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Fresh Cut Versus Stored Cut Paraffin-Embedded Tissue: Effect on Immunohistochemical Staining for Common Breast Cancer Markers

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Abstract

The proper handling of unstained paraffin slides for immunohistochemistry has been a matter of debate, with several studies demonstrating loss of antigenicity with prolonged storage at room temperature, 4°C, and –20°C. The purpose of this study was to determine if long-term storage of unstained slides at –80°C would impact the staining intensity and expression distribution of markers used to molecularly subtype breast cancer specimens (estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), cytokeratin 5 (CK5), epidermal growth factor receptor (EGFR) and Ki67). The staining pattern of previously unstained breast tumor slides (n=39–64) stored at –80°C for a minimum of 9.93 years (avg. 12.8 years) was compared to the staining pattern of fresh cut slides from the same tumors. The Allred scoring method was used to score ER (0–2=negative, 3–8=positive), CK5 (0–4=positive) and EGFR (0–4=positive). ASCO/CAP guidelines were used to assess HER2 (0/1+, 2+ or 3+). Ki67 scores were determined based on the proportion of cells stained of any intensity, with 20% staining used as a cut-off. Agreement was assessed using concordance rates and chance-corrected agreement statistics. The chance-corrected agreements were as follows: 0.94 (38/39) for ER, 0.92 (53/55) for CK5, 0.87 (61/64) for EGFR, 0.87 (37/39) for HER2 and 0.67 (46/54) for Ki67. Long-term storage of cut unstained slides at –80°C does not significantly impact the scoring interpretation of ER, CK5, EGFR and HER2.

Keywords

formalin-fixed paraffin-embedded tissue; storage; antigenicity; breast cancer; subtyping

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INTRODUCTION

Formalin-fixation paraffin-embedding (FFPE) is the most common method of tissue preparation in surgical pathology. When paraffin blocks are sectioned, tissue slides can be used for a variety of purposes such as special stains, *in situ* hybridization and immunohistochemistry to study morphology, DNA aberrations and protein expression respectively. The advent of tissue microarrays (TMAs) has permitted the creation of slides that contain large tumor collections, having the advantage of reduced costs, resources, and tissue usage.

The quality of immunohistochemistry performed on paraffin slides is highly dependent on antigen preservation. While slides cut and processed fresh from paraffin blocks that were stored at room temperature maintain antigenicity for decades, many studies have demonstrated a loss of antigenicity, or epitope instability, in stored unstained paraffin slides.¹⁻⁴ This is problematic for multiple reasons: (1) numerous laboratories store unstained slides for eventual use as positive controls for diagnostic purposes, (2) the creation of multiple cut slides prevents repeated “facing” of the paraffin block, which would prevent tissue loss of rare specimens or of TMAs, and (3) the creation and distribution of unstained slides permits multicenter collaboration for molecular epidemiological and translational research.

The cause of antigenicity loss of stored paraffin slides is unknown; however, multiple sources have been proposed. A rapid decline in antigenicity is seen with slide storage at high temperatures.^{5, 6} Blind C. *et al.* demonstrated that photo-oxidation, secondary to UVA exposure, and dry heat increased the rate of stored slide antigenicity decline.⁷ Xie R. *et al.* suggested that hydrolysis of proteins due to inadequate dehydration of the tissue during tissue processing may be an important source of reduced immunoreactivity over time.⁸

Several methods have been proposed to improve long-term storage of unstained paraffin slides. The most widely recognized is applying a fine layer of paraffin after slide drying.^{2, 9, 10} The additional coating of paraffin behaves like a seal and may be infused with wax-soluble antioxidants (i.e. butylated hydroxytoluene, butylated hydroxyanisole) to further reduce oxidation effects. Despite its potential, some have demonstrated that removing the paraffin layer, which is approximately 2 mm thick, is challenging and often results in patchy immunohistochemical staining⁷, while others reported poor antigen preservation.⁵ In addition, one group demonstrated that the infusion of the paraffin with wax-soluble antioxidants actually reduced the antigen preservation effects of the paraffin.²

DiVito *et al.* described the use of a nitrogen dessication chamber to reduce antigenicity loss.² After three months storage in a nitrogen dessication chamber, immunohistochemistry for estrogen receptor (ER), pan-cytokeratin and Ki67 was more highly preserved compared to slides stored in room air with a non-significant decline of ER staining compared to fresh cut slides. Both pan-cytokeratin and Ki67 demonstrated significantly reduced staining, having 54% and 59%, respectively, of the staining compared to fresh cut slides alone.² In addition, this protocol requires the purchase and maintenance of special equipment with unique monitoring requirements.

