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The FOXP1 Winged Helix Transcription Factor Is a Novel Candidate Tumor Suppressor Gene on Chromosome 3p[1]

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ABSTRACT

The JC12 monoclonal antibody recognizes a previously unknown nuclear protein that showed a restricted distribution in normal tonsil and was also overexpressed in a subset of diffuse large B-cell lymphomas. Using this reagent, we expression cloned cDNAs encoding its antigenic target and identified this protein as a novel putative transcription factor, FOXP1. The FOXP1 protein sequence contains predicted domains characteristic of transcription factors, including a winged helix DNA-binding motif, a second potential DNA-binding motif, a C_2H_2 zinc finger, nuclear localization signals, coiled-coil regions, PEST sequences, and potential transactivation domains. The *FOXP1* gene has been mapped to chromosome 3p14.1, a region that commonly shows loss of heterozygosity in a wide range of tumors and which is reported to contain a tumor suppressor gene(s). Using tissue arrays and immunohistochemistry, we demonstrate that both the *FOXP1* mRNA and protein are widely expressed in normal tissues. The levels of *FOXP1* mRNA were compared in paired normal and tumor tissues (from the same patient) using a tissue array containing cDNAs extracted from 68 samples taken from kidney, breast, prostate, uterus, ovary, cervix, colon, lung, stomach, rectum, small intestine, and from nine cancer cell lines. Differences in *FOXP1* mRNA expression between normal and tumor samples were observed in 51% of cases. Most striking was the comparative loss of expression in 73% of colon tumors and comparative overexpression of *FOXP1* mRNA in 75% of stomach tumors. Analysis of the *FOXP1* mRNA expression in normal tissues (not taken from cancer patients) indicated that loss of *FOXP1* expression may occur in some histologically normal tissues adjacent to tumors. Immunohistochemical analysis of FOXP1 protein expression was performed on 128 solid tumors, including 16 renal, 9 breast, 12 lung, 20 colon, 21 stomach, 10 head and neck, 35 prostate, and 5 pancreatic cases. Complete loss of expression, increased expression, and cytoplasmic mislocalization of the predominantly nuclear FOXP1 protein were frequently observed in neoplastic cells. Our study identifies *FOXP1* as a new candidate tumor suppressor gene localized to the chromosome 3p14.1 region.

INTRODUCTION

Transcription factors play a central role in regulating an organism's normal development because the gene regulation in developmental processes occurs primarily at the transcriptional level (1). The *forkhead* domain was defined in 1990 by the homology between the DNA-binding domains of *HNF*-3[3] and the *Drosophila forkhead* gene (2–5). Proteins containing such domains are now grouped into the "winged helix" family, named after the three-dimensional structure of the DNA-binding domain when bound to DNA (6). Members of this family take part in a wide range of normal developmental events, including the control of cellular differentiation and proliferation, pattern formation, and signal transduction (7, 8).

In addition to their normal roles, members of this family participate in mammalian oncogenesis. *Qin* is a retrovirally transduced murine oncogene (9), and at least three winged helix genes are involved in chromosomal translocations in human malignancies. *AFX* and *AF6q21* have both been identified fused to *MLL* in cases of acute leukemia (10, 11). *FKHR* fuses to members of the paired box (or PAX) transcription factor family, *PA×3* and *PA×7*, as a result of two translocations associated with alveolar rhabdomyosarcoma (12, 13).

More recently, this family of transcription factors has been identified as additional targets for the PI(3)K/PKB signaling pathway (reviewed in Ref. 14). Overexpression of the AFX-like (FOXO family) *forkhead* transcription factors has been shown to cause growth suppression in a variety of cell lines, mediating cell cycle regulation by Ras and PKB through $p27^{Kip1}$ (15). The authors of this study suggested that disruption of the PI(3)K/PKB/*forkhead* transcription factor pathway during tumorigenesis may override a cell cycle block rather than bestow protection from apoptosis. They concluded that inactivation of these proteins is an important step in oncogenic transformation (15).

Knowledge of chromosomal deletions has made a significant contribution to the detection of tumor suppressor genes, and the Mitelman Database of Chromosome Aberrations in Cancer (2001)[4] provides a useful site for searching for the genetic abnormalities associated with a chromosomal locus. Deletions at chromosome 3 have been reported to be the third most common of all known deletions in human tumors (16). Studies using cytogenetic and LOH techniques have identified four regions at 3p that may contain tumor suppressor genes. They involve chromosome 3 bands p12, p14.2, p21.3, and p25 (reviewed in Refs. 17 and 18). There is considerable interest in the identification of potential tumor suppressor genes within these regions. Some candidates include the *FHIT* gene, which spans the fragile site, FRA3B, at 3p14.2; the DNA mismatch repair gene *MLH1*, which resides at 3p21.3-p23; the DNA repair gene *XPC* located at 3p25; and the *VHL* tumor suppressor gene at 3p25-p26.

Here we report the identification of a novel winged helix transcription factor, FOXP1, using a new monoclonal antibody (JC12). The *FOXP1* gene has been mapped to chromosome 3p14.1, a chromosomal locus shown to be disrupted in a range of solid tumors. Characterization of both *FOXP1* mRNA and protein expression has shown that this molecule is widely expressed in normal tissues, and that its expression is frequently altered in solid tumors.

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[3] The abbreviations used are: HNF, hepatocyte nuclear factor; DLBCL, diffuse large B-cell lymphoma; LOH, loss of heterozygosity; PI(3)K, phosphatidylinositol 3 kinase; PKB, protein kinase B; RCC, renal cell carcinoma; VHL, von Hippel-Lindau; NLS, nuclear localization signal; PIN, prostate intraepithelial neoplasia; CMV, cytomegalovirus; MTE, multiple tissue expression; cdk, cyclin-dependent kinase; S/T, serine/threonine; SCC, squamous cell carcinoma.

[4] Internet address: http://cgap.nci.nih.gov/Chromosomes/Mitelman.

MATERIALS AND METHODS

Antibodies. Mouse monoclonal antibody JC12 (isotype IgG2a) was raised as described previously (19) during a fusion intended to make antibodies against a peptide (NAAAESRKGQERFNC) from the *Bcl-X* gene. The peptide had been coupled to purified protein derivative by the manufacturer (Severn Biotech, Kidderminster, United Kingdom).

Immunological cDNA Library Screening. Two cDNA libraries in lambda ZAP Express (Stratagene, Cambridge, United Kingdom) made from testis or circulating blood nucleated cells were screened with antibody JC12 for protein expression in *Escherichia coli* as described previously (20). The positive clones were excised *in vivo* from the lambda ZAP Express vector (Stratagene), following the manufacturer's instructions, to yield plasmids pAB195–200 containing the cDNA inserts in plasmid pBK-CMV. The pBK-CMV vector contains a bacterial promoter enabling prokaryotic or eukaryotic expression of the cloned cDNA fused in frame with β-galactosidase.

