Original Article

Blood Oxygenation Level-dependent Magnetic Resonance Imaging of Breast Cancer: Correlation with Carbonic Anhydrase IX and Vascular Endothelial Growth Factor

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Abstract

Background: Blood oxygenation level-dependent magnetic resonance imaging (BOLD-MRI) is a functional MRI technique which involves using the paramagnetic properties of deoxyhemoglobin to image the local tissue oxygen concentration. The purpose of this study was to investigate whether BOLD-MRI could evaluate hypoxia and angiogenesis of breast invasive ductal carcinoma (IDC).
Methods: Ninety-eight female patients with IDC were retrospectively included in this research. All patients underwent breast BOLD-MRI at 3.0 T before surgery. R2* values of BOLD-MRI were measured. The expression of carbonic anhydrase IX (CA IX) and vascular endothelial growth factor (VEGF) was analyzed by immunohistochemistry. Spearman's correlation analysis was used to correlate R2* value with CA IX and VEGF levels.
Results: Heterogeneous intensity on BOLD-MRI images was the main finding of IDCs. The mean R2* value was 52.8 ± 18.6 Hz. The R2* values in patients with axillary lymph node metastasis were significantly higher than the R2* values in patients without axillary lymph node metastasis ($t = 2.882$, $P = 0.005$). R2* values increased with CA IX level and positively correlated with the level of CA IX ($r = 0.616$, $P < 0.001$); however, R2* value had no significantly correlation with the level of VEGF ($r = 0.110$, $P = 0.281$).
Conclusion: BOLD-MRI could noninvasively evaluate chronic hypoxia of IDC, but not angiogenesis.

Key words: Angiogenesis; Blood Oxygenation Level-dependent Magnetic Resonance Imaging; Carbonic Anhydrase IX; Hypoxia; Invasive Ductal Carcinoma; Vascular Endothelial Growth Factor

Introduction

Hypoxia and angiogenesis are the basic characters of breast cancer. Angiogenesis can induce capillary growth into the tumor which supplies required nutrients, leading to tumor expansion and metastases. The essential role of angiogenesis in tumor growth was first proposed by Judah Folkman, who described tumors as "hot and bloody.[1]" Hypoxic tumor tissue may cause resistance to radiation therapy and some forms of chemotherapy and has been shown to be associated with malignant progression, increased incidence of metastases, and poor prognosis.[2] Evaluation of hypoxia and angiogenesis in tumor will benefit most to hypoxia-modifying and anti-angiogenesis treatments.

Carbonic anhydrase IX (CA IX) is a membranously located metalloenzyme involved in pH homeostasis with the influence on regulation of cell proliferation, oncogenesis, and tumor progression. CA IX is an indicator of hypoxia in the tumor and its expression is associated with more aggressive forms of breast cancer.[3,4] Vascular endothelial growth factor (VEGF) has been demonstrated to be a major contributor to angiogenesis.[5] VEGF-targeting therapies have shown significant benefits and been successfully integrated in routine clinical practice for other types of cancer. CA IX and VEGF have been used as biomarker of hypoxia and

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angiogenesis. Currently, magnetic resonance imaging (MRI) is the best modality for assessing morphology and function of tumor.[6] Dynamic contrast-enhanced MRI (DCE-MRI) has been used to detection of angiogenesis and hypoxia of cancer and requires intravenous injection of an exogenous contrast agent such as gadolinium.[7,8] Blood oxygenation level-dependent (BOLD) MRI, first described by Ogawa and Lee,[9] is a functional MRI technique which involves using the paramagnetic properties of deoxyhemoglobin to image the local tissue oxygen concentration.[10,11] BOLD-MRI contrast is sensitive to pO_2 in tissues adjacent to perfused vessels, so we speculate that BOLD-MRI could noninvasively evaluate hypoxia and angiogenesis. The aim of this study was to investigate whether BOLD-MRI correlates with CA IX and VEGF in breast invasive ductal carcinoma (IDC).

Methods

Patients' selection

This study has been approved Ethics Committee of Beijing Chaoyang Hospital. Informed consent of each patient has been obtained, and the investigations have been conducted according to the *Declaration of Helsinki*. From January 2013 to July 2015, 98 women with breast IDCs were retrospectively included in this research. Breast MRI was obtained in all patients within one week before surgery. All included patients had no previous excisional biopsy or chemotherapy before MRI. Patients who did not undergo BOLD-MRI before surgery or were older than 75 years were excluded. The clinical information including patients' age and body mass index (BMI), tumor sizes, histological grade, and axillary lymph node metastases was referenced to the medical records.

