Title: Relative Abundance of Full-Length and Truncated FOXP1 Isoforms are Associated with NFκB Activity in Follicular Lymphoma

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FOXP1 is a transcriptional repressor that has been proposed to repress the expression of some NFκB-responsive genes. FOXP1 protein is expressed in the majority of Follicular Lymphoma (FL) cases, but its function and possible role in tumorigenesis has not yet been investigated. Recently, truncated isoforms of FOXP1 have been found to be expressed in Diffuse Large B-cell Lymphoma and were proposed to have an oncogenic effect by de-repressing NFκB-associated genes. Here we have used a novel qPCR-based assay to investigate the relative abundance of full-length and truncated FOXP1 isoforms in FL samples, and gene-expression microarrays to investigate their effect on NFκB-associated genes. We have shown that increasing relative abundance of truncated FOXP1 isoforms is associated with increased expression of a large number of NFκB-associated genes in FL, which was accompanied by a reduced response to first-line chemotherapy. 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. The association between relative abundance of truncated and full-length FOXP1 isoforms with chemotherapeutic response warrants confirmation in a large prospective study.

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 (Gandhi and Marcus, 2005). 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 (Brown et al., 2005), 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) (Barrans et al., 2004; Banham et al., 2005). However, its role in FL, and possible relation to prognosis, has not yet been investigated.

FOXP1 has been described as both a tumor suppressor candidate, due to deletion of its locus in multiple solid tumor types (Alimov et al., 2000; Banham et al., 2001), as well as a potential oncogene, due to its over-expression in other tumors sometimes associated with genetic translocation (Wlondarska et al., 2005). 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 (Wang et al., 2003). The murine *Foxp1* gene also contains an NFAT binding sequence in its promoter region. This presents *FOXP1* as a possible negative feedback mechanism to regulate NFκB signalling, which would represent a tumor-suppressor role. However, Brown *et al.* (2008) have recently described 7 NH₃-terminally truncated isoforms of *FOXP1* by bioinformatics, and found two of these (isoforms 3 and 9) to be expressed in DLBCL samples. One cell-of-origin (COO) subtype of DLBCL, activated B-cell like (ABC) DLBCL (Shipp et al., 2002), is characterized by constitutive NFκB activity. Truncated isoforms of *FOXP1* were observed to be associated with this subtype (Brown et al., 2008), 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 (Pajer et al., 2006). It may also provide some explanation for the contradictory findings regarding the prognostic capacity of Foxp1 protein levels in DLBCL (Brown et al., 2005; Barrans et al., 2004; Hans et al., 2004) and its association with the ABC subtype (Brown et al., 2008; Hans et al., 2004). This is because the JC12 antibody employed for these investigations cannot differentiate between isoforms, and it is likely to be the balance between tumor suppressing and oncogenic forms of *FOXP1*, rather than absolute Foxp1 protein abundance as assessed by IHC staining, that is informative with regard to overall *FOXP1* function and patient prognosis.

Here we have developed a novel quantitative real-time PCR (qPCR) 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 patients treated at a single institution in order to assess its function and prognostic worth. In order to investigate the relationship between *FOXP1* isoforms and NFκB signalling, microarrays were employed to interrogate the expression of NFκB-associated genes. Using this approach we found that increasing relative abundance of truncated *FOXP1* isoforms was associated with poorer response to chemotherapy in FL patients, although this remains to be verified in a larger uniform population. Furthermore, we found that the relative abundance of full-length and truncated isoforms of *FOXP1* 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.

Methods:

Patient Samples and RNA Extraction

Diagnostic fresh-frozen lymph node specimens from 12 FL patients were obtained from the Australian Leukemia and Lymphoma Group Tissue Bank and Bio Options bio-repository service (Los Angeles, CA). Clinical information, including response to first-line treatment, was provided for 11 of these samples (Supplementary Table 1). Patients were classified as either achieving a complete response (CR) or non-complete response (N-CR; partial response or stable disease) according to the Cheson criteria

(Cheson et al., 2007). 10mg of tissue was homogenized using a rotor-strator homogenizer in 1mL Trizol. Chloroform was added according to the manufacturers protocol, and the phases separated by centrifugation. The upper aqueous phase was removed and total-RNA purified from it using a Micro-to-Midi RNA purification kit (Invitrogen) according to the manufacturers protocol. RNA integrity was analysed with an Agilent Bioanalyser 2100 using the NanoChip protocol.

