Targeted exome sequencing and chromosomal microarray for the molecular diagnosis of nevoid basal cell carcinoma syndrome

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1. Introduction

Nevoid basal cell carcinoma syndrome (NBCCS; MIM# 109400), also known as Gorlin syndrome, is an autosomal dominant disorder exhibiting multiple BCCs, palmar-plantar pits, keratocystic odontogenic tumors (KCOTs), calcification of the falx cerebri, and skeletal abnormalities [1]. *PTCH1* (MIM# 601309), mapped to 9q22.32 and consisting of 23 exons encoding 1447 amino acids, has been identified as a main causative gene for NBCCS [2,3].
PTCH1 protein is a receptor for sonic hedgehog (Shh), and activation of the Shh pathway due to inactivation of PTCH1 protein may be relevant to NBCCS phenotypes [2].

Although we had performed polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP) analysis and Sanger sequencing in 20 cases of NBCCS following the previous report [4], we failed to detect any PTCH1 mutations in 8 of 20 cases (40%) of NBCCS by the analyses. As the detection rates of germline PTCH1 mutations in cases of NBCCS were reported to be 40–75% by conventional methods, such as PCR–SSCP analysis, PCR-based direct sequencing of all exons, or denaturing high performance liquid chromatography [5–7], our detection rate of 60% was similar to those results. This failure to detect mutations may be due to copy number alterations (CNAs) of the regions over one exon of PTCH1 [8,9], the presence of mutations located outside exons and exon-intron boundaries of PTCH1, or mutations in other candidate genes associated with the Shh pathway, such as mutations within introns or regulatory regions of PTCH1, or mutations in other candidate genes except PTCH2 and SUFU [10–12].

Recently, next-generation sequencing (NGS) has been adopted in clinical testing for patients with congenital diseases suspected to be genetic in origin including NBCCS, because single-nucleotide variations (SNVs) and small insertions/deletions (indels) as well as gross CNAs involved in a defect of disease-causing genes can be simultaneously screened using whole exome sequencing or targeted exome sequencing (TES) in a time- and cost-effective manner [9]. In this study, we performed a comprehensive TES-based genetic analysis using a multi-gene panel, including PTCH1, PTCH2, SUFU, and other shh-related genes except SMO and STK36 in 8 cases with no mutations detected by previous PCR–SSCP analysis and 2 recently enrolled cases of NBCCS (Fig. 1), gross deletions detected in PTCH1 by TES were examined in detail by chromosomal microarray (CMA) analysis.

2. Materials and methods

2.1. Cases analyzed by TES analysis

We analyzed 10 Japanese cases with a clinical diagnosis of NBCCS based on the presence of diagnostic criteria of Kimonis (Table 1, Supplementary Fig. S1) [13]. In 8 cases, no PTCH1 mutations were detected by our previous PCR–SSCP analysis (Fig. 1). In the remaining 2 recent new cases, we performed TES analysis without PCR–SSCP analysis in advance. The molecular diagnosis was performed using genomic DNA extracted from the patient’s whole blood after obtaining informed consent. This study was approved by the ethical committees of Tokushima University.

2.2. TES by NGS and data analyses

We used MiSeq bench-top sequencer (Illumina, San Diego, CA, USA) to perform NGS with a TruSight One Sequencing Panel (Illumina), which provides for the simultaneous targeted sequencing of the exon regions of 4813 clinically relevant genes. The alignments of sequencing reads to the human reference genome (GRC37/hg19), duplicate read removal, local realignment around indels, base quality score recalibration, variant calling and annotation were performed as previously described [14]. To identify single nucleotide variants (SNVs), we excluded sequence variants with minor allele frequencies (>0.01) included in various human genome variation databases, as previously described [14] and an integrative Japanese Genome Variation Database (iJGVD, https://ijgvd.megabank.tohoku.ac.jp) [15]. To complement the SNVs and indel analyses, detection of CNAs using TES data with a resolution of a single exon to several exons, depending on the size of exons, was performed using DNAcopy (R/Bioconductor; http://bioconductor.org) [16] and eXome-Hidden Markov Model v1.0 (XHMM, https://atgu.mgh.harvard.edu/xhmm/) as previously described [17].

