A20 inactivation in ocular adnexal MALT lymphoma

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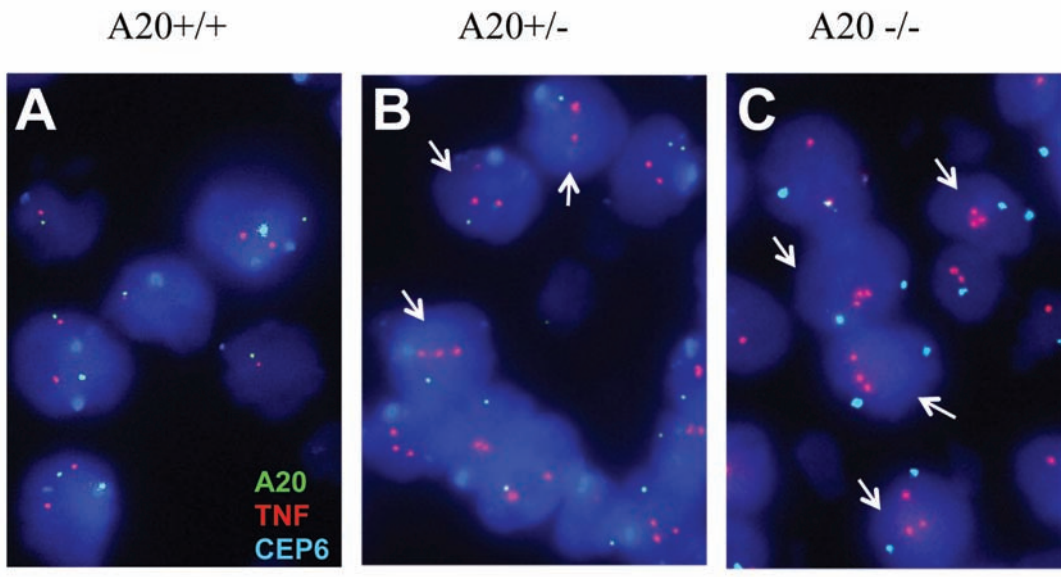
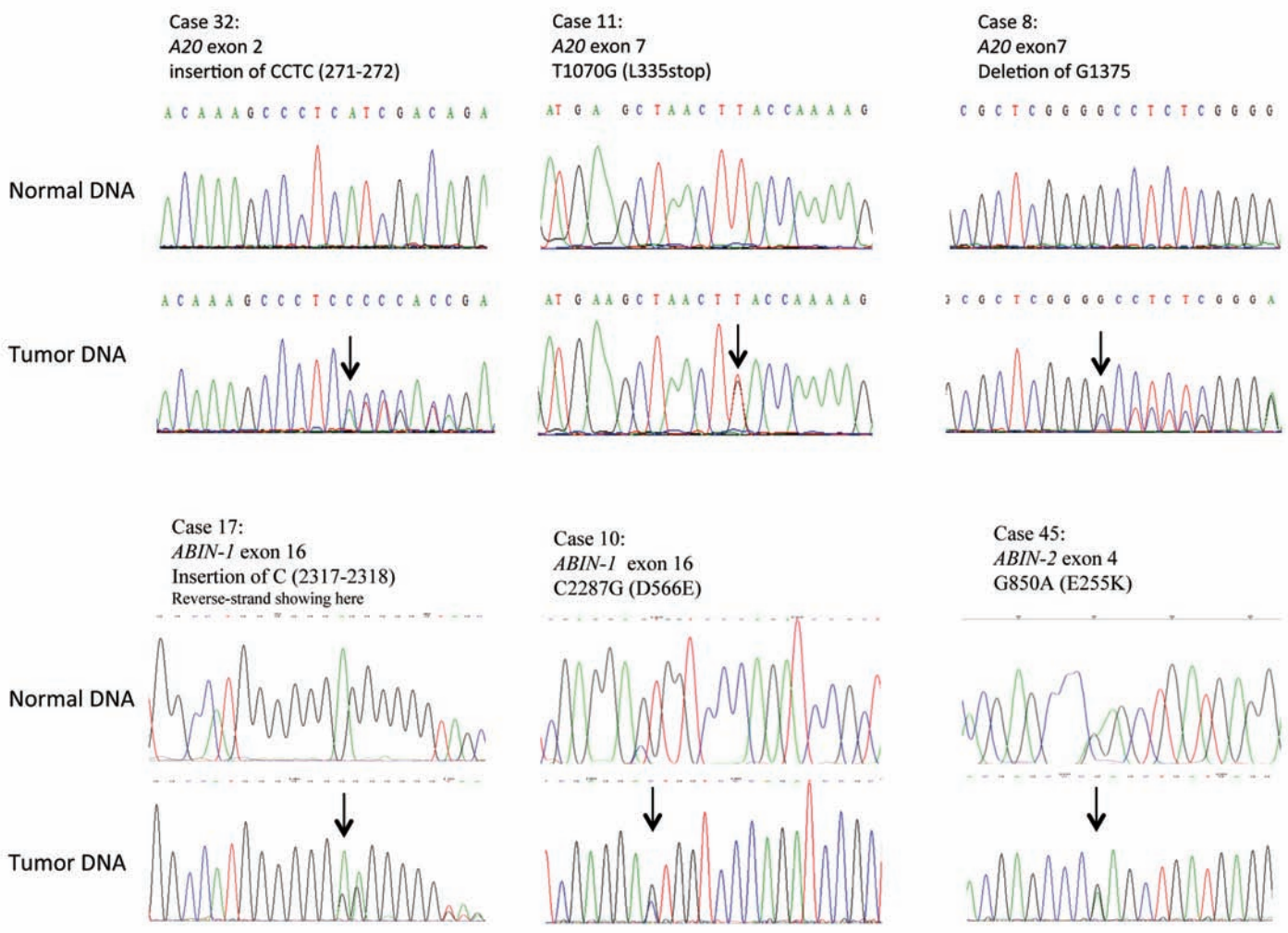
Citation: Bi Y, Zeng N, Chanudet E, Huang Y, Hamoudi RA, Liu H, Dong G, Watkins AJ, Ley SC, Zou L, Chen R, Zhu X, and Du M-Q. A20 inactivation in ocular adnexal MALT lymphoma. Haematologica 2012;97(6):926-930. doi:10.3324/haematol.2010.036798

