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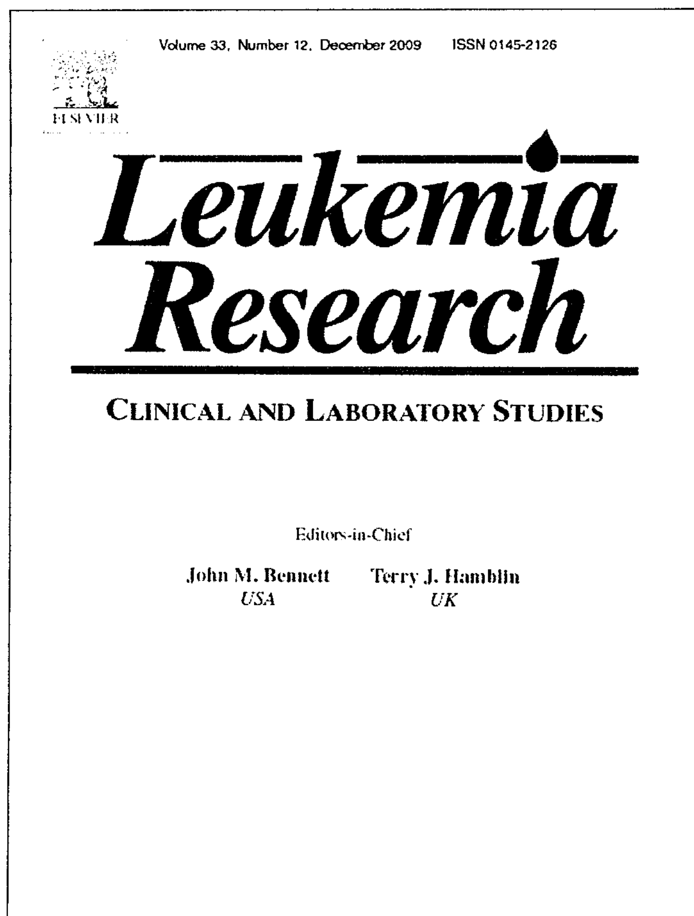

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Brief communication

Relative abundance of full-length and truncated FOXP1 isoforms is associated with differential NFκB activity in Follicular Lymphoma

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ABSTRACT

FOXP1 is a transcriptional repressor that has been proposed to repress the expression of some NFκB-responsive genes. Furthermore, truncated forms of FOXP1 have been associated with a subtype of Diffuse Large B-cell Lymphoma characterised by constitutive NFκB activity, indicating that they may inhibit this repression. We have shown that FL tumors have increased relative abundance of truncated FOXP1 isoforms and this is associated with increased expression of NFκB-associated genes. Our results provide strong evidence that relative FOXP1 isoform abundance is associated with NFκB activity in FL, and could potentially be used as a marker for this gene signature.

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1. Introduction

Follicular Lymphoma (FL) is the most common type of indolent non-Hodgkin's Lymphoma (NHL) in adults. Despite recent advances, the disease remains incurable [1]. The FL International Prognostic Index (FLIPI) uses clinical criteria to stratify patients into prognostic groupings, but there remains a large amount of heterogeneity in disease outcome within FLIPI sub-categories. In order to more accurately predict the clinical course of FL, molecular markers that relate to disease behaviour need to be elucidated and their role defined. One potential candidate is FOXP1, a transcriptional repressor belonging to the FOX family of forkhead box/winged-helix domain-containing proteins. It is expressed in the majority of FL tumors [2], and strong immunohistochemical (IHC) staining for FOXP1 has been associated with poor outcome in another common type of NHL, Diffuse Large B-cell Lymphoma (DLBCL) [3,4]. However, the role of FOXP1 has not yet been investigated in FL.

