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Strategies for H-score normalization of preanalytical technical variables with potential utility to immunohistochemical-based biomarker quantitation in therapeutic response diagnostics

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Abstract. Digital quantitative immunohistochemical analysis of protein biomarker expression offers a broad dynamic range against which clinical outcomes may be measured. Semi-quantitative expression data represented as an H-score is produced by computer generated average intensity of positive staining given weight by the percentage of cells showing positive staining. While patient H-scores vary for biological reasons, variation may also arise from preanalytic technical issues, such as differences in fixation protocols. In this study, we present data on two candidate calibrator nuclear-localized proteins, SNRPA and SnRNP70, with robust and consistent expression levels across breast cancers. Quantitative expression measurement of these two candidate biomarkers may potentially be used to eliminate the effect of differences in preanalytic processing of specimens by normalizing H-scores derived from test biomarkers of interest. To examine the effects of preanalytical fixation variation on biomarker quantitation and potential utility of candidate calibrators to address such issues, 6 surgically-resected human breast cancer patient specimens were divided into 6 portions and fixed under distinct conditions (fixation following resection in formalin for 2 hr, 8 hr or 48 hr, or held overnight at 4°C in buffered saline prior to formalin fixation for 2 hr, 8 hr, or 48 hr). We find H-score variation between fixation conditions within individual patient's tumors that were stained for XPF, ATM, BRCA1, pMK2 and PARP1. Most interestingly, detectable expression of SNRPA and SnRNP70 is covariant to test biomarkers under distinct fixation conditions and so these hold the potential for serving as calibration standards for general antigen preservation and reactivity.

Keywords: IHC, theranostics, biomarker, fixation, preanalytical variation

1. Introduction

IHC Biomarker expression levels of FFPE-derived sections have been reported to discriminate therapeutic response in a variety of solid tumor malignancies [1–6]. Creating accurate marker-specific IHC assay cut-off values for prediction of therapeutic response is of great interest, but potential technical issues remain that may hinder correct ascertainment of accurate IHC expression levels of individual biomarkers within patients. One of these potential obstacles may be the effect of variability in time to fixation and formalin fixation time on the protein expression assayed by IHC as scored by digital quantitative image analysis systems [7–11].

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While the field has generally employed subjective classification by trained pathologists using standard 1+, 2+, 3+ scoring strategies, advances in digital pathology and image analysis software offer the potential for more objective and quantitative analysis using specific computer scoring algorithms [12–14]. Assays for other molecular analytes utilize standard calibrators to assess overall sample quality and integrity (e.g. RNA employing actin or tubulin measurements). To date, IHC suffers from a lack of such technical standardizing controls. As we move from more subjective assessments of IHC-stained samples to computer assisted quantitative ranking within patient cohorts, corrective criteria for eliminating technical variations need to be applied for establishing models with greatest sensitivity and specificity.

Here we show the variation of patient H-scores derived from tumors that were divided and fixed under different conditions allowing for the possibility of less precise placement within ordered patient population continuums. We have examined two candidate IHC calibrators and show that their expression is covariant with test biomarkers based on the slope of H-scores across various fixation conditions. These criteria are hallmarks of their potential utility as candidate calibrators in normalizing quantitative IHC.

2. Methods

2.1. Patients and tumor samples

Female invasive ductal breast carcinoma tumor samples from 6 individual patients were surgically resected (Pantomics Contract Research Services, Richmond, CA) following guidelines set forth by IRB approval. Patient and individual specimen data is provided in Table 1. Each tumor specimen was divided into 6 parts and each part was fixed in 10% neutral buffered formalin for 2, 8 or 48 hrs, or was held for 24 hours at 4°C in saline before fixation. Specimens were paraffin-embedded and 4 um sections were taken from blocks. All slides were IHC stained within 2 weeks of sectioning.

