移植した。コントロールとして無処置表皮細胞および無処置線維芽細胞の皮膚とした。

3） VII型コラーゲンの基底膜への供給の検討

無免疫動物に人工皮膚移植後、3週、6週、9週で構築された皮膚を生検し、VII型コラーゲンのモノクローナル抗体であるLH7.2を用いて蛍光抗体法にて検討した。

4） Anchoring fibrils形成の検討

構築された皮膚を生検し、電顕学的に基底膜でのanchoring fibrilsの形成を検討した。

C. 研究結果

1） レトロウィルスを用いてHallopeau-Siemens劣性栄養障害型表皮水疱症患者細胞への正常VII型コラーゲン遺伝子の導入

全くVII型コラーゲンを発現していないHallopeau-Siemens劣性栄養障害型表皮水疱症患者の表皮細胞と線維芽細胞に、レトロウィルスを用いて正常なVII型コラーゲン遺伝子を導入した。導入したそれぞれの細胞は、ウェスタンブロットにて、同等量のVII型コラーゲンの発現を確認した。

2） VII型コラーゲンの基底膜への供給の検討

作成した人工皮膚を無免疫動物に移植し、構築された皮膚を生検し、VII型コラーゲンの発現を蛍光抗体にて検討した所，3週、6週、9週いずれの週においても、無処置表皮細胞と遺伝子導入線維芽細胞を組み合わせた人工皮膚移植による皮膚が最も基底膜に強くVII型コラーゲンが強く沈着していることが認められた（図1A）。さらに、基底膜に沈着したVII型コラーゲンを数値化すると、有意に無処置表皮細胞と遺伝子導入線維芽細胞の人工皮膚移植において、VII型コラーゲンの発現が高いことが確認された（図1B）。

3） Anchoring fibrils形成の確認

人工皮膚の移植により構築された皮膚において、anchoring fibrilsの形成を確認するために、電顕を施行した所、遺伝子導入表皮細胞の皮膚においても、遺伝子導入線維芽細胞の皮膚においても、anchoring fibrilsの形成を確認した。また、無処置の皮膚においてはanchoring fibrilsが形成されなかった（図2）

D. 考察

生体のヒトの皮膚においては、表皮細胞の方が線維芽細胞よりもVII型コラーゲンの発現量が多く、基底膜に存在するVII型コラーゲンの多くは、表皮細胞から供給されていることは知られている。そこで、線維芽細胞が表皮細胞と同じVII型コラーゲンの量を発現することが出来れば、どちらの細胞がより効果的に基底膜にVII型コラーゲンを供給できるかを比較検討できることになる。そこで、今回、レトロウィルスを用いて表皮細胞および線維芽細胞にVII型コラーゲン遺伝子の導入により、同等の遺伝子を導入することを可能とし、同等のVII型コラーゲン蛋白を発現させた。その細胞を用いて、人工皮膚を作成、基底膜に沈着したVII型コラーゲン蛋白の発現を解析した。

今回は，実際の遺伝子治療を想定して，劣性栄養障害型表皮水疱症患者から得られた表皮細胞と線維芽細胞に我々が確立した

レトロウイスル法にて，VII型コラーゲンの遺伝子を導入した。劣性栄養障害型は全くVII型コラーゲンの発現がない重症型のHallopeau-Siemens 型と少しは発現のある非Hallopeau-Siemens型に分類されるが，今回はHallopeau-Siemens 型患者の細胞を使用した。その細胞を用いて、遺伝子導入を行い、人工皮膚を作成したところ、無処置表皮細胞と遺伝子導入線維芽細胞を組み合わせた人工皮膚が最も基底膜に強くVII型コラーゲンが強く沈着していることが認められた（図１Ａ)。よって、線維芽細胞に遺伝子を導入した方がより基底膜にVII型コラーゲンを供給出来ることが判明した。また、電子顕微鏡学的所見においても、Anchoring fibrilsの形成を確認し、正常の皮膚と同等の基底膜を作成できることが可能であることが示唆された。

このように、培養が容易である線維芽細胞を標的細胞として遺伝子治療に用いることが、より効果的な治療法に成り得ることが考えられる。

E. 結論

今回我々は、線維芽細胞が表皮細胞より基底膜にVII型コラーゲンを供給することが可能であることを示した。よって、栄養障害型表皮水疱症の遺伝子治療における標的細胞としては，表皮細胞よりも線維芽細胞の方が有力候補となることが示唆された。

F. 健康危険情報

特になし

G. 研究発表

１）Goto M, Sawamura D, Ito K, Abe M, Nishie W, Sakai K, Shibaki A, Akiyama M, Shimizu H. Fibroblasts show more potential target cells than keratinocytes for *COL7A1* gene therapy of dystrophic epidermolysis bullosa. J Invest Dermatol, Apr;126(4):766-72, 2006.

