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The FOXP1 transcription factor is expressed in the majority of follicular lymphomas but is rarely expressed in classical and lymphocyte predominant Hodgkin's lymphoma

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Summary

The expression of the *FOXP1 forkhead*/winged helix transcription factor has been investigated in normal and neoplastic lymphoid tissues using the FOXP1-specific monoclonal antibody, JC12. Using single and double immunoenzymatic staining, FOXP1 expression has been studied in a series of classical and lymphocyte predominant Hodgkin's lymphomas, follicle centre lymphomas and Hodgkin's lymphoma-derived cell lines. Neoplastic cells in the majority of classical and lymphocyte predominant Hodgkin's lymphoma were FOXP1-negative. In two cases of classical Hodgkin's lymphoma, the tumour cells showed mislocalisation of FOXP1 to the cytoplasm, while in one case of lymphocyte predominant Hodgkin's lymphoma, and in the Hodgkin's lymphoma cell line KMH2, scattered tumour cells showed nuclear expression of FOXP1. In contrast, the tumour cells in the majority of follicle centre lymphomas showed strong nuclear FOXP1 reactivity. Double labelling studies of lymphoid tissue indicated that a variable proportion of CD20-positive germinal centre B cells express FOXP1 while the vast majority of CD30-positive cells are FOXP1-negative. The heterogeneity of FOXP1 expression in germinal centre-derived lymphomas, may have more to do with the transforming events underlying these distinct types of lymphoma than with their common origin.

Introduction

The cellular derivation and phenotype of the tumour cells in classical Hodgkin's lymphoma (HL) has puzzled scientists for many years. Although it is now widely recognised that the malignant Hodgkin and Reed-Sternberg cells (HRS) in classical HL (cHL) derive in most instances (approximately 97%) from B-lymphocytes (Küppers *et al.* 1994, Marafioti *et al.* 2000), detection of T-cell receptor rearrangement has also been demonstrated in a small proportion of cases (Muschen *et al.* 2000, Seitz *et al.* 2000). However, the fact remains that HRS cells do not express typical B-cell markers such as immunoglobulin, CD20 or CD79a. The ambiguous phenotype of HRS cells, which are characterised by the expression of the lymphocyte-activation marker CD30 and by CD15, prompted several groups to investigate the mechanisms underlying the loss of their B-cell identity. Recent studies have demonstrated that an absence or reduced expression of transcription factors (e.g. BOB-1, OCT-2 and PU-1) (Stein *et al.* 2001, Re *et al.* 2001, Marafioti *et al.* 2002), controlling the expression of B-cell molecules such as immunoglobulin, CD20, CD79a and J-chain (Henderson & Calame 1998), is a common finding in HRS cells. While gene expression profiling studies have reported that HL cell lines have decreased mRNA levels for nearly all established B lineage-specific genes (Schwering *et al.* 2003). These features may contribute to their aberrant phenotype. An opposite scenario was found in lymphocyte predominant HL (LPHL), where the tumour cells (called popcorn or L&H cells) retain the characteristics of B cells. Molecular studies using single cell PCR have shown that the L&H cells represent monoclonal populations derived from germinal centre B cells with ongoing somatic mutation in approximately half of the cases (Bräuninger *et al.* 1997, Marafioti *et al.* 1997).

We have produced a monoclonal antibody, JC12, that recognises a nuclear protein, subsequently identified by expression cloning as FOXP1, a member of the *forkhead* or winged helix family of transcription factors (Banham *et al.* 2001). JC12 labelling showed a restricted

distribution of the FOXP1 protein in normal tonsil, staining mantle zone lymphocytes, numerous cells in the interfollicular area together with a variable proportion of cells (usually less than 50%) in the germinal centre. In the current study, we explored the expression pattern of the FOXP1 protein in a series of cHL, LPHL and follicle centre lymphoma (FCL) cases as well as in HL-derived cell lines.