2.2. Tissue microarrays and immunohistochemistry

Four micron sections from breast cancer microarray TMA 1503 (Pantomics, Richmond, CA) and six individual divided patient samples (36 sections in total) were stained using an automated stainer (Ventana, Tucson, AZ). Sections plus positive control multi-tumor blocks containing representative 0, 1+, 2+ and 3+ breast tissues for each antibody were stained with six biomarkers (ATM, BRCA1, XPF, PARP1, SNRPA and SnRNP70) using optimized protocols. pMK2 was stained manually with a 2 hour primary antibody incubation. Table 2 indicates the specific biomarkers tested, the antibodies utilized, and relevant information with regard to antigen retrieval processes that were employed. Initially, slides were baked at 60°C for 30 minutes in an oven incubator and barcode labels containing specific experimental information were created and placed on each slide. All slides were deparaffinized and following antigen retrieval, incubation was with specified antibodies at optimized dilutions. Detection was via an HRP-conjugated secondary antibody (16 min) and DAB. Slides were counterstained with

Table 1

Patients' breast cancer tumors information. Clinical information is shown for the six patients' tumor specimens utilized in this study. All specimens were surgically resected from female patients and upon histologic examination were classified as invasive ductal carcinomas

Case	20101720	1003043	1003079	1003353	20102383	1003982
Age	46	60	47	56	57	58
Histology	Inv Duct Carc	Inv Duct Carc	Inv Duct Carc	Inv Duct Carc	Inv Duct Carc	Inv Duct Carc
Grade	II	II	II	II	II	II
T	2	2	3	2	2	2
N	1	2	1	0	1	1
M	0	0	0	0	0	0
Nodal Status	2/7 nodes+	13/18 nodes+	3/19 nodes+	0/17 nodes+	1/9 nodes+	3/13 nodes+
ER	ND	ND	–	+	ND	–
PR	ND	ND	–	–	ND	–
p53	ND	ND	+++	–	ND	+
cErbB2	ND	ND	–	–	ND	–
Ki-67	ND	ND	+	–	ND	+

Table 2
IHC Staining for Specific Antibodies. Ab-specific staining conditions are listed for each biomarker assessed as part of this study. The optimal conditions for these Abs on the indicated platforms have been previously optimized for signal to noise as well as dynamic expression ranges in study–derived patient specimens as well as control cell-lines

Antibody	ATM	BRCA1	XPF	pMK2	PARP1	SNRPA	SNRNP70
Ab Vendor	Epitomics	BioCare	Abcam	Cell Signal Technology	AbD Serotec	Abnova	GenWay
Ab Clone	Y170	Ms110	SPM228	27B7	A6.4.12	NA	NA
Ab Stock Conc (μg/ml)	533	33	200	100	1000	1000	1000
Final Ab Dil	1 : 2000	1 : 40	1 : 250	1 : 100	1 : 625	1 : 15000	1 : 125
Ab Time/Temp of Incubation	60 min, 37°C	60 min, RT*	60 min, 37°C	120 min, RT*	60 min, 37°C	60 min, 37°C	60 min, 37°C
Antigen Retrieval Condition /Time	CC1**, Standard (64 min)	RiboCC***, Mild (32 min)	CC1**, Standard (64 min)	10 mM Citrate, pH 6.0 (30 min)	RiboCC***, Standard (64 min)	CC1**, Mild (32 min)	CC1**, Mild (32 min)
Staining Method	Ventana Autostainer	Ventana Autostainer	Ventana Autostainer	Manual	Ventana Autostainer	Ventana Autostainer	Ventana Autostainer

*RT - Room Temperature (21°C+/- 0.5°C); **CC1 - Ventana Antigen Retrieval Cell Conditioning Buffer 1 (Tris/Borate/EDTA Buffer pH8.0); ***RiboCC - Ventana Antigen Retrieval Citrate Buffer pH6.0

hematoxylin and bluing reagent for 4 minutes each. Slides were then washed in water, dehydrated, and cover-slipped.

