SUPPLEMENTARY APPENDIX

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Supplementary methods

DNA extraction

QIAamp DNA Mini kit (Qiagen, Courtaboeuf, France) was used to extract DNA from PBMC and from tumor tissues: thin slices (3-4 µm) were performed if at least 50% of tumor cells were present on the section; core sectioning was performed if less than 50% of lymphomatous cells were present. A hematopathologist (TL) confirmed presence of tumor cells at both sample ends. Tumor samples required pre-treatment to remove paraffin. These additional steps consisted of incubation with proteinase K in lysis buffer overnight at 56°C and under shaking at 1,400 rpm, then centrifugation for 14 minutes at 14,000 rpm. Agarose gel electrophoresis was performed to verify the absence of DNA degradation. All *TNFAIP3* PCR products and β2 microglobulin, which is widely expressed among B cells and used as a positive control, were evaluated using agarose gel electrophoresis before sequencing.

Sanger sequencing of *TNFAIP3*

Sequencing of the 8 coding exons (2 to 9) of *TNFAIP3*, as well as exon/intron junctions, exon 1 (which is not coding), part of intron 5 and part of the 3' intronic region of the *TNFAIP3* gene was performed on germline and lymphoma DNA using the Sanger method, resulting in 13 PCR fragments 300 to 500 base pairs (bp) in length (supplementary figure 1). PCR was performed using the Hotstart Taq DNA polymerase kit (Qiagen) following manufacturer's recommendations. PCR was performed with 20 ng DNA, 1 µM each forward and reverse primer (see below), 1X buffer, 125 µM dNTPs, 3 mM MgCl2 and 2 Units Qiagen HotStar Taq in a 25 µl reaction. Reactions were amplified for 35 (when using PBMC DNA) or 40 cycles (when using lymphoma DNA) (94°C for 15 min followed by 35 or 40 denaturation cycles at 94°C for 30 s and annealing at 60°C for 30s and extension at 72°C for 1 min). For exon 1: 40 cycles with 50 ng DNA, without added $MgCl_2$ and with 10 % DMSO were performed.

PCR Primers

	Primer for 5'-3'	Primer rev 5'-3'	Ampl Size (bp)	Software
Exon 1	CAGTCTGCAGTCTTCGTGGCGG	GAGCGCTGCAAAACCAACGCC	405	NCBI
Exon 2	AGAGGAGTCGTATTAAAGTCAG	TAAAGCTTCATGAATGGGGATC	447	Oligo6
Exon 3	GGCTGGTTTATTCTGAAAACCTT	CTTCGCTTAGCCAAATTCATGT	399	Primer 3
Exon 4	CAGAATGACTTTTTAGTACAGGGAGT	CTTTGGCAGTGTGGTTTTCTC	300	Primer 3
Exon 5	CACTGCCAAAGTTCAGGTAACA	AACCAAGCAAGTCACAGAACAA	394	Primer 3
.Intron 5	GCTTTGGCCTCTCAAATTTAC	GGTGGATCTTATTGGTTAAAGTCC	300	Primer3
Exon 6	TTTGTTTTCCCATTTGGGTTTA	GGTGGCTGAGGTTAAAGACAGT	392	Primer 3
Exon 7	TTGTTCTATGAGCTAATGATG	AGTTCAGCTTTGGGAGTTTG	361	Oligo6
	ATGGATGTAAAATGTGAAACGC	CCTCTGCTGTAGTCCTTTTG	493	Oligo6
	CGGATTTTGTGAACGTTGCC	ACAAAACCCCTTCTGGACAG	487	Oligo6
Exon 8	AGGCTGGCCTAATCTGTATTTG	TTGTCACTGTCGGTAGAAAACG	370	Primer 3
Exon 9	TTTCATTGTGCTCTCCCTAAG	CAGAGGATAGCACCATGATG	434	Oligo6
TT>A	TCCTTTCTTTATAGGGTGGAG	CAAAAGGGTAAGAGGGGGC	221	Oligo6
β2m	TACAGACAGCAAACTCACCC	TGGAGAAGGGAAGTCACGG	399	Oligo6

Capillary electrophoresis of these fragments was performed on an ABI3130 xl sequencer (Applied Biosystems, Bedford, USA). Sequence data was aligned to the *TNFAIP3* human reference sequence available on a public database (NCBI). All chromatograms produced by Sanger sequencing were manually inspected and compared to the reference sequence in order to determine which germinal or lymphoma samples contained variants, and if the variants were indeed lymphoma mutations or germline variants. *TNFAIP3* sequence analyses were interpreted by two independent readers, one using a manual reading method and the other using Genalys software.

