FEBS Letters 580 (2006) 4073–4080

A novel cervical cancer suppressor 3 (CCS-3) interacts with the BTB domain of PLZF and inhibits the cell growth by inducing apoptosis

Seung Bae Rho, Young Gyo Park, Kyoungsook Park, Seung-Hoon Lee[1], Je-Ho Lee*

Molecular Therapy Research Center, Sungkyunkwan University, Samsung Medical Center Annex 8F, 50, Ilwon-Dong, Kangnam-Ku, Seoul 135-710, South Korea

Received 18 May 2006; revised 22 June 2006; accepted 22 June 2006

Available online 30 June 2006

Edited by Jesus Avila

Abstract **Promyelocytic leukemia zinc finger protein (PLZF) is a sequence-specific, DNA binding, transcriptional repressor differentially expressed during embryogenesis and in adult tissues. PLZF is known to be a negative regulator of cell cycle progression. We used PLZF as bait in a yeast two-hybrid screen with a cDNA library from the human ovary tissue. A novel cervical cancer suppressor 3 (CCS-3) was identified as a PLZF interacting partner. Further characterization revealed the BTB domain as an interacting domain of PLZF. Interaction of CCS-3 with PLZF in mammalian cells was also confirmed by co-immunoprecipitation and in vitro binding assays. It was found that, although CCS-3 shares similar homology with eEF1A, the study determined CCS-3 to be an isoform. CCS-3 was observed to be downregulated in human cervical cell lines as well as in cervical tumors when compared to those from normal tissues. Overexpression of CCS-3 in human cervical cell lines inhibits cell growth by inducing apoptosis and suppressing human cyclin A2 promoter activity. These combined results suggest that the potential tumor suppressor activity of CCS-3 may be mediated by its interaction with PLZF.**

Keywords: Cervical cancer suppressor 3; PLZF; Cyclin A2; Cell cycle arrest; BTB/POZ domain

1. Introduction

Promyelocytic leukemia zinc finger protein (PLZF) is a sequence-specific DNAbinding protein containing nine C-terminal C_2H_2 zinc finger motifs and an N-terminal BTB (broad complex, tramtrack, bric-a-brac)/POZ (Pox virus and zinc finger) [1–4]. PLZF functions as a transcriptional regulator for cell cycle progression by binding to the promoter of target genes, such as those for cyclin A and the interleukin-3 receptor α subunit [5–8]. It also interacts with nuclear co-repressor proteins such as N-CoR, SMRT, and mSin3A, which in turn interact with histone deacetylases (HDAC1) [9–12].

PLZF is expressed during early embryogenesis of the axial skeleton, liver, and heart. It serves as a regulator for Hox gene expression, which is utilized as a growth-inhibitor and pro-apoptotic factor in limb budding. In the hematopoietic system, PLZF is expressed in bone marrow, early myeloid cell lines, and peripheral blood mononuclear cells, as well as in the ovary, and at lower levels, in the kidney and lungs [5,13].

The BTB/POZ domain of PLZF is an evolutionarily conserved protein–protein interaction domain found in essential transcriptional regulators involved in a variety of important developmental processes, such as in homeostasis, neoplasia, apoptosis, cell growth, proliferation, differentiation, nuclear sub-localization, and transcriptional repression during pattern formation in embryogenesis [14–21]. The BTB/POZ domain is also strongly implicated in the regulation of gene expression through the local chromatin conformation. The BTB/POZ motif, downregulates transcription by interacting with specificity protein 1 (Sp1) zinc finger and by also interfering with DNA binding activity of Sp1 [22]. Another BTB/POZ protein, B-cell lymphoma 6 (Bcl-6), is rearranged in a large diffuse cell known as Hodgkin's lymphoma, as well as in AIDS-associated lymphoma, and is also upregulated during myogenesis [23–25]. These reports suggest a critical role played by the BTB/POZ motif.

To begin to characterize the PLZF-dependent pathway, we used a yeast two-hybrid system to screen a human ovary cDNA library for novel PLZF binding proteins. We identified human cervical cancer suppressor 3 (CCS-3), a component of cervical tumor suppression. CCS-3 was found to have similarities to eukaryotic elongation factor 1A (eEF1A) which is implemented in cellular processes including the organization of cytoskeleton, oncogenic transformation, signal transduction, and nuclear export of tRNA [26–28]. The mapping studies reveal the BTB domain as being a PLZF interacting domain. Overexpression of CCS-3 and PLZF in human cervical cell lines inhibits cell growth by inducing apoptosis as well as suppressing the promoter activity of human cyclin A2. These results suggest that the cell cycle arrest function of CCS-3 may be involved as a tumor suppressor for cervical tumors.

2. Materials and methods

2.1. Yeast two-hybrid analysis

We constructed the LexA-human PLZF fusion protein and used it as bait to screen the binding proteins from the human ovary cDNA library in which proteins were expressed as B42 fusion proteins. The

*Corresponding author. Fax.: +82 2 3410 6829.
E-mail address: jeholee@unitel.co.kr (J.-H. Lee).

[1] Present address: Department of Life Science, Yong-In University, 470 Sanga-dong, Yongin 449-714, Kyunggido, South Korea.

Abbreviations: CCS-3, cervical cancer suppressor 3; PLZF, promyelocytic leukemia zinc finger protein; BTB, broad complex tramtrack bric-a-brac; Sp1, specificity protein 1; Bcl-6, B-cell lymphoma 6; ONPG, *O*-nitrophenyl β-D-galactopyranoside; FITC, annexin V-fluorescein isothiocyanate; PI, propidium iodide

0014-5793/$32.00 © 2006 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2006.06.047

resulting plasmid pLexA-PLZF was introduced into yeast strain EGY48 [*MATa*, *his3*, *trp1*, *ura3-52*, *leu2::pLeu2-LexAop6*/pSH18-34 (*LexAop-lacZ* reporter)] by a modified lithium acetate method [29]. The cDNAs encoding B42 fusion proteins were introduced into competent yeast cells that already contained pLexA-PLZF and the transformants, tryptophan prototrophy (plasmid marker), were selected for on a synthetic medium (Ura^-, His^-, Trp^-) containing 2% glucose. We tested their interactions with pLexA-PLZF on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside as described. Then, β-galactosidase activity was measured by adding 140 μl of 4 mg/ml *O*-nitrophenyl β-D-galactopyranoside (ONPG) [30,31].

