Inadequate Formalin Fixation Decreases Reliability of p27^{Kip1} Immunohistochemical Staining: Probing Optimal Fixation Time Using High-density Tissue Microarrays

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Immunohistochemical analysis of molecular targets in clinical tissues is increasingly becoming central to our ability to render diagnoses, to predict prognosis, to select patients for appropriate therapies, and to provide surrogate end points for therapeutic monitoring. For example, reduction of immunohistochemical staining for the cyclin-dependent kinase inhibitor p27^{Kip1} has been proposed as a potential prognostic biomarker in prostate, breast, and gastrointestinal tumors. We observed that with our standard formalin fixation in rapidly processed (same-day) radical prostatectomy specimens, there is often a gradient of p27^{Kip1} staining in normal prostate epithelium, with more staining near the periphery and less staining toward the center of the sample. This raised the hypothesis that the reliability of staining for p27^{Kip1} is decreased in inadequately fixed tissues. The implications of this, if true, are that many studies using p27^{Kip1} for prognostic purposes may be subject to unpredictable artifacts, and hence unreliable results, if the fixation of the specimens is not well controlled. The objectives of the present study were (1) to formally test the hypothesis that inadequate fixation time is responsible for apparent loss of p27^{Kip1} nuclear staining and (2) to test a recently proposed method for improving the uniformity of immmunohistochemical staining using formalin injection. Prostate tissue sections from radical prostatectomy specimens were either processed immediately (zero time fixation) or fixed for 1, 2, 3, or 8 days in 10% neutral buffered formalin before processing into paraffin. To assure identical antigen retrieval and immunohistochemical staining conditions for specimens fixed for different lengths of time, 2 high-density tissue microarrays (TMAs), containing 564 tissue samples (0.6 mm in diameter) were constructed. Based on an estimate of the percentage of nuclei in normal prostatic epithelial secretory cells with strong staining, quality of p27^{Kip1} staining was graded in a blinded fashion with respect to fixation time. There was a significant increase in the percentage of cores that were scored as "strong" as fixation time increased from 0 (same-day processing) to 1 or more days ($P <$ .0001). Interestingly, even at 8 days of fixation, there was excellent staining that was superior to the same-day processing. Based on these results, we conclude the following: (1) for large clinical specimens that have been fixed briefly to decrease diagnostic turn-around time, the reliability of interpretation of immunohistochemical staining may be quite limited; (2) for p27^{Kip1}, decreased antigen staining as a result of the widely held concept of "overfixation" is much less of a problem than "underfixation"; (3) formalin injection produces a marked improvement in staining for several markers, including p27^{Kip1}; and (4) high-density TMAs, which assure identical test conditions, provide an excellent platform on which to evaluate the effects of tissue fixation on immunohistochemical staining. HUM PATHOL 33:756–760. Copyright 2002, Elsevier Science (USA). All rights reserved.

Key words: **formaldehyde fixation, p27,^{Kip1} prostatic neoplasms, tissue microarrays.**

Abbreviations: **TMA, tissue microarray; PBS, phosphate-buffered saline; GSTP1, π class glutathione S transferase.**

The localization of protein expression in human clinical tissue samples promises to enhance our ability to render diagnoses, to predict prognosis, to select patients for appropriate therapies, and to provide surrogate end points for therapeutic monitoring. Yet, the widespread adoption of immunohistochemical staining to aid clinical decisions and research studies is not without potential pitfalls. Several problems regarding the ability to interpret immunohistochemical staining on archival tissue have been addressed in the literature.[1-4] Although some of the difficulties stem from loss of antigenicity caused by prolonged storage of paraffin blocks or unstained slides,[3,4] most of these problems are related to issues of fixation.[1,2] The majority of pathology specimens, both archival and those currently being accrued, are subjected to preservation of morphology using formaldehyde-based fixatives (10% buffered formalin). Major problems identified with formaldehyde based fixatives, which work by producing protein-protein and protein-nucleic acid cross-links, have been raised. These problems generally fall into the categories of inadequate fixation ("underfixation") or prolonged fixation ("overfixation").[1] Both of these problems are thought to contribute to variability in the staining of tissues across a given slide. "Underfixation" results from the fact that formalin diffuses slowly into many tissues and requires sufficient time to chemically cross-link targets (generally 24 to 48 hours for larger specimens). In terms of "underfixation," it has been suggested that there may be a gradation of immunohistochemical staining such that there is strong staining