2.3. Image acquisition/management and algorithm-based analyses

Stained TMA and whole section slides were converted into digital format using Scanscope XT Slide scanner (Aperio Technologies, USA). This system combines a linear array detector with high performance optomechanics to digitize an entire slide at high resolution with 20X objective within minutes. Tumor regions were annotated and scored with user-defined image analysis macros from Aperio's Image Analysis Kit. Variation in staining as defined by a multiplicative formula for computer generated H-score values was compared between the various conditions of time to fixation and formalin fixation times.

$$\text{H-score}(0-300\ \text{scale}) = \mathbf{3}^{*}(\%\ \mathbf{at\ 3+}) + \mathbf{2}^{*}(\%\ \mathbf{at\ 2+}) + \mathbf{1}^{*}(\%\ \mathbf{at\ 1+})$$

While the whole section contains both tumor and normal tissue, 3 regions of interest (ROIs) were selected, analyzed and averaged as best representative of tumor present within the tissue for targeted assessment rather than a much more time consuming automated macro scoring of the whole section, only a portion of which is applicably tumor. Staining was conducted on serial sections from patient tumor fragments. In the case of TMAs, H-scores from 2 individual cores per patient were averaged. A core or ROI was considered informative if fifty or greater tumor cells were present for analysis.

2.4. Candidate calibrator identification

A search was instituted to identify candidate calibrators for IHC based staining that would serve to control for technical variation due to tissue processing and stability. Figure 1 illustrates that approx 1300 genes/products were considered [15, 16]. 200 consistently expressed housekeeping genes were nominated for further in silico analyses and attrition of 123 was based on inconsistent protein expression levels across a wide variety of tumor types and normal human tissues (Human Protein Atlas and GeneCards) yielding a candidate list of 77. A second level criteria for selection was based on our principal interest in nuclear-localized DNA damage and repair enzymes, and so focus was narrowed to best candidates with the same localization as biomarkers to which they would serve as calibrators. Dominantly cytoplasmic-localized proteins

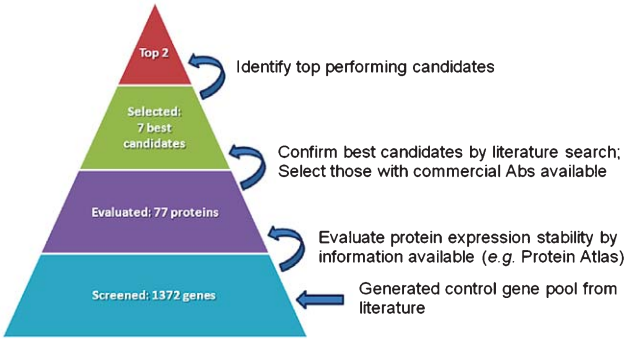

Fig. 1. Flowchart for identification of best candidate calibrator biomarkers. The schema by which candidate calibrators were identified and vetted is depicted. From a pool of over 1300 candidate genes, literature searches and survey of open access sources such as Protein Atlas and GeneCards stratified the search to an assessable number for laboratory investigation and characterization.

were eliminated. Also, only proteins with commercially available antibodies were nominated for further study. Collectively, these added elimination criteria resulted in refinement to seven candidate proteins and their expression levels were examined in breast cancer tissue-microarrays (TMAs). Two candidates were identified from this group, ribonucleotide binding proteins SnRNP70 and SNRPA, which had optimal H-score consistency among total patients examined.

3. Results

3.1. *Consistent expression levels for candidate calibrators in breast tumors comprising a commercially available TMA*

SNRPA and SnRNP70 display narrow ranges of expression across a patient group derived from a commercially available breast cancer TMA (Fig. 2). For comparison purposes, the dynamic range of a test