A third method to reduce antigenicity loss involves sealing slides with vacuum packing and a dessicant (Drierite).⁸ After 3 months, proteins were found to be less degraded and had strong aquaporin immunohistochemistry regardless of storage temperature.⁸ Unfortunately, this protocol has not been tested on human specimens or with antibodies used clinically.

A universally accepted cause of decreased antigenicity over time is storage temperature, with many demonstrating increased preservation as storage temperature decreases.^{3, 5, 6} Grabau *et al.* demonstrated that storage for 3 years at -80°C resulted in minimal to no antigenicity loss.¹¹ Interestingly, no study except one¹, has attempted to look at antigen degradation effects in slides stored for periods longer than a few years.

Immunohistochemistry (IHC) can be used to separate breast tumors into molecular subgroups which differ in terms of prognosis and response to treatment.^{12–14} Large international breast cancer research collaborations, including the Breast Cancer Family Registry¹⁵ described below, rely on archived FFPE tissue for IHC analyses. These consortia would highly benefit from studies that would demonstrate reliable storage methods for sectioned paraffin tissues such that the specimens may be preserved without sacrificing IHC accuracy.

The purpose of this study was to determine whether the IHC result for the markers commonly used for molecular subtyping of breast cancer (ER, Human epidermal growth factor receptor 2 (HER2), cytokeratin 5 (CK5), epidermal growth factor receptor (EGFR), Ki67) would be similar for IHC performed on unstained slides stored for numerous years at -80°C compared to IHC performed on fresh cut slides.

MATERIAL AND METHODS

Room temperature-stored paraffin blocks from 67 invasive breast carcinomas that had been resected at Ontario hospitals and were participating in the Ontario Familial Breast Cancer Registry¹⁶ were retrieved for analysis. 61 tumors were diagnosed as invasive ductal carcinomas, 4 tumors were diagnosed as invasive lobular carcinomas, 1 tumor was diagnosed as a micropapillary carcinoma and 1 tumor was diagnosed as a tubulolobular carcinoma. For long-term storage, 4- μm sections were cut from the paraffin blocks, placed in an opaque, light resistant slide box, and were stored in a Sanyo -80°C (VIP Series) ultra low MDF-U73V freezer. The slides were stored for an average of 153.87 months (12.8 yrs, SD=0.92, Range (9.93, 14.00)) before being stained in this study. The original paraffin blocks were stored at room temperature. Both stored slides and fresh cut 4- μm sections from the original paraffin blocks were used for IHC. Primary antibodies were as follows: ER (6F11, 1/40 dilution, Vector (Burlington, ON); HER2 (4B5, Ventana (Roche, Tucson, AZ); CK5 (XM26, 1/100 dilution, Biocare Medical (Markham, ON); EGFR (31G7, 1/25 dilution, Invitrogen (Burlington, ON); Ki67 (MIB1, 1/200 dilution, DAKO (Glostrup, Denmark). ER, Ki67 and CK5 were pretreated with TRIS HCl Buffer (pH 9) and underwent microwave antigen retrieval in a Micromed T/T Mega Microwave Processing Lab Station (ESBE Scientific, Markham, Ontario, Canada). EGFR was pretreated with pepsin for 10 minutes at 37°C . All primary antibodies except HER2 were incubated on slides for one hour at room temperature. Visualization was accomplished using an avidin/biotin peroxidase system

(VECTASTAIN Elite ABC Kit, Vector Labs) with diaminobenzidine (Betazoid DAB Chromogen, Biocare Medical) used as chromogen and Mayer's hematoxylin as counterstain. IHC was performed using the Labvision 720 Immunostainer (Fisher Scientific). HER2 IHC was performed using the PATHWAY Ventana System (clone 4B5; Roche) according to manufacturer's instructions and processed using the Ventana Benchmark Ultra Immunostainer. In total, 39 tumors were used for ER analysis, 55 tumors were used for CK5 analysis, 64 tumors were used for EGFR analysis, 39 tumors were used for HER2 analysis, and 54 tumors were used for Ki67 analysis.

Scoring Systems and Statistical Analysis

The Allred scoring method was used to score ER (0,2=negative, 3–8=positive), CK5 (4=positive) and EGFR (4=positive). The 4 cutoff used to define positivity for CK5 and EGFR was based on our previous studies.^{17,18} ASCO/CAP guidelines were used to assess HER2 (0/1+, 2+ or 3+). All slides were reviewed by a breast pathologist (AMM) who was blind to slide preparation. The Ki67 proliferation index was determined by selecting a random area within the tumor and assigning a percentage based on the proportion of cells stained. 20% staining was used as a cut-off to separate high versus low proliferation. Agreement between dichotomized scores of each marker obtained from the pairs of stored and fresh cut slides was assessed using concordance rates and the chance-corrected agreement statistic, kappa.¹⁹ For continuous markers, agreement was assessed by Concordance Correlation Coefficient (CCC).²⁰ Lattice graphs were plotted to display the agreements. Statistical analyses of agreement were performed using SAS 9.2 software (SAS Institute, Inc.). Lattice graphs were made using R statistical software, version 3.3.1 (<http://www.r-project.org/>).