Methods

Tissue samples

Thirty-seven cHL, 8 cases of LPHL and 13 FCL, diagnosed according to the WHO classification (Jaffe *et al*. 2001) were obtained from the archive of the Department of Pathology at the John Radcliffe Hospital, Oxford, UK and from the University Hospital Merkur, Zagreb, Croatia. In addition, one reactive tonsil and one lymph node were also included in this study. Samples were fixed in buffered formalin and embedded in paraffin according to routine procedures (Biddolph & Gatter 2000).

Cell lines

The Jurkat cell line was obtained from the Sir William Dunn School of Pathology, Oxford, UK. The KMH2 and the L428 (HL-derived) cell lines were purchased from the DSMZ cell collection (Braunschweig, Germany). The cells were maintained in culture in RPMI 1640 containing 10% foetal calf serum (Invitrogen, Paisley, Scotland) at 37 °C in 5% CO_2. Aliquots of each cell line used in this study were centrifuged, washed in PBS and fixed for 48 h in PBS/formalin buffer at room temperature and cell pellets were embedded in paraffin wax.

Immunohistochemistry

Four-micro metre sections were cut from paraffin blocks and captured on electrically charged slides (Snowcoat X-tra^{TM}, Surgipath, USA). The production of the FOXP1-specific monoclonal antibody, JC12, and the conditions used for antigen retrieval and immunostaining paraffin embedded tissues with the hybridoma supernatant have been described previously (Banham *et al*. 2001). Antibody binding was detected by indirect immunoperoxidase (Envision Kit, Dako Cytomation, Glostrup, Denmark). Sections were then counterstained with hematoxylin (Gill No. 3, Sigma, UK) and mounted in Aquamount (BDH, UK). The immunostaining results were reviewed by the authors (including a qualified pathologist, TM), and in each stained section, reactive lymphocytes (e.g. mantle zone cells), vessels and epithelium served as positive internal controls.

Double immunoenzymatic labelling

Double enzymatic immunostaining for FOXP1 (JC12) and CD30 (Ber-H2, Dako Cytomation) was performed on tonsil and lymph node together with 9 cases of cHL. Furthermore, paraffin sections from tonsil and lymph node, together with 4 cases of LPHL were also double immunostained for FOXP1 (JC12) and CD20 (L26, Dako Cytomation). The details of the methodology used for the double immunostaining has been described previously (Marafioti *et al*. 2003).

Results

Lymphoid tissue sections

JC12 reactivity was assessed on sections of reactive tonsil and lymph node. As previously described in tonsil (Banham *et al*. 2001), nuclear FOXP1 expression was observed in mantle zone cells and in several lymphoid and non-lymphoid cells in the interfollicular areas, whereas only a proportion of germinal centre cells were positive. Double immunoenzymatic labelling of tonsil to investigate the co-expression of both FOXP1 and CD20 demonstrated variable expression levels of FOXP1 in both germinal centre B cells and in interfollicular large B cells (Figure 1). Furthermore, CD30-positive lymphoid cells in tonsil and lymph node (localised in either germinal centres or the interfollicular areas) were predominantly FOXP1-negative (Figure 1), with the exception of occasional FOXP1/CD30-positive cells lying in the interfollicular area.

cHL

Reactivity of the JC12 antibody was investigated on paraffin sections from a total of 45 cases of HL. There was no FOXP1 expression detected in the HRS cells in 35 of the 37 cases of cHL studied. The exceptions were 2 cases that showed cytoplasmic/cell membrane positivity (Figure 2). These data were confirmed, for each case, by double immunoenzymatic staining for both CD30 and FOXP1 showing the absence of FOXP1 expression within the CD30-positive HRS cells (Figure 2).

Cell lines

Heterogeneous results were observed in the two cHL-derived cell lines. Nuclear FOXP1 expression in a proportion of cells was seen in the KMH2 cell line whereas L428 was consistently FOXP1-negative (Figure 2). The Jurkat T-cell line, included as a staining control, showed weak nuclear FOXP1 positivity in scattered cells (Figure 2).

