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Deregulation of JAK2 signaling underlies primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma

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Haematologica 2022
Volume 107(3):702-714

ABSTRACT

Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma (pcAECyTCL) is a rare variant of cutaneous T-cell lymphoma with an aggressive clinical course and a very poor prognosis. Until now, neither a systematic characterization of genetic alterations driving pcAECyTCL has been performed, nor effective therapeutic regimes for patients have been defined. Here, we present the first high-resolution genetic characterization of pcAECyTCL by using whole-genome and RNA sequencing. Our study provides a comprehensive description of genetic alterations (i.e., genomic rearrangements, copy number alterations and small-scale mutations) with pathogenic relevance in this lymphoma, including events that recurrently impact genes with important roles in the cell cycle, chromatin regulation and the JAK-STAT pathway. In particular, we show that mutually exclusive structural alterations involving *JAK2* and *SH2B3* predominantly underlie pcAECyTCL. In line with the genomic data, transcriptome analysis uncovered upregulation of the cell cycle, JAK2 signaling, NF-κB signaling and a high inflammatory response in this cancer. Functional studies confirmed oncogenicity of *JAK2* fusions identified in pcAECyTCL and their sensitivity to JAK inhibitor treatment. Our findings strongly suggest that overactive JAK2 signaling is a central driver of pcAECyTCL, and consequently, patients with this neoplasm would likely benefit from therapy with JAK2 inhibitors such as Food and Drug Adminstration-approved ruxolitinib.

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Received: October 19, 2020.

Accepted: March 24, 2021.

Pre-published: April 1, 2021.

https://doi.org/10.3324/haematol.2020.274506

Introduction

Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma (pcAECyTCL) is a rare variant of cutaneous T-cell lymphoma (CTCL) still regarded as a provisional entity by the World Health Organization (WHO) and characterized by an abrupt onset and a highly aggressive clinical course.[1,2] pcAECyTCL presents primarily in the skin with widespread plaques and tumors, often with hemorrhagic ulcerations and necrosis; however, dissemination to extracutaneous sites (especially the central nervous system, lungs, oral cavity and testes) is not uncommon.[3,4] Malignant T cells causing pcAECyTCL typically express CD3, CD7, CD8, CD45RA, TCR-βF1, T-BET and one or more cytotoxic markers (e.g., granzyme B, perforin, TIA-1), which strongly suggests that neoplastic cells in this lymphoma derive from CD8+ T cells.[2,3] Effective therapeutic regimes for pcAECyTCL are currently lacking, and consequently, patients have a poor prognosis with a median overall survival of 12 months.[1]

Thus far the study of the pathogenetic basis of this malignancy has been marginal due to its rarity. Recently, a study performed on tumors from 20 patients defined the copy number alteration (CNA) profile of pcAECyTCL by using array-based comparative genomic hybridization,[5] and before this, two clinical case reports

included the evaluation of CNA in single patients by using array-based methods as well.[6,7] Recurrent CNA uncovered by these studies include losses within 1p, 9p, 13q and 16p as well as gains within 7q, 8q and 17q, with loss of the region containing *CDKN2A/B* being the most frequent CNA.[5] However, aside from the aforementioned chromosomal imbalances, causative genetic changes in pcAECyTCL remain unknown.

Here, we present the first high-resolution genomic analysis of pcAECyTCL using whole-genome sequencing (WGS) and RNA sequencing (RNA-seq). We describe for the first time a number of genomic rearrangements, CNA and small-scale mutations with pathogenic relevance in this lymphoma. In particular, our results suggest that overactivation of JAK2 signaling due to oncogenic changes in *JAK2* and *SH2B3*, two genes with key roles in this signaling pathway, underlie predominantly pcAECyTCL. These findings have important implications for patient standard of care.

Methods

Patient selection and sequencing

Frozen tumor biopsies (≥70% tumor cells) from 12 patients with pcAECyTCL (*Online Supplementary Figure S1*; *Online Supplementary Table S1*) were subjected to WGS. Six samples of this cohort (i.e., AEC2-4/6/8/12) were additionally subjected to RNA-seq. Sequencing, data processing and DNA/RNA analyses are described in the *Online Supplementary Appendix* (*Online Supplementary Figures S2* and *S3*; *Online Supplementary Tables S2 to S9*). Diagnosis was performed by an expert panel of dermatologists/pathologists in accordance with the WHO-EORTC classification for primary cutaneous lymphomas.[1,2] Patient material was approved by the Institutional Review Boards of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and Leiden University Medical Center. Informed consent was obtained from patients in accordance with the declaration of Helsinki.

Validation of structural genomic alterations and small-scale mutations

Select rearrangements, interstitial deletions and single nucleotide variants (SNV) were validated by Sanger sequencing, droplet digital polymerase chain reaction (ddPCR) and/or fluorescence *in situ* hybridization (FISH). Details of the validation experiments are included in the *Online Supplementary Appendix* (*Online Supplementary Figures S4 to S9*; *Online Supplementary Table S10*).

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections were immunohistochemically stained with primary antibodies against phospho-STAT3 (Cell Signaling Technology, Cat.No. 9145) or phospho-STAT5 (Cell Signaling Technology, Cat.No. 9359) using Dako REAL detect system (Dako, Cat.No. K5005), counterstained in Mayer's hematoxylin solution and coverslipped using Vectamount (Vector Laboratories, Cat.No. H5000).

Cell culture, fusion gene construction and viral transduction

Ba/F3 cells (DSMZ, Cat.No. ACC-300) were used for functional experiments. Parental Ba/F3 cells were cultured in RPMI-1640 (10% heat-inactivated fetal bovine serum, 10 ng/mL interleukin-3 [IL3]) at 37° C with 5% CO_2 in a humidified atmosphere. *JAK2* fusions (i.e., *TFG-JAK2*, *PCM1-JAK2*, *KHDRBS1-JAK2*) and control genes (i.e., eGFP, *TFG-MET*) were constructed and inserted into a lentiviral vector using the method described by Lu *et al.*[8] Primers, templates and vectors used for fusion gene construction are detailed in the *Online Supplementary Appendix* (*Online Supplementary Tables S11 to S13*). Lentiviral particles were produced in HEK-293T cells, quantified by p24 enzyme-linked immunosorbent assay (ELISA), and transduced into Ba/F3 cells at MOI-9 with lipofectamine. Successfully transduced Ba/F3 cells were selected with puromycin (2.5 μg/mL) for 3 days.

Ba/F3 cell viability and inhibitor assays

Cell viability of parental and transduced Ba/F3 cells was determined 7 days after IL3 withdrawal by MTT assay (Promega, Cat.No. G4000). Inhibitor assays were performed by treating IL3-independent Ba/F3 cells expressing fusion genes with ruxolitinib or AZD1480 at seven different concentrations for 72 hours and measuring cell viability by MTT assay.

Western blots

The effect of JAK1/2 inhibitors ruxolitinib and AZD1480 on JAK2 and STAT5 phosphorylation was evaluated by western blotting. Cells were washed to remove traces of serum and incubated with inhibitor for 90 minutes. Cells were lysed in SDS lysis buffer containing protease inhibitors and separated by SDS-PAGE. Antibodies employed were anti-JAK2 (Abcam, Cat.No. ab108596), anti-phospho-JAK2 (Cell Signaling Technology, Cat.No. 3776), anti-STAT5 (Cell Signaling Technology, Cat.No. 94205), anti-phospho-STAT5 (Cell Signaling Technology, Cat.No. 9351) and anti-GAPDH (Cell Signaling Technology, Cat.No. 2118).

