Monitoring gastric lymphoma in peripheral blood by quantitative IgH allele-specific oligonucleotide real-time PCR and *API2-MALT1* PCR

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Summary

Gastric extranodal marginal zone lymphoma (EMZL) often shows prolonged localised disease, but the present study demonstrated the presence of tumour cells in peripheral blood (PB) of low stage patients. We studied the presence of tumour cells in PB in gastric lymphoma patients harbouring or lacking t(11;18)(q21;q21), by real-time immunoglobulin (Ig)H allele-specific oligonucleotide-polymerase chain reaction (ASO-PCR) and *API2-MALT1* PCR. Tumour cells were exclusively detected in PB of t(11;18)(q21;q21)$^{+}$-EMZL patients. The presence of tumour cells in PB and gastric biopsy follow-up samples showed a good correlation in these patients, suggesting clinical relevance for monitoring of tumour cells in PB of gastric t(11;18)(q21;q21)$^{+}$-EMZL patients.

Keywords: extranodal marginal zone lymphoma, monitoring gastric lymphoma, translocation t(11;18)(q21;q21), *API2-MALT1*, allele-specific-polymerase chain reaction.

Although gastric extranodal marginal zone lymphoma (gastric EMZL) often shows an indolent clinical behaviour, involvement of bone marrow (15–20%), other mucosa-associated lymphoid tissue (MALT) sites (30–50%) (van Krieken *et al*, 1989; Thieblemont *et al*, 2000) and peripheral blood (PB) (Bertoni *et al*, 2000) of patients with stage I tumours has been reported. *Helicobacter pylori* eradication results in lymphoma regression in 70–80% of the low-grade cases, but remission may occur after several months, necessitating prolonged follow-up. The translocation t(11;18)(q21;q21), which results in the *API2-MALT1* fusion (Dierlamm *et al*, 1999), is found in 20–30% of gastric EMZL (Schreuder *et al*, 2003) and has shown to be predictive for unresponsiveness to *H. pylori* eradication treatment for all stages of gastric EMZL (Liu *et al*, 2002).

Prospective quantitative monitoring of minimal residual disease in PB has been described for other non-Hodgkin lymphomas (Mandigers *et al*, 2001), but the clinical relevance of tumour cells in PB follow-up samples in gastric EMZL patients is presently unknown. We therefore applied both quantitative allele-specific oligonucleotide-polymerase chain reaction (ASO-PCR) and *API2-MALT1* PCR analysis for the detection and monitoring of tumour cells in PB follow-up samples in patients harbouring or lacking t(11;18)(q21;q21)$^{+}$-EMZL.

Study design

Patients selection and sample preparation

Gastric biopsies and PB samples (20 ml) were collected from eight gastric lymphoma patients taken at diagnosis and during a follow-up period of 20–70 months (Table I). Six patients harboured EMZL, one patient a diffuse large cell lymphoma with accompanying EMZL and one patient a Burkitt-like

Table I. Clinical characteristics and results of molecular follow-up of biopsy and peripheral blood samples in t(11;18)(q21;q21)-positive and negative lymphoma patients 1–8.

					Biopsy			Peripheral	Blood	Amplicon ratio PB/biopsy‡	*n* tumour cells /1·105 PBMC§
Case	Sex/ age	Follow-up (months)	Histological/clinical diagnosis	Treatment	CDR3-PCR	t(11;18)	ASO-PCR	t(11;18)	ASO-PCR	ASO-PCR	ASO-PCR
1	F/56	In. diagn.	Gastritis		P						
		4	EMZL	a-Hp	P + M			+	ne		
		7	EMZL		M	pos	+	nt	+	$8{\cdot}1\ 10^{-3}$	567
		11	EMZL					nt	+	$1{\cdot}3.\ 10^{-4}$	9
		17	EMZL					+	+	$7{\cdot}4{\cdot}10^{-5}$	5
		24	EMZL					+	+	$4{\cdot}1{\cdot}10^{-3}$	287
		30	hCR	CT/RT				−	−		
2	M/76	In. diagn.	EMZL		M						
		2	EMZL	a-Hp		pos	+	+	+	$1{\cdot}7{\cdot}10^{-2}$	1190
		22	hCR	RT							
		72	cCR					+ †	−		
3	F/58	In. diagn.	EMZL		M						
		2	EMZL	a-Hp		pos	+	nt	−		
		22	hCR	RT							
		50	cCR					+ †	−		
4	M/50	In. diagn.	EMZL *		M						
		2	hCR			neg	+	−	−		
5	M/59	In. diagn.	EMZL + DLBCL		M	neg					
		0,5	EMZL + DLBCL		M		+	−	−		
		7	HCR	CT				nt	−		
		19	HCR					nt	−		
6	M/44	In. diagn.	Burkitt-like	CT	M						
		3	Burkitt-like	CT	M	neg	+	−	+	$7{\cdot}1.\ 10^{-4}$	50
		19	HCR					−	+	$2{\cdot}0{\cdot}10^{-3}$	140
7	M/77	In. diagn.	EMZL		M	pos*	nt	+	nt		
		1·5	EMZL	a-Hp							
		2·5	HCR	RT				+	nt		
8	F/27	In. diagn.	EMZL	CTRT	M						
		8	remission								
		38	EMZL					−	nt		
		40	EMZL	Resect/surgery		pos*	nt				
		132	hCR					−	nt		

