Clinical quantitation of diagnostic and predictive gene expression levels in follicular and diffuse large B-cell lymphoma by RT-PCR gene expression profiling

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Running title: Lymphoma gene signatures from globally amplified polyA cDNA
Abstract

Recent microarray gene expression profiling studies have identified gene signatures predictive of outcome, so-called “Indicator” genes, for diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). However, measurement of these genes in routine practice remains difficult. We applied real-time PCR, to polyA cDNAs prepared from 106 archived human frozen lymph nodes (63 of FL, 25 of DLBCL, 10 reactive lymph nodes and 4 cases with paired samples of FL (4) and subsequent DLBCL (4)). Reverse transcription and polyA RT-PCR was performed and resultant cDNA probed by real-time PCR for 36 candidate Indicator genes, selected from microarray studies. Nine genes showed statistically significant different expression between FL and DLBCL, including Cyclin B, COL3A1, NPM3, H731, PKC.B1, OVGL, ZFPC150, HLA-DQ-a, and XPB. Of these, Cyclin B, NPM3, and COL3A1 were higher in DLBCL. Six genes showed statistically significant higher expression in the neoplastic nodes compared to reactive nodes, namely PKCB-1, BCL-6, EAR2, ZFX, Cyclin B, YY.1. High levels of YY.1 were associated with a shorter survival interval in both FL and DLBCL. The method is simple, sensitive and robust, facilitating routine use and may be used as a platform for clinical measurement of prognostic gene signatures.
**Introduction**

Microarray gene expression profiling has identified gene signatures, or “Indicator” genes, predictive of outcome in many cancer types including diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL).\(^1\,^2\,^3,^4\) There is now an urgent need to translate these signatures to clinical use. However, gene microarrays rely on relatively large amounts of fresh starting tissue obviating measurement of Indicator genes in routine practice \(^1\) and there is a need for development of another, simple, robust, relatively inexpensive and sensitive method for their translation to clinical use. To test the use of Indicator genes as a diagnostic tool, specifically in lymphoma, we have developed a simple, practical polyA PCR based method for analysis of Indicator profiles in DLBCL and FL, using very small tissue samples.

PolyA PCR co-ordinate 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.\(^5,^6,^7\) PolyA PCR enables global mRNA amplification from picogram amounts of RNA and has been routinely used to analyse expression in small samples including single cells.\(^8\) The polyA cDNA pool generated is also indefinitely renewable and as such represents a “molecular block”.\(^9,^10\) The polyA cDNA can be then be assayed for the expression of particular genes, either by hybridization with cDNA microarrays\(^9\) or by real-time PCR. Real-time PCR measurement however, enables more precise quantitation of the expression levels of specific Indicator genes. As such it is better suited for measurement in the clinical arena, whilst also focussing on diagnostically relevant Indicator genes. This approach thereby enables gene signatures to be detected within very small amounts of starting material.\(^11\)

In this study, PolyA RT-PCR was applied to RNA extracted from archived human frozen lymph nodes and the resultant cDNA analysed by TaqMan\(^\text{TM}\) real-time PCR for 36
Indicator genes (abstracted from\(^2,3,4\)) in order to find prognostic and diagnostic genes within this set of microarray identified candidate genes.

**Material and methods**

**Clinical samples**

One hundred and eleven archived human frozen lymph nodes (Ln), (with at least 5 years follow up) with a diagnosis of follicular lymphoma or diffuse large B-cell lymphoma were obtained, with informed consent, from the archives of the Christie Hospital NHS Trust, Manchester, UK. This work was approved by the Central Manchester Multicentre Research Ethical Committee (MREC). The samples were selected from the archive on a sequential chronological basis, taking all samples for which consent was given, in the archive from 1995 backwards to 1989. These were initially assessed for suitability on the basis of biopsy size, amount of necrosis and confidence of diagnosis. All cases unlikely to have sufficient material for analysis, with more than 10% necrosis or with uncertain or complex / mixed diagnoses were excluded. Following this initial selection 111 cases were available for study and paraffin embedded sections from these cases were reviewed by two pathologists (RJB & LPM). In five of the cases the diagnosis was revised to Mantle Cell Lymphoma and excluded from the study. The remaining 106 cases were classified into the following groups (N refers to numbers of frozen samples):

- **Group 1**: Follicular lymphoma WHO (grade 1 or 2) without evidence of subsequent transformation (FL) (number = 63)

- **Group 2**: Diffuse large B- cell lymphoma arising de novo without any evidence of pre-existing follicular lymphoma, (DLBCL) (number = 25).

