Genotype–Phenotype Correlation of TRPV3-Related Olmsted Syndrome

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We have previously shown that gain-of-function variations in transient receptor potential vanilloid-3 (TRPV3) underlay Olmsted syndrome, a rare hyperkeratotic skin channelopathy. In this study, we attempt to establish a genotype–phenotype correlation in Olmsted syndrome, which has been unclear owing to the rarity and heterogeneity of the condition. We identified five previously unreported TRPV3 variations (R416Q, R416W, L655P, W692S, and L673F) and three recurrent variations (G568D, G568V, and L673F) in nine unrelated patients. Seven variants were expressed in human embryonic kidney 293 cells, and channel behavior was characterized electrophysiologically, with results compared with the clinical severity. These variant TRPV3 channels, in either homomeric or heteromeric form, exhibited differentially elevated basal open probability, increased voltage sensitivity, and cytotoxicity. Functional changes were particularly pronounced in variants corresponding to severer Olmsted syndrome (e.g., L673F and W692S) but not in mild Olmsted syndrome variants (e.g., R416Q). Interestingly, the extent of functional rescue by wild-type TRPV3 in vitro was also consistent with the clinical severity of the variants. These findings, in combination with all reported cases, indicate a preliminary genotype–phenotype correlation, that is, variations in the S4–S5 linker and transient receptor potential domain of TRPV3 significantly enhance channel function, causing severe phenotype, whereas other variations appear to exert milder effects on channel function and disease phenotype.


INTRODUCTION

Olmsted syndrome (OS) is a rare genodermatosis characterized principally by palmoplantar keratoderma (PPK) and periorificial hyperkeratosis (Mevorah et al., 2005). Keratoderma is usually progressive with mutilation, leading to disability of the hands and feet ranging from flexion deformities to autoamputation of the digits. Periorificial hyperkeratosis can be found around the mouth, anus, perineum, nostrils, and ear meatus, with a predilection for the mouth corners. Other frequently reported features include alopecia, perifollicular keratosis, nail dystrophy, and leukokeratosis (Mevorah et al., 2005; Tang et al., 2012). Some patients experience intolerant itching and/or pain sensation in hyperkeratotic areas. Histologically, OS is characterized by psoriasiform epidermal hyperplasia with inflammatory infiltration in the upper dermis, for instance, massive mast cell infiltration in the superficial dermis (Lin et al., 2012; Mevorah et al., 2005). We have previously demonstrated that gain-of-function variations in TRPV3, encoding a transient receptor potential (TRP) vanilloid-3 (TRPV3) channel protein mainly expressed in keratinocytes, cause OS (Lin et al., 2012). In vitro studies revealed that the variant TRPV3 channels were constitutively open, causing significant death of variant-expressing mammalian cells through possible calcium overload (Xiao et al., 2008). Correspondingly, a significantly higher percentage of apoptotic keratinocytes was detected in skin lesions of patients with OS, suggesting that hyperkeratosis in OS might be due to increased apoptosis of keratinocytes when expressing hyperactive variant TRPV3 channels. Apart from TRPV3, two other genes have later been identified as causing OS: MBTPS2 for X-linked recessive OS (Haghighi et al., 2013; Wang et al., 2014) and PERP for autosomal-dominant OS (Dai et al., 2020; Duchatelet et al., 2019).

TRPV3 is a nonselective cation channel that has a tetrameric architecture with four subunits symmetrically arranging around an aqueous pore (Zubcevic et al., 2018). Each subunit has six transmembrane domains (S1–6), with the N-terminus and C-terminus located intracellularly. The N-terminus of TRPV3 consists of an ankyrin repeat domain, a linker domain, and a pre-S1 helix, whereas the C-terminus contains a TRP domain, which is proposed to interact with the linker...
between S4 and S5 (Singh et al., 2018; Zubcevic et al., 2018). When activated by agonists such as 2-aminoethoxydiphenyl borate (2-APB), the pore between S5 and S6 opens and becomes permeable to cations with a higher selectivity for divalent ions such as calcium and magnesium ions.

