ORIGINAL ARTICLE

Comparison of gene-expression profiles in parallel bone marrow and peripheral blood samples in acute myeloid leukaemia by real-time polymerase chain reaction

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Background: Gene signatures (*Indicator* genes) in bone marrow that provide more precise prognostication in haematological malignancy have been identified by microarray expression studies. It would be beneficial to measure these diagnostic signatures in peripheral blood.

Aims: To determine the degree of correspondence of gene expression for a set of *Indicator* genes between bone marrow and peripheral blood in acute myeloid leukaemia (AML).

Methods: Parallel bone marrow aspirate and peripheral blood samples were obtained from 19 patients diagnosed with AML and mononuclear cells isolated from both sample types. mRNA was globally amplified by polyadenylated real-time polymerase chain reaction (polyA RT-PCR); the expression of 15 AML *Indicator* genes, identified from previous microarray studies, was measured by RT-PCR. All values were normalised to the mean expression of three housekeeping genes (*IF2-β*, *GAP* and *RbS9*) and were statistically compared using SPSS software.

Results: No significant difference in expression between bone marrow and peripheral blood was observed for 10 of the genes (*leptin receptor*, *CD33*, *adipsin*, *proteoglycan 1*, *MB-1*, *cyclin D3*, *hSNF2b*, *proteasome iota*, *HkrT-1* and *E2A*), indicating its possible use in monitoring disease activity in peripheral blood samples, whereas *c-myc*, *HOXA9*, *LYN*, *cystatin c* and *LTC4s* showed significantly different expression between bone marrow and peripheral blood samples.

Conclusion: These results indicate a possible use for the method in monitoring AML in peripheral blood by RT-PCR measurement of *Indicator* genes. In addition, the initial use of polyA PCR facilitates translation to very small clinical samples, including fractionated cell populations, of particular importance for monitoring haematological malignancy.

Diagnosis and monitoring of the acute leukaemias, acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL), is currently achieved at the level of cell morphology, protein expression and cytogenetics.¹ Recent studies have shown the ability to further stratify disease on the basis of expression of gene signatures, *Indicator* genes, identified by microarray profiling.² These studies hold the promise of more specific diagnosis, prognostication and the development of tailored treatment,³ and a practical method is now required for the measurement of these gene sets in routine clinical practice.

However, use of cDNA arrays in routine clinical practice is limited by cost and the need for relatively large amounts of RNA. We have used a global amplification approach known as polyadenylated polymerase chain reaction (polyA PCR) to overcome these limitations. PolyA PCR coordinately amplifies cDNA copies of all polyadenylated mRNAs, thereby generating a PCR product (polyA cDNA) whose composition reflects the relative abundance of all expressed genes in the starting sample, and which can be applied to very small samples, including single cells.⁴ Real-time polymerase chain reaction (RT-PCR) measurement using gene-specific primers and probes of the expression levels of specific *Indicator* genes then allows gene signatures to be detected in the polyA cDNA, thereby enabling expression profiling of very small amounts of starting material.⁵ We have tested use of the method by measuring levels of gene expression in bone marrow from patients with acute leukaemia for 17 *Indicator* genes selected from a previous microarray study by Golub

*et al.*⁶ most ($n = 15$) of which showed similar expression in AML and ALL to that reported by Golub *et al.*,⁶ suggesting diagnostic utility.⁷ The aim of our study was to determine the degree of correspondence of gene expression between bone marrow and peripheral blood for the same set of *Indicator* genes as a first step towards their use for diagnosis using peripheral blood rather than bone marrow samples.

