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Identification of Overexpressed Receptor in Small Cell Lung Cancer (SCLC) and Distribution of Non-Functionalized Core-Shell Magnetic Nanoparticles in an Animal Model of Small Cell Tumor Cancer

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Abstract

The Small Cell Lung Cancer (SCLC) is a lethal malignancy associated with a poor prognosis worldwide. SCLC accounts for 15%-20% of diagnosed lung cancers. It is different (pathological, molecular, biological, and clinical) from other lung cancers, provoking a low quality of life in patients. To this end, alternative diagnosis and treatment methods are nowadays mandatory.

This work contributes to those studies based on nanomedicine for diagnosis and treatment of SCLC by using core-shell magnetic nanoparticles functionalized with a linker and drugs. In particular, this contribution aims, on the one hand, at identifying the overexpressed receptor in tumorous cells to select the best target for the functionalization of Core-Shell Nanoparticles (CSNPs) and, on the other hand, at evaluating the residence time distribution of non-functionalized core-shell magnetic nanoparticles, *in vivo*. The latter focuses on assessing the performance of nanoparticles as a contrast-based diagnostic method during the Enhanced Permeation and Retention (EPR) effect; Magnetic Resonance Images (MRI) are used to characterize the residence distribution time of the nanoparticles *in vivo*.

Keywords: Small Cell Lung Cancer (SCLC); Transferrin receptor (CD71 receptor); Magnetic Resonance Images (MRI); Core-shell magnetic nanoparticles

Introduction

Lung cancer is, nowadays, responsible for around 1.8 million deaths, about 18.4% in 2018 [1]. Although it comprises only approximately 11.6% of new cancer cases, this is, worldwide, one of the main malignancies causing cancer-related deaths [2]. Approximately 75%-85% of lung cancers are caused by carcinogens present in tobacco smoke, whereas 15% to 25% of lung cancer cases occur in lifetime never-smokers [3].

The two main types of lung cancer, identified based on histologic, clinical, and neuroendocrine characteristics, are Non-Small Cell Lung Cancer (NSCLC), representing 80%-85% of cases, and Small Cell Lung Cancer (SCLC), representing 15%-20% of cases [4]. NSCLC and SCLC differ molecularly, with many genetic alterations, exhibiting subtype specificity [5]. SCLC is a malignant epithelial tumor classified as a high-grade neuroendocrine carcinoma tumor that usually appears in the central airways with a high growth rate and early development of metastases [6].

The traditional treatments used to deal with this type of pathologies are radiotherapies, chemotherapies, and surgeries [7]. Nevertheless, they, in most of the patients, tend to be ineffective presenting side effects, which lead to a low quality of life [8]. In this scenario, the academy and pharmaceutical industry conduct their research to propose alternative diagnostic methods and treatments [9]. Nanomedicine seems a promising technology to diagnose but also treat lung cancer in general but SCLC in particular [10].

The design bases of cancer nanomedicine, in particular for solid tumors, are the Enhanced Permeation and Retention (EPR) effect and the cancer cell-specific affinity targeting [11]. Their appropriate design, but as well as the study of their impact on cancer nanomedicine extends the therapeutic window by enhancing efficacy and reducing toxicity [12]. For instance, more accumulation in target sites, but thereby less exposure to other organs is expected by the EPR effect. In this regard, large circulation contributes to accumulation by the EPR effect. In this regard, studies evaluating passive and active targeting of SCLC and those describing the *in vivo* fate of nanoparticles are necessary to extend nanomedical to other health scales.

This work contributes to those studies evaluating new diagnosis and treatment methods based on nanomedicine for lung cancer. The two aims of this research are in this perspective: To identify the overexpressed receptor in tumorous cells to select the best target for the functionalization of Core-Shell Nanoparticles (CSNPs); and to evaluate the distribution of non-functionalized core-shell magnetic nanoparticles *in vivo*.

The latter relates to the EPR effect to use these nanoparticles as a contrast-based diagnostic method.