Expression of the JC12 Antigen in Transfected Cells. Plasmids pAB195–200 were transfected into the COS-1 monkey fibroblast cell line using the DEAE dextran method (21). Cells were used for transfection at 75% confluence, and 5 μg of plasmid DNA was used for each 25 cm^2 flask. After 3 days in culture, the cells were recovered by EDTA treatment, and cytocentrifuge preparations were made for immunocytochemical staining (22).

DNA Sequencing. DNA sequencing was performed using either M13 universal and reverse primers or internal oligonucleotides, a Cy5 Autoread sequencing kit, and an ALF Express DNA sequencer (Pharmacia, St. Albans, Herts, United Kingdom). Sequence for the full-length *FOXP1* cDNA encoded by plasmid pAB195 has been deposited in GenBank under accession no. AF146696. The cDNA sequences for plasmids pAB196 and pAB199 and pAB200, which were commercially sequenced by MWG Biotech (Milton Keynes, United Kingdom), have been assigned accession nos. AF146697, AF146698, and AF275309, respectively.

Bacterial Protein Expression and Detection by Western Blotting. The *E. coli* strain XLOLR (Stratagene), containing either plasmids pAB195–200 or the empty vector pBK-CMV, was induced to express recombinant proteins for 3 h at 37°C by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside as described previously (20). Protein extracts were prepared, resolved by SDS-PAGE electrophoresis, and Western blotted using the JC12 antibody and an enhanced chemiluminescence kit (Amersham) as described previously (20).

Preparation of Tonsil Extracts. Nuclear and cytoplasmic fractions of tonsil cells were prepared using an ABC buffer system. Briefly, cells were lysed for 2 min at 4°C in buffer A [50 mM NaCl, 10 mM HEPES (pH 8.0), 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% NP40, and protease inhibitors]. The lysate was microfuged at 6000 rpm for 3 min, and the supernatant (cytoplasmic fraction) was transferred to a clean tube. The pellet was washed three times by gently overlaying with buffer B [25% glycerol, 50 mM NaCl, 10 mM HEPES (pH 8.0), 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and protease inhibitors] and then removing the buffer. Nuclear proteins were extracted in buffer C [350 mM NaCl, 25% glycerol, 10 mM HEPES (pH 8.0), 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and protease inhibitors] for 20 min at 4°C. After centrifugation at 13,000 rpm for 20 min, the supernatant (nuclear fraction) was removed to a clean tube.

Hybridization to Clontech's (Palo Alto, CA) Expression Arrays. The 1.9-kb *Eco*RI fragment containing the NH_2 terminus of the *FOXP1* gene was labeled with ^{32}P using the High Prime Labeling Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The cDNA probe was then hybridized to Clontech's human MTE array and matched tumor/normal expression array following the manufacturer's instructions. Additional information about the cases and tissues on these arrays can be obtained from the Clontech Web site.[5]

Cell Lines and Tissue Samples. The COS-1 cell line was obtained from the Sir William Dunn School of Pathology, Oxford, United Kingdom. Cells were cultured in RPMI 1640 containing 10% FCS (Life Technologies, Inc.) at 37°C in 5% CO_2. Tissues were obtained from the Histopathology Department at the John Radcliffe Hospital, Oxford, United Kingdom; the Hospital Clinic, University of Barcelona, Spain; and the Pathology Department of the Haemek Medical Center, Israel.

Immunostaining of Tissue Sections. Tissues were dewaxed and then subjected to antigen retrieval by microwaving for 2 × 4 min in 50 mM Tris and 1 mM EDTA (pH 9.0). Immunostaining was carried out using the DAKO Envision system (DAKO Ltd., Cambridgeshire, United Kingdom) with antibody JC12 (diluted 1/80 in PBS containing 10% normal human serum). The stained sections were counterstained with hematoxylin (Gill's No.3; Sigma Chemical Co.) and mounted in Aquamount (Merck/BDH). The immunostaining results were reviewed by qualified pathologists, and cases which had no immunostaining in either the normal or neoplastic cells were discarded as noninformative.

RESULTS

Preparation of the JC12 Antibody. The monoclonal antibody JC12 is secreted by a cloned hybridoma obtained from a mouse immunized with a peptide sequence from the apoptosis gene *Bcl-X*. However, antibody JC12 did not recognize the Bcl-X peptide or immunostain cells transfected with *Bcl-X* cDNA (data not shown). We therefore assume that the immunogen used is irrelevant to the antibody's specificity and that the JC12 antibody is a murine autoantibody, because it is reactive with mouse tissue (data not shown).

Expression Cloning of the Gene Encoding the Protein Recognized by Antibody JC12. Six cDNA clones were isolated by the bacterial expression cloning technique using the JC12 antibody: two from a testis cDNA library (pAB195 and pAB199) and four from a blood cDNA library (pAB196–8 and pAB200). The proteins encoded by these six plasmids were expressed in *E. coli*, and all were recognized by antibody JC12 when tested by Western Blotting (Fig. 1*A*). In contrast, the antibody did not recognize any bacterial proteins or the β-galactosidase expressed by the "empty" vector pBK-CMV. Four cDNAs (pAB195, pAB196, pAB199, and pAB200) were selected for further characterization as their protein products were recognized by the JC12 antibody when eukaryotically expressed in transfectanted COS cells. Partial sequencing of the remaining plasmids pAB197 and pAB198 showed that these encoded a putative secreted protein, SIG11 (AF072733).

Sequencing of cDNAs and Identification of an Open Reading Frame. All four plasmid clones were sequenced and found to contain regions from the same gene. The three smaller clones were NH_2-terminally truncated and contained internal deletions when compared with the cDNA in plasmid pAB195, which contained a full-length cDNA clone. The deletions in pAB196 and pAB199 may be the result of alternative splicing events because the 5′ exon-intron sequence of pAB196 (AG/gt) represents the consensus splice junction sequence, and the 3′ sequence (ag/GA) is close to the consensus sequence ag/GC

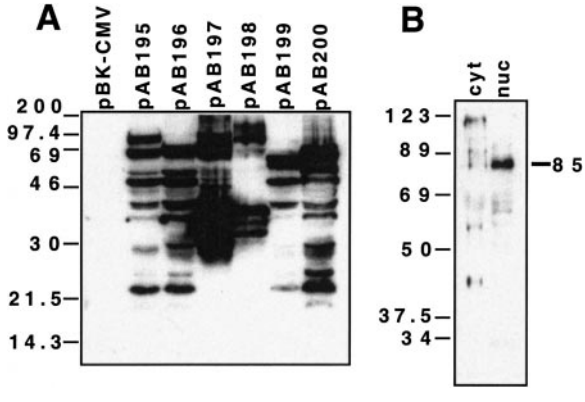


Fig. 1. Western blotting of recombinant proteins and cell lysates with monoclonal antibody JC12. *A*, antibody JC12 reactivity with recombinant proteins expressed from each of the isolated cDNA clones. There is no reactivity with the pBK-CMV empty vector. In *B*, the antibody detects an M_r 85,000 protein in nuclear (*nuc*) tonsillar extracts.