Validation of mutations and losses of heterozygosity (LOH) by pyrosequencing

All SNPs, mutations or LOH found within *TNFAIP3* exons and exon/intron junctions with the Sanger method were confirmed on independent PCR products by pyrosequencing. Genotyping by pyrosequencing was performed using standard protocols[1,2]. Regions of interest for validation were amplified using 20 ng of DNA and 5 to 7.5 pmol of forward and reverse primer, one of them being biotinylated. Sequences for oligonucleotides for PCR amplification and pyrosequencing are available upon request. Reaction conditions were 1x

HotStar Taq buffer supplemented with 1.6 mM $MgCl_2$, 100 µM dNTPs and 2.0 U HotStar Taq polymerase (Qiagen) in a 25 µl volume. The PCR program consisted of a denaturing step of 15 min at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at the respective annealing temperature and 20 s at 72°C, with a final extension of 5 min at 72°C. 10 µl of PCR product were rendered single-stranded as previously described[1,2] and 4 pmol of the respective sequencing primer were used for analysis. Quantitative DNA methylation analysis was carried out on a PSQ 96MD system with the PyroGold SQA Reagent Kit (Qiagen) and results were analyzed using the Q-CpG software (V.1.0.9, Biotage AB).

Measurement of *TNFAIP3* mRNA level by quantitative Reverse Transcription PCR

Two protocols of quantitative RT PCR were followed.

To compare *TNFAIP3* mRNA levels according to genotype, A20 was quantified with primer pairs specific for A20 or the housekeeping gene huRLPO (Human acidic ribosomal protein) in a BioRad Real-Time PCR system using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche) following manufacturer's recommendations. q-RT-PCR was performed with 1 µl DNA, 0.5 µM each forward and reverse primer, 1X buffer and MgCl2 at 2.25 mM in a 10 µl reaction. Reactions were amplified as followed: 95°C for 10 min followed by 40 denaturation cycles at 95°C for 5 s, annealing at 60°C for 5 s and extension at 72°C for 10 s.

To confirm expression of the A20 variants, RNA was extracted from transfected cells and 500 ng of RNA were treated or not with DNAse I (Biolabs). RNA was then reverse-transcribed or not using the High Capacity cDNA reverse transcription kit and samples were used for qRT-PCR using the Power SYBR® Green PCR Master Mix (Applied Biosystems) with a 300 nM final primer concentrations carried out on a StepOne™ Real-Time PCR System (Applied Biosystems). Relative expression within a given sample was calculated as a ratio (amol of A20/fmol of 18S).

Primers for quantitative Reverse Transcription PCR

	Primer for 5'-3'	Primer rev 5'-3'	Amplicon size
A20	TGGCACAACTCATCTCATCAA	GCCATTTCTTGTACTCATGCTG	113 bp
huRPLP0	GAGAAACTGCTGCCTCATATC	CATGTCCCTGATCTCAGTGAG	78 bp

