bjh research paper

t(11;18)(q21;q21) of mucosa-associated lymphoid tissue lymphoma results from illegitimate non-homologous end joining following double strand breaks

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Received 11 December 2003; accepted for publication 9 February 2004
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Summary

t(11;18)(q21;q21) is the most frequent chromosomal aberration specifically associated with mucosa-associated lymphoid tissue (MALT) lymphoma. The translocation fuses the *API2* gene to the *MALT1* gene and generates a functional API2-MALT1 transcript. The breakpoint of the fusion gene is well characterized at the transcript level but poorly understood at the genomic level and the mechanism underlying the translocation is unknown. We identified the genomic breakpoint in 19 t(11;18)-positive MALT lymphoma cases by polymerase chain reaction and sequencing and analysed the junctional sequences. The breakpoints were scattered in intron 7 and exon 8 of the *API2* gene, and introns 4, 6, 7 and 8 of the *MALT1* gene. Comparative sequence analysis between the *API2-MALT1* fusion on der(11) and the *MALT1-API2* fusion on der(18) showed extensive alterations including deletions, duplications and non-template-based insertions at the fusion junctions in all cases examined. An extensive sequence search failed to reveal any known sequence motifs that might be associated with chromosomal recombination or any novel consensus sequences at or near the breakpoints on both der(11) and der(18) except in one case, in which *Alu* repeats spanned the breakpoint of the *MALT1-API2* fusion. Our results suggest that t(11;18) may result from illegitimate non-homologous end joining following double strand breaks.

Keywords: mucosa-associated lymphoid tissue lymphoma, t(11:18) (q21;q21), double strand break, non-homologous end joining.

Mucosa-associated lymphoid tissue (MALT) lymphoma is a distinct subtype of low-grade marginal zone B-cell lymphoma arising from extranodal sites such as the stomach, lung, salivary gland, thyroid, conjunctiva skin, etc. (Isaacson *et al*, 2001; Du & Isaacson, 2002). Interestingly, these organs are normally devoid of any native lymphoid tissue and the lymphoma at these sites arises from the MALT acquired as a consequence of a chronic inflammatory or auto-immune disorder. Notably, gastric MALT lymphoma is invariably preceded by *Helicobacter pylori*-associated chronic gastritis, while salivary gland and thyroid MALT lymphomas are commonly associated with lymphoepithelial sialadenitis and Hashimoto's thyroiditis respectively. Antigenic and immunological stimulation thus plays a critical role in the genesis and expansion of the lymphoma clone. Genetically, MALT lymphoma is characterized by three specific chromosomal translocations, namely t(11;18)(q21;q21)/API2-MALT1 (Auer *et al*, 1997; Ott *et al*, 1997), t(1;14)(p22;q32)/IgH-BCL10 (Wotherspoon *et al*, 1992) and t(14;18)(q32;q21)/IgH-MALT1 (Sanchez-Izquierdo *et al*, 2003; Streubel *et al*, 2003). Among these, t(11;18) is the most frequent but occurs at dramatically variable frequencies in MALT lymphoma of different sites, from 0–1% in the thyroid and salivary gland to 30–40% in the stomach and lung (Ye *et al*, 2003). These findings suggest that the occurrence of the translocation is heavily influenced by the nature of premalignant diseases associated with MALT lymphoma. In gastric MALT lymphoma, t(11;18) has been shown to be significantly associated with infection by CagA-positive strains of *H. pylori*, further implicating a role of aetiological factors in the occurrence of the translocation (Ye *et al*, 2003).

doi:10.1111/j.1365-2141.2004.04909.x

t(11;18) causes reciprocal fusions of *API2-MALT1* on der(11) and *MALT1-API2* on der(18) (Akagi *et al*, 1999; Dierlamm *et al*, 1999; Morgan *et al*, 1999). As only the *API2-MALT1* fusion transcript is consistently expressed in MALT lymphoma with t(11;18), this fusion product is assumed to be oncogenic (Dierlamm *et al*, 1999; Uren *et al*, 2000; Lucas *et al*, 2001). The fusion junctions at the transcript level have been well characterized. On the *API2* gene, they are always downstream of the third baculovirus inhibitors of apoptosis repeats (BIR) domain but upstream of the C-terminal RING domain, whereas on the *MALT1* gene, they are consistently upstream of the C-terminal caspase-like domain (Fig 1). Thus, the resulting *API2-MALT1* fusion transcript always comprises the N-terminal *API2* with three intact BIR domains and the C-terminal *MALT1* region containing an intact caspase-like domain (Ott *et al*, 1997; Akagi *et al*, 1999; Dierlamm *et al*, 1999, 2000; Baens *et al*, 2000a; Kalla *et al*, 2000; Liu *et al*, 2000, 2001, 2002; Motegi *et al*, 2000; Nakamura *et al*, 2000; Remstein *et al*, 2000; Ye *et al*, 2003). The specific selection of these domains of the *API2* and *MALT1* genes to form a functional fusion product strongly suggests their importance and synergy in oncogenesis.

The mechanisms underlying the occurrence of t(11;18) are unknown. Most lymphoma-associated chromosomal translocations involve the antigen receptor locus and are believed to be the result of illegitimate recombination involving the VDJ recombination machinery (Willis & Dyer, 2000; Kuppers & Dalla-Favera, 2001; Marculescu *et al*, 2002). t(11;18) is one of the few lymphoma-associated chromosomal translocations that do not involve the antigen receptor locus (Morris *et al*, 1994) and thus it is particularly interesting to investigate the mechanisms underlying the translocation. So far two studies have examined this by sequence analysis of the genomic breakpoints of t(11;18). One study examined five cases and did not reveal any sequence motifs including the VDJ recombination heptamer/nonamer at the breakpoints, which are known to be involved in chromosomal translocations, and concluded that the API2-MLT fusion might result from a non-homologous end joining event after multiple double-strand breaks (Baens *et al*, 2000b). In contrast, the other study investigated a single case and identified a VDJ heptamer sequence at the *API2* breakpoint on der(11) and proposed a role of VDJ recombination in t(11;18) (Sato *et al*, 2001). To further understand the mechanism underlying the occurrence of t(11;18), we characterized the junction sequence of both *API2-MALT1* and *MALT1-API2* fusions in 19 t(11;18)-positive MALT lymphomas.