MATERIALS AND METHODS

Sample acquisition

Parallel bone marrow aspirate and peripheral blood samples, both collected from each patient at the same time, were obtained, with informed consent, from 19 patients with AML, after treatment (10 men, 9 women; age range 20–72 years, mean age 45 years). Table 1 gives the clinical details for each patient, including the blast count in the parallel bone marrow and peripheral blood samples in each case; full details were not available for one patient. Table 1 also details the age and cytogenetic abnormality for each patient at the time of primary diagnosis of AML and at the time of sampling of parallel bone marrow aspirate and peripheral blood samples. For all but one of the patients, samples were collected during routine monitoring while the patients were in remission. All

Abbreviations: AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; Mhouse, mean of the three housekeeping genes; polyA PCR, polyadenylated polymerase chain reaction; RbS9, ribosomal protein S9; RT-PCR, real-time polymerase chain reaction

Table 1 Clinical details of patients and samples

Sex	Age at primary Dx	Primary Dx	Cytogenetics at Dx	Age at sampling	PB blasts (%)	BM blasts (%)	Cytogenetics at sampling	Treatment stage at sampling
M	42	AML	Tri 8	43	0	2	Normal, 100% female donor	1st CR, 11 months after allogeneic sibling transplant
F	45	AML M2	Complex	45	0	0	Normal	1st CR, just before SCT
M	17	AML	Normal, bilineage on relapse	20	0	80	Failed, 58% donor	3rd CR 6 months after MUD
M	62	AML M2		63	0	3	Normal	2 nd CR
M	54	AML M5	Normal	54	0	2	Normal	1st CR
M	36	AML M4eo	Inv 16	37	0	0	Normal	In 2nd CR, just before allogeneic sibling transplant
M	64	AML	Normal	64	0	0	Normal	1st CR, after 1st chemotherapy
M	27	AML M4eo	Inv 16	29	0	0	Normal, 100% female donor	2nd CR, after allogeneic SCT
M	25	ALL	Ph+	26	9	96	6/20 cells Ph+	Relapse 9 months after SCT, started on Glivec
M	71	AML M2	t(8;21)	72	0	2	Normal	1st CR, after 1st chemotherapy
M	36	AML M4	Inv 16	38	0	1	Normal	2nd CR, after 2nd chemotherapy but before auto SCT
F	32	AML M2	t(8;21)	32	0	1	Normal	1st CR, after 2nd chemotherapy
M	24	AML, refractory	Monosomy 7, t(x;3)	25	0	1	Normal, 100% donor	In CR, 6 months after MUD transplant
F	55	APML	t(15;17)	56	0	0	Normal	After completion of 4 × chemotherapy and before starting treatment
F	48	AML M2	Normal	63	0	0	Normal, 100% donor	2nd CR, 11 months after mini sibling transplant
M	46	APML	t(15;17)	47	0	0	Normal	1st CR, after 2 × chemotherapy but before maintenance
M	37	AML	Normal	40	0	2		1st CR, after 4 × chemotherapy
M	56	AML, refractory	Del 7q	57	0	0	Normal, 8% donor	Short CR

AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; APML, acute promyelocytic leukaemia; chemo, chemotherapy; CR, complete remission; Dx, diagnosis; MUD, matched unrelated donor; SCT, stem cell transplant.

bone marrow aspirates were collected in Hank's buffered saline solution with heparin, whereas peripheral blood samples were collected in tubes containing standard ethylenediaminetetraacetic acid. Mononuclear cells were isolated immediately from both samples by density-gradient centrifugation and used for RNA extraction.

Global amplification of polyadenylated mRNAs (polyA RT-PCR)

Total RNA was extracted from all the samples, using an RNeasy mini kit (Qiagen, Valencia, California, USA). Global amplification of cDNA corresponding to all expressed genes (polyA PCR) was carried out as previously reported.^{4 5 8}

