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A20 is targeted by promoter methylation, deletion and inactivating mutation in MALT lymphoma

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Four recurrent chromosomal translocations, namely t(11;18)(q21;q21)/*API2-MALT1*, t(1;14)(p22;q32)/*BCL10-IGH*, t(14;18)(q32;q21)/*IGH-MALT1* and t(3;14)(p13;q32)/*FOXP1-IGH*, have been described in extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma). The oncogenic products of the first three translocations are believed to exert their oncogenic activity through activation of the transcription factor NF-κB, whereas the role of FOXP1 in lymphomagenesis remains to be investigated.[1] These translocations occur at variable incidences in MALT lymphomas of different sites, and are rare or absent in the ocular adnexa, salivary glands and thyroid.[1]

To characterize the genetic makeup of MALT lymphoma lacking the above chromosomal translocations, we investigated the genomic profiles of translocation negative MALT lymphomas of the ocular adnexa and lung by array- comparative genomic hybridization (array-CGH) and identified *A20* as the target of 6q23.3 deletion and *TNF* locus as a potential target of 6p21 gain exclusively in ocular adnexal cases.[2] Subsequent fluorescence *in situ* hybridization screening showed that *A20* deletion occurred preferentially in MALT lymphomas of the ocular adnexa, salivary glands and thyroid. In ocular adnexal cases, in which clinical information was available, *A20* deletion was significantly associated with adverse clinical parameters, and this association was independent of the presence of other genetic abnormalities.[2] Interestingly, among the 12 cases showing *A20* deletion, 3 displayed a homozygous deletion, indicating complete inactivation of the gene. While preparing our manuscript, four independent studies reported biallelic inactivation of *A20* by mutation and/or deletion in 67/381 (17.5%) B-cell lymphomas, including MALT lymphoma, diffuse large B-cell lymphoma and Hodgkin lymphoma.[3–6] *A20*, also known as TNF α-induced protein 3 (*TNFAIP3*), is a well-known negative regulator of the NF-κB activation pathway and can attenuate the NF-κB activity triggered by signaling from TNF and Toll-like receptors.[7] In view of these findings, *A20* could potentially act as a tumor suppressor gene. Nonetheless, it remains to be investigated whether *A20*, like other tumor suppressor genes, is also targeted for inactivation by promoter methylation and whether *A20* abnormalities impact on clinical presentation and treatment response. In this study, we investigated *A20* genetic and epigenetic abnormalities, and also examined the clinical impact of *A20* inactivation in MALT lymphoma.

A total of 17 MALT lymphomas with available adequate DNA samples or tissue materials were screened for mutation in the *A20* coding sequence by PCR and sequencing using DNA samples extracted from microdissected tumor cells of formalin-fixed paraffin-embedded tissue biopsies (Supplementary Materials and methods; Supplementary Table S1). They included seven cases showing *A20* hemizygous deletion with or without *TNF* locus gain, four cases with *TNF* locus gain only and a further six cases without these abnormalities.[2] A total of 4 mutations were detected in 3 (17.6%) of the 17 cases examined. All the mutations were confirmed by sequencing at least two independent PCR products from both orientations and excluded from the known polymorphisms. Cases 9 and 12 showed one nonsense mutation each, whereas case 16 showed one nonsense mutation and a 2 bp deletion (frozen tissue was not available, thus not possible to further investigate whether these mutations occurred in one or both alleles) (Table 1). Case 9 showed both *A20* mutation and hemizygous deletion, whereas cases 12 and 16 showed *A20* gene mutation but not deletion.

A20 promoter methylation was investigated in a total of 27 MALT lymphomas of the ocular adnexa (25), salivary glands (1) and thyroid (1) (Supplementary Materials and methods). Genomic DNA was treated by bisulfite to convert unmethylated cytosine to uracil, while keeping methylated cytosines intact, and then analyzed by pyrosequencing to assess methylation of 18 consecutive CpG positions in the *A20* promoter region. High efficiency of bisulfite treatment was demonstrated by internal conversion controls (Figure 1). The reliability of the assay was further ascertained by reproducible results from independent bisulfite treatments and pyrosequencing experiments. *A20* promoter methylation was seen in 7 (26%) of the 27 MALT lymphomas investigated, and they included 5 ocular adnexal cases and both extra-ocular cases (Table 1; Figure 1). Of these seven cases, six showed a similar methylation pattern, with prominent methylation at the 7th, 8th and 9th CpG sites. Remarkably, *A20* promoter methylation was significantly associated with *A20* hemizygous deletion ($P=0.011$, Fisher's exact test), being found in 5/8 (62%) cases with hemizygous deletion, but only in 2/19 (11%) cases with an intact *A20* (Table 1). None of the four cases with *A20* promoter methylation, which were also investigated for mutation, showed *A20* gene mutation. The single case (no. 9) that harbored both *A20* gene deletion and nonsense mutation showed no evidence of *A20* promoter methylation. Thus, it appeared that *A20* promoter methylation and gene mutation are mutually exclusive. To our knowledge, this is the first comprehensive analysis