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Supported by grants from Public Health Services NIH/NCI Specialized Program in Research Excellence (SPORE) in Prostate Cancer, Johns Hopkins (P50CA58236), and The University of Michigan (P50CA69568).

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0046-8177/02/3307-0012$35.00/0
doi:10.1053/hupa.2002.126187

near the periphery of a slide and less staining in the middle. This has been interpreted to indicate that adequate fixation occurred near the outside of the specimen, but that the inside of the specimen was preserved only by coagulative fixation from ethanol, which would occur during tissue processing.[1] In terms of "overfixation," it has been widely held that prolonged fixation in formalin results in loss of antigen staining with many antigens.[1]

P27Kip1 is a cyclin-dependent kinase inhibitor whose expression is decreased in several cancer types, including endocrine neoplasms; lymphomas; and gastric, colorectal, and mammary carcinomas.[5] In prostate tissue, p27Kip1 is expressed nearly uniformly in the luminal secretory cells, is downregulated in carcinoma, and has been proposed as a potential biomarker whose immunohistochemical detection may be useful in prediction of prognosis.[6-13] Regarding our radical prostatectomy specimens, we have noticed that the nuclei of secretory luminal cells in normal prostate epithelium stain nearly uniformly strongly for p27Kip1 in regions of the slide near the periphery, but nuclear staining is markedly decreased in many of the epithelial cells as the observer looks toward the center of the slide. This problem is particularly troublesome because in prognostic studies using cancer specimens, p27Kip1 is downregulated and what is graded is a loss or decrease in nuclear staining. We hypothesized that this gradation of staining was the result of lack of sufficient formalin fixation of the tissue toward the center of the block resulting from insufficient time for adequate aldehyde cross-linking ("underfixation"). If decreased staining is occurring in some specimens because of an artifact of fixation, then this could markedly impact the reliability of the staining interpretation and hence the validity of the results of many studies. In the present study, therefore, we tested the hypothesis that underfixation results in artifactual loss of p27Kip1 nuclear staining. We chose to use tissue microarrays[14] to provide uniform antigen retrieval and staining conditions. We obtained freshly prepared, cut sections of prostate tissue from radical prostatectomies and divided these into several pieces that were fixed in formalin for various lengths of time. We then constructed a high-density tissue microarray (TMA) from these specimens and sections were stained for p27Kip1. Finding that, indeed, inadequate time of fixation produced loss of p27Kip1 staining using our standard approach of radical prostatectomy processing, we tested a recently presented method of formalin injection[5] to determine whether this method would allow us to retain our rapid turnaround and provide tissues deemed adequate for immunohistochemical staining.

MATERIALS AND METHODS

Specimen Handling and TMA Construction

In our standard approach to radical prostatectomy sectioning, the gland is inked, and the specimens are fixed for 1 to 4 hours in formalin by simply placing the specimen in a minimum of 15 volumes of 10% neutral buffered formalin. Prostate specimens are then sectioned from apex to base into 3- to 5-mm slices, and each slice is further sectioned into halfs or, more commonly quadrants. Tissues are submitted into standard-sized cassettes, and are placed onto our automated tissue processors the same day (day 0). This results in a total fixation time of approximately 5 hours before commencing tissue dehydration.

