Early growth response-1 mediates downregulation of telomerase in cervical cancer

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Early growth response (Egr)-1 is a transcription factor that triggers transcription of downstream genes within 15–30 min of various stimulations. These genes are expressed rapidly through specific promoter activation and mediate cell growth and angiogenesis. Following the previous computational identification of a site that was thought to be an Egr-1 consensus binding site at –273 to –281 in the human telomerase reverse transcriptase (hTERT) promoter region, the present study was conducted to evaluate the role of Egr-1 in the regulation of hTERT and telomerase in uterine cervical cancer. First, the expression of Egr-1 and hTERT at the mRNA level was examined in cervical cancer tissues. Egr-1 and hTERT were expressed much higher in cervical cancer tissues than in the normal cervix. However, a negative correlation was noted in the expression between Egr-1 and hTERT. By luciferase assay using hTERT promoter constructs, hTERT transcriptional activation was shown to be inhibited when Egr-1 was overexpressed. Furthermore, Egr-1 overexpression decreased hTERT protein production as well as hTERT mRNA as observed by western blotting analysis and real-time reverse transcription–polymerase chain reaction, respectively. The present study suggests that Egr-1 plays an important regulatory role in the transcriptional activation of hTERT. (*Cancer Sci* 2008; 99: 1401–1406)

Early growth response (Egr) transcription factors are a group of immediate–early genes that trigger *de novo* transcription within 15–30 min of diverse external stimuli, including serum, phorbol esters, hypoxia, and several growth factors.[1–8] They are reported to transactivate genes that mediate cell growth and angiogenesis.[3,4] Egr-1 is a phosphorylated intranuclear protein consisting of 533 amino acids and a type of zinc finger transcription factor that belongs to the *WT1* gene family.[3,9,10] Egr-1 protein binds to a specific DNA sequence, CGCCCCCGC, in the presence of zinc ions. More than 40 genes have been identified as being targeted by Egr-1.[11–14] Most of these genes regulate enzymatic homeostasis and encode proteins that play important roles in the developmental environment and physiological process.

Telomerase is an enzyme that plays an extremely important role in oncogenesis.[15,16] It is believed to rewind the mitotic clock that leads to aging and to enable permanent cell division. Telomeres and telomerase have drawn considerable attention since the 1990s as molecular targets in aging and carcinogenesis.[15–18] One of the most important requirements for unlimited cell reproduction is to restore telomeres or to activate telomerase, which immortalizes cells. Only cells with telomerase activity express human telomerase reverse transcriptase (hTERT), and telomerase activity appears when the *hTERT* gene is introduced into normal somatic cells that do not possess the activity.[19–22] Various binding and regulatory elements are present on *hTERT* promoters that increase or suppress the enzymatic expression.[23–28] Interestingly, Takakura *et al*. have found a WT1 binding site on the promoter of hTERT at the region between –273 and –281.[29] WT1 competitively inhibits transcription activation through Egr-1 by apparently binding to the same motif as the Egr-1 protein.[8] In the present study, we evaluated the effect of Egr-1 on hTERT transcription in cervical cancer, and propose a hypothesis in which Egr-1 plays a role in the regulation of hTERT and telomerase activity.

Materials and Methods

Experimental samples. Samples from cervical cancer and normal cervix were obtained with informed consent from patients undergoing surgery or biopsy. The protocols used here have been approved by the ethics commitees of the respective institutions where the study was carried out and conform to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). Sample tissues were minced finely into small pieces with scissors, washed in 0.9% sterile saline to avoid contamination of red blood cells that could interfere with polymerase chain reaction (PCR), snap frozen, and stored at –80°C until use in the telomeric repeat amplification protocol assay and reverse transcription (RT)-PCR.

Cell culture. CaSki and SiHa cells, all of which were derived from cervical cancer and obtained from the American Type Culture Collection, were grown in RPMI-1640 supplemented with 10% fetal bovine serum in the presence of 5% CO_2.

