Oncogenic Mutations and Gene Fusions in CD30-Positive Lymphoproliferations and Clonally Related Mycosis Fungoides Occurring in the Same Patients

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The emergence of a common progenitor cell has been postulated for the association of CD30-positive lymphoproliferative disease (LPD) and mycosis fungoides (MF) within the same patient. Up to now, no comprehensive analysis has yet addressed the genetic profiles of such concurrent lymphoma subtypes. We aimed to delineate the molecular alterations of clonally related CD30-positive LPD and MF occurring in the same two patients. We analyzed the molecular profile of 16 samples of two patients suffering both from CD30-positive LPD and MF being obtained over a time course of at least 5 years. To detect oncogenic mutations, we applied targeted sequencing technologies with a hybrid capture-based DNA library preparation approach, and for the identification of fusion transcripts, an anchored multiplex PCR enrichment kit was used. In all samples of CD30-positive LPD and MF, oncogenic fusions afflicting the Jak/signal transducer and activator of transcription signaling pathway were present, namely *NPM1–TYK2* in patient 1 and *ILF3–JAK2* in patient 2. Additional signal transducer and activator of transcription 5A gene *STAT5A* mutations exclusively occurred in lesions of CD30-positive LPD in one patient. CD30-positive LPD and MF may share genetic events when occurring within the same patients. Constitutive activation of the Jak/signal transducer and activator of transcription signaling pathway may play a central role in the molecular pathogenesis of both entities.

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INTRODUCTION

During the last decade, a significant progress has been achieved to decipher the molecular pathogenesis of cutaneous lymphomas (Chevret and Merlio, 2016; da Silva Almeida et al., 2015; Damsky and Choi, 2016; McGirt et al., 2015; Prasad et al., 2016; Ungewickell et al., 2015; Wang et al., 2015; Woollard et al., 2016). Shared molecular profiles with convergent genetic aberrations in pivotal oncogenic pathways could be identified in mycosis fungoides (MF) or Sézary syndrome (Bastidas Torres et al., 2018b; Chang et al., 2018). Increasing insight into key genetic drivers does not only help to better understand the pathogenesis of cutaneous lymphomas but also opens the field for better prognostic algorithms and paves the way for the development of novel patient-specific targeted treatment options.

Until recently, only limited data had been available on the molecular pathogenesis and the key drivers of cutaneous CD30-positive lymphoproliferative diseases (LPDs) (Karai et al., 2013). The spectrum of these disorders includes lymphomatoid papulosis (LyP) and primary cutaneous anaplastic large cell lymphomas (cALCLs) (Benner and Willemze, 2009). As a unifying oncogenic mechanism in both entities, rearrangements involving the *DUSP22/IRF4* locus have been identified in 5% of LyP (Karai et al., 2013) and in up to 30% of cALCL (Feldman et al., 2009; Wada et al., 2011). Moreover, similar *DUSP22* rearrangements were also detected in 18% of CD30-positive transformed MF (Pham-Ledard et al., 2010). We recently deciphered further common molecular mechanisms of transformation occurring in up to 50% of CD30-positive LPDs by detecting highly recurrent activating hotspot mutations and oncogenic fusion transcripts that directly affect the Jak and signal transducer and activator of transcription (STAT) signaling pathway (Maurus et al., 2020). Motivated by these findings, we analyzed the molecular profile with respect to TCR rearrangement, oncogenic mutations, as well as gene fusions in two patients who each presented with concomitant or sequentially occurring distinct lesions of both CD30-positive LPD as well as MF. During a disease course of at least 5 years and a remarkably long follow-up of over 10 years in total, we were able to include

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Abbreviations: cALCL, cutaneous anaplastic large cell lymphoma; LPD, lymphoproliferative disease; LyP, lymphomatoid papulosis; MF, mycosis fungoides; PMID, PubMed ID; STAT, signal transducer and activator of transcription

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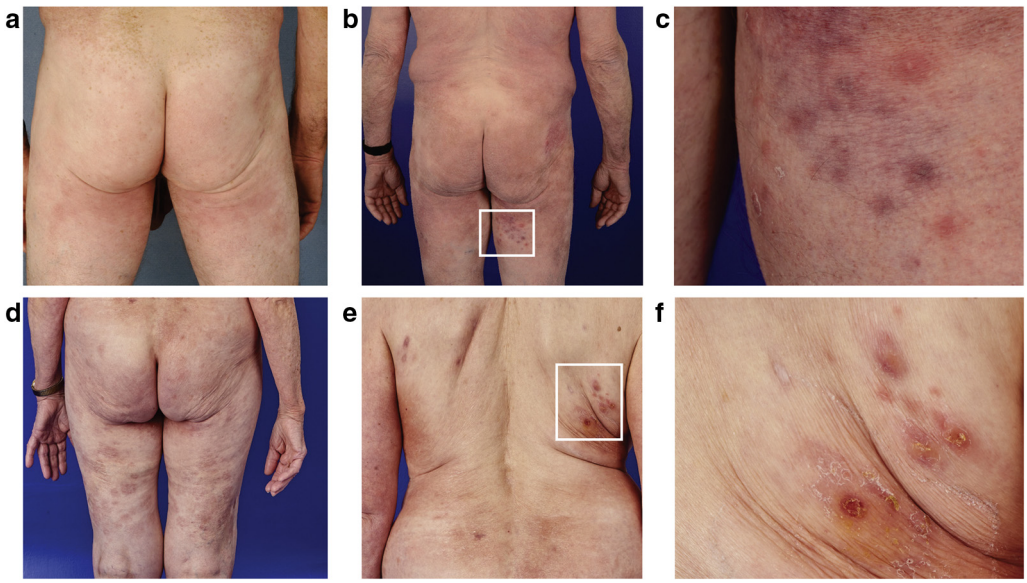

Figure 1. Representative clinical images of patients 1 and 2. (**a–c**) Patient 1. (**a**) Localized patches histologically proven as MF at the back, buttocks, and both thighs (date of presentation: 2012). (**b**) Slowly progressive patches of MF (date of presentation: 2017) with agminated self-healing papules corresponding to LyP lesions. Close-up view of the indicated cut-out in **c**. (**d–f**) Patient 2. (**d**) Disseminated long-standing patches and (**e**) spontaneously regressing papules at the trunk and extremities (date of presentation: 2019). Close-up view of the indicated cut-out in **f**. Patients consented to the publication of their images. LyP, lymphomatoid papulosis; MF, mycosis fungoides.

sequentially obtained biopsy samples (n = 16) of these two patients into our genetic analysis with a corresponding well-documented clinicopathological correlation of each assessed lesion.

RESULTS

To decipher the molecular characteristics of CD30-positive LPDs linked to MF, we performed different genetic screening analyses of 16 samples of two well-characterized patients (Figure 1 and Table 1).