- **Group 3**: Paired frozen samples of pure follicular lymphoma WHO (grade 1 or 2) (frozen tissue available) and subsequent transformed diffuse large B-cell lymphoma (frozen tissue available) [FL(F) pre t-DLBCL (F)] (number = 4); these were matched pairs with samples in group 4.
Group 4: Paired frozen samples of diffuse large B-cell lymphoma (frozen tissue available) for which there had been a previous biopsy showing pure follicular lymphoma WHO (grade 1 or 2) (frozen tissue available) [DLBCL (F) post FL (F) (number = 4); these were matched pairs with samples in group 3.

Group 5: Reactive lymph nodes (RLN) (number = 10).

The groups and clinical data are summarised in table 1.

Table 1

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Table 1: Groups, clinical data and samples used in the study (F=frozen tissue, t= transformed,), detail of composition of each group are detailed in the above text

**Extraction of RNA and global amplification of Poly Adenylated mRNAs (PolyA RT-PCR)**

The lymph nodes were homogenised using a Mixer Mill MM 300 (Qiagen, Crawley, West Sussex, UK). Total RNA was then extracted an RNeasy™ mini kit (Qiagen), as recommended by the manufacturer; DNase was used to remove the contaminating genomic
DNA, and PolyA RT-PCR carried out. Global amplification of cDNA corresponding to all expressed genes (polyA PCR), was carried out as previously reported.

**Specific RT PCR**

Taqman™ PCR primers and probes were designed for 36 Indicator genes and 4 housekeeping genes using Primer Express Software (Applied Biosystems, CA, USA) and are listed in Table S1. All PCR primer pairs were designed for mRNA sequence within 300bp of the 3’ end of each Indicator gene (Table S1 in supplemental data) and were tested in PCR reactions carried out in 25 µl containing 1ng PolyA cDNA, 0.33 µmol/L of each oligonucleotide, 0.5 units Ex-Taq polymerase (TaKaRa), and 0.25 µmol/L dNTPs in the buffer supplied by the manufacturer. PCR was performed using the following thermal cycle: 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C and one minute at 72°C.

**Taqman™ real-time quantitative PCR**

For each gene, Taqman™ PCR was applied to 1ng PolyA cDNA from each sample and to 10 microlitres of serially diluted human genomic standards, using a Taqman™ Gold kit. All samples were analysed using an ABI Prism 7700 sequence detection system. The copy number of each gene was determined by reference, after normalisation to Mhouse (below), of the real-time PCR expression level to human genomic DNA standards.

**Normalisation**

The expression levels of four housekeeping genes (IF2-beta, GAP, human ribosomal protein S9 mRNA and Beta actin) were measured by RT-PCR in each sample. Copy numbers obtained for the mean (Mhouse) of the four housekeeping genes (IF2-b, GAP, RbS9 and Beta actin) in each sample were divided by the highest Mhouse in all samples resulting in a normalisation correction factor. Following real time PCR amplification and quantification of the selected genes, this factor was then used for normalisation of expression levels of each of the 36 genes measured. Specifically the expression level of each gene (Ct value) was quantified at least twice against a standard curve obtained from a serial dilution of human
sonicated DNA. For each gene an equation, formulated from the best standard curve, was used to calculate copy number. The following specific example is given for GAP where 
\[
\text{Copy number} = 10^A((A-38.325)/-3.64) \times B, \quad (A= \text{Mean of Ct values}, \ B= \text{Dilution factor}, \ S\text{lope}= 3.64, \ Y-\text{Intercept}= 38.325).
\]
Finally, copy numbers obtained for the mean of the IF2-b, GAP, RbS9 and beta actin housekeeping genes (Mhouse) expression levels were divided by the highest mean value obtained in the experiment, resulting in a correction factor. All data was then normalised using this factor.