2.2. In vitro pull-down assay

cDNA encoding human PLZF was subcloned into the *Eco*RI and *Xho*I restriction sites of the pGEX4T-1 (Amersham Biosciences, Uppsala, Sweden) to generate glutathione *S*-transferase (GST) fusion protein (GST-PLZF). The human CCS-3 was ligated into pET29a (Novagen, Madison, WI) using *Eco*RI and *Xho*I to generate a histidine fusion protein (His-CCS-3). GST and histidine fusion proteins were purified using either a GST column (Amersham) or a Ni^{2+} column (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocols. The purified GST-PLZF (2 μg) was mixed with His-CCS-3 (2 μg). The mixtures were then added to 20 μg of GST column matrix (glutathione sepharose 4B) and incubated at 25 °C for 30 min. The slurry was pelleted by centrifugation and washed. The pellet of the gel matrix was re-suspended in 20 μl elution buffer (10 mM glutathione, 50 mM Tris–HCl, pH 8.0) and incubated at 25 °C for 20 min to elute the bound GST fusion proteins. The eluted proteins were separated by SDS–PAGE and the proteins were detected by Coomassie staining.

2.3. Subcloning of deletion mutants of PLZF

Three deletion mutants (BTB domain) of PLZF were isolated by PCR using the combination of the following primers PLZF(BTB)-F1, 5′-CGGGAATTCATGGATCTGACAAAAATG-3′; PLZF(BTB)-R1, 5′-ATTCTCGAGTCACTTCAGGCACTGTTC-3′; PLZF(RD2)-F2, 5′-ATTGAATTCACCAAGGCTGCAGTGGAC-3′; PLZF(RD2)-R2, 5′-ATTCTCGAGTTAGCCAGCCTCAGCTGG-3′. PLZF(ZF)-F3, 5′-CGGGAATTCAGCTACATCTGCAGTGAG-3′; PLZF(ZF)-R3, 5′-ATTCTCGAGTCACACATAGCACAGGTA-3′. PCR products spanning each fragment were cloned into the *Eco*RI and *Xho*I restriction sites of the pGilda. Each constructed plasmid was introduced into yeast EGY48 expressing either CCS-3 or PLZF hybrid protein.

2.4. Co-immunoprecipitation

cDNA encoding human PLZF was isolated by PCR using a specific template which was then cloned into pEGFPC1 (Clontech) and digested with *Bgl*II and *Eco*RI (pEGFPC1-PLZF). The human CCS-3 cDNA was ligated into pcDNA4/HisMax (Invitrogen) using *Eco*RI and *Xho*I (pcDNA4/HisMax-CCS-3). For co-immunoprecipitation, SiHa cancer cells were co-transfected with the cDNA constructs of pEGFPC1-PLZF and pcDNA4/HisMax-CCS-3 using FuGENE6 (Roche Applied Science, Basel, Switzerland). As a negative control, pEGFPC1-PLZF and an empty vector pcDNA4/HisMax was also co-transfected. Three days after transfection, cells were harvested by trypsinization and centrifugation. Cell pellets were washed in PBS, re-suspended in cell lysis solution (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, and 200 μg/ml PMSF). Lysates were incubated with anti-GFP antibody (Zymed) and precipitated with protein A–agarose. The precipitated proteins were resolved by SDS–PAGE, and determined using the ECL system (Amersham).

2.5. RT-PCR

The normal cells consisted of MRC-5 and IMR-90, which are derived from the fibroblasts cell line. Also included as a normal cell, HEK-293, which belongs to the human embryonic kidney cell line. SiHa and HeLa, cervical cancer cells, which originated from the human cervical cancer lines, were used for the study. A total of 8 patients with cervical carcinoma were treated in the Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea. The cervical carcinoma tissues being studied are squamous carcinomas. After cell lysis with TRIZOL Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), the cell extracts were incubated, with the addition of chloroform, for 5 min at 30 °C. After vortexing, the mixtures were centrifuged at 14000 rpm for 15 min. We collected the upper layer and added isopropanol for RNA precipitation. After washing with 100% ethanol, 1 μg of RNA dissolved in distilled water was used. cDNA was prepared by random priming 500 ng of total RNA using a first-strand cDNA Synthesis kit and then carried out with specific primers for CCS-3. Furthermore, the PCR product sequences were analyzed, and were found to be identical to the sequences of the CCS-3 and non-CCS-3 gene, respectively.

2.6. MTT assays

The relative rate of cell proliferation was quantified by MTT assays. Briefly, SiHa cervical cancer cells were grown in a DMEM medium containing 10% FBS. Cells were seeded at a density of 3×10^3 cells per well on 96-well plates. Three days after transfection, fresh medium containing 10% FBS, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-^{2}H-tetrazolium bromide (MTT) solution (Sigma, 5 μg/ml) were added to each well. Each well was then incubated for an additional 4 h at 37 °C. The amount of MTT-formazan was determined through absorbance using a microculture plate reader at 540 nm. Each sample was assayed in triplicate and independent experiments were repeated three times.

2.7. Apoptosis assays

SiHa cervical cancer cells were plated onto 4-chamber slides and then transfected with Mock (an expression vector only without insert) or CCS-3 cDNAs for 24 h using Effectene as described above. Cells were incubated with FITC-labeled Annexin V and propidium iodide (PI) for 15 min according to the supplier's protocol (Boehringer Mannheim, Mannhein, Germany) and then analyzed on a FACS Vantage (Becton Dickinson, San Jose, USA). For the evaluation of nuclear morphology, cells were fixed in methanol and stained with 2 μg/ml of DAPI (Boehringer Mannheim) at 37 °C for 15 min, and were washed twice with PBS and examined with the fluorescence microscope.

2.8. Cell cycle analysis

SiHa cells transfected with Mock or CCS-3 expression vector was fixed with ice-cold 70% ethanol, centrifuged for 5 min at $1000 \times g$, and re-suspended in PBS containing 5 mM EDTA and RNase A (50 μg/ml). After incubation for 30 min at 37 °C, the cells were stained with PI (50 μg/ml) and analyzed by flow cytometry with FACS.

2.9. Luciferase reporter gene assays

All construct (IL-3R-Luc and pCyclinA2-Luc) for the promoter-reporter assay were kindly provided by Dr. Inpyo Choi (Korea Research Institute of Bioscience and Biotechnology, Taejon, Republic of Korea) [32]. SiHa cells plated on 60-mm dishes were transfected with the indicated plasmids using Effectene (Qiagen, Hilden, Germany). After lysis, the cell extracts were incubated with the luciferase substrate for 30 min at room temperature. Then, a 5 μl aliquot of each sample was transferred into the luminometer plate, and the luciferase activity was measured with the Luciferase Assay Systems (promega, Madison, WI, USA).

