ICAM-1 as a molecular target for triple negative breast cancer

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Triple negative breast cancers (TNBCs) have a high mortality rate owing to aggressive proliferation and metastasis and a lack of effective therapeutic options. Herein, we describe the overexpression of intercellular adhesion molecule-1 (ICAM-1) in human TNBC cell lines and tissues, and demonstrate that ICAM-1 is a potential molecular target and biomarker for TNBC therapy and diagnosis. We synthesized ICAM-1 antibody-conjugated iron oxide nanoparticles (ICAM-IONPs) as a magnetic resonance imaging (MRI) probe to evaluate tumor targeting. Quantitative analysis of ICAM-1 surface expression predicted the targeting capability of ICAM-IONPs to TNBC cells. MRI of the TNBC xenograft tumor after systemic administration of ICAM-IONPs, coupled with iron quantification and histology, demonstrated a significant and sustained MRI contrast enhancement and probe accumulation in tumors with ICAM-1 overexpression relative to control. Identification of ICAM-1 as a TNBC target and biomarker may lead to the development of a new strategy and platform for addressing a critical gap in TNBC patient care.

Significance

Triple negative breast cancers (TNBCs) have a poor prognosis (5-y survival of 74.5%) among all breast cancer patients (5-y survival of greater than 95%) because of the aggressiveness of the disease and the lack of targeted therapeutics. We show that intercellular adhesion molecule-1 (ICAM-1) is differentially expressed in human TNBC tumor tissues by immunohistochemistry and in human TNBC cell lines via quantification of gene and protein expression. Iron oxide nanoparticles functionalized with ICAM-1 antibody (ICAM-IONP) were synthesized as MRI probes. An in vivo signal enhancement of 2.6-fold for ICAM-IONPs was measured relative to controls, demonstrating that ICAM-1 is a potential diagnostic and therapeutic target for TNBC treatment.


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specificity, ICAM-1 was chosen for further evaluation as a target for TNBC.

Although its role as a TNBC target and biomarker has yet to be fully investigated, ICAM-1 expression has been shown to associate with aggressive tumor phenotypes in breast cancer, prostate cancer, and myeloma (20, 21). Evidence suggests that ICAM-1 triggers multiple cell-signaling pathways that promote cancer cell proliferation, migration, resistance to apoptosis, and development of cell adhesion molecule-induced drug resistance (20, 21).

To validate that ICAM-1 is highly overexpressed in TNBC tumors, immunohistochemistry was conducted by using 149 human breast tumor tissues representing different ER/PR/HER2 status along with 144 human normal tissues of 20 different organs. ICAM-1 staining in TNBC tissues was stronger and present in more cells (Fig. 1 B and E) compared with non-TNBC tissues (Fig. 1 C and F) and normal breast mammary epithelium (Fig. 1 D and G). TNBC exhibited a significant increase in ICAM-1 expression compared with various other subtypes of breast cancers and normal epithelium (Fig. 1 H). ICAM-1 expression is absent in normal human breast, cerebrum, colon, esophagus, kidney, liver, ovary, pancreas, prostate, rectum, skin, small intestine, and uterine cervix, but positive ICAM-1 staining was observed in normal spleen, lung, lymph node, thymus, testis, and bone (Fig. S2). These findings correlate with the Human Protein Atlas database (www.proteinatlas.org), revealing that the ICAM-1 expression in normal organs is substantially less than that of EGFR, another generally accepted TNBC target. The finding that ICAM-1 is overexpressed in 26 human TNBC tissues provides clinical evidence supporting ICAM-1 as a potential molecular target for TNBC.