Although TRPV3-related OS is very rare with only 38 cases reported to date, the identification of an increasing number of variations across different domains of TRPV3 and the recent expansion of the OS phenotypic spectrum (Wilson et al., 2015) have made it possible to investigate the genotypic–phenotype correlation. In this study, we aim to establish whether there is a correlation between genotypic and functional aspects of TRPV3 variants and OS phenotype. To that end, we enrolled nine unrelated OS cases with varying clinical severity, from whom five previously unreported and three recurrent TRPV3 variations are identified. A number of variants, located in different domains of TRPV3, are expressed in human embryonic kidney (HEK293) cells, and their functional properties are extensively characterized, with results compared with their corresponding clinical severity.

RESULTS
Identification of different TRPV3 variations in nine patients with OS with varying clinical severity
We enrolled nine OS cases, all of whom were Chinese except patient 7, who was Italian Caucasian. Patients 2, 3, 7, and 9 exhibited mild, focal, and nonmutating PKP, whereas the others had much more severe, diffuse, and mutilating PKP. Patients 1, 2, and 3 had a positive family history (Supplementary Figure S1), and the rest was sporadic. No abnormalities in hematological, biochemical, or immunological tests were noted. Detailed clinical features are listed in Table 1, and representative phenotypes of OS are shown in Figure 1a–c.

Sanger sequencing targeting the TRPV3 gene in these patients revealed five previously unreported variations (R416Q, R416W, L655P, W692S, and L694P) and three recurrent variations (G568D, G568V, and L673F), all in the heterozygous state (Supplementary Figure S2). All variations segregated perfectly with phenotype in those patients with a positive family history and were absent in their healthy parents and 200 unrelated healthy individuals.

Homomic TRPV3 channels carrying OS variations displayed gain-of-function properties
From a total of 21 TRPV3 variations hitherto known to cause OS, we selected seven variations for functional studies; these variations were representative of different mutated TRPV3 domains and a spectrum of clinical severity. These include R416Q/W in the linker domain of N-terminus, W521S in the S2–3 linker, which causes recessive OS, G573A/V in the S4–5 linker, L673F located between S6 and the TRP domain, and W692S in the TRP domain. In order to determine the functional consequence of these variations, the variants along with wild-type (WT) TRPV3 were transiently expressed in HEK293 cells, and TRPV3 channel currents were recorded using patch-clamp electrophysiology with inside-out configuration. WT TRPV3 channels generated weak voltage-dependent outward currents and showed a robust response to 300 μM 2-APB (Figure 2a and c). R416Q, R416W, and W521S variant channels showed larger voltage-dependent currents, although they retained the strong outward rectification characteristic seen in WT channels (Figures 2a and c and Supplementary Figure S3a and b). The 2-APB also induced strong activation. By contrast, the voltage-dependent currents of G573A, G573V, L673F, and W692S variant channels lost the strong outward rectification characteristic, resulting in a nearly linear current–voltage relationship (Figures 2a and b and Supplementary Figure S3a) (Lin et al., 2012). This is usually seen in fully opened TRPV3 channels (Lin et al., 2012). Moreover, these variant channels generated large basal currents and had a negligible further response to 2-APB (Figures 2c and d and Supplementary Figure S3b), suggesting high spontaneous activities. To confirm this observation further, we performed single-channel recordings to test the open probability (Po) of R416Q, L673F, and W692S variant channels without extrinsic activation, namely the basal Po. These channels retained their single-channel conductance but had a clearly increased basal Po without 2-APB activation (Figure 2e and f). R416Q variant, which caused mild OS, had an increased basal Po compared with the WT but not to a level comparable with that of L673F and W692S variants, which have a Po of nearly 0.8. Given that markedly increased basal Po leaves less room for channels to reach a full opening, these results may explain the weak responses of L673F and W692S variants to 2-APB activation.

