Effect of an *EDA-A1* gene mutant on the proliferation and cell cycle distribution of cultured human umbilical vein endothelial cells

KE LEI[1], LUNCHANG WANG[1], BING MA[2], PING SHI[2], LONGJIANG LI[3], TUANJIE CHE[4] and XIANGYI HE[5]

Departments of [1]Stomatology and [2]Respiratory Medicine, Central Hospital of Guangyuan, Guangyuan, Sichuan 628000; [3]State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, Sichuan 610041; [4]Institute of Cell Biology, School of Life Science; [5]Department of Prosthodontics, School of Stomatology, Lanzhou University, Lanzhou, Gansu 730000, P.R. China

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Abstract. Ectodysplasin (*EDA*) gene mutation is associated with hypohidrotic ectodermal dysplasia (HED). The aim of this study was to investigate the effect of ectodysplasin, transcript variant 1 (*EDA-A1*) on the proliferation and cell cycle of ECV304 human umbilical vein endothelial cells (HUVECs). Recombinant eukaryotic expression vectors containing mutant (M) and wild-type (W) *EDA-A1* coding sequences, pcDNA3.1 (-)-EDA-A1-M and pcDNA3.1 (-)-EDA-A1-W, respectively, were transfected into ECV304 cells. The *EDA-A1* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR), and the protein was detected by western blotting. The *EDA-A1* gene and protein were detected in ECV304 cells transfected with pcDNA3.1 (-)-EDA-A1-M and pcDNA3.1 (-)-EDA-A1-W, but not in ECV304 cells transfected with empty plasmid or cells that had not undergone transfection. Compared with the control group, the *EDA-A1* gene mutant significantly decreased the proliferation of ECV304 cells and its inhibition rate was 45.70% ($P<0.01$), whereas the wild-type *EDA-A1* gene did not cause such growth inhibition ($P>0.05$). A significant increase of the fraction of cells in the G_0/G_1 phase of the cell cycle was observed in the ECV304 cells of the mutant group compared with wild type group, with an increase in the S phase population and a concomitant reduction in the G_2/M phase population ($P<0.05$). These results indicate that compared with the wild-type gene, transfection with a mutant *EDA-A1* gene inhibited the proliferation and cell cycle of cultured HUVECs.

Correspondence to: Dr Ping Shi, Department of Respiratory Medicine, Central Hospital of Guangyuan, 16 Jingxiangzi Street, Guangyuan, Sichuan 628000, P.R. China
E-mail: pingshier@163.com

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Introduction

Hypohidrotic ectodermal dysplasia (HED), also known as anhidrotic ectodermal dysplasia (AED) or Christ-Siemens-Touraine Syndrome, is an X-linked recessive genetic disease (1). HED is a rare congenital genetic disorder with a birth incidence of 1/100,000-1/10,000 (2). It is characterized by the diminution or absence of eccrine sweat glands, oligodontia and peg-shaped teeth, and hair that is sparse and fine (1,3). Previous study indicates that X-linked HED (XLHED) is caused by mutations of the ectodysplasin A (*EDA*) gene at Xq12-13.1 (4).

The *EDA* gene encodes the protein ectodysplasin A, a member of the tumor necrosis factor (TNF) ligand family, which is associated with NF-κB signaling mechanisms (5,6). Bayés *et al* (7) indicated that the *EDA* gene (GenBank Gene ID: 1896) has a variant 1 transcript (EDA-A1) with a full length of 5,296 bp (NM_001399.4, GI: 54112099), which has an open reading frame of 1,176 bp, and encodes a protein with 391 amino acids. Our previous clinical and molecular study of a family with XLHED, it was showed that a missense mutation of *EDA-A1* (907A→C; A907C) would cause the change of a glutamine residue to a proline residue (Gln306Pro), and eukaryotic expression vectors carrying mutant *EDA-A1* (pcDNA3.1 (-)-EDA-A1-M) and wild-type *EDA-A1* (pcDNA3.1 (-)-EDA-A1-W) were constructed (8).

