High levels of BACH2 associated with lower levels of BCL2 transcript abundance in t(14;18)(q21;q34) translocation positive Non-Hodgkin's Lymphoma

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The t(14;18)(q21;q34) BCL2 translocation is a common genetic alteration in Follicular and Diffuse Large B-cell Lymphoma. However, it is not invariably associated with BCL2 gene over-expression due to undefined mechanisms that regulate expression from the proximal immunoglobulin heavy-chain (IgH) promoter. The BACH2 transcriptional repressor is able to modulate activity of this promoter. Here we have shown that, in tumor samples with BCL2 translocation, those with high levels of BACH2 had significantly lower BCL2 transcript abundance compared to those with low levels of BACH2. This indicates that BACH2 may be partially responsible for regulation of BCL2 expression from the t(14;18)(q21;q34) translocation.

Introduction

The t(14;18)(q21;q34) translocation is found in 85-90% of Follicular Lymphoma (FL) and 12-30% of Diffuse Large B-cell Lymphoma (DLBCL) tumors [1]. This aberration places the anti-apoptotic BCL2 oncogene under control of the immunoglobulin heavy-chain (IgH) promoter. Due to the activity of the Ig-promoter in B-cells, this translocation should result in the up-regulation of BCL2 expression, a phenotype associated with resistance to apoptosis chemotherapy. However, BCL2 protein abundance is only increased in approximately 50% of cases with the t(14;18)(q21;q34) translocation, indicating that other factors may control the expression of BCL2 from this aberrant locus.

The BACH2 gene encodes a transcriptional repressor that forms a co-repressor complex with small *MafK* proteins and binds MARE elements such as the HS1, HS2, HS3 and HS4 elements of the IgH locus [2], which have been shown to mediate BCL2 expression from the t(14;18)(q21;q34) translocation [3]. It is expressed at high levels in immature B-cells, translocates into the nucleus in response to reactive oxygen species (ROS), and is hypothesized to function in normal differentiating B-cells by preventing premature expression of immunoglobulins [2]. Transcript levels of BACH2 are used for the classification of DLBCL into COO subtypes [4], but have also been described as an independent prognostic factor [5]. In a Japanese study, immunohistochemical staining for BACH2 revealed that it is expressed in 30-33% of DLBCL cases, and patients with strong expression had a better prognosis than those with weak or variable expression [6]. However, BACH2 has not yet been investigated in FL patients or Caucasian DLBCL patients.

Due to the role of BACH2 in silencing transcription from the IgH locus, we hypothesised that BACH2 may regulate BCL2 expression in non-Hodgkin's Lymphoma (NHL) patients with the t(14;18)(q21;q34) translocation. In order to investigate this hypothesis a PCR-based assay was used to detect this translocation in FL and DLBCL tumor samples. Gene transcript abundance of BACH2 and BCL2 were also measured by quantitative real-time PCR (qPCR). Through this we have shown that patients carrying the translocation and expressing high levels of the BACH2 gene transcript have a significantly lower gene transcript abundance of BCL2, compared to those carrying the translocation and expressing low levels of BACH2. This suggests that BACH2 may have a role in regulating the expression of BCL2 from this translocation.

Materials and Methods

Tumor Samples and Nucleic Acid Extraction

Fresh-frozen diagnostic lymph-node specimens were obtained for seven FL and seven DLBCL samples from the Australian Leukemia and Lymphoma Group tissue bank (Queensland, Australia). RNA was extracted from 10mg of tissue from each sample by a combined TRIZOL/column purification protocol. Briefly, tissue was homogenized in 1mL of TRIZOL (Invitrogen) using a rotor-strator homogenizer and the phases separated according to the manufacturers protocol. The aqueous phase was then mixed with and equal volume of 70% ethanol and purified using the Micro-to-Midi RNA purification system (Invitrogen). Integrity of all RNA samples was interrogated with an Agilent BioAnalyser 2100 using the RNA Nano LabChip protocol. DNA was extracted by homogenizing 10mg of each sample in 1mL of

phosphate-buffered saline, followed by purification using a DNeasy Tissue Kit (Qiagen).