We further characterized and quantified ICAM-1 gene and surface protein expression in nine different human cell lines: three TNBC, four non-TNBC, and two nonneoplastic lines (Fig. 1 S).
Because TNBCs are more prevalent in women under 50 y of age, African American women, and individuals carrying the BRCA1 gene mutation (1), we analyzed ICAM-1 levels in seven breast cancer cell lines—derived from patients of African American and Caucasian origin, of ages spanning 32 to 69 y, and with wild-type and mutant BRCA1 gene status—relative to nonneoplastic, human mammary epithelial cells MCF10A and AG11132 (as shown in Fig. 1). TNBC cells MDA-MB-231, MDA-MB-436, and MDA-MB-157 exhibited 13.9-, 11.6-, and 9.9-fold higher ICAM-1 gene expression, respectively, than MCF10A (even higher fold relative to AG11132). In non-TNBC cells, MCF7 and MDA-MB-361 showed elevated ICAM-1 gene expression relative to nonneoplastic cells, but at markedly lower levels than TNBC cells.

Consistent with ICAM-1 gene expression levels, TNBC cells exhibited between 8- and 25-fold higher ICAM-1 surface protein levels than non-TNBCs and normal cells (Fig. 1). Compared with the surface protein density of HER2, a clinical target and biomarker for breast cancer, the ICAM-1 surface protein density (751,000–2,350,000 molecules/cell) on TNBC cells was comparable to the HER2 surface density (875,000–5,020,000 molecules/cell) measured on HER2-positive breast cancer cells (MDA-MB-361 and SKBR3). Immunofluorescent staining of ICAM-1 overexpression in TNBC cells revealed greater ICAM-1 surface staining on TNBCs (Fig. 1 J–L) relative to non-TNBCs (Fig. 1 M–P) and nonneoplastic cells (Fig. 1 Q and R).

Furthermore, ICAM-1 was localized largely on the TNBC cell membranes, suggesting that it might be recognized and bound by targeted therapeutic agents. Based on the above results, we concluded that ICAM-1 expression is not ubiquitous in all breast cancers. ICAM-1 is overexpressed on the surface of TNBC cells and thus may be used as a target and biomarker for TNBC-targeted therapy.

Characterizations of TNBC-Targeted Iron Oxide Nanoparticles. To test ICAM-1 targeting for TNBC, we synthesized a TNBC-targeted MRI probe using magnetic iron oxide nanoparticles (IONPs) conjugated with ICAM-1 antibodies (ICAM-IONPs). ICAM-1 antibodies were conjugated covalently to casein-coated IONPs via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) chemistry (see schematic illustration in Fig. 2A) (22). Similarly, casein-coated IONPs were conjugated with Hereceptin (humanized anti-HER2 antibody; HER2-IONPs) or nonspecific IgG (IGG-IONPs) as controls for evaluation of targeting specificity, efficiency, and delivery. The morphology and monodispersity of ICAM-IONPs were examined by transmission electron microscopy (TEM; Fig. 2B) and dynamic light scattering (DLS; Fig. 2Q). ICAM-IONPs, with a core diameter of 15 nm, had a mean hydrodynamic radius of 36.6 ± 5.6 nm and a zeta potential of −41.4 ± 3.2 mV. The antibody density for ICAM-IONP, IGG-IONP, and HER2-IONP was determined experimentally using FITC-labeled antibodies as ligands (Fig. 2Q). Approximately two or three antibody molecules were conjugated to each particle. The ICAM-IONPs obtained showed no cytotoxicity (Fig. 2 N–P).

Effective targeting of TNBC cells via the ICAM-1 antibody first was evaluated in vitro by the binding and uptake of FITC-labeled ICAM-IONPs, IGG-IONPs, and HER2-IONPs. Our normalized fluorescent intensity data demonstrated that TNBC cells exhibited 2.4- to 4-fold greater binding to ICAM-IONPs than IGG-IONPs or HER2-IONPs because of the abundance of ICAM-1 expression (Fig. 2C). Quantitative analysis of ICAM-1 surface protein expression was directly correlated with increased binding of ICAM-IONPs. In comparison, HER2-IONPs showed positive targeting to HER2-positive cells but failed to target TNBC cells because of their HER2 deficiency. Using a Prussian blue staining assay to examine the presence of iron oxide, we further confirmed the strong and specific binding of ICAM-IONPs to TNBC cells (Fig. 2 E–G and Fig. S3) compared with minimal binding to non-TNBC (Fig. 2 H–K) and nonneoplastic cell lines (Fig. 2 L and M). We observed analogous patterns of binding between ICAM-IONPs and HER2-IONPs to both ICAM-1- and HER2-overexpressing cell lines, respectively. Blocking of ICAM-1 with free ICAM-1 antibody inhibited ICAM-1 binding and uptake by TNBC to levels commensurate with IGG-IONPs (Fig. 2D). These results indicate that ICAM-IONPs exhibit ICAM-1 targeting activity and specificity.