Patients and methods

Patients

Nineteen cases of t(11;18)-positive MALT lymphoma with fresh-frozen tumour tissues were retrieved from Department of Histopathology, University College London, London and the Groupe d'Etude des Lymphomes Digestifs, France. They

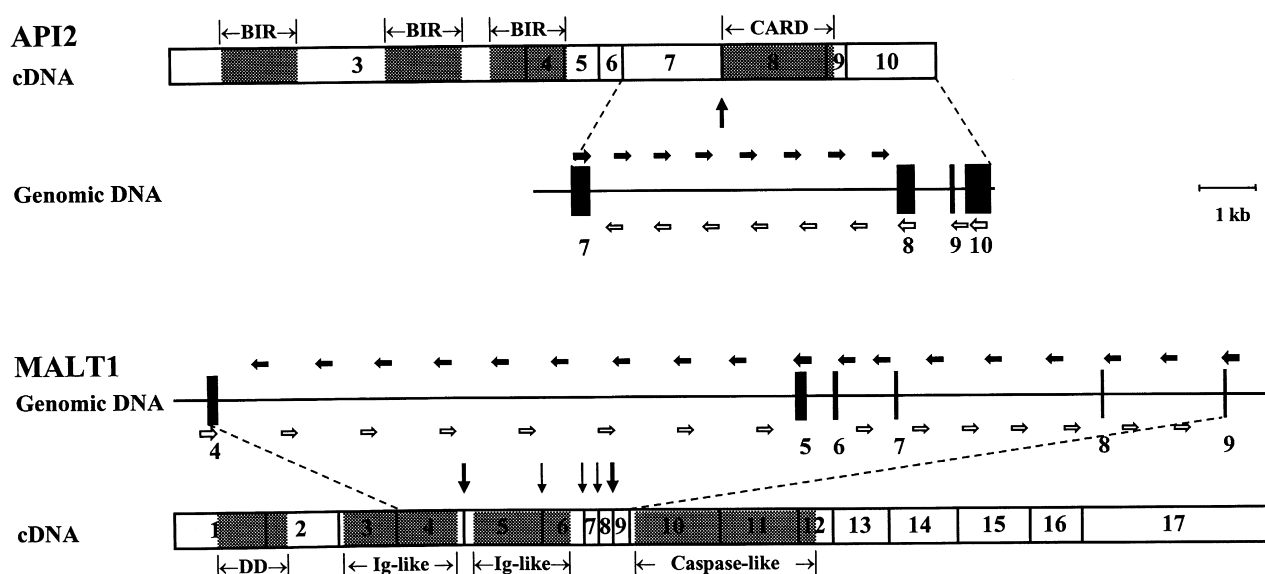

Fig 1. Schematic illustration of the structure of the *API2* and *MALT1* transcripts and genomic regions spanning the breakpoints observed. The *API2* and *MALT1* transcripts were depicted according to GenBank accession numbers NM001165 and AF130356, respectively, with exons numbered, domains shaded and fusion junctions indicated by vertical arrows. The regions of the *API2* (AP001167) and *MALT1* (AC104365) genes spanning the breakpoints are drawn to scale to show the position of the primers used for amplification of the genomic fusion on der(11) (filled horizontal arrows) and der(18) (unfilled horizontal arrows). The primers positioned to exons are used for primary long distance PCR and those positioned to introns are used for nested PCR to narrow down the fragment containing the fusion junction. BIR, baculovirus inhibitors of apoptosis (IAP) repeat; CARD, caspase recruitment domain; DD, death domain.

included 16 from stomach, four from lung and two from small intestine (Table I). t(11;18) and the *API2-MALT1* fusion junction at the transcript level in these cases were identified previously by reverse transcription polymerase chain reaction (RT-PCR) and sequencing (Table I) (Liu *et al*, 2001, 2002).

Amplification and sequencing of t(11;18) genomic fusions

High molecular DNA was extracted from frozen tissues using the Wizard® genomic DNA purification kit (Promega, Southampton, UK). To amplify the fusion genes on der(11), a long-distance PCR was performed for each case using TaKaRa LA Taq™ (TaKaRa Biomedicals, Otsu, Japan) followed by multiple nested PCRs (Fig 1; Table S1). In our previous study, RT-PCR demonstrated that the fusion junctions of the *API2-MALT1* transcript were at the 3′-end of exon 7 on the *API2* gene in all 19 cases but varied at the 5′-end of exons 5 (11 cases), 8 (six cases) and 9 (two cases) on the *MALT1* gene (Fig 1; Table I) (Liu *et al*, 2001, 2002). The genomic breakpoint was assumed to be in the respective introns: i.e. intron 7 of the *API2* gene and introns 4, 7 and 8 of the *MALT1* gene (Fig 1). The primary PCR was therefore performed using a sense primer to the exon 7 of the *API2* gene and an antisense primer to the exon 5 of the *MALT1* gene in cases with putative breakpoints in intron 4 or an antisense primer to the exon 9 in those with putative breakpoints in introns 7 and 8. The PCR was carried out in a 50 μl reaction mixture containing 250 ng template DNA and 2·5 U TaKaRa LA Taq™ with a two-step cycling protocol comprising an initial denaturation at 94°C for 1 min, 30 cycles of 94°C for 20 s and 65°C for 15 min, and a final extension at 72°C for 10 min. According to genomic sequences of the *API2* (GenBank accession no. AP001167) and *MALT1* (AC104365) genes, the maximum expected size of the genomic fusion flanked by these primers was less than 13·5 kb, a size well within the maximal amplicon (up to 40 kb) allowed by the TaKaRa LA Taq™. The amplified products, which varied in sizes depending on the position of the breakpoint, were then used as templates for multiple nested PCRs with a series of sense primers to the intron 7 of the *API2* gene and antisense primers to the corresponding introns of the *MALT1* gene in order to narrow down the region containing the breakpoint (Table S1). The intronic primers were designed with an average interval of 1 kb. Following various combinations of *API2* and *MALT* primers, the amplified fusion gene fragments of <1 kb were subjected to direct sequencing on both orientations using dRhodamine dye terminators on an ABI Prism 377 sequencer (PE Applied Biosystems, Foster City, CA, USA).

