

# Investigation of Breast Cancer Microstructure and Microvasculature from Time-Dependent DWI and CEST in Correlation with Histological Biomarkers

Yuko Someya (✉ [someyayuko@kuhp.kyoto-u.ac.jp](mailto:someyayuko@kuhp.kyoto-u.ac.jp))

Kyoto University

**Mami Iima**

Kyoto University

**Hirohiko Imai**

Kyoto University

**Akihiko Yoshizawa**

Kyoto University Hospital

**Masako Kataoka**

Kyoto University

**Hiroyoshi Isoda**

Kyoto University

**Denis Le Bihan**

NeuroSpin/Joliot, CEA-Saclay Center, Paris-Saclay University

**Yuji Nakamoto**

Kyoto University

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## Research Article

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# Abstract

We investigated the associations of time-dependent DWI, non-Gaussian DWI and CEST parameters with histological biomarkers in a breast cancer xenograft model. 22 xenograft mice (7 MCF-7 and 15 MDA-MB-231) were scanned at 2 diffusion times ( $T_d = 2.5/5$  ms) with 11 b values (0–600 s/mm<sup>2</sup>), and 9/27.6 ms with 17 b values (0–3000 s/mm<sup>2</sup>). The apparent diffusion coefficient,  $ADC_{0-600}$ , shifted ADC,  $sADC_{200-1500}$  and the ADC change with  $T_d$ , as well as IVIM and non-Gaussian diffusion parameters were estimated. CEST images were acquired and the amide proton transfer signal intensity (APT SI) were measured. The  $sADC_{200-1500}$  change and combined ADC change ( $ADC_{0-600, 2.5\text{ ms}}$  with  $sADC_{200-1500, 27.6\text{ ms}}$ ) was significantly larger for MCF-7 group, and the combined ADC change was positively correlated with  $Ki67_{max}$  and APT SI.  $ADC_0$  decreased significantly in MDA-MB-231 group and K increased significantly with  $T_d$  in MCF-7 group. APT SI and cellular area had a moderately strong positive correlation in MDA-MB-231 and MCF-7 tumors combined, and there was a positive correlation in MDA-MB-231 tumors. There was a significant negative correlation between APT SI and the Ki-67-positive ratio in MDA-MB-231 tumors and when combined with MCF-7 tumors. The associations of combined ADC change and APT SI with Ki-67 parameters indicate that the  $T_d$ -dependent DW and CEST parameters are useful to predict the histological markers of breast cancers.

## Introduction

MR imaging can provide anatomical and functional information about both normal tissues and diseased ones, such as tumors<sup>1</sup>. Among the quantitative MRI methods available, diffusion-weighted imaging (DWI) and chemical exchange saturation transfer (CEST) imaging might be useful to reveal the microstructure and microvasculature within cancer lesions<sup>1-3</sup>. Although histological assessment from biopsy remains the basis for determining treatment, many cancers are very heterogeneous, and biopsy often fails to capture their heterogeneity, as the amount of the tumor obtained with biopsy is very limited. Whole tumor mapping using MRI might provide additional information about the correlations between heterogeneity and histology, which could be useful for evaluating the extent of tumors in the preoperative state or for decision making about neoadjuvant cancer treatment. Regarding breast tumors, which are the leading cause of cancer death among women worldwide<sup>4</sup>, imaging biomarkers with greater specificity to tumor biology like proliferation activity have been desired to facilitate selection of treatment options<sup>5</sup>.

Apparent diffusion coefficient (ADC) values obtained from DW images have been widely used in oncologic imaging. In various tumors, ADC has been recognized as a sensitive surrogate for elevated cellularity, contributing to reduced motion of water<sup>6</sup>. However, the association of ADC values with tumor proliferative markers, such as Ki-67 expression, in breast cancer is still controversial<sup>7,8</sup>. Lately, the diffusion time ( $T_d$ ) has been identified as an important acquisition parameter that can influence ADC values<sup>9</sup>. Recent investigations have emphasized the importance of  $T_d$  because diffusion is hindered by many obstacles in biological tissues, decreasing the level of water molecule displacement compared with

free diffusion paths. The ADC value decreases when  $T_d$  gets longer, as water molecules have a greater probability of interacting with microscopic tissue features such as cell membranes and fibrous tissues. Several studies have reported that various types of malignant tumors revealed the decrease in ADC values with long  $T_d$  <sup>10-13</sup>.

There have been explorations of several non-Gaussian DWI and intravoxel incoherent motion (IVIM) parameters that are useful for the differentiation of malignant and benign breast lesions <sup>14-17</sup>. Non-Gaussian DWI, which can be investigated using multiple  $b$  values (including high  $b$  values), is more sensitive to water diffusion hindrance by tissue constitutive elements (e.g., cell membranes, fibers). In contrast, IVIM MR imaging reflects both the diffusion of the water molecules and the perfusion (i.e., pseudo-random flow of blood in capillary networks). Perfusion-related parameters, including the pseudo-diffusion coefficient ( $D^*$ ) and the perfusion fraction ( $f$ ), may predict tumor microvasculature. Regarding tumor angiogenesis, a correlation between microvessel density (investigated by CD31 staining) and IVIM parameters has been observed in various types of cancers <sup>18-20</sup>.

In this study we have also used a “shifted ADC” (sADC) which is calculated using 2 key  $b$  values (here 200 and 1500  $s/mm^2$ ) to enhance a combined sensitivity to both diffusion and non-Gaussian diffusion, as well as IVIM effects <sup>21</sup>.

CEST has been increasingly considered as a promising molecular imaging method that can evaluate the presence of low concentrations of molecules other than water <sup>22</sup>. CEST might provide complementary molecular information related to cancer metabolism <sup>23</sup>, and its utility has been explored in oncology, including breast cancer xenograft <sup>24,25</sup> and human breast cancer <sup>26-28</sup> studies. Amide protons from mobile proteins and peptides are typical endogenous CEST agents. Several investigations have reported that amide proton transfer (APT) imaging might be useful to predict tumor proliferation indices such as the Ki-67 index <sup>28-31</sup>.

Our purpose was to investigate the associations of time-dependent DWI, non-Gaussian DWI, IVIM, and CEST parameters obtained from 7T MRI of a murine breast xenograft model with histological biomarkers.

## Results

### Time-dependent DWI and CEST

The DWI parameters, which were dependent on  $T_d$  in the two tumor xenograft models, are provided in Table 1. The  $T_d$  dependence of the DWI parameters is shown in Figs. 1 and 2. Both  $ADC_{0-600}$  and  $sADC_{200-1500}$  exhibited a tendency to decrease when the  $T_d$  value increased (Fig. 1).

