Brief Report

Targeted sequencing approach to identify genetic mutations in Nasu-Hakola disease

Jun-ichi Satoh*, **Motoaki Yanaizu, Youhei Tosaki, Kenji Sakai, Yoshihiro Kino**

Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, Tokyo, Japan.

Summary	**Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder characterized by sclerosing leukoencephalopathy and multifocal bone cysts, caused by a loss-of-function mutation of either *TYROBP (DAP12)* or *TREM2*. TREM2 and DAP12 constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Premortem molecular diagnosis of NHD requires genetic analysis of both *TYROBP* and *TREM2*, in which 20 distinct NHD-causing mutations have been reported. Due to genetic heterogeneity, it is often difficult to identify the exact mutation responsible for NHD. Recently, the revolution of the next-generation sequencing (NGS) technology has greatly advanced the field of genome research. A targeted sequencing approach allows us to investigate a selected set of disease-causing genes and mutations in a number of samples within several days. By targeted sequencing using the TruSight One Sequencing Panel, we resequenced genetic mutations of seven NHD cases with known molecular diagnosis and two control subjects. We identified homozygous variants of *TYROBP* or *TREM2* in all NHD cases, composed of a frameshift mutation of c.141delG in exon 3 of *TYROBP* in four cases, a missense mutation of c.2T>C in exon 1 of *TYROBP* in two cases, or a splicing mutation of c.482+2T>C in intron 3 of *TREM2* in one case. The results of targeted resequencing corresponded to those of Sanger sequencing. In contrast, causative variants were not detected in control subjects. These results indicate that targeted sequencing is a useful approach to precisely identify genetic mutations responsible for NHD in a comprehensive manner.**

Keywords: DAP12, Nasu-Hakola disease, targeted sequencing, TREM2, TYROBP

1. Introduction

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL; OMIM 221770), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts (*1,2*). Although NHD patients are clustered in Japan and Finland, approximately 200 NHD cases are presently reported worldwide.

**Address correspondence to:*
Dr. Jun-ichi Satoh, Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo, Japan.
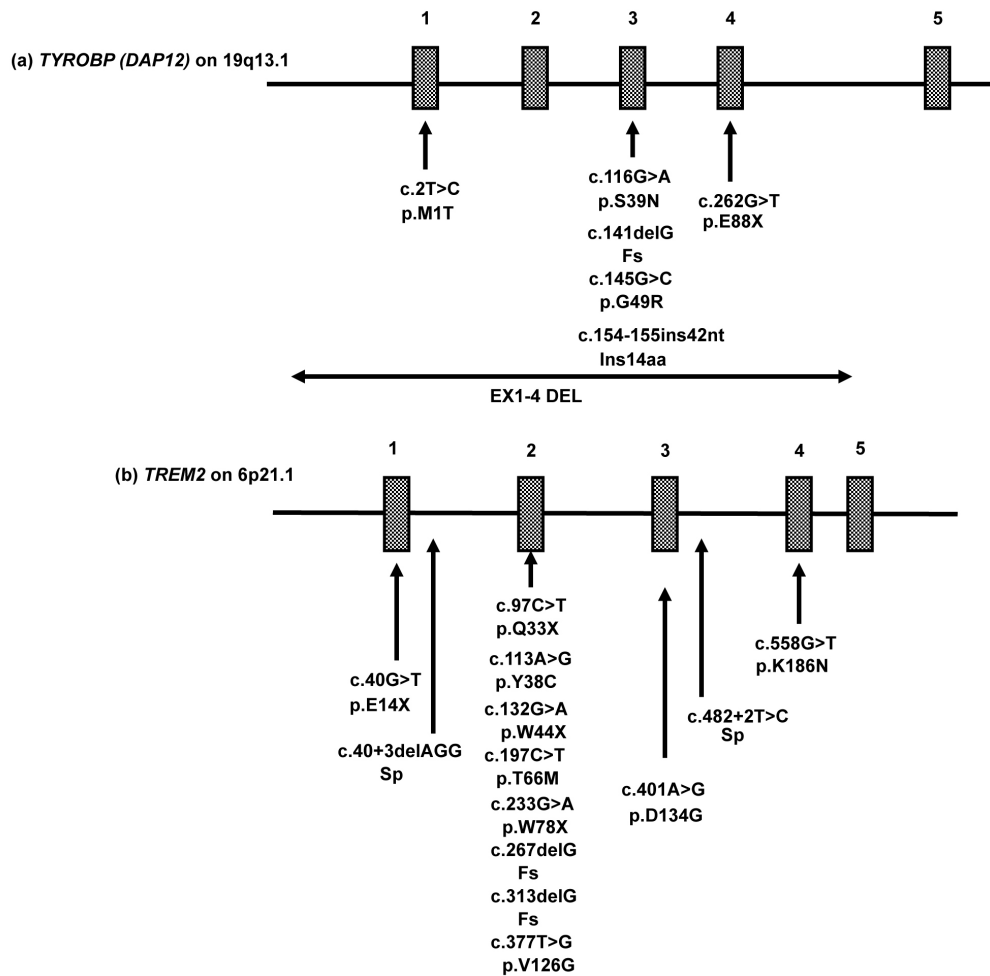E-mail: satoj@my-pharm.ac.jp

