Letters to the Editor

CORRESPONDENCE RE: GERADTS J, WILENTZ RE, ROBERTS H. IMMUNOHISTOCHEMICAL DETECTION OF THE ALTERNATE *INK4A*-ENCODED TUMOR SUPPRESSOR PROTEIN P14^{ARF} IN ARCHIVAL HUMAN CANCERS AND CELL LINES USING COMMERCIAL ANTIBODIES: CORRELATION WITH P16^{INK4A} EXPRESSION. MOD PATHOL 2001;14:1162–68.

To the Editor: We were interested in the technique for the immunohistochemical detection of p14ARF (ARF) in archival human cancers suggested by Geradts *et al.* We have also tested a panel of anti-p14 ARF antibodies, including the Neomarkers 14P02, Santa Cruz C-18 and 4C6 from Gordon Peters, on paraffin-embedded cell lines and human tissues. We similarly conclude that optimal ARF detection in archival paraffin-embedded tissue sections requires longer primary antibody incubation times (overnight at 4° *versus* 1 hour at room temperature) and higher antibody concentrations than formalin-fixed, paraffin-embedded cell lines.

The authors demonstrated ARF localization in the nucleus, nucleolus, and cytoplasm using the antibody 14PO2 (Neomarkers). On the basis that cytoplasmic staining was evident in cell lines where ARF is deleted, the authors disregarded cytoplasmic staining as nonspecific.

Recent *in vitro* evidence (1) suggests that ARF may associate with a cytoplasmic protein Pex19p, with resultant inactivation of wild-type p53. This demonstrates the importance of detecting cytoplasmic ARF in tumors. In a recent series of 50 breast cancers, we demonstrated strong nuclear ARF expression in 42% and strong cytoplasmic ARF expression in 16% of cases (2). The antibody used was a mouse monoclonal 4C6, a gift from Gordon Peters (ICRF, London), and at a concentration of 200 μg/mL did not show cytoplasmic staining of the negative control cell line MCF-7.

Recent evidence that HER2/*neu* expression may reduce ARF binding to Mdm2 (3) suggests that the ARF pathway may have significant clinical and therapeutic implications. We recommend that the authors should not dismiss cytoplasmic ARF on the pretext of the 14PO2 antibody, while the functional significance of the subcellular localization of ARF remains to be established.

Sarah Vestey,

Zoë Winters
Division of Surgery
University of Bristol
Bristol Royal Infirmary
Bristol, UK

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In reply: Thank you for giving us the opportunity to respond to the letter by Sarah Vestey and Zoë Winters, who commented on our recent paper in *Modern Pathology* (1). These investigators performed immunohistochemical p14^{ARF} studies using 14P02, the monoclonal antibody from NeoMarkers featured in our article, as well as two other antibodies that we did not test. They refer to their p14^{ARF} staining results in a series of 50 breast carcinomas that were published in abstract form only (2). They agree with our observation that paraffin-embedded tissues require a higher primary antibody concentration compared to cell buttons. Vestey and Winters cite evidence that, under certain conditions, p14^{ARF} may be localized to the cytoplasm (3). They also observed strong cytoplasmic staining in 8 of 50 breast cancers (2). This staining pattern reportedly was not observed in the MCF-7 cell line using the same antibody, and it is implied that therefore the cytoplasmic staining must reflect genuine presence of the antigen. In their letter, Vestey and Winters state that we "disregarded cytoplasmic staining as nonspecific" and suggest that we (and, presumably, other investigators) "should not dismiss cytoplasmic ARF on the pretext of the 14P02 antibody, while the functional significance of the subcellular localization of ARF remains to be established."

It appears that the letter by Vestey and Winters was prompted by an overinterpretation of carefully worded statements in our paper (1). It is true that (i) we disregarded cytoplasmic staining in the cells of interest because (ii) this type of reactivity could be

found in cells known not to express $p14^{ARF}$. From the latter observation we drew the inevitable conclusion that, under our assay conditions, cytoplasmic staining may be nonspecific, the important qualifier being MAY. We also stated that the significance of the variable subcellular staining patterns was unclear. We do not dispute that, in some instances, cytoplasmic reactivity on a $p14^{ARF}$ stain may indeed reflect presence of the protein in that compartment. However, we know of no way to reliably distinguish this from nonspecific cytoplasmic staining. In general, we subscribe to the view that paraffin section immunohistochemistry is not a good technique to prove subcellular localization of a particular antigen, especially in the cytoplasm; immunofluorescence and cellular fractionation studies seem to be more reliable in this regard. In fact, we require this kind of corroborating evidence before we score any novel immunohistochemical marker.

In our paper, we expressed the hope that new second-generation anti-$p14^{ARF}$ antibodies may be more sensitive and specific. This turned out to be the case with anti-$p16^{INK4a}$ antibodies. We no longer routinely use any of the anti-p16 antibodies from our earlier studies whose immunohistochemical properties we previously described in detail (4). Newer anti-p16 antibodies produce significantly better signal-to-noise ratios (1, 5, 6). It is conceivable that mouse monoclonal 4C6 is a novel anti-$p14^{ARF}$ antibody with improved specificity and sensitivity. It is not commercially available, and we did not have an opportunity to test it. We have not seen any data on the immunohistochemical performance characteristics of this antibody. The breast cancer study by Vestey and Winters was presented at the 2001 San Antonio Breast Cancer Symposium (2) but has not been published in a peer-reviewed journal, and at this point there is insufficient evidence to suggest that antibody 4C6 is superior to 14P02. Ideally, the immunohistochemical stains obtained with any novel antibody should be directly compared to those produced by previously tested ones. Moreover, it cannot necessarily be assumed that the same antibody turns out to be optimal for different laboratories, although it is likely that ultimately the antibody with the best performance characteristics will find the widest use.

In conclusion, we stand by our position that, with the anti-$p14^{ARF}$ antibodies we tested, cytoplasmic reactivity is uninterpretable because it may or may not indicate the presence of functional protein. Until an antibody becomes available that reliably distinguishes cytoplasmic $p14^{ARF}$ from nonspecific background, we shall continue to focus on the presence or absence of nuclear/nucleolar immunoreactivity. On behalf of all of the authors,

Joseph Geradts, M.D.
Department of Pathology and Laboratory Medicine
Roswell Park Cancer Institute
Buffalo, New York

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