RESULTS

Lattice plots demonstrating the concordance between the scores accrued from stored and fresh cut slides are presented in Figures 1 to 3. The concordance rates were 0.97, 0.94, 0.96, 0.95 and 0.85 for ER, HER2, CK5, EGFR, and Ki67 respectively (Table 1). The chance-corrected agreements were almost perfect for ER, HER2, CK5, and EGFR with high estimated kappa values as follows: 0.94 for ER, 0.86 for HER2, 0.92 for CK5, and 0.87 for EGFR (Table 1). The chance-corrected agreement for dichotomized Ki67 was only moderate (kappa of 0.67) and the concordance rate was 0.85 (46/54) (Table 1). This is similar to the CCC calculated for continuous Ki67 (0.85 (0.76, 0.91); Table 1). Example immunohistochemical sections for which there was discordance between stored and fresh cut slides are shown in Figure 4. The discordant tumor for ER was called negative on the stored slide but positive on the fresh cut slide (Fig. 1A; Fig. 4). For HER2, two discordant tumors were called negative on fresh cut slides but were called equivocal on stored slides (Fig 1B; Fig. 4). The two discordant tumors for CK5 were called positive on stored slides but negative on fresh cut slides (Fig. 2A; Fig. 4). For EGFR, two discordant tumors were called positive on stored slides but negative on fresh cut slides (Fig. 4) and one tumor was called negative on the stored slide but positive on the fresh cut slide (Fig. 2B; Fig. 4). For Ki67, 7 tumors were called high (≥ 20%) on stored slides but low on fresh cut slides (Fig. 4), and 1 tumor was called low (<20%) on stored slides but high on fresh cut slides (Fig. 3). No

major differences were noted between the staining patterns (location of staining or intensity) for any of the markers examined.

DISCUSSION

With advancements in diagnostic histopathology and molecular research, there is an increasing need to identify appropriate, long-term storage solutions for FFPE sections. In particular, protocols that preserve staining for IHC markers that are used for the molecular sub-classification of breast cancers would be useful for large, multicenter tissue microarray studies that may rely on archived tissue. We describe here that long-term storage of unstained, uncoated paraffin slides at -80°C produces IHC results for ER, CK5, EGFR, and HER2 which are highly comparable to the results produced from fresh cut slides. The few discrepant tumors were different for each marker and therefore specific pre-analytical variables which may have predisposed a particular tumor to abnormal antigen staining patterns after storage were not identified. Although using a 20% Ki67 cutoff to separate high and low proliferative activity produced a lower chance-corrected agreement compared to the other markers, the stored and fresh cut score values were still highly comparable for the most part. Our findings are in agreement with Grabau *et al.* who documented good antigen preservation for ER, EGFR and HER2 in FFPE breast tissue sections stored for 3 years at -80°C .¹¹

There are several limitations of this study that require discussion. First of all, our cohort was small ($n=39-64$) with a predominance of ER positive and HER2 negative tumors. There were few tumors that were weakly ER positive, therefore it is difficult to comment on the reliability of prolonged -80°C slide storage for identifying tumors which have weak ER expression. In addition, since only two HER2 positive cases were included in this study, it is also difficult to draw general conclusions on the reliability of using stored slides for HER2 testing. Since the scores were determined by a single pathologist visually, there is inherent variability in the assessment of staining patterns that can be influenced by tumor heterogeneity as opposed to alterations in antigenicity. Future research could take advantage of whole slide computerized image analysis to determine if the few discrepant cases were in fact due to differences in staining.

