negative activity, moderate level, and high level. Cell lines such as HepG2, Hep3B, PLC/PRF/5, SNU-182, SNU-354, Colo205 and HCT116 had low to negative level of endogenous E2F transcriptional activity. On the other hand, SNU-368, SNU-387, SNU-449, SNU-484, SNU-638, SW480, SW620, DLD1 and HCT15 cells showed a moderate level, while strong E2F transcriptional activities were observed in SNU-475 and SNU-216 cells. The negative E2F activity observed in several cancer cells might be due to the active repression of universal transcription factors by the pRB-E2F complex. Thus, although several cancer cells maintained the functional status of E2Fs as the active repressors of universal transcription factors, most of the cancer cells had the repression lifted and showed a significant level of E2F-mediated transcriptional activity. These results suggest that the relief of E2F from pRB-mediated check and its subsequent activation is a general phenomenon accompanying the human carcinogenesis.

Key Words: E2F transcription factor, Transcriptional activity, Rb, Cancer cells

INTRODUCTION

Accurate control of the cellular proliferation process is necessary for normal development and for prevention of proliferative diseases such as cancer.[1)] The cell cycle is a highly controlled process that is affected by both positive and negative growth regulatory signals during the G1 stage.[2)] These signals mainly operate by controlling the transcriptional activity of the crucial cellular factor, E2F.[3,4)] To date, six different E2Fs have been identified, E2F-1 to -6, each of which can hetero-

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dimerize with DP-1 or -2.[5,6] These transcription factors play pivotal roles in cell cycle progression and entry into the S stage by regulating the activity of the promoters containing their binding sites.[1,4] The cellular genes controlled by E2Fs encode nucleotide biosynthesis enzymes such as thymidine kinase (tk), DNA polymerase α and proliferating cell nuclear antigen (PCNA), and cell cycle regulators including cdc2, cdk2, cdk4, cyclin A, D and E, retinoblastoma tumor suppressor protein (pRB) and E2F itself.[1,4,7,8]

It is well known that the E2Fs are bound and regulated by pRB.[9] This protein is a transcriptional repressor and a member of multigene family, containing two additional related proteins, p107 and p130.[10~12] It also plays an important role as a regulator of cell cycle, apoptosis and differentiation by interacting with at least 110 cellular proteins.[11,13] Functional inactivation of pRB family proteins, their structural dysfunctions, and abnormal expression of RB gene are associated with development of a variety of human cancers.[14~18] Several viral oncoproteins can also trigger cancer development by disrupting E2F-pRB interaction.[19~21]

In this study, we investigated the endogenous E2F transcriptional activities of human hepatocellular, gastric and colorectal cancer cells using a synthetic E2F-responsive reporter vector. As the result we could classify the cells into three categories according to the endogenous E2F transcriptional activity, i.e., low level or negative activity, moderate level, and high level.

MATERIALS AND METHODS

1) Cell culture

Human hepatocellular cancer (HCC) cell lines, such as HepG2, Hep3B and PLC/PRF/5 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), 2.0 mM L-glutamine (Gibco BRL) and 10% fetal bovine serum (FBS, HyClone) at 37°C in an incubator flushed continuously with 5% CO_2. Among the SNU series cell lines, which were established from the primary tumors of Korean patients cells,[22,23] six SNU HCC cell lines (SNU-182, -354, -368, -387, -449 and -475) and three gastric cancer (GC) cells (SNU-216, -484 and -638) were used in this experiment. These cell lines and six colorectal cancer (CRC) cells (SW480, SW620, DLD1, HCT15, HCT116 and Colo205) were cultured in RPMI1640 supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% FBS at 37°C in an incubator flushed continuously with 5% CO_2.

2) Vector construction

For quantitative estimation of the endogenous E2F transcriptional activities in HCC, GC and CRC cells, β-galactosidase reporter (β-Gal, LacZ) vectors were used. p(E2F)$_6$/Min-LacZ was constructed by inserting 6 copies of consensus E2F binding sites upstream of the minimal promoter of HSV-tk gene in pMin-LacZ vector. pSp1/Min-LacZ and p(E2F)$_6$/Sp1/Min-LacZ were the same as pMin-LacZ and p(E2F)$_6$/Min-LacZ except that they contained an additional binding site for Sp1 and CTF between the E2F binding sites and the minimal promoter sequence. HβA-LacZ, which expresses β-Gal gene under the control of human β-actin promoter, was used as the positive control.

