Short Technical Report

Protein Expression Analysis Using Quantitative Fluorescence Image Analysis on Tissue Microarray Slides

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ABSTRACT

We have developed a tissue microarray (TMA)-based quantitative fluorescence image analysis (QFIA) method in which protein markers on TMA sections were labeled by immunofluorescence using tyramide signal amplification and a quantitative fluorescence detection system. Using this method, BRCA1 protein expression patterns were studied in the TMA sections of cell lines with known levels of BRCA1 expression and in a small group of human tissue samples obtained from sporadic epithelial ovarian cancers, their corresponding adjacent dysplastic fields, and distant non-tumor areas. We detected distinctive BRCA1 expression patterns in high-grade and low-grade sporadic epithelial ovarian cancers and their associated adjacent dysplastic fields. However, such patterns of expression could not be adequately detected by traditional immunohistochemical staining methods. TMA-QFIA provides a sensitive, automated, and quantitative measurement of protein expression on archived tissue and cell samples and will be a useful tool for protein-level molecular profiling analyses.

INTRODUCTION

The recently developed tissue microarray (TMA) method provides a high-throughput tool for the study of gene dosage and protein expression patterns in a large number of archived tissues. This technique can be used for rapid, parallel, and comprehensive molecular profiling of cancer, without exhausting limited tissue resources (5). However, there are currently no automated quantitative methods available for protein-level expression analysis in situ on TMA sections constructed from paraffin-embedded tissue blocks. Such methods are needed to increase assay speed and to improve measurement accuracy and reproducibility by removing the categorical scoring limitations and biases inherent in conventional manual immunohistochemical (IHC) evaluation. Controlled quantitative data are also more easily standardized and shared among investigators. In addition, a continuous value scale may elucidate levels of expression with small but biologically meaningful differences that might not otherwise be revealed by categorical manual scoring systems. Our well-established automated quantitative fluorescence image analysis (QFIA) technique has been previously demonstrated as a system that simultaneously allows quantitative measurements of multiple cellular protein biomarkers on individual cells in cytological specimens (3,6). The purpose of this study was to determine whether QFIA might

also provide a powerful tool for the quantitative analysis of protein markers on TMA sections derived from archived tissue samples.

To test this possibility, we studied the differential results between the instrument-based TMA-QFIA method and the conventional TMA-IHC staining method on BRCA1 protein expression in archived paraffin-embedded materials. BRCA1 is an important tumor suppressor gene that is involved in the development of breast and ovarian cancers. Although mutations in this gene are rarely seen in sporadic cancers, it has been hypothesized that altered expression of this gene by non-mutational, epigenetic mechanisms such as DNA hypermethylation may be a more common event (1,9). This hypothesis has not received general acceptance because of the variable results obtained by conventional techniques including IHC and Western analysis (8).

In this study, BRCA1 protein expression was measured using the TMA-QFIA technique on TMA sections constructed from tissues in vivo that had been collected from patients both with and without ovarian cancers. Cell lines with known levels of BRCA1 protein expression confirmed by Western analysis were tested in a cell array and included in the tissue array to provide reference controls for the tissue samples. Since the fields of a cancer-bearing organ may show molecular changes that recapitulate the process of carcinogenesis, tissues from areas adjacent to and

distant from tumors (the adjacent dysplastic and distant normal fields, respectively) were also analyzed in the ovarian cancer patients. Since the carcinogenic process may involve changes in the expression levels of candidate proteins that are subtle and/or below the threshold of detection by conventional methods, valuable information may be obtained only when a quantitative and sensitive detection method is used, as demonstrated by our TMA-QFIA technique.

MATERIALS AND METHODS

Tissues, Cell Lines, and TMA Preparation

Archival formalin-fixed and paraffin-embedded tissues from eight cases of sporadic epithelial ovarian cancers lacking the BRCA1 mutation and three non-ovarian cancer controls were obtained from the Department of Pathology and Laboratory Medicine, University of California at Los Angeles. We reviewed the histologic slides from the cases selected for the study to confirm the tissue diagnosis, note the quality of preservation (e.g., fixation), and localize morphologically appropriate regions of tumor and adjacent and distant fields from which to sample. The adjacent precancerous dysplastic fields included polypoid lesions with nuclear atypia that lacked obvious invasion and distant fields that included normal epithelial tissues of the fallopian tube, inclusion cyst, and/or ovarian surface. Three ovarian cancer cell lines (3AO, MLV5, and MLV3) that were kindly provided by Dr. WenXing Zheng's laboratory at the University of Southern California and a breast cancer cell line (MCF7) that is known to express high levels of BRCA1 protein (8) were also employed in the study. These cell lines were cultured in MEM medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acid, 1 mM sodium pyruvate, and 10% FBS at 37°C in a humidified, 5% CO₂ culture chamber. The cell lines were fixed in 10% formalin and processed in the same manner as standard archival tissues, which produced paraffin-embedded cell-pellet blocks. These cell-pellet blocks were used to make a cell line

TMA and were added to a separate TMA containing human tissue samples.

