Panel Sequencing Shows Recurrent Genetic FAS Alterations in Primary Cutaneous Marginal Zone Lymphoma

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Primary cutaneous marginal zone lymphoma (PCMZL) represents an indolent subtype of non-Hodgkin lymphoma that is clinically characterized by slowly growing skin tumors with a very low propensity for systemic dissemination. The underlying genetic basis of PCMZL has not been comprehensively elucidated. To gain deeper insight into the molecular pathogenesis of PCMZL, we performed hybridization-based panel sequencing of 38 patients with well-characterized PCMZL. In 32 of the 38 patients, we identified genetic alterations within 39 selected target genes. The most frequently detected alterations (24/38 patients, 63.2%) affected the FAS gene, of which 22 patients harbored alterations, which affect the functionally relevant death domain of the apoptosis-regulating FAS/CD95 protein in a dominant-negative manner. In addition, we identified highly recurrent mutations in three other genes, namely SLAMF1, SPEN, and NCOR2. Our molecular data suggest that apoptosis defects provide the molecular basis of the observed clinical features of PCMZL, which commonly presents with only slowly growing skin tumors, reflecting its invariably indolent behavior. From a diagnostic point of view, highly recurrent FAS mutations in PCMZL presumably separate this indolent lymphoma entity from pseudolymphoma, and this adds adjunctive discriminatory features at a molecular level.

INTRODUCTION

Primary cutaneous marginal zone lymphoma (PCMZL) belongs to the group of indolent B-cell non-Hodgkin lymphomas of the extranodal or mucosa-associated lymphoid tissue, according to the current World Health Organization/European Organisation for Research and Treatment of Cancer classification (Swerdlow et al., 2016; Willemze et al., 2005). Clinically, this subtype of lymphoma is characterized by slowly growing skin nodules with a typically low tendency for spontaneous regression but an excellent therapeutic response to skin-directed treatment strategies and extraordinarily rare systemic dissemination. Hence, most patients present with an unrestricted overall survival (Swerdlow, 2017). Most PCMZL with plasmacytic differentiation exhibit a class-switched phenotype showing preferential expression of IgG and less often IgA, in contrast to the mostly non–class-switched, IgM-positive noncutaneous marginal zone lymphomas (MZLs) (Brenner et al., 2013).

The molecular basis for tumor development and progression of PCMZL is not well understood. Genetic hallmarks of noncutaneous MZL, such as chromosomal translocations of MAL1, BCL2, or BCL10, as well as (aberrant) somatic hypermutations affecting immunoglobulin genes and protooncogenes such as PIM1 or cMYC, have been described only sporadically in PCMZL (Deutsch et al., 2009; Palmedo et al., 2007). These genetic alterations may convey both proliferative and anti-apoptotic effects in the tumor cells.

Although recent mutational profiling of aggressive cutaneous B-cell lymphoma subtypes such as primary cutaneous diffuse large B-cell lymphomas has shown crucial genetic aberrations in key signaling cascades such as the B-cell receptor– and toll-like receptor–dependent pathways (Koens et al., 2014; Menguy et al., 2016; Pham-Ledard et al., 2012), pathognomonic driver mutations in PCMZL have not been identified until recently. However, we recently described activating oncogenic mutations of MYD88 in a subset of IgM-restricted PCMZL by applying next-generation sequencing (NGS) techniques (Wobser et al., 2017). Somatic mutations in the MYD88 gene are crucially involved in the molecular pathogenesis of different subtypes of B-cell non-Hodgkin lymphoma such as cutaneous and noncutaneous diffuse large B-cell lymphomas and Waldenström macroglobulinemia. With less frequency, similar activating mutations in

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MYD88 have also been described in noncutaneous MZL. The increasing knowledge about such molecular aberrations and their consecutive biological effects has already been implemented within the clinical routine. Besides more accurate diagnostic subtype attribution and better prognostic stratification, the identification of cardinal mutations allows the application of more specific pharmacological approaches and, thus, has revolutionized the therapeutic armamentarium in hemato-oncology (Sehn and Gascoyne, 2015).

