Fixation Delay Adversely Impacts Androgen Receptor Staining

Bora Gurel[1], Tsuyoshi Iwata[1], Susan Dalrymple[1], Toby Cornish[1], Kristen Lecksell[1], John T. Isaacs[4,5], Angelo M. De Marzo[1-5]

Departments of Pathology[1], Urology[2] and Oncology[3], The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins[4] and The Brady Urological Research Institute[5], The Johns Hopkins University, School of Medicine, Baltimore,

Abstract

Background: Autopsy studies (AS) can be of utility in assessing protein expression and the results can shape clinical medicine. For example, the only method to obtain large numbers of metastatic prostatic adenocarcinoma (CaP) tissues is to perform an autopsy. It is widely held that virtually all men with late stage CaP who do not have predominant neuroendocrine (pNE) features, harbor an intact androgen receptor (AR) axis. This renders all non-pNE cases as eligible to receive novel anti-AR agents currently in clinical testing. A recent AS, however, reported that 20% of men dying of CaP had no AR IHC staining. If this is correct, then such men would receive no benefit from novel AR-targeted therapies. Yet, even with a rapid autopsy, the average interval between patient death and the start of autopsy is 3 hours. Thus, it is not clear whether the absence of AR staining is related to epitope instability resulting from delayed fixation.
Design: To study the effects of fixation delay on IHC, we xenografted LnCaP and LAPC-4 CaP cells into atyhmic nude mice. After a period of 2 months, the mice were sacrificed and subjected to a 0, 1, 3, 20, 24 or 48-hour delay before harvesting of xenografts for standard formalin fixation. Tissues were then processed routinely and stained with a panel of antibodies recognizing AR and other targets in distinct cellular compartments. Only non-autolyzed tissue was examined. Computerized image analysis was used to measure staining intensities and the fraction of positive cells.
Result: With increasing delay in fixation, a significant stepwise drop was observed in AR and NKX3.1 staining (% mean AR positive staining: 68.7% vs. 14.56%; median intensity: 172.6 vs. 151.2, 0 vs 24 hrs respectively, $p<0.0001$; % mean NKX3.1 positive stainng: 67.7% vs. 21.7%, 0 vs. 24 hrs respectively, $p<0.0001$). AMACR staining exhibited a converse stepwise increase in intensity. No significant change in staining was observed with Fibrillarin or CK8.
Conclusion: A delay in fixation does adversely impact the IHC staining for AR. Loss of staining was not, however, universally found--the effect was antibody/antigen specific. The delay before formalin fixation must be considered when interpreting IHC stains, especially in autopsy material, and tests should be run to determine whether the specific analyte in question is stable to delayed fixation.

Introduction

- Immunohistochemical methods are frequently employed to assess expression levels and expression patterns of a multitude of proteins
- Autopsy material represents a considerable source of specimen material for immunohistological and molecular studies
- The only method to obtain large amounts of metastatic prostatic adenocarcinoma specimens is to perform an autopsy
- A recent autopsy reported that 20% of men who had died of prostate adenocarcinoma did not exhibit androgen receptor (AR) staining by immunohistochemistry (*Cancer Res. 2004 Dec 15;64(24):9209-16*)
- The average interval between patient death and the start of the autopsy is 3 hours
- It is not clear whether the absence of AR staining is due to epitope instability resulting from delayed fixation

Materials and Methods

- 5 athymic nude mice were xenografted with LNCaP and LAPC-4 prostate adenocarcinoma cells
- After 2 months, the mice were sacrificed and subjected to either formalin or 95% ethanol (EtOH) fixation after a delay of 0, 1, 3, 24 or 48 hours
- Tissues were stained immunohistochemically with two different anti-androgen receptor(AR) antibodies, AMACR, CK8, C-MYC, Fibrillarin, H2AX, NKX 3.1 and Phospho-S6. A routine Hematoxylin-Eosin (H&E)stain was also performed
- Immunohistochemical staining was first manually assessed, followed by computerized image analysis of selected stains.
- Staining percentage was manually assessed as the percentage of cells showing any staining
- Immunohistochemical staining was analyzed using FRIDA (Framework for Image Dataset Analysis), our custom open source image analysis software (Fig. 2)
- The FRIDA image analysis process is based upon the creation of "masks"
  - "Color masks" select only pixels of a certain color
  - "Lasso masks" define a region of interest
  - "Meta masks" are generated using Boolean logic operators
- FRIDA generates a number of data points for every spot analyzed:
  - The area (number of pixels) matching the values defined by a mask
  - The mean intensity value of the pixels in a mask
  - The sum of the intensity values for each pixel in a mask
- A staining percentage, defined as the ratio of brown pixels to total nuclear area; and a staining score, the sum of all intensities of brown staining divided by total nuclear area, was generated for every stain analyzed