Functional analysis of wild-type and A20 mutants

A gene reporter assay was used to assess the function of the identified abnormalities of *TNFAIP3*. The pGL4.32 [luc2P/NF-κB-RE/Hygro] plasmid (Promega, Charbonnières-les-Bains, France), which drives luciferase expression in response to NF-kB activation, was used as a reporter gene system. HEK 293T cells (6,000 cells/well) were seeded 72 hours before testing in high-glucose Dulbecco's minimal essential medium (Invitrogen, Cergy Pontoise, France) containing 2 mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10% fetal calf serum at 37°C in 96-well plates. Twenty-four hours before testing, the cells were cotransfected in serum free OptiMEMTM™ medium (Invitrogen), using Lipofectamine 2000 as recommended by the manufacturer (Invitrogen) with the plasmids coding for the wild-type or A20 mutants, the luc2P/NF-κB-RE/Hygro plasmid and the β-galactosidase plasmid (pMIR Report-β-gal control vector, Applied Biosystems). Two mutated sequences of *TNFAIP3* were tested: the first one contained the rs2230926 minor allele resulting in the p.F127C A20 variation; the second one contained an insertion of two guanines at position 138196024 (exon 3) leading to a 122 amino acid truncated protein instead of a 790 amino acid protein. On the test day, TNFα was added to the culture medium at 10 ng/ml. After 6 hours of incubation cells were harvested and assayed for β-galactosidase and luciferase activities using a spectrophotometer (Victor, Perkin Elmer, Courtaboeuf, France) and a luminometer (Tristar, Berthold, Thoiry, France), respectively. To standardize the transfection efficiency, the relative light units obtained in the luciferase assay were divided by the optical density obtained in the β-galactosidase assay. A total of 8 transfection experiments in HEK 293T cells were performed with the wild type A20, 7 experiments with the rs2230926G variant and 3 experiments with the GG insertion (New 3).

Western Blot analysis of A20 protein variants

Total protein extracts were prepared from transfected-HEK 293T cells. Briefly, cells were lysed as described previously [3]. Then 40 µg of protein was submitted directly to SDS-PAGE. After protein blotting on an Odyssey nitrocellulose membrane (LI-COR, Lincoln, NE, USA), blots were incubated for 1 hour at room temperature in TBS/0.1% Tween 20/5% BSA, then incubated overnight at 4°C with rabbit anti-human *TNFAIP3* antibody (1:5000, clone EPR2663, Clinisciences, Nanterre, France) and mouse anti α-Tubulin (1:5000, Sigma-Alrich) antibodies. After extensive washes, blots were incubated with an IRDye 800-conjugated affinity purified anti-rabbit IgG second antibody (1:10,000; Perbio Science, Bezons, France) and an IRDye 680-conjugated affinity purified anti-mouse IgG second antibody (1:10,000; Perbio Science) for 1 hour at room temperature. After extensive washes, proteins were visualized with an Odyssey Fc apparatus (LI-COR). A20 infrared fluorescence was normalized to α-Tubulin as determined by densitometry using the Image Studio software (LI-COR).

