FOXP3 immunohistochemistry on formalin-fixed paraffin-embedded tissue: poor correlation between different antibodies

Y L Woo, J Sterling, R Crawford, S H van der Burg, N Coleman, M Stanley

ABSTRACT
Since its original description, there has been a substantial output of publications related to the FOXP3 gene. The FOXP3 protein, a member of the forkhead/winged-helix family of transcriptional regulators is a nuclear product and is not expressed in the cell cytoplasm or on the cell surface. Expression of this single transcription factor causes a developmental switch in naïve T cells to a suppressor cell phenotype, more commonly referred to as regulatory T cells (Tregs). Tregs have been intensively studied in various autoimmune diseases, infections and different cancers. An increasing choice of commercially available monoclonal antibodies targeting FOXP3 is now available. This report describes the experience of using two commonly used monoclonal FOXP3 antibodies on formalin-fixed paraffin-embedded sections of different organs, including the cervix and vulva. The antibodies targeting different FOXP3 epitopes unexpectedly resulted in significantly different staining patterns. This phenomenon has not been previously reported and is likely to be an important observation.

The Foxp3 gene was first identified when its product was found to be abnormal in mice with the X linked disorder Scurfy. Subsequently, the FOXP3 gene was found to be mutated in the human X linked disorder IPEX syndrome (a recessive disorder characterised by the neonatal onset of insulin-dependent diabetes mellitus, infections, enteropathy, thrombocytopenia and anaemia, other endocrinopathy, eczema and cachexia). Mutations in Foxp3 and FOXP3 cause severe, multi-organ autoimmunity in both mouse and humans. The FOXP3 gene has been identified as the critical gene that controls the differentiation of naïve T cells into regulatory T cells (Tregs) and FOXP3 protein is considered a lineage marker of Tregs. As described recently, ‘There is currently a growth industry in ascribing all manner of T cell regulation to this Treg subpopulation. …The claims for a role of Treg cells in every ill known to mouse and humans, from cancer to autoimmunity, need critical evaluation…’. The function and role of Tregs are often inferred from investigations to detect the presence of the cells using commercially available monoclonal antibodies that are specific for FOXP3.

Here, unexpected immunohistochemistry results utilising two different monoclonal antibodies against FOXP3 on formalin-fixed paraffin-embedded sections of different tissues are reported.

METHODS
Materials and antibody
As part of an investigation into the profile of local tissue infiltrating lymphocytes in different grades of cervical intraepithelial neoplasia, two commercially available anti-FOXP3 antibodies (ab22510 and ab20034, Abcam, Cambridge, UK) were used for immunohistochemistry on 5 μm sections of formalin-fixed paraffin-embedded uterine cervical tissue. In total, more than 250 sections of the cervix were stained. As a negative control, no primary antibody was applied to sections, and tonsillar tissue was used as a positive control. After initial results, other slides were used to compare the staining patterns derived from the two antibodies: normal colon and spleen, and vulval skin affected by intraepithelial neoplasia.

Immunohistochemistry
Immunohistochemistry was undertaken in a laboratory with extensive experience in immunohistochemistry and cytochemistry. Multiple 5 μm serial sections from selected formalin-fixed, paraffin-

Figure 1  Example of a cervical section stained with ab22510. The area with the punctate staining pattern is magnified.

Figure 2  (A) Cervical specimen stained with ab20034. (B) Serial section stained with ab22510; the punctate staining pattern is magnified.
embedded blocks were cut onto aminopropyltriethoxysilane-coated slides. The tissues were dewaxed in xylene and rehydrated. Heat-induced antigen retrieval in citrate buffer (3 min in a pressure cooker) was followed by blockade of endogenous peroxidase activity with hydrogen peroxide. The sections were incubated with 10% goat serum in phosphate-buffered saline to reduce non-specific binding and background staining. The conditions for use of the primary mouse monoclonal antibodies were optimised, and the antibodies were used at 1:10 dilution (ab22510) and 1:40 (ab20034) dilution. They were applied onto the slides and incubated overnight at 4°C.

