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Potential use of spectral image analysis for the quantitative evaluation of estrogen receptors in breast cancer

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Summary. Evaluation of estrogen receptor (ER) content is an important factor in the choice of therapy and prognosis of breast cancer patients. In this study, we demonstrate a new spectral image analysis technique for objective and quantitative evaluations of stained specimens. The SpectraCube™ system was used to analyze nuclear antigens in thirteen cases of breast cancer stained by the immunoperoxidase method with hematoxylin counterstain. Spectral imaging segregated the spectrum of diaminobenzidine (DAB) from the background color of hematoxylin and a spectral index was calculated. The spectral index essentially agreed with the pathologist's index (on a scale of 0 to 3) in seven out of the thirteen cases. A substantial number of ER positive pixels was detected in the two cases scored as 0 by the pathologist's index. In a test case scored as 1 by the pathologist's index we detected a significant number of pixels, representing 47% of the nuclei, with DAB-intensity values higher than the cut-off value of 1.2. These data suggest that spectral image analysis is a sensitive method providing intensive information with high reproducibility. Our spectral imaging method is highly flexible, enabling the user to define the spatial resolution of the analyzed specimen by choosing the number of pixels per one nucleus.

Key words: Spectral image analysis, Quantification, Breast cancer, Prognostic factors, Estrogen receptors

Introduction

Evaluation of hormone receptor expression in tumor cell nuclei is an integral part of routine breast cancer diagnosis (Lehr et al., 1998). Diagnosis is determined as a function of a number of factors such as TNM (tumor node metastasis) stage; tumor type and differentiation; patient age; hormonal, cytotoxic and immune therapeutic modalities, and the duration of tamoxifen treatment. In addition, the estrogen (ER) and progesterone (PR) receptor status of the carcinoma provides important information for prognosis and choice of therapeutic approach (Bejar et al., 1998). Patients classified as ER-positive/PR-positive are treated with tamoxifen, which has proven to produce a favorable response and survival advantage (Tonetti and Jordan, 1997). Additionally, tamoxifen is the only drug known to reduce the incidence of collateral disease and produces relatively few harmful side effects (Tonetti and Jordan, 1997). However, in routine breast cancer diagnosis, receptor expression is often quantified in arbitrary scores with high inter- and intraobserver variability. Therefore, a question is raised as to whether pathologists' subjective impressions of negative versus positive ER states - and their readings of grades of positivity - are reliable in determining appropriate oncological management.

Immunohistochemical assays are most commonly used today to stain ER. These methods are quick, inexpensive and allow hormone receptor determination even if only small amounts of tumor tissue are available. Nevertheless there is no consensus regarding the method for scoring the results. Although the immunohistochemical technique is easy to standardize, its interpretation relies solely on subjective visual estimates, yielding only qualitative or at best semiquantitative results (Baddoura et al., 1991; Lehr et al., 1998). Most pathologists distinguish between positive and negative results based on the percentage of positive tumor cells, the cutoff being arbitrarily defined between 5 and 45% (Battifora et al., 1990; Raymond and Leong, 1990; Molino et al., 1995; Veronese et al., 1995; Pertschuk et al., 1996). The most commonly used method for semiquantification of the hormone-receptor status is the so-called H-score. The H-scores take into consideration both the color intensity of the immunoreactive neoplastic cell nuclei and the percentage of positive cells in histological sections of paraffin-embedded tissue stained with anti-hormone-receptor antibody by one of the commercially available immunohistochemical kits. Still, most of these scoring systems are cumbersome to

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perform and are subject to high interobserver variability.