Detection of the t(14;18)(q21;q34) Translocation

The t(14;18)(q21;q34) translocation was detected using a nested-PCR assay previously described by Poeat and Sklar (1997). Briefly, 1µg of DNA is first amplified with outer primers specific for the BCL2 major break region (mbr-O), the BCL2 minor cluster region (mcr-O) and a degenerate immunoglobulin heavy-chain joining (J_H) region primer (Jh_O). This amplification was performed using a high processivity *Taq* DNA polymerase (BIO-X-ACT™ Long; Bioline), with the accompanied buffer, 1mM $MgCl_2$, 10uM of each primer, and 10µM of each dNTP. The second-round amplification was performed using 1µL of a 1:10 dilution of the first-round product and utilized nested primers for the same regions (mbr-I, mcr-I, Jh-I). The reaction was performed using the same reaction components but substituting normal-processivity *Taq* DNA polymerase (Invitrogen) and the corresponding reaction buffer. Primer sequences and cycling conditions were as previously described [6]. In order to increase sensitivity, the products of the second-round reaction were detected with an Agilent BioAnalyser using the DNA-1000 LabChip protocol.

cDNA Synthesis and Quantitative Polymerase Chain Reaction

Complimentary-DNA (cDNA) was reverse transcribed in triplicate reactions for each sample from 1µg of total-RNA using the Superscript III Reverse Transcription System (Invitrogen). The products from each set of triplicate reactions were then pooled. qPCR was performed with a RotorGene 6000 (Corbett Research) using SYBR Green chemistry, and normalized to the reference gene RPL13A. Gene-specific

primers for RPL13A, BACH2 and BCL2 can be seen in table 1. Reactions for each gene were performed in triplicate for each sample and consisted of 1X iQ SYBR Green Supermix (BioRad), 10µM of each primer and 1uL of cDNA. Cycling conditions consisted of an initial denaturation at 95°C for 10min followed by 40 cycles of 95°C for 20s, 60°C for 20s and 72°C for 30s. Melt curves were analyzed for each reaction to ensure amplification specificity and the absence of genomic DNA contamination. Relative expression of BACH2 and BCL2 was found by normalizing to Universal Human Reference RNA (Stratagene) using the $2^{-\Delta\Delta Ct}$ method as previously described [7]. Differences in BCL2 transcript abundance between groups was assessed using the independent-sample T-test.

Table 1: qPCR primers utilized in this investigation.

Gene	Forward Primer	Reverse Primer	Amplicon Size
RPL13A	ATCTTGTGAGTGGGGCATCT	CCCTGTGTACAACAGCAAGC	108bp
BCL2	GCTGAAGATTGATGGGATCG	TACAGCATGATCCTCTGTCAAG	80bp
BACH2	CACATTCAGTGCCAAGTGCT	ACCCCTCTTTGTCCAAGTCC	80bp

Results

Prevalence of the t(14;18)(q21;q34) Translocation

The t(14;18)(q21;q34) translocation was detected in a total of 57% (8/14) of NHL tumor samples. This included 71% (5/7) of FL cases and 43% (3/7) of DLBCL cases. The amplicon produced by the second-round reaction varied in size between patients due to variation in the position of BCL2 and J_H break-points. However, there was a clear distinction in amplicon sizes between two main groups of patients with the translocation; those with amplicon sizes of 58-60bp and those with amplicon sizes of 185-201bp (figure 1). The no-template control exhibited an amplicon at 18bp, which

