B. 研究方法

難治性疾患克服研究事業の対象疾患は指定 130 疾患と多岐にわたり個々の疾患の克服研究を進める上で有用な研究試料も多種類にわたる。疾患の性質により、研究資源として収集される生体試料は多種類に及ぶ。しかし、基本は血液などの採取しやすい試料が大勢を占めると考えられる。そこで、まず血液から得られる試料の基本操作方法や自動システム、分離された細胞・血漿・DNA などの分離試料の分注凍結保存を行うに当たり、2 次元バーコードチューブ（2D チューブ）システムを導入し、電子制御によりヒューマンエラーをできる限り回避するシステム構築を行う。

疾患研究に必要とされる試料への研究者側の要請も研究の方向性により多岐にわたると考えられるが、収集研究班へのアンケート調査を行うことにより収集対象試料や研究の目的を調査する。

本事業の特徴の一つに研究試料の収集と提供の両面において倫理問題、権利問題などの社会的法的問題が存在しており、医学研究倫理の専門家を加え、患者試料の収集において倫理問題に適切に対応することとし、難病研究資源バンク事業において特異的な医学研究倫理の問題や、試料収集研究に当たる研究班の優先性の問題などの法的、社会的問題について検討を行う。

C. 研究結果

本年度事業としては、患者試料収集の具体的な見通しを立てることを目的として平成 21 年 9 月から 11 月に全収集研究班（53 班）を対象にアンケート調査を行い 34 班より回答を得（回答率 64.1%）た。結果の概略を図 1～5 に示す。集計の概略としては、1）目的とする収集試料の種類は様々だが DNA の割合は高い、2）半数が臨床検査時に試料収集を考えている、3）既存収集試料の IC 取得の状況は半数でとられている、4）3 分の 1 弱がゲノム解析を収集の目的としている、5）試料の利用に関しては半数が研究班内での試料利用と共同研究利用に限定する、といった内容であった。

寄託された試料の基本操作方法や自動システム、分離された細胞・血漿・DNA などの分離試料の分注凍結保存を確実にする電子システム構築については、図 6、7 に示すように難病資源バンク内でのみ接続可能な室内 LAN によって管理し担当者がログインしたうえで記録入力を行い、2D チューブ保管管理システムと連動してヒューマンエラーをできる限り回避するシステム構築を行った。

本研究事業においては、難治性疾患という機微に触れる疾患を対象とする事業であること、また試料提供者（患者）の権利保護や試料を利用した公平な研究機会確保を行うための倫理的・法的・権利調整問題、また診断を行う臨床医の権利保障など、多岐にわたる課題の克服が重要な課題として位置づけられる。

基盤研においては、これまでも生物資源研究事業の中でヒト細胞の受け入れや分譲などについて組織内部および外部の研究倫理審査委員会においてヒト試料の研究利用に対応してきた実績と理論的研究の経験を有している。基盤研において難病研究資源バンク専任の医学研究倫理審査委員会を組織し患者試料受け入れにかかわる倫理申請に特化して審査する体制を構築した。

図 1

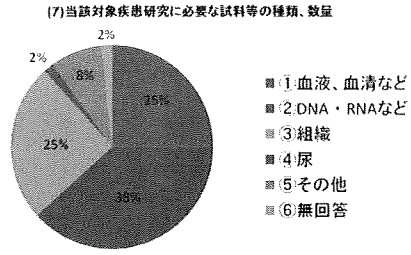


図 2

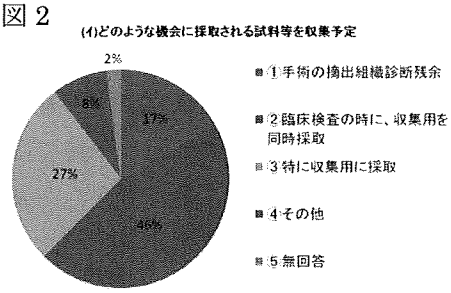
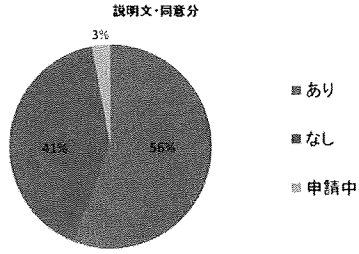


図 3



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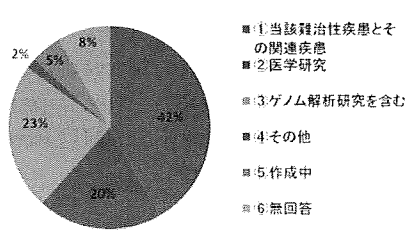


図 5

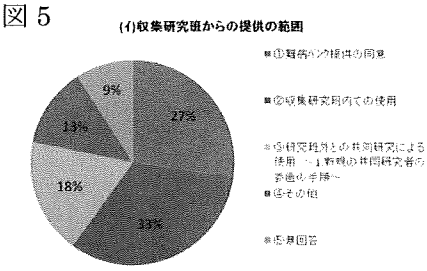


図 6

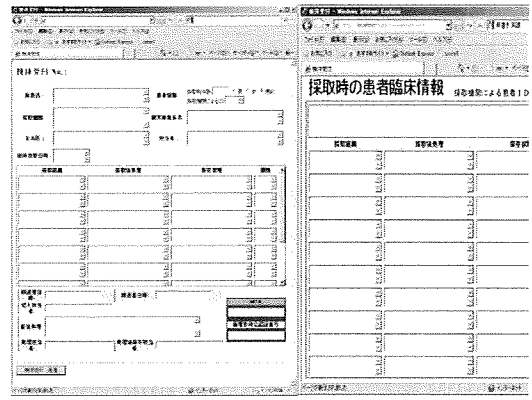
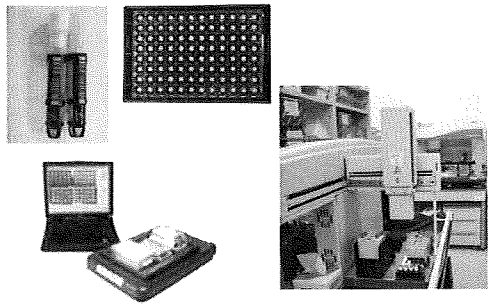


図 7

2Dバーコードシステムによる試料管理



D. 考案

本分担研究では「難治性疾患対策研究、生体試料等の収集に関する研究」により収集された患者試料を集中化して品質管理を行う「難病研究資源バンク」を創設し、統合的管理・支援体制を整備して、品質管理された試料を基礎研究機関に提供し、難治性疾患克服研究のより一層の効率的推進を図ることを目的としている。当初の事業予定では、疾患患者資料の収集が直ちに開始されることを想定していたが、収集研究班へのアンケート調査、また昨年 9 月に行われた事業説明会で各収集研究班の担当者との直接の話し合い、および直接、収集研究班担当者への訪問調査討議などを行い、患者試料収集に係る単純ではない問題点が明確となった。収集研究班の目的試料の種類も異なっており、個々の収集研究班に対して倫理申請上の必要事項など個別の対応を取りながら事業を進めた。