[5] Internet address: http://www.clontech.com.

for the 3′ exon-intron junction. The splice sites in the pAB199 clone are 5′ TC/aa and 3′ ag/AA. These do not correspond as closely to the consensus sequences described above, but they may still represent exon-intron boundaries, because only 5% of donor sites have been reported to match the strict consensus (23). The cDNA insert in plasmid pAB195 contained the longest cDNA insert and encoded the largest molecular weight protein (M_r ~89,000) among the four clones that were positive by immunostaining. This value (M_r 89,000) is compatible with the molecular weight (M_r 85,000) of the molecule recognized by monoclonal antibody JC12 in a tonsil nuclear extract (Fig. 1*B*).

A potential open reading frame of 677 amino acids, bordered by stop codons at both the 5′ and 3′ ends, was encoded by nucleotides 249-2279 of the pAB195 cDNA sequence. There were two potential in-frame methionine ATG initiation codons, the first of which had purines in both positions −3 and +4, whereas the second only had a purine at −3.

The JC12 Antigen Represents a Novel Winged Helix Protein, FOXP1. The full-length sequences from clone pAB195 were used to BLAST search the nonredundant National Center for Biotechnology Information database. Two cDNA sequences (AF151049 and NM_016477) and one genomic sequence (AC069298), all of which had been submitted recently, were found to encode the COOH terminus of pAB195. However our sequence represents the first description of the full-length cDNA for this gene and its corresponding polypeptide. At the time that the pAB195 cDNA sequence was originally isolated, the closest match was glutamine (Q)-rich factor 1, a partial cDNA (accession no. A49395) cloned from a murine B-cell leukemia (BCL1; Ref. 24).

The mouse and human proteins share 98% identity over the 84 amino acid sequence submitted to the database, differing in only a single conserved amino acid substitution. This homology defined the protein recognized by antibody JC12 as belonging to the HNF 3/*forkhead* domain family of winged helix proteins (24). In line with a new unified nomenclature for the winged helix/forkhead transcription factors (25), the protein encoded by pAB195 has been assigned the name FOXP1 by Dr. Daniel Martínez on behalf of the HUGO Nomenclature Committee.

Sequence Analysis of the FOXP1 Protein. Fig. 2*A* shows the FOXP1 amino acid sequence, and the schematic diagram in Fig. 2*B* illustrates the location of predicted domains in the polypeptide and the position of the internal deletions in pAB196 and pAB199. The FOXP1 protein also contains a second nucleic acid-binding domain with amino acids 308–331 matching the Cys_2-His_2 zinc-finger consensus Cx{2,4}C × 3(L,I,V,M,F,Y,W,C)x8Hx{3,5}H. This additional nucleic acid-binding domain is also present in two other winged helix transcription factors closely related to FOXP1, namely the human JM2 (FOXP3) protein (NM_014009) and the *Drosophila* CG16899 gene product (AE003684).

The FOXP1 protein also contains potential transactivation domains which are found in many transcriptional regulatory proteins. These activation domains can be grouped into categories based on their amino acid content, including acidic, glutamine-, proline-, serine-, and threonine-rich domains, and have been shown to function both *in vitro* and *in vivo* by interaction with the basal transcription machinery (26, 27). The NH_2 terminus of FOXP1 contains two glutamine-rich domains (aa 55–77 and aa 110–194) and a S/T-rich region between aa 244 and 268. The COOH terminus has two S/T/P-rich regions (aa 387–431 and aa 613–626) and an acidic domain at the extreme COOH terminus (aa 637–677). Significantly both glutamine-rich re-

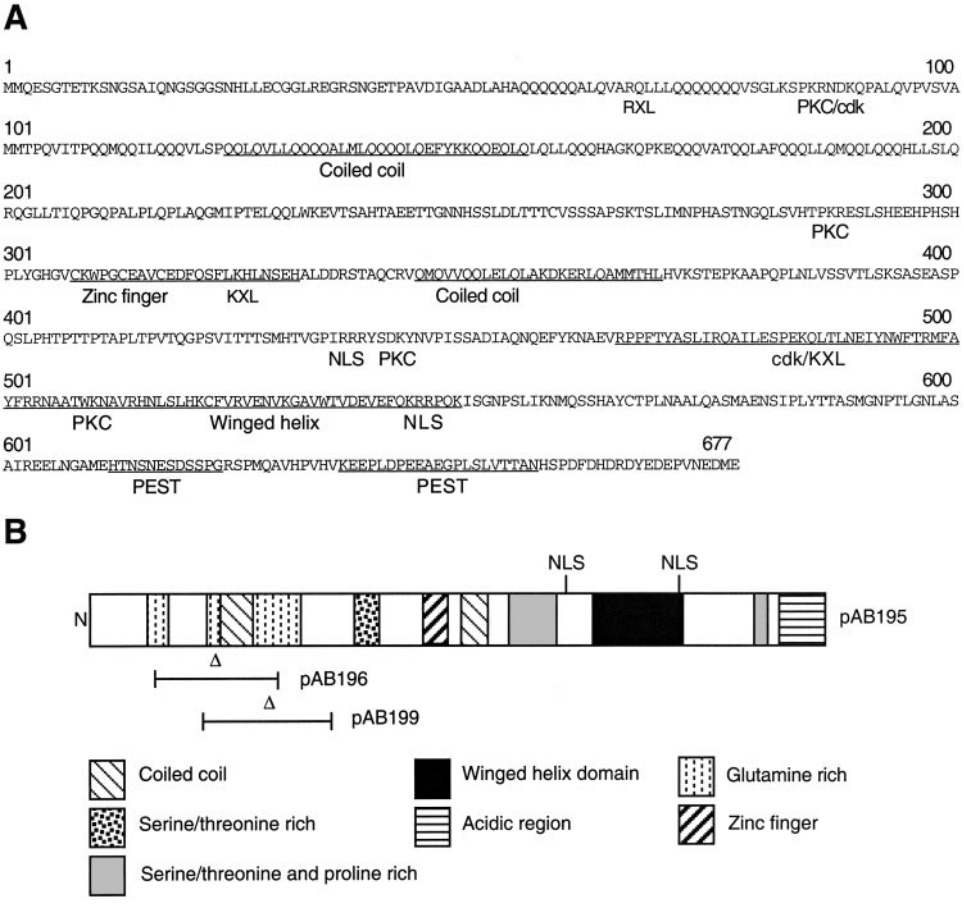


Fig. 2. Sequence analysis of the FOXP1 protein. *A*, amino acid sequence of the predicted protein product encoded by the cDNA in plasmid pAB195. Phosphorylation sites for protein kinase C (*PKC*), cdk protein kinase (*cdk*), and NLS are indicated underneath the protein sequence. *B*, schematic diagram of the FOXP1 protein indicating the location of predicted domains and motifs. The internal deletions within the proteins encoded by plasmids pAB196 and pAB199 are indicated by *bars*.

gions contain adjacent bulky hydrophobic groups that have been shown to be important in proteins, such as Sp1 (28) and Oct-2 (29), for the function of these transactivation domains.

