Introduction

Protein and glycoprotein biomarkers in tissue diagnosis and treatment of varied human cancers rely on the use of formalin-fixed and paraffin embedded (FFPE) tissue based immunohistochemistry. There are known drawbacks to the formalin-fixed tissue based immunohistochemical method including tissue fixatives [1], length of fixation, age of fixed tissues [2] and slides before staining, antigen loss, technical issues of antigen retrieval, antibody types and manual scoring of the stain, varied cut-off values and false positives [3]. The introduction of the high-throughput method of tissue microarrays [4], and its use in validating other methods for cancer biomarker selection has also spurred the need for automated image analysis and quantitation methods for scoring of stains [5-9]. Tissue microarrays can be used to validate cDNA microarray profiling findings [10] but the immunohistochemical data may need to be as rigorously processed or normalized [11] as for other high-throughput data sets. For example, a meta-analysis of melanoma biomarkers showed a wide variation of cut-off points for the expression of multiple markers and their utility in determining survival [12] which creates some difficulty in comparing outcome analysis. Furthermore markers to differentiate pulmonary from breast cancer used 7 antibodies but did not normalize the intensity scores before data analysis [13]. Other high-throughput methods such as oligonucleotide (DNA) microarrays [14], quantitative real-time polymerase chain reaction (qTR-PCR) and quantitative proteomics [15] and array comparative genomics (CGH) ([16]) that have data standards and methods of analysis including normalization [17]. Efforts to standardize immunohistochemical data sets are

Original Article
Quantitative analysis of p53 expression in human normal and cancer tissue microarray with global normalization method

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Abstract: Tissue microarray based immunohistochemical staining and proteomics are important tools to create and validate clinically relevant cancer biomarkers. Immunohistochemical stains using formalin-fixed tissue microarray sections for protein expression are scored manually and semi-quantitatively. Digital image analysis methods remove some of the drawbacks of manual scoring but may need other methods such as normalization to provide across the board utility. In the present study, quantitative proteomics-based global normalization method was used to evaluate its utility in the analysis of p53 protein expression in mixed human normal and cancer tissue microarray. Global normalization used the mean or median of β-actin to calculate ratios of individual core stain intensities, then log transformed the ratios, calculate a mean or median and subtracted the value from the log of ratios. In the absence of global normalization of p53 protein expression, 44% (42 of 95) of tissue cores were positive using the median of intensity values and 40% (38 of 95) using the mean of intensities as cut-off points. With global normalization, p53 positive cores changed to 20% (19 of 95) when using median of intensities and 15.8%(15 of 95) when the mean of intensities were used. In conclusion, the global normalization method helped to define positive p53 staining in the tissue microarray set used. The method used helped to define clear cut-off points and confirmed all negatively stained tissue cores. Such normalization methods should help to better define clinically useful biomarkers.

Keywords: Global normalization, immunohistochemistry, cancer biomarkers, p53 protein
Global normalization method, immunohistochemistry and p53 protein expression

proposed (MISFISHIE), but as yet do not embrace normalization standards. The recent consensus draft from the Food and Drug Administration (FDA) group defines biomarkers as “objectively measured and evaluated” marker for disease detection, classification, predictive and for treatment outcome and the need for comparable analytical protocols and validation [18].

Normalization in high throughput (TMA) immunohistochemical studies can accommodate the technical aspects of immunohistochemical staining, image acquisition, antibody types and dilution, temporal changes in protein content in cells, and variations in protocols. Normalization is important in data sets derived from protein or antibody microarrays to accommodate the many variables from platform, slide to slide variables [19]. Liquid chromatography/mass spectrometry (LC-MS) data requires normalization methods to reduce false positives [20]. Normalization of data in western blotting and qRT-PCR use endogenous reference genes and proteins, such as β-actin, as controls to define relative expression levels of target genes or proteins [21] and is centered on their relative stability in the cell and their functional needs. There are many endogenous controls and arguments abound as to which and when to use [22].

The objective of the study was to test a proteomics data normalization method on the expression of p53 obtained by digital image analysis [23]. Digital image analysis method was preferred so that similar measurement criteria were used for all samples. The p53 oncoprotein was chosen for its functions as tumor suppressor, transcription factors and its myriad interactions with other proteins; p53 is also mutated in many cancers, touted as a biomarker for diagnosis of cancer and target for use in therapy [24, 25]. This means that its expression in both normal, proliferating and cancer tissues is dependent on the level of stress in the cell and hence subject to more variations and more dynamic [26]. The p53 protein expression in breast cancer was concordant with its mutation status [27]. The protein p53 interacts with cell death and survival proteins in the cytoplasm including autophagy proteins [28], responds to endoplasmic reticulum stress, and thus not exclusive to the nucleus in function [29].