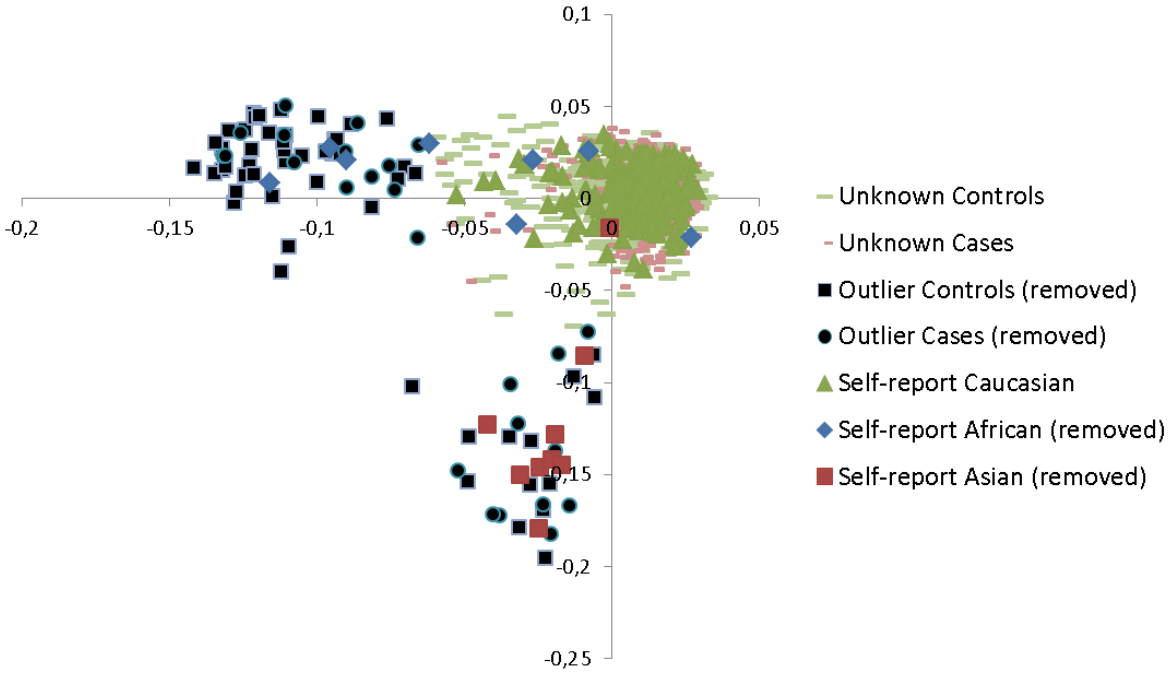
Immunocytochemistry

HEK 293T Cells were seeded in 24-well plates and processed for immunocytochemistry. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized for 15 min with a 0.25% solution of Triton X-100 diluted in PBS. Cells were then incubated with primary antibody (anti FLAG M2 (Sigma) 0.5µg/ml or IgG 0.5µg/ml) overnight at 4 C. After several washes with PBS, Tween 0.1%, the cells were incubated with the appropriate fluorochrome-coupled secondary antibody (Alexa 555; Goat anti mouse; 1/500; Invitrogen) for 45 min. Nuclear counterstaining