学位論文（博士）

Different degree of loss-of-function among four missense mutations in the *EDAR* gene responsible for autosomal recessive hypohidrotic ectodermal dysplasia may be associated with the phenotypic severity

（常染色体潜性（劣性）遺伝の低汗性外胚葉形成不全症の原因となる *EDAR* 遺伝子の４つのミスセンス変異の機能喪失の程度の違いは、表現型の重症度と相関している可能性がある）

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令和４年１１月

学位論文の関連論文の研究背景及び要旨

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〔題名〕

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（常染色体潜性（劣性）遺伝の低汗性外胚葉形成不全症の原因となる *EDAR* 遺伝子の 4 つのミスセンス変異の機能喪失の程度の違いは、表現型の重症度と相関している可能性がある）

The Journal of Dermatology（令和 4 年 9 月 30 日掲載受理）

〔研究背景〕

低汗性外胚葉形成不全症（hypohidrotic ectodermal dysplasia: HED）は、乏毛症、乏歯症、低汗症を 3 徴候とする稀な症候群である。また、本疾患の患者は、鞍鼻、眼周囲色素沈着、耳介低位などの特徴的な顔貌異常を示す。HED の遺伝形式は多くが X 連鎖潜性（劣性）だが、常染色体顕性（優性）または常染色体潜性（劣性）遺伝形式を示す家系が存在することも知られている。X 連鎖潜性（劣性）遺伝の HED は ectodysplasin A（*EDA*）遺伝子の変異によって、常染色体遺伝の HED は ectodysplasin A receptor（*EDAR*）または EDAR-associated death domain（*EDARADD*）遺伝子のいずれかの変異によって発症する。*EDA* 遺伝子がコードする ectodysplasin-A1（EDA-A1）は、tumor necrosis factor（TNF）リガンドスーパーファミリーに属する。EDAR は TNF 受容体スーパーファミリーに属し、N 末端の細胞外領域にリガンド結合ドメイン、細胞内領域には death domain（DD）をそれぞれ有している。EDAR は EDA-A1 の特異的な受容体であり、EDARADD は EDA-A1 のアダプター蛋白として機能する。EDA-A1 は EDAR の細胞外領域に結合し、EDARADD は細胞内で EDAR とお互いの DD を介して結合する。さらに、EDARADD は TNF receptor-associated factor 6（TRAF6）などのシグナル伝達物質と相互作用することで、最終的に NF-κB の活性化につながる。

EDAR 遺伝子の変異については、潜性（劣性）変異では発現や機能が失われ、顕性（優性）変異では野生型 EDAR 蛋白に対して dominant negative 効果を示すことが報告されている。しかし、現在までに、遺伝子型と表現型との明確な相関関係は明らかにされていない。

〔要旨〕

本研究では、特定の遺伝子型と表現型との関連を明らかにするために、*EDAR* 遺伝子の潜性（劣性）変異に着目し、その特徴を詳細に検討した。具体的には、過去に EDAR の DD 内に同定された潜性（劣性）遺伝形式を示す 4 種類のミスセンス変異（p.R358Q、p.G382S、p.I388T、p.T403M）について、培養細胞での過剰発現系で一連の解析を実施した。これらの変異の中で、p.R358Q は EDARADD との結合能を失い、下流の NF-κB 活性を低下させることが知られており、機能喪失の

陽性対照として用いた。

まず、細胞溶解液を用いた western blot 法では、p.R358Q および p.T403M 変異型 EDAR 蛋白は野生型 EDAR 蛋白よりも発現量が減衰し、より大きい分子量を示した。一方で、p.G382S および p.I388T 変異型 EDAR 蛋白は野生型 EDAR 蛋白と同様の発現パターンを示した。また、各 EDAR 蛋白の細胞内での局在を解析するために実施した蛍光免疫染色法では、野生型 EDAR 蛋白と同様に p.G382S および p.I388T 変異型 EDAR 蛋白は細胞質内に局在が認められたが、p.R358Q と p.T403M 変異型 EDAR 蛋白は細胞膜に発現していた。これらの結果から、変異型蛋白間で発現パターンが異なることが示された。続いて行った NF-κB レポーターアッセイでは、すべての変異型 EDAR 蛋白が NF-κB の活性化を抑制したが、p.R358Q と p.T403M 変異型 EDAR 蛋白に比べ、p.G382S と p.I388T 変異型 EDAR 蛋白による抑制効果は軽微であった。EDAR と EDARADD の結合を検討した共免疫沈降法では、p.R358Q と p.T403M 変異型 EDAR 蛋白は EDARADD との結合能を完全に喪失していたが、p.G382S と p.I388T 変異型 EDAR 蛋白は、ある程度結合能を維持した。これらの解析で、p.G382S 変異型 EDAR の機能喪失の程度は最も軽度と考えられた。

過去の研究で、野生型 EDAR は TRAF6 とは直接結合しないことが報告されており、本研究で実施した野生型 EDAR と TRAF6 間の共免疫沈降法でも同様の結果が得られた。しかしながら、驚くべきことに、本研究で解析した全ての変異型 EDAR 蛋白は TRAF6 と直接結合する性質を示した。

