Quantitative Analysis of the Decay of Immunoreactivity in Stored Prostate Needle Biopsy Sections

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Key Words: Prostate; Prognostic marker; Immunohistochemistry; Antigenicity; Paraffin slides; Method

Abstract

Application of immunohistochemistry to assess the presence of prognostic tissue markers is used widely. The quantitation of these markers may be hampered by a time-related loss of antigenicity in formalin-fixed paraffin-embedded tissue stored on glass slides. Potential loss of immunohistochemical staining intensity was studied on prostatic needle biopsy sections stored for a maximum of 4 years with antibodies against p27kip1, CD-44s, MIB-1, and androgen receptor (AR). In benign tissue, the positive/total ratio for p27kip1 was determined, while CD-44s staining intensity was assessed semiquantitatively. For MIB-1 and AR, nuclear staining intensity was assessed using computed image analysis. An exponential and significant decay of immunoreactivity was seen for p27kip1, CD-44s, MIB-1, and AR, with half-lives of 587 days, 214 days, and 290 days for p27kip1, MIB-1, and AR, respectively. Immunohistochemical assessment of prognostic tissue markers on stored slides must be considered with care in research and clinical settings.

The application of immunohistochemistry to study the expression of tissue markers on formalin-fixed paraffin-embedded tissue is widespread, and the prognostic role of some of these markers is used as a diagnostic and therapeuetic decision-making tool in several cancers. The need for additional prognostic tumor markers is a drive for considerable research efforts in many institutions. To evaluate the prognostic importance of a tissue marker by immunohistochemistry, well-fixed and adequately processed and preserved tissue material is a prerequisite for the prevention of false-positive and false-negative staining outcomes. In an effort to stain specific markers of interest in prostate needle biopsy sections, we happened to notice a potential loss of immunoreactivity over time in formalin-fixed paraffin-embedded tissue stored on glass slides.

In most clinical and research settings, it is a common practice to store precut unstained tissue sections on glass slides for reasons of direct access to positive control slides. Second, tissue specimens are stored on glass slides for retrospective studies in case there is too little tissue material left in the paraffin blocks after completion of routine diagnostics. This holds particularly true for prostate and mamma 18-gauge needle biopsy specimens.

Several authors have reported a loss or, occasionally, an increase of antigenicity in paraffin sections stored on glass slides. Unfortunately, the results of these studies lack general applicability because of their use of highly unorthodox tissue-fixation methods. Therefore, we wanted to repeat these studies on loss of immunostaining intensity on tissue specimens fixed and processed according to an on-time method, commonly applied in the majority of pathology laboratories. The potential loss of immunostaining intensity of 4 prognostic tissue markers for prostate
cancer was studied on tissue sections of prostatic needle biopsies stored for a maximum of 4 years.5-13 The expression of antigens in stored slides was compared with that in freshly cut paraffin blocks. By using this approach, we demonstrated an exponential decay of immunoreactivity throughout the years of all 4 studied prognostic tissue markers.

Materials and Methods

Tissue Specimens

From 1994 through 1998, all prostate needle biopsy specimens were fixed routinely in 10% buffered formalin at pH 7.4, embedded in paraffin, cut into 4-µm tissue sections, and mounted on glass slides. Unstained tissue sections representative of the detected prostate cancer were stored for later use on glass slides coated with amino-alkylsilane (AAS), in a dark environment at room temperature. To evaluate loss of immunostaining, we immuno histochemically stained and assessed a series of 7 slides for the storage years 1994 to 1998 with antibodies against the nuclear cell-cycle marker MIB-1 (Immunotech, Marseille, France) and p27kip1 (Novocastra, Newcastle upon Tyne, England), the cell-cell adhesion protein CD-44s (Bender MedSystems, Vienna, Austria), and the androgen receptor (AR) (clone F39.4.1).14 For each marker, batched series, including freshly cut specimens of prostate biopsies from 1994, were immunostained.

Immunostaining

After deparaffinization through xylene and 100% ethanol, endogenous peroxidase activity was blocked by immersing the slides for 20 minutes in a 3% hydrogen peroxide–methanol bath. The slides were placed in a 10-mmol/L concentration of citrate buffer, pH 6.0. Antigen retrieval was performed in a microwave oven at 700 W for 15 minutes. After cooling, the slides were placed in a Sequenza immunostaining system (Shandon, Runcorn, England) and preincubated with 10% normal goat serum (DAKO, Glostrup, Denmark) in phosphate-buffered saline–bovine serum albumin, 5%. Then, the slides were incubated overnight at 4°C with the primary antibody MIB-1 at an optimal dilution of 1:3,000, p27kip1 at 1:40, anti-AR at 1:200, and anti–CD-44s at 1:20 in phosphate-buffered saline–bovine serum albumin, 5%. For all immunostaining procedures, the conventional avidin-biotin complex method was applied. Briefly, a 30-minute incubation with the biotinylated goat-antimouse antibody (Biogenex, San Ramon, CA) was followed by a 30-minute incubation with the streptavidin-peroxidase complex (Biogenex). Subsequently, antibody-antigen binding was visualized with diaminobenzidine hydrochloride (Fluka, Neu-Ulm, Germany) with a 0.08% solution of hydrogen peroxide, and the CD-44s–stained and p27kip1–stained specimens were counterstained lightly with Mayer hematoxylin, dehydrated, and covered. No counterstaining was performed for MIB-1 and AR.