Clinically, patients show pathological bone fractures during the third decade of life, and a frontal lobe syndrome during the fourth decade of life, followed by progressive dementia and death until the fifth decade of life (*3*). Pathologically, the brains of NHD patients exhibit extensive demyelination, accumulation of axonal spheroids, microglia activation and intense astrogliosis predominantly in the white matter of frontal and temporal lobes and the basal ganglia (*4*).

NHD is caused by various homozygous loss-of-function mutations located in one of two genes, TYRO protein tyrosine kinase-binding protein (*TYROBP*), alternatively named DNAX-activation protein 12 (*DAP12*) on chromosome 19q13.1 or triggering receptor expressed on myeloid cells 2 (*TREM2*) on chromosome 6p21.1 (*5,6*). TREM2 and DAP12 constitute a receptor/ adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia

Figure 1. Twenty different mutations causative of NHD. NHD-causing mutations in **(a)** *TYROBP (DAP12)* or **(b)** *TREM2* are shown with genetic and protein definitions and the position of exons.

(*7*). Premortem diagnosis of NHD requires genetic analysis of both *TYROBP* and *TREM2*. Up to the present, a panel of 20 different NHD-causing mutations was reported (Figure 1) (*8,9*). Due to genetic heterogeneity, it is often difficult to identify the exact mutation responsible for NHD. Furthermore, homozygous or compound heterozygous mutations, comprised of c.40+3delAGG, p.Q33X, p.Y38C, p.T66M, p.D86V and p.W198X in *TREM2*, cause frontotemporal dementia (FTD)-like syndrome without bone involvement (*10,11*). Importantly, accumulating evidence indicates that heterogeneous variants of p.R47H and p.R62H in *TREM2*, confer a substantial risk for development of late-onset Alzheimer's disease (LOAD) (*11,12*).

Recently, the revolution of the next-generation sequencing (NGS) technology has had a great impact on the field of genome research. Targeted sequencing with focused gene panels, which are designed to include genomic regions causative of genetic diseases, allows us to investigate rapidly and efficiently a selected set of candidate genes in many samples (*13*). By using the TruSight One Sequencing Panel, we resequenced genetic mutations of seven NHD cases with known

molecular diagnosis.

2. Materials and Methods

2.1. *Ethics*

The Human Research Ethics Committee (HREC) of the Meiji Pharmaceutical University (MPU) approved this study (No. 1904, 2015), which follows the Ethical Guidelines for Analytical Research on the Human Genome/Genes, Japan. Written informed consent was taken from all participants.