また、基盤研究所内部での難病研究資源バンク専属の医学研究倫理審査委員会の組織化のため基盤研医学研究倫理員会の承認を受け、設立することができた。システム整備に関しては、患者試料を処理・検査・保管管理のための施設・機器の整備を行うとともに、コード管理システムの構築を行った。

E. 結論

本研究事業の目的は「難治性疾患対策研究、生体試料等の収集に関する研究」により収集された患者試料を集中化して品質管理を行う「難病研究資源バンク」を創設し、統合的管理・支援体制を整備して、品質管理された試料を公平に基礎研究機関に提供し、難治性疾患克服研究のより一層の効率的推進を図ることにある。収集研究班へのアンケート調査および収集研究班担当者との討議により患者試料収集に係る単純ではない問題点を明確にすることができた。また基盤研究所内部での難病研究資源バンク専属の医学研究倫理審査委員会を設立することができた。システム整備に関しては、患者試料を処理・検査・保管管理のための施設・機器の整備を行うとともに、コード管理システムの構築を行った。

F. 健康危険情報

特になし

G. 研究発表

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H 知的財産権の出願・登録状況 (予定を含む)

1 特許取得 特になし

2. 実用新案登録 特になし

3. その他 特になし

研究成果の刊行に関する一覧表

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doi: 10.1111/j.1399-0004.2009.01257.x

Short Report

Identification of 11 novel mutations in *USH2A* among Japanese patients with Usher syndrome type 2

Nakanishi H, Ohtsubo M, Iwasaki S, Hotta Y, Mizuta K, Mineta H, Minoshima S. Identification of 11 novel mutations in *USH2A* among Japanese patients with Usher syndrome type 2.
Clin Genet 2009: 76: 383–391. © John Wiley & Sons A/S, 2009

Usher syndrome (USH) is an autosomal recessive disorder characterized by retinitis pigmentosa and hearing loss. USH type 2 (USH2) is the most common type of USH and is frequently caused by mutations in *USH2A*, which accounts for 74–90% of USH2 cases. This is the first study reporting the results of scanning for *USH2A* mutations in Japanese patients with USH2. In 8 of 10 unrelated patients, we identified 14 different mutations. Of these mutations, 11 were novel. Although the mutation spectrum that we identified differed from that for Caucasians, the incidence of mutations in *USH2A* was 80% for all patients tested, which is consistent with previous findings. Further, c.8559-2A>G was identified in four patients and accounted for 26.7% of mutated alleles; it is thus a frequent mutation in Japanese patients. Hence, mutation screening for c.8559-2A>G in *USH2A* may prove very effective for the early diagnosis of USH2.

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Key words: hearing loss – retinitis pigmentosa – Usher syndrome – *USH2A*

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Usher syndrome (USH) is an autosomal recessive disorder characterized by retinitis pigmentosa (RP) and hearing loss (HL), with or without vestibular dysfunction. USH is the most common cause of combined deafness and blindness in industrialized countries, with a general prevalence of 3.5–6.2 per 100,000 live births (1–6). USH is clinically and genetically heterogeneous and can be classified into three clinical subtypes on the basis of the severity and progression of HL and the presence or absence of vestibular dysfunction. USH type 1 (USH1) is characterized by congenital severe-to-profound HL and absent vestibular response; USH type 2 (USH2), by stable congenital moderate-to-severe HL and normal vestibular response; and USH type 3 (USH3), by progressive HL and variable vestibular response (7–10).

USH2 is the most common type, accounting for >50% of USH cases (5). Three causative genes have been identified for USH2: *USH2A* (11, 12), *USH2C* (current HUGO symbol, *GPR98*) (13), and *USH2D* (*DFNB31*) (14). *USH2A*, which encodes the protein usherin, accounts for approximately 74–90% of USH2 cases (15–17). Usherin is a large protein comprising many functional domains; two isoforms of usherin (isoform A and full-length isoform B) have been reported (Fig. 1) (11, 12, 18).

Mutation analysis of *USH2A* for the full-length coding region consisting of exons 1–73 in European Caucasian patients has revealed a frequent mutation – p.Glu767fs (c.2299delG) in exon 13 (19–21). We screened Japanese patients for mutations in *USH2A* and found a frequent mutation (c.8559-2A>G) and 11 novel mutations. This is the first report of mutation screening in Japanese USH patients.

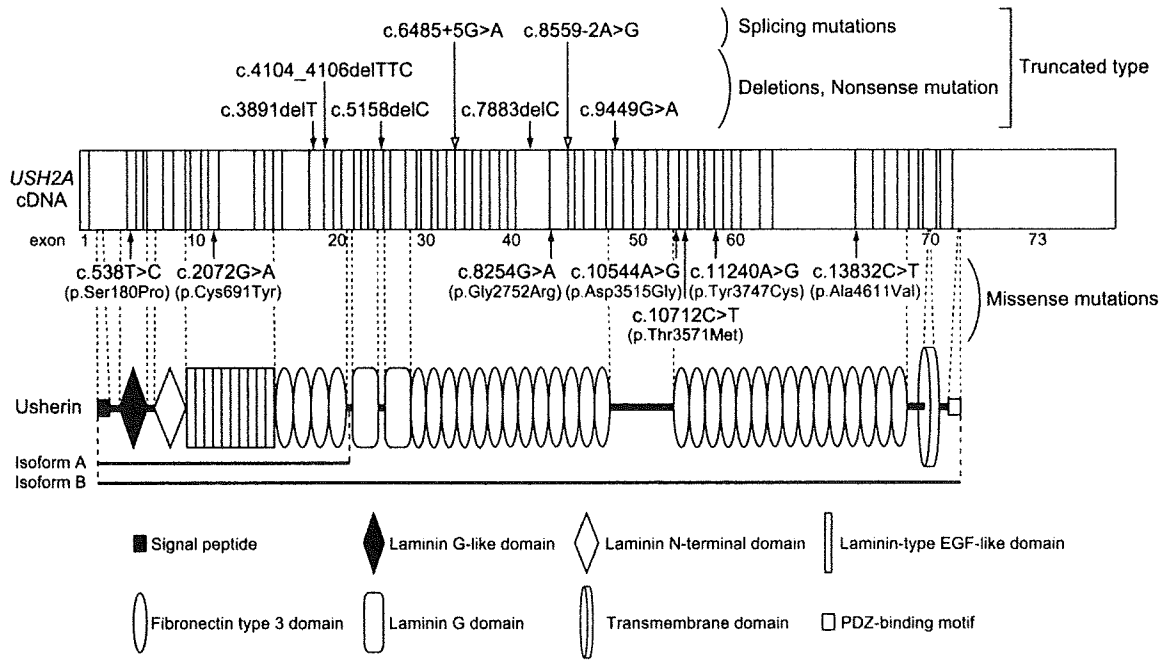

Fig. 1. Schematic distribution of mutations identified in *USH2A*. Upper, *USH2A* cDNA with exon boundaries. Lower, usherin domains encoded by *USH2A*. All mutations were widely distributed throughout almost the entire *USH2A* without any clear hot spot. The open arrows indicate mutations in introns, and closed arrows, mutations in exons.