Quantitation

Staining in the benign prostatic glands was assessed blindly. For p27kip1- and CD-44s–stained slides, this was performed by 2 independent observers (A.N.V. and T.H.v.d.K.). For p27kip1, 400 nuclei were counted, and a positive/total ratio was calculated. For CD-44s, the membranous staining was scored semiquantitatively as follows: 0, absent; +, weak, only in basal cells; ++, moderate, in basal cells and sporadically in luminal cells; ++++, intense, in basal cells and most luminal cells. For MIB-1 and AR, nuclear staining intensity was assessed by a single observer (A.N.V.) using a computer video-image analysis program (KS 400, Kontron Elektronik, GmbH, Eching, Germany). For each slide, 20 randomly selected color video images of 512 · 512 pixels with a resolution of 0.4348 µm per pixel were recorded. For all nuclei above a prefixed threshold, the inverse mean density was measured by the computer program. The detection of the immunoperoxidase product was enhanced by omission of the hematoxylin counterstaining.

Statistical Methods

The chi-square test was used to determine the significance of differences in the CD-44s score in the different storage years. The Mann-Whitney U test was used to assess differences in mean positive/total ratio for p27kip1, absolute immunostaining intensity, and number of detected nuclei per slide for MIB-1 and AR. The level of significance was set at .05. For p27kip1, MIB-1, and AR, the difference between day of storage and day of immunostaining was calculated, and a decay curve of relative antigen expression was generated by using the formula:

\[ y_t = 100 \cdot 2^{(-\alpha x_t)} + a_0 \]

in which \( y_t \) stands for the relative antigen expression as a percentage, \( x_t \) for days of storage before immunostaining, and \( \alpha \) and \( a_0 \) are parameters assessing the curvature and horizontal asymptote of the exponential decay curve. Using this formula, the day of immunostaining (\( x_0 = 0 \)) stands for a relative antigen expression of 100% (\( y_0 = 100 \)). A half-life \( (x_{1/2}) \) of antigen expression was calculated by replacing factor \( x_t \) with 1/\( \alpha \).

Table I shows the exact figures for \( \alpha \) and \( a_0 \) for the different tumor markers.
Results

For p27kip1, there was a gradual but consistent decrease of mean positive/total ratio throughout time varying from 75.9% ± 12.49% in 1998 to 11.96% ± 30.85% in 1994 (Table 2) with a calculated half-life of p27kip1 antigen expression of 587 days. The freshly cut specimens had a mean ratio of 45.1% ± 30.6%. This was not statistically different from the slides stored in 1998 (Mann-Whitney U test). The mean interobserver variance was 6.3% per slide.

Similarly, for CD-44s, a significant and continuous decay was recorded throughout time (Table 3) (P < .01). In these slides, the discrepancy in interobserver slide assessment of more than 1 digit was noted in only 2 (6%) of 36 slides. A significant loss of nuclear immunostaining intensity was seen throughout time for MIB-1 as was quantitated by computer-assisted image analysis (Table 2; P < .001), while the number of separately detected nuclei per slide could be kept relatively constant per storage year.

For AR, the total number of detected nuclei per storage year and the mean number of detected nuclei per slide decreased significantly with time (Table 2; P < .001), but in the remaining detected nuclei, the mean measured immunostaining intensity was not different by storage year. The half-life of immunohistochemical staining intensity for MIB-1 was calculated as 214 days, and for AR, the half-life of proportional nuclear immunopositivity was 290 days (Figure 1). For both MIB-1 and AR immunostaining intensity and detection of positive controls, the freshly cut specimens of the paraffin blocks for 1994 were comparable with the immunostaining results of the slides stored in 1998 (Table 2), indicating maintenance of antigenicity of tissue stored in paraffin blocks.

Discussion

Immunohistochemical assessment of tissue markers in (pre)malignant tissue is used widely, and it is presumed that the assessment of these markers will continue to have a

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<tr>
<th>Table 1</th>
<th>Constant Variables for the Tumor Markers p27kip1, MIB-1, and Androgen Receptor (AR)*</th>
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<tbody>
<tr>
<td></td>
<td>alpha</td>
</tr>
<tr>
<td>p27kip1</td>
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<tr>
<td>MIB-1</td>
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<td>AR</td>
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*alpha and a0 are parameters assessing the curvature and horizontal asymptote of the exponential decay curve.

