義はないとする報告が相次いでいる[30)-33)]。現状ではネフローゼの病因としてのsuPARについては否定的と考えざるを得ない。

CD80(B7-1)

一般的な抗原提示において，異物は樹状細胞やマクロファージなどの抗原提示細胞によって取り込まれ抗原として提示される。T細胞はT細胞受容体(T cell receptor: TCR)を介して抗原提示細胞上の抗原情報を認識し，自ら活性化して分裂したりエフェクター細胞へと分化する。この情報交換の際，T細胞は抗原提示細胞と強固に接着し，その接着面に“免疫シナプス”を形成する。CD80(B7-1)は膜蛋白質であり，活性化B細胞と抗原提示細胞に発現する。CD80はTCRの活性化に呼応してCD4+T細胞上のCD28と結合し，T細胞の増殖を促進する。このようにCD80とCD28の間の相互作用/共刺激シグナル伝達はT細胞とB細胞または抗原提示細胞間での重要な情報伝達を担っており，獲得免疫応答を調整している。一方，負の補助刺激受容体であるCTLA-4もCD80をリガンドとして結合するが，その親和性がCD28とCD80の結合の数十倍と高いためCD28とCD80の結合，すなわちCD28シグナルを強力に阻害する。

糸球体上皮細胞が刺激を受け傷害されるとCD80を発現するようになることが動物実験により明らかにされた[34)]。またMCNSの再発時に尿中のCD80濃度が上昇するが，これは寛解期やFSGS患者ではみられないことから，CD80の発現変化はMCNSで特異的にみられる現象である可能性も示唆されていた[35)]。MCNS患者の血清を培養ポドサイトに添加するとCD80の発現が上昇することがin vitroでも示されており[36)]，MCNSとCD80発現量との間には密接な関連があると考えられる。このようにまず血清中の何らかの刺激によるCD80の発現誘導を1st hit，その後のCD80のシグナルを抑制するCTLA4の発現低下を2nd hitとし，それらの結果CD80の恒常的に活性が上昇することがMCNSの原因であるとするtwo-hit仮説も提唱され始めている[37)]。

アバタセプトはCTLA4とIgGのキメラタンパク質であり，CD80と結合することによりCD80-CD28シグナルを抑制し免疫応答を減弱させる。そこでアバタセプトにより糸球体上皮細胞上のCD80を抑制することが蛋白尿の減弱につながるのか，最近複数のグループにより検討が行われた。Yuらは5人のFSGS患者(4人はリツキシマブ抵抗性，1人はステロイド抵抗性ネフローゼ症候群)にアバタセプトを投与し，いずれの患者においてもネフローゼレベルの蛋白尿が改善したと報告した[38)]。一方で，Garinらはアバタセプトが MCNS患者において一時的な蛋白尿抑制効果を持ったのに対して，FSGS患者では尿中CD80抗原が減少するにもかかわらず，蛋白尿に変化がなかったと報告している[39)]。また別のグループからアバタセプトがFSGS患者の蛋白尿への効果は乏しいことが報告されている[40)]。ネフローゼ症候群におけるCD80の作用，およびアバタセプトの効果については今後の症例の集積が必要である。

ポドサイト因子

先天性ネフローゼ症候群やステロイド抵抗性ネフローゼ症候群では原因遺伝子が明らかになりつつある(表2)。これらは発症年齢により，先天性ネフローゼ症候群として生後早期に症状の出るもの(*NPHS1*, *NPHS2*, *NPHS3*, *CD2AP*, *MYO1E*, *PTPRO*)，常染色体優性遺伝の形式をとる成人発症のもの(*TRPC6*, *ACTN4*, *INF2*)，他臓器症状を伴うもの(*WT1*, *LAMB2*, *LMX1B*, *MYH9*)，と便宜上3つに大きく分類することができる。これらの遺伝子産物のうち*LAMB2*は基底膜成分であるLaminin β2をコードするが，その他の多くの遺伝子は糸球体上皮細胞に強く発現する蛋白質をコードしているため，これらの疾患はポドサイト病(Podocytopathy)の一病態ととらえることができる。なお，欧米の検討では生後1年以内に発症する乳児ネフローゼ症候群の3分の2は4つの遺伝子変異で説明され(*NPHS1*-24%，*NPHS2*-38%，*LAMB2*-5%，*WT1*-3%)，2歳未満発症のステロイド抵抗性ネフローゼ症候群/先天性ネフローゼ症候群においては現在判明しているうちの24遺伝子の変異が9割近くに見出されることも報告されている[41)]。ステロイド抵抗性ネフローゼ症候群においても2000名を超える症例の解析から既知の27遺伝子で説明される症例が3割に及ぶことが報告されている[42)]。