Materials and methods

Subjects

Ten unrelated Japanese patients from various regions throughout Japan were referred to Hamamatsu University School of Medicine for genetic diagnosis of USH. All patients met the following criteria for USH2: RP, congenital moderate-to-severe sensorineural HL, and normal vestibular function (7). The clinical evaluation of the affected patients consisted of elicitation of medical history, and ophthalmological and audiovestibular examinations. A medical history included the following information: age at diagnosis of HL, nature of HL, age at onset of night blindness, and age at diagnosis of RP.

Ophthalmological evaluation consisted of best-corrected visual acuity measurement, slit-lamp microscopy, ophthalmoscopy, Goldmann perimetry, and electroretinography (ERG). Visual fields were evaluated by Goldmann perimetry of both eyes, and the isopters for the V/4e, III/4e, and I/4e test targets were measured. ERG was performed according to International Society for Clinical Electrophysiology of Vision (ISCEV) protocol (22).

Auditory examinations consisted of otoscopy, pure-tone audiometry (125–8000 Hz), and tympanometry. Severity of HL was classified using the pure-tone average (PTA) over 500, 1000, 2000, and 4000 Hz in the better-hearing ear as follows: normal hearing, <20 dB; mild HL, 21–40 dB; moderate HL, 41–70 dB; severe HL, 71–90 dB; and profound HL, >91 dB. The PTA over 500, 1000, 2000, and 4000 Hz was measured in each ear, and the loss in PTA over 5–10 years was used to categorize progression of HL as follows: no progression, <10 dB loss; mild progression, 10–15 dB; moderate progression, 15–25 dB; severe progression, 25–35 dB; and rapid progression, ≥35 dB loss (23). Serial audiograms taken <5 years apart were excluded from the evaluation of progression.

Vestibular function was evaluated on the basis of the medical history concerning childhood motor development and the results of caloric tests. Caloric stimulation of each ear was performed with cold water (20°C, 5 ml) and the results were classified according to the peak slow-phase velocity as follows: normal, ≥20°/s; canal paresis, <20°/s.

A set of 135 control samples was obtained from Japanese individuals with no visual or hearing impairment and used to assess the frequency of nucleotide sequence variants. The institutional review board of Hamamatsu University School of Medicine approved this study, and written informed consent was obtained from all participants prior to enrollment.

Mutation analysis

Genomic DNA was extracted from peripheral lymphocytes by using standard procedures. All 73 exons of *USH2A* and their flanking sequences were amplified by polymerase chain reaction (PCR). The PCR products were purified with Wizard SV gel and PCR Clean-up System (Promega, Madison, WI) or treated with Exonuclease I and Antarctic Phosphatase (New England Biolabs, Ipswich, MA). Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3100 autosequencer (Applied Biosystems, Foster City, CA). The PCR primers used to amplify 19 exons and sequencing primers used for 2 exons were newly designed (Table S1, Supporting information); the other primers have been previously described (12, 15). Using direct sequencing or restriction enzyme-based assay, Japanese control chromosomes were tested for all the novel mutations identified during the mutation analysis.

Prediction of splice scores

Using the Splice Site Prediction by Neural Network software (http://www.fruitfly.org/seq_tools/splice.html), we predicted the degree to which each sequence variation located within or close to a splice site influenced the splicing (24). This software determines whether an applied sequence contains a splice donor or acceptor site. If appropriate candidate sequences are identified for these sites, the predicted probability is given as a score from 0 to 1, with 1 indicating the highest probability.

Results

Mutation analysis

Mutation analysis of *USH2A* in 10 unrelated Japanese patients revealed 14 different probable pathogenic mutations in 8 patients (Tables 1 and 2). Of these, 11 mutations were novel (Table 2). Splicing mutation c.8559-2A>G was identified in 4 of 10 patients and accounted for 4 of the 15 mutated alleles detected (26.7%); the other mutations were seen in 1 patient each. Thus, c.8559-2A>G occurs frequently in Japanese patients. Mutations were widely distributed throughout almost the entire *USH2A*, without any apparent hot spot (Fig. 1). The only exception was exons 70–73, which encode a transmembrane domain or intracellular region; no mutations were identified in this sequence. This finding is consistent with those of previous reports (12, 19–21, 25–29). In five of the eight patients (C712, C116, C152, C452, and C557), two probable pathogenic alleles were identified and confirmed to be on different chromosomes by using parent or sibling samples (Table 1). For two patients (C237 and C212), segregation analysis could not be performed due to difficulties in collecting samples from the patients' families. We therefore could not confirm whether the two mutations (c.8559-2A>G and p.Trp3150X) in patient C237 were located on different chromosomes, although both were considered pathogenic. Similarly, we were unable to assign the three sequence alterations identified in patient C212 (p.Cys691Tyr, p.Gly2752Arg, and p.Tyr3747Cys) to different chromosomes and to determine which of these alterations were pathogenic. Segregation analysis revealed that the 2 sequence alterations in patient C332 were on the same chromosome. In this patient, p.Ser1369del appeared to be pathogenic and p.Ala4611Val on the same chromosome not to be. The other mutation remained undetected. None of these 14 mutations were found in the Japanese controls (Table 2).

We detected two splicing mutations, c.6485+5G>A and c.8559-2A>G, in patient C152. Neither of these was detected in 270 control chromosomes (Tables 1 and 2). Using a computer program (Splice Site Prediction by Neural Network), *in silico* analysis was performed to predict the degree to which these nucleotide substitutions in splice sites affect splicing. In the case of c.6485+5G>A, the score for the splicing donor site of the normal allele was 0.93, while that for the mutant allele was 0.45. In the case of c.8559-2A>G, the score for the normal allele was 0.99, but the mutant allele was not recognized as a splicing acceptor site. These results support the notion that these splicing mutations are pathogenic.