F, female; M, male; in. diagn., initial diagnosis; M, monoclonal; P, polyclonal; hCR, histological complete remission; cCR, clinical complete remission; DLBCL, diffuse large B-Cell lymphoma; RT, radiotherapy; CT, chemotherapy; BMT, bone marrow transplantation; a-*H.p*, triple *Helicobacter pylori* eradication therapy; ne, not evaluable; nt, not tested; ASO-PCR, allele-specific oligonucleotide-polymerase chain reaction.

* derived from the conjunctiva.

†negative after 40 cycles of PCR, positive after 50 cycles of PCR.

‡ratio of ASO-PCR amplification signal in PBMC follow-up sample and ASO-PCR amplification signal of biopsy sample taken at initial diagnosis.

§The number of tumour cells in PB per $1{\cdot}10^5$ PBMC. For example, in the PB sample taken 7 months after diagnosis in case 1, $8{\cdot}1\ 10^{-3} \times 8{\cdot}0\ 10^4 \times 0{\cdot}7 = 454$ cells ($=$ 567 cells/$1{\cdot}0\ 10^5$ PBMC) were detected; $8{\cdot}1\ 10^{-3}$ equals the ratio ASO-PCR amplification in PBMC to biopsy sample, $8{\cdot}0\ 10^4$ is the number of PBMC equivalent to 0·5 μg DNA (Willems *et al*, 2000) and 0·7 indicates 70% of tumour cells in biopsy sample. The percentage of tumour cells in biopsies of cases 1–3 and 6 was 70%.

lymphoma as classified according to the World Health Organization classification (Jaffe *et al*, 2001). Peripheral blood mononuclear cells (PBMC) obtained by density gradient centrifugation were washed, counted and cryopreserved as described previously (Willems *et al*, 2000). DNA and RNA were extracted from PBMC and biopsies using the QIAamp Blood kit (Qiagen, Hilden, Germany) and the RNAzol B isolation kit (CAMPRO-Scientific, Veenendaal, The Netherlands), respectively.

Real time IgH ASO-PCR and API2-MALT1 PCR

Quantitative real-time ASO-PCR detection of tumour-specific immunoglobulin heavy chain (*IGH*) sequences in 0·5 μg DNA

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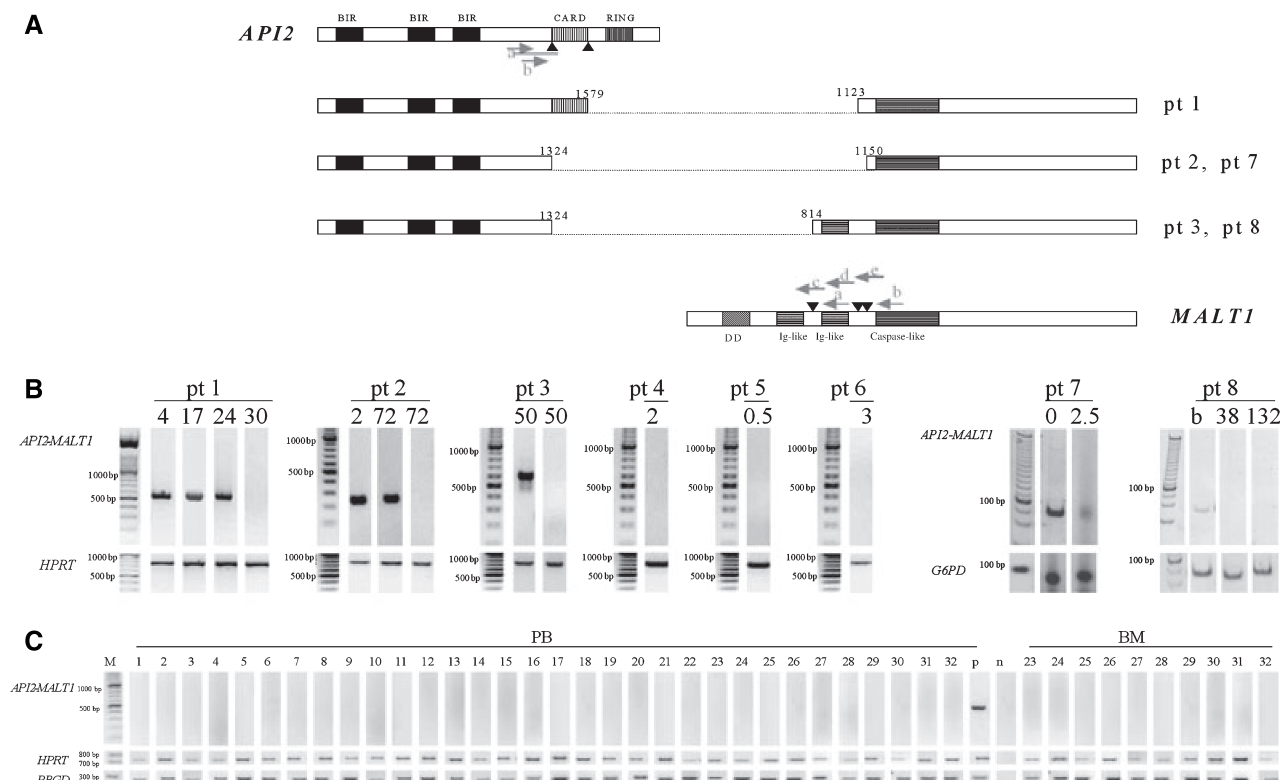