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<th>Table 2</th>
<th>Immunostaining Assessment of Tissue Markers p27kip1, MIB-1, and Antiandrogen Receptor (AR)</th>
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<tr>
<td>Storage Year</td>
<td>No. of Slides Stained</td>
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<td>1995</td>
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<td>1996</td>
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<td>1997</td>
<td>7</td>
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<tr>
<td>1998</td>
<td>7</td>
</tr>
<tr>
<td>1994*</td>
<td>4</td>
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* Positive control; freshly cut specimen.
† P < .01, Mann-Whitney U.
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<th>Table 3</th>
<th>Immunostaining Assessment of CD-44s*</th>
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<tr>
<td>Storage Year</td>
<td>Immunostaining Intensity of CD-44s</td>
</tr>
<tr>
<td>1994</td>
<td>0</td>
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<tr>
<td>1995</td>
<td>3</td>
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</tr>
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<td>1998</td>
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* Data are given as number of slides. 0, absent; +, weak, expression only in basal cells; ++, moderate, expression in basal cells and sporadically in luminal cells; ++++, intense expression in basal cells and in most luminal cells (P < .01, chi-square).
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Figure 1: Exponential decay curve of immunoreactivity for p27kip1, MIB-1, and the androgen receptor (AR). On the y-axis is the relative antigen expression (%), and on the x-axis, the time of storage before immunostaining (y). For MIB-1, the relative antigen expression stands for absolute immunostaining intensity, and for p27kip1 and AR, this stands for the proportion of immunopositive cells. For MIB-1, a half-life of immunostaining intensity was calculated as 214 days, and for AR and p27kip1, the half-life of proportional immunopositivity was 290 and 587 days, respectively.

Obviously, the time course of loss of immunoreactivity depends on the antigen and probably also on the affinity of the antibody and the type of tissue. Whether a tissue marker presents itself with loss of absolute immunostaining intensity or with loss of relative immunopositivity depends on its frequency of expression in benign tissue in combination with its ease of detection as a separate object by the naked eye or by a computer program.

MIB-1 is a cell proliferation marker and is expressed by few cells in benign prostate tissue. Therefore, MIB-1-positive nuclei were detected easily against a white background by the naked eye and the computer program. With time, all MIB-1 immunopositive nuclei will be detected as separate objects on stored slides, although with a decreasing immunostaining intensity. Figure 1 shows that the MIB-1 immunostaining intensity reaches an asymptote of relative antigen expression after slide storage.

The AR and p27kip1, on the other hand, are expressed in most nuclei in benign prostate tissue. Whereas the staining assessment of p27kip1 was done with the naked eye, the immunoreactivity of AR was assessed using a computer program, which was set to detect all AR-positive nuclei above a certain detection threshold. We observed that for AR, more positive nuclei could be detected with the naked eye than could be detected by the computer program. Therefore, it is assumed that only nuclei with an AR expression that exceeded background staining were detected as separate measurable objects by the computer program. With loss of immunoreactivity through time, the proportion of AR-positive nuclei and, thus, the mean number of detected nuclei per slide will decrease (Figure 1). Of course, the exact total of cells susceptible for detection could not be obtained to calculate a positive/total ratio, as was done for p27kip1, but the expected number of cells susceptible for detection was kept relatively constant by recording an exact number of 20 video images per slide.

A cause for the observed decay in immunoreactivity on stored slides cannot be given with certainty. It seems clear that the composition of tissue fixation is of utmost importance in the prevention of loss of immunoreactivity in stored slides, since Jacobs et al reported a significant decrease of immunostaining of p53, factor VIII, estrogen receptor, and Bcl-2 within 12 weeks of slide storage in breast carcinoma specimens after fixation in 10% buffered formalin supplemented with 70% alcohol. Bertheau et al reported a loss or even an increase of antigenicity in stored slides of different tissue origins after fixation in 10% formalin and postfixation with Bouin solution. Furthermore, oxidation of the antigen and masking of the antigen may underlie this loss of immunoreactivity. In our study, antigenicity of p27kip1, MIB-1, and AR was preserved when sections of long-term stored paraffin blocks were freshly cut. These results are consistent with those of Manne et al, who
observed no temporal decline in p53 and Bcl-2 expression after long-term storage of paraffin blocks. Despite their unorthodox tissue fixation, Jacobs et al showed that coating the surface of the tissue sections with a paraffin coat to diminish contact with the ambient atmosphere did not significantly prevent loss of immunoreactivity for p53 in breast carcinoma. This result suggests that antigen degradation may be prevented only by embedding the tissue specimen deep in the paraffin block and by proper tissue fixation and processing. Whether storage of tissue slides in a low-oxidative environment, ie, in N2, at different storage temperatures, or under a paraffin coat will diminish the destruction of the antigen by oxidation and, thereby, diminish decay of immunoreactivity is under further study. Alternative methods to retrieve immunoreactivity of the antigen after its decay may be an optimized microwave antigen retrieval or antigen amplification method, such as the tyramide signal amplification (TSA) method.

In this article, we emphasized the pitfalls that may occur in the assessment of immunohistochemically stained, formalin-fixed, paraffin-embedded tissue stored on glass slides at room temperature, whether for research or clinical settings. Storage of tissue material on glass slides for future use can cause unreliable immunostaining results for an indefinite number of antigens, while this immunoreactivity is maintained when tissue is archived in paraffin blocks.

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References