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Response of MCF7 Human Breast Cancer to Tamoxifen: Evaluation by the Three-Time-Point, Contrast-enhanced Magnetic Resonance Imaging Method

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Abstract
Variations in the cellular volume fraction and in the microvascular permeability of MCF7 human breast tumors were used to assess response to tamoxifen. These pathophysiological features were mapped by applying the three-time-point, contrast-enhanced, high resolution magnetic resonance imaging method (H. Degani et al., Nat. Med., 3: 780–782, 1997). Short-term treatment with tamoxifen caused a highly significant increase in the fraction of pixels displaying intermediate contrast agent clearance pattern and a significant increase in the fraction of pixels displaying high rate of contrast agent entrance. These changes resulted from a marked rise in the extracellular volume fraction, indicating increased necrosis, and from an augmentation in the microvascular permeability, predominantly in the vicinity of the high extracellular volume fraction areas, as a result of stress-induced angiogenesis.

Introduction
In the course of endocrine therapy or chemotherapy of breast cancer, it might take several months before a reliable objective assessment of response or resistance can be achieved. It is therefore important to develop methods that can evaluate tumor response to therapy at an early stage and thereby improve breast cancer management. It is now well established that changes in tumor vascular pathophysiology might indicate a failure for response to therapy (1, 2). The microcirculation of breast tumors in the course of therapy was characterized recently by using positron emission tomography and color Doppler imaging (3–5). Contrast-enhanced MRI3 has been also evaluated as a method for monitoring the success rate of therapy (6, 7). Variations in contrast enhancement are derived from differences in the density and permeability of the microvascular system and in the extent of interstitial space accessible to the contrast agent. Image analysis of dynamic contrast-enhanced MRI was based on kinetic models and on correlation with histological findings (8, 9). These analyses provided estimations of the capillary surface area times permeability (termed here microvascular permeability) and of the EVF (10–13). In most dynamic studies, the recording parameters have been optimized to increase temporal resolution at the expense of spatial resolution. However, because tumors are usually highly heterogeneous, high spatial resolution is necessary for characterizing their pathophysiological features (14, 15). We have recently developed a new algorithm that analyzes contrast-enhanced images recorded at high spatial and temporal resolution. This algorithm maps the microvascular permeability and the extracellular volume fraction per pixel (15, 16). We further developed a new method that uses high spatial resolution and three time points along the enhancement curve (termed the 3TP method) and provides estimations of the microvascular permeability and the EVF per pixel (17).

In this study, we describe the application of the 3TP method to map the microvascular permeability and the EVF of MCF7 human breast tumors implanted in nude mice. Specifically, we examined whether tamoxifen induces significant changes in these parameters and whether these changes can indicate response to tamoxifen. We have found that tamoxifen treatment led to a marked increase in the EVF of the tumors and to increased microvascular permeability in regions surrounding the high EVF loci. These changes can be detected and measured by the new 3TP method to assess response to tamoxifen.

Materials and Methods

MCF7 Tumors. MCF7 cells were cultured routinely under standard conditions and were prepared for inoculation as described previously (18). Approximately 105 cells/ml were injected s.c. into the flank of CD1-NU female, athymic mice, 6–8 weeks of age (n = 18). Prior to the injection, a pellet of 17β-estradiol (0.72 mg/pellet, 60 days release time; Innovative Research, Sarasota, FL) was implanted underneath the skin of the back. Tamoxifen treatment for 1 or 2 weeks (n = 9) was initiated when the tumors reached a size of ~1 cm3 by removing the estrogen pellet and implanting a tamoxifen pellet (5 mg/pellet, released, 45 days release time; Innovative Research). In control tumors (n = 9), a new estrogen pellet was implanted after 60 days. During the MRI experiments, mice were anesthetized by an i.p. injection with sodium pentobarbital (0.06 mg/g weight; CTS, Ltd., Kiriat Malachi, Israel). All tumors were processed for histological studies at the end of the experiment and were sectioned in planes closely parallel to the planes of the MR images, as described previously (18).
**In Vivo \(^1\)H Imaging.** MR images were recorded with a 4.7 Tesla, Biospec spectrometer (Bruker, Karlsruhe, Germany) equipped with a \(^1\)H radio frequency coil 7.5 cm in diameter. The protocol of each experiment included: (a) imaging with a multislice SpE, \(T_2\)-weighted sequence, with an echo time (TE) and a repetition time (TR) of 68 and 2400 ms, respectively, accumulating four averages; (b) imaging of a selected slice from the center of the tumor using a \(T_1\)-weighted, SpE sequence with TE of 15 ms and TR of 250 or 400 ms and a time resolution of 1 or 1.5 min, respectively, accumulating two averages; and (c) injection of 0.4 mmol/kg weight of (NMG)\(_2\)GdDTPA into the tail vein of the mouse (0.1 ml in \(<5 \) s), followed by imaging the same central slice with the same \(T_1\)-weighted sequence as in (b), in a serial manner. All images were recorded at the same spatial resolution with a slice thickness of 1 mm and an in-plane resolution of 195 × 390 \(\mu\)m. The overall imaging time of each tumor lasted for about 1 h.