Fig 1. Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *API2-MALT1* fusion transcripts in biopsy and peripheral blood (PB) samples from gastric lymphoma patients. (A) Structure of the *API2* and *MALT1* cDNA and the different fusion cDNAs detected in gastric lymphoma biopsies of patients (pt) 1–3, 7 and 8. PCR was performed on PB-cDNA samples of cases 1–6 with sense primer *API2*a (5′-ATTA-ATGCTGCCGTGGAAAT-3′) and antisense primers *MALT1a* (5′-GGATTCAGAGACGCCATCAACAC -3′) and *MALT1b* (5′-GGTGCTCCCGGTAATTCATA-3′) as previously reported (Schreuder *et al*, 2003) and on PB samples of cases 7 and 8 with sense primer *API2*b (5′-GGAAGAGGAGAGAGAAAGAGCA-3′) and *MALT1c* (5′-CCAAGACTGCCTTTGACTCT-3′) *MALT1d* (5′-GGATTCAGAGACGCCATCAA-3′) and *MALT1e* (5′-CCAAAGGCTGGTCAGTTGTTT-3′) according to Liu *et al* (2002). Primers were used in PCR's and for bidirectional sequence analysis of the fragments. Sequences are based on National Centre for Biotechnology Information (NCBI) accession numbers NM001165 (*API2*) and AF130356 (*MALT1*). Arrows depict breakpoints in *API2* (bp 1324 or 1579) and *MALT1* (bp 814, 1123 or 1150). Arrowheads indicate positions of the primers used. BIR, baculovirus; CARD caspase recruitment domain; DD, death domain; RING, really interesting new gene (domain). (B) *API2-MALT1* PCR in PBMC samples in gastric lymphoma patients case 1–8. Examples of PCR fragments using primer *API2a-MALT1b* (cases 1–6) and *API2b-MALT1e* (cases 7 and 8). Breakpoints: case 1, *API2*-1579 *MALT1*-1123 (546 bp fragment); cases 2 and 7 *API2*-1324 *MALT1*-1150 (265 bp fragment); cases 3 and 8 *API2*-1324 *MALT1*-814 (601 bp fragment). Control reactions using primers for the housekeeping genes hypoxanthine phosphoribosyl transferase (*HPRT*), porphobilinogen deaminase (*PBGD*) and glyseraldehyde-3-phosphate dehydrogenase (*G6PD*) were run in parallel pt = patient, b = biopsy. Lane numbers indicate time (in months) of follow-up PBMC samples. Additional lanes 72 (patient 2) and 50 (patient 3) indicate repeat PCR experiments on PB samples. (C) Control reactions: *API2-MALT1* PCR in PB or BM samples of gastric EMZL patients stage Ie lacking t(11;18)(q21;q21) (lanes 1–6), healthy individuals (lanes 7–13), patients with non-malignant colorectal disorders (lanes 14–19), patients without malignancy or colorectal disorders (lanes 20–32). *P* = t(11;18)(q21;q21) positive (case 3), *n* = negative control (water).