ステロイド感受性ネフローゼ症候群(SSNS)やMCNSでは遺伝的な背景がどの程度発症に関与しているのだろうか？ ステロイド感受性ネフローゼ症候群の同胞内での発症は3%とされており，家族的な発症自体は稀である[43)]。確かにSRNSに比べてSSNSでは既知の遺伝子異常の頻度は極めて低く，例えば38名のSSNSの解析では遺伝子異常を有するものは見つからなかった[44)]。しかし病理学的にMCNSとされている症例においても遺伝子変異が原因となる症例も存在する。例えば本邦の蛋白尿を繰り返す兄弟において軽微なNephrinの異常が

表2　ネフローゼ症候群の遺伝因子

	遺伝子	遺伝子座	遺伝形式	OMIM	腎組織	蛋白	役割
1. 主に小児期に発症するもの	*NPHS1*	19q13.1	AR	602716	FSGS/MGC	Nephrin	スリット膜成分
	NPHS2	1q25-31	AR	604766	FSGS/MGC	Podocin	スリット膜成分
	NPHS3	10q23-24	AR	610725	DMS/FSGS	PLCε1	?
	WT1	11p13	AD(女性)	256370	DMS/FSGS	WT1	転写因子
	CD2AP	6p12.3	AR	607832	FSGS	CD2AP	スリット膜成分
	PTPRO	12p12	AR	600579	FSGS/MGC	GLEPP1	頂端面膜蛋白
	MYO1E	15q22.2	AR	601479	FSGS	Myosin1e	足突起—細胞骨格
	ARHGDIA	17q25.3	AR	601925	DMS/FSGS	RhoGDI	細胞骨格
	ADCK4	19q13.2	AR	615567	FSGS	ADCK4	酵素
	EMP2	16p13.13	AR	602334	MCD	EMP2	膜蛋白
	CRB2	9q33.3	AR	609720	FSGS	Crumbs2	膜蛋白
	COQ2	4q21-22	AR	609825	FSGS/CG	COQ2	ミトコンドリア酵素
	LMX1B	9q34.1	AD	161200	FSGS/MCNS	LMX1b	転写因子
2. 主に成人発症するもの	*INF2*	14q32.33	AD	613237	FSGS	INF2	足突起—細胞骨格
	ACTN4	19q13	AD	604638	FSGS	α-actinin4	足突起—細胞骨格
	ARHGAP24	4q21	AD	610586	FSGS	Filgap	Rho 蛋白質
	ANLN	7p14.2	AD	616027	FSGS	Anilin	足突起—細胞骨格
	TRPC6	11q21-22	AD	603652	FSGS	TRPC6	スリット膜成分
3. 他臓器症状を伴うもの							
眼異常	*LAMB2*	3p21	AR	609049	DMS/FSGS	Laminin β2	基底膜成分
性分化異常，Wilms 腫瘍	*WT1*	11p13	AD	194080	DMS	WT1	転写因子
性分化異常	*WT1*	11p13	AD	136680	FSGS	WT1	転写因子
爪，骨変形	*LMX1B*	9q34.1	AD	161200	FSGS/MCNS	LMX1b	転写因子
脳室拡大	*CRB2*	9q33.3	AR	609720	FSGS	Crumbs2	膜蛋白
骨形成不全，免疫不全	*SMARCAL1*	2q35	AR	606622	FSGS	SMARCAL1	細胞骨格
神経症状	*COQ2*	4q21-22	AR	609825	FSGS/CG	COQ2	ミトコンドリア酵素
神経症状，難聴	*COQ6*	14q24.3	AR	614647	FSGS	COQ6	ミトコンドリア酵素
神経症状	*PDSS2*	6q21	AR	610564	FSGS	DLP1	ミトコンドリア酵素
神経症状	*MTTL1*	ミトコンドリア		590050	FSGS	MTTL1	ミトコンドリア酵素
神経症状	*SCARB2*	4q21.1	AR	602257	FSGS	LIMPII	リソソーム
表皮水疱症，幽門閉鎖	*ITGB4*	17q25.1	AR	147557	FSGS	Integrin β4	基底膜/細胞接着
表皮水疱症，難聴	*CD151*	11p15.1	AR	602243	基底膜異常	Tetraspanin	基底膜/細胞接着
早老症	*ZMPSTE24*	1p34	AR	606480	FSGS	STE24	?
間質性肺疾患，表皮水疱症	*ITGA3*	17q21.33	AR	605025	FSGS	Integrina3	基底膜/細胞接着
血小板異常	*MYH9*	22q11.2	AD	160775	FSGS	Myosin Heavy Chain 9	足突起—細胞骨格
4. 疾患関連遺伝子	*GPC5*	13q31.3		602446	NS	GPC5	?
	HLADQA1	6p21.32		146880	SSNS	HLA-DQA1	T 細胞シグナル
	PLCG2	16q23.3		600220	SSNS	Phospholipase C γ2	Ca シグナル
	APOL1	22q12.3		603743	FSGS	Apolipoprotein L-1	?
	MYO1E	15q22.2		601479	FSGS	Myosin1e	足突起—細胞骨格
	CUBN	10p13		602997		Cubilin	受容体