H. 知的財産の出願・登録状況

特になし。

図とその説明

図１（Ａ）　遺伝子導入細胞を用いた人工皮膚の移植による経時的蛍光抗体所見。Kera は遺伝子導入表皮細胞と無処置線維芽細胞の人工皮膚を移植、Fib は無処置表皮細胞と遺伝子導入線維芽細胞の人工皮膚を移植、Cont はいずれの細胞も遺伝子を導入しない人工皮膚の移植。

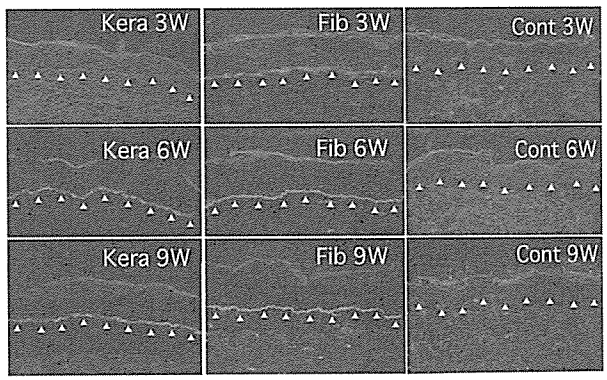


図２（Ｂ）　遺伝子導入細胞を用いた人工皮膚の移植による蛍光抗体所見の基底膜に沈着した VII 型コラーゲンの量。Ｋは表皮細胞にのみ遺伝子を導入した人工皮膚から構築された皮膚、Ｆは線維芽細胞にのみ遺伝子を導入した人工皮膚、Ｃは遺伝子を全く導入しない人工皮膚。

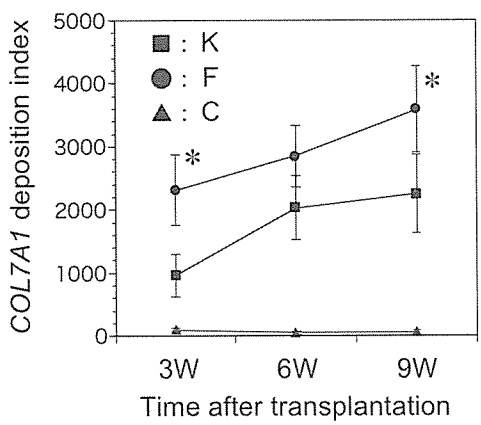
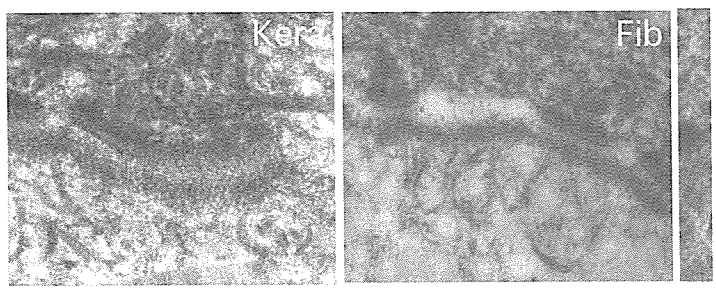


図２　人工皮膚の移植による電子顕微鏡学的所見。Kera は遺伝子導入表皮細胞と無処置線維芽細胞の人工皮膚を移植した基底膜の電顕像、Fib は無処置表皮細胞と遺伝子導入線維芽細胞の人工皮膚を移植した基底膜の電顕像、Cont はいずれの細胞も遺伝子を導入しない人工皮膚の移植による基底膜の電顕像。



厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書
毛包上皮の幹細胞を標的とした遺伝子導入、毛包再構成システムに関する研究

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研究要旨

毛包上皮への遺伝子導入は、皮膚疾患の遺伝子治療のみならず、全身性疾患の遺伝子治療の方法としても、非常に魅力的なアプローチ法である。今回、我々は、遺伝子治療の効果を高めるために、毛包上皮の幹細胞を標的とした遺伝子導入法を試みた。毛包上皮における持続的な、安定した導入遺伝子の発現を実現するため、レポーター遺伝子を含むレトロウイルスベクターを培養毛包幹細胞に導入した。さらに、それらの細胞を毛乳頭細胞と混合し、免疫不全マウスのファイブに移植した。その結果、我々は、導入遺伝子を有する毛包付属器の再構成に成功した。再構成皮膚において、毛包上皮、皮脂腺、毛包間表皮を含む全ての上皮成分に導入遺伝子の発現が確認された。さらに、導入遺伝子の発現は、移植後、６ヶ月を経過しても継続して認められた。この毛包上皮の幹細胞を標的とした遺伝子導入と毛包の再構成システムは、信頼性の高い遺伝子機能解析や遺伝子治療に、広く応用可能な方法と考えられる。

A. 目的

毛包上皮の幹細胞は、皮膚疾患の場合だけでなく、全身性の疾患に対しても、その遺伝子治療の標的として理想的である。その理由としては、毛包は、身体表面に位置しており、比較的安全に遺伝子の導入が行えること、毛包上皮は、その毛髪の産生能からも予想される通り、非常に大きな蛋白産生能力を有していること等が挙げられる。本研究では、この遺伝子治療の標的としての優れた資質を有している毛包上皮を標的として、遺伝子導入を行うシステムを構築することを目的とした。

単に、毛包上皮に遺伝子を導入したのでは、導入遺伝子の発現は、毛包上皮のターンオーバーによって、すぐに失われてしまう。そこで、我々は、毛包の上皮細胞の幹細胞を生体から取り出し、選択的に幹細胞に遺伝子を導入し、その導入遺伝子を有する幹細胞から、毛包を再構成することを試みた。この方法によって、導入された遺伝子は、安定して、持続的に発現する事になる。

