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Analysis of hidradenitis suppurativa–linked mutations in four genes and the effects of PSEN1-P242LfsX11 on cytokine and chemokine expression in macrophages

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Abstract

Hidradenitis suppurativa (HS), or acne inversa, is a chronic inflammatory skin disorder characterized clinically with acne-like lesions in apocrine gland–bearing skin, follicular occlusion and recurrent inflammation. Thirty-four unique mutations in patients with HS have been found in three genes encoding the γ-secretase complex: nicastrin (NCSTN), presenilin 1 (PSEN1), presenilin enhancer 2 (PSENEN) and in POGLUT1, an endoplasmic reticulum O-glucosyltransferase involved in Notch signaling. We have carried out a system review and have performed a functional analysis of the 34 unique reported HS-linked mutations in *NCSTN*, *PSEN1*, *PSENEN* and *POGLUT1*. We have also examined the effects of the HS-linked PSEN1-P242LfsX11 mutation on cytokine and chemokine expression in macrophages. Mutations in *NCSTN* are predicted to cause loss of function, to result in loss of transmembrane (TM) domain, to affect NCSTN substrate recruitment sites, to cause loss or creation of new ligand binging sites and to alter post-translational modifications and disulfide bonds. PSEN1-P242LfsX11 occurs at the opposite side of TM5 from Alzheimer's disease–linked PSEN1 mutations. All of the *PSENEN* mutations occur on TM regions that are predicted to disrupt membrane function. *POGLUT1* mutations lead to an early termination of protein synthesis and are predicted to affect ligand binding function. In addition, PSEN1-P242LfsX11 mediates cytokine and chemokine expression and prolongs tumor necrosis factor α production on the inflammatory processes in THP-1 cells and phorbol-12-myristate-13-acetate–differentiated macrophages in response to lipopolysaccharide stimulation. These *in silico* analyses are instructive for functional studies of the HS-linked mutations. The PSEN1-P242LfsX11 mutation mediates cytokine and chemokine expression in macrophages.

Introduction

Hidradenitis suppurativa (HS), or acne inversa, is a chronic inflammatory skin disorder. It was estimated that the prevalence of HS varies from 0.05–4% in different populations or patient cohorts. A recent population study of the 47 690 patients with HS showed that the overall of sex- and age-adjusted HS population prevalence was 0.10%, or 98 per 100 000 persons in the United States. There were approximately three times the number of female HS patients (73.8% women) compared to male patients (26.2% men). The population prevalence of African American and biracial patients was more than 3- and 2-fold greater (1) than the overall population. The clinical

characteristics of HS include acne-like lesions in apocrine gland–bearing skin, follicular occlusion and progressive scarring from recurrent inflammation. Treatments include antibiotics, anti-inflammation regiments, acne washes and medicines and surgical procedure such as carbon dioxide laser excision and marsupialization (2). However, the disease progression in severe HS patients who have poor response to treatments often causes keloids, contractures and immobility, severely affecting the quality of HS patients' lives (3).

The etiology of HS is unclear. One-third of the HS patients are reported to have a family history, and the pattern of inheritance suggests a single-gene disorder inherited as an autosomal dominant trait (4). Thirty-four unique mutations in patients with familial or sporadic HS have been found in genes encoding three of the four genes comprising the γ-secretase complex: nicastrin (*NCSTN*), presenilin 1 (*PSEN1*), presenilin enhancer 2 (*PSENEN*) (5) and recently, in POGLUT1, an endoplasmic reticulum (ER)

O-glucosyltransferase involved in Notch signaling (6). However, how these HS-linked genetic mutations lead to HS pathogenesis remains unknown.

In silico programs have emerged as a convincing approach to study the structure and function of variants and mutations. We have carried out a system review and performed a functional *in silico* analysis of the 34 reported HS-linked mutations in *NCSTN*, *PSEN1*, *PSENEN* and *POGLUT1*. Notably, studies have demonstrated alterations in the immune responses in HS patients (7), and HS patients have chronic or frequent bacterial infections when circulating blood monocytes mature into tissue macrophages, which actively participate in the inflammatory processes (3). The bacterial endotoxin lipopolysaccharide (LPS) has been shown to stimulate host macrophages to produce inflammatory cytokines. Human monocytic THP-1 cells differentiate into macrophages by exposure to phorbol-12-myristate-13-acetate (PMA). Here we report the effect of

Table 1. Mutation spectrum of *NCSTN*, *PSEN1*, *PSENEN* and *POGLUT1* in HS patients