AR：常染色体劣性遺伝，AD：常染色体優性遺伝
DMS：びまん性メサンギウム硬化症，FSGS：巣状分節性糸球体硬化症，CG：虚脱性糸球体腎症，MGC：微小糸球体病変

報告されている[45]。また Nail-Patella 症候群の原因遺伝子 *LMX1B* の変異が腎外症状を呈さずに蛋白尿のみを呈する患者において見出されている[46]。さらに，幼少期発症の家族性 SSNS の解析により EMP2 の遺伝子変異が見出されている[47]。EMP2 は糸球体上皮細胞や内皮細胞に発現しており，膜蛋白質のカベオリンの発現を制御しており，その変異が上皮細胞の形態変化の原因となると考えられる。またその他の遺伝子異常として，SRNS の原因として見出された kidney ankyrin repeat-containing protein 1(KANK)1，2，4 の変異が SSNS/MCNS 患者にも見出されている[48]。これらの事実から，糸球体上皮細胞機能に影響する遺伝子変異は必ずしも先天性ネフローゼ症候群や SRNS という病像を呈さずに，SSNS/MCNS の原因ともなりうることが示唆される。

ある遺伝子変異の有無によって病気の発症が決定されるような原因遺伝子のみではなく，感受性遺伝子と呼ばれるような，発症のリスクをあげる遺伝子多型についても大規模な検討が始まっている。複数の感受性遺伝子を

原因とする疾患の場合，それぞれの遺伝子がもつ発症リスクの大きさは「オッズ比」であらわされる。具体的には，感受性遺伝子をもたない人にくらべて，感受性遺伝子をもつ人のリスクが何倍になるかという数値で表される。ゲノムワイド関連解析(Genome Wide Association Study: GWAS)は，ヒトの全ゲノム中に1000万種以上もあるといわれるSNPs(一塩基多型：ゲノム上で一塩基だけが他のものに置き換わっている変異のうち，特定の集団の1%以上にみられるものをいう)の代表的なものをマーカーとして使い，特定の個人が全ゲノム中にどのようなSNPsをもつのかを網羅的に検討するものである。本邦の200例弱の後天性ネフローゼ症候群を対象としたGWASが行われ，Glypican-5をコードする*GPC5*のイントロンにおけるSNP多型がネフローゼ症候群の発症と相関することが報告された。Glypican-5は糸球体上皮細胞に発現しているが，糸球体上皮細胞特異的にGlypican-5をノックダウンしたマウスでは実験的な蛋白尿発症に抵抗性を示すことから，この遺伝子の発現量により糸球体上皮の傷害への感受性が規定されると考えられている[49]。また，200例ほどの小児期発症ステロイド感受性ネフローゼ症候群のGWAS解析では，6番染色体上のHLA-DQA1の多型の割合がSSNSで有意に上昇していた(オッズ比2.1)[50]。これらの多型そのものだけでは疾患の発症する訳ではないが，こういった複数の感受性遺伝子の関与がネフローゼ発症のリスクに大きく影響する可能性がある。今後広まると考えられるこれらの巨視的ゲノム解析は感受性遺伝子の動態を明らかにするのみならず，特定の民族や人種などの集団にみられる遺伝的な構造を明らかにするためにも有効である。

なぜ未だにMCNSの原因がわからないのか？

ネフローゼ症候群については古くは15世紀から記載があり，これまで述べた通り1970年代からその原因について様々な検討が行われてきた。先天性ネフローゼ症候群やステロイド抵抗性ネフローゼ症候群については遺伝子異常が明らかになり，多くの部分が糸球体上皮細胞異常で説明されることが判明しているが，MCNSについては現在に至ってもまだ不明な部分が多い。この理由には以下のような様々な可能性が考えられる。

(1)現在解析されている血中因子(サイトカイン等を含む)以外の因子による
(2)原因(遺伝的，免疫的背景あるいは循環因子)が単一ではない
(3)複数の因子の組み合わせによって発症する(例：糸球体上皮細胞因子+免疫因子，T細胞因子+B細胞因子，1st hit+2nd hitなど)
(4)遺伝的な複数の因子の複合した結果発症する(Double Heterozygosityなど)

また，ネフローゼ症候群の原因解析に関する研究の困難な理由も様々挙げられる。

(1)信頼できるin vitro再現系，動物モデルが確立されていない
(2)ステロイド等の免疫抑制薬の投与が行われるため，治療後の血液あるいは尿サンプルを解析に含めるとその影響を無視できない
(3)小児特有の疾患であり，FSGSを含めた内科領域の研究に比べて研究者および研究費が少ない
(4)原因究明を目的とした大規模なレジストリーが行われていない

今後の検討としてはこれらの問題点を踏まえ，ゲノム/エピゲノム/プロテオーム/トランスクリプトームなどの網羅的解析を大規模なコホートを用いて行う必要があると考えられる。また家族歴のある患者の遺伝的背景を明らかにすることが，より一般的な特発性ネフローゼ症候群の発症原因に迫る契機となるかもしれない。