B. 研究方法

１）ラット毛包のバルジ由来の毛包上皮の幹細胞の培養系の確立

ラット髭毛包のバルジ部位より、microdissection 法により、毛包上皮の幹細胞と考えられる細胞を採取し、培養した。それらの培養細胞について、細胞動態的検討を加えた。

２）培養ラットバルジ幹細胞における遺伝子発現の検討

培養ラットバルジ由来毛包上皮の幹細胞から、mRNA を抽出し、Agilent 社製の Rat oligo microarray を用いて、幹細胞と考えられる培養細胞の遺伝子発現パターンを検討した。

３）ラット毛包のバルジ由来幹細胞に対す

る遺伝子導入、毛包再構成実験

上記にて、得られたラット毛包上皮幹細胞に対して、GFP、あるいは、LacZ の reporter gene をコードするレトロウイルスベクターを遺伝子導入した。それら、導入遺伝子を有する毛包上皮幹細胞を、未処理のラット毛乳頭細胞を混合して、SCID マウスの背部に移植した。その後、経時的に、移植部皮膚を採取し、組織学的に観察すると同時に、導入遺伝子の発現状況を検討した。

C. 研究結果

1） ラット毛包のバルジ由来の毛包上皮の幹細胞の培養系の確立

ラット髭毛包のバルジ部位より、microdissection 法により得られた細胞からの培養系は、それ以外の外毛根鞘由来の細胞、表皮由来の細胞と比べて、特に増殖能が優れており、有為に、colony forming unit 数が多かった。これらのデータは、このバルジ由来の細胞集団が、毛包上皮の幹細胞に富む集団であることを裏付けていた。

2）培養ラットバルジ幹細胞における遺伝子発現の検討

培養ラットバルジ由来毛包上皮の幹細胞からの mRNA を、Agilent 社製の Rat oligo microarray を用いて、検討した結果、procollagen type II, alpha I, insulin-like growth factor-binding protein, fibroblast growth factor, matrix metalloproteinase 3 等の遺伝子の発現が上昇していた。

3）ラット毛包のバルジ由来幹細胞に対する遺伝子導入、毛包再構成

遺伝子を導入された毛包上皮幹細胞から、移植後、3 週間で、毛包付属器の再構成が観察された。再構成毛包は、ほぼ、正常な形態を有し（図 1）、それらの上皮成分には、導入された reporter gene の発現が確認された。その後、経時的に、reporter gene の発現を観察した結果、再生毛包上皮には、移植後、6 ヶ月後にも、導入遺伝子の発現が確認された。

D. 考察

今回、我々が分離、培養した毛包上皮の幹細胞集団において発現が上昇していた遺伝子は、procollagen type II, alpha I, insulin-like growth factor-binding protein, fibroblast growth factor, matrix metalloproteinase 3, ornithine decarboxylase 1 等であった。これらの中で、procollagen type II, alpha I, insulin-like growth factor-binding protein, fibroblast growth factor は、以前から幹細胞において発現の上昇が報告されている遺伝子であり、本研究で、我々が分離、培養した上皮細胞集団が、毛包上皮の幹細胞に富む集団であったことを裏付けるデータであった。

本研究の結果から、毛包上皮の幹細胞から再構成された皮膚においては、毛包付属器のみならず、毛包周囲の毛包間表皮にも導入遺伝子の発現が見られた。また、導入された遺伝子について、細胞移植後半年以上経過しても、その発現が認められた。このように長期に渡り、導入遺伝子の発現が見られた理由は、本研究での方法で、高率に幹細胞に遺伝子が導入されていたためと考えられる。今後、バルジ部の毛包上皮細胞を採取後、幹細胞マーカーを用いて、FACS を行い、さらに、採取細胞中の幹細胞の密度を高くすれば、幹細胞への遺伝子導入効率を上げることができると考えられた。

E. 結論

今回の研究から、バルジ部の毛包上皮の幹細胞を採取し、培養系を確立し、遺伝子を導入後、毛包付属器を再構成した場合、その上皮組織からは、導入遺伝子の発現は、移植後、６ヶ月を経過しても継続して認められることが確認された。この毛包上皮の幹細胞を標的とした遺伝子導入と毛包の再構成システムは、信頼性の高い遺伝子機能解析や遺伝子治療に、広く応用可能な方法と考えられる。

F. 健康危険情報

特になし

G. 研究発表

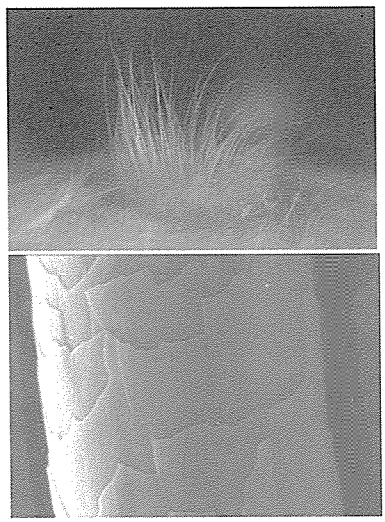
論文１）Sugiyama-Nakagiri Y, Akiyama M, Shimizu H. Hair follicle stem cell-targeted gene transfer and reconstitution system. Gene Ther 13, 732-737, 2006.

