Simultaneous Measurement of Nucleolin and Estrogen Receptor in Breast Cancer Cells by Laser Scanning Cytometry

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Abstract. Background: The purpose of the study was to test the feasibility of laser scanning cytometry (LSC) to simultaneously measure estrogen receptor (ER) and nucleolin (NU) expression in the nuclei of the same individual breast cancer cells. Materials and Methods: Cancer cells from 64 breast tumors were labeled with anti-NU and biotinylated anti-ER antibodies, then with secondary FITC-conjugated antibody and streptavidin-APC conjugate, respectively, and measured by LSC. The expression of NU in the nucleus and NU aggregates (NUA), number of NUA, nuclear and NUA areas and ER expression were assessed for each cell. Results: ER-bound APC fluorescence correlated with nuclear NU (r=0.65; p<0.001) and NUA-bound FITC fluorescence (r=0.59; p<0.001). Good correlation was found between percentages of ER-positive cells in LSC and by image analysis in paraffin-embedded sections (r=0.59, p<0.001). Conclusion: ER and NU expression can be measured simultaneously in the same nuclei of breast cancer cells.

Breast cancer comprises 18% of all female cancers, thus being the commonest female malignancy worldwide (1). Estrogen receptor (ER) is a transcription factor which, via binding to the specific DNA target called estrogen responsive element, regulates transcription of many genes related to cell proliferation (2). ER assessment is helpful in selecting patients who will most likely benefit from endocrine therapy, and provides reliable prognostic information (1, 3, 4). Nucleolin (NU) is a major nucleolar protein of growing eukaryotic cells located in the nucleolus and nucleus (5). It is involved in pre-rRNA transcription synthesis and maturation, processing and ribosome assembly, shuttling of ribonucleoprotein between nucleus and cytoplasm, as well as induction of chromatin decondensation by binding to histone H1 and unwinding of RNA/DNA duplexes in the 5’ to 3’ direction (5,6). To date no reports on analysis of NU in breast cancers have been published. The purpose of the study was to test the feasibility of laser scanning cytometry (LSC) to simultaneously measure ER and NU expression in the nuclei of the same individual human breast cancer cells.

Materials and Methods

The samples of breast cancer cells were obtained by fine-needle aspiration biopsy of mastectomy specimens with invasive ductal breast carcinomas from 64 women, who underwent surgery in the Regional Cancer Hospital, Szczecin, Poland. The age of the patients ranged from 21-81 years (mean 58 years). None of these patients was treated with preoperative chemo- or radiotherapy.

A suspension of cells was deposited on glass slides by cytocentrifugation at 1000 RPM for 5 minutes (Shandon, Inc., Pittsburg, PA, USA). The cells were fixed in 1% paraformaldehyde on ice for 15 minutes followed by 80% ethanol at -20°C overnight. The cells were rinsed twice in phosphate-buffered saline (PBS), permeabilized in 0.1% Triton-X for 5 minutes on ice and labeled with mouse anti-human nucleolin antibody (#sc-8031 Santa Cruz Biotechnology, USA; diluted 1:20 in PBS, at 4°C, overnight), followed by F(ab')2 fragments of FITC-conjugated goat anti–mouse secondary antibody (#F0479 DAKO Corp., USA; diluted 1:20 in PBS, at room temperature for 30 minutes). Then the cells were rinsed in PBS, incubated with biotinylated mouse anti-human estrogen receptor · antibody (#E7107 DAKO Corp.; diluted 1:30 in PBS, at 4°C for 30 minutes) followed by streptavidin-APC conjugate (#554067 PharMingen, BD Bioscience, USA; diluted 1:20, at 4°C for 30 minutes). Nuclei were counterstained with 5 Ìg/ml of propidium iodide (PI) in the presence of 100 Ìg/ml of RNase A (PI/RNAse A) for 30 minutes. To prevent drying, small pieces (1 cm2) of polyethylene foil (Parafilm, American National Can, Neenah, WI, USA) were placed on the slides over the cells. Incubations were carried out in a humid chamber in the dark. Finally, the cells were coverslipped and subjected to measurement by LSC. Slides prepared from the same sample and treated according to the described procedure but with respective isotype antibodies served as negative controls.

