Jpn. J. Cancer Res. 87, 1204–1209, December 1996

Failure to Detect Mutations in the Retinoblastoma Protein-binding Domain of the Transcription Factor E2F-1 in Human Cancers

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The functions of the transcription factor E2F-1 are regulated by the RB protein through the RB-binding domain of E2F-1 and this factor is considered to be an important molecule that functions downstream of the RB protein. In order to determine whether E2F-1 that cannot bind to RB might be associated with various human cancers, we searched for mutations in the RB-binding domain of E2F-1 using samples of DNA from various clinical specimens obtained from 406 cancer patients (with lung, pancreatic, stomach, colon, esophageal, and hepatic cancers) by analysis of polymerase chain reaction-mediated single-strand conformational polymorphism. No mutations or deletions were detected in genes for E2F-1 from any of the tumor tissues examined. These results suggest that a mutation or deletion in E2F-1 that might affect binding of the RB protein is not involved in human cancers.

Key words: Retinoblastoma protein — E2F-1 — Mutation — Carcinogenesis — PCR-SSCP

The retinoblastoma gene (RB gene) encodes a tumor-suppressor protein, which blocks progression through the cell cycle of cells in the G1 phase.[1,2] The RB protein can bind to a number of cellular proteins through the "pocket" region of the RB protein.[3] Interaction of the RB protein with cellular proteins could suppress the function of such proteins and thereby interrupt the cell cycle. Several oncoproteins encoded by DNA tumor viruses, such as E1A of adenovirus, the large T antigen of simian virus 40 and E7 of human papillomavirus, can also bind to the RB protein through the "pocket" region of the RB protein.[4,5] Mutations in the RB gene have been found in a variety of human tumor tissues.[6–8] Most such mutations were located in the pocket region of the RB protein.[7] These observations suggest that mutations in the RB protein might prevent the binding of certain cellular proteins, with resultant transformation of cells.[9]

The transcription factor E2F-1 is one of the cellular proteins that can bind to the RB protein.[10,11] E2F-1 is involved in regulation of the expression of various genes and protooncogenes, such as the gene for dihydrofolate reductase, the gene for DNA polymerase α, *cdc2*, the gene for cyclin A, the RB gene, the gene for thymidine kinase, and c-*myc* and B-*myb*, which contain a putative binding site for E2F-1 in their respective promoter regions.[12–19] Interaction of the RB protein with E2F-1 inhibits the transcriptional activity of E2F-1.[20–22] Overexpression of E2F can overcome the induction of G1 arrest by the wild-type RB protein.[23] Thus, E2F-1 seems likely to be an important factor that acts downstream of the RB protein.[24] It is possible that a mutation that prevents binding of E2F-1 to the RB protein might be linked to carcinogenesis. Therefore, we examined whether any point mutations in the RB-binding region of E2F-1 could be detected in a variety of human cancerous tissues by PCR-SSCP analysis.[25]

MATERIALS AND METHODS

Preparation of DNA Samples of high-molecular-weight DNA were prepared from specimens of cancerous tissues from a total of 406 patients, as follows: lung, 291; pancreas, 22; stomach, 43; colon, 21; esophagus, 5; and liver, 24. DNA was obtained by treatment of samples with proteinase K and extraction with a mixture of phenol and chloroform as described elsewhere.[26] The cancerous tissues contained less than 10% contamination of normal cells as evaluated from a histological study.

PCR-SSCP analysis Oligonucleotide primers were synthesized in a DNA/RNA synthesizer (model 394; Applied Biosystems, Foster City, CA) and purified with Oligonucleotide Purification Cartridges (Applied Biosystems) and by polyacrylamide gel electrophoresis. The designations, nucleotide sequences, and nucleotide positions in the sequence of E2F-1 cDNA of the primers were as follows: U4-1, TGCGGGCTCCCGTGGACGA, 1229–1247; and D2-1, AGGGGGGTGAGGTCCCCAAA, 1420-1439. The sequences of mutated primers were U4-2, TGCGGGCTACCGTGGACGA; and D2-2, AGGGTGGTGAGGTCCCCAAA. The 5′ ends of primers

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Abbreviations: RB, retinoblastoma; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism.

were labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham, Little Chalfont, Buckinghamshire, UK) and polynucleotide kinase (10 units; Toyobo Inc., Osaka) for PCR-SSCP. The reaction mixture for PCR, in a volume of 5 μl, contained 50 ng of a sample of genomic DNA, 0.125 pmol of labeled primers, PCR buffer, 62.5 μM dNTPs and Taq polymerase (0.0625 units, Takara Inc., Kyoto). Amplification was performed for 30 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min in a Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). Then 10-fold diluted PCR products were heated with 95% formamide, 20 mM EDTA, 0.015% xylene cyanol and bromophenol blue at 95°C for 3 min, and 1 μl of the solution was applied to a 5% non-denaturing polyacrylamide gel (acrylamide : N,N'-methylene-bisacrylamide, 99 : 1, w/w) with or without 10% glycerol. Electrophoresis was performed at a constant voltage of 1,400 V for approximately 2 h with cooling by a fan. The gel was dried on filter paper and exposed to X-ray film.

RESULTS

For detection of mutations in the RB-binding domain of E2F-1 by PCR-SSCP analysis, we chose the primers described in "Materials and Methods" and determined

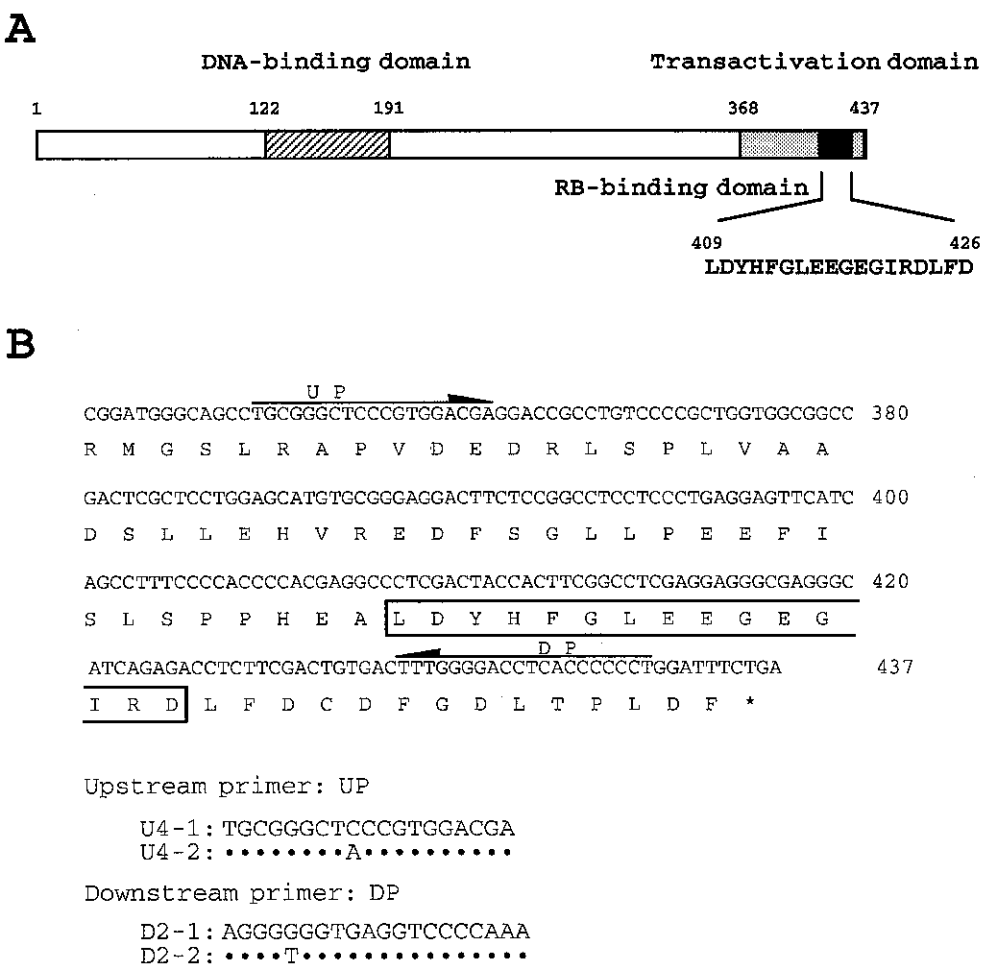

Fig. 1. The region of the gene for E2F-1 that was subjected to PCR-SSCP analysis and the primers used in this study. A, Structure of the E2F-1 protein. The DNA-binding domain, the transcription-activation domain and the RB-binding domain are depicted schematically. B, The DNA sequence encoding the RB-binding domain of E2F-1 and the primers used for PCR-SSCP analysis. U4-1 and D2-1 have the wild-type sequence. U4-2 and D2-1 have sequences with a single-base mutation in each, and were used in the control experiment. The rectangle indicates the RB-binding domain.