3. Results

3.1. Identification of CCS-3 as a PLZF binding protein and mapping of the interaction region

The human ovary cDNA library, fused to the gene for the transcription activator pJG4-5, was introduced into yeast cells containing pLexA-PLZF as bait. Approximately 3.9×10^6 independent transformants were pooled and re-spread on the selection media (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose to induce the expression of cDNA. If a B42-tagged protein interacts with PLZF, it will activate the transcription of the *LEU2* gene and allow the host cells to grow on a synthetic

medium lacking leucine. Among the 15 colonies obtained on the selection media, a total of 6 colonies showed galactose dependency. The plasmids were isolated from the selected yeast cells and were then introduced into *Escherichia coli* KC8 to isolate the plasmids carrying pJG4-5-cDNA inserts. The plasmids were then isolated by the plasmid marker *trp* in the *E. coli* host, and the purified plasmids were sequenced. A homology search in GenBank using the BLAST program revealed that all six plasmids encoded CCS-3 (GenBank Accession No. AF322220).

To identify the CCS-3 binding region of PLZF, cDNA constructs containing three PLZF deletion mutants were designed as shown in Fig. 1A. In the two-hybrid system, the full-length human CCS-3 cDNA and either a plasmid containing a full-length human PLZF cDNA (Full, Fig. 1A) or a plasmid containing three truncated mutant forms (BTB, RD2, and ZF, Fig. 1A) of cDNAs were co-transformed into EGY48 yeast cells. Cells containing only full-length PLZF cDNA construct and BTB-PLZF grew on the Ura, His, Trp and Leu deficient plates, whereas yeast cells transformed with both deletion mutant colonies (RD2, and ZF) failed to grow (Fig. 1A). To confirm these results, we determined the binding activity of these constructs by measuring the relative expression level of β-galactosidase. As shown in Fig. 1A, β-galactosidase assay results confirmed that these mutants (RD2, and ZF) could not bind to CCS-3.

The interaction between PLZF and CCS-3 was further examined by GST pull-down analysis and co-immunoprecipitation. For GST pull-down analysis, the GST-PLZF fusion protein was mixed with His-CCS-3, and the mixture was then purified employing the GST column matrix. The eluted proteins were separated by gel electrophoresis and the proteins were detected by Coomassie staining. As shown in Fig. 1B, GST-PLZF was co-purified with His-CCS-3 at an approximate molar ratio of 1:1. As positive controls, GST only (lane 1), GST-PLZF-Full (lane 2), GST-PLZF-BTB (lane 3), and His-CCS-3 (lane 4) were purified using either a GST column matrix or a Ni^{2+} column and loaded each 200 ng on the gel. For co-immunoprecipitation, cDNA constructs of PLZF (pEGFPC1-PLZF) and CCS-3 (pcDNA4/HisMax-CCS-3), or pEGFPC1-PLZF and pcDNA4/HisMax (vector only) were co-transfected into SiHa cells. Subsequently, immunoprecipitation was performed using anti-GFP antibody with lysates from both transfected cells. After immunoprecipitation, the precipitated proteins were immunoblotted using anti-His antibody or anti-PLZF antibody. As shown in Fig. 1C, pcDNA4/HisMax-CCS-3 was co-immunoprecipitated with pEGFPC1-PLZF (lane 2 in upper panel), whereas no interaction was observed between pcDNA4/HisMax (vector only) and pEGFPC1-PLZF (lane 1 in upper panel). An immunoblotting using anti-PLZF antibody confirmed that an equal amount of PLZF was precipitated in both samples (middle panel). Whole cell lysates from both samples were contained the equivalent proteins when we immunoblotted using anti β-actin antibody (lower panel).

3.2. Sequence comparison of CCS-3

Due to the lack of information on CCS-3, we identified the amino acid sequence of CCS-3, and performed an amino acid alignment with the NCBI database (Fig. 2A). With the findings,

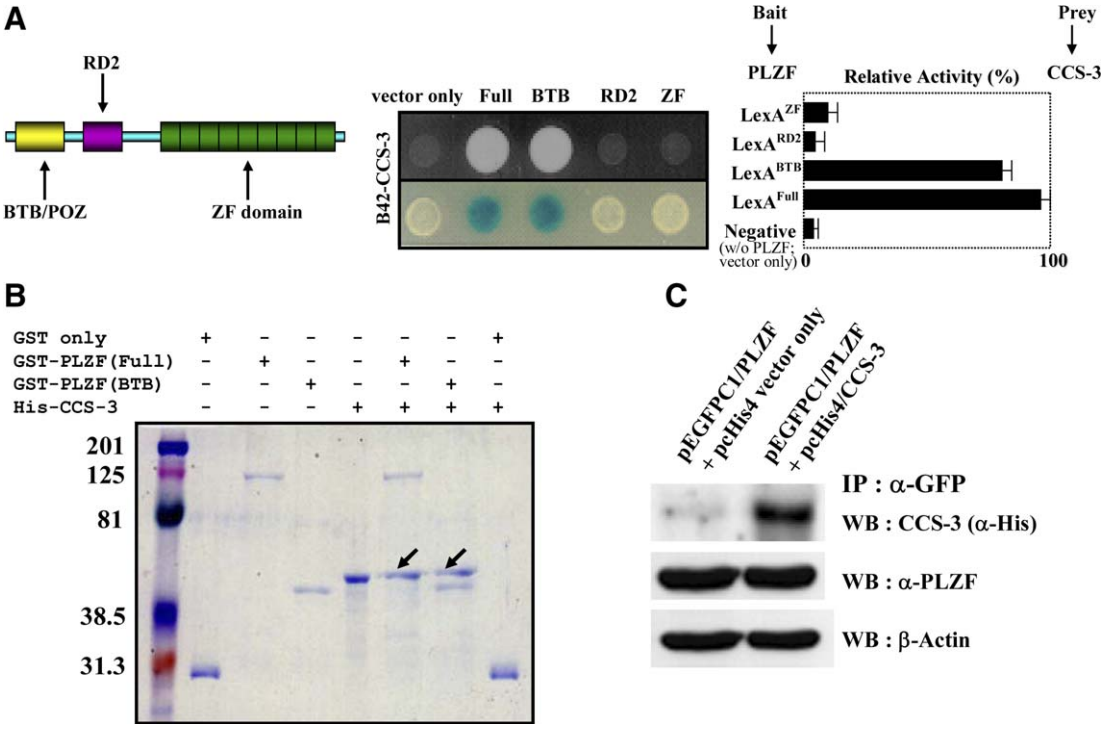

Fig. 1. PLZF interacts with CCS-3. (A) The structure is a representation of PLZF. Identification of the binding domain of PLZF that is responsible for interaction with CCS-3 by two-hybrid assay. The positive interactions were determined by cell growth on leucine-depleted medium. Quantitation of binding strength by ONPG assays to measure the levels of β-galactosidase activity. (B) Interaction between PLZF and CCS-3 were confirmed by in vitro binding assay. The GST-PLZF(full) and GST-PLZF(BTB) fusion proteins were mixed with His-CCS-3. The mixtures were subjected to glutathione affinity purification. (C) Co-immunoprecipitation PLZF with CCS-3. Lane 1, lysate from pEGFPC1-PLZF and pCDNA/His (vector only) co-transfectant; lane 2, lysate from pEGFPC1-PLZF and pCDNA/His-CCS-3 co-transfectant. IP means immunoprecipitation and WB means immunoblotting with indicated antibodies.

