Treatment of Invasive Brain Tumors Using a Chain-like Nanoparticle

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Abstract

Glioblastoma multiforme is generally recalcitrant to current surgical and local radiotherapeutic approaches. Moreover, systemic chemotherapeutic approaches are impeded by the blood–tumor barrier. To circumvent limitations in the latter area, we developed a multicomponent, chain-like nanoparticle that can penetrate brain tumors, composed of three iron oxide nanospheres and one drug-loaded liposome linked chemically into a linear chain-like assembly. Unlike traditional small-molecule drugs or spherical nanotherapeutics, this oblong-shaped, flexible nanochain particle possessed a unique ability to gain access to and accumulate at glioma sites. Vascular targeting of nanochains to the αvβ3 integrin receptor resulted in a 18.6-fold greater drug dose administered to brain tumors than standard chemotherapy. By 2 hours after injection, when nanochains had exited the blood stream and docked at vascular beds in the brain, the application of an external low-power radiofrequency field was sufficient to remotely trigger rapid drug release. This effect was produced by mechanically induced defects in the liposomal membrane caused by the oscillation of the iron oxide portion of the nanochain. In vivo efficacy studies conducted in two different mouse orthotopic models of glioblastoma illustrated how enhanced targeting by the nanochain facilitates widespread site-specific drug delivery. Our findings offer preclinical proof-of-concept for a broadly improved method for glioblastoma treatment.

Introduction

The invasive forms of brain tumor, such as glioblastoma multiforme (GBM), are terminal upon diagnosis and no new protocols have been developed in more than 30 years. Current approaches for the treatment of glioma are limited in their effectiveness, because GBM tumors are characteristically diffuse, highly invasive, nonlocalized, and drug penetration across the blood–tumor barrier (BTB) is poor for most chemotherapeutic agents (1, 2). Today, systemic chemotherapy is not the primary treatment of choice for brain tumors due to the presence of the BTB. In an effort to avoid having to penetrate the BTB, implantable biodegradable drug depots within a brain tumor are currently being used in clinical practice to localize a chemotherapeutic and allow for controlled drug delivery (3). However, this method relies on drug diffusion from a central core. As a result, drug usually cannot reach the tumor periphery where the most aggressive cells persist. Therefore, the ideal drug delivery system should be based on systemic intravascular administration, which utilizes the tumor’s own blood supply for transport allowing for drug delivery throughout the tumor and its invasive sites.

Notably, nanoparticles have shown promise, because they can be designed not only to carry a range of cytotoxic drugs, but also to “smuggle” the drug into intracranial tumors such as gliomas. For example, although the potent chemotherapeutic drug doxorubicin (DOX) exhibits insignificant penetration of the BTB, it was demonstrated in patients with glioblastomas that long circulating liposomal nanoparticles containing doxorubicin achieved a 13- to 19-fold higher accumulation of doxorubicin in brain cancerous lesions compared with the normal brain (4). However, even though the BTB compromises the impermeable nature of the blood-brain barrier (BBB), blood vessels are not nearly as leaky as the angiogenic vessels observed in other cancer types (5). Thus, nanoparticles exhibit relatively low penetration into gliomas with a patchy, near-perivascular distribution, resulting in failure to deliver drugs to the difficult-to-reach invasive sites of brain tumors (6).

To circumvent the limitations of today’s drugs in treating invasive brain tumors, a multicomponent, flexible chain-like nanoparticle was developed. The nanoparticle, termed nanochain, is composed of three iron oxide nanospheres and one...
drug-loaded liposome chemically linked into a linear, chain-like assembly (Fig. 1A and B; refs. 7, 8). The multicomponent nature and shape of the particle result in two unique features that facilitate effective treatment of difficult-to-treat brain tumors using a low dose of cytotoxic drugs as illustrated in Fig. 1C (7, 9, 10). Contrary to traditional small-molecule drugs or more contemporary nanotherapeutics, the nanochain particle possesses a unique ability to gain rapid access to and be deposited at brain tumor sites. The nanochains are capable of transporting their drug cargo to brain tumors via highly specific vascular targeting of the vascular bed associated with the primary tumor mass and its invasive sites. Numerous studies have shown that αvβ3 integrin is highly overexpressed on brain tumors’ vascular bed, which has led to clinical trials testing integrin antagonists as antiangiogenic agents for patients with GBM (11–16). Furthermore, αvβ3 integrin is minimally expressed on normal resting blood vessels (17, 18). Notably, RGD-targeted nanoparticles are rapidly internalized by endothelial cells via the αvβ3 integrin receptor (13, 14, 19, 20). Hence, the nanochain utilizes a cyclic RGD peptide as a ligand to target the αvβ3 integrin receptor on the endothelium of angiogenic blood vessels of brain tumors. The size, shape, and flexibility of the nanochains significantly increase the margination of the particles toward the blood vessel walls in microcirculation (i.e., continuous scavenging of vascular walls), and targeting avidity of nanoparticles (i.e., latching on vascular target) due to geometrically enhanced multivalent attachment on the vascular target (9).