結　び

特発性ネフローゼ症候群の治療の中心は依然としてステロイドを基礎とした免疫抑制療法であり，多彩な副作用が問題となる。ネフローゼ症候群の原因解明の最終的な目標は原因を元にした特異的な治療や予防法を見出すことであり，今後の検討がステロイドに代わる新たな創薬など革新的治療法の開発につながることが期待される。

「日本小児腎臓病学会の定める基準に基づく利益相反に関する開示事項はありません。」

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Pathogenic mechanism of childhood idiopathic nephrotic syndrome

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Pathogenic mechanism of childhood nephrotic syndrome has been investigated for decades, and congenital nephrotic syndrome and early onset steroid resistant nephrotic syndrome has been found to mainly be attributed to podocyte dysfunction. Although many hypotheses regarding the pathogenesis of minimal change nephrotic syndrome (MCNS) including immunological abnormalities and podocyte dysfunction have been proved to be valid, the common definitive cause of MCNS has not been identified. Here, different hypotheses regarding the cause of MCNS are reviewed, and the difficulty in resolving the mechanism is discussed.

Key words: idiopathic nephrotic syndrome, minimal change nephrotic syndrome, podocyte

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Biallelic Mutations in Nuclear Pore Complex Subunit *NUP107* Cause Early-Childhood-Onset Steroid-Resistant Nephrotic Syndrome

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The nuclear pore complex (NPC) is a huge protein complex embedded in the nuclear envelope. It has central functions in nucleocytoplasmic transport, nuclear framework, and gene regulation. Nucleoporin 107 kDa (NUP107) is a component of the NPC central scaffold and is an essential protein in all eukaryotic cells. Here, we report on biallelic *NUP107* mutations in nine affected individuals who are from five unrelated families and show early-onset steroid-resistant nephrotic syndrome (SRNS). These individuals have pathologically focal segmental glomerulosclerosis, a condition that leads to end-stage renal disease with high frequency. *NUP107* is ubiquitously expressed, including in glomerular podocytes. Three of four *NUP107* mutations detected in the affected individuals hamper NUP107 binding to NUP133 (nucleoporin 133 kDa) and NUP107 incorporation into NPCs in vitro. Zebrafish with *nup107* knockdown generated by morpholino oligonucleotides displayed hypoplastic glomerulus structures and abnormal podocyte foot processes, thereby mimicking the pathological changes seen in the kidneys of the SRNS individuals with *NUP107* mutations. Considering the unique properties of the podocyte (highly differentiated foot-process architecture and slit membrane and the inability to regenerate), we propose a "podocyte-injury model" as the pathomechanism for SRNS due to biallelic *NUP107* mutations.

Introduction

Nephrotic syndrome (NS) is a renal disease caused by disruption of the glomerular filtration barrier, which results in massive proteinuria, hypoalbuminemia, and dyslipidemia. Idiopathic NS occurs in 16/100,000 children.[1] Most children with idiopathic NS respond well to steroids, but 10%–20% of affected children are categorized as having steroid-resistant NS (SRNS).[2–6] SRNS is a clinically and genetically heterogeneous renal disorder that might have an immunological, structural, or functional etiology.[2,5,7–9] Higher rates of genetic delineation are expected in early-onset SRNS.[7] Clinical differences in SRNS have been suggested to depend on its age of onset.[7] Current medical management and prognosis in NS are based largely on the histological diagnosis. Effective SRNS treatments are not well established, and renal transplantation is eventually required. Importantly, 63%–73% of those with childhood-onset SRNS show pathologically focal segmental glomerulosclerosis (FSGS),which carries a great risk of progression to end-stage renal disease (ESRD).[1,6,8,10] To date, at least 27 genes are associated with SRNS, thereby expanding our knowledge of the pathomechanisms involved in SRNS and podocyte development and function.[11] Although SRNS is the leading cause of ESRD in children worldwide, approximately 70% of those with childhood-onset SRNS are genetically uncharacterized.[7,11] We describe here an additional genetic cause of early-onset SRNS and propose its possible pathomechanism.

Material and Methods

Human Subjects

A total of 18 families (10 with affected siblings and 8 with a single affected individual) who lack any known genetic causes of SRNS (in 27 known genes) were recruited to this study. They presented with non-syndromic early-onset SRNS with onset ages between 1 and 11 years. The clinical aspects of 7 of the 18 families have

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been described previously.[12] Affected individuals were resistant to standard steroid therapy but were partially responsive to immunosuppressive drugs. At least ten affected individuals in eight families underwent renal transplants and have had no recurrence of SRNS to date. All samples were collected after written informed consent was obtained. The study protocol was approved by the institutional review boards of Yokohama City University School of Medicine, Kansai Medical University, RIKEN, Tokyo Women's Hospital, and Kobe University.