培養細胞での過剰発現系においては、機序は不明だが EDAR 蛋白を含む種々の TNF 受容体が細胞質内に発現する傾向を示すことが知られていたことから、p.R358Q および p.T403M 変異型 EDAR 蛋白の細胞膜への局在は異常な発現パターンと考えられる。NF-κB レポーターアッセイおよび共免疫沈降法の結果から、各変異型 EDAR 蛋白と EDARADD の親和性は NF-κB 活性低下の程度と強く相関することが示唆された。今回解析した 4 種類のミスセンス変異は、いずれも EDAR の機能や構造に重大な影響を与えると複数のデータベースで推測されていたが、各データベースのスコアは 4 つの変異の間で非常に類似していた。すなわち、現在の予測ツールの解析能力には限界があり、本研究のように実際に発現・機能解析を行う重要性がハイライトされたといえる。

4 種類の変異型 EDAR 蛋白に共通する唯一の現象は、野生型 EDAR 蛋白が EDARADD を介して間接的に TRAF6 と相互作用するのに対し、TRAF6 と直接結合することである。これは、変異型 EDAR 蛋白が EDAR、EDARADD、TRAF6 からなる正しい蛋白複合体を形成できないことを示唆しており、*EDAR* 遺伝子変異に起因する HED の鍵となっている可能性があるが、本現象の病的意義を解明するためには今後のさらなる検討を要する。

本研究で得られた結果に基づき、各変異を機能喪失の度合いで評価した。R358Q と T403M を「重度」、p.I388T を「中等度」、p.G382S を「軽度」とした。各変異を報告した文献に提示されていた表現型と比較検討した結果、*EDAR* 遺伝子変異の機能喪失の程度が HED の重症度と相関している可能性が示唆された。

Received: 15 August 2022 | Accepted: 30 September 2022

DOI: 10.1111/1346-8138.16610

ORIGINAL ARTICLE

The Journal of Dermatology

Different degree of loss-of-function among four missense mutations in the *EDAR* gene responsible for autosomal recessive hypohidrotic ectodermal dysplasia may be associated with the phenotypic severity

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Funding information
Grant-in-Aid for Scientific Research (C), Grant/Award Number: KAKENHI 18K08269; Japan Society for the Promotion of Science (JSPS); Ministry of Health, Labor and Welfare, Japan, Grant/Award Number: 22FC1015

Abstract

Hypohidrotic ectodermal dysplasia is a rare condition characterized by hypohidrosis, hypodontia, and hypotrichosis. The disease can show X-linked recessive, autosomal dominant or autosomal recessive inheritance trait. Of these, the autosomal forms are caused by mutations in either *EDAR* or *EDARADD*. To date, the underlying pathomechanisms or genotype–phenotype correlations for autosomal forms have not completely been disclosed. In this study, we performed a series of in vitro studies for four missense mutations in the death domain of EDAR protein: p.R358Q, p.G382S, p.I388T, and p.T403M. The results revealed that p.R358Q- and p.T403M-mutant EDAR showed different expression patterns from wild-type EDAR in both western blots and immunostainings. NF-κB reporter assays demonstrated that all the mutant EDAR showed reduced activation of NF-κB, but the reduction by p.G382S- and p.I388T-mutant EDAR was moderate. Co-immunoprecipitation assays showed that p.R358Q- and p.T403M-mutant EDAR did not bind with EDARADD at all, whereas p.G382S- and p.I388T-mutant EDAR maintained the affinity to some extent. Furthermore, we demonstrated that all the mutant EDAR proteins analyzed aberrantly bound with TRAF6. Sum of the data suggest that the degree of loss-of-function is different among the mutant EDAR proteins, which may be associated with the severity of the disease.

KEYWORDS
death domain, EDAR, EDARADD, hypohidrotic ectodermal dysplasia, TRAF6

1 | INTRODUCTION

Hypohidrotic ectodermal dysplasia (HED) is a rare syndrome that is characterized by abnormal development of hair, teeth, and eccrine sweat glands, leading to show hypotrichosis, hypodontia, and hypohidrosis, respectively.[1] Affected individuals with HED also exhibit characteristic facial features, such as saddle nose, periorbital pigmentation, and low-set ears. In most cases, HED shows an X-linked recessive inheritance pattern (Online Mendelian Inheritance in Man [OMIM] 305100), while autosomal dominant (OMIM 129490) or recessive (OMIM 224900) forms of the disease are also known. The genetic basis of HED has already been disclosed. X-linked HED is caused by mutations in ectodysplasin (*EDA*) gene,[2] and autosomal forms of HED result from mutations in either ectodysplasin A receptor (*EDAR*) or EDAR-associated death domain (*EDARADD*) genes.[3,4] The longest product encoded by the *EDA* gene, known as ectodysplasin-A1 (EDA-A1), belongs to the tumor necrosis factor (TNF) ligand superfamily and is believed to be crucial for ectodermal

development.[5–7] EDAR is a type I transmembrane protein and a member of the TNF receptor superfamily with a ligand-binding domain in the N-terminal extracellular region, as well as a potential death domain in its intracellular region.[8] Importantly, EDAR has been shown to be a specific receptor of EDA-A1,[9] and EDARADD functions as an adaptor protein of EDAR.[4] EDA-A1 binds to its receptor EDAR, which subsequently associates with EDARADD via their death domains.[10] In addition, EDARADD further binds to other components for the signal transduction, such as TNF receptor associated factor 6 (TRAF6), which finally leads to the downstream activation of NF-κB.[11,12]

Regarding mutations in the *EDAR* gene, it has been reported that recessive mutations lead to loss of expression or function, while dominant ones show a dominant-negative effect against wild-type (Wt) EDAR protein.[13–18] To date, however, clear genotype–phenotype correlations have not been characterized. We postulate that each mutation can show difference in the degree of abnormalities, which may be associated with the severity of the disease.