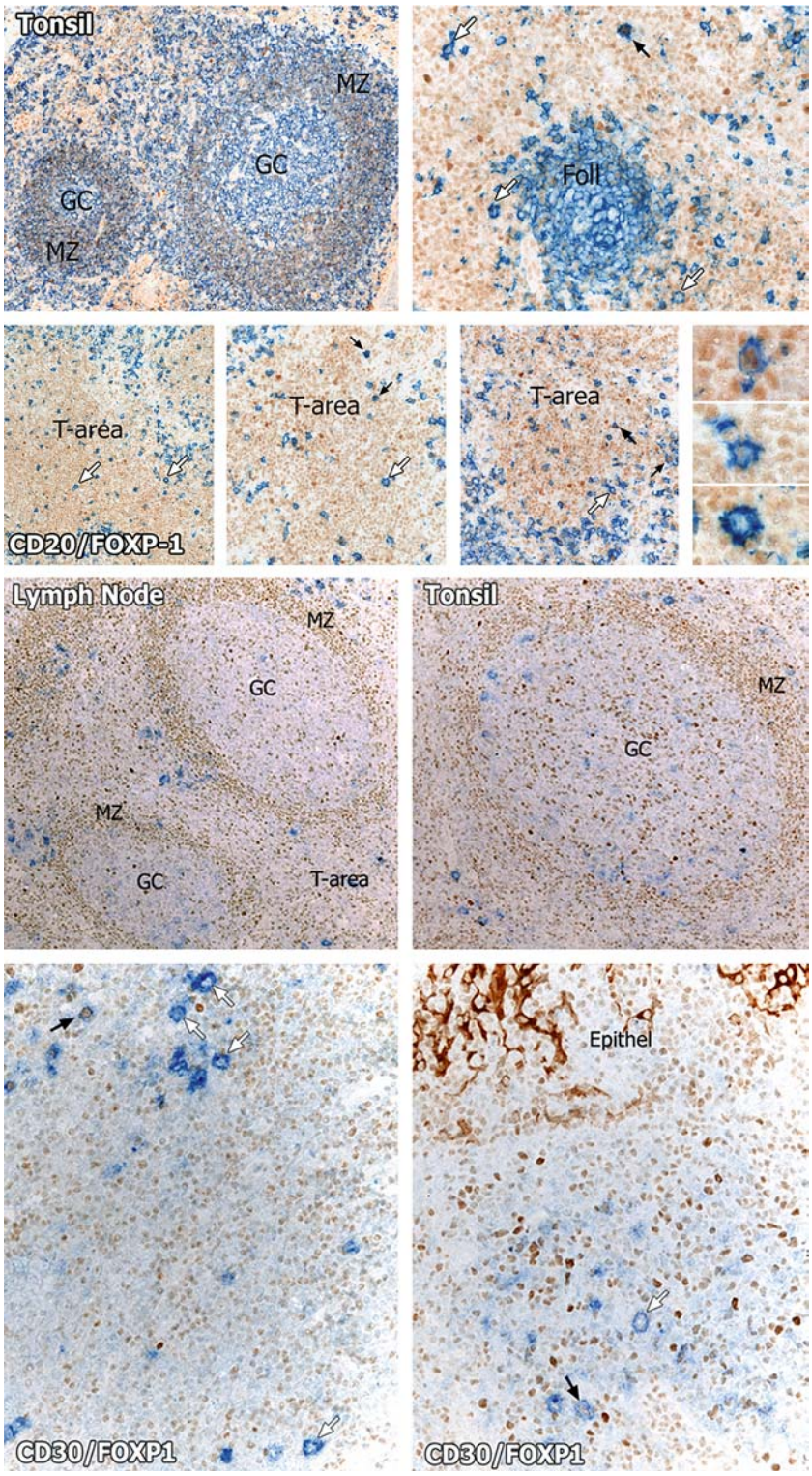

Figure 1. Normal lymphoid tissue double stained for FOXP1 and either CD20 or CD30. The top panels represent tonsil double stained for the expression of FOXP1 protein (brown) and CD20 (blue). White arrows correspond to CD20-positive/FOXP1-negative cells while black arrows indicate double CD20/FOXP1-positive cells. The FOXP1 protein is expressed by the majority of the mantle zone (MZ) B cells but shows heterogenous expression in B cells in the germinal centre (GC), surrounding follicles (Foll) and in the T-cell rich area (T-area). The lower four panels illustrate lymph node and tonsil double stained for FOXP1 (brown) and CD30 (blue). The white arrows correspond to CD30-positive/FOXP1-negative cells while the black arrows indicate the occasional double positive cell in the interfollicular areas.