For the current study, fresh radical prostatectomy specimens (n = 23) were inked and sectioned into tissue quadrants. Portions of specimens were cut into sections 3- to 5-mm thick and were placed into 10% neutral buffered formalin. Tissue sections were then either processed immediately (less than 5-hour total fixation time and designated as day 0) or fixed for 1, 2, 3, 7, or 8 days in 10% buffered formalin at room temperature before processing on a commercial automated tissue processor. Blocks were cut, and sections were stained with hematoxylin and eosin for histological confirmation of normal prostate epithelial tissue. Regions of normal-appearing epithelium were circled on the glass slides. These slides were then used as templates for construction of two high-density TMAs containing 564 samples (0.6 mm in diameter) using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD) as previously described.[14] The TMA block was sectioned at 4 μm.

Immunohistochemical Staining

Manual immunohistochemistry was performed with the ChemMate Detection System (Ventana, Tucson, AZ). After paraffin removal and hydration was performed, slides were paired to form the capillary gap and immersed in 10 mmol citrate buffer (Vector, Burlingame CA; pH, 6.0). Slides were then steamed for 14 minutes (Black and Decker Handy Steamer Plus, Towson, MD) to induce epitope retrieval. Slides then were placed into phosphate-buffered saline (PBS) with Tween (Sigma Chemical, St. Louis, MO) for 5 minutes at room temperature. Endogenous peroxidase was quenched by immersion in 3% hydrogen peroxide for 7 minutes at room temperature. Slides were rinsed twice with PBS and Protein Blocker (ChemMate Universal Detection System, Ventana, Tucson, AZ) was applied for 5 minutes. After they were rinsed twice in PBS, the slides were incubated with primary antibodies ([1] p27Kip1, mouse monoclonal antibody, 1:1600; Transduction Labs, Lexington KY; [2] glutathione *S* transferase [GSTP1]; rabbit polyclonal, 1:2000; DAKO, Carpinteria, CA; [3] 34βE12, mouse monoclonal antibody, 1:50; Enzo Biochem, Farmingdale, NY; [4] Ki-67, mouse monoclonal, clone MIB-1, 1:100; Zymed, South San Francisco, CA) either overnight at 4°C (p27Kip1, GSTP1) or for 45 minutes at room temperature (Ki-67). Slides were rinsed twice with PBS and then incubated with secondary biotin-labeled antibodies for 30 minutes at room temperature. Slides were rinsed twice with PBS, and avidin-biotin complex–horseradish peroxidase (ABC-HRP) was applied for 30 minutes at room temperature. Slides were washed twice in PBS and incubated in peroxide/diaminobenzidine (DAB) as substrate/chromagen per the manufacturer's instructions (ChemMate). Slides were washed twice in PBS, counterstained with hematoxylin for 30 seconds, rinsed in deionized water, dehydrated through graded ethanols, cleared in xylene, and mounted. Controls for p27Kip1 consisted of human tonsil tissue as described.[9]

TMA Sample Image Acquisition and Management

Digital images were acquired using the Bacus Labs Incorporated Imaging System (BLISS, Bacus Laboratories, Lombard, IL) as previously described.[16] In this system, a low power scan of the entire stained TMA slide is obtained automatically. The operator then indicates through the software the number and location of the array spots. All array spots are then automatically scanned at full resolution using a 20x Zeiss Plan-Apochromat objective (Zeiss Corp, Thornwood, NY). Each array spot is imaged as 6 individual 640 × 480–pixel images that the software automatically "tiles" into a single composite image. The composite images are stored in a file containing the embedded x, y coordinates from the tissue array spot.[16,17] The files are then imported into a relational database and related by their x, y coordinates to the specimen from which they were derived.

Images of each TMA spot, which were blinded with respect to fixation status, and samples were graded based on the estimated percentage of strong nuclear staining in luminal epithelial cells. A score of "1" was assigned for strong nuclear staining in 0% to 25% of the epithelium; a score of "2" was assigned for 26% to 50%; a score of "3" was assigned for 51% to 75%, and a score of "4" was assigned for 76% to 100%. In a subset of specimens, two pathologists separately scored the samples in a blinded fashion, and interrater reliability was assessed. All images were scored using a computer monitor, and images were transmitted over the Internet.

