Evaluation of the theranostic properties of gadolinium-based nanoparticles for head and neck cancer

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Abstract

Background: The aim of the study was to evaluate the benefits of the combination of Gadolinium-based nanoparticles AGuIX and radiotherapy on the recurrence free survival after tumor resection in a head and neck animal orthotopic model.

Methods: Human head and neck CAL33 orthotopic tumors were implanted in female NMRI nude mice. The biodistribution of AGuIX was studied by fluorescence imaging. Tumor resection was performed 19 days after tumor implantation. Radiotherapy was performed 23 days after resection (10 Gy), 1 hour after AGuIX IV injection.

Results: After systemic administration, AGuIX passively accumulated in the orthotopic tumors. After tumor surgery, the combination of AGuIX with radiotherapy significantly improved the recurrence free survival and the median survival time (196 days) compared to irradiated only mice (75 days).

Conclusion: This study demonstrated the improvement of the recurrence free survival following combination of AGuIX injection with radiotherapy after Head and neck tumor resection.

Key words: gadolinium, head and neck cancer, nanoparticles, orthotopic animal model, radiotherapy

1 | INTRODUCTION

Head and neck cancer (HNC) is the sixth most common type of cancer worldwide, with 600,000 new cases and 300,000 deaths each year.¹ Despite technological and medical advances, most of the patients are diagnosed at a late stage, and the 5-year survival rates are about 34% for pharyngeal and oral cavity cancers and 55% for laryngeal cancers.²

Surgery, radiotherapy, and chemotherapy are the three main treatments of HNC. Surgery is considered as the main treatment and could be completed by radiotherapy or radiochemotherapy in case of incomplete resection and/or lymphovascular and perineural invasion and lymph node capsular invasion, to lower the risk of recurrence. Concomitant chemotherapy could potentiate radiotherapy. Currently, platinum-based chemotherapy is indicated in association with radiotherapy in case of risk of relapse.³ Radiotherapy is designed to apply ionizing radiation at a sufficiently high cytotoxic dose to kill cancer cells. It should be focused on the tumor to minimize damages to the surrounding healthy tissues.

Over the past two decades, the development of nanoparticles for medical applications was a growing field, especially to improve medical imaging techniques and therapeutic protocols.⁴,⁵ These theranostic agents, nanometric in size and composed of materials of various chemical natures (metals, metal
oxides, silicates lipids, etc), combine diagnostic and therapeutic capabilities. In particular, the gadolinium-based nanoparticles, named AGuIX (NHTheraguix, Crolles, France), were developed via an original top-down process. They are composed of a polysiloxane matrix bearing chelating species of 1, 4, 7, 10-tetraazacyclododecanec-1, 4, 7, 10-tetraacetic acid (DOTA) at the surface. The DOTA ligands chelate the Gd\(^{3+}\) ions, allowing their use as a MRI contrast agent.\(^5\)\(^-\)\(^7\) The DOTA ligands can also complex radionuclide to perform positron emission tomography (PET) and single-photon emission computed tomography (SPECT). Moreover, primary amine functions readily enable dye covalent conjugation to the polysiloxane matrix for fluorescence imaging: a near-infrared dye (cyanine 5.5) can be grafted onto the nanoparticles to permit fluorescence imaging\(^8\) and therefore near-infrared (NIR) optical imaging-guided surgery.

In addition, these gadolinium-based nanoparticles exhibit high radiosensitizing properties in vivo and in vitro, related to their high atomic number,\(^9\)\(^-\)\(^14\) that render them helpful therapeutic agents for radiotherapy.\(^5\)\(^,\)\(^15\)\(^-\)\(^17\) Despite a rapid blood and renal clearance as a result of their small size (<6 nm), these particles significantly accumulate within the tumors by a passive targeting corresponding to the enhanced permeability and retention (EPR) effect.\(^18\)\(^,\)\(^19\) An active targeting can be combined to the passive targeting, by grafting on the surface of the nanoparticles, ligands specific for cancer cells, and/or microenvironment cells receptors. It should provide a more efficient accumulation of these particles in the tumor region.\(^20\)

In a previous study, we have developed an optimized experimental xenograft orthotopic animal model of HNC.\(^21\) In this model, tumor fragments were implanted directly in the oral cavity of the mice. This head and neck orthotopic animal model was reproducible and representative of the complete disease process.

The present study aims to study the biodistribution of fluorescent gadolinium-based nanoparticles AGuIX in this head and neck orthotopic animal model by in vivo fluorescence imaging. Afterward, we evaluate the radiosensitizing effect of the nanoparticles on the recurrence free survival rate of mice.

2 | MATERIALS AND METHODS

2.1 | Gadolinium-based nanoparticles

The gadolinium-based nanoparticles AGuIX were provided by the start-up Nano-H (Saint Quentin Fallavier, France). They consisted of a polysiloxane core surrounded by gadolinium chelates (DOTA) covalently grafted to the inorganic matrix. When needed to perform biodistribution studies by optical imaging, Cyanine 5.5 (Cy 5.5) near-infrared dye was covalently grafted onto nanoparticles.