Plasmid constructions. pGL3-1375-luc, pGL3-378-luc, pGL3-776-luc, pGL3-1175-luc, and pGL3-181-luc are hTERT promoter–luciferase reporter plasmids in which a 181-bp sequence upstream of the transcription start site of hTERT (designated nt +1) and a 77-bp sequence (5′-untranslated region) downstream of the transcription start site were cloned into pGL-Basic (Promega, Madison, WI, USA) at *Mlu*I and *Bgl*II sites.[29] The reporter plasmid pGL1375mt-luc contained three mutations in the putative binding site of Egr-1 at –273 to –281, which were introduced by PCR-based site-specific mutagenesis. An Egr-1 expression plasmid was prepared by subcloning *Egr-1* into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) using the *Eco*RI site.

Transfection and luciferase assays. Each cell line was seeded at 2×10^4 cells/35-mm dish and incubated overnight at 37°C in a 5% CO_2 incubator. For each transfection, 0.5–3.0 mg of empty vector or expression vector and 0.1–0.3 μg of promoter–luciferase DNA were mixed in 0.2 mL Opti-MEM (Invitrogen), and a precipitate was formed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations. The cells were washed with Opti-MEM, and complexes were applied to the cells. After transfection (24 h), cells were harvested and extracts prepared with Glo Lysis buffer (Promega). Luciferase activity was measured in extracts from triplicate samples using the Bright-Glo Luciferase Assay System (Promega).

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Real-time RT-PCR analysis. Total RNA was isolated using Isogen reagent (Nippon Gene, Tokyo, Japan) and quantified by A_{260}/A_{280} measurement using an Ultraspec 3000 (GE Healthcare Biosciences, Tokyo, Japan). Total RNA (5 μg) was reverse transcribed into cDNA. Real-time PCR was carried out for quantitative estimation, with the 20-μL PCR reactions containing 5 mmol/L $MgCl_2$, 2 μL SYBR Green Mastermix (Roche Diagnostics, Tokyo, Japan), 5 μL 1:10 diluted cDNA, and 0.3 μmol/L of both forward and reverse primers. Egr-1 was amplified using the following primer pair: 5′-GAG CAC CTG ACC ACA GAG T-3′ and 5′-GGA-GAA GCG GCC AGT ATA G-3′. *hTERT* was amplified using the following primer pair: 5′-CCT CTG TGC TGG GCC TGG ACG ATA-3′ and 5′-ACG GCT GGA GGT CTG TCA AGG TAG-3′. The reactions were then cycled in a LightCycler (Roche Diagnostics) with the following parameters: denaturation for one cycle at 95°C for 10 s, and 45 cycles (temperature transition of 20°C/s) of 95°C for 0 s, 50°C for 10 s, and 72°C for 15 s. The mRNA level of each PCR product was estimated by the LightCycler software (Roche Diagnostics) and normalized to the β-actin mRNA level.[25]

Western blotting analysis. CaSki and SiHa cells were seeded at 2.0×10^6 cells/150-mm dish, incubated overnight at 37°C, and transfected with 15 μg of either empty vector (pcDNA3.1) or Egr-1 expression vector using Lipofectamine 2000 for 24 h as described above. Subsequently, cells were harvested and lysed on ice for 30 min in lysis buffer (10 mmol/L Tris [pH 8.0], 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 400 mmol/L NaCl, 10% glycerol, 0.5% NP-40, 0.5 mmol/L sodium fluoride, and 1 mmol/L dithiothreitol [DTT]) containing complete protease inhibitor mixture (Roche Diagnostics). The lysates were subjected to centrifugation at 14800*g* for 15 min, and the soluble fraction was collected. Protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). Equal amounts of protein (40 mg) were loaded onto a 4–12% sodium dodecylsulfate–polyacrylamide gel and subjected to electrophoresis at 200 V for 50 min. Proteins were transferred onto a polyvinylidene difluoride membrane and probed with anti-hTERT antibody (H-231; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Egr-1 antibody (588; Santa Cruz Biotechnology) and anti-actin antibody (C4; Roche Diagnostics). The same blot was probed with different antibodies after stripping the membrane. Each protein was detected by horseradish peroxidase-conjugated secondary antibody coupled with enhanced chemiluminescence western blotting detection reagents (GE Healthcare Biosciences). The intensity of each band was normalized to the intensity of the β-actin band. Normal TIG3 cells were used as a hTERT-negative control.