H. 知的財産の出願・登録状況

特になし。

図とその説明

図１、上：マウス背部に、再構成された毛包。下：hair shaft の走査電顕像。正常ラット髭に近い形態を呈していた。



厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書
造血幹細胞由来表皮細胞の遊走機序に関する研究

分担研究者　阿部理一郎　北海道大学・北海道大学病院皮膚科　講師

研究要旨

これまで再生医学領域の研究により、種々の体細胞成分が骨髄幹細胞から分化可能であることが明らかとなった。骨髄由来幹細胞が表皮細胞に分化しうることも予想されている。よって、皮膚基底膜構造タンパク欠損疾患である表皮水疱症に対して、同種骨髄移植によりドナー骨髄由来表皮細胞を分化させ、欠損構造タンパクを産生させることで根治させることを目的として検討を行った。本年度の研究で、これまでの検討で確認した骨髄由来表皮細胞の割合を増加させるために、骨髄由来表皮細胞特異的遊走因子を同定した。さらに実際に、この因子が、生体内における骨髄由来表皮細胞の割合を増加させることも明らかにした。加えて、骨髄由来表皮細胞増加の創傷治癒への影響も検討した。

A. 目的

これまで再生医学領域の研究により、種々の体細胞成分が骨髄幹細胞から分化可能であることが明らかとなった。骨髄幹細胞から表皮細胞への分化の可能性も示唆されているが、なお確定はえられていない。一方先天的酵素欠損症（Hunter 症候群など）は同種骨髄移植によりドナー由来細胞から欠損酵素を供給する治療が試みられ臨床的にも有用であることが証明されている。しかしながら同種骨髄移植を用いた、構造タンパク欠損症に対する治療法の研究はなされておらず、定説もない。そこで、国の稀少難治性疾患に指定されている皮膚基底膜構造タンパク欠損疾患（基底膜構造タンパク欠損により、微細な外力でびらん、潰瘍をきたす重症難知性疾患）である表皮水疱症に対して、同種骨髄移植により構造タンパク欠損症を根治させることを目的として、検討を行った。

B. 研究方法

①骨髄由来表皮細胞特異的遊走因子の同定：

皮膚再生の現象の場である、創傷治癒過程の皮膚における細胞遊走因子のスクリーニングを行い、候補となる遊走因子を数種同定した。同定したそれぞれの遊走因子において、骨髄幹細胞への遊走惹起能を in vitro で検討した。さらに皮膚創傷部位への骨髄由来表皮細胞遊走への、それぞれの遊走因子の影響を検討した。

②骨髄由来表皮細胞増加の創傷治癒への影響の検討：

骨髄由来表皮細胞増加の、皮膚再生過程に対する寄与を解析するため、上記同定遊走因子を皮膚創傷部位に投与することによる、創傷治癒への影響を検討した。

C. 研究結果

①骨髄由来表皮細胞特異的遊走因子の同定：

現在までの検討で、皮膚創傷部位に特異的に発現し、かつ骨髄幹細胞の遊走を特異的に誘導する、骨髄由来表皮細胞特異的遊走因子を同定した。この同定因子は生体内

においても骨髄由来表皮細胞の数を増加させた。

②骨髄由来表皮細胞増加の創傷治癒への影響の検討：

上記同定遊走因子を皮膚創傷部位に投与することにより、創傷治癒が有意に促進した。

D. 考察

本年度の研究で、骨髄由来表皮細胞の割合を増加させるために、骨髄由来表皮細胞特異的遊走因子を同定した。さらに実際に、この因子が、生体内における骨髄由来表皮細胞の割合を増加させることも明らかにした。加えて、骨髄由来表皮細胞増加の創傷治癒への影響も検討し、創処治癒を促進させることを明らかにした。

今後は現在までの成果を、臨床応用に近づけるべく、表皮構造タンパク欠損マウス（VII型コラーゲンノックアウトマウス）への正常マウスから骨髄移植療法を行う。

E. 結論

今回の研究で、骨髄由来表皮細胞特異的遊走因子を同定した。加えて、骨髄由来表皮細胞増加の創傷治癒への影響も検討し、創処治癒を促進させることを明らかにした。

F. 健康危険情報

特になし

G. 研究発表

研究成果の刊行に関する一覧表にまとめた

H. 知的財産の出願・登録状況

特になし

Ⅳ　研究成果の刊行に関する一覧表

IV　研究成果の刊行に関する一覧表

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Ⅴ　研究成果の刊行物・別刷

ORIGINAL ARTICLE

Compound Heterozygous Mutations Including a *De Novo* Missense Mutation in *ABCA12* Led to a Case of Harlequin Ichthyosis with Moderate Clinical Severity