The same strategy was applied to amplification and sequencing of the reciprocal fusion on the der(18) using different sets of primers designed from the same regions (Fig 1; Table S1).

Sequence analysis of the genomic fusion junctions of t(11;18)

A comprehensive analysis of nucleotide sequence at and around the fusion junctions was performed using following

Table I. Characteristics of the fusion junctions of t(11;18) at transcript and genomic level.

		Fusion junction on transcript		Fusion junction on der(11)				Fusion junction on der(18)			Deletion (−) or duplication (+)	
Case no.	Tumour site	API2	MALT1	PCR size (bp)	API2	MALT1	Insertion	API2	MALT1	Insertion	API2	MALT1
1	Lung	3′-Ex 7	5′-Ex 5	9782	In 7	In 4		In 7	In 4		−86	−130
2	Stomach	3′-Ex 7	5′-Ex 5	9536	In 7	In 4		In 7	In 4		−1223	+135
3	Stomach	3′-Ex 7	5′-Ex 5	10 540	In 7	In 4		In 7	In 4		−64	−20
4	Stomach	3′-Ex 7	5′-Ex 5	11 466	Ex 8	In 4						
5	Stomach	3′-Ex 7	5′-Ex 5	6959	In 7	In 4		In 7	In 4		−1186	−95
6	Stomach	3′-Ex 7	5′-Ex 5	7503	In 7	In 4		In 7	In 4		−645	−92
7	Lung	3′-Ex 7	5′-Ex 5	8164	In 7	In 4						
8	Lung	3′-Ex 7	5′-Ex 5	3425	In 7	In 4	'aa'	In 7	In 4		−25	−90
9	Stomach	3′-Ex 7	5′-Ex 5	2723	In 7	In 4		In 7	In 4	't'	−611	−207
10	Stomach	3′-Ex 7	5′-Ex 5	5684	Ex 8	In 4		Ex 8	In 4		+108	+105
11	Stomach	3′-Ex 7	5′-Ex 5	2890	In7	In 4	'agaggaata'	In 7	In 4	'aa'	−15	−17
12	Stomach	3′-Ex 7	5′-Ex 8	10 274	Ex 8	In 6						
13	Stomach	3′-Ex 7	5′-Ex 8	7156	In 7	In 6		In 7	In 8		−2166	+3320
14	Lung	3′-Ex 7	5′-Ex 8	8496	In 7	In 7						
15	Stomach	3′-Ex 7	5′-Ex 8	4382	In 7	In 7						
16	Stomach	3′-Ex 7	5′-Ex 8	4031	In 7	In 7	't'					
17	Stomach	3′-Ex 7	5′-Ex 8	4031	In 7	In 7	't'	In 7	In 4		−2012	−10 585
18	Small intestine	3′-Ex 7	5′-Ex 9	5655	In 7	In 8		In 7	In 8		−140	−160
19	Stomach	3′-Ex 7	5′-Ex 9	1519	In 7	In 8		In 7	In 8		−149	+241

MALT, mucosa-associated lymphoid tissue; PCR, polymerase chain reaction; Ex, exon; In, intron.

computer programs. Basic Local Alignment Search Tool (BLAST) 2 sequence was used to identify the intron-exon boundaries of the *API2* and *MALT1* genes and to search for sequence identities between the two genomic regions (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Dialign and Bioedit were used to align sequence fragments spanning breakpoints to search for consensus sequence at or near the breakpoints (http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/dialign_submit; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). RepeatMasker was used to search for repetitive elements in the *API2* and *MALT1* genes (http://repeatmasker.org/). Fuzznuc was used to search for known sequence patterns or motifs at or near the breakpoints (http://www.hgmp.mrc.ac.uk/Software/EMBOSS/Apps/). Multiple EM for Motif Elicitation (MEME) was used to search for novel conserved regions at or near the breakpoint (http://meme.sdsc.edu/meme/website/intro.html) and Motif Alignment and Search Tool (MAST) was employed to search databases for the identified motifs (http://meme.sdsc.edu/meme/website/intro.html).

In order to assess whether the distribution of the breakpoint observed was deterministic or stochastic, comparison was made between the observed breakpoints and 'those' randomly generated by computing. One hundred sets of 19 random 'breakpoints' were generated from the intron 7 and exon 8 of the *API2* gene and from introns 4 to 8 of the *MALT1* gene using the Research Radomizer software (http://www.randomizer.org/form.htm) and the data obtained were normalized by ensuring that the ranking order between the observed and experimental points on both the genes was the same. The correlation coefficient was then calculated using the Minitab (http://www.minitab.com) as a measure to assess the 'goodness-of-fit' between the observed breakpoints and those generated by the computer program.

Results

Genomic breakpoints on both the API2 and MALT1 gene were scattered

The genomic *API2-MALT1* fusions of up to 11·5 kb on der(11) were amplified by long-distance PCR in all 19 cases analysed (Table I). Subsequent multiple nested PCRs followed by sequencing allowed identification of the genomic breakpoints in all cases and their distribution is shown in Fig 2A. In the majority of cases, the genomic breakpoints on both the *API2* and *MALT1* genes were in the corresponding introns as predicted by the fusion junctions at the transcript level (Table I; Fig 1) (Liu *et al*, 2001, 2002). However, in three cases (cases 4, 10 and 12), the genomic breakpoints on the *API2* gene were in exon 8, although the fusion points at the transcript level were at the 3′-end of exon 7. Similarly, the genomic breakpoints on *MALT1* were in intron 6 in two cases (cases 12 and 13) whereas their fusion points at the transcript level were at the 5′-end of exon 8. These findings strongly indicate that the residual *API2* exon 8 and *MALT1* exon 7 in the *API2-MALT1* fusion were spliced out during RNA processing in order to generate a functional transcript.