Here, we aimed to investigate the genetic features of PCMZL in more detail by focusing on its underlying mutational landscape. Using ultradeep NGS, we analyzed a panel of 39 tumor-relevant genes that had been selected for their well-defined roles in B-cell development and lymphomagenesis.

RESULTS

Panel sequencing unravels frequent genetic alterations in PCMZL

We used hybridization-based panel sequencing to gain deeper insight into the molecular pathogenesis of PCMZL and to identify tumor drivers. In a cohort of 38 PCMZL patients, we detected 77 single nucleotide variants (SNVs), small deletions, and potential splice site variants (Figure 1). Overall, 32 patients harbored at least one genetic alteration, and 27 patients showed more than one variant. In six patients, no genetic alteration was detectable by our applied panel of genes. In 20 of the 39 genes of the customer-designed panel (see Supplementary Table S1 online), no mutation was found (see Supplementary Table S2 online).

Recurrent mutations are detected in four genes with aberrations in FAS, making this the most common genetic event in PCMZL

The NGS approach identified several recurrent alterations. The most frequently mutated gene was FAS, an apoptosis-inducing cell surface death receptor of the TNF receptor superfamily. We showed that this gene was affected in 10 of 38 patients by using stringent filter settings. Four of 10 FAS mutations were located within the effector domain, that is, the death domain. Five mutations were considered to affect splice sites of exon 8, because they were located in close proximity (±5 base pairs) to the coding sequence of the 25-base pair spanning exon. The alternative splicing of exon 8 caused a frameshift deletion of FAS mRNA encoding for a truncated FAS protein lacking the death domain (E218Mfs*4). One additional FAS mutation mapped to exon 4 and did not cluster with the other detected and described SNVs. In summary, 9 of 10 detected molecular alterations within the FAS gene lead to disrupted protein function by affecting the crucial death domain and are predicted to convey anti-apoptotic properties.

The second frequently mutated gene was SLAMF1, a self-ligand receptor of the signaling lymphocytic activation molecule family, which was altered in 9 of 38 patients. Whereas all 10 FAS-mutated patients each harbored only a single FAS frameshift deletion, missense, or splice site mutation, three of the nine SLAMF1-mutated patients exhibited two missense mutations in the SLAMF1 gene. In two of these patients, the missense mutations affected the same allele, because they were detected exclusively in the same reads. In patient 35, the mapping of the two missense mutations to cis- or trans-alleles was not feasible, because the two mutations were mainly covered by two different hybridization probes. All detected SLAMF1 SNVs were localized in the SLAM domain. In contrast to this clustered occurrence of mutations within the SLAMF1 gene in PCMZL, in other tumor entity SLAMF1 SNVs were rather continuously distributed over the whole protein sequence, according to cBioPortal entries (http://www.cbioportal.org/) (Cerami et al., 2012; Gao et al., 2013).

The third and fourth most common genetic events in our PCMZL cohort were mutations of SPEN and NCOR2, which were detected in 7 and 5 of 38 patients, respectively. Both genes code for proteins that act as transcriptional co-repressors and histone deacetylase-interacting factors and that orchestrate diverse cellular events such as metabolic state or epigenetic regulation within cancer cells (Battaglia et al., 2010). In patient 22, we identified one missense and one nonsense mutation in NCOR2. Although SPEN and NCOR2 mutations appeared to be mutually exclusive, in 10 of 12 patients aberrations within these two genes were detected together, with alterations affecting other genes of the panel in variable combinations (Figure 1). In contrast to FAS and SLAMF1 mutations, SPEN and NCOR2 SNVs were evenly distributed over the whole protein, showing no hotspot regions. However, three of seven SPEN mutations showed the same variant at glycine 1259, which was altered to a serine (Figure 2). To our knowledge, this putative hotspot variant has not been reported in any other tumor entity; however, it is predicted to be benign by several prediction tools (Sorting Intolerant from Tolerant [i.e., SIFT], http://sift.bii.a-star.edu.sg/ and Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/ [Adzhubei et al., 2010; Kumar et al., 2009]).