Table 1

MRI parameters for the MCF-7 and MDA-MB-231 groups.  $P < 0.05^*$ ,  $P < 0.01^{**}$ , considered as statistically significant

	$T_d$	MCF-7	MDA-MB-231	<i>P</i> -value
<b>DWI parameters</b>		N = 7	N = 15	
ADC <sub>0-600</sub> (*10 <sup>-3</sup> mm <sup>2</sup> /s)	2.5ms	1.10 ± 0.09	0.99 ± 0.08	<b>0.011*</b>
	5ms	0.93 ± 0.17	0.84 ± 0.06	0.091
	9ms	0.81 ± 0.21	0.79 ± 0.23	0.448
	27.6ms	0.65 ± 0.16	0.68 ± 0.23	0.837
ADC <sub>0-600</sub> change (%) between 2.5ms and 27.6ms		41.5 ± 12.7	31.3 ± 21.0	0.210
sADC <sub>200-1500</sub> (*10 <sup>-3</sup> mm <sup>2</sup> /s)	9ms	0.68 ± 0.09	0.62 ± 0.10	0.066
	27.6ms	0.48 ± 0.07	0.53 ± 0.12	0.490
sADC change (%) between 9ms and 27.6ms		29.6 ± 4.4	14.6 ± 15.8	<b>0.002**</b>
combined ADC change between ADC <sub>2.5ms</sub> and sADC <sub>27.6ms</sub> (%)		56.7 ± 4.4	46.4 ± 12.4	<b>0.026*</b>
<b>Non-Gaussian diffusion parameters</b>		N = 7	N = 15	
ADC <sub>0</sub> (*10 <sup>-3</sup> mm <sup>2</sup> /s)	9ms	0.84 ± 0.15	0.82 ± 0.17	0.546
	27.6ms	0.72 ± 0.19	0.68 ± 0.16	0.546
K	9ms	0.47 ± 0.27	0.76 ± 0.28	<b>0.005**</b>
	27.6ms	0.85 ± 0.15	0.92 ± 0.22	0.332
<b>IVIM parameters</b>		N = 15	N = 7	
<i>f</i> (%)	9ms	6.7 ± 5.6	5.8 ± 4.8	0.582
	27.6ms	7.9 ± 5.6	5.2 ± 5.6	0.267

	$T_d$	MCF-7	MDA-MB-231	<i>P</i> -value
$D^*$ (*10 <sup>-3</sup> mm <sup>2</sup> /s)	9ms	7.11 ± 3.59	6.10 ± 1.89	0.891
	27.6ms	5.70 ± 2.02	5.73 ± 2.37	0.989
<b>CEST parameters</b>		N = 5	N = 12	
APT SI (%)		6.48 ± 3.59	3.58 ± 1.91	0.113

The  $ADC_{0-600}$  values at  $T_d$  values from 2.5 ms to 9 ms, and  $sADC_{200-1500}$  values at  $T_d$  values of 9 ms were lower in the MDA-MB-231 group than the MCF-7 group, whereas the  $ADC_{0-600}$  and  $sADC_{200-1500}$  values at  $T_d = 27.6$  ms were compatible for the two groups. The  $sADC_{200-1500}$  change and the combined ADC change in the MCF-7 group were significantly larger than those in the MDA-MB-231 group ( $29.6 \pm 4.4\%$  vs.  $14.6 \pm 15.8\%$ ,  $P < 0.01$ ;  $56.7 \pm 4.4\%$  vs.  $46.4 \pm 12.4\%$ ,  $P < 0.05$ ; respectively). The  $ADC_{0-600}$  change had no significant difference between two xenograft model ( $41.5 \pm 12.7\%$  in the MCF-7 group vs.  $31.3 \pm 21.0\%$  in the MDA-MB-231 group;  $P = 0.21$ ).

The MDA-MB-231 group had a significantly higher K value than the MCF-7 group at  $T_d = 9$  ms ( $0.47 \pm 0.27$  vs.  $0.76 \pm 0.28$ ,  $P < 0.01$ ). However, K had no significant  $T_d$  dependence in either xenograft model. There was no significant difference in any other IVIM or non-Gaussian diffusion parameters between the two different types of tumor xenografts (Table 1). In the MDA-MB-231 group, the  $ADC_0$  value was significantly lower with increased  $T_d$  ( $P < 0.01$ ) and, in the MCF-7 group, the  $ADC_0$  value insignificantly decreased with increased  $T_d$  ( $P = 0.16$ ). The K value was significantly higher in the MCF-7 group ( $P < 0.05$ ) with increased  $T_d$ , and K in the MDA-MB-231 group insignificantly increased ( $P = 0.06$ ) with increased  $T_d$  (Fig. 2).

We conducted CEST imaging on 5 and 12 mice with MCF-7 and MDA-MB-231 tumors, respectively. There was no significant difference of APT SI between the two different types of tumor xenografts, as shown in Table 1.

APT SI had a significant positive correlation with combined ADC change ( $R = 0.57$ ,  $P < 0.05$ ) in mixing MDA-MB-231 and MCF-7 tumors (Fig. 3). The  $sADC_{200-1500}$  change had a tendency to correlate positively with APT SI, but not significantly ( $R = 0.48$ ,  $P = 0.052$ ). The  $ADC_{0-600}$  change had no correlation with APT SI ( $R = 0.20$ ,  $P = 0.45$ ).

## Association between MRI (DWI and CEST) parameters and histological biomarkers

The association between DWI parameters and histological biomarkers is summarized in Fig. 4. In the MCF-7 group, the combined ADC change had a positive correlation with Ki-67 LI, but the correlation was

not significant ( $R = 0.64$ ,  $P = 0.14$ , Fig. 4A). There was a positive correlation between the combined ADC change and  $Ki-67_{max}$  ( $R = 0.82$ ,  $P < 0.05$ , Fig. 4B). In the MDA-MB-231 group, the Ki-67 index revealed abnormally high expression and had a small standard deviation (SD). Hence, there was no correlation between the combined ADC change and the Ki-67 tumor proliferation index. Each ADC value using a different  $T_d$  had no significant correlation with the Ki-67 index. There was no significant association between MVD and  $f$  with  $T_d$  values of either 9 or 27.6 ms. However, at  $T_d = 27.6$  ms, a weak trend was observed between them ( $R = 0.63$ ,  $P = 0.13$  for MCF-7 and  $R = 0.31$ ,  $P = 0.26$  for MDA-MB-231).

The relationships between CEST parameters and histological biomarkers are summarized in Fig. 5. A positive correlation between APT SI and cellular area was shown in MDA-MB-231 tumors and in MDA-MB-231 and MCF-7 tumors combined ( $R = 0.54$ ,  $P = 0.07$  and  $R = 0.60$ ,  $P = 0.01$ , respectively). APT SI had no significant correlation with the Ki-67 index. APT SI had a significant negative correlation with the Ki-67-positive ratio in MDA-MB-231 tumors and in MDA-MB-231 and MCF-7 tumors combined ( $R = -0.59$ ,  $P = 0.04$  and  $R = -0.59$ ,  $P = 0.01$ , respectively).