DNA Extraction

Peripheral-blood leukocytes or saliva from affected individuals and their families was collected. Genomic DNA was extracted with a QIAamp DNA Blood Max Kit (QIAGEN) or Oragene DNA (DNA Genoteck) according to the instructions of each manufacturer.

Whole-Exome Sequencing and Informatics Analyses

Whole-exome sequencing (WES) was performed on affected individuals (one individual from each family) and their parents when the samples were available, as reported previously.[13] In brief, 3-μg samples of genomic DNA were sheared with the Covaris S2 system (Covaris); genome partitioning was performed with SureSelect Human All Exon V5 (Agilent Technology) according to the manufacturer's instructions. Prepared samples were run on a HiSeq 2000 instrument (Illumina) with 101-bp paired-end reads and 7-bp index reads. The sequence reads were mapped to the human reference sequence (GRCh37) by Novoalign 3.00. Next, PCR duplication and variant calls were processed by Picard and the Genome Analysis Toolkit. Ten of the 18 families have multiple affected children, suggesting the autosomal-recessive model, in which homozygous or compound-heterozygous variants are focused in each affected individual. Genetic variants in exons and canonical splice sites (±2 bp) with a minor allele frequency (MAF) of >0.005 in the NHLBI Exome Sequencing Project Exome Variant Server (EVS), Exome Aggregation Consortium (ExAC) Browser, Human Genetic Variation Database (HGVD, which is a public exome database for the Japanese population), or in-house Japanese exome data (n = 575) were removed from the candidates. Genes that harbor recessive variants detected commonly in two or more probands were selected. Candidate recessive variants were checked in each family by Sanger sequencing for confirmation that such variants co-segregated with the disease.

Haplotype Analysis

To determine the haplotype associated with c.2492A>C (p.Asp831Ala), which was found commonly in the five families, we amplified samples of genomic DNA or whole-genome-amplified DNA with 13 microsatellite markers (*D12S364*, *S12S310*, *D12S1617*, *D12S345*, *D12S85*, *D12S368*, *D12S83*, *D12S326*, *D12S351*, *D12S346*, *D12S78*, *D12S79*, and *D12S86*) from the ABI PRISM Linkage Mapping Set (Life Technologies).The PCR products were run on a 3500xl Genetic Analyzer (Life Technologies) and analyzed with GeneMapper 5 software (Life Technologies). Additionally, informative SNPs were chosen from the WES data for each affected individual and used thereafter for constructing haplotype blocks.

Expression of Human *NUP107*

NUP107 (nucleoporin 107 kDa; GenBank: NM_020401.2; MIM: 607617) expression in human embryos and adults was checked by a TaqMan Gene Expression Assay with two probe sets (Hs00914854_g1 and Hs00220703_m1 from Life Technologies) internally standardized by beta actin (Life Technologies). cDNA from human fetal and adult tissues was purchased from Clontech. qPCR was performed by a Rotor-Gene Q instrument (QIAGEN), the data from which was analyzed by the ΔΔCt method with Rotor-Gene 6000 Series software (QIAGEN). The experiments were done in duplicate. The expression level of each tissue represents the mean value of the duplicates.

Histopathology and Transmission Electron Microscopy on Samples from Individuals with Early-Onset SRNS

We stained 3-μm-thick sections cut from paraffin-embedded biopsied kidney tissues with H&E, periodic acid-Schiff stain, and periodic acid methenamine silver stain according to standard methods. For transmission electron microscopy, 1-mm renal-biopsy specimen cubes were fixed in 2% phosphate-buffered glutaraldehyde (pH 7.3) at room temperature, dehydrated in an alcohol gradient, and embedded in Epon-Araldite resin. Sections of 1-μm thickness were cut with an ultra-microtome (Ultracut UCT, Leica), stained with toluidine blue, and examined with a light microscope. Ultrathin sections (60–90 nm) stained by lead citrate were examined with a JEM1011 transmission electron microscope (JEOL). The TUNEL method was used to detect apoptotic cells on tissue sections with an in situ apoptosis detection kit (Takara) according to the manufacturer's instruction.

Immunofluorescence Microscopy

We deparaffinized and rehydrated 3-μm-thick paraffin sections of a necropsy specimen and then autoclaved them in target retrieval solution (S1700, Dako) for 15 min at 105°C. The sections were subjected to immunofluorescence labeling with primary antibodies including rabbit anti-NUP107 mAb (1.5 μg/ml, EPR12241, ab182559, Abcam), mouse anti-WT1 mAb (1:100, WT49, NCL-L-WT1-562, Leica), and mouse anti-Ezrin mAb (1:500, 3C12, E8897, lot 102K4824, Sigma-Aldrich). Normal rabbit and mouse immunoglobulins (IgGs) (sc-2027 [lot L1212] and sc-2025 [lot H1512], respectively, Santa Cruz) were used for negative controls. The CSAII kit (K1497, DAKO) was used for signal amplification of WT1, and other primary antibodies were visualized with Alexa555-conjugated anti-rabbit (1 μg/ml) or Alexa647-conjugated anti-mouse IgG (2 μg/ml) secondary antibodies (A21429 or A21236, respectively, Life Technologies), and then samples were mounted with ProLong Gold antifade reagent (P36930, Life Technologies). Single optical sections were acquired at 16-bit data depth with a confocal microscope system (AxioImager.Z1 microscope with LSM 700 laser scanner, Carl Zeiss) equipped with a C-Apochromat water immersion objective (40×, 1.2 numerical aperture [NA], Carl Zeiss); images were arranged with Photoshop CS5 (Adobe Systems).