Magnetic resonance imaging protocol

Breast MRI was performed at a 3.0 T clinical MRI system (Trio Tim, Siemens, Erlangen, Germany) using the phased array breast surface coil in the prone position. The MRI images were performed using the following sequences: The axial T2-weighted turbo spin-echo sequences and T1-weighted fast low-angle shot gradient-echo sequences, then the axial BOLD-MRI with a multiple gradient-recalled-echo sequence with repetition time/echo time of 124 ms/3.31–39.05 ms, 10 echo train length, field of view = 280 mm, matrix size = 256 mm × 256 mm, flip angle = 15°, voxel size = 1.5 mm × 1.1 mm × 5.0 mm, average = 4, slice thickness = 5.0 mm with a 1-mm slice gap, voxel size = 1.5 mm × 1.1 mm × 4.0 mm, and bandwidth = 260 Hz per pixel.

Magnetic resonance imaging evaluation

After imaging, breast BOLD-MR images were transferred to AW4.4 workstation (GE Healthcare, USA). R2* values of BOLD were analyzed using a FuncTool-R2 Star software (Advantage Workstation 4.4 GE Healthcare, Milwaukee, WI, USA). One radiologist with 10-year experience in breast MRI analyzed R2* value without knowing other clinical information. A region of interest with an area of 10 ± 4 mm^2 was placed in the lesions. To eliminate bias, the cystic or necrotic portions of the tumor causing T2 shine-through effect, shown as high-signal intensity on T2-weighted image, were excluded. The three measurements were averaged and used as the R2* values.

Immunohistochemistry

Immunohistochemical staining of CA IX and VEGF was, respectively, performed on 5 μm formalin-fixed, paraffin-embedded tissue sections. Antigen retrieval with Tris-EDTA (pH 9.0) in a pressure cooker was performed at 95°C for 3 min. Five percent hydrogen peroxide was used for 10 min to block the endogenous peroxidase. After blocking with 2% normal horse serum for 30 min, the sections were incubated with a rabbit polyclonal antibody anti-CA IX (Abcam, Cambridge, UK, 1:1000 dilution) or a rabbit polyclonal antibody anti-VEGF (Abcam, Cambridge, UK; 1:400 dilution) for 90 min at room temperature. And biotin/avidin system using the VECTASTAIN Elite ABC kit (Vector Laboratories, California, USA) was used for this immunoperoxidase procedure. Finally, diaminobenzidine tetrahydrochloride solution (Vector Laboratories, California, USA) was used as a substrate. According to the manufacturer's recommendations, nuclei were counterstained with hematoxylin. Known positive tissue specimens were used as positive controls for CA IX and VEGF (lung cancer). Negative control slides without primary antibodies were included for each staining. Scoring was performed by an expert with 11-year experience with breast disease who was blinded to other clinical information. The staining for CA IX and VEGF was scored in a semi-quantitative method[12] by estimating the percentage area of tumor cells that had been stained: negative for <1%, weakly positive for 1–10%, moderately positive for 10–50%, and strong positive for >50% immunostaining.

Statistical analysis

Analyses were performed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA). All data are expressed as means ± standard deviation (SD) unless otherwise specified. Normal distribution of R2* value was tested using the Kolmogorov-Smirnov test. ANOVA and least significant difference *t*-test were used to compare R2* values among different groups and Pearson or Spearman's correlation analysis was used to correlate R2* values with the CA IX and VEGF expression. Two-sided $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of patients

Ninety-eight female patients (age range, 28–74 years; mean age 51.2 ± 13.5 years; median, 52 years) with 98 lesions were included in this study. Axillary lymph node metastases were detected in 48 patients. The mean diameter of the lesions was 16.5 ± 7.2 mm, with a median of 18 mm (range, 8–33 mm).

Among 98 lesions, 56 lesions were ≤20 mm in size and 42 lesions were 20–50 mm. Histopathological analysis revealed that 4 lesions were in Grade I, 62 lesions were in Grade II, and 32 lesions in Grade III.

R2* value of blood oxygenation level-dependent magnetic resonance imaging

On BOLD-MRI, IDCs were heterogeneous intensity [Figures 1 and 2]. The mean R2* value of 98 lesions was 52.8 ± 18.6 Hz. Kolmogorov-Smirnov test ($Z = 0.093$, $P = 0.094$) suggests that the samples follow a normal distribution. There was no significant correlation between the R2* values and age ($r = 0.073$, $P = 0.478$). As shown in Table 1, there was no significant correlation between the R2* values, BMI, or histological grade. The R2* values of tumor with the size ≤20 mm were significantly less than the R2* values of tumor with the size >20 mm. And the R2* values in patients with axillary lymph node metastasis were significantly more than the R2* values in patients without axillary lymph node metastasis.