was performed with 0.5 µg/mL 4,6-diamidino-2-phenylindole (DAPI), and coverslips were mounted on slides with ProLong Gold mounting medium (Invitrogen). For standard microscopy, fluorescent cells were observed with an Olympus Provis AX70 (Rungis, France), and images were acquired at ×40 magnification with Qcapture Pro version

5.1 (Q Imaging Inc., Surrey, British Columbia, Canada) using an Evolution VF Monochrome camera (Media Cybernetics Inc, Bethesda, Maryland).

Supplementary figures

A



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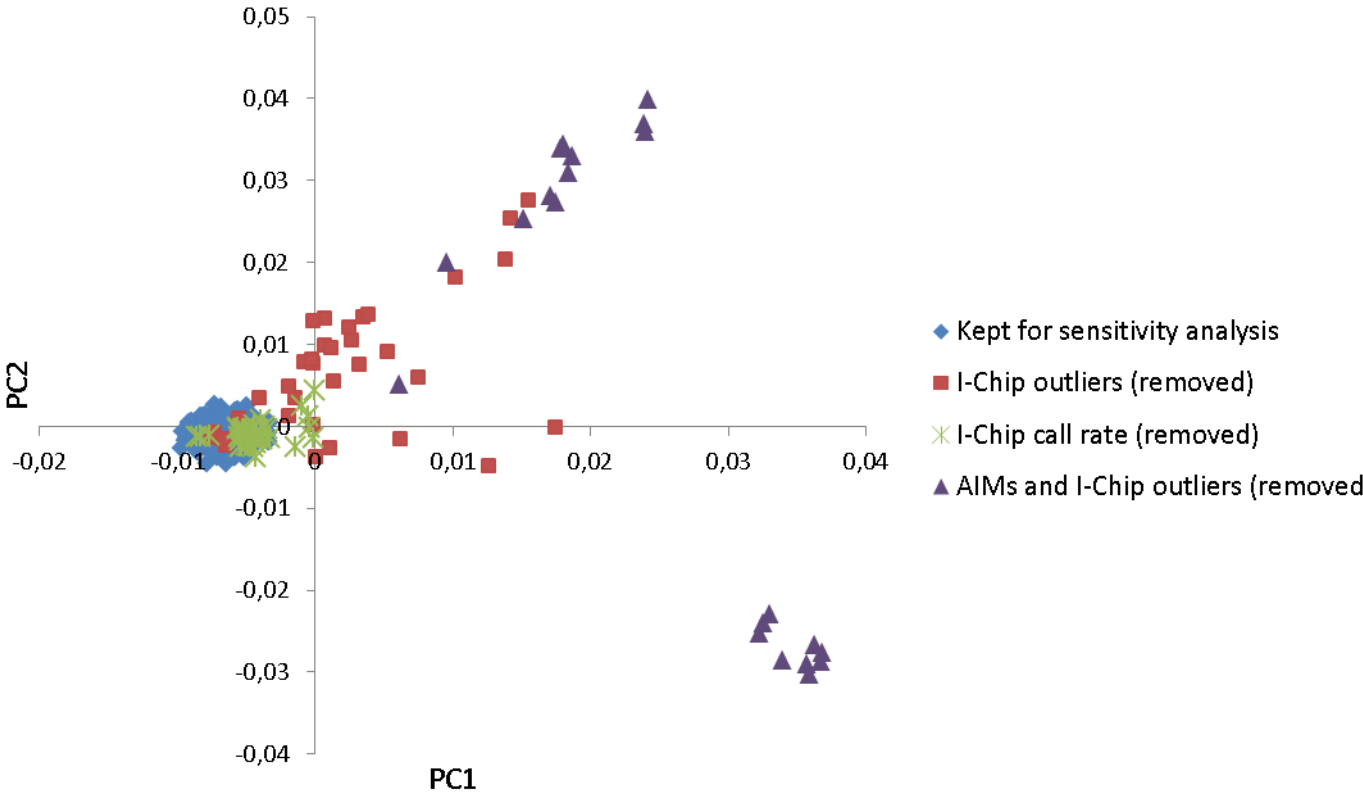