samples was performed on a ABI/Prism 7700 system according to Willems *et al* (2000). ASO complementary to the CDRI (sense) and CDR3 (antisense) regions of the *IGH* sequences of malignant clones were designed, and internal FR-VH gene-family specific consensus probes were chosen. The specificity of each IgH ASO-PCR was determined using lymphoma biopsy DNA and normal PBMC-DNA as positive and negative controls, respectively. ASO-PCR reactions were carried out in triplicate and signals were normalised to β-actin PCR signals. Serial dilutions of DNA from a lymphoma biopsy were used as calibrator samples. RT-PCR and *API2-MALT1* PCR on frozen lymphoma tissue RNA samples (cases 1, 4 and 5) and all PBMC-RNA samples were carried out as previously described (Schreuder *et al*, 2003). *API2-MALT1* reverse transcription (RT)-PCR on RNA isolated from paraffin-embedded lymphoma tissue and PB whole cell lysates (cases 7 and 8) was performed according to Liu *et al* (2002). PB cDNA samples from seven healthy individuals, six EMZL patients lacking t(11;18) or 19 patients with non-malignant gastrointestinal disease served as negative controls. PCRs were repeated seven times (Limpens *et al*, 1995), with 50 cycles each.

Results and discussion

We studied the presence of tumour cells in PB follow-up samples of eight gastric lymphoma patients, using tumour

specific *IGH* sequences (cases 1–6) or *API2-MALT1* RNA transcripts (cases 1–3, 7 and 8) as the marker (Table I). Tumour cells were detected by ASO-PCR in PB samples of 2 t(11;18)(q21;q21)-EMZL patients (cases 1 and 2) and a Burkitt-like lymphoma patient (case 6). *API2-MALT1* PCR showed PB involvement in four of the five t(11;18)(q21;q21)$^+$-EMZL patients (cases 1–3, 7; Table I; Fig 1).

To our knowledge, this is the first report on specific quantitative detection of tumour cells in PB follow-up samples of EMZL patients. Our real-time ASO-PCR assay detected between five and 1190 circulating tumour cells in a background of $1{\cdot}10^5$ PBMC. *API2-MALT1* PCR appeared to be even more sensitive, as tumour cells were detected in follow-up PB samples of cases 2 and 3 exclusively with this technique (Table I, Fig 1). These data stress the requirement for sensitive techniques for the detection of low numbers of circulating neoplastic cells in PB of gastric EMZL patients and may explain the negative results in previous study using Southern blot analysis (Liang *et al*, 1990). *API2-MALT1* PCR is more practical than ASO-PCR, but its application in molecular diagnostics is restricted to 20–30% gastric EMZL patients that harbour t(11;18)(q21;q21).

Notably, a good correlation between the presence of tumour cells in PB and gastric biopsies during follow-up was observed in t(11;18)(q21;q21)$^+$-EMZL patients with PB involvement. Furthermore, in these patients histological complete remission was accompanied by a decrease in number of circulating tumour cells in PB to either undetectable (case 1) or low levels (cases 2, 3 and 7). *Helicobacter pylori* eradication therapy did not influence tumour cell numbers in PB. However, radiotherapy; resulted in a decrease of circulating tumour cells in PB to very low or near undetectable levels (cases 1–3 and 7). In one patient, treated with systemic chemotherapy combined with radiotherapy, no tumour cells were detected in PB at complete remission. The clinical significance (of small numbers) of tumour cells in PB in patients with histological regression is currently unknown and long-term follow-up data are required to determine whether eradication in PB is recommendable.

This study demonstrated that patients with clinically localised disease showed PB involvement at presentation, as reported previously (Bertoni *et al*, 2000). We found PB involvement was associated (exclusively) with gastric EMZL patients harbouring t(11;18)(q21;q21). These data and the recent finding that t(11;18)(q21;q21) is associated more often with disseminated gastric lymphomas (Liu *et al*, 2001), suggest that gastric t(11;18)$^+$-EMZL is rather a systemic disease.

We did not detect cells carrying t(11;18)(q21;q21) in PB of individuals lacking the translocation (Fig 1C), suggesting t(11;18)(q21;q21) does not occur in PB outside the context of diagnosed t(11;18)(q21;q21)$^+$EMZL. The data presented in this study suggest monitoring of lymphoma cells by *API2-MALT1* PCR in gastric t(11;18)(q21;q21)$^+$-EMZL patients is clinically significant. However, studies including more patients with prolonged follow-up are necessary to determine whether this practical and patient-friendly method, is an alternative to endoscopic bioptic follow-up in gastric t(11;18)(q21;q21)$^+$-EMZL patients.

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