Both patients suffer from MF and CD30-positive LPD

Patient 1 presented at the time of the first diagnosis with a long-standing MF stage IB with patches and plaques. He additionally developed at different time points during the further clinical course (total follow-up since the date of the first diagnosis is 41 years) small regressing papules being diagnosed as LyP. Whereas all MF samples were CD30 negative on histology with small epidermotropic CD4-positive lymphocytes as per definition, the LyP lesions showed strong expression of CD30 by the large neoplastic blasts (LyP type A). Close clinicopathological correlation yielded a clear-cut diagnosis for each lesion of patient 1 with an unequivocal attribution to the respective lymphoma subtypes (Figure 2). Samples for this analysis (n = 8) were obtained during a time course of 7 years (2012−2019).

Patient 2 was initially diagnosed with small-cell CD30-negative CD4-positive MF in stage IA. During the further disease course, the patient developed papules being compatible with LyP. In this patient, two lesions (samples 10 and 15) had to be designated as quite ambiguous or borderline on histological and/or clinical grounds. First, one larger papular lesion in patient 2 (sample 10) had been completely excised shortly after the occurrence, and therefore, no data on potential self-healing tendency could be obtained. This lesion was hence classified somehow with caution as LyP type C taking into account the possible differential diagnosis of transformed CD30-positive MF or cALCL, especially with regard to sheet-like CD30-positive atypical blasts in the dermis and the further disease course exhibiting quite extensive involvement of a draining lymph node. Secondly, one biopsy of an ulcerated small plaque (sample 15) in close proximity to spontaneously regressing

Table 1. Patient Characteristics

Patient Characteristics	Patient 1	Patient 2
Sex	M	F
Year of birth	1944	1939
Age at diagnosis, y	35	67
Date of primary diagnosis	1979	2006
Lymphoma subtype at primary diagnosis	MF stage IB	MF stage IA
Date of the first occurrence of LyP lesions	2002	2018
Sequential treatment	Topical steroids, PUVA, IFN, bexarotene, MTX	Topical steroids, PUVA, bexarotene, MTX, local irradiation, brentuximab
Current treatment	Topical steroids	Brentuximab
Extracutaneous manifestations	No	Yes (inguinal lymph node)
Follow-up, y	41	14
Final status	Alive with lymphoma (MF with patches/ plaques <10% BSA)	Alive with lymphoma (MF with patches/ plaques <10% BSA)

Abbreviations: BSA, body surface area; F, female; LyP, lymphomatoid papulosis; M, male; MF, mycosis fungoides; MTX, methotrexate; PUVA, psoralen plus UVA.

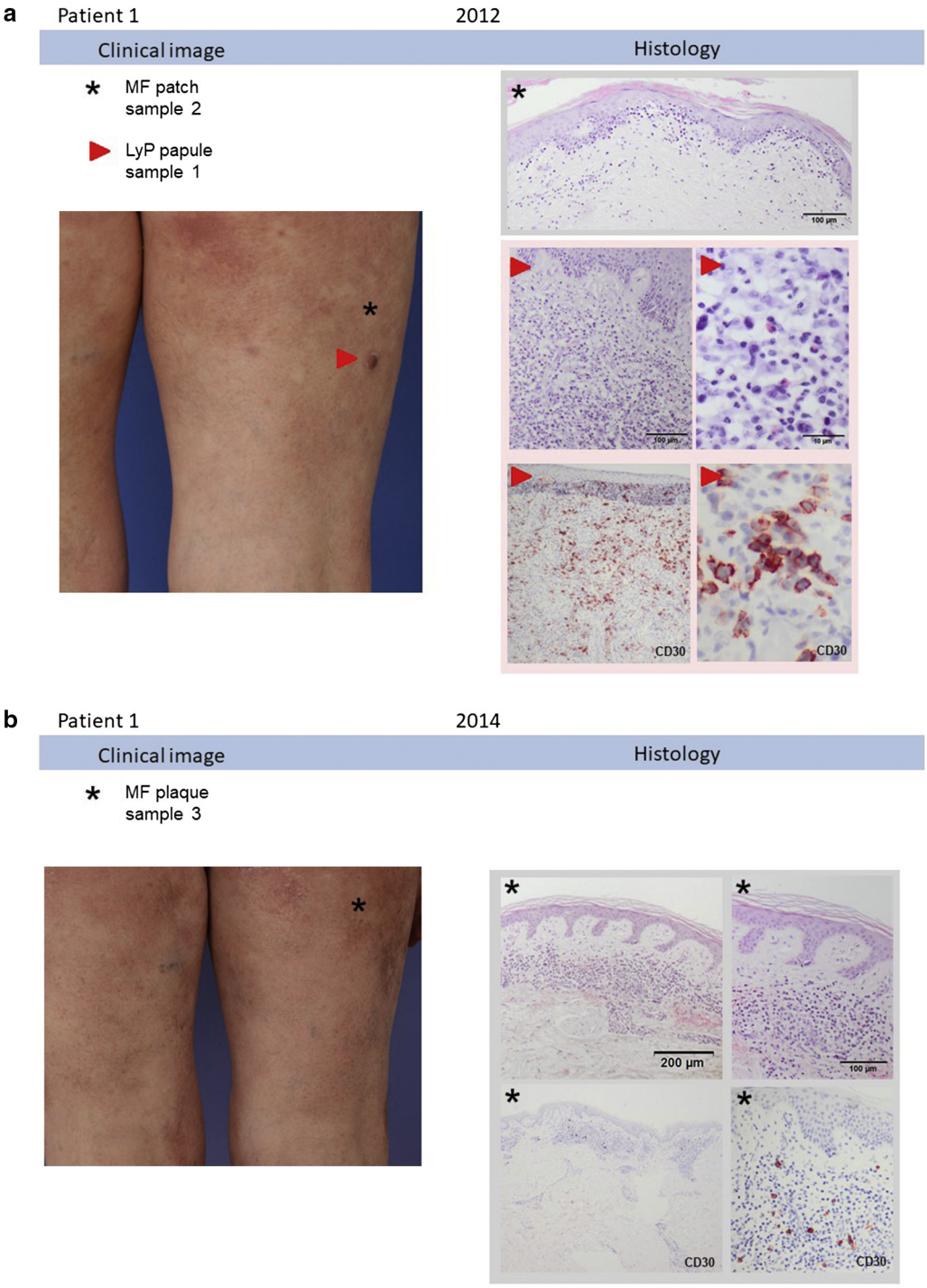

Figure 2. Representative clinical and histological images for each biopsy. Clinical features, area of biopsy taken for routine histology (FFPE), and further genetic analysis (FFPE, cryopreserved tissue) and photomicrographs of corresponding histological sections (H&E staining, CD30 staining) are illustrated for each patient over the disease course. Lesions of MF (patches, plaques) with no or faint/low CD30 expression of small neoplastic lymphocytes are indicated by an asterisk. All skin lesions with papular/nodular morphology and all lesions with large CD30-positive blasts on histology are indicated by a red arrow. Strong CD30 expression was found with variable frequency depending on LyP subtype (A vs. C). The lymph node showed focal nodular, sheet-like CD30-positive lymphoma infiltration rather than diffusely scattered lymphoma cells. Patients consented to the publication of their images. cALCL, cutaneous anaplastic large cell lymphoma; DD, differential diagnosis; FFPE, formalin-fixed, paraffin-embedded; LyP, lymphomatoid papulosis; MF, mycosis fungoides; NA, not available; tMF, transformed mycosis fungoides.

classical LyP papules exhibited on histology small epidermotropic lymphocytes with a faint expression of CD30 in about 20% of lymphoma cells. For this lesion, diagnosis of partially CD30-positive MF was favored over a possible differential diagnosis of LyP type D. However, all other lesions were clearly attributable to either MF or CD30-positive LPD (Figure 2). The analyzed lesions (n = 8) of patient 2 were obtained during a time course of 5 years (2015–2020).