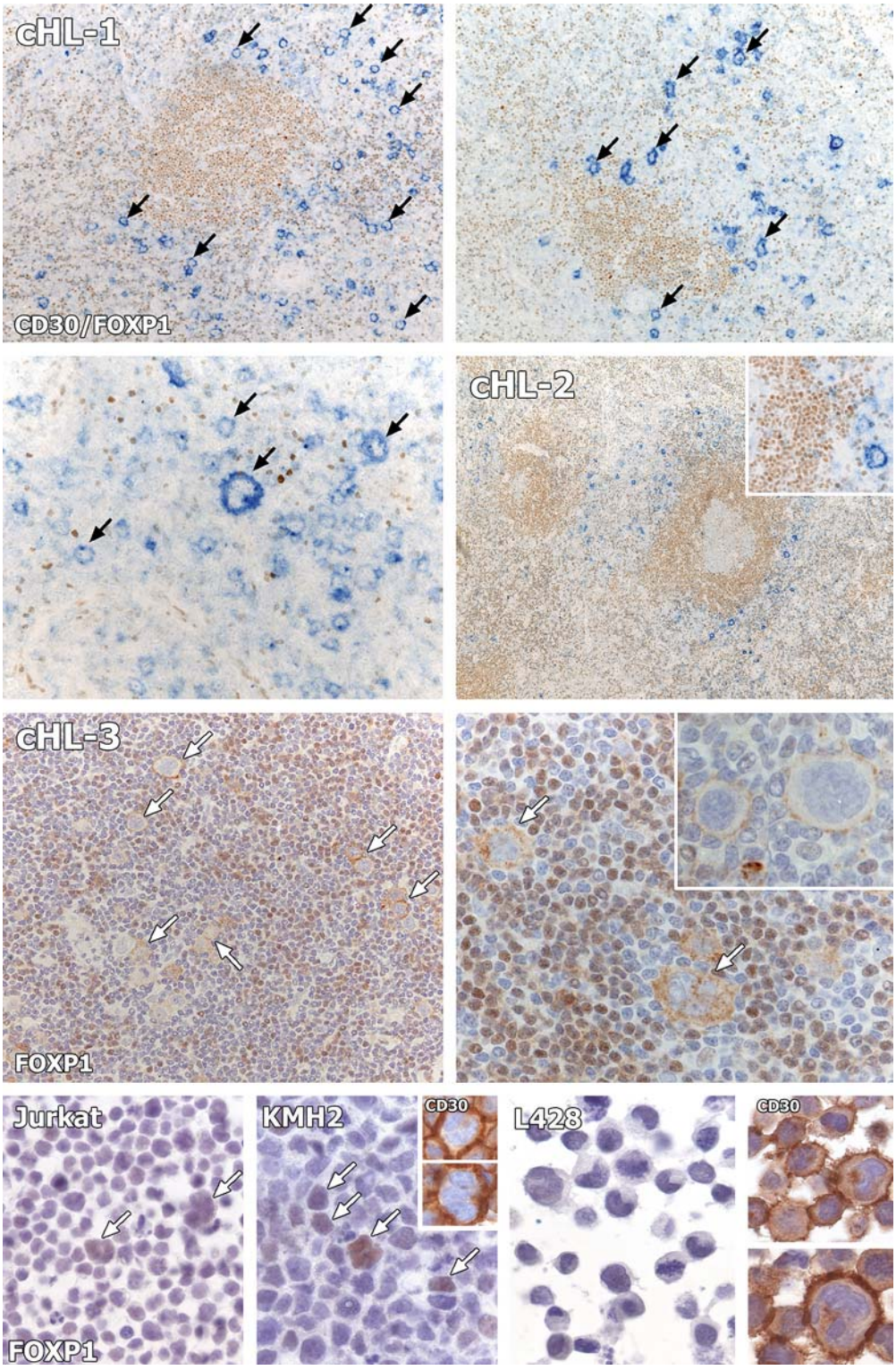

Figure 2. Expression of the FOXP1 protein in cHL and in cell lines. The top four panels represent cases of cHL double stained for the expression of FOXP1 (brown) and CD30 (blue). The CD30-positive tumour cells in both cHL-1 and cHL-2 are FOXP1-negative (black arrows). The third case, cHL-3, is stained only for the expression of FOXP1 to demonstrate the cytoplasmic and membrane localisation of the protein in the tumour cells (white arrows). The surrounding lymphocytes show the normal nuclear FOXP1 expression pattern. Weak nuclear expression of the FOXP1 protein was observed in a proportion of cells in the Jurkat T-cell line (included here as a control for processing and staining) and in the KMH2 cHL cell line (white arrows), while cells of the L428 cHL line were FOXP1-negative. The CD30 positivity of the cHL cell lines was confirmed as illustrated.

LPHL

A similar pattern of JC12 reactivity to that identified in cHL was observed among the 8 cases of LPHL. The L&H cells were FOXP1-negative in 7 out of 8 cases. In the remaining case scattered large cells with nuclear FOXP1 positivity were observed (Figure 3). As in each case a conspicuous number of medium to large cells expressed FOXP1, we decided to perform double immunoenzymatic labelling for FOXP1 and CD20 to