3) Transient transfection

To explore the promoter activities of reporter vectors, $1.5 \sim 2.5 \times 10^5$ cells/well were transiently transfected in a 6-well plate with 1 μg of each LacZ vector and 0.2 μg of internal control plasmid (pGL2, Promega) using LipofectAMINE Plus Reagent (Invitrogen) according to supplier's protocol. After transfection, the cells were incubated in a 5% CO_2 incubator for 3 hr, added with FBS to bring the final serum concentration to 10% and incubated further for 48 hr before harvesting for luciferase and β-Gal assays.

4) Cell extraction and luciferase assay

Harvested cells were washed in PBS and resuspended in 50 μl of ice-cold 250 mM Tris (pH 7.8)/1 mM DTT. Cells were lysed by three freeze-thaw cycles using liquid nitrogen and a 37°C water bath. The cell lysate was centrifuged at 12,000 rpm for 15 min at 4°C to pellet

debris. The luciferase activity was measured using a 10 μl aliquot of cell lysate mixed with 350 μl of solution A (25 mM glycyglycine (pH 7.8), 2 mM ATP and 10 mM $MgSO_4$). The luciferase light units were recorded for 30 sec in the integration mode in a luminometer (Berthold Lumat LB9501, Wildbad), which automatically injected 100 μl of solution B (60 μg of D-luciferin/ml of 5 mM glycyglycine (pH 7.8)).

5) β-Galactosidase Assay

The β-Gal activity in the cell lysate was determined as followings. Forty microliter of the lysate was mixed with 800 μl of chilled solution I (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$ and 50 mM β-mercaptoethanol). At 30 sec intervals, 200 μl of solution II (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 2 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG, Aldrich) was added to this mixture and, after brief vortexing, the samples were incubated at 37°C. When the reaction mixture developed appropriate level of yellow color, 500 μl of 1 M Na_2CO_3 was added at 30 sec intervals to stop the reaction, and the absorbance of the resulting mixture was measured at 420 nm.

RESULTS

1) Examination of the endogenous E2F transcriptional activity in hepatocellular carcinoma cells

To determine the endogenous E2F transcriptional activity in HCC cells, each β-Gal reporter vector was co-transfected with pGL2 internal control vector into