Taqman RT-quantitative PCR

Golub *et al*⁶ identified 50 *Indicator* genes discriminatory between AML and ALL, from which 15 *Indicator* genes had been chosen for measurement by RT-PCR in bone marrow aspirate samples from patients with AML and ALL in our previous study.⁷ These genes included genes highly expressed in AML—*cystatin c*, *leptin*, *CD33*, *HOXA9*, *adipsin*, *proteoglycan 1*, *LTC4s* and *LYN*, and genes highly expressed in ALL—*c-myc*, *MB-1*, *cyclin D3*, *hSNF2*, *proteasome iota*, *HkrT-1* and *E2A*. Taqman PCR primers and probes were designed for each gene, using Primer Express Software (Perkin Elmer Applied Biosystems, Foster City, California, USA), and are listed in table 2. For each gene, Taqman PCR was applied to 1 ng of polyA cDNA from each sample and to 10 µl of serially diluted human genomic standards, using a Taqman Gold kit.⁵ All samples were analysed with an ABI Prism 7700 sequence detection system (Perkin Elmer Applied Biosystems). The copy number of each gene was determined by reference, after normalisation to the mean of the three housekeeping genes (Mhouse; below), of the RT-PCR expression level to the human genomic DNA standards.⁵

Normalisation

The expression levels of three housekeeping genes (*IF2-β*, *GAP* and human ribosomal protein S9 (*RbS9*) mRNAs) were measured by RT-PCR in each sample. Copy numbers obtained for the Mhouse (*IF2-β*, *GAP* and *RbS9*) in each sample were divided by the highest Mhouse in all samples, resulting in a normalisation correction factor. After real-time PCR amplification and quantification of the selected genes, this factor was used for normalisation of expression levels of each of the 15 genes measured.

Statistical analysis

The data were not normally distributed and non-parametric tests were used. Statistical analysis of the expression levels of the 15 genes in the two groups (bone marrow aspirate *v* peripheral blood) was carried out using the Mann–Whitney U test with $p \leq 0.05$ for significance. All the tests were carried out using SPSS software. As the expression levels for all the genes have a wide range, the natural logarithm was used to plot all values because it allows a wide range of values to be visually compared. The ranking of all the expression levels of *Indicator* genes between the paired peripheral blood and bone marrow aspirate samples was analysed and presented as the mean rank statistical difference.

Cluster analysis

Unsupervised cluster analysis of the normalised gene expression values for each sample was carried out using Cluster and TreeView from the Eisen laboratory (<http://rana.lbl.gov/EisenSoftware.htm>), with the protocols detailed with the software. Clustering was carried out on the gene expression values from all 38 samples for all genes, and separately for those showing significant difference in expression between bone marrow and peripheral blood samples with the Mann–Whitney U test.

