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False-positive results in diagnostic immunohistochemistry are related to horseradish peroxidase conjugates in commercially available assays☆

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ABSTRACT

False-positive results in diagnostic immunopathology can lead to unnecessary treatments. The purpose of this study was to do a side-by-side comparison of 10 different antibodies commonly used in the clinical laboratory altering only the horseradish peroxidase (HRP) conjugate. The automated Leica BOND-MAX platform was used to study serial sections from 203 tissues including controls compared in a blinded fashion using the HRP conjugates from Leica (Refine HRP), Ventana Medical Systems (Ultraview HRP), and Enzo Life Sciences (Polyview HRP). False-positive results, defined as signal from cases known to not contain the target, were noted in 23 (13%) of 171 cases with the Leica HRP, 62 (36%) of 171 cases with the Ventana HRP, and no cases with the Enzo HRP. Each data set was performed simultaneously allocating 1 tray for each of the 3 different HRP conjugates. HER2/neu analysis from triple-negative breast cancers were scored as positive by immunohistochemistry in 6 (24%) of 25 cases using either the Refine or Ultraview HRP and in 0 of 25 cases with the Enzo conjugate. It is concluded that false-positive results in a wide spectrum of diagnostic immunopathology tests can occur from 13% to 36% of cases with commonly used commercial assays based on the HRP conjugate.

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1. Introduction

Perhaps the most substantial advance in the field of diagnostic surgical pathology over the last 25 years has been the predominance of immunohistochemistry in making definitive diagnoses and treatment decisions [1-8]. This advance, in turn, is strongly related to the improvements in generating antigen-specific antibodies, in understanding the role of specific proteins in many different diseases, and in the automation of the assay allowing much higher throughput. To cite a few examples, the diagnosis of the mesothelioma as well as the site of metastatic disease of unknown primary has been revolutionized by immunohistochemistry [1,3,5,7,8]. Plaza et al [7] were able to characterize 118 tumors to soft tissues as metastatic and not primary sarcomas based on the immunohistochemistry profile. The clinical significance of immunohistochemistry results is underscored by studies showing that breast cancers that are HER2/neu positive and treated with herceptin and docetaxol will have an overall response rate of 61% as compared with 34% if the latter drug alone is used [4]. Still, it is well documented that herceptin can cause serious adverse effects including heart damage in up to 30% of patients and pulmonary edema, and thus, it is important that only women with HER2/neu amplified breast cancers receive the treatment [4].

False-positive and false-negative results in immunohistochemistry have been recognized since the methodology began [9-13]. Even a few years ago, it was reported that about 20% of ER and PR immunohistochemistry results for breast cancer in the diagnostic laboratory were inaccurate [11]. It is well documented that variables such as type and pH of the fixative, time of fixation, and time interval after tissue procurement before fixation can be correlated with either poor signal (false negative) or high background (false positive) [9,13]. These issues have been mitigated by the near universal use of rapid fixation in 10% buffered formalin for small surgical biopsies. However, other variables such as the pretreatment conditions (protease vs antigen retrieval), tissue quenching, antibody concentration, incubation time of the primary antibody, and the detection kit can also correlate with both false-negative and false-positive results [9,13-15]. The most commonly used catalytic enzyme in diagnostic immunohistochemistry is horseradish peroxidase (HRP). Thus, ultimately, false-positive results reflect the inappropriate activity of the peroxidase in cells that do not contain the antigen of interest.

There are many commercially available kits and automated platforms used in diagnostic immunohistochemistry. Two of the most widely used are from Leica Biosystems and Ventana Medical Systems. The purpose of this article was to compare the secondary HRP conjugate from Leica, Buffalo Grove, IL, Ventana, Tuscon, AZ, and Enzo Life Sciences, Farmingdale, NY in a side-by-side comparison on the Leica BOND-MAX.

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2. Materials and methods

2.1. Case selection

Each sample was from a surgical biopsy ranging in size from 5 to 20 mm that was fixed immediately after excision in 10% neutral-buffered formalin for 7 to 24 hours, then embedded into paraffin. When indicated, 5-mm cores of the larger biopsies were prepared as tissue microarrays. For each tissue, the histologic diagnosis was confirmed using hematoxylin and eosin (H&E) staining. The pathology reports, including all immunohistochemistry and ancillary tests, were reviewed to confirm the diagnosis.

The study focused on the following commonly encountered diagnostic decisions: melanoma vs nevus, cervical intraepithelial neoplasia (CIN) vs a benign mimic of CIN, leiomyoma vs leiomyosarcoma, small cell carcinoma of the lung vs non–small cell cancer, and the ER/PR/HER2/neu status of breast carcinomas.

2.2. Diagnostic criteria of false-positive and false-negative results plus signal

Extensive clinical-pathologic diagnoses were available for each case, and pathology reports were reviewed to confirm the diagnosis. In this manner, false positives for individual cases were determined by the presence of a 3,3′-diaminobenzidine (DAB) precipitate in cells known to not have the target of interest. For example, a triple-negative breast cancer case was scored as a false-positive result for HE2/neu if complete membrane staining was observed in more than 10% of the tumor cells. Similarly, if a breast cancer case was known to be HER2/neu amplified, then the HER2/neu test was scored as a false negative if less than 10% of the tumor cells showed complete membrane staining.