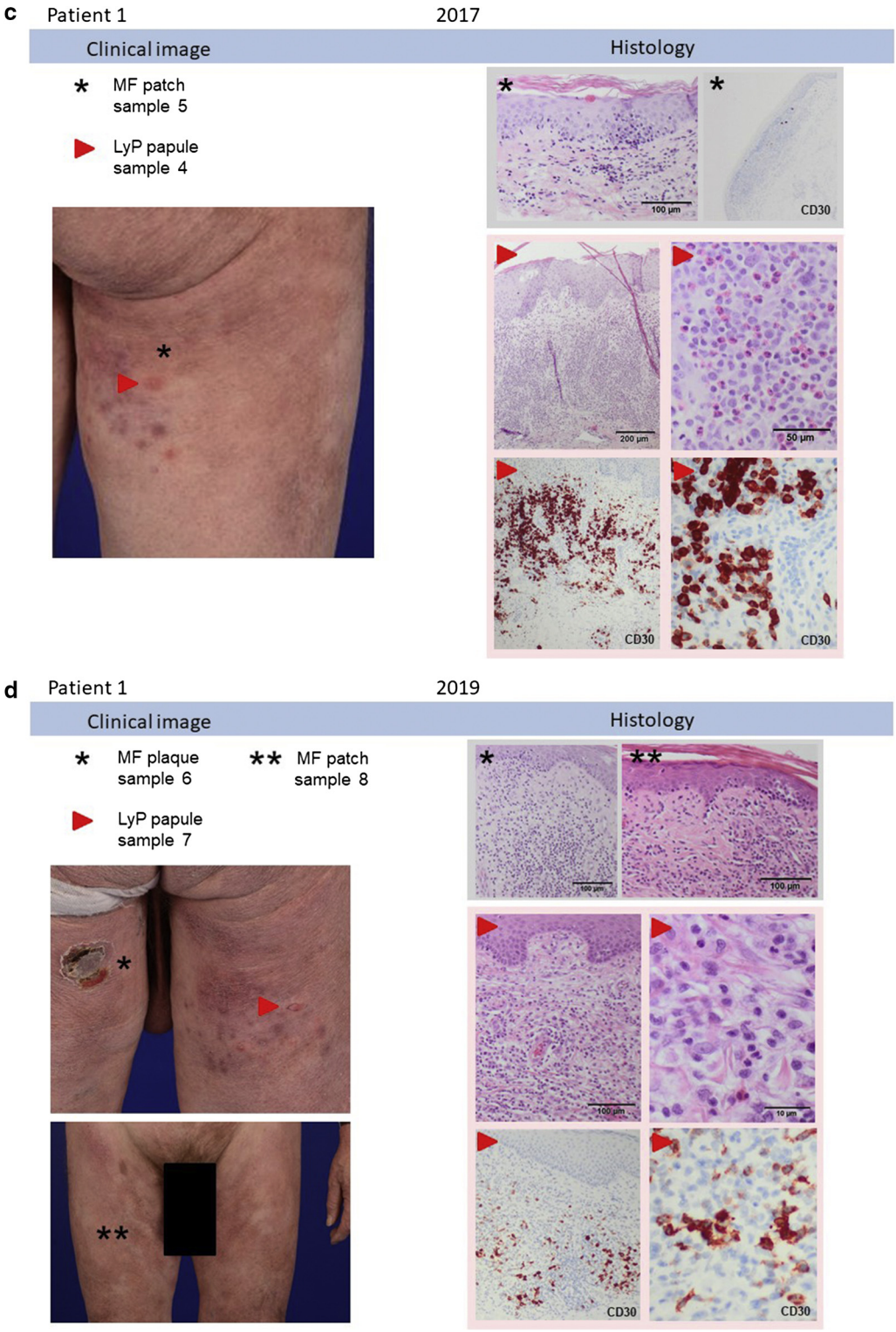

Figure 2. (*continued*).

Clonality analysis displays cognitional monoclonal T-cell populations in MF and CD30-positive LPD within the same patient

Concordant monoclonal amplificates were detected in each patient across all lymphoma subtypes (Table 2). These findings imply the close clonal relationship between MF and CD30-positive LPD within each of the two investigated patients.

RNA panel sequencing reveals oncogenic Jak fusions, including *TYK2* and *JAK2*

To determine pathogenic fusion transcripts, several samples of both patients were screened with Archer FusionPlex Pan-Heme panel (ArcherDX, Boulder, CO) comprising 199 genes being implicated in lymphomagenesis. In both examined patients, Jak fusions were detected (Table 2).