Blood oxygenation level-dependent magnetic resonance imaging and carbonic anhydrase IX expression

As demonstrated in Figures 1 and 2, CA IX was predominantly stained in the cytoplasm but slightly in the plasma membranes.[13,14] Immunostaining demonstrated negative CA IX expression in 12 lesions, weakly positive in 17 lesions, and moderately and strongly positive in 28 and 41 lesions. Table 2 demonstrates that there were significant differences in the R2* values with deferent CA IX levels ($F = 13.915$, $P < 0.001$). R2* values significantly increased with CA IX levels, but R2* values in negative-CA IX group is similar to R2* values in weakly positive group. Spearman correlation analysis showed the R2* values positively correlated with CA IX ($r = 0.696$, $P < 0.001$).

Blood oxygenation level-dependent magnetic resonance imaging and vascular endothelial growth factor expression

As shown in Figures 1 and 2, VEGF staining was predominantly in the cytoplasm. Immunostaining demonstrated 8 lesions in negative VEGF expression, 18 lesions in weakly positive expression, 34 and 38 lesions in moderately and strongly positive expression. Spearman correlation analysis suggested a weak positive correlation between VEGF and CA IX expression ($r = 0.291$, $P = 0.004$). ANOVA analysis [Table 3] revealed that there were not significant differences in the R2* values among four VEGF levels ($F = 1.895$, $P = 0.136$), but the R2* values in the moderately positive group were significantly higher than R2* values in the weakly positive group ($t = 2.407$, $P = 0.020$). Spearman correlation analysis showed that the R2* value did not significantly correlate with VEGF levels ($r = 0.110$, $P = 0.281$).

Discussion

In this study, we studied the correlation of BOLD-MRI with CA IX and VEGF in breast IDC and we found R2* values of BOLD-MRI positively correlated with CA IX but not with VEGF.

As the intrinsic hypoxia markers, CA IX is quite important in cancer progression and its abundance in tumors has prognostic and predictive value.[3,4,11] Molecular imaging

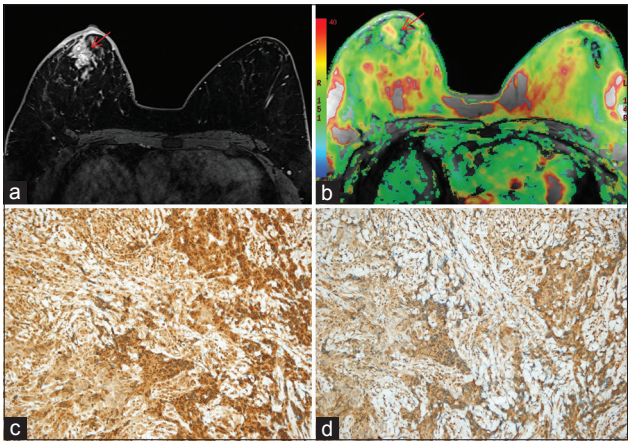

Figure 1: Magnetic resonance imaging and immunostaining in a 48-year-old female with right invasive ductal carcinoma in Grade III. (a) Contrasted-magnetic resonance imaging showing an irregular mass with the heterogeneous enhancement in the right breast (red arrow). (b) Blood oxygenation level-dependent magnetic resonance imaging showing the mass with heterogeneous intensity (red arrow) with a mean value of 65.3 Hz. (c) Immunostaining of carbonic anhydrase IX showing the strong positive expression of carbonic anhydrase IX in invasive ductal carcinoma (original magnification ×200). (d) Immunostaining of vascular endothelial growth factor showing the moderate positive expression of vascular endothelial growth factor in invasive ductal carcinoma (original magnification ×200).