The FOXP1 protein sequence was analyzed with the PSORT II program (30), and two potential NLSs were identified within the FOXP1 protein, namely, the PIRRRYS sequence between aa 434 and 440 and the KRRP sequence between aa 543 and 546.

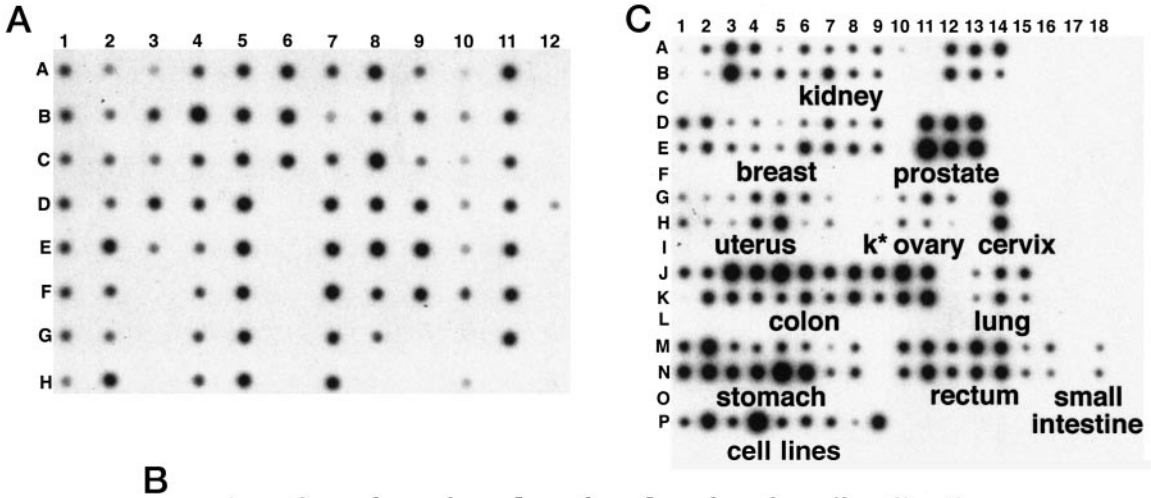
Analyses using the PESTfind program predicted that the FOXP1 protein contains two potential PEST sequences (31) in the acidic region near its COOH terminus (aa 612–623 and 636–656). A recent report shows that there is often an overlap between acidic activation domains and destruction elements (as occurs in FOXP1) in a number of transcription factors which are subject to ubiquitin-mediated proteolysis (32).

Another interesting feature of the FOXP1 protein is the prediction that two regions (aa 124–155 and aa 344–369) have the potential to form coiled coils (33). These motifs mediate protein-protein interactions and act as dimerization motifs in a number of transcription factors and other proteins (reviewed in Ref. 34).

There are a number of potential cyclin-cdk phosphorylation sites within the FOXP1 protein, comprising a S/T-P phosphoacceptor site and a preference for a basic residue at position +3 (where S/T is position 0; Refs. 35 and 36). Physical association with the cdk kinase may also play a role in establishing substrate specificity, and cyclin-cdk2 complexes bind stably to a number of cell cycle regulatory proteins. The ZRXL sequence (where Z and X are typically basic) has been identified as the cyclin-cdk2 binding motif in a number of these proteins, including E2F1, p107, and p21 (37–39). In both p45/Skp2 and pRB, the sequence KXL is used instead of RXL (40, 41). Both potential RXL and KXL motifs were present within the FOXP1 protein sequence (Fig. 2). The FOXP1 protein also contains a recognition site for the p70S6-kinase (RRYS), which is itself regulated by the PI(3)K.

Expression of the FOXP1 mRNA in Normal and Neoplastic Tissues. To determine the expression levels of *FOXP1* mRNA in normal tissues, the pAB195 cDNA was used to probe a human MTE array (Fig. 3, *A* and *B*). The normal tissues on this array come from nondiseased victims of sudden death/trauma and are pooled from a number of individuals. Loading of the cDNAs is normalized for three housekeeping genes to enable quantitative comparisons between gene expression in different tissues.

The *FOXP1* mRNA was found to be widely expressed in normal



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney	*E. coli* rRNA
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver	*E. coli* DNA
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen	Poly r(A)
F	temporal lobe	hippo-campus		ventricle, right	ilocecum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus	human C_0t -1 DNA
G	p. g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone morrow	ovary		colorectal adeno-carcinoma, SW480	fetal lung	human DNA 100 ng
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549		human DNA 500 ng

* paracentral gyrus

Fig. 3. Analysis of the expression of the *FOXP1* mRNA in human tissues. *A*, hybridization of the *FOXP1* cDNA to a MTE array. *B*, legend to the MTE array. *C*, hybridization of the *FOXP1* cDNA to a matched tumor/normal expression array. Tissue sources for cDNAs on the array are as follows: normals row *A*/tumors row *B*, kidney 1–14; normals row *D*/tumors row *E*, breast 1–9, prostate 11–13; normals row *G*/tumors row *H*, uterus 1–7, k* kidney 8, ovary 10–12, cervi × 14; normals row *J*/tumors row *K*, colon 1–11, lung 13–15; normals row *M*/tumors row *N*, stomach 1–8, rectum 10–16, small intestine 18. Row *P* human cancer cell lines: 1 HeLa, 2 Daudi, 3 K562, 4 HL-60, 5 G361, 6 A549, 7 MOLT-4, 8 SW480, and 9 Raji.