Candidates for pathogenic variants were confirmed by Sanger sequencing. The pathogenicity of missense variants was assessed using tools for prediction of possible impact of amino acid substitution on the structure and function of human protein such as MutationTaster (http://www.mutationtaster.org/index.html), PolyPhen-2 version 2.2.2r398 (http://genetics.bwh.harvard.edu/pph2/index.shtml), and PROVEAN tool (http://provean.jcvi.org/). Identified alterations were also evaluated by comparison with known alterations reported in mutation databases such as the Human Genome Mutation Database (HGMD) professional 2016.1 (http://www.hgmd.cf.ac.uk/ac/index.php) and ClinVar (http://www.ncbi.nlm.nih.gov/clinvar).

Fig. 1. Flow chart of this study.
2.3. Chromosomal microarray (CMA) analysis

CNA validation and detailed mapping of altered regions were performed using the CytoScan HD array platform (Affymetrix, Santa Clara, CA, USA), which provides 750,000 polymorphic (single-nucleotide polymorphism, SNP) and 1950,000 non-polymorphic (CNA) markers as described previously [9,17]. The raw data were analyzed using Affymetrix Chromosome Analysis Suite (ChAS) Software and the output data were interpreted with the UCSC Genome Browser (http://genome.ucsc.edu). The disease-related genes were selected based on the information from Online Mendelian Inheritance in Man (OMIM, http://www.omim.org/).

3. Results

All 10 cases satisfied clinical diagnostic criteria of NBCCS (Table 1). Out of 10 cases, specific SNVs or small indels of PTCH1 causing inferred amino acid changes were identified in 4 cases, whereas CNAs within or around PTCH1 were found in 3 cases (Fig. 1, Table 2). SNVs or CNAs were detected by TES in 5 of 8 cases, in which possible causative SNVs were not detected by previous PCR–SSCP analysis (Fig. 1, Table 2). In the remaining 3 cases, however, no possible disease-causing genetic alterations were detected in PTCH1, PTCH2, SUFU, and other shh-related genes except SMO and STK36, which are not included in TruSight One.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Major criteria</th>
<th>Minor criteria</th>
<th>Other clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>M</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>F</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>F</td>
<td>Dozens</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>M</td>
<td>Face, abdomen,</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>F</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>F</td>
<td>Forehead, axilla,</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>62</td>
<td>F</td>
<td>Forehead, axilla,</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>41</td>
<td>F</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>35</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>M</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 1
Clinical features of NBCCS cases in this study.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>PTCH1 mutation detected by PCR–SSCP analysis</th>
<th>Altered gene(s) detected by TES</th>
<th>Mutation of PTCH1</th>
<th>Type</th>
<th>Nucleic acid change</th>
<th>Amino acid change</th>
<th>Exon</th>
<th>Mutation-Taster prediction (score)</th>
<th>PolyPhen-2 prediction (score)</th>
<th>PROVEAN tool prediction (score)</th>
<th>Reported as pathogenic alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>No</td>
<td>Gross deletion</td>
<td>All</td>
<td>NA</td>
<td>NA</td>
<td>14</td>
<td>Disease causing (1)</td>
<td>NA</td>
<td>Neutral (-2.287)</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>No</td>
<td>Nonsense</td>
<td>c.2198C&gt;G</td>
<td>p.S733X</td>
<td>14 Disease causing (1)</td>
<td>NA</td>
<td>Neutral (-2.287)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>PTCH1</td>
<td>Gross deletion</td>
<td>12–15</td>
<td>NA</td>
<td>NA</td>
<td>9</td>
<td>Disease causing (1)</td>
<td>Probably damaging (0.996)</td>
<td>Deleterious (-5.048)</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>No</td>
<td>PTCH1</td>
<td>Missense</td>
<td>c.1311G&gt;T</td>
<td>p.S438I</td>
<td>9 Disease causing (1)</td>
<td>NA</td>
<td>Neutral (-2.287)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>No</td>
<td>No</td>
<td>Gross deletion</td>
<td>All</td>
<td>NA</td>
<td>NA</td>
<td>12</td>
<td>Disease causing (1)</td>
<td>NA</td>
<td>Deleterious (-12.476)</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>No</td>
<td>PTCH1</td>
<td>2-bp deletion</td>
<td>c.3487_3488delinsAATGGGCC</td>
<td>p.G1163delinsNGP</td>
<td>21 Disease causing (0.999)</td>
<td>NA</td>
<td>Deleterious (-5.048)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>PTCH1</td>
<td>Gross deletion</td>
<td>All</td>
<td>NA</td>
<td>NA</td>
<td>12</td>
<td>Disease causing (1)</td>
<td>NA</td>
<td>Deleterious (-12.476)</td>
<td>No</td>
</tr>
</tbody>
</table>

PCR–SSCP, polymerase chain reaction–single strand conformation polymorphism; NT, not tested; NA, not available.
sequencing panel, even though these 3 cases satisfied the same diagnostic criteria as the other 7 cases (Table 1).