Electrophoretic mobility shift assays. Oligonucleotides containing the Egr-1 consensus DNA-binding site and the WT1 binding site from the hTERT promoter were purchased as single-stranded DNA from Operon Biotechnologies (Tokyo, Japan). Double-stranded oligonucleotides, in buffer containing 10 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, and 1 mmol/L EDTA, in molar amounts of the complementary oligonucleotides were mixed in a 1.5-mL microcentrifuge tube and placed in a heat block at 95°C. The sequences of the complementary pairs were 5′-AGC GCC CGC GCG GGC GGG-3′ with 5′-GGA TCC AGC GGG GGC GAG CGG GGG CGA-3′ (Egr-1), 5′-GGA TCC AGC TAG GGC GAG CTA GGG CGA-3′ (Egr-1mt), 5′-AGC GCC CGC GCG GGC GGG-3′ (hTERT-WT-1), and 5′-GAG CGG CGC GCA AAC GGG GAA GC-3′ (hTERT-WT-1mt). Each oligonucleotide was synthesized commercially (Operon Biotechnologies). The heat block was allowed to cool down to room temperature, and the samples were desalted on a G-25 microspin column (GE Healthcare Biosciences). The double-stranded oligonucleotides were end-labeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P] ATP. For electrophoretic mobility shift assays (EMSA), end-labeled double-stranded oligonucleotides, at 5000 c.p.m., were incubated with 2 mL Egr-1 protein prepared using the TnT Quick Coupled Transcription/Translation System (Promega) at room temperature (22°C) for 30 min in the presence of a binding buffer containing 10% glycerol, 20 mmol/L HEPES (pH 7.5), 25 mmol/L KCl, 2 mmol/L DTT, 2 mmol/L $MgCl_2$, 0.4% NP-40, and 1 mg sheared salmon sperm DNA. When competition assays were carried out, an unlabeled Egr-1 consensus sequence oligonucleotide and hTERT-WT1 were incubated with the protein and buffer for 5 min before the addition of each labeled oligonucleotide. For supershift assays, 0.2 mg Egr-1 antibody (588; Santa Cruz Biotechnology) was incubated with the binding mixtures for 5 min before the addition of labeled oligonucleotide. Samples (20 mL) were loaded onto a 5% non-denaturing polyacrylamide gel and subjected to electrophoresis at 150 V for 1 h using 0.5× Tris-borate EDTA (1 mmol/L Tris-borate EDTA: 89 mmol/L Tris, 8 mmol/L boric acid and 2 mmol/L EDTA [pH 8.3]) as running buffer. After electrophoresis, gels were exposed to XAR film (Kodak) with intensifying screens at –80°C.

Results

Egr-1 and hTERT expression in cervical cancer. The expression of hTERT and Egr-1 mRNA was examined in cervical cancer from 18 patients and normal cervix from 13 patients who suffered from uterine myoma or prolapse of the uterus. Triplicate measurements were carried out. hTERT and Egr-1 mRNA levels were much higher in cervical cancer than in normal tissues ($P < 0.1$; Fig. 1a). As shown in Figure 1b, however, in cervical cancer, the plot of Egr-1 mRNA level on the *x*-axis and hTERT mRNA level on the *y*-axis revealed that the expression of these mRNAs in cervical cancer tissues were negatively correlated ($P < 0.01$). Namely, the higher the relative Erg-1 mRNA level, the lower the relative hTERT mRNA level.