| ID	Mutation category	Nucleotide change	Amino acid change	TM	Ethnic origin	Reference
NCSTN						
1	**Missense**	c. 223G>A	p.V75I	Yes	Chinese	(13)
2		c.553G>A	p.D185N	Yes	Caucasian	(9)
3		c.632C>G	p.P211R	Yes	Chinese	(14)
4		c.647A>C	p.Q216P	Yes	Chinese	(13)
5		c.944C>T	p.A315V	Yes	Chinese	(15)
6		c.1229C>T	p.A410V	Yes	Chinese	(16)
7	**Nonsense**	c.349C>T	p.R117X	No	Chinese, Caucasian, African	(5,16,17)
8		c.477C>A	p.C159X	No	Chinese	(18)
9		c.497C>A	p.S166X	No	Chinese	(19)
10		c.1258C>T	p.Q420X	No	Chinese	(54)
11		c. 1300C>T	p.R434X	No	Caucasian	(20)
12		c. 1695T>G	p.Y565X	No	Chinese	(14)
13		c.1702C>T	p.Q568X	No	Caucasian, Japanese	(55)
14		c.1799delTG	p.L600X	No	Indian	(21)
15	**Frameshift**	c.210_211delAG	p.T70fsX18	No	Chinese	(10)
16		c.487delC	p.Q163SfsX39	No	Chinese	(5)
17		c.687insCC	p.C230PfsX31	No	Indian	(21)
18		c.1752delG	p.E584DfsX44	No	Chinese	(5)
19		c.1768A>G	p.590AfsX3	No	Caucasian	(20)
20	**Splice site**	c.582+1delG	p.F145fs_X54	No	Japanese	(55)
21		c.996+7G>A	p.L282_G332del	Yes	Caucasian	(9)
22		c.1101+1G>A	p.E333_Q367del	Yes	Caucasian	(22)
23		c.1101+10A>G	p.E333_Q367del	Yes	African	(9)
24		c.1352+1G>A	p.Q393fs_X9	No	Chinese	(10)
25		c.1551+1G>A	p.A486_T517del	No	Chinese	(5)
PSEN1						
26	**Frameshift**	c.725delC	P242LfsX11		Chinese	(5)
PSENEN						
27	**Frameshift**	c.66delG	p.F23LfsX46		Chinese	(5,23)
28		c.66_67insG	p.F23VfsX98		Caucasian	(9)
29		c.279delC	p.P94SfsX51		Chinese	(5)
30	**Nonsense**	c.168T>G	p.Y56-101Pdel		Caucasian	(24)
31	**Splicing**	c.167-2A>G	p.G55-101Pdel		Chinese	(25)
32	Missense	c.194T>G	p.L65R		Chinese	(25)
POGLUT1						
33	**Nonsense**	c.814C>T	p.R272*		Caucasian	(26)
34	**Splicing**	c.430-1G>A	p.K246_392Ldel		Caucasian	(6)
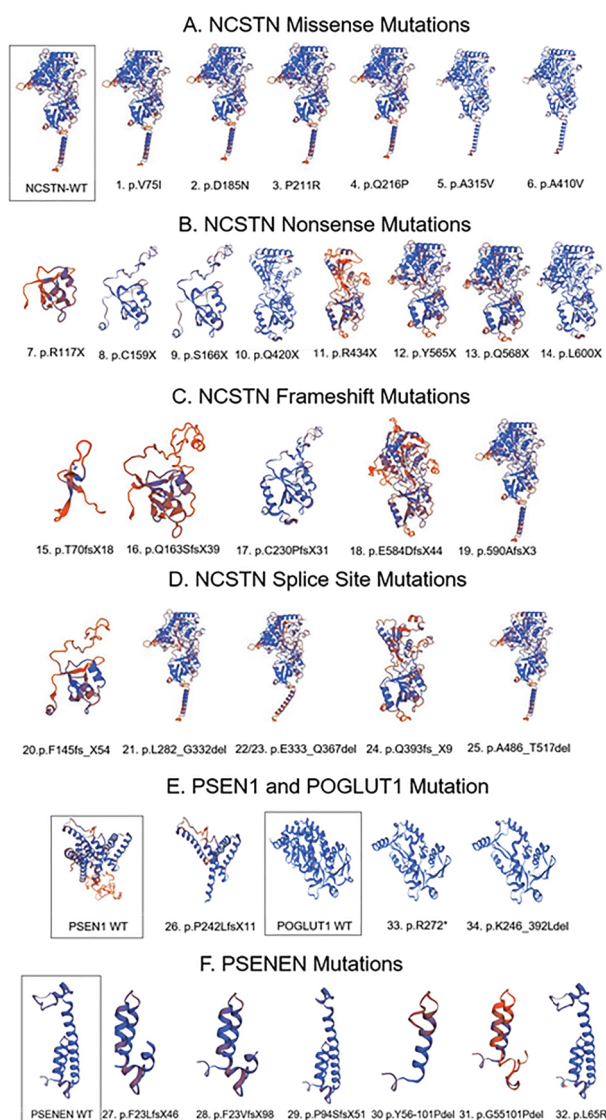
Figure 1. 3D structures of 34 unique HS-linked mutations in *NCSTN*, *PSEN1*, *PSENEN* and *POGLUT1* by SWISS-MODEL. Control WT protein is framed. Most of the HS-linked nonsense, frameshift and splice site mutations resulted in marked 3D structure change, consistent with loss of function. A C-terminal end frameshift mutation NCSTN-E584DfsX44 resulted in striking 3D structural change, while another nearby downstream frameshift mutation p.590AfsX3 (six amino acids apart) caused only minor 3D change.

overexpression of wild-type (WT) *PSEN1* (*PSEN1-WT*) and the HS-linked *PSEN1-P242LfsX11* mutation on the inflammatory processes in THP-1 cells and PMA-differentiated macrophages in response to LPS.

Results

Mutation spectrum of NCSTN, PSEN1, PSENEN and POGLUT1 in HS patients and functional *in silico* analyses

A total of 34 unique mutations have been identified in familial or sporadic HS patients with a diversity of mutation types in Caucasian, Chinese, Japanese, Indian or African ethnic origin (Table 1). Of these, a vast majority (74%, 25/34) of the mutations were in *NCSTN* (six missenses, eight nonsenses, five frameshifts and six in splice sites resulted in frameshift or in-frame deletions). A single frameshift *PSEN1-P242LfsX11*

mutation was detected in *PSEN1* (5). Six mutations were found in *PSENEN* (18%, 6/34) (three frameshifts, one nonsense, one splicing, one missense). Two mutations were in *POGLU1* (one nonsense, one splicing). NCSTN-R117X and Q568X were identified in more than one ethnic population and multiple families; the rest of HS-linked mutations are private to each HS family or subject. NCSTN-c.1799delTG is a two-base deletion that leads to a nonsense change L600X, while two splicing site mutations in NCSTN, c.582+1delG p. F145fs_X54 and c.1551+1G>A p.A486_T517del, result in frameshifts, while the other four splicing mutations cause in-frame deletions (Table 1).