However, even after successful targeting to brain tumors, the drug molecules must spread to all the cancer cells, especially the hard-to-reach ones, resulting in widespread anticancer activity throughout the entire volume of tumors. Although nanoparticles typically release their content slowly, drug release from nanochains can be remotely triggered due to mechanically induced defects of the liposomal membrane caused by the oscillation of the iron oxide portion of the nanochain in the presence of an radio frequency (RF) field (7). Two hours later, after nanochains slip from the blood stream and dock on the vascular bed of brain tumors, a low-power RF field (10-kHz frequency; 5 mT amplitude) is applied outside the body. The field causes the nanochain to vibrate, breaking open the drug-loaded liposome and spreading cytotoxic drugs to the entire volume of glioma sites (7, 21).

In contrast with delivery of cancer drugs via passive intratumoral accumulation, our strategy utilizes the overexpressed αvβ3 integrin receptor as a docking site to establish well-distributed drug reservoirs on the brain tumor vasculature, which can subsequently spread free drug in the tumor interstitium using an RF field as an external trigger. In this study, we show that the synergy of nanochain’s enhanced targeting and widespread drug delivery capabilities facilitates enhanced treatment of brain tumor sites, which are otherwise inaccessible by conventional therapies.

**Materials and Methods**

**Materials**

The primary antibody for the specific endothelial antigen CD31 was purchased from BD Biosciences Pharmingen. Secondary antibodies and cell culture media were obtained from Invitrogen. Cross-Linked Ethoxylate Acrylate Resin (CLEAR) resin, reaction vessels, other accessories for solid-phase chemistry, and the cyclo (Arg-Gly-Asp-D-Phe-Cys) or c(RGDfC) peptide were purchased from Peptides International Inc. The cross-linkers 3,3’-Dithiobis(sulfosuccinimidylpropionate: DTSSP) and sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylic acid (sulfo-SMCC), and the cleaving agent Tris[2-carboxyethyl] phosphine (TCEP) were obtained from Thermo Fisher Scientific. Polyethylene glycol (PEG) conjugates were purchased from Laysan Bio. General solvents and chemicals were obtained from Thermo Fisher Scientific. Doxorubicin was obtained from Sigma.

**Synthesis and characterization of nanoparticles**

The nanochains were fabricated using a two-step solid-phase chemistry based on a previously published method (7, 8). More details on the synthesis of the nanochain particles can be found in previous publications (7, 8, 21).

**Tumor model**

All animal procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Case Western Reserve University (Cleveland, OH). The CNS-1 rodent glioma tumor model was used for these studies. Five- to 8-week-old NIH athymic nude mice (20–25 g each) were housed in the Athymic Animal Core Facility at Case Western Reserve University according to institutional policies. CNS-1 cells were
infected with GFP encoding lentivirus, harvested for intracranial implantation by trypsinization, and concentrated to 1 × 10^7 cells/ml in PBS. Mice were anesthetized by intraperitoneal administration of 50 mg/kg ketamine/xylazine and fitted into a stereotaxic rodent frame. Cells were implanted at AP = +0.5 and ML = −2.0 from bregma at a rate of 1 μL/minute in the right striatum at a depth of −3 mm from dura. A total of 200,000 cells were implanted per mouse. Similar procedures were used for the 9L glioma model.