In addition to the probable pathogenic mutations listed in Table 2, 43 sequence alterations were identified in 10 patients (Table 3). These alterations were predicted to be non-pathogenic for various reasons. Some of these have been reported as polymorphisms in previous reports, and most newly identified alterations within the exons, except for c.5142T>C (p.Asn1741Asn), were also found in the control chromosomes. The exception, c.5142T>C, was not detected in any of the 64 control chromosomes and has not been reported previously, but was considered benign because it was identified in patient C237 together with c.8559-2A>G and p.Trp3150X (see above). The 3 novel intronic sequence alterations in or close to splicing donor/acceptor sites (c.848+5G>A, c.4758+3A>G, and c.15298-24T>C) identified in the patients' chromosomes were also detected

Table 1. Clinical information of patients in whom probable pathogenic mutations were identified

Patient	Age	Sex	Mutations		Age (years)[a]			Visual acuity		Visual field	ERG	Fundus of the eye	Cataracts	Severity of HL	Progression of HL		Caloric test
			Allele 1	Allele 2	HL	NB	RP	Right	Left						Right	Left	
Compound heterozygotes[b]																	
C712	24	F	p.Ser180Pro	p.Leu1720X	3	13	21	0.8	0.7	25-65° with relative scotomas (III/4e)	Extinguished	Typical RP	No	Severe	No	No	Normal
C116	40	M	p.Gln1298ArgfsX12	p.Pro2628GlnfsX13	6	13	25	0.03	0.03	10° (V/4e)	NA	Typical RP	No	Moderate	No	No	Normal
C152	47	F	c.6485 + 5G>A	c.8559 – 2A>G	6	14	26	0.2	0.03	10° (V/4e)	Extinguished	Typical RP	Both eyes[e]	Moderate	Mild	No	Normal
C452	32	F	c.8559 – 2A>G	p.Asp3515Gly	6	17	18	1.2	1.2	20-30° (III/4e)	Extinguished	Typical RP	No	Moderate	No	No	Normal
C557	50	M	c.8559 – 2A>G	p.Thr3571Met	7	16	28	HM	HM	5° (V/4e)	Extinguished	Typical RP	Both eyes	Moderate	NA	NA	Normal
C237	22	M	c.8559 – 2A>G; p.Trp3150X		3	13	16	0.6	0.7	<5° (V/4e)[d]	Extinguished	Typical RP	No	Severe	Severe	Severe	Normal
C212	33	F	p.Cys691Tyr; p.Gly2752Arg; p.Tyr3747Cys		6	12	26	0.5	0.4	10-15° with residual temporal field (III/4e)	Extinguished	Typical RP	No	Moderate	NA	NA	Normal
Heterozygote[c]																	
C332	31	F	p.Ser1369del; p.Ala4611Val		4	17	30	0.9	0.9	10-15° (III/4e)	Extinguished	Typical RP	No	Moderate	NA	NA	Normal

HL, hearing loss; NB, night blindness; RP, retinitis pigmentosa; ERG, electroretinography; HM, hand movements; NA, data not available.

[a]Age at diagnosis of HL, age at onset of NB, and age at diagnosis of RP were shown.

[b]In five patients (C712, C116, C152, C452, and C557), using parent or sibling samples, it was confirmed that the pair of mutations were on the different alleles. For 2 patients (C237 and C212), no such segregation analysis was performed because samples from family members were not available.

[c]For C332, 2 detected changes were on the same allele and another pathogenic allele remained undetected.

[d]V/4e isopter could not be detected on Goldmann perimetry.

[e]Cataract of the left eye was removed at 38 years of age.

Table 2. Probable pathogenic mutations identified in Japanese USH2 patients examined in this study

Mutation type	Nucleotide change	Predicted translation effect	Exon/Intron number	Domain	Number of alleles	Alleles in control chromosomes	Reference
Nonsense	c.9449G>A	p.Trp3150X	Exon 48		1	0/114	27
Deletion	c.3891delT	p.Gln1298ArgfsX12	Exon 18	FN3[b]	1	0/130	This report
	c.4104_4106delTTC	p.Ser1369del[a]	Exon 19	FN3	1	0/130	This report
	c.5158delC	p.Leu1720X	Exon 25		1	0/114	This report
	c.7883delC	p.Pro2628GlnfsX13	Exon 41	FN3	1	0/130	This report
Splicing	c.6485 + 5G>A		Intron 33		1	0/270	This report
	c.8559 – 2A>G		Intron 42		4	0/270	27
Missense	c.538T>C	p.Ser180Pro	Exon 3	LamGL[c]	1	0/270	This report
	c.2072G>A	p.Cys691Tyr	Exon 12	EGF_Lam[d]	1	0/270	This report
	c.8254G>A	p.Gly2752Arg	Exon 42	FN3	1	0/270	This report
	c.10544A>G	p.Asp3515Gly	Exon 53	FN3	1	0/260	This report
	c.10712C>T	p.Thr3571Met	Exon 54	FN3	1	0/270	19, 20
	c.11240A>G	p.Tyr3747Cys	Exon 58	FN3	1	0/270	This report
	c.13832C>T	p.Ala4611Val[a]	Exon 64	FN3	1	0/270	This report

[a]These 2 variants are allelic.
[b]FN3, fibronectin type 3 domain; [c]LamGL, laminin G-like domain; [d]EGF_Lam, laminin-type EGF-like domain.

in the control chromosomes. Both c.848+5G>A and c.4758+3A>G were analyzed using the *in silico* method described above. The scores for the normal and mutant alleles of c.848+5G>A were 1.00 and 0.96, respectively, and those for c.4758+3A>G were 0.98 and 0.73, respectively. As the differences between the scores were small, these alterations may not affect splicing.

Seven missense mutations were detected in the patients (Table 2). None of these were identified in at least 260 Japanese control alleles. All amino acid residues affected by these mutations were compared with those encoded by orthologous genes of various vertebrates (dog, rat, mouse, chicken, and zebrafish); the residues were completely conserved among these species (data not shown). Conversely, most synonymous and non-synonymous sequence alterations in coding regions predicted to be benign were not evolutionarily conserved (Table 3), suggesting that these alterations are non-pathogenic.

Clinical findings

All 8 patients in whom at least 1 mutant allele was detected had developed night blindness at ages 12–17 years (mean ± SD, 14.4 ± 2.0 years) and had been diagnosed with RP by ophthalmologists at ages 16–30 years (23.8 ± 4.9 years, Table 1). In all patients, visual fields were symmetrically constricted, pigmentary degeneration was typical for RP with peripheral bone-spicule pigmentation, and standard combined ERGs were extinguished (Table 1, Fig 2a). The current visual field ranged from constriction to 25–65° with relative scotomas (III/4e) in patient C712 to severe concentric constriction in patient C237, in whom V/4e isopter could not be detected on Goldmann perimetry. The best-corrected visual acuity ranged from 1.2 to hand movements. Two subjects (C152 and C557) reported having cataracts (25%); patient C557 had had a cataract in the left eye removed.

The patients were diagnosed with hearing impairment by otorhinolaryngologists at ages 3–7 years (5.1 ± 1.6 years, Table 1). All patients wore hearing aids and had intelligible speech. Tympanometry yielded normal results, consistent with the clinical findings of a normal tympanic membrane and middle ear cavity. Audiograms showed bilateral moderate-to-severe sensorineural HL with a typical slope toward high frequencies (mean hearing level, 62.2 ± 13.8 dB; Table 1, Fig. 2b). Two patients (C152 and C237) complained of progression of HL. Audiograms of patient C152 showed mild progression in the right ear and those of patient C237 revealed severe progression in the both ears (Table 1, Fig. 2c). They had no history of noise exposure or ototoxic medication.