**Image Analysis.** Image analysis was performed on a Dec-3000 work station (model 600; Digital Equipment Co., Maynard, MA). Two algorithms were developed and applied to analyze the time evolution of contrast enhancement at pixel resolution:

(a) The algorithm of the 3TP method (17). This algorithm is based on recording contrast-enhanced images at three time points: the first time point \(t_0\) is recorded before contrast agent administration, and the second \(t_1\) and third time \(t_2\) are recorded after the administration of the contrast agent to the blood circulation. These time points are selected on the basis of a model as described below. The intensities of each pixel, at the three time points, serve to determine the entrance (wash-in) and clearance (wash-out) patterns of the contrast agent between the intravascular and extravascular compartments as follows. The relative values of the two post contrast MRI intensities \((I(t_1), I(t_2))\) are coded by color hues according to: (i) slow wash-out with \(I(t_1) = I(t_2)\) coded red; and (ii) moderate wash-out with \(I(t_1) = I(t_2)\) coded green; (iii) fast wash-out with \(I(t_1) > I(t_2)\) coded blue. The initial rate \((I(t_1) - I(t_0))/I(t_0)\), which determines the wash-in rate, is coded by color intensity. Thus, for each wash-out pattern coded by color hue, there is a range of wash-in rates coded by color intensity. The final output includes color hue and color intensity coded images and a calibration map that relates the color hue and color intensity in the 3TP image to microvascular permeability and EVF values, as will be described below.

(b) The algorithm of the detailed kinetic method that applies a nonlinear curve-fitting program to analyze time-dependent, contrast-enhanced intensities (15). The resultant output includes two separate maps of microvascular permeability and EVF.

Both algorithms are based on the same physiological model, presented previously by Tofts and Kermode (12). This model assumes that the microvascular volume is relatively small and can be neglected. In addition, there is a fast equilibration of the contrast agent in the intravascular compartment and rate-limiting reversible permeation from the intravascular to the extracellular volume. The following equation was used for the nonlinear curve-fitting algorithm and for constructing the 3TP calibration map:

\[
\text{Enhancement}(t) = \frac{I(t) - I(t_0)}{I(t_0)} = 1 - \exp[-R_1C_i(t)TE]\left[1 - 2\exp\left(-TR/TE\right)^2\left(1/T_{1\alpha} + R_1C_i(t)\right)\right] e^{-TR/T_{1\alpha}}
\]

where \(R_1 = 4.3 \text{ s}^{-1}\text{mm}^{-1}\) and \(R_2 = 4.9 \text{ s}^{-1}\text{mm}^{-1}\) are the coefficients for the respective change in plasma \(T_1\) and \(T_2\) relaxation times with GdDTPA concentration, and \(T_{1\alpha} = 2.7 \text{ s}\) is the longitudinal relaxation time of tumor tissue (15). \(C_i(t)\), the mean concentration of the contrast agent per unit volume is given by:

\[
C_i(t) = D\left(b_2\exp\left(-m_1t\right) + b_3\exp\left(-m_2t\right) + b_4\exp\left(-Ktv_i\right)\right)
\]

where \(b_1 = Ka_1/Kv_1 - m_1\), \(b_2 = Ka_2/Kv_1 - m_2\), and \(b_3 = -(b_1 + b_2)\). \(D = 0.4 \text{ mmol/kg weight}\) is the GdDTPA dose, and \(a_1 = 4.3 \text{ kg/liter}, a_2 = 3.2 \text{ kg/liter}, m_1 = 0.43 \text{ min}^{-1},\) and \(m_2 = 0.057 \text{ min}^{-1}\) are pharmacokinetic parameters determined previously for nude mice (15). \(K\) and \(v_i\), the microvascular permeability and the EVF, respectively, were calculated by the fitting algorithm (15) or estimated by the 3TP method from the corresponding calibration map (17).

Image analysis based on fitting the dynamic data to Eq. 1 yielded maps of microvascular permeability and EVF. The EVF relates to the cellular volume fraction (CVF) by: \(CVF = 1 - \text{EVF}\). Assessment of the quality of the fitting for each pixel was obtained by calculating the proportion of variability \(R^2\), as described previously (15). Pixels in regions where the program failed to fit the enhancement curves to the model-based equation were selected out and marked black in the resultant maps derived from the image analysis.