With respect to clinical correlation, more detailed clinical information about the presence, number, extent, and frequency of cutaneous/extracutaneous relapses was analyzed for 15 representative patients. The median follow-up time for patients with FAS mutations (n = 8) was 84.5 (range 23–191) months. The median follow-up time for patients without FAS mutations (n = 7) was 74 (range 4–145) months. All patients with proven FAS mutations showed at least one cutaneous relapse during the course of their disease, and more than 50% of nonmutated patients remained free of disease after primary therapy. However, there was no difference in the total amount of cutaneous relapses per patient, that is, the occurrence of new skin lesions during the clinical course (median = 3 relapses). Moreover, the presence of additional mutations such as in SPEN, SOCS1, or MYD88 did not correlate with disadvantageous clinical course. Extracutaneous dissemination of PCMZL was seen in only one nonmutated patient with locoregional nodal involvement, as assessed by radiographic (ultrasound or computed tomography) staging (not histologically proven), who achieved persistent complete remission after rituximab therapy. When last seen, all analyzed patients were in complete remission.

Because the high frequency and the obvious clustering at functionally relevant hotspot regions of alterations within the FAS and SLAMF1 genes are likely to contribute to the molecular pathogenesis and tumorigenesis of PCMZL, we further
addressed these molecular aberrations in more detail in subsequent analyses.

**FAS mutations occur at variable frequencies but invariably lead to disruption of the death domain**

The described FAS mutations occurred in many of the 10 patients with remarkably low frequencies. In order to not miss the detection of additional low-frequency FAS alterations in other samples, we reduced our initially applied filter stringencies (see Materials and Methods section) from 15 to 5 reads for the alternative variant. Furthermore, we decreased the alternative read frequency for FFPE material from 5% to 2%, comparable to fresh-frozen material. This filter adaption showed that 14 further patients were harboring FAS alterations detected with modified filter settings (alternative variant coverage ≥ 5x, frequency ≥ 2%). Triangles indicate patients harboring an activating MYD88 mutation (exclusively in IgM-restricted cases). Only genes of the panel with at least one SNV are depicted; the most frequently mutated genes are listed in descending order. IHC, immunohistochemistry; Pat.-ID, patient identification; PCMZL, primary cutaneous marginal zone lymphoma; SNV, single nucleotide variant.

To validate these FAS variants, confirmatory Sanger sequencing of cDNA samples of the patients was performed (see Supplementary Figure S1 online). This approach allows the validation of the low-frequency splice site defects, which would not have been detected by sequencing of genomic DNA. In addition, Sanger sequencing of cDNA confirms an actual expression of the detected FAS variants. We were able to obtain qualitative, analyzable sequences for 17 patients and could confirm all of the detected FAS variants, including three frameshift deletions, two point mutations within the death domain, and 11 potential exon 8 splice sites. Four of 11 splice site mutations were located one or two base pairs upstream of exon 8 in intron 7, whereas six other splice sites were located one, two, or five base pairs downstream of exon 8 in intron 8 (transcript variant NM_000043) (Figure 3a).