Figures 6 and 7 show representative whole tumor histological images (H&E staining, cellular area map, Ki-67-positive cell map, and CD31 staining), MR images (T2WI, sADC map ( $T_d = 9$  ms), sADC map ( $T_d = 27.6$  ms), the sADC change map, and APT imaging map), and H&E staining on high power magnification. Large sADC change and high APT SI were evident in the viable tumor regions.

## Histopathologic features

The histopathological features of MDA-MB-231 and MCF-7 xenograft models are summarized in Supplementary Table S1 online. The cell size of the MDA-MB-231 tumors was smaller than that of the MCF-7 group ( $12.2 \pm 0.3 \mu\text{m}$  vs.  $15.2 \pm 0.7 \mu\text{m}$ ,  $P < 0.05$ ), and the cellular area of the MDA-MB-231 group was lower than that of the MCF-7 group ( $51.3\% \pm 22.7\%$  vs.  $71.8\% \pm 12.2\%$ ,  $P < 0.05$ ). In immunohistochemical evaluation, Ki-67 LI,  $Ki-67_{max}$ , and MVD were significantly higher in the MDA-MB-231 group than the MCF-7 group ( $75.5\% \pm 3.3\%$  vs.  $58.8\% \pm 11.5\%$ ,  $80.7\% \pm 3.9\%$  vs.  $63.9\% \pm 10.0\%$ , and  $44.1 \pm 15.9$  vs.  $23.5 \pm 6.8$ , respectively;  $P < 0.01$ ). The Ki-67-positive ratio, Ki-67 positive area divided by cellular area, and indices using whole-slide imaging were also significantly higher in the MDA-MB-231 group than the MCF-7 group ( $60.2\% \pm 20.4\%$  vs.  $41.5\% \pm 12.5\%$ ,  $P < 0.01$ ). A strong positive correlation between cellular area and Ki-67 positive area was found in the MDA-MB-231 group ( $R = 0.74$ ,  $P < 0.01$ ), but not in the MCF-7 group ( $R = 0.41$ ,  $P = 0.32$ ).

## Discussion

In this study, we investigated the association between DW parameters measured at different  $T_d$  values (including ADC, sADC, IVIM, and non-Gaussian DWI), CEST parameters, and histopathological features, especially tumor proliferative markers, using murine xenograft models of human breast cancer. We found that the combined ADC change using different  $T_d$  values had a significant positive correlation with APT SI. Previous studies have reported that time-dependent DWI or CEST imaging might provide information

about tumor microstructure indicating tumor proliferation in oncologic imaging<sup>28-32</sup>. Comparisons of APT SI and IVIM parameters in cervical cancers<sup>33</sup>, gliomas<sup>34</sup>, and hepatocellular carcinomas<sup>35</sup>, as well as comparisons of APT SI and diffusion kurtosis imaging in rectal carcinomas<sup>36</sup>, have been reported. However, the relationship between time-dependent DWI parameters, as tissue level markers, and CEST parameters, as molecular level markers, is still unknown, to the best of our knowledge.

We used two different human breast cancer cell lines: an estrogen-dependent tumor cell line (MCF-7) and an aggressive, triple negative breast tumor cell line (MDA-MB-231). The MDA-MB-231 tumor cells showed smaller cell sizes, larger necrotic areas, higher Ki-67 expression, and greater MVD, suggesting histopathological malignancy, which is consistent with previous studies<sup>37-41</sup>. Although these histopathological features appear very different, single measurements of diffusion, IVIM, and CEST quantitative parameters were not useful to distinguish between the two different tumor types. Only ADC values at the shortest  $T_d$  (2.5 ms using OGSE), sADC change and combined ADC change showed statistically significant differentiation between the two xenograft groups.

The observed decrease in ADC and sADC values with  $T_d$  in breast tumors was in agreement with the literature, as were the results of our previous investigation<sup>11,32,42</sup>. This confirms the hypothesis that diffusion hindrance in tumors increases when the  $T_d$  value increases, as more molecules might hit the increased number of obstacles such as cell membranes. Our results also confirm that ADC value is a  $T_d$ -dependent quantitative marker and thus that standardization of acquisition parameters in diffusion MRI is important, especially for multicenter studies<sup>43</sup>. Indeed, although the  $T_d$  settings available on clinical MRI scanners used to be similar, the availability of new, stronger gradient hardware now allows access to a broad range of  $T_d$  values while maintaining high b values. In general, the previous studies implicated that the ADC values obtained at very short  $T_d$  might diminish the contrast for differentiation between lesion types (i.e., malignant vs. benign lesions) because of a reduced sensitivity to diffusion hindrance<sup>11,32</sup>. Our study revealed that only ADC values at the shortest  $T_d$  (2.5 ms using OGSE) can distinguish between two xenograft models. The ADC results obtained at short  $T_d$  values might capture the movement of water molecules in all tissue compartments on the cellular and subcellular scales, whereas the ADC acquired at longer  $T_d$  values might increase the diffusion MRI signal's emphasis on the extracellular space. In this study, we used a tumor model that mixed cellular and necrotic components in various proportions and ROIs on the whole tumor. The variation of ADC values is particularly large in the MDA-MB-231 group with a large necrotic area. As the  $T_d$  is increased, the water molecule displacement becomes larger, and then ADC values at long  $T_d$  values may indicate an increase in SD, reflecting the heterogeneity of the tissue. We considered that the ADC values at  $T_d = 2.5$ ms, which captures the diffusion motion on a small scale, captures the small cell size (many cell membranes) of the MDA-MB-231 tumors, resulting in significantly lower ADC values in the MDA-MB-231 groups. These results also suggest that setting an appropriate  $T_d$  is troubling, however the comparison of ADC values between short and long  $T_d$  values might be simple useful parameters to reveal additional information about micro tissue structure, such as cell size, cell density, or cell membranes<sup>44</sup>.

A previous study that compared between benign and malignant tumors revealed greater ADC change in malignant than benign lesions<sup>32,42</sup>. In this study, we assumed that more malignant pathological features like active proliferation, tight structure, and small cell spacing hinder the diffusion of water molecules inside tissue and ultimately decrease the ADC value in MDA-MB-231 tumors. However, in this study, the more aggressive MDA-MB-231 tumors had less sADC change and combined ADC change than the MCF-7 tumors. The presence of a larger necrotic area because of MDA-MB-231's aggressiveness is assumed to be one of the causes of the lower sADC change and combined ADC change observed in those tumors. There should be almost no structure in necrotic areas, which means that diffusion should be less hindered and almost free. Indeed, the areas with small sADC changes corresponded to the necrotic areas in some tumors, as shown in Figs. 6 and 7. Investigation of  $T_d$ 's dependence on tumor characterization provides new insight to supplement standard ADC values, and maps of ADC changes were useful for highlighting tumor features such as heterogeneity, as shown in this study. Furthermore, our result that the ADC change, calculated using standard ADC values with b values of 0 and 600 s/mm<sup>2</sup>, were not able to distinguish between two different xenografts is an interesting point. The shifted ADC change, excluding the signal of b value of 0 and using relatively high b value, is suggested to reflect non-Gaussian diffusion effect and to be more useful to estimate the tumor microstructure, because the behavior of DW signals is non-Gaussian, especially in highly restricted tissues such as cancers with cell proliferation.