Several attempts were made in order to employ image analysis techniques in the process of receptor quantitation. Bejar et al. (1998) measured the optical density of the nuclei in the digitized image file of the field and presented it in arbitrary gray-level units. The units were then divided into three equal intervals and the percentage of cell nuclei in each interval was determined. Lehr et al. (1998) used image analysis software to determine the region of interest of each nucleus in the image and the mean value of grayscale units. However, both procedures ignore the contribution of the natural color of the specimen to the stain intensity of the nuclei. Furthermore, most receptor staining procedures apply an additional stain for the counter staining of the examined specimen, in order to differentiate between tissue and subcellular domains. The contribution of the background stain is thus extremely significant and may lead to inaccurate, and perhaps false, results.

The development of spectral imaging for biological and medical applications introduced both fields to large amounts of information extracted from a single image (Rothmann et al., 1998a). Spectral imaging, as the terminology suggests, combines spectroscopy and imaging. In dramatic contrast to conventional microscopy in which fluorochrome discrimination is based on the measurement of a single intensity through a specific optical filter, spectral imaging allows one to measure and analyze the full spectrum of light at all pixels of an image. Spectral imaging has opened up new possibilities for the analysis of tumors (Malik et al., 1998), tissue-sections (Rothmann et al., 1998b), cells (Rothmann et al., 1997) and chromosomes (Schrock et al., 1996; Veldman et al., 1997). The SpectraCube™ system combines spectral imaging with microscopy and is based on Fourier transform multi-pixel spectral imaging. The light intensity at any wavelength is measured from multiple points of an image and stored in a spectra cube file whose appellate signifies the two spatial dimensions of a flat sample (x and y) and the third dimension, the spectrum, representing the light intensity for every wavelength. The digitized data of the cube can be subjected to further analysis by mathematical methods.

In this study we demonstrate a new generation technique for objective, quantitative and accurate evaluations of immunohistochemical hormone-receptor expression. Spectral imaging is applied in order to achieve a complete discrimination between background coloring and the specific staining of hormone receptors.

Materials and methods

Immunohistochemical staining

A total of 13 cases of infiltrating duct carcinoma of the breast were selected from a series of cases, submitted during the last quarter of 1998 to the department of Pathology, Sheba Medical Center, Tel-Hashomer, Israel. Formalin-fixed paraffin-embedded tissues were cut to 4 micron thick sections and stained, under standard conditions by NexES (Ventana Medical Systems, Tucson, AZ), using diaminobenzidine (DAB) as a substrate for the immunoperoxidase technique. Sections were counter-stained with hematoxylin. Two independent pathologists determined the status of ER and PR in 13 cases of breast carcinoma. The intensity of the brown reaction product in the nuclei was graded from 0 to 3. Unstained nuclei were graded 0 while dark brown nuclei were graded 3. The intensity index was defined as the weighted score of the combined grades of color intensity multiplied by the percentage of positive nuclei.

Spectral image analysis

Systematic random sampling (Gundersen and Jensen, 1987) was performed on the stained breast carcinoma specimens (Fig. 1A) followed by spectrally resolved imaging using the SpectraCube™ (Applied Spectral Imaging, Migdal HaEmek, Israel) (Garini et al., 1996). Measurements were made using a x40 objective on 10 viewing fields of 220x220 pixels. During the measurements all the parameters of the microscope remained constant. Light intensity at each wavelength in the range of 450-850 nm was recorded for 104 pixels from each field and represented as transmitted light spectra. The spectra were stored in a cube file. The spectrum of a control specimen stained only for estrogen receptor and the spectrum of a control specimen stained only by the background stain were recorded and stored as reference spectra (Fig. 1B). Each reference spectrum was assigned an arbitrary color.

Quantification of estrogen receptors

A quantification procedure was developed in order to digitize the spectral information from the specimen, distinguish the background from the ER signal and calculate the ER index (Fig. 1). We employed a spectral classification algorithm, which operates on the spectral image and calculates the degree of similarity between each reference spectrum and the spectrum of each pixel composing an image using linear combination (Fig. 1D). In this algorithm, the pixel spectrum is expressed as a weighted sum of the pre-defined reference spectra. The weights are the spectral coefficients combined to best fit the pixel's spectrum. The linear combination finds how much of each reference spectrum must be added to best match the pixel's spectrum. Given spectrum S at pixel x,y, the linear combination algorithm will find the set of coefficients wk such that

$$Sx,y = Rk\ wk + e$$

Where e is as small as possible.