One patient harboring a potential splice site mutation (c.676+5G>C) did not show the frameshift caused by alternative splicing of exon 8 at the cDNA level as determined by Sanger sequencing, although a different patient with the c.676+5G>A base exchange expressed the exon 8 deleted transcript. Additionally, eight FAS wild-type PCMZL samples and five cutaneous pseudolymphoma samples were also validated for exon 8 splice site defects, and as expected, none of the samples expressed the p.E218Mfs*4 coding transcript. To additionally confirm the presence of the alternative p.E218Mfs*4 coding transcripts, we performed RNA sequencing analyses of six patients (patients 4, 6, 7, 10, 19, and 29) who harbor splice site mutations. In fact, the exon 8 deleted transcripts were detected in patients 4 and 6 (Figure 3c). Moreover, this transcript was not detectable in any of the four patients (2, 5, 15, and 24) harboring death domain-affecting substitutions in exon 9. However, in all
**Figure 3. Recurrent somatic FAS alteration in PCMZL.** (a) Location of all mutations within the FAS gene affecting the death domain (transcript variant NM_000043) and protein (UniProtKB-P25445). Red indicates validation by Sanger sequencing. (b) Left: Sanger sequencing of genomic DNA of patient 4 with the c. 676+1G>A mutation (*) detected by panel sequencing. Right: Sanger sequencing of cDNA of patient 4 displaying FAS wild-type transcript and p.E218Mfs*4 encoding transcript with an exon 8 deletion. The mutation frequency was 2% in the panel sequencing approach. (c) Snapshot of exons 7–9 of the FAS gene of patient 4 (Integrated Genomics Viewer). Red boxes indicate the lack of exon 8 in the p.E218Mfs*4 encoding transcripts. (d) Number and type of all discovered FAS mutations in PCMZL affecting the death domain (DD). ex, exon; PCMZL, primary cutaneous marginal zone lymphoma.
The Mutational Landscape of Cutaneous Marginal Zone Lymphoma

Table 1. FAS alterations detected in PCMZL

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<th>Sanger</th>
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</table>

Abbreviations: AA, amino acid; ID, identification; n.a., not analyzable because of low RNA/cDNA quality; n.d., not done; PCMZL, primary cutaneous marginal zone lymphoma; WT, wild type.

Recurrent mutations in SLAMF1 are located in the extracellular domain of the respective protein

The detected SLAMF1 variants are all clustered in the SLAM domain, which in turn is localized to the extracellular part of this receptor. The structural model of the extracellular domain and the so far only described ligand of the receptor, the measles virus hemagglutinin, show that the SLAMF1 mutations determined in PCMZL are not overlapping with the binding interface (Figure 4). Because the localization of the dimerization domain of SLAMF1 receptors is described to widely concur with the SLAMF1-measles virus hemagglutinin binding interface, it is not tempting to assume that the SLAMF1 missense mutations may result in disruption of dimerization or ligand binding (Hashiguchi et al., 2011).

DISCUSSION

Recent whole genome, whole exome, and panel sequencing analysis of cutaneous T-cell lymphomas provided crucial clues for the underlying molecular aberrations affecting apoptosis, cytokine and T-cell receptor signaling, and epigenetic regulation as possible drivers for mycosis fungoides and Sézary syndrome, the most common cutaneous T-cell lymphomas (Choi et al., 2015; da Silva Almeida et al., 2015; McGirt et al., 2015; Ungewickell et al., 2015). Moreover, the resulting deeper knowledge of mutations in genes encoding proteins involved in chromatin remodeling and histone modification, as well as in intracellular signaling, has provided a rationale for further evaluating inhibitors such as histone deacetylase inhibitors or JAK/STAT inhibitors in preclinical and clinical settings (Damsky and Choi, 2016). Until now, comparable efforts addressing the mutational landscape of cutaneous B-cell lymphomas and especially of the indolent subtype PCMZL have not been undertaken.

Therefore, we aimed to widen our molecular approach by taking advantage of ultra-deep NGS targeting 39 potential key oncogenic genes involved in PCMZL lymphomagenesis.

The main finding of our study was the identification of recurrent alterations of the FAS gene in nearly two thirds of all PCMZL patients. All but one of these mutations affected the intracellular death domain of the respective protein.

patients the coverage was too low to allow quantitative assessment (see Supplementary Tables S2 and S3). Furthermore, these validation approaches showed that the high-sensitivity target enrichment system allows very sensitive NGS analyses, because splice site alterations with very low sensitivity target enrichment system allows very sensitive NGS analyses, because splice site alterations with very low frequencies were confirmed by Sanger sequencing and RNA sequencing (see example with 2% allele frequency in Figure 3b and c).