Motor developmental delay was not reported, and all patients started walking before 18 months of age. The caloric test was normal in eight patients (Table 1). These results indicate that all patients had normal vestibular function, although additional evaluations (rotary chair test) with potentially greater sensitivity for detecting subtle vestibular dysfunction were not performed.

Table 3. Presumed non-pathogenic alterations found in *USH2A* in 10 patients

Nucleotide change	Predicted translation effect	Exon/Intron number	Domain	Conservation in h/d/r/m/c/z species[a]	Number of alleles	Alleles in control chromosomes	Reference
c.373A>G	p.Thr125Ala	Exon 2		T/T/T/V/S/S	2		15
c.504G>A	p.Thr168Thr	Exon 3	LamGL[c]	T/T/T/T/A/S	2		35
c.573A>G	p.Val191Val	Exon 3	LamGL	V/V/V/V/V/Q	1	2/66	This report
c.848 + 5G>A		Intron 5			1	6/270	This report
c.879T>G	p.Leu293Leu	Exon 6		L/L/L/F/F/F	1	2/66	27
c.1419C>T	p.Thr473Thr	Exon 8	LamNT[d]	T/T/S/S/T/S	3		15
c.1644 + 34C>A		Intron 9			17		15
c.3157 + 35A>G		Intron 15			3		15
c.3812 − 8T>G		Intron 17			3		15
c.4457G>A	p.Arg1486Lys	Exon 21		R/R/R/R/S/F	17		15
c.4758 + 3A>G		Intron 22			1	2/34	This report
c.5142T>C	p.Asn1714Asn	Exon 25		N/Q/E/E/S/E	1	0/64	This report
c.6317T>C	p.Ile2106Thr	Exon 32	FN3[e]	I/T/T/T/V/N	8		19
c.6506T>C	p.Ile2169Thr	Exon 34	FN3	I/V/L/L/L/V	14		19
c.7300 + 43C>T		Intron 38			2		This report
c.8624G>A	p.Arg2875Gln	Exon 43	FN3	R/L/L/L/L/L	1		19
c.8656C>T	p.Leu2886Phe	Exon 43	FN3	L/F/F/F/F/F	1		19
c.8681 + 120G>A		Intron 43			1		21
c.9056 − 157C>T		Intron 45			2		This report
c.9343A>G	p.Thr3115Ala	Exon 47		T/T/I/I/A/T	2		21
c.9595A>G	p.Asn3199Asp	Exon 49		N/D/D/D/D/N	2		21
c.10232A>C	p.Glu3411Ala	Exon 52		E/A/A/A/A/S	14		19
c.10388 − 27T>C		Intron 52			14		19
c.11231 + 45C>T		Intron 57			7		This report
c.11504C>T	p.Thr3835Ile	Exon 59	FN3	T/A/A/S/D/D	2		21
c.11602A>G	p.Met3868Val	Exon 60	FN3	M/V/V/V/L/I	1		19
c.11711 + 71A>T		Intron 60			11		This report
c.12066 + 73A>G		Intron 61			3		This report
c.12608A>G	p.Gln4203Arg	Exon 63	FN3	Q/Q/R/T/R/E	2	9/220	This report
c.12612A>G	p.Thr4204Thr	Exon 63	FN3	T/T/T/T/A/D	11		25
c.12666A>G	p.Thr4222Thr	Exon 63	FN3	T/A/G/A/T/S	2		19
c.13191G>A	p.Glu4397Glu	Exon 63	FN3	E/E/E/E/E/E	8		19
c.13478G>A	p.Arg4493His	Exon 63	FN3	R/R/R/R/R/R	1	2/98	This report
c.14481C>T	p.Ala4827Ala	Exon 66		A/A/A/A/A/S	3	4/66	This report
c.14513G>A	p.Gly4838Glu	Exon 66	FN3	G/Q/Q/Q/R/I	3	4/66	This report
c.14543G>A	p.Arg4848Gln	Exon 66	FN3	R/Q/Q/H/Q/E	3	4/66	This report
c.15076A>G	p.Lys5026Glu	Exon 70		K/E/G/E/E/G	3	4/66	This report
c.15298 − 24T>C		Intron 71			3	4/66	This report
c.15592 − 194A>T		Intron 72			4		This report
c.15634A>G	p.Ser5212Gly	Exon 73		S/S/S/S/S/-[f]	3	4/66	This report
c.*49A>C[b]		Exon 73			3	4/66	This report
c.*131G>A		Exon 73			1	3/66	This report
c.*191T>G		Exon 73			3	4/66	This report

[a]h/d/r/m/c/z denotes human/dog/rat/mouse/chicken/zebrafish usherin orthologs, respectively.
[b]A-to-C substitution located 49 nucleotides downstream of the termination codon. According to nomenclature guidelines for the description of sequence variations (http://www.hgvs.org/mutnomen/).
[c]LamGL, laminin G-like domain; [d]LamNT, laminin N-terminal domain; [e]FN3, fibronection type 3 domain.
[f]Ser5212 in human usherin was not detected in the usherin ortholog in zebrafish.

Discussion

This is the first study to analyze mutations in *USH2A* among Japanese patients. We detected 14 different mutations; p.Glu767fs, the most prevalent mutation in European Caucasians that accounts for approximately 30% of mutated alleles (19–21), was not detected. Furthermore, p.Thr3571Met was the only mutation that was common between Japanese and other populations, including European, French Canadian, Jewish, and Palestinian. This mutation has been found in Spanish and French patients (12, 19–21, 25, 26, 28, 29). In contrast, p.Trp3150X and c.8559-2A>G identified in this study have been reported in Chinese