Egr-1 decreases endogenous hTERT expression. Next, in order to determine whether Egr-1 regulates hTERT transcription, the effect of Egr-1 on hTERT expression was examined in cervical cancer cell lines cotransfected with hTERT promoter and Egr-1-expression plasmids. For the cotransfection assays, the cervical cancer cell lines CaSki and SiHa were cotransfected with Egr-1-expression plasmid (pcDNA3.1-Egr-1) or empty vector (pcDNA3.1) and the pGL3-1375-luc construct containing the full-length proximal promoter. In both cotransfected cell lines, Egr-1 reduced the hTERT promoter activity to 12.5–20% when the Egr-1-expression plasmid ranged from 20 to 100 ng in cotransfection (Fig. 2). Here, dose dependence was observed in the decrease of hTERT promoter activity due to Egr-1. Based on these findings, an attempt was made to determine whether the endogenous cellular *hTERT* gene is regulated by Egr-1 as was indicated in the cotransfection system. CaSki and SiHa cell lines were transfected with pcDNA3.1-Egr-1 or control pcDNA3.1, total RNA and protein was extracted 24 h after transfection, and western blot analysis and real-time RT-PCR were carried out. Erg-1 was shown to decrease hTERT protein and mRNA levels in both Egr-1-transfected cell lines (Fig. 3). WT1 competitively inhibits transcription activation through Egr-1 by apparently binding to the same motif as the Egr-1 protein,[8] and it is known that the hTERT promoter has one WT-1 binding site.[30] From the above findings, Egr-1 appears to decrease the expression of hTERT by interacting with the *hTERT* regulation region.

Identification of an Egr-1 cis-acting element in the *hTERT* promoter region. It was now of considerable interest to identify a possible regulatory element in the hTERT transcriptional region that is responsive to Egr-1. CaSki and SiHa cell lines were cotransfected with hTERT reporter constructs with and without WT1 binding sites in the promoter region and pcDNA3.1-Egr-1.

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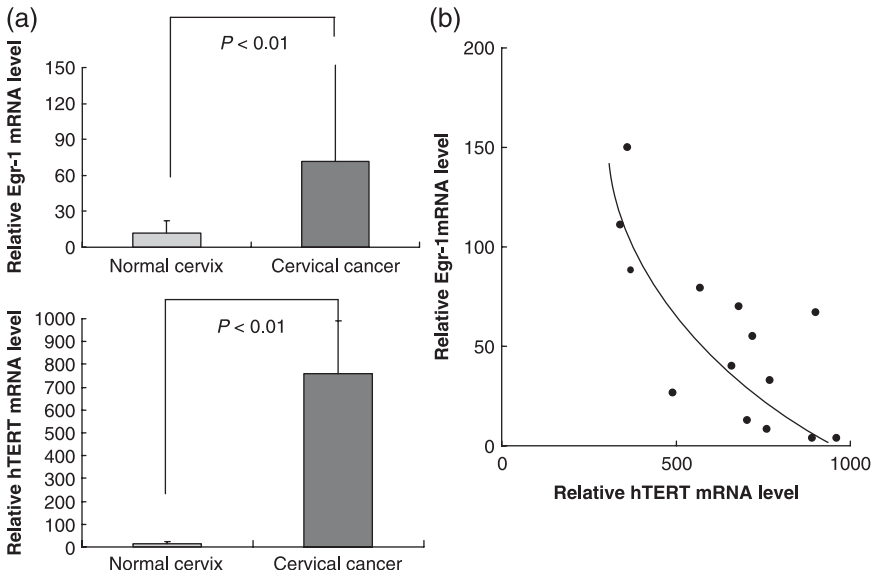

Fig. 1. (a) Determination of human telomerase reverse transcriptase (hTERT) and early growth response (Egr)-1 expression by real-time reverse transcription–polymerase chain reaction. Cervivical cancers (n = 18), and normal cervical epithelia (n = 13). Bars are ±SD in triplicate assays. (b) Relationship between the expression of hTERT and Egr-1 mRNA in the cervical cancers. *X*-axis, relative Egr-1 mRNA; *y*-axis, relative hTERT mRNA. Egr-1 and hTERT mRNA levels were negatively correlated (P < 0.01).