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Harlequin ichthyosis (HI) is one of the most devastating genodermatoses. Recently, *ABCA12* mutations were identified as the cause of HI. A newborn Japanese male demonstrated the typical features of HI. The patient was treated with oral etretinate and his general condition has been good (now aged 1.5 years). This patient with moderate clinical severity was compound heterozygous for a novel *de novo* missense mutation 1160G>A (S387N) in exon 10 and a maternal deletion mutation 4158_4160delTAC (T1387del) in exon 28 of *ABCA12*. T1387del was a deletion of a highly conserved threonine residue within the first adenosine 5′ triphosphate-binding domain and is thought to seriously affect the function of the ABCA12 protein. Conversely, the residue 387 is located outside the known active sites of ABCA12 and S387N is predicted not to lead to a serious functional deficiency in ABCA12. Electron microscopy revealed abnormal lamellar granules in the granular layer cells and a moderate number of lipid vacuoles in the cornified cells. Disturbed glucosylceramide transport was confirmed in the cultured keratinocytes from the patient. No *de novo* mutation in *ABCA12* has yet been reported either in HI or lamellar ichthyosis. The present case suggested that a *de novo ABCA12* mutation might underlie HI.

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INTRODUCTION

Harlequin ichthyosis (HI) (MIM no. 242500) is a severe and often fatal congenital ichthyosis with an autosomal recessive inheritance pattern (Williams and Elias, 1987; Akiyama, 1999; Judge *et al.*, 2004).

In 2005, *ABCA12* mutations were identified in HI families (Akiyama *et al.*, 2005; Kelsell *et al.*, 2005). The pathomechanisms of HI became known when functional defects in the lipid transporter ABCA12 were shown to cause abnormal lipid transport via lamellar granules (LGs) in the keratinocytes, resulting in malformation of the patient's intercellular lipid layers of the stratum corneum (Akiyama *et al.*, 2005). However, genotype/phenotype correlations in HI cases with *ABCA12* mutations have yet to be fully elucidated (Akiyama, in press a).

In the study of this case, we have found a compound heterozygous *ABCA12* combination of mutations, a novel *de novo* missense mutation, S387N, in exon 10 and a maternal deletion mutation T1387del in exon 28 in a newborn HI baby. In the majority of HI patients, deletion or truncation mutations seriously affecting ABCA12 function were found. However, in the present case, one mutation was a missense mutation located in the cytoplasmic region of ABCA12 polypeptide, not in the transmembrane domain or the adenosine 5′ triphosphate (ATP)-binding cassettes. The nature and site of mutations might be the key to the relatively moderate phenotype of this HI patient.

In addition, the novel mutation S387N was the first reported *de novo* mutation in *ABCA12*. Our case suggested the possibility that *de novo* mutations can cause the occurrence of HI in non-consanguineous families.

RESULTS

Clinico-pathological features of the patient

The patient was a newborn Japanese male. He was born at 33 weeks and 3 days pregnancy by premature, vaginal delivery (body weight 1,876 × *g*). He was the first child of non-consanguineous healthy parents. There was no family history of congenital ichthyosis. At birth, the patient had presented with severe hyperkeratosis over his entire body, severe ectropion, eclabium, and malformed pinnae (Figure 1). The patient's clinical features at birth were characteristic of typical HI. He was admitted into a neonatal intensive care unit and had oral etretinate treatment (1 mg/kg/day). At this time, light microscopy of the lesional skin sample from the patient's trunk showed marked hyperkeratosis with only a

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Abbreviations: ATP, adenosine 5′ triphosphate; HI, harlequin ichthyosis; LG, lamellar granule

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Figure 1. **The patient showed a typical clinical phenotype of HI in the neonatal period.** The entire body surface was covered with thick plate-like scales and fissures.

small number of parakeratotic cells. At the age of 1 year, the patient showed dark brownish, thick scales over the entire body surface including the face, palms, and the soles.

ABCA12 mutation analysis

Mutation analysis of the 53 exons including the intron–exon boundaries of the entire *ABCA12* gene revealed a 1160G>A transition in exon 10 and one previously reported deletion mutation 4158_4160delTAC in exon 28 in each allele in a compound heterozygous fashion in the patient (sequence according to Lefèvre *et al.* (2003)) (GenBank accession NM 173076) (Figure 2). The mutation 1160G>A transition was a novel missense mutation that changed a serine residue of codon 387 to an asparagine residue (S387N). This missense mutation S387N was not found in either in the parent's (father or mother), although the patient's mother was heterozygous for the deletion mutation 4158_4160delTAC (Figure 2). Thus, the missense mutation S387N was thought to be a *de novo* mutation and the deletion mutation was a maternal mutation. Paternity testing by microsatellite marker analysis proved the father was the patient's true genetic father (data not shown). These mutations were not found in 200 normal, unrelated Japanese alleles (100 normal unrelated Japanese individuals) by sequence analysis, and were unlikely to be a polymorphism (data not shown). The deletion mutation 4158_4160delTAC led to an in-frame deletion of a highly conserved threonine residue at codon 1387 (T1387del) within the first ATP-binding