Apart from cases 16 and 17, which shared the same breakpoints on both the *API2* and *MALT1* genes, and cases 5 and 6, which shared the same breakpoint on the *MALT1* gene only, all the remaining cases ($n = 15$) had different breakpoints that appeared to be distributed randomly on both the genes. The breakpoints did not show any tendency of clustering and there was no significant correlation in distribution of the breakpoints between the two genes ($P > 0{\cdot}05$).

Using the same PCR strategy, the *MALT1-API2* fusion on der(18) was amplified in 13 of 19 cases and their breakpoints were characterized. Comparison of the breakpoints identified from the *MALT1-API2* fusion on der(18) with those observed from the *API2-MALT1* fusion on der(11) revealed deletions, insertions and/or duplications of both the *API2* and *MALT1* gene sequences in all 13 cases (detailed below). Thus, the breakpoints recorded from the *MALT1-API2* fusion on der(18) were different from those obtained on the *API2-MALT1* fusion on der(11) (Fig 2B). Based on the breakpoints from the *MALT1-API2* fusion, three pairs of cases (cases 2 and 3, 5 and 6, 13 and 18) had identical breakpoints on both the *MALT1* and *API2* genes, although the breakpoints for each pair were different. The cases with the same breakpoints had been independently analysed and the possibility of PCR contamination was excluded. The remaining seven cases showed different breakpoints scattered on both genes. Statistical analysis failed to show any significant correlation in distribution of the breakpoints between the two genes ($P > 0{\cdot}05$).

The breakpoints observed above from both the *API2-MALT1* and *MALT1-API2* fusions were also different from those reported previously (Baens *et al*, 2000b; Sato *et al*, 2001).

Comparison of the breakpoints identified from the *API2-MALT1* and *MALT1-API2* fusions with 100 sets of computer-generated random 'breakpoints' from the same breakage region of both the *API2* and *MALT1* genes showed a significant correlation between the sets of observed data and sets of computer-generated data for both genes ($r > 0{\cdot}95$ and $P<0{\cdot}001$ in each case), suggesting that the observed breakpoints may have occurred in a random fashion.

Extensive sequence alterations occurred at the fusion junction of t(11;18)

Comparative sequence analysis between the *API2-MALT1* fusion on der(11) and the *MALT1-API2* fusion on der(18) revealed extensive alterations at the fusion junctions including deletions, duplications and non-template-based insertions in all 13 cases examined (Table I; Fig 3). Deletions ranged from 15 to 10 585 bp and occurred in 12 cases, affecting both the *API2* and *MALT1* genes in nine cases and only the *API2* gene in the remaining three cases. In one case (case 17), deletion involved a coding exon of the *MALT1* gene, while in the remaining cases, deletions were restricted to the intronic