Results

JAK2 fusions are prominent in a complex landscape of rearrangements

The analysis revealed a heterogeneous and complex landscape of genomic rearrangements (total events, 426; range, 10-65; mean/patient ± standard deviation [SD], 36±21) (Figure 1; Figure 2A; *Online Supplementary Figure S3*). Fifty-three percent of events were interchromosomal (range/patient, 27-80%) (Figure 2B). The majority of rearrangements (77%) disrupted either one or two annotated genes, while the rest (23%) disrupted nongenic regions (Figure 2C). Four patients, AEC6, AEC7, AEC10 and AEC12, displayed complex rearrangements (chromothripsis/chromoplexy-like) affecting chromosomes 13, 10, 1/9/12 and 4, respectively (Figure 2D; *Online Supplementary Figure S3* and *S10*). We observed a total of 305 rearranged genes, 59 of which are implicated in neoplasms at present (*Online Supplementary Table S14*). Gene ontology analysis revealed that rearranged genes encode principally (n_{genes}=91 of 305) proteins with roles in signal transduction (i.e., hydrolases, transferases, enzyme modulators, receptors) and transcriptional regulation (i.e., transcription factors, chromatin regulators) (Figure 2E; *Online Supplementary Tables S15* and *S16*). Out of seventeen recurrently rearranged genes detected in our cohort ($n_{patients}$ =2 or 3) (*Online Supplementary Table S17*), six are established cancer genes with important functions in the regulation of the cell cycle (i.e., *MYC*, *RB1*), chromatin remodeling (i.e., *BAZ1A*) and the JAK-STAT pathway (i.e., *JAK2*, *PTPRC*, *SH2B3*) (Figure 1; *Online Supplementary Figures S4* and *S5*).

The JAK-STAT pathway, a frequent driver of hematological neoplasms, was the only cytokine-elicited signal transduction pathway impacted by rearrangements in pcAECyTCL. Fusion genes involving *JAK2* were detected

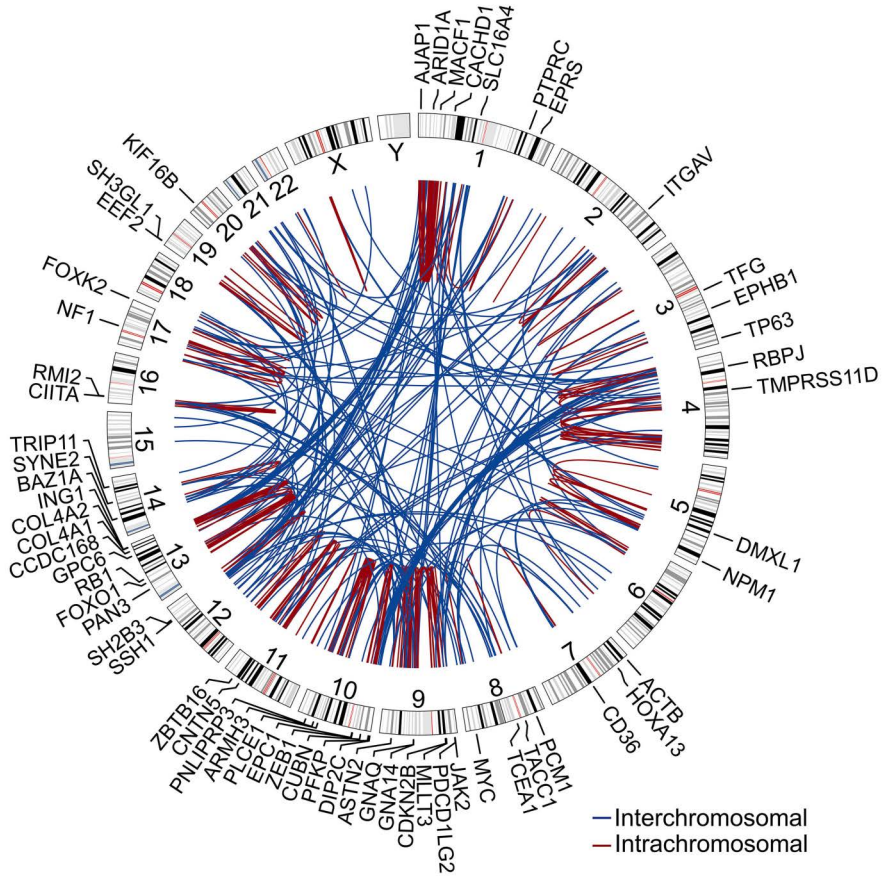

Figure 1. Landscape of genomic rearrangements in pcAECyTCL. Circos plot showing 426 genomic rearrangements detected in twelve pcAECyTCL genomes by whole-genome sequencing (WGS). The outer ring shows rearranged genes with established roles in cancer. The area at the center of the plot contains arcs representing interchromosomal (blue) and intrachromosomal (red) events. The ring between the gene labels and the arcs contains human chromosome ideograms arranged circularly end to end. pcAECyTCL: primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma.

in three of 12 patients (i.e., AEC4: *KHDRBS1-JAK2*; AEC9: *PCM1-JAK2*; AEC11: *TFG-JAK2*) (Figure 2F to H). These events fused the tyrosine kinase domain of JAK2 with one or more oligo/dimerization domains from the fusion partner (i.e., AEC4: Qua1 domain, AEC9: coiled-coil domains, AEC11: PB1 domain) (Figure 2F to H). The resulting chimeric proteins are predicted to self-oligo/dimerize and become activated without the need of cytokine-mediated receptor stimulation, ultimately overactivating JAK2 signaling. Of note, two of three patients carrying JAK2 fusions carried *MYC* fusions as well (i.e., AEC4: *ACTB-MYC*, AEC9: *NPM1-MYC*) (*Online Supplementary Figure S4*). Interestingly, apart from acquiring the ability to self-activate, *JAK2* fusions under the transcriptional control of their partner's promoter may also experience augmented expression in comparison to wild-type *JAK2*, as evidenced in patient AEC4 (Figure 2I). In contrast, rearrangements involving *PTPRC* and *SH2B3*, each observed in two of 12 patients, disrupted these two negative regulators of the JAK-STAT pathway.

JAK2 signaling inhibitor SH2B3 is focally deleted in pcAECyTCL

The most frequent broad chromosomal imbalances ($n_{patients} \geq 4$; >3 Mb) were deletions within 1p, 8p, 9q, 10p, 11q and 13q and gains within 7q, 8q, 17q and 21q (Figure 3A; Figure 4A). We identified 24 recurrent focal (≤ 3 Mb) minimal common regions (MCR) shared by CNA between patients ($n_{patients} \geq 3$; deletions: 19, gains: 5) (*Online Supplementary Table S5*), 12 of which contained cancer genes predominantly involved in the cell cycle, chromatin regulation and the JAK-STAT pathway (Figure 4A).

The most common focal MCR involving cancer genes was deletion at 9p21.3 (10 patients), which included cell cycle regulators *CDKN2A/B* (*Online Supplementary Figure S7*). Of note, *CDKN2A/B* were found to be inactivated by interstitial deletions, unbalanced rearrangements, SNV and presumably even the action of long non-coding RNA *ANRIL* (*CDKN2B-AS1*)[9] (Figure 4A; *Online Supplementary Figure S11*). Five of 12 patients had deletions at 1p36.11 and 13q14.11, which contained chromatin remodeler *ARID1A* and candidate cancer gene *ELF1*,[10] respectively. Deletions at 1p36.32-p36.33, 1p36.22 and 12q24.12, observed in four of 12 patients, involved tumor suppressors *TNFRSF14*, *MIR34AHG* and *SH2B3*, respectively. Finally, three of 12 patients had deletions at 4q13.1-q13.2, 10p11.22, 11q14.2, 16p13.13 and 19p13.3, which contained tumor suppressors *EPHA5*, *EPC1* (alongside *ZEB1*), *EED*, *SOCS1* and *STK11*, respectively. On the other hand, gain at 17q21.31 (four patients), which enclosed *ETV4*, was the only recurrent ($n_{patients} \geq 3$) focal gain containing a cancer gene.