Expression Vectors

Mammalian expression vectors were prepared with the Gateway system (Life Technologies). The *NUP107* open reading frame was amplified by PCR with human cDNA derived from a human lymphoblastoid cell line. The PCR product was introduced into the Gateway pDONR221 vector (Life Technologies), and its sequence was confirmed by Sanger sequencing. For mutagenesis, a QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used. After confirming appropriate mutagenesis, we performed LR recombination to create a mammalian expression

vector (pcDNA-DEST53, Life Technologies) to produce N-terminally GFP-fused NUP107 proteins. Among four *NUP107* mutations observed in this cohort, c.969+1G>A was mimicked by c.969_970insTAG, which created the nonsense codon just after the mutation (p.Asp324*). Whereas two truncating mutations (c.969+1G>A and c.1079_1083delAAGAG [p.Glu360Glyfs*6]) are thought unlikely to be present in vivo because of nonsense-mediated decay, these constructs were used as controls for the binding loss, given that C-terminally truncated proteins reportedly lose the NUP107-NUP133 interaction.[14]

Cell-free Protein Synthesis and In Vitro Pull-Down Assays

In vitro transcription and cell-free protein synthesis were performed as described previously.[15,16] In vitro transcription templates for wild-type or mutant *NUP107* were amplified by slit-primer PCR. For generation of transcription templates, the first PCR was performed with 50 ng/μl of each plasmid, 100 nM of the S1 common primer (5′-CCACCCACCACCACCAACAAAAAAG CAGGCTATG-3′), and 100 nM of the vector-specific reverse primer (5′-ATCTTTTCTACGGGGTCTGA-3′). The second PCR was performed with the first PCR product as a template with 100 μM of the SPu primer (5′-GCGTAGCATTTAGGTGACACT-3′), 100 μM of the vector-specific reverse primer (5′-ACGTTAAGGGATTTTGGT CA-3′), and 1 μM of either the deSP6-E02-FLAG-tagged primer or the biotin-ligation site (bls) primer for the addition of the nucleotide sequences of the FLAG tag or the bls tag, respectively (FLAG tagged: 5′-GGTGACACTATAGAACTCACCTATCTCTCTACACAAA ACATTTCCCTACATACAACTTTCAACTTCCTATTATGGACTACAA GGATGACGATGACAAGCTCCACCCACCACCACCAATG-3′; bls tagged: 5′-GGTGACACTATAGAACTCACCTATCTCTCTACACAAA ACATTTCCCTACATACAACTTTCAACTTCCTATTATGGGCCTGA ACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAACTCC ACCCACCACCACCAATG-3′).

An ENDEXT Wheat Germ Expression Kit (CellFree Sciences) was used for cell-free protein synthesis according to the manufacturer's instructions for the bilayer translation method. Biotinylated proteins were produced as described previously.[17]

Biotinylated wild-type or altered NUP107 was mixed with FLAG-NUP133 (nucleoporin 133 kDa; GenBank: NM_018230.2; MIM: 607613) in lysis buffer containing 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2% Triton X-100, 1 mM DTT, and 10 mg/ml BSA. After incubation for 1 hr at 26°C, streptavidin MagneSphere beads (Promega) were added, and the mixture was incubated for 30 min at room temperature. After three washes with lysis buffer, bound proteins were eluted from the beads with 20 μl of 2× SDS sample buffer. Bound proteins were separated by SDS-PAGE followed by immunoblotting with an anti-FLAG antibody (Sigma-Aldrich) or a Streptavidin-HRP conjugate (GE Healthcare). Proteins on the blot were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and FluorChem FC2 (Alpha Innotech) in accordance with the protocol from each manufacturer.