Gain-of-function properties of TRPV3 variants can be differentially rescued by heteromeric assembly with WT TRPV3, alleviating cytotoxicity to varying degrees
The majority of patients with OS are heterozygotes for TRPV3 variations, in which the variant allele plays a dominant role over the coexpressed WT allele, leading to OS phenotype. This implies that the variant TRPV3 subunits coassemble with WT TRPV3 subunits in vivo to form heteromeric channels that are likely to be overall hyperactive. To determine the extent of functional changes caused by TRPV3 variant subunits through heteromeric assembly, HEK293 cells were cotransfected with WT and variant TRPV3 cDNAs at a 1:1 ratio, which mimicked the genetic background of patients with OS heterozygous for TRPV3 variations. Overall, heteromeric channels formed by R416Q and WT had similar functional behavior to those formed by R416Q alone (Figure 3). Heteromeric channels formed by G573A and WT along with those of W692S and WT generated large voltage-dependent currents with little 2-APB sensitivity, which were nearly identical to their homomeric counterparts. This suggested that the coassembly with WT subunits was insufficient to rescue the effect of G573A and W692S variations (Figures 3a–d and Supplementary Figure S3). By contrast, heteromeric channels formed by L673F and WT displayed reduced voltage sensitivity, stronger response to 2-APB activation, and markedly lower basal Po than homomeric L673F channels, suggesting a partial rescue by WT subunits (Figures 2e and f and 3e and f). Interestingly, the presence of WT subunits resulted in a nearly complete rescue of the hyperactivity in W521S variant channels (Supplementary Figure S3).

As we previously showed elevated apoptosis of keratinocytes as a mechanism by which gain-of-function TRPV3 variations caused OS (Lin et al., 2012), we measured cell death in HEK293 cells expressing each of the three TRPV3
Table 1. Characteristics of Patients with OS with TRPV3 Variations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (PPK, y)</th>
<th>Onset Age (PPK, y)</th>
<th>Age of Visit (y)</th>
<th>PPK</th>
<th>Periorificial Keratoderma</th>
<th>Hair Abnormalities</th>
<th>Nucleotide (Amino Acid)</th>
<th>Exon</th>
<th>Family History</th>
<th>Lesional Itch</th>
<th>Lesional Pain</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>1</td>
<td>25</td>
<td>++; asymmetric; diffuse and mutilating PPK in the left sole and hand; focal, nonmutilating PPK in the right side</td>
<td>–</td>
<td>–</td>
<td>c.1246C&gt;T (p.R416W)</td>
<td>10</td>
<td>+AD</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>5</td>
<td>40</td>
<td>++; asymmetric; focal and nonmutilating PPK in the right hand and foot; left side normal</td>
<td>–</td>
<td>–</td>
<td>c.1246C&gt;T (p.R416W)</td>
<td>10</td>
<td>+AD</td>
<td>+</td>
<td>+++/Δ</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>8</td>
<td>28</td>
<td>++; symmetric; focal and nonmutilating</td>
<td>+; corners of mouth, coccygeal region; +; lusterless and coarse c.1247G&gt;A (p.R416Q)</td>
<td>10</td>
<td>+AD</td>
<td>+</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>15 d</td>
<td>7</td>
<td>++++; symmetric; diffuse and mutilating</td>
<td>++++; corners of the mouth and right ear, perianal area; +; partial white hair</td>
<td>c.1703G&gt;T (p.G568V)</td>
<td>13</td>
<td>–</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>5</td>
<td>16</td>
<td>++++; symmetric; diffuse and mutilating PPK in the planta; focal and nonmutilating PPK in the palms</td>
<td>++++; coccygeal region</td>
<td>–</td>
<td>c.1703G&gt;A (p.G568D)</td>
<td>13</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
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<tr>
<td>6</td>
<td>F</td>
<td>1</td>
<td>35</td>
<td>++; symmetric; diffuse and nonmutilating; constriction digital bands</td>
<td>–</td>
<td>–</td>
<td>c.1964T&gt;C (p.L655P)</td>
<td>15</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>2</td>
<td>8</td>
<td>++; symmetric; focal and nonmutilating</td>
<td>–</td>
<td>+++; thin, coarse, and unmanageable hair with mild hypotrichosis</td>
<td>c.2017C&gt;T (p.L673F)</td>
<td>15</td>
<td>–</td>
<td>++</td>
<td>+++/Δ</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>1.5</td>
<td>7</td>
<td>+++; symmetric; diffuse and nonmutilating; mild bone absorption of the terminal digits</td>
<td>+++; perianal area; +++; moderate hypotrichosis, easily broken; eyebrows and eyelashes affected</td>
<td>c.2075G&gt;C (p.W692S)</td>
<td>15</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>1</td>
<td>7</td>
<td>++; symmetric; focal and nonmutilating PPK in both feet; mild PPK in the terminal digits of both hands</td>
<td>+; corners of the mouth</td>
<td>–</td>
<td>c.2081T&gt;C (p.L694P)</td>
<td>15</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
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</tbody>
</table>