Remarkably, deletions at 12q24.12 were strikingly focal in all affected patients (20 Kb – 457 Kb), leading to the loss of one or more functional domains of SH2B3 (i.e., DD, PH, SH2 domains) in these individuals (Figure 3B and C; *Online Supplementary Figure S5*). *SH2B3* (LNK) encodes an adaptor protein that antagonizes JAK2 signaling as part of a negative feedback loop in various hematopoietic cell types (e.g., erythroid progenitors, hematopoietic stem cells, megakaryocytes, pre-B cells, etc.) by suppressing the kinase activity of JAK2 through its SH2 domain.[11] Of note, structural alterations involving *JAK2* and *SH2B3* were mutually exclusive in our cohort, affecting altogether seven of 12 patients. In addition, we investigated the possibility of *SH2B3* silencing by promoter hypermethylation

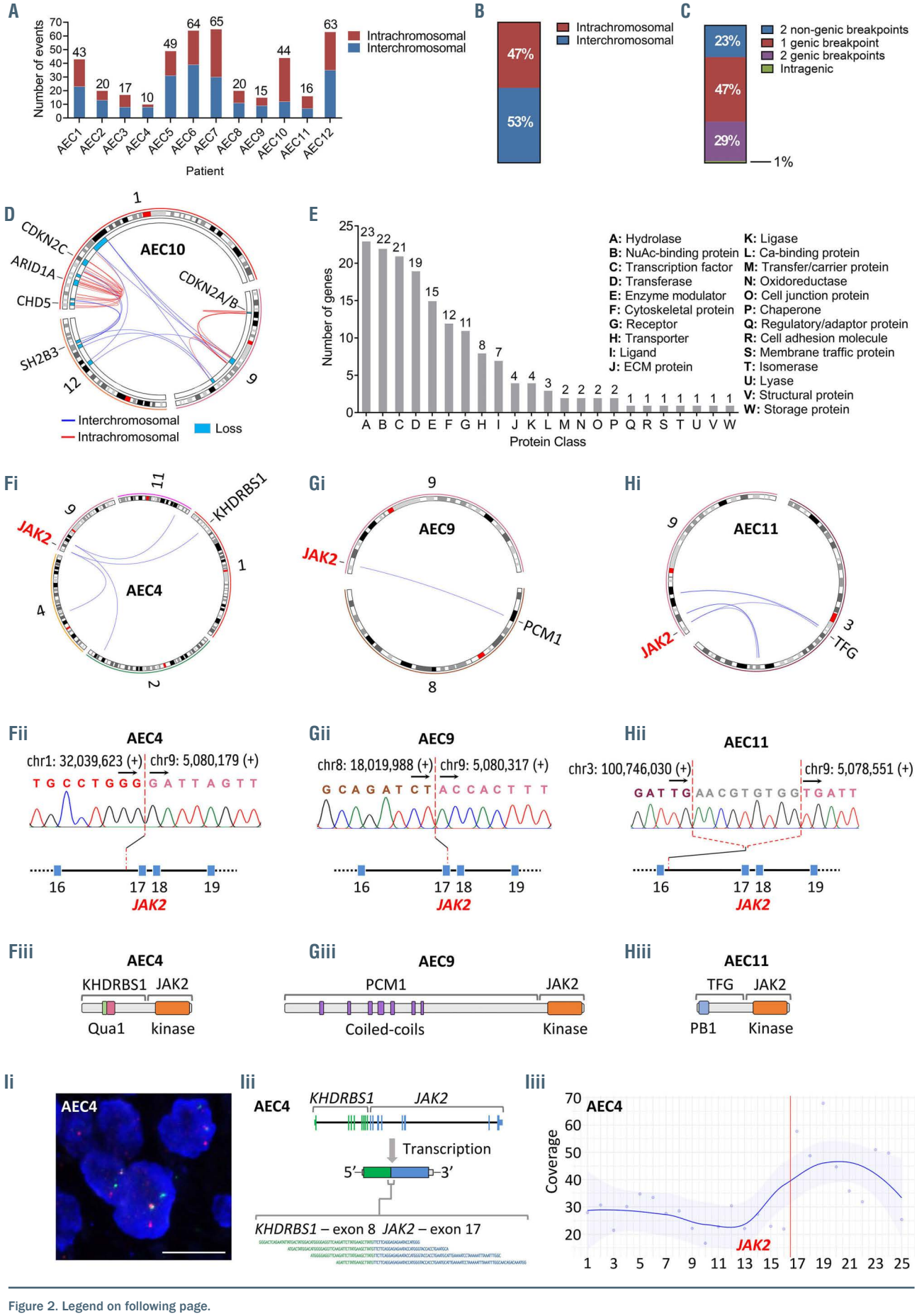

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Figure 2. *JAK2* fusions are recurrent in a complex landscape of rearrangements. (A) Number of genomic rearrangements per patient. The distribution of inter- and intrachromosomal rearrangements per patient is shown too. (B) Distribution of inter- and intrachromosomal rearrangements (cohort). (C) Distribution of genomic rearrangements based on the type of DNA sequences (genic, nongenic) involved in the event (cohort). (D) Circos plot showing a chromoplexy-like event in patient AEC10 that mediated the loss of multiple genomic regions in chromosomes 1, 9 and 12, several of which enclosed established tumor suppressor genes. (E) Distribution of rearranged genes according to the protein class their encoded proteins belong to. (F, G and H) Genomic rearrangements generated self-activating *JAK2* fusions in pcAECyTCL as evidenced in patients (F) AEC4, (G) AEC9 and (H) AEC11. (i) Circos plots showing interchromosomal rearrangements involving chromosome 9 in patients with pcAECyTCL. *JAK2* rearrangements were the common denominator between chromosome 9 events observed in these individuals. (ii) Validation of translocation breakpoints at *JAK2* by Sanger sequencing in pcAECyTCL patients. Breakpoints occurred between exon 16 and exon 17 in all cases. (iii) Rearrangements involving *JAK2* led to the formation of fusion genes encoding the tyrosine kinase domain of *JAK2* and the oligo/dimerization domains of the fusion partners (*KHDRBS1*: Qua1 domain, *PCM1*: coiled-coil domains, *TFG*: PB1 domain), conferring the resulting chimeric protein the ability to self-activate. (I) In addition to acquiring self-activation ability, *JAK2* fusions can also experience increased expression in comparison to wild-type *JAK2*. (i) Image of break-apart fluorescence *in situ* hybridization (FISH) analysis showing a *JAK2* rearrangement in patient AEC4. Scale bar, 10 μm. (ii) Active expression of fusion gene *KHDRBS1-JAK2* in patient AEC4 was detected by RNA sequencing (chimeric reads shown in diagram). (iii) Plot showing mean read coverage across all exons of *JAK2* in patient AEC4. RNA expression between exon 17 and exon 25, the part of *JAK2* under the transcriptional control of *KHDRBS1*'s promoter and encoding its tyrosine kinase domain, is considerably higher compared to RNA expression between exon 1 and exon 16. The red line indicates the breakpoint position. pcAECyTCL: primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma.

Table 1. Identical variants in JAK and STAT proteins reported in other hematological malignancies.

Gene	Variant	Type	Neoplasm	Effect	Functionally validated	Affected patient
JAK2	p.L393V	Germline	PV[15,28]	Slightly HS	Yes[15]	AEC3
JAK3	p.M511I	Somatic	T-PLL, AML, JMML, NKTCL[13,27,28]	GoF	Yes[13]	AEC12
JAK3	p.R657W	Somatic	T-ALL[12,28]	GoF	Yes[12]	AEC5
STAT5B	p.S434L	Somatic	T-ALL[17]	Unknown	No	AEC8
STAT5B	p.N642H	Somatic	T-ALL, T-LGL, T-PLL, NKTCL, EATL[14,27,28]	GoF	Yes[14]	AEC1, AEC6
STAT5B	p.Y665F	Somatic	T-LGL, T-PLL, ALCL ALK–, NKTCL[14,27,28]	GoF	Yes[14]	AEC7
STAT5B	p.P702S	Somatic	T-PLL[16]	Possibly GoF	No	AEC2

GoF: gain-of-function; HS: hypersensitive; ALCL ALK–: ALK– anaplastic large cell lymphoma; AML: acute myeloid leukemia; EATL: enteropathy-associated T-cell lymphoma; JMML: juvenile myelomonocytic leukemia; PV: polycythemia vera; T-ALL: T-cell acute lymphoblastic leukemia; T-LGL: T-cell large granular lymphocytic leukemia; T-PLL: T-cell prolymphocytic leukemia; NKTCL: extranodal natural killer T-cell lymphoma.

in our patients using methylation-specific melting curve analysis (MS-MCA) and found no evidence of this inactivation mechanism (*Online Supplementary Figure S9*).