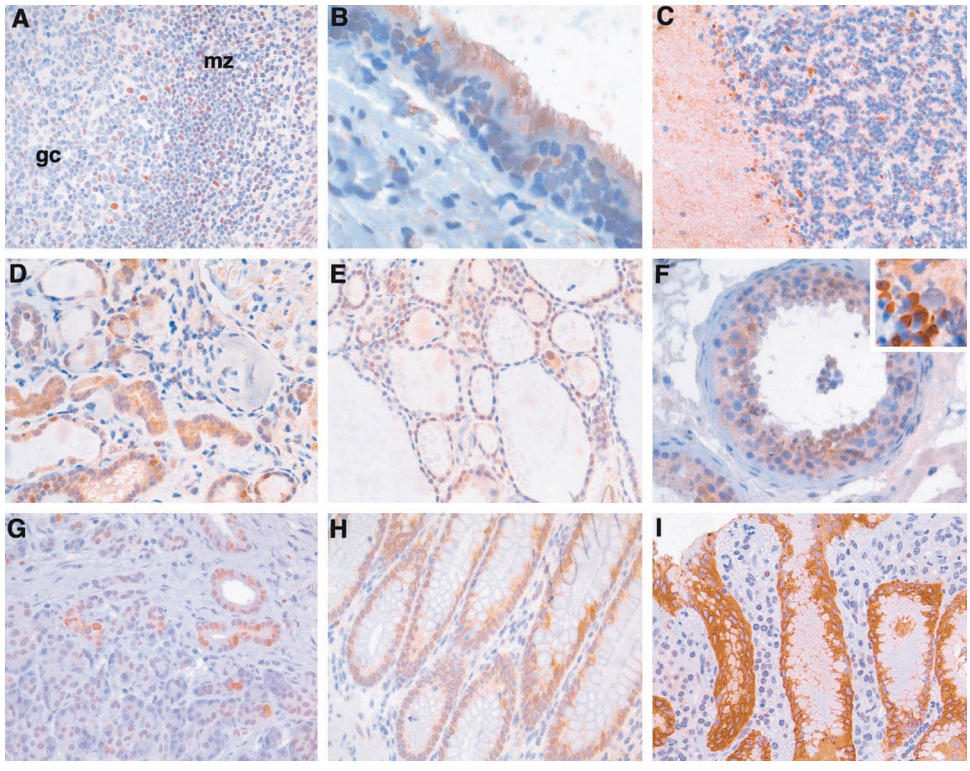


Fig. 4. Immunohistochemical staining of FOXP1 protein in normal tissues. In *A*, tonsil shows positive nuclear JC12 staining in some cells in the germinal center (*gc*) and the majority of cells in the mantle zone (*mz*). *B*, bronchial epithelium from the lung, showing nuclear staining of epithelial cells. *C*, cerebellum showing nuclear expression in approximately half of the cells in the granular layer and weak cytoplasmic staining. *D*, kidney showing cytoplasmic positivity in the proximal tubules and heterogeneous nuclear expression in the distal tubules and collecting ducts. *E*, heterogeneous nuclear staining in thyroid. *F*, testis showing both nuclear and cytoplasmic staining with a capped localization on the spermatids (visible in the high-power *inset* from a frozen section). *G*, nuclear labeling of exocrine acini and ductal epithelial cells in the pancreas. *H*, colon showing weak to moderate predominantly nuclear staining of cells in the base of crypts. *I*, fundic-type mucosa from the stomach showing strong cytoplasmic staining of foveolar epithelial lining while nuclear staining is visible in small lymphocytes in the lamina propria.

tissues, and no tissues lacked expression of this gene. Higher levels of mRNA were seen in some samples, particularly those from lymphoid and gastrointestinal tissues. Furthermore, the *FOXP1* mRNA was widely expressed in fetal tissues, in addition to those from adults, indicating that this molecule has a role throughout development. The majority of the tissues showed higher levels of *FOXP1* mRNA expression than was found in the cancer cell lines.

The same *FOXP1* fragment was also used to probe a matched tumor/normal expression array (Fig. 3*C*). Differential *FOXP1* mRNA expression between tumor and normal tissues was observed in 35 of 68 of the cases on the array. The most striking examples comprised the decreased levels of *FOXP1* mRNA in colon tumor samples, compared with their normal counterparts, and a trend for higher levels of *FOXP1* mRNA in the stomach tumor samples. Prostate carcinomas showed some evidence of increased expression of the *FOXP1* mRNA. In most tissues, including kidney, breast, uterus ovary, and lung, the normal tissue samples taken from cancer patients expressed relatively lower levels of *FOXP1* mRNA than would have been predicted based on the expression levels seen on the normal tissue array.

Expression of the FOXP1 Protein in Normal Tissues. Immunohistochemical staining of paraffin-embedded tissues with the JC12 monoclonal antibody was used to investigate the expression of the FOXP1 protein in normal human tissues. The FOXP1 protein is widely expressed in normal human tissues, including tonsil (Fig. 4*A*), bronchial epithelium (Fig. 4*B*), cerebellum (Fig. 4*C*), kidney (Fig. 4*D*), thyroid (Fig. 4*E*), pancreas (Fig. 4*G*), colon (Fig. 4*H*), spleen, blood, thymus, skin, and ovary, having a predominantly nuclear distribution. In most tissues, only a proportion of cells express the FOXP1 protein, *e.g.*, in reactive tonsil, many of the mantle zone B cells expressed the FOXP1 protein, whereas only a variable proportion (<50) of the germinal center cells expressed this protein. However, cytoplasmic labeling was also seen in some cells, particularly in epithelial tissues, including stomach (Fig. 4*I*) and colon (Fig. 4*H*), in lung macrophages and in spermatocytes (Fig. 4*F*). In testis, the spermatid nuclei also showed an unusual "capped" immunostaining pattern for FOXP1 protein (Fig. 4*F* high-power *inset*). In normal colonic mucosa, the cytoplasmic and nuclear JC12 staining showed a reciprocal distribution, with weak to moderate predominantly nuclear expression in the basal part of the crypts and strong, almost exclusively cytoplasmic labeling of cells toward the surface epithelium.

Expression of the FOXP1 Protein in Solid Tumors. Immunohistochemical staining of paraffin-embedded tumor biopsies with antibody JC12 was used to investigate the expression of FOXP1 protein in human malignancies.

RCC. Nuclear staining was seen in 7 of 10 cases of nonpapillary RCC (Fig. 5, *A* and *B*). In the remaining three cases, one showed only weak nuclear FOXP1 expression, one showed exclusively cytoplasmic expression (Fig. 5*E*), and one showed both nuclear and cytoplasmic expression. One renal chromophobe tumor showed high levels of both nuclear and cytoplasmic expression (Fig. 5*C*), whereas two cases showed only weak nuclear expression (Fig. 5*D*). Cytoplasmic expression was observed in the one papillary RCC case tested (Fig. 5*F*). Of the clear cell tumors known to have mutations in the *VHL* tumor suppressor gene or clinical features of *VHL* disease, three of four showed nuclear expression.

Breast Tumors. Moderate to strong nuclear staining for FOXP1 (Fig. 5*G*) was observed in three of nine cases of breast cancer (of which two were *in situ* tumors); the remaining six cases were ductal tumors, of which five showed only heterogeneous weak nuclear positivity (Fig. 5*H*), and one was negative (Fig. 5*I*).

Lung Carcinomas. Weak nuclear FOXP1 expression was seen in two of six small cell lung carcinoma cases (Fig. 5, *J* and *K*), and only very weak patchy nuclear staining was observed in the remaining four cases, which were largely negative (Fig. 5*L*). The intensity of the weak nuclear expression in the former two cases was comparable with that seen in the adjacent bronchial epithelium (*bottom right*, Fig. 5*J*). In contrast, none of the non-small cell lung carcinomas exhibited nuclear JC12 staining, two of six showed patchy cytoplasmic labeling (Fig. 5*M*), and four of six cases were negative (Fig. 5*N*).