Human umbilical vein endothelial cells (HUVECs) are cells derived from the endothelium of veins from the umbilical cord. They are used as a laboratory model system for the study of the function and pathology of endothelial cells (9). In the present study, the effects of transfection with the *EDA-A1* gene and its mutant on the proliferation, cell cycle and protein expression of HUVECs were investigated.

Materials and methods

Cell culture. The ECV304 HUVECs were provided by Professor Chunming Wang (Lanzhou University, Lanzhou, China). The cells were cultured in RPMI-1640 (Huamei Biotechnique Co., Ltd., Shanghai, China). The medium included 10% fetal bovine serum (FBS; Evergreen Biological

Engineering Materials, Co. Ltd., Hangzhou, China) and 100 U/ml penicillin/streptomycin (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA). The cells were maintained in a humidified incubator in an atmosphere containing 5% CO_2 at 37˚C, and subjected to digestion with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) overnight. Cells were maintained at $2x10^5$-$1x10^6$ cells/ml. An Olympus IX70 inverted microscope (Olympus Corporation, Tokyo, Japan) was used for the observation of cell morphology.

Plasmid extraction. The eukaryotic plasmids, pcDNA3.1 (-)-EDA-A1-M and pcDNA3.1 (-)-EDA-A1-W, were constructed as previously described (8). Plasmid DNA was extracted using Plasmid Extraction kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol, and 3 μl DNA was subsequently diluted to 1 ml with sterile deionized water. Absorbance (A) values at 260 and 280 nm were measured using a UV spectrophotometer (UV-2401, Shimadzu Corpoartion, Kyoto, Japan). The plasmid DNA concentration was calculated as follows: Plasmid DNA concentration (μg/μl) = A260 x dilution factor x 50/1,000. The plasmid DNA (positive recombinants and empty control) was precipitated with ethanol. Each DNA pellet was then resuspended in sterile deionized water.

Cell transfection. Transfection of the ECV304 cells was performed using the calcium phosphate co-precipitation method, according to the protocol provided with the Effectene Transfection Reagent kit (Qiagen GmbH, Hilden, Germany). Transfection was carried out when the cell density had reached 70% confluence after 24 h of cell-passaging. Cells were transferred into a complete medium (CM) 2 h prior to transfection. Then, 2.5 μg plasmid DNA was slowly added to 2 M $CaCl_2$ and allowed to stand for 10 min. The DNA-$CaCl_2$ solution was slowly added dropwise to 2X HEPES-buffered saline (HeBS), containing 280 mM NaCl, 1.5 mM Na_2HPO_4, and 50 mM HEPES (pH 7.05), and allowed to stand for 30 min until tiny particles precipitated. The precipitate was uniformly dropwise added to the cells (70% confluence; ~$2x10^5$ cells/ml) in the culture flasks. After a 12-h growth at 37˚C in a humidified incubator containing 5% CO_2, cells were washed twice with HeBS, followed by culturing in CM. Empty vector-transfected cells were used as the control group.

Reverse transcription-polymerase chain reaction (RT-PCR). To semi-quantitatively analyze the expression levels of *EDA-A1* in cells, RT-PCR analysis was performed. Total RNA was extracted from the cells from each group after culturing for 48 h, using a reverse transcription (RT) kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Primers for *EDA-A1* were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The primers used were as follows: *EDA-A1* (408 bp), forward: 5'-CGC AGG ATC CAT GGG CTA CCC GGA GGT-3', and reverse: 5'-ATT AAG CTT GCC AAG CGG GCA CCA GGG AGA C-3'; β-actin (230 bp), forward: 5'-TTCACAGGCAGGACAGAAGA-3', and reverse: 5'-TTGAAGGTCGCAGAGTTCCT-3'. The 50-μl PCR reaction system comprised cDNA template (2 μl), 1X PCR Buffer (5 μl), deoxynucleotide (dNTP; 1 μl), primer (forward and reverse, 1 μl), Taq DNA polymerase (1 μl) and ddH_2O (39 μl). RT-PCR was performed in a thermal reactor (Thermocycler, Takara Bio Inc., Otsu, Japan). Products were subjected to electrophoresis (1.5% agarose gel, 120 V, 90 mA).