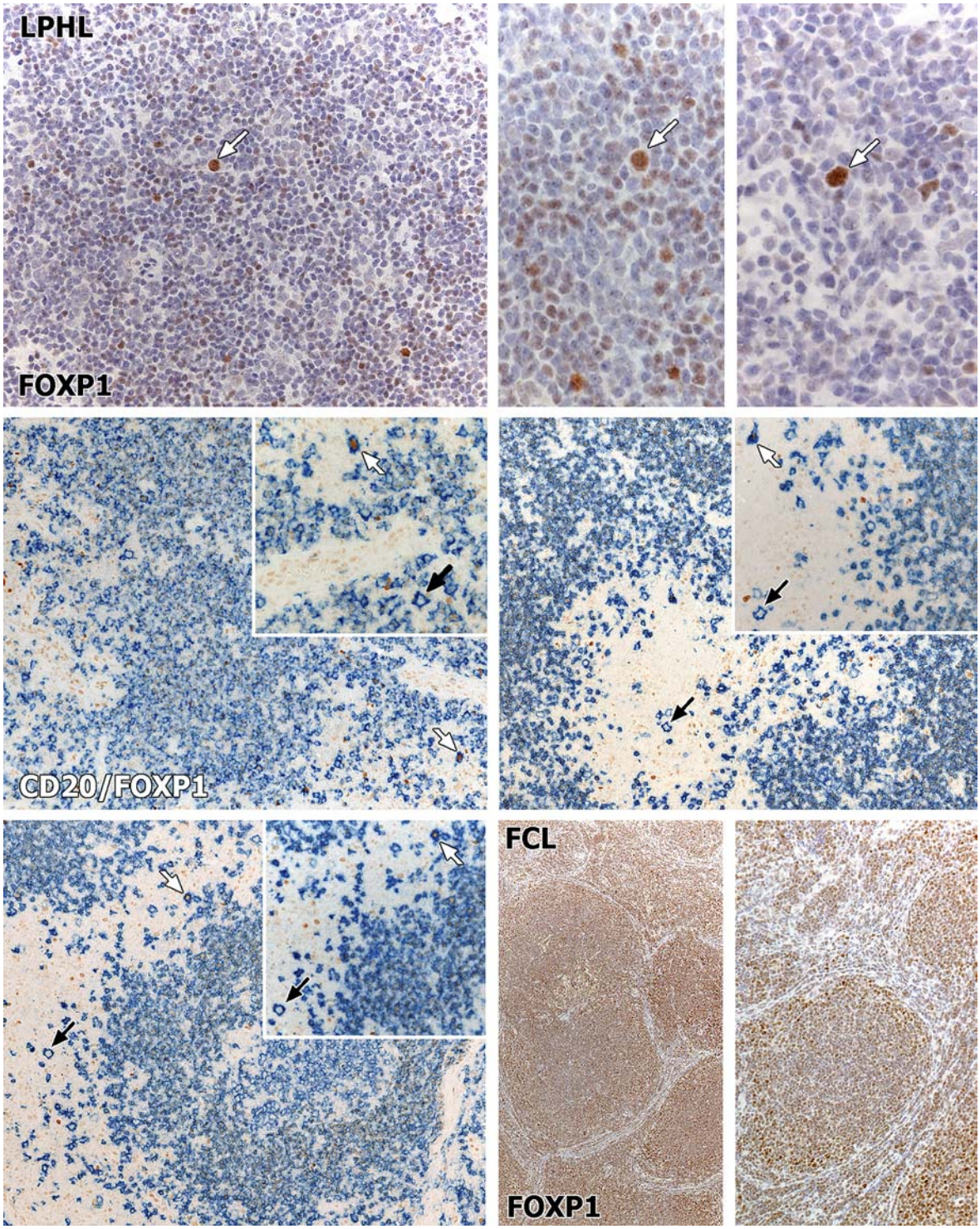

Figure 3. Expression of the FOXP1 protein in LPHL and in FCL. The top row of panels illustrate nuclear FOXP1 expression in the only FOXP1 positive case of LPHL. White arrows indicate FOXP1 positive L&H cells. The middle and bottom left panels illustrate this case double stained with CD20 (blue) and FOXP1 (brown). The FOXP1 positive and negative L&H cells are indicated by white and black arrows respectively. Two cases of FCL stained for the expression of FOXP1 are illustrated at the bottom right.

explore the nature of the large FOXP1-positive cells. The large CD20-positive tumour cells were FOXP1-negative with the exception of one case that exhibited scattered double FOXP1/CD20-positive and FOXP1-negative/CD20-positive L&H cells (Figure 3). Most of the additional large FOXP1-positive cells present were epithelioid cells and macrophages.

FCL

The expression of FOXP1 was also investigated in 13 cases of FCL. Surprisingly, in 11 cases that were diagnosed according to the WHO classification (Jaffe *et al.* 2001) as FCL, mostly of grade 1 and/or 2, all the tumour cells showed nuclear FOXP1 expression (Figure 3). However, the intensity of FOXP1 expression varied among the neoplastic cells being strong in the large cells and moderate in the small. In contrast, the remaining 2 cases, which were of grade 2 and 3, did not express FOXP1.

Discussion