We have performed a functional *in silico* analysis of the HS-linked mutations using a variety of programs. Of the HS-linked mutations in *NCSTN*, *PSEN1*, *PSENEN* and *POGLUT1*, there are 29% (10/34) nonsense, 26% frameshift (9/34) and 24% splicing site changes (8/34). By SWISS-MODEL, most of the HS-linked nonsense, frameshift and splice site mutations resulted in marked 3D structure changes (Fig. 1). Notably, a C-terminal end

Table 2. Predictions of functional effects of the *NCTSN* and *POGLUT1* missense mutations

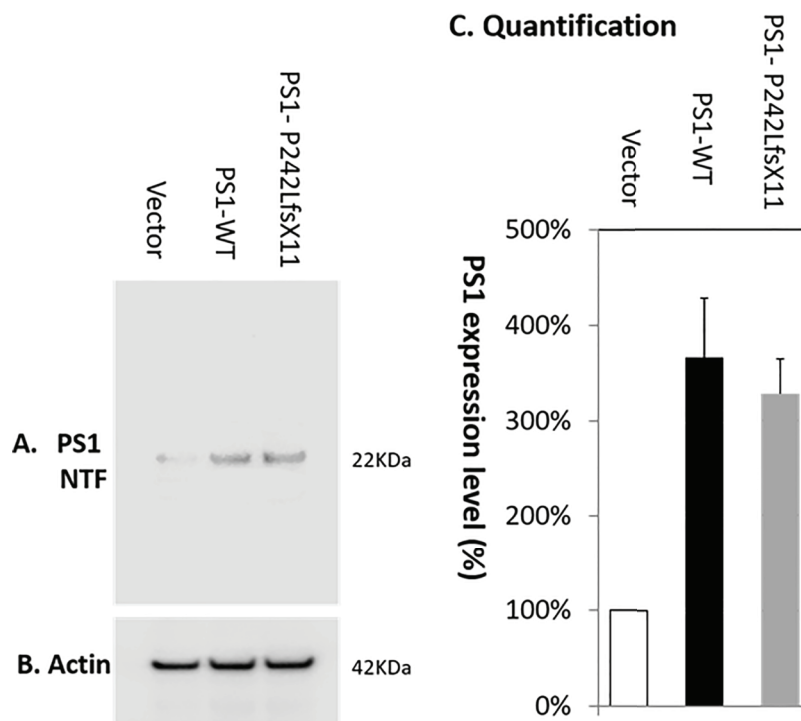| ID	Gene	Mutations	PolyPhen-2	SNP&Go	Proven
1	*NCSTN*	p.V75I	Probably damaging	Neutral	Neutral
2		p.D185N	Benign	Neutral	Neutral
3		p.P211R	Probably damaging	Disease	Deleterious
4		p.Q216P	Probably damaging	Disease	Deleterious
5		p.A315V	Benign	Neutral	Neutral
6		p.A410V	Benign	Neutral	Neutral
32	*PSENEN*	p.L65R	Possibly damaging	Disease	Deleterious

Figure 2. Western blotting quantification of PS1 expression in PMA-differentiated macrophages. Compared to vector control (100%), overexpression of PSEN1-WT or PSEN1- P242LfsX11 led to a significantly increased expression of PS1-WT (384 ± 11%) and PS1- P242LfsX11 (328 ± 11%), respectively. (**A**) Anti-PS1 N-terminal antibody detected the expression of a PS1 N-terminal product (25 kDa). (**B**) Anti-actin antibody detected the even expression level of β-actin (42 kDa). (**C**) Quantification of PS1 expression level using Image Studio Version 5.2.

frameshift mutation NCSTN-E584DfsX44 resulted in a striking 3D structural change (Fig. 1), while another nearby downstream frameshift mutation p.590AfsX3 (six amino acids apart) caused only a minor 3D change (Fig. 1).

By PolyPhen-2, SNP & Go and Proven prediction, among six *NCSTN* missense mutations, NCSTN-P211R and Q216P were most deleterious; V75I is probably damaging by PolyPhen-2; and D185N, A315V and A410V are predicted to have benign or neutral effects (Table 2). Sixty percent (15/25) of NCSTN mutations are nonsense or frameshift mutations that cause a truncation of the protein product. Structurally, NCSTN contains a large extra cellular domain and a single transmembrane (TM) (8), which is located at amino acid position 670–692 by Transmembrane Region Structure (THMEM). Forty percent (10/25) of *NCSTN* mutations (six missense mutations and four splicing site mutations) retain the TM region, while 60% (15/25) of other *NCSTN* nonsense, frameshift mutations and c.582+1delG (9) and c.1352+1G>A (experimental confirmed) (10) lose the TM domain to become cytosolic proteins that cannot enter the cell to initial signaling (Table 1). Among four splicing site mutations that do not affect TM regions, three potentially affect two key NCSTN

substrate recruitment sites: Gry333 and Tyr337. p. L282_G332del occurs next to the residue of NCSTN substrate recruitment site G333, and E333_Q367del and E333_Q367del completely abolish two NCSTN substrate recruitment sites Gry333 and Tyr337 (8), which suggests that these *NCSTN* mutations affect important substrate recruitment structures. Fifty percent (3/6) of the *NCSTN* splicing site mutations affect substrate recruitment.