Statistical Analysis

Data were recorded directly into Microsoft Access 2000 (Microsoft Corp, Redmond, WA). Statistical analysis was carried out using STATA 6.0 for Microsoft Windows (Stata Corp, College Station, TX).

RESULTS

The gradation of staining seen with the standard sections from a radical prostatectomy sample sectioned and cut using our routine protocol of same-day tissue processing is illustrated in Fig 1. As can be seen, the nuclear staining is more uniform and intense toward the outside of the gland, with only very weak nuclear staining toward the inside. We describe this as "outside-in gradation" in staining. Tissues were fixed for different lengths of time to evaluate the hypothesis that the lack of strong staining toward the center of the specimen was caused by inadequate fixation. These tissues were used to construct 2 TMAs. The majority (75%) of TMA cores (n = 423 of 564 total spots) contained adequate normal prostate epithelial tissue for evaluation. Those considered inadequate for p27^{Kip1} scoring contained either predominantly stromal tissue or too little tissue. Overall, 258 of 423 (61%) evaluated tissue cores were considered to have "strong" staining for p27^{Kip1} such that they contained greater than 50% (staining scores of "3" or "4") of epithelial cells with strong nuclear staining (Table 1). There was an increase in the frequency of spots that were scored as "strong" as the specimens were fixed longer (Fig 2). Interestingly, there was little decrement in staining with prolonged fixation such that even at 8 days of fixation, the score was higher than that at day 0 and equal to that of day 2. Because there was a leveling off of the improved staining, with little apparent decrement, and because it is generally impractical for surgical specimens to be fixed longer than 2 days, statistical analysis was carried out on spots that were grouped into the following 3 categories: those fixed for 0 days, those fixed for 1 day, and those fixed for 2 or more days. There was a significant increase in the percentage of cores that were scored as "strong" (Pearson χ^2, 56.8; $P < .0001$; Table 1) as fixation time increased from 0 to 1 or more days.

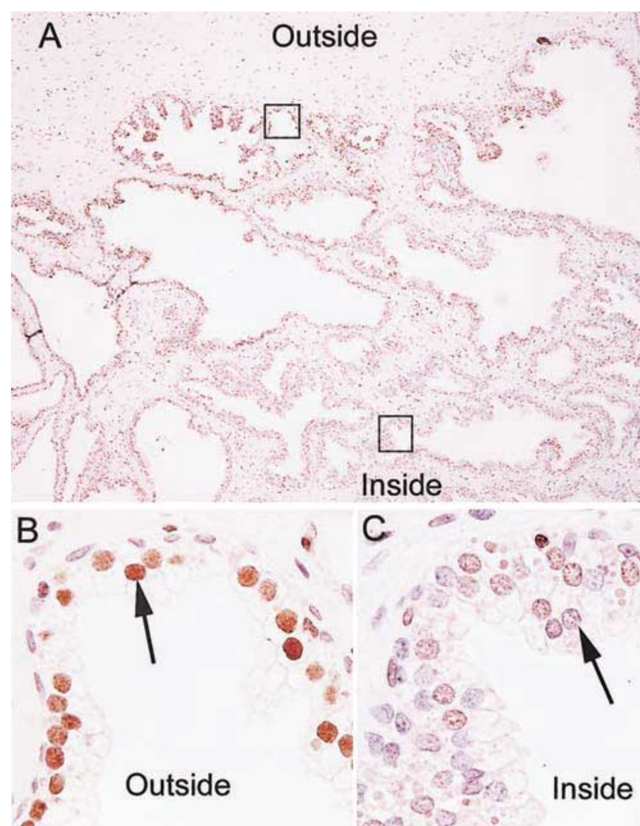

FIGURE 1. "Outside-in" gradation of staining for p27^{Kip1} in normal prostate epithelium. (A) Low-power view of staining near periphery of the specimen. Area marked "outside" is within 0.5 mm of the inked surgical margin. Note strong nuclear staining toward the outside of the gland and weak staining toward the inside. (B) Boxed area from (A) at higher power showing strong nuclear staining of secretory luminal cells (arrow) near outside of gland. (C) Boxed area from (A) at higher power showing very weak/negative nuclear staining in secretory luminal cells (arrow) toward the inside of the specimen. (anti-p27^{Kip1}; original magnification (A) ×40; (B, C) ×400.)