A Novel qPCR Assay for Relative Quantitation Full-Length and Truncated FOXP1 Isoform Transcripts

Assays were designed to be specific for exons 1 (N-terminal) and 20 (C-terminal) of the *FOXP1* gene (Figure 1). The N-terminal assay thus only quantitates isoforms 1 and 2. The C-terminal assay quantitates all isoforms except for isoform 2. Expression of isoform 2 has not been demonstrated in these malignancies, so its expression is not expected to have an effect on the results obtained. Movement of the C-terminal assay qPCR threshold cycle (C_T) is thus only influenced by the abundance of both full-length and truncated isoforms, whereas the N-terminal assay C_T is influenced only by the abundance of full-length isoforms. Changes in the abundance of full-length isoforms would therefore alter C_T values of both the N-terminal and C-terminal assays and cause no change to the resulting ΔC_T ($\Delta C_T = \text{C-terminal } C_T - \text{N-terminal } C_T$). In contrast, changes in the abundance of truncated isoforms would alter only the C-terminal assay C_T , resulting in an increased ΔC_T value with increasing relative abundance of truncated isoforms and decreasing ΔC_T with decreasing relative abundance of truncated isoforms. Differences in ΔC_T between groups was defined by independent-sample T-tests. See supplementary information for detailed qPCR methods and primer sequences.

Figure 1

Gene Expression Microarray Analysis

Whole-genome gene-expression analysis was performed using Illumina Sentrix Human-6 Expression Beadchips (v2.0). Labelling, washing, hybridization and scanning were performed according to manufacturer's protocol at the Australian Genome Research Foundation (Victoria, Australia). For detailed methods, see supplementary information. NF κ B associated genes were determined according to a

literature search (Supplementary Data). Normalised probe fluorescence intensity was used as the measure of transcript abundance for NFκB-associated genes. Increased relative abundance of truncated *FOXP1* isoforms was hypothesized to be associated with increased NFκB signalling, so a one-tailed Pearson's test was employed to determine significance of correlations using SPSS software. Because NFκB activity is associated with a DLBCL subtype with poorer outcome, increases in *FOXP1* ΔC_T and expression of NFκB-associated were hypothesised to be associated with the non-CR group of patients. Significance of associations was testing with one-tailed independent samples T-tests using SPSS software.

Results:

Efficacy of the FOXP1 Isoform qPCR Assay

Our novel qPCR assay was able to detect both transcripts for both truncated and full-length *FOXP1* isoforms in all FL samples in this investigation. *FOXP1* ΔC_T values ranged between 11.51, representing a high relative abundance of truncated *FOXP1* isoform transcripts, and 6.13, representing a low relative abundance of truncated *FOXP1* isoform transcripts. Importantly, there was no association between RNA integrity and *FOXP1* ΔC_T (p=0.94), indicating this value is not simply a by-product RNA degradation.

FOXP1 Isoforms are Associated with NFκB Signalling in FL

From microarray analysis of FL samples, 68% (76/111) of genes associated with NFκB signalling showed significant positive correlations (R range 0.517 to 0.845, p<0.05) with *FOXP1* ΔC_T value in FL samples (Figure 2). Of these, 68% (52/76) were significant at p<0.01. 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). In addition, *IFNA1* showed a significant negative correlation (R=-0.763, p=0.002). Among the genes with significant correlations with *FOXP1* ΔC_T were important markers of NFκB activity; *NFKB1*, *NFKB2*, *NFKBIA*, *RELA* (Figure 3).

Figure 2 and 3

Association between FOXP1 ΔC_T and Chemotherapeutic Response

Two patients were excluded from this analysis of response to first-line chemotherapy because clinical data was not available for one patient and the other was treated with a ‘watch-and-wait’ approach. Expression of only 2.7% (3/111) of NF κ B-associated genes (*CASP1*, $p=0.04$; *RHOC*, $p=0.02$; *IRAK1*, $p=0.01$) were significantly different in sample from FL patients with CR compared to samples from those with N-CR. However, *FOXP1* ΔC_T values were found to be significantly ($p = 0.01$) lower in FL patients with a complete response to chemotherapy (mean $\Delta C_T = 10.47$, $n = 5$) compared to those that did not have a complete response (N-CR; mean $\Delta C_T = 7.64$, $n = 5$).