Materials and Methods

SCLC cell line H69AR (ATCC® CRL-11351), used as tumorous cell line, was cultured in an RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine (2 mM), and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin); and a non-tumorous lung cell line MRC5 (ATCC® CCL171) was used as control. The latter cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic, in a 5% CO_2 humidified incubator at 37˚C.

The identification of the overexpressed receptor in SCLC accounted for the extraction of membrane protein. The extraction was isolated with Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Scientific™) with protease inhibitor cocktail added. Equal amounts of protein were prepared for proteomic studies. Spectrometric data were obtained in a Synapt G 2-Si mass spectrometer (Waters Corporation).

Based on spectrometric analysis results, it was performed a Western Blotting analysis to confirm the overexpressed receptor in SCLC. Equal amounts of protein were prepared and run in SDS/PAGE gel; proteins were transferred onto a nitrocellulose membrane. The membrane was incubated overnight with goat anti-CD71 (Santa Cruz Biotechnology, sc-32272), and a monoclonal anti-β-actin at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The bands containing the proteins were visualized on x-ray film (Kodak) using an enhanced chemiluminescence (Western Lightning Plus-ECL, PerkinElmer, Inc.) kit. Densitometric analysis of Western blot bands was performed using the software image studio TM version 5.2.5.

Along with analyzes mentioned above, *in vivo* studies were performed; SCLC subcutaneous tumors were generated in nude mice (Foxn1nu: Nu/Nu), and nanoparticles were inoculated in the tail by intravenous injection to analyze their distribution employing Magnetic Resonance Imaging (Varian MRI 7 Tesla). Preparation of magnetic nanoparticles A simple co-precipitation method was used for the synthesis of iron oxide nanoparticles.

50 ml of 1.5 M NaOH was used as a reducing agent. A volume of 10 mL of deionized water was degassed with nitrogen gas and then 1 mM $FeCl_2.4H_2O$, 2 mM $FeCl_3 6H_2O$, 0.85 ml HCl was added. Then the solution was mixed and then the NaOH solution was added under constant stirring with a nitrogen atmosphere at 80°C for 1.5 h. Black solid powders formed which were separated using a magnet and washed with distilled water and ethanol 3 times each. The black precipitate was separated and washed to be used for the synthesis of core-shell NPs.

Preparation of Fe_3O_4 iron oxide seed solution

Iron oxide seed solution was prepared using 5 ml of Au Fe_3O_4 solution and then diluted with 25 ml of distilled water. 5 ml of dimethyl sulfoxide (DMSO, 0.2% w/v) added for the removal of agglomeration in the particles, and stirred under N_2 atmosphere. The temperature was maintained at 80°C throughout the experiment for 3 h.

Preparation of the gold seed solution

The gold seed solution was initially prepared by mixing 1 ml of CTAB (0.1 M) and 2 ml of AA (1 mM) together and then added to 50 ml of $HAuCl_4$ solution (0.3 M).

Synthesis of core/shell nanoparticles Au-Fe_3O_4

The iron oxide seed solution and the gold seed solution were mixed, thus synthesizing core-shell nanoparticles of Au-Fe_3O_4. Initially, the violet gold seed solution was added drop wise to the brown iron oxide solution. This mixture was kept under constant stirring for 6 h until the brown solution turned dark purple.

Along with the analyzes mentioned above, *in vivo* studies were performed; SCLC subcutaneous tumors were generated in nude mice (Foxn1nu: Nu/Nu), and nanoparticles were inoculated by intravenous tail injection to analyze their distribution employing Magnetic Resonance Imaging (Varian MRI 7 Tesla).

Results and Discussion

Based on the proteomic analysis, it was found that transferrin receptor protein (also known as CD71) is overexpressed in SCLC displays the western blot analysis, indicating that the transferrin receptor was highly up-regulated in SCLC, presenting a change ten times more compared with the standard lung cell line. This result is in line with literature reporting that CD71, (a homodimeric type II membrane glycoprotein, ~95 kDa) binds to and assists entry of its ligand into cells for the delivery of iron **(Figure 1)**.