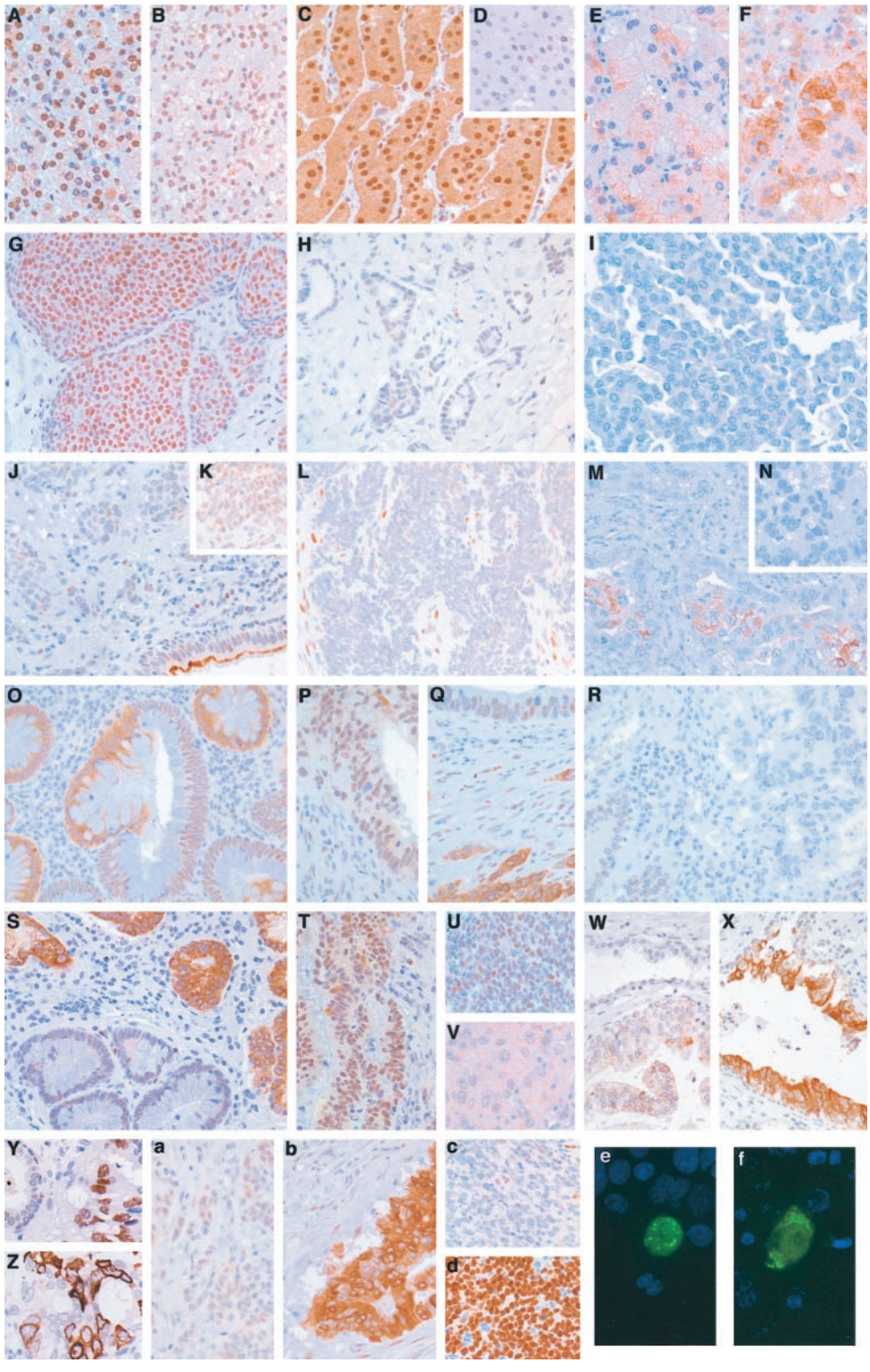


Fig. 5. Immunohistochemical staining of FOXP1 protein in neoplastic tissues. Renal clear cell carcinomas without (*A*) and with (*B*) VHL disease showing nuclear positivity with antibody JC12. Renal chromophobe tumors showing either strong cytoplasmic and nuclear labeling (*C*) or weak heterogeneous nuclear staining (*D*). Renal clear cell (*E*) and papillary tumors (*F*) with cytoplasmic positivity. Breast carcinoma *in situ* (*G*) and breast ductal carcinoma (*H*) showing nuclear labeling while the breast ductal carcinoma in *I* was negative. Small cell lung carcinomas showing normal (*J* and *K*) to weak (*L*) nuclear staining. Note the curious para-apical distribution of the FOXP1 protein in the bronchial epithelium (*J*). Patchy cytoplasmic labeling of a squamous non-small cell lung tumor (*M*). Negative non-small cell lung adenocarcinoma (*N*). The transition from normal (*left*) to neoplastic epithelium (*right*) from a tubulovillous colon adenoma (with foci of *in situ* carcinoma, not illustrated; *O*). Heterogeneous nuclear and cytoplasmic JC12 staining in a well-differentiated colon adenocarcinoma with mucinous differentiation (*P* and *O*) containing ribbons of poorly differentiated neoplastic cells (*Q*, *bottom*). *R*, poorly differentiated colon adenocarcinoma exhibiting no labeling of the neoplastic cells (*right*), while the base of nonneoplastic crypts have weak nuclear positivity. *S*, stomach base of foveolae with intestinal metaplasia showing loss of cytoplasmic staining. *T*, well-differentiated stomach adenocarcinoma, intestinal type, with strong nuclear and weak cytoplasmic labeling. Head and neck tumors showing nuclear labeling in an undifferentiated carcinoma (*U*) and cytoplasmic labeling of an SCC case (*V*). Prostate biopsy showing PIN with weak cytoplasmic positivity (*bottom*) and negative normal glands (*W*, *top*). Intense cytoplasmic staining in intraductal spreading of an otherwise infiltrating prostate carcinoma (*X*). Variation within the same prostate carcinoma case showing strong nuclear (*Y*) and heterogeneous strong cytoplasmic or negative (*Z*) labeling in different areas of the tumor. Pancreatic tumors showing either weak nuclear (*a*) or strong cytoplasmic labeling (*b*) of the neoplastic cells. DLBCLs (*c* and *d*) showing largely negative (*c*) or strong (*d*) nuclear labeling of the tumor cells. Note the positive normal lymphocytes in the negative case (*c*). Immunofluorescent staining for FOXP1 (*green*) in COS cells transfected with either pAB195 (*e*) or pAB196 (*f*) and counterstained with 4′,6-diamidino-2-phenylindole (*blue*).

