Effect of mutant eda-a1 gene on huvecs

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

Running title: The effect of mutant *EDA-A1* gene on HUVECs.

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Highlights:

1. *EDA-A1* gene mutant significantly decreased proliferation of human umbilical vein endothelial cells(HUVECs).
2. HUVECs of mutant group were blocked at G_0/G_1 and S phase.
3. HUVECs of wild group accumulated in S phase and decreased in G_2/M phase.

Abstract

Background: To investigate the effect ofectodysplasin A gene (*EDA-A1*) on proliferation and cell cycle ofhuman umbilical vein endothelial cells (HUVECs) and explore the possible mechanism underlying this process.

Methods : Recombinant eukaryotic expression vectorspcDNA3. 1(-)- *EDA -A1* -M/W (mutant, M; wild, W) containing the coding sequence of *EDA-A1* -M/W were transfected into HUVECs. *EDA-A1* -M/W genes were amplified by reverse transcription polymerase chain reaction (RT-PCR), and the proteins were detected by western blot. Then MTT assay for cell proliferation of HUVECs in each group was performed and cell cycle was detected using flow cytometry.

Results : The *EDA-A1* gene and protein were detected respectively by RT-PCR and western blot in HUVECs transfected with pcDNA3. 1(-)- *EDA-A1* -M/W, but not in HUVECs transfected with empty plasmid pcDNA3. 1(-) (control group) and cells without transfection. Compared with control group, *EDA-A1* gene mutant significantly decreased proliferation of HUVECs and the inhibition rate was 45. 70% (P <0. 01), whereas wild *EDA-A1* gene did not cause such growth inhibition (P> 0. 05). A significant increase of the G_0/G_1 and S fraction was seen in the HUVECs of mutant group, compared withwild group with an accumulation in S phase and a concomitant decrease in G_2/M phase population (P <0. 05).

Conclusion : Compared with the wide-type, the mutant *EDA-A1* gene could inhibit the proliferation and cell cycle of the HUVEC.

Key words: *EDA-A1* gene; Mutant; Human umbilical vein endothelial cell; Cell cycle; Proliferation

Introduction

Hypohidrotic ectodermal dysplasia (HED), also called anhidrotic ectodermal dysplasia (AED) or Christ-Siemens-Touraine Syndrome, is a kind of X-linked recessive genetic disease (XLHED) (1). HED is a rare congenital genetic disorder with a birth incidence of 1/100, 000-1/10, 000 (2, 3). It is characterized by the diminution or absence of eccrine sweat glands, oligodontia and peg shaped teeth and sparse hair (1, 4). Previous study indicates that XLHED is caused by the ectodysplasin A gene (*EDA-A1*) mutant (5).

EDA-A1 , a major causative gene of HED, locates in Xq12-13. 1 and encodes a novel tumornecrosis factor (TNF) ligand family protein ectodysplasin A (EDA-A1) and this protein is associated with the nuclear factor-κB (NF-κB) signaling mechanisms (5-9). Bayes M *et al* . (10) indicates that the full-length of *EDA-A1* is 5296bp (http://www. ncbi. nlm. nih. gov/, AH007059, Gene ID 4007891), the open reading frame (ORF) of *EDA-A1* is 1176bp, and it encoding the protein with 391 amino acids (EDA-A1, GeneID1896). Studies showed the combination of EDA-A1 and ectodysplasin receptor (EDAR) could promote programmed cell death and active the signaling of NF-κB (8, 11). Recently, the related research on HED are mostly for mutation analysis of *EDA-A1* , and more than 100 mutations in the *EDA* gene have been reported to cause XLHED up to now (12, 13). However, there have few reports relating to the function of mutant *EDA-A1* , and the exact pathological mechanism of mutant *EDA-A1* on HED is still unclear.

In the present study, *EDA-A1* mutant (pcDNA3. 1 (-)- *EDA-A1* -M) and wild type (pcDNA3. 1(-)-EDA-A1-W) eukaryotic expression vector that we used were constructed in our previous study (14). Then the function of transfected *EDA-A1* and its mutant for cell proliferation and cell cycle ofHUVECs were analyzed. The aim of this study was to investigate the effect of *EDA-A1* on proliferation and cell cycle of HUVECs and explore the possible mechanism underlying this process.