2.2. *Targeted resequencing*

All patients showed clinical characteristics of NHD, such as multiple bone cysts and diffuse leukoencephalopathy. Before targeted sequencing, the causative mutations were characterized by Sanger sequencing of the *TYROBP* or *TREM2* gene. Fifty ng of genomic DNA extracted from peripheral blood mononuclear cells (PBMC) was processed for DNA library preparation by using MiSeq Reagent Kit V3 (Illumina, San Diego,
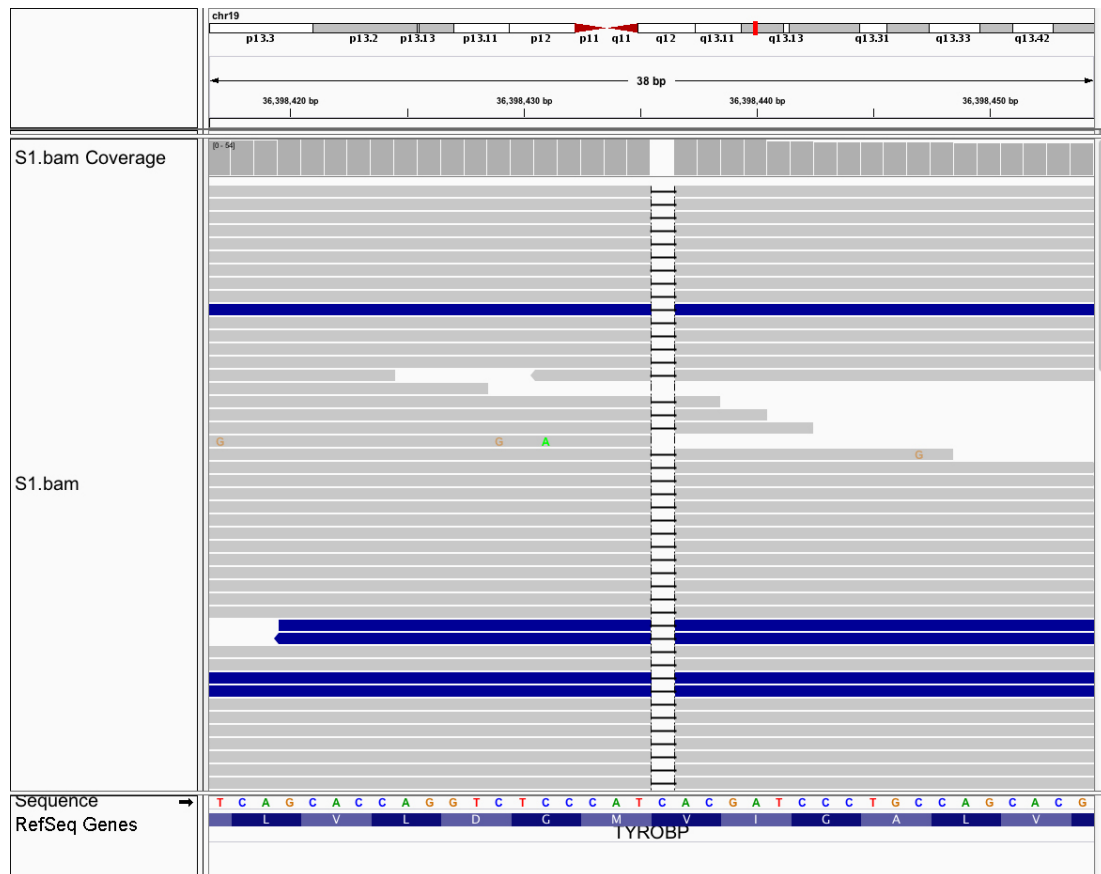
Figure 2. A frameshift mutation of c.141delG in exon 3 of *TYROBP*. By targeted resequencing, we identified a frameshift mutation of c.141delG in exon 3 of *TYROBP*, causing premature termination at amino acid residue 52 in four NHD cases. The genetic mutation was visualized by importing the sequence alignment data into IGV.