Colon Tumors. The positive tumors showed heterogeneous FOXP1 expression patterns with nuclear staining and, to a lesser extent, cytoplasmic labeling being observed with antibody JC12. The staining pattern was variable both when comparing different cases and when comparing areas within a single case. There was a trend toward decreased cytoplasmic FOXP1 expression in the carcinomas (18 of 20) in comparison with nonneoplastic mucosa. The majority of the colon carcinomas (15 of 20) also showed weak or absent nuclear labeling for FOXP1. The transition from normal epithelium to neoplastic epithelium in a tubulovillous adenoma shows a loss of cytoplasmic and increased nuclear staining of the neoplastic cells (Fig. 5*O*). Variation within a single case is shown for an adenocarcinoma with mucinous differentiation, which shows nuclear labeling in a well-differentiated neoplastic gland (Fig. 5*P*), whereas ribbons of poorly differentiated neoplastic cells show only strong cytoplasmic staining (Fig. 5*Q*). Six cases of colon cancer were negative when immunostained for FOXP1 expression (Fig. 5*R*).

Stomach Adenocarcinomas. Surgically resected stomach adenocarcinomas (21) were immunostained for FOXP1. The most obvious difference between normal and tumor cells was the weaker cytoplasmic staining of malignant cells, which was also observed in metaplastic cells (Fig. 5*S*, *bottom*), compared with normal foveolar cells, which showed strong cytoplasmic staining and only weak, if any, nuclear staining (Fig. 4*I*). In contrast, 7 of 21 (33%) of the adenocarcinomas showed moderate to strong nuclear positivity. Four cases were negative, six showed weak, and three cases showed moderate to strong nuclear labeling (Fig. 5*T*). Three cases showed weak, and four cases showed moderate to strong nuclear and cytoplasmic labeling, whereas only one case was exclusively cytoplasmically stained.

Head and Neck SCCs. Two of the 10 cases tested showed moderate levels of nuclear FOXP1 staining. Of the remainder, one case was negative, five cases had weak nuclear positivity (Fig. 5*U*), and two cases showed exclusively cytoplasmic immunostaining (Fig. 5*V*). The two well-differentiated SCC cases both showed only very weak nuclear staining confined to a subpopulation of tumor cells.

Prostate Carcinoma. In normal prostate, inflamed glands (surrounded by lymphocytes) and secretory epithelial cells overlying transitional metaplasia were consistently positive for FOXP1. Cases of prostate carcinoma (35) were immunostained for FOXP1; 9 cases were negative, whereas intense labeling, stronger than in normal cells, and either nuclear or cytoplasmic was evident in 5 cases. PIN, a form of preinvasive lesion, was frequently more positive than the surrounding normal glands (Fig. 5*W*). Again, variation was observed within individual cases, *e.g.,* some parts of the tumor showed nuclear positivity (Fig. 5*Y*), whereas neoplastic cells in other areas were either negative or showed strong cytoplasmic staining (Fig. 5*Z*).

Pancreatic Adenocarcinoma. Immunostaining of five samples identified two cases with only weak nuclear expression (Fig. 5*a*) when compared with normal tissue (Fig. 4*G*), whereas three cases showed very intense immunostaining for FOXP1, which was predominantly cytoplasmic (Fig. 5*b*).

Tissues Adjacent to Tumors. We frequently found loss of FOXP1 immunostaining in nonmalignant cells adjacent to or within tumors, particularly normal lymphocytes and endothelial cells, which in other cases, provided clear internal positive controls.

Expression of the FOXP1 Protein in Eukaryotic Transfectants. The cDNAs encoding FOXP1 (pAB195, pAB196, pAB199, and pAB200) were transfected into COS cells. Cytospin preparations of these cells were then immunostained for FOXP1 expression with antibody JC12. The proteins expressed from pAB195, pAB199, and pAB200 were detected in the nucleus. The full-length FOXP1 protein encoded by pAB195 was most strongly expressed and was often observed to localize to discrete sites within the nucleus (Fig. 5*e*). In contrast, the protein expressed by the pAB196 cDNA, which lacks portions of the FOXP1 NH_2 terminus but retains both NLSs, was predominantly localized in the cytoplasm of transfected cells.

DISCUSSION

This study was initiated after the observation that a new monoclonal antibody (JC12), of unknown specificity, showed nuclear labeling with a restricted distribution in reactive human tonsil and stronger labeling in four cases of DLBCL (Fig. 5, *c* and *d*). Expression cloning of the gene encoding the target of the JC12 antibody identified a new member of the winged helix transcription factor family, named *FOXP1*. The full-length *FOXP1* cDNA (encoded by plasmid pAB195) has two potential in-frame methionine codons at the start of the predicted open reading frame and multiple stop codons upstream, indicating that we have cloned the full-length gene. We propose that the second methionine codon has better Kozak consensus (GCCA/GCCATGG) than the first because of the occurrence of A at position −3 and G at position −6 and is therefore more likely to represent the start site for translation (42). Furthermore, two other winged helix genes, *WIN* (43) and *AF6q21* (44), have two possible ATG initiation codons, and both use the second to initiate translation.

The predicted FOXP1 protein sequence contains two potential nucleic acid-binding motifs, one of which is a *forkhead*, or winged helix, domain. This domain exhibits only 36% homology to the DNA-binding domain from the original member of the family *HNF-3α* (45). However, this domain has been shown to be capable of binding DNA (24) in the homologous mouse Foxp1 [glutamine (Q)-rich factor 1] protein which differs from that of the human FOXP1 winged helix domain by only a single conservative amino acid change.

The FOXP1 protein contains a second potential DNA-binding domain, a Cys_2-His_2 zinc finger. Two additional winged helix proteins, FOXP3 and the *Drosophila* CG16899 gene product, also contain both these domains, and they are the most closely related proteins to FOXP1 revealed by database searches. Mutations in the *FOXP3* gene have been shown recently to cause the fatal lymphoproliferative disorder of the scurfy mouse (46) and the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome in humans (47, 48).

The FOXP1 protein contains other regions (characteristic of both the winged helix family and transcription factors in general) that may regulate transcription or direct nuclear localization. Although there is no single definitive structural motif for a NLS (reviewed in Refs. 49 and 50), they are typically short sequences, usually rich in lysine and arginine residues, and often contain proline. The sequence KRRP occurs in FOXP1 at the end of the second wing of the *forkhead* domain in a region shown to encode a NLS in other family members, *e.g.,* HNF-3 (51). Although the second NLS in many other winged helix genes is found in helix one of the *forkhead* domain (51), this region is not conserved in FOXP1. The NLS is predicted to occur upstream of this domain as is found in the *FKHL15* winged helix gene (52).

As mentioned previously, two *FOXP1* cDNAs isolated during expression cloning (pAB196 and pAB199) contained in-frame deletions within their 5′ region that may be the result of alternative splicing. Both variant proteins would lack the NH_2-terminal coiled-coil domain and most or all of the second glutamine-rich domain, regions that may be important for mediating protein-protein interactions or transcriptional regulation, respectively. There are many known examples of proteins with different biological activities generated from a single gene by alternative splicing, including splice variants for a number of other winged helix genes (53–55). It is thus possible that these variants are translated *in vivo* to yield proteins that differ in their function(s) from the full-length FOXP1 protein.