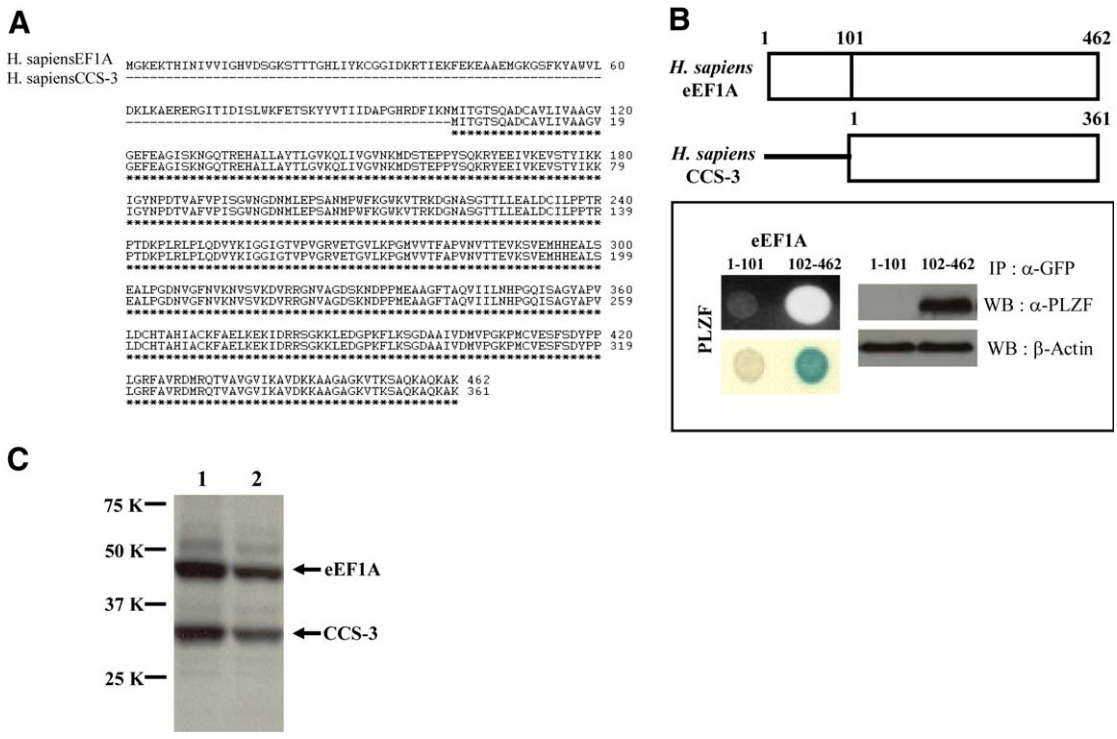

Fig. 2. Alignment of CCS-3. (A) Alignment of amino acid sequence of the CCS-3 with *H. sapiens* eEF1A. (B) The differences between the two were found using co-immunoprecipitation analysis, where CCS-3′s N-terminal region appeared to contain a 101 amino acid deletion when compared to eEF1A. (C) Western blot analysis of eEF1A or CCS-3 in human HEK 293 and MRC-5. Lane 1, lysate from HEK 293 (normal human embryonic kidney cell lines); lane 2, lysate from MRC-5 (normal fibroblasts cell lines). Western blot analysis was conducted by employing eEF1A polyclonal antibody, which has 163–462 amino acids corresponding to an epitope at its C-terminus.

we were able to identify that CCS-3 and eEF1A shared similar homology. The differences between the two were found using co-immunoprecipitation analysis, where CCS-3′s N-terminal region appeared to contain a 101 amino acid deletion when compared to eEF1A. We believe the 101 amino acids that do not appear on CCS-3 are the amino acids that produce eEF1A's functions. Since eEF1A and CCS-3 conduct opposite functions, more research must be conducted on the N-terminal regions of both in order to formulate function and mechanism (Fig. 2B). In order to identify whether CCS-3 is an isoform of eEF1A, the use of the cell lines HEK 293, human embryonic kidney cell, and MRC-5 were observed through western blotting. Western analysis was conducted by employing eEF1A polyclonal antibody, because this antibody contains 163–462 amino acids corresponding to an epitope at its C-terminus. As seen in Fig. 2C, two bands were identified, the top being eEF1A and the bottom being the detection of a smaller protein. Through the results found we were able to determine that eEF1A was not a partial cDNA clone, but instead was found to be an isoform, with a deletion of 101 amino acids at the N-terminal end of eEF1A.

3.3. CCS-3 gene expression

To examine the expression of CCS-3, total RNA was used to determine the level of CCS-3 expression. Total RNA was isolated from several cervical cells from both normal and tumor tissues. In contrast to the high level of CCS-3 mRNA in normal human cell lines (HEK293, MRC5, IMR90), CCS-3 mRNA level was very low to undetectable in human cervical cancer cells (SiHa, HeLa) (Fig. 3A). From the analyses of 8 different patient samples, we also observed the cancer-specific reduction of CCS-3 in 7 cases (Fig. 3B and data not shown). All of these results suggest that low CCS-3 expression may be associated with human cervical cancer.

3.4. CCS-3 induces apoptosis in human cancer cells

To investigate the effects of CCS-3 in human cancer cells, CCS-3 overexpression was induced in SiHa cervical cancer cells. Transient overexpression of CCS-3 caused a change in cell morphology (Fig. 4A) and dramatically reduced in cell growth (Fig. 4B). To confirm that the reduction in cell numbers represented apoptosis, we examined the morphology of the DAPI-stained nuclei in SiHa cells overexpressing control vector and full-length CCS-3. Full-length, CCS-3-overexpressing cells showed fragmented nuclei, which are characteristic of apoptosis, transfectants containing only control vector did not show any signs of DNA fragmentation (Fig. 4C). Apoptosis was also examined by FACS analysis after double staining with Annexin V-fluorescein isothiocyanate (FITC) and PI. CCS-3-overexpressing cells showed strikingly similar characteristics of apoptotic cells, notably a low forward scatter and high side scatter profile (Fig. 4D). In the control vector culture, 93.31% of the cells were viable, while 2.84% were in early apoptosis and 3.85% were in the late or final stages of apoptosis ($P < 0.01$) (left panel). Initial analysis of the SiHa cells induced with CCS-3 during 12 and 24 h showed no significant modifications in relation to the control culture (data not shown). However, in SiHa cells treated with CCS-3 during 72 h, 48.52% of the cells were viable, while 37.39% were in early apoptosis and 14.09% were in the late or final stages of apoptosis (right panel). These results suggest that CCS-3 overexpression induce apoptosis in cervical carcinoma cells.