CA, USA) and target enrichment by the TruSight One Sequencing Panel that contains coding regions of 4,813 genes associated with known clinical phenotypes (Illumina). The genes were selected from the Human Gene Mutation Database (HGMD; *http://www.hgmd. cf.ac.uk*), the Online Mendelian Inheritance in Man database (OMIM; *http://omim.org/search/advanced/ entry*), the GeneTests database (*http://www.genetests. org*), the Illumina TruSight Exome content set, and other available sequencing panels. The full list of the genes is available online (*http://www.illumina.com/content/ dam/illumina-marketing/documents/products/gene_lists/ gene_list_trusight_one.zip*). The concentration of target DNA was quantified by Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The prepared library was loaded on the Flowcell of MiSeq (Illumina) for sequencing.

2.3. *Processing of sequencing data*

The sequencing data, composed of 150 bp paired-end reads, were processed for MiSeq Reporter (Illumina), which is endowed with Burrows-Wheeler Aligner (BWA) for alignment on a reference sequence of hg19 and Genome Analysis Toolkit (GATK) for variant calling. Then, the Variant Call format (VCF) file was

processed for analyzing on VariantStudio (Illumina), which contains annotation databases, such as the ClinVar database (ClinVar; *http://www.ncbi.nlm.nih.gov/clinvar*), OMIM, the dbSNP database (dbSNP; *http://www.ncbi. nlm.nih.gov/SNP*), the Polymorphism Phenotyping v2 database (Polyphen-2; *http://genetics.bwh.harvard.edu/ pph2*), and the Sorting Intolerant from Tolerant database (SIFT; *http://sift.jcvi.org*). The genetic mutations were visualized by importing a BAM format file into Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA, USA).

3. Results and Discussion

Variant calling data enclosed in a VCF file were filtered by VariantStudio under the condition of homozygous mutations, quality value > 1000, read depth > 30, and only variants with ClinVar annotation. Pathogenic and probably pathogenic mutations with ClinVar significance were selected. Benign mutations on PolyPhen-2 and tolerated mutations on SIFT were excluded. Finally, we were able to narrow down the set of disease causing-mutations in all NHD cases, composed of a frameshift mutation of c.141delG in exon 3 of *TYROBP* (*14*), causing premature termination at amino acid residue 52 in four cases (Figure 2), a missense mutation of c.2T>C
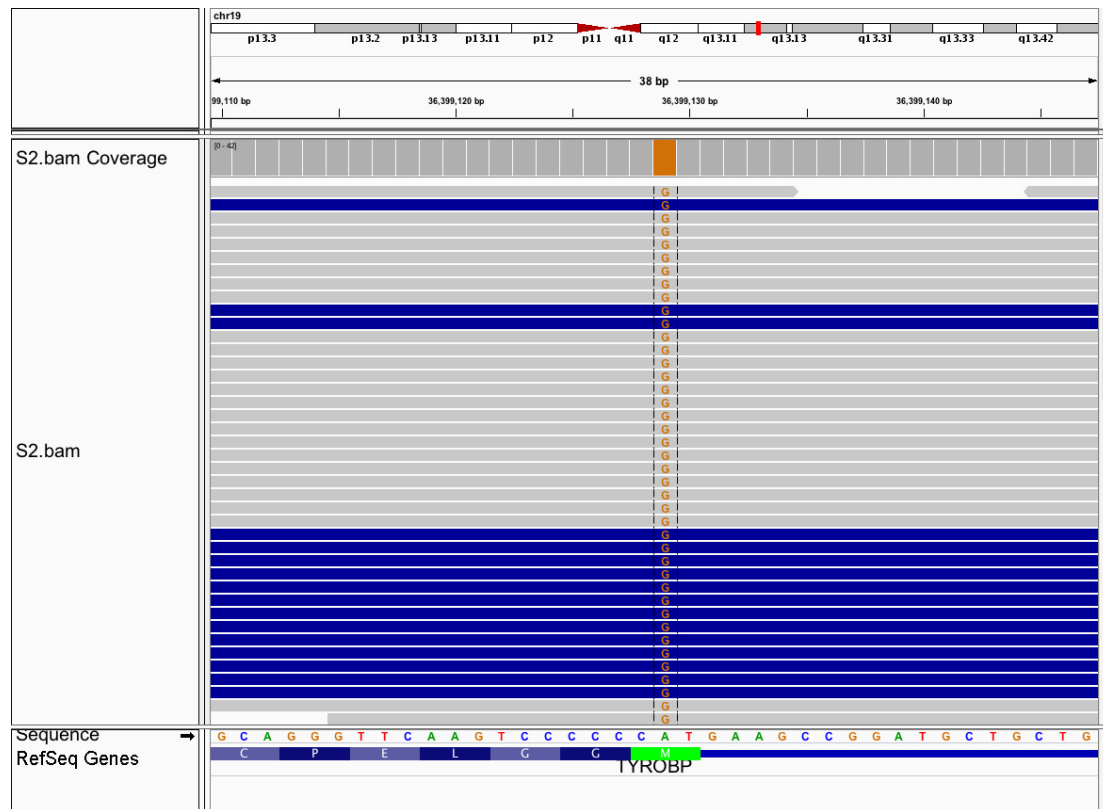
Figure 3. A missense mutation of c.2T>C in exon 1 of *TYROBP*. By targeted resequencing, we identified a missense mutation of c.2T>C at the initiation codon in exon 1 of *TYROBP* in two NHD cases. The genetic mutation was visualized by importing the sequence alignment data into IGV.