Western blot analysis. In order to prepare cell lysates, cells were collected and cell extracts were prepared using radioimmunoprecipitation assay buffer, according to the manufacturer's protocol (Biotek Corporation, Beijing, China). Cell lysates were collected following centrifugation at 9,500 x g for 15 min at 4˚C, and were subsequently transferred to clean microcentrifuge tubes. For western blot analysis, proteins were extracted from the cells in each group. Proteins were collected following cell lysis. The Bradford protein assay (10) was used to confirm the protein content. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a 0.45-μm pore size nitrocellulose (NC) membrane (RPN303E; Amersham; GE Healthcare Life Sciences, Chalfont, UK). Membranes were blocked with Tris-buffered saline (TBS; Boster Biological Technology, Ltd., Wuhan, China) containing 5% milk and 0.5% Tween for 1 h (37˚C), and then washed three times with 0.1 M TBS (pH 7.6) with 0.1% Tween (TBST). Anti-EDA-A1 rabbit anti-serum polyclonal antibody was obtained by custom rabbit immunization using purified FLAG-EDA as the immunogen. Then, the NC membrane was treated with TBST solution (containing 2% milk, 1:200 dilution of anti-EDA-A1 rabbit anti-serum) for 1 h at 37˚C, and washed with TBST three times. Following incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (1:2,000; A6154; Sigma-Aldrich, St. Louis, MO, USA), the expression levels of the target protein were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA)

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for the detection of cell proliferation. To determine the proliferation of each group of ECV304 cells, an MTT assay was performed. The 24-h-infected and uninfected cells were seeded into a 96-well plate with an inoculation density of 5,000 cells/well and incubated at 37˚C. After 12 h, 100 μl serum-free Dulbecco's modified Eagle's medium was added to each well. After 72 h, 20 μl MTT was added to each well and incubation was continued at 37˚C for 4 h. Then, the medium was removed and the precipitate was dissolved in dimethylsulfoxide. The absorbance (optical density, OD) at 560 nm was measured using a SpectraMax 190 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The inhibition rate of cell growth was calculated (n=10) on the basis of the experimentally measured OD value.

Cell cycle analysis. Flow cytometry was used to investigate the cell cycle. Following incubation for 48 h, the cells were collected and washed with cold phosphate-buffered saline. The washed cells were fixed in 70% cold ethanol with incubation overnight at 4˚C. To stain the cells, propidium iodide (PI) solution was added. A flow cytometer (Coulter Epics XL; Beckman Coulter, Inc., Brea, CA, USA) was used to analyze the samples. CellQuest 6.0 software (BD Biosciences, San

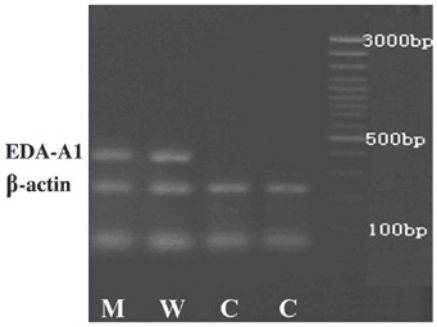

Figure 1. Detection of mRNA expression of the *EDA-A1* gene in ECV304 cells transfected with wild-type (W) and mutant (M) *EDA-A1* gene or empty vector control (C) by reverse transcription-polymerase chain reaction. EDA-A1, ectodysplasin A, transcript variant 1.