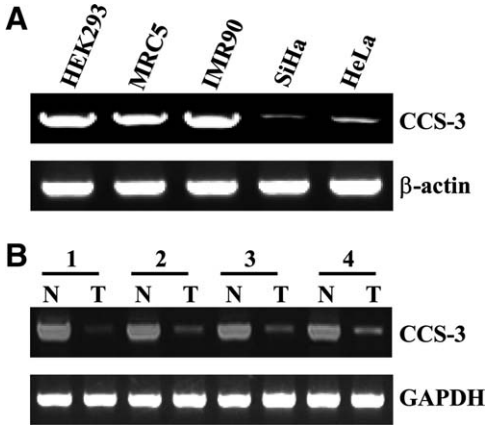

Fig. 3. Expression of CCS-3 in human cancer cell lines and patient tissues. (A) RT-PCR was employed, to examine by semi-quantitative analysis, the expression level of CCS-3 in different normal (HEK293, MRC5, IMR90) and cervical cancer cell lines (SiHa, HeLa). (B) The expression status of CCS-3 in the normal and cancerous regions was isolated from human cervical cancer patients. Total RNAs were isolated from the normal and cancer regions in the patient's cervix, and RT-PCR was conducted to compare the CCS-3 expression levels using specific primers for CCS-3. Among the eight informative samples, the results of only four cases are shown. The β-actin cDNA probe was used to evaluate the amount of mRNA loaded in each lane.

3.5. CCS-3 functioned as a transcriptional repressor

To explore the functional relation between the interactions of CCS-3 and PLZF in transcriptional activity, we performed a luciferase assay with a reporter construct (pIL-3-Luc) containing four copies of the PLZF binding site. We found that CCS-3 specifically repressed this reporter gene about twofold (Fig. 5A). The transcriptional repression was dependent on the amount of plasmid transfected, and when both CCS-3 and PLZF were expressed, there was an additive effect on repression (Fig. 5B). Cyclin A2 expression is essential for cell cycle progression in both normal and cancer cells. CCS-3 also suppressed the promoter activity of human cyclin A2 which embraces the PLZF-response factors (Fig. 5C). In addition, when SiHa cells were treated with TGF-β1, the activity of cyclin A2 promoter was decreased, while the expression of CCS-3 and PLZF was increased (Fig. 5D). These combined results suggest that CCS-3 functions as a transcriptional repressor and is required for the transcriptional effects of PLZF.

4. Discussion

Tumor transformation arises as a result of an accumulation of genetic and epigenetic alterations to proto-oncogenes and tumor suppressor genes. The genes that are altered during tumorigenesis chiefly affect a variety of biologic pathways such as apoptosis, cell-cycle, and the differentiation of the cancer cells [32–34]. In particular, one or more factors in the pRb regulatory pathway, including p53, p16, cyclin D, or pRb itself, may be inactivated in almost 50–100% of human tumors [35,36]. For example, the p53 tumor suppressor gene plays a critical role in maintaining cellular homeostasis and tumor-free survival of an organism by modulating cell-cycle progression or apoptosis [37,38]. Normal cells and tissues are tightly regulated by the balance between cellular proliferation and death by extracellular signals, and this is mediated by the activation of cell-cycle factors. The diversity of transcription factor oncogenes implicated in the human lymphoma and in leukemia is striking, although their essential functions can be traced to the fundamental steps of cell growth, development, or survival [39].

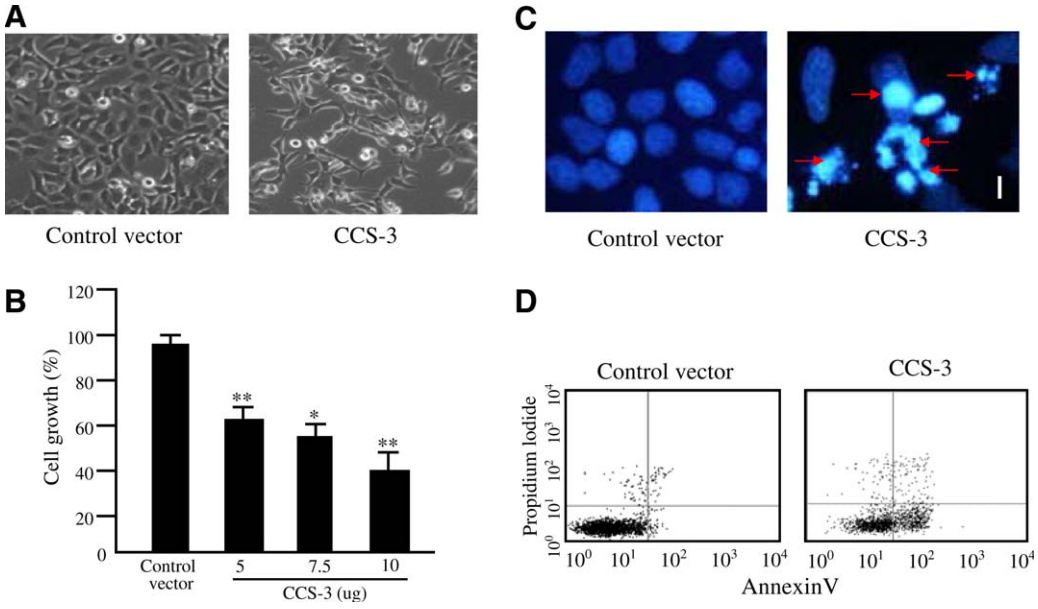

Fig. 4. CCS-3 induces apoptosis in human cancer cells. (A) Cells were transfected with control vector or CCS-3 expression vector and cell morphology was observed by light microscopy. (B) Cell growth was determined by MTT colorimetric assay with the indicated expression vectors in SiHa cells. (C) SiHa cells transfected with the indicated expression vectors were stained with DAPI to examine the nuclei and then examined by fluorescence microscopy. Arrows indicate the observed DNA fragmentations. Size bar, 20 μm. (D) Apoptotic cell death by CCS-3. Transfected SiHa cells with the indicated expression vectors were incubated with FITC-labeled Annexin V and PI for 15 min and then analyzed by FACS. Data represent means ± S.D. from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with control alone.