Histologic evaluation
Once the appropriate tumor sizes were established (∼8 days after tumor inoculation), the animals were injected intravenously with the nanochain particles at a dose of 0.5 mg/kg doxorubicin. At 2 hours after injection, the animals were exposed to the RF field for 60 minutes. Animals were euthanized 24 hours after injection, and organs were extracted and analyzed histologically for location of brain tumor (CNS-1-GFP cells). Serial tissue sections of 12-micron thickness were stained for the specific endothelial antigen CD31 and with the nuclear stain DAPI. The tissue sections were imaged at ×5, ×10, and ×20 magnification on the Zeiss Axios Observer Z1 motorized FL inverted microscope. To evaluate the spread of drugs in relation to the location of nanochains, we used Prussian blue to stain iron. Direct fluorescence (red) imaging was used for doxorubicin. Histologic sections were imaged on a Zeiss Axios Observer Z1 motorized FL inverted microscope. To obtain an image of the entire section, a montage of each section was made using the automated tiling function of the microscope.

Survival study
Once the tumors were established (∼5 days; histologically confirmed), the animals were injected intravenously with the nanochain at a dose of 0.5 mg/kg doxorubicin. At 2 hours after injection, a 60-minute application of the RF field (amplitude B = 2 mT, frequency f = 10 kHz, RF power = 3–5 Watts) was employed using a custom-made solenoid (N = 105 turns, inner diameter = 2.8 cm; ref. 7). During this procedure, the animals were anesthetized through the administration of inhalant isoflurane. Two subsequent treatments were applied at time intervals of 2 days at the same doxorubicin dose followed by exposure to RF following identical protocol to the first cycle (if applicable). Following the same schedule for the treatments and RF application, control groups included animals treated with nanochains (no RF), liposomal doxorubicin with RF, free doxorubicin, and saline. After tumor inoculation, mice were monitored daily for any abnormal symptoms. The well-being of the animals took priority in decisions about euthanasia or other interventions. When animals showed a 10% loss of body weight, they were euthanized in a CO₂ chamber. Although the 10% weight loss was the primary endpoint criterion for the vast majority of the animals, two animals in the CNS-1 glioma model, one animal in the doxorubicin-treated group, and one animal in the nanochain-treated group (+RF), had to be euthanized because the animals suffered from inactivity and lethargy. At the terminal point, the tumor mass at the primary site had grown significantly with a final size of about 1.8 mm. Although the final size of the tumor at the primary site does not indicate the degree of tumor invasiveness, we histologically observed a considerable number of distant sites with dispersive brain tumor cells in the case of the CNS-1 glioma model. Time of death was determined to be the following day.

Statistical analysis
Means were determined for each variable in this study and the resulting values from each experiment were subjected to one-way ANOVA with post hoc Bonferroni test (SPSS 15). A P value of less than 0.05 was used to confirm significant differences. Normality of each dataset was confirmed using the Anderson–Darling test.

Results

Synthesis and characterization of nanochains
To fabricate the multicomponent nanochain particles, a step-wise solid-phase chemistry approach was developed. In the first step (Fig. 2A), amine-PEG functionalized iron oxide nanospheres (hydrodynamic diameter of 27 nm) were conjugated onto amine-functionalized CLEAR resin via a homo-bifunctional cross-linker reactive toward primary amines containing a disulfide bridge. The iron oxide nanospheres were allowed to bind to the solid support and then cleaved off using a reducing agent. The thiolytic cleavage liberated the iron oxide nanosphere from the solid support converting the amines to a different chemical functionality (thiol group) on the portion of the nanosphere’s surface that was linked to the resin. The second step (Fig. 2B) used the same type of resin and the modified nanospheres were introduced in a step-by-step manner using a hetero-bifunctional cross-linker for conjugation between primary amine and sulfhydryl groups. As a final component, an amine functionalized doxorubicin-loaded liposome with a hydrodynamic diameter of 35 nm was added. It should be noted that each step included multiple washing cycles to remove any unbound nanospheres and excess reagents from the nanoparticle–resin complex. In the end, the thiol of the cysteine residue of the (RGDfC) peptide was used to conjugate the targeting ligand to the remaining amine-terminated PEG on the surface of nanochains. Finally, the αvβ3 integrin-targeting nanochains were cleaved off the resin and recovered.