Material and Method

Cell culture

HUVECs were kindly provided by professor Wang chunming (Lanzhou University, China). HUVECs were cultured in RPMI-1640 (Huamei Company, Shanghai, China) Medium. The medium were consisted of 10% fetal bovine serum (FBS) (Evergreen Company, Hangzhou) and 100U/ml penicillin/streptomycin. All these cells were maintained in humidified incubator of 5% CO_2 at 37â„ ƒ (0. 25% trypsin digestion overnight). Inverted microscope was used for the cell morphology investigation. All the experiments were performed at least in triplicate and repeated at least twice.

Plasmid extraction

EDA-A1 mutant (pcDNA3. 1(-)- *EDA-A1* -M) and wild type (pcDNA3. 1 (-)-EDA-A1-W) eukaryotic expression vector that we used were constructed in our previous study (14). Totally 3μl mutant (M) and Wild-type (W) plasmid DNA was extracted respectively from transfected HUVECs, followed by the sterile deionized water diluted to 1ml. The values of A260nm and A280nm were measured by UV spectrophotometer. Plasmid DNA concentration (μg / μl) = A260 × dilution factor × 50/1000. The plasmid DNA (positive recombinants and empty control) was precipitated by ethanol. Then the DNA pellet was resuspended in sterile deionized water.

Cell transfection

Cell transfection was carried out according to the instructions of QIAGEN-Effectene Transfection Reagent Kit (QIAGEN). Transfection was carried out when the cell density was up to 70% after 24 hour-cellpassaging. Cells were transferred into a complete medium (CM) 2 hours before transfection. Totally

2. 5μg mutant (M) and Wild-type (W) plasmid DNA was slowly added to the 2 M $CaCl_2$ solution (stand for 10 minutes). DNA-$CaCl_2$ solution was slowly added dropwise to the 2 ×HeBS (stand for 30 minutes) until the precipitation of tiny particles. The precipitate was uniformly dropwise added to the culture flasks. After a 12 hours growth under standard conditions, cells were washed 2 times with HeBS, followed by the cultured in CM. HUVECs transfected with empty vector were used as the control group.

Semi-quantitative real-time PCR

To identify the expression levels of *EDA-A1* in HUVECs, semi-quantitative real-time PCR (SqRT-PCR) analysis was performed. Total RNA was extracted from cultured cells in each group (cultured for 48 hours) by using reverse transcription (RT) kit (Fermentas Company), followed by the *EDA-A1* primers designation (Primer Premier 5. 0 software) and synthesis (Shanghai Biological Engineering Company). The primers used were as follows, *EDA-A1* (408bp): 5’- CGC AGG ATC CAT GGG CTA CCC GGA GGT -3’ (forward) and 5’- ATT AAG CTT GCC AAG CGG GCA CCA GGG AGA C -3’ (reverse), β-actin (230bp): 5’- ACG CAT TTG GTC GTA TTG GG-3’ (forward) and 5’- TGA TTT TGG AGG GAT CTC GC-3’ (reverse). The 50μl PCR reaction system were: cDNA template (2μl), 10 × PCR Buffer (5μl), dNTP (1μl), primer (up and downstream, 1μl), Taq DNA polymerase (1μl), ddH_2O (39μl). Products were subjected to electrophoresis (1. 5% agarose gel, 120V, 90mA).

Western blot analysis

For Western blot analysis, proteins were extracted from HUVECs in each group. Proteins were collected after cell lysis. Protein concentration was

determined using the Bradford dye-binding method (15). The proteins were separated by SDS-PAGE and transferred tothe 0. 45μm pore size nitrocellulose (NC) membrane (RPN303E, Amersham Company). NC membranes were blocked with TBS buffer (5% milk and 0. 5%-Tween) for 1 hour (37°C). Then, the membrane was incubated overnight at 4â„ ƒ with the rabbit antibodies EDA-A1 and β-actin (1: 200 dilution with TBST solution), followed by incubation at room temperature for 1h with an anti-rabbit secondary antibody (Sigma). Finally, the expression levels of the target proteins were visualized withchromogenic substrate.