is present a high proportion of samples without a translocation amplicon and is assumed to be due to primer-dimerisation as a result of the large size of the primers.
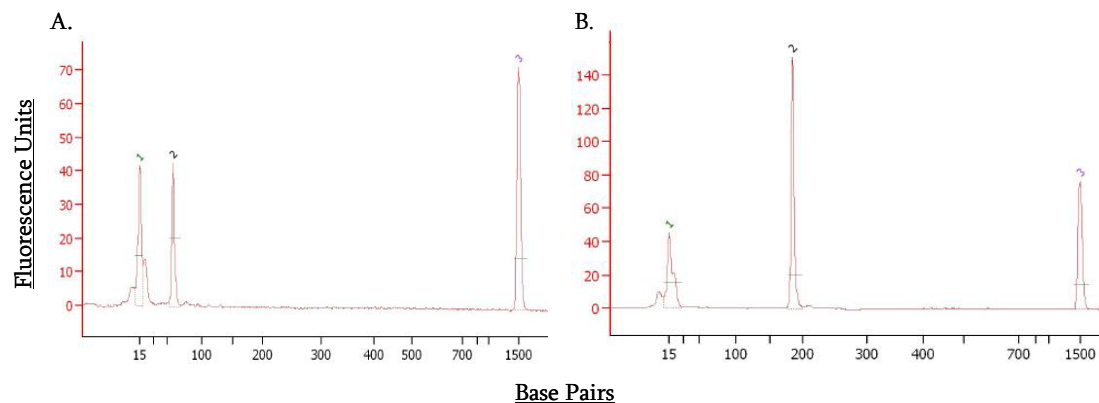

Figure 1: BioAnalyser electrophoretograms showing examples of the small (A) and large (B) translocation amplicons in from two NHL patients. Peaks labeled 1 and 3 are the BioAnalyser size markers, while the peaks labeled 2 are the translocations amplicons with sizes of 58bp and 185bp for A and B respectively.

BACH2 and BCL2 Gene Expression in Non-Hodgkin's Lymphoma Tumor Samples

There was a large degree of heterogeneity in BCL2 and BACH2 gene expression between patients. Samples carrying the translocation had higher average BCL2 gene transcript abundance, but this was not significantly different from those samples without the translocation (p=0.14). On average BACH2 was more highly expressed in samples without the translocation compared to those with the translocation, but again this was not statistically significant (p=0.47). There was no significant difference in either BCL2 (p=0.67) or BACH2 (p=0.94) between FL and DLBCL samples.

BCL2 expression from the t(14;18)(q21;q34) Translocation may be regulated by BACH2

Of the eight patients carrying the t(14;18)(q21;q34) translocation, the four patients with the highest relative expression of BACH2 were classified as 'high BACH2'

(mean relative BACH2 expression = 2.25) expressers and the four patients with the lowest relative expression of BACH2 were classified as 'low BACH2' expressers (mean relative BACH2 expression = 11.78); the difference in relative BACH2 transcript abundance between these two groups is statistically significant ($p<0.01$). Low BACH2 expressers with the translocation had significantly higher relative BCL2 transcript abundance compared to high BACH2 expressers with the translocation (Figure 1; $p=0.02$). Furthermore, in those samples with no detectable t(14;18)(q21;q34) translocation, there was no significant difference in BCL2 gene transcript abundance ($p=0.17$) between high BACH2 expressers (mean relative BACH2 expression = 2.99) compared to low BACH2 expressers (mean relative BACH2 expression = 18.11).
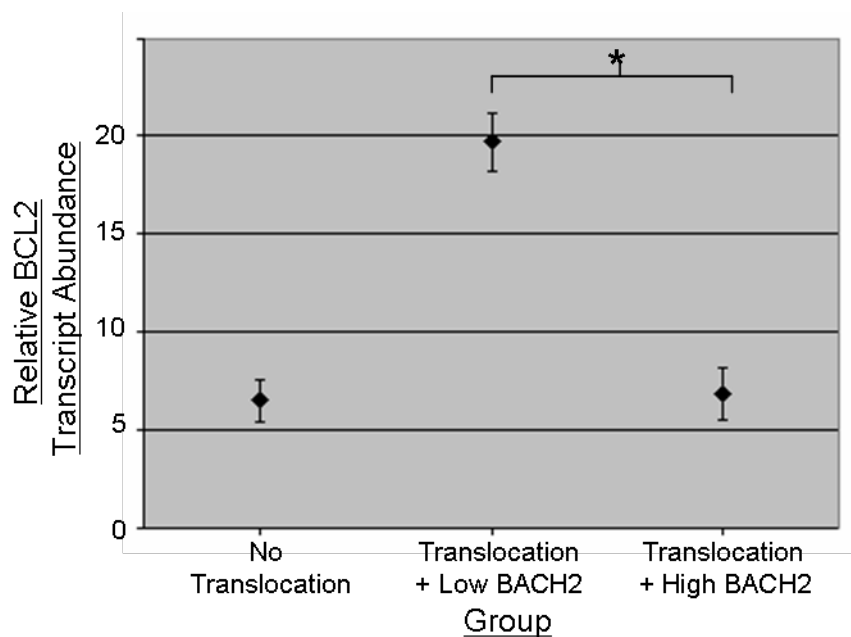