Pathogenic small-scale mutations in JAK-STAT pathway genes predominate in pcAECyTCL

The discovery of recurrent structural alterations affecting principally genes involved in the cell cycle, chromatin regulation and the JAK-STAT pathway (via JAK2) prompted us to search for pathogenic indels and SNV in exonic sequences of genes with roles in the aforesaid cellular processes and additional signal transduction pathways (i.e., MAPK, NF-κB, PI-3-K/Akt and T-cell receptor [TCR] pathways) (*Online Supplementary Table S7*).

Besides the seven patients with structural alterations impacting the JAK2-SH2B3 signaling axis, four additional patients were found to carry *bona fide* gain-of-function SNV either in *JAK3* (i.e., AEC5: p.R657W[12]; AEC12: p.M511I[13]) or *STAT5B* (i.e., AEC1 and AEC6: p.N642H[14]). Also, patient AEC3 bore a germline SNV in *JAK2* (p.L393V[15]) which has been reported to render JAK2 slightly hypersensitive to cytokine stimulation (EPO ligand) (Figures 4A, 5A and B; Table 1). Moreover, two patients with *JAK2* fusions and three patients with *SH2B3* deletions also carried SNV affecting conserved residues in *STAT3* (i.e., AEC4: p.H19R; AEC9: p.G604A) and *STAT5B* (i.e., AEC2: p.P702S[16]; AEC7: p.Y665F[14]; AEC8: p.S434L[17]), respectively (*Online Supplementary Figure S6*). Similarly, three patients carrying (putative) gain-of-function SNV in *JAK* or STAT genes also had indels leading to premature stop codons either in *SH2B3* (i.e., AEC2: p.L201Sfs*78; AEC6: p.V35Afs*154) or *SOCS1* (i.e., AEC5: p.S71Rfs*14) (Figures 4A and B, 5A).

Overall, nine of 12 patients had either structural or small-scale genetic alterations impacting the JAK2-SH2B3 signaling axis whereas the remaining three patients carried pathogenic indels/SNV in other JAK-STAT pathway genes (Figures 4A and B, 5C). In addition, cancer genes involved in the cell cycle (i.e., *TP53*) and chromatin regulation (i.e., *ARID1A, KMT2D, NCOR1*) were found to be recurrently impacted either by truncating mutations (i.e., nonsense, frameshift) or SNV predicted as deleterious (Figure 4A). We also observed 34 additional patient-specific small-scale mutations of unknown significance in reputable cancer genes (*Online Supplementary Table S6*).

Transcriptome analysis uncovers upregulation of JAK2 signaling in pcAECyTCL

At present it is widely accepted that malignant T cells in pcAECyTCL derive from $CD8^+$ T cells;[1] however, to date no specific $CD8^+$ T-cell subtype has been proposed as the cell of origin of this lymphoma. Since a hallmark of malignant T cells in pcAECyTCL is their distinctive epidermotropism,[3,4] we compared gene expression in pcAECyTCL with gene expression in normal skin-resident $CD8^+$ T cells, which are characterized by a marked preferential tropism to the epidermal layer of the skin.[18] This analysis identified 1,603 differentially expressed (DE) genes (1,076 upregulated, 527 downregulated, false discovery rate [FDR] <0.01) in the disease (Figure 6A; *Online Supplementary Table S8*). We next performed gene set enrichment analysis (GSEA) using annotated gene sets from MSigDB to search for deregulated pathways/processes. Upregulated canonical signaling profiles included the JAK-STAT pathway (via STAT3, and to a lesser extent, via STAT5) and the TNF-α/NF-κB pathway. In addition, pcAECyTCL was characterized by the upregulation of the cell cycle (i.e., E2F targets, G2/M checkpoint, mitotic spindle) and high inflammatory response (Figure 6B; *Online Supplementary Table S18*).

Further examination of DE genes involved in the JAK-STAT pathway revealed that JAK2 signaling was specifi-

cally deregulated in pcAECyTCL. Upregulated genes included among others *JAK2* itself, components of type I and II cytokine receptors that signal predominantly via JAK2 (i.e., *IFNGR2*, *IL12RB2*) and established enhancers of JAK2 signaling (i.e., *PTPN11*, *SH2B1*). In contrast, downregulated JAK-STAT pathway genes included *PTPRC* and genes encoding receptors exclusively associated with signal transduction via JAK1, JAK3 or TYK2 (Figure 6C).

In order to validate JAK-STAT pathway activation in pcAECyTCL, we investigated the presence of activated STAT proteins (pSTAT3 and pSTAT5) by immunohistochemistry (IHC) in eight sequenced patients with available tumor tissue. Robust activation of JAK-STAT signaling (via STAT3, STAT5 or both) was confirmed in all evaluated patients (Figure 6D).

JAK2 fusions identified in pcAECyTCL confer cytokine-independent survival ability to cells

In order to validate the predicted effects of the *JAK2* fusions found in pcAECyTCL (i.e., *PCM1-JAK2*, *KHDRBS1-JAK2*, *TFG-JAK2*) on cell survival, we engineered these fusion genes into murine pro-B Ba/F3 cells which die in the absence of exogenous IL3 (Figure 7A). Because self-oligo/dimerizing JAK2 fusions were predicted to activate downstream STAT proteins without the need of upstream cues elicited by cytokine stimulation,