All measurements were performed using LSC (Compucyte Corp., USA) equipped with argon-ion and helium-neon (HeNe) lasers. The contouring parameter was set on PI-red nuclear fluorescence. Cell properties were recorded using a 40x objective lens. FITC-green fluorescence (peak emission: 520 nm) and PI-red
fluorescence (peak emission: 610 nm) excited by argon laser (488 nm) and APC-far red fluorescence (peak emission: 660 nm) excited by HeNe laser (633 nm) were measured in up to 10,000 cells. WinCyte 3.4 software was used to measure NU and ER-associated fluorescence parameters within the nuclear area depicted by the red integration contour of PI. The fluorescence in situ hybridization (FISH) function of the WinCyte 3.4 served for detection of NU aggregates (NUA) of area greater than 1 µm².

The following parameters of each individual cell were calculated: (1) NU fluorescence within the nucleus, which reflected the NU content in the whole nucleus, (2) NU fluorescence within all NUA of a cell, (3) NU fluorescence per NUA of a cell, (4) NU fluorescence in the remaining karyoplasm (NU fluorescence within all NUA of the cell subtracted from NU fluorescence within nucleus), (5) number of NUA, (6) nuclear area, (7) area of NUA, (8) ratio of NU fluorescence over nuclear area, (9) ratio of NUA fluorescence over NUA area, (10) ER fluorescence within nucleus and (11) percentage of ER-positive cells.

Figure 1 presents a schematic approach to the ER and NU measurements and data analysis used in our study. Figure 1 A shows the real-time scanned cell image. The threshold contour (th) was set on PI-red fluorescence. ER-bound long red fluorescence and NU-bound green fluorescence, as well as number of NUA (FISH green spots) and NU fluorescence of NUA, were measured within the integration contour (int). Background fluorescence (bkg) was automatically subtracted from cell fluorescence. Analysis of the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean value ± SE</th>
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<tbody>
<tr>
<td>NU fluorescence within nucleus</td>
<td>319032 ± 18556 *</td>
</tr>
<tr>
<td>NU fluorescence within all NUA of a cell</td>
<td>181471 ± 12528 *</td>
</tr>
<tr>
<td>NU fluorescence per NUA of a cell</td>
<td>131971 ± 9117 *</td>
</tr>
<tr>
<td>NU fluorescence within the remaining karyoplasm</td>
<td>144818 ± 13727 *</td>
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<tr>
<td>Number of NUA</td>
<td>1.4 ± 0.03</td>
</tr>
<tr>
<td>Nuclear area</td>
<td>77.7 ± 2.68 µm²</td>
</tr>
<tr>
<td>NUA area</td>
<td>5.8 ± 0.14 µm²</td>
</tr>
<tr>
<td>Ratio of NU fluorescence to nuclear area</td>
<td>4271 ± 265</td>
</tr>
<tr>
<td>Ratio of NUA fluorescence to NUA area</td>
<td>30008 ± 1558</td>
</tr>
<tr>
<td>ER fluorescence within nucleus</td>
<td>327812 ± 27598 *</td>
</tr>
<tr>
<td>% of ER-positive cells</td>
<td>48.4 ± 3.2 %</td>
</tr>
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* arbitrary units
**NU fluorescence within all NUA of a cell subtracted from NU fluorescence within nucleus
PI-red maximal pixel in relation to the nuclear area made it possible to discriminate single cells for further analysis (dot-plot B). ER-bound integrated long red fluorescence and the percentage of ER-positive cells (dot-plot C), as well as parameters related to NU fluorescence such as NU fluorescence integrated separately over the nucleus (dot-plot D) and over NUA, the number of NUA and their area (dot-plots E, F), were analyzed in gates set above respective controls.

Estimation of the percentage of ER-positive breast cancer cells was conducted in parallel by the automated image analysis (IA) system Leica Q600 QWin (Leica Cambridge Ltd., UK). Formalin-fixed and paraffin-embedded sections were incubated with anti-estrogen receptor α antibody (#M7047 DAKO Corp.) and visualized with streptavidin-biotin DAKO LSAB + System, HRP (#K0690 DAKO Corp.). At least 1000 cells from areas with the highest frequency of ER-positive cells were examined using 400x magnification. The cut-off value for ER-negative tumors was set at 10% of positively-stained nuclei.