In summary, all collected FAS sequencing data showed a very high FAS mutation rate in PCMZL. In total, 24 of 38 (63.2%) sequenced PCMZL samples showed a FAS alteration as assessed with reduced filter stringencies. Twenty-two of these FAS alterations unite different types of mutations, including six missense mutations within the death domain, premature stop gains due to frameshift deletions (four patients), nonsense mutations (one patient), and the splice site mutations deleting exon 8 (11 patients), which in turn all affect and disrupt the intracellular death domain of the death receptor (Table 1 and Figure 3d).
Regarding recent large-scale mutational profiling studies of systemic B-cell lymphomas, FAS mutations have been detected in diffuse large B-cell lymphomas, follicular lymphoma, and nodal MZL but to a much lower frequency. Additionally, FAS alterations in these lymphoma subtypes occur with irregular distribution in the FAS gene and do not cluster within a mutational hotspot region as observed in this study (Krysiak et al., 2017; Morin et al., 2016; Spina et al., 2016).

Usually, FAS wild-type proteins form a homotrimer in response to the binding of a homotrimer of FAS ligands. Subsequently, FADD is recruited to FAS. This in turn results in the recruitment of further binding partners such as CASP8, thereby activating the caspase cascade and further downstream apoptotic signals (Figure 5a) (Li et al., 1998; Waring and Mullbacher, 1999). Several mutations affecting the death domain of FAS (including our FAS variants) have already been extensively studied in vitro. The vast majority of these characterized FAS mutants convey a resistance to FAS-mediated apoptosis (Fisher et al., 1995; Hsu et al., 2012; Jackson et al., 1999). The diminished ability of altered FAS to transmit apoptotic signals is attributed to disrupted FADD and CASP8 recruitment, because even one defective FAS protein in the trimer is sufficient to disrupt normal function of the receptor complex. Accordingly, these mutations act in a dominant negative manner, and the remaining wild-type proteins are sequestered (Jackson et al., 1999; Kuehn et al., 2011).

We assume that FAS alterations in PCMZL similarly disrupt apoptotic signaling via the FAS receptor. These molecular findings fit well with the indolent biological and clinical behavior of PCMZL that is reflected by slowly growing papules and tumors of limited size without tendency for ulceration. This clearly contrasts the common clinical presentation of cutaneous diffuse large B-cell lymphomas, which mostly show rapid growth of large and ulcerating tumors, most likely due to the effect of activating mutations in oncogenes driving enhanced proliferation such as activating MYD88 mutations.

The detection of FAS alterations in PCMZL not only provides insight into its molecular pathogenesis, but with respect to diagnostic issues adds adjunctive discriminatory features at a molecular level to better separate this indolent lymphoma entity from reactive inflammatory skin infiltrates and pseudolymphoma.

To our knowledge, there is no evidence that alteration of FAS may lead to enhanced resistance to skin-directed (radiotherapy) or systemic (anti-CD20 antibodies) therapy. Hence, it is assumed that PCMZL patients harboring FAS mutations do not exhibit any disadvantageous overall prognosis. However, the presence of FAS mutations correlated with a higher tendency for skin relapses compared with wild-type patients in our limited patient collective. Further studies are required to address this observation in a preferably prospective setting and within a larger patient cohort.

Besides FAS mutations, we also detected genetic aberrations primarily in three additional genes (SLAMF1, SPEN, and NCOR2). The role of mutations in SLAMF1, SPEN, and NCOR2 with respect to oncogenesis and the molecular pathogenesis of lymphoma is widely unexplored. Moreover, the detected variants within these genes are uncharacterized and therefore remain to be elucidated. SPEN and NCOR2 can take part in the same transcriptional repressive complex within the NOTCH signaling pathway and might therefore have similar effects on this pathway if they are mutated (Figure 5b) (Bray, 2016; Kopan and Ilagan, 2009). This idea is supported by the fact that in our patients’ mutations affecting SPEN and NCOR2 occur mutually exclusive.

