Recurrent presence of the PLCG1 S345F mutation in nodal peripheral T-cell lymphomas

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Supplementary data.

MATERIALS AND METHODS

PATIENT SAMPLES.

The series included 101 formalin-fixed, paraffin-embedded (FFPE) PTCLs samples including 60 AITL and 41 PTCL-NOS. Clinical data for the patients and some mutational and GEP data have been reported in two previous studies (1, 2). Diagnostic criteria were based on the WHO classification (3). The diagnosis was confirmed by two pathologists (SMR-P and MAP). The samples were provided by the Biobanks of the Centro Nacional de Investigaciones Oncológicas (Madrid, Spain), Hospital Universitario Marqués de Valdecilla (Santander, Spain), Hospital Universitario 12 de Octubre (Madrid, Spain), Complejo Hospitalario Universitario de Vigo (Pontevedra, Spain) and Fundación Jiménez Díaz (Madrid, Spain). The project was supervised by the Ethical Committee of the Hospital Carlos III.

DNA EXTRACTION.

We extracted DNA from tumoral FFPE samples using a QIAamp® DNA FFPE Tissue kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's protocol.

DETECTION OF PLCG1 MUTATION BY qPCR.

For the *PLCG1* mutation c.1034T>C screening, primers and LNA-probes were designed by TIB- Molbiol (Berlin, Germany) to detect the point mutation by LNA-clamped real-time PCR. Primers, probes and conditions are described in Vaque *et al* (4). For further validation of the presence of this PLCG1 mutation, a new qBiomarker Somatic Mutation Assays, based in allelic discrimination assessed by qPCR, was designed and validated by SabBioscence-Qiagen (SMPH221535AR) for this specific mutation and tested in Vaque *et al* (4). Both are semi-quantitative methods that have sensitivity

enough to detect mutations present in as low as 10% of alleles in DNA obtained from FFPE material.

Detection of PLCG1 mutation by LNA-clamped real-time PCR. The probes for The LNA-mediated clamped real-time PCR were designed by TIB- Molbiol (Berlin, Germany): Fw: 5'-TGACCATACCTACCTGCCTCT-3'; Rv: 5'-CACTGGGGAGCAACATCA-3', Sensor 5'-LCRed640- GGACTCACTGAAGAACTGGTCCC -PH, Aṅchor 5 -GCAGCGAGCATAGGCTTCCAAGG-FL, PLCG1 WT-specific LNA-containing oligonucleotide 5'-TCACTGGAGAACTG-NH2. Both the Sensor and the LNA clamp are phosphorylated at the 3̓-end so that they cannot function as primers. The reaction was done in a LightCycler II (Roche Applied Science, Indianapolis, IN). The results were analyzed using LightCycler™ software 3.5 (Roche Applied Science).

Detection of PLCG1 mutation by alleleic discrimination by qPCR. Allele specific amplification is based on the discrimination by Taq polymerase between a match and a mismatch at the 3' end of the PCR primer. Positive and negative samples controls were include in all the assays. The mutation status was determined according to manufacturer's instructions. Those samples with $Ct^{REF} > 35$ were discarded. Samples were considered as mutated if ΔCt^{TEST} ($Ct^{MUT} – Ct^{REF}$) < 5.

Each sample was tested by duplicate (for each test) at least in two independent experiments for both methods. For further confirmation, selected cases were sequenced by fragment collection after qPCR genotyping with LNA-clamped qPCR to improve the sensitivity of detection by Sanger sequencing. Only those cases found to be positive by both techniques were considered as mutated. All experiments were done blinded with respect to the clinical data.

STATISTICAL ANALYSIS.

To assess associations between categorical variables, we used the chi-square contingency test with Yates' correction, or Fisher's exact test, as appropriate. Disease-specific survival was defined as the period from the date of diagnosis to death from the tumor. Kaplan–Meier survival analyses were carried out for lymphoma-specific survival, using the long-rank test to examine differences between groups. Estimates

were considered statistically significant for two-tailed probabilities of $p<0.05$. All analyses were carried out with SPSS 19.0 (SPSS Inc., Chicago, IL).