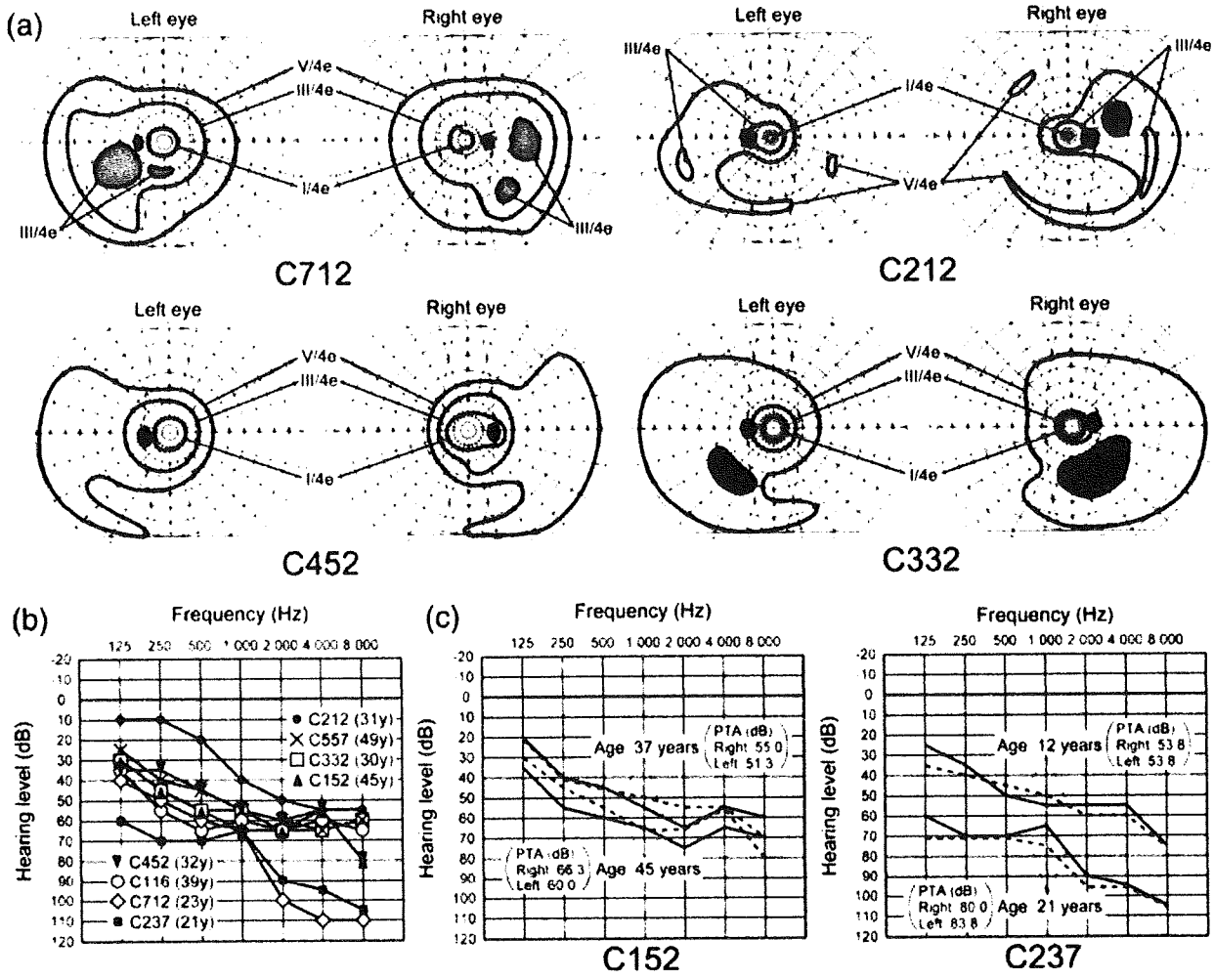

Fig. 2. (a) Goldmann kinetics of four patients (C712, C452, C212, and C332) with test targets V/4e, III/4e, and I/4e. In four cases visual fields were symmetrically constricted. The black and gray areas denote absolute and relative scotomas, respectively. (b) Overlapped audiograms for the better-hearing ears of eight patients with *USH2A* mutations. All patients showed moderate-to-severe hearing loss with a sloping audiogram. The age at which audiogram was taken is shown in parentheses ('y' stands for years). (c) Pure-tone audiograms of two patients (C152 and C237). Audiograms of patient C152 showed mild progression in the right ear and those of patient C237 revealed severe progression in the both ears. The continuous lines indicate audiograms of the right ear, and dashed lines, audiograms of the left ear. PTA, pure-tone average (500, 1000, 2000, and 4000 Hz).

patients (27). These results indicate that the mutation spectrum for *USH2A* among Japanese patients differs from that among the above-mentioned non-Asian populations. The Japanese and Chinese mutation spectra may resemble each other, but an accurate comparison could not be made because the number of Chinese patients (three families) analyzed was insufficient (27).

We found at least 1 mutated allele in 8 of 10 USH2 patients. This frequency (80%) is similar to that in European, Jewish, and French-Canadian patients (19–21, 26, 29), indicating that mutation screening for *USH2A* is also highly sensitive for diagnosing USH2 among Japanese patients. Moreover, we found that 40% of Japanese USH2 patients display at least one c.8559-2A>G mutation, which accounts for 26.7% of mutated alleles. Thus, this seems to be a frequent mutation among Japanese USH2 patients, although the number of patients examined was small. During early childhood when the symptoms of USH2 have not yet developed, ophthalmological examinations including ERG are required to distinguish USH2 from nonsyndromic deafness (30). Mutation screening for *USH2A*, specifically targeting c.8559-2A>G, may enhance the efficiency and practicability of early diagnosis in Japanese patients.

Among the eight patients in whom probable pathogenic mutations were identified, only patient C212 showed three mutations (Table 1). We could not determine which mutations were allelic and pathogenic. Of the three mutations in C212, only p.Cys691Tyr was situated in the isoform A-covered region; the others (p.Gly2752Arg and p.Tyr3747Cys) were found in the isoform B-specific region. Dreyer et al. previously described that in patients with mutations in both alleles of the isoform B-specific region, both mutations are never of the missense type, and at least one mutation is of the truncated type (nonsense, deletion, insertion, or splicing) (21). We reviewed other studies and our present results (C152, C452, and C557) and found that the finding of Dreyer et al. is correct in almost all cases (12, 19–20, 25–29). This suggests that at least one of p.Gly2752Arg and p.Tyr3747Cys is not pathogenic and therefore that p.Cys691Tyr in the isoform A-covered region is pathogenic. Additionally, Cys691 is known to be an important residue in the consensus sequence of the laminin-type EGF-like domain (31).

A few points should be noted regarding the clinical variations among the study patients. The severity of HL ranged from moderate to severe, and two young patients (C712 and C237) showed severe HL. Further, progression of HL, which has been previously reported in *USH2A* patients (23, 32), was observed in two of the study patients (C152 and C237). Visual function also varied among the study patients. Although visual function tended to deteriorate with age in all patients, the visual field of patient C237 was severely constricted for his age when it was compared to the visual fields of the other study patients and of *USH2A* patients in previous reports (9, 28). Such variations in the phenotype of this disease, especially those observed in patient C237, may suggest the involvement of genetic modifiers or environmental factors – a notion that is consistent with the published literature (33, 34).

In conclusion, mutation screening of *USH2A* in 10 Japanese patients revealed 14 different mutations, confirming that the mutation spectrum in Japanese patients differs from the previously reported spectra in patients of other ethnicities, including Caucasian, Jewish, and Palestinian. Among these 14 mutations, c.8559-2A>G was detected in 4 of 10 patients, indicating that this is a frequent mutation in Japanese patients. Mutation screening for c.8559-2A>G in *USH2A* may be very effective for the early diagnosis of USH2. Further analysis is necessary to obtain a more precise mutation spectrum and to identify other frequent mutations for effective and practical mutation tests.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Nucleotide primers designed for PCR and sequencing of *USH2A*.