Calibration maps of the 3TP method were obtained by a separate computer program that initially calculated the enhancement \((I(t_1)/I(t_0) - 1)\) at the two post-contrast time points \((i = 1, 2)\) for each pair of \(K\) and \(v_i\) in the range 0–0.1 min\(^{-1}\) and 0–1, respectively, using the values given above of the MRI and pharmacokinetic parameters in Eq. 1. Following this calculation, a color hue/intensity-coded distribution within a range of \(K\) and \(v_i\) values was constructed according to the 3TP method, yielding the desired calibration map (Fig. 1). The color intensities in the 3TP calibration maps ranged between 0 and 255, with the fastest
initial rate scaled to 255. The three time points that provide the best resolution for estimating $K$ and $v_1$ are obtained by an iterative process aimed at reaching a map in which the $K$-$v_1$ plane is divided between the three colors to approximately three equal areas, as demonstrated in Fig. 1.

Results

Mice inoculated with MCF7 cells developed tumors of about 1 cm³ in size, within 6 weeks. Eighteen tumors in 18 mice were scanned by MRI once or twice as follows. Nine tumors growing in the presence of estrogen were imaged once and were then removed for histology. Five tumors were imaged once after tamoxifen treatment and were then removed for histology. Four tumors were imaged twice, before tamoxifen treatment and after the treatment, and were then removed for histology.

Analysis of the dynamic contrast-enhanced MRI data by the kinetic fitting algorithm and by the 3TP algorithm yielded maps of microvascular permeability and EVF, as described in "Materials and Methods." An example of the microvascular permeability and EVF maps derived by applying both algorithms is demonstrated in Fig. 2, along with typical contrast enhancement curves in selected pixels (Fig. 2D). The kinetic fitting and the 3TP analysis show the heterogeneity in the distribution of microvascular permeability and EVF. The light green pixels in the 3TP image (e.g., pixel #1 in Fig. 2, A and D) reflect, according to the calibration map, pixels with lower microvascular permeability and lower EVF values compared with pixel #1. Here too, the kinetic fitting analysis confirmed these results (Fig. 2, B–D). The dark red pixels in the 3TP image (e.g., pixel #4 in Fig. 2, A and D) reflect, according to the calibration map, pixels with a very low microvascular permeability relative to that of pixel #1 and lower EVF, as confirmed in the microvascular permeability and EVF maps (Fig. 2, B–D). As expected, in necrotic regions devoid of capillaries, the enhancement curves could not be fitted to Eq. 1, which assumes the presence of leaky capillaries in each pixel (Ref. 15; e.g., pixel #5 in Fig. 2, B–D). These areas were enhanced with a slow wash-out pattern indicative of high EVF and low microvascular permeability (Fig. 2, A and D). It is important to note that these nonvascularized regions are usually found in the vicinity of regions with high microvascular permeability and high EVF (light green in the 3TP images); therefore, their enhancement is mainly due to diffusion of the contrast agent through the extracellular space of the nearby vascularized regions.

The 3TP analysis of tumors imaged before and after initiation of tamoxifen treatment showed clearly alterations resulting from the treatment (Fig. 3 and Table 1). Before tamoxifen treatment, dark blue, dark green, and dark red pixels appeared in inner viable regions indicating low EVF values with varying values of microvascular permeability, and part of the tumor was not enhanced at all, as shown in Fig. 3A. In the outer rim of the tumors, the predominant coloring of light green pixels indicated...
high microvascular permeability and high EVF (Fig. 3A). After tamoxifen treatment (1 or 2 weeks), notable changes were observed in the 3TP images of the same tumor (Fig. 3C). All pixels were enhanced, and a substantial increase in the fraction of pixels with higher EVF and higher vascular permeability (coded in light green) was observed, primarily in internal parts of the tumor. These changes were correlated with the gross histopathological features observed in $T_2$-weighted images. Before treatment, tumors were mostly composed of viable tissue with few necrotic loci, indicated in the $T_2$-weighted images by gray and bright pixels, respectively (Fig. 3B). After treatment, an increase in the fraction of necrosis (fraction of bright pixels) was observed (Fig. 3D; Ref. 18). The correlation between the $T_2$-weighted image and the 3TP image also showed the presence of pixels with high microvascular permeability surrounding necrotic regions that demonstrated high EVF.