Moreover, *NCSTN* mutations also led to altered post-translational modifications. Y565X occurs on a tyrosine phosphorylation site, and R434X occurs on a glycosylation site. NCSTN-R434X disrupts the protein immediately before Asn435, one of the two NCSTN prominent glycans Asn55 and Asn435 (8). Twenty-one percent (5 of 24) of the *NCSTN* mutations, NCTSN-P211R, L600X, C230PfsX31, P590AfsX3 and F145fs_X54, occur at cysteine residues participating in disulfide bonds (11,12). Six potential NCSTN ubiquitination sites are predicted: K78, T127, K386, K403, K591 and K597. Six residues in NCSTN undergo sumoylation: G146, S341, K386, P423, T459 and D476. NCSTN-P590AfsX3 occurs immediately before the predicted ubiquitination site K591 and abolishes two ubiquitination sites—K591 and K597. F145fs_X54 abolishes sumoylation site

Table 3. Differential expression of genes in cells overexpressing *PSEN1* or *PSEN1-P242LfsX11* in response to LPS in PMA-differentiated macrophages by PCR Array analyses

Genes	Description	Fold change	Gene function
PSEN1 versus control overexpressed			
TNFα [a]	Tumor necrosis factor α	4.90	Multifunctional proinflammatory cytokine, apoptosis
CCL13	Chemokine (C-C motif) ligand 13	2.45	Inducing chemotaxis, chronic inflammation
CXCL13	Chemokine (C-X-C motif) ligand 13	2.11	Lymphoid chemokine, chronic inflammation
CCL19	Chemokine (C-C motif) ligand 19	2.04	Lymphoid chemokine, chronic inflammation
PSEN1 versus control under-expressed			
IL12A	Interleukin 12A	−3.34	IL12 activate and link the innate and acquired immune responses
IL16	Interleukin 16	−3.18	Binds and signals through CD4 receptor
LIF	Leukemia inhibitory factor	−3.14	Mediates cell proliferation, differentiation and survival
IL12B	Interleukin 12B	−2.96	Acts on T and natural killer cells
IL1B	Interleukin 1B	−2.64	A subunit for IL12 and IL23; IL23 mediates late-stage inflammation
BMP2	Bone morphogenetic protein 2	−2.64	Regulates the process of hair follicle regeneration
CSF2	Colony stimulating factor 2	−2.52	Proliferation and differentiation of hematopoietic progenitor cell
CCL22	Chemokine (C-C motif) ligand 22	−2.16	T-cell differentiation
IL23A	Interleukin 23A	−2.09	IL23 mediates late-stage inflammation
IL10	Interleukin 10	−2.05	Arrests and reverses the chronic inflammatory response
PSEN1-P242LfsX11 versus control overexpressed			
CX3CL1	Chemokine (C-X3-C motif) ligand 1	4.43	The molecular control of leukocyte traffic at the endothelium
TNFSF11	Tumor necrosis factor 11	2.55	Regulates dendritic cell and osteoclast function; T-cell activation
IL11	Interleukin 11	2.52	The pleiotropic effects on hematopoietic cells
CCL17	Chemokine (C-C motif) ligand 7	2.19	Recruits selected subsets of leukocytes in inflammation
TNFα [a]	Tumor necrosis factor α	2.00	Multifunctional proinflammatory cytokine; apoptosis
PSEN1-P242LfsX11 versus control under-expressed			
CSF2	Colony stimulating factor 2	−3.50	Proliferation and differentiation of hematopoietic progenitor cell
IL5	Interleukin 5	−2.71	Growth and differentiation factor for B cells and eosinophils
BMP2	Bone morphogenetic protein 2	−2.52	Regulates the process of hair follicle regeneration
IL12B	Interleukin 12B	−2.10	IL12 activate and link the innate and acquired immune responses
CXCL1	Chemokine (C-X-C motif) ligand 1	−2.09	Directs and confines neutrophil influx to sites of injury
ADIPOQ	Adiponectin	−2.05	Attenuate the inflammatory response
IFNG	Interferon γ	−2.05	Critical for innate and adaptive immunity against bacterial infections
IFNA2	Interferon α2	−2.05	Reduces inflammation
MSTN	Myostatin	−2.05	Regulates embryonic development and maintains tissue homeostasis
THPO	Thrombopoietin	−2.05	The megakaryocyte proliferation and lineage
XCL1	Chemokine (C motif) ligand 1	−2.05	Induces the migration of cells expressing XCR1, including lymphocytes
NODAL	Nodal homolog	−2.05	A long-range signaling molecule
IL4	Interleukin 4	−2.05	B-cell stimulatory factor; T-cell and mast cell growth factor activities
IL21	Interleukin 21	−2.05	Stimulates B-cell proliferation
IL3	Interleukin 3	−2.05	The proliferation of hematopoietic cell
IL2	Interleukin 2	−2.05	The T-cell growth factor maintaining the immune system
IL17A	Interleukin 17A	−2.05	Stimulates cells to produce inflammatory mediators including IL1
BMP7	Bone morphogenetic protein 7	−2.05	Osteoblast differentiation in pluripotential and mesenchymal stem cells
CCL11	Chemokine (C-C motif) ligand 11	−2.05	Recruitment of leukocytes to inflammatory lesions
LIF	Leukemia inhibitory factor	−2.02	Mediates cell proliferation, differentiation and survival

[a] *TNFα exhibited overexpression, and LIF, IL12B, BMP2 and CSF2 exhibited under-expression in both PSEN1 and PSEN1-P242LfsX11 groups.*

G146. Both NCSTN-E333_Q367del and E333_Q367del abolish sumoylation site S341. NCSTN-T70fsX18 and R117X abolish all the ubiquitination and sumoylation sites, and C159X and S166X abolish four of the six ubiquitination sites and five of the six sumoylation sites.