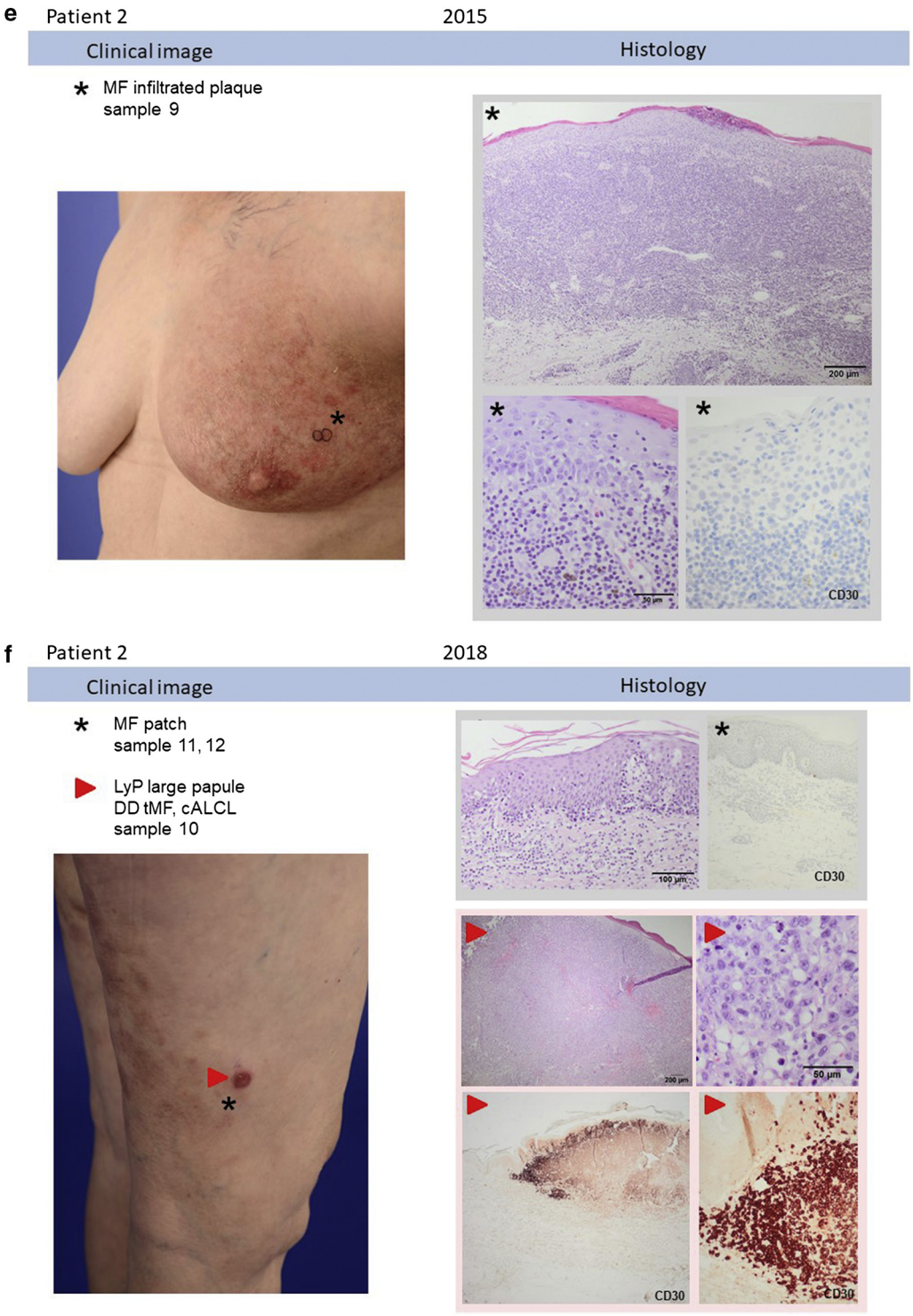

Figure 2. (*continued*).

Patient 1 showed an *NPM1–TYK2* (breakpoints according to hg19: chr5:170827929 and chr19:10468814) fusion transcript in the sequenced samples of both MF and CD30-positive LPD. To verify the presence of this oncogenic transcript in all tissue samples across all entities of patient 1, confirmatory PCR detection for the *NPM1–TYK2* transcript was conducted. All evaluated samples were positive for this transcript.

Patient 2 showed a different Jak fusion transcript, namely *ILF3–JAK2* (breakpoints according to hg19: chr19:10794646 and chr9:5080229) in the analyzable sequenced samples (9, 13, and 14). Samples 10–12 and 16 failed owing to low RNA quality extracted from archived formalin-fixed paraffin-embedded material. Confirmatory *ILF3–JAK2* PCR analyses affirmed the presence of this fusion transcript in all assessable lesions of both CD30-positive LPD and MF.

No *DUSP22* rearrangements (investigated by FISH analysis) or *ALK* fusions/expression were detected in any samples obtained from the two patients.

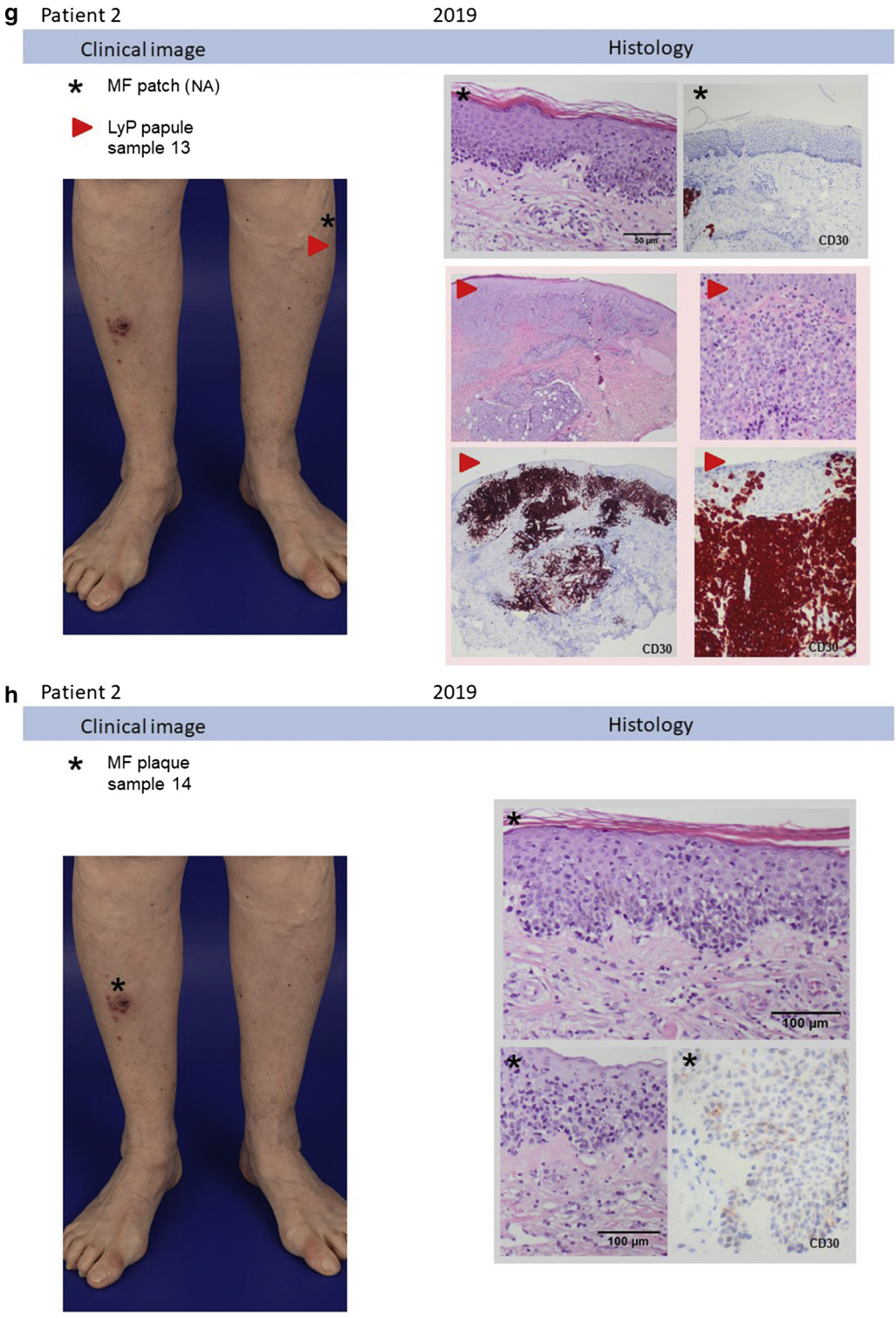

Figure 2. (*continued*).

