SHORT REPORT

Development of immunoglobulin variable heavy chain gene consensus probes with conjugated 3' minor groove binder groups for monitoring minimal residual disease in childhood acute lymphoblastic leukaemia

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Aims: To develop immunoglobulin heavy chain variable (VH) gene probes that are shorter and more flexible in position for monitoring minimal residual disease (MRD) in childhood leukaemia (ALL), using minor groove binder (MGB) technology.

Methods: All VH germline sequences registered in the database were aligned and the consensus regions were determined. The reliability of the MGB probes was compared with non-MGB probes in all 24 cases of ALL.

Results: Ten MGB probes (16 to 18 mers) were designed that enabled all the germline sequences on the database to be analysed, whereas the conventional non-MGB probes (21 to 27 mers) did not allow the analysis of four of the VH1 and five of the VH3 germline sequences. The sequencing results in five of the 24 cases of ALL were not matched to the non-MGB probes.

Conclusions: MGB technology allows shorter probes to be designed, enabling MRD to be detected in childhood ALL. This would provide a considerable reduction in cost for a large MRD study.

Real time quantitative polymerase chain reaction (RQ-PCR) is an attractive approach for the quantitative evaluation of minimal residual disease (MRD) in bone marrow and peripheral blood stem cells, monitoring tumour cell reduction in the early response to primary treatment, or predicting disease relapse during long term follow up, because of its high sensitivity, reproducibility, and simplicity.¹⁻³ In childhood acute lymphoblastic leukaemia (ALL), consensus strategies, using a small number of fluorogenically labelled germline probes and an allele specific oligonucleotide (ASO) RQ-PCR method targeting immunoglobulin heavy chain (IgH) gene rearrangement, have been established and used for evaluating the efficacy of various treatment regimens. These consensus strategies have contributed to a considerable reduction in cost and labour during large MRD studies of ALL.

Donovan and colleagues³ designed IgH variable region (VH) gene family consensus fluorogenically labelled probes (non-MGB) because clonal IgH sequences of ALL are typically in the germline configuration. In a recent study, we assessed MRD in 24 cases of childhood ALL (VH1, two cases; VH2, one case; VH3, 15 cases; VH4, four cases; VH6, one case; and VH7, one case), including those detailed in a previous report,⁴ using ASO RQ-PCR with the non-MGB probes designed by Donovan *et al*,³ and found that in five cases the sequences were

not completely matched to the non-MGB probes in the VH1 or VH3 families (VH1-24, one case; VH1-46, one case; VH3-72, two cases; VH3-73, one case). In fact, four germline sequences in the VH1 family (VH1-f, VH1-18, VH1-24, and VH1-46) and five germline sequences in the VH3 family (VH3-d, VH3-15, VH3-49, VH3-72, and VH3-73) registered in the V BASE directory of human Ig genes (<http://www.mrc-cpe.cam.ac.uk/DNAPLOT.php>) were not completely matched to the non-MGB probes of Donovan *et al* (VH1, VH3A, and VH3B) (fig 1). Therefore, patient specific probes were designed in these cases.

"Minor groove binder probes show positional flexibility"

Recently, minor groove binder (MGB) conjugated DNA probes have been developed and used for 5' nuclease PCR assays. These form extremely stable duplexes with single stranded DNA targets, allowing the use of shorter probes for hybridisation based assays.⁵ Their shorter length resulted in better sequence specificity of the MGB probes and lower fluorescent background staining as a result of a reduction in non-specific probe hybridisation in comparison with ordinary DNA probes (non-MGB probes), and the use of internal non-fluorescent quencher dye instead of ordinary 3' quencher dye (TAMRA; tetramethylrhodamine). Because of the high melting temperature (T_m) requirements of PCR, non-MGB probes vary substantially in length from 14 to 40 mers, depending on the GC content of the amplified DNA fragment, whereas the MGB probes vary from 12 to 20 mers. Therefore, MGB probes show positional flexibility. The aim of our present study was to design MGB probes corresponding to all germlines in each VH family.