Abbreviations: AD, autosomal dominant; F, female; M, male; OS, Olmsted syndrome; TRPV3, transient receptor potential vanilloid-3; PPK, palmoplantar keratoderma.
The following symbols are used: –, absent/normal; +, present; ++, mild; ++++, moderate; ++++++, severe; Δ, erythermalgia.
variants (R416Q, L673F, and W692S). Consistent with our previous results, significantly increased apoptosis was seen in cells expressing L673F or W692S but not in those expressing R416Q, which displayed an apoptotic rate comparable to the WT level (Figure 4a and b). The cytotoxicity in cells expressing the L673F variant was partially alleviated by coexpression with WT TRPV3, indicating that the hyperactivity of L673F variant channels could be partially rescued by WT subunits (Figure 4a and b) in accordance with the electrophysiological results (Figures 2e and f and 3e and f). However, such a rescuing effect was absent in cells coexpressing the W692S variant and WT.

Validation of functional properties of heteromeric variant channels using concatemers incorporating variant and WT TRPV3

Our functional data obtained from cells cotransfected with variant and WT TRPV3 constructs indicated that there was a correlation between channel properties and their corresponding clinical severity. For example, channels formed by the severe OS variant W692S demonstrated robust basal activation with a negligible response to 2-APB (Figures 2c and 3c). Nevertheless, the measurement of 2-APB response may be confounded by variation-induced cytotoxicity, which can cause downregulation of channel expression (Li et al., 2014; Loukin et al., 2011). To clarify further the functional properties of heteromeric channels, we made concatemers in which cDNA for WT TRPV3 was covalently linked with that for WT TRPV3 or variants, including R416Q, L673F, and W692S, in a head-to-tail fashion (Figure 5a). Transfection with these concatemers did not cause dramatic cell death (data not shown), suggesting that in this condition, the WT subunit was sufficient to rescue cells from cytotoxic effects caused by the variants. Thus, the expression of these variants should no more be affected by the cytotoxicity.

We then tested the electrophysiological properties of the concatemers. First, the WT concatemer showed very low basal currents and yielded robust currents to 2-APB activation, with properties similar to those observed upon expression of monomeric WT TRPV3 subunits (Figure 5b and d). For the concatemer containing the L673F variant, outwardly rectifying currents were observed. Interestingly, the concatemer containing the W692S variant exhibited channel opening even at very negative potentials, whereas remarkable inactivation was observed at more positive potentials (Figure 5b and c). We also applied 2-APB to the variant...
concatemers, which, overall, were found to elicit comparable or larger currents than the WT concatemer (Figure 5d and e). Consistent with voltage-dependent activation, the concatemer containing W692S generated massive inward currents at negative potentials when activated by 2-APB (Figure 5b and d). Although the concatemers only mimicked the TRPV3 channels containing two WT and two variant subunits (which are not fully representative of in vivo scenario where one, two, or three variant subunits can be present), these findings clearly confirmed the functional results obtained from cells cotransfected with variant and WT TRPV3, further supporting the correlation between the variant channel properties and the corresponding clinical severity in our patients.