Targeted DNA panel sequencing shows *STAT5A* mutations in CD30-positive LPD but not in CD30-negative MF of the same patient

After having detected the unifying genetic events in lesions of CD30-positive LPD and MF within these two patients, that is, shared T-cell clonality and oncogenic gene fusions afflicting the Jak gene, we further scrutinized the samples by deep sequencing. Our aim was to identify further molecular aberrations that could differentiate LyP from MF and thus may explain the distinct biological behavior and different histological and clinical phenotypes of these specific lymphoma subtypes.

We used a custom-designed DNA panel comprising 40 genes that are associated with T- and B-cell development and

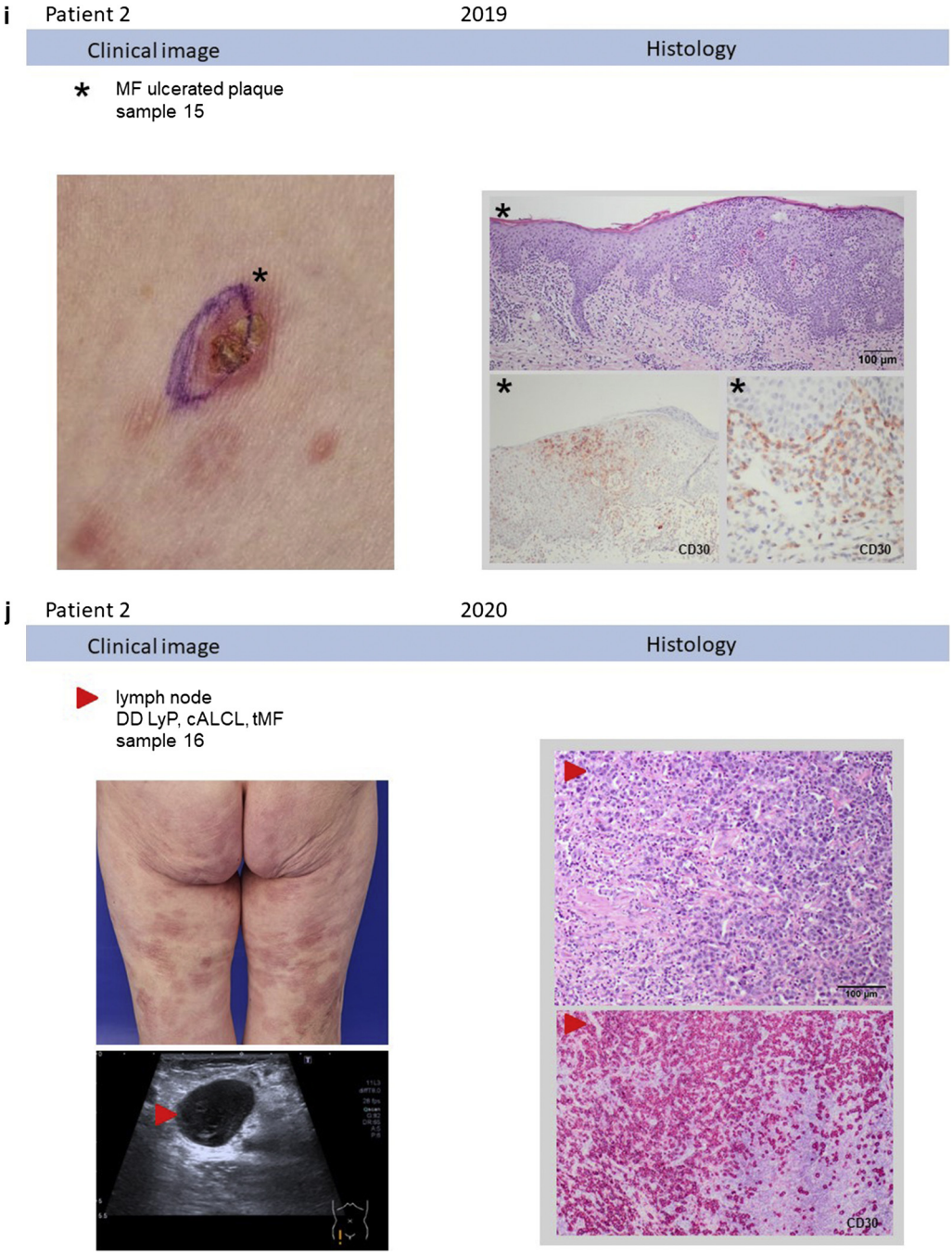

Figure 2. (*continued*).

lymphomagenesis as described elsewhere (Maurus et al., 2020). An overview of all detected mutations is shown in Table 2. No relevant germline mutations were identified.

In patient 1, we detected in all investigated samples of LyP (samples 1, 4, and 7) an identical *STAT5A* mutation (c.1297G > A, p.E433K, NM_003152.3). This hitherto unreported *STAT5A* mutation is located within the DNA-binding domain of the STAT5A protein, thus probably harboring the potential to activate the Jak/STAT signaling pathway. Of note, these *STAT5A* mutations were not present in any of the analyzed/evaluable MF lesions of patient 1. Hence, the exclusive presence of *STAT5A* mutations in CD30-positive LPD lesions but not in MF lesions within this same patient represents the cardinal discriminatory molecular feature in our applied genetic approach between these two lymphoma subtypes. For all other genes included in the panel, we obtained wild-type sequences for both lymphoma entities in patient 1.

In all evaluable samples (n = 6) of patient 2, the same oncogenic *DNMT3A* mutation (c.2186G > A, p.R729Q, NM_02255.4) could be detected in addition to the shared *ILF3–JAK2* fusion mentioned earlier. This *DNMT3A* mutation is predicted to be pathogenic and has already been described in other hematologic neoplasms (Ley et al., 2010). The