It was unexpected that for the majority of mismatched cases (13 out of 18, 72%) the discrepancy involved a positive staining result for the stored slide and a negative staining result for the fresh cut slide. This is particularly noticeable for Ki67 where the majority of the discrepancies were due to increased staining on the stored slides compared to the fresh cut slides. Although only a very small number of cases ($n=5$) were used, Grabau *et al.* noted the same phenomenon for their study of Ki67 which used the same clone, antigen retrieval method, and storage temperature as was used in our study.¹¹ Bertheau *et al.* observed an unexpected increase in antigenicity of vimentin in years old breast cancer slides which prompted them to propose that it was antigenicity alteration, not antigenicity loss, that occurred with prolonged slide storage.¹

The reason that frozen stored slides have increased antigenicity compared to fresh cut slides is unclear; however, two theories deserve mentioning. Research by Sompuram *et al.* using

phage display suggested that antigen retrieval worked to increase antigenicity by breaking cross-links of the antigenic protein to neighboring irrelevant proteins and allowing the primary antibody to recognize the linear epitope sequence in the tissue.²¹ It is possible that the “freeze-thaw” cycle that results when removing slides from storage could disrupt formaldehyde-induced cross-linking, hydrogen bonding and electrostatic interactions that mask epitopes. Freeze-thaw cycles could therefore interrupt the antigen-masking induced by formaldehyde fixation. Alternatively, it is possible that the low storage temperatures are enough to preserve antigenicity and that the variability of “room temperature” may cause a loss of antigenicity, particularly on the cut surface of stored blocks that are exposed to room air. It is important to note for future research that Ki67 may be overestimated for FFPE tissues that have been stored for a prolonged period at -80°C .

Despite the limitations of our study, we have demonstrated that FFPE sections can be stored at -80°C with minimal alteration to IHC results for common breast tissue markers. It is promising that there was good concordance between stored and fresh cut slides despite the length of storage and before the institution of protocols to limit pre-analytical variables (e.g., ischaemic time and length of fixation). As epitope instability is antigen and antibody clone dependent, future studies should explore the effects of long-term storage on other prognostic and predictive markers. In addition, research which compares very long-term storage (i.e. greater than 10 years) at temperatures less extreme than -80°C (e.g. -20°C) may help to identify more cost-effective storage options.

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Figure 1.

A. Estrogen Receptor (ER) lattice plot comparing the IHC Allred score for breast cancer tumor slides stored at -80°C to fresh cut slides. An Allred score ≥ 3 was called positive. Bolded and italicized tumors are those for which there was a discrepancy between scores (positive/negative) accrued by the two processing methods. **B.** Human epidermal growth factor receptor 2 (HER2) lattice plot comparing the HER2 score (0/1+, 2+, 3+) of breast cancer tumor slides stored at -80°C to fresh cut slides. Bolded and italicized tumors are those for which there was a discrepancy between scores (Negative, Equivocal, Positive) accrued by the two processing methods.

Figure 2.

A. Cytokeratin 5 (CK5) lattice plot comparing the IHC Allred score of breast cancer tumor slides stored at -80°C to fresh cut slides. An Allred score ≥ 4 was called positive. Bolded and italicized tumors are those for which there was a discrepancy between scores (positive/negative) accrued by the two processing methods. **B.** Epidermal Growth Factor Receptor (EGFR) lattice plot comparing the IHC Allred score of breast cancer tumor slides stored at -80°C to fresh cut slides. An Allred score ≥ 4 was called positive. Bolded and italicized tumors are those for which there was a discrepancy between scores accrued by the two processing methods.

Figure 3.

Ki67 lattice plot comparing the proliferation index for breast cancer tumor slides stored at -80°C to fresh cut slides. Ki67 was called high if there was a 20% proliferation index. Bolded and italicized tumors are those for which there was a discrepancy between scores (high/low) accrued by the two processing methods.

Figure 4.

Example FFPE cases for which the immunohistochemical staining patterns for ER, CK5, EGFR, HER2 and Ki67 differed when comparing fresh cut tumor slides to tumor slides stored at -80°C . 200 $\times$ magnification. ER was called positive on the fresh cut slide and negative on the stored slide. CK5 was called negative on the fresh cut slide and positive on the stored slide. EGFR was called negative on the fresh cut slide and positive on the stored slide. HER2 was called negative on the fresh cut slide and equivocal (2+) on the stored slide. Ki67 was called negative on the fresh cut slide and positive on the stored slide.

Ki67 had a proliferation index of 8% on the fresh cut slide and a proliferation index of 30% on the stored slide. DAB chromogen, hematoxylin counterstain.

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Table 1

Results of Agreement

Marker	# of Agreements	# of Comparisons	% Agreement	Measure of Agreement	
				Kappa	95% CI
ER	38	39	97	0.94	(0.83,1.00)
HER2	37	39	94	0.86	(0.68,1.00)
CK5	53	55	96	0.92	(0.82,1.00)
EGFR	61	64	95	0.87	(0.72,1.00)
Ki67*	46	54	85	0.67	(0.46,0.87)

* cut-point 20%; For continuous Ki67 the concordance correlation coefficient with 95% CI is 0.85 (0.76, 0.91)