\textbf{Statistical analysis}

The data was not normally distributed and therefore non-parametric tests were used. Statistical analysis of the expression levels of the 36 genes in the six groups was performed using the Mann Whitney test and Kruskal-wallis with a \(p \leq 0.05\) for statistical significance. All the tests were performed using SPSS software v13 (Woking, Surrey, UK). As the expression levels for all the genes falls across a large range, the a log2 scale was used to plot all values to aid visual comparison. The ranking of the expression levels of \textit{Indicator} genes between samples was presented as the mean rank statistical difference.\(^{10}\) Kaplan-meir survival analysis using a log rank test was also performed.

\textbf{False discovery analysis}

Since multiple statistical tests were performed, False Discovery Rate analysis was performed to identify genes significant after accounting for multiple testing. Specifically, the siggenes package in bioconductor\(^{13}\) (Gentleman \textit{et al}, 2004), was used to estimate False Discovery Rate (FDR) by applying the SAM method\(^{14}\) on the log2 data, in the usual way. Local FDR was not computed, because it was considered to be unreliable due to the relatively small number of transcripts available to compute the underlying distributions. Instead, gene lists were generated at FDRs of approximately 5, 8 and 11% for each
comparison; membership to each of these sets is reported in the results. \( \Delta \) and corresponding FDR rates are as follows: comparison 1; \( \Delta = 0.65 \); FDR=5.5\%; \( \Delta = 0.50 \); FDR=8.0\%; \( \Delta = 0.1 \); FDR=11.0\%; comparison 2; \( \Delta = 1.0 \); FDR=6.0\%; \( \Delta = 0.80 \); FDR=8.6\%; \( \Delta = 0.2 \); FDR=11.8\%; comparison 3 pt1; no suitable values of \( \Delta \) found, pt2: \( \Delta = 1.5 \); FDR=4.1\%
Results

PolyA cDNA was generated from mRNA extracted from all the 106 archived human frozen lymph nodes. The copy number for each gene in each sample was reproducible over at least duplicate tests demonstrating reliability of the method. Specific PCR of these polyA cDNAs was positive for each of the selected Indicator genes, and bands of appropriate sizes were present in all samples, demonstrating presence of the target transcript in the polyA cDNA (data not shown). There was no statistically significant difference in the value of Mhouse for the different diagnostic groups (data not shown). Following normalisation of the data the comparisons detailed below were made:

1 Comparison of expression between FL (group 1), DLBCL (group 2) and RLN (group 5)

Ten genes, namely PKC.B1, OVGL, ZFPC150, BCL6, XPB, EAR.2, ZFX, NPM3, CYCLIN B, YY.1 showed statistically different expression between reactive, FL and DLBCL samples, of which PKC (P ≤ 0.004), OVGL (P ≤ 0.025), ZFPC150 (P ≤ 0.031), BCL-6 (P ≤ 0.001), XPB (P ≤ 0.048), EAR.2 (P ≤ 0.032), ZFX (P ≤ 0.004), were upregulated in FL compared to reactive Ln, whilst NPM3 (P ≤ 0.052), YY.1 (P ≤ 0.031) were upregulated in FL and DLBCL compared to reactive Ln, and cyclin B (P ≤ 0.012) was upregulated in DLBCL compared to FL and reactive LN, samples (figure 1). Comparison between both FL and DLBCL (n=88) with reactive Ln (n=10), identified six genes showing statistically significant difference, specifically PKCB-1 (P ≤ 0.042), BCL-6 (P ≤ 0.001), EAR2 (P ≤ 0.041), ZFX (P ≤ 0.007), cyclin B (P ≤ 0.045), YY.1 (P ≤ 0.020) (ACTA, and HSF were close to significance at p ≤ 0.053), each of which showed higher expression in the neoplastic nodes (figure 2). With a False Discovery Rate (FDR) of 5.5%, 3 of these genes were found to be differentially expressed, (BCL6, YY1,ZFX), at 8.0%, 6 of them (PKCB1,BCL6,XPB,ZFX,CYCLINB,YY1) and at 11%, 9 of them (PKCB1, OVGL, ZFPC150, BCL6, XPB, ZFX,NMP3,CYCLINB,YY1).
Comparison of expression between FL (group 1) and DLBCL (group 2)