Immunoprecipitation

The cell lysate used for immunoprecipitation was prepared according to a method reported previously[18,19] with a slight modification. In brief, HeLa cells were transfected with the wild-type or altered N-terminally GFP-fused NUP107 construct by Viafect (Promega) according to the manufacturer's instructions. The cells were lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.4), 400 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM DTT supplemented with complete proteinase inhibitor cocktail (Roche Diagnostics GmbH), and PhosSTOP (Roche Diagnostics); sonicated; and then incubated for 30 min at 4°C. For debris removal, the crude lysate was centrifuged at 20,630 × *g* for 20 min at 4°C. After collection, the supernatant was diluted 3.75× in dilution buffer (10 mM Tris-HCl [pH 7.4], 2 mM EDTA, 1 mM DTT, complete proteinase inhibitor cocktail, and PhosSTOP). For immunoprecipitation of the GFP-fused NUP107, mouse anti-GFP antibody (11-814-460-001, Roche Diagnostics) and Protein G Sepharose beads (17-0618-01, GE Healthcare) were added. After incubation for 2 hr at 4°C, the beads were washed with wash buffer (lysis buffer diluted 3.75× in dilution buffer). After the protein-bound beads were boiled, they were run on an SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore). Membranes prepared in this manner were incubated in 0.2% Casein in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for blocking. The membrane was probed with rabbit anti-GFP primary antibody (598, MBL) diluted at 1:1,000 and mouse anti-NUP133 (M00055746-M01, Abnova) diluted at 1:500 followed by secondary antibodies HRP-rabbit anti-rat IgG (A5795, Sigma-Aldrich) and HRP-goat anti-mouse IgG (170-6516, Bio-Rad) both diluted at 1:3,000 with 0.2% Casein in TBS-T. For obtaining protein signals, Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used as a chemiluminescence substrate.

Subcellular Localization of NUP107

HeLa cells cultured in DMEM (Life Technologies) containing 10% fetal bovine serum (Sigma-Aldrich) at 37°C in an atmosphere of 5% CO_2 on poly-L-lysine-coated coverslips (Wako) were transfected with the wild-type or altered N-terminally GFP-fused NUP107 vector with the use of Viafect (Promega). After incubation for 48 hr, the cells were washed with pre-warmed PBS at 37°C and then fixed with pre-warmed 2% paraformaldehyde (Wako) in PBS at 37°C for 10 min. The cells were treated with 0.5% Triton X-100 in PBS for 2.5 min and then incubated with 5% normal goat serum (NGS, Merck Millipore) in PBS for 1 hr. After blocking, the cells were reacted with the primary antibody (MAb414 [mouse anti-nuclear pore complex (NPC) proteins], MMS-120P, Covance) diluted at 1:3,000 in 1% NGS in PBS for 2 hr, washed with PBS, and then reacted with the secondary antibody (Alexa Fluor 594 goat anti-mouse IgG, A11032, Life Technologies) in 1% NGS in PBS for 2 hr. After staining, the cells were mounted in paraphenylenediamine solution (80% glycerol in PBS and 1 mg/ml paraphenylenediamine, 11873580001, Roche Diagnostics). Images were captured with a DeltaVision microscope (Applied Precision) equipped with a Plan Apo objective lens (100×, 1.35 NA, Olympus) and a Cool Snap HQ2 CCD camera (Photometrics).

Zebrafish Knockdown by Microinjection of Morpholino Oligonucleotides

The antisense morpholino oligonucleotides (MOs) for *nup107* translation blocking (TB) (5′-AAGTCTGACTCCATTCCATATT GTC-3′)[20] and for *nup107* splice blocking (SB) (5′-ATACATTTA AGCTCACCTCTCTGAC-3′) and a standard MO control (5′-CCT CTTACCTCAGTTACAATTTATA-3′) obtained from Gene Tools were injected into 1- to 2-cell-stage embryos, each at a final concentration of 0.25 mM. The experiment was authorized by the Institutional Committee for Fish Experiments at the National Research Institute of Fisheries Science.

RNA Isolation and RT-PCR Analysis

Total RNA was extracted from embryos at 24 hr post-fertilization (hpf) with TRIzol reagent according to the manufacturer's (Life Technologies) protocol. Double-stranded cDNA was synthesized with M-MLV reverse transcriptase (Promega) and then amplified by PCR with ExTaq (Takara). For detecting the splicing mutation (caused by the MO injections) in *nup107* exon 24, the following primers were used: 5′-TGAACTGTCCTCCGGTGAAG-3′ (forward) and 5′-TGCGATGATGTCAGCAAGAC-3′ (reverse). For the PCR amplifications, the initial denaturing step at 94°C for 5 min was followed by 29 cycles of 30 s at 94°C, 30 s at 61°C, 30 s at 72°C, and a final extension of 7 min at 72°C. PCR products were separated on 3% agarose gels.

