Analysis of ZAP70 expression in adult acute lymphoblastic leukaemia by real time quantitative PCR

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Abstract

Background: ZAP70 gene expression is associated with poor prognosis in B-cell lymphoproliferative disorders especially chronic lymphocytic leukaemia (CLL) but its role in adult B-ALL has not been established. On diagnostic samples from 76 patients with adult ALL (65 with B-ALL and 11 with T-ALL) ZAP70 mRNA expression levels were studied by real time-quantitative PCR (RT-qPCR) analysis.

Findings: A broad distribution of ZAP70 expression was observed in ALL, ranging from 0.002 to 5.3 fold that of the ZAP70 positive Jurkat reference cell line. No association was observed between expression levels and the presence of specific cytogenetic abnormalities. Five cases, including one case of T-ALL, had ZAP70 expression above the level of the Jurkat reference cell line.

Conclusions: Our results confirm the frequent expression of ZAP70 in adult ALL. Limited comparisons made did highlight poor-risk patients with high ZAP70 expression, but due to lack of clinical information on patient samples we were unable to directly assess the impact on disease prognosis. ZAP-70 may be an important laboratory assay in adult ALL and further studies are warranted to study a potential correlation with cytogenetic and other genetic markers.

Keywords: ZAP70, ALL, RT-qPCR

Background

Zeta associated protein tyrosine kinase (ZAP70) is a 70kD molecule associated with the ζ-chain of the CD3 T-cell receptor (TCR) complex [1]. On formation of the immunological synapse, immune receptor tyrosine-based activation motifs (ITAM) in the CD3 and ζ-chains are activated by phosphorylation. The ζ-chains become docking sites for ZAP70 thereby activating the MAP kinase, calcium/calcineurin and protein kinase C signalling pathways. Thus ZAP70 plays a major part in lymphocyte signal transduction resulting in cell differentiation and proliferation [2].

ZAP70 gene expression plays a critical role in the transition of pre-B to pro-B cells within the bone marrow. Genetic inactivation of the ZAP70 gene results in failure of pre-B cell receptor (pre-BCR) induced differentiation, proliferation and heavy chain exclusion, and a failure to progress beyond the pre-B cell stage [3]. ZAP70 is not expressed in normal mature B cells derived from bone marrow, peripheral blood, or tonsil [4]. ZAP70 expression is also observed in a large proportion of patients with chronic lymphocytic leukaemia (CLL) where it has been associated with poor clinical outcome in several studies [5]. More recently, ZAP70 protein has been detected in a wide variety of additional B-cell lymphoproliferative disorders including mantle cell lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma [6,7].

While several groups, including our own, have demonstrated ZAP70 mRNA expression in paediatric pre-B ALL [8], there have been no previous studies that have used RT-qPCR to determine ZAP70 expression in adult cases of ALL and then correlated the results with cytogenetic abnormalities at diagnosis. In the present work we have used RT-qPCR analysis to determine the expression of ZAP70 mRNA levels in 76 adult patients with B-ALL or T-ALL at the time of diagnosis. We show that ZAP70 gene expression is detected in almost all cases with a broad range of
expression levels across the cohort. No association was observed between ZAP70 expression and cytogenetic abnormalities identified in these patients.

**Results**

In total we examined 76 cases of ALL, of which 65 were of B-cell origin (B-ALL) and 11 of T-cell origin (T-ALL). ZAP70 mRNA expression was measured as fold increase/ decrease relative to expression levels in the reference ZAP70 positive Jurkat cell line Table 1. A broad range of distribution of ZAP70 mRNA expression (range 0.002-5.36; median 0.169; mean 0.306) (Figure 1a) was observed. As expected, ZAP70 expression was seen in all cases of T-ALL (range 0.09-2.955; median 0.233; mean 0.504) (Figure 1b). Overall, 5 cases (4 B-ALL, 1 T-ALL) expressed ZAP70 at higher levels than the reference ZAP70 positive Jurkat cell line (Figure 1b).

We then searched for potential correlations between ZAP70 expression and known genetic abnormalities within the B-ALL tumours. 53/65 (82%) in the B-ALL group had cytogenetic changes such as t(9;22) (n = 18), 9p abnormality (n = 12), t(1;19) TCF3-PBX1 gene fusion, (previously called E2A-PBX1) (n = 3) and a range of other abnormalities, including Burkitt lymphoma, hyperdiploidy, hypodiploidy, monosomy 7 and 12p abnormality (n = 20). 12 cases of B-ALL had no observable cytogenetic abnormality. No association was observed between the level of ZAP70 expression and individual cytogenetic subgroups (Figure 1c), although we noted a statistically insignificant trend towards increased levels of ZAP70 mRNA in cases with monosomy 7 and 12p abnormalities (data not shown). No association was found between ZAP70 mRNA expression and the ZAP70 copy numbers based on cytogenetic data (Additional file 1).