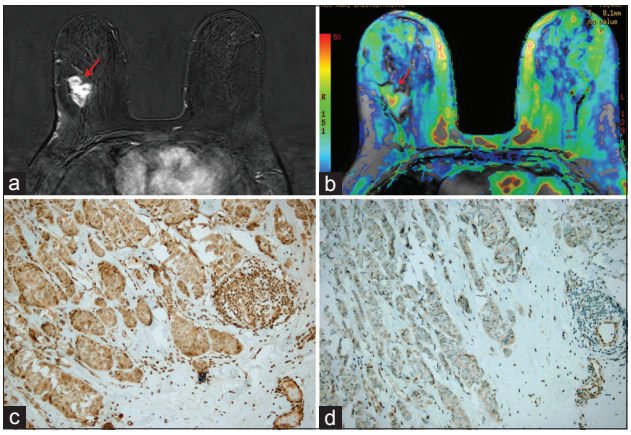

Figure 2: Magnetic resonance imaging and immunostaining in a 36-year-old female with right invasive ductal carcinoma in Grade II. (a) Contrasted-magnetic resonance imaging showing an irregular mass with the heterogeneous enhancement in right breast (red arrow). (b) Blood oxygenation level-dependent magnetic resonance imaging showing the mass with heterogeneous intensity (red arrow) with a mean value of 52.1 Hz. (c) Immunostaining of carbonic anhydrase IX showing the moderate positive expression of carbonic anhydrase IX in invasive ductal carcinoma (original magnification ×200). (d) Immunostaining of vascular endothelial growth factor showing the weak expression of vascular endothelial growth factor in invasive ductal carcinoma (original magnification ×200).

Table 1: Correlation between R^2* value and prognostic factors

Prognostic factors	*N*	R^2* (Hz)	*t*	*P*
BMI (kg/m²)				
≤24.0	59	52.7 ± 18.7	$F = 0.193$	0.793* ($t = 0.381$)
24.1–28.9	25	51.4 ± 19.8	($P = 0.825$)	0.538† ($t = -0.028$)
≥29.0	14	55.4 ± 17.1		0.630‡ ($t = 0.239$)
Tumour Size (mm)				
≤20.0	56	47.1 ± 18.1	−4.051	0.000
20.1–50.0	42	61.7 ± 16.0		
Histological grade				
I	4	49.7 ± 17.5		
II	62	55.1 ± 17.7	0.416	0.678§
III	32	53.4 ± 16.4		
Lymph node metastasis				
Positive	48	58.1 ± 16.7	2.882	0.005
Negative	50	47.6 ± 19.1		

BMI: ANOVA $F = 0.064$, $P = 0.940$; LSD-*t* test: *BMI ≤24.0 kg/m² and BMI 24.1–28.9 kg/m²;†BMI 24.1–28.9 kg/m² and BMI ≥29.0 kg/m²; ‡BMI ≤24.0 kg/m² and BMI ≥29.0 kg/m²; Histological grade: Mann–Whitney U test: §grade II and grade III.

Table 2: Correlation between R^2* value and Carbonic anhydrase IX expression

CA IX expression	*N*	R^2* value Mean ± Std (Hz)	*t* value (*P*)
Negative	12	37.4 ± 9.23	$t = -1.106$ ($P = 0.916$)*
Weakly positive	17	37.9 ± 15.7	$t = -4.814$ ($P < 0.001$)†
Moderately positive	28	54.5 ± 11.0	$t = -4.395$ ($P < 0.001$)‡
Strong positive	41	63.3 ± 19.2	$t = -3.967$ ($P < 0.001$)§
F (*P*)		13.915 ($P < 0.001$)	

R^2* value, LSD-*t* test: *Negative group and weakly positive group; †Weakly group and strong group, ‡Negative group and moderately positive group; §Negative group and strong positive group; Weakly positive group and moderately positive group: $t = -4.235$, $P<0.001$; moderately group and strong group: $t = -2.118$, $P = 0.038$.

Table 3: Correlation between R^2* value and Vascular endothelial growth factor expression

VEGF expression	*N*	R^2* value Mean ± Std (Hz)	*t* value (*P*)
Negative	8	56.1 ± 18.0	$t = 1.502$ ($P = 0.146$)*
Weakly positive	18	45.4 ± 18.9	$t = -1.882$ ($P = 0.065$)†
Moderately positive	34	56.4 ± 16.5	$t = -0.060$ ($P = 0.952$)‡
Strong positive	38	54.8 ± 19.8	$t = 0.162$ ($P = 0.872$)§
F (*P*)		1.895 (0.136)	

R^2* value, LSD-*t* test: *Negative group and weakly positive group; †Weakly group and strong group; ‡Negative group and moderately positive group; §Negative group and strong positive group; Weakly positive group and moderately positive group: $t = -2.407$, $P = 0.020$; moderately group and strong group: $t = 0.377$, $P = 0.707$.