Each reference spectrum is given a different weight

wk in this sum, in an attempt to match the pixel's spectrum Sx,y as closely as possible. Each pixel x,y is given the color of the reference spectrum Rk with the largest coefficient wk.

The classification was performed on normalized spectra in order to neutralize the effect of intensity differences between the reference and the pixel's spectra. The data was stored in a cube file where two dimensions are of the flat sample (x and y), and the third dimension is the spectral coefficient divided into two bands, one of the hormone-receptor spectrum and the other of the background spectrum. The spectral coefficients of the hormone receptor spectrum were normalized to 1. The multiplication (Fig. 1E) of the relative contribution of the hormone-receptor spectrum wR1 by the intensity of each pixel Ix,y (from the negative image of the original grayscale image, Fig. 1C) revealed the absolute net intensity of the ER staining for every pixel (Fig. 1F).

For each case, positive and negative controls were determined from a control sample with a previously established diagnosis, which was processed and stained with the specimen. Each file, representing a single field in the specimen, was opened and processed as follows: The total intensity and the number of significant pixels (intensity value higher than the lowest control value) were calculated. The sum of intensity of all the fields of a specimen was divided by the total number of significant pixels. The procedure is summarized in

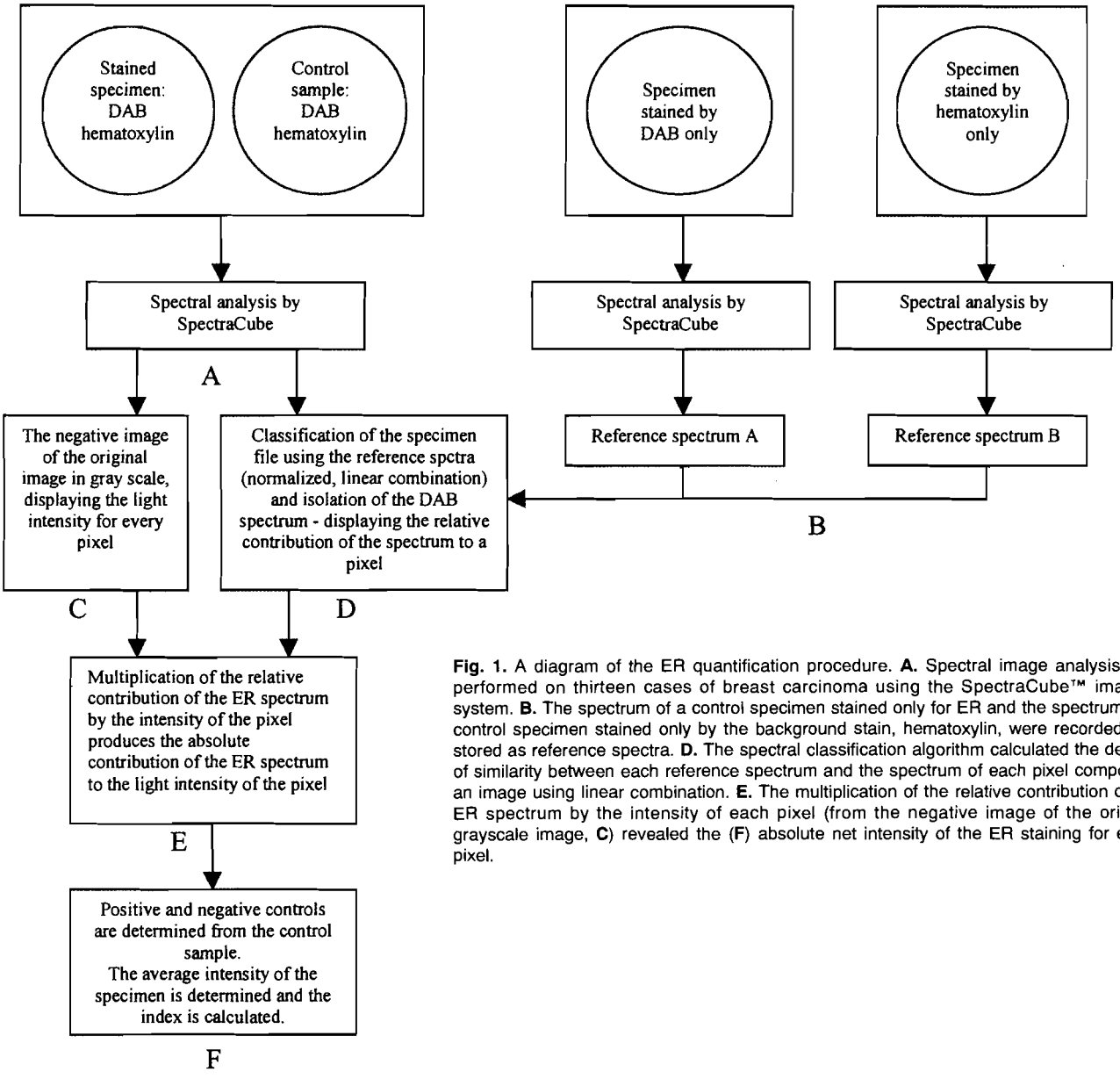
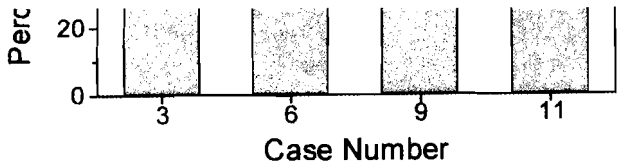