A strong positive correlation between  $Ki-67_{max}$  and combined ADC change was found in MCF-7 tumors ( $R = 0.82, P < 0.05$ ). Cells with high Ki-67 expression tend to have high cellular proliferation and tight tissue structure, which hinders diffusion of water molecules inside and between these cells. No statistically significant correlation between tumor proliferative markers and combined ADC changes was found in MDA-MB-231 tumors. The MDA-MB-231 cell line has been known to express very high levels of Ki-67: almost 100% of these cells stain positively for Ki-67<sup>37</sup>, in contrast to human breast cancer. We also found in our study that MDA-MB-231 cell lines had abnormal expression and a small standard deviation of Ki-67 LI ( $75.5\% \pm 3.3\%$ ), so caution is required when extrapolating results obtained with xenograft models to clinical cancer when considering the association between the Ki-67 marker and MR parameters. Indeed, our single measurement of the ADC value for each  $T_d$  value had no correlation with any tumor proliferative biomarkers. It has often been found that ADC values are linked to both cell density and proliferation rate (measured by Ki-67) in tumors<sup>45,46</sup>. However, ADC can also be affected by other tissue characteristics, such as vascularity and extracellular water diffusivity. The rate of change of ADC with  $T_d$  appears to be more promising than the ADC value itself for highlighting differences in the tumor environment. Furthermore, the differences between ADC values observed in various tissue types at different  $T_d$  values might reflect functional differences in diffusion hindrance (e.g., related to membrane permeability to water<sup>44</sup>) in addition to microstructure (related to cell geometry)<sup>47,48</sup>.

The tendencies of decreased  $ADC_0$  and increased K with  $T_d$  were stronger at  $T_d = 9$  ms than 27.6 ms in both xenograft models, further confirming the increase in diffusion hindrance. No significant differences of  $f$ ,  $D^*$ , or the time dependence of IVIM parameters were found between the two tumor groups. The differences between various IVIM models (the exponential model and sinc model)<sup>49,50</sup> might explain



some time-dependence of IVIM parameters. In this study, we used an exponential model that is valid as long as  $T_d$  is sufficiently long, but there is a general consensus that this model might not be accurate at small time scales, explaining the moderate correlation between  $f$  and MVD found only at long  $T_d$  (27.6 ms) and not at short  $T_d$ . IVIM MRI might provide quantitative parameters of angiogenesis, like  $f$ , which should increase with the proliferation of neovascularity in some tumors. Tumor angiogenesis is essential for tumor growth and metastasis, and the establishment of a noninvasive imaging tool is essential to obtain the degree of angiogenesis noninvasively. As  $f$  is expected to be useful as an indicator of the degree of angiogenesis, further investigation with a larger group of subjects is desirable.

There was no significant association between APT SI and Ki-67 LI in our study. One reason for this result might be the abnormally high rate of Ki-67-positive cells, which was found especially in the MDA-MB-231 model. Second, Ki-67 LI was calculated as the mean positive cell count in the cellular area, and it therefore could not be directly compared with the mean APT SI value, which was calculated from the ROI covering the whole tumor including necrosis. Although several previous studies have reported positive correlations between APT SI and Ki-67 LI in gliomas<sup>29</sup>, meningiomas<sup>30</sup>, and rectal carcinomas<sup>31</sup>, no correlation was found in rectal adenocarcinomas<sup>51</sup>. There have been few studies on the utility of APT imaging in the differential diagnosis of different breast tumors<sup>26,28</sup>, and further investigation will be required to determine whether there is an association between APT SI and Ki-67 LI.

The positive correlation found between cellular area and APT SI (as shown in Fig. 5) is consistent with the general view that mobile protons in the cytoplasm (and mobile proteins and peptides in tumors<sup>52</sup>) are the major sources of APT signals. Malignant tumors with high Ki-67 expression have higher cellularity and increased cytosolic protein in the cytoplasm, resulting in higher APT SI<sup>3</sup>. In contrast, in our study, a negative association was observed between the Ki-67 positive rate and APT SI (Fig. 5). The motivations for establishing the Ki-67 positive ratio (i.e. the ratio of Ki-67-positive area divided by the cellular area) were to reduce bias in ROI selection and to analyze the value equivalent to the conventional Ki-67LI in ROIs covering the whole tumor using Halo. Because Ki-67 is usually localized to the nucleus when detected by immunohistochemistry (an unusual cell membrane and cytoplasmic pattern of Ki-67 reactivity has been rarely described<sup>53</sup>), a large Ki-67-positive ratio means a small cytoplasmic area. In Jiang's report<sup>54</sup>, primary central nervous system lymphomas (PCNSL) had significantly lower APT SI than high-grade gliomas, and this finding was hypothetically attributed to higher nucleus–cytoplasm ratios (N/C) in PCNSLs, which would reduce the amount of cytoplasmic protein. No significant correlation between APT and the Ki-67 positive ratio was observed in MCF-7 cells, which have a small N/C and varying Ki-67 positive cell rates. The decreased APT SI signal in MDA-MB-231 tumors may have been caused by high N/C, but this interpretation is not straightforward.

A moderate positive correlation between combined ADC change and APT SI was found in our study, suggesting a link between tumor microstructure at the cellular scale and molecular status. Both parameters have been reported to reflect the tumor proliferative potential, as shown by Ki-67 expression, reflecting pathological malignancy, although diffusion MRI and CEST rely on totally different

mechanisms. The sADC change maps and APT maps (Figs. 5 and 6) look very similar, including a signal decrease in the necrotic area. Because the ADC values using 2 b values did not correlate significantly with APT SI, ADC change using multiple  $T_d$  values may be useful as additional information to conventional DW parameters for distinguishing and evaluating two different types of malignancies, as in the present study.

The limitations of this study include that the number of mice was relatively small and that xenograft models were used. Xenograft models provide a whole organism environment for tumor growth, but using immunocompromised mice and differences associated with the implantation site might be limitations. For example, the observed aberrant expression of Ki-67 is different from that in human breast cancer. Thus, we cannot directly extrapolate our results to humans. Further investigation is warranted to verify the associations of DWI and CEST parameters with histopathological biomarkers. Then, in this study, we are not able to compare simply between  $ADC_{0-600}$  change and  $sADC_{200-1500}$  change because the using  $T_d$  to calculate these change values were different. The maximal b value in OGSE was  $600 \text{ s/mm}^2$  for maintaining the same TE as those in PGSE, hence the  $sADC_{200-1500}$  change (high key b value of  $1500 \text{ s/mm}^2$ ) was calculated at  $T_d = 9 \text{ ms}$  and  $27.6 \text{ ms}$ .