TISSUE MICROARRAY CONSTRUCTION AND IMMUNOHISTOCHEMICAL ANALYSIS.

Representative areas from the FFPE lymphomas samples were carefully selected on H&E-stained sections and three 1-mm-diameter tissue cores were obtained from each specimen. The tissue cores were precisely arrayed in a new paraffin block using a tissue microarray (TMA) workstation (Beecher Instruments, Silver Spring, MD).

TMA sections were stained by the Endvision method with a heat-induced antigen-retrieval step for CD3, CD30, NFATc1, Ki67, p-ERK antibodies and NF-KB subunits for the classic and alternative NF-KB pathways, p50 and p52 respectively. Cases were considered positive for each marker following previously reported cut-off values for each (1, 2, 4). Reactive tonsil tissue was included as a control. The primary antibodies were omitted to provide negative controls (Supplementary Table 2).

Supplementary References

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SUPPLEMENTARY TABLES

Supplementary Table 1. Univariate analysis of clinical and molecular parameters and mutational status of the *PLCG1* gene in the cohort of 41 patients with PTCL-NOS

Clinical parameters				
	Number of cases (%)	**WT**	**MUT**	**p**
N	**41**	**35**	**6**	
Sex	**38**	**32**	**6**	0.374
Male	19 (50%)	17 (53.1%)	2 (33.3%)	
Female	19 (50%)	15 (46.9%)	4 (66.7%)	
Age at diagnosis	**37**	**31**	**6**	0.804
<60 years	14 (37.8%)	12 (38.7%)	2 (33.3%)	
≥60 years	23 (62.2%)	19 (61.3%)	4 (66.7%)	
IPI	**35**	**29**	**6**	0.701
Low risk	14 (40%)	12 (41.4%)	2 (33.3%)	
Low-intermediate risk	6 (17.1%)	5 (17.2%)	1 (16.7%)	
High-intermediate risk	9 (25.8%)	8 (27.6%)	1 (16.7%)	
High risk	6 (17.1%)	4 (13.8%)	2 (33.3%)	
PIT	**29**	**24**	**5**	0.189
Low risk	5 (17.2%)	5 (20.8%)	0 (0%)	
Low-intermediate risk	11 (37.9%)	10 (41.7%)	1 (20%)	
High-intermediate risk	6 (20.8%)	5 (20.8%)	1 (20%)	
High risk	7 (24.1%)	4 (16.7%)	3 (60%)	
ECOG	**32**	**26**	**6**	0.753
<1	23 (71.9%)	19 (73.1%)	4 (66.7%)	
≥1	9 (28.1%)	7 (26.9%)	2 (33.3%)	
Treatment	**33**	**29**	**4**	0.400
CHOP/CHOP-LIKE	29 (87.9%)	26 (89.7%)	3 (75%)	
Others	4 (12.1%)	3 (10.3%)	1 (25%)	
Response	**33**	**27**	**6**	0.082
CR	19 (57.6%)	17 (63%)	2 (33.3%)	
PR	8 (24.2%)	7 (25.9%)	1 (16.7%)	
No response	6 (18.2%)	3 (11.1%)	3 (50%)	
Recurrence	**31**	**25**	**6**	0.569
No	23 (74.2%)	18 (72%)	5 (83.3%)	
Yes	8 (25.8%)	7 (28%)	1 (16.7%)	
State of the patient	**36**	**30**	**6**	0.032
Dead	22 (61.1%)	16 (53.3%)	6 (100%)	
Alive	14 (38.9%)	14 (46.7%)	0 (0%)	