FOXP1 has been described as both a tumor suppressor candidate, due to deletion of its locus in multiple solid tumor types [5,6], as well as a potential oncogene, due to its over-expression in other tumors sometimes associated with genetic translocation [7]. Investigations into FOXP1 have yielded some interesting speculations regarding its role in malignancies. FOXP1 is known to repress transcription of some NFAT- and NFκB-responsive genes, such as IL-2, which contain forkhead (FKH) consensus sequences in their promoter region proximal to NFκB binding sites [8]. However, 7 NH_3-terminally truncated isoforms of FOXP1 have recently been described [9], 3 of which were found to be expressed in DLBCL samples. Truncated isoforms were particularly associated with a subtype of DLBCL that is characterised by constitutive NFκB activity [9], suggesting that they may have an inhibitory effect on the repressor activities of full-length isoforms and promote NFκB signalling. This proposed alternate role for full-length and truncated FOXP1 isoforms is in line with observations that have described FOXP1 as both a tumor suppressor gene and an oncogene [10].

Immunohistochemical (IHC) analysis of FOXP1 employs the JC12 antibody, which is unable to differentiate between isoforms. We have therefore developed a novel quantitative real-time PCR (qRT-PCR) assay to measure the relative abundance of full-length and truncated isoforms of FOXP1 at the transcript level. We have used this assay to measure the relative abundance of FOXP1 isoforms in tumor samples from a cohort of FL patients and compared measurements to those derived from control hyperplastic lymphoid tissue (HLT). These measurements were then correlated with microarray-based expression measurements of NFκB-associated genes. Using this approach we found that the FL tumors have increased relative

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abundance of truncated isoforms of FOXP1 compared to control HLT, and this correlated with the transcript abundance of a large number of NFκB-associated genes. This suggests that FOXP1 may function in regulating NFκB activity in FL.

2. Methods

Diagnostic fresh-frozen lymph node specimens from 20 FL patients (9 Grade I, 6 Grade II and 5 Grade III) were obtained from the Australian Leukemia and Lymphoma Group Tissue Bank and Bio Options bio-repository service (Los Angeles, CA). For use as control HLT, tonsil tissue was obtained from 12 cancer-free tonsillitis patients. DNA and total-RNA were extracted from each sample (see Supplementary Methods), and RNA integrity was analysed with an Agilent Bioanalyser 2100 using the NanoChip protocol. Immunohistochemical assessment of FOXP1 protein abundance was also conducted on neoplastic follicular regions of all FL samples with available tissue sections (2 × Grade I, 1 × Grade II and 2 × Grade III) using the JC12 antibody (see Supplementary Methods).

The relative abundance of full-length and truncated isoforms of FOXP1 was assessed in each sample using novel qRT-PCR assays. Assays were designed to be specific for exons 1 (N-terminal), 6a (Isoform-2) and 20 (C-terminal) of the FOXP1 gene (Supplementary Fig. 2). The relative abundance of full-length and truncated isoforms was therefore given by the N-terminal C_T normalised to the C-terminal C_T (FOXP1 ΔC_T), with increasing FOXP1 ΔC_T corresponding to increasing abundance of truncated isoforms. Measurements of the absolute FOXP1 transcript abundance were given by the C-terminal C_T normalised to the RPL13A C_T (C-terminal ΔC_T). Measurements of isoform-2 transcript abundance were given by the exon 6a C_T normalised to the RPL13A C_T (Isoform-2 ΔC_T). Differences in ΔC_T values between groups were defined by independent-sample T-tests. For detailed explanation and methods of qRT-PCR methods see supplementary information.

NFκB-associated genes were determined according to a literature search (Supplementary Data) and their expression determined in all FL samples using whole-genome gene-expression analysis performed with Illumina Sentrix Human-6 Expression Beadchips. Normalised probe fluorescence intensity was used as the measure of transcript abundance for NFκB-associated genes. Increased relative abundance of truncated FOXP1 isoforms was hypothesised to be associated with increased NFκB signalling, so a one-tailed Pearson's test was employed to determine significance of correlations using SPSS software. Whole-genome single nucleotide polymorphism (SNP) microarray analysis was performed also performed on all 20 FL samples using Affymetrix 250K Sty SNP Microarrays. Amplification, labelling, fragmentation, hybridisation and scanning were performed according to the manufacturer's protocol. DNA copy number analysis was performed using the Copy Number Analysis Tool (CNAT) associated with Affymetrix GeneChip Genotyping Software (GTYPE, verson 4.0), in comparison to SNP array data from 24 healthy controls.