Table 2. Genetic Data and Phenotypic Findings

Sample	Date of Biopsy	Lesion Type	Diagnosis	Large Cell Morphology	CD30 Positivity > 10%	Gene	Mutation (cDNA)	Mutation (Protein)	Fusions	DUSP22	Clonality (TCRG)
Patient 1											
1	2012	Papule	LyP	Yes	Yes	*STAT5A*	c.1297G > A	p.E433K	*NPM1–TYK2*	Neg	Monoclonal 220 bp
2	2012	Patch	MF	No	No	Failed			*NPM1–TYK2*	Neg	Monoclonal 220 bp
3	2014	Plaque	MF	No	No	*STAT5A* wt			*NPM1–TYK2*	Neg	Monoclonal 220 bp
4	2017	Papule	LyP	Yes	Yes	*STAT5A*	c.1297G > A	p.E433K	*NPM1–TYK2*	Neg	Monoclonal 220 bp
5	2017	Patch	MF	No	No	Failed			*NPM1–TYK2*	Neg	Failed
6	2019	Plaque	MF	No	No	*STAT5A* wt			*NPM1–TYK2*	Neg	Failed
7	2019	Papule	LyP	Yes	Yes	*STAT5A*	c.1297G > A	p.E433K	*NPM1–TYK2*	Neg	Monoclonal 220 bp
8	2019	Patch	MF	No	No	*STAT5A* wt			*NPM1–TYK2*	Neg	Monoclonal 220 bp
Patient 2											
9	2015	Infiltrated plaque	MF	No	No	*PLCG1*	c.1034C > T	p.S345F subclonal 2%	*ILF3–JAK2*	NA	Monoclonal 162 bp
						DNMT3A	c.2186G > A	p.R729Q			
10	2018	Large papule	LyP, DD cALCL, tMF	Yes	Yes	Failed			Failed	Neg	Monoclonal 162 bp
11	2018	Patch	MF	No	No	Failed			Failed	Neg	Monoclonal 162 bp
12	2018	Patch	MF	No	No	*PLCG1* wt			Failed	Neg	Monoclonal 162 bp
						DNMT3A	c.2186G > A	p.R729Q			
13	2019	Papule	LyP	Yes	Yes	*PLCG1*	c.2254C > G	p.L752V	*ILF3–JAK2*	Neg	Monoclonal 162 bp
						DNMT3A	c.2186G > A	p.R729Q			
14	2019	Plaque	MF	No	No	*PLCG1*	c.2254C > G	p.L752V	*ILF3–JAK2*	Neg	Monoclonal 162 bp
						DNMT3A	c.2186G > A	p.R729Q			
15	2019	Ulcerated plaque	MF	No	Yes (faint)	*PLCG1* wt			*ILF3–JAK2*	Neg	Monoclonal 162 bp
						DNMT3A	c.2186G > A	p.R729Q			
16	2020	Lymph node	LyP, DD cALCL, tMF	Yes	Yes	*PLCG1* wt			Failed	Neg	Monoclonal 162 bp
						DNMT3A	c.2186G > A	p.R729Q			

Abbreviations: cALCL, cutaneous anaplastic large T-cell lymphoma; DD, differential diagnosis; FFPE, formalin-fixed paraffin-embedded; LyP, lymphomatoid papulosis; NA, not available; Neg, negative; tMF, transformed mycosis fungoides; wt, wild type.

Failed implied that the analysis failed owing to limited DNA and RNA quality from archived FFPE material.

presence of *DNMT3A* mutations was irrespective of lymphoma subtype and corresponding phenotype (large cell morphology, CD30-expression).

Albeit with a lower frequency, also *PLCG1* mutations (c.1034C > T, p.S345F and c.2254C > G, p.L752V) could be detected in patient 2. To our astonishment, these *PLCG1* mutations were present both in samples obtained from the lesions of CD30-positive LPD (n = 1) and those obtained from CD30-negative MF (n = 2) (sample 9: c.1034C > T, p.S345F; sample 13, 14: c.2254C > G, p.L752V, NM_002660.3). The oncogenic *PLCG1* p.S345F mutation has already been described in MF, has been functionally analyzed, and thus was designated as pathogenetically relevant (Vaqué et al., 2014). The second *PLCG1* mutation detected in our study (*PLCG1* p.L752V) lacks up till now any entry in relevant databases and is devoid of further functional characterization. However, a different mutation (*PLCG1* p.L752Q) that affects the same codon as *PLCG1* p.L752V has been predicted to be pathogenic according to the cosmic database. This fact suggests a potential relevance/pathogenicity of the variant (p.L752V) in the samples of patient 2 detected in this study. With respect to the two different lymphoma subtypes in this patient, we could not identify any mutation being exclusively present in only one versus the other lymphoma subtype as was the case in patient 1.

DISCUSSION

The concomitant or sequential association of LyP with additional solid or hematologic tumors—mainly cutaneous lymphomas (MF, cALCL) as in our two patients—has been known for decades and has been recently reassured by three larger retrospective studies (Cordel et al., 2016; Melchers et al., 2020a, 2020b, 2020c). Nevertheless, these lymphoma subtypes are rare disease entities, especially when occurring in combination within the same patient, and research data beyond clinical registries are mainly limited to case reports or small case series.

Owing to overlapping T-cell clones in lesions of LyP, cALCL, and/or MF within the same patient, the origin of a common progenitor cell has already been postulated before. Molecular workup of such cases was hitherto merely limited to PCR analysis of the *TCR* gene (Basarab et al., 1998; de la Garza Bravo et al., 2015; Stowman et al., 2016; Zackheim et al., 2003). Being in line with these previous publications, in our patients, an identical T-cell clone could be identified in all evaluable samples of the skin and lymph nodes in each of the two patients, respectively.

The first study published last year taking advantage of a more sophisticated approach recently addressed this issue by means of targeted next-generation sequencing and comparative genomic hybridization of samples taken from clonally related lesions of LyP and cALCL in one single patient (Xerri et al., 2019). However, in this specific case, despite a shared T-cell clone, rather distinct genetic profiles were described in each of these two entities (Xerri et al., 2019). This implies early divergence of respective subtypes from a common precursor cell in this patient. However, the presence of gene fusions—as addressed by our approach—was not the scope of this previous analysis.

By our meticulous genetic analysis of sequential biopsies (n = 16) of two different patients, we could observe several shared molecular aberrations comprising fusions and mutations in lesions of CD30-positive LPD and MF. Moreover, the pattern of the detected genetic events remained quite stable over time and was conserved in lesions occurring later on during clinical course over a follow-up of >5 years. Hence, divergent from the report mentioned earlier (Xerri et al., 2019), our findings suggest a rather late divergence of a common progenitor cell retaining its genetic profile even over decades. This was especially true for the detected gene fusions *NPM1–TYK2* and *ILF3–JAK2*, which were invariably present in all evaluable samples of each patient, respectively.

The identified fusion transcripts follow a classical mechanism of genomic translocations by fusing (i) effector domains of oncogenic driver genes with (ii) ubiquitously and strongly expressed genes of central cellular processes. In this case, we found the functionally relevant kinase domains of Jak (*JAK2* and *TYK2*) being fused to widely expressed RNA-binding proteins, namely the already described *NPM1* gene (Velusamy et al., 2014) and *ILF3*. This mechanism ensures the constitutive expression of the Jak effector domains exhibiting their oncogenic downstream signaling characteristics. Thus, these fusions are likely to represent an early common molecular event for lymphomagenesis of both lymphoma entities and thus might represent possible therapeutic targets, for example, by applying Jak inhibitors.