MTT assay for cell proliferation detection

To determine the proliferation of HUVECs in each group, the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay was performed. The 24 hours-transfected and untransfected cells were seeded into 96-well plate with inculation density of 5000 cells/well and incubated at 37â„ ƒ. After 12 hours, 100 μl serum-free DMEM was added in each well. After 72 hours, 20 μl MTT was added into each well to continue incubation at 37â„ ƒ(4 hours). Then, the medium was removed and the precipitation was dissolved in DMSO. The absorbance at 560 nm was measured by SpectraMax 190 microplate reader (Moteular Devices Company) for colorimetric analysis. Inhibition rate of cell growth was calculated (n= 10) based on the experimentally measured absorbance value (OD value).

Cell cycle analysis

Flow cytometry was used to detect the cell cycle. After incubation for 48 h, the cells were collected and washed with cold PBS. The washed cells were

fixed in 70% cold ethanol with incubation overnight at 4â„ ƒ. To stain the cells, prodium iodide (PI) solution was added. Flow cytometer (Coulter Epics XL, Beckman Coulter Company) was used to analyze the samples. Cell Quest software was used to analyze the cell percentage of G0 / G1 phase, S phase, and G2 / M phase.

Statistical analysis

All assays were performed in triplicate and datawere expressed as mean values ±s. d. The SPSS 13. 0 software employing ANOVA was used to analyze all data which expressed as mean±SD. P values less than 0. 05 was considered as significantly different.

Results

EDA-A1 expression pattern in HUVECs influenced by plasmid-mediated transfection

To identify the expression level of *ED1-A1* in HUVECs transfected with vector pcDNA3. 1(-)- *EDA-A1* -M or pcDNA3. 1(-)- *EDA-A1* -W, the RNA samples with an OD260/OD280 ration of 1. 8-2. 0 were chosen for RT-PCR. The HUVECs with pcDNA3. 1(-)- *EDA-A1* -M or pcDNA3. 1(-)- *EDA-A1* -W transfection showed a band nearly 400 bp compared with control using semi-quantitative PCR and primers specific to *EDA-A1* (Figure 1). Additionally, β-actin band between 200 bp and 300 bp have been seen in all the groups. Then, EDA-A1 protein expression in HUVECs were detected by western blot. Figure 1 shows that the EDA-A1 protein was expressed in the transfected cells with pcDNA3. 1(-)- *EDA-A1* -M or pcDNA3. 1(-)- *EDA-A1* -W vector, however, it could not be

achieved in control group. In conclusion, the *EDA-A1* was expressed in HUVECs after exogenous delivered of *EDA-A1* , but not in the un-treated control cells.

Overexpression of EDA-A1 affects HUVECs proliferation

To elucidate the effect of *EDA-A1* on HUVECs proliferation, the MTT assays were performed. As shown in Figure 2, the HUVECs viability at 96 h transfection was decreased significantly in the mutant group by comparison with wild type and control. The proliferation of mutant group cells was suppressed by 45. 7% compaired to control, while the wild type group was suppressed by 16. 0% (Table 1, Figure 3).

EDA-A1 overexpression regulates the cell cycle of HUVECs

To determine the role of plasmid-mediated *EDA-A1* transfection in cell cycle of HUVECs, the flow cytometry was used (Figure 4). We observed that 25. 45 ± 1. 89 % cells were arrested at G0/G1 phase of cell cycle in the mutant group compared with 20. 37 ± 0. 6% and 20. 30 ± 0. 68% cells in wild type and control groups, respectively (Table 2). During S phase, both mutant and wild type groups showed significantly higher cell percentages (14. 80 ± 1. 45% and 12. 4 0 ± 1. 75%) than that of control (8. 55 ± 0. 57%). However, both transfection groups had lower cell percentages than control in G2/M phase. The lowest cell percentage with 62. 15 ± 1. 94% was showed in the mutant group during S phase. We could conclude that the cell cycle distribution in G0/G1, S, and G2/M of HUVECs were regulated by *EDA-A1* overexpression.

Discussion

HED characterized by impaired development of hair, eccrine sweat glands and teeth is caused by mutations in the EDA-A1 gene (3, 16). Recently, the related research on HED are focused on the mutation analysis of EDA-A1, however, the exact pathological mechanism of HED caused by mutant EDA-A1 is still unclear (17). In this study, we investigated the effect of HED related gene EDA-A1 on proliferation and cell cycle of HUVECs. The results showed that mutant EDA-A1 gene significantly decreased proliferation of HUVECs ($P < 0.01$). Moreover, a significant increase of the G0 /G1 and S fraction was seen in the HUVECs of mutant group.