In conclusion, the associations of combined ADC change with different  $T_d$  values, API SI, and histopathological parameters such as Ki-67 expression and cellularity were found. These results indicate that the  $T_d$ -dependent DWI and CEST parameters are useful for investigating the microstructure of breast cancers. Furthermore, diffusion parameter values, especially sADC, are dependent on  $T_d$ , confirming that this  $T_d$  dependence is strongly associated with the degree of diffusion hindrance, which increases with the  $T_d$ . These results indicate the importance of reporting  $T_d$  in future studies.

## Methods

### Cell culture and animal experiments

All animal experiments were performed in accordance with national guidelines and the Regulation on Animal Experimentation at Kyoto University and in compliance with the ARRIVE guidelines, and approved by the Kyoto University Animal Care Committee. Two human breast cancer cell lines (MCF-7 and MDA-MB-231) purchased from American Type Culture Collection, Manassas VA, USA were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin solution at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .

MCF-7 and MDA-MB-231 cells ( $1 \times 10^6/\mu\text{L}$ ) were subcutaneously inoculated into the right hindlimbs of 7 and 15 immunodeficient mice (ICR nu/nu 6–8-week-old females; Charles River Laboratories Japan, Yokohama, Japan), respectively. A  $17\beta$ -estradiol pellet (0.18 mg, 60-day release; Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously into the neck to promote optimal tumor growth of the estrogen receptor-positive MCF-7 cells. During all procedures, the mice were anesthetized

with 2% isoflurane (Wako Pure Chemical Industries, Osaka, Japan). Xenografts were allowed to grow for 7–11 weeks to develop tumors of suitable size.

## MRI of mice

We imaged 22 xenograft mice (7 MCF-7 and 15 MDA-MB-231) on a 7T MRI scanner (Bruker Biospec, Ettlingen, Germany) using a  $^1\text{H}$  quadrature transmit/receive volume coil. The mice were anesthetized with 1–3% isoflurane in air and were kept still inside the magnet using ear bars and a bite bar connected to a nose cone. Respiration and rectal temperature were continuously monitored using an MR-compatible monitoring system (Model 1025, SA Instruments, Inc., Stony Brook, NY, USA). The rectal temperature was maintained at 34–37°C.

## Acquisition of DW images and parameters

The single-shot echo-planar imaging (SE-EPI) acquisition parameters were: TR = 2500 ms, TE = 57 ms, 8 averages, 4 segments, resolution =  $250 \times 250 \mu\text{m}^2$ , matrix size =  $100 \times 100$ , field of view =  $25 \times 25 \text{mm}^2$ , and slice thickness = 1.5 mm. DWI were obtained using oscillating gradient spin-echo (OGSE)<sup>55</sup> and classical pulsed gradient spin-echo (PGSE) sequences. Four different  $T_d$  values were used: 2.5 ms and 5 ms in OGSE and 9 ms and 27.6 ms in PGSE. We used 11 and 17 b values for OGSE ( $0\text{--}600 \text{s/mm}^2$ ) and PGSE ( $0\text{--}3000 \text{s/mm}^2$ ), respectively. The acquisition time for each  $T_d$  was 14.4 and 22.4 minutes for OGSE and PGSE, respectively.

A shifted ADC (sADC) value was calculated using b values of 200 and  $1500 \text{s/mm}^2$  as:

$$sADC = \ln S(Lb)/S(Hb)/(Hb - Lb) \quad [1]$$

where Lb and Hb are a “low key b value” and a “high key b value,” respectively<sup>21</sup>.

The  $ADC_{0-600}$  change,  $sADC_{200-1500}$  change and a combined ADC change between the different ADC and sADC values obtained at different diffusion times were calculated as:

$$ADC_{0-600} \text{ change } (\%) = (ADC_{t=2.5ms} - ADC_{t=27.6ms})/ADC_{t=2.5ms} \times 100 \quad [2]$$

$$sADC_{200-1500} \text{ change } (\%) = (sADC_{t=9ms} - sADC_{t=27.6ms})/sADC_{t=9ms} \times 100 \quad [3]$$

$$\text{combined ADC change } (\%) = (ADC_{t=2.5ms} - sADC_{t=27.6ms})/ADC_{t=2.5ms} \times 100 \quad [4]$$

The rationale for the combined ADC change was to optimize both effects of the diffusion time and non-Gaussian diffusion.

The signals acquired with each  $T_d$  at  $b > 500 \text{s/mm}^2$  (free from perfusion-related IVIM effects) were first fitted using the non-Gaussian diffusion kurtosis model<sup>56</sup>:

$$S(b) = [ [S_{diff} \exp[-bADC_0 + K(bADC_0)^2/6] ]^2 + NCF]^{1/2} \quad [5]$$

where  $S(b)$  is raw signal intensity,  $S_{diff}$  is the theoretical signal that would be obtained at  $b = 0$  s/mm<sup>2</sup> accounting for only the tissue diffusion component, and NCF (noise floor correction factor) characterizes the “intrinsic” Rician noise contribution observed at low signal intensities within amplitude-reconstructed MR images. The noise floor was estimated from the average image background noise across runs<sup>56</sup>.

As a second step, the fitted diffusion signal component was subtracted from the measured signal acquired with  $b < 500$  s/mm<sup>2</sup>, and the remaining signal was fitted using the perfusion-related IVIM model to obtain estimates of the flowing blood fraction  $f$  (which represents the microvascular flowing volume fraction) and the pseudo-diffusion  $D^*$  (which represents perfusion-related incoherent microcirculation):

$$S_{ivim}(b) = S_{oivim} \exp(-bD^*) \quad [6]$$

And

$$f = S_{oivim} / (S_{oivim} + S_{diff}) \quad [7]$$

where  $S_{ivim}$  is the raw signal intensity after the diffusion component has been removed, and  $S_{oivim}$  is the theoretical signal from perfusion-related IVIM at  $b = 0$  s/mm<sup>2</sup>. For the non-Gaussian and IVIM parameters, two  $T_d$  values (9 ms and 27.6 ms) from PGSE were used.