In this study, we focus on recessive mutations in the *EDAR* gene and attempt to characterize them in detail to reveal the association with the phenotypes. We tested a total of four missense mutations, p.R358Q, p.G382S, p.I388T, and p.T403M, within the death domain of EDAR, all of which have been identified in families showing an apparent autosomal recessive inheritance pattern.[15,17,19–22] Of these, we have chosen the mutation p.R358Q as a positive control for the loss-of-function, as it has clearly been demonstrated to lose the affinity to EDARADD and show markedly reduced activation of the downstream NF-κB.[17]

2 | METHODS

2.1 | Generation of expression vectors

Expression vectors for an N-terminal Flag-tagged full-length wild-type (Wt) EDAR (pCXN2.1-Flag-EDAR-Wt), an N-terminal myc-tagged intracellular region (IC) of Wt-EDAR (pCXN2.1-myc-EDAR-IC-Wt), an N-terminal Flag- or myc-tagged Wt-EDARADD (pCXN2.1-Flag-EDARADD-Wt and pCXN2.1-myc-EDARADD-Wt, respectively), and an N-terminal hemagglutinin (HA)-tagged Wt-TRAF6 (pCXN2.1-HA-TRAF6-Wt) were previously generated.[18,23] The nucleotide sequences for EDAR-IC were cut out from the pCXN2.1-myc-EDAR-IC-Wt vector with restriction enzymes *EcoR*I and *Kpn*I, and were suncloned into pCMV-Tag2A vector (Agilent Technologies), which expresses an N-terminal Flag-tagged protein. The generated vector was designated as pCMV-Tag2A-EDAR-IC-Wt. Subsequently, the Flag-EDAR-IC sequences were cut out from the pCMV-Tag2A-EDAR-IC-Wt vector with *Not*I and *Kpn*I, and were subcloned into pCXN2.1 vector (pCXN2.1-Flag-EDAR-IC-Wt).[24] Using the QuikChange site-directed mutagenesis kit (Agilent Technologies), the mutations c.1073G>A (p.R358Q), c.1144G>A (p.G382S), c.1163T>C (p.I388T), and c.1208C>T (p.T403M) were introduced into the pCXN2.1-Flag-EDAR-Wt, pCXN2.1-Flag-EDAR-IC-Wt, and pCXN2.1-myc-EDAR-IC-Wt vectors. The nucleotide sequences of all the generated vectors were confirmed by Sanger sequencing.

2.2 | Cell culture and western blots (WBs)

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were seeded in six-well dishes the day before transfection. One microgram of vectors for an N-terminal Flag-tagged full-length EDAR (Wt or mutants) or an empty pCXN2.1 vector were transfected into each well using Lipofectamine 2000 (Life Technologies) according to manufactures instructions. 24 h after the transfection, the cells were harvested and homogenized in lysis buffer (20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 10% Glycerol, 2 mM EDTA, 0.5% Triton X, and 1X Protease Inhibitor Cocktail (Takara Bio Inc.)). Total cell lysates were collected by centrifugation at 15 000 *g* for 10 min at 4°C. The lysates were mixed with 4XLDS sample buffer and 10× reducing agent (Life Technologies), incubated at 75°C for 10 min, and then loaded to 4%–12% NuPAGE gels (Life Technologies). WBs were performed as described previously.[25] The primary antibodies used were mouse monoclonal anti-DDDDK (Flag; diluted 1:5000; MBL International), rabbit polyclonal anti-EDAR (diluted 1:2000),[17] and mouse monoclonal anti-beta-actin (diluted 1:3000; Sigma-Aldrich).

2.3 | Indirect immunofluorescence (IIF)

HEK293T cells were plated in eight-well chamber slides (Nalge Nunc International) on the day before transfection. In line with the manufacturer's instructions, 100 ng of vectors for an N-terminal Flag-tagged full-length EDAR (Wt or mutants) or an empty pCXN2.1 vector were transfected with Lipofectamine 2000 (Invitrogen), and were further cultured for 30 h. IIF was performed as described previously.[25] The primary antibodies used were rabbit polyclonal anti-EDAR (diluted 1:500)[17] and mouse monoclonal anti-pan-cadherin (diluted 1:100; Abcam). The secondary antibodies used were Alexa Fluor 594 goat anti-rabbit IgG (diluted 1:500; Life Technologies) and Alexa Fluor 488 goat anti-mouse IgG (diluted 1:500; Life Technologies). The nuclei were couterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The images were analyzed with a confocal laser scanning microscope (Olympus Fluoview FV3000; Olympus).

2.4 | NF-κB reporter assay

HEK293T cells were seeded in 12-well dishes the day before transfection. 50 ng of pNF-κBLuc vector (Clontech) was

transfected into each well along with 25 ng of vectors for an N-terminal Flag-tagged full-length EDAR (Wt or mutants), an N-terminal Flag-tagged EDARADD, or an empty pCXN2.1 vector using Lipofectamine 2000 (Life Technologies). 50 ng of a construct for β-galactosidase reporter (Promega) was also transfected for normalization of transfection efficiency. The cells were lysed 30 h after the transfection and the signals were assayed as described previously.[26] The results represent triplicate determination of a single experiment that is representative of a total of three similar experiments. The data were presented as means ± SEM, and were analyzed with Dunnett's multiple-comparison test. $p < 0.05$ was considered statistically significant.