We also determined false-positive rates for groups of tissues by using as a reference point the published results for the expression of a given protein. For example, a cutoff value of 3.6 for the Ki-67 index has been shown to differentiate uterine leiomyoma vs leiomyosarcoma [16]. Similarly, the Ki-67 index for mesothelial derived inclusion ovarian cysts is very low (<0.1) [17].

2.3. Immunohistochemistry protocol

Each antibody (either mouse or rabbit) was optimized with known positive controls using a previously published protocol [13,14]. The following antibodies were used in this study: HMB45 (RTU, Enzo), chromogranin (RTU BioGenex), melanin-A, S-100, HER2/neu, ER, Ki-67 (each RTU, Ventana), and p16 (RTU CINtec), Ki-67 (Dako, 1:200), and HER2/neu (1:1500, Dako). For each antibody, optimal pretreatment consisted of antigen retrieval for 30 minutes using the Leica AR #2 solution, except for HER2/neu where 10 minutes of pretreatment in this solution was optimal.

The incubation time for the primary antibody was 60 minutes. The incubation time for the HRP conjugates was 13 minutes, and DAB incubation time was 10 minutes. A given experimental set consisted of 3 serial sections in the 3 separate trays of the Leica BOND-MAX, where the protocols were identical except for the HRP conjugate source. In this manner, variables such as the primary antibody, lot number for the detection kit, and antigen retrieval solution/time were identical for the slides tested for the Enzo, Ventana, and Leica HRPs. The HRP polymer from Leica was from the Bond Polymer Refine Detection system (catalogue #DS 9800). The Ventana HRP came from the UltraView Universal DAB detection kit (catalogue #760-500). The Enzo HRP polymers were the antirabbit (PolyView Plus HRP—catalogue #ENZ-ACC 103-0150) and the antimouse conjugates (catalogue #ENZ-ACC 104-0150).

2.4. Quantification of the data

Each immunohistochemistry result was scored blinded to the source of the HRP conjugate. The coding included the diagnosis of each case and the primary antibody. In this way, it could be determined which cell type(s) should show a signal, the specific cell localization of the signal (cytoplasmic, cell membrane, or nuclear), and which cell(s) would not contain the target of interest. A case was scored as a false negative if less than 10% of the target cells showed a signal. A case was scored as a false positive if more than 10% of nontarget cells showed a signal. The Ki-67 index was obtained by scoring at least 250 target cells.

2.5. Statistical analyses

Statistical analysis was done using the InStat Statistical Analysis Software (GraphPad Software, La Jolla, CA) (version 3.36) and a paired *t* test (also referred to as a "repeated-measure *t* test") testing the null hypothesis that the probability that the false-positive rate for a given HRP conjugate was equivalent to the probability with a different conjugate. The null hypothesis was rejected if the significance level was less than 5%.

3. Results

3.1. Human papillomavirus–positive CIN 1 lesions vs human papillomavirus–negative mimics of CIN

The study began comparing CIN 1 lesions to mimics of CIN. It is well documented that CIN 1 represents productive infection by human papillomavirus (HPV). Thus, these lesions contain high copy of HPV DNA that is easily detected by in situ hybridization [13]. Although the histologic changes induced by the virus that include disorganized squamous cell growth, variability in nuclear size, shape, and chromaticity, and variable-sized perinuclear halos may be diagnostic, other non-HPV conditions such as reactive squamous metaplasia can mimic the disease [13].

Mimics of CIN upon cervical biopsy for an atypical Papanikolaou test can occur in up to 30% of such biopsies. Most diagnostic pathology laboratories use p16 and Ki-67 immunohistochemistry testing to differentiate CINs from their mimics [13,18]. Thus, we studied 28 cervical biopsies in which 14 were determined to be CIN 1 by H&E examination and a positive HPV DNA in situ result. The other 14 cases were equivocal on H&E examination and were HPV DNA negative and, thus, were determined to be mimics of CIN (Fig. 1).

Each of these 28 tissues was tested for p16 and Ki-67 using the 3 different HRP conjugates. The results are summarized in Table 1. Note that each of the CIN 1 lesions was strongly positive for p16 and Ki-67 with each of the 3 HRP conjugates. Also note that the mimics of CIN 1 were scored as positive for both p16 and Ki-67 in 4, and 7 of 14 cases using the Enzo, Leica, and Ventana HRP conjugate, respectively. Representative data are presented in Fig. 1. Thus, although false-negative results were not evident, false-positive results were evident in 29% and 50% of the negative for CIN biopsies using the Leica and Ventana HRP conjugate, respectively.

3.2. Breast carcinomas: ER, PR, and HER2/neu status.

Next a series of breast carcinomas were studied. These included 50 breast cancers where the ER/PR status had already been determined. These 50 cases included 25 triple-negative (ER, PR, HER2/neu negative) breast cancers and 25 tubular carcinomas (or well-differentiated breast cancers with tubular features) where each tumor, as expected, was strongly ER+/PR+ and HER2/neu negative. Ten breast cancers documented to be HER2/neu amplified by fluorescence in situ hybridization served as the positive control for this immunohistochemistry test.

The Enzo, Leica, and Ventana HRP conjugate correctly identified the 10 HER2/neu-positive breast cancers as amplified for this oncogene (Fig. 2). Table 2 contains a summary of the data for the 50 cases. Note

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