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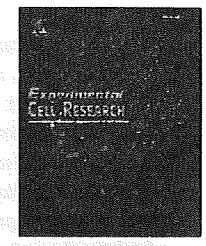
101種類のヒトiPS作製(図5)を発表した論文



Research Article

Mesenchymal to embryonic incomplete transition of human cells by chimeric OCT4/3 (POU5F1) with physiological co-activator EWS

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ABSTRACT

POU5F1 (more commonly known as OCT4/3) is one of the stem cell markers, and affects direction of differentiation in embryonic stem cells. To investigate whether cells of mesenchymal origin acquire embryonic phenotypes, we generated human cells of mesodermal origin with overexpression of the chimeric *OCT4/3* gene with physiological co-activator EWS (product of the *EWSR1* gene), which is driven by the potent *EWS* promoter by translocation. The cells expressed embryonic stem cell genes such as *NANOG*, lost mesenchymal phenotypes, and exhibited embryonal stem cell-like alveolar structures when implanted into the subcutaneous tissue of immunodeficient mice. Hierarchical analysis by microchip analysis and cell surface analysis revealed that the cells are subcategorized into the group of human embryonic stem cells and embryonal carcinoma cells. These results imply that cells of mesenchymal origin can be traced back to cells of embryonic phenotype by the *OCT4/3* gene in collaboration with the potent cis-regulatory element and the fused co-activator. The cells generated in this study with overexpression of chimeric OCT4/3 provide us with insight into cell plasticity involving OCT4/3 that is essential for embryonic cell maintenance, and the complexity required for changing cellular identity.

Introduction

Somatic stem cells have been shown to have a more flexible potential, but the conversion of mesenchymal cells to embryonic stem (ES) cells has still been a challenge and requires gene transduction [1–4]. This phenotypic conversion requires the molecular reprogramming of mesenchyme. Mesenchymal stem cells or mesenchymal progenitors have been isolated from adult bone marrow [5], adipose tissue [6], dermis [7], endometrium [8], menstrual blood [8], cord blood [9,10], and other connective tissues [11]. These cells are

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capable of differentiating into osteoblasts [12], chondrocytes [13], skeletal myocytes, adipocytes, cardiomyocytes [14,15], and neural cells [16]. However, most of the differentiation capability is limited to cells of mesodermal origin. This is in contrast to ES cells derived from the inner cell mass of the blastocyst that differentiate into cells of three germ cell layers. ES cells are pluripotent and immortal, and, therefore, ES cells provide an unlimited number of specialized cells.

Embryonic and adult fibroblasts have been induced to become pluripotent stem cells (iPS cells) or ES-like cells by defined factors including POU5F1 (also known as OCT4/3) [1–3]. OCT4/3 protein, a member of the POU family of transcription factors, is related to the pluripotent capacity of ES cells, and is thus a distinctive marker to identify primordial germ and embryonic stem cells [17–21]. OCT4/3 is down-regulated during oogenesis and spermatogenesis [22]. Furthermore, knocking out the *OCT4/3* gene in mice causes early lethality because of lack of inner cell mass formation [23], and OCT4/3 is critical for self-renewal of ES cells [24]. During human development, expression of OCT4/3 is found at least until the blastocyst stage [25] in which it is involved in gene expression regulation. OCT4/3 functions as a master switch in differentiation by regulating cells that have, or can develop, pluripotent potential by activating transcription via octamer motifs [26].

The EWS gene was originally identified at the chromosomal translocation, and fused with the ets transcription factors in Ewing sarcoma, as is the case of other sarcomas [27–30].

We report here the generation of human cells that overexpress the *OCT4/3* gene with physiological co-activator EWS (translation product of the *EWS* gene). In this study we show that the cells of mesenchymal origin overexpressing OCT4/3 can be traced back to cells with an embryonic phenotype.

Materials and methods

Cell culture

GBS6 cells were generated from primary or first passage cells of a pelvic tumor [31], and cultured in tissue culture dishes (100 mm, Becton Dickinson) in the G031101 medium (Med Shirotori, Tokyo). All cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. When the cultures reached subconfluence, the cells were harvested with Trypsin–EDTA Solution (cat# 23315, IBL) at 0.06% trypsin, and replated at a density of 5×10^5 in a 100 mm dish. Medium changes were carried out twice weekly thereafter. Both H4-1 and Yub10F were human bone marrow cells. The 3F0664 were human bone marrow-derived mesenchymal cells and were purchased from Lonza (PT-2501, Basel, Switzerland). The H4-1, Yub10F and 3F0664 cells were cultured in the mesenchymal-stem-cell-growth (MSCG)-Medium-BulletKit (PT-3001, Lonza). The NCR-G1 (a human yolk sac tumor line), NCR-G2 (a human embryonal carcinoma cell line from a testicular tumor), NCR-G3 (a human embryonal carcinoma cell line from a testicular tumor) and NCR-G4 (a human embryonal carcinoma cell line) were cultured in the G031101 medium as previously described [32]. In an experiment to inhibit cell adhesion, GBS6 and NCR-G3 cells were treated with anti-human E-cadherin, monoclonal (Clone HECD-1) (M106, TAKARA BIO INC.) at 100 μg/mL. Treatment with the demethylating agent, 5′-aza-2′-deoxycytidine (5azaC; A2385, SIGMA), was performed on GBS6 cells. GBS6 cells were treated with 3 μM of 5azaC for 24 h, and then cultured without treatment for 24 h. The 5azaC-traeated GBS6 cells were described as "GBS6-5azaC". MRC-5 human fetal lung fibroblasts were maintained in POWEREDBY10 medium (MED SHIROTORI CO., Ltd, Tokyo, Japan). We used these cells at between 17 and 25 PDs for the infection of the retroviral vectors. 293FT cells were maintained in DMEM containing 10% FBS, 1% penicillin and streptomycin. iPS cells were maintained in iPSellon medium (007001, Cardio) supplemented with 10 ng/mL recombinant human basic fibroblast growth factor (bFGF, WAKO, Japan). For passaging, iPS cells were washed once with PBS and then incubated with Dulbecco's Phosphate-Buffered Saline (14190-144, Invitrogen) containing 1 mg/mL Collagenase IV (17104-019, Invitrogen), 1 mM $CaCl_2$, 20% Knockout Serum Replacement (KSR) (10828-028, Invitrogen), and 0.05% Trypsin–EDTA Solution (23315, IBL) at 37 °C. When colonies at the edge of the dish started dissociating from the bottom, DMEM/F12/collangenase was removed. Cells were scraped and collected into 15 mL conical tubes. An appropriate volume of the medium was added, and the contents were transferred to a new dish on irradiated MEF feeder cells. The split ratio was routinely 1:3.

G-banding karyotypic analysis and spectral karyotyping (SKY) analysis

Metaphase spreads were prepared from cells treated with Colcemid (Karyo Max, Gibco Co. BRL, 100 ng/mL for 6 h). We performed a standard G-banding karyotypic analysis on at least 50 metaphase spreads for each population. SKY analysis was performed on metaphase-transduced cells according to the kit manufacturer's instruction (ASI, Carlsbad, CA) and a previously published method [33].