2.5 | Co-immunoprecipitation (co-IP) assays

HEK293T cells were plated in six-well dishes the day before transfection. Expression vectors for EDAR-IC, EDARADD, and TRAF6 (1.0 μg each) were transfected with Lipofectamine 2000 (Life Technologies). The total amount of vectors in each transfection was adjusted with an empty pCXN2.1 vector. 24 h after the transfection, total cell lysates were extracted from the cells as described above. The samples were incubated with mouse monoclonal anti-DDDDK (Flag) agarose gels (MBL International) for 2 h at 4°C. The agarose beads were washed with lysis buffer four times. The precipitated proteins were eluted in 4XLDS sample buffer and 10× reducing agent (Life Technologies), incubated at 75°C for 10 min. Electrophoresis on 4%–12% NuPAGE gels (Life Technologies) and WBs were performed. The primary antibodies used were rabbit polyclonal anti-myc (diluted 1:1000; MBL International), rabbit polyclonal anti-DDDDK (Flag; diluted 1:1000; MBL International), and rabbit polyclonal anti-HA (diluted 1:3000; Abcam).

3 | RESULTS

3.1 | Expression patterns were different among the mutant EDAR proteins

We initially overexpressed Wt or the four mutant EDAR proteins in HEK293T cells and analyzed their expression by WBs with anti-Flag and anti-EDAR antibodies. The EDAR proteins carried a Flag-tag at the N-terminus (Figure 1a), and thus the anti-Flag antibody recognized only the premature forms before the signal peptide was removed, while the anti-EDAR antibody was expected to detect mainly the mature forms (Figure 1a). In WBs with the anti-Flag antibody, all the EDAR proteins showed a single fragment, 55 KDa in size, but the expression levels of R358Q- and T403M-mutant EDAR proteins were lower than Wt-EDAR protein (Figure 1b). WBs with the anti-EDAR antibody revealed a fragment, 52 KDa in size, in all the EDAR proteins analyzed, while a larger fragment, around 60 KDa in size, was also detected in R358Q- and T403M-mutant EDAR proteins, suggesting that some aberrant modifications occurred in these two mutants (Figure 1b).

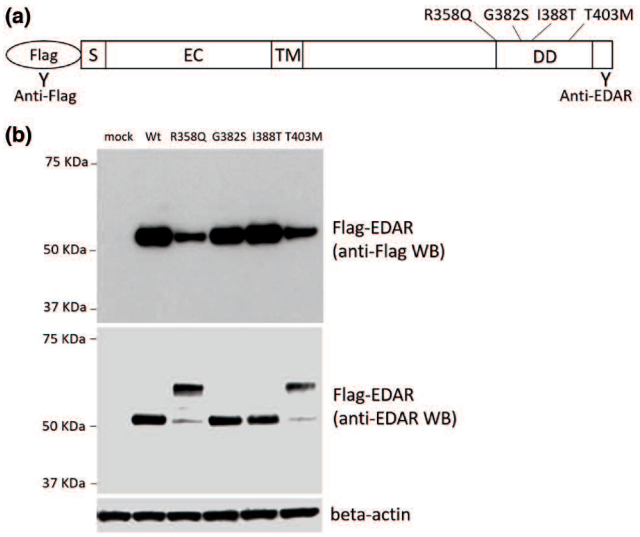

FIGURE 1 Schematic representation of EDAR protein and the results of western blots. (a) Schematic representation of EDAR protein and position of the four missense mutations analyzed in this study. The epitope of anti-Flag and anti-EDAR antibodies is also indicated. S, signal peptide; EC, extracellular domain; TM, transmembrane domain; DD, death domain. (b) Expression vectors for an N-terminal Flag-tagged wild-type (Wt) and the four mutant EDAR (Flag-EDAR) were transfected into HEK293 cells, and the expression patterns were analyzed by western blots (WBs) with anti-Flag and anti-EDAR antibodies. WB with anti-beta-actin antibody was performed as a control.

3.2 | Distinct subcellular localization between the mutant EDAR proteins

We then performed IIF studies to analyze subcellular localization of Wt and the four mutant EDAR proteins in HEK293T cells, which showed that Wt-EDAR protein was predominantly expressed within the cytoplasm (Figure 2). Similarly, p.G382S- and p.I388T-mutant EDAR proteins also showed an intracellular localization (Figure 2). In contrast, p.R358Q- and p.T403M-mutant EDAR proteins were clearly expressed at the cell membrane (Figure 2). The results revealed two distinct expression patterns between the mutant EDAR proteins.