Forkhead proteins comprise a transcription factor family found exclusively in animals and fungi. The members of this family display remarkable functional diversity and are involved in a number of different biological processes, for example; development, oncogenesis, signal transduction, cell cycle regulation, control of metabolism and chromatin remodelling (recent review (Carlsson & Mahlapuu 2002)). The FOXP subfamily was first described in 2001 and currently comprises four members, FOXP1-4, each of which contain both a C-terminal *forkhead* domain together with an N-terminal zinc finger motif. Although most *forkhead* proteins act as transcriptional activators the FOXP1, FOXP2 and FOXP3 proteins have been shown to repress transcription (Schubert *et al.* 2001, Shu *et al.* 2001). Relatively little is yet known about the function of these proteins. However, mutations in FOXP2 have been linked to an inherited speech and language defect (Lai *et al.* 2001), while mutation or loss of the FOXP3 protein (scurfin) results in a fatal lymphoproliferative disease, immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome (Bennett *et al.* 2001, Brunkow *et al.* 2001, Wildin *et al.* 2001). Recently, FOXP3 has been shown to have an important role in both T-cell activation and T-cell development (Khatti *et al.* 2001, Schubert *et al.* 2001) and is directly involved in generating $CD4^+CD25^+$ regulatory T cells (Fontenot *et al.* 2003, Hori *et al.* 2003, Khattri *et al.* 2003). Studies on the murine Foxp1 protein have indicated that members of this family can heterodimerize and that FOXP1 can transcriptionally repress the IL-2 promoter (Wang *et al.* 2003). The expression of FOXP1 has been investigated in malignant tissues (Banham *et al.* 2001) and recently, *FOXP4* mRNA expression was shown to be down regulated in kidney tumours (Teufel *et al.* 2003).

