Mutated JAK kinases and deregulated STAT activity are potential therapeutic targets in cutaneous T-cell lymphoma

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Supplementary information

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MATERIALS AND METHODS

Clinical Samples: Samples from 46 patients diagnosed with mycosis fungoides (MF) and Sézary syndrome (SS) were collected from the following Spanish institutions: H.U. 12 de Octubre, H.U. Puerta de Hierro-Majadahonda, Fundación Jimenez Diaz, MD Anderson Cancer Center, and H. Gregorio Marañon (Madrid), Complexo Hospitalario Universitario de Vigo (Pontevedra), H. Nuestra Señora de la Candelaria, (Tenerife), H.U. Gran Canaria Dr. Negrin (Gran Canaria), H. General de Elche, H. Vega Baja de Orihuela (Alicante), H. General Universitario de Valencia (Valencia), H. Virgen de la Salud (Toledo), H. Virgen de la Arrixaca (Murcia), H. de la Línea de la Concepción (Cádiz), H. Reina Sofia (Córdoba), H.U. Príncipe de Asturias (Asturias), H.U. Marqués de Valdecilla (Santander), Hospital de Plasencia (Cáceres). Collections were supervised by the local Ethics Committees. Mutational data of 11 of these patients have been previously reported [1].

Diagnostic criteria were based on World Health Organization–European Organization for Research and Treatment of Cancer classification [2]. The study design, patient information sheet, and informed consent form were all approved by the Ethics Committee of each hospital and centrally reviewed by the HUMV Ethics Committee. This study was conducted in accordance with the principles of the Declaration of Helsinki.

Unless otherwise stated, the samples used were obtained from formalin-fixed paraffin-embedded (FFPE) tissue biopsies with a minimum of 50% of tumoral cells in the infiltrate. The proportion of neoplastic cells was assessed taking atypical morphology and abnormal immunophenotypes into account. A TCR rearrangement study showed

clonality in all cases. Matched non-tumoral DNA was extracted from FFPE normal adjacent tissue or saliva, or peripheral blood neutrophils when available. In the latter case, a TCR rearrangement study was performed to rule out the possibility of contamination with malignant lymphocytes. Of all cases with mutations in *JAK* kinases (table 1) only the case number 2 (carrying the mutation JAK1-I597M) lacked non-tumoral DNA. DNAs from two cell lines derived from CTCLs (MyLa and HuT-78) were also analyzed using NGS.

Genomic DNA sample quality test: Genomic DNA was extracted by standard methods as previously described [1]. The quantity and integrity of purified DNA was assessed by Qubit 2.0 fluorometric quantitation (Life Technologies) using the Qubit® dsDNA BR Assay Kit (Life Technologies) and by capillary electrophoresis in a 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) using High Sensitivity DNA reagents and chip kits (Agilent Technologies) following the manufacturer's instructions.

Cell lines: Human HuT-78, and HH cell were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and MyLa cells from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). All cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Invitrogen Corporation, USA) in a humidified atmosphere at 37°C with 5% CO_2.

Sequencing: Data were obtained by independent ultrasequencing methods as follows: 1) specific pseudokinase domain PCR-based amplification protocol for *JAK1*, *JAK2* and *JAK3* genes (named PsTKd-PCR hereafter), followed by specific indexing and sequencing using MiSeq (Illumina), and 2) a targeted gene-enrichment kit (HaloPlex, Agilent Technologies) coupled to Ion Torrent sequencing. This work also includes previously published mutational data of *JAK* genes obtained independently by our group [1]. Sequencing using MiSeq was performed at IMEGEN (Valencia, Spain) whereas that using Ion-PGM was conducted at NIMGenetics (Madrid, Spain)

1) *PsTKd-PCR library preparation and sequencing with MiSeq.* Coding_exons from the pseudotyrosine kinase region of the *JAK1*, *JAK2* and *JAK3* genes were

amplified using Pfu DNA polymerase (Promega Corporation) combined with the specific oligonucleotides and indexed as follows: all amplicons from the same patient were mixed in a tube. 500 ng of each DNA sample was repaired using NEBNext: Ultra End Repair/dA Tailing Module kit (Biolabs) and linked to a pair of adapters, the 3' and 5' ends, respectively. A pair of indexing primers was then bound to the adapters to allow subsequent identification of each sample. Finally, a pair of indexing primers was bound to the adapters to allow each sample to be identified. DNA was purified with Agencourt AMPure XP beads (Beckman Coulter) and 4 ng of each DNA was sequenced by next generation sequencing on a MiSeq Personal platform (Illumina). Primers for amplification and sequencing were designed using ExonPrimer (http://ihg.gsf.de/ihg/ExonPrimer.html), checked with the USCS Genome Browser PCR *in silico* tool, and purchased from Eurofins (Fisher Scientific) (details available upon request). They were validated by an independent PCR-based approach using a different high-fidelity polymerase (Herculase II Fusion Enzyme with dNTP combo; Agilent Technologies) coupled to a similar but independent indexing and sequencing protocol, as described above.