TNBC-Targeted MRI of TNBC Xenograft Tumor with ICAM-IONPs. We then examined the ability of ICAM-IONPs for targeted imaging
of TNBC tumors in vivo by MRI using a xenograft TNBC mouse model. MDA-MB-231 cells were implanted s.c. in immunodeficient athymic nude mice. MRI was performed on three groups of tumor-bearing mice injected i.v. with IGG-IONP, HER2-IONP, or ICAM-IONP when tumors reached 1 cm³ in volume. Each group was scanned before injection of the imaging probes (pre-injection) and 24 h and 48 h post injection with a set of MRI sequences, including T₁, T₂-weighted spin echo imaging, and T₂ relaxometry. The T₂-weighted images presented in Fig. 3A show decreased signals in the regions of the tumor as the result of enhanced T₂ contrast from uptake of IONP probes in tumors. Quantification of MRI signals in three groups demonstrated a 10% (IGG-IONP), 17% (HER2-IONP), and 26% (ICAM-IONP) signal drop at 24 h after administration of the probes, which lasted at least 48 h (Fig. 3B). ICAM-IONPs significantly improved MRI contrast by actively targeting the TNBC tumor via ICAM-1 binding. It is worth noting that HER2-targeted MRI probes were reported to change the MRI signal by 18–46% in HER2-positive breast tumors (23, 24), comparable to the ICAM-1–targeted MRI probe demonstrated in TNBC tumors in the current study.

The biodistribution and tumor accumulation of MRI probes were evaluated by quantifying iron in collected organs and tissue. Fig. 3C shows comparative iron accumulation in seven organs harvested from mice at 48 h after a single tail vein administration of IGG-IONPs, HER2-IONPs, and ICAM-IONPs. Correlating with the in vivo MRI results, the iron accumulation of ICAM-IONPs in TNBC tumors was 3.7- and 2.1-fold higher than that of IGG-IONPs and HER2-IONPs with reference to untreated tumors, respectively (Fig. 3C, Inset). Histological analysis was performed to further confirm the targeting of ICAM-IONPs to the tumor and the observed MRI contrast change in vivo (Fig. 3D). TNBC tumor sections were stained with ICAM-1 antibody, HER2 antibody, hematoxylin and eosin (H&E), and Prussian blue. Consistent with the MRI findings, tumors from mice receiving ICAM-IONPs showed a high level of ICAM-1 expression and strong Prussian blue staining of IONPs. In contrast, Prussian blue staining was low in tumors receiving IGG-IONPs or HER2-IONPs. Low HER2 surface expression in TNBC tumors did not result in significant HER2-IONP accumulation. Thus, results from the in vivo MRI experiments suggest that the uptake of ICAM-IONPs is driven by ICAM-1 expression on TNBCs.

Discussion

It is noteworthy that our discovery of ICAM-1 as a TNBC target and biomarker also reveals promising functions of this well-characterized receptor, which may be explored for clinical applications. ICAM-1 plays an important role in inflammation. Logically, anti-ICAM-1–targeted interventions were developed for the treatment of chronic inflammatory disorders (25, 26). The role of ICAM-1 in oncology also has been under intense investigation. ICAM-1 up-regulation is observed in several types of cancers associated with advanced disease, poor survival, and resistance to chemotherapy (20, 21). Treatment with a human ICAM-1 antibody has demonstrated potent macrophage-dependent anti-myeloma activity in vivo (27). ICAM-1 cross-linking leads to phosphorylation of proteins, cytoskeletal modifications, and gene regulation governing cell shape, recruitment, and migration (28).