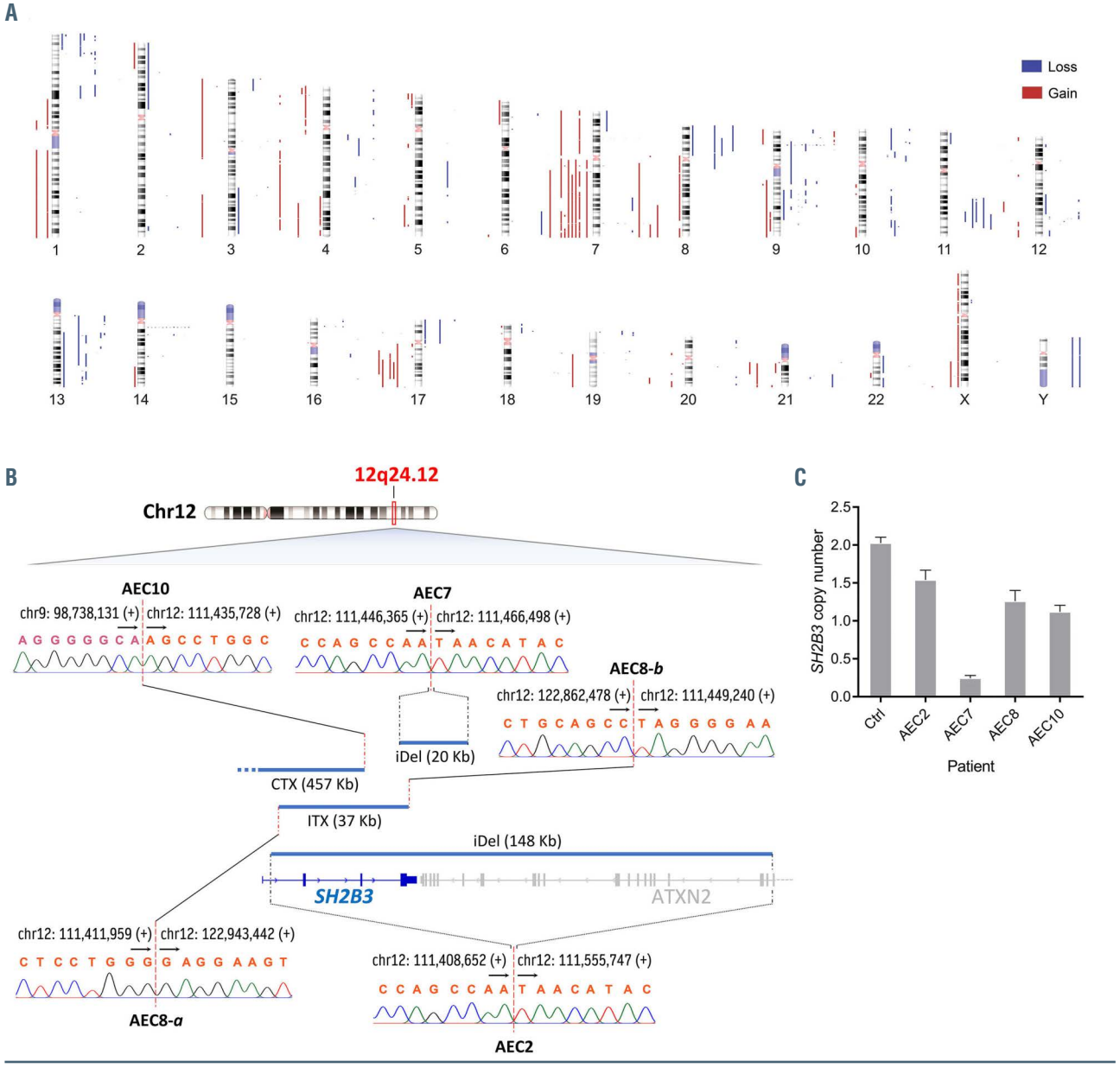

Figure 3. Landscape of copy number alterations reveals focal *SH2B3* inactivation in pcAECyTCL. (A) Human chromosome ideograms showing regions of gain and loss detected through whole-genome sequencing (WGS) in twelve primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma (pcAECyTCL) genomes. Blue bars to the right of the chromosomes depict regions of loss whereas red bars to the left of the chromosomes depict regions of gain. (B) Deletions at 12q24.12 (blue bars), where *SH2B3* resides, were the most focal (<500 Kb) copy number alteration (CNA) events in pcAECyTCL. Inactivation of *SH2B3* was mediated by interstitial deletions and unbalanced rearrangements. Breakpoints of structural alterations at 12q24.12 in all affected patients were validated by Sanger sequencing. Genomic coordinates of breakpoints according to reference genome GRCh38. Arrows indicate the direction towards which genomic coordinate numbers increase. Plus (+) and minus (-) signs specify strand polarity. CTX: interchromosomal rearrangement; ITX: intrachromosomal rearrangement; iDel: interstitial deletion. (C) Copy number losses involving *SH2B3* in patients with pcAECyTCL were validated by droplet digital polymerase chain reaction. Ctrl: control CD8+ T cells.

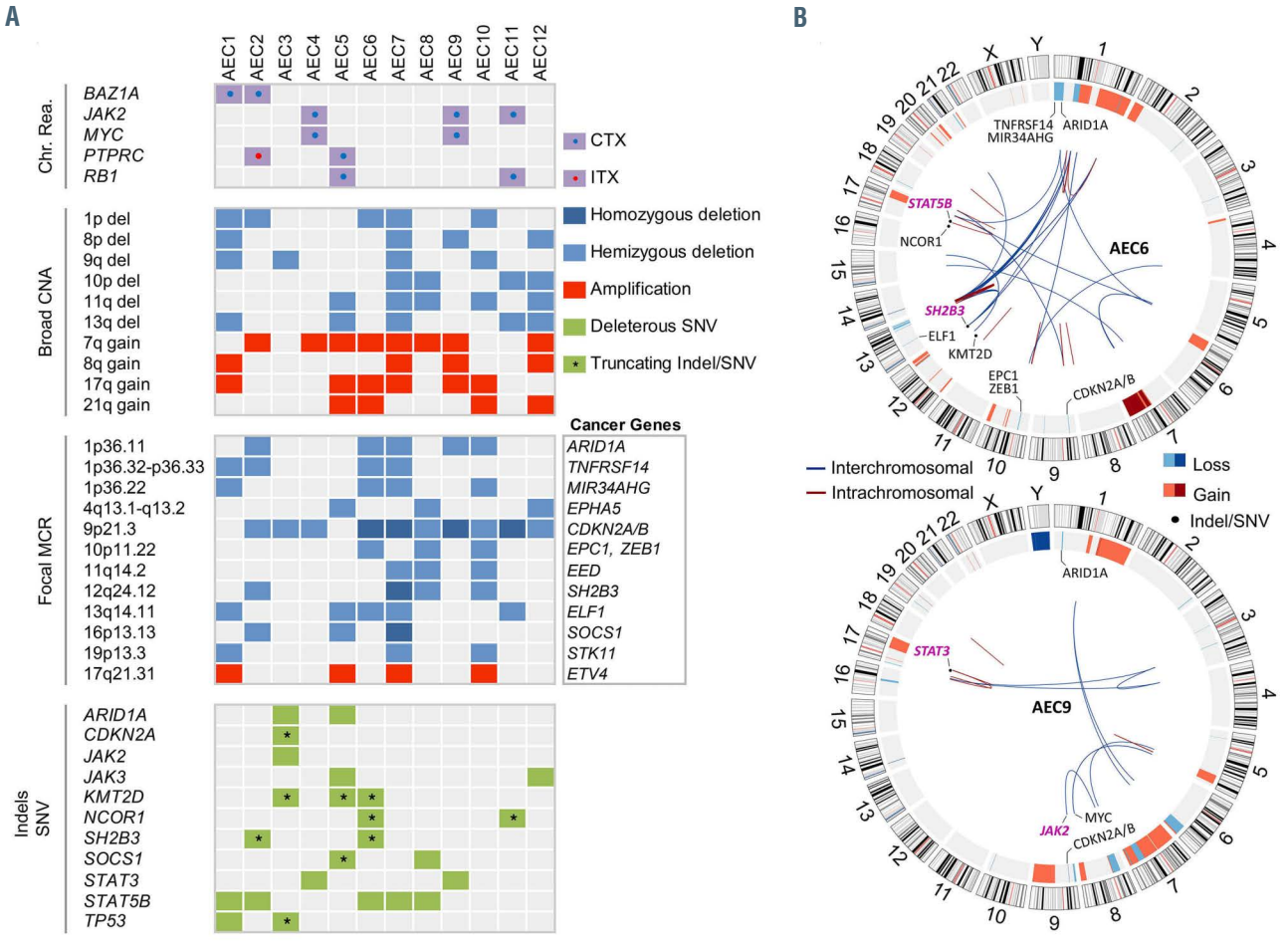

Figure 4. Distribution of recurrent chromosomal rearrangements, copy number alterations and deleterious indels/single nucleotide variants in pcAECyTCL. (A) First panel: recurrent chromosomal rearrangements impacting cancer genes. Second panel: recurrent large-scale copy number alterations (CNA) (>3 Mb). Third panel: focal minimal common regions (MCR) (≤ 3 Mb) shared by CNA; *bona fide* cancer genes residing within focal MCR are specified. Fourth panel: Indels and single nucleotide variants (SNV) in cancer genes leading to protein truncations, reported as pathogenic in literature or predicted as disease-causing (SIFT and PolyPhen-2). Only genes altered in more than one patient are indicated. CTX: interchromosomal rearrangement; ITX: intrachromosomal rearrangement. (B) Circos plots showing genetic alterations in patients AEC6 and AEC9. Despite inter-patient heterogeneity, molecular abnormalities affecting genes with roles in the cell cycle, chromatin regulation and JAK2 signaling (genes in light purple) were recurrent in primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma (pcAECyTCL).

these chimeric proteins were expected to increase survival of Ba/F3 cells in the absence of IL3. Seven days after IL3 withdrawal, survival of Ba/F3 cells expressing each of the three engineered JAK2 fusions was noticeably higher ($P<0.05$, student's *t*-test) than survival of the parental Ba/F3 cells (wild-type control) and Ba/F3 cells expressing eGFP (negative control) (Figure 7B).

We next evaluated the effect of FDA-approved JAK1/2 inhibitor ruxolitinib on each of the three IL3-independent cell lines carrying *JAK2* fusions. Ruxolitinib inhibited the growth of all cell lines in a dose-dependent manner (Figure 7C and D) with half maximal inhibitory concentration (IC_{50}) values in the low nanomolar range (9–15 nM), in concordance with the reported inhibitory activity of this drug.[19] Since fusion partners *PCM1* and *TFG* have extensively been proven by others to confer chimeric kinases (including JAK2) the ability to trans-autophosphorylate via self-oligo/dimerization,[20-25] we carried on further validation with *JAK2* fusion containing novel kinase fusion partner *KHDRBS1*. For extra verification, we treated Ba/F3 cells expressing *KHDRBS1-JAK2* with inhibitor AZD1480, which has higher specificity for JAK2 than ruxolitinib,[26] and confirmed that cytokine-independent survival of these cells depends on JAK2 signaling (Figure 7D). Finally, we corroborated by western blotting that growth inhibition exerted by ruxolitinib and AZD1480 was accompanied by a dose-dependent inhibition of JAK2 and STAT5 phosphorylation in Ba/F3 cells driven by *KHDRBS1-JAK2* (Figure 7D).