A single frameshift *PSEN1-P242LfsX11* mutation is identified in *PSEN1*, which is predicted to truncate the PS1 proteins after the fifth TM domain at the cytosolic region of the N-terminal, which markedly alters the 3D structure of PS1 (Fig. 1). Western blotting quantification of PS1 expression in PMA-differentiated

macrophages detected a 25 kDa PS1 C-terminal fragment (Fig. 2). Compared to vector control, overexpression of PSEN1-WT or PSEN1-P242LfsX11 led to a significantly increased expression of PS1-WT (384 ± 17%) and PS1-P242LfsX11 (328 ± 11%), respectively.

PSENEN contains three TMs, at amino acid positions 18–38, 60–80 and 85–101. The PSENEN N-terminus is cytoplasmic, followed by two short helices that dip into the membrane (8). All the *PSENEN* mutations occur on TM regions: frameshift mutations F23LfsX46 and F23VfsX98 delete all three TM regions, while

Table 4. Time-dependent TNFα levels in the culture media in THP-1 cells and PMA-differentiated macrophages in response to LPS stimulation

Cells	Group/mean ± SEM (pg/ml)	0 h	4 h	6 h	8 h	24 h	P ANOVA time
	Control	51.5 ± 17.7	1125.9 ± 95.9	1279.9 ± 31.9	1041.5 ± 67.0	309.0 ± 23.7	<0.001
	PSEN1-WT	58.1 ± 19.2	957.5 ± 19.6	1235.5 ± 29.6	1215.5 ± 18.4	425.2 ± 17.4	<0.001
	PSEN1-P242LfsX11	104.0 ± 21.4	1314.8 ± 106.6	1555.5 ± 167.7	1343.6 ± 72.6	314.2 ± 21.1	<0.001
THP-1	P PSEN1-WT versus Ctrl (Fold change)	NS	NS	NS	0.01 (1.2)	<0.001 (0.98)	
	P P242LfsX11 versus Ctrl (Fold change)	0.04 (2.0)	NS	NS	0.003 (1.3)	NS	
	P P242LfsX11 versus PSEN1-WT (Fold change)	NS	0.002 (1.4)	0.04 (1.3)	0.05 (1.1)	<0.001 (0.74)	
	Control	481.0 ± 68.8	758.0 ± 67.7	1190.3 ± 153.6	1075.5 ± 157.9	653.8 ± 77.9	<0.001
	PSEN1-WT	690.9 ± 29.7	782.9 ± 86.8	849.7 ± 60.1	1105.0 ± 99.5	1102.3 ± 176.7	<0.001
PMA- differentiated macrophages	PSEN1-P242LfsX11	252.2 ± 29.1	1563.7 ± 205.1	2065.3 ± 274.3	2067.5 ± 279.5	2087.9 ± 352.9	<0.001
	P PSEN1-WT versus Ctrl (Fold change)	0.006 (1.4)	NS	0.03	NS	0.01 (1.7)	
	P P242LfsX11 versus Ctrl (Fold change)	0.003 (0.50)	0.0008 (2.1)	0.006 (1.7)	0.003 (1.9)	0.001 (3.2)	
	P P242LfsX11 versus PSEN1-WT (Fold change)	<0.001 (0.37)	0.001 (2.0)	<0.001 (2.4)	0.002 (1.87)	0.03 (1.89)	

P94SfsX51 disrupts TM 3. Nonsense Y56-101Pdel and c.167-2A>G splicing site mutations lead to similar disruptions of TM 2 and 3. The missense mutation PSENEN-L65R lies in the second TM region and is predicted to be deleterious by all three programs (Table 2).

POGLUT1 is located in the lumen of the ER. Both POGLUT1-R272* and C.430-1G>A, K246* lead to an early termination of protein synthesis. POGLUT1-R272* is located in the C-terminal domain and results in a truncated form of POGLUT1 with partial loss of the C-terminal domain. The splicing site c.430-1G>A mutation was identified in exon 4 of the *POFUT1* gene in patients with HS and Dowling–Degos disease (DDD) syndrome, which potentially generates aberrant splicing with loss of functionality (6). POGLUT1 is predicted to possess 17 ligand binding sites of interactions with chain A. Hydrogen bonds include A.Y117, A.S152, A.R158, A.R158, A.D196, A.V197, A.V197, A.L199, A.V214, A.A215, A.A215, A.S217, A.F218, A.R219, A.R219 and salt bridges, A.R158 and A.R219. Either POGLUT1- c.430-1G>A (K246*) or R272* completely abolished ligand binding function and shows significant alteration of global quality estimate by Qualitative Model Energy Analysis values: POGLUT1-WT: −71; POGLUT1- c.430-1G>A (K246*): 0.90; and R272* 0.45, indicating a greater deviation in mutant forms from the POGLUT1-WT.