Fig. 1. A diagram of the ER quantification procedure. **A.** Spectral image analysis was performed on thirteen cases of breast carcinoma using the SpectraCube™ imaging system. **B.** The spectrum of a control specimen stained only for ER and the spectrum of a control specimen stained only by the background stain, hematoxylin, were recorded and stored as reference spectra. **D.** The spectral classification algorithm calculated the degree of similarity between each reference spectrum and the spectrum of each pixel composing an image using linear combination. **E.** The multiplication of the relative contribution of the ER spectrum by the intensity of each pixel (from the negative image of the original grayscale image, **C**) revealed the (**F**) absolute net intensity of the ER staining for every pixel.



of cases 1, 6, 9, 10, 11, 12 and 13; in cases 2, 4, 7 and 8, the pathologist's index declared a higher value. **C.** Pixel-by-pixel analysis of cases 9 and 11 revealed a substantial number of positive ER values, while in case 3 the positive ER values were low. In case 6 which was scored as 1 by both the pathologist's index and the compatible spectral index, the analysis detected a large number of pixels (representing 47% of the nuclei) with intensity values higher than the conventional cut-off value of 1.2 (dark gray).

Figure 2.

Results

Spectral quantification of ER receptors of breast carcinoma tumors and the control tumor revealed two distinct groups of specimens differing by their DAB-intensity levels. Although all the specimens were prepared in the same medical center on the same date, five specimens were highly stained while the other eight were stained lightly (Fig. 3A). However, since all the data were digitized it was possible to correct the DAB-intensity according to the control values.