with a fluorescent antibody targeting CA IX can successfully detect hypoxic ductal carcinoma *in situ* of the breast.[15] The primary source of contrast in BOLD-MRI is endogenous, paramagnetic deoxyhemoglobin which increases the MR transverse relaxation rate (R2* value) of water in the blood and surrounding tissues, thus BOLD-MRI contrast is sensitive to pO_2 in tissues adjacent to perfused vessels.[16] Rakow-Penner *et al*.[17] suggest that BOLD contrast can consistently be detected in the healthy breast using a robust protocol including a single-shot fast spin-echo (SSFSE) sequence and a gradient-echo (GRE) pulse sequence. We found IDC on BOLD-MRI had a relative heterogeneous R2* intensity. We speculated that the heterogeneous R2* intensity was caused by the heterogeneous distribution of oxygen pressure and neovessels in IDC.[2] Our results showed the mean R2*values in IDC were similar with the baseline R2* value in chemically induced rat mammary tumors.[18] Liu *et al*.[19] demonstrated that R2* value correlated with hypoxia inducible factor-1α. And our results showed the R2* values in IDC increased with the CA IX level and positively correlated with the CA IX levels. These suggest that BOLD-MRI can be used to predicting the hypoxic status of IDC. Moreover, we noticed there was no significantly difference of R2* values in CA IX-negative group and CA IX-weakly positive group. We speculated that this may be because of small included numbers in each group or pO_2 between these two groups was similar. IDC often was heterogeneous intensity on BOLD-MRI; however, relatively higher intensity in normal breast parenchyma and the much lower intensity in the fatty tissue of breast were often observed. This can be explained by the normal breast parenchyma maybe having better tissue oxygenation than the tumor. However, it is unknown whether the low signal of breast fatty tissue is caused by the low blood supply and low pO_2.

Recently, there have been increased interests in using MRI to assess tumor angiogenesis. Zhang *et al*.[20] suggest that DCE-MRI parameters help predict rectal carcinoma angiogenesis measured by VEGF expression. However, Su *et al*.[8] did not found any correlation of DCE-MRI parameters with microvessel density and VEGF for assessment of angiogenesis in breast cancer. Our results demonstrated a positive correlation between VEGF and CA IX expression in breast IDC and the R2* value in the moderately positive group is significantly higher than R2* value in the weakly positive group, but no significant correlation was found in R2* value and VEGF level. This may be because the multi-echo GRE sequence of BOLD-MRI decouples the effect of flow perfusion from deoxyhemoglobin.[2] Since R2* values correlate with CA IX, but not with EGFR, we think that BOLD-MRI is more sensitive to diffusion-related (chronic) hypoxia than to perfusion-related (acute) hypoxia.

Breast IDC is a heterogeneous tumor that has variable behavior, outcome, and treatment response. Prognostic

factors are quite important information for the evaluation and treatment to IDC. Yanai *et al*.[21,22] suggested that higher BMI might influence aggressive tumor characteristics in women with breast cancer. However, no correlation between BMI and R2* value was found in our patients. Tumor size is a valuable prognostic factor of breast cancer. Razek *et al*.[23] showed ADC of IDC negatively correlated with tumor size. Our results showed that compared with IDC in size ≤20 mm, R2* values in IDC in size >20 mm significantly increased. We thought this was because the tumor with the larger size may be the more hypoxia. The histological grade is useful in clinical practice to assess the behavior of neoplasia. McPhail and Robinson[18] showed that there was no significant correlation between tumor grade and tumor R2* value in rat mammary tumors. In our research, R2* value in Grade II was similar to Grade III. The presence of metastasis in axillary lymph node is the most important single predictor of long-term survival in patients with breast cancer. Razek *et al*.[23] showed lower ADC values correlated with positive lymph node metastasis. We found that R2* value in patients with axillary lymph node metastasis is significantly higher than R2* without axillary lymph node metastasis. This predictive model may decrease the need for surgical staging of axilla in patients with breast cancer.

There are several limitations in this research. Only IDC is included in this research; the relation between R2* value and hypoxia, angiogenesis in breast cancer with other histological types such as lobular carcinoma, requires a further study. We did not measure pO_2 with the Eppendorf polarographic electrode, so the correlation of R2* values and pO_2 is not clear. In the case of hypoxia, it is important to remember that if the tumor stays hypoxic throughout the course of a treatment, in the follow-up study, we will observe the prognostic potential of R2* with respect to outcome of neoadjuvant chemotherapy in patients with breast cancer.

In conclusion, for IDC, R2* values of BOLD-MRI were related to CA IX levels, but not VEGF. BOLD-MRI could detect chronic hypoxia in breast IDC.

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Conflicts of interest

There are no conflicts of interest.

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