TMA construction was carried out with a technique from Kononen et al. (5) that uses a precision arraying instrument (Beecher Instruments, Silver Spring, MD, USA). In this study, we obtained 2–4 representative tissue samples, when available, from each of the three areas (tumor, adjacent precancerous lesion, and normal distant field) from each case and from the three normal control cases. A total of five consecutive 4- μ m-thick array sections were used; one was stained with hematoxylin and eosin (H&E) to verify the continued integrity and grading of the arrayed sample, one each for BRCA1 analysis with immunofluorescence-based QFIA and conventional IHC staining, and one each to serve as negative controls for these techniques.

Western Analysis

Total protein was isolated from tissue by incubation with 2 $\times$ lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 0.5% NonidetTM P-40, 1 mM PMSF, 1 μ g/mL aprotinin) on ice for 30 min with periodic vortex mixing. The proteins were separated by 7%–8% SDS-PAGE and transferred onto a Hybond[®] ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). They were visualized on the membrane by staining with Ponceaus solution. Immunodetection was performed as follows: blocking with 5% milk in TBS-T; incubation with the primary BRCA1 antibody (Ab-1) at a 1:1000 dilution in 1% milk in TBS-T with 0.1% sodium azide overnight at 4°C; incubation with secondary antibody (mouse IgG HRP conjugated 1:3000) for 1 h at room temperature; chemiluminescence detection with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences); and 24-min washes with TBS-T between each step.

Immunofluorescence Labeling and QFIA

The paraffin-embedded TMA sections (4- μ m-thick) were dewaxed, deparaffinized, and rehydrated, followed by heat-antigen retrieval in 0.01 M cit-

rate buffer, pH 6.0, for 25 min, followed by cooling at room temperature for 15 min. The assay was performed using an automatic staining machine (model 6000; BioGenex, San Ramon, CA, USA). We used a three-step immunofluorescence procedure using the tyramide signal amplification labeling protocol (NEN[®] Life Science Products, Boston, MA, USA) and Texas Red[®] as a fluorochrome to label the BRCA1 protein. While numerous antibodies against BRCA1 were commercially available, we used the monoclonal antibody Ab-1 (clone MS110), since previous studies (8) demonstrated that this was the most specific antibody as a primary labeling reagent for BRCA1. The slides were incubated sequentially with protein blocking solution (30 min), avidin and biotin blocking solutions (10 min each), BRCA1 primary antibody (1:100 for 30 min), biotin-conjugated mouse anti human IgG (1:200 for 30 min), streptavidin-HRP (1:100 for 30 min), tyramide amplification reagent (1:50 for 7 min), and streptavidin-Texas Red (1:500 for 30 min), with multiple washes between these steps. After labeling with BRCA1, we dual-labeled the slides with Hoechst 33258, which is a highly specific DNA staining solution. The stained slide was placed on a QFIA stage (Marzhauser Precision Stage) that can simultaneously mount eight slides. The entire slide was scanned, sequentially measuring each array tissue spot one at a time.

A detailed description of the QFIA procedure can be found elsewhere (3,6,7). Briefly, QFIA entails two parts, image analysis and quantitative fluorescence. To isolate our feature of interest, the nuclei, both morphometry and direct nuclear staining were used as image analysis techniques, thereby reducing errors caused by the binding of fluorochrome in areas other than the nuclei. A targeted protein concentration may be related to the intensity of the epi-illumination-excited fluorescence in a stoichiometrically labeled sample. Automated TMA scanning, image acquisition, and analysis were performed with custom-designed QFIA software on a microscope system (Carl Zeiss, Thornwood, NY, USA). This software also contained an algorithm for background subtraction (3). The image of each array

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spot was captured by a television camera selected and calibrated to have an extremely linear response over wide ranges of light intensities. The image was then encoded into digital signals and analyzed for feature extraction. Quality control and accuracy were maintained by providing standardization using a stable phosphor particle with a fluorescent output that is linearly related to the exciting light intensity as an external standard for cytofluorescence (4). In this study, the mean fluorescence intensity (average gray mean, AGM, of 2–4 array spots) of the isolated epithelial cell nuclei was measured to reflect the level of protein expression. A corresponding array omitting the primary antibody but with the same concentration of nonimmune mouse IgG (Dako, Glostrup, Denmark) served as a negative control for the QFIA analysis.