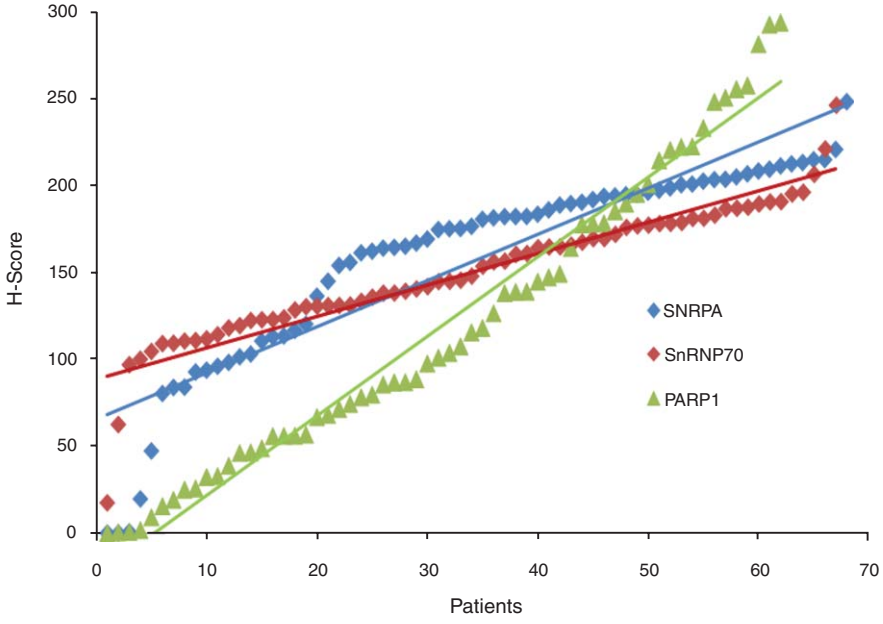

Fig. 2. Dynamic range of expression for 2 candidate calibrators, SnRNP70 and SNRPA, relative to test biomarker PARP1. Ordered patient ranking is depicted for H-scores derived from human breast cancer TMA staining and scoring along with added trend lines.

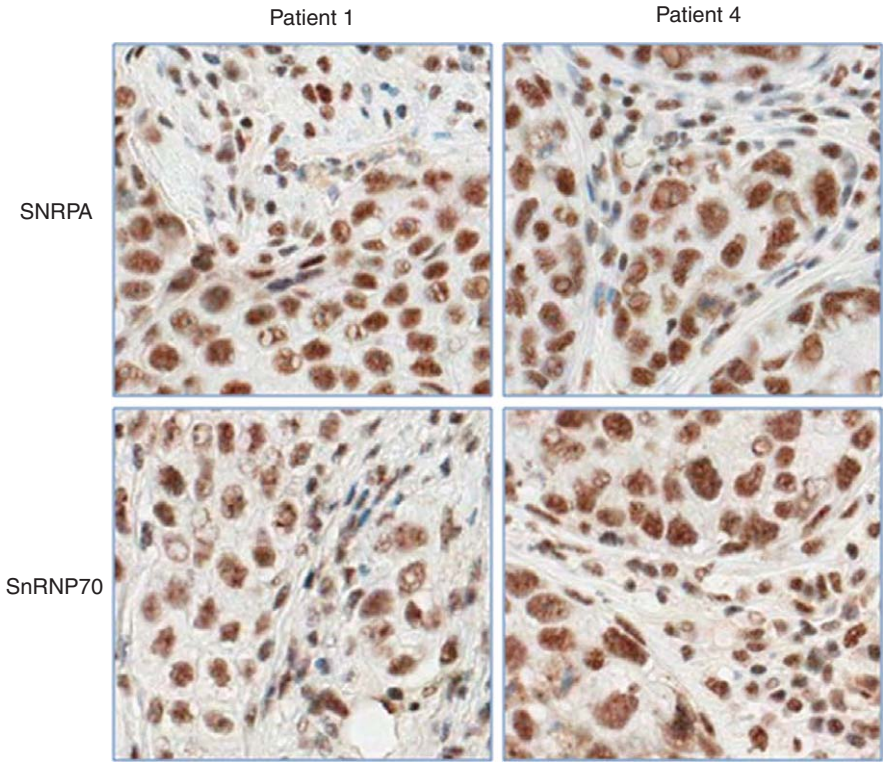

Fig. 3. Representative IHC staining for candidate calibrators in FFPE-derived human breast cancer tissue. SNRPA and SnRNP70 IHC staining is shown for breast cancer –derived sections for patients 1 and 4 from this study (fixed for 48 hours without delay following surgical resection). Note that the nuclear-specific staining (devoid of cytoplasmic background stain) is consistent for each candidate calibrator across patients tested for any given set of fixation conditions.

biomarker of interest is also displayed to illustrate the consistency of H-scores for candidate calibrators across all patients. Note the narrow range of expression for patient specimens comprising the TMA population for SnRNP70 and SNRPA (%CV = 24.7 and %CV = 36.5, respectively) relative to a test biomarker PARP1 (%CV = 69.6) that is expected to yield a more broad dynamic range (% coefficient of variation (%CV) = (Standard deviation (SD)/Mean) X 100.) Fig. 3 shows representative staining from two breast cancer patients utilized in this study. Note IHC staining for expression is robust with ideal signal to noise and staining is specifically restricted to the nucleus for each of the two biomarkers.