Because of the simplified purification procedure and easy handling of multiple reaction vessels, the solid-phase–based synthesis enables us to manufacture large amounts of nanochains that exhibited a high degree of uniformity. On the basis of analysis of multiple TEM images (minimum count was 200 particles), the majority of the particles (>85%) is composed of nanochains with three iron oxide spheres and one liposome (7, 21). As shown in Fig. 2C, the hydrodynamic size of the nanochain particle and each constituent nanosphere, as measured by dynamic light scattering (DLS), verified the TEM findings. Because of the high intraliposomal space available for drug encapsulation and the efficient remote loading technique (22), the entrapment efficiency was very high (drug cargo: 6.8 × 10⁻³ ng doxorubicin per particle). To determine the contribution of blood plasma to leakage of doxorubicin, Fig. 2D shows an in vitro stability test. The dialysis curve plateaued after approximately 3 hours, and the nanochain exhibited a leakage of approximately 6.5% of the total encapsulated doxorubicin after 24 hours, which was comparable with the leakage of a typical 100-nm liposome.

Vascular targeting of brain tumors
To evaluate the nanochain’s ability at targeting brain tumors, we compared the αvβ3 integrin-targeting nanochains with αvβ3 integrin-targeting 30-nm liposomes, their nontargeting variant and free doxorubicin. For this study, we selected...
the orthotopic CNS-1 glioma model. Recent reviews have compared the most commonly used rodent glioma models (e.g., C6, 9L) in terms of pathologic and genetic similarities with the human disease (23, 24). The CNS-1 model is one of the few models that recapitulate the microenvironment of the human disease and display several histologic features and diffuse growth and invasive pattern similar to human GBM. In addition to their ability to express several glioma markers, a three-dimensional cryo-imaging technique showed that the rodent CNS-1 glioma cells is a valid system to study the highly dispersive nature of glioma tumor cells along blood vessels and white matter tracts in vivo (25).

The animals were injected with the various doxorubicin formulations via tail vein at a dose of 0.5 mg/kg doxorubicin 8 days after orthotopic tumor inoculation. The animals were transcardially perfused with heparinized PBS 24 hours after intravenous administration, and brains were retrieved and analyzed for doxorubicin content following an established protocol (26). At this time point, doxorubicin was undetectable in blood circulation. We predominantly collected the tumor mass at the primary site because it was very challenging to identify the margins of the invasive sites of the tumor. Vascular targeting of nanochains resulted in a 4.7% of the administered dose being localized in brain tumor (Fig. 3A), which was 18.6-fold higher than free doxorubicin. Free doxorubicin and nontargeted liposomes exhibited very low accumulation (<1% of injected dose) at brain cancer sites. The targeted liposomes and nontargeted nanochains displayed an accumulation of 1.45% and 1.75% of the injected dose, respectively, in brain tumors. As expected, the levels of doxorubicin in normal brain were undetectable in the case of all the formulations.

After evaluating the significant targeting enhancement of doxorubicin to brain tumors using nanochains, we used noninvasive imaging to assess the therapeutic efficacy of the nanoparticle with or without the RF application. By infecting the CNS-1 cells with GFP encoding lentivirus, the expression of GFP of the CNS-1 cells enabled in vivo imaging using a Maestro fluorescence imaging system (Supplementary Fig. S1A). As a metric of the response of brain tumors to various treatments (n = 5 in each group), quantification of fluorescence signal was used (Fig. 3B). Because of the emission of GFP in the green zone of the visible spectrum, the depth-dependent attenuation of the fluorescence signal primarily by the skull (and the brain tissues) does not allow accurate quantification of the number of CNS-1 glioma cells in the case of in vivo imaging (Supplementary Fig. S1B). However, the relative differences of fluorescence signal among the various treatments can be used as a comparison in a semiquantitative manner. At 8 days after orthotopic tumor inoculation, the animals were intravenously injected with nanochains at a dose of 0.5 mg doxorubicin per kg of body weight. A 60-minute application of the RF field (amplitude B = 5 mT, frequency f = 10 kHz) was used at the point of maximum accumulation of the nanochain in brain tumors (2 hours after injection; ref. 7). Previous in vitro studies identified that a 60-min application of the RF field at the selected operating conditions guarantees approximately 100% release of nanochain’s drug cargo (7). Maestro imaging was performed...
before and 48 hours after injection to allow for the cytotoxic effects of doxorubicin to occur. Figure 3B shows that the signal of the untreated animals doubled within 48 hours. Although free doxorubicin decelerated the disease, the brain tumor continued growing as indicated by the increase of fluorescence signal. Even with the nanochain’s enhanced targeting efficacy, the nanochain therapy (without RF) resulted in a similar anticancer effect to free doxorubicin. However, animals treated with nanochain followed by application of the RF field exhibited a substantial decrease of fluorescence signals, which indicates the additional therapeutic effects of the RF-triggered release. Furthermore, no temperature increase of the brain tissue was observed due to the RF field (Supplementary Fig. S2), indicating that the triggered release mechanism of drug from the nanochains is not based on hyperthermia.