In this study, the global normalization (central tendency) method was used in the analysis of p53 expression staining in human normal and cancer tissue microarrays (TMAs). The immunostaining for β-actin in a similar TMA set was used as the endogenous control and for calculating the p53 to β-actin ratios before applying normalization method. The number of p53 positive tissue cores changed from 44% (42 of 95) to 20% (19 of 95; using median intensities for all calculations) and 15.8% (15 of 95; using mean intensities for calculations). This also affected sensitivities, specificities, positive and negative likelihood for p53. The introduction of similar methods in studies aiming to validate or create new biomarkers may help adoption of markers for clinical and diagnostic use.

Materials and methods

Antibodies

The antibodies for the study were (a) p53 rabbit monoclonal antibody #1026-1 from Epitomics Inc (b). β-Actin, rabbit monoclonal antibody from Cell Signaling Biotechnology-New England Biolabs Inc #4970. Antibodies were used as suggested by the manufacturers and used at dilution of 1/50 after optimization.

Tissue microarrays (TMAs)

The MUT951 9x12 (96 cores) normal and cancer array from Pantomics Inc (California, USA). The cores are derived from separate sources and are treated as individual cases and not as duplicates or quadruplicates from a single source.

Immunohistochemistry

All immunohistochemical stains were carried out on the Ventana Discovery XT (Ventanna AZ USA) using the DAB Map protocol and included standard deparaffinization, blocking endogenous peroxidase and biotin, incubation with primary and secondary antibodies and development with DAB (di-aminobenzidine). The tissue arrays were stored at 4C and heated to 60C for 1 hour before use. The slides were counterstained with hematoxylin.

Scanning of TMA cores and quantitative immunohistochemical analysis

The TMA sets were scanned using Aperio Scanscope (Aperio, Calif USA). The TMA tissue cores were individually copied, labeled and
stored in a separate folder as tiff files.

The tissue microarray cores were individually analyzed using the NIH Image J (v1.42) plug-in deconvolTMA (and its dependent G. Landini’s Color Deconvolution v1.3 [30, 31]. The cores were stored in separate folders for p53 and β-actin. The steps involved annotation and markup of individual cores, and analysis of individual core tissues. The data sets obtained included tissue area, brown area, brown ID, mean, standard deviation (sd), and median of intensities for each tissue core. The mean and median for median or mean intensities for the set were used as cut-off for positive stain without ratio. The mean or median of mean and median intensities of β-actin was used for normalizing the mean of individual cores and then calculating the mean ratio for cut-off.

Global normalization

The normalization method adopted was previously used for quantitative proteomics and appeared simple and easy to use [23]. The ratios obtained by dividing β-actin mean or median intensities were log transformed, then a mean or median calculated and then subtracted from individual log ratio values; only values above 0 were used as positive for p53.

Statistical analysis

Statistical analyses for all data were performed using Gretl (v1.8).

Results

The mean of the median intensity values for β-actin was 16.78 and the mean of the mean values was 18.4. Figure 1 (A-D) illustrates the negative and positive tissue cores for p53 and β-actin. Figure 2 (A, B, C) shows comparison of median and mean intensities and scatter plots of mean and median (ratios) intensities.

The data for MUT951 TMA are shown Tables 1 (A, B, C, D). In using the median intensity values for calculating ratios and applying global normalization, the total number of p53 positive tissue cores changed from 42 of 95 when no ratio and normalization was used (44%) to 19 of 95 (20%) after global normalization. In addition, using the mean of the ratios alone for cut-off also reduced the number of p53 positive cores (35 of 95, 37%).

When the calculations of ratios, log transformations and global normalization were based on the mean of the mean intensities, 15 of 95 (15.8%) tissue cores were positive for p53 after
global normalization.

Discussion

In this study using human mixed normal and cancer tissue microarrays, the introduction of a global normalization method removed false positives. The ratio method alone also confirmed all negatives in normal tissues. The ratio method alone could be deemed suitable for manual or semiquantitative scoring [32-37] and could improve clinical utility of biomarkers. As shown in this study, the ratio method is still not adequate to remove false positives. The removal of positive status in the majority of normal and benign tumor tissues means that excessive p53 in benign or normal tissues should alert one to other possibilities such as mutations in closely related gene networks or in p53 itself [24, 38].

The digital image acquisition and analysis also suffers from use of different algorithms from different manufacturers [39] and some other method of normalization will be necessary to compare results from different algorithms. Furthermore, there is a growing interest and use of different methods of image processing and analysis for dia-aminobenzidine-based (DAB) immunohistochemical staining such as spectral imaging and analysis [40], color deconvolution [30, 31, 41], Hue-Saturation-Intensity [42], normalized RGB [43] and CMYK [44].