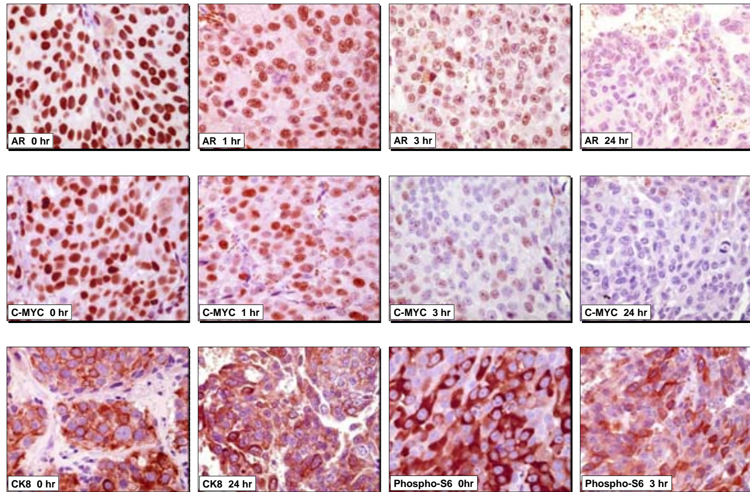

Fig. 1. AR, C-MYC, CK8 and Phospho-S6 staining in freshly fixed tissue and after fixation delay. The reduction in AR and C-MYC staining is evident even after 3 hours. Conversely, CK8 staining is not altered after 24 hours. Phospho-S6 staining persists after 3 hours.

Results

Analysis of Immunohistochemical Stains

- In formalin-fixed tissues, AR-1, AR-2, C-MYC, and NKX 3.1 stains showed a stepwise decrease in both the percentage of cells stained and the staining intensity (Fig. 1, 3 and 4)
- In contrast, staining with AMACR and H2AX showed a stepwise increase with delayed fixation (Fig. 3 and 4)
- CK8, and Fibrillarin staining was stable throughout fixation delay, and interestingly, Phospho-S6 staining also remained remarkably stable for the first 3 hours (Fig. 1 and 3)
- In contrast to formalin fixation, there was little or no staining with AR-1, AR-2, C-MYC and H2AX in EtOH-fixed tissues, while CK-8, Fibrillarin , NKX 3.1 and Phospho-S6 staining remained comparable to formalin-fixed tissues

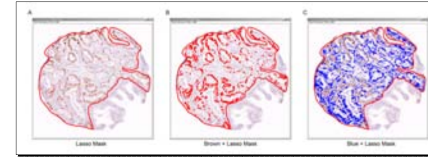

Fig. 2. The FRIDA image analysis process. A. Creation of the "Lasso" mask, enabling the user to disregard the benign prostatic epithelium in the TMA spot. B. And C. "The "Meta-Masks" created by combining the lasso and color masks

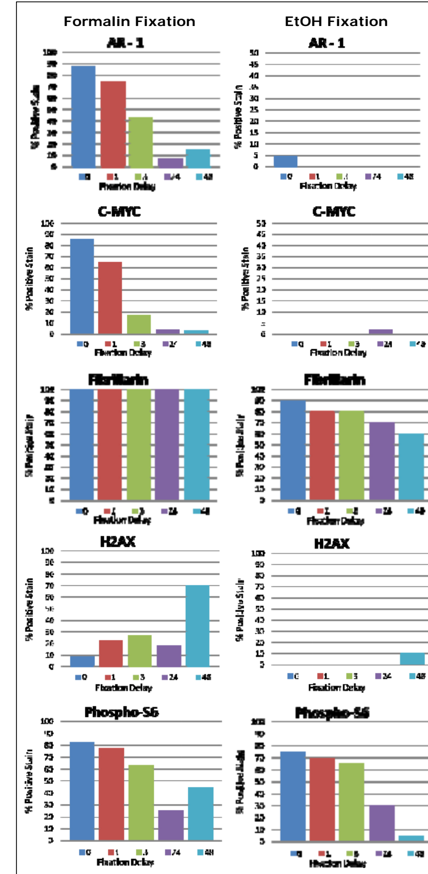

Fig. 3. Manual analysis of stains. The left hand column denotes formalin-fixed tissues, whereas tissues in the right hand column were fixed in EtOH.

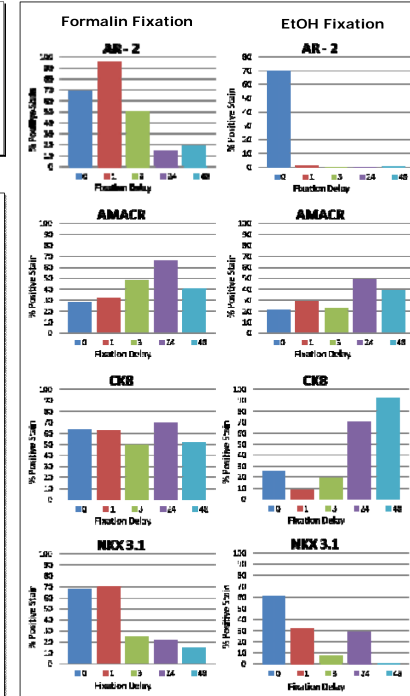

Fig. 4. Computerized image analysis results of selected stains. Compare the formalin-fixed tissues on the left to the EtOH-fixed tissues on the right.

Summary and Conclusions

- AR staining by two different anti-AR antibodies was observed to decrease in a stepwise fashion with increasing delay in fixation
- The staining of some proteins, such as C-MYC and NKX 3.1 also decreased, while others increased or did not change
- The choice of fixation agent also greatly influences the quality of IHC staining
- The delay before fixation and the fixative of choice must be considered when interpreting IHC stains, especially in autopsy material, and tests should be run to determine whether the specific analyte in question is stable to delayed fixation