Taken together, our finding that ICAM-1 is a promising TNBC target and biomarker may lead to an effective ICAM-1 targeting strategy for imaging and treatment of TNBC. Previous studies in wound healing, rheumatoid arthritis, and acute stroke demonstrated that enlimomab (anti-ICAM-1 antibody) was well tolerated by different patient groups, indicating that it may be safe and well tolerated in humans (29–31). Although the ICAM-1 antibody did not affect TNBC cell proliferation in vitro (Fig. S4), we observed that the ICAM-1 antibody significantly reduced TNBC cell migration (Fig. 3E and F). Greenwood et al. (32) reported that an ICAM-1 antibody blockade resulted in ICAM-1 molecules lacking cytoplasmic tails that could not activate Rho proteins. Similar antitumor activity of the ICAM-1 antibody or siRNA was observed in several human cancers (20, 21, 27).

In summary, our results demonstrate the identification of ICAM-1 as an efficient TNBC molecular target based on the in vitro evaluation of its TNBC-specific molecular profile and preclinical in vivo ICAM-1–targeted molecular MRI in a TNBC tumor model. The findings provide a rationale for further preclinical and clinical evaluation and development of ICAM-1–targeted treatments for TNBC.

Materials and Methods

Complete details of materials are provided in SI Materials and Methods.

PCR Array. The human signaling PathwayFinder RT² Profiler PCR Array was used to screen possible TNBC targets in MDA-MB-231, MCF7, and MCF10A cells. First, each cell line was incubated at 5 × 10⁵ cells per well in a six-well cell culture plate overnight. One microgram RNA of each cell line was

Fig. 3. In vivo MR detection of TNBC using ICAM-1 targeting MRI probes. (A) Color maps of T₂-weighted MR images of a mouse implanted with the TNBC cell line MDA-MB-231 at different time points (pre, 24 h, and 48 h) after injection of IGG-IONP, or HER2-IONP, or ICAM-IONP. (B) Quantification of the MRI signal enhanced by IGG-IONP, HER2-IONP, and ICAM-IONP at 24 h and 48 h. Significant R₂ signal changes were observed with time after ICAM-IONP treatment. *P < 0.05, **P < 0.01. (C) Whole-body distribution of iron accumulation of IGG-IONP, HER2-IONP, and ICAM-IONP in liver (L), spleen (S), kidney (K), heart (H), lung (LU), muscle (M), brain (B), and tumor (T). (D) Histology for MDA-MB-231 tumor accumulation of IGG-IONP, or HER2-IONP, or ICAM-IONP. Tumors were sectioned and stained with H&E, Prussian blue agent, ICAM-1 antibody, and HER2 antibody. In Prussian blue staining, the sections in blue suggest areas of IONP localization. The strongest blue staining is present in the ICAM-IONP group (bottom row, second column).

Scale bar represents 50 μm. (E) Representative micrographs depicting three TNBC cells (MDA-MB-231, MDA-MB-436, and MDA-MB-157) incubated with nonspecific IgG or ICAM-1 antibody after transmigrating through 8-μm pores of a transwell membrane. Images taken were on the reverse side of the membrane facing the lower chamber. (F) ICAM-1 antibody reduces the migration of TNBC cells. Nonspecific IgG is used as control. Cells were treated with 10 μg/mL ICAM-1 antibody or nonspecific IgG. *P < 0.05, ***P < 0.001.
converted to cDNA using the RT² First Strand Kit according to the manufacturer's instructions. Diluted cDNA was added to the RT² SYBR Green/Fluorescein qPCR Mastermix. The human signaling pathwayFinder RT² Profiler PCR Array was loaded with 25 μL well of cDNA–Mastermix according to the PCR protocol provided by the manufacturer. Results were analyzed using the RT² Profiler PCR Array Data Analysis Template v3.0.