Figure S1: Principal components analyses

Principal components analyses (PCA) of A) all discovery cohort subjects, using 47 Ancestry-Informative Markers (AIMs), and B) subset of cases for sensitivity analyses, using Immunochip markers. Subjects are plotted by top two principal components (PC1 and PC2). After outlier removal, PC1 and PC2 values were used in logistic regressions to adjust for residual ancestry differences.

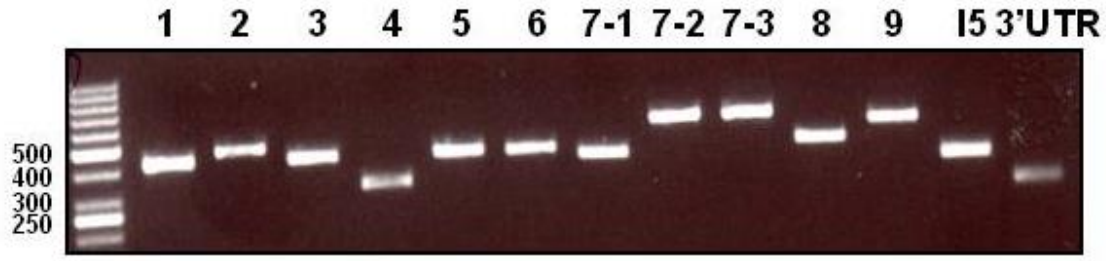

Figure S2: the 13 PCR products generated by *TNFAIP3* exon sequencing

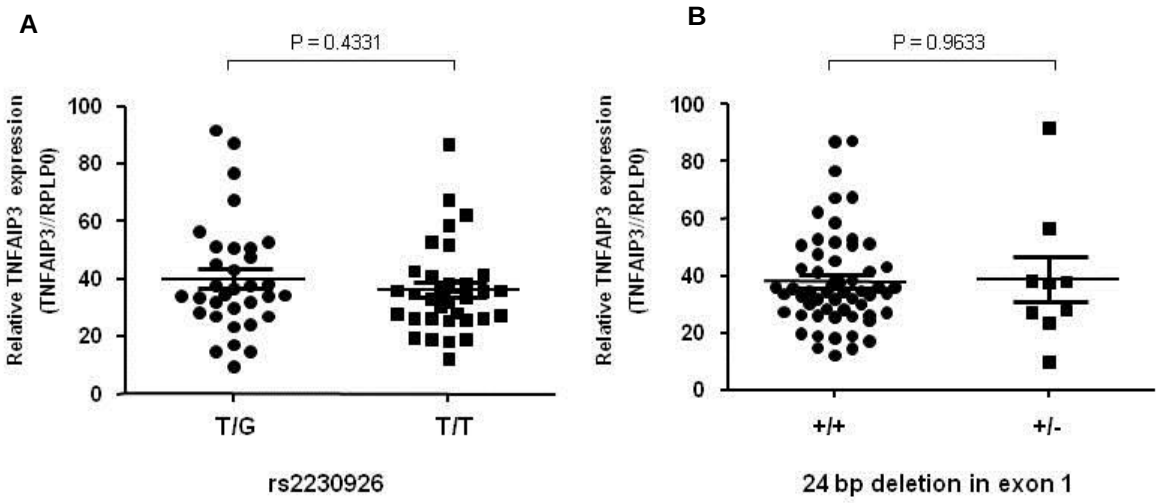

Figure S3: *TNFAIP3* mRNA expression depending on the germline abnormalities.

TNFAIP3 mRNA level was assessed among pSS patients according to their genotype (i.e., rs2230926 (A) and New 1 deletion (B)) and irrespective of their lymphoma status. Thirty three and thirty four pSS patients harboring the rs2230926G (T/G) and rs2230926T alleles (T/T), respectively, were tested (A). Nine and fifty eight pSS patients harboring the 24 bp deletion (+/-) or not (+/+) were tested (B).

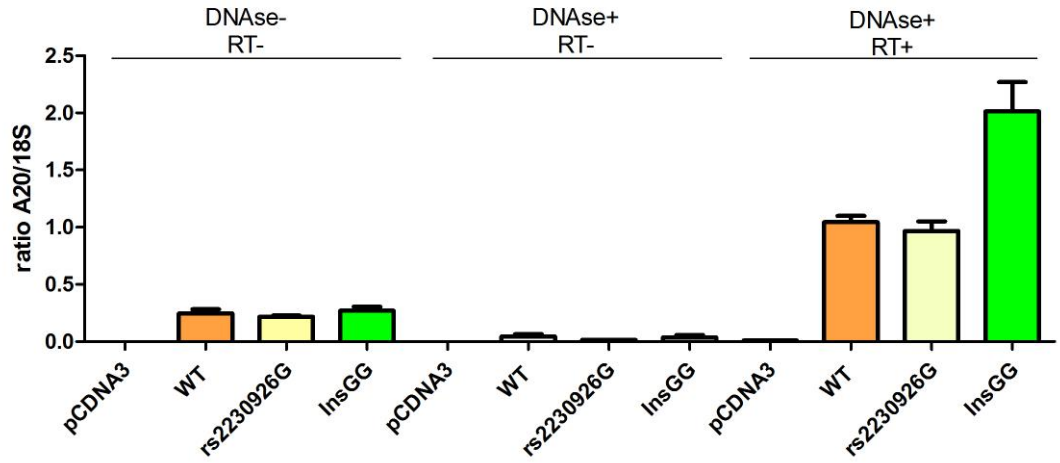

Figure S4: Expression of A20 transcripts by transfected HEK293T cells. Plasmids encoding either wild type A20 (WT) or A20 mutants (rs2230926G or InsGG) were transfected into HEK 293T cells pre-stimulated with TNF([10 ng/ml] for 6 hours). Different pre-treatments were performed before PCR: DNAse- RT- (without DNAse treatment and without reverse transcription (RT)), DNAse+ RT- (with DNAse treatment and without RT) and DNAse+ RT+ (with DNAse treatment and with RT). For each condition, several samples were tested: pcDNA3 (empty vector), WT (transfection with A20 WT), rs2230926G (transfection with A20 rs2230926G) and InsGG (transfection with A20 InsGG). Results are expressed as mean of 3 triplicates ±SEM. The level of A20 cDNA was normalized to that of 18S.

Supplementary table

Table S1: Characteristics of the 44 pSS patients with lymphoma.