2) *HaloPlex enrichment and sequencing with Ion-PGM*. Target enrichment was performed using Agilent Technologies' HaloPlex system. In all, 635 target regions including the coding exons plus 10 flanking bases of 45 genes previously found mutated in CTCL [1] were submitted for DNA capture probe design using the Agilent SureDesign web-based application (manuscript in preparation). Only the data related to *JAK1*, *JAK2* and *JAK3* were considered in this work. The target regions were captured according to manufacturer's instructions (HaloPlex Target Enrichment System Protocol for Ion Torrent) with reagents from the custom design HaloPlex Target Enrichment kit (Agilent Technologies). Briefly, 400 ng of genomic DNA was digested with the specific cocktail of restriction enzymes provided in the kit. Digested DNA was then hybridized to a probe for target enrichment, indexed and captured. Each DNA was then amplified by PCR at T_m = 60°C, for 18 cycles, using a Herculase II Fusion Enzyme kit (Agilent Technologies). Next, amplified target libraries were purified using an Agencourt AMPure XP Kit (Beckman Coulter Genomics), following the manufacturer's guidelines, and quantified. Template dilutions were calculated after library concentrations were

normalized to ~100 pM using the Ion Library Equalizer kit (Life Technologies). Library templates were clonally amplified using the Ion One Touch 2™, following the manufacturers' protocol. Recovered template-positive ion sphere particles (ISPs) were subjected to enrichment according to the manufacturer's protocol. Samples were subjected to the standard Ion-PGM 200 Sequencing v2 protocol using Ion 318 v2 chips (Life Technologies). Up to five samples were loaded per Ion 318 v2 chip due to variable coverage uniformity. Samples analyzed using this protocol were validated by Sanger sequencing. The sequence of the oligonucleotides used in this process are available upon request.

Western blot: Cells were lysed and subjected to 10% acrylamide SDS-PAGE, using standard procedures, followed by transfer onto a nitrocellulose support membrane (Immobilon, Millipore) and western blot. All primary antibodies were diluted 1/1,000 unless otherwise stated, and all secondary antibodies were diluted 1/20,000. The following antibodies were used: anti-α-tubulin (sc-23948, Santa Cruz), monoclonal, anti-phospho-STAT1 (Tyr701) (58D6) rabbit mAb (Ref.9167, Cell Signaling), anti-phospho-STAT3 (Tyr705) (D3A7) XP® rabbit mAb (Ref.9145, Cell Signaling), anti-phospho-STAT5 (Tyr694) (C11C5) rabbit mAb (Ref.9359, Cell Signaling), anti-STAT1 (Ref.9172, Cell Signaling), anti-STAT3 (Ref.4904, Cell Signaling), anti-STAT5 (Ref.9358, Cell Signaling), anti-PARP (H250, sc-7150, Santa Cruz), goat anti-mouse IgG, DylightTM800 and goat anti-rabbit IgG, DylightTM800 (Thermo scientific). Data were collected using an Odyssey Infrared imaging system (Li-Cor).

Cell viability: For drug cytotoxicity experiments, CTCL cell lines were seeded in 96-well plates at a density of 10,000 cells per well, and the inhibitor INCB018424 was added over a range of doses for 48 hours, using dimethylsulfoxide (DMSO) as a control up to a maximum concentration of 20 µl/ml. Cell viability was measured as the intracellular ATP content, using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), as indicated by the manufacturer. All experiments were done in triplicates, and all numerical data were expressed as the mean and standard error of the mean (SEM).

Flow cytometry analysis: The distribution of cells among the phases of the cell cycle were evaluated using propidium iodide (PI, Sigma-Aldrich, St Louis, MO, USA) and Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Technologies-Thermo Fisher Scientific) according to the manufacturer's instructions. Induction of apoptosis was evaluated using FlowCellect Annexin Red Kit (EMD Millipore Corporation, Billerica, MA, USA), according to the manufacturer's instructions. Data were collected using a FACS-Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using CellQuest Pro software (BD Biosciences).