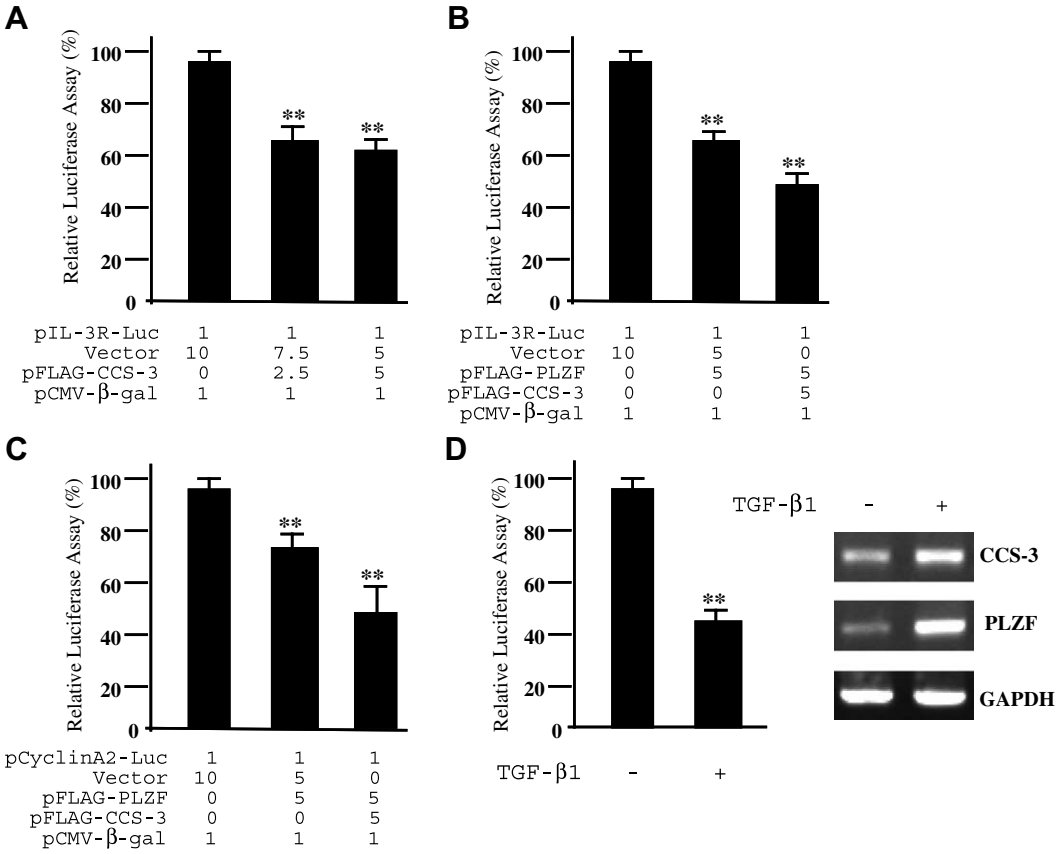

Fig. 5. CCS-3 enhances transcriptional repression mediated by PLZF. (A and B) Dose-dependent transcriptional repression of CCS-3 on the pIL-3-Luc activity. (C) Dose-dependent transcriptional repression of CCS-3 on the cyclin A2-Luc activity. The indicated amounts (μg) of each plasmid were transiently transfected in SiHa cells. At 48 h after transfection, the cells were assayed for luciferase activity. Data represent means ± S.D. from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with control alone. (D) Effects of TGF-β1/CCS-3 on PLZF activity. SiHa cells transfected with luciferase-reporter plasmid bearing human cyclin A2 promoter were incubated in the presence of 1.5 ng/ml TGF-β1 for 24 h, and harvested for luciferase activity (left), for RT-PCR analysis of CCS-3 (right).

PLZF is a transcription factor containing nine C-terminal Cys2-His2 zinc finger motifs related from *Drosophila* to *Homo sapiens* and containing an N-terminal BTB/POZ protein–protein interaction domain. The domain inhibits the binding of transcription factors, including RARα, to DNA when linked *cis*, suggesting that PLZF-RARα may act by sequestering RxR or by sequestering other retinoid receptors within inactive multimeric complexes [1,40]. PLZF is expressed in multiple tissues during early development. It is also expressed by early hematopoietic progenitor cells with a speckled nuclear distribution and is downregulated during myeloid differentiation [13].

The mechanism of transcription repression of PLZF has been shown to occur via the binding of the sequence-specific DNA promoter followed by recruitment of corepressor proteins, N-coR and SMRT, mSIN3A and HDAC1, members of a multi-protein complex of histone deacetylases. ETO nuclear factor interacts with PLZF and can potentiate transcriptional repression by PLZF [19]. Recently, VDUP1 upregulated by TGF-β1, was shown to inhibit the cell-cycle by recruiting co-repressor complex including FAZF, PLZF, and HDAC1 [34].

eEF1A plays many important roles in eukaryotic cells aside from its central role of translation. eEF1A forms complexes with other components of the cell such as actin and tubulin [26,41,42]. The role of EF1A in oncogenesis has been investigated in prostate carcinoma as well [43]. Also, it has been shown that eEF1A protein is over-expressed in metastatic cells as compared to non-metastatic cells, and eEF1A from metastatic cells contain a lower affinity for F-actin [44]. The importance of eEF1A in our research lies in eEF1A's reported ability to be involved in apoptosis. eEF1A expression is upregulated by p53 transcription factor when located in erythroleukemic cell lines of an apoptotic phenotype expressed only in the temperature sensitive mutant p53 gene [45,46].

In this study, we provided evidence that CCS-3 has anti-tumor activity when associated as a component of transcriptional co-repressors. We have examined the expression pattern of CCS-3 in various tissue and cell lines. A semi-quantitative RT-PCR analysis demonstrated that CCS-3 expression was dramatically reduced in tumor tissues but not in normal tissues. We identified CCS-3, a component of the cervical tumor suppressor, as a novel PLZF binding protein. The interaction between PLZF and CCS-3 could be the first example of a regulation mechanism the PLZF protein, which may have a role in preventing nuclear translocation of PLZF. Another important function of CCS-3 is its role in cell cycle regulation. Overexpression of CCS-3 and PLZF in human cervical cancer cell lines inhibits cell growth and suppresses cyclin A2 promoter activity. This suppressive effect may be due to the recruitment of the transcriptional repressor factor. Our result

on the CCS-3 binding assay with PLZF the yeast two-hybrid system revealed that the critical PLZF domain for binding CCS-3 resided within the BTB/POZ domain. The domain is known as a transcriptional repressor.