One of the major challenges for any in situ quantitative fluorescence-based analysis is nonspecific background and autofluorescence. Our QFIA software encompasses three steps to minimize

these problems. (i) The autofluorescence of the entire slide (the areas without the array spot) was subtracted automatically before the measurement by switching the excitation from the labeled fluorochrome (in this case Texas Red, with $560 \text{ nm} \pm 40$) to an unlabeled fluorochrome (e.g., BODIPY® with excitation of $485 \text{ nm} \pm 22$). The assumption is that the nonspecific autofluorescence can be excited at a different wavelength, while the specific fluorescence should not. This approach has been validated previously (3,7). (ii) For each array spot, epithelial cells were specifically selected by manually drawing these regions of interest for measurement so that no fluorescence contribution from non-epithelial cells would be included. To do this, the captured images were reviewed on the monitor one spot at a time, and an outline was drawn around the epithelial area using a light pen. We then measured all of the cells within the drawn area. The total number of cells was determined by nuclear staining, and it may vary among spots,

depending on the type of sample. A minimum of 50 epithelial cells per spot was deemed sufficient for measurement. (iii) The background fluorescence of each array spot on a negative control section was subtracted from the corresponding spot on the measured section.

Traditional IHC Analysis

After deparaffinization and antigen retrieval, the slides were then placed on an autostainer and incubated sequentially with either primary antibody (1:100 dilution of mouse monoclonal anti-BRCA1, Ab-1), or with same concentration of nonimmune mouse IgG as a negative control, followed by HRP conjugates of rabbit anti-mouse immunoglobulins (1:100 dilutions; Dako). The peroxidase enzyme was then localized with 3, 3'-diaminobenzidine, tetrahydrochloride and hydrogen peroxide. Formalin-fixed, paraffin-embedded tissue samples known to express high levels of BRCA1 were included in each run as positive control slides.

Figure 1. BRCA1 expression in cell lines. (a) Western analysis. The values given represent the results of densitometric measurements (with a scale from 0 to 225). (b) Texas Red-labeled BRCA1 on a cell array block (400× magnification). (c) Results of TMA-QFIA analysis (mean + 1 SD of the three experiments). QFIA measures the maximal pixel fluorescence intensity of each cell and these are averaged across all cells, producing an AGM. The AGM fluorescence intensity is shown plotted against various concentrations of the primary antibody. (A) MCF7, (B) MLV5, (C) MLV3, and (D) 3AO.

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RESULTS AND DISCUSSION

We first studied the expression of BRCA1 in three ovarian cancer cell lines (3AO, MLV5, and MLV3) and one breast cancer cell line (MCF7) using Western analysis. The purpose was to determine whether we could detect the known differential protein expression patterns of BRCA1 among these cell lines, thereby determining whether these cell lines could serve as controls for the analysis of human tissue samples. Next, we tested whether BRCA1 protein expression could be detected by quantitative immunofluorescence image analysis on TMA sections constructed from paraffin-embedded blocks of the cell culture models, which had been processed in the same manner as the archival tissues. Finally, we tested whether BRCA1 protein expression could be detected by quantitative immunofluorescence image analysis on

TMA sections constructed from paraffin-embedded archival ovarian cancer tissue blocks. The ovarian cancer cell lines were also included in these later TMA sections as controls.