Molecular parameters				
	Number of cases (%)	**WT**	**MUT**	**p**
NFATc1	**39**	**33**	**6**	0.123
Negative	15 (38.5%)	11 (33.3%)	4 (66.7%)	
Positive	24 (61.5%)	22 (66.7%)	2 (33.3%)	
P50	**41**	**35**	**6**	0.192
Negative	8 (19.5%)	8 (22.9%)	0 (0%)	
Positive	33 (80.5%)	27 (77.1%)	6 (100%)	
P52	**40**	**34**	**6**	0.729
Negative	11 (27.5%)	9 (26.5%)	2 (33.3%)	
Positive	29 (72.5%)	25 (73.5%)	4 (66.7%)	
P-ERK	**41**	**35**	**6**	0.633
Negative	31 (75.6%)	26 (74.3%)	5 (83.3%)	
Positive	10 (24.4%)	9 (25.7%)	1 (16.7%)	
CD30	**38**	**32**	**6**	0.030
Negative	31 (81.6%)	28 (87.5%)	3 (50%)	
Positive	7 (18.4%)	4 (12.5%)	3 (50%)	
Ki67	**40**	**34**	**6**	0.847
Negative	28 (70%)	24 (70.6%)	4 (66.7%)	
Positive	12 (30%)	10 (29.4%)	2 (33.3%)	
CD3	**39**	**33**	**6**	0.164
Negative	2 (5.1%)	1 (3%)	1 (16.7%)	
Positive	37 (94.8%)	32 (97%)	5 (83.3%)	

WT: wild type; MUT: mutated; IPI: International Prognostic Index; PIT: Prognostic Index for PTCL; ECOG: Eastern Cooperative Oncology Group; CHOP: cyclophosphamide, vincristine, doxorubicin, prednisone; CR: total response; PR: partial response.

Supplementary Table 2. Univariate analysis of clinical and molecular parameters and mutational status of the *PLCG1* gene in the cohort of 60 patients with AITL.

Clinical parameters				
	Number of cases (%)	**WT**	**MUT**	**p**
N	**60**	**53**	**7**	
Sex	**60**	**53**	**7**	0.055
Male	41 (68.3%)	34 (64.2%)	7 (100%)	
Female	19 (31.7%)	19 (35.8%)	0 (0%)	
Age at diagnosis	**59**	**52**	**7**	0.057
<60 years	16 (27.1%)	12 (23.1%)	4 (57.1%)	
≥60 years	43 (72.9%)	40 (76.9%)	3 (42.9%)	
IPI	**52**	**45**	**7**	0.765
Low risk	14 (26.9%)	11 (24.4%)	3 (42.9%)	
Low-intermediate risk	16 (30.8%)	14 (31.1%)	2 (28.6%)	
High-intermediate risk	12 (23.1%)	11 (24.4%)	1 (14.3%)	
High risk	10 (19.2%)	9 (20%)	1 (14.3%)	
PIT	**47**	**40**	**7**	0.946
Low risk	6 (12.8%)	5 (12.5%)	1 (14.3%)	
Low-intermediate risk	17 (36.2%)	15 (37.5%)	2 (28.6%)	
High-intermediate risk	16 (34%)	13 (32.5%)	3 (42.9%)	
High risk	8 (17%)	7 (17.5%)	1 (14.3%)	
ECOG	**51**	**44**	**7**	0.325
<1	37 (72.5%)	33 (75%)	4 (57.1%)	
≥1	14 (27.5%)	11 (25%)	3 (42.9%)	
Treatment	**52**	**46**	**6**	0.400
CHOP/CHOP-LIKE	34 (65.4%)	31 (67.4%)	3 (50%)	
Others	18 (34.6%)	15 (32.6%)	3 (50%)	
Response	**48**	**42**	**6**	1.000
CR	32 (66.7%)	28 (66.7%)	4 (67.7%)	
PR	8 (16.7%)	7 (16.7%)	1 (16.7%)	
No response	8 (16.7%)	7 (16.7%)	1 (16.7%)	
Recurrence	**44**	**39**	**5**	0.947
No	27 (61.4%)	24 (61.5%)	3 (60%)	
Yes	17 (38.6%)	15 (38.5%)	2 (40%)	
State of the patient	**57**	**50**	**7**	0.805
Dead	35 (61.4%)	31 (62%)	4 (57.1%)	
Alive	22 (38.6%)	19 (38%)	3 (42.9%)	