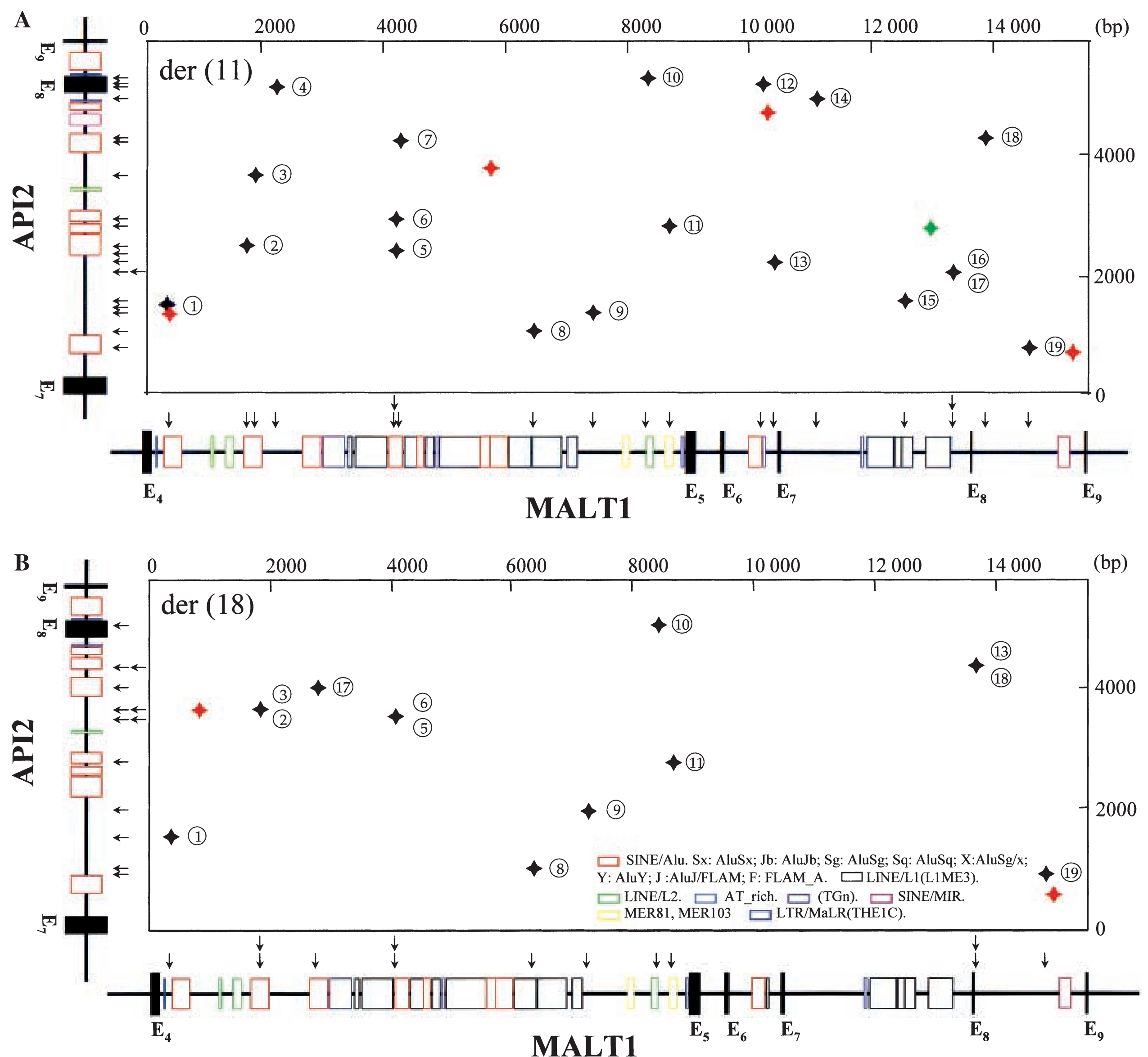

Fig 2. Distribution of the genomic breakpoints of t(11;18) observed on der(11) (A) and der(18) (B). The breakpoint in each case (circled number) is plotted as a co-ordinate against its position (indicated by arrows) on the *API2* (*y*-axis) and *MALT1* (*x*-axis) genes drawn to scale. Solid black boxes represent exons and their numbers are denoted. Open coloured boxes show repetitive elements and an insert illustrates the type of repeat. Breakpoints in red are those identified by Baens *et al* (2000b) and those in green were identified by Sato *et al* (2001).

region. Duplications ranging from 105 to 3320 bp were found in four cases, affecting both the *API2* and *MALT1* genes in one case and only the *MALT1* gene in the remaining three cases. Non-template insertions ranging from 1 to 9 bp were observed in five cases (Tables I and S2). In one case (case 11), insertion occurred at both the junctions of the *API2-MALT1* and *MALT1-API2* fusions, while in the remaining four cases, insertion took place at the junction of either of the two fusions. Interestingly, non-template insertion was mutually exclusive of duplication in all cases. It was also noted that 19 junctions including 11 on der(11) and eight on der(18) had mono (eight junctions, six of them sharing a 't' nucleotide), di (five junctions), tri (two junctions), or tetro (two junctions) nucleotides or 16 bp nucleotides (case 17, Figs 3 and 4) common to the sequences of both genes at the breakpoint. The precise fusion points in these cases, therefore, could not be identified (Table S2).

Absence of consensus sequence motifs at or near the breakpoint of t(11;18)

To gain insight into the molecular mechanism of the translocation, sequence motifs known to be associated with chromosomal rearrangement including VDJ recombination

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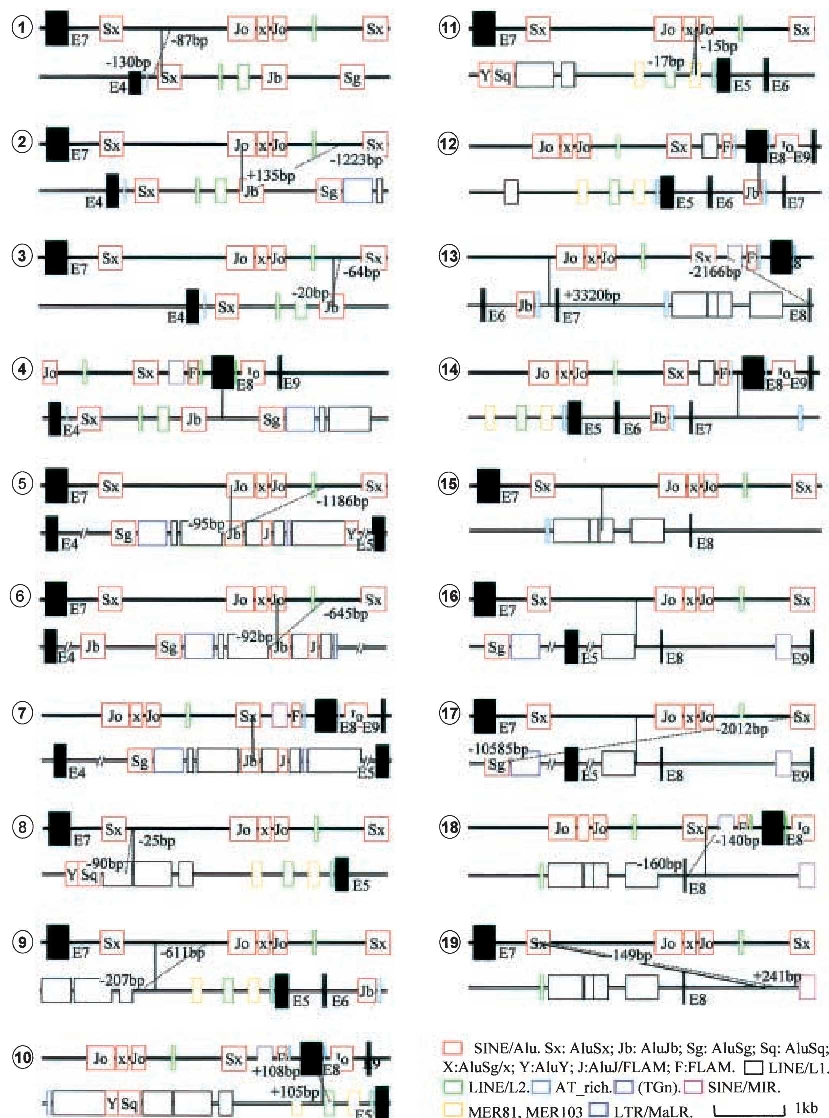

Fig 3. Schematic illustration of breakage and exchange of the *API2* and *MALT1* gene sequences in t(11;18). The regions of the *API2* (top) and *MALT1* (bottom) genes spanning the breakpoints are aligned for each case. The joining sites on der(11) are indicated by a solid vertical line, while those on der(18) are indicated by a dotted line. The size of DNA fragments deleted (−) or duplicated (+) is indicated. No fusion junctions on der(18) were identified in cases 4, 7, 12, 14, 15 and 16. Filled black boxes represent exons and their numbers are denoted. Open coloured boxes represent the position and size of the repetitive elements and an insert indicates the type of repeat. The subfamily of *Alu* repeats is also indicated.