To determine the interrater reliability of the staining results, a nested set (n = 117) of the spots was evaluated separately by two observers. Overall, the agreement for the 4-tiered staining system was 74.1% (expected agreement, 58.1%; $\kappa = 0.383$; $z = 7.59$; $P < .00001$). When the scoring was categorized using a 2-tiered system, as either strong or not (corresponding to a score of "3" to "4" for strong and "1" to "2" for not

Table 1. Scoring of p27^{Kip1} on Tissue Microarray Spots Stratified by Fixation Time

Fixation Time (d)	IHC Score				
	1	2	3	4	Total
0	29	22	15	26	92
1	19	24	30	34	107
2	10	7	42	2	61
3	20	16	40	27	103
7	4	6	28	1	39
8	1	7	13	0	21
Total	83	82	168	90	423

Abbreviation: IHC, immunohistochemical.

strong), the agreement was improved (agreement, 92.3%; expected agreement, 74.53%; κ = 0.689; $P <$ .00001).

Unfortunately, in most surgical pathology practices, pressure to decrease turnaround time of specimen reporting precludes prolonged (multiday) fixation. Recently, Ruijter et al indicated that vigorous injection of intact prostatectomy specimens with formalin, followed by microwave treatment, resulted in improved immunohistochemical staining for E-cadherin[15]. To determine whether this method would enhance the staining for p27^{Kip1} in specimens processed with short fixation times (ie, day 0 fixation as indicated above), we processed a group of prostatesctomy specimens in this way and compared the staining with that of the standard method of no injection and same-day processing. After staining specimens using this approach, we noted excellent staining with only minimal "outside-in gradation" in most but not all sections. Using the standard fixation method, 4 of 12 standard tissue sections were scored as strong staining, whereas with the injection method, 41 of 45 sections were scored as strong staining. The difference between

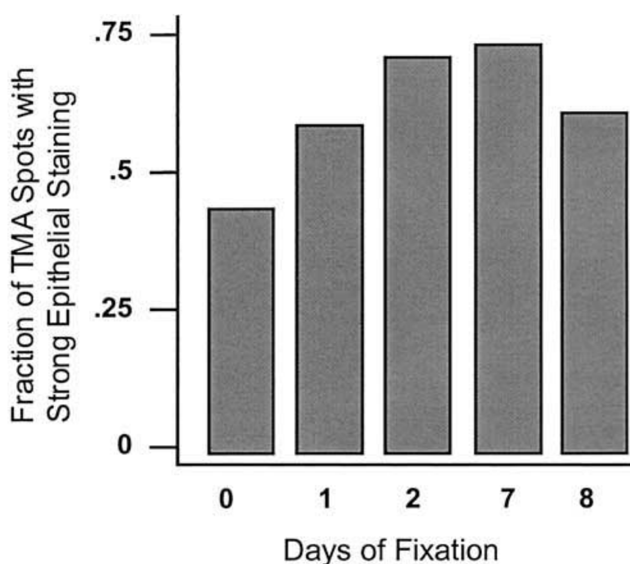

FIGURE 2. Fraction of strongly staining prostate epithelium in TMA spots in relation to time of fixation.

Table 2. Scoring of p27^{Kip1} Using Standard Sections

Fixation	IHC Score			
	2	3	4	Total
Noninjected (no.)	8	4	0	12
Injected (no.)	4	3	38	45
Total (no.)	12	7	38	57

Abbreviation: IHC, immunohistochemical.

the staining for p27^{Kip1} using the 2 methods of fixation was highly statistically significant (Table 2; $P <$ .0001, Fisher exact test.).