## Acquisition of CEST images and parameters

CEST slices were obtained at the region of maximum tumor width. CEST images were acquired using the rapid acquisition with relaxation enhancement (RARE) sequence with a single continuous wave saturation pre-pulse. The acquisition parameters were: TR = 5000 ms, effective TE = 12 ms, RARE factor = 16, a centric ordered phase encoding, matrix size = 96×96, field of view = 25×25 mm<sup>2</sup>, and slice thickness = 2 mm. The saturation parameters were: saturation time = 1 s, saturation RF power = 5.9 μT, saturation offset frequencies with respect to water resonance = 41, range = ± 5 ppm, and 0.25 ppm steps. The acquisition time for each saturation offset was 30 seconds, and the total acquisition time was 20.5 minutes. Reference images were also acquired at a saturation frequency of 40 ppm. The water frequency offset caused by B0 inhomogeneity was corrected using the water saturation shift referencing (WASSR) method<sup>57</sup>. The magnetization transfer ratio asymmetry ( $MTR_{asym}$ ) values were calculated as:

$$MTR_{asym}(\alpha ppm) = [S_{(-\alpha ppm)} - S_{(+\alpha ppm)}] / S_0 \quad [8]$$

where  $S_0$  and  $S(\pm\alpha ppm)$  are the MRI signals without and with a saturation RF pulse, respectively. The APT signal intensity was defined as the  $MTR_{asym}$  value at 3.5 ppm.

## Image and histopathological analysis

Regions of interest (ROIs) were drawn on T2WI as a reference containing the whole tumor region using a freehand tool, and the ROIs were then copied to the corresponding DWI and APT images to obtain their signal intensity. The mean values of the diffusion parameters were retrieved for each ROI. MRI data analysis was performed using code developed in MATLAB (Mathworks, Natick, MA, USA).

The tumor specimens were formalin-fixed and paraffin-embedded, and then sectioned for hematoxylin and eosin (H&E) stains, which were performed in a routine manner. Immunohistochemical staining for Ki-67 (1:100 dilution, Leica Biosystems, NCL-L-Ki67-MM1, Novocostra, Newcastle, UK) and CD31 (1:100 dilution, Cell Signaling Technology, #77699, MA, USA) was performed using the Ventana Discovery XT Autostainer (Tucson, Arizona, USA). With regard to Ki-67 staining, the Ki-67 proliferation index was assessed using only the areas of tissue containing the highest concentrations of cells. The Ki-67 proliferation index was evaluated using the percentage of immunoreactive cells in the area of observation under high power magnification (HPF,  $\times 200$ ). Five values were averaged to determine the final Ki-67 labeling index (Ki-67 LI), which were calculated in a routine manner via microscopic estimation, and the Ki-67<sub>max</sub> was determined as the maximum of five values. With regard to CD31 staining, the number of all vessels in 10 HPF ( $\times 40$ ), was added after pre-scanning at low magnification ( $\times 10$ ) to choose the area with the impression of the highest vessel profile number ("hot spot") in the solid area. The result, microvessel density (MVD), was calculated as the mean count of vessels in the HPF viewing range.

The tumor specimens were scanned, converted to whole-slide imaging, and analyzed using automated histology image analysis software (Halo, Indica labs, Corrales, NM, USA). Four areas (cellular, necrotic, muscle, and stromal areas) were separated and quantified using a pattern recognition algorithm. Cellular area was defined as a percentage of the total area. The Ki-67-positive cell map over the whole tumor was also generated using Halo, and the Ki-67-positive area was defined as a percentage of the total area. The Ki-67 positive ratio was defined as the Ki-67-positive area as a percentage of the cellular area. Cell size was defined as the mean diameter of 10 cells observed in a square ROI of size  $200 \times 200 \mu\text{m}$ . Three square ROIs were drawn in representative viable areas of the tumor, and cell size was averaged over these ROIs.

## Statistical analysis

The estimated parameter values were expressed as mean  $\pm$  SD. Between-group differences were compared using the Wilcoxon rank sum test. Spearman's rank correlation coefficients were used to evaluate the correlations between each parameters. *P* values of less than 0.05 were considered statistically significant. All statistical analysis was performed using Medcalc (version 11.3.2.0, Mariakerke, Belgium) and R (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria) statistical software.

## Declarations

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## Author contributions statement

Y.S. and M.I. conceived the study and conducted the experiments. Y.S., M.I. Hirohiko Imai and D.L.B. analyzed the MRI data. Histopathological evaluation were performed by Y.S., M.I., and A.Y. Y.S., M.I., Hirohiko Imai, A.Y., M.K., Hiroyoshi Isoda, D.L.B., Y.N contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

## Additional information

The authors declare no competing interests.

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## Figures

Figure 1

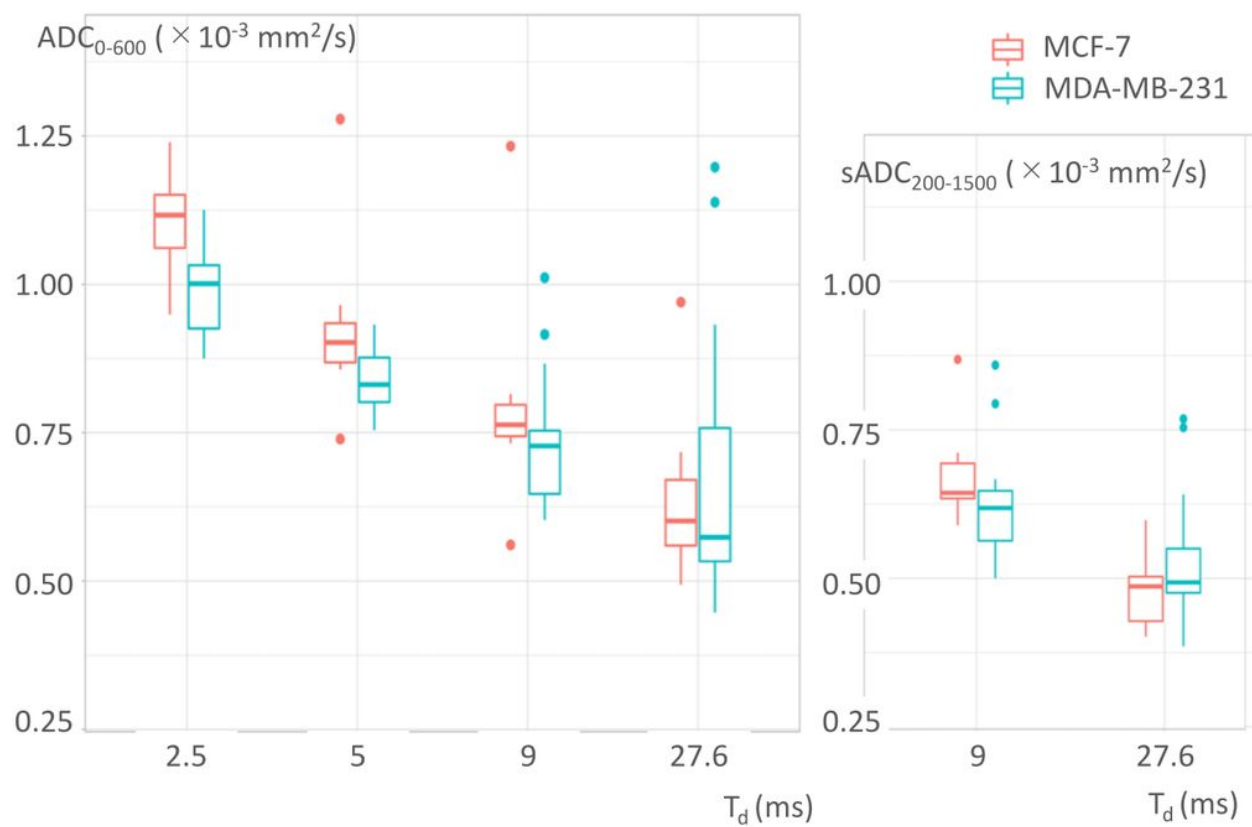


Figure 1

Box-whisker plots of ADC<sub>0-600</sub> and shifted ADC<sub>200-1500</sub> (sADC<sub>200-1500</sub>) against diffusion time.