3.2. Preanalytical fixation differences and effect on driver biomarker H-scores

In addition to consistency of expression across samples that comprise a study population, a favorable characteristic for an IHC candidate calibrator is covariance with the technical variable for which the candidate calibrator aims to normalize. To that end, we sought to investigate the amount of technical variation that could arise in test biomarkers upon IHC analysis of individual tumors that were divided and fixed under different conditions. Figure 4 indicates staining for a single patient tumor that was divided and fixed under different conditions prior to staining for BRCA1 and phosphoMAPKAP kinase2. The level of fixation has a profound impact on the level of staining (48 hours displays much greater antigen detection sensitivity than 2 hour fixation) and thus when an algorithm is applied to a test specimen set, ordering could be affected by lack of uniformity due to technical handling consideration.

In addition to BRCA1 and phosphoMAPKAP kinase 2, XPF, SnRNP70, SNRPA, ATM and PARP-1 nuclear localized IHC signals were assessed in 6 individual patient tumors for 6 individual fixation conditions (immediate immersion for 2 hr, 8 hr, or 48 hr in neutral buffered formalin or held overnight at 4°C

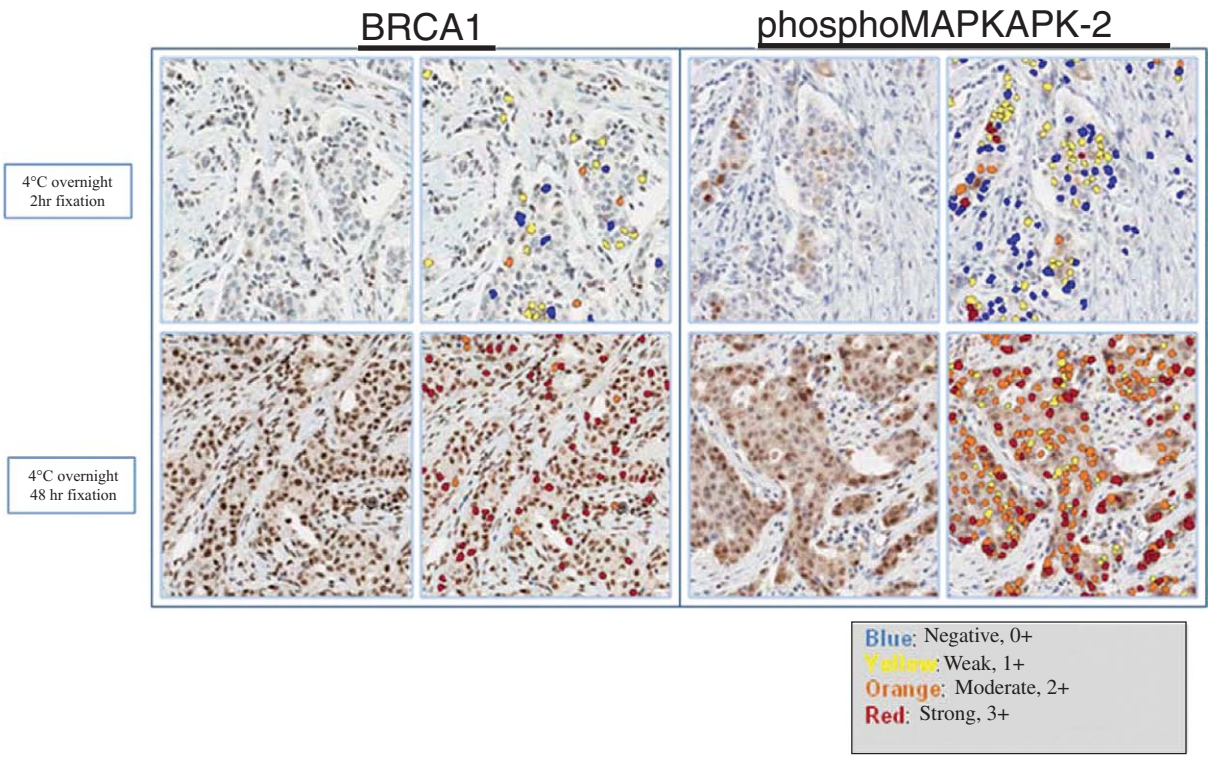

Fig. 4. IHC staining for BRCA1 and pMK2 from separately fixed samples of an FFPE-derived breast cancer patient specimen. (for each depicted biomarker, left panel = IHC staining, right panel = IHC staining with Aperio-based macro scoring).