Nine genes showed statistically significant difference in expression between FL (n=63) and DLBCL (n=25) in frozen samples, of which cyclin B (p≤0.025), COL3A1 (p≤0.025) and NPM3 (p≤0.024) were upregulated in FL, and H731 (p≤0.02), PKC.B1 (p≤0.01), OVGL (p≤0.019), ZFPC150 (p≤0.030), HLA-DQ-a (p≤0.041) and XPB (p≤0.024) in DLBCL (figure 3). With an FDR of 6.0%, 6 of these genes were found to be differentially expressed (H731, PKCB1, OVGL, ZFPC150, HLA-DQ-a, XPB), the same 6 at 8.6%, (H731, PKCB1, OVGL, ZFPC150, HLA-DQ-a, XPB) and at 11.8%, 9 (CYCLINB, COL3A1, NPM3, H731, PKC.B1, OVGL, ZFPC150, HLA-DQ-a and XPB).

Comparison of expression between; i) matched samples of FL before (group 3) and after (group 4) transformation to DLBCL, and ii) FL before transformation (group 3) and FL without subsequent transformation (group 1).

Only one gene, PKCG (p≤0.029), showed statistically significant different expression between matched cases of FL before transformation (group 3) and after transformation to DLBCL (group 4) (figure 4a). It was not possible to choose an appropriate value for delta to yield reasonable FDRs, probably a consequence of the small number of replicates in the comparison. Conversely, 2 genes showed statistically significant different expression in cases of FL that either did (group 3) or did not (group 1) go on to transform, namely ACTA (p≤0.046) and HLA-DQ-a (p≤0.029) (figure 4b); at an FDR of 4.5%, one gene (HLA-DQ) was identified.

Comparison of expression between cases of FL and DLBCL dead or alive after follow-up

YY.1, showed statistically significant (p≤0.047) upregulation in those patients with FL dead (n=47) compared to those alive (n=16) after follow up (Figure 5a). Several genes, namely, HSP40 (p≤0.007), MINOR (p≤0.020), H731 (p≤0.010) and YY.1 (p≤0.003) showed
statistically significant difference in expression between patients with DLBCL dead (n=22) and alive (n=3) after follow up (Figure 5b).

5 Survival analysis for all cases of FL and DLBCL

For both FL and DLBCL, the data for each gene was grouped into 4 quartiles for use in Kaplan-meir survival analysis including all cases, both dead and alive (Figure 6a). In FL, three genes showed statistically significant association with survival, namely XPB, TNF and YY.1, and specifically high levels of YY.1 were associated with shorter survival interval (Figure 6b). In DLBCL, three genes showed statistically significant association with survival, namely YY.1, TNF, and HLA-DQ-a. High levels of YY.1 and low levels of HLA-DQ-a and TNF were associated with shorter survival interval (Figure 6c).
Discussion

Recent gene expression profiling has identified gene signatures predictive of outcome, so-called *Indicator* genes, for diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL). However, measurement of *Indicator* genes in routine practice remains difficult. We have demonstrated utility of real-time PCR measurement of *Indicator* genes in globally amplified polyA cDNA as a practical method for their clinical analysis. PolyA PCR enables global mRNA amplification from picogram amounts of RNA and the polyA cDNA pool generated is indefinitely renewable, representing a “molecular block”. Real-time PCR measurement of the expression levels of specific *Indicator* genes then allows gene signatures to be detected in the polyA cDNA. In this project we applied real-time PCR for 36 *Indicator* genes, to polyA cDNAs prepared from 106 archived human frozen lymph nodes; specifically 63 cases of FL, 25 of DLBCL, 10 reactive lymph nodes and 4 cases with paired samples of FL and subsequent DLBCL were analysed. PolyA RT-PCR was performed on extracted RNA and the resultant cDNA probed for 36 candidate *Indicator* genes (selected from 2,3,4), by real-time PCR with quantification against human DNA, and normalisation to the mean of four housekeeping genes.