**Immunohistochemical Staining.** One hundred forty-nine cases of human breast cancer tissue and 144 cases of human normal tissue microarray samples were evaluated for ICAM-1 expression as described previously (33, 34). Immunohistochemical staining was performed by using paraffin-embedded human breast cancer tissue microarrays (BR15083, BR1505, and T088) and normal tissue microarrays (BN00011 and BN1002a). The individual tissue cores in the microarrays were scored by a surgical pathologist, with no knowledge of sample identity, for no staining (0), weak staining (1), moderate staining (2), or strong staining (3). Photomicrographs were taken on an Olympus BX41 microscope by using an Olympus Q-Color5 digital camera (Olympus America Inc.).

**Cell Culture.** Three human TNBC cell lines (MDA-MB-231, MDA-MB-436, and MDA-MB-157), four human non-TNBC cell lines (MCF7, HCC1500, SKBR3, and MDA-MB-361), and two nonneoplastic mammary epithelial cell lines (AG11132 and MCF10A) were studied. MDA-MB-231, MDA-MB-436, MDA-MB-157, MCF7, HCC1500, SKBR3, MDA-MB-361, and MCF10A were available through the American Type Culture Collection; AG11132 was obtained from Coriell Institute. MDA-MB-157, MDA-MB-436, MDA-MB-361, and MDA-MB-11132 were cultured in DMEM, and HCC1500 was cultured in Roswell Park Memorial Institute (RPMI)-1640, SKBR3 in McCoy-5A, AG11132 in Mammary Epithelial Cell Basal Medium, and MCF10A in DMEM/F12 (1:1) medium, with all recommended supplements, respectively. All cells were maintained at 37 °C in a humidified incubator with 5% (vol/vol) CO₂.

**Quantification of ICAM-1 Gene Expression.** The gene expression level of ICAM-1, breast cancer ICAM-1 cell lines was characterized using quantitative RT-PCR (qRT-PCR). MDA-MB-231, MDA-MB-436, MDA-MB-157, MCF7, HCC1500, MDA-MB-361, SKBR3, AG11132, and MCF10A cells were cultured at 5 × 10⁶ cells per well in a six-well cell culture plate overnight. Cells were removed from each well by incubating with a trypsin/EDTA solution for 3 min. The cells were washed with PBS three times. RNA was extracted, purified using the Qiagen RNAeasy Mini Kit, and quantified using a SpectraMax 384 UV-visible spectrophotometer (Molecular Device). Reverse transcription was conducted using the Applied Biosystems TaqMan RT protocol. Detection and quantification of mRNA were performed with the StepOnePlus Real-Time PCR System (Applied Biosystems). All PCR samples were referenced to the gene expression of GAPDH.

**Quantification of ICAM-1 Surface Expression.** Breast cancer cell ICAM-1 surface protein expression was evaluated by a BD FACScalibur flow cytometer (BD Biosciences) as described previously (35, 36). Quantification of the ICAM-1 protein expression was evaluated by a BD FACSCalibur flow cytometer (BD Biosystems TaqMan RT protocol. Detection and quantification of mRNA were performed as described previously and quantified using a SpectraMax Plus 384 UV-visible spectrophotometer (Molecular Device). Reverse transcription was conducted using the Applied Biosystems TaqMan RT protocol. Detection and quantification of mRNA were performed with the StepOnePlus Real-Time PCR System (Applied Biosystems). All PCR samples were referenced to the gene expression of GAPDH.

**Characterization of ICAM-IONPs.** The morphology and size of ICAM-IONP nanoparticles were studied by using TEM (Hitachi H-7500; accelerating voltage, 75 kv). The TEM samples were prepared by dropping diluted nanoparticle solutions on a carbon-coated copper grid and were air dried. The hydrodynamic size and surface charge of IONPs in aqueous solution were evaluated using a DLS instrument (Malvern Zetasizer Nano S-90) equipped with a 22-mW He-Ne laser operating at 632.8 nm. FITC-conjugated ICAM-IONPs, or IGG-IONPs, or HER2-IONPs (ICAM-IONP-FITC, or IGG-IONP-FITC, or HER2-IONP-FITC) also were prepared to evaluate the antibody densities on obtained MRI probes. FITC-conjugated IgG, or HER2 antibody, or ICAM-1 antibody was used in the synthesis by replacing their non-fluorophore-tagged forms. Other conditions were kept the same during the synthesis. Antibody density on each type of MRI probe was calculated by using a FITC standard concentration curve.