at the initiation codon in exon 1 of *TYROBP* (*14*) in two cases (Figure 3), or a splicing mutation of c.482+2T>C at the splice-donor consensus site in intron 3 of *TREM2* (*15*) in one case (Figure 4). The entire process was completed within a couple of days. The results of targeted resequencing corresponded completely to those of Sanger sequencing. Causative variants were not detected in control subjects.

In the present study, by targeted sequencing on a commercially available focused gene panel, we resequenced *TYROBP* or *TREM2* mutations in seven NHD cases with known molecular diagnosis. We identified genetic variants in all seven NHD cases, which were previously validated by Sanger sequencing, indicating that this approach is highly reliable and reproducible. Thus, targeted sequencing provides a rapid, convenient, high throughput, and comprehensive platform for molecular diagnosis of NHD in a clinical setting. Furthermore, targeted sequencing is capable of detecting novel unpredicted variants located in focused genes, including noncausative but disease-modifying ones. In contrast, Sanger sequencing of individual genes and exons is often laborious with low throughput, although it does not require expensive NGS machines. With respect to the disadvantage, the results of targeted sequencing are derived exclusively from a limited set of focused genes. Furthermore, this approach could not detect noncoding or deep intronic mutations and

copy number variations. However, targeted sequencing produces a much smaller dataset size, compared with that of whole genome or whole exome sequencing, making interpretation of the data more concise (*16*).

A recent study sequenced genomic DNA of 79 patients with sporadic inclusion body myositis (sIBM) on a panel of 38 target genes, and identified 27 rare coding missense variants with those including disease-causing mutations in the valosin containing protein (*VCP*) gene, suggesting that targeted sequencing is a clinically meaningful approach for genetic evaluation of sIBM (*17*). More recently, targeted sequencing with the TruSight One sequencing panel, causative mutations were found in six of 17 families of pediatric patients with various genetic diseases, such as Sotos syndrome, Joubert syndrome, and neurofibromatosis type 1A (*16*). In different studies, targeted sequencing with the TruSight One Sequencing Panel showed somatic mosaicism of a disease-causing mutation in a patient with CDKL5-related encephalopathy (*18*), and served as a prenatal diagnostic tool for LAMA2-related muscular dystrophy (*19*). Furthermore, targeted sequencing with the TruSight One Sequencing Panel identified three distinct rare homozygous and compound heterozygous variants causative of nephronophthisis-related ciliopathy (NPHP-RC), an autosomal recessive cystic kidney disease, in three patients (*20*).