**DISCUSSION**

Variations in *TRPV3*, *MBTPS2*, or *PERP* have been implicated in the pathogenesis of OS (Dai et al., 2020; Lin et al., 2012; Wang et al., 2014). In TRPV3-related OS, additional clinical features have been reported, including immunological abnormalities and erythromelalgia (Danso-Abbeam et al., 2013; Duchatelet et al., 2014a, 2014b). Because erythromelalgia is noted in two of our patients (patients 2 and 7) and six previously reported patients with OS (Cao et al., 2016; Duchatelet et al., 2014a, 2014b; Ni et al., 2016; Wilson et al., 2015), one may suspect that erythromelalgia is likely to represent an important feature of TRPV3-related OS rather than just a coincidental condition. Clinical presentation of the eight patients varies greatly, suggesting the involvement of modifier genes and/or environmental factors. Because erythromelalgia is not yet a well-established feature of OS, the severity of PPK, periorificial keratoderma, and hair abnormality may still be used primarily in the clinical classification of OS and erythromelalgia, or the degree of lesional itch and pain is also informative in subsequent classification. Because erythromelalgia can be triggered by warmth, it is reasonable to speculate that gain-of-function variations in thermosensitive TRPV3 may lower the temperature-gating

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**Figure 2. Homomeric TRPV3 channels carrying OS variations exhibited a gain-of-function phenotype.** (a) Representative inside-out recordings of homomeric WT and variant channels. (b) Ratios of current amplitude at $-100$ mV to that at $+100$ mV. (c) Representative currents of WT or variant channels activated by 300 μM 2-APB at ±80 mV. 130 mM Ba$^{2+}$ was used to assess leak currents. (d) Ratios of basal current amplitude to current amplitude evoked by 2-APB. (e) Representative single-channel currents at −80 mV in the absence (basal level, upper panels) or presence (lower panels) of 300 μM 2-APB. (f) Basal $P_o$ of the WT and variant channels in the condition without 2-APB activation. $n = 3$ for WT; $n = 4$ for all variants. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. 2-APB, 2-aminoethoxydiphenyl borate; Ba$^{2+}$, barium ion; C, closed state; n.s., not significant; O, open state; OS, Olmsted syndrome; pA, picoampere; $P_o$, open probability; s, second; TRPV3, transient receptor potential vanilloid-3; WT, wild-type.
threshold, leading to pain and hypersensitivity to innocuous stimuli (Luo and Hu, 2014; Moore et al., 2018). Another possible explanation is the interaction between TRPV3 and other ion channels, such as sodium channel 1.7, whose genetic variants underlie inherited erythromelalgia (Yang et al., 2004). Future investigation will address these speculations.

It remains challenging to make the clinical diagnosis of OS with atypical or mild features, thereby necessitating the importance of TRPV3 variation testing in these patients. In general, typical (severe) cases should at least have mutilating PPK, periorificial keratoderma, and alopecia, whereas atypical cases lack one or more of the above (Supplementary Figure S4). We have reviewed all TRPV3-related OS cases reported to date and found that variations located at residues G573 and W692 tend to cause typical (severe) OS, whereas variations at other reported residues seem to associate with the atypical OS (Supplementary Table S1). In this study, we further classify atypical OS into the moderate and mild OS on the basis of the degree of PPK, periorificial keratoderma, hair, and sensory abnormalities (e.g., nonmutilating PPK with or without periorificial lesions and hypotrichosis) (Supplementary Figure S4). To gain functional insights into the TRPV3 variants responsible for individual forms of OS, we selected seven TRPV3 variants representing different clinical forms and structure domains. Our extensive functional characterization of the physiologically relevant form of the variants (i.e., heteromeric variant TRPV3 channels) reveals a positive correlation between the region of variation, variant channel properties, and OS phenotype.