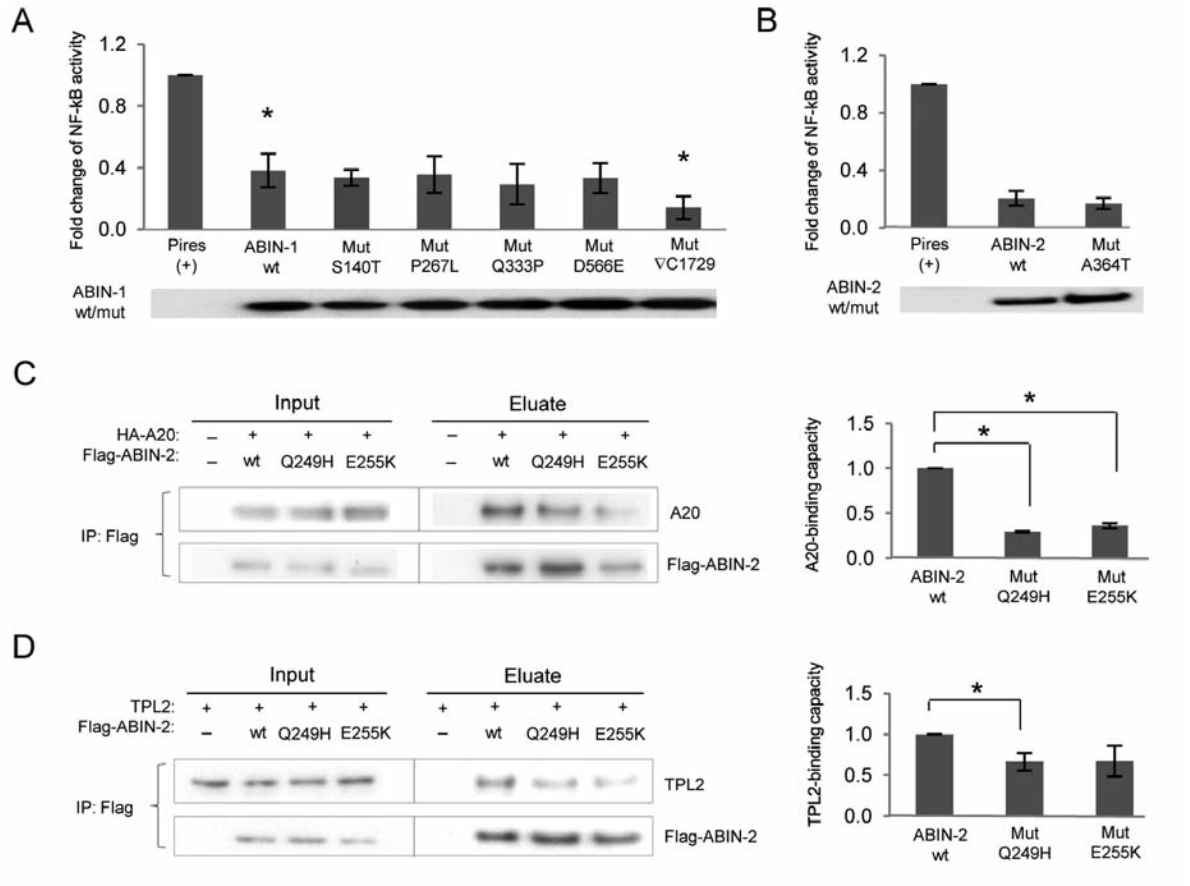
Online Supplementary Figure S1. Examples of *A20* deletion and *TNFA/B/C* gain in ocular adnexal MALT lymphoma. Three color FISH for investigation of copy number changes at the A20 (RP11-356i2 labeled with spectrum green) and *TNFA/B/C* (bPG296P20 labeled with spectrum orange) loci, together with centromeric probe CEP6 (spectrum aqua). Cell nuclei showing copy number abnormalities are indicated by arrows. (A) A case showing no A20 and *TNF/AB/C* copy number changes. (B) A case showing *A20* heterozygous deletion and *TNFA/B/C* gain. (C) A case with A20 homozygous deletion and TNFA/B/C gain.