By analyzing variations in the percentage of each color and in the intensity distribution of the colors, before and after treatment, it was possible to evaluate the statistical significance of the changes and define the parameters that indicate response to tamoxifen. The results are summarized in Fig. 4. Tamoxifen treatment led to a highly significant increase ($P < 0.0006$) in the fraction of pixels showing intermediate wash-out rate (green) from 28.0% ± 2.8% to 54.5% ± 6.8% (mean ± SE; Fig. 4A). Another significant change ($P < 0.02$) was a reduction in the percentage of nonenhancing pixels from 28.1% ± 4.5% to 10.4% ± 5.4%. Paired $t$ test of tumors that were followed before and after tamoxifen treatment ($n = 4$) also indicated a significant increase ($P < 0.02$) in the percentage of pixels with intermediate wash-out (green) pattern and a significant reduction ($P < 0.003$) in the percentage of nonenhancing pixels (Table 1A). For example, in a tumor followed before and 2 weeks after tamoxifen treatment, the percentage of green pixels increased from 30.9% to 62.6%, while the percentage of black pixels decreased from 17.7% to 0.2%. The percentage of pixels that show slow wash-out (red) or fast wash-out (blue) did not change significantly by the treatment.

In addition to the changes in the wash-out patterns (color hue), tamoxifen treatment led to changes in the wash-in rates coded by color intensity (Fig. 4B and Table 1B). Analysis of the wash-in rates showed significant shifts to higher intensities as a result of tamoxifen treatment. The intensities were divided into four ranges (Fig. 4B), and the numbers of pixels in each range changed significantly ($P < 0.0001$ to $P < 0.003$) after tamoxifen treatment. Analysis of the wash-in rate distribution for the intermediate wash-out pattern (intensity distribution of green pixels) that became dominant after the treatment also indicated significant shifts to higher intensities for the four ranges of intensities ($P < 0.0001$ to $P < 0.015$; Fig. 4C and Table 1C).

According to the calibration map (Fig. 1), the changes in color intensity reflect a shift to higher EVF and higher microvascular permeability. In pixels that exhibited either the slow wash-out pattern (red) or the fast one (blue), a trend of faster wash-in rates was observed too but in a less pronounced manner. Thus, from the changes in the extent of clearance and in the rates of entrance of the contrast agent, it can be concluded that tamoxifen treatment increased the fraction of tumor with high EVF and increased the fraction of tumor with high microvascular permeability. We also noted from the distribution of the color hue/color intensity in the 3TP images that pixels with high microvascular permeability and high EVF are distributed in the tumor in a specific manner, localized predominantly around the necrotic regions.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Distribution of color hue and color intensity derived from 3TP images of MCF7 tumors ($n = 4$) obtained before and 1-2 weeks after tamoxifen treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean fraction of area (% ± SE), before tamoxifen</td>
</tr>
<tr>
<td>A. Color</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>25.8 ± 5.8</td>
</tr>
<tr>
<td>Green</td>
<td>52.8 ± 7.2</td>
</tr>
<tr>
<td>Blue</td>
<td>15.1 ± 2.8</td>
</tr>
<tr>
<td>Black</td>
<td>31.2 ± 8.9</td>
</tr>
<tr>
<td>B. Intensity</td>
<td></td>
</tr>
<tr>
<td>52–102</td>
<td>37.3 ± 1.8</td>
</tr>
<tr>
<td>103–153</td>
<td>23.1 ± 5.4</td>
</tr>
<tr>
<td>154–204</td>
<td>4.8 ± 1.5</td>
</tr>
<tr>
<td>205–255</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>C. Green pixel intensity</td>
<td></td>
</tr>
<tr>
<td>52–102</td>
<td>12.1 ± 2.5</td>
</tr>
<tr>
<td>103–153</td>
<td>11.2 ± 3.6</td>
</tr>
<tr>
<td>154–204</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>205–255</td>
<td>2.2 ± 0.8</td>
</tr>
</tbody>
</table>

* A statistically significant difference as indicated by paired $t$ test: $^a P < 0.02$; $^b P < 0.003$; $^c P < 0.04$; $^d P < 0.007$; and $^e P < 0.06$. / Black, nonenhancing pixels.
Fig. 4  Mean color hue and color intensity distribution in 3TP images of MCF7 tumors in the presence of estrogen (n = 13) and following tamoxifen treatment (n = 9). A, color hue distribution (data are means; bars, SE). B, color intensity distribution (data are means; bars, SE). C, intensity distribution for the green pixels (data are the means; bars, SE). Black pixels, nonenhancing pixels.