The ability to create meaningful predictive and prognostic markers is not enhanced by observer variations in scoring and variations in scoring systems [45, 46]. In a study comparing five different methods for DAB-immunohistochemistry stained tissues [43], there were differences in classification errors. These dilemmas may be reduced by use of appropriate proteomic normalization methods.

The use of ratio and normalization method is necessary for estimating the amplification of Her-2 determined by fluorescent-insitu hybridization (FISH [47]. Furthermore TMA are used in validation of other high-throughput methods [48 -50] and correlates well with conventional slide immunohistochemical staining [51]. So that should enable introduction of simple and easily applicable normalization methods in IHC–based tissue microarray studies.

The use of p53 for diagnosis of cancer and biomarker of cancer progression [52] and as a
Global normalization method, immunohistochemistry and p53 protein expression

**Table 1.** Quantitative Analysis of p53 staining in MUT951 TMA Comparing Non-ratio and Ratio Plus Global Normalization. Sensitivity, specificity, likelihood of positive and negative tests using p53 to separate cancer from non-cancer tissue cores were as follows Sensitivity=52%, Specificity=68%, LR positive=1.6, LR negative=0.71.

(A) p53 (No ratio and using the mean of median values as cut-off; mean=10.344)

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (23)</td>
<td>18 (78.3%)</td>
<td>5 (21.7%)</td>
<td>23</td>
</tr>
<tr>
<td>Cancer (60)</td>
<td>29 (48.3%)</td>
<td>31 (51.7%)</td>
<td>60</td>
</tr>
<tr>
<td>Adenomas/Meningioma (12)</td>
<td>6 (50%)</td>
<td>6 (50%)</td>
<td>12</td>
</tr>
</tbody>
</table>

(B) p53 Ratio and Global Normalization( using positive values after subtraction of the mean of log of ratios). Sensitivity=28%, Specificity=94%, LR positive=4.7. LR negative=0.06

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (23)</td>
<td>22 (94.5%)</td>
<td>1 (4.5%)</td>
<td>23</td>
</tr>
<tr>
<td>Cancer (60)</td>
<td>43 (71.7%)</td>
<td>17 (28.3%)</td>
<td>60</td>
</tr>
<tr>
<td>Adenoma/Meningiomas (12)</td>
<td>11 (91.7%)</td>
<td>1 (8.3%)</td>
<td>12</td>
</tr>
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</table>

(C) p53 staining analysis using mean of the mean intensities for ratio and global normalization (mean of mean intensities=11.68)

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (23)</td>
<td>4 (17%)</td>
<td>19 (83%)</td>
<td>23</td>
</tr>
<tr>
<td>Cancer (60)</td>
<td>31 (52%)</td>
<td>29 (48%)</td>
<td>60</td>
</tr>
<tr>
<td>Adenoma (12)</td>
<td>3 (25%)</td>
<td>9 (75%)</td>
<td>12</td>
</tr>
</tbody>
</table>

(D) p53 staining status after global normalization using mean of the mean intensities (mean of log ratios = -0.30085)

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (23)</td>
<td>1 (4%)</td>
<td>22 (96%)</td>
<td>23</td>
</tr>
<tr>
<td>Cancer (60)</td>
<td>14 (23%)</td>
<td>46 (77%)</td>
<td>60</td>
</tr>
<tr>
<td>Adenoma (12)</td>
<td>0</td>
<td>12 (100%)</td>
<td>12</td>
</tr>
</tbody>
</table>

therapeutic target [53] may need integration of all methods of gene expression including validation by immunohistochemistry [17]. The choice of methods of validation and cut-off points of relative protein content will be important for translating high-throughput data sets and studies into clinical use [18, 54, 55]. In addition, methods of determining positive protein expression should enable correlation with genomics data as gene expression also reflects possible structural alterations while protein content is also subject to protein life-cycle effects, and gene copy number variations [56, 57].

In the TMA sets used for the study, β-actin stain was not universally present and probably reflects the minimum detectable by the method (unlike Western blotting that detects in nanograms of protein). This variable expression of β-actin in its use as endogenous control, though similar variabilities are noted with endogenous control ([58]) can be redeemed by using more than one endogenous control. In normalizing western blotting of proteins such as p53 and estrogen receptor-β, both tubulin and β-actin are used as both are reliable housekeeping genes [59, 60].

The use of global normalization method could make high-throughput TMA studies better understood for clinical use and improve agreements between studies.
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Global normalization method, immunohistochemistry and p53 protein expression


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Global normalization method, immunohistochemistry and p53 protein expression