Figure 3. Gain-of-function phenotype in three OS variants can be differentially rescued by heteromeric assembly with WT TRPV3. (a) Representative inside-out recordings of heteromeric variant TRPV3 channels. (b) Ratios of current amplitude at −100 mV to that at +100 mV. (c) Representative currents of heteromeric variant TRPV3 channels activated by 300 μM 2-APB at ±80 mV. 130 mM Ba2+ was used to assess leak currents. (d) Ratios of basal current amplitude to current amplitude evoked by 2-APB. (e) Representative single-channel currents at −80 mV in the absence (basal level, upper panels) or presence (lower panels) of 300 μM 2-APB. (f) Basal Po of the heteromeric variant TRPV3 channels in the condition without 2-APB activation. n = 4. *P < 0.05; **P < 0.001. 2-APB, 2-aminoethoxydiphenyl borate; Ba2+, barium ion; C, closed state; n.s., not significant; O, open state; OS, Olmsted syndrome; pA, picoampere; Po, open probability; s, second; TRPV3, transient receptor potential vanilloid-3; WT, wild-type.
The R416Q variant located in the linker domain is identified (patient 3) and functionally characterized in this study. Consistent with the milder OS phenotype it causes, the channels formed by this variant have functional properties similar to those in WT TRPV3 channels, including a strong outward rectification, high sensitivity to 2-APB, slightly increased basal $P_o$, and very mild cytotoxicity in vitro. The R416 residue has been reported as a shared functional element important for activation and assembly of TRPV1-4 channels, including 2-APB-mediated activation (Luo and Hu, 2014; Singh et al., 2018; Zubcevic et al., 2018), heat sensing (Yao et al., 2011), and protein folding, assembly, and trafficking (Garcia-Elias et al., 2015; Liao et al., 2013).

**Figure 4. Differential cytotoxicity induced by the expression of TRPV3 variants.** (a) HEK293 cells transfected with WT TRPV3, homomeric TRPV3 variants (R416Q, L673F, W692S), or heteromeric TRPV3 variants (L673F + WT and W692S + WT) were stained with Hoechst 33342 (Hoechst, blue) and PI (red). Representative images in each experimental condition are shown. Bar = 50 μm. (b) Quantification of cell death rates, that is, red nuclei/blue nuclei × 100%. Data are averaged from three independent experiments. *$P < 0.05$; **$P < 0.01$. HEK, human embryonic kidney; n.s., not significant; PI, propidium iodide; TRPV3, transient receptor potential vanilloid-3; WT, wild-type.
dimensional model of human TRPV3 (PDB 6MHO), R416 is located relatively far away from the pore domain (Supplementary Figure S6b–c), and its variation is expected to impact channel gating indirectly, causing a less dramatic change in the overall channel activity (Singh et al., 2019; Zubcevic et al., 2018). This is in keeping with the milder OS phenotype in our patients harboring variation at this residue.

Of particular interest is the L673F variant, which has been reported to cause OS without periorificial keratoderma and is thus classified as atypical OS. By reviewing four OS cases with L673F variation (Duchatelet et al., 2014b; Takeichi et al., 2017), the severity seems to vary between patients, especially the degree of PPK and pain sensation. Our functional studies show that although the L673F variation disrupts TRPV3 channel function (with reduced response to 2-APB, increased basal Po, and cytotoxicity), the variational effects can be partially rescued by incorporation of WT TRPV3 subunits, which is not the case for variants on G573 and W692 residues corresponding to clinically severe OS (see details below). Interestingly, an adjacent variation, M672I, has also been reported in a relatively moderate OS case (Ni et al., 2016). These mutated residues, M672 and L673, lie in the C-terminal end of S6 in a stretch of five residues (Asn-Met-Leu-Ile-Ala) with a high degree of evolutionary conservation across species in all TRP genes (Supplementary Figure S5). They are proposed to play a critical role in TRP channel gating (Duchatelet et al., 2014b; Ni et al., 2016). Recently, L673 has been shown to locate in the last helical turn of the inner helix of TRPV3, facing the aqueous hydrophilic environment of the inner pore (Supplementary Figure S6c) and contributing to a potential pore-obstructing hydrophobic gate with I674 residue (Singh et al., 2018; Zubcevic et al., 2018). It is likely that when all the four TRPV3 subunits undergo L673F substitution for a larger phenylalanine side chain (Duchatelet et al., 2014b), there would be inadequate space to fit the intramolecular constriction in the closed-state conformation of the TRPV3 pore, forcing the channel to remain open. This could explain the partial rescuing effect by WT TRPV3 because the incorporation of WT subunits in heteromeric channels could restore the space for conformational change during channel gating.