Table 1 *A20* abnormalities and clinicopathological correlation in MALT lymphoma

Case[a]	Anatomical site	Stage at diagnosis[b]	Sex	Age	Etiology[c]		A20[d]			TNF[d] locus copy number	Treatment (response)	Follow-up period (m)	Lymphoma relapse
					Infectious	Autoimmune	Gene copy number	Mutation	Methylation				
1	Ocular	—	M	74	None	None	1	No	Yes	2	RT (CR)	10	Axillary LN (10 m)
2	Ocular	1	F	60	None	None	1	No	Yes	2	RT (CR)	20	Preauricular and submandibular LN (8 m), soft tissue at neural foramen (16 m)
3	Ocular, LN	3	M	84	None	None	1	No	Yes	2	Leukeran (PR)	28	Alive with disease
4	Ocular	—	F	87	None	Ulcerative colitis	0	—	—	3	—	—	—
5	Ocular	1	M	51	None	None	2	—	Yes	3–4	Excision + RT (CR)	73	None
6	Ocular	—	M	69	None	None	2	—	Yes	2	—	—	—
7	Ocular	1	F	84	CPS	None	1	No	No	2	RT (CR)	12	None
8	Ocular, LN	3	M	66	None	None	1	No	No	4	RT (CR)	96	Submandibullar LN (72 m)
9	Ocular	—	—	—	CPN	—	1	C1777T, Gln593Stop	No	3	—	—	—
10	Ocular	1	M	62	None	None	2		No	3–4	NA (CR)	242	Opposite orbit (31 m)
11	Ocular	1	M	68	None	None	2	No	No	4–5	RT (CR)	30	None
12	Ocular	1	M	64	None	None	2	C811T, Arg171Stop	No	4	RT (CR)	22	None
13	Ocular	1	F	60	HSV1/2	None	2	No	No	3	RT (CR)	120	Submandibular LN (108 m)
14	Ocular	—	F	71	ADV8	—	2	No	No	4	—	—	—
15	Ocular	4	M	49	None	None	2	—	No	2	RT (PR)	52	Lip (10 m), eyelid and LG (28 m)
16	Ocular	1	M	55	Hepatitis C	None	2	G460T, Glu162Stop, ΔCT1877-8	No	2	RT (CR)	11	None
17	Ocular	NA	F	44	None	None	2	—	No	2	RT (CR)	6	None
18	Ocular	1	F	76	None	None	2	No	No	2	RT (CR)	12	None
19	Ocular	1	F	79	None	None	2	—	No	2	RT (CR)	84	Opposite orbit (72 m)
20	Ocular	1	F	65	None	Sarcoidosis	2	No	No	2	RT (CR)	96	None
21	Ocular	1	F	89	CPN	None	2	—	No	2	RT (CR)	72	None
22	Ocular	—	M	66	None	None	2	No	No	2	—	—	—
23	Ocular	—	M	80	None	None	2	No	No	2	—	—	—
24	Ocular	1	F	58	None	None	2	—	No	2	—	—	—
25	Ocular	1	F	79	None	—	2	—	No	2	—	—	—
26	Ocular	—	F	37	None	—	2	No	No	2	—	—	—
27	Salivary glands	—	F	44	—	Sjögren's syndrome, arthritis	0	—	—	2	—	36	Cervical LN (18 m), systemic follicular lymphoma[e] (24 m), bone marrow (36 m)
28	Salivary glands	—	M	6	—	—	1	No	Yes	4	—	—	—
29	Thyroid	—	F	62	—	—	1	—	Yes	2	—	—	—
30	Liver	—	M	66	—	—	0	—	—	3–4	—	—	—

ADV8, adenovirus type 8; CPN, *Chlamydia pneumoniae*; CPS, *Chlamydia psittaci*; CR, complete response; HSV1/2, herpes simplex virus type 1 and 2; LN, lymph node; m, months; MALT, mucosa-associated lymphoid tissue; NS, not specified; PR, partial response; RT, radiotherapy; —, unavailable.