Statistical analysis was done using the non-parametric R-Spearmann test included in Statistica 5.0 software. P values less than 0.05 were considered statistically significant.

Results

Nucleolin and ER were visualized in the same nuclei of breast cancer cells (Figure 2). NU-bound FITC staining was present as green spots of various sizes located in the nucleus, as well as orange staining uniformly dispersed throughout the remaining karyoplasm due to overlap of green FITC fluorescence over a vivid red fluorescence of PI.

Table I shows mean values (± SE) of all parameters assessed in 64 cases of invasive ductal breast cancers. NU fluorescence measured within all NUA correlated with the area of NUA (r=0.84; p<0.001). ER fluorescence correlated with nuclear NU (r=0.65; p<0.001) and NUA fluorescence (r=0.59; p<0.001). A correlation was found between ER fluorescence in the nucleus and the percentage of ER-positive cells as measured by LSC (r=0.43; p<0.001). When tumors with less than 11.6% of ER-positive cells (a median of ER-positive cells assessed by IA) were considered as ER-negative (n=12), a good correlation was found between the percentages of ER-positive cells measured by LSC and by IA (r=0.59, p<0.001).

Discussion

Several authors have reported on the results of measurements of ER or NU stained with fluorochrome-labeled antibodies and recorded by flow cytometry or LSC, however to date no such experiments have been performed concurrently in the same individual cells (7-9). The simultaneous measurement of two or more different cell constituents by LSC requires appropriate selection of fluorochromes with the least possible overlapping spectra. Selection of PI, FITC and APC does not require any compensation, which could potentially affect the data interpretation. NU and ER fluorescence-associated parameters have been measured rapidly (up to 10 cells/s) and with high sensitivity (10). The ratio of mean area of NUA over mean nuclear area indicates that approximately one-fourteenth of the nucleus is occupied by the NUA. The mean integrated NU fluorescence measured within all NUA of a cell divided by the mean NU fluorescence within the nucleus shows that approximately half of NU fluorescence is preferentially localized within the aggregates. The concentration of NU in aggregates (ratio of NUA fluorescence to NUA area) is 15-fold higher than in the remaining karyoplasm. Since, on one hand, NU is the main constituent of nucleoli (5) and the concentration of NU in aggregates (as compared to the remaining karyoplasm) is high and, on the other hand, NUA morphologically resemble nucleoli, from a practical point of view NUA can
be considered as nucleoli. NU is also one of the major components associated with the AgNORs (11-13). The mean number of NUA (1.4±0.03) in our study was comparable to the lower value of AgNORs reported in invasive breast cancers (range 1.74-6.2 per cell) (11, 12). This may have resulted from technical limitations of the LSC software. The problem of the underestimation of the number of nucleoli has been extensively discussed by Gorczyca et al. (7). Each NUA is contoured only when the FISH green spot within the nucleus has an integral green fluorescence greater than the threshold set at 900 and the area of this spot is greater then 1 µm². The latter limitation is the lowest possible value to set up by the WinCyte software. NUA smaller then 1 µm² or with lower green fluorescence intensity are not contoured. The increase of the threshold level of the FISH contour caused underestimation of the number of NUA due to loss of less intense ones. On the other hand, the decrease of the threshold level also caused loss of NUA due to coalescence of closely lying spots. A modification of the software and a change of the objective lens to a higher magnification (60x), combined with a change of the WinCyte software to recognize this lens may provide more precise estimates of the number of the NUA.

The good correlation between the percentage of ER-positive cells measured by LSC and by IA in formalin-fixed paraffin-embedded sections (r=0.59, p<0.001) and the 11.6% cut-off value for LSC-determined ER-negative tumors, corresponding to 10% of the cut-off value for IA, suggests that these two methods may be equivalent in pathologic practice.

We conclude that LSC can simultaneously measure two parameters in the same cancer cell nuclei and therefore can be useful to assess the heterogeneity of breast cancer cells in relation to ER and NU. Because NU and ER are involved in the regulation of cell proliferation, the combined assessment of ER status and nucleolin by LSC may provide additional information on the proliferative potential of individual breast cancer cells. This method may also be adapted to concurrently analyze other proteins of, for example, prognostic value in human breast cancer.

Acknowledgements

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References