In patient 1, the cardinal unifying genetic aberration was an *NPM1–TYK2* fusion both in samples of LyP and MF mirroring this feature as the basic transforming mechanism for both lymphoma entities. Whereas *NPM1–TYK2* fusions have already been described in CD30-positive LPD, up till now, no similar gene fusions had been described in MF or Sézary syndrome. In MF, a rather complex and heterogeneous landscape of interchromosomal and intrachromosomal rearrangements has been observed. Various translocations resulting in deletion of tumor suppressors such as *SOCS1* or *HNRNPK* have recently been described in MF, which also mediate enhanced Jak signaling (Bastidas Torres et al., 2018a).

Of note, in patient 1, the LyP lesions could be unequivocally differentiated from MF lesions by clinical and histological/immunophenotypical findings and as well by discriminatory genetic features: additional *STAT5A* mutations were invariably present only in samples of LyP but not in samples of clonally related MF. Such a co-occurrence of fusions as well as mutations within the same pathway is a quite remarkable phenomenon. The detected *STAT5A* mutation (p.E433K) is located within the DNA-binding domain of the STAT5A protein with a proposed capacity to activate the Jak–STAT signaling pathway. This obvious molecular black-and-white pattern allows us to hypothesize that an aberrantly activated Jak–STAT pathway on the ground of a molecular two-hit-mechanism—oncogenic fusions followed by additional somatic mutations—may drive a common progenitor cell of patient 1 to a neoplastic lymphoma cell of LyP with consecutive distinct biological, histological, and clinical features.

Similar to patient 1, analogous rearrangements of genes of the Jak/STAT family, namely *ILF3–JAK2* fusions, were also observed in evaluable lesions of patient 2. In addition, all evaluable lesions of both CD30-positive LPD and MF of

patient 2 exhibited the same *DNMT3A* mutation (p.R729V) being predicted to impair the catalytic methyltransferase activity. As far as we know, alterations of the *DNMT3A* gene have not been described in LyP or cALCL. In contrast, recurrent alterations of epigenetic regulators, including the *DNMT3A* gene (mutations, deletions), have been identified with variable frequencies in systemic anaplastic large cell lymphoma (Di Napoli et al., 2018), MF, peripheral T-cell lymphomas, and myeloid neoplasms (Chang et al., 2018; Choi et al., 2015; Damsky and Choi, 2016; Palomero et al., 2014) with prognostic and therapeutic implications (Ley et al., 2010; Park et al., 2020).

In contrast to universally present *DNMT3A* mutations in all samples, only 50% of the analyzed samples of patient 2 also showed *PLCG1* mutations. Of note, these additional single nucleotide variants occurred irrespective of lymphoma subtype or phenotype. Moreover, no further mutations of Jak–STAT–related genes were present in samples obtained from this patient. Corresponding to these molecular data, differentiation between LyP and MF in patient 2 was not as clear cut as in patient 1: clinical, histological, and immunophenotypical findings suggested, for example, for samples 10 and 15 rather a continuum of these lymphoma subtypes with categorization as borderline as already discussed in the literature (Bekkenk et al., 2000; Fauconneau et al., 2015; Gao et al., 2021; Kadin et al., 2014). Until now, no reliable immunohistochemical or molecular marker has been established for daily routine to clearly differentiate CD30-positive MF from CD30-positive LPD. Hence, final diagnosis still relies on close clinicopathological correlation and meticulous monitoring of these patients (Bekkenk et al., 2000; Eberle et al., 2012; Fauconneau et al., 2015; Kadin et al., 2014; Kempf et al., 2011; Lezama and Gratzinger, 2018; Pham-Ledard et al., 2010; Vergier et al., 2000) as outlined in Figure 2.

Interestingly, the activating *PLCG1* S345F mutation occurred subclonally in sample 9 but was not detectable anymore in later biopsies. Instead, the patient acquired an alternative *PLCG1* mutation (p.L752V) in lymphoma lesions evolving during the further clinical course (13 and 14), thus underlining the general importance of *PLCG1* alterations for lymphomagenesis. Single nucleotide variants in *PLCG1* leading to constitutive activation of the nuclear factor of activated T-cells pathway have been designated as indicators of progression and higher tumor stage in MF (Pham-Ledard et al., 2010; Tensen, 2015; Vaqué et al., 2014). In CD30-positive LPD, mutations in *PLCG1* have not been described yet.

In conclusion, CD30-positive LPD and MF harbor unifying molecular aberrations (Kempf et al., 2018; Willemze and Meijer, 2003). Our data on two patients presented in this paper further substantiate the concept that oncogenic fusions together with somatic mutations in *JAK/STAT* genes play a key role in the pathogenesis of CD30-positive LPD. Additional underlying molecular and epigenetic mechanisms that drive the final phenotype of the common precursor cell to evolve into skin manifestations corresponding to either LyP, cALCL, or MF still remain to be elucidated and will be the scope of further investigation.

MATERIALS AND METHODS

Patient characteristics

Within a retrospective setting, two well-characterized patients suffering from both CD30-positive LPD and MF were retrieved from the clinical and histological archives of the Department of Dermatology, University Hospital Würzburg (Germany), and sequential biopsy samples were analyzed. Two further cases also suffering from both LyP and MF were omitted from further investigation because in these two cases, the lesions of the different lymphoma subtypes were clinically located in close relation at the skin, and microdissection of biopsy samples was not possible to discern with certainty the respective histological subtypes for further genetic workup. A close correlation of clinical features (Figure 1) with corresponding histopathological findings as well as appropriate staging examinations were applied to establish a final diagnosis of CD30-positive LPD or MF with respective disease stage attribution (Olsen et al., 2007). Subtype-specific treatment followed national and international guidelines (Dippel et al., 2017; Kempf et al., 2011; Willemze et al., 2019).

Fresh-frozen cryopreserved tissue was available from both patients (five samples in total; samples 4, 9, and 13–15). To enlarge the number of samples for this study, we further analyzed formalin-fixed, paraffin-embedded material of the two patients (11 samples in total; samples 1–3, 5–8, 10–12, and 16). Data on histological and immunophenotypical features of each lesion of our analyzed patients are included in Table 2.

Approval of the entire study was obtained from the Ethics Committee at the Medical Faculty of the University of Würzburg. Informed written consent was obtained from all patients before analysis. Patients consented to the publication of their images.

DNA and RNA extraction, cDNA synthesis, and hybridization-based panel sequencing

Samples were processed as previously described (Maurus et al., 2020). For all lesions, which were in close proximity to each other at the skin, microdissection was performed to clearly differentiate between LyP lesions (samples 1, 4, 10) and cutaneous manifestations of associated MF (samples 2, 5, 11, 12). Read statistics are depicted in Supplementary Table S1.

Data analysis quality trimming. An initial quality assessment was performed using FastQC, version 0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and low-quality reads were trimmed from 151 bps paired-end reads using TrimGalore, version 0.4.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), powered by Cutadapt, version 1.8 (https://cutadapt.readthedocs.io/en/stable/).