Statistical analysis: Unless otherwise specified, all experiments were done independently in triplicate and all numerical data were summarized as the average and SEM using GraphPad PRISM. Levels of significance are indicated as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

CASE	JAK status	Sex/Age at diagnosis:	MF clinical lesions	LCT (months)	Tumors (months)	Erythroderma (months)	Lymph Node involvement (months)	Exitus	Exitus (cause)	Follow up (months)
1	JAK1 MT	F, 58	T	NO	YES(80)	NO	YES(101)	YES	Lymphoma	105
2	JAK1 MT	M, 83	E	NO	NO	YES(8)	YES(10)	YES	Others	39
3	JAK1 MT	F, 61	T	UNK	UNK	UNK	UNK	UNK	-	UNK
4	JAK1 MT	F, 75	E	NO	NO	YES	YES(28)	NO	-	44
5	WT	F, 84	T	NO	YES(14)	NO	NO	NO	-	16
6	WT	M,54	E, T	NO	YES(24)	YES(24)	NO	YES	Lymphoma	31
7	JAK3 MT	M, 49	T	YES(164)	YES(164)	NO	YES(180)	NO	-	276
8	JAK3 MT	F, 68	E	NO	NO	YES(13)	YES(28)	NO	-	35
9	JAK3 MT	F, 71	SS	NO	NO	YES	NO	YES	Lymphoma	104
10	WT	F, 42	E, T	NO	YES(72)	YES(69)	NO	YES	Lymphoma	73
11	WT	F, 57	T	NO	YES(23)	NO	NO	NO	-	33
12	WT	M, 64	T	YES(44)	YES	NO	NO	YES	Lymphoma	55
13	WT	M, 48	Fl, T	YES(151)	YES(129)	NO	No	NO	-	151
14	WT	F, 87	T	UNK	YES(122)	NO	YES(124)	YES	Lymphoma	132
15	WT	F, 54	E, Fl, T	YES(144)	YES(151)	YES(13)	NO	NO	-	233
16	WT	F, 26	T	YES(116)	YES(71)	NO	NO	YES	Unk	75
17	WT	M, 43	Fl, T	YES(121)	YES(92)	NO	NO	NO	-	142
18	WT	M, 81	E	NO	NO	YES(42)	NO	NO	-	72

19	WT	F, 40	T	YES(260)	YES(248)	NO	YES(248)	YES	Lymphoma	275
20	WT	M, 83	P/P	NO	NO	NO	NO	UNK	-	120
21	WT	F, 35	T	YES(132)	YES(142)	NO	NO	NO	-	141
22	WT	M, 65	Fl	NO	NO	NO	NO	YES	Others	54
23	WT	M, 60	T, E, FL	YES(60)	YES(59)	YES(65)	YES	YES	Lymphoma	UNK
24	WT	F, 18	T	NO	YES(36)	NO	YES(95)	YES	Lymphoma	83
25	WT	F, 57	SS	UNK	UNK	UNK	UNK	UNK	-	UNK
26	WT	M,41	SS	UNK	UNK	UNK	UNK	UNK	-	UNK
27	WT	F, 57	T	YES(5)	YES(56)	NO	YES(78)	YES	Lymphoma	83
28	WT	M, 78	SS	NO	NO	YES	UNK	YES	Others	100
29	WT	M, 69	E, Fl, T	YES(50)	YES(50)	YES(2)	NO	YES	Lymphoma	68
30	WT	M, 45	T , Fl	NO	YES(81)	NO	NO	NO	-	65
31	WT	M, 33	T	YES(322)	YES(317)	NO	NO	YES	Lymphoma	221
32	WT	M, 34	T , Fl	YES(63)	YES(48)	NO	NO	YES	Lymphoma	76
33	WT	F, 69	T	YES(40)	YES(40)	NO	NO	NO	-	76
34	WT	M, 45	FI	NO	YES(8)	NO	NO	NO	-	8
35	WT	M, 48	FI	YES(78)	YES(369)	YES(46)	YES	UNK	-	62
36	WT	F, 29	P/P	YES(162)	YES(165)	NO	YES(216)	YES	Lymphoma	238
37	WT	M, 53	P/P	YES(156)	YES(144)	YES(156)	NO	NO	-	240
38	WT	F, 46	P/P	YES(63)	YES(53)	NO	NO	NO	-	74
39	WT	F, 68	UNK	UNK	UNK	UNK	UNK	UNK	-	UNK
40	WT	F, 29	T	YES(16)	YES	NO	YES	NO	-	UNK

41	WT	F, 61	SS	UNK	NO	YES	YES	NO	-	UNK
42	WT	F, 42	E	UNK	YES	YES(8)	YES(8)	YES	Lymphoma	12
43	WT	F, 56	E	UNK	UNK	YES	YES	YES	Unk	UNK
44	WT	M, 64	E	UNK	UNK	UNK	YES	YES	Lymphoma	36
45	WT	M, 56	E	UNK	NO	YES(2)	YES(5)	UNK	-	UNK
46	WT	M, 64	E	UNK	NO	YES	YES	YES	Others	13

Supplementary table 1: Clinical characteristics of the patient series: E: erythrodermic MF, F: female, Fl: Foliculotropic MF, L: Lymphoma, M: male, ND: No Data, O: Other cause, P/P: Classical MF, patch/plaque stage, SS: Sézary syndrome, T: Classical MF with tumours, LCT: Large cell transformation, UNK: Unknown.

SUPPLEMENTARY REFERENCES

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