[a]All cases were negative for translocations involving *MALT1*, *BCL10* and *FOXP1*, except case 22 that harbored t(1;14)(p22;q32)/*BCL10-IGH*.[2]

[b]Clinical staging was carried out by careful clinical examination and computerized tomography (CT) or magnetic resonance imaging (MRI) scan.

[c]Infectious status were available from previous studies and clinical history as detailed in Supplementary Information.

[d]The results on *A20* and *TNF* genes copy number changes were obtained from a previous study.[2]

[e]Follicular lymphoma was clonally linked to MALT lymphoma and both lymphomas harbored t(14;18)(q32;q21)/*IGH-BCL2*.

of *A20* promoter methylation by pyrosequencing in lymphoma. While revising our manuscript, Honma *et al*. reported *A20* methylation in 10 of 24 cases of activated B-cell like diffuse large B-cell lymphoma and 3 of 8 cases of mantle cell lymphoma by methylation-specific PCR analysis of a single CpG site upstream of the κB-biding sites.[8]

To investigate further the impact of *A20* deletion and promoter methylation on its transcript expression, we measured the *A20* mRNA expression in cases with adequate tissue materials by real-time quantitative RT–PCR. RNA was extracted from microdissected tumor cells of formalin-fixed paraffin-embedded tissues in eight cases (Supplementary Materials and methods; Supplementary Table S2). They included three cases without any *A20* abnormalities, one case with hemizygous deletion, three cases with *A20* hemizygous deletion and promoter methylation and one case with homozygous *A20* deletion. Real-time quantitative RT–PCR was performed with two sets of *A20* primers, along with GAPDH and 18S rRNA as reference control. Results from both sets of *A20* primers were similar and showed a trend of correlation between the extent of *A20* abnormalities and the level of *A20* mRNA expression. The lowest expression was seen in the cases with complete *A20* inactivation either by homozygous deletion or hemizygous deletion plus promoter methylation, whereas the highest expression was found in the cases with intact *A20* (Supplementary Figure S1). Although the number of comparable cases allowing direct analysis of the impact of *A20* promoter methylation on its transcript expression was small, the cases with both hemizygous deletion and promoter methylation did show a lower *A20* transcript expression than the case with only *A20* hemizygous deletion. These preliminary results are in line with the expected role of promoter methylation in transcriptional silencing of the remaining *A20* allele.

Thus, concurrent *A20* hemizygous deletion and promoter methylation or mutation, as well as homozygous *A20* deletion, could result in complete *A20* gene inactivation, whereas *A20* hemizygous deletion or promoter methylation most likely lead to partial *A20* inactivation. In keeping with this notion, there was a significant correlation between the extent of *A20* abnormalities and clinicopathological presentations in ocular adnexal MALT lymphoma. Clinicopathological data were available in 17 cases (follow-up: 6–242 months, median 30 months, Table 1). Most of these patients were treated by radiotherapy. The case (no.16) that harbored two mutations was excluded from clinical correlation analyses, as it was not possible to determine whether the mutations affect one or both alleles, thus define whether *A20* was completely or partially inactivated. Both *A20* complete and partial inactivation were associated with concurrent involvement of different adnexal tissues or distant spread at diagnosis ($P=0.016$, $P=0.047$, respectively, Fisher's exact test). Importantly, *A20* complete inactivation was significantly associated with a shorter lymphoma-free survival ($P<0.001$, Figure 2), whereas cases with partial inactivation shared a similar