heptamer (5′-CACWGTG) and nonamer (5′-ACAAAAACC) (Tycko & Sklar, 1990; Cayuela *et al*, 1997; Kuppers & Dalla-Favera, 2001; Marculescu *et al*, 2002), immunoglobulin (Ig) class switch pentamer (5′-GRGST) (Dalla Favera *et al*, 1983; Jeffs *et al*, 1998; Kuppers & Dalla-Favera, 2001), eukaryotic topoisomerase II cleavage site (5′-RNYNNCNNGYNGKTN YNY) (Domer *et al*, 1995; Felix *et al*, 1995; Obata *et al*, 1999), chi-like octomer (CCWSYVK) (Wyatt *et al*, 1992), translin binding sequence [5′-ATGCAG-N(0–4 bp)-GCCCWSSW or 5′-GCNCWSSW N(0–2 bp)GCCCWSSW] (Aoki *et al*, 1995; Hosaka *et al*, 2000), DNase I hypersensitive site (5′-CAC-TTAAGCTGTGTACTCCCAT) (Forrester *et al*, 1990), purine–pyrimidine sequences, polypurine and polypyrimidine and palindromes (Boehm *et al*, 1989) were searched in the 100 bp sequence each side of the fusion junction in all cases using the Fuzznuc program (Table S2). The analysis did not reveal the presence of any of the above sequence motifs at or near the breakpoint in each case, suggesting that these sequence motifs were not involved in t(11;18).

We next examined whether repetitive sequences including short interspersed repetitive elements (SINEs; *Alu* and MIR), long interspersed repetitive elements (LINEs; LINE 1, LINE 2 and L3/CR1), long terminal repeat (LTR) elements (MaLRs, ERVL, ERV_class I and ERV_class II) and DNA elements (MER1_type and MER2_type) were implicated in the occurrence of t(11;18). The frequencies of these repetitive sequences

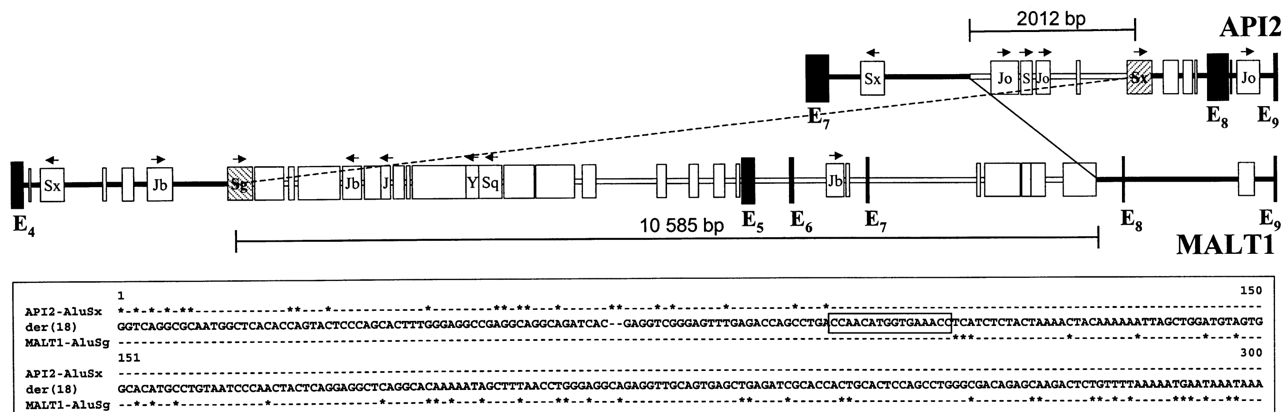

Fig 4. Illustration of an *Alu*-involved recombination of t(11;18) in case 17. Top: partial genomic structure of the *API2* and *MALT1* genes shows breakage and joining on der(11) (solid line) and der(18) (dotted line). The joining on der(18) occurs between the *Alu*Sx on the *API2* and *Alu*Sg on the *MALT1*, resulting in deletions of a 2012 bp fragment on the *API2* gene and a 10 585 bp fragment on the *MALT1* gene. Solid black boxes represent exons and open boxes represent repetitive elements with *Alu* repeats indicated. Horizontal arrows indicate the orientation of *Alu* repeats. Bottom: alignment of 300 bp *Alu* repeats from parental genes and the der(18) fusion gene; the resulting *Alu* fusion on der(18) maintains the characteristics of an *Alu* repeat.

between the regions involved in chromosomal breaks and the rest of the *API2* or *MALT1* gene were compared using RepeatMasker. Only the *Alu* and LINE1 repeats were found to be relatively rich in the breakpoint region of the *API2* and *MALT1* gene respectively. *Alu* repeats accounted for 29% of the intron 7 sequence but only 16% of the rest of the intronic sequence of the *API2* gene, while LINE1 repeats contributed to 25% of introns 4–8 but 13% of the rest of the intronic sequence of the *MALT1* gene.

The breakpoints on the *API2* and *MALT1* genes derived from both *API2-MALT1* and *MALT1-API2* fusions were analysed against the location of the above repetitive sequences (Figs 2 and 3). There was no correlation between the position of the breakpoints and the location of SINE/Mer, LINE, LTR and DNA elements in all cases. However, breakpoint was within an *Alu* repeat of both participating genes on der(11) in four cases (cases 2, 5, 6 and 7) and on der(18) in one case (case 17). In four of these five cases, the *Alu* repeats involved were either in opposite orientation (cases 2, 5 and 6) or from different subfamilies (case 7), suggesting that the chromosomal recombination in these cases was unlikely to be mediated by *Alu* repeats. In the remaining case (case 17), the two *Alu* repeats involved, *Alu*Sx from chromosome 11 and *Alu*Sg from chromosome 18, were in the same orientation and shared 77% of sequence homology (Fig 4). The recombination occurred within a 16 bp consensus fragment between the two parental *Alu* repeats, resulting in deletions of a 2012 bp fragment from chromosome 11 and a 10 585 bp fragment from chromosome 18.