The Western analysis showed that the MLV5, a cell line from a borderline ovarian serous cystadenoma, and MCF7, a known high expresser for BRCA1 (8), had strong BRCA1 expression. In contrast, 3AO, an aggressive and high-grade ovarian carcinoma cell line, and MLV3, a benign serous cystadenoma cell line, had much less BRCA1 expression (Figure 1a). Cell arrays were then labeled with the same anti-BRCA1 monoclonal antibody we used for the Western analysis, using Tyramide Signal Analysis coupled with a three-step immunofluorescence protocol. The section was analyzed by QFIA. Figure 1b presents the actual images of labeling for each cell line, and Figure 1c presents the fluorescence intensity mea-

sured by QFIA for various antibody concentrations and different cell lines (average + 1 SD of all three independent experiments). Figure 1b shows that BRCA1 labeling on the cell array appeared specific, with an exclusively nuclear stippling pattern. The levels of BRCA1 expression were equally strong for the MCF7 and MLV5 cells (Figure 1c). This finding was concordant with the Western analysis. However, while the Western analysis did not show a visibly discernible difference in expression between the 3AO and MLV3 cells, the QFIA demonstrated a much lower expression of BRCA1 in 3AO than in MLV3 cells (Figure 1, b and c), which is a finding more consistent with the previous observation of a decreased BRCA1 expression in high-grade ovarian cancers (9). Since this observation from Western analysis and TMA-QFIA has been independently repeated three times, it is less likely that this difference

Figure 2. Representative images of a TMA section and BRCA1 labeling on benign and tumor cores. A portion of the H&E-stained ovarian TMA section including three cell lines (3AO, MLV5, and MLV7) is shown at the top of the figure (20× magnification). Representative images of BRCA1 with immunofluorescence-based QFIA (400× magnification) and IHC-labeling (200× magnification) in benign and malignant cores are shown.

Figure 3. BRCA1 fluorescence intensity measured by QFIA (AGM of 2–4 array cores). Well-differentiated tumors (grade 1) are considered low grade and moderately differentiated and poorly differentiated tumors (grades 2 and 3, respectively) are considered high-grade. Morphologically normal fallopian tube, inclusion, cyst, or ovarian surface epithelium is used as matching “normal” tissue for these evaluations. Core spot histologic diagnoses are depicted as symbols. The numbers below the symbols correspond to categorical IHC scores.

represents a false positive caused by nonspecific tyramide amplification, although one cannot entirely rule out such a possibility. Nevertheless, these observations demonstrated that it was feasible to perform BRCA1 protein expression analysis on TMA sections constructed from cells and, by extension, archived paraffin-embedded tissue blocks using the TMA-QFIA technique. In addition, the cell-to-cell variation of BRCA1 expression depends on the cell type. For both high (MCF7 and MLV5) and low (3AO) expressers, the expression is rather homogeneous with the cv of all measured spots falling below 10%. More variation is seen in the intermediately expressing cell line MLV3, with a cv range from 10% to 15%.

We then analyzed the test array composed of the three control ovarian cell lines and archived ovarian tissue samples obtained from eight patients with sporadic papillary serous ovarian cancers (five high-grade, two low-grade, and one low-malignant-potential lesion), and three non-ovarian cancer normal tissue controls. Figure 2 shows a portion of the H&E-stained test array (2a), representative images of a benign inclusion cyst and malignant tumor stained for BRCA1 either by QFIA or IHC. In benign glands, we saw an intense staining of BRCA1 in the epithelial cells lining the glands. Some of the stromal cells also showed significant staining. The BRCA1 staining (Texas Red) was mainly seen in the nuclei (outlined by blue fluorescence, which corresponds to DNA labeling by Hoechst 33258). However, in high-grade solid tumor areas, the BRCA1 staining was rather weak. Analysis of replicate TMA spots of each case showed that 74% had an AGM cv of less than 10% among the spots. The disparity of the measurements between the replicate spots in the remaining 26% of cases may be the result of expression heterogeneity and/or technical factors inherent to antibody-based analysis. The median value of the AGM from replicated spots of each case was used for subsequent analysis. Figure 3 summarizes the QFIA and IHC results obtained from the TMA sections. The BRCA1 labeling intensities in benign ovarian surface epithelial cells from the three normal controls and distant non-

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tumor fallopian tube epithelial cells obtained from tumors patients were clustered in a narrow QFIA intensity zone, which extended only 30 AGM (from 89 to 119 AGM) and fell intermediately between the low-grade and high-grade tumor sample regions. Compared with the corresponding non-tumor distant (normal) areas for each individual case, the high-grade tumors and most of their adjacent dysplastic fields had a clearly decreased BRCA1 expression. In contrast, the BRCA1 expression in the low-grade tumors and their adjacent dysplastic fields did not decrease; instead, it tended to increase slightly to above the normal range or remain within it. These patterns of expression are consistent with findings reported by others using RT-PCR techniques (9). The full meaning of these observations should be carefully examined in larger-scale studies. However, the finding of similar patterns of expression in dysplastic

fields and their corresponding low- and high-grade tumors appear to support the “two-pathway” ovarian cancer theory, a hypothesis derived from clinical observations that low- and high-grade tumors might develop and progress through distinctively different pathways (2). With IHC analysis, these distinctive patterns of expression in the dysplastic fields were less obvious.