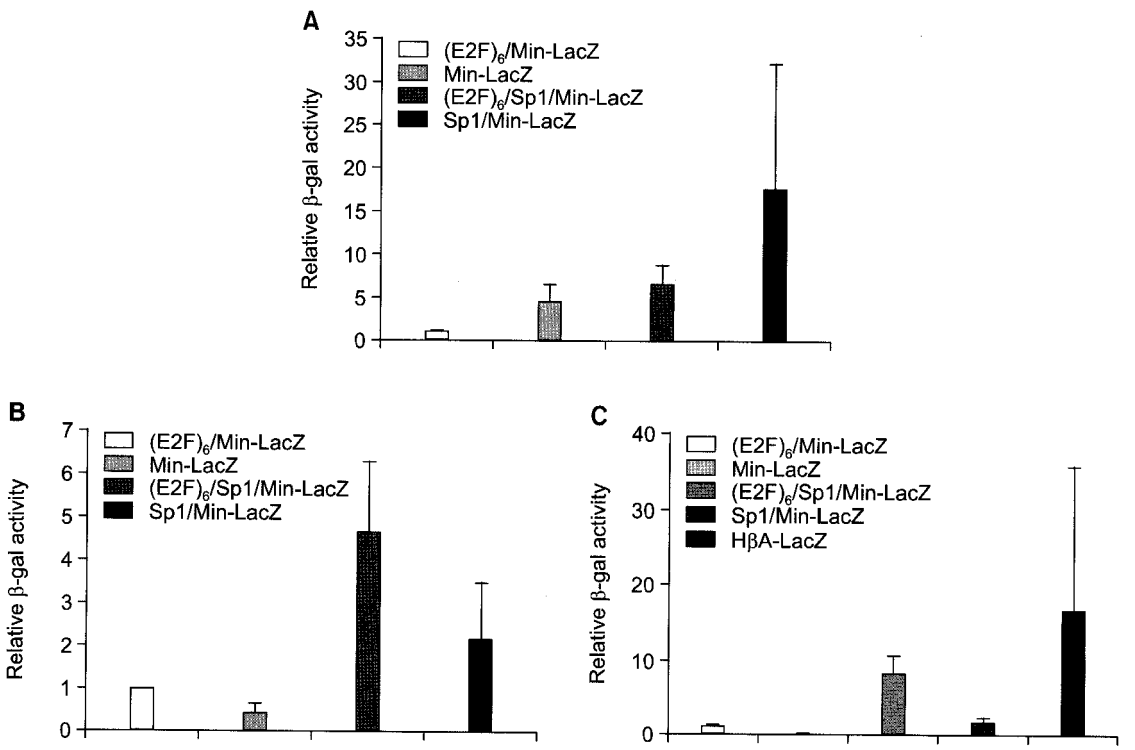

Fig. 1. The endogenous E2F transcriptional activity of hepatocellular carcinoma cells. To measure the E2F-mediated transcription activity, 1 μg of p(E2F)6/Min-LacZ, pMin-LacZ, p(E2F)6/Sp1/Min-LacZ or pSp1/Min-LacZ vector was introduced into HepG2 (A), SNU-368 (B) and SNU-475 cells (C). 0.2 μg of pGL2 vector was co-transfected into each cell as the internal control. β-Gal activities in the cell lysate was measured by reading OD at 420 nm and finally normalized for the transfection efficiency. HβA-LacZ vector was used as the positive control.

these cells. The reporter activities expressed in each cell were estimated by β-Gal and luciferase assays using the lysate of transfected cells, and then the β-Gal values were normalized for transfection efficiency by compensating for the luciferase activity. Finally, the endogenous E2F transcriptional activity of each cell was determined by calculating the reporter activity ratios between the E2F-responsive vector and the corresponding parental vector, i.e., p(E2F)$_6$/Min-LacZ to pMin-LacZ and p(E2F)$_6$/Sp1/Min-LacZ to pSp1/Min-LacZ. The E2F-responsive reporter vectors p(E2F)$_6$/Min-LacZ and p(E2F)$_6$/Sp1/Min-LacZ were derived from pMin-LacZ and pSp1/Min-LacZ, respectively, by inserting 6-mer E2F binding sites. p(E2F)$_6$/Sp1/Min-LacZ contains binding sites for universal transcription factors such as Sp1 and CTF, and thus can measure the synergism or antagonism between them and E2Fs.

In HepG2 cells, the activity ratio of p(E2F)$_6$/Min-LacZ to pMin-LacZ and p(E2F)$_6$/Sp1/Min-LacZ to pSp1/Min -LacZ was 0.23 and 0.37, respectively, indicating that in these cells the endogenous E2F transcriptional activity was nullified by pRB (Fig. 1A). Especially, the negativity of p(E2F)$_6$/Sp1/Min-LacZ to pSp1/Min-LacZ in HepG2 cells suggests that E2Fs, by complex formation with pRB, even worked as active repressors for the transcription mediated by universal transcription factors such as Sp1. Similarly, PLC/PRF/5 (0.43, 0.19) and Hep3B (1.74, 0.48) cells showed negative levels of E2F transcriptional activity (data not shown). In contrast, SNU-182 (0.90, 1.31) and SNU-354 cells (1.22, 1.09) barely exhibited any endogenous E2F transcriptional activity (data not shown). Since the ratio of p(E2F)$_6$/Sp1/Min-LacZ to pSp1/Min-LacZ in these cells is close to unity, the E2F-mediated active repression on universal transcription factors also appears to haven been relieved. The reporter activity ratio in SNU-368 cells was 2.00, 2.15, respectively, indicating that they have moderate level of E2F activity (Fig. 1B). A similar profile of endogenous E2F activity was observed in SNU-449 (1.64, 1.57) cells (data not shown). The activity ratio of reporter vectors was particularly high in SNU-475 cells (11.22, 5.57), where the absolute level of E2F activity approached that of β-actin (Fig. 1C). After all, SNU-475 cells showed the highest endogenous E2F activity among the 9 HCC cells tested (Table 1). In conclusion, we could observe three categories of cells where the endogenous E2F function is repressed, unactivated or activated, respectively.