3.3 | All the four mutant-EDAR proteins showed reduction in activation of the downstream NF-κB

To investigate how the *EDAR* gene mutations would affect the downstream signaling, we performed NF-κB reporter assays. As the first step, we simply overexpressed Wt- or mutant-EDAR in HEK293T cells. As compared with Wt-EDAR, all the four mutant EDAR showed statistically lower luciferase activity (Figure 3a). The reduction was especially obvious in p.R358Q- and p.T403M-mutant EDAR (Figure 3a). We subsequently performed NF-κB reporter assays under the condition that EDARADD-expression vector was co-transfected. When EDARADD and Wt-EDAR were

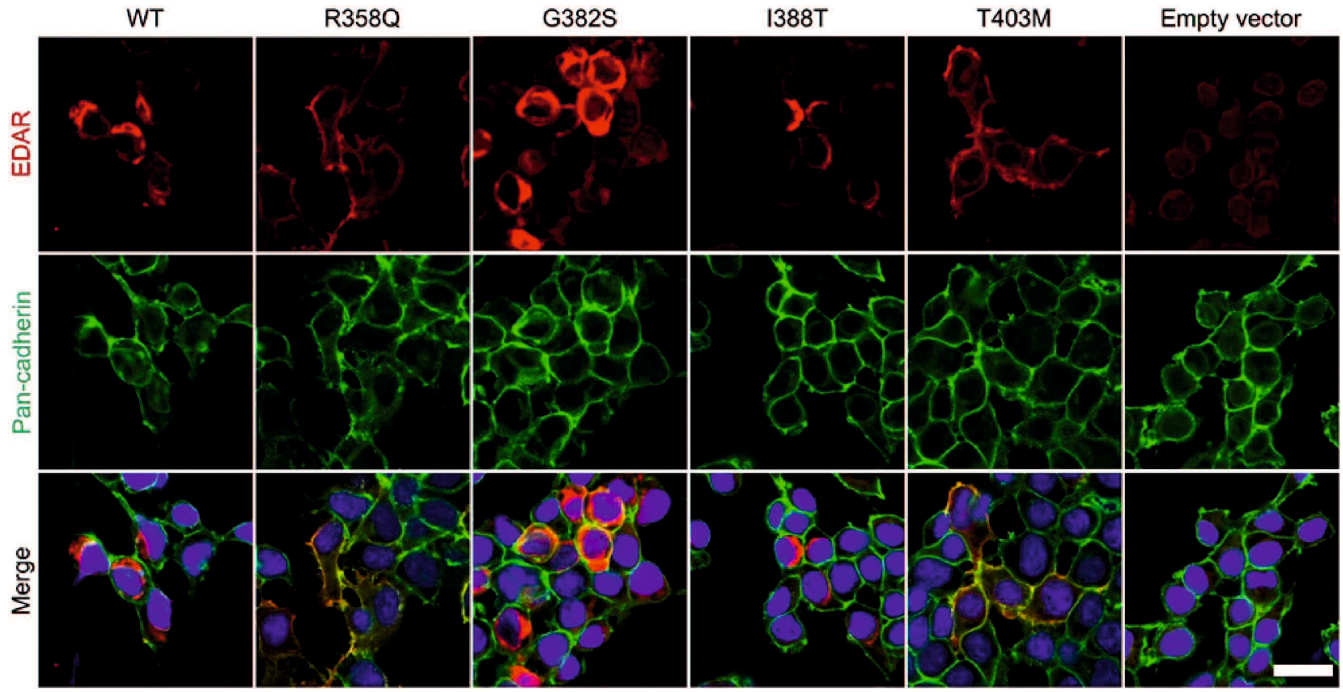

FIGURE 2 Subcellular localization of wild-type and the four mutant EDAR proteins. Expression vectors for an N-terminal Flag-tagged wild-type (Wt) and the four mutant EDAR were transfected into HEK293 cells, and indirect immunofluorescence was performed with anti-EDAR and anti-pan-cadherin antibodies. Wt-, p.G382S-, and p.I388T-mutant EDAR proteins were expressed within the cytoplasm, while p.R358Q- and p.T403M-mutant EDAR proteins were at the cell membrane. Counterstaining with DAPI is shown in blue. Scale bar: 20 μm.