The reported HS patients who carry a mutation in *NCSTN*, *PSEN1*, *PSENEN* or *POGLUT1* display severe and typical symptoms of HS lesions (13,9,14–16,5,17–21,10,22–26,6). While mutations in *NCSTN*, *PSEN1* and *PSENEN* occur in patients with HS only, HS patients who are carrying a *POFUT1* mutation also have DDD syndrome (6).

Overexpression of PSEN1-WT or PSEN1-P242LfsX11 increased cytokine expressions in response to LPS stimulation

Compared to control, PSEN1-WT expression was increased by 297 ± 19% and PSEN1-P242LfsX11 expression was increased by 214 ± 31% as detected by RT-PCR 24 h after transfection. Overexpression of PSEN1-WT or PSEN1-P242LfsX11 was also validated by western blotting quantification of PS1 expression in PMA-differentiated macrophages. Compared to vector control, overexpression of PSEN1-WT or PSEN1- P242LfsX11 led to a significantly increased expression of PS1-WT (384 ± 17%) and PS1-P242LfsX11 (328 ± 11%), respectively, as detected with an anti-PS1 N-terminal antibody (Fig. 2). A 25 kDa PS1 N-terminal product was detected in PMA-differentiated macrophages following overexpression of either PSEN1-WT or PSEN1-P242LfsX11. Overexpression of PSEN1-WT or PSEN1-P242LfsX11 resulted in over- and under-expressed genes encoding both cytokines and chemokines (Table 3). Overexpression of PSEN1-WT or PSEN1-P242LfsX11 led to approximately four and five genes significantly overexpressed, with only the concordant-expressed gene encodes the pro-inflammatory cytokine tumor necrosis factor α (*TNFα*) in either the PSEN1-WT or the PSEN1-P242LfsX11 group. In contrast, *LIF*, *IL12B*, *BMP2* and *CSF2* were concordantly underexpressed (Table 3).

To validate the observed increase in *TNFα* expression, time-dependent TNFα levels in the culture supernatant of THP-1 cells or PMA-differentiated macrophages at 0, 4, 6, 8 and 24 h after LPS exposure were analyzed by ELISA. Consistent with the increased *TNFα* expression detected by PCR Array analysis, TNFα levels were statistically significantly increased from 4 h with peak level at 6–8 h after LPS exposure in either THP-1 cells or PMA-differentiated macrophages by analysis of variance (ANOVA) and student's *t*-test (Table 4). Compared to control, overexpression of either PSEN1-WT or PSEN1-P242LfsX11 significantly increased TNFα levels at 6, 8 or 24 h. PSEN1-P242LfsX11 led to a 3.2-fold increase of TNFα at 24 h. Moreover, overexpression of PSEN1-P242LfsX11 led to a statistically significant 1.9–2.4-fold higher TNFα level than overexpression of PSEN1-WT from 4 h through 24 h after LPS stimulation in PMA-differentiated macrophages. In addition, at 24 h after LPS stimulation, the TNFα level declined in control but remained at peak in either PSEN1-WT or PSEN1-P242LfsX11 PMA-differentiated macrophages. This indicates that overexpression of PSEN1 or PSEN1-P242LfsX11 not only increased but also prolonged TNFα production in response to LPS stimulation.

Discussion

We have performed an extensive analysis of 34 HS-linked mutations in *NCSTN*, *PSEN1*, *PSENEN* and *POGLUT1* in HS patients. The results of this analysis are an important instructive tool for functional studies of these HS-linked mutations.

NCSTN, *PSEN1* and *PSENEN* encode the components of the γ-secretase complex (5), and *POGLUT1* is involved in Notch signaling (6). γ-secretase–deficient mice mimic histological

features found in HS patients, including follicular keratinization (5). NCSTN knockdown in HaCaT cells impaired γ-secretase activity and proliferation and differentiation of keratinocytes. Expression levels of several γ-secretase substrates involved in the Notch pathway were significantly attenuated in NCSTN-silencing HaCaT cells and the lesion of the HS patient. Phosphoinositide 3-kinase (PI3K), AKT and its activated form pAKT were markedly elevated in NCSTN-silencing HaCaT cells (18). This indicates that HS-linked *NCSTN* mutations may impair proliferation and differentiation of keratinocytes mainly through the Notch and PI3K/AKT signaling pathways (18).

Mutations in *NCSTN* are predicted to cause loss of function as a result of frameshift and premature translation termination, to result in loss of TM domain, to affect NCSTN substrate recruitment sites, to cause loss or creation of new ligand binging site and to alter post-translational modifications and disulfide bonds (11,12), all of which support the notion that the *NCSTN* mutations result in significantly reduced levels of NCT and reduced γ-secretase–mediated processing of Notch and signaling in the skin (27). However, testing four *NCSTN*–missense mutations, V75I, D185N, P211R and Q216P, for their effects on mediating Notch processing and signaling demonstrated the vague role of HS-linked *NCSTN* mutations in HS pathogenesis. The NCSTN-V75I, D185N and P211R mutants can function in Notch signaling *in vivo*; in contrast, mutant Q216P failed to rescue Notch processing and nuclear signaling (28). This suggests that NCSTN-V75I, D185N and P211R mutations have a significant role in the pathogenesis of the disease but through mechanism(s) other than impaired signaling by Notch 1 (28). The C-terminal end frameshift mutation NCSTN-E584DfsX44 resulted in a striking 3D structural change, suggesting that this mutation is likely located at a critical site for NCSTN conformation. Ubiquitiation and sumoylation are involved in post-translational modification. A large number of *NCSTN* mutations affect predicted ubiquitiation and sumoylation sites, suggesting that post-translational modification might contribute to HS pathogenesis.