The spectral data obtained represented the DAB-intensities of the ER staining for every pixel of the field. The values of DAB-intensities varied from level 0 to 255 on a continuous scale. Thus, the spectral method presented large amounts of data, which could be translated to any other scaling method. In order to display the spectral results in the common pathologist's scale (0 to 3), we used the pathologist's index for the control specimen to calculate the compatible spectral index of the specimen (Fig. 3B). As shown in Figure 3B, while the compatible spectral index essentially agreed with the pathologist's index of cases 1, 6, 9, 10, 11, 12 and 13, some conflict was observed in the other cases. In

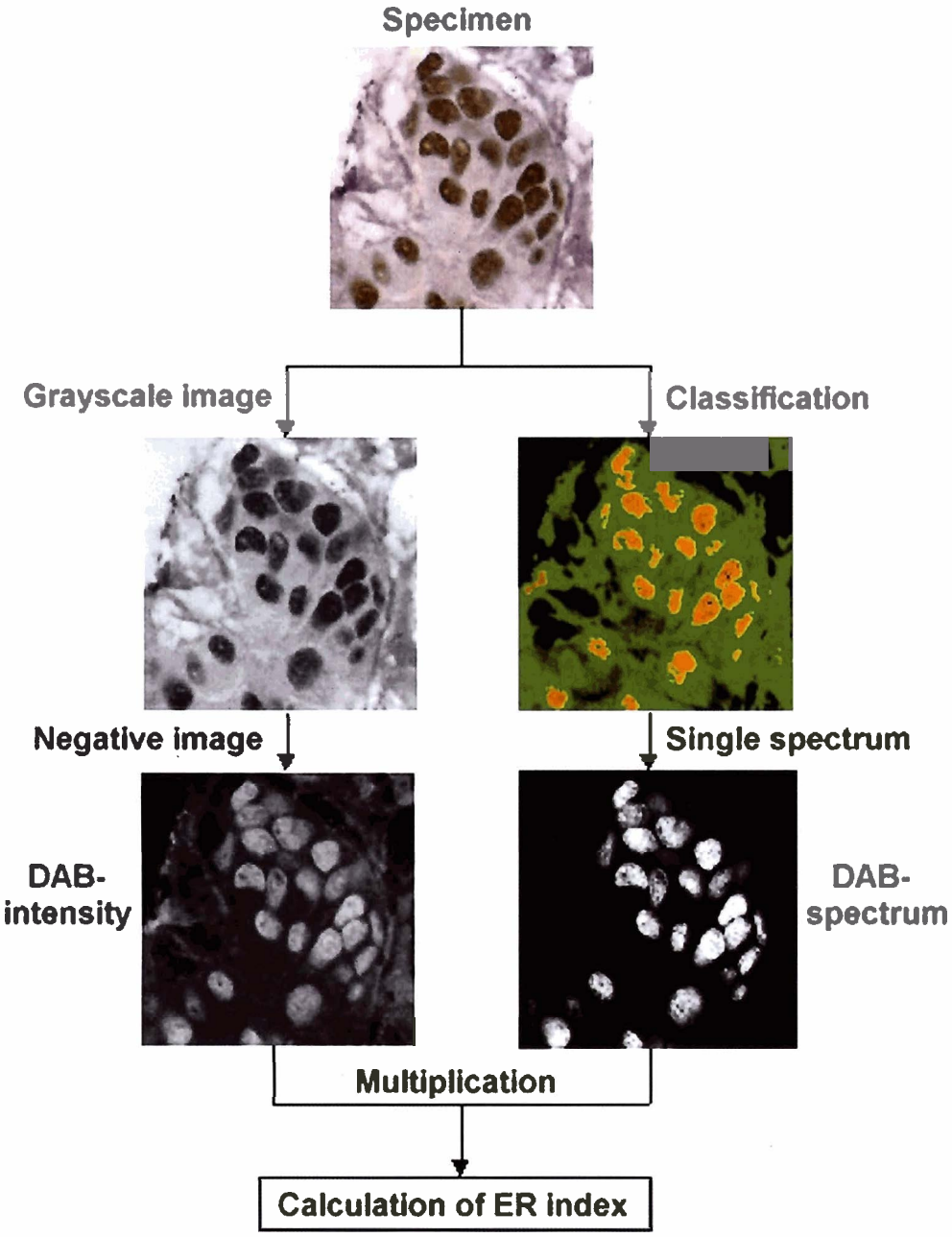
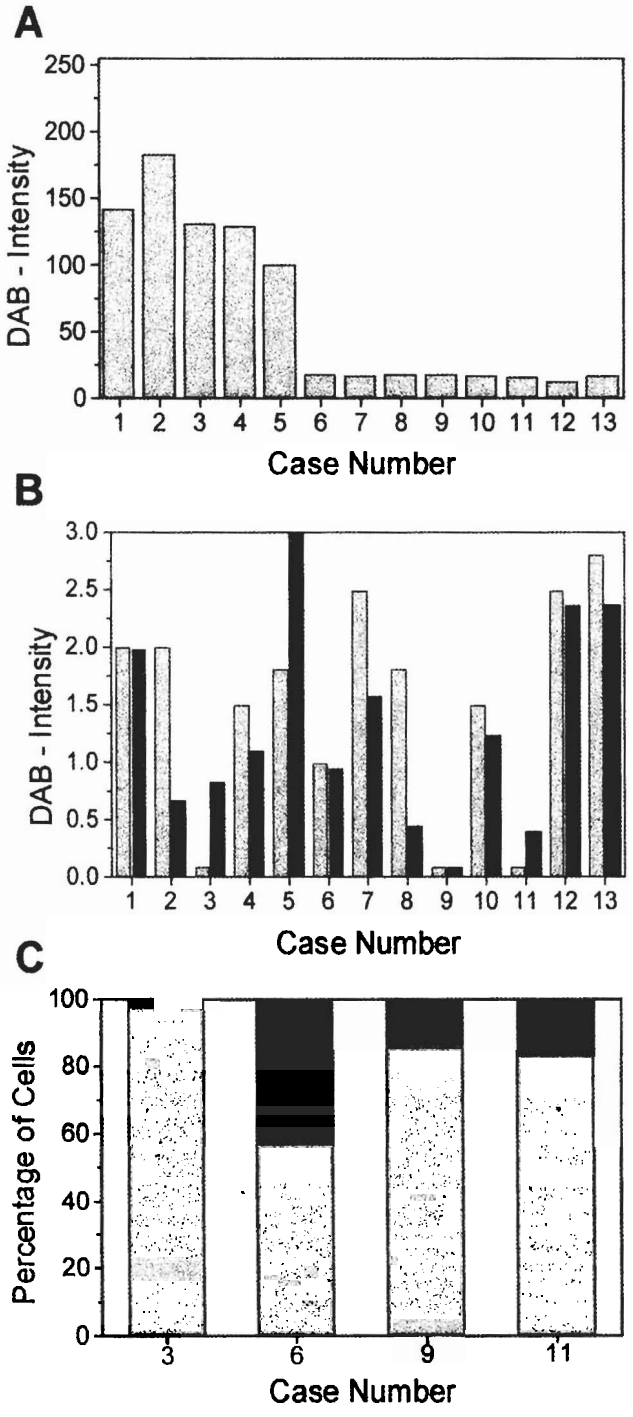