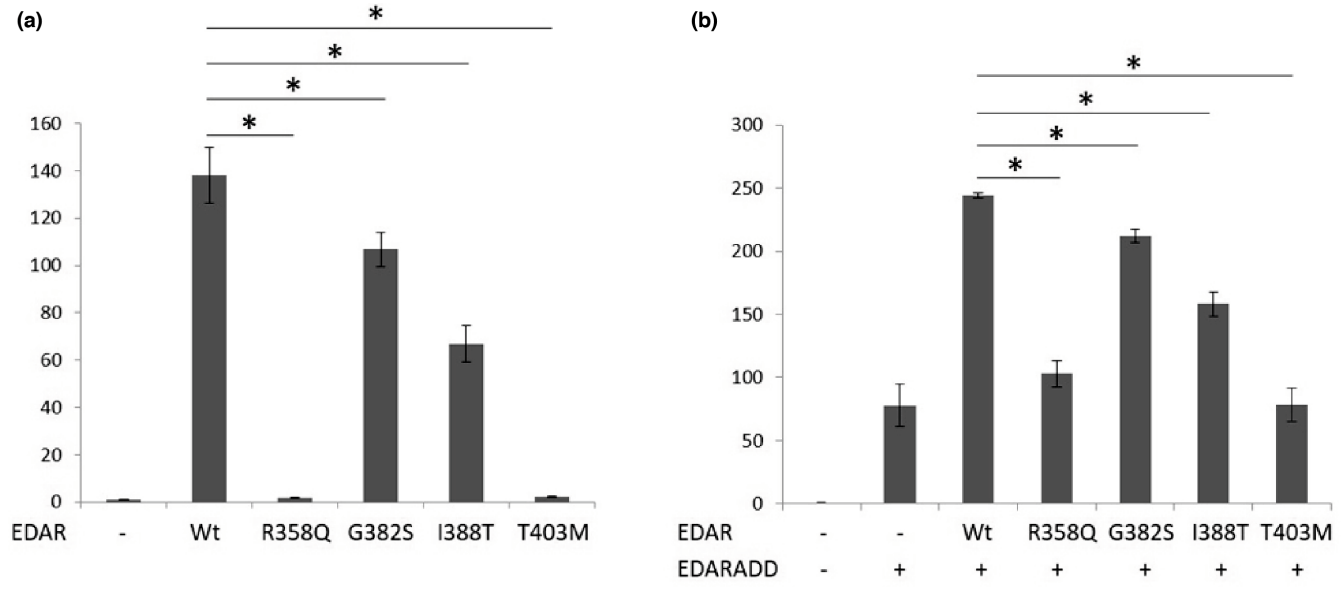

FIGURE 3 All the for mutant EDAR proteins showed reduced activation of NF-κB. (a) When wild-type (Wt) or the mutant EDAR proteins were overexpressed in HEK293T cells, Wt-EDAR markedly upregulated the luciferase activity, while the activation by all the four mutant EDAR proteins was statistically lower than that by Wt-EDAR. In particular, p.R358Q- and p.T403M-mutant EDAR proteins markedly reduced the activity. (b) On the condition that EDARADD was co-overexpressed, p.R358Q- and p.T403M-mutant EDAR proteins significantly reduced the luciferase activity, while the reduction by p.382S- and p.I388T-mutant EDAR was moderate, as compared to Wt-EDAR. The data were analyzed with Dunnett's multiple-comparison test. $p < 0.05$ was considered statistically significant (asterisks).

co-overexpressed, the luciferase activity was markedly upregulated (Figure 3b). This ability of upregulation maintained to some extent in G382S- and I388T-mutant EDAR proteins, which, nonetheless, was statistically lower than Wt-EDAR (Figure 3b). By contrast, R358Q- and T403M-mutant EDAR proteins only weakly upregulated the luciferase activity (Figure 3b). The results suggest that the degree of loss-of-function appears to vary among the mutant EDAR proteins.

3.4 | Mutant EDAR showed reduced or loss of affinity to EDARADD

In order to investigate physical interaction between EDARADD and the mutant-EDAR, we conducted co-IP assays on the condition that myc-tagged intracellular domain of EDAR (myc-EDAR-IC) and Flag-tagged EDARADD (Flag-EDARADD) were co-overexpressed in HEK293T cells. While the Wt-EDAR-IC was co-immunoprecipitated with Flag-EDARADD, R358Q- and T403M-mutant EDAR were not at all (Figure 4). Co-IP of I388T-mutant EDAR protein was weakly detected, while that of G382S-mutant EDAR protein was relatively abundant, of which level was slightly less than that of Wt-EDAR (Figure 4).

3.5 | Aberrant interaction between the mutant EDAR and TRAF6

It is known that TRAF6 is an important component for downstream of the EDA-A1/EDAR/EDARADD signaling.[11] We firstly analyzed if Wt-EDAR was capable of directly binding with TRAF6. Co-IP assays showed that Wt-EDAR-IC did not bind with TRAF6 without EDARADD, whereas TRAF6 was co-immunoprecipitated with Wt-EDAR when EDARADD was co-overexpressed (Figure 5a). The result indicates that EDAR does not have an affinity with TRAF6, while it indirectly interacts with TRAF6 via EDARADD. We subsequently tested the interaction between TRAF6 and the mutant EDAR. Surprisingly, co-IP assays demonstrated that TRAF6 was co-immunoprecipitated with all the four mutant EDAR-IC even without EDARADD (Figure 5b), suggesting that the mutant EDAR proteins were capable of directly binding with TRAF6.

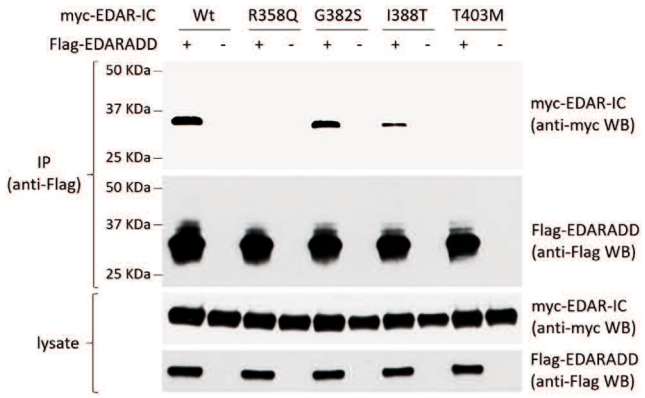

FIGURE 4 The mutant EDAR proteins showed reduced or abolished affinity with EDARADD. An N-terminal myc-tagged intracellular domain (IC) of wild-type (Wt) or the mutant EDAR proteins (myc-EDAR-IC) were overexpressed with an N-terminal Flag-tagged EDARADD (Flag-EDARADD), and immunoprecipitation (IP) with anti-Flag antibody was performed, which was followed by western blots (WBs) with anti-myc and anti-Flag antibodies. While Wt-EDAR-IC was efficiently co-immunoprecipitated with Flag-EDARADD, either p.R358Q-EDAR-IC or p.T403M-EDAR-IC was not. Co-IP of p.I388T-EDAR-IC was markedly reduced, and that of p.G382S was slightly reduced, as compared with Wt-EDAR-IC.