Table 2 List of TaqMan primers and probes

Gene (human)	Accession no	1 = 5'-(FAM)-probe-(TAMARA)-3'
		2 = 5'-primer -3'
		3 = 5'-primer -3'
<i>IF2-β</i>	U23028	1 = TGGAGTTGGGATGTGGAAGTG 2 = CTGCCGGCCTGCTTAG 3 = TCCTCCCTAGGCAGAAGCTTTGTCCAT
<i>GAP</i>	J04038	1 = CATGGCCTCCAAGGAGTAAG 2 = GGGACTCCCCAGCAGTGA 3 = ACCAGCCCCAGCAAGAGCACAAGA
<i>RbS9</i>	U14971	1 = GAGACGACGAGGAGGAGGATTA 2 = GCAGGAAAACGAGACAATCCA 3 = TCCACCTGTCCCTCTGGGCTG
<i>c-myb</i>	M15024	1 = GGGAGACAGAACTGTGGTTGAT 2 = CAAAAGTTCAGTGTGGCCA 3 = CCAGTCACTGCCTTAAGAACATTGATGCA
<i>proteasome iota</i>	X59417	1 = GCAGAGAGAGACTAAACATTGTCGT 2 = TCCATGATGTTTGTGTTGTACCT 3 = TTTACCAGATCCGTGATGCCACTTACCTG
<i>MB-1</i>	M80462	1 = GGTGTCCACTCTTCTCCCTC 2 = GAGCCTGGGAAAGGCAGC 3 = AAAGTCCCCACCTCTTAACCTAATCCC
<i>cyclin D3</i>	M92287	1 = ATGCCCTCTCTCCATTGT 2 = AGCCACCTCCAGGGCATC 3 = CCTCTGCCCATCTCTCCAGCAG
<i>SNF2</i>	D26156	1 = AAA TAC AGT AGG AGG AGT GGA CGG 2 = TGCCTCCGAGTTTG 3 = ACGCACATGCATCCACACTTAACAGT GAC
<i>HkrT-1</i>	S50223	1 = AGCCTCGTAAACCCGGG 2 = GGAGACCAAGCCACAGACTTCC 3 = AAGACTCACTTACCCTCTTGAAAGCTGGTACAGA
<i>E2A</i>	M31523	1 = TGCCTCAAGATGGTTTCAAAA 2 = AGGACAGGTCTCTGAATCCACC 3 = CTGGATGATTGGGACTTTAAACGACCCTC
<i>LTC4</i>	NM_000897	1 = ACGGAGCCGGGAAAGAAG 2 = GGTCACTAGAACTTAAATGATAGAGACTAGGAT 3 = CCGGAGCCTCCAGCTGCC
<i>LYN</i>	M16038	1 = TGGATTCTCCACTCAGTTGCAA 2 = GGCTGCACTCAGATGTTGTCAA 3 = ACTTGTCTCAGCAGCTGGTAATCTGTCTC
<i>HOXA9</i>	XM_088184	1 = TGAATGCTGATTGTAACGGAGTTAA 2 = CCCAGATTCAAGGATACAAATCT 3 = AATAAATTATACACTATGAAACGCCATTGG GCTACTG
<i>CD33</i>	M23197	1 = ATCTGTGCTCTTTCATTGCTAAGTG 2 = TACCTACTCAGAGAGAAGAAAATGGAG 3 = ATGATGTCACACAAGCTCTTAACCTTCCATG
<i>adispin</i>	M84526	1 = GGGCTGAAGGTCAGGGT 2 = AACCAAGTGCAGGAGTGGATG 3 = ACCCAAGCAACAAAGTCCCGAGCA
<i>leptin</i>	Y12670	1 = CCAAGAGCCTCAACATTTCTAG 2 = CATCAAAATGCTCTGGGTCCA 3 = CTTATTAGAAATGCAGAATCTGAAGCCCCAC
<i>cystatin C</i>	M27891	1 = TCACCTCTATGCACACCTCCC 2 = TGTCTGTCTCTGGTGCAAG 3 = ACCCTGGACTGGTGGCCCC
<i>proteoglycan 1</i>	X17042	1 = TGTGTTTGCAGAGCTAGTGGATG 2 = TGAAAATACACAGATATCCAGGAAAAA 3 = ACAGTTGTGCTGTCAATACCT

FAM, 6-carboxyfluorescein; *RbS9*, ribosomal S9; TAMRA, 6-carboxytetramethylrhodamine.

RESULTS

PolyA real-time polymerase chain reaction

All 38 samples of total RNA prepared from the 19 paired samples of human bone marrow and peripheral blood were subjected to polyA RT-PCR, yielding polyA cDNA in the expected size range of 100–700 bp.

Comparative gene expression pattern of housekeeping genes in parallel bone marrow and peripheral blood samples

Figure 1 shows the mean expression levels for the three housekeeping genes (*IF2-β*, *GAP* and *RbS9*) in the bone marrow aspirate and peripheral blood samples, with the Mhouse. All three genes were expressed in both samples and

all showed higher mean expression in the peripheral blood than in the bone marrow, particularly *RbS9*. The mean expression levels of *GAP* and *RbS9* were much higher than that of *IF2-β* (fig 1).