We have demonstrated that *FOXP1* mRNA and protein are widely expressed in normal human tissues. Both the levels of mRNA and protein, together with the subcellular localization of the FOXP1 protein, vary between different tissues. The level of protein expression also varies within the same cell type and between different cell types within the same tissue, *e.g.,* in secondary lymphoid follicles, the majority of mantle zone B cells are positive, whereas the percentage of germinal center B cells expressing FOXP1 is very variable (usually between 10 and 50%). The different patterns of subcellular localization of the FOXP1 protein are likely to be biologically significant, and regulation of transcription factor function by subcellular compartmentalization has been widely reported, *e.g.,* the nuclear trafficking of the p53 tumor suppressor protein (reviewed in Ref. 56). This regulatory mechanism allows transcriptional machinery to respond rapidly to cell signaling pathways. The AFX family of winged helix transcription factors are also regulated by changes in subcellular localization,

because these proteins are exported from the nucleus after activation of the PI(3)K pathway through their phosphorylation by PKB/Akt (57, 58). However, the FOXP1 sequence does not contain any potential PKB phosphorylation sites, although we did identify a recognition site for the p70S6-kinase (RRYS), which is itself regulated by the PI(3)K.

EST sequences corresponding to the COOH terminus of the *FOXP1* sequence contain the mapped sequence tag sts-W89007 localizing the gene between markers D3S1261 and D3S1604, which approximates to chromosome bands 3p12.3–3p14.1 (59), and the National Center for Biotechnology Information locus link information for *FOXP1* states that this gene has been cytogenetically mapped to 3p14.1. Deletions of chromosome 3 have been reported to be the third most common of all known deletions in human tumors (16). Studies using cytogenetic and LOH techniques have identified regions on 3p that may contain tumor suppressor genes (reviewed in Refs. 17, 18, and 60). In contrast, a survey of 3p deletions in hematological malignancies concluded that these anomalies were not a common feature in this type of neoplasia. When they did occur, most were secondary aberrations and were more distal (3p25–3p26) than those seen in solid tumors (61). This may be significant in relation to the overexpression of the FOXP1 protein that we have observed in cases of DLBCL, and we are currently investigating the expression of the FOXP1 protein in hematopoietic malignancies.

On the basis of the association between the chromosome localization of the *FOXP1* gene and the presence of an unidentified tumor suppressor gene in this region, we investigated the expression of FOXP1 in solid tumors. Differential expression of the *FOXP1* mRNA between tumor and adjacent normal tissue was frequently observed in tumors, particularly stomach and colon. One limitation of this technique is that it cannot distinguish between closely related or polymorphic mRNAs of different sizes. Furthermore, tumor samples usually contain some normal tissue so that a negative tumor can give a false-positive signal. It is possible that the lower levels of *FOXP1* expression (when compared with the relative levels on the normal tissue expression array) in a number of normal tissues adjacent to tumors may indicate that these histologically normal tissues already have alterations in the expression of this gene. This explanation is supported by data from a number of studies that have identified allelic loss at either 3p or 3p12–14 in premalignant lesions from a range of solid tumors (62–69) and in histologically normal tissues, *e.g.*, in bronchial (70) and nasopharyngeal epithelia (71). Changes in the expression of the *FOXP1* mRNA may therefore be an early event in tumor formation, and our finding that immunostaining identified changes in FOXP1 protein expression in gastric intestinal metaplasia and PIN supports this hypothesis.

Immunohistochemical analysis of FOXP1 protein in 128 cases of solid tumors identified a variety of different staining patterns, as has been seen with other tumor suppressor genes, such as p53 and $p27^{Kip1}$. Nuclear, nuclear and cytoplasmic, exclusively cytoplasmic, and complete loss of expression were observed in a range of tumor types. Particularly prevalent was a heterogeneous pattern of FOXP1 expression, both in terms of the proportion of positive cells, intensity of staining, and the proteins subcellular localization, even within a single case. The immunostaining data were consistent with the *FOXP1* mRNA expression profiles obtained from the matched tumor/normal array experiment.

Mutations in the *VHL* gene at 3p25 cause a predisposition to the development of a number of different tumor types, including renal tumors. In addition, the *VHL* gene is also mutated in 57% of sporadic RCC (72). Three of the four cases of clear-cell RCC known to have either mutations in the *VHL* tumor suppressor gene or VHL syndrome expressed nuclear FOXP1 protein. A genetic locus, *NRC-1*, within chromosome 3p12 has already been shown to functionally mediate tumor suppression independently of *VHL* mutation in RCC (73), whereas loss of heterozygosity in a region containing the FOXP1 locus at 3p13–14 has been widely reported in RCC (summarized in Ref. 74). It is possible that loss of *FOXP1* expression may either represent or contribute to an additional *VHL* independent pathway in renal tumor formation.

Immunostaining of COS cells transfected with the FOXP1 cDNAs suggested one mechanism by which the FOXP1 protein could be localized to the cytoplasm. The FOXP1 protein containing an NH_2-terminal deletion encoded by the pAB196 cDNA was predominantly cytoplasmic in location despite containing both potential nuclear localization sites. Thus, a region in the FOXP1 NH_2 terminus is important for its nuclear retention, and alternative splicing may be one mechanism by which the subcellular localization of this protein is regulated.

In an abstract describing preliminary experiments, we reported apparent colocalization of the FOXP1 protein with the $p27^{Kip1}$ and $p21^{Waf1}$ proteins in double immunofluorescent labeling experiments performed on COS transfectants (75). However, we have not subsequently confirmed this finding, which appeared to have been an immunostaining artifact caused by antibody JC12 (IgG2a), binding both IgG_{2a} and IgG1 secondary antibodies.

While this manuscript was under review, a paper was published describing the cloning and characterization of the mouse *Foxp1* and *Foxp2* genes (76). The human and mouse proteins share 97% identity, with the exception of a stretch of 30 glutamine residues present in the NH_2 terminus of the mouse Foxp1 sequence. This study characterized the expression of *Foxp1* mRNAs and demonstrated that Foxp1 acts as a transcriptional repressor of the lung-specific mouse CC10 and human SP-C promoters (76). The transcriptional repression domain contained the novel zinc-finger motif, located between amino acids 251 and 490, NH_2-terminal to the winged-helix/forkhead DNA binding domain (76). These data suggest that FOXP1, like Foxp1, may function as a transcriptional repressor and may also have an important role in spatially restricting the expression of genes in lung epithelium.

In conclusion, our initial study of FOXP1 indicates that this novel transcription factor, the gene for which maps to a region exhibiting LOH in human cancer, shows abnormal expression patterns in solid tumors. However, additional studies are required, *e.g.*, identification of loss of function mutations, inactivation in familial and sporadic tumors, and rescue of the tumor phenotype by the wild-type allele, to confirm its biological role as a tumor suppressor gene.

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