in saline prior to fixation for 2 hr, 8 hr, or 48 hr. The H-scores derived from each of the biomarkers generally increased with fixation time. Most interesting here is that the H-score increase with fixation time was consistent for several test biomarkers (ATM, XPF, phosphoMAPKAP kinase2) as well as candidate calibrators SNRPA and SnRNP70 (Fig. 5). Another confirming positive trait for their potential utility as IHC calibrators is that the trendline (slope) for different test biomarkers is indistinguishable between SNRPA and SnRNP70.

While a general trend was observed for all markers relative to fixation conditions, variation was not uniform across all markers (Fig. 5). PARP1 and BRCA1 were shown to be the most variable relative to formalin fixation time, with scores widely ranging from quantitative image analysis. The observation that the trendline (slope) of variation across the different fixation conditions was less similar for BRCA1 and PARP1 may be indicative of a more limited utility for calibrators in removing the technical noise for all test biomarkers with equal efficiency.

A comparison of average H-scores for the patient group ($n = 6$) categorized by individual fixation conditions is depicted in Fig. 6. Consistent across different fixations, SNRPA and SnRNP70 displayed more consistent H-score averages than other biomarkers tested as evidenced by a lower %CV. Again, this limited variation relative to other test biomarkers is a hallmark of potential utility as a candidate calibrator analyte. While ATM, phosphoMAPKAP kinase 2, SNRPA, SnRNP70 and XPF showed less dramatic variation among fixation conditions than PARP1 or BRCA1, enhanced antigen detection and H-scores consistently trended higher with longer fixation times.

4. Discussion

The DNA repair nuclear localized proteins examined in this study display substantial variation in computer generated H-scores under varying fixation conditions. These data indicate that uniform fixation