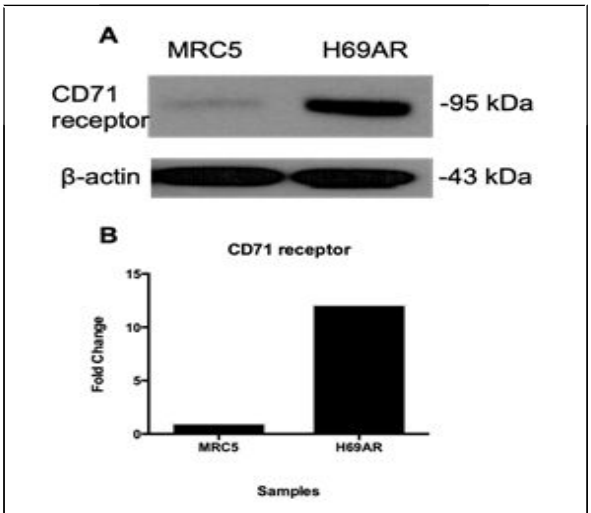

Figure 1: a) Transferrin Receptor Western Blot analysis of non-tumoral and tumoral Lung cell lines and; b) The densitometry analysis of transferrin bands.

MRI results elucidated how the NPs were distributed and their residence time distribution in the mouse model. NPs mainly

located at the kidney; besides, at 8 hours, the largest concentration of NPs was observed, and at 24 hours, NPs had left the mouse model through excreted urine. CSNPs were found present in a high level near the tumor area, which was attributed to the Enhanced Permeability and Retention (EPR) effect which is widely considered to be a major driving force for nanoparticles to reach and accumulate in the tumor, through either passive or active targeting **(Figure 2)**.

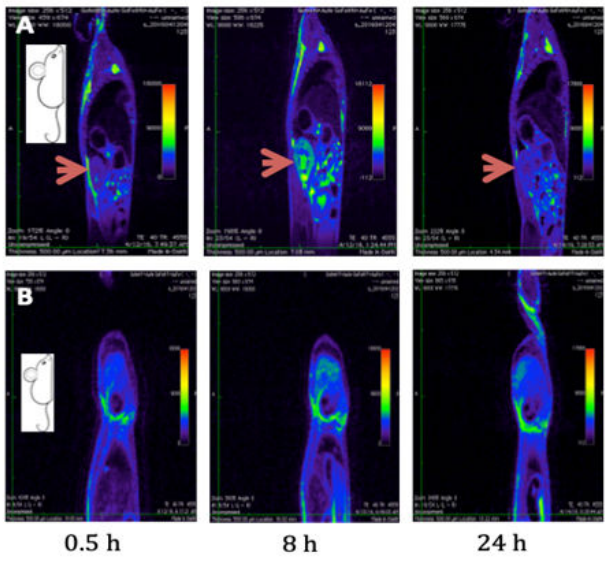

Figure 2: MRI Analysis, nanoparticles signal shows in green; a) Image of the mice showing the kidney area and; b) Image of the mice showing the kidney tumor area.

Conclusion

The functionalization of the nanoparticles used in nanomedicine is based on coupling chemotherapeutic drugs and ligands that allow a higher affinity for tumor cells. In the case of SCLC, it has been defined that the target protein is the transferrin receptor, which would be valuable for the therapeutic effect to have a more significant impact on the tumor cells by decreasing the damage in healthy cells to some extent.

In another aspect, it was determined that the nanoparticles used in this nanotherapeutic system are appropriately designed to be used as a diagnostic method by MRI analysis since they are quickly accumulated in the tumor area. The last due to their physicochemical characteristics and the EPR effect. In this first phase we evaluate the permanence of non-functionalized magnetic nanoparticles, and at a later stage, and once the target is known on the cancer cell, it is to functionalize the core-shell magnetic nanoparticles, *in vivo* and evaluate the therapeutic effect.

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