Online Supplementary Figure S2. Examples of *A20*, *ABIN-1/2* mutation detected in ocular adnexal MALT lymphoma.



Online Supplementary Figure S3. Functional characterization of *ABIN-1* and *ABIN-2* mutations. (A and B) NF-κB reporter assay demonstrates that none of the ABIN-1/2 mutants/variants investigated shows any evidence of impaired ability in suppression of TNFα induced NF-κB activation in HEK293 cells. (C and D) In comparison to the wild-type ABIN-2, both the E255K and Q249H variants show defective binding to A20 and TPL2. The data that correspond to the NF-κB reporter assays represent 3 independent experiments, whereas the immunoprecipitation experiment was performed twice for A20 and three times for TPL2. WT: wild-type; mut: mutant; * $P<0.05$.



Online Supplementary Table S1. Primers and PCR conditions used for amplification of the A20, ABIN-1 and ABIN-2 coding exons.

Genes	Exon	Primer name	Sequence (5'-3')	Amplicon size (bp)	PCR condition
A20	E2	Forward	GATCAAACACTGGGGTTTCC	446	PCR was carried out in a 10 µL reaction mixture with 10ng template DNA and AmpliTaq Gold (Applied Biosystems) master mix according to the manufacturer's instructions. The PCR conditions were 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 45 s, touch-down annealing from 65 to 60°C (1°C 1ess every 2 cycles) for 45 s, and extension at 72°C for 1 min.
		Reverse	GCTATCACCCAGGCAAAAGA		
	E3	Forward	ACCATTCAGTCCCCTAGAATAGC	433	
		Reverse	AGTATGCTTCGCTTAGCCAAAT		
	E4-5	Forward	GGGAGTACAGGATACATTCAAGC	592	
		Reverse	GCAAAAAGGAAAACCCTGATG		
	E6	Forward	TGGCCTTGTTTAGTAGAATACTGTTT	393	
		Reverse	TCCTCTCTAACCATGCACAAGA		
	E7	Forward	TTGTGTGTGATTTTGTGTATTCTCAT	502	
		Reverse	CTGCACTTCATGGCAGTGGT		
	E7	Forward	GCCCTTTTCTGTTCAGTGAG	570	
		Reverse	AGGAACAAAACCCCTTCTGG		
	E8	Forward	GGGTGACCCCTATGTGGTACT	292	
		Reverse	AGAAAACGCTCCAGCAAAAA		
	E9	Forward	GTGCTCTCCCTAAGAAATGTGAG	466	
		Reverse	CACCCTTAAGCCCACTGTTG		