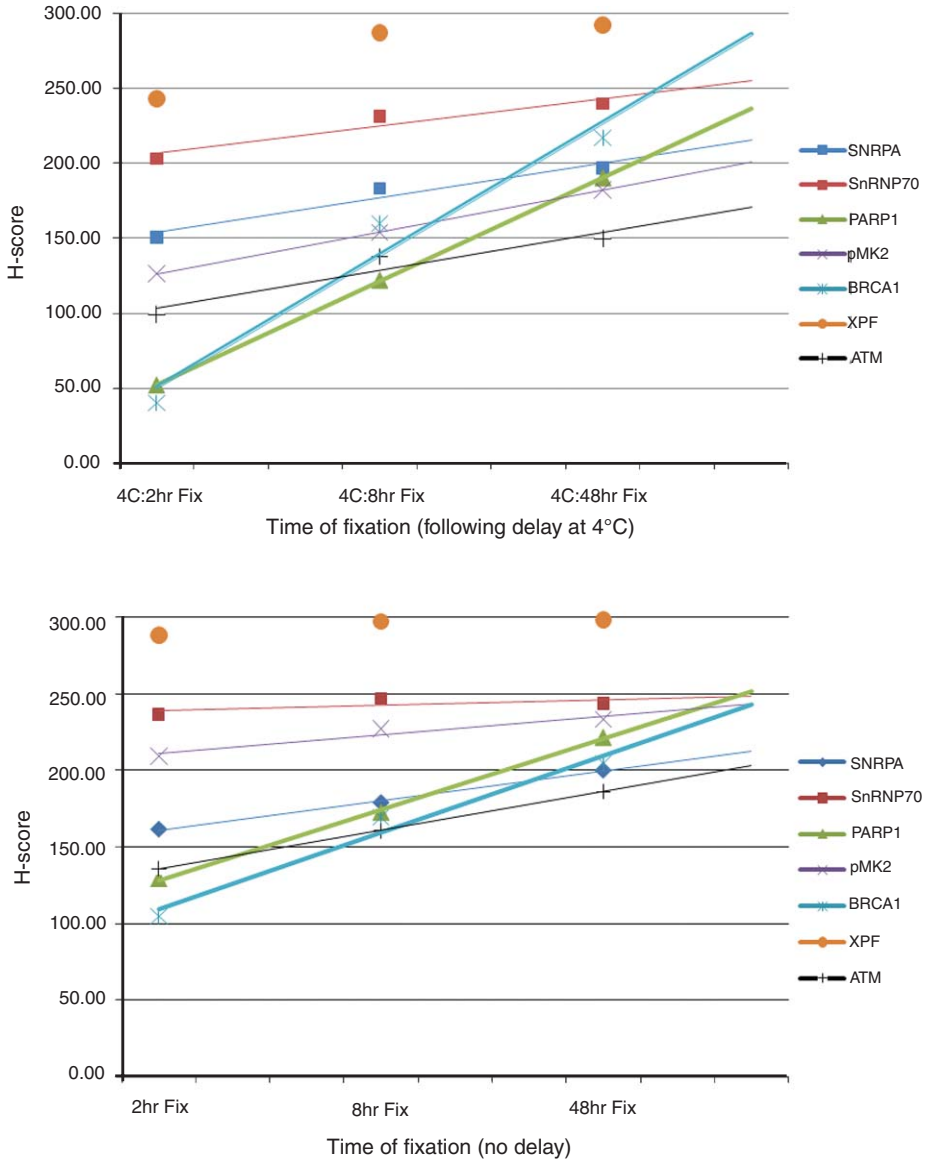

Fig. 5. Biomarkers co-vary in H-score relative to fixation conditions. Samples were either fixed for the indicated time or held overnight at 4°C prior to fixation for indicated times. Trend lines are added for comparison of different biomarkers H-scores across the different fixations.

will eliminate one source of variation for IHC results, and is a necessary precursor to proper patient ranking in studies designed to develop diagnostic algorithms to identify chemotherapy responders and non-responders with high sensitivity and specificity.

In attempting to normalize for pre-analytical technical variation, one would not anticipate that a calibrator that was more or less sensitive to technical variation relative to the biomarker for which it would serve as standard would be the ideal candidate. Rather, the best calibrator would be one whose measured expression moves commensurately by technical variation to the marker for which it normalizes. The data presented here are consistent with both SnRNP70 and SNRPA as being covariant relative to the test markers that have been examined here as measured by trend lines H-scores across different fixation conditions for these breast cancer patient specimens.

	4C:2hr Fix						
	SNRPA	SnRNP70	PARP1	pMK2	BRCA1	XPF	ATM
Mean	150.65	203.30	51.85	126.38	40.39	243.20	99.12
Std Dev	23.47	28.53	42.33	65.82	30.47	60.68	73.15
% CV	15.58	14.04	81.63	52.08	75.43	24.95	73.80

	4C:8hr Fix						
	SNRPA	SnRNP70	PARP1	pMK2	BRCA1	XPF	ATM
Mean	183.45	231.29	121.81	154.00	159.81	287.28	137.76
Std Dev	13.64	16.43	96.39	76.67	50.45	13.90	62.37
% CV	7.43	7.10	79.13	49.79	31.57	4.84	45.28

	4C:48hr Fix						
	SNRPA	SnRNP70	PARP1	pMK2	BRCA1	XPF	ATM
Mean	196.99	239.95	190.05	182.25	216.94	292.02	149.84
Std Dev	19.28	24.97	76.24	38.67	35.12	11.85	65.78
% CV	9.79	10.40	40.12	21.22	16.19	4.06	43.90