3. Results

The effect of isoform-2 transcripts on N-terminal assay measurement, and hence the ability of FOXP1 ΔC_T values to truly reflect the relative abundance of full-length and truncated isoforms, was assessed by quantification of isoform-2 in all FL and control HLT samples. FOXP1 isoform-2 was detected at low levels in all control HLT samples, with isoform-2 ΔC_T values ranging between 16.15 and 20.46 (mean = 17.85, SD = 1.31). In contrast, 50.0% of FL samples had undetectable levels of isoforms-2, and samples with detectable levels had isoform-2 ΔC_T values ranged from 19.33 to 27.83 (mean = 23.19, SD = 3.82). This corresponded to a significant decrease in isoform-2 transcript abundance in FL samples to 0.05-fold of that in control HLT samples ($p = 0.017$). Expression of isoform-2 significantly correlated with the expression of only 4 NFκB-associated genes (IL8, $p < 0.001$; IL1A, $p = 0.026$; IKBKE, $p = 0.029$; TRIF, $p = 0.044$), corresponding to the number of associations expected by chance.

Absolute FOXP1 transcript abundance, as determined by the C-terminal assay normalised to the internal reference gene, was measured in order to assess its association with protein levels and its impact on the expression of NFκB-associated genes. The absolute abundance of FOXP1 transcripts was lower in FL samples (mean = 3.84, SD = 2.54) than control HLT samples (mean = 3.97, SD = 0.34), but this difference was not statistically significant ($p = 0.436$). Furthermore, no significant correlations were found between C-terminal ΔCT values and the expression of NFκB-associated genes. FOXP1 protein was detectable by IHC in all FL samples, but 60% (3/5) of samples showed scores of 0 (staining in <10% of nuclei). The remaining samples showed scores of 2 and 3 (staining in 31–50% or >50% of nuclei respectively). This is in line with that previously observed by Banham et al. [4], who noted nuclear, nuclear and cytoplasmic, solely cytoplasmic, and complete lack of staining in a variety of tumors. With the addition of IHC and qRT-PCR data from 5 DLBCL samples (Green et al., unpublished data), FOXP1 C-terminal ΔC_T values and FOXP1 IHC score showed a significant negative correlation ($p = 0.037$). It should be noted that ΔC_T values have an inverse relationship with expression, so a negative correlation with ΔC_T values corresponds to a positive correlation between protein and absolute transcript abundance.