**In Vivo Nanoparticle Probe Binding.** Quantitative analysis of ICAM-IONP-FITC binding to TNBCs (MDA-MB-231, MDA-MB-436, MDA-MB-157) was conducted using flow cytometry. Non-TNBCs (MCF7, HCC1500, MDA-MB-361, and SKBR3) and nonneoplastic cells (AG11132 and MCF10A) were selected as controls. Cells were cultured for 2 h at RT with 1 μg/mL FITC, and MDA-MB-361, and MDA-MB-11132 were cultured in DMEM, and HCC1500 was cultured in Roswell Park Memorial Institute (RPMI)-1640, SKBR3 in McCoy-5A, AG11132 in Mammary Epithelial Cell Basal Medium, and MCF10A in DMEM/F12 (1:1) medium, with all recommended supplements, respectively. All cells were maintained at 37 °C in a humidified incubator with 5% (vol/vol) CO₂.

**Prussian Blue Staining.** The nine cell lines (× 10⁶ cells) in Fig. 15 were seeded separately in a Lab-Tek II Chamber Slide System with 1 mL medium overnight at 37 °C. After medium was removed, cells were rinsed with PBS three times and fixed with 4% formaldehyde in PBS at RT for 10 min, followed by washing with PBS then soaking in working solution composed of 10% potassium ferrocyanide (II) trihydrate and 20% HCl solution (vol:vol 1:5) for 1 hour at 4 °C. After being washed with PBS, slices were counterstained with nuclear fast red for 5 min. Blue dots representing the remaining IONPs in organs were investigated with a Leica TCS SP5 confocal fluorescence microscope (Leica Microsystems).

**In Vivo MRI.** Animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Emory University. Breast tumors were established s.c. by injecting 5 × 10⁶ MDA-MB-361 cells into the fourth mammary fat pad of athymic nude mice (Charles River; n = 5 for each group). Tumors were developed for 5–7 wks until they were at least 1 cm³ in volume. In vivo MRI was performed on the tumor-bearing mice in three groups, which were injected i.v. with IGG-IONP, HER2-IONP, and ICAM-IONP (at the dosage of 20 mg Fe/kg mouse weight), respectively. Images were obtained at pre, 24 h, and 48 h post injection with a 3 T MRI scanner (Siemens Healthcare) with fast spin echo and multiecho time (TE) sequence for T₂-weighted MRI. The imaging parameters were as follows: repetition time (TR) of 3,200 ms, TE of 86 ms, 320 × 128 matrix, 120 × 60-mm² field of view, 150° flip angle, and 1.00-mm slice thickness for T₂-weighted imaging, TR of 3,710 ms and 20 different TEs, starting at 12 ms with 12-ms increments for multi-TE imaging. To quantify the signal intensity for tumor, regions of interest (ROIs) were drawn around the whole tumor at the same slice with the same imaging depth. The pixel intensity was calculated and normalized to the area of ROIs by ImageJ software.

**Histology.** The organs (liver, spleen, kidney, lung, heart, and muscle) and tumor samples were collected at 48 h after injection. The panthelothric colorimetric method was used to determine the iron concentration in organs after they were digested in concentrated HNO₃. Pathologies of MDA-MB-231 tumors with IGG-IONP, or HER2-IONP, or ICAM-IONP were investigated by H&E staining, Prussian blue staining, and ICAM-1 and HER2 immunohistological staining. All staining was performed for the tumor slices following the standard protocol.

**Statistical Analysis.** Quantitative data are presented as means ± SD. Differences were compared using an unpaired t test. P values <0.05 were considered statistically significant.
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