On the second day, the slides were washed with Tris-buffered saline containing 0.05% Tween. This was followed by incubation with secondary biotinylated goat anti-mouse antibody at room temperature. The colour change reaction was performed with horseradish peroxidase (HRP) labelled streptavidin biotin complex (SABC) using diaminobenzidine (DAB) as substrate. The slides were then counterstained with Harris Haematoxylin, dehydrated, and cleared in xylene using a Leica Autostainer XL (Leica

Figure 3  (A, C, E and G) Sections stained with ab20034. (B, D, F and H) Sections stained with ab20051. (A and B) cervix; (C and D) vulva; (E and F) spleen; (G and H) colonic mucosa. Serial sections are used for comparing the staining with the two antibodies.
Instruments, Nussloch, Germany). Coverslips were applied with DPX mountant (CV5000 Coverslipper; Leica Instruments).

The numbers of immunophenotyped cells in the stained sections were counted by visual inspection. For each section, photographic images of each microscopic field were obtained (magnification, ×100).

RESULTS
Tissue samples of cervical intraepithelial neoplasia grades 1–3 were initially stained with ab22510. An unusual punctate pattern of staining in the nucleus was consistently observed. Furthermore, the staining also appeared to be within the nucleus of the epithelial cells (figs 1 and 2). This unexpected result prompted a comparative trial of another anti-FOXP3 antibody, ab20034, which produced, unexpectedly, a significantly different staining pattern (fig 2). To investigate this phenomenon further, serial slices of formalin-fixed paraffin-embedded sections of other tissue types were studied. Interestingly, the initial observation was not only confined to the cervix but also seen in colonic tissue, normal spleen and specimens of vulval intraepithelial neoplasia. These represented columnar epithelium, lymphoid organ and also other stratified squamous epithelium (fig 3). In all the specimens, the cells that were positively stained were better defined with ab20034. The margins were clearer, and the punctate pattern that was noted using ab22510 was not observed.

DISCUSSION
Over the last 5 years, there has been an annual doubling of publications relating to FOXP3, while those on regulatory T cells have been even more substantial. The results of many of these studies have relied on the use of both ab20034 and ab22510.5–8

How are the two mouse monoclonal antibodies different? ab20034 is raised against FOXP3 fusion protein (uncharacterised epitope), while ab22510 is raised against a synthetic peptide derived from residues 418–431 of the C terminus of human FOXP3. The sequence of this immunogen is SQRRSPR-CSNPTGQP (Abcam, personal communication). A protein BLAST search of this sequence revealed that this immunogen was unique to foxp3 (mouse) and FOXP3 (humans). Therefore, although it is possible, these antibodies are highly unlikely to cross-react with other proteins.

In a recent study where the expression of FOXP3 was studied on different populations of T cells using monoclonal antibodies derived from different cell clones (259D, 206D, 236A/E7 and PCH101), different staining patterns were also observed.9 When seven different monoclonal antibodies were tested,10 all of them were found to recognise the FOXP3 protein in routinely fixed tissues and labelled FOXP3 transfectants. However, some monoclonal antibodies gave less non-specific background staining than others, whereas some showed stronger labelling of positive cells. In that study, it was noted that the monoclonal antibody from clone 256A/E7, which is equivalent to ab20034, was found to be most suitable for immunohistochemistry on paraffin-embedded sections, consistent with the observations made in this study. This suggests that different monoclonal antibodies to FOXP3 do not produce identical staining patterns.

It is not the purpose of this report to speculate on the reasons behind the significant differences observed in the staining patterns obtained using ab20034 and ab22510. It does however emphasise the point that not only does the interpretation of FOXP3-positive cells in tissue need to be critically evaluated, but that also the techniques used to study Tregs require greater standardisation.

Take-home messages
- Regulatory T cells (Tregs) play an important role in inflammatory conditions.
- Tregs can be detected in tissue using antibodies directed against FOXP3 protein.
- Commercially available anti-FOXP3 antibodies are raised against a variety of epitopes; in immunohistochemistry they can give very different patterns of subcellular localisation of the marker protein.

Funding: This project was funded by Cancer Research UK. Yin Ling Woo is a Cancer Research UK Gordon Hamilton Fairley Clinical Fellow.

Competing interests: None.

Ethics approval: Ethics committee approval was obtained for this study.

REFERENCES