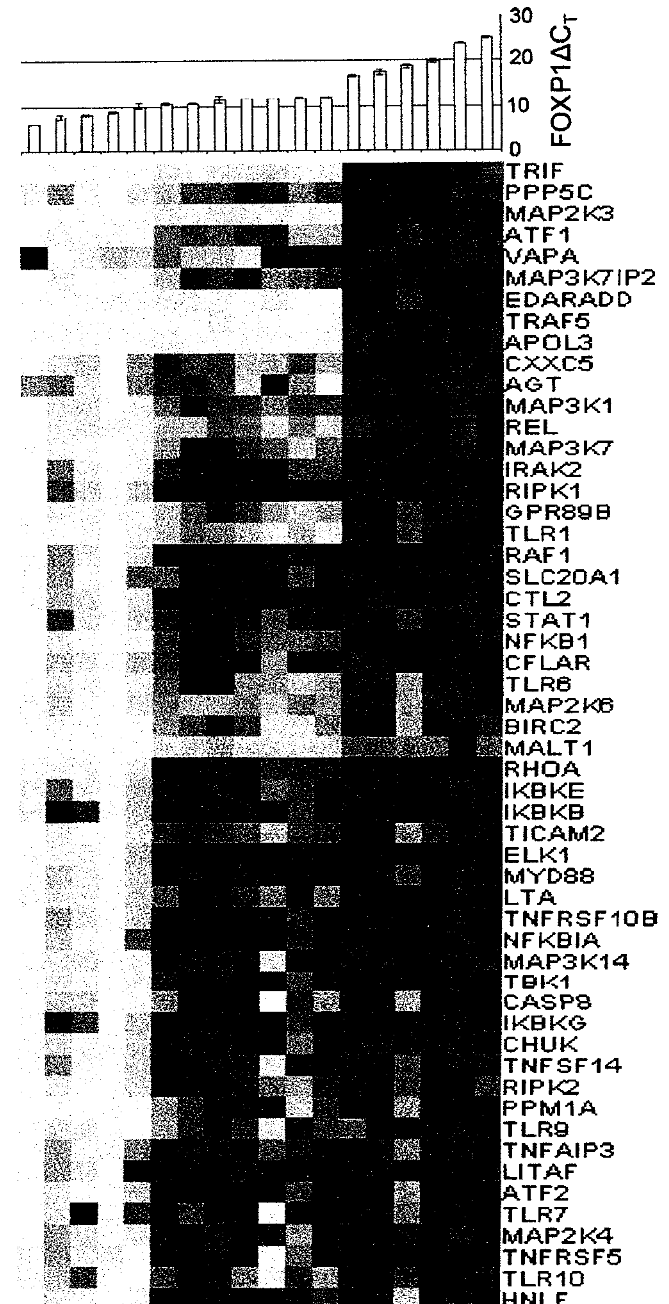

Fig. 1. Heat-map of NFκB-responsive genes with highly significant ($p < 0.01$) correlations with FOXP1 ΔC_T value showing increasing expression with increasing abundance of truncated FOXP1 isoforms.