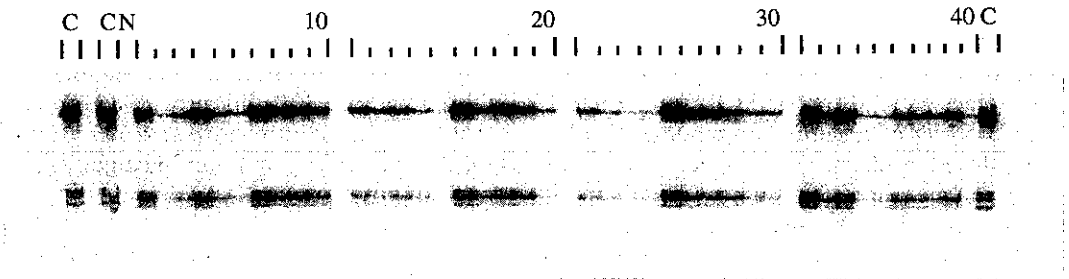

Fig. 2. PCR-SSCP analysis of the region of the E2F-1 gene that encodes the RB-binding domain. The samples of DNA prepared from specimens of human cancer tissues were analyzed by the PCR-SSCP method with the primers U4-1 and D2-1 (lanes 1 to 40). A sample of DNA prepared from HeLa cells, which have the wild-type gene for E2F-1, was used as a control template in lane C. No DNA was added to the reaction mixture in lane N.

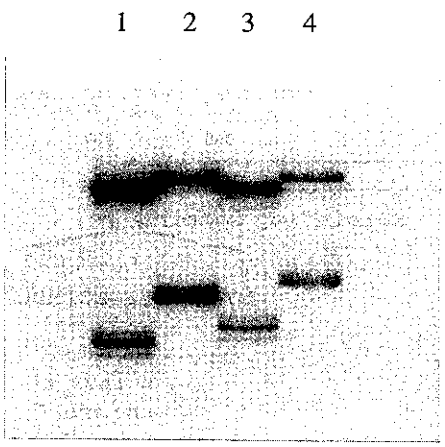

Fig. 3. The control experiment for the PCR-SSCP analysis of the RB-binding domain of E2F-1 using mutated primers. PCR-SSCP analysis of the region of the gene for E2F-1 that encodes the RB-binding domain using DNA prepared from HeLa cells was performed with wild-type and with mutated primers. The combinations of primers used are shown below the photograph of the gel.

the optimal conditions for PCR. The region encoding the RB-binding domain of E2F-1 was amplified without any spurious reaction products. With the U4-1 and D2-1 primers, a DNA fragment of 211 bp that included the region encoding the RB-binding domain (residues 409–426; Fig. 1A) was amplified by PCR. We examined mutations in this region using DNA from cancerous tissues of 406 patients (lung, 291; pancreas, 22; stomach, 43; colon, 21; esophagus, 5; and liver, 24). Some of the results of PCR-SSCP analysis are shown in Fig. 2. When DNA was prepared from HeLa cells, which have the wild-type sequence of E2F-1, and used as a control template, two main bands corresponding to the separated complementary strands and a few minor bands were observed (Fig. 2, lane C). These accessory bands were considered to have been derived from the same fragments and formed other conformations. No bands with abnormal mobility were detected with any of the 406 samples of DNA examined in this study (Fig. 2, lanes 1–40). To confirm that single-base mutations could be detected under our conditions, PCR-SSCP analysis was performed with primers with a single-base mutation and a sample of DNA prepared from HeLa cells. When the mutated primers U4-2 or D2-2 (Fig. 1B) were used, both bands were shifted (Fig. 3, lanes 2 and 3) as compared with the bands obtained by PCR with the wild-type primers U4-1 and D2-1 (Fig. 3, lane 1). When two mutated primers were used, the bands exhibited yet another pattern (Fig. 3, lane 4). Thus, it was evident that even a single-base substitution in the sequence could be detected under our conditions. We attempted PCR-SSCP analysis under other conditions (using gels with 10% glycerol) but, again, no bands with abnormal mobility were detected (data not shown). Furthermore, we performed a direct sequencing of the PCR products in 9 patients selected at random, but could not detect any sequence difference. These results strongly suggest that there were no mutations in the RB-binding domain of E2F-1 in any of the samples examined in this study.