Table 1. Classification of hepatocellular, gastric and colorectal cancer cell lines based on the level of endogenous E2F transcriptional activity.

E2F transcriptional activity	Cell lines	Origin
High level	SNU-475	Liver
	SNU-216	Stomach
Intermediate level	SNU-368	Liver
	SNU-387	Liver
	SNU-449	Liver
	SNU-484	Stomach
	SNU-638	Stomach
	SW480	Colon
	SW620	Colon
	DLD 1	Colon
	HCT15	Colon
Low level or negative activity	HepG2	Liver
	Hep3B	Liver
	PLC/PRF/5	Liver
	SNU-182	Liver
	SNU-354	Liver
	HCT 116	Colon
	Colo 205	Colon

2) Examination of endogenous E2F transcriptional activity in gastric cancer cells

We also estimated the endogenous E2F transcriptional activities in GC cells derived from Korean patients, i.e., SNU-216, -484 and -638. In SNU-484 cells, the activity ratio of p(E2F)$_6$/Min-LacZ to pMin-LacZ, and p(E2F)$_6$/Sp1/Min-LacZ to pSp1/Min-LacZ was 2.63 and 1.91, respectively, indicating that they had moderate E2F transcriptional activities (Fig. 2A). A similar result was observed in SNU-638 (1.83, 2.95)(data not shown). Interestingly, the SNU-216 cells showed an extraordinarily high endogenous E2F activity (45.77 and 28.55,

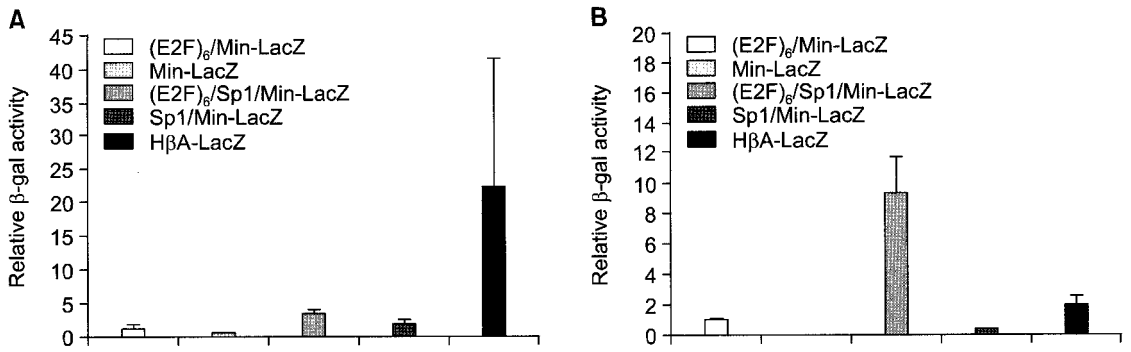

Fig. 2. The endogenous E2F transcriptional activity of gastric cancer cells. To measure the E2F-mediated transcription activity, SNU-484 (A) and SNU-216 cells (B) were transfected with E2F-responsive reporter vectors or control plasmids as outlined in Fig. 1 legend.

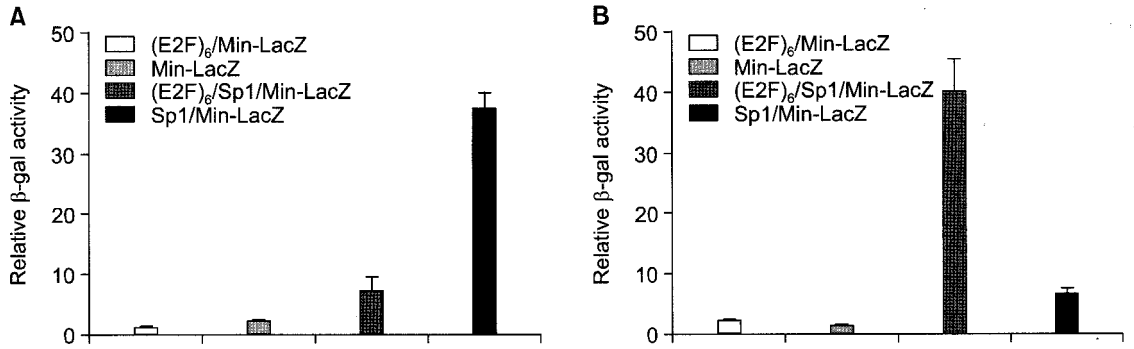

Fig. 3. The endogenous E2F transcriptional activity of colorectal cancer cells. To measure the E2F-mediated transcription activity, Colo205 (A) and SW480 cells (B) were transfected with E2F-responsive reporter vectors or control plasmids as outlined in Fig. 1 legend.