Patients Identification	Age at diagnosis of pSS	anti SSA/anti SSB	RF	Age at diagnosis of lymphoma	Histology	Localization	Grade
1G	52	1	0	63	MALT	stomach	IV
2G	60	1	1	70	MALT	secondary salivary glands	IV
3G	14	1	1	18	MALT	parotid gland	IE
4G	48	1	1	49	MALT	ocular adnexal	IE
5G	75	0	0	75	MALT	cavum	IV
6G	29	1	1	39	MALT	parotid gland	IE
7G	64	1	1	64	MZL	nodal	IV
8G	53	0	1	53	DLCL	skin	IE
9G	50	1	0	59	MALT	parotid gland	IE
10G	43	1	1	43	MALT	parotid gland	IV
11G	49	1	1	49	MALT	parotid gland	IE
12G	58	0	0	58	lymphocytic lymphoma	lymph nodes	IV
13G	72	0	1	72	MALT	cavum	IV
14G	53	1	0	53	MZL	splenic	IS
15G	58	1	1	58	MALT	parotid gland	IE
16G	55	1	0	58	MALT	parotid gland	IE
17G	51	0	0	51	MALT	secondary salivary glands	IE
18G	55	1	0	55	MALT	parotid gland	IE
19G	54	1	1	59	MALT	parotid gland	IE
20G	28	1	1	33	MALT	parotid gland	IE
21G	59	0	1	63	MALT	secondary salivary glands	IE
22G	48	1	1	56	MZL	nodal	IV
23G	41	1	0	48	MZL	nodal	IV
24G	34	1	1	39	MALT	parotid gland	IE
25G	65	0	1	65	MZL	splenic	IS
26G	32	0	1	41	EBV-associated B cell lymphoproliferative disorder	splenic and lymph nodes	IV
27G	70	1	1	79	DLCL	lymph nodes	III
28G	65	0	1	69	MALT	lung	IE
29G	49	1	1	60	MALT	lung	IV
30G	50	1	1	52	MALT	stomach	IE
31G	41	0	0	54	MALT	skin	IE
32G	54	0	0	58	DLCL	lymph nodes	IV
33G	47	1	1	61	MALT	parotid gland	IE
34G	59	0	0	59	fungoid mycosis	skin	IE
35G	62	0	1	62	MALT	parotid gland	IE
36G	44	1	1	52	MALT	parotid gland	IV
37G	25	1	1	42	MALT	cavum	IE
38G	57	1	1	78	DLCL	lymph nodes	IV
39G	54	0	1	57	MALT	secondary salivary glands	IV
40G	53	1	0	57	HL	lymph nodes	IV
41G	44	1	1	54	DLCL	lymph nodes	I
42G	84	0	0	86	MZL	circulating	I
43G	69	0	0	69	follicular lymphoma	mediastinum	IV
44G	17	1	1	33	MALT	thymus	IE

Anti SSA/ anti SSB: Positivity for anti Sjögren's Syndrome A and/or B (1) or negativity (0); RF: Positivity for Rheumatoid Factor (1) or negativity (0); MALT: Mucosa Associated Lymphoid Tissue, DLCL: diffuse large cell lymphoma, MZL: Marginal Zone Lymphoma

Table S2: Clinical characteristics of the 20 pSS patients with lymphoma and both germline and somatic sequence data available and the entire cohort of pSS patients with lymphoma

	Patients with germline and somatic sequence data	Entire cohort
Number of patients	n=20	n=44
Median age at diagnosis	55,5	53
Anti SSA+ n (%)	13 (65)	28 (64)
RF+ n (%)	16 (80)	29 (66)
Median age at diagnosis of lymphoma	59	57,5
MALT lymphoma n (%)	13 (65)	27 (61)

Supplementary References

1. Dejeux E, Olaso R, Dousset B, et al. Hypermethylation of the IGF2 differentially methylated region 2 is a specific event in insulinomas leading to loss-of-imprinting and overexpression. Endocr Relat Cancer 2009;16:939-52.
2. Tost J, Gut IG. DNA methylation analysis by pyrosequencing. Nat Protoc 2007;2:2265-75.
3. Viengchareun S, Bouzinba-Segard H, Laigneau JP, et al. Prolactin potentiates insulin-stimulated leptin expression and release from differentiated brown adipocytes. J Mol Endocrinol 2004;33:679-91.