**Histologic evaluation**

After evaluating the targeting capabilities and anticancer effects of the nanochain in a macroscopic manner, we assessed the localization of nanochain in invasive glioma and the degree and topology of doxorubicin delivery with or without the application of the RF field. Histologic analysis was performed on separate groups of mice 24 hours after injection (n = 3). A representative image of a brain section is shown in Fig. 4A (left) displaying the presence of the primary tumor and clusters of invasive cancer cells dispersed in the brain. Bright field microscopy of the same histologica section stained with hematoxylin-eosin is shown in Supplementary Fig. S3. Most notably, application of the RF field resulted in widespread delivery of doxorubicin at both the primary and invasive sites of brain tumor (of Fig. 4A, right). We then focused on an invasive site (shown by the yellow circle in Fig. 4A) to assess the ability of the nanochain to deliver drugs to dispersing glioma cells. Imaging at higher magnification of this invasive site showed that nanochains were selectively localized on the endothelium associated with cancerous sites (Fig. 4B, left). Notably, application of the RF field facilitated the spreading of doxorubicin to distant cells away from nanochain deposits (Fig. 4B, right). Although the RF-triggered release resulted in widespread delivery of doxorubicin to the target site (Fig. 4C), no spread of doxorubicin was observed in the case of nanochain-treated animals that were not exposed to RF, since the fluorescence signal of intraliposomal doxorubicin is quenched (Fig. 4D). The in vivo anticancer effects of the nanochain treatment followed by RF observed in histology are consistent to cell cytotoxicity studies (Supplementary Fig. S4). Although 30-nm liposomal doxorubicin, 100-nm liposomal doxorubicin, and nanochain without RF exhibited moderate cytotoxicity (less than 30% relative cytotoxicity), RF-triggered release of doxorubicin from nanochain had significant cytotoxic effects (65% cytotoxicity), which was comparable with the effect of free doxorubicin (75% cytotoxicity), indicating the release of free doxorubicin molecules from the nanochain particles upon application of the RF field.

**Survival studies**

The therapeutic effect of the nanochain treatment was determined in two orthotopic glioma models by measuring survival times. Control treatments included free doxorubicin followed by RF and nanochain without RF. The treatments were administered three times (Fig. 5A), each at a dose of 0.5 mg/kg doxorubicin. Targeted nanochains exhibited a 2.6-, 3.2-, and 6-fold higher...
deposition in brain tumors than nontargeted nanochains, targeted liposomes, and nontargeted liposomes, respectively (Fig. 5B). Since the targeting efficiency of the nanochains to brain tumors was found to be significantly higher than those formulations, we chose to only assess the effect of targeted nanochains with or without the combination of the RF field on the survival rate. Not surprisingly, the free doxorubicin treatment had negligible therapeutic benefits in the CNS-1 model. On the other hand, even with their enhanced targeting capability, nanochains (without RF) exhibited a moderate effect. However, the survival time of the nanochain-treated animals followed by RF was significantly increased (25 ± 3 days; mean ± SD) when compared with the nanochain-treated group without RF (16 ± 1 days), doxorubicin-treated group (10 ± 3 days), and the untreated group (9 ± 1 days). In comparison with standard chemotherapy (i.e., doxorubicin), the 2.5-fold increase in survival of the nanochain-treated animals followed by RF is highly significant considering the highly invasive nature of the CNS-1 model.