Fig. 2B). Thus, in these cells the activity of p(E2F)$_6$/Sp1/Min-LacZ was even higher than that of HβA-LacZ. None of the GC cells tested in this experiment showed a negative E2F activity.

3) Examination of endogenous E2F transcriptional activity in colorectal cancer cells

Finally, we estimated the endogenous E2F transcriptional activities in various CRC cells. In Colo205 cells, the activity ratio of p(E2F)$_6$/Min-LacZ to pMin-LacZ and p(E2F)$_6$/Sp1/Min-LacZ to pSp1/Min- LacZ was 0.50 and 0.19, respectively (Fig. 3A). This result suggests that like in HepG2 cells the E2F transcription activity was nullified by pRB in these cells and that the E2Fs acted as active repressors on universal transcription factors. Similarly, HCT116 cells had a negative level of E2F activity (0.47, 0.29) (data not shown). In contrast, in SW480 cells the reporter activity ratio was 1.90 and 6.02, respectively, indicating that they have moderate E2F transcription activity (Fig. 3B). We also observed similar reporter activity ratios in SW620 (2.52, 5.23), DLD1 (1.76, 2.37) and HCT15 (1.41, 2.07) cells (data not shown). Since the ratio of p(E2F)$_6$/Sp1/ Min-LacZ to pSp1/Min-LacZ is significantly larger than that of p(E2F)$_6$/Min-LacZ to pMin-LacZ in SW480 and SW620 cells it appears that some degree of synergism occurred between E2Fs and the universal transcription factors in these cells.

DISCUSSION

Genetic analysis in a variety of human tumors has revealed that some of the molecules most often altered in the cancer are those involved in the control of G1/S transition of cell cycle.[4,24~28] Especially, cdk-cyclin D/INK4/pRB/E2F pathway has been found to be altered in over 80% of human cancers, either by mutations within the genes encoding these proteins or in their upstream regulators.[25,27] For example, there are activating mutations of oncogenes such as cyclin D1 or cdk4 genes, or inactivating mutations in tumor suppressor genes including the cdk4 inhibitors.[4,27] In addition, viral oncoproteins increase E2F transcriptional activity in host cells through interactions with pRB and induce various cancers.[19~21,25]

Through the reporter vector systems used in this study, we previously confirmed negligible levels of endogenous E2F transcriptional activity in normal cells such as NIH/3T3 and mouse embryonic fibroblast, and dramatically high levels of endogenous E2F transcriptional activity in the cells harboring a DNA tumor viral genome, such as 293 (adenovirus E1A), HeLa and C3 (HPV E7), and COS-7 (SV40 T) (Manuscript in preparation). Based on these results, we estimated the endogenous E2F transcriptional activities of various cancer cells that are frequently used in cancer studies or established from the primary tumors of Korean patients. We could classify these cells into three categories according to the level of endogenous E2F activity, i.e., low level or negative activity, moderate level, and high level (Table 1). Five HCC cells such as HepG2, Hep3B, PLC/PRF/5, SNU-182 and SNU-354, and two CRC cells such as Colo205 and HCT116 showed a negative level or null activity of endogenous E2F transcriptional activity. Three HCC cells including SNU-368, -387 and -449, two GC cells such as SNU-484 and -638, and four CRC cells such as SW480, SW620, DLD1 and HCT15 had moderate level, while SNU-475 and SNU-216 cells showed a high level of E2F transcriptional activity. Among these categories, those cells belonging to moderate to high level may carry mutations in pRB gene or the regulators of E2F-pRB pathway, or express transforming oncogenes of a DNA tumor virus. It is interesting that several cancer cells maintained the functional status of E2Fs as the active repressors of universal transcription factors just like what is observed in normal differentiated cells. However, most of the cancer cells had the pRB/E2F-mediated active repression lifted and showed a significant level of E2F-mediated transcriptional activity. Thus, these results demonstrate that the relief of E2F from pRB-mediated check and its subsequent activation is a general phenomenon accompanying the human carcinogenesis.