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) (Li et al., 2004). Truncated isoforms of *FOXP1* not only lack their N-terminal coiled-coil domain, which may mediate such interactions, but also lack most of or their entire second poly-glutamine domain. The length of these domains has been shown to correlate with transcriptional repressor activity of the Androgen receptor, and the poly-glutamine domain of murine Foxp1 has been shown to strongly regulate transcriptional repressor function (Wang et al., 2003). Truncated Foxp1 isoforms have also been shown to be predominantly localized to the cytoplasm, despite the maintenance of their nuclear localization signal (Banham et al., 2001). Expression of truncated isoforms may therefore result in loss of *FOXP1* repressor function by one or both of two alternate mechanisms. 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 repressor function of full-length isoforms by competitive binding of target sequences. Alternatively, truncated isoforms may interact with full length isoforms as well as other FOXP subfamily proteins, and sequester them to the cytoplasm via an unknown mechanism. In either scenario it is the relative abundance of full-length and truncated isoforms, rather than the absolute *FOXP1* abundance (that is assessed by IHC staining with the JC12 antibody), that would yield the ultimate effect on transcriptional repression.

We have provided evidence that the relative abundance of full-length and truncated isoforms of *FOXP1* is significantly associated with NFκB activity in FL samples. An increasing abundance of truncated isoforms positively correlated with the abundance of a large number of NFκB-associated genes, including markers of NFκB activation such as *NFKB1*, *NFKB2*, *NFKBIA* and *RELB*. This suggests that, in FL, full-length *FOXP1* may function as transcriptional repressors of NFκB signalling, as previously hypothesised. Furthermore, truncated isoforms of *FOXP1* may play an oncogenic role by inhibiting this repression and allowing increased NFκB signalling. This is in line with a recent investigation in which two truncated isoforms of *FOXP1* (isoforms 3 and 9) were associated with the ABC subtype of DLBCL, which is characterised by constitutive NFκB activity (Brown et al., 2008).

The qPCR assay in this investigation does not allow full elucidation of the complexity of *FOXP1* function in FL. This assay provides a single measure for the transcripts abundance of all truncated isoforms. We were therefore unable to determine which truncated specific isoforms were responsible for associations with NFκB signalling, and whether these corresponded to the isoforms associated with the ABC subtype of DLBCL. Further insight into the complexity of the action of *FOXP1* isoforms is provided by the significant negative correlation found between *FOXP1* ΔC_T and *IFNA* expression. The opposite trend observed for this gene compared to other NFκB-associated genes may, for example, be due to different FOXP-family protein heterodimers mediating *IFNA* expression. In order to fully define the role of individual *FOXP1* isoforms in FL, further investigation with more isoform-specific techniques is required.

Constitutive activation of NFκB is associated with the ABC subtype of DLBCL and predicts a poor outcome. It is therefore likely that increased relative abundance of truncated *FOXP1* isoforms in FL, and the accompanied increase in NFκB signalling would also be associated with a poor outcome. We have provided some evidence of this; samples from patients who did not achieve CR to first-line chemotherapy had significantly higher *FOXP1* ΔC_T values in comparison to samples from patients who did achieve CR. No association was seen between therapeutic response and expression of the majority of NFκB-associated genes in this investigation. This suggests the global NFκB signature, rather than individual genes, is informative with

regard to disease behaviour, and *FOXP1* Δ CT may be able to be employed as a marker for this signature. However, these conclusions are based upon a small cohort of patients and require validation in a prospective investigation utilising a large uniform cohort.

In conclusion we have associated the relative abundance of full-length and truncated *FOXP1* isoforms with the transcript abundance of a large number of NF κ B-associated genes in FL tumor samples. This supports the hypothesis that *FOXP1* isoforms may play an important role in regulating NF κ B signalling. Due to the lack of variability in Foxp1 IHC staining between FL patients, relative *FOXP1* isoform abundance measured by qPCR could potentially be used as a marker for NF κ B activity in this disease, and a possible pharmacogenomic indicator of patients who may benefit from therapy with modulation of NF κ B.

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