Comparison of Indicator gene expression levels in parallel bone marrow and peripheral blood samples

C-myb, *HOXA9*, *LYN*, *cystatin c* and *LTC4s* showed a significant difference in expression in the bone marrow and peripheral blood samples, whereas the other genes did not. The expression values for the significantly different genes are shown in box plots (fig 2). The levels of expression of *HOXA9* and *LTC4s* in the bone marrow samples were significantly higher in the bone marrow than in the peripheral blood,

Figure 1 Comparative gene expression pattern of *IF2-β*, *GAP* and *RbS9* housekeeping genes in parallel bone marrow and peripheral blood samples. The mean expression levels of the three housekeeping genes, measured by RT-PCR in polyA cDNAs prepared from parallel human bone marrow and peripheral blood samples, are shown as millions of copy number for each gene, with the mean copy number for the three housekeeping genes. BM, bone marrow, PB, peripheral blood, Mhouse, mean of three housekeeping genes.

whereas by contrast, the levels of expression of *cystatin c* and *LYN* were significantly lower in the bone marrow than in the peripheral blood.

Figure 3 shows the mean ranks for the *Indicator* genes between the two samples, which indicate divergence of those genes with significant difference between the samples and convergence of the remainder.

Cluster analysis

Cluster analysis carried out in the space of all genes showed no clear clustering of the samples, although the housekeeping genes (*IF2-β*, *GAP* and *RbS9* mRNAs) clustered together (fig 4A). Conversely, unsupervised clustering in the space of those genes showing significant difference in expression between bone marrow and peripheral blood samples (*c-myb*, *HOXA9*, *LYN*, *cystatin c* and *LTC4s*) showed clear clustering of the bone marrow and peripheral blood samples, confirming that these genes differ between the two samples (fig 4B). Clustering in the space of those genes with no significant difference in expression between bone marrow and peripheral blood showed no clear grouping of the two samples (fig 4C).

DISCUSSION

Several microarray studies have identified gene signatures for haematological malignancies in general, and for acute

Figure 2 Expression levels of genes with significant difference between bone marrow and peripheral blood. Box plots show the values for the genes for which there was a significant difference in the level of expression between the bone marrow and peripheral blood samples. BM, bone marrow; LN, natural logarithm; PB, peripheral blood.

Figure 3 Mean ranks for the *Indicator* genes in acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) in each of the bone marrow and peripheral blood samples. Mean ranks (y axes) for each gene, calculated using the Mann–Whitney U test in the bone marrow and peripheral blood samples, sorted on the basis of mean ranks for bone marrow, are shown for (A) genes expressed at high level in AML and (B) genes expressed at high level in ALL. BM, bone marrow; PB, peripheral blood; proteogly, proteoglycan; proteoso, proteosome. LN, natural logarithm.

leukaemia in particular.^{2,3} These promise more specific diagnosis, prognosis and treatment, with expected improvements in patient survival,³ and a practical method for measurement of these gene signatures in routine clinical practice is urgently required.

We have used a global amplification approach known as polyA PCR to overcome these limitations. PolyA PCR coordinately amplifies cDNA copies of all polyadenylated mRNAs, thereby generating a PCR product (polyA cDNA), the composition of which reflects the relative abundance of all genes expressed in the starting sample, and which can be applied to very small samples, including single cells.⁴ The expression profiles of specific *Indicator* genes can then be measured by RT-PCR using gene-specific primers and probes, thereby enabling expression profiling of very small amounts of starting material.⁵ We have tested the use of the method in bone marrow samples from AML and ALL, showing its ability to distinguish between these two groups using *Indicator* genes identified from independent microarray studies.^{6,7} Specifically, for 17 *Indicator* genes showing differential expression between AML and ALL by microarray analysis,⁶ all but two genes measured by RT-PCR showed expression in AML and ALL similar to that in the microarray study.⁷ Following on from this work, the aim of our study was to determine the degree of correspondence of gene expression for the same set of *Indicator* genes between bone marrow and peripheral blood samples as a first step towards their use for diagnosis using peripheral blood either together with, or instead of, bone marrow samples.