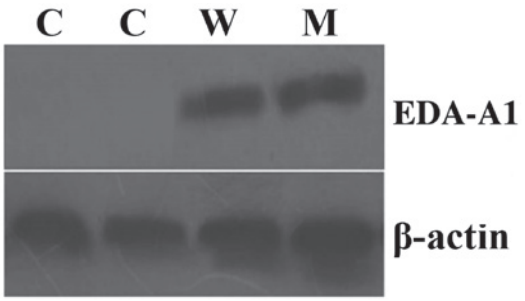

Figure 2. Detection of the expression of EDA-A1 protein in ECV304 cells transfected with wild-type (W) and mutant (M) *EDA-A1* gene or empty vector control (C) by western blotting. EDA-A1, ectodysplasin A, transcript variant 1.

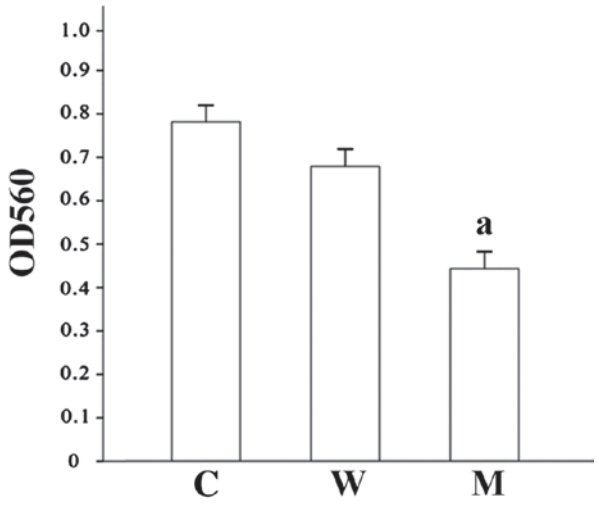

Figure 3. OD560 values of ECV304 cells transfected with wild-type (W) and mutant (M) *EDA-A1* gene or empty vector control (C) after culturing for 96 h. [a]P<0.01 vs. the control group. OD560, optical density at 560 nm; EDA-A1, ectodysplasin A, transcript variant 1. n=10 per group.

Jose, CA, USA) was used to analyze the percentage of cells in the G_0/G_1, S and G_2/M phases (n=5).

Statistical analysis. SPSS statistical analysis software, version 13.0 (SPSS, Inc., Chicago, IL, USA) was used to conduct analysis of variance testing for all data, which are expressed as the mean ± standard deviation. P-values less than 0.05 were considered to indicate a statistically significant difference.

Table I. OD560 value of ECV304 cells transfected with the *EDA-A1* gene following 96-h culture.

Group	OD560	Inhibition rate (%)
Control	0.79±0.037	2.5
Wild type	0.68±0.016	16.0
Mutant	0.44±0.033[a]	45.7[a]

[a]P<0.01 vs. the control group. OD560, optical density at 560 nm; EDA-Al, ectodysplasin A, transcript variant 1.

Results

EDA-A1 expression pattern in ECV304 cells is influenced by plasmid-mediated transfection. To determine the expression level of *ED1-A1* in the transfected ECV304 cells, the RNA samples with an OD260/OD280 ratio of 1.8-2.0 were selected for RT-PCR. The ECV304 cells transfected with pcDNA3.1 (-)-EDA-A1-M or pcDNA3.1 (-)-EDA-A1-W showed a band at ~400 bp that was not observed for the empty vector-transfected control cells when examined using semi-quantitative PCR and primers specific to *EDA-A1* (Fig. 1). Additionally, a β-actin band between 200 bp and 300 bp was observed for all groups. Then, EDA-A1 protein expression levels in the ECV304 cells were detected by western blotting. Fig. 2 shows that the EDA-A1 protein was expressed in the cells infected with pcDNA3.1 (-)-EDA-A1-M or pcDNA3.1 (-)-EDA-A1-W vector, but was not expressed in the control group. In conclusion, *EDA-A1* mRNA and protein was expressed in ECV304 cells following the exogenous delivery of *EDA-A1*, but not in control cells.