In summary, we have identified CCS-3 as a novel binding partner for PLZF via yeast two-hybrid screening. We have also mapped the critical molecular domains required for this interaction. Overexpression of CCS-3 in human cervical cell lines inhibits cell growth by inducing apoptosis and suppressing human cyclin A2 promoter activity. Transfection experiments indicate that CCS-3 may bind PLZF and prevent nuclear translocation. These combined results suggest that the cell cycle arrest of CCS-3 may be involved in the transcriptional repression pathway in cervical tumors.

Acknowledgements: We thank Drs. S.A. Martinis and S.R. Blanke (Department of Biochemistry, University of Illinois at Urbana-Champaign, USA) and Richard Yoo (University of Washington) for critical readings of the manuscript. This research was supported by the Science Research Center (Molecular Therapy Research Center) grant from the Korea Science and Engineering Foundation.

References

[1] Bardwell, V.J. and Treisman, R. (1994) The POZ domain: a conserved protein–protein interaction motif. Genes Dev. 8, 1664–1677.
[2] Li, J.Y., English, M.A., Ball, H.J., Yeyati, P.L., Waxman, S. and Licht, J.D. (1997) Sequence-specific DNA binding and transcriptional regulation by the promyelocytic leukemia zinc finger protein. J. Biol. Chem. 272, 22447–22455.
[3] Sitterlin, D., Tiollais, P. and Transy, C. (1997) The RAR alpha-PLZF chimera associated with acute promyelocytic leukemia has retained a sequence-specific DNA-binding domain. Oncogene 14, 1067–1074.
[4] Parrado, A., Robledo, M., MoyaQuiles, M.R., Marin, L.A., Chomienne, C., Padua, R.A. and AlvarezLopez, M.R. (2004) The promyelocytic leukemia zinc finger protein downregulates apoptosis and expression of the proapoptotic BID protein in lymphocytes. Proc. Natl. Acad. Sci. USA 101, 1898–1903.
[5] Ball, H.J., Melnick, A., Shaknovich, R., Kohanski, R.A. and Licht, J.D. (1999) The promyelocytic leukemia zinc finger (PLZF) protein binds DNA in a high molecular weight complex associated with cdc2 kinase. Nucleic Acids Res. 27, 4106–4113.
[6] Yeyati, P.L., Shaknovich, R., Boterashvili, S., Li, J., Ball, H.J., Waxman, S., NasonBurchenal, K., Dmitrovsky, E., Zelent, A. and Licht, J.D. (1999) Leukemia translocation protein PLZF inhibits cell growth and expression of cyclin A. Oncogene 18, 925–934.
[7] Melnick, A., Ahmad, K.F., Arai, S., Polinger, A., Ball, H., Borden, K.L., Carlile, G.W., Prive, G.G. and Licht, J.D. (2000) In-depth mutational analysis of the promyelocytic leukemia zinc finger BTB/POZ domain reveals motifs and residues required for biological and transcriptional functions. Mol. Cell. Biol. 20, 6550–6567.
[8] Kang, S.I., Chang, W.J., Cho, S.G. and Kim, I.Y. (2003) Modification of promyelocytic leukemia zinc finger protein (PLZF) by SUMO-1 conjugation regulates its transcriptional repressor activity. J. Biol. Chem. 278, 51479–51483.
[9] Hong, S.H., David, G., Wong, C.W., Dejean, A. and Privalsky, M.L. (1997) SMRT corepressor interacts with PLZF and with the PML-retinoic acid receptor alpha (RARalpha) and PLZF-RARalpha oncoproteins associated with acute promyelocytic leukemia. Proc. Natl. Acad. Sci. USA 94, 9028–9033.
[10] David, G., Alland, L., Hong, S.H., Wong, C.W., DePinho, R.A. and Dejean, A. (1998) Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. Oncogene 16, 2549–2556.
[11] Grignani, F., Matteis, S.D., Nervi, C., Tomassoni, N.L., Gelmetti, V., Cioce, M., Fanelli, M., Ruthardt, M., Ferrara, F.F., Zamir, P.G., Seiser, C., Lazar, M.A., Minucci, S. and Pelicci, P.G. (1998) Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. Nature 391, 815–818.
[12] Guidez, F., Ivins, S., Zhu, J., Soderstrom, M., Waxman, S. and Zelent, A. (1998) Reduced retinoic acid-sensitivities of nuclear receptor corepressor binding to PML- and PLZF-RARalpha underlie molecular pathogenesis and treatment of acute promyelocytic leukemia. Blood 91, 2634–2642.
[13] Reid, A., Gould, A., Brand, N., Cook, M., Strutt, P., Li, J., Licht, J., Waxman, S., Krumlauf, R. and Zelent, A. (1995) Leukemia translocation gene, PLZF, is expressed with a speckled nuclear-pattern in early hematopoietic progenitors. Blood 86, 4544–4552.
[14] Horowitz, H. and Berg, C.A. (1996) The Drosophila pipsqueak gene encodes a nuclear BTB-domain-containing protein required early in oogenesis. Development 122, 1859–1871.
[15] Dent, A.L., Shaffer, A.L., Yu, D., Allman, D. and Staudt, L.M. (1997) Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science 276, 589–592.
[16] Lai, Z.C. and Li, Y. (1999) Tramtrack69 is positively and autonomously required for Drosophila photoreceptor development. Genetics 152, 299–305.
[17] Staudt, L.M., Dent, A.L., Shaffer, A.L. and Yu, X. (1999) Regulation of lymphocyte cell fate decisions and lymphomagenesis by BCL-6. Int. Rev. Immunol. 18, 381–403.
[18] Barna, M., Hawe, N., Niswander, L. and Pandolfi, P.P. (2000) Plzf regulates limb and axial skeletal patterning. Nat. Genet. 25, 166–172.
[19] Melnick, A.M., Westendorf, J.J., Polinger, A., Carlile, G.W., Arai, S., Ball, H.J., Lutterbach, B., Hiebert, S.W. and Licht, J.D. (2000) The ETO protein disrupted in t(8;21)-associated acute myeloid leukemia is a corepressor for the promyelocytic leukemia zinc finger protein. Mol. Cell. Biol. 20, 2075–2086.
[20] UsuiAoki, K., Ito, H., UiTei, K., Takahashi, K., Lukacsovich, T., Awano, W., Nakata, H., Piao, Z.F., Nilson, E.E., Tomida, J. and Yamamoto, D. (2000) Formation of the male-specific muscle in female Drosophila by ectopic fruitless expression. Nat. Cell Biol. 2, 500–506.
[21] Melnick, A., Carlile, G., Ahmad, K.F., Kiang, C.L., Corcoran, C., Bardwell, V., Prive, G.G. and Licht, J.D. (2002) Critical residues within the BTB domain of PLZF and Bcl-6 modulate interaction with corepressors. Mol. Cell. Biol. 22, 1804–1818.
[22] Lee, D.K., Suh, D., Edenberg, H.J. and Hur, M.W. (2002) POZ domain transcription factor, FBI-1,represses transcription of ADH5/FDH by interacting with the zinc finger and interfering with DNA binding activity of Sp1. J. Biol. Chem. 277, 26761–26768.
[23] Ye, B.H., Lista, F., LoCoco, F., Knowles, D.M., Offit, K., Chaganti, R.S. and DallaFavera, R. (1993) Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. Science 262, 747–750.
[24] Gaidano, G., LoCoco, F., Ye, B.H., Shibata, D., Levine, A.M., Knowles, D.M. and DallaFavera, R. (1994) Rearrangements of the BCL-6 gene in acquired immunodeficiency syndrome-associated non-Hodgkin's lymphoma: association with diffuse large-cell subtype. Blood 84, 397–402.
[25] LoCoco, F., Ye, B.H., Lista, F., Corradini, P., Offit, K., Knowles, D.M., Chaganti, R.S. and DallaFavera, R. (1994) Rearrangements of the BCL6 gene in diffuse large cell non-Hodgkin's lymphoma. Blood 83, 1757–1759.
[26] Negrutskii, B.S. and El'skaya, A.V. (1998) Eukaryotic translation elongation factor 1 alpha: structure, expression, functions, and possible role in aminoacyl-tRNA channeling. Prog. Nucleic Acid Res. Mol. Biol. 60, 47–78.
[27] Ejiri, S. (2002) Cloning and expression of Bombyx mori silk gland elongation factor 1gamma in *Escherichia coli*. Biosci. Biotechnol. Biochem. 66, 558–565.
[28] Calado, A., Treichel, N., Muller, E.C., Otto, A. and Kutay, U. (2002) Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. EMBO J. 21, 6216–6224.
[29] Ito, H., Fukada, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153, 163–168.
[30] Rho, S.B., Lee, K.H., Kim, J.W., Shiba, K., Jo, Y.J. and Kim, S. (1996) Interaction between human tRNA synthetases involves