Fig. 2. A summarizing diagram of the ER quantification procedure. A breast carcinoma specimen stained for ER is analyzed using the SpectraCube™. Spectral classification reveals a spectral-map where the color of each pixel represents the reference spectrum it best matches. The relative contribution of the DAB-spectrum is multiplied by the negative image of the original specimen. The output data is processed to generate a spectral index.

cases 2, 4, 7 and 8, the pathologist's index declared a much higher value than the value obtained by the spectral quantification. In cases 3 and 5 the spectral analysis detected more ER than the amount determined by the pathologist.

The most intriguing and critical results were those of cases 3, 6, 9 and 11. The pathologist's index for these cases was lower than the conventional cut-off value of 1.2 determined for tamoxifen therapy. Cases 3, 9 and 11, initially scored as 0 by the pathologist's index, were analyzed using the spectral data in order to determine the number of pixels displaying ER values higher than the cut-off value. The number of pixels was then divided by the number of pixels of the average nucleus in the field to produce the percentage of cells displaying positive, above threshold ER values. As shown in Figure 3C, cases 9 and 11 contained a substantial number of positive ER values while in case 3 the positive ER values were low. The most significant result was obtained for case 6 which was scored as 1 by both the pathologist's index and the compatible spectral index. However, analysis of each single pixel detected a large number of pixels, representing 47% of the cells, with intensity values higher than the cut-off value.



Discussion

In this study, we aimed to develop a new spectral analysis technique for the evaluation of hormone receptors, free of all the pitfalls of the common, subjective evaluation method. Several studies have discussed the question as to what degree do pathologists reliably and consistently recognize negative scores and three grades of ER status. Bejar et al. (1998) has indicated that a pathologist's skill in consistently discriminating between negative and weakly positive scores is dubious at best. The human brain can discriminate up to 64 gray levels, in contrast to the 256 levels of light intensity detected by computer-assisted image analysis. In addition, humans have difficulties in quantifying exactly what they see. Recognition of shifts from negative (0) to weakly positive (1) grades is neither concise nor reproducible. The same holds true for distinctions between grades 1 and 2 and between grades 2 and 3 (Bejar et al., 1998).

The lack of consistency among the cutoff values chosen by laboratories to define an estrogen receptor negative result also complicates the interpretation of the data (Kinsel et al., 1989; Tesch et al., 1993; Detre et al., 1995; Osborne, 1998). Using immunohistochemical analysis, some laboratories include tumors in which 10 or even 20 percent of the cells contain estrogen receptors among those designated estrogen-receptor-negative (Osborne, 1998). Unless those laboratories have justified their cutoff values with clinical follow-up data, many

Fig. 3. A. Spectral quantification of ER receptors in control breast carcinoma tumor. The specimens prepared under standard conditions reveal two distinct levels of DAB-intensities presented in arbitrary units (AU). **B.** The pathologist's index on a scale of 0 to 3 (light gray) and the compatible spectral index (dark gray) which were calculated for each case. As shown, the compatible spectral index agreed with the pathologist's index of cases 1, 6, 9, 10, 11, 12 and 13; in cases 2, 4, 7 and 8, the pathologist's index declared a higher value. **C.** Pixel-by-pixel analysis of cases 9 and 11 revealed a substantial number of positive ER values, while in case 3 the positive ER values were low. In case 6 which was scored as 1 by both the pathologist's index and the compatible spectral index, the analysis detected a large number of pixels (representing 47% of the nuclei) with intensity values higher than the conventional cut-off value of 1.2 (dark gray).

patients may be misclassified as having estrogen-receptor-negative tumors and thus may not be offered tamoxifen treatment (Osborne, 1998). This may explain why tamoxifen was found beneficial in women with estrogen receptor negative tumors (Osborne, 1998). Other data suggest that tumors with any detectable level of estrogen receptors (with even 1% of cells staining positive) should be considered positive (Knight et al., 1980).

A vast number of studies has been dedicated to reducing the effect of environmental conditions on the scoring procedure by developing an objective and quantitative evaluation method (Rostagno et al., 1994; Lehr et al., 1998). Image analysis has introduced several advantages to the quantitation of hormone receptor status and the pathological diagnosis (el-Badawy et al., 1991; Rostagno et al., 1994; Cavaliere et al., 1996; Cohen, 1996; Bejar et al., 1998; Kirkegaard et al., 1998; Lehr et al., 1998). Data is collected and digitized from several fields in the specimen enabling mathematical manipulations and processing of the information obtained. Pathological practice dictates the rules and conditions for decision-making and final scoring. This process can be computerized to utilize the digitized data and to produce an objective, unbiased result. The result obtained by computerized image analysis is presented on a continuous scale providing the pathologist with a more accurate information regarding the specimen. Automation of all the steps leading to the diagnostic result could yield an easy-to-use system with rapid performance.