	2hr Fix						
	SNRPA	SnRNP70	PARP1	pMK2	BRCA1	XPF	ATM
Mean	161.50	236.51	129.10	209.24	104.90	288.17	135.88
Std Dev	35.65	23.31	81.95	44.24	57.19	14.85	48.90
% CV	22.07	9.86	63.48	21.14	54.51	5.15	35.99

	8hr Fix						
	SNRPA	SnRNP70	PARP1	pMK2	BRCA1	XPF	ATM
Mean	179.11	247.16	172.54	227.20	169.33	297.29	160.51
Std Dev	25.81	28.18	66.02	44.24	69.80	2.02	49.74
% CV	14.41	11.40	38.27	19.47	41.22	0.68	30.99

	48hr Fix						
	SNRPA	SnRNP70	PARP1	pMK2	BRCA1	XPF	ATM
Mean	200.38	243.86	221.63	233.38	204.65	298.27	186.28
Std Dev	27.72	19.32	59.38	32.88	52.65	2.35	28.63
% CV	13.84	7.92	26.79	14.09	25.73	0.79	15.37

	All Fixation Conditions						
	SNRPA	SnRNP70	PARP1	pMK2	BRCA1	XPF	ATM
Mean	178.68	233.68	147.83	188.74	149.34	284.37	144.90
Std Dev	7.51	4.83	18.82	17.02	14.50	21.84	15.88
% CV	4.21	2.07	12.73	9.02	9.71	7.68	10.96

Fig. 6. Variation as measured by mean H-score and %CV for given biomarkers and different fixation conditions. The H-scores for each of 6 patients for a given biomarker were averaged per fixation and Std Dev and %CV calculated. Additionally all fixation conditions for all patients were averaged to gain average %CV for a given biomarker.

The manner in which a candidate calibrator would be employed to improve test data sets upon which therapeutic models of response and resistance remains an open question that demands further testing. One could simply use an H-score cut-off value as a quality metric for patient inclusion. As these marker standards are expected to be expressed at a reasonably constant level across a patient population, samples that do not achieve a reasonable H-score potentially could be considered technically compromised. Thus, for these patient specimens a calibrator is technically irrelevant to improving the data set and the specimen removed as uninformative. Additionally, one could normalize patient driver biomarker H-scores by attenuated ratiometric scoring with the calibrator H-score (Test biomarker H-score/calibrator biomarker H-score). Optimally, one could use both a cut-off criteria for inclusion combined with an attenuated ratiometric score for those samples that achieve an acceptable calibrator H-score. Currently we are testing this in solid tumor chemotherapeutic response and resistance models and find that by eliminating patients with H-score<60, fewer than 3% of patients for each biomarker are affected. Preliminary results also show that R^2 values average 0.85 for biomarker expression assessments of patient rank ordering agreement before and after calibration range. Thus, ratiometric scoring may result in modest reordering of patients but the attenuated dataset is not dissimilar entirely to the parent set. (Pierceall and Ward, unpublished observations).

While we have chosen to focus on fixation as a technical variable that warrants normalization in IHC-based studies, other technical preanalytical factors may also be addressed by the development and application of such candidate calibrators. Age of tissue and/or section and conditions under which specimens are stored (including variant ambient temperature) has been identified as a source of potential antigen reactivity signal loss [17–19]. An additional source of scoring variation may include photo-oxidation following exposure to light [20]. Lastly, the candidate calibrators discussed here address important issues not only in biomarker modeling and applied predictive theranostics but also in establishing interlaboratory reliability [21] when comparing and evaluating patient status relative to standard tumor markers including but not limited to HER2, ER, and PR.

The added value of candidate calibrators would be realized when patient samples are diagnostically assessed against already postulated predictive models. As patient specimens are likely from multiple medical treatment centers, the variation in fixation or technical processing protocols is likely to be extensive, again highlighting the important potential utility in promoting interlaboratory agreement. Thus, proper placement within the predictive model is likely to vary with diagnostic material source. Calibration of test patient H-scores for given biomarkers would allow proper theranostic assessment to be based on biologic variation with minimal technical confounder effects.

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