Figure 2

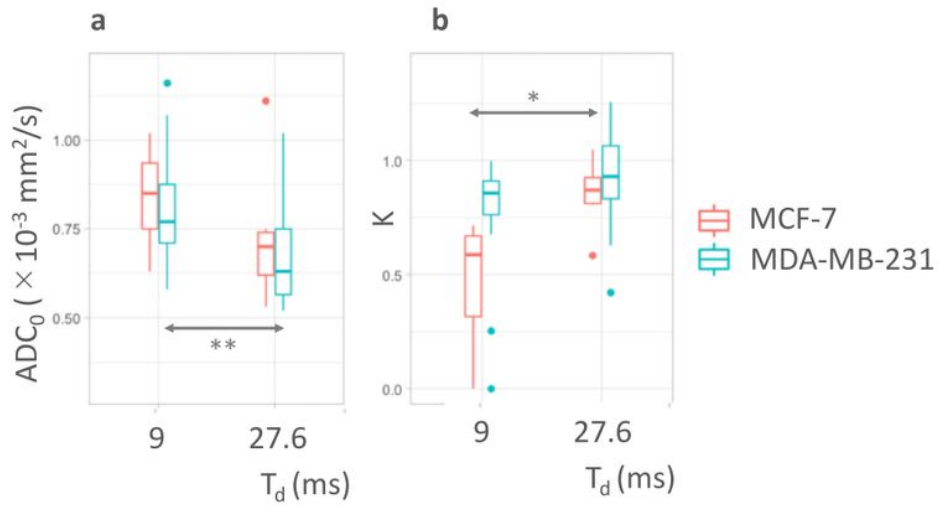


Figure 2

Box-whisker plots of (A) ADC<sub>0</sub>, and (B) K parameters against diffusion time. \* P < 0.05, \*\* P < 0.01. ADC<sub>0</sub> in the MDA-MB-231 group decreased significantly (P < 0.01), and ADC<sub>0</sub> in MCF-7 group decreased (P = 0.16), with increased diffusion time. K in the MCF-7 group increased significantly (P < 0.05) and K in the MDA-MB-231 group increased (P = 0.06) with increased diffusion time.

Figure 3

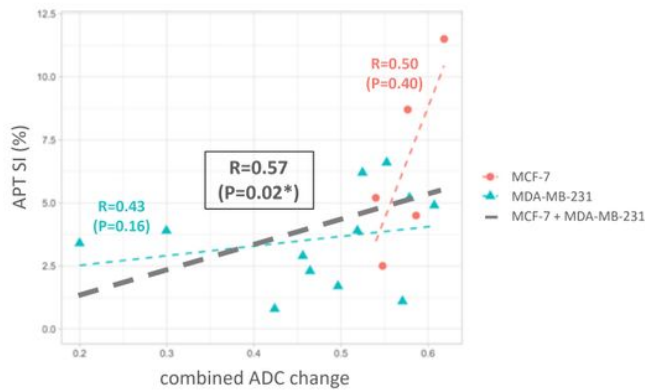


Figure 3

Scatter plots of combined ADC change against APT SI. The Spearman's rank correlation coefficients R, the corresponding P values in each tumors are shown in the figure. The black dashed line indicates the relationship between combined ADC change and APT SI in the entire tumors (mixing MCF-7 and MDA-MB-231 groups).

Figure 4

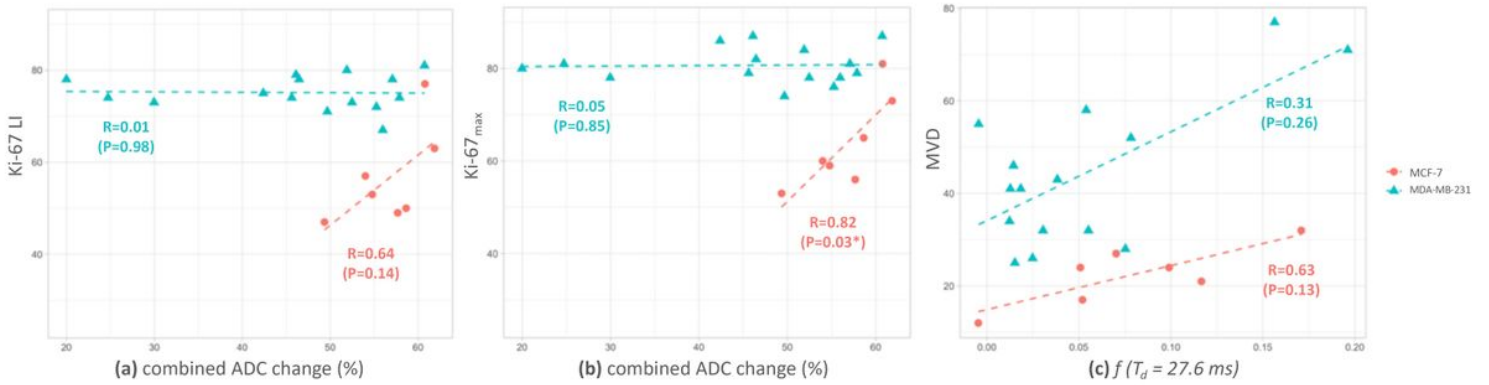


Figure 4

Scatter plots of (a) combined ADC change against Ki-67 LI, (b) combined ADC change against Ki-67<sub>max</sub>, and (c)  $f(T_d = 27.6 \text{ ms})$  against MVD. The Ki-67 indices in the MDA-MB-231 tumors revealed abnormally high expression, as shown in Figures 4a and 4b.