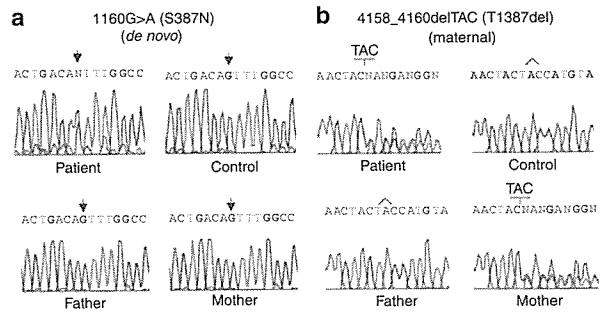

Figure 2. **Compound heterozygous mutations of ABCA12 in the patient.** (**a**) Direct sequencing revealed a heterozygous 1160G>A transition (a missense mutation S387N) in exon 10 of *ABCA12* of the patient, but not in his parents or normal control samples. (**b**) A heterozygous deletion mutation 4158_4160delTAC (T1387del) was found in exon 28 of *ABCA12* of the patient and his mother, but not in his father or in normal controls.

domain of ABCA12. Thus, the deletion mutation is thought to seriously affect either the function or specific critical structures of the ABCA12 protein (Akiyama *et al.*, 2005).

Ultrastructure of the patient's skin

Electron microscopy revealed that, in the keratinocyte cytoplasm of the granular layer, no normal LGs were apparent. Lipid droplets, vacuoles, and multivesiculated bodies were seen in keratinocytes in the keratinized and granular cell layers (Figure 3a and b). The number of abnormal lipid droplets in the keratinized stratum corneum cells was smaller than that in skin samples from HI patients harboring the homozygous splice acceptor site mutation IVS23-2A>G of *ABCA12* (Akiyama *et al.*, 2005) (Figure 3c), although the number of lipid vacuoles was larger than those in lamellar ichthyosis patients with *ABCA12* mutations (data not shown). Cornified cell envelope formation was normal in the granular and cornified layer cells (Figure 3h).

Distribution of ABCA12 and glucosylceramide in patient's skin

Immunofluorescence studies revealed that ABCA12 was positive in the upper epidermal layers, mainly in the granular layers, of normal human skin (Figure 4c). In the epidermis of the present patient, reduced ABCA12 immunostaining was seen in the upper epidermis (Figure 4a), compared with seriously reduced immunolabeling in epidermal keratinocytes from the HI patient harboring a homozygous splice acceptor site mutation IVS23-2A>G (Figure 4b) (Akiyama *et al.*, 2005). These findings confirmed that the present patient expresses a significant amount of mutated ABCA12 protein in his epidermis.

Immunofluorescent staining showed that glucosylceramide, a major lipid component of LGs (Vielhaber *et al.*, 2001; Ishida-Yamamoto *et al.*, 2004) and an essential component of the epidermal permeability barrier (Holleran *et al.*, 1993), was sparsely distributed in the patient's upper epidermis (Figure 4a), compared with a restricted, intense distribution in the granular layers of normal skin (Figure 4c). The condensed glucosylceramide staining was not seen in the patient's granular layers (Figure 4a).

Abnormal glucosylceramide transport in patient's cultured keratinocytes

Culture of the patient's keratinocytes under high-Ca^{2+} conditions (2.0 mM) induced a large number of cells to express condensed glucosylceramide staining around the nuclei, and this glucosylceramide failed to become localized to the periphery of the keratinocyte cytoplasm (Figure 5a). Culture of normal human keratinocytes in high-Ca^{2+} conditions exhibited a more diffuse glucosylceramide staining throughout the cytoplasm (Figure 5b).

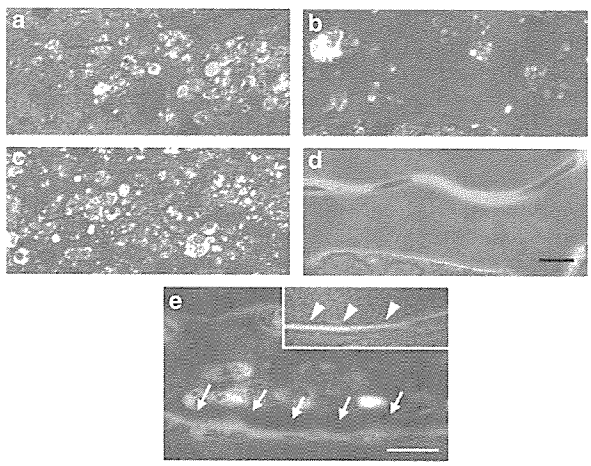

Figure 3. Abnormal LGs in the granular layer cells and accumulation of lipid droplets in the stratum corneum cells of the patient's epidermis. (**a**) Abnormal LGs in the granular layer cells of the patient. (**b**) Abnormal lipid droplets accumulated in the cornified cells in the patient's epidermis, although the numbers and amounts of lipid droplets was smaller than that seen in an HI patient harboring a homozygous splice acceptor site mutation IVS23-2A>G of *ABCA12* (**c**). (**d**) No lipid droplets were observed in control normal human stratum corneum cells. (**a**, **b**) the present patient; (**c**) control typical HI patient reported previously (Akiyama *et al.*, 2005); (**d**) normal control human skin. (**e**) Cornified cell envelope (arrows) with normal thickness was seen in the cornified layer cells in the present HI patient. Inset: the cornifed layer cell of the control lamellar ichthyosis patient with transglutaminase 1 gene mutations showed malformed, thin cornified cell envelope (arrowheads). Bars = 200 nm.