Discussion

The dynamics of contrast agent distribution in a tissue can be analyzed, explicitly or approximately, using model-based equations. Furthermore, to resolve the heterogeneity of the spatial distribution of a contrast agent in a tissue, it is necessary to record and analyze the dynamic data at high spatial resolution. Here we demonstrate that it is possible to characterize the dynamics of contrast enhancement at high spatial resolution with a limited number of time points (e.g., three) using a model-based method. We also show how to estimate at pixel resolution the microvascular permeability and EVF and how to use these pathophysiological features to assess early response to tamoxifen therapy.

The relatively slow kinetics of contrast enhancement in MCF7 human breast cancer implanted in nude mice enabled us to acquire images at both high temporal resolution and high spatial resolution. A model-based fitting algorithm was then applied to analyze the kinetic data yielding the best-fitted microvascular permeability and EVF within the fitting accuracy ($R^2$). The 3TP method estimated the microvascular permeability and the EVF from the wash-out behavior (color hue) and wash-in rate (color intensity) in each pixel, using the same model. The comparison between the values obtained by the fitting algorithm and the 3TP estimates served to validate the 3TP method.

The model assumes that the enhancement in each pixel is due to the presence of permeable capillaries. The kinetic algorithm based on this model failed to analyze signal enhancement of pixels in necrotic regions, which are devoid of capillaries. These regions can be enhanced through diffusion of contrast agent in the extracellular space from neighboring pixels with highly permeable vessels. The diffusion process in a pixel with high EVF dictates a slow wash-out pattern. Indeed, the enhancement pattern in necrotic regions exhibited a slow wash-out pattern, which according to the 3TP method is indicative of high EVF (coded red). Thus, although the fitting algorithm failed to fit the enhancement curves in necrotic pixels, the 3TP method described the entrance and clearance patterns of the contrast agent.

The most striking change following tamoxifen treatment was the increase in the fraction of tumor with both high microvascular permeability and high EVF. These regions were intratumoral and localized in the vicinity of necrosis. This increase in microvascular permeability in regions surrounding necrosis was viewed by the 3TP analysis as a marked increase in the percentage of light green pixels. The increased wash-in rate in these boundary regions led to increased diffusion from their extracellular volume to the extracellular volume of nearby pixels and to a reduction in the percentage of nonenhancing (black) pixels in the tumor.

Previous imaging studies of breast cancer response to chemotherapy, using contrast-enhanced MRI, positron emission tomography, or color Doppler flow imaging, demonstrated response by a decrease in the wash-in rate and in the extent of contrast enhancement (3-5, 7, 19). It is evident from our results that response to therapy with a cytostatic drug such as tamoxifen evokes specific physiological changes that overall show an opposite trend: increase in the wash-in rate of contrast enhancement and a higher fraction of enhancement. Unlike chemotherapeutic drugs that are cytotoxic and kill proliferating cells, including endothelial cells, tamoxifen acts as a cytostatic drug that inhibits growth but does not directly kill endothelial cells. Thus, processes that follow tamoxifen treatment, but are not hormonally regulated (i.e., not induced by estrogen or inhibited...
by tamoxifen) such as hypoxia-induced angiogenesis, can occur in the presence of tamoxifen. The induction of hypoxia by tamoxifen was demonstrated recently in MCF7 tumors (20). We propose that the "apparent" higher perfusion in response to tamoxifen therapy was not a result of improved vascularity but originated from an induction of growth of permeable vessels by stress-hypoxia-induced angiogenesis. We have described previously the sequence of events following tamoxifen treatment of MCF7 tumors (15, 16, 18). Initially tamoxifen appeared to inhibit endothelial cell growth, possibly via inhibiting the secretion of angiogenic growth factors (18) or by an hormone-independent mechanism (21). This inhibition diminishes neo-vascularization and impairs nutrient and oxygen delivery, leading to enhanced necrosis. Consequently, cells in the border of necrotic-hypoxic regions evoke stimulation of angiogenesis and formation of highly permeable microvessels (16). The phenomenon of stress-hypoxia-induced angiogenesis at the vicinity of necrosis has been shown to occur in other types of tumors, and its mechanism via up-regulation of vascular endothelial growth factor has been demonstrated previously (22, 23).

In summary, we have found a congruency between the kinetic fitting analysis and the 3TP analysis of contrast-enhanced MRI, both based on the same physiological model (12). We have used the 3TP method to evaluate response of breast cancer to hormonal therapy. The interpretation of the 3TP images indicated marked changes in the extent and distribution of the vascular permeability and in the EVF, as a result of tamoxifen treatment.

References
Response of MCF7 human breast cancer to tamoxifen: evaluation by the three-time-point, contrast-enhanced magnetic resonance imaging method.


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