Overexpression of EDA-A1 affects ECV304 cell proliferation. To elucidate whether *EDA-A1* has an effect on ECV304 cell proliferation, MTT assays were performed. As shown in Fig. 3, the ECV304 cell viability at 96 h infection was decreased significantly in the mutant group by comparison with that in the wild type and control groups. The proliferation of mutant group cells was suppressed by 45.7% relative to control, while the proliferation of the wild type group was suppressed by 16.0% (Table I, Fig. 3).

EDA-A1 overexpression regulates the cell cycle of ECV304 cells. To determine the effect of plasmid-mediated *EDA-A1* infection on the cell cycle of ECV304 cells, flow cytometric analysis was conducted (Fig. 4). It was observed that 25.45±1.89% of cells were arrested at the G_0/G_1 phase of the cell cycle in the mutant group, compared with 20.37±0.68 and 20.30±0.68% of cells in the wild type and control groups, respectively (Table II). During the S phase, the mutant and wild type groups showed significantly higher cell percentages (12.40±1.75 and 14.80±1.45%, respectively) than the control group (8.55±0.57%). However, the two *EDA-A1*-transfected

Table II. Effect of *EDA-A1* gene transfection on the cell cycle of ECV304 cells.

Group	G_0/G_1 phase	S phase	G_2/M phase
Control	20.30±0.68	8.55±0.57	71.15±0.57
Wild type	20.37±0.68	14.80±1.45[a]	64.83±0.85[a]
Mutant	25.45±1.89[a,b]	12.40±1.75[a]	62.15±1.94[a]

[a]P<0.05 vs. the control group; [b]P<0.05 vs. the wild type group. EDA-A1, ectodysplasin A, transcript variant 1.