EDA-A1 protein, a type â…¡ transmembrane protein, is one of the TNF ligand family members involved in ectodermal development (18). EDA-A1 contains a TNF-like domain (aa: 245–391), a collagen domain, and a furin protease recognition sequence (7, 8, 19-21). The TNF-like domain is necessary and sufficient for receptor molecule EDAR binding (22, 23). Furthermore, EDA-A1 has been shown to specifically bind to EDAR, which could promote programmed cell death and active the signaling of NF-κB (8, 11). In our study, the reason why EDA-A1 mutant could inhibit the proliferation and block the cell cycle progression in G0/G1 phase and S phase of HUVECs might be the change of protein spatial configuration and biological activity that caused by the EDA-A1 gene mutation and the changed protein could not combined with EDAR and thus inhibit the signaling of NF-κB. Maria et al. found that HED was related with the blocked signaling pathway of NF-κB (9). Pascal et al. found that point mutations in the TNF-like domain of EDA-A1 strongly decreased EDAR binding to EDA-A1 by altering the folding of EDA

(21). Moreover, the substitution of Gln306 with Pro in our study was found to be located in the TNF-like domain of EDA-A1 and may influence the epithelial signaling pathway required for the normal ectodermal development through altering the topology of EDA, which is consistent with previous study.

HUVECs are cells derived from the endothelium of veins from the umbilical cord, and they are often used as a laboratory model system for the study of the function and pathology of endothelial cells (24). Some studies showed that during vascular development and pathological angiogenesis, the maintenance of blood vessel homeostasis and its functional execution depend on the integrity of vascular endothelium, which is affected by proliferation, migration and apoptosis of endothelial cells (25, 26). Furthermore, Jie et al. showed that recovery of injured endothelial cells through regulated endothelial cell proliferation plays significant roles in thrombosis disease (27). In our study, mutant EDA-A1 decreased the proliferation of HUVECs, therefore, we suspected that pathological mechanism underlying HED caused by EDA-A1 may be the growth inhibit of endothelial cells which could lead to the defection of eccrine sweat glandsis. Despite of all results mentioned above, there were still some limitations in the present study, whether the EDA-A1 mutant blocked the combination of EDA-A1 with EDAR required further experiment.

In conclusion, our study revealed EDA-A1 gene mutant could inhibit the proliferation and cell cycle of HUVECs. We explored the mechanism of HED caused by mutant EDA-A1. The substitution of Gln306 with Pro may influence the epithelial signaling pathway required for the normal ectodermal development through altering the topology of EDA, which could impair the

binding of EDA-A1 to EDAR and further inhibit the signaling of NF-κB. Our finding broadens the spectrum of EDA-A1 mutations and may help to understand the molecular basis of XLHED and aid genetic counseling.

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Conflict of interest

The authors have declared that no competing interests exist.

Authors’ contributions

KL and LW participated in the design of this study, and they both performed the statistical analysis. BM and TC carried out the study, together with PS, collected important background information, and drafted the manuscript. LL and XH conceived of this study, and participated in the design and helped to draft the manuscript. All authors read and approved the final manuscript.

Figure legends:

Figure 1 Detection of mRNA expression of *EDA-A1* gene in ECV304 cells by RT-PCR: M: mutant group; W: wild group; C: control group.

Figure 2 Expression of ECV304 cells transfected with *EDA-A1* gene and mutant: M: mutant group; W: wild group; C: control group.

Figure 3 OD560 value of ECV304 cells transfected with *EDA-A1* gene after cultured for 96h: M: mutant group; W: wild group; C: control group; a: compared with the control group, P <0. 01.

Figure 4 The effect of *EDA-A1* gene mutant on cell cycle in ECV304 cells.

Table 1 OD560 value of ECV cells transfected with *EDA-A1* gene after cultured for 96h

Group	OD560	Inhibition rate (%)
Control	0. 79 ± 0. 037	2. 5
Wide type	0. 68 ± 0. 016	16. 0
Mutant	0. 44 ± 0. 033 [a]	45. 7 [a]

Note: a: compared with control group, P <0. 01.

Table 2 Effect of *EDA-A1* gene mutant on cell cycle in ECV304 cells

Group	G_0/G_1 phase	S phase	G2/M phase
	(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. 4 0±1. 75 [a]	62. 15±1. 94 [a]

Note: a: compared with control group, P <0. 05; b: compared with wild group, P <0. 05