Discussion

This study describes the first high-resolution genetic profiling of pcAECyTCL using next-generation sequencing. The landscape of structural genomic alterations of pcAECyTCL was characterized by considerable genomic instability and inter-patient heterogeneity. Most rearrangements (328 of 426) identified in pcAECyTCL disrupted annotated genes, and approximately one-third of all rearranged genes (91 of 305) were found to play roles in signal transduction and transcriptional regulation. In addition, four of 12 patients experienced chromothripsis/chromoplexy-like events which mediated the deletion of relevant tumor suppressors (e.g., *CDKN2C*, *CHD5*, *FAS*, *PTEN*, etc.). In full agreement with previously published

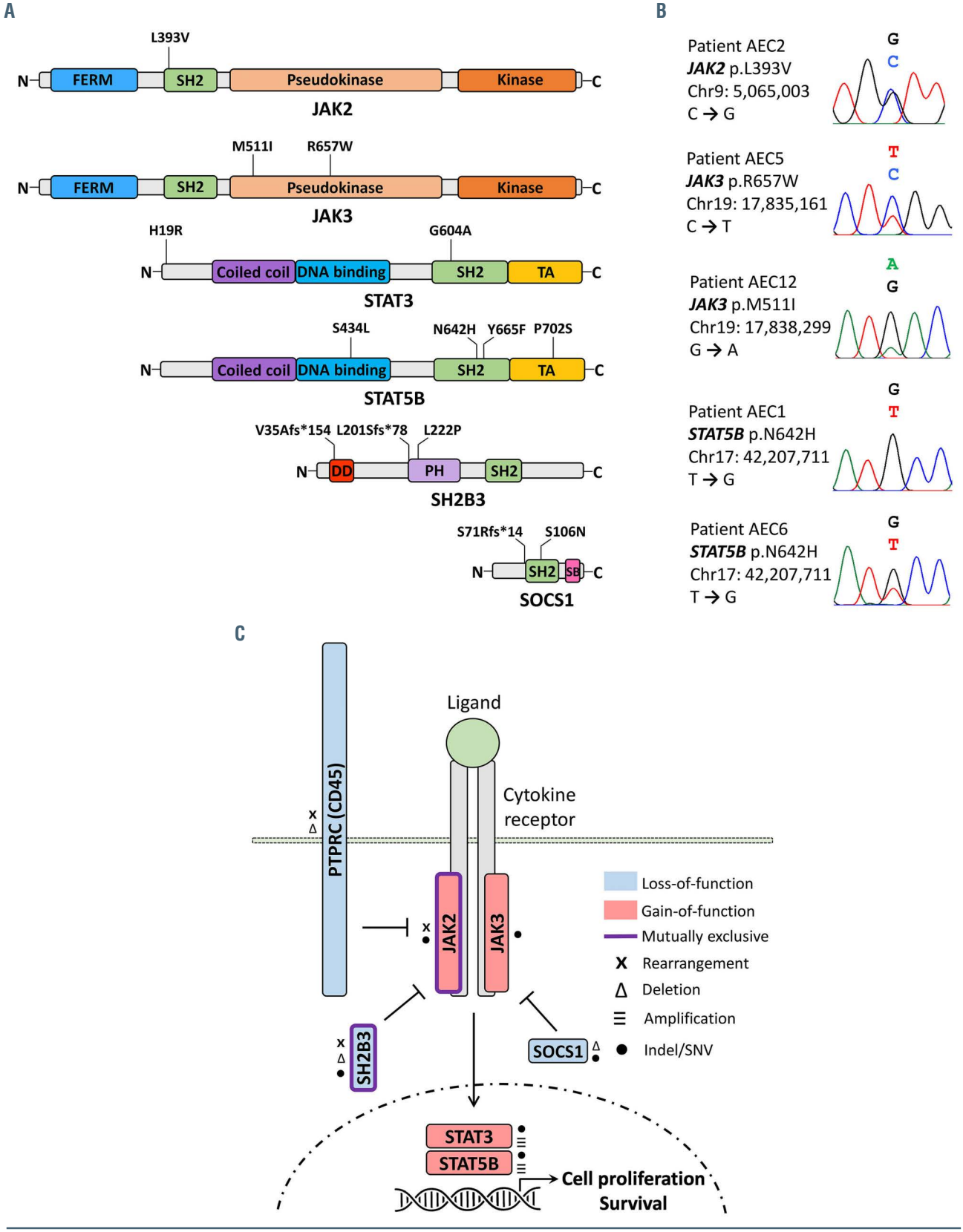

Figure 5. Small-scale mutations in genes of the JAK-STAT pathway are predominant in pcAECyTCL. (A) Diagrams showing deleterious indels and single nucleotide variants (SNV) in *JAK2*, *JAK3*, *STAT3*, *STAT5B*, *SH2B3* and *SOCS1* detected in primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma (pcAECyTCL) by whole-genome sequencing (Table 1). (B) Sanger chromatograms confirming presence of *bona fide* pathogenic SNV in patients with pcAECyTCL. (C) Summary of genetic alterations affecting members of the JAK-STAT pathway in pcAECyTCL.

