Novel Urokinase Plasminogen Activator Receptor (uPAR) Targeted Probe and Its in vivo MRI of 4T1 Xenograft Breast Cancer Model

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Urokinase plasminogen activator (uPA) is a protease regulating matrix degradation, cell motility, metastasis and angiogenesis. Cellular receptor of uPA (uPAR) is highly expressed in breast cancer cells, intra-tumoral fibroblasts and endothelial cells. We have developed an uPAR-targeted MRI probe that is consisted of size uniformed magnetic iron oxide (IO) nanoparticles conjugated with amino-terminal fragments (ATF, 135 amino acids) of uPA as the cell targeting moiety for uPAR. Our results of *in vivo* molecular MRI of mice bearing xenograft breast tumor showed uPAR-targeted ATF-IO nanoprobe has high specificity to the uPAR+ cancer cells and induced MRI contrast in the tumor with uPAR overexpression.

MATERIAL AND METHODS

Magnetic iron oxide nanoparticle Size uniform and tunable IO nanoparticles were formed from the reaction of iron oxide powder and mixture of oleic acid and octadecene at 300 °C and collected as precipitate by adding chloroform and acetone into the reaction mixture. Amphiphilic triblock polymer with carboxylate groups were used to coat and stabilize the IO nanoparticles. The presence of carboxylate groups on the IO nanoparticles allows coupling of Ni-NTA for conjugating His-tagged ATF peptides. For this study, nanoparticles with a core size of 9 nm were used. With triblock polymer coating (~10 nm thick), our preparation of magnetic iron oxide nanoparticle has a surface charge of -36 mV (ζ) and R_2 of 120 (S^{-1}. mM^{-1}) at 3T.

ATF and ATF-IO conjugates Gene construct for mouse ATF (1-135 aa) was cloned into pET bacteria-expressing plasmid and produced the His-tagged ATF peptides in E. coli. His-tagged ATF peptides were then purified using a Ni-protein purification column. ATF peptides then were further conjugated to the surface of IO nanoparticles through reaction of His-tag to Ni-NTA (**Fig. 1**). Near infrared (NIR) fluorescent molecule Cy5.5 was cross-linked to the ATF-IO nanoparticles for tracking MRI probe with NIR imaging.

In vitro and vivo MRI Various experiments were performed to examine the target specificity of ATF-IO to the different cell lines with different level of uPAR expression, using multi-echo T_2 measurement of cell samples incubated with ATF-IO nanoparticles. To test specificity and sensitivity of ATF-IO nanoprobe for imaging of the uPAR+ tumor *in vivo*, we used mice bearing mouse mammary tumors derived from 4T1 cell line. The development of uPAR(+) tumor in mice was followed using bioluminescence imaging (BLI) before MRI. Mice bearing uPAR(+) tumor were injected with a calculated dosage of ATF-IO nanoparticle dispersion through the tail vein. T1 weighted gradient echo imaging and multi-echo T2 relaxometry mapping were performed before and after i.v. administration of ATF-IO nanoparticles or IO nanoparticles alone as the control. Images were acquired using a 3T scanner (Philips Intera) with a customized rodent coil. Region of interest (ROI) analyses were performed in the areas of tumor, liver, kidney, spleen, brain and muscle to obtain the IO nanoparticles induced contrast change. uPAR targeted binding of ATF-IO nanoparticles were validated by histological Prussian Blue staining for iron and NIR confocal fluorescence microscopy in vitro or NIR imaging in vivo with Cy5.5.

RESULTS

ATF-IO nanoparticles exhibited highly specific binding to the tumor cells with uPAR overexpression in vitro compared to those of uPAR(-) cells. Furthermore, ATF-IO nanoparticles showed receptor mediated intracellular internalization in confocal fluorescence microscopy. uPAR targeted MRI in vivo revealed that ATF-IO nanoparticles were accumulated in the subcutaneously (s.c.) developed 4T1 mouse mammary tumors, evidenced by marked signal decreases in various areas of the tumor in T2 weighted spin echo imaging (**Fig. 2C**) and regional T2 reduction in the T2 map. The ROI analysis showed that the level of signal change post injection of ATF-IO nanoparticles was the most significant in the tumor compared to that of the tumor from mice received unconjugated IO particles, suggesting that ATF-IO nanoparticles were preferential accumulated in the uPAR (+) tumor areas. T2 and T2* induced MRI signal decrease was less in liver and spleen of mice injected with ATF-IO nanoparticles than that of animals received unconjugated IO particles. It is likely that liver and spleen uptake of the nanoparticles was reduced when using ATF-IO nanoparticles. The tumor specific accumulation of ATF-IO nanoparticles was confirmed by in vivo NIR imaging using Cy5.5 cross-linked ATF-IO nanoparticles (**Fig. 2D**). This observation was further validated by the positive Prussian blue staining of tumor tissues collected from mice received ATF-IO nanoparticles (**Fig. 3**) and Prussion blue staining was negative in that received unconjugated-IO nanoparticles. In summary, we demonstrated that uPAR targeted ATF-IO nanoprobe has sufficient sensitivity for *in vivo* molecular MRI and the conjugation of ATF to IO nanoparticle is stable under the condition of *in vivo* site specific delivery of the imaging probe.

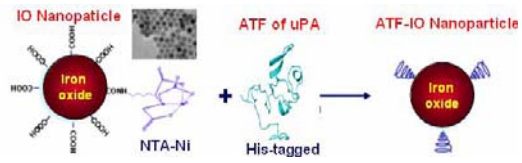

Fig.1 ATF peptide of uPAR from mouse breast cancer cells can be prepared with histidine-tag and conjugated to IO nanocrystals via –COOH of surface coating polymer.

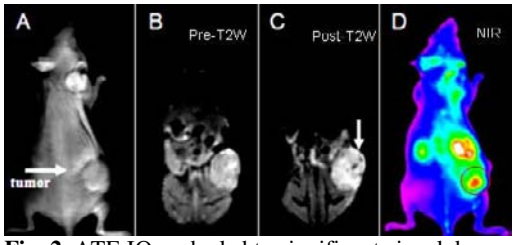

Fig. 2. ATF-IO probe led to significant signal drops in the tumor (arrow indicated) in T2W images and confirmed by NIR imaging using dual labeled Cy5.5-ATF-IO nanoparticles.

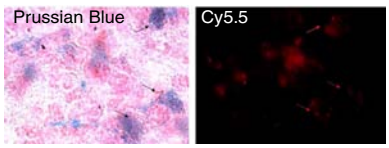

Fig 3. Co-localized positive Prussian blue staining (left) and NIR confocal fluorescence microscopy confirmed the uPAR targeted ATF-IO nanoparticles accumulated in the tumor.