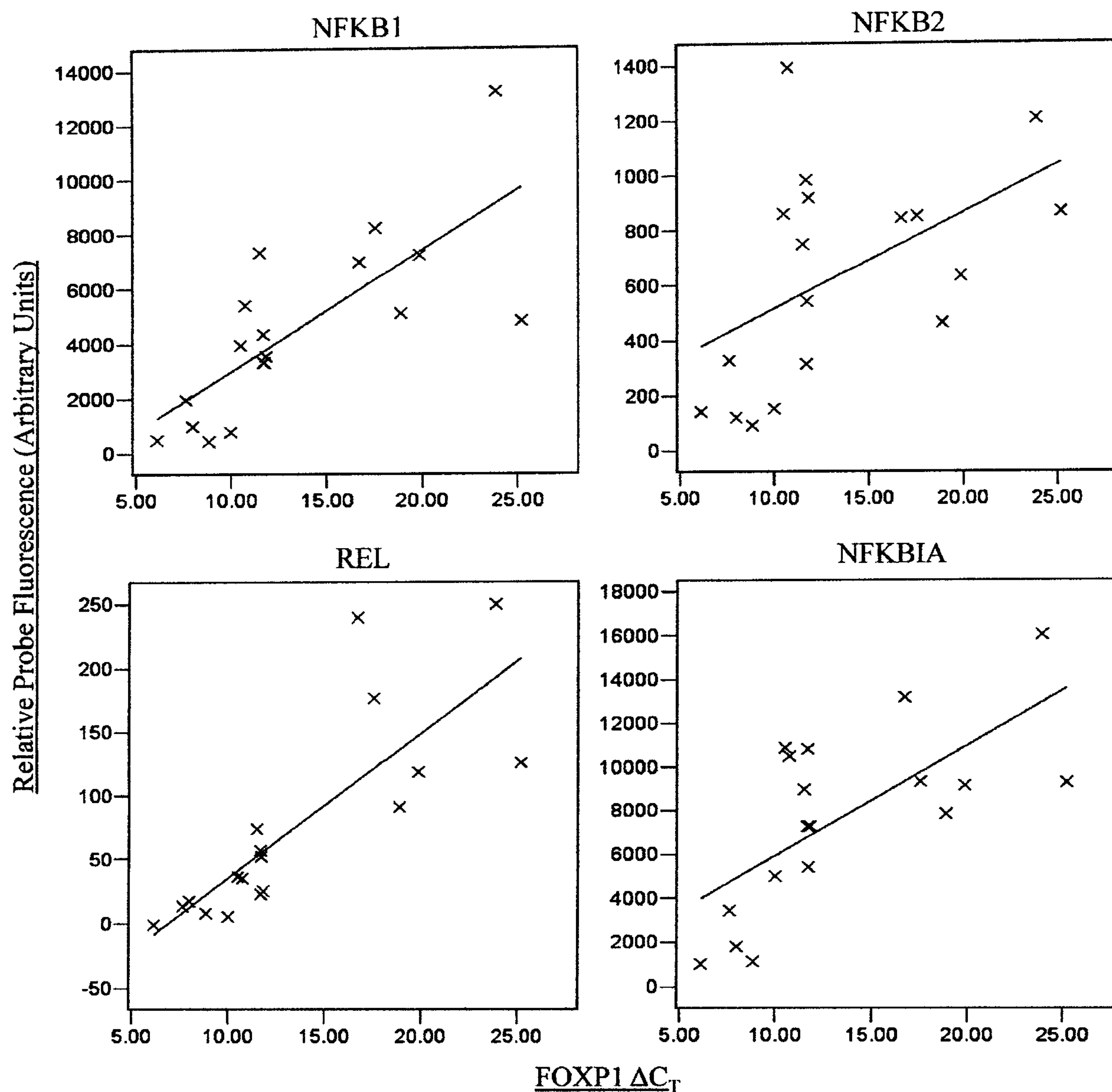

Fig. 2. Trends of increasing expression of central regulators of NFκB signalling with increasing FOXP1 ΔC_T value.

Full-length transcripts were detected by the N-terminal assay in 95% (19/20) of FL and in all HLT samples. The FOXP1 ΔC_T values obtained for FL samples were significantly higher in FL samples (mean = 18.38, SD = 6.11) compared to control HLT samples (mean = 13.88, SD = 1.57; $p = 0.025$). Importantly, qRT-PCR measurements of absolute FOXP1 transcript abundance showed a highly significant correlation with gene-expression microarray-based measurements ($R = 0.952$, $p < 0.001$), and there was no association between RNA integrity and FOXP1 ΔC_T ($p = 0.94$), indicating this value is not simply a by-product of RNA degradation. Before correlating FL FOXP1 ΔC_T values with the expression of NFκB-associated genes, DNA copy number of 2p16.1 was assessed in all samples. One sample showed DNA amplification between SNPs at physical positions of 60,880,316 and 61,007,156; a region mapping over the REL locus. Because of the confounding effect that this could have on NFκB activity, this sample was removed from downstream analysis.

From microarray analysis of FL samples, 61% (83/137) of genes associated with NFκB signalling showed significant positive correlations (R range 0.418–0.959, $p < 0.05$) with FOXP1 ΔC_T value in FL samples (Fig. 1). Of these, 72% (60/83) were significant at $p < 0.01$. Significant correlations included 56% (19/34) of NFκB-responsive genes, 63% (20/32) of genes with a role in activating the NFκB pathway, 66% (27/41) of genes that were positive regulators of the NFκB pathway and 57% (17/30) of other genes associated with the NFκB pathway. The significant positive correlation of these genes indicates increased transcript abundance with increasing relative abundance of truncated FOXP1 isoforms compared to full-length isoforms (Supplementary Information). Among the genes with significant correlations with FOXP1 ΔC_T were important markers of NFκB activity: NFKB1, NFKB2, NFKBIA, and RELA (Fig. 2).

4. Discussion

The N-terminal region of FOXP1 has been proposed to act as a complex docking site for transcriptional co-repressors, such as carboxyl-terminal binding protein-1 (CtBP-1) [11]. Truncated iso-

forms of FOXP1 not only lack their N-terminal coiled-coil domain, which may mediate such interactions, but also lack most of or the entire second poly-glutamine domain—the length of which has been shown to correlate with transcriptional repressor activity [8]. The loss of function associated with N-terminal coiled-coil and poly-glutamine domains, but the maintenance of DNA-binding activity associated with C-terminal winged-helix and forkhead domains, suggests that truncated FOXP1 isoforms may inhibit the

plete response, comprehensive analysis of this trend was inhibited due to the unavailability of the relevant prognostic clinical data for the remaining samples.

In conclusion, we have found that truncated isoforms of FOXP1 are highly expressed in FL. This relative abundance of full-length and truncated FOXP1 isoforms in each FL tumor sample correlated with the transcript abundance of a large number of NFκB-associated. This finding requires validation in a large cohort