To summarize, our targeted ultradeep NGS analysis showed frequent and recurrent molecular alterations in PCMZL. Among these mutated genes, which orchestrate intracellular networks of apoptosis control, epigenetic regulation, and intracellular signaling, dominant-negative FAS alterations were the key molecular finding. These dominant-negative mutations were shown to cluster within narrow hotspot regions of the FAS gene and are predicted to convey apoptosis defects by afflicting the death domain.

MATERIALS AND METHODS

Patients and tissue

The study included 38 patients with a clear-cut diagnosis of PCMZL. Approval of the entire study was obtained from the ethics committee at the Medical Faculty of the University of Würzburg, Germany, and the study was conducted in accordance with the Declaration of Helsinki principles. All patients of the lymphoma clinic provided written informed consent to the study.

Clinical, histological, and immunophenotypical features of all analyzed patients were consistent with PCMZL according to the current International Society for Cutaneous Lymphomas and World Health Organization/European Organisation for Research and Treatment of Cancer recommendations on the diagnosis and classification of cutaneous lymphomas (Senff et al., 2008; Willemze and Meijer, 2006). Histopathological examinations were done by using hematoxylin and eosin- and Giemsa-stained slides and a standard panel of immunohistochemical antibodies; in addition, immunohistochemical staining for heavy chains (IgA, IgG, IgG4, and IgM) was performed. Although 36 patients had plasmacytic differentiation with immunohistochemically detectable light and heavy chain restriction, two patients did not show any plasmacytic differentiation,
and therefore heavy or light chain expression was not detectable by immunohistochemistry. Moreover, five cutaneous pseudolymphomas with features of lymphadenosis cutis benigna served as controls for determination of alternative FAS exon 8 splicing by Sanger sequencing.

Histological diagnosis of PCMZL and pseudolymphomas were confirmed by three dermatopathologists (MW, EG, and AR). Fresh-frozen cryopreserved tissue was available from 11 patients (patients 1–11). To enlarge the number of PCMZL patients for this study, we further analyzed formalin-fixed paraffin-embedded (FFPE) material from an additional 28 patients (patient 12–38).

Gene panel
The 39 target genes of the panel were selected based on multiple aspects, including our own data from previous whole-exome sequencing analyses of four PCMZL patients matched to corresponding blood samples (K Maurus et al., unpublished data) and available publications about genetic alterations in related B-cell lymphoma subtypes, such as nodal MZL or ocular MZL (Jung et al., 2017; Spina et al., 2016) (see Supplementary Table S1). Moreover, interacting genes belonging to cardinal pathways or signaling networks of B-cell lymphomagenesis were especially addressed.

DNA extraction
Genomic DNA from PCMZL patients was obtained from either 10 μm cryopreserved or FFPE sections using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) for cryopreserved material and the GeneRead DNA FFPE Kit (Qiagen) for FFPE material. The amount of tumor infiltrate in the respective cryopreserved specimens was reviewed in a hematoxylin and eosin staining from the corresponding sections. DNA quantitation was assessed by either Qubit double-stranded DNA broad range assay for cryopreserved material or quantitative PCR for FFPE material (TaqMan RNase P Detection Reagents Kit, Life Technologies, Darmstadt, Germany).

RNA extraction and cDNA synthesis
Additionally, RNA was extracted from the same FFPE and cryopreserved material with the RNeasy FFPE Kit and the QiAamp RNA Blood Mini Kit (Qiagen), respectively. Subsequently, RNA was transcribed into cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) using the random hexamer primer protocol.