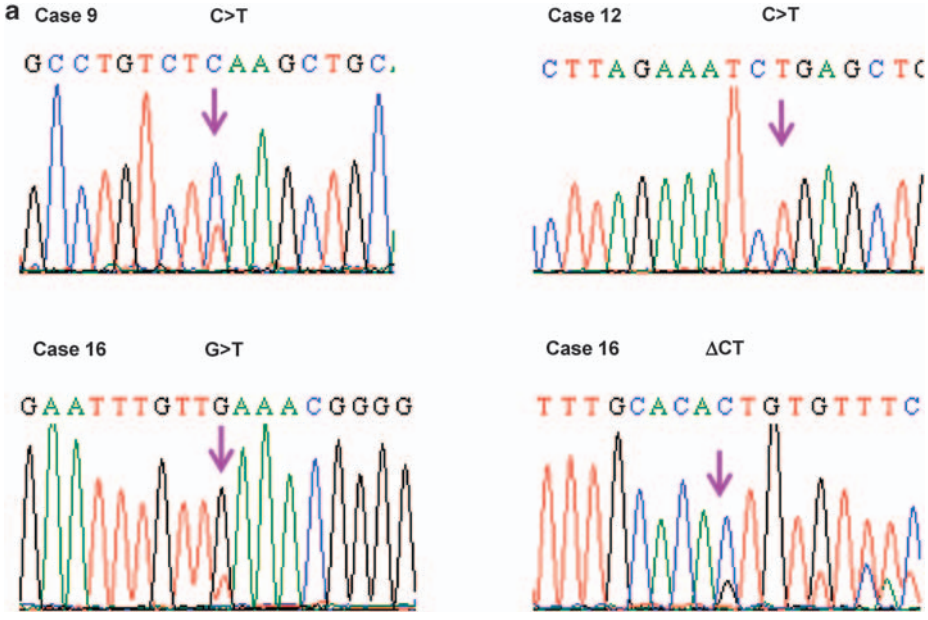

Figure 1 Screening of *A20* gene mutation and promoter methylation by DNA sequencing and pyrosequencing respectively. (**a**) Examples of *A20* gene mutations. All mutations including nucleotide substitutions in the three cases generate or lead to a stop codon. (**b**) Upper panel: *A20* promoter sequence. The κB-binding sites are shown in green boxes and the sequence of the first exon appears in the red text. A 222 bp fragment was examined on a PyroMark MD platform (Biotage). The positions of PCR and pyrosequencing primers for the bisulfite converted sequence are, respectively, indicated by a solid underline (forward primer $^{5'}$GGGGTAAAGTAGATTG$^{3'}$, reverse biotinylated primer $^{5'}$CCCAAATCCTAATCAAAAC$^{3'}$) and a dotted underline ($^{5'}$GTAGTTTGTAGTTTT$^{3'}$ and $^{5'}$GTTAAGAGAGATTATATTTTTAGT$^{3'}$). The CpG sites successfully investigated are shown in bold. Lower panel: examples of pyrosequencing. The top panel shows methylation at the CpG sites 6, 7, 8 and 9 in an ocular adnexal MALT lymphoma (case 3) with a hemizygous *A20* deletion. The bottom panel displays no methylation at these CpG sites in an ocular adnexal MALT lymphoma without *A20* deletion (case 10). All results were confirmed in two independent bisulfite treatments and pyrosequencing experiments. The *y* axis represents the signal intensity in arbitrary units, whereas *x* axis shows the dispensation order. The CpG sites are highlighted in gray. The expected intensities are shown as gray histograms. The percentage of methylation at individual CpG positions is shown at the top of the pyrogram. The cut-off values used to define methylation is 20%, based on the mean + 3 s.d. of the percentages of CpG methylation from normal lymphoid tissue and MALT lymphoma cases clearly lacking evidence of methylation. The efficiency of the bisulfite conversion was assessed for each sample by dispensing a cytosine (C) after a thymine (T) converted from a non-methylated C and no signal is seen for the C residue (highlighted in yellow), indicating complete conversion.