Nine genes showed statistically significant different expression between FL and DLBCL, including cyclin B, COL3A1, NPM3, H731, PKC.B1, OVGL, ZFPC150, HLA-DQ-a, and XPB. Of these, cyclin B, a cell cycle gene, NPM3, nucleolar phosphoprotein, and COL3A1 were higher in DLBCL. Interestingly, both cyclin B and NPM3 have been associated with mitogenesis in tumours in other studies, concordance with upregulation in DLBCL which is a more aggressive tumour, with a higher cell growth fraction, than FL. Interestingly, Okuda et al 15 demonstrated that NPM1, a nucleolar phosphoprotein, is a target of another cell cycle protein, CDK2/cyclin E, and the same association may explain the upregulation of both cyclin B and NPM3 in DLBCL. The upregulation of Col3A1 in DLBCL is harder to explain, though this has also been reported for advanced compared to local ovarian carcinoma16,
though this probably reflects tumour associated fibroplasias, which may not be relevant for lymphomagenesis. Of the genes downregulated in DLBCL, of particular interest was the reduction in expression of XPB, a DNA helicase showing lower expression in more aggressive splenic marginal zone lymphoma.\textsuperscript{17} Of the remaining genes, HLA-DQ-a and OVGL, both downregulated in DLBCL, were also downregulated in cases of FL with foci of transformation, indicating a possible role in the process of transformation. Mutations in the HLA class II genes leading to loss of expression of HLA-DQ have been reported in DLBCL\textsuperscript{18}, whilst the importance of immune cells in outcome of FL has also been recognised\textsuperscript{19}, supporting such a role. OVGL has been associated with ovarian cancer\textsuperscript{20} but its role in lymphoma is obscure; it was over expressed in cases of DLBCL refractory to treatment\textsuperscript{4}.

Six genes showed statistically significant different expression between the neoplastic (FL and DLBCL) and reactive LNs. Of these two, PKCb-1 and ZFX, also distinguished between FL and DLBCL. ZFX is a zinc-finger binding protein present in prostatic adenocarcinoma\textsuperscript{21}, consistent with our finding of upregulation in the neoplastic nodes. Of greater interest however, is the finding of upregulation of cyclin B and Bcl-6 in the neoplastic nodes, cell cycle and anti-apoptotic genes that have been associated with malignancy in many tissues, and particularly in lymphoma, in previous studies. Ear2 encodes a protein with homology to a steroid receptor, and binds to the TGACCT direct-repeat motif\textsuperscript{22}, but is role in lymphogenesis is unclear. YY.1 (Ying-Yang1) is a zinc-finger protein reported to positively regulate IL-4 gene expression in lymphocytes\textsuperscript{23}. It has also been associated with prostatic cancer, but interestingly in this study was also up regulated in cases of FL dead after follow-up. YY.1 inhibits FAS-induced apoptosis and its down-regulation increased sensitivity of an NHL B-cell line to Rituximab\textsuperscript{24}, whilst the same group also show that the regulation of Fas resistance by NF-kappa B is mediated via YY.1 expression and activity.\textsuperscript{25}

In support of this possible role, YY.1 was also up regulated in those cases of DLBCL dead
after follow up, though the numbers of patients alive with DLBCL after follow up were low.

Whilst distinction between FL and DLBCL can already be made by conventional morphological and immunohistochemical means, prediction of outcome of FL is less easy, though recent microarray studies have identified an immune signature associated with survival. This signature has not yet been validated in further studies, though YY.1 was associated with death in this study. FL often leads to death following transformation to DLBCL and a signature predictive of this at initial presentation would be clinically useful as it could direct more aggressive treatment or inform closer follow up. In this study we identified several genes that may fulfil this role. Specifically, ACTA, HLA-DQ-a were significantly upregulated in cases of FL that subsequently transformed to DLBCL. However, the number of cases of FL with subsequent transformation was low and these results therefore require validation in a prospective study. Considering the process of transformation in the matched cases of FL with subsequent DLBCL, PKCG, which encodes for protein kinase C-gamma, was up regulated upon transformation. It has not been associated with cancer, though PKC-beta over expression is associated with decreased survival in DLBCL. 26