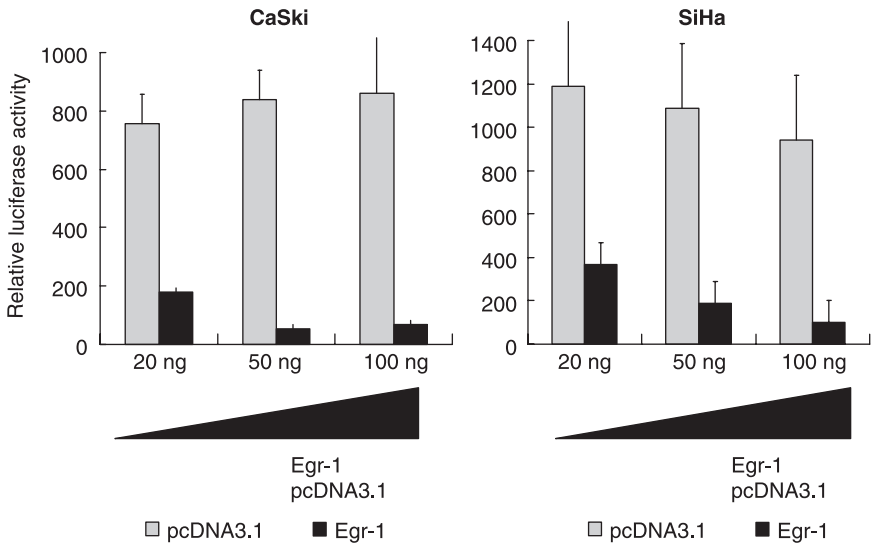

Fig. 2. Early growth response (Egr)-1 transactivates human telomerase reverse transcriptase (*hTERT*) promoter activity. CaSki and SiHa cells were transfected with pGL3-1375-luc and 20 ng, 50 ng, or 100 ng Egr-1 expression construct or empty vector, pcDNA3.1. The empty vector pcDNA3.1 was used to keep total plasmid DNA constant. Luciferase assays were carried out after 24 h, and enzymatic activities reported in relative luminescence units. Bars, ±SD in triplicate assays.

Reporter luciferase was assayed 24 h later. As shown in Figure 4, pGL3-1375-luc containing the region –1375 through –16 was affected by Egr-1. The results were similar when the other plasmids pGL3-378-luc, pGL3-776-luc, and pGL3-1175-luc were used, whereas pGL3-181-luc missing the WT1-binding site gave different results. Computer-based analysis of this region showed the presence of GCGGGGGCC, which is thought to be a consensus Egr-1 binding site and is also found in the WT1 site at –281 to –273 (Fig. 4).[29] The reporter construct pGL3-1375 Egr-1mt-luc, in which GGG in the consensus sequence was replaced with AAA, was prepared. In a cotransfection assay with this plasmid, Egr-1 did not alter the reporter enzyme activity (Fig. 5), indicating that this Egr-1–WT1 site (at –281 to –273) in the hTERT promoter is an essential regulatory element for hTERT transcription.

Egr-1 interacts with the WT-1 binding site in the *hTERT* promoter region. EMSA were carried out to determine whether Egr-1 interacts directly with the WT-1 binding site in the hTERT promoter region. Double-stranded oligonucleotides corresponding to the sequence of the WT-1 binding site (hTERT-WT1) were incubated with Egr-1 protein prepared in an *in vitro* transcription and translation system and subjected to electrophoresis (Fig. 6). DNA–protein complexes were formed when end-labeled hTERT-WT1 was incubated with Egr-1-programmed rabbit reticulocyte lysates. These DNA–protein complexes were not observed in unprogrammed lysates (Fig. 6b). The migration of these complexes was specifically delayed by anti-Egr-1 antibody. Addition of a 50-fold molar excess of cold Egr-1 consensus oligonucleotides markedly reduced this DNA–protein complex formation, whereas neither cold Egr-1mt nor hTERT-WT1mt without the consensus sequence bound (Fig. 6b). Additionally, for confirmation of Egr-1 binding to hTERT-WT1, competition assays were carried out using end-labeled Egr-1 consensus oligonucleotides and unlabeled hTERT-WT1 or Egr-1mt. hTERT-WT1 efficiently competed with the wild-type binding site for Egr-1 when added at a 50–200-fold molar excess, whereas such binding competition was not observed in mutant Egr-1mt (Fig. 6a). These results clearly show that Egr-1 binds to hTERT-WT1.