While a relatively continuous distribution pattern of ZAP70 mRNA levels was seen across the B-ALL cohort, the level of expression was markedly increased in 4 cases, with values ranging from 1.1-5.4 (mean 2.4). Of these B-ALL patients, two had complex cytogenetics whilst the other two had t(9;22) translocations thus predicting poor outcomes in all four cases [9]; however the remaining 16 patients who carried a t(9;22) cytogenetic abnormality had ZAP70 expression levels within the main distribution. Similar to CLL patients where a high ZAP70 expression level is associated with a poor prognosis, ZAP70 mRNA expression may be relevant to the prognosis of patients with B-ALL.

**Discussion**

This is the first distribution profile for ZAP70 mRNA expression in adult B-lineage ALL patients by RT-qPCR. The results demonstrate a broad range of expression and a markedly increased expression in a small proportion of cases (6%). Chiaretti et al. [10] used microarray analysis to determine ZAP70 mRNA expression in 95 adult ALL cases followed by immunoblotting to confirm protein expression. In their study, relatively high ZAP70 expression levels were observed in patients with the t(1;19) TCF3-PBX1 gene rearrangement but similar high ZAP70 levels were not seen in our cohort. CDKN2A, a tumour suppressor gene on chromosome 9, can be inactivated by deletion, mutation or methylation. Its role in B-ALL is currently under dispute and has been identified by some groups to have a prognostic role in childhood and adult ALL [11-15]. We analysed patients with 9p abnormalities, without t(9;22), t(1;19), t(8;14), and found no association with ZAP70 mRNA levels.

Although the biochemical basis for the correlation between ZAP70 expression and poor prognostic aggressive

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**Table 1 Patient demographic and cytogenetic data**

| Type          | Cytogenetics          | Cases | M/F | Age (yr) | ZAP70 expression | Associated abnormalities |
|---------------|-----------------------|-------|-----|----------|------------------|-------------------------|
| T-ALL         |                       | 11    | 6/5 | 44.7; (19-56) | 0.363; 0.252; (0.000-2.955) | 9p abnormality (n = 5) 6q deletion (n = 2) (10;14) (n = 1) |
| B-ALL         |                       | 65    | 36/29 | 46.0; (18-79) | 0.332; 0.185; (0.002-5.360) |
| B-ALL         | t(9;22)               | 18    | 10/8 | 44.6; (23-70) | 0.440; 0.191; (0.010-5.360) | Hyperdiploid (n = 2), Monosomy 7 (n = 2) 9p abnormality (n = 3)12p gain (n = 2) t(1;19) (n = 1) |
| B-ALL         | t(1;19) TCF3-PBX1     | 3     | 2/1  | 43.3; (42-52) | 0.219; 0.210; (0.193-0.254) | 9p abnormality (n = 2) 6q deletion (n = 2) |
| B-ALL         | 9p abnormality        | 12    | 8/4  | 45.2; (18-72) | 0.180; 0.176; (0.004-0.535) | Monosomy 7 (n = 2) RUNX1 (n = 2) 12p abnormality (n = 3) 6q deletion (n = 2) |
| B-ALL         | Burkitt lymphoma      | 6     | 3/3  | 44.7; (22-78) | 0.159; 0.168; (0.005-0.350) | 12p abnormality (n = 1) 9p abnormality (n = 2) |
| B-ALL         | Hyperdiploid          | 6     | 5/1  | 45.9; (22-76) | 0.232; 0.184; (0.004-1.383) |
| B-ALL         | Monosomy 7            | 4     | 1/3  | 44.9; (37-58) | 0.177; 0.182; (0.015-0.399) | 12p abnormality (n = 1) |
| B-ALL         | Hypodiploid           | 3     | 1/2  | 43.7; (18-79) | 0.173; 0.182; (0.067-0.290) | 12p abnormality (n = 1) |
| B-ALL         | MLL/AFF1              | 1     | 1    | 30.0 | 0.089 | 12p abnormality |
| B-ALL         | Normal 46, XX or 46, XY | 12   | 6/6  | 46.7; (18-82) | 0.140; 0.100; (0.002-0.291) |

* Mean patient age in years (range).

**ZAP70 RNA expression was quantified by RT-qPCR and the normalised values expressed relative to that seen in Jurkat control cells. Data shown are mean, median and range for each sub-group.**
disease in CLL is unknown, an association with enhanced signal transduction through the pre-BCR complex and phosphorylation of phosphotyrosine phosphatase has been observed [16]. Unfortunately clinical information on patients recruited into this study could not be obtained, though the cytogenetic information on patients with high $ZAP70$ mRNA levels suggests possible inferior outcomes. This potential association of high $ZAP70$ mRNA levels with inferior outcomes may not be independent, as the cytogenetic findings in the four patients with the highest $ZAP70$ expression, i.e. complex or t(9;22), also predict a poor prognosis. However cytogenetic assessment can be difficult [17] and therefore $ZAP70$ mRNA expression levels may have a role as an alternative prognostic marker in patients with adult B-ALL. This in turn may allow escalation or de-escalation of therapeutic strategies as well as the possibility of using the $ZAP70$-specific inhibitor, Piceatannol [18] as an