Kaplan-Meir survival analysis identified a small number of genes associated with survival interval in both FL and DLBCL. Of these high levels of YY.1 were associated with a shorter survival interval in both FL and DLBCL. This is of particular interest given the reported role of YY.1 in producing resistance to Rituximab due to downregulation of FAS–induced apoptosis.25 This was reported for a cell culture study and has not been shown in clinical studies, though the result in this project suggests a possible detrimental role for YY.1 clinically and that it may act as a predictor of Rituximab response. Upregulation of HLA-DQ-a was associated with longer survival interval, in agreement with its role as a positive predictor of survival in the work of Rosenwald et al. 3 TNF showed differential association with survival interval in FL and DLBCL in which high levels were associated with short and long survival
intervals respectively; why this is so is unclear, though it may reflect fundamentally different responses of the immune system to either indolent (FL) or aggressive (DLBCL) disease. Comparison of the gene expression level in patients with either FL or DLBCL alive or dead at the end of the study interval confirmed the importance of YY.1 as a predictor of outcome. Interestingly however, the other genes discriminatory between patients dead and alive at the end of the study period differed from those predictive of length of survival by Kaplan-Meir survival, though the genes in each analysis were statistically significant. This indicates a bi-modal survival model in which patients with a lower survival chance express certain genes, namely, at higher levels than those with a higher survival chance, since the genes expressed at higher levels in those dead of disease do not match to those predictive of length of survival, with resultant divergence of survival lines in Kaplan-Meir analysis into two broad groups, one with long survival and one with relatively short survival. This suggests that FL comprises two, at least, groups, one in which there is relatively short survival and another of long term survivors; this matches clinical experience.

This project 1) demonstrates the possibility of using polyA PCR for global amplification of clinical samples and real-time PCR to measure diagnostically informative gene expression profiles in the resultant polyA cDNA “molecular block” & 2) identifies novel prognostic markers for FL and DLBCL. The method is simple, sensitive and robust, allowing translation into routine clinical use, and whilst lymphoma represents a relatively small group of cancer patients the generic nature of microarray gene profiles for cancer subtypes will facilitate simple extension of the method to other patient groups.
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The authors declare that there is no conflict of interest.
References


Figure 1: Expression levels (log 2) of genes with statically significant difference between FL (group 1), DLBCL (group 2) and reactive lymph nodes (group 5); for each gene box plots are shown in upper panel and mean rank statistic in lower panel.
Figure 2: Expression levels (log 2) of genes with statically significant difference between FL (group 1) and DLBCL (group 2) with reactive LN (group 5); for each box plots are shown in upper panel and mean rank statistic in lower panel.
Figure 3: Expression levels (log 2) of genes with statically significant difference between FL (group 1) and DLBCL (group 2); for each box plots are shown in upper panel and mean rank statistic in lower panel.
Figure 4: Expression levels (log 2) of genes with statically significant difference between a) samples of FL before (group 3 / FL pre t-DLBCL) and after (group 4 / DLBCL post FL) transformation and, b) between FL before transformation (group 3 / FL pre t-DLBCL) and FL without subsequent transformation (group 1 / FL); for each box plots are shown in upper panel and mean rank statistic in lower panel.
Figure 5: Expression levels (log 2) of genes with statically significant difference between cases of a) FL and b) DLBCL dead or alive after follow-up; for each for each box plots are shown in upper panel and mean rank statistic in lower panel.
Figure 6: Kaplan-Meir survival analysis of: a) FL vs DLBCL; b) FL (group 1) based on gene expression level, data shown for significant indicator genes, namely XBP, TNF and YY.1. The data for each gene was grouped into 4 quartiles for use in Kaplan-Meir survival analysis and these are indicated in the survival curves as 1 = 1st quartile, 2 = 2nd quartile, 3 = 3rd quartile & 4 = 4th quartile; 1st quartile used for lower end of gene expression for each gene.
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