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REFERENCES

1) Black AR, Azizkhan-Clifford J. Regulation of E2F: a family of transcription factors involved in proliferation control. *Gene* 1999; 237: 281-302.
2) Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999; 13: 1501-1512.
3) Muller H, Helin K. The E2F transcription factors: key regulators of cell proliferation. *Biochim Biophys Acta* 2000; 1470: M1-12.
4) Trimarchi JM, Lees JA. Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* 2002; 3: 11-20.
5) Mundle SD, Saberwal G. Evolving intricacies and implications of E2F1 regulation. *FASEB J* 2003; 17: 569-574.
6) Helin K. Regulation of cell proliferation by the E2F transcription factors. *Curr Opin Genet Dev* 1998; 8: 28-35.
7) Yamasaki L. Growth regulation by the E2F and DP transcription factor families. *Results Probl Cell Differ* 1998; 22: 199-227.
8) DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by the E2F1 transcription factor include DNA

synthesis- and G1/S-regulatory genes. *Mol Cell Biol* 1995; 15: 4215-4224.

9) Helin K, Lees JA, Vidal M, Dyson N, Harlow E, Fattaey A. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. *Cell* 1992; 70: 337-350.

10) Grana X, Garriga J, Mayol X. Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene* 1998; 17: 3365-3383.

11) Mulligan G, Jacks T. The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet* 1998; 14: 223-229.

12) Stevaux O, Dyson NJ. A revised picture of the E2F transcriptional network and RB function. *Curr Opin Cell Biol* 2002; 14: 684-691.

13) Morris EJ, Dyson NJ. Retinoblastoma protein partners. *Adv Cancer Res* 2001; 82: 1-54.

14) Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y, Yamamuro T. Mutation spectrum of the retinoblastoma gene in osteosarcomas. *Cancer Res* 1994; 54: 3042-3048.

15) Liu Y, Heyman M, Wang Y, Falkmer U, Hising C, Szekely L, Einhorn S. Molecular analysis of the retinoblastoma gene in primary ovarian cancer cells. *Int J Cancer* 1994; 58: 663-667.

16) Ozen H. Bladder cancer. *Curr Opin Oncol* 1998; 10: 273-278.

17) Bieche I, Lidereau R. Loss of heterozygosity at 13q14 correlates with RB1 gene underexpression inhuman breast cancer. *Mol Carcinog* 2000; 29: 151-158.

18) Azechi H, Nishida N, Fukuda Y, Nishimura T, Minata M, Katsuma H, Kuno M, Ito T, Komeda T, Kita R, Takahashi R, Nakao K. Disruption of the p16/cyclin D1/retinoblastoma protein pathway in the majority of human hepatocellular carcinomas. *Oncology* 2001; 60: 346-354.

19) Nevins JR. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 1992; 258: 424-429.

20) Nevins JR. Cell cycle targets of the DNA tumor viruses. *Curr Opin Genet Dev* 1994; 4: 130-134.

21) Ewen ME. Regulation of the cell cycle by the Rb tumor suppressor family. *Results Probl Cell Differ* 1998; 22: 149-179.

22) Park JG, Lee JH, Kang MS, Park KJ, Jeon YM, Lee HJ, Kwon HS, Park HS, Yeo KS, Lee KU. Characterization of cell lines established from human hepatocellular carcinoma. *Int J Cancer* 1995; 62: 276-282.

23) Park JG, Yang HK, Kim WH, Chung JK, Kang MS, Lee JH, Oh JH, Park HS, Yeo KS, Kang SH, Song SY, Kang YK, Bang YJ, Kim YH, Kim JP. Establishment and characterization of human gastric carcinoma cell lines. *Int J Cancer* 1997; 70: 443-449.

24) Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; 100: 57-70.

25) Nevins JR. The Rb/E2F pathway and cancer. *Hum Mol Genet* 2001; 10: 699-703.

26) Classon M, Harlow E. The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* 2002; 2: 910-917.

27) Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* 2002; 1602: 73-87.

28) Chau BN, Wang JY. Coordinated regulation of life and death by RB. *Nat Rev Cancer* 2003; 3: 130-138.