3.1. Causative SNVs and indels identified in cases with NBCCS by TES

3.1.1. Case 3

In case 3, we identified a nonsense mutation in \textit{PTCH1} (p.S733X) caused by a 1 base substitution at nucleotide 2198, which was predicted to be a loss-of-function mutation, likely leading to an aberrant mRNA targeted for degradation via nonsense-mediated decay (NMD) or resulting in a truncated protein.

3.1.2. Case 7

In case 7, we identified a missense mutation in \textit{PTCH1} (p.S438I) caused by a 1 base substitution at nucleotide 1313 in exon 9. This mutation was predicted to be “deleterious” and “damaging” by PROVEAN tool and PolyPhen-2, respectively. The same alteration was detected in the affected daughter, although genetic analysis has never been performed in the affected son (Supplementary Fig. S1). Therefore, we concluded this alteration to be responsible for NBCCS, even though this mutation has not been reported in HGMD or ClinVar.

3.1.3. Case 9

In case 9, we identified an in-frame indel, a 2-bp deletion and 8-bp insertion (c.3487_3488delinsAATGGGCC) in exon 21, resulting in the inclusion of two amino acids (p.G1163delinsNGP) within the transmembrane domain. This mutation was predicted as “deleterious” by PROVEAN tool. Therefore, we concluded this alteration to be responsible for NBCCS, even though this mutation has not been reported in HGMD or ClinVar.

3.1.4. Case 10

In case 10, we identified a possibly deleterious frameshift mutation (p.T557Sfs*69) creating a new stop codon at codon 625 by a 2-bp deletion at nucleotides 1670-1671 in exon 12. As this mutation was previously reported to be causative for NBCCS by Takahashi et al. [18], we concluded this alteration to be responsible for NBCCS.

3.2. Causative CNAs identified in cases with NBCCS by TES

In 3 cases, we were unable to identify any causative SNVs or indels within the possible target genes, but rather detected entire or intragenic \textit{PTCH1} deletions resulting in loss-of-function of this gene by TES analysis (Table 2, Fig. 2). All these deletions failed to be detected by previous PCR–SSCP analysis. As TES is able to detect CNAs only on targeted exons and unable to correctly determine an altered copy number, CMA analysis was subsequently performed to validate these deletions and to determine copy number and detailed genomic location of the deleted regions (Fig. 3A–C).

3.2.1. Case 2 and case 8

In cases 2 and 8, we detected gross hemizygous deletions including the entire \textit{PTCH1} gene. In case 2, we identified an approximately 4.06 Mb deletion (chr9:97,848,293–101,911,452) containing 90 target exons including all exons of \textit{PTCH1} by TES. Hemizygous deletion was confirmed by CMA, and the precise size of the deletion determined by CMA was approximately 4.3 Mb (chr9:97,637,037–101,936,873) containing 50 RefSeq genes (24 disease-related genes) from C9orf3 to TGFBR1 (Fig. 3A). In case 8, we identified an approximately 1.22 Mb deletion (chr9:97,848,293–99,064,223) containing 50 target exons including all exons of \textit{PTCH1} by TES. Hemizygous deletion was confirmed by CMA, and the precise size of the deletion determined by CMA was approximately 2.22 Mb (chr9:97,470,295–99,685,607) containing 25 RefSeq genes (9 disease-related genes) from C9orf3 to LOC441454 (Fig. 3B). These alterations have not been reported in HGMD or ClinVar. Array-based karyotype results of cases 2 and 8 were arr[hg19] 9q22.32(97,637,037_101,936,873) \times 1 and arr[hg19] 9q22.32(97,470,295_99,685,607) \times 1, respectively.