Target enrichment
The HaloPlexHS Target Enrichment System (Agilent Technologies, Santa Clara, CA) was used for library preparation strictly according to the manufacturer’s protocol. For the amplification of the captured libraries, 24 cycles were used. Determination of the size and quality of the libraries was performed using the Bioanalyzer high-sensitivity DNA chip (Agilent Technologies). Quantitation of the libraries was measured in a Qubit double-stranded DNA HS assay (Life Technologies).

Next-generation sequencing
In a 150-base pair paired-end sequencing approach, the libraries were sequenced on the MiSeq platform (Illumina, San Diego, CA).

Quality trimming
Quality assessment was performed using FastQC, version 0.11 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and low-quality reads below a phred quality score of 30 and a length of 30 base pairs were trimmed with TrimGalore, version 0.4.0, powered by Cutadapt, version 1.8 (Martin, 2011).

Sequence alignment and variant calling
Read alignment of the trimmed reads and somatic SNV and small insertion/deletion calling was performed with SureCall, version 3.0.1.4 (Agilent Technologies), using the HaloPlexHS method with the following adjustments: the minimum allele frequency for calling a variant was set to 2%, and the minimum read depth for sample point mutations was set to 20.

Additionally, to improve the identification of small insertions/deletions, Scalpel, version 0.5.3 (Fang et al., 2016), an independent read alignment against the UCSC hg19 human reference genome, was performed using BWA mem, version 0.7.12 (Li and Durbin, 2009), with default parameters. Sorted BAM files were created using Picard, version 1.125, and an index was created using SAMtools, version 1.3.
(Li et al., 2009). To account for the PCR duplicates, a de-duplication step was added using the tool provided by Agilent, version 1.0. Read statistics are given in Supplementary Table S4 online.

Somatic variants were reported if (i) the variant led to a protein alteration or affected a splice site, (ii) the variant was very rare in the general population and reported with a minor allele frequency of less than 0.5% in the 1000 Genomes and dbSNP databases, and (iii) the variant was covered by at least 20 reads and the alternative allele was covered by at least 15 reads and comprised at least 2% for cryopreserved tumors (patients 1–11) and 5% for FFPE samples (patients 12–38), unless stated otherwise in the text or figure legends.

All variants were visually examined using the Integrative Genomics Viewer, version 2.3.68 (Robinson et al., 2011).

RNA sequencing
RNA sequencing was performed in 10 patients (patients 4, 6, 7, 10, 19, and 29 harboring FAS splice site mutations and patients 2, 5, 15, and 24 who harbor mutations in the death domain). Libraries were prepared with a SMARTer Stranded Total RNA-Seq Kit, version 2, Pico Input Mammalian kit according to manufacturer's specifications (Takara Bio, Mountain View, CA). Sequencing was performed in 75-base pair paired-end mode on the NextSeq500 platform (Illumina).

Quality assessment and adapter clipping, as well as quality trimming, were performed as described. Reads with a minimum length of 20 nucleotides were then mapped against the ENSEMBL human reference genome (GRCh37.75) using STAR, version 2.5.2b (Dobin et al., 2013). SAM to BAM conversion, as well as sorting and indexing of the BAM files, was performed using SAMtools, version 1.3 (Li et al., 2009). Variants and transcripts were visualized and counted using the Integrative Genomics Viewer, version 2.3.68 (Robinson et al., 2011).

Sanger sequencing
PCR reactions to amplify the FAS target regions from cDNA were performed with a 5°C touchdown protocol using a recombiant Taq DNA Polymerase (Invitrogen, Carlsbad, CA). (Oligos are listed in Supplementary Table S5 online). Amplificates were directly sequenced in two directions with the BigDye Terminator, version 1.3, Cycle Sequencing Kit and analyzed with the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Finally, sequences were monitored using the ChromasPro software, version 1.6 (Technelysium, South Brisbane, Australia).

CONFLICT OF INTEREST
The authors state no conflict of interest.

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.2018.02.015.

REFERENCES