**P < 0.01. 2-APB, 2-aminoethoxydiphenyl borate; n.s., not significant; pA, picoampere; pF, picofarad; s, second; TRPV3, transient receptor potential vanilloid-3; WT, wild-type.

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Figure 5. Functional characterization of concatemers incorporating WT and variant TRPV3. (a) Models of WT and variant (R416Q, L673F, W692S) concatemers. (b) Representative inside-out recordings of WT and variant TRPV3 concatemers. (c) Ratios of current amplitude at −100 mV to that at +100 mV. (d) Representative currents of WT and variant TRPV3 concatemers activated by 2-APB at ±80 mV. (e) Current density at −80 mV. n = 3 for WT; n = 4 for all variants. **P < 0.01. 2-APB, 2-aminoethoxydiphenyl borate; n.s., not significant; pA, picoampere; pF, picofarad; s, second; TRPV3, transient receptor potential vanilloid-3; WT, wild-type.
Residues in the S4–5 linker and TRP domain of TRPV3 are hotspots for OS-related variations (Supplementary Table S1), especially the G573 residue on the S4–5 linker, where G573A, G573C, G573S, and G573V substitutions have been reported. G573C/S are recurrent variations found in seven patients hitherto with typical (severe) OS phenotype. These variants form constitutively active channels with weak or no response to 2-APB (Lin et al., 2012). This is also the case in G573A/V variants found in this study (Lin et al., 2012), which indicates that the highly conserved G573 residue plays an important role in TRPV3. Similar to W692 residue that is situated in the TRP domain, W692C/G substitutions have been reported to cause severe OS and dramatically affect channel properties in vitro (Lin et al., 2012; Ni et al., 2016). In this study, we expand the knowledge by characterizing the previously unreported W692S variant, demonstrating significant cell death that cannot be rescued by coexpression with WT TRPV3. Because G573 lies directly above W692 and both surround the pore domain in the three-dimensional structure (Supplementary Figure S6d), variations of these residues are predicted to directly alter channel gating through structural rearrangements, leading to severe OS phenotype (Duchatelet et al., 2014a; Eytan et al., 2014; Ni et al., 2016; Singh et al., 2019). Nevertheless, we also identify a previously unreported L694P variation in the TRP domain, which, paradoxically, causes a milder phenotype (patient 9). Future research is warranted to address this phenotypic discrepancy. Because W692 and L694 surround the R693 residue that is known to involve 2-APB binding (Singh et al., 2018; Zubcevic et al., 2018), this may explain the loss of 2-APB sensitivity in W692S-variant channels.

The G568 residue, which lies at the junction between the S4 helix and S4–5 linker, appears to be another variation hotspot, with three variations (G568C, G568D, and G568V) reported thus far (Cao et al., 2016; Choi et al., 2018; Duchatelet et al., 2014a; Nagai et al., 2017; Wilson et al., 2015). Interestingly, the G568C variation has been reported as being recessive (Duchatelet et al., 2014a) or semidominant (Cao et al., 2016) in separate studies. The gain-of-function property of the G568C variant can be partially rescued by coexpression with WT TRPV3, suggesting that it causes a less drastic functional consequence than G573 variant. Consistently, patients reported to date have milder hair abnormalities or nonpersistent perionifolial lesions, although erythromelalgia may be present (Cao et al., 2016; Duchatelet et al., 2014a).

The W521S variant, which lies in the S2–3 linker of TRPV3, has been reported in a patient with autosomal-recessive OS (Eytan et al., 2014). Our electrophysiological experiments reveal a gain-of-function nature of the variant channels, whose functional abnormalities are nearly completely rescued by the incorporation of WT TRPV3 subunits. This clearly indicates that the W521S variation in the heterozygous state does not exert significant pathogenic effects on protein function, whereas it does in the homozygous state, in agreement with the previous clinical observation that patients with this heterozygous variation displayed no cutaneous phenotype (Eytan et al., 2014).