To further examine whether t(11;18) might be mediated by an unknown novel sequence motif, we compared the 200 bp sequence fragment spanning the junction of both the *API2-MALT1* and *MALT1-API2* fusions in all cases (Table S1) using Dialign and Bioedit programs but failed to identify any putative consensus sequence. We also separately searched these sequence fragments, the genomic region of *API2* exons 6–7 and *MALT1* exons 4–9 as well as the entire *API2* and *MALT1* genes for palindromic sequences, tandem sequences and novel homologous sequences using MEME. These analyses did not reveal the consistent presence of unknown consensus sequence patterns or motifs at or near the breakpoints, nor was there any significant intra- and inter-chromosomal homology other than the presence of the repetitive sequence as discussed above.

Discussion

Genomic breakpoints on both the API2 and MALT1 gene are scattered

Guided by the known breakpoints of the *API2-MALT1* fusion transcripts, we have amplified the genomic sequence of both the *API2-MALT1* and *MALT1-API2* fusions by long distance PCR and studied the junction sequence in 19 cases of t(11;18) positive MALT lymphoma. The observation of high frequencies of deletions and duplications in both the *API2* and *MALT1* genomic sequences strongly indicates that multiple double strand DNA breaks must have occurred during the translocation process. The observed breakpoints on the *API2* gene were scattered in the intron 7 and exon 8, while those on the *MALT1* gene were distributed in the introns 4, 6, 7 and 8. Despite that recurrent breakpoints were seen in two cases, in general, the breakpoints observed from both fusions appeared to be distributed irregularly and this was also supported by computer modelling. Random distribution of breakpoints was observed in a common reciprocal translocation t(11;22) in Ewing's tumours (Zucman-Rossi *et al*, 1998), albeit the mechanisms underlying such random DNA double strand breaks are unclear. In view of the evidence that the breakpoints

observed were scattered, multiple and were not associated, at least in the majority of cases, with any putative sequence motifs that might play a role in chromosomal recombination, the double strand breaks probably did not result from a regulated cellular event.

Double strand breaks can be induced by exogenous DNA damaging agents such as ionizing radiation or by endogenous metabolic products such as free radicals generated by oxidative respiration. Although the exact cause and nature of the double strand breaks in the present cases are yet unknown, data emerging suggest that the occurrence of t(11;18) is influenced by aetiological factors associated with MALT lymphoma. The translocation occurs at dramatically variable frequencies in MALT lymphoma of different sites, from 0–1% in the thyroid and salivary gland to 30–40% in the stomach and lung (Ye *et al*, 2003). In gastric MALT lymphoma, the translocation has been shown to be significantly associated with infection of CagA positive strains of *H. pylori* (Ye *et al*, 2003), which is more virulent in the induction of inflammatory responses including generation of genotoxic chemicals such as oxygen reactive species. Oxygen reactive species are known to cause a range of DNA damages, particularly double strand breaks (Aman, 1999; Ferguson & Alt, 2001), it is tempting to speculate that the double strand breaks and deletion of the *API2* and *MALT1* gene sequence may be related to genotoxic insults caused by inflammatory responses in premalignant lesions associated with MALT lymphoma.

t(11;18) may result from non-homologous end joining following double strand breaks

To understand the mechanisms underlying the chromosomal recombination following DNA double strand breaks, we systematically searched for known recombination signals and putative sequence motifs that may associate with chromosomal recombination at or near the breakpoints. We examined all the known recombination signals including VDJ recombination heptamer and nonamer (Tycko & Sklar, 1990; Cayuela *et al*, 1997; Kuppers & Dalla-Favera, 2001; Marculescu *et al*, 2002), Ig class switch pentamer (Dalla Favera *et al*, 1983; Jeffs *et al*, 1998; Kuppers & Dalla-Favera, 2001), eukaryotic topoisomerase II cleavage site (Domer *et al*, 1995; Felix *et al*, 1995; Obata *et al*, 1999), chi-like octomer (Wyatt *et al*, 1992), translin binding sequence (Aoki *et al*, 1995; Hosaka *et al*, 2000), DNase I hypersensitive site (Forrester *et al*, 1990), purine-pyrimidine sequences, polypurine and polypyrimidine and palindromes (Boehm *et al*, 1989) and did not find any association between these sequence motifs and the breakpoints observed. Furthermore, our extensive computer-aided sequence search failed to reveal any unknown consensus sequence patterns or motifs that might be implicated in the chromosomal recombination at or near the breakpoints observed. Our results are in line with the findings by Baens *et al* (2000b) who studied five cases of t(11;18)-positive MALT lymphoma but differ from the observation by Sato *et al* (2001) who studied a single case. The latter study showed the presence of a VDJ recombination heptamer sequence motif at the *API2* breakpoint and a partially matched sequence at the *MALT1* breakpoint on der(11). None of the breakpoints observed in the present study and that by Baens *et al* (2000b) was at or near the VDJ heptamer and, moreover, a sequence search revealed that there is only one VDJ recombination heptamer site in the breakpoint region of the *API2* gene but none in the breakpoint region of the *MALT1* gene. Taken together, the data reported so far do not support a role of the specific sequence-mediated chromosomal recombination in t(11;18).

We next investigated the putative role of repetitive sequences including SINEs, LINEs, LTR elements and DNA elements in t(11;18), as they have been shown to be implicated in gene rearrangement and chromosomal translocation in human tumours (Onno *et al*, 1992; Rudiger *et al*, 1995; Jeffs *et al*, 1998; Hill *et al*, 2000). With the exception of one case (case 17) in which *Alu* repeat might be implicated in the *MALT1-API2* fusion on der(18), there was no evidence of involvement of the above repetitive sequences in both the *API2-MALT1* and *MALT1-API2* fusions in the remaining cases. Thus, homologous recombination is less likely to be involved in t(11;18), at least in the majority of cases.