ABIN-1	E2	Forward	GGCATTGACAGACTAGAGCTTC	363	PCR was carried out in a 10 µL reaction mixture with 5-10 ng template DNA and AmpliTaq Gold 360 (Applied Biosystems) master mix plus GC-enhancer according to the manufacturer's instructions. The PCR conditions were 95°C for 10 min to activate the enzyme, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 58-65°C (depending on the primer set) for 20 s, and extension at 72°C for 30-45 s (depending on the amplicon size).
		Reverse	ACTTGGGCAGAGGCATATG		
	E3	Forward	GGGAGAAGCAGCACACTG	272	
		Reverse	GAAGGGAGTTCACTGTGAAGG		
	E4	Forward	CTCCCATTCTCCCACACTC	310	
		Reverse	GACCTCAACCCTCTTTCTTC		
	E5	Forward	GCTTTGTGATCTCATGTGAGATG	327	
		Reverse	GGGATCTGAACAGGTTCTGTG		
	E6	Forward	GAGGAAGCAGAGGGAGAATG	346	
		Reverse	GAGCAGGAAGGTGGGAAG		
	E7	Forward	CCCACCATCCTCTAGCTCAG	252	
		Reverse	CCAGAAATCAGTGCTGCAC		
	E8	Forward	CAACTCATGCGATAGTGAGC	332	
		Reverse	GAACCTTCTACCACTGGCAC		
	E9	Forward	GGTCTTAGAGGAGCACCGAG	236	
		Reverse	AGCCTTTGTGCTGCTGGT		
	E10	Forward	TAGCCACTATTCACACACACC	265	
		Reverse	CCCCAAGGTTCAAAGCTG		
	E11	Forward	CCTCTTTAGCAGCTTTGAACC	375	
		Reverse	GCTTGTTTGCTCCACAGAAC		
	E12	Forward	CCAGGCAGGACAGAGAATC	379	
		Reverse	CCTGAGTCACTCCCAGTGTG		
	E13	Forward	GAAGGAGTTCCCTGAGGATG	387	
		Reverse	GGTTATGGTGCTGGAAAGG		
	E14	Forward	AGGAGGCATGGGAGTCTG	251	
		Reverse	GAGGACAGGCCAGTTGC		
	E15	Forward	CCAGAGGGAAGCATCAG	259	
		Reverse	CACACACTGTGCATCCATC		
	E16	Forward	AGAGGGTGATGAGATGGGT	381	
		Reverse	CGGTGCTGTTTAGTTCAGAAG		
	E17	Forward	GCCTCAGATTCCTTACCTGTG	366	
		Reverse	CCTCCACCAAGAGCAGAAAC		
	E18	Forward	ACTGTTCCTGCACTGCATTC	378	
		Reverse	CTCTCTCCACTCAGCAGCA		
ABIN-2	E1	Forward	CGGGCGCGGAAGTTGC	405	Identical to ABIN-1 PCR conditions
		Reverse	TCGCTCACCCACCCAGGA		
	E2	Forward	GCATCCACGCCAGGTCTTC	441	
		Reverse	CGATGCTCCCAGCACACAG		
	E3	Forward	GGACGCAGTGATAATCTGTG	329	
		Reverse	CCAGTCTCTAGGGACTGTGTG		
	E4	Forward	CTCTCACTCAGCGAATCACTG	423	
		Reverse	CCATGATTTCGGCCACCAC		
	E5	Forward	GAAGGCTTGGTGGCACTG	314	
		Reverse	GGCAGACACAGAAAGGCTC		
	E6	Forward	GCAGGAAGCAAAGTGAGG	453	
		Reverse	CTGTCCCTGAGGGCAGCTG		