In summary, the nine unrelated OS cases presented in this study largely expand the OS genotypic and phenotypic spectrums. Our functional investigation provides detailed channel properties for R416Q, L673F, and W692S variants that correspond to mild, moderate, and severe OS, respectively. These findings, together with those from previously reported TRPV3-related OS cases, have established a preliminary genotype–phenotype correlation; that is, variations in the S4–5 linker and the TRP domain of TRPV3 tend to affect the channel properties profoundly, causing severe clinical manifestations. In contrast, variations in other domains appear to have a lesser impact on TRPV3, leading to milder OS.

**MATERIALS AND METHODS**

**Patients and genotyping**

This study was approved by the Clinical Research Ethics Committee of the Peking University First Hospital (Beijing, China) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the participants. The patient or his or her parents consented to the publication of the image. Nine patients with clinically confirmed OS were recruited. Peripheral blood samples were obtained, and genotyping was performed as previously described (Lin et al., 2012; Wilson et al., 2015). The clinical features of the patients are listed in Table 1.

**TRPV3-expressing vectors**

Human TRPV3 cDNA was cloned into the pCMV6-AC-GFP vector (OriGene, Rockville, MD). With the recombinant plasmid used as a template, TRPV3 variants containing desired point variations were generated using a Site-Directed Fast Mutagenesis System (TransGen Biotech, Beijing, China). Primers used in this process are listed in Supplementary Table S2.

**Cell culture and transient transfection**

HEK293 cells were cultured in a DMEM containing 10% fetal bovine serum (Gibco, Darmstadt, Germany) and 1% penicillin and/or streptomycin at 37 °C with 5% carbon dioxide. Transient transfection was performed using Lipofectamine 2000 as per the manufacturer’s instructions (Invitrogen, Carlsbad, CA). For cotransfection experiments, equal amounts of WT and variant TRPV3 plasmids were used.

**Electrophysiology**

Electrophysiological experiments were performed at 24–48 hours after transfection. Currents were recorded using a HEKA EPC10 amplifier with PatchMaster software (HEKA, Lambrecht, Germany) in inside-out configuration. Patch pipettes were pulled from borosilicate glass and fire polished to have a resistance of approximately 2 MΩ. Membrane potential was held at 0 mV unless otherwise stated. For voltage-dependent activation, patches were held at 0 mV, stepped from −160 mV to 240 mV in an increment of 20 mV for 300 milliseconds, and then stepped to −100 mV for 100 milliseconds. Current amplitudes were analyzed at ±100 mV. For 2-APB−induced activation, currents were elicited by a protocol consisting of a 300-millisecond step to +80 mV, followed by a 300-millisecond step to −80 mV at 1-second intervals. Both pipette and bath solutions contained 130 mM sodium chloride, 0.2 mM EDTA, and 3 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffered to pH 7.4 with sodium hydroxide). All chemicals, including 2-APB, were purchased from Sigma (St. Louis, MO). All experiments were conducted at 22 °C.

**Cell death assay**

HEK293 cells were washed three times with PBS and incubated with Hoechst 33342 and/or propidium iodide in PBS for 10 minutes. Cells
were imaged and counted in five random fields under a fluorescence microscope (Olympus IX71, Tokyo, Japan) equipped with a digital camera. Nuclei of total cells and dead cells were indicated by positive Hoechst 33342 and propidium iodide signals, respectively. The rate of cell death is determined by averaging the percentage of dead cells (propidium iodide–positive nuclei)/Hoechst 33342–positive nuclei × 100% in each experimental condition.

Data analysis
Data are expressed as the mean ± SEM. Statistical significance was evaluated using unpaired two-tailed Student’s t-test for comparison between two samples. *P < 0.05; **P < 0.01; ***P < 0.001.

Data availability statement
No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST
The authors state no conflicts of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: YY; Formal Analysis: WZ, LH, XC; Funding Acquisition: YY; Investigation: XC, JZha, ML, HW, JZhan, QC, CF, LD; Methodology: WZ, LH, XC, ML, HW, QC; Resources: YY, XW, LT, ZL; Supervision: YY; Writing - Original Draft Preparation: WZ, LH, XC, ZL; Writing - Review and Editing: ML, YY

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.06.035.

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