4 | DISCUSSION

In this study, we performed in vitro analyses to characterize four missense mutations in the *EDAR* gene which have previously been identified in families with HED showing an autosomal recessive inheritance trait.[15,17,19–22] We detected that these mutant EDAR proteins showed different behaviors in cultured cells. Concerning the subcellular localization, Wt-, p.G382S- and p.I388T-mutant EDAR proteins were mainly expressed within the cytoplasm, while p.R358Q- and p.T403M-mutant EDAR proteins preferentially localized at the cell membrane (Figure 2), which might reflect different expression patterns in WBs (Figure 1b). It is known that many types of TNF receptors including EDAR tend to be expressed within the cytoplasm in IIF staining although the significance or the mechanism for this phenomenon remains unknown.[27–30] Interestingly, a dominantly-inherited mutant EDAR has been shown to be expressed at the cell membrane,[30] similar to p.R358Q- and p.T403M-mutant EDAR. Therefore, in IIF studies in cultured cells, it can be considered that the localization at the cell membrane is an abnormal expression pattern.

In NF-κB reporter assays, p.R358Q- and p.T403M-mutant EDAR showed markedly reduced activation of NF-κB, while the reduction was moderate in p.I388T and mild in p.G382S, respectively (Figure 3a,b). In co-IP assays, p.R358Q- and p.T403M-mutant EDAR completely abolished the affinity with EDARADD, and p.I388T-mutant EDAR only weakly bound with EDARADD (Figure 4). In contrast, p.G382S-mutant EDAR maintained a strong affinity with EDARADD, as compared with the other mutant EDAR proteins (Figure 4). The results of co-IP were consistent with those of NF-κB reporter assays, and thereby, the degree of reduction of NF-κB activity is highly correlated with the affinity between each mutant EDAR and EDARADD.

The missense mutations analyzed in this study (p.R358Q, p.G382S, p.I388T, and p.T403M) were all a non-conservative amino acid substitution within the death domain of EDAR protein (Figure 1a), and several databases predicted that all the four mutations would severely affect the function and/or the structure of EDAR (Table 1). The score in each database was quite similar among the four mutations (Table 1), suggesting that the prediction tools did not precisely reveal the actual consequences resulting from the mutations. It also highlighted the importance of experiments in wet laboratories like ours.

The only common phenomenon among all the four mutant EDAR proteins was that they obtained an ability to bind directly with TRAF6, while the Wt-EDAR interacted indirectly with TRAF6 through EDARADD (Figure 5a,b). The results suggest that the mutant EDAR would fail to form a correct protein-complex composed of EDAR, EDARADD, and TRAF6, which might be a "key" abnormality for HED resulting from the *EDAR*-gene mutations. In order