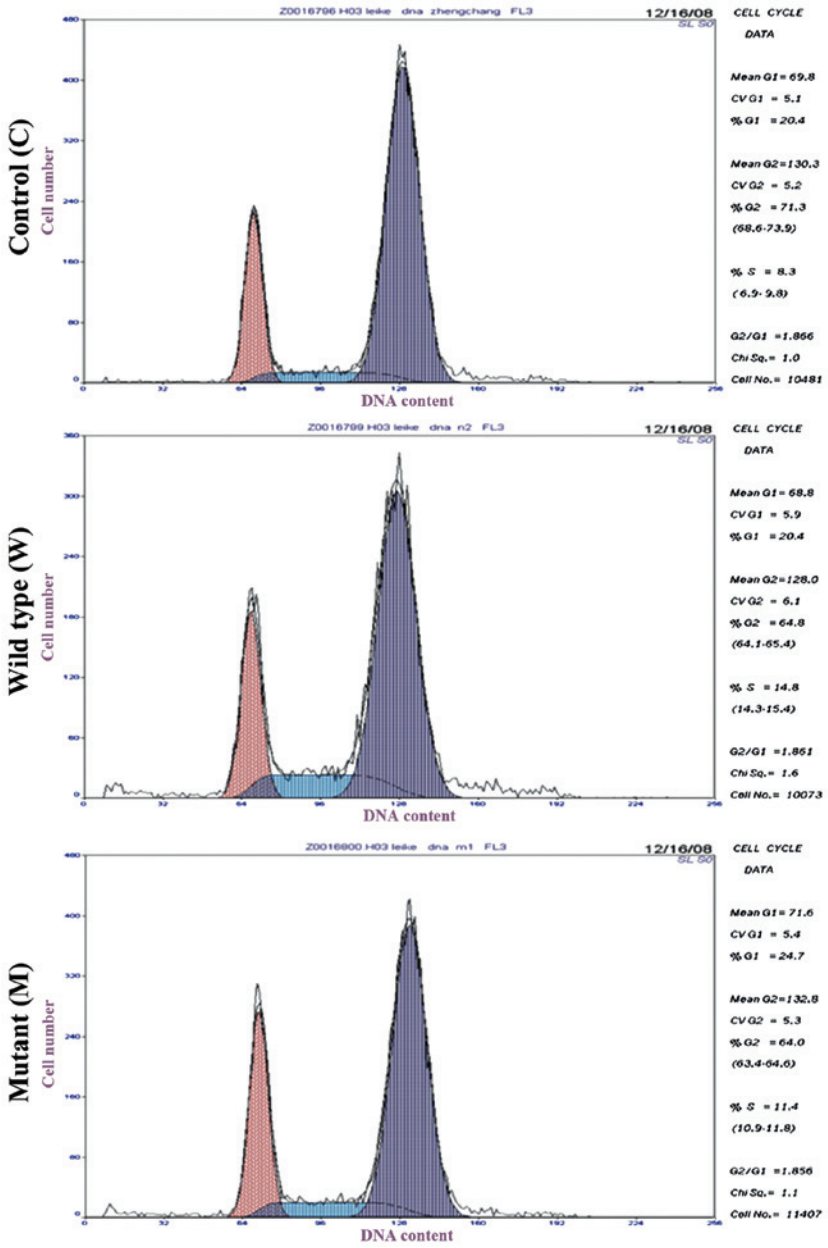

Figure 4. Effect of *EDA-A1* gene transfection on the cell cycle of ECV304 cells as shown by flow cytometry. EDA-A1, ectodysplasin A, transcript variant 1.

groups had lower cell percentages than the control in the G_2/M phase. The lowest cell percentage in the G_2/M phase was 62.15±1.94% in the mutant group. It may be concluded that the cell cycle distribution in the G_0/G_1, S and G_2/M phases of ECV304 cells were regulated by *EDA-A1* overexpression.

Discussion

Until now, the exact pathological mechanism of HED has remained unclear. In the present study, the effect of a HED-associated gene (*EDA-A1*) on the proliferation and cell

cycle of ECV304 cells was investigated. The results indicated that mutant and wild-type *EDA-A1* genes might have distinct biological functions affecting the proliferation and cell cycle distribution of cultured HUVECs.

EDA-A1, which is a variant of the major causative gene of HED (*EDA*), is located on chromosome Xq12-13.1 and encodes a protein containing 391 amino acids (4,11). The EDA-A1 protein, a type II transmembrane protein, is a member of the TNF ligand family. It consists of a short extracellular domain, a transmembrane region, a collagen area, and a TNF ligand subunit (6,11-13). The combination of EDA-A1 and the ectodysplasin receptor can promote programmed cell death and activate NF-κB signaling (11,14).

Currently, the research relating to HED mostly comprises case reports and mutation analysis; however, few studies have reported on the function of the *EDA-A1* gene, particularly in cell activity. Immunohistochemical analysis of human MCF-7 and COS-1 cells transfected with pCMV5-EDA-A2 identified strong signals at the cell surface for some transfected cells, and changes in the cell morphology of MCF-7 were found to be associated with the expression of EDA-A2 (7). Another previous study demonstrated that pIRES2-EGFP-EDA eukaryotic plasmids could be successfully transfected into dental pulp mesenchymal cells and stably expressed (15). In the present study, compared with the control group, the proliferation of ECV304 cells in the mutant group was decreased significantly, and cell growth was inhibited. This may be due to a change in the spatial configuration and biological activity of the EDA-A1 protein caused by the *EDA-A1* gene mutation. However, the reduction of cell proliferation of the ECV304 cells transfected with wild-type *EDA-A1* was not significant.

The cell cycle, which consists of a series of highly ordered phases (G_1, S, G_2 and M), is important to both normal and cancer cells (16). The actions of antitumor agents are also characterized by an association with cell cycle phase (17). The results of the present study indicated that the *EDA-A1* gene mutant had an impact on the cell cycle, and blocked cell cycle progression in the G_0/G_1 and S phases. However, the present study was limited, since it lacked transfection experiments with oral-related cell lines.

In conclusion, the present study revealed the inhibitory effect of an *EDA-A1* gene mutant on the proliferation of ECV304 cells. The aim of our future research is to focus on the transfection of the *EDA-A1* gene in other oral cavity-related cell lines, and to further elucidate the effect of the *EDA-A1* gene on tooth, jaw and craniofacial development.

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