Online Supplementary Table S2. Primers used for quantitative RT-PCR.

Gene name	Size of amplicon	Primers spanning different exons*	Size of amplicon	Accession Number
18SrRNA	F 5' TGACTCAACACGGGAAACC R 5' TCGCTCCACCAACTAAGAAC	No	114bp	NR_003286
BCL2	F 5' TTGCTTTACGTGGCCTGTTTC R 5' GAAGACCCTGAAGGACAGCCAT	No	94bp	NM_000633
TLR6	F 5' AACAAGTACCACAAGCTGAAG R 5' CTCTAATGTTAGCCCAAAAGAG	No	100bp	NM_006068
CCR2A	F 5' GCGTTTAATCACATTCGAGTGTTT R 5' CCACTGGCAAATTAGGGAACAA	No	77bp	NM_000647
CD69	F 5' CCACCAGTCCCCATTTCTCAA R 5' TTGGCCCACTGATAAGGCAAT	E2 – E3	125bp	NM_001781

*Where possible, one of the primer pair was designed to span an exon-exon junction to prevent amplification from genomic DNA.

Online Supplementary Table S3. Summary of *A20*, *ABIN-1* and *ABIN-2* genetic abnormalities in ocular adnexal MALT lymphoma.

Case N.	Age/Sex	Side	Sites involved	*TNF* loci Gain	*A20* deletion	A20 Methylation	*A20* mutation (NM_006290; NP_006281)	*ABIN-1* mutation (NM_006058; NP_006049)	*ABIN-2* mutation (NM_024309; NP_077285)
1	63/F	Single	Orbit	(+)	Homo	(-)	(-)		
2	64/M	Single	Orbit	(-)	Heter	(-)	Exon 5, A799T (R245X) truncation		
3	50/M	Single	Orbit	(-)	Heter	(-)	Exon 4, C625T (Q187X) truncation		
4	41/M	Single	Conjunctiva	(-)	Heter	(-)	Exon 7, C1560A (C498X) truncation		
5	43/M	Single	Orbit	(-)	Heter	(-)	Exon 7, G1348T (E428X) truncation		
6	49/M	Single	Orbit	(+)	Homo	(-)	(-)		
7	82/M	Single	Orbit + Conjunctiva	(-)	Heter	(-)	Exon 7, C1573T (Q503X) truncation		
8	54/M	Single	Orbit	(-)	(-)	(+)	Exon 7, ΔG (1375) frameshift		
9	75/M	Single	Orbit	(+)	(-)	(-)	Exon 7, G1433T (G456V if in wild-type allele); ΔG (1434) frameshift		
10	79/M	Single	Orbit	(-)	(-)	(-)	Exon 4, T647C (I194T); Exon 6, ΔT (891) frameshift	Exon 16, C2287G (D566E): germline mutation	
11	68/M	Single	Orbit	(-)	(-)	(-)	Exon 6, Δ5bp (1037-1041) frameshift; Exon7, T1070G (L335X) truncation truncation		
12	58/M	Single	Orbit + Conjunctiva	(-)	(-)	(-)	Exon 3, Δ19bp (402-420) frameshift; Exon 3, T425G (L120W)		
13	72/F	Single	Orbit + Conjunctiva	(-)	(-)	(-)	Exon 2, insertion A (360-361); Exon 6, insertion TGTT (895-896) frameshift		
14	55/F	Bilateral	Orbit + Conjunctiva	(-)	(-)	(-)	Exon 6, Δ5bp (1037-1041) frameshift; Exon 7, C1573T (Q503X) truncation		
15	82/F	Single	Conjunctiva	(+)	(-)	(-)	Exon 3, Δ84bp (403-486); Exon 7, C1534T (Q490X) truncation	Exon 8, C1376T (R263W)	
16	54/M	Single	Orbit	(-)	(-)	(-)	Exon 2, ΔC (334) frameshift		
17	71/M	Single	Orbit + Conjunctiva	(-)	(-)	(-)	Exon 4, insertion A (576-577) frameshift	Exon 16, insertion C (2317-2318), frameshift: somatic mutation	Exon 4, G850A (E255K): germline mutation; Exon 6, G1177A (A364T): germline mutation
18	58/F	Single	Orbit + Conjunctiva	(-)	(-)	(-)	Exon 4, insertion A (576-577) frameshift		
19	31/M	Single	Orbit	(-)	(-)	(-)	Exon 7, Δ5bp (1310-1314) frameshift		
20	58/M	Single	Orbit	(-)	(-)	(-)	Exon 2, ΔA(307) frameshift		
21	72/M	Single	Conjunctiva	(+)	(-)	(-)	Exon 7, Δ20bp (1583-1602) frameshift		Exon 4, G834T (Q249H): germline mutation
23	41/M	Single	Orbit	(-)	(-)	(-)	Exon 7, C1573T (Q503X) truncation		
24	62/M	Single	Orbit	(-)	(-)	(-)	Exon 6, Δ11bp (904-914) frameshift		