Figure 2: Mean BCL2 expression (+/- Standard Error) in groups categorized by the presence of the t(14;18)(q21;q34) translocation and BACH2 expression. *Statistically significant.

Discussion

The BCL2 gene is an important regulator of apoptosis and is recurrently targeted by chromosomal translocations in FL and DLBCL. This places the BCL2 gene under

control of the IgH promoter, which is highly active in the B-cells from which these malignancies originate. BCL2 is expressed at high levels in some patients and is associated with decreased overall survival and progression-free survival [1]. However, BCL2 overexpression is not always the result of translocation, and BCL2 translocation does not always result in its overexpression. This indicates that there are multiple mechanisms in place that regulate the abundance of BCL2, both within and outside the context of the t(14;18)(q21;q34) translocation. In order to predict the effect of this highly prevalent translocation, and its possible anti-apoptotic implications, these regulatory mechanisms must be further defined.

The BACH2 gene may be partially responsible for the regulation of BCL2 expression from the t(14;18)(q21;q34). This gene encodes a transcription factor capable of repressing transcription from the IgH locus. High expression of BACH2 has been described as a positive prognostic factor in DLBCL [5], and knock-out of BACH2 expression is lymphomagenic in the Raji cell-line [8]. However, in neither of these cases was the cellular role of BACH2 defined. It is therefore possible that the association between high BACH2 expression and favourable prognosis in DLBCL may be partially due to a role in regulating expression from oncogene-IgH translocations.

Here we examined the expression of BCL2 in FL and DLBCL samples in relation to the t(14;18)(q21;q34) translocation and BACH2 expression, and have provided evidence for this hypothesis. In tumor samples containing this translocation, BCL2 gene transcript level was inversely associated that of BACH2. This indicates that BACH2 may have a role in silencing BCL2 expression from this translocation.

Chemotherapeutic agents that cause the production of ROS may therefore be an attractive chemotherapy for patients with this translocation because they may result in increased nuclear translocation of BACH2 and subsequently increase its repressor activity on the aberrant loci. It may also be beneficial to explore the role of BACH2 in regulating expression of other oncogenes, such as BCL6 or c-MYC, that have been translocated to immunoglobulin loci.

Regulation of the expression from oncogene-IgH translocations may not be the only function of BACH2 that is associated with a favourable prognosis. This is indicated by the increased chemotherapeutic response of Raji cells when BACH2 expression is restored [9], despite no translocations being present in this cell-line. Furthermore, the lack of a significant correlation between BACH2 expression and BCL2 expression in samples with the t(14;18)(q21;q32) translocation indicates that there may be other factors associated with BCL2 abundance in these situation. This may include the activity of factors such as p53, which represses expression from wild-type BCL2 genes. In order to fully define the role of BACH2 in regulating expression from BCL2 translocations, these findings must be validated in a cohort with a larger sample size.

In conclusion, we have provided evidence that BACH2 may regulate BCL2 expression from the t(14;18)(q21;q34) translocation. Chemotherapeutic agents that induce ROS could potentially be pharmacogenomically targeted towards patients with this aberration in order to increase BACH2 repressor activity and decrease apoptosis-resistance associated with BCL2 overexpression.

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