Image analysis of the DAB-stained specimen, whether analyzed manually or by computerized methods, measures the level of nuclear staining while disregarding the possible contribution of the illuminating light source of the microscope to the diagnosis. The level of ER in a specific specimen could vary from one diagnostic determination to the other due to the use of different intensity levels of illumination. In addition, most receptor staining procedures apply an additional stain for the counter staining of the examined specimen. The contribution of the background staining is thus extremely significant and may lead to inaccurate, and perhaps false, results.

Spectral image analysis enables the differentiation of distinct color constituents according to their unique spectra. Rostagno et al. (1994) based their analysis of estrogen receptors on the different light absorption spectra of the chromogen diaminobenzidine and Harris's hematoxylin coloration when exposed to light of differing wavelengths. The SpectraCube™ system, used in the present study, offers some new advantages to spectral imaging by enabling the measurement and analysis of the full spectrum (450-850nm) of light at all pixels of an image. The spectral image presents a three dimensional array of data which combines precise spectral information with two-dimensional spatial correlation. The analysis of a spectral image creates a unique database, which enables the extraction of features and the evaluation of quantities from multi-point spectral information, impossible to obtain otherwise.

In the present study, we used the SpectraCube™ for the evaluation of the ER status in thirteen cases of breast carcinoma. The ER spectrum was distinguished from other colored elements in order to obtain a net contribution of hormone-receptor staining. Variability in DAB-intensities of the control samples was revealed by spectral analysis. These distinctions, hardly apparent to the human eye, can lead to a false negative result for lightly stained receptors and affect the clinicians' choice of treatment. This situation was resolved by the correction of the light intensity values of the specimens by the previously scored control samples. The calculation of the spectral index included the segregation of the ER spectrum from other spectral elements and the calculation of the average DAB-intensity per specimen. The net spectral index of ER was based solely on the DAB-intensity with the exclusion of any environmental noise such as the intensity of the light source and the counter-staining. Comparison between the pathologist's index and the calculated spectral index was achieved by calculation of a compatible spectral index based on the same pathologist's scale (0 to 3) used previously to score the 13 cases. The compatible spectral index essentially agreed with the pathologist's index in 7 out of the 13 cases. However, the conflict observed in the rest of the cases may be the result of the different approaches used by the two methods. While the pathologists consider a whole nucleus as the basic unit for scoring, the spectral image analysis relates to each individual pixel in a 'positive' nucleus composed of 500050 pixels. The DAB-intensity in each pixel is a function of the local concentration of ER. In order to extract more information from the spectral database of each specimen, we applied a pixel-by-pixel analysis. We detected a substantial number of ER positive pixels in the 2 cases scored as 0 by the pathologist's index. In addition, a significant number of pixels (representing 47% of the nuclei) with DAB-intensity values higher than the conventional cut-off value of 1.2 was discovered in a case scored as 1 by the pathologist's index. These pixels, either distributed over the specimen or locally positioned, are equal to a number of highly stained nuclei and thus may affect the choice of treatment. In addition, these results indicate that a decision-support system based on a single factor is neither sufficient nor reliable.

Our spectral quantification method is extremely flexible, enabling the user to determine the desired spectral resolution or the number of pixels per one nucleus and the number of analyzed fields per one specimen. The highly intensive information obtained is digitized, allowing automated calculation with high reproducibility. The analysis does not require any special preparation of the specimen beyond the routine immunohistochemical staining and thus is easy to master. Spectral imaging succeeded in segregating DAB and hematoxylin yielding the pure value of ER. The

ability of spectral imaging to segregate distinct spectra could contribute greatly to the analysis of multi-stained specimens.

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