RT-PCR

The cDNAs were synthesized with an aliquot (5 μg) of each total RNA using Oligo-(dT)20 primer (18418-020, Invitrogen) and SuperScript III Reverse Transcriptase (18080-044, Invitrogen). Both the RNA strand of an RNA–DNA hybrid and single-stranded DNA were degraded by RNaseH (18021-071, Invitrogen). For the thermal cycle reactions, cDNA was amplified by T3 Thermocycler (Biometra, Goettingen, Germany) under the following reaction conditions: 30 cycles of a PCR (94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) after an initial denaturation (94 °C for 1 min). Primer sets used for PCR reactions are described in Tables 1 and 2. As the same amount of cDNA template was used in all reactions, in comparison to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) standard, the expression levels were evaluated. The controls consisted of reactions without reverse transcriptase in the process of cDNA synthesis.

Table 1 – PCR primers to detect the chimeric *EWS-OCT4/3* gene and untranslocated *OCT4/3* gene.

Symbol	Name	Sequence
A	EWS exon6-F	5′ TTA GAC CGC AGG ATG GAA AC 3′
B	EWS ex6 intron-F	5′ GTG GGG TTC ACT AT 3′
C	POU5F1-1a-F	5′ GAT CCT CGG ACC TGG CTA AG 3′
D	POU5F1-2-F	5′ CTT GCT GCA GAA GTG GGT GGA GGA A 3′
E	POU5F1-1a-R	5′ TCA GGC TGA GAG GTC TCC AA 3′
F	POU5F1-3-R	5′ CTG CAG TGT GGG TTT CGG GCA 3′

Table 2 – PCR primers for detection of gene transcripts.

Name	Sequence	Size (bp)
Nanog	Forward: 5′ AGT CCC AAA GGC AAA CAA CCC ACT TC 3′ Reverse: 5′ ATC TGC TGG AGG CTG AGG TAT TTC TGT CTC 3′	164
Sox2	Forward: 5′ ACC GGC GGC AAC CAG AAG AAC AG 3′ Reverse: 5′ GCG CCG CGG CCG GTA TTT AT 3′	253
UTF1	Forward: 5′ ACC AGC TGC TGA CCT TGA AC 3′ Reverse: 5′ TTG AAC GTA CCC AAG AAC GA 3′	230
GAPDH	Forward: 5′ GCT CAG ACA CCA TGG GGA AGG T 3′ Reverse: 5′ GTG GTG CAG GAG GCA TTG CTG A 3′	474

Immunoblot analysis

Whole lysates of GBS6 or NCR-G3 cells were loaded on 10% SDS/PAGE (40 μg total protein/lane) and transferred to a nitrocellulose membrane. The blots were probed with antibodies against anti-Oct3/4 (C-20 for the C-terminus of OCT4/3 of human origin; sc-8629, Santa Cruz), developed with polyclonal rabbit anti-goat Immunoglobulins/HRP antibody (P0160; Dako), and detected by chemiluminescence following the manufacturer's protocol (ECL Western Blotting Analysis System, Amersham).

Flow cytometric analysis

Cells were stained for 30 min at 4 °C with primary antibodies and immunofluorescent secondary antibodies. The cells were then analyzed on a Cytomics FC 500 (Beckman Coulter, Inc., Fullerton, CA, USA) and the data were analyzed with the FC500 CXP Software ver.2.0 (Beckman Coulter, Inc., Fullerton, CA, USA). Antibodies against human CD9 (555372, PharMingen), CD13 (IM0778, Beckman), CD14 (6603511, Beckman), CD24 (555426, PharMingen), CD29 (6604105, PharMingen), CD31 (IM1431, Beckman), CD34 (IM1250, Beckman), CD44 (IM1219, Beckman), CD45 (556828, PharMingen), CD50 (IM1601, Beckman), CD55 (IM2725, Beckman), CD59 (IMK3457, Beckman), CD73 (550257, PharMingen), CD81 (555676, PharMingen), CD90 (IM1839, Beckman), CD105 (A07414, Beckman), CD106 (IM1244, Beckman), CD117 (IM1360, Beckman), CD130 (555756, PharMingen), CD133 (130-080-801, Miltenyi Biotec), CD135 (IM2234, Beckman), CD140a (556002, PharMingen), CD140b (558821, PharMingen), CD157 (D036-3, IBL), CD166 (559263, PharMingen), CD243 (IM2370, Beckman), ABCG2 (K0027-3, IBL),

Table 3 – Expression of human ES cell-associated genes.

A

Cell		OCT4/3	SOX2	NANOG	UTF1	TDGF1	ZIC3	DPPA4	MYC	KLF4
GBS6	Flags	P	A	A	A	A	A	A	P	A
	Raw	2493	56	9	15	23	19	9	3261	171
GBS6-5azaC	Flags	P	A	A	A	A	A	A	P	A
	Raw	6620	146	19	28	20	15	11	1359	102
NCR-G1	Flags	A	A	A	A	P	P	A	A	A
	Raw	46	67	11	19	5180	2349	97	157	91
NCR-G2	Flags	P	A	P	A	P	P	P	A	A
	Raw	2093	120	3972	166	2154	389	873	160	84
NCR-G3	Flags	P	P	P	P	P	P	P	P	P
	Raw	14338	1239	14925	9208	11207	5294	4036	1086	1151
NCR-G4	Flags	P	P	P	P	P	P	P	P	A
	Raw	10602	352	9469	1684	9830	2741	2138	746	151
H4-1	Flags	A	A	A	A	A	A	A	P	P
	Raw	83	13	11	17	40	7	2	1635	489
3F0664	Flags	A	A	A	A	A	A	A	P	P
	Raw	56	63	14	34	21	21	22	735	832
Yub10F	Flags	A	A	A	A	A	A	A	P	A
	Raw	19	49	9	63	12	12	4	680	9

B

Gene symbol	Probe set ID	Gene name
POU5F1	208286_x_at	Oct4/3
SOX2	213721_at	Sox2
NANOG	220184_at	Nanog; Nanog homeobox
UTF1	208275_x_at	Utf1; undifferentiated embryonic cell transcription factor 1
TDGF1	206286_s_at	Tdgf1; teratocarcinoma-derived growth factor 1
ZIC3	207197_at	Zic3; odd-paired homolog
DPPA4	219651_at	Dppa4; developmental pluripotency associated 4
MYC	202431_s_at	c-myc
KLF4	220266_s_at	Klf4; Kruppel-like factor 4

A. Gene expression was examined with the Human Genome U133A Probe array (Affymetrix). Raw data values (*Raw*) for each gene expression are shown. *Flags*: Gene expression was judged to be "*P* (present)" or "*A* (absent)" in each cell by the GeneChip Analysis Suite 5.0 computer program. GBS6-5azaC: GBS6 cells were exposed to 3 μM 5′-aza-2′-deoxycytidine for 24 h, and then cultured without any treatment for 24 h.
B. Gene names for each symbol.