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25	63/M	Single	Orbit	(-)	(-)	(-)	Exon 3, insertion A (472-473) frameshift		
26	75/F	Single	Conjunctiva	(-)	(-)	(-)	Exon 4, G557A (W164X) truncation		
27	76/M	Single	Orbit	(-)	(-)	(-)	Exon 5, ΔA(799) frameshift		
28	76/M	Single	Lachrymal gland	(+)	(-)	(-)	Exon 7, T1551A (C495X) truncation		
29	64/F	Single	Orbit	(-)	(-)	(-)	Exon 6, Δ5bp (1037-1041) frameshift		
30	60/M	Single	Orbit + Conjunctiva	(-)	(-)	(-)	Exon-2 and intron-2 splicing site, ΔTAAGA(361+2_+6)		
31	53/M	Single	Orbit + Conjunctiva	(-)	(-)	(-)	Exon 2, Δ5bp (348-352) frameshift		
32	41/F	Bilateral	Lachrymal Gland	(+)	(-)	(-)	Exon 2, insertion CCTC (271-272) frameshift		
33	50/M	Single	Orbit	(-)	(-)	(-)	Exon 5, G757A (G231R): germline mutation		
34	71/F	Single	Orbit + Conjunctiva	(+)	Heter	(-)	(-)		
35	52/M	Single	Orbit	(-)	Heter	(-)	(-)		
36	50/M	Single	Conjunctiva	(-)	(-)	(-)	(-)	Exon 8, C1389T (P267L): germline mutation	
37	65/M	Bilateral	Orbit + Conjunctiva	(-)	(-)	(-)	(-)	Exon 8, C1376T (R263W)	
38	76/M	Single	Orbit	(-)	(-)	(-)	(-)	Exon 10, A1587C (Q333P)	
39	45/M	Single	Orbit + Conjunctiva	(-)	(-)	(-)	(-)	Exon 5, G1008C (S140T): germline mutation	
40	64/M	Single	Orbit	(-)	(-)	(-)	(-)		Exon 6, G1177A (A364T): somatic mutation
41	52/M	Single	Orbit + Conjunctiva	(-)	(-)	(-)	(-)		Exon 4, G834T (Q249H): germline mutation
42	50/F	Single	Lachrymal gland	(-)	(-)	(-)	(-)		Exon 4, G834T (Q249H): germline mutation
43	52/M	Single	Orbit + Conjunctiva	(-)	(-)	(-)	(-)		Exon 4, G834T (Q249H): germline mutation
44	77/M	Single	Orbit	(-)	(-)	(-)	(-)		Exon 4, G850A (E255K); Exon 6, G1177A (A364T)
45	62/M	Single	Orbit	(-)	(-)	(-)	(-)		Exon 4, G850A (E255K): germline mutation; Exon 6, G1177A (A364T): germline mutation
46	72/M	Bilateral	Lachrymal gland	(-)	(-)	(-)	(-)		Exon 4, G834T (Q249H): germline mutation
47	24/F	Bilateral	Conjunctiva	(-)	(-)	(-)	(-)		Exon 4, G850A (E255K): germline mutation

Homo: homozygous deletion; Heter: heterozygous deletion; Δ: deletion; ∇: insertion.

Online Supplementary Table S4. Incidence of *ABIN-1* and *ABIN-2* novel non-synonymous polymorphisms detected in Chinese patients with ocular adnexal MALT lymphoma and a Han Chinese population.*

Gene	Nucleotide change	Amino acid alteration	Incidence in ocular adnexal MALT lymphoma	Incidence in Han Chinese population#	
ABIN1	G1008C	S140T	0.95%	0/81	0%
ABIN1	C1376T	R263W	1.9%	1/89	1.1%
ABIN1	C1389T	P267L	0.95%	1/88	1.1%
ABIN1	A1587C	Q333P	0.95%	0/100	0%
ABIN1	C2287G	D566E	0.95%	0/36	0%
ABIN2	G834T	Q249H	4.8%	3/62	4.8%
ABIN2	G850A	E255K	3.8%	0/77	0%
ABIN2	G1177A	A364T	3.8%	1/67	1.5%

**The incidences of* ABIN-1 *and* ABIN-2 *polymorphisms in Chinese patients with ocular adnexal MALT lymphoma are derived from the present study, while those in a Han Chinese population are from the analyses of sequence data from the 1000 Genome Project.*

 # The next generation sequence data from the 1000 Genome Project.[1] The sequence files in BAM format for Han Chinese population were retrieved online (ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/pilot_data/data/ and ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/data/). The BAM files were aligned using BWA or MAQ aligners.[2,3] Samtools software suite running on Linux was used to identify the BAM sequence files that contained sequences covering the polymorphism sites.[2] The sequence files were visualized using IGV[4] and for each polymorphism site the sequence coverage and Phred scores were recorded. A filter of 3 or more sequence coverage and Phred score of 10 or more for each base were used to select individuals and sequences that were adequate for the calculation of the incidence of the polymorphism, as described previously.[5] A total of 133 sequence files mapping to the polymorphisms found in this study were retrieved: 94 Southern Han Chinese (CHS), 30 Beijing Han Chinese (CHB) and 9 Denver Chinese (CHD). Only the individuals with sequence data that passed the filtering criteria (coverage ≥ 3; Phred score ≥ 10) for each polymorphism site[5] were included in the calculation of the incidence of the polymorphism.

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