Read alignment. The trimmed reads were mapped to the human reference genome (hg19) using BWA MEM, version 0.7.17 (PubMed ID [PMID]: 19451168), and sorted and indexed using Picard, version 1.125 (available online at http://broadinstitute.github.io/picard/), and SAMtools, version 1.3 (PMID: 19505943), using htslib, version 1.3. Local insertion–deletion realignment was executed with GATK, version 3.5 (PMID: 20644199).

According to the manufacturer's instructions, a deduplication step was added using the AgilentMBCDedup tool, version 1.0, provided by Agilent (Santa Clara, CA). GATK, version 3.5, was also used for coverage calculations.

Somatic variant calling. MuTect1, version 1.1.4 (PMID: 23396013); VarScan2, version 2.4.1 (PMID: 22300766); and Scalpel, version 0.5.3 (PMID: 27854363), were used to identify somatic single nucleotide variants and small somatic insertions or deletions (Supplementary Table S2). All variants were annotated with ANNOVAR, version 2019-10-24 (PMID: 20601685). Variants were considered somatic if they have an impact on the protein sequence or if they affect a splice site, if they are rare in the population (below a frequency of 2% in 1000g2015aug_all, ExAC_nontcga_ALL, gnomAD_exome_ALL, and gnomAD_genome_ALL), if the position is covered by at least 20 reads and the alternative allele is covered by at least 8 reads, and if they comprised at least 2% and are absent in the matched normal blood sample.

All variants were visually examined using the Integrative Genomics Viewer, version 2.3.68 (Thorvaldsdóttir et al., 2013). In addition, all detected variants were checked in all corresponding samples of the same patient.

RNA fusion sequencing

We used an anchored multiplex PCR-based next-generation sequencing assay, including 199 genes related to lymphoid and myeloid malignancies, which allowed us to identify any fusion affecting these genes. RNA libraries were prepared of the following material: patient 1 samples 1, 3, and 4 and patient 2 samples 9–14 using the Archer FusionPlex Pan-Heme Kit strongly according to the Archer FusionPlex Protocol for Illumina. Libraries were sequenced on the NextSeq 500 platform (Illumina, San Diego, CA). Sequencing data were analyzed with the Archer Analysis software, version 6.2.3, using default parameters for quality assessment and fusion calling/annotation. Failed samples are mentioned in Table 2 and Figure 3 and were excluded from interpretation. In addition, to the Archer analysis pipeline, data were analyzed with an alternative tool. Therefore, adapters and low-quality reads were trimmed with TrimGalore, version 0.4.0, powered by Cutadapt, version 1.8. For fusion detection, Arriba, version 1.1.0, (https://github.com/suhrig/arriba), which is based on the STAR aligner, version 2.5.4b (https://github.com/alexdobin/STAR), was used with default settings. Strong fusions were considered for confirmatory PCR analyses.

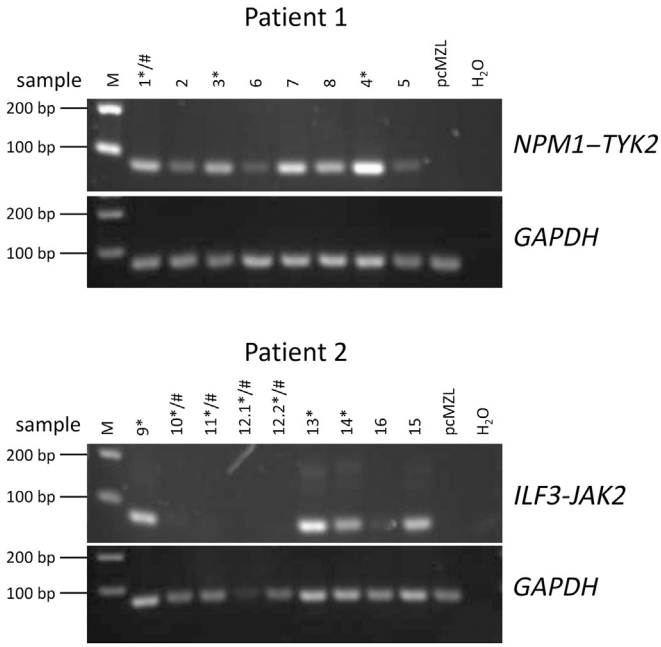

Figure 3. Gel electrophoresis of fusion transcripts. Gel electrophoresis of patients 1 and 2 for fusion transcript detection with asterisk (*)-marked samples was investigated by targeted RNA fusion sequencing with Archer FusionPlex Pan-Heme panel, with the hash (#)-marked samples indicating failed default quality parameters of targeted RNA fusion sequencing analyzed by the Archer Analysis software, version 6.2.3; pcMZL served as a neutral FFPE RNA/cDNA control; 12.1/12.2 depict a duplicate of the same sample. Sample 16 (FFPE) was excluded from interpretation owing to a weaker GAPDH signal and an ambiguous *ILF3–JAK2* fusion signal. M represents 100 bp ladder. FFPE, formalin-fixed paraffin-embedded; MF, mycosis fungoides; pcMZL, primary cutaneous marginal zone lymphoma.

PCR fusion analysis

The fusion target regions detected by targeted RNA sequencing were amplified from cDNA of all analyzed patient samples using DreamTaq-DNA Polymerase (Invitrogen, Carlsbad, CA). In addition, a GAPDH amplificate was generated to check for RNA/cDNA quality. For the amplification of the *NPM1–TYK2* and the *ILF3–JAK2* fusions, the following primers were used:

ACTCCAGCCAAAAATGCACAAA (forward) and CTCAGCTTGATGAAGGGGCT (reverse) for *NPM1–TYK2* (59 °C annealing temperature, amplificate length 75 bp), GCTATGGGTACGGAGGCAAC (forward) and TCAGGTGGTACCCATGGTATTC (reverse) for *ILF3–JAK2* (60 °C annealing temperature, amplificate length 73 bp), and

CCGCATCTTCTTTTGCGTCG (forward) and ATCCGTTGACTCCGACCTTC (reverse) for *GAPDH* (60 °C annealing temperature, amplificate length 78 bp). Amplificates were separated on a 2% agarose gel (Figure 3).

DUSP22/IRF4 FISH screening

Paraffin sections were treated with the VP2000 processor (Abbott, Chicago, IL) and stained with the Kreatech IRF4/DUSP22 (6p25) Break FISH probe (Leica Biosystems, Wetzlar, Germany) for 16 hours at 37 °C. Stained slides were analyzed by fluorescent microscopy.

TCRG rearrangement analysis

T-cell clonality was analyzed by PCR amplification using the Biomed-2 primers for *TCRG* (van Dongen et al., 2003).

Data availability statement

Data sets related to this article can be found at https://www.ebi.ac.uk/ega/datasets, hosted at the European Genome-Phenome Archive (EGAS00001005094).

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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.xjidi.2021.100034.

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