MATERIALS AND METHODS

DNA was isolated from bone marrow samples with a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) and amplified by PCR to determine the VH sequences, as described previously.^{6,7} Each PCR product was ligated to pGEM-T Easy Vectors (Promega, Madison, Wisconsin, USA) and transformed into DH5 α competent cells (Toyobo, Tokyo, Japan). Thirty subcloned colonies were chosen at random, and plasmid DNA was purified. Sequence analysis was

Abbreviations: ASO RQ-PCR, allele specific oligonucleotide real time quantitative polymerase chain reaction; ALL, acute lymphoblastic leukaemia; IgH, immunoglobulin heavy chain; MRD, minimal residual disease; MGB, minor groove binder; T_m, melting temperature; VH, IgH variable region

Figure 1 Alignment of (A) immunoglobulin heavy chain variable region 1 (VH1) and (B) VH3 family sequences registered in the V BASE database. Dashes indicate identity with the germline sequence of VH1-02 or VH3-07, respectively, and differences are indicated by capital letters. The locations of the consensus minor groove binder (MGB) probes (MGB-VH1A, MGB-VH1B, MGB-VH3A, MGB-VH3B, and MGB-VH3C) are enclosed in boxes with solid lines. The locations of the non-MGB probes (VH1 and VH3A) are enclosed by dotted lines. Seven VH1 germline sequences could be analysed with the non-MGB probe (upper columns), but four (lower columns) could not. In the VH3 family, nine germlines (lower columns) could not be analysed with the non-MGB VH3A probe. Four and nine germline sequences not analysed with the non-MGB probes could be analysed with the MGB probes (MGB VH1A and VH1B, and MGB VH3A-C, respectively).

performed to determine the clone specific VH region sequences.

All germline sequences of the VH families registered in the V BASE database were aligned, and attempts were made to synthesise consensus germline MGB probes in each family using the Primer Express v1.5 software package (Applied Biosystems, Foster City, California, USA). MGB probes were prepared with a 5' reporter dye (6-FAM; 6-carboxyfluorescein), an internal quencher dye (non-fluorescent quencher), and 3'-MGB. The Tm of the probes was approximately 70°C, which is about 10°C above the Tm of the matching primers, to ensure proper hybridisation to the target sequence.

The RQ-PCR assay was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems). The reaction mixture contained 300 ng of template DNA, 200 nmol/litre of each primer, 5µM probe, and 25 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) in a final volume of 50 µl. The cycling programme consisted of 50 cycles of a two step PCR consisting of denaturation for 15 seconds at 95°C and

combined annealing/extension for one minute at 60°C. Assay specific standard curves for each set of primers and a probe were constructed by plotting the threshold cycle against the known copy number of each positive control plasmid. The plasmid concentration was determined spectrophotometrically, and the copy number was calculated. Plasmids were diluted in a precise series, ranging from 5 pg to 0.005 fg (from 2×10^6 to two copies). For normalisation of each target, the copy number of β actin was used as an internal control.⁸

RESULTS

Three MGB probes were designed with VH3 rearrangements in the region of sequence homology (fig 1; table 1). These covered all VH3 germline sequences registered in the V BASE database, whereas non-MGB VH3 probes (VH3A and VH3B by Donovan and colleagues³) were not applicable in three of our 15 VH3 cases. In the VH1 family, two MGB probes were designed in the region of sequence homology (fig 1; table 1). Thus, it was possible to analyse all sequences of the VH1 region registered in the V BASE database with either the MGB-VH1A or MGB-VH1B probe. In comparison with unmodified probes (21 to 27 mers), MGB probes were shorter (16 to 18 mers), but had a higher Tm ($\sim 70^\circ\text{C}$).

Because the nominated germline sequences of VH2, VH4, VH5, VH6, and VH7 were almost identical, short regions of sequence identity appropriate for the design of a single probe were found, as with the non-MGB probes designed by Donovan and colleagues³ (table 1). All MGB probes were tested in quantitative reactions on serially diluted IgH plasmids from patients, and each gave a characteristic logarithmic amplification plot (data not shown).

We are currently conducting a prospective MRD study to evaluate the practical usefulness of a RQ-PCR assay using our consensus germline probes. The copy number for the target before and after treatment was almost identical between the MGB and non-MGB probes. The study review board permitted us to present MRD data for 18 of 24 patients examined (table 2). One of the patients (number 18 in table 2), whose sequences were matched to the MGB-VH1A probe but not the VH1 non-MGB probe, is shown in fig 2.

DISCUSSION

In our study, we designed 10 novel MGB germline probes, and all IgH gene rearrangements registered in the V BASE database could be detected with one of the probes. It was possible to use the probes to assess MRD in patients with four VH1 and five VH3 germlines that could not be evaluated using the non-MGB probes of Donovan *et al.*³ Furthermore, the cost of MGB probes is almost equal to that of non-MGB probes, so that the reduction in probe numbers leads to a cost benefit.