Figure 5

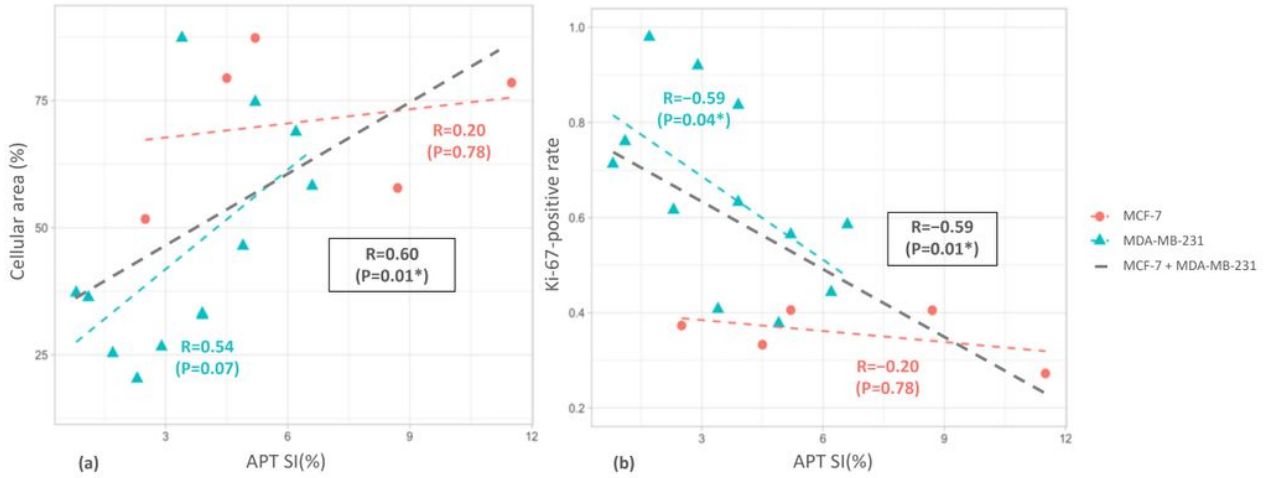


Figure 5

Scatter plots of (a) APT SI against Cellular area, (b) APT SI against Ki-67-positive ratio. The Spearman's rank correlation coefficients  $R$ , the corresponding  $P$  values in each tumor are shown in the figure. The black dashed line indicates the relationship between APT SI and histological biomarkers in the entire tumors (mixing MCF-7 and MDA-MB-231 groups).

Figure 6

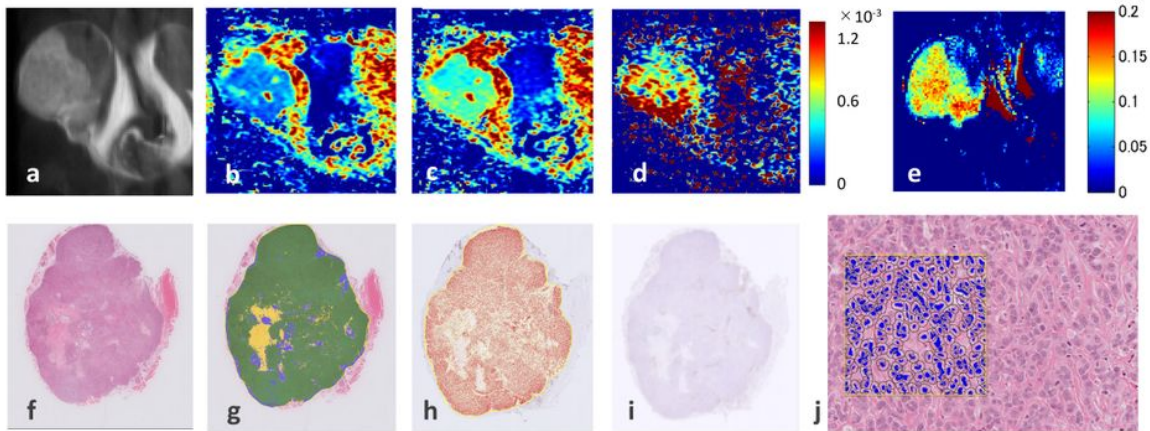


Figure 6

Representative case of MCF-7 tumor. MR images: (a) T2WI, (b) sADC map (diffusion time,  $T_d = 9$  ms), (c) sADC map ( $T_d = 27.6$  ms), (d) sADC change map, (e) APT imaging map, and whole tumor histological images: (f) H&E staining, (g) cellular area map, (h) Ki-67-positive cell map, (i) CD31 staining. (j) H&E staining on high power magnification ( $\times 400$ ). Yellow line squares with 200  $\mu\text{m}$  sides were drawn on Figure 6j. The decrease of ADC with  $T_d$  is evident. The dorsal part of the tumor shows high sADC change and APT SI.

Figure 7

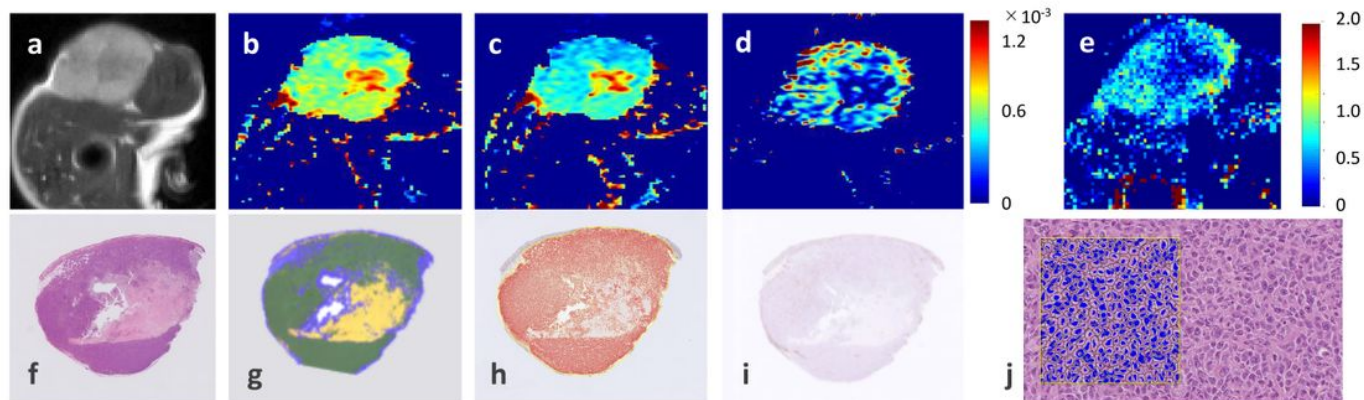


Figure 7

Representative case of MDA-MB-231 tumor. MR images: (a) T2WI, (b) sADC map (diffusion time,  $T_d = 9\text{ms}$ ), (c) sADC map ( $T_d = 27.6\text{ms}$ ), (d) sADC change map, (e) APT imaging map, and whole tumor histological images: (f) H&E staining, (g) cellular area map, (h) Ki-67-positive cell map, (i) CD31 staining. (j) H&E staining on high power magnification ( $\times 400$ ). Yellow line squares with  $200\ \mu\text{m}$  sides were drawn on Figure 7j. The decrease of ADC with  $T_d$  is evident. Central necrosis is apparent in regions with low sADC change and APT SI.

## Supplementary Files

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