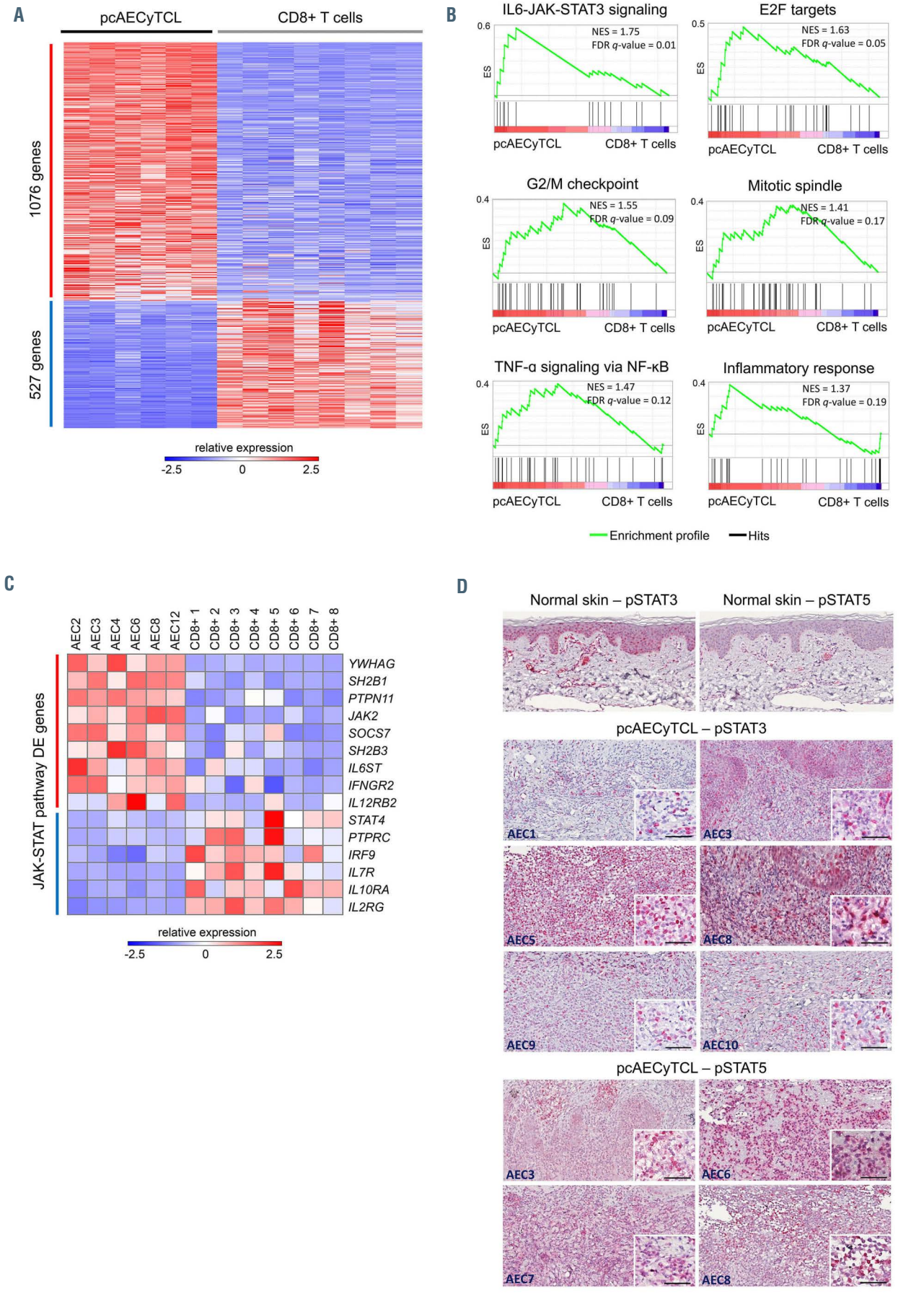

Figure 6. Legend on following page.

Figure 6. RNA sequencing supports upregulation of JAK2 signaling in pcAECyTCL. (A) Heat map showing 1,603 differentially expressed genes (1,076 upregulated, 527 downregulated, false discovery rate [FDR] <0.01) in pcAECyTCL when compared to skin-resident CD8+ T cells. (B) Gene set enrichment analysis (GSEA) uncovered upregulation of the JAK-STAT pathway, the cell cycle (E2F targets, G2/M checkpoint, mitotic spindle), the NF-κB pathway and high inflammatory response in pcAECyTCL. NES: normalized enrichment score; FDR *q*-value: false discovery rate *q*-value. (See the *Online Supplementary Table S18* for a complete list of GSEA signatures) (C) Examination of differentially expressed genes involved in the JAK-STAT pathway revealed that *JAK2* itself, enhancers of JAK2 signaling and components of cytokine receptors that signal predominantly via JAK2 are upregulated in primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma (pcAECyTCL). (D) Activation of the JAK-STAT pathway (via STAT3 and/or STAT5) in pcAECyTCL was confirmed by immunohistochemistry (IHC) on tumor tissue from sequenced patients (i.e., AEC1/3/5-10). Neoplastic cells exhibited activated STAT3 and/or STAT5 in the nucleus. Normal skin (control) displayed STAT3 activation in keratinocytes and endothelial cells as well as STAT5 activation in melanocytes and endothelial cells. Scale bar, 50 μm.

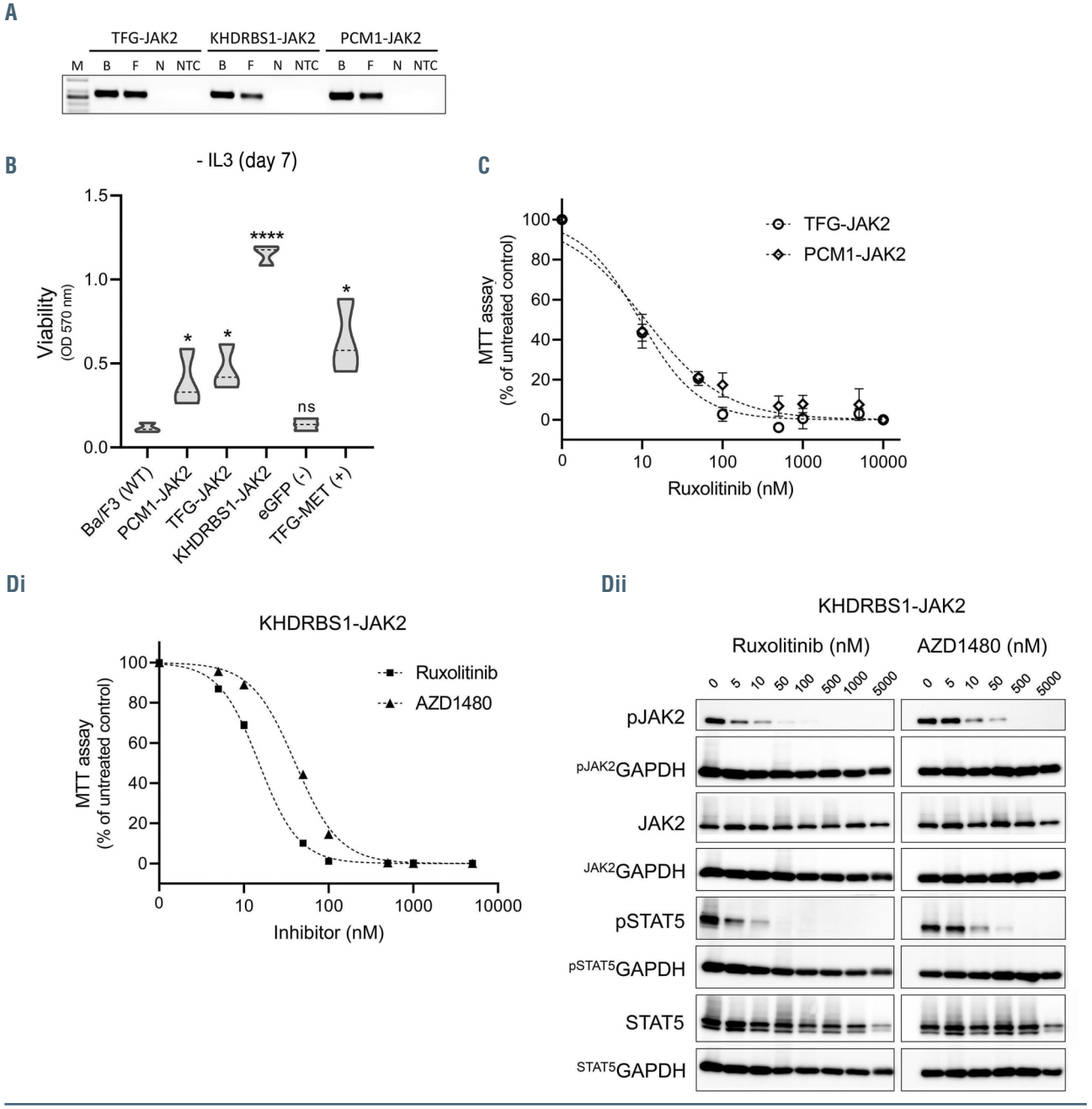

Figure 7. Oncogenicity validation of *JAK2* fusions identified in pcAECyTCL. (A) Expression of *JAK2* fusions in transduced Ba/F3 cells was verified by reverse transcriptase polymerase chain reaction. M: molecular-weight marker; B: Fusion DNA in backbone (positive control); F: cDNA from Ba/F3 cells transduced with *JAK2* fusion gene; N: cDNA from Ba/F3 cells transduced with eGFP gene (negative control); NTC: non-template control (H.O). (B) Violin plots showing viability of Ba/F3 cells expressing fusion genes *KHDRBS1-JAK2*, *PCM1-JAK2* or *TFG-JAK2* seven days after interleukin-3 (IL3) withdrawal (mean OD, n=3). Viability of all cell lines expressing *JAK2* fusions was noticeably higher (P<0.05, student's *t*-test) compared to wild-type and negative control cells. Control samples: parental Ba/F3 cells (wild-type control), Ba/F3 cells expressing eGFP (negative control), Ba/F3 cells expressing fusion gene *TFG-MET* (positive control). *P<0.05; ****P<0.0001. (C) Dose-response curves of Ba/F3 cells expressing *PCM1-JAK2* (half maximal inhibitory concentration [IC_{50}]=11 nM) or *TFG-JAK2* (IC_{50} =9 nM) when exposed to various concentrations of JAK1/2 inhibitor ruxolitinib (mean OD; error bars, standard deviation [SD], n=3). (D) Validation experiments with JAK2 fusion containing novel kinase fusion partner *KHDRBS1*. (i) Dose-response curves of Ba/F3 cells expressing KHDRBS1-JAK2 when exposed to various concentrations of JAK1/2 inhibitors ruxolitinib (IC_{50}=15 nM) or AZD1480 (IC_{50}=40 nM) (mean OD; error bars, SD, n=3). (ii) Western blot analysis of Ba/F3 cells expressing *KHDRBS1-JAK2* showed a dose-response reduction in phosphorylation of JAK2 and STAT5 with increasing concentrations of ruxolitinib or AZD1480.

data,[5] we found that gains within 7q and 17q as well as losses within 1p and 13q were the most common large-scale chromosomal imbalances.