Although recurrent breakpoints were seen in a few cases, the breakpoints of t(11;18) in general appeared to be scattered and were not commonly associated with any sequence motif that may be potentially associated with chromosomal recombination. The translocation may result from illegitimate non-homologous end joining following double strand breaks. The molecular mechanisms underlying t(11;18) are thus different from those responsible for the antigen receptor-associated chromosomal translocations, in which the VDJ recombination machinery is implicated at least in the double stand break at the antigen receptor locus and the direct recombination between the oncogene and antigen receptor gene (Jaeger *et al*, 1993; Welzel *et al*, 2001).

Non-homologous end joining following double strand breaks is implicated in chromosomal translocations of soft tissue sarcomas and leukaemias (Super *et al*, 1997; Zucman-Rossi *et al*, 1998; Gillert *et al*, 1999; Richardson & Jasin, 2000; Elliott & Jasin, 2002). These translocations are commonly featured by sequence alterations at the fusion junctions including deletions, duplications, non-template-based insertions and inversions, similar to those of t(11;18) observed in the present study. Whereas inversion was not observed in our series, insertion, deletion and/or duplication of various sizes were found in every case at one or both breakpoint junctions. In addition, the sharing of 1–4 bp nucleotides between the *API2* and *MALT1* genes at the breakpoint junctions was frequently seen (19 of 32 junctions, or 60%), which is a key feature of non-homologous end joining known as microhomology (Lieber, 1998). While two DNA ends that do not share sequence homology can still be joined, the frequency of joining between two DNA ends with microhomologous sequence is much higher than random positioning of the join site (Lieber,

1998). These findings suggest that there may be common mechanisms underlying the various translocations of different diseases. Data emerging indicate that defects in molecules participating in non-homologous end joining promote the development of lymphomas and soft tissue sarcomas that harbour chromosomal structural abnormalities including translocations, deletions and duplications. Impairment of various non-homologous end joining components appears to confer different susceptibility to the development of lymphomas or soft tissue sarcomas (Sharpless *et al*, 2001; Zhu *et al*, 2002).

Evidence of selection to express functional API2-MALT1 transcripts

As discussed above, t(11;18) appears to be the result of non-homologous end joining following double strand DNA breaks. A crucial question raised here is why the breakpoints observed are clustered in the regions of intron 7 and exon 8 of the *API2* gene and introns 4, 6, 7 and 8 of the *MALT1* gene. It is possible that the clustering reflects the presence of fragile sites in these regions. It has also been shown recently that the formation of specific translocations in human lymphomas such as t(14;18)(q32;q12) in follicular lymphoma is determined in part by spatial proximity of translocation-prone gene loci (Roix *et al*, 2003). Although the spatial organization of the *API2* and *MALT1* loci is not known yet, the breakpoint pattern observed indicates the involvement of selection of t(11;18) that is capable of generating a functional *API2-MALT1* fusion product and gives a clonal advantage. There are several strands of evidence to support this notion.

First, the *API2-MALT1* fusion transcript is always in frame despite that the genomic fusion occurs between various introns or exons of the two genes leading to a reading frame shift in some cases. This is best illustrated when the *API2-MALT1* genomic fusion involves the exon 8 of the *API2* gene as shown in the present study. In such cases, the residual exon 8 is invariably spliced out during the RNA processing, resulting in the expression of a functional fusion transcript. Secondly, the *API2-MALT1* fusion always comprises the N-terminal *API2* with three intact BIR domains and the C-terminal *MALT1* region containing an intact caspase-like domain. The specific selection of these domains indicates their importance and synergy in oncogenesis. It has been shown that both the API2 and MALT1 proteins were unstable, while the API2-MALT1 product was stable. The domains responsible for protein instability, the C-terminal of API2 and the N-terminal of MALT1, are lost by the translocation (Izumiyama *et al*, 2003). In addition, the API2-MALT1 fusion product, but not the wild type API2 or MALT1, has been shown to activate nuclear factor κB (NFκB), a transcription factor for a number of cell survival and growth related genes (Barkett & Gilmore, 1999). The BIR domain is believed to mediate the self-oligomerization of the fusion product, while the caspase-like domain is required for activating the IκK complex, consequently triggering a cascade of events leading to NFκB activation (Lucas *et al*, 2001; McAllister-Lucas *et al*, 2001). Furthermore, the deletions observed, although occurring extensively at the breakpoints of both the *API2* and *MALT1* genes, appear to always spare the domains critical for the function of the API2-MALT1 fusion product. In case 17, deletion involved the *MALT1* exons 5–7 but not its downstream exons that constitute the caspase-like domain. In a further six cases in which the genomic *MALT1-API2* fusion was failed to be amplified despite readily amplification of the *API2-MALT1* fusion, the deletions are anticipated to be beyond the region targeted by the primers used and may involve the entire C-terminal *API2* gene and/or the entire N-terminal *MALT1* gene. A cryptic deletion of a chromosomal 11 fragment including the entire C-terminal *API2* has been found previously in one of the two t(11;18)-positive MALT lymphomas (Dierlamm *et al*, 1999).

In summary, the genomic breakpoints of t(11;18) are generally not associated with any known or putative sequence motif that may associate with chromosomal recombination. Extensive sequence alterations including deletions, duplications and non-template-based insertions occur commonly at the fusion junction. t(11;18) may result from illegitimate non-homologous end joining following double strand breaks.

Supplementary material

The following material is available at http://www.blackwell-publishing.com/products/journals/suppmat/bjh/bjh4909/bjh4909sm.htm

Table S1. Primers used for primary and nested PCR and sequencing of t(11;18) breakpoint junctions on der(11) and der(18).

Table S2. Partial genomic junction sequence of *API2-MALT1* on der(11) and *MALT1-API2* on der(18).

Acknowledgments

This study is supported by a research grant from Leukemia Research Fund, UK.

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