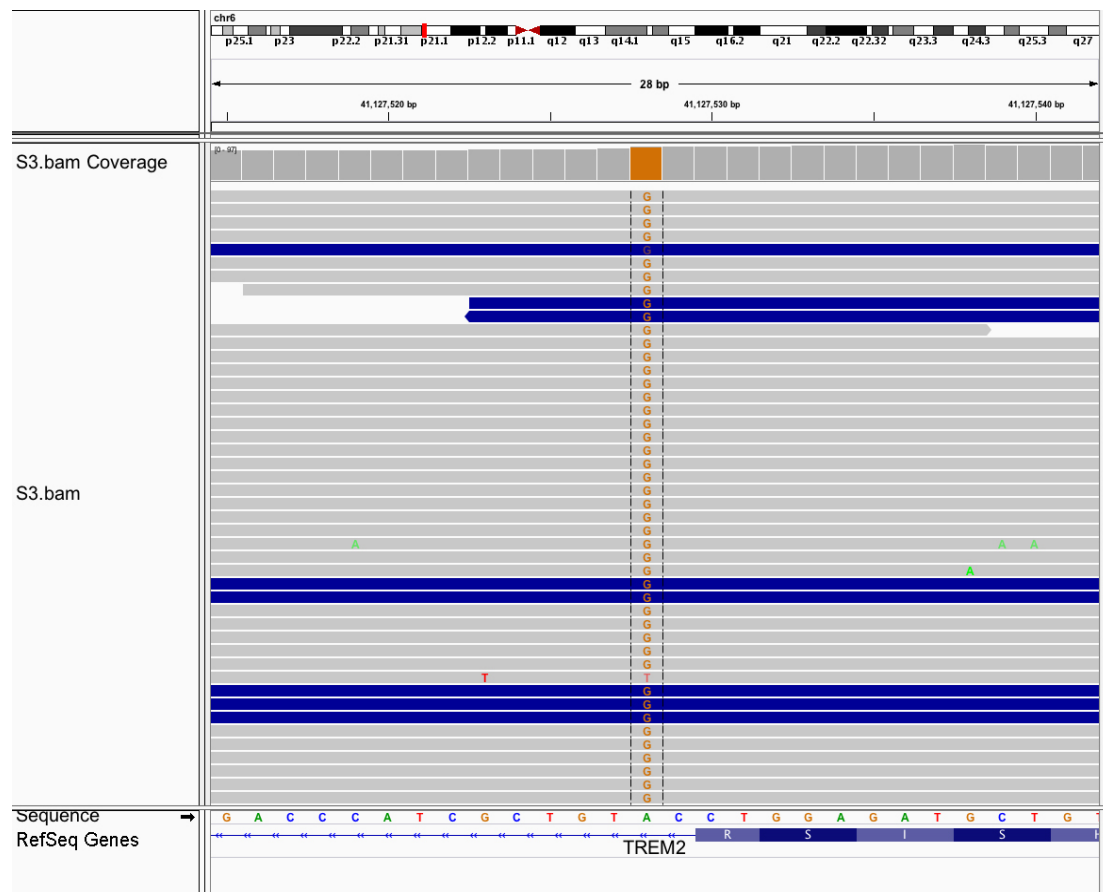In conclusion, targeted sequencing is a useful

Figure 4. A splicing mutation of c.482+2T>C in intron 3 of *TREM2*. By targeted resequencing, we identified a splicing mutation of c.482+2T>C at the splice-donor consensus site in intron 3 of *TREM2* in one NHD case. The genetic mutation was visualized by importing the sequence alignment data into IGV.

approach for the exact molecular diagnosis of rare Mendelian diseases, including NHD, by investigating genetic mutations in a comprehensive manner.

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