If these results are true for p27^{Kip1} staining, what about other immunohistochemical biomarkers? We have seen this pattern of "outside-in gradation" with a number of biomarkers. One of these is the pi-class GSTP1. Staining for GSTP1 is absent in the majority of prostate cancer specimens, but is present in the majority of benign prostate epithelial glands, with staining most often occurring in the basal cells.[18-20] As opposed to p27^{Kip1} staining, this marker shows predominantly cytoplasmic localization. Using standard sections from the formalin-injected cases we also found strong and consistent staining for GSTP1 (n = 25). To better determine how general the formalin injection method may be, we stained adjacent sections (n = 25) with both anti–Ki-67 and antibasal cell-specific cytokeratins (34βE12). Staining for these later markers, which are used commonly in surgical pathology, was also excellent (data not shown). Thus, injection of prostatectomy samples with formalin, followed by microwave treatment, resulted in high-quality staining for 4 of 4 immunohistochemical markers tested.

To determine whether microwaving is important in the process of enhanced antigen staining or whether the injection of the prostates with formalin alone is adequate, we compared staining in prostates that were either injected plus microwaved with those that were injected alone. For staining with all 4 markers, no difference was detected using several sections from each prostate. Therefore, we conclude that microwaving does not contribute to the increased quality of immunohistochemical staining and that the process of saturating the prostate tissue with formalin by injection is sufficient to obtain the improved staining.

Because we are postulating that inadequate time of fixation is occurring in large tissue sections, it would follow that prostate needle biopsies might stain well for p27^{Kip1}, even after relatively brief fixation. Indeed, when we examined 20 recent prostate needle biopsies specimens that were processed the same day as the were obtained, all showed near-uniform strong staining in the luminal cells in normal-appearing epithelium (data not shown).

DISCUSSION

In this study, we show that immunohistochemical staining against p27^{Kip1} protein using radical prostatectomy specimens in formalin-fixed tissue may mistakenly appear to be downregulated as a result of an artifact relating to inadequate tissue fixation. Contrary to popular dogma in the pathology field, prolonged fixation, even up to 8 days, produced no decrement in staining for p27^{Kip1}. Although TMAs are not necessary to carry out studies on the effects of fixatives on immunostaining, they do allow for near-uniform antigen retrieval and immunostaining reaction conditions as well as high throughput analysis; thus, we submit that TMAs are an excellent platform to evaluate the effects of tissue fixation on immunohistochemical staining. These results have implications for both diagnostic and research specimens for retrospective and prospective study of prognostic markers. Additionally, these findings suggest that for those institutions, or commercial ventures, about to undertake large-scale production of TMAs for genomic research, attention should be paid to adequacy of the fixation of the tissues.

We also report that for prostatectomy specimens, the specimens can be processed the same day to decrease turn-around time, if the specimens are first subjected to vigorous formalin injection. Although we did not find it necessary to subject the specimens to microwaving, we did find that microwaving them after injection improves the ability to section the gland, because it results in a firming effect (HF and AMDM, unpublished observations). Thus, we recommend that the injection and microwaving technique be used for those who wish to process radical prostatectomy samples within 1 day. Perhaps formalin injection into other solid organs should be studied to determine if improved immunohistochemical staining could be attained after short processing.

The current results indicate that for p27^{Kip1}, a simple antigen-retrieval technique (steaming in citrate buffer) allows recovery of staining even after prolonged fixation (8 days) such that there appears to be more of a problem with underfixation rather than overfixation. Several studies in the literature describe the potential use of immunohistochemical staining for p27^{Kip1} for aiding in the prediction of biochemical recurrence of prostate cancer, independent of other established risk factors such as Gleason score and pathological stage.[7,8,10,12] Our results would suggest that if specimens in different institutions are fixed for different lengths of time in formalin, then appropriate care should be taken when attempting to use p27^{Kip1}. This study also suggests that other putative biomarkers being considered for implementation into clinical practice should undergo a similar evaluation to determine the effect of formalin fixation. The TMA is an excellent method to conduct such validation studies.

Acknowledgment. The authors thank Geert van Leenders for illustrating, first-hand, the method of formalin injection of radical prostatectomy specimens.

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