Molecular parameters				
	Number of cases (%)	**WT**	**MUT**	**p**
NFATc1	**56**	**50**	**6**	0.154
Negative	13 (23.2%)	13 (26%)	0 (0%)	
Positive	43 (76.8%)	37 (74%)	6 (100%)	
P50	**57**	**50**	**7**	0.078
Negative	16 (28.1%)	16 (32%)	0 (0%)	
Positive	41 (71.9%)	34 (68%)	7 (100%)	
P52	**57**	**50**	**7**	0.111
Negative	24 (42.1%)	23 (46%)	1 (14.3%)	
Positive	33 (57.9%)	27 (54%)	6 (85.7%)	
P-ERK	**60**	**53**	**7**	0.570
Negative	40 (66.7%)	36 (67.9%)	4 (57.1%)	
Positive	20 (33.3%)	17 (32.1%)	3 (42.9%)	
CD30	**56**	**49**	**7**	0.004
Negative	46 (82.1%)	43 (87.8%)	3 (42.9%)	
Positive	10 (17.9%)	6 (12.2%)	4 (57.1%)	
Ki67	**60**	**53**	**7**	0.048
Negative	50 (83.3%)	46 (86.8%)	4 (57.1%)	
Positive	10 (16.7%)	7 (13.2%)	3 (42.9%)	
CD3	**55**	**48**	**7**	0.444
Negative	4 (7.3%)	3 (6.2%)	1 (14.3%)	
Positive	51 (92.7%)	45 (93.8%)	6 (85.7%)	

WT: wild type; MUT: mutated; IPI: International Prognostic Index; PIT: Prognostic Index for PTCL; ECOG: Eastern Cooperative Oncology Group; CHOP: cyclophosphamide, vincristine, doxorubicin, prednisone; CR: total response; PR: partial response.

Supplementary Table 3. Panel of antibodies used in this series.

Antibody	***Clone***	***Source***	***Cut-off value for positivity***
CD3 FLEX	Rabbit polyclonal	DAKO	>10%.
CD30	CONGD/B9	Monoclonal CNIO	>10%.
NF-k B p50	Rabbit mono	Gene Tex	>10%
NF-k B p52	Mouse mono	Upstate	>10%
Ki67 FLEX	MIB-1	DAKO	>85%
NFATc1	7A6	BD Biosciences	>10%.
p-ERK	20G11	Cell Signaling Technologies	>10%.

Supplementary Table 4. Clinical and pathological characteristics of PLCG1 mutated cases, including NFAT, p50 and CD30 immunohistochemical data.

CASE	DX	SEX	AGE	STAGE	TREATMENT	STATUS	OS (m)	NFATc1	p50	CD30
P1	AITL	M	40	IV-B	ND	Dead	1	pos	pos	pos
P2	AITL	M	76	III-B	CVP	Dead	1	pos	pos	pos
P3	PTCL-NOS	F	81	II-B	CHOP	Dead	12	neg	pos	pos*
P4	PTCL-NOS	F	42	IV-B	Allogeneic transplant	Dead	44	pos	pos	neg
P5	AITL	M	27	IV-A	CHOP	Alive	10	pos	pos	pos
P6	PTCL-NOS	M	75	III-B	CHOP	Dead	4	pos	pos	pos
P7	AITL	M	44	III	CHOP	Dead	24	pos	pos	neg
P8	AITL	M	58	III	VAPAC	Dead	95	pos	pos	neg
P9	AITL-LIKE	F	69	II-B	CHOP	Dead	4	neg	pos	neg
P10	PTCL-NOS	M	81	II-B	ND	Dead	36	neg	pos	pos
P11	PTCL-NOS	F	72	III-B	ND	Dead	6	neg	pos	neg
P12	AITL	M	99	II-A	Gemcitabine, Oxaliplatin, Rituximab	Alive	14	pos	pos	pos
P13	AITL	M	42	IV-B	CHOP	Alive	33	ND	pos	neg

DX: Diagnosis; OS: Overall Survival; m: months; M: male; F: female; CHOP: cyclophosphamide, vincristine, doxorubicin, prednisone; CVP: cyclophosphamide, vincristine, prednisone; VAPAC: vincristine, cytosine arabinoside, prednisone, doxorubicin, cyclophosphamide; ND: no data; pos: positive; neg: negative; pos*: CD30>80%