Others have recently described an ASO primer approach using consensus JH probes.^{1,2} We tested the ASO primer

Table 1 Nucleotide sequences of germline consensus minor groove binder probes designed in our study	
Probe	Sequences (5'-3')
MGB-VH1A	CTGCTCAGCTCCATGTAG
MGB-VH1B	CTCAGCTCCATGTAGGC
MGB-VH2	CCACAGGGTCCATGTTG
MGB-VH3A	CTGGAGATGGTGAATC
MGB-VH3B	CTTGAGATGGTGAATCTG
MGB-VH3C	CTCTGGAGATGGTGAACC
MGB-VH4	CTCAGCTTCAGGGAGAAC
MGB-VH5	CTGCTCCACTGCAGGTAG
MGB-VH6	CAGCTGCAGGGAGAAGTC
MGB-VH7	CGTGCTGACAGAGGTGTC

Table 2 Results of the RQ-PCR assays using an MGB probe and a non-MGB probe in 18 patients with ALL

Patient	Normalised values of PCR target detected by MGB probe* (those by non-MGB probe)				
	Initial diagnosis	15 days	1 month	3 months	4 months
1	42700 (38000)	0.34 (-)	- (-)	- (-)	1.36 (0.74)
2	17500 (11700)	17.6 (15.4)	- (-)	- (-)	-
3	38200 (29400)	14060 (16500)	1280 (687)	- (-)	- (-)
4	27100 (30300)	33400 (34800)	- (-)	- (-)	-
5	15300 (9050)		336 (425)	- (-)	-
6	8700 (10700)	870 (440)			
7	3160 (320)	35.0 (57.0)			
8	7700 (4100)	1.42 (0.73)			
9	69500 (68000)		8550 (8400)	5.84 (9.10)	
10	3350 (4540)		210 (180)		
11	29000 (32800)		- (-)	- (-)	
12	19800 (20800)		10.5 (7.6)	- (-)	
13	4840 (4290)		- (-)	- (-)	- (-)
14	2330 (2480)		- (-)	- (-)	-
15	770 (535)		- (-)	- (-)	- (-)
16	23000 (18300)		- (-)	- (-)	
17	1250 (1430)		6200 (530)	350 (340)	
18	12830 (11250)	17.4 (12.4)	- (-)	4.9 (0.74)	

ALL, acute lymphoblastic leukaemia; blank, not determined; MGB, minor groove binder; RQ-PCR, real time quantitative polymerase chain reaction; -, not detected.
*The normalised values of targets were expressed as the ratio of the target copy number for each 10⁵ cell equivalent from the integrated standard curves of human β actin, as described previously.⁴

A

B

C

Figure 2 (A) Positions of primers/minor groove binder (MGB) probe sequences in a patient with acute lymphoblastic leukaemia (number 18 in table 2) with the VH1 family. The location of the MGB-VH1A probe is circled. The VH1 non-MGB probe location is circled with a dotted line. Arrows indicate both forward and reverse primers. (B) An amplification plot showing plasmids that matched perfectly with the MGB-VH1A probe. (C) Standard curve of plasmids perfectly matched to the MGB VH1A probe. Arrows indicate the copy numbers of the target IgH gene in bone marrow specimens obtained at initial diagnosis and after 15 days of treatment.

approach using a consensus MGB JH probe, and we expect that a far simpler technique would be available for a large scale clinical study.

“It was possible to use the probes to assess minimal residual disease in patients with four VH1 and five VH3 germlines that could not be evaluated using the non-minor groove binder probes of Donovan *et al*”

In conclusion, an ASO RQ-PCR assay with MGB probes developed for the detection of MRD in childhood ALL was

shown to be a feasible technique for the identification of patients at risk of relapse, because of the shorter length of the probes and their positional flexibility. The assay was easier to use and had better specificity and cost performance than assays using conventional probes.

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Take home messages

- Minor groove binder probe technology allows shorter probes to be designed, which also have more positional flexibility
- These probes enable minimal residual disease to be detected in childhood acute lymphoblastic leukaemia and can therefore identify those patients at risk of relapse
- The assay is easier to use and had better specificity and cost performance than assays using conventional probes

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