PSEN1 was a major locus for early-onset familial Alzheimer's disease (FAD) (5). A single frameshift PSEN1-P242LfsX11 mutation was detected in familial HS patients (5). More than 185 missense or inframe deletion mutations and promoter variants in *PSEN1* have previously been found in patients with FAD (http://www.alz.org/) and sporadic dilated cardiomyopathy (29), indicating the pleiotropic nature of the presenilins (30). AD-associated *PSEN1* mutations alter the γ-secretases cleavage of β-APP to increase Aβ 42/40 ratio, resulting in Aβ plague formation and related AD pathology (5). Overexpression or silencing of presenilin caused cardiac dysfunction in *Drosophila* (31). Overexpression of PSEN1-P242LfsX11 in zebrafish embryos enhanced Notch signaling but did not affect γ-secretase cleavage of APP (32), which suggests that the involvement of the *PSEN1* mutation in HS pathogenesis also has a mechanism independent of γ-secretase activity. Notably, in AD patients, only one side of each TM helix in PS1 is affected; the hot spots of Leu219, Glu222, Leu226, Ser230, Met233 and Phe237 are placed on the same side of TM5 (8), while the HS-linked PSEN1-P242LfsX11 is on the other side of TM5 in PS1. This distribution or structure of AD-linked PSEN1 mutation is significantly different from HS-linked PSEN mutations that may indicate functional importance.

POGLUT1 is an ER O-glucosyltransferase that adds glucose moieties to serine residues in EGF-like repeats, such as those found in the *trans*-activating NOTCH intracellular domain (33). Mutations in *POGLUT1*, including W4X, R218X, R279PfsX3 and R279W, have been previously described in unrelated Caucasian patients with DDD4, an abnormally dark skin coloring condition

(hyperpigmentation) (26,34,35). Mutations in *POGLUT1* caused an ∼50% weaker POGLU1 expression in a patient's lesional skin compared to controls by immunohistologic staining for POGLUT1 (35). In addition, a missense mutation in *POGLUT1* was identified in patients with muscular dystrophy. Muscles from patients demonstrated decreased Notch signaling, dramatic reduction in satellite cell pool and a muscle-specific α-dystroglycan hypoglycosylation not present in patients' fibroblasts, suggesting a Notch-dependent pathomechanism for this novel form of muscular dystrophy (33). Mutations in *PSENEN* are also identified in DDD patients (24). Evidence has suggested the association between decreased Notch activity and *POFUT1* mutations (36). The finding of POGLUT1 mutations in patients with HS–DDD syndrome indicates that aberrant Notch signaling is involved in both HS and DDD pathogenesis. Notably, mutations in POGLUT1 and NCSTN are linked to dysregulation of Notch signaling that might also contribute to small vessel disease, as well as vascular cognitive impairment (37), forming the basis for developing new therapies to control neurodegeneration in Alzheimer's patients.

An increasing amount of evidence suggests the presence of systemic inflammation associated with HS, and bacteria are likely a second driver of inflammation. Our findings demonstrate that overexpression of PSEN1-WT or PSEN1-P242LfsX11 altered four or five cytokine and chemokine expression profiles in PMA-differentiated macrophages. Overexpression of presenilin WT exerts a dominant negative effect when expressed at high levels (38). The truncated PS1 protein resulting from PSEN1-P242LfsX11 led to higher TNFα expression than PS1-WT and four different cytokine or chemokine gene expressions. The mechanisms are unclear, and it might be related to noted different structures as the PSEN1-P242LfsX11 mutation resulted in a truncated PS1 protein that did not affect γ-secretase activity as much as the PS1-WT. The increased expression of proinflammatory *TNFα* and the decreased expression of *LIF*, *IL12B*, *CSF2*, *BMP2* and other genes associated with the overexpression of PSEN1-P242LfsX11 may promote inflammatory processes, impair the activation/maintenance of immune cells and reduce hair follicle regeneration. Of concordantly under-expressed genes, *LIF* and *CSF2* are essential for the proliferation and differentiation of hematopoietic progenitor cells into granulocytes and macrophages (39,40); IL12 is critical for the activation and maintenance of immune responses (41); and *BMP2* regulates stem cell activation in the process of hair follicle regeneration in the dermis (42). Finally, the altered cytokine and chemokine gene expression profile may contribute to the pathogenesis in HS patients with the PSEN1-P242LfsX11 mutation. This indicates that overexpression of PSEN1-P242LfsX11 not only increased but also prolonged TNFα production in response to LPS stimulation.