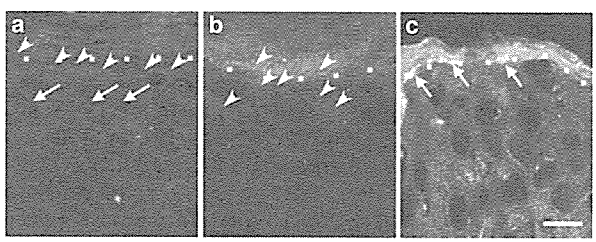

Figure 4. Abnormal ABCA12 immunostaining and disturbed distribution of glucosylceramide in the patient's epidermis. (**a**) In the patient's upper epidermis, weak ABCA12 immunostaining (red: arrows) was diffusely seen in the keratinocyte cytoplasm. Glucosylceramide labeling (green: arrowheads) was also diffusely observed in the keratinocytes of upper epidermis. (**b**) In the control HI patient carrying a homozygous splice acceptor site mutation IVS23-2A>G of *ABCA12*, ABCA12 immunolabeling (red) was very weak, although diffuse glucosylceramide immunolabeling (green: arrowheads) was seen in the keratinocyte cytoplasm of upper epidermis. (**c**) In normal control human epidermis, both ABCA12 (red) and glucosylceramide (green) labelings overlapped, resulting in a yellow color in the granular layer (arrows). Dotted line; granular layer–cornified layer interface. ABCA12, tetramethylrhodamine isothiocyanate (red); glucosylceramide, FITC (green); nuclear staining, TO-PRO (blue). Bar = 10 μm.

DISCUSSION

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families, encoding a highly conserved group of proteins involved in energy-dependent active transport (Higgins, 1992; Allikmets *et al.*, 1996; Dean *et al.*, 2001; Borst and Elferink, 2002) and, recently, this superfamily has been highlighted in the dermatology fields (Uitto, 2005). The ABCA subfamily is suggested to work in lipid transport (Weng *et al.*, 1999; Hayden *et al.*, 2000; Orso *et al.*, 2000; Schmitz and Langmann, 2001; Peelman *et al.*, 2003) and has received considerable attention (Klein *et al.*, 1999) because mutations in these genes have been implicated in several human genetic diseases (Allikmets *et al.*, 1997a, b; Brooks-Wilson *et al.*, 1999; Rust *et al.*, 1999; Oram, 2002). Keratinocyte LGs are known lipid-transporting organelles and LG contents are secreted into the intercellular space, forming an intercellular lipid layer between the granular layer cells and keratinized cells in the stratum corneum. Our previous study (Akiyama *et al.*, 2005) has clearly demonstrated that ABCA12 functions in the transport of endogenous lipid to the keratinocyte cell periphery via LGs.

The abnormal LGs in the granular layer keratinocytes and a lack of extracellular lipid lamellae reflect the defective lipid transport via LGs and the malformation of intercellular stratum corneum lipid layer in HI (Akiyama, in press a). In 2005, *ABCA12* mutations that seriously affect its function were shown to cause a loss of the skin lipid barrier, leading to HI (Akiyama *et al.*, 2005). In addition to HI with defective lipid layers in the stratum corneum, ichthyosis syndromes are also thought to share similar pathomechanisms (Akiyama, in press b). For example, Dorfman–Chanarin syndrome (neutral lipid storage disease) showed malformation of LGs and

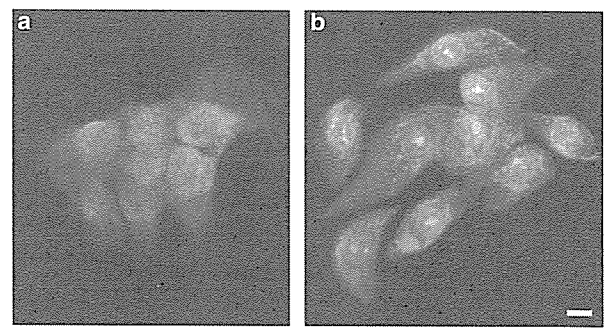

Figure 5. Altered glucosylceramide distribution in the patient's cultured keratinocytes. (**a**) In keratinocytes cultured from the patient, glucosylceramide labeling (green) was observed mainly in the perinuclear area of cytoplasm, suggesting disturbed glucosylceramide transport. (**b**) In normal cultured human keratinocytes, glucosylceramide (green) was seen diffusely in the cytoplasm to the cell periphery. Glucosylceramide, FITC (green); nuclear staining, propidium idodide (red). Bar = 10 μm.

defective lipid production in LGs caused by a deficiency in the CGI-58 protein that is thought to be involved in the pathogenesis of this form of ichthyosis (Akiyama *et al.*, 2003). In Sjögren–Larsson syndrome harboring fatty aldehyde dehydrogenase (FALDH) gene (*ALDH3A2*) mutations, defective LG formation was reported as one sign of a putative pathogenetic mechanism (Shibaki *et al.*, 2004). Recently, mutations in a new gene, *FLJ39501*, encoding a cytochrome *P*450, family 4, subfamily F, polypeptide 2 homolog of the leukotriene B4-ω-hydroxylase (CYP4F2) were reported to underlie lamellar ichthyosis cases linked to chromosome 19p12-q12 (Lefèvre *et al.*, 2006). These facts further support the idea that abnormal LG lipid contents and defective intercellular lipid are prevailing concepts of pathogenetic mechanisms in the ichthyoses.