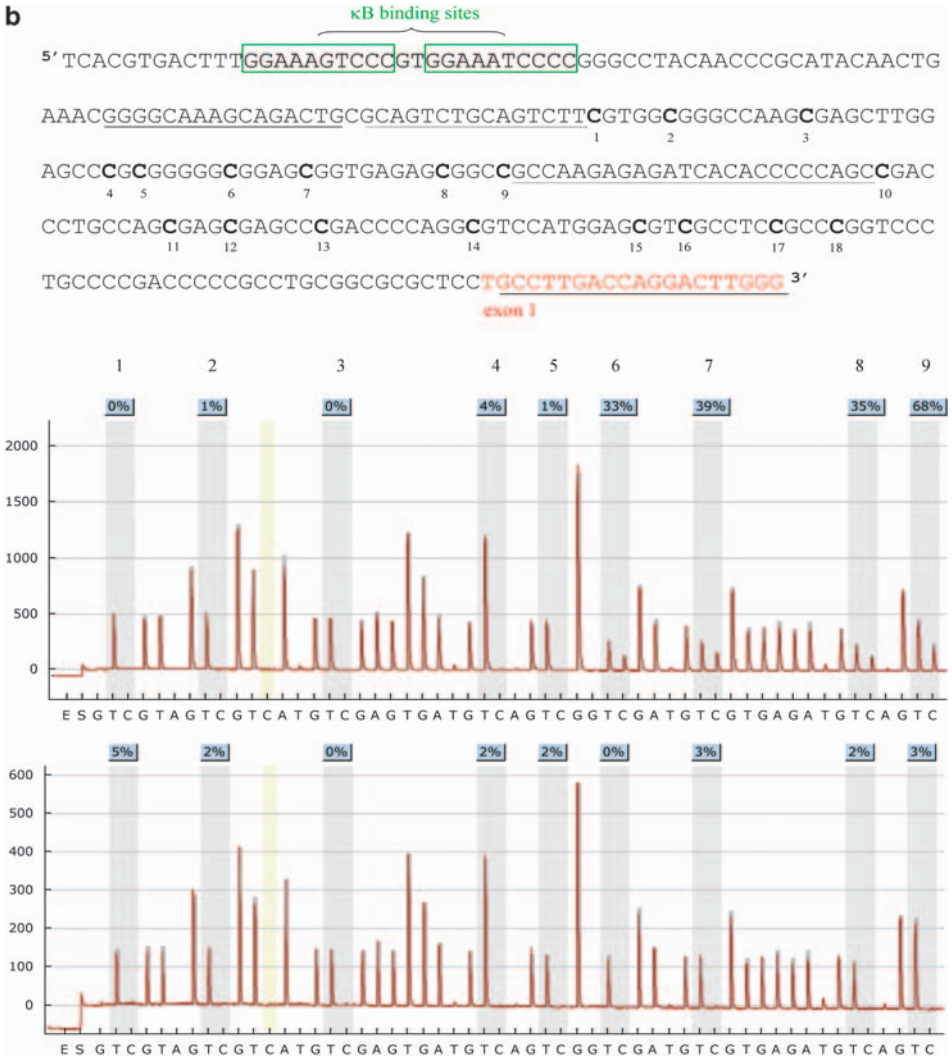

Figure 1 Continued.

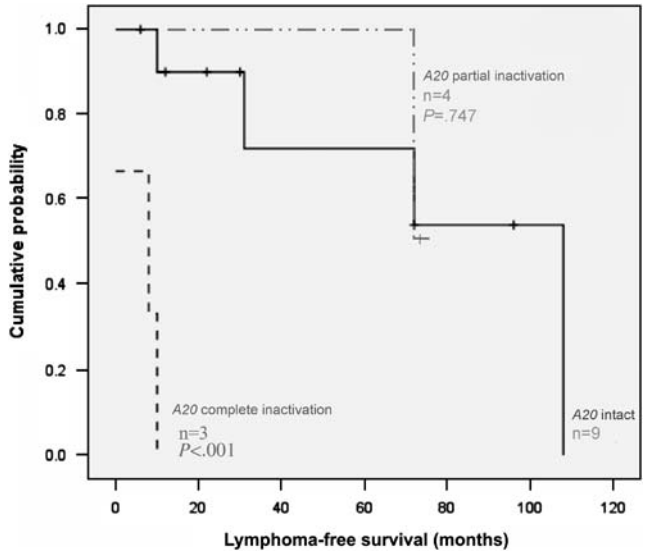

Figure 2 Clinical impact of A20 inactivation on lymphoma-free survival in ocular adnexal MALT lymphoma. Kaplan–Meier estimates of lymphoma-free survival according to *A20* gene status, using log-rank test for comparison (event 'relapse' right-censored, Statistical Package for Social Sciences SPSS UK version 13). Complete inactivation is defined by the presence of both *A20* hemizygous deletion and promoter methylation (three cases), whereas partial inactivation corresponds to the presence of *A20* hemizygous deletion (two cases) or promoter methylation (one case) or mutation (one case).

profile to those without *A20* abnormalities. Although these findings await confirmation by study of large cohort of cases, the observation highlights for the first time the importance of complete inactivation of the *A20* gene in lymphoma development.

Our findings showed, for the first time, that promoter methylation was an alternative mechanism for *A20* inactivation in MALT lymphoma, in addition to gene deletion and mutation reported very recently.[3–6,8] The biallelic inactivation by promoter methylation and deletion, hemizygous deletion and mutation, homozygous deletion is in line with the Knudson's two-hit hypothesis on the inactivation of tumor suppressor genes. As expected, re-expression of wild-type A20 in cell lines with biallelic inactivation of the *A20* gene induced apoptosis and cell growth arrest and these effects depended on its negative regulation of NF-κB pathway.[5,6,8] Together, these findings indicate that A20 is a new tumor suppressor in lymphoma. In view of the critical role of A20 as a central negative regulator of NF-κB activation pathway and the diverse functions of NF-kB in B- and T-cell development and biology, the extent of A20 involvement in various lymphoma subtypes remains to be investigated.

Conflict of interest

The authors declare no conflict of interest.

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