Discussion

Egr-1 is defined as a transcription factor that accumulates rapidly in the nucleus upon stimulation by mitogens, a variety of cytokines, and cellular stress including hypoxia.[3,4] It affects growth and apoptosis, and is involved in remodeling of various

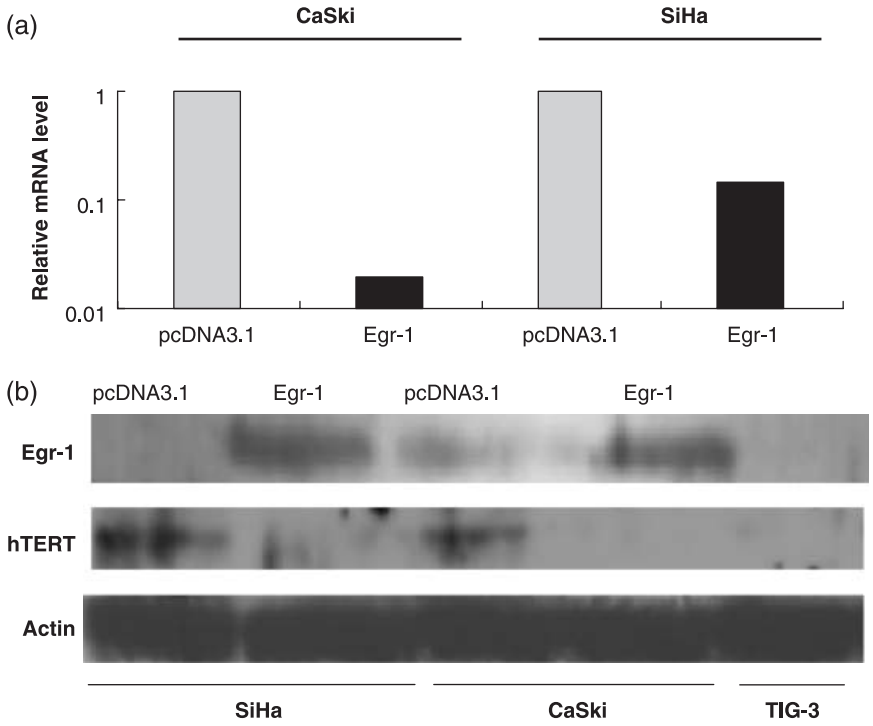

Fig. 3. (a) Human telomerase reverse transcriptase (hTERT) mRNA levels in CaSki and SiHa cells transfected with early growth response (Egr)-1. CaSki and SiHa cells were transfected with plasmid constructs expressing Egr-1 and pcDNA3.1 using Lipofectamine 2000. Total RNA isolated after transfection was subjected to real-time reverse transcription–polymerase chain reaction. (b) Egr-1 decreases endogenous hTERT protein. hTERT levels in CaSki and SiHa cells transfected with Egr-1 are shown. CaSki and SiHa cells were transfected with plasmid constructs expressing Egr-1 and pcDNA3.1 using Lipofectamine 2000. Cell lysates were prepared and subjected to western blot analysis, using antibodies to hTERT, Egr-1, and β-actin. Equal amounts of cellular protein (40 μg) were loaded. In addition, normal TIG3 cells were used as a hTERT negative control.