Elevated expression of TNFα has been identified in skin lesions of patients with HS, suggesting a key role for HS pathogenesis (43). The role of Th17 cells and enhanced expression of IL-17 and IL-1β have been explored (44). The analysis on lesional HS biopsy samples showed a clustering of all TH1/TH17-associated cytokines (IL-17, interferon γ, IL-12, IL-23, IL-32, IL-1β, TNFα) around overall lesional inflammation, highlighting the importance of the TH1/TH17 cytokines in HS pathogenesis (44). Macrophages are enriched in HS infiltrates and release numerous pro-inflammatory cytokines such as IL-23 and IL-1β and TNFα, exacerbating the inflammation and contributing to the pathogenesis of HS. Dysregulated matrix metalloproteases 2 and 9 overexpression, toll-like receptor upregulation, impaired Notch signaling, NLRP3 inflammasome upregulation and dysregulated keratinocyte function have also been shown to be associated

with HS (45). Obesity and smoking contribute to macrophage dysfunction and correlate with HS incidence (45). Moderate to severe HS patients who received a TNFα inhibitor infliximab showed a 50% decline from the baseline HS Severity Index score (46). In addition, patients who were administered a TNFα inhibitor adalimumab by injection every 2 weeks experienced a decrease in Sartorius score after 6 weeks (47). It was reported that two Phase III clinical trials of adalimumab for treatment of HS patients compared to placebo resulted in significantly higher clinical response rates in both trials at 12 weeks (48). Our findings provide mechanistic support for these clinical trial data. HS patients with a *PSEN1* mutation may benefit greatly from TNFα-inhibiting agents such as infliximab, adalimumab, rituximab and ustekinumab, in particular after anti-inflammatory regimens fail to control the disease process. In contrast, administration of the TNFα modulator etanercept in AD patients demonstrated no apparent effect on cognitive functioning though TNFα has been implicated in the pathogenesis of AD (49,50). Human-induced pluripotent stem cell (iPSC) can modulate inflammatory responses in animal models of atherosclerosis (51), inflammatory bowel disease (52), periodontal disease (53) and so on, suggesting that iPSCs may be a new optional treatment for HS. In addition, it is of importance to explore the functional effects of iPSCs in HS inflammatory pathogenesis and compare the functional effects of HS-linked mutations with AD-linked mutations in iPSC-derived neurons, astrocytes and microglia.

In summary, we have provided functional *in silico* analyses of HS-linked genetic mutations and also found that the PSEN1-P242LfsX11 mutation mediates cytokine and chemokine expression in LPS-stimulated macrophages. Further functional studies are warranted to understand the role of HS-linked genetic mutations in the disease etiology and pathogenesis.

Materials and Methods

Identification of reported HS-linked genetic mutations and functional *in silico* analysis

We performed an NCBI-Pubmed search using the terms 'hidradenitis suppurativa' and 'mutation', and all reported studies were retrieved (13,9,14–16,5,17–21,10,22–26,6).

Functional *in silico* analysis of the HS-linked mutations was carried out by using the following programs: SWISS-MODEL (https://swissmodel.expasy.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), PROVEAN (http://provean.jcvi.org/index.php), SNP&GO (http://snps.biofold.org/snps-and-go/snps-and-go.html), TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), NetGlycate (http://www.cbs.dtu.dk/services/NetGlycate/), UniProt (https://www.uniprot.org/uniprot/) and SUMOplot Analysis Program (http://www.abgent.com/sumoplot) to predict the mutational impact on the biological function of NCSTN, PSEN1, PSENEN and POGLUT1, as well as on 3D structure, protein stability, TM domain, ligand binding, glycation, phosphorylation, ubiquitination, sumoylation and glycosylation.

Cloning, THP-1 and PMA-differentiated macrophages cell culture

We obtained PSEN1-WT cDNA by using RT-PCR amplification of human brain mRNA and *PSEN1-P242LfsX11* cDNA by site-directed mutagenesis (QuikChange® Site-Directed Mutagenesis Kit, Stratagene) and subcloned PSEN1-WT or PSEN1-P242LfsX11 cDNA into pcDNA3.1 vector (Life Technologies). The constructs were sequenced on an ABI 3730 DNA analyzer (Thermo Fisher Scientific, Inc) and validated by comparison with the *PSEN1* NCBI-GenBank sequence (accession number NM_000021).

THP-1 cells (TIB-202, ATCC) were cultured using the standard ATCC culture condition. THP-1 differentiation into macrophages was achieved by addition of 100 nM PMA for 72 h in culture media. THP-1 cells and PMA-differentiated macrophages were transfected with PSEN1-WT-, PSEN1-P242LfsX11- pcDNA3.1 or control pcDNA3.1 vector, respectively, using the Effectene® Transfection Reagent (Qiagen, CA). Cells were harvested 24 h after transfection. Total RNA was extracted using RNeasy® Mini Kit, and cDNA was synthesized using the RT² First Strand Kit (Qiagen). The expression of PSEN1-WT or PSEN1-P242LfsX11 in THP-1 cells was validated using real-time RT-PCR (Qiagen) on a CFX96 Real-time PCR analyzer (BIORAD). The level of overexpression of PSEN1-WT or PSEN1-P242LfsX11 in PMA-differentiated macrophages was detected by western blotting with an anti-PS1 N-terminal antibody. PS1 expression level was quantified using Image Studio Version 5.

LPS stimulation and Human Cytokines & Chemokines RT² Profiler PCR Array

PMA-differentiated macrophages were treated with LPS (100 ng/mL) at 24 h after transfection. At 6 h after LPS treatment, the expression of 84 key secreted proteins central to the immune response in PMA-differentiated macrophages was examined and analyzed using the Human Cytokines & Chemokines RT² Profiler PCR Array (Qiagen). Differentially expressed genes were defined as those that were over- or under-expressed, exceeding 2-fold as compared to control in repeated experiments with a statistically significant value ($P < 0.05$). To validate increased TNFα expression, time-dependent TNFα levels in the culture supernatant of THP-1 cells or PMA-differentiated macrophages at 0, 4, 6, 8 and 24 h after LPS exposure were analyzed by ELISA using the Human TNFα Single-Analytic ELISA Kit (Qiagen).

Conflict of Interest statement. None declared.

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