To do this we measured, by RT-PCR, the expression levels of 15 *Indicator* genes for AML in 19 parallel bone marrow and peripheral blood samples. Each of these sample pairs was harvested from patients treated for AML, all but one of whom was in remission at the time of sampling. The 15 genes measured were selected from genes discriminatory between AML and ALL.⁶ Although all the paired bone marrow and peripheral blood samples analysed in our study were from patients with AML, the seven *Indicator* genes for ALL were included, as patients were recruited to the study sequentially and it was not possible to predict in advance the number of patients presenting with either AML or ALL.

Comparison of the median levels of expression for each gene between the two sample types showed significant differences in the level of expression for 5 of the 15 genes measured—namely, *c-myb*, *cystatin c*, *HOXA9*, *LTC4s* and *LYN*, whereas the remaining 10 genes (*adipsin*, *CD33*, *proteoglycan*, *proteosome iota*, *leptin*, *cyclin D3*, *E2A*, *SNF2*, *Hkrt-1* and *MB-1*) showed no significant difference in expression levels between the two sample types. This indicates that the expression level for these 10 genes in the peripheral blood reflects their expression in the bone marrow, suggesting therefore that the gene-expression profile in the bone marrow can be judged by peripheral blood analysis. Most samples used were from patients in remission. Notwithstanding this, there remained a good correlation between the expression levels for these 10 genes between the two sample types. Such a correlation was therefore not a feature merely of high blast counts, and suggests that the level of these 10 genes in the peripheral blood reflects that in the bone marrow even at low blast counts. This may facilitate monitoring of acute myeloid leukaemia, particularly if the genes measured are associated with other known molecular markers of minimal residual disease.

Cluster analysis confirmed the results of the Mann–Whitney U analysis by an independent, unsupervised approach. Specifically, those genes showing significant difference in expression between the two sample types were able to separate the two types by unsupervised clustering, whereas no distinction between the two types was seen by clustering in the space of those genes showing no significant difference between bone marrow and peripheral blood samples. Conversely, the housekeeping genes clustered together, showing equivalence of their expression across all samples, thereby validating their use.

Of interest is the fact that of the five genes showing a difference between the two sample types (*cyclin D3*, *proteoglycan*, *LYN*, *c-myb* and *HOXA9*), only one, *c-myb*, is ALL specific, whereas the remaining four were all drawn from the list of genes up regulated in AML compared with ALL. This is of relevance considering that all the samples analysed were from patients with AML, in whom it may be hypothesised that genes predictive of ALL would probably not be altered from normal, either in the bone marrow or in the peripheral blood. Conversely, for the other four genes, difference in expression level between the peripheral blood and bone marrow may be due to the particular involvement of these genes in the pathobiology of AML, resulting in their differential expression between bone marrow and circulating leukaemic cells.

The results show the utility of the polyA PCR method for generation of polyA cDNA from bone marrow and peripheral blood. This aspect of the method renders it applicable to very small clinical samples, by virtue of the amplification by polyA PCR. The *Indicator* genes measured showed similarity of expression between bone marrow and peripheral blood for most (10 of 15) of the genes, indicating the possible use of the method for monitoring of acute myeloid leukaemia in the peripheral blood by RT-PCR measurement of AML *Indicator* genes.

Figure 4 Unsupervised cluster analysis for (A) all genes, (B) genes showing either the presence or the absence of significant differences in expression between bone marrow and peripheral blood and (C) genes with significant differences in expression between bone marrow and peripheral blood. Distinction between the bone marrow and peripheral blood samples is present only for clustering with the genes, showing difference in expression between the two samples, which shows clustering into two groups (labelled 1 and 2 in B), one composed mainly of bone marrow and one mainly of peripheral blood samples. Clustering in the space of all genes or genes without differences between the two samples failed to distinguish the two sample types, as shown by the presence of a dominant cluster (labelled 3 and 4 in (A) and (C), respectively). The normalised gene expression values for each sample were processed using the Cluster and TreeView software from the Eisen laboratory (<http://rana.lbl.gov/EisenSoftware.htm>).

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