Furthermore, no disappearance of bands was observed in the analysis of any of the samples, indicating that loss of both alleles of the E2F-1 gene, or at least of the RB-binding region, had not occurred in the cancerous tissues that we examined.

DISCUSSION

E2F-1 was originally described as a cellular factor that specifically binds to the promoter region of the adenovirus E2 gene.[27] Later, it was shown that transfection of NIH 3T3 cells with the gene for E2F-1 results in the anchorage-independent growth of the cells[28] and that E2F-1 cooperates with an activated *ras* gene to induce the formation of morphologically transformed foci of primary rat embryo fibroblasts, an indication that the gene for E2F-1 behaves as an oncogene.[29] A mutant of E2F-1 defective in the RB-binding domain has greater transforming activity than the wild-type E2F-1, suggesting that E2F-1 that cannot bind the RB protein might be the form that is active in oncogenesis.[29] Accordingly, we expected that mutations of E2F-1 in the RB-binding domain might be found in some human clinical cancers.

We analyzed that part of the gene for E2F-1 that corresponded to the RB-binding region in various human cancer tissues from 406 patients by PCR-SSCP analysis, but no abnormal bands were detected. PCR-SSCP analysis has been used successfully for detection of various aberrations in DNA, including point mutations, and it has been found to be especially useful for the analysis of a large number of samples. However, this method is strongly dependent on the precise experimental conditions. In order to exclude the possibility of false-negative results, we performed the PCR-SSCP assay using single-base-mutated primers as a control experiment, and shifted bands were observed as compared with the bands obtained with the wild-type primers. Furthermore, we performed PCR-SSCP analysis under other conditions, namely, with 10% glycerol in the separation gels. However, we failed to detect any band with abnormal mobility. These results support our hypothesis that there were no mutations or deletions in the RB-binding domain of E2F-1 in the samples from all 406 patients in this study. Mutation of E2F-1 that prevents binding of RB seems, therefore, not to be involved in carcinogenesis in most human cancers. However, we cannot rule out the possibility that a mutation in E2F-1 might be associated with a small population of specific cancers, since the number of samples was relatively small in this study.

As a simple explanation for our observation that no mutation in E2F-1 had occurred in the human cancers that we examined, we can postulate that the chromosomal loci of the E2F-1 gene might not have contained fragile sites that have a tendency to induce multiple genetic alterations.[30] By contrast, it was recently reported that an "E2F-1 knockout" mouse developed tumors and exhibited a maturation stage-specific deficiency in thymocyte apoptosis.[31, 32] Although the overexpressed gene for E2F-1 in cultured cells behaves like an oncogene,[29] E2F-1 also functions to suppress the formation of tumors and to regulate apoptosis *in vivo*. Therefore, because of the multiplicity and complexity of the functions of E2F-1, cells having a mutation in E2F-1 might not expand clonally or might be eliminated by apoptosis.

Multiple genetic alterations caused by point mutation, deletion, recombination and/or amplification of an oncogene, a tumor-suppressor gene or some other genes, whose functions are involved in cell proliferation, are considered to be a direct reflection of the progression or malignancy of human tumors.[33–35] Point mutation in *ras* genes or allelic loss and mutation of p53, RB or $p16^{INK4}$ have frequently been found in many human cancers.[36–39] However, such genetic alterations have not been found in certain other oncogenes, such as *waf*-1[40] and c-*raf*-1[41] in clinical cancer specimens. Thus, investigations of mutations in many kinds of gene and the correlation of each mutation with malignancy in human clinical cancers are very important in studies of carcinogenesis.

ACKNOWLEDGMENTS

The authors are very grateful to Dr. Takao Sekiya of the National Cancer Center Research Institute for his kind advice on the PCR-SSCP method and to Dr. Eisaku Yoshida of Banyu Tsukuba Research Institute for helpful discussions.

(Received July 29, 1996/Accepted September 12, 1996)

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