To illustrate the therapeutic efficacy of the nanochain treatment, we also used the 9L model, which is an aggressive but not invasive glioma model. As expected, the therapeutic effect of the free doxorubicin treatment was negligible as indicated by the survival time being similar to the untreated group. Although 100% of the untreated and doxorubicin-treated mice died within 28 days, 40% of the nanochain-treated group followed by RF was still alive after 77 days (Fig. 5C). At the terminal point, post portem measurements showed that the tumor at the primary site was 1.8 ± 0.17 mm (mean ± SD) in size for both animal models.

The average weight progression for each group is shown in Supplementary Fig. S5. Because of the highly selective deposition of the nanochain particles at brain tumors and the subsequent efficient spreading of drug, this significant therapeutic outcome was achieved at a very low dose (i.e., 0.5 mg/kg), which is 10- to 20-fold lower than the typical clinical regimens of liposomal doxorubicin.

**Discussion**

Invasive brain tumors are recognized as one of the deadliest forms of cancer. This stems from the fact that radiation therapy...
 weeks compared with placebo (27). On the other hand, systemic administration of nanoparticles has the potential to facilitate access to the entire brain tumor by utilizing the tumor’s own blood supply.

Historically, attempts to improve nanoparticle homing to brain tumors have relied on the EPR effect followed by targeting of various receptors to direct the nanoparticle–drug complex into brain tumor cells. However, even though the BTB slightly compromises the nature of BBB, brain tumors consist of blood vessels that are not as leaky as the angiogenic vessels observed in other cancer types. This results in low penetration of nanoparticles into the brain tumor interstitium, resulting in failure to reach the majority of the primary tumor mass and especially its invasive sites (6). Furthermore, the EPR effect is typically noticeable at the core regions of a brain tumor, while it is attenuated at the invasive sites of brain tumors with dispersing cancer cells. This is due to the fact that the BBB of invasive sites has a very high likelihood to remain intact. Importantly, it is not uncommon to find dispersing brain tumor cells as far away as 4 cm from the primary site (28). Along these lines, the nanochain-based therapy exploits the tumor vasculature as a docking site for the nanoparticles followed by RF-triggered drug release to spread the drug throughout the primary tumor and its invasive sites, which are nearly inaccessible by today’s systemic therapeutics.

Although various receptors have been exploited to target nanoparticles to cancer cells (e.g., folate, EGF receptors; refs. 29–34), our work suggests that αvβ3 integrin-mediated vascular targeting provides highly selective targeting of brain tumors. In fact, the nanochains have been specifically designed to target the tumor vasculature (9). The particle shape governs the navigation of circulating nanoparticles through different biologic processes, including targeting of difficult-to-reach cancer sites (35). One of the pivotal steps dictating the transport of flowing nanoparticles is their margination toward the blood vessel walls. Contrary to spherical nanoparticles, nanoparticles with geometrical asymmetry (e.g., oblong shape) are subjected to torques, resulting in tumbling and rotation, which increase the lateral drift of nanoparticles toward the blood vessel walls in microcirculation (36–38). Furthermore, the particle shape also governs the targeting avidity of nanoparticles using receptor-ligand systems. Compared with nanoparticles, oblong-shaped nanoparticles exhibit enhanced targeting avidity due to geometrical enhanced multivalent docking. Indeed, within 24 hours after injection, integrin-targeting of nanochains resulted in a 4.2% of the administered dose being localized in brain tumor, which was 3-fold higher than integrin-targeting liposomes. Regarding the in vivo fate of the remaining nanochains, our previous publication evaluated the organ distribution of nanochains, which was comparable with the behavior of standard 100-nm PEGylated liposomes (7). At 24 hours after injection, the nanochains were mostly found in the reticuloendothelial organs (liver and spleen). Even though such a significant portion of the injected dose accumulated at the tumor site, the histologic studies show that no spread of doxorubicin in the brain tumor was observed in the absence of the external stimulus (i.e., RF field). Not surprisingly, even with the enhanced targeting, the nanochain treatment (without RF) provided only modest benefits in terms of prolonged survival. This is primarily related to the drug release profile from nanoparticles. Although free drug in its molecular form quickly spreads within the tumor interstitium (39–41), nanoparticles, without any triggering mechanism, release their content at a
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M.A. Griswold reports receiving commercial research grant from Siemens Healthcare (MRI Business Unit). No potential conflicts of interest were disclosed by the other authors.

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