In the present case based on the reported amino-acid sequence (Annilo *et al.*, 2002), the deletion mutation 4158_4160delTAC led to an in-frame deletion of a threonine residue at codon 1387 (T1387del) within the first ATP-binding domain of ABCA12 protein (Figure 6). This threonine residue is a highly conserved residue between diverse species and is thought to be important in the function of ABCA12 (Akiyama *et al.*, 2005). Indeed, a compound heterozygous patient with this deletion mutation and a separate *ABCA12* truncation mutation was previously reported as a typical HI newborn who died 15 days after birth (Akiyama *et al.*, 2005).

The novel *de novo* mutation S387N in the present patient is located outside all of the known ABCA12 active transporter sites, within the cytoplasmic domain at N-terminus of ABCA12 polypeptide (Figure 6). Both serine and asparagine are neutral amino acids with small side chains and this missense mutation would be expected to not significantly affect the conformation of ABCA12 molecule. Thus, one would predict the *de novo* missense mutation S387N not to lead to a serious ABCA12 functional loss. Considering the nature and site of the *ABCA12* mutations in this case, we suggest that a combination of the deletion mutation and the missense mutation leads to the current patient's HI phenotype with a moderate clinical severity.

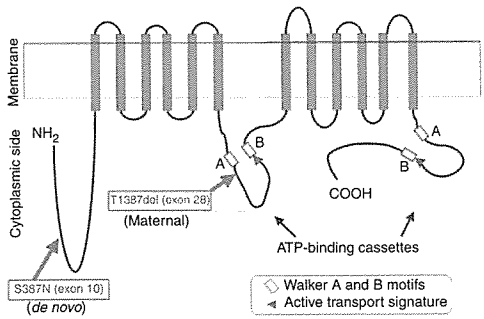

Figure 6. Schematic sequential arrangement of the domain structures of ABCA12 protein and the position of mutations in the present HI patient. Mutations in the present HI patient are marked by red arrows. Note that the *de novo* missense mutation S387N is located in the intracytoplasmic region between the N-terminus and the first transmembrane domain, not in any active sites, and the other, deletion mutation T1387del is within the first ATP-binding cassette, which is thought to be important for ABCA12 lipid transporter activity.

In type 2 lamellar ichthyosis, a relatively mild form of congenital ichthyosis, all five reported *ABCA12* mutations were missense mutations that resulted in only one amino-acid alteration (Lefèvre *et al.*, 2003). Conversely, in our previous study on HI families, no *ABCA12* missense mutations were identified and most of the defects led to severe truncation of ABCA12 peptide, affecting important nucleotide-binding fold domains and/or transmembrane domains (Akiyama *et al.*, 2005). The other, non-truncation mutations in HI were deletion mutations affecting highly conserved *ABCA12* sequences (Akiyama *et al.*, 2005). Thus, it was thought that only truncation or deletion mutations in conserved regions, which seriously affect the function of the ABCA12 transporter protein, can lead to the HI phenotype.

In an additional series of HI patients, most *ABCA12* mutations were homozygous truncation mutations (Kelsell *et al.*, 2005). Only one mutation in one HI patient was a missense mutation and the patient was heterozygous for a truncation mutation and the missense mutation.

Recently, the prognosis of newborns affected with HI has improved, owing to better targeted oral retinoid treatment. More than half of HI newborns including cases with a serious functional loss of ABCA12 survive beyond the perinatal period (Akiyama *et al.*, 2005; Akiyama, in press a). Thus, it is difficult to discuss genotype/phenotype correlations and quality of life prognosis for *ABCA12* mutations in HI.

Most families with HI or lamellar ichthyosis caused by *ABCA12* mutations were consanguineous and, in those families, causative *ABCA12* mutations were homozygous mutations inherited with an autosomal recessive trait. No *de novo* mutation has yet been reported in *ABCA12*, as far as we know (Lefèvre *et al.*, 2003; Akiyama *et al.*, 2005; Kelsell *et al.*, 2005). The present novel missense mutation S387N in our case was the first *de novo* mutation reported in *ABCA12*. The genetic information of whether the causative mutations are inherited or *de novo* is very important for genetic counseling in severe genetic disorders such as HI. Indeed, in the present family, the parents had requested a prenatal diagnosis of HI for their subsequent pregnancy. However, we have demonstrated that the missense mutation was a *de novo* mutation and that the patient's father was not a carrier of any *ABCA12* mutation. Thus, the family could be spared of the worry of an unnecessary HI prenatal diagnosis for the subsequent pregnancy almost, although we cannot completely exclude the possibility that the father has a mosaic mutation affecting his germ line. The present case suggested that, in non-consanguineous HI families, a *de novo ABCA12* mutation might be one source of mutations and underlines that the detection of causative *ABCA12* mutations is essential for the genetic counseling for HI.

MATERIALS AND METHODS

Mutation detection

Mutational analysis was performed in the affected baby and the parents. Briefly, genomic DNA isolated from peripheral blood was subjected to PCR amplification, followed by direct automated