Histopathology and Transmission Electron Microscopy of Zebrafish

Larvae injected with control MO, *nup107*-TB MO, and *nup107*-SB MO at 5.5 days after fertilization were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C overnight. After fixation, the samples were washed three times with 0.1 M cacodylate buffer for 30 min each and then postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for 3 hr. The samples were dehydrated in graded ethanol solution (50%, 70%, 90%, and 100%), infiltrated with propylene oxide (PO) two times for 30 min each, immersed in a 70:30 mixture of PO and resin (Quetol-812, Nisshin EM) for 1 hr, and then kept in an open-capped tube so that volatile PO would evaporate overnight. The samples were transferred to fresh 100% resin and polymerized at 60°C for 48 hr. The polymerized resins were cut into semi-thin (1.5-μm) sections with an Ultracut UCT (Leica) and then stained with 0.5% toluidine blue. Ultra-thin (70-nm) sections were cut on an Ultracut UCT (Leica) ultramicrotome and mounted on copper grids. The sections were stained with 2% uranyl acetate at room temperature for 15 min, washed with distilled water, and stained with lead stain solution (Sigma-Aldrich) at room temperature for 3 min. The grids were observed with a transmission electron microscope (JEM-1400Plus, JEOL) at 80 kV.

Molecular-Dynamics Simulation of the p.Asp831Ala Substitution in NUP107

Molecular-dynamics (MD) simulations of the wild-type and p.Asp831Ala Nup107 were carried out with the program package GROMACS (Groningen Machine for Chemical Simulation) version 5.0 with the Optimized Potentials for Liquid Simulations all-atom force field based on the local Møller-Plesset perturbation theory (OPLS-AA/L).[21] The starting structure of NUP107 was extracted from the crystal structure of the NUP107-NUP133 complex (PDB: 3CQC). The missing regions in NUP107 were modeled with the Phyre2 modeling server,[22] and the p.Asp831Ala substitution was introduced with FoldX software.[23] The wild-type and altered NUP107 molecules were solvated with simple-point-charge water molecules in a cubic box extending at least 1.0 nm from the protein surface. Sodium ions were added to neutralize the systems, which were then subjected to energy minimization for 50,000 steps by steepest descent. The minimized systems were then equilibrated by position-restrained MD simulation for soaking the water molecules in the macromolecules in two steps as follows: an NVT ensemble (constant number of particles, volume, and temperature) for 100 ps and an NPT ensemble (constant number of particles, pressure, and temperature) for 4,000 ps each at 310 K. The well-equilibrated systems were then subjected to MD simulations for 30 ns each at 310 K without any restrictions. In all simulations, for maintaining a constant temperature of 310 K, temperature coupling using velocity rescaling with a stochastic term[24] was employed with a coupling constant τ of 0.1 ps. Van der Waals interactions were modeled with 6–12 Lennard-Jones potentials with a 1.4-nm cutoff. Long-range electrostatic interactions were calculated with the particle-mesh Ewald method[25] with a 1.4-nm cutoff for the real-space term. Covalent bonds were constrained with the LINCS algorithm.[26]

Results

Pathogenic Mutations Detected by WES

To identify the genetic cause of early-onset SRNS, we performed WES on 18 probands. Because we found multiple affected siblings in ten families, we speculated on an autosomal-recessive inheritance pattern for SRNS and focused on the recessive variants shared by two or more families with well-performed WES data (Tables S1–S3, S4, and S5). Biallelic mutations in *NUP107*, which encodes NUP107, were common in five families, and the mutation co-segregated perfectly with the affected state in all five families (Figure 1A, Table 1, and Figure S1). None of the other families in our cohort had any pathological variants in *NUP107* or any other known genes associated with SRNS, as listed in Table S6.

We identified a total of four *NUP107* mutations, including two missense mutations (c.469G>T [p.Asp157Tyr] and c.2492A>C [p.Asp831Ala]), one 5-bp deletion (c.1079_1083delAAGAG [p.Glu360Glyfs*6]), and one splice-donor-site mutation (c.969+1G>A) (Table 2). Heterozygous c.2492A>C was common in all five families. The two missense mutations altered evolutionally conserved amino acids (Figure S2) and were predicted to be pathogenic by web-based programs PolyPhen-2 and MutationTaster (Table 2). Furthermore, p.Asp831Ala resides within the Nup84-Nup100 domain (Figure S3). The 5-bp deletion was subjected to nonsense-mediated mRNA decay and probably led to a lack of protein synthesis (Figure S4). The splicing mutation (c.969+1G>A) causes a loss of the intrinsic splicing donor site (Figure S5). All four variants were examined in the EVS, ExAC Browser, HGVD, and in-house Japanese exome database (n = 575). The c.1079_1083delAAGAG variant was observed at frequencies of 0.0000083 in the ExAC Browser and 0.0008696 in the in-house Japanese exome data. Another variant, c.2492A>C, was observed at a frequency of 0.0013587 only in HGVD, but not in the EVS, ExAC Browser, or in-house Japanese exome data (Table 2). The other mutations (c.469G>T and c.969+1G>A) were never observed in any of four variant databases. Among 881 *NUP107* variants registered in the ExAC Browser, a total of 31 variants with a MAF $\geq$ 0.005 were in non-coding regions (intronic but not in canonical acceptor or donor sites or UTRs) or were synonymous variants (Table S7). Furthermore, 36 loss-of-function variants in *NUP107* are not homozygous (all heterozygous; Table S8). Therefore, this genetic