Figure 1 $ZAP70$ expression in adult ALL patients. (a) $ZAP70$ RNA expression in adult ALL patients quantified by RT-qPCR. (b) $ZAP70$ expression in combined adult B-ALL and T-ALL patient cohorts. (c) $ZAP70$ expression in adult B-ALL patients classified into different cytogenetic groups. The cytogenetic group classified as others includes Burkitt lymphoma, hyperdiploid, hypodiploid, monosomy 7 and MLL/AFF1. In each panel, $ZAP70$ mRNA levels were normalised to GAPDH expression and then reported as a fold increase/decrease relative to $ZAP70$ expression in the Jurkat cell line (set as an arbitrary value of 1.0); n = number of patients.
adjuvant in ALL therapy. Large cohort studies in adult ALL patients addressing the role of ZAP70 mRNA expression levels in association with cytogenetic and other genetic markers are hence warranted.

Methods

Patient samples

Bone marrow specimens were collected at disease presentation from adult ALL patients attending various centres in West Midlands, UK and referred to the West Midlands Regional Genetics Laboratory between 1998 and 2005, as part of routine genetic analysis of the leukaemias. Bone marrow mononuclear cells (BMMC) were isolated either by Ficoll density centrifugation or by red cell lysis using Erythrocyte Lysis Buffer (Qiagen, Crawley, UK). BMMC were lysed and total RNA was recovered in 60 μl RNAse free water using QiAamp spin columns (QiAamp RNA Mini Kit, Qiagen, Crawley, UK). Excess material after diagnostic testing was stored within the ethically approved Central England Haematology Research Biobank (REC reference: 09/H0405/12). Anonymised cDNA samples from the biobank were used for the ZAP70 study. Samples included in the study were from consecutive cases where sufficient excess material remained in patients older than 16.

Routine cytogenetics, FISH and endpoint RT-PCR

Cytogenetic analysis was performed as part of routine analysis using standard methods (G-banding). FISH analysis for MLL rearrangements, BCR-ABL1 gene fusions, and ETV6-RUNX1 gene fusion was routinely performed on all ALL samples [17], using commercial probes following manufacturer’s protocols (Vysis LSI MLL, Dual Color, Break Apart Rearrangement Probe; Vysis LSI BCR/ABL Dual Color, Dual Fusion Translocation Probe; Vysis LSI ETV6/TEL/RUNX1(AML1) ES Dual Color Translocation Probe Set) (Abbott Molecular, Illinois, USA). In addition, other FISH probes were used variably due to suspicion on G-band analysis and changes in routine FISH screening policies during the period when the samples were collected (Vysis LSI p16 (9p21) SpectrumOrange/CEP 9 SpectrumGreen Probe; MYC Break Apart FISH Probe Kit; IGH/MYC/CEP 8 TriColor DF FISH Probe Kit; LSI IGH Dual Color, Break Apart Rearrangement Probe; CEP4 and CEP10 probes (Abbott Molecular, Illinois, USA); and Dako SIL-TAL1 FISH DNA Probe, Sub-Deletion Signal (Dako, Glostrup, Denmark)). End-point RT-PCR for BCR-ABL1 transcripts was performed routinely using standard procedures [19].

Quantification of ZAP70 expression by RT-qPCR

Relative quantitation of ZAP70 mRNA expression was performed using an ABI 7700 Sequence Detection System (Applied Biosystems UK) and analysed using SDS software 1.7. ZAP70 transcripts were detected using the following primers and probe: forward primer 5’-CGCTGCACAA GTTCCCTGGT-3’, reverse primer 5’-GACACCTGGTG GCAGAGCT-3’, Taqman probe 5’-(FAM)-CATTGCTGTCACAGGATCTCTCCCCTCT-(TAMRA)-3’. GAPDH transcripts, which served as an internal control, were quantified using a commercial assay (Applied Biosystems, UK). PCR amplification and data normalisation were performed as previously described [8]. These normalised ZAP70 to GAPDH ratios were then calculated as a fold change relative to the ZAP70 to GAPDH ratio of the ZAP70 positive Jurkat T-cell line, defined as having an expression level of 1.0. All test samples were run in duplicate (mean values used) and template-negative samples served as control and were always negative.

Statistical analysis

Statistical analysis was performed by group comparison using either a two-sample test or ANOVA and correlation analysis was evaluated with Pearson coefficient, using GraphPad Prism 4 and SPSS software.

Additional file

Additional file 1: Table S1. ZAP70 expression data and cytogenetic analysis of individual ALL samples.

List of abbreviations

ALL: Acute lymphoblastic leukaemia; CLL: Chronic lymphocytic leukaemia; ZAP70: Zeta associated protein70.

Competing interests

The authors declare no competing interests.

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Author’s contributions

PM and MG conceived and co-ordinated the study. GC and AB carried out molecular genetic studies. GC, AB and MG analysed the data. GC drafted the manuscript. PM, MG and FW participated in study design and helped in drafting the manuscript. All authors read and approved the final manuscript.

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