repeated sequence elements. Proc. Natl. Acad. Sci. USA 93, 10128–10133.
[31] Rho, S.B., Chun, T., Lee, S.H., Park, K. and Lee, J.H. (2004) The interaction between E-tropomodulin and thymosin β-10 rescues tumor cells from thymosin β-10 mediated apoptosis by restoring actin architecture. FEBS Lett. 557, 57–63.
[32] Han, S.H., Jeon, J.H., Ju, H.R., Jung, U., Kim, K.Y., Yoo, H.S., Lee, Y.H., Song, K.S., Hwang, H.M., Na, Y.S., Yang, Y., Lee, K.N. and Choi, I. (2003) VDUP1 upregulated by TGF-β1 and 1,25-dihydorxyvitamin D3 inhibits tumor cell growth by blocking cell-cycle progression. Oncogene 22, 4035–4046.
[33] Ross, D.T., Scherf, U., Eisen, M.B., Perou, C.M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S.S., VandeRijn, M., Waltham, M., Pergamenschikov, A., Lee, J.C., Lashkari, D., Shalon, D., Myers, T.G., Weinstein, J.N., Botstein, D. and Brown, P.O. (2000) Systematic variation in gene expression patterns in human cancer cell lines. Nat. Genet. 24, 227–235.
[34] Corn, P.G. and El-Deiry, W.S. (2002) Derangement of growth and differentiation control in oncogenesis. BioEssays 24, 83–90.
[35] Clurman, B.E. and Roberts, J.M. (1995) Cell cycle and cancer. J. Natl. Cancer Inst. 87, 1499–1501.
[36] Sherr, C.J. (1996) Cancer cell cycles. Science 274, 1672–1677.
[37] Vousden, K.H. and Lu, X. (2002) Live or let die: the cell's response to p53. Nat. Rev. Cancer 2, 594–604.
[38] Park, K., Kim, K., Rho, S.B., Choi, K., Kim, D., Oh, S.H., Park, J., Lee, S.H. and Lee, J.H. (2005) Homeobox Msx1 interacts with p53 tumor suppressor and inhibits tumor growth by inducing apoptosis. Cancer Res. 65, 749–757.
[39] Rabbitts, T.H. (1991) Translocations, master genes, and differences between the origins of acute and chronic leukemias. Cell 67, 641–644.
[40] Licht, J.D., Shaknovich, R., English, M.A., Melnick, A., Li, J.Y., Reddy, J.C., Dong, S., Chen, S.J., Zelent, A. and Waxman, S. (1996) Reduced and altered DNA-binding and transcriptional properties of the PLZF-retinoic acid receptor-alpha chimera generated in t(11;17)-associated acute promyelocytic leukemia. Oncogene 12, 323–336.
[41] Bektas, M., Nurten, R., Gurel, Z., Sayers, Z. and Bermek, E. (1994) Interactions of eukaryotic elongation factor 2 with actin: a possible link between protein synthetic machinery and cytoskeleton. FEBS Lett. 356, 89–93.
[42] Condeelis, J. (1995) Elongation factor 1 alpha, translation and the cytoskeleton. Trends Biochem. Sci. 20, 169–170.
[43] Gopalkrishnam, R.V., Su, Z.Z., Goldstein, N.I. and Fisher, P.B. (1999) Translational infidelity and human cancer: role of the PTI-1 oncogene. Int. J. Biochem. Cell. Biol. 31, 151–162.
[44] Edmonds, B.T., Wyckoff, J., Yeung, Y.G., Wang, Y., Stanley, E.R., Jones, J., Segall, J. and Condeelis, J. (1996) Elongation factor-1 alpha is an overexpressed actin binding protein in metastatic rat mammary adenocarcinoma. J. Cell Sci. 109, 2705–2714.
[45] Kato, M.V., Sato, H., Nagayoshi, M. and Ikawa, Y. (1997) Upregulation of the elongation factor-1alpha gene by p53 in association with death of an erythroleukemic cell line. Blood 90, 1373–1378.
[46] Kato, M.V. (1999) The mechanisms of death of an erythroleukemic cell line by p53: involvement of the microtubule and mitochondria. Leukemia Lymphoma 33, 181–186.