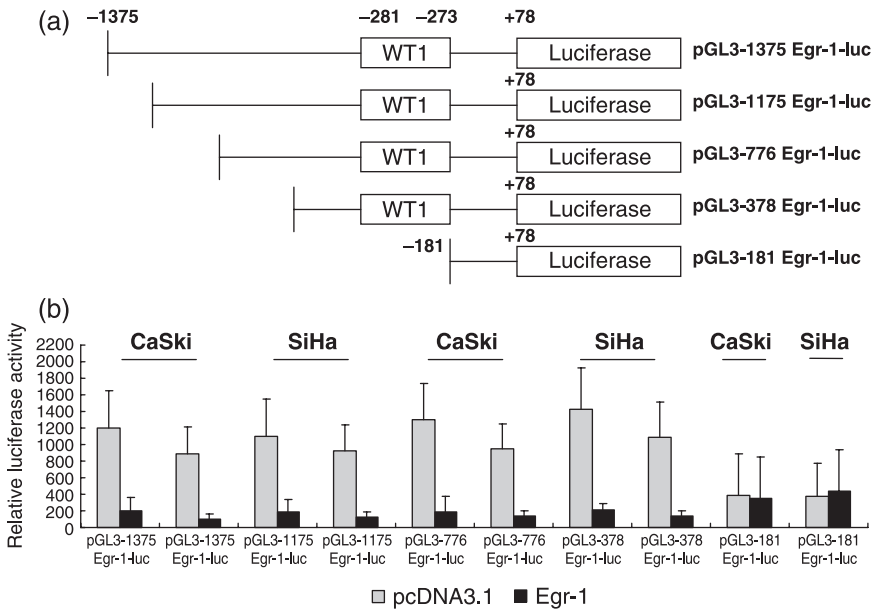

Fig. 4. Human telomerase reverse transcriptase (hTERT) reporter constructs with and without the WT1-binding site and early growth response (Egr)-1 effect. (a) pGL3-1375-luc, pGL3-1175-luc, pGL3-776-luc, and pGL3-378-luc contained the WT1 site, pGL181-luc did not contain the WT1 site. (b) Relative reporter luciferase activities in CaSki and SiHa cell lines cotransfected with hTERT reporter constructs and pcDNA3.1-pcDNA3.1 plasmid.

types of cells as well.[30,31] However, the effect of Egr-1 expression in cancers appears to be a complicated issue. Some reports have shown that Egr-1 induces cell arrest against tumor transformation,[32,33] whereas others have reported that Egr-1 inhibits apoptosis and enhances tumor growth.[5,34]

In the present study, it was shown that hTERT and Egr-1 were expressed at significantly higher mRNA levels in cervical cancer than in the normal cervix, suggesting that Egr-1 plays a specific role in biological events in cervical oncogenesis. In addition, Egr-1 mRNA showed a tendency to increase in cervical cancer with higher squamous cell carcinoma related antigen concentrations. It was difficult to evaluate the significance of these differences from the small number of patients enrolled in the present study. However, our results suggest that Egr-1 expression is related to the progression of tumors and prognosis. The finding that Egr-1 and hTERT showed a negative correlation in their mRNA expression is particularly interesting. It suggests that Egr-1 inhibits the expression of telomerase. Our computer analysis indicated that there is an Egr-1 motif in the hTERT promoter. In fact, it has been shown here that not only hTERT promoter activity but also its mRNA expression and protein production decreased significantly when Egr-1 was expressed excessively by Egr-1 expression constructs transfected in cervical cancer cell lines. WT1 is a transcription factor similar to Egr-1, acts as a tumor-suppressor gene, and is believed to bind competitively with Egr-1 to a specific DNA sequence motif in the hTERT promoter

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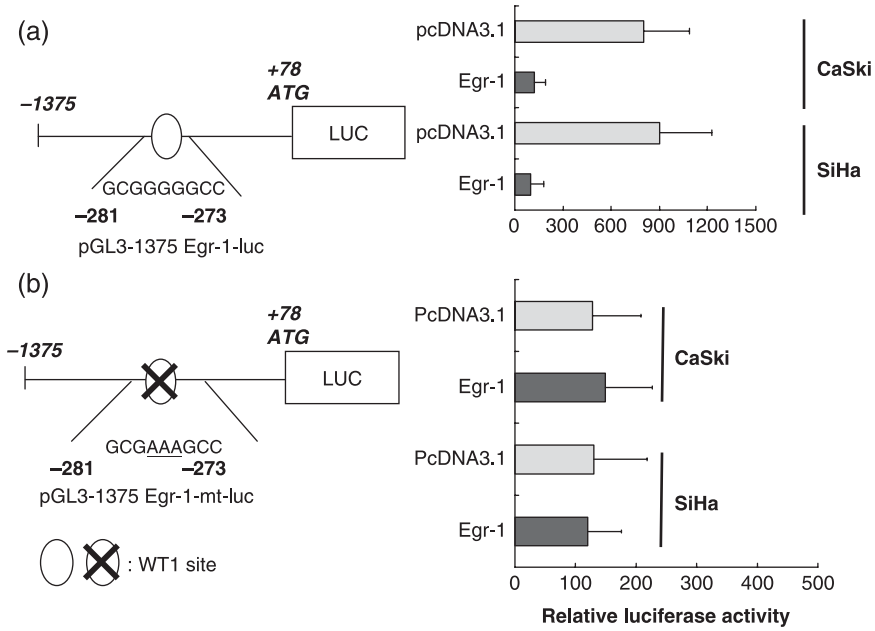

Fig. 5. Effect of early growth response (Egr)-1 on pGL3-1375-luc reporter gene espression. (a) pGL3-1375-luc reporter construct and (b) pGL3-1375 Egr-1 mt-luc with GGG replaced with AAA in the Egr-1-binding site are represented. CaSki and SiHa cells were transfected with pGL3-1375-luc and pGL3-1375 Egr-1 mt-luc and 2 μg pcDNA3.1-Egr-1 or control pcDNA3.1. Luciferase assays were carried out after 24 h. Bars show ±SD in triplicate assays.