Preliminary studies have shown that the FOXP1 protein is heterogeneously expressed within normal B-cell subsets and is very highly expressed in some cases of diffuse large B-cell lymphoma (DLBCL) (Banham *et al.* 2001). DLBCL represent a heterogeneous group of lymphomas and studies based on microarray gene expression profiling have demonstrated the existence of clinically and phenotypically different subcategories (Alizadeh *et al.* 2000, Rosenwald *et al.* 2002). Additional data from this study has been reported, including the finding that *FOXP1* mRNA expression is up-regulated during normal B-cell activation (induced by anti-IgM and/or CD40L) and, among DLBCL, is expressed in a poor prognosis activated B-cell-like subtype (Shaffer *et al.* 2002). These data are consistent with preliminary studies of FOXP1 protein expression in DLBCL and in DLBCL-derived cell lines (Banham *et al.* 2002). Interestingly, although gene expression profiles of cHL cell lines were recently shown to cluster as a distinct entity, they were most similar to those of cell lines derived from activated B-cell-like DLBCL (Küppers *et al.* 2003). The variable expression of FOXP1 in germinal centre B cells and in DLBCL, together with its reported association with B-cell activation, prompted our investigation of the expression of this protein in HL.

Initially we examined FOXP1 expression among the normal B-cell population. Staining for FOXP1 in combination with CD30 revealed that the vast majority of CD30-positive cells lying in the rim of follicle centres and/or in the T-cell-rich areas were FOXP1-negative. The exceptions were single, small to medium lymphocytes localized in the T-cell areas that were double CD30/FOXP1-positive. The lack of FOXP1 protein in normal, activated (CD30-positive) B cells was surprising following reports that *FOXP1* mRNA expression increased during normal B-cell activation (Shaffer *et al.* 2002). In contrast, mantle cells and up to 50% of germinal centre cells (mainly localized in the dark zone) were CD20/FOXP1-positive. In the interfollicular T-cell-rich areas there were many single nuclear FOXP1-positive cells (mostly T cells) while the CD20-positive interfollicular large B cells expressed FOXP1 in a heterogeneous manner. This scenario is consistent with observations of lymphomas reported in the current study.

HRS cells are characterised by the expression of CD30 and were FOXP1-negative in most of the cases studied. Two cases of cHL showed cytoplasmic/membrane FOXP1 expression, while scattered nuclear FOXP1 expression was observed in the cHL-derived cell line, KMH2. FOXP1 expression in non-malignant germinal centres is restricted to the nucleus. Cytoplasmic mislocalisation is a well established mechanism for inactivating the function of nuclear transcription factors and we hypothesise that the normal nuclear functions of

the FOXP1 protein are likely to be blocked when it is present in the cytoplasm. Interestingly, in contrast to HRS cells, L&H cells of LPHL are CD30-negative and retain expression of B-cell markers, yet are also largely FOXP1-negative. Overall, FOXP1 expression and/or nuclear function is rare in both cHL and LPHL, but it appears that this is not associated with defects in B-cell related gene expression nor with the expression of CD30.

To determine whether the lack of FOXP1 expression was a reflection of the germinal centre origin of HL, we extended the investigation to include cases of FCL. Surprisingly, 11 of the 13 FCL cases showed nuclear FOXP1 expression in the tumour cells. The presence or absence of FOXP1 expression in germinal centre-derived lymphomas might be a reflection of the normal heterogeneity of its germinal centre expression, or there may be distinct FOXP1-positive and negative subpopulations giving rise to different lymphomas. Studies of monoclonal immunoglobulin gene rearrangements in FCL and HL-derived from the same patient, have indicated that these originated from a common germinal centre precursor B-cell (Marafioti *et al*. 1999) although the two diseases show different phenotypes. These data indicate that FOXP1 expression patterns are not solely due to the germinal centre origin of these lymphomas, but suggests that they may be influenced by later transforming events. Further studies will be required to determine whether this differential expression of FOXP1 contributes to the clinical behaviour and biology of these diseases.

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