Our analysis identified a group of *bona fide* oncogenes and tumor suppressors with central roles in the cell cycle (i.e., *CDKN2A/B*, *MIR34AHG*, *MYC*, *RB1*, *TP53*), chromatin regulation (i.e., *ARID1A*, *BAZ1A*, *EED*, *EPC1*, *KMT2D*, *NCOR1*, *ZEB1*) and the JAK-STAT pathway (i.e., *JAK2*, *JAK3*, *PTPRC*, *SH2B3*, *SOCS1*, *STAT3*, *STAT5B*) whose copy number, sequence organization and/or nucleotide composition were found to be recurrently altered in our pcAECyTCL cohort. Genetic alterations involving JAK-STAT pathway genes were the most notable due to their predominance, likely proliferation-promoting effects and known causative roles in hematological cancers. A subset of SNV affecting JAK-STAT pathway genes in pcAECyTCL have confirmed oncogenic activity in other T-cell lymphomas (Table 1).[27,28]

JAK2 and *SH2B3*, which govern the activation and termination of JAK2 signaling in normal hematopoietic cells, respectively, underwent mutually exclusive alterations in nine of 12 patients from our cohort. Mutations in these two genes are associated with BCR-ABL1– myeloproliferative neoplasms (MPN), a group of myeloid malignancies driven by overactive JAK2 signaling.[11,29] However, unlike BCR-ABL1– MPN where *JAK2* and *SH2B3* are mainly affected by pathogenic SNV and/or indels, these two genes experienced predominantly structural alterations in pcAECyTCL. On one hand, *JAK2* formed fusion genes encoding self-activating chimeras. On the other hand, *SH2B3* was inactivated by focal interstitial deletions and unbalanced rearrangements. The previous suggests that pcAECyTCL is mainly driven by aberrant JAK2 signaling resulting from oncogenic changes leading to JAK2 overactivation or SH2B3 deficiency. Moreover, we demonstrated that *JAK2* fusions found in pcAECyTCL promote cytokine-independent cell survival and their oncogenic activity was shown to be successfully inhibited by ruxolitinib. Of note, *JAK2* fusions functionally analogous to the ones identified in pcAECyTCL have been previously described and confirmed as oncogenic in other hematological malignancies (e.g., B- and T-cell acute leukemias, MPN).[20] Also, recurrent deletion of *SH2B3* has been reported in an aggressive subtype of B-cell precursor acute lymphoblastic leukemia.[30]

We found that genetic alterations involving *JAK2* and *SH2B3* co-existed with SNV predicted or confirmed as pathogenic in *STAT3* or *STAT5B* in six of nine affected patients. Previous functional *in vitro* studies with cell lines have suggested that mutations in STAT proteins (especially dimerization-enhancing SNV) observed in T-cell lymphomas operate as aberrant amplifiers of upstream signals from cytokines, overactive receptors or deregulated JAK proteins, rather than as initiators of deregulated JAK-STAT signaling themselves.[27] In this scenario, mutations in STAT3/5B would contribute to pcAECyTCL progression by making the pre-existing overactive JAK2 signaling more robust and severe. However, recent evidence derived from a murine model suggests that at least gain-of-function mutation STAT5B (p.N642H), one of the most common pathogenic SNV in human T-cell lymphomas,[31] is sufficient by itself to promote the development of neoplasms primarily derived from mature $CD8^+$ T cells.[32] Remarkably, malignant $CD8^+$ T cells in these animals showed preferential migration to the skin, lung and the central nervous system, all of which are commonly affected body sites in pcAECyTCL.[32] Consistent with this evidence, patient AEC1, the only individual in our cohort who had a single JAK-STAT pathway gene mutated, carried the STAT5B (p.N642H) mutation biallelically.

Several pathogenetic features found in pcAECyTCL have also been reported in mycosis fungoides (MF) and/or Sézary syndrome (SS). Genetic alterations common to pcAECyTCL, MF and SS include recurrent inactivation of *ARID1A*, *CDKN2A*, *CDKN2B*, *NCOR1*, *PTPRC*, *TP53* and *ZEB1* as well as occasional activating mutations in *JAK3*, *MYC* and *STAT3*.[33-39] Other genetic alterations observed in pcAECyTCL have been found before either in MF (e.g., *SOCS1* and *STK11* inactivation) or SS (e.g., *RB1* inactivation, *STAT5B* mutations).[33,34,39] By contrast, *JAK2* fusions and *SH2B3* inactivation have not been reported in other CTCL variants to the best of our knowledge and appear to be characteristic features of pcAECyTCL.

In agreement with the recurrent genetic alterations involving the JAK2-SH2B3 signaling axis observed in pcAECyTCL, transcriptome analysis revealed upregulation of JAK2 signaling. *SH2B1* and *PTPN11*, which encode two proteins with the ability to enhance JAK2 signaling,[40,41] stood out among upregulated JAK-STAT pathway genes. Adaptor protein SH2B1 has been proven to bind to JAK2 and stimulate its kinase activity.[42] Similarly, phosphatase PTPN11 (SHP-2) has been shown to positively regulate JAK2-mediated STAT5 phosphorylation.[43] In contrast, phosphatase PTPRC (CD45), whose expression has been shown to attenuate JAK2 signaling in hematopoietic and lymphoma cells,[44,45] was downregulated in pcAECyTCL. Yet, the exact molecular interactions underlying the action of these three regulators of JAK2 signaling remain to be fully elucidated.

Transcriptome analysis also revealed upregulation of the cell cycle, the TNF-α/NF-κB pathway and a high inflammatory response in pcAECyTCL. Notably, the co-activation (crosstalk) of JAK-STAT signaling (especially via STAT3) and NF-κB signaling is a well-documented phenomenon in cancer, and it has been shown to promote a pro-oncogenic inflammatory microenvironment in the tumor.[46] For instance, aberrant JAK2 signaling (via STAT3) in MPN promotes chromatin changes that induce NF-κB signaling; and the resulting combined action of these two pathways, appear to drive the characteristic chronic inflammatory state observed in these neoplasms.[47] Our data, in line with the previous, suggest that co-activation of JAK2 signaling and NF-κB signaling operates in pcAECyTCL as well, and their joint action might be responsible for the inflammatory state detected in pcAECyTCL tumors.

Taken together, our findings strongly suggest that overactivation of JAK2 signaling plays a pivotal role in the pathogenesis of pcAECyTCL. Therefore, patients with this lymphoma would likely benefit from treatment with JAK2 inhibitors (e.g., FDA-approved ruxolitinib). In addition, the potential combination of JAK2 inhibitors with NF-κB inhibitors (e.g. bortezomib,[48] dimethyl fumarate[49]) represents an attractive possibility since targeting both pathways might have a synergistic effect and reduce the chance of resistance acquisition.

Disclosures

No conflicts of interest to disclose.

Contributions

ANBT, DF, RW, MV, EB and CPT conceptualized and designed the project; ANBT and CPT wrote the manuscript; ANBT, DC and HM performed the bioinformatic analyses; ANBT, JO, DF and LV performed the experiments; ANBT and DC produced figures and tables; ANBT analyzed the results and interpreted the data; DF, LV, RW, MV and EB provided valuable biological specimens; ANBT, DC, JO, DF, HM, LV, RW, MV, EB, and CPT revised and approved the final manuscript.

Acknowledgements

The authors thank Tim van Groningen and Yixin Luo for providing valuable technical support.

Funding

This study was funded by the Dutch Cancer Society (KWF, grant UL2013-6104) and Associazione Amici di Sabrina Fadini Onlus (A.S.F.O., grant 0800000-PR-LUMC).

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