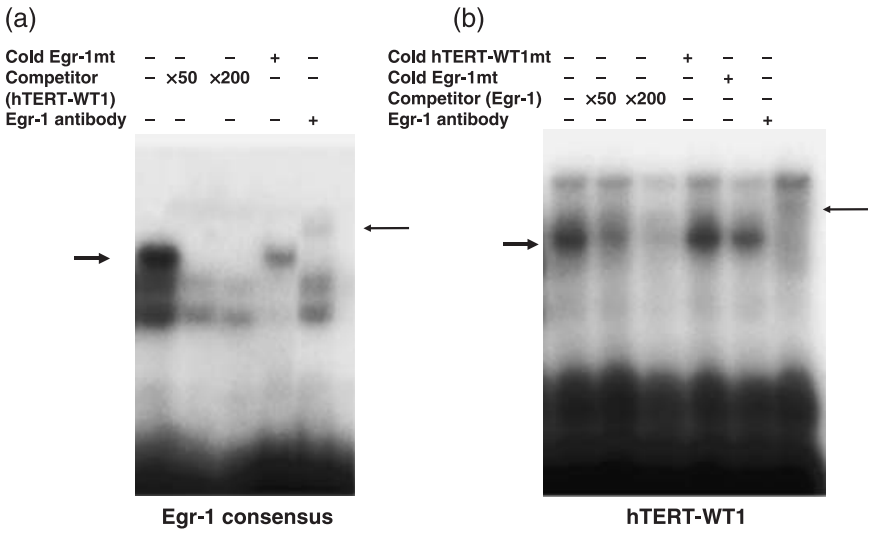

Fig. 6. Analysis of early growth response (Egr)-1 interaction with WT1 response element in the human telomerase reverse transcriptase (hTERT) promoter. (a) Egr-1 consensus oligonucleotide was end-labeled with ^{32}P and used in the binding reaction with Egr-1 as above. For the supershift assay, anti-Egr-1 antibody was added to the binding reaction. For competitor assays, a 50-fold and a 200-fold molar excess of WT1 and hTERT consensus oligonucleotides were added to the binding reaction. (b) Oligonucleotides containing the WT1 binding site of the hTERT promoter were labeled with ^{32}P and used in binding reactions with Egr-1 prepared in rabbit reticulocyte lysates. For competitor assays, a 50-fold molar excess of Egr-1 consensus oligonucleotide was used. Shifted and supershifted bands as well as the labeled probe are indicated.

region. It is unclear, however, how WT1 protein competes with Egr-1 protein in the binding, and remains to be studied further. As there are reports that Egr-1 has antitumor effects,[14] Egr-1 may play a role in regulating hTERT promoter activity in terms of tumorigenesis. Egr-1 should be a candidate for functionally targeting the arrest of cell growth and apoptosis, and once clarified, the functional mechanism involved here is expected to make a considerable contribution in the development of new therapies for cervical cancer that molecularly target Egr-1.[35,36]

In conclusion, we have evaluated the hTERT promoter activity affected by Egr-1 in cervical cancer cell lines in an attempt to clarify the mechanism by which hTERT is regulated, and have shown that hTERT expression is downregulated with the concomitant expression of Egr-1. Egr-1 is involved directly in the reduction of hTERT transcription through a regulatory element to which Egr-1 binds. The findings obtained in the present study corroborate the hypothesis that Egr-1 plays a significant role in the progression of tumors.[5,6,30] They also suggest a new mechanism by which telomerase is regulated in tumors, and shed light on adaptive responses of tumor cells.[37]

Acknowledgments

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