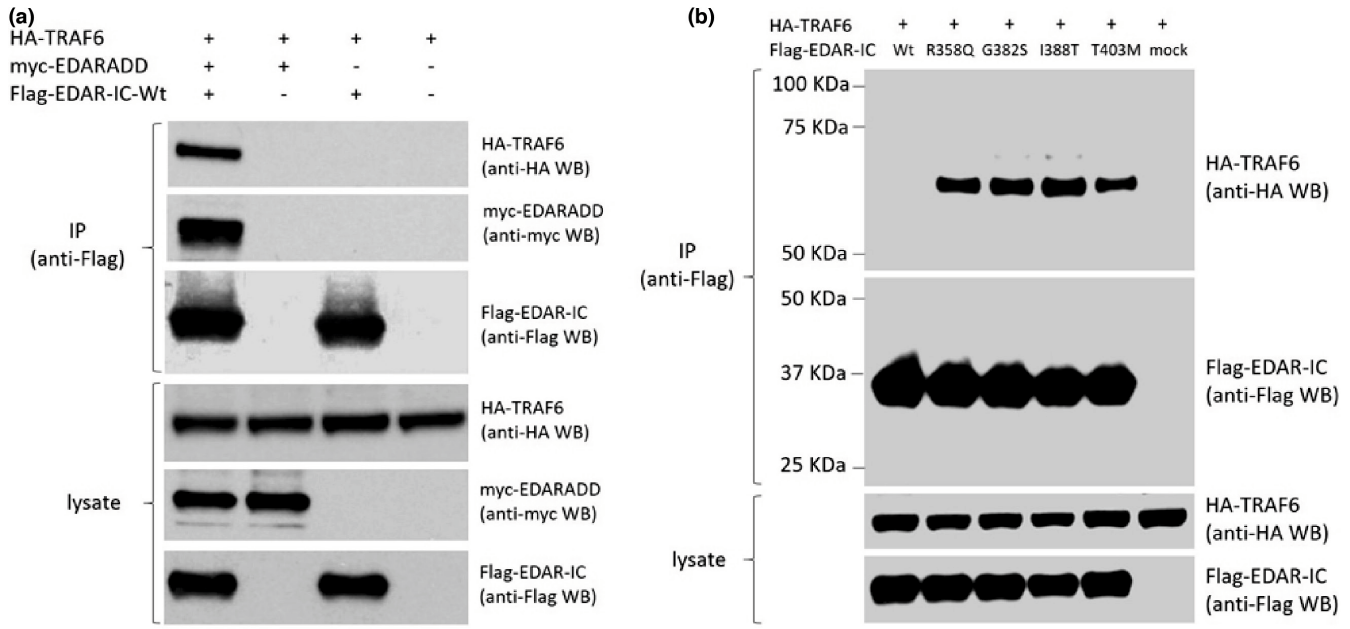

FIGURE 5 All the mutant EDAR proteins directly bound with TRAF6, while wild-type EDAR did not. (a) An N-terminal HA-tagged TRAF6 (HA-TRAF6) and an N-terminal Flag-tagged intracellular domain (IC) of wild-type (Wt) EDAR (Flag-EDAR-IC-Wt) were co-transfected either with or without an N-terminal myc-tagged EDARADD (myc-EDARADD), and immunoprecipitation (IP) with anti-Flag antibody was performed, which was followed by western blots (WBs) with anti-HA, anti-myc, and anti-Flag antibodies. When myc-EDARADD was co-overexpressed, HA-TRAF6 was co-immunoprecipitated with Flag-EDAR-IC-Wt (lane 1). By contrast, HA-TRAF6 was not co-immunoprecipitated without EDARADD (lane 3). (b) Wt- or the mutant Flag-EDAR-IC proteins were overexpressed with HA-TRAF6, and IP with anti-Flag antibody was performed, which was followed by WBs with anti-HA and anti-Flag antibodies. While HA-TRAF6 was not co-immunoprecipitated with Wt-EDAR protein (lane 1), it was clearly co-immunoprecipitated with all the four mutant EDAR proteins (lanes 2–5).

TABLE 1 Prediction for the four *EDAR* gene mutations analyzed in this study

Mutation		SIFT	PolyPhen	REVEL	MetaLR
p.R358Q	Score	0.030	0.992	0.740	0.732
	Prediction	Deleterious	Probably damaging	Likely disease causing	Damaging
p.G382S	Score	0.000	1.000	0.792	0.714
	Prediction	Deleterious	Probably damaging	Likely disease causing	Damaging
p.I388T	Score	0.000	0.998	0.812	0.733
	Prediction	Deleterious	Probably damaging	Likely disease causing	Damaging
p.T403M	Score	0.000	0.999	0.746	0.738
	Prediction	Deleterious	Probably damaging	Likely disease causing	Damaging

TABLE 2 Characteristics of the mutant EDAR proteins analyzed in this study

	p.R358Q	p.G382S	p.I388T	p.T403M
Expression level on western blots	Reduced	Similar to wild-type	Similar to wild-type	Reduced
Expression pattern on western blots	Larger size	Similar to wild-type	Similar to wild-type	Larger size
Localization	Cell membrane	Cytoplasm (Similar to wild-type)	Cytoplasm (Similar to wild-type)	Cell membrane
Activation of NF-κB	Markedly reduced	Slightly reduced	Moderately reduced	Markedly reduced
Affinity to EDARADD	Abolished	Slightly reduced	Markedly reduced	Abolished
Aberrant affinity to TRAF6	+	+	+	+
Degree of loss-of-function	Severe	Mild	Moderate	Severe

Abbreviations: WBs, western blots; WT, wild-type.

to further disclose the pathomechanisms related to this phenomenon, we performed additional NF-κB reporter assays on the condition that TRAF6 was co-overexpressed. However, we were unable to obtain any significant findings because TRAF6, by itself, highly activated the downstream NF-κB with or without EDAR/EDARADD (data not shown).

Based on the results obtained in this study, we evaluated each mutation in terms of the degree of loss-of-function: R358Q and T403M as "severe", p.I388T as "moderate", and p.G382S as "mild", respectively (Table 2). The most important question is whether our results are correlated with the severity in phenotypes or not. We (Y.S and R.H.) have previously experienced a Japanese HED patient with bi-allelic *EDAR*-gene mutation p.R358Q.[17] The patient definitely showed severe phenotypes with extremely-sparse hairs, only few teeth, and diminished sweating.[17] Similarly, the mutation p.T403M also resulted in a severe type of HED according to a report by others.[22] Concerning the mutations p.G382S and p.I388T, patients with either mutation showed less severe hair symptoms.[20,21] In addition, a patient with the mutation p.G382S did not seem to have apparent saddle nose or low-set ears.[20] Although an accurate evaluation for the phenotypes is difficult because of lacking detailed clinical features in the reference papers,[15,19–22] there is a possibility that the degree of loss-of-function of the *EDAR*-gene mutations is associated with determining the severity of HED.

Our study not only underscored crucial roles of EDAR in ectodermal development, but also provided precious information regarding the genotype–phenotype correlation for HED.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research (C) (KAKENHI 18K08269) from the Japan Society for the Promotion of Science (JSPS) to Y.S., and the "Research on Measures for Intractable Diseases" Project: matching fund subsidy (22FC1015) from the Ministry of Health, Labor and Welfare, Japan.

CONFLICT OF INTEREST

None declared.

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How to cite this article: Yagi S, Yasuno S, Ansai O, Hayashi R, Shimomura Y. Different degree of loss-of-function among four missense mutations in the *EDAR* gene responsible for autosomal recessive hypohidrotic ectodermal dysplasia may be associated with the phenotypic severity. J Dermatol. 2022;00:1–8. https://doi.org/10.1111/1346-8138.16610