3.2.2. Case 5

In case 5, we detected an approximately 8.92 kb intragenic deletion of \textit{PTCH1} from exon 12 to exon 15 (chr9:98,229,388–98,238,306) by TES. Hemizygous deletion within \textit{PTCH1} of approximately 7.93 kb (chr9:98,227,309–98,235,234) was confirmed by CMA. Array-based karyotype results of case 5 was arr[hg19] 9q22.32(98,227,309_98,235,234) \times 1, although the location and size of the deleted region may be underestimated or overestimated due to uneven coverage of probes on CMA (Fig. 3C). These alterations have not been reported in HGMD or ClinVar.

4. Discussion

In this study, we applied TES using a TruSight One Sequencing Panel based on NGS technology to simultaneously investigate the
sequence and copy number status of all exons of candidate disease-causing genes, including known three causative genes, PTCH1, PTCH2, and SUFU, in 10 cases with a clinical diagnosis of NBCCS. In 2 cases (cases 9 and 10) that have not been analyzed by PCR–SSCP analysis, we detected small PTCH1 indels. In 8 cases (cases 1–8) where we were not able to detect any PTCH1 mutations by previous PCR–SSCP analysis and Sanger sequencing, PTCH1 small deletions/insertions were identified by NGS and Sanger sequencing in 2 cases (cases 3 and 7). This is because the causative exons have not been checked due to no obvious pattern differences by PCR–SSCP analysis. In 3 cases (cases 2, 5 and 8), we identified gross deletions, which were not detected by conventional methods. However, no mutation was detected within possible disease-causing genes in shh pathway even by TES analysis in 3 cases (cases 1, 4 and 6), although SMO and STK36, which have never been identified as causative genes for NBCCS, are not included in our sequencing panel. In these cases, mutations may exist outside of the analyzed regions such as those within introns or regulatory regions of the candidate genes. In addition, it is difficult to exclude the possibility of somatic mosaicism occurred in causative genes, although only one case of possible type 2 mosaicism of PTCH1 in NBCCS has been reported [19] and none of our mutation-negative cases showed signs of segmental distribution of BCCs, pits, and so on. Further examinations using additional techniques, such as whole genome sequencing and ultra deep sequencing or digital PCR, are needed to detect variations in these mutation-negative cases.

Although there was no significant difference in the major clinical features of NBCCS among the 10 cases, many atypical clinical features were observed in case 2 out of 2 cases (cases 2 and 8) in which entire PTCH1 deletions were detected. The clinical features of case 2 included severe mental retardation, WEST syndrome (epilepsy), autism, hydrocephalus, hypotonia, inguinal

Fig. 3. Image of deleted regions detected by chromosomal microarray analysis. (A) An approximately 4.3 Mb deletion containing genes from C9orf3 to TGFBR1, including full-length PTCH1, was detected in case 2. (B) An approximately 2.22 Mb deletion containing genes from C9orf3 to LOC441454, including full-length PTCH1, was detected in case 8. (C) The deletion size was approximately 7.93 kb within PTCH1 in case 5.
hernia, epicanthic folds, low set ears, short neck, scoliosis, and strabismus. On the other hand, case 8 presented typical clinical features of NBCCS and no atypical phenotype.

As C9orf3 at the proximal site of the deletion was common to both case 2 and case 8, the distal region of the deletion that consisted of approximately 2.25 Mb (chr9: 99,685,608–101,936,873) containing 25 RefSeq genes (15 disease-related genes) from NUTM2G to TGFBR1 may have contributed to the development of the atypical phenotype only observed in case 2. Similar phenotypes with interstitial 9q22.3 microdeletion have been reported as microdeletion 9q22.3 syndrome [20–22]. Among them, 2 cases reported by Redon et al. [20] closely resembled case 2 in terms of both clinical features and deletion sites, suggesting that genes involved in the genotype-phenotype correlation in these two reported cases and case 2 locate within this 2.25 Mb deleted region.

Among 25 RefSeq genes (15 OMIM genes) from NUTM2G to TGFBR1, we take note of TGFBR1 with particular interest. TGFBR1 encodes the type 1 transforming growth factor beta (TGF-β) receptor. TGF-β superfamily signaling pathways are ubiquitous and essential regulators of cellular and physiological processes [23]. Heterozygous loss-of-function mutations in TGFBR1 are reported to cause multiple developmental anomalies referred to as Loeys–Dietz syndrome [24]. This syndrome exhibits widespread alterations by next-generation sequencing in Gorlin syndrome, PLoS ONE 10 (2015) e0140480.