Several issues should be considered when one compares the results obtained from TMA-QFIA and manual categorical IHC. The first issue with IHC is that a wide range of perceived intensity values are grouped into a limited range of scoring categories. For example, in this study, tissue spots with an IHC score of 1 had TMA-QFIA AGM values ranging from 30 up to 110, looking across all cases. Within a case, the maximal IHC static range covered from 44 to 101 AGM (case 10). Thus, information may be lost as

the result of the IHC categorical grouping. In addition, “gray zones” of scoring between categories (such as between 0–1 and 1–2) add to scoring ambiguities. For example, one case (case 4) demonstrated reversed IHC versus QFIA staining orders because staining intensities for both the normal and low-grade tumors were within the gray zone of IHC (score 1–2), which was clearly separable by QFIA. The second issue is the sensitivity of detection. In our limited study, the TMA-QFIA appears to be able to detect subtle expression abnormalities that may not be detectable by manual IHC. For example, using IHC analysis, three of the four high-grade cases would fall in the normal IHC range. However, using QFIA, all high-grade tumors fell in a region significantly below the normal range, as expected. This is further exemplified by findings from the adjacent dysplastic lesions in which a heteroge-

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neous BRCA1 expression pattern can be detected by QFIA but not by IHC analysis. Overall, three out of four dysplastic lesions adjacent to high-grade tumor tissue also showed a significantly decreased expression by QFIA compared with the normal range; but, using IHC analysis, three out of four were in the normal range. Another issue is that TMA-QFIA provides objective instrument-based measurements, whereas manual IHC analysis is subjective and prone to intra- and inter-observer variations. All these factors may contribute to the differences in the observations made using TMA-QFIA and IHC analysis, particularly for detecting changes at the low end of the target-protein expression level and QFIA spectrum (i.e., AGM<50) and for detecting subtle changes of expression.

While this study used a specific image analysis system, the procedures and software developed can be applied to other more advanced fluorescence-based imaging systems such as laser scanning cytometry. In addition, the immunofluorescence-based TMA-QFIA method could potentially analyze multiple protein and DNA (e.g., fluorescence in situ hybridization) markers simultaneously at the single-cell level through multi-labeling approaches through which intracellular marker expression interrelationships can be analyzed. As shown in this study, BRCA1 was double-labeled with Hoechst 33258. The second labeling not only facilitated the defining of areas to be measured on each spot but also measured DNA ploidy, which provides another valuable tumor marker by itself (6).

Traditionally, the immunofluorescence assay is unsuitable for paraffin sections because of the high level of background fluorescence. However, with the combination of the tyramide signal amplification method using multiple blocking treatments before the incubation of primary antibody and the unique background subtraction algorithms of our QFIA software, we demonstrated that specific labeling and measurements could be achieved on TMA sections in this study. This is particularly true when the measured cellular protein marker (in this case BRCA1) is double-labeled with a DNA-specific stain (as we used in this study), since the

second labeling can be used to outline the area to be measured for each individual cell. Another inherent problem in measuring the constituents of the cells in histologic sections is that most of the cells are transected at different levels. Because only a fraction of a cell or nucleus is assayed, such measurements alone provide limited information about the quantity of the measured constituent per cell. However, with simultaneous DNA staining (Hoechst 33258), one could potentially develop a ratiometric analysis approach, which would provide an estimate of measured nuclear constituents per unit of DNA. Such normalization allows for the comparisons of marker expression in sectioned cells of different thickness but may be problematic with abnormal ploidy states, which are rather common in tumor cells.

The TMA protocol is designed as a platform for analyzing large numbers of tissues in a high-throughput manner. Our study shows the potential application of combining TMA with QFIA for measuring protein expression in situ, particularly for the detection of subtle expressional abnormalities that may not be detectable by traditional IHC methods. The automated image capture and fluorescence-based measurement algorithm of TMA-QFIA are significant technological advances toward fully automated protein expression analysis. While this is a small-scale study, the TMA-QFIA technique should translate well to studies involving a large number of samples.

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