2.2 | Cell line

This study was conducted using two human head and neck squamous cell carcinoma cell lines: CAL 33-Luc cell line (kind gift of Dr BOZEC [Antoine-Lacassagne Center, Nice, France]) derived from a tongue carcinoma and stably transfected with the pLenti-Luciferase vector, and SQ20B cell line derived from a laryngeal carcinoma (Institut Albert Bonniot, Grenoble, France).

Cells were maintained in a monolayer culture in Dulbecco’s Modified Eagle’s medium supplemented with 10% of heat-inactivated fetal bovine serum (v/v) in a humidified incubator (Sanyo, Japan) at 37\(^\circ\)C in an atmosphere containing 5% CO\(_2\). The subculturing was made twice a week.

2.3 | Head and neck orthotopic tumor model

Animal experiments were conducted in accordance with protocols approved by the Ethical Committee of Grenoble. Female athymic NMRI nude mice (5-6 weeks old) were purchased from Janvier laboratories (Le Genet sur Isle, France). The mice were housed five per cage with food and water ad libitum and were maintained under specific pathogen-free conditions.

Anesthesia was induced through isoflurane 4% in a mixture of N\(_2\)/O\(_2\) [80:20 for induction and 2% thereafter] and ketamine (0.1 mg/g) associated with medetomidin (0.2 μg/g) for intraperitoneal injection of 5 × 10\(^6\) CAL33-Luc cells suspended in 200 μL of phosphate-buffered saline in both right and left flanks for heterotopic establishment of tumors. Tumor growth was followed macroscopically. After 2 weeks, mice were anesthetized as described previously, and the tumors were excised by direct approach. The mice were then sacrificed by cervical dislocation. For orthotopic tumors establishment, mice were anesthetized with an intraperitoneal injection of medetomidin (0.2 μg/g)/ketamine (0.1 mg/g) associated with a subcutaneous injection of buprenorphone (0.1 mg/kg). A tumor fragment of 0.5 mm\(^3\), obtained from the subcutaneous tumors, was implanted through a small incision on the inner aspect of the mouse’s left cheek. The incision was closed using PDS (polydioxanone 6.0 monofilament synthetic absorbable suture). Animal weight and tumor growth were monitored three times a week, using a caliper, and once a week by bioluminescence imaging after intraperitoneal injection of 150 mg/kg of Luciferin (Promega, Charbonnières, France). Head and neck orthotopic tumors developed over a period of 19 days. The same protocol was used for orthotopic SQ20B tumor model, to obtain two head and neck tumor models from different histological origin.

2.4 | Tumor imaging study

This study was performed on CAL33-Luc and SQ20B orthotopic tumor models, to evaluate the biodistribution of the gadolinium-based nanoparticles in the both models. After tumor growth, the gadolinium-based nanoparticles labeled with cyanine 5.5 were injected into the tail vein of
anesthetized mice (200 µL at [Gd\(^{3+}\)] approximately 20 mmol/L). A total of 12 mice were included for the tumor imaging study, 9 mice bearing CAL33-Luc orthotopic head and neck tumors (n = 3 per timepoint, that is, 1 hour 30 minutes, 5 hours, and 24 hours), and 3 SQ20B bearing orthotopic head and neck tumor. The evaluation of the nanoparticles’ biodistribution was performed by in vivo fluorescence imaging (Optimal Platform, Grenoble, France). Fluorescence imaging was acquired prior injection, 30 minutes, 1 hour 30 minutes, 3 hours, 5 hours, and 24 hours after injection, with a 660-nm light-emitting diodes.

Two-dimensional-fluorescence images were acquired using a back-thinned charge-coupled device camera at −80°C (ORCAII-BT-512G, Hamamatsu, Massy, France) fitted with a high-pass filter RG9 (Schott, Jena, Germany). Image display and analysis were performed using the Wasabi software (Hamamatsu, Massy, France). For the follow-up of the fluorescence signal in function of time on whole-body images, a region of interest (ROI) was drawn on the tumor region and on the skin of each mouse. The number of photons collected into each ROI was then used to calculate the tumor/skin fluorescence ratios. This ratio allowed us to subtract the skin fluorescence from the measured fluorescence. At the end of the experiment, mice were sacrificed and dissected for imaging of organs and plasma. Semiquantitative data were obtained from the fluorescence images by drawing ROIs on the different organs. The noise was quantified on each image and subtracted from the fluorescence signal. For dissected organs, the results of fluorescence quantifications were expressed as a number of photons per pixel per unit of time exposure.

Fluorescence microscopy of the frozen tumors was carried out using a laser-scanning confocal microscope (LSM 710, Zeiss, Jena, Germany) with a ×100 objective. Tumor sections were covered with 4',6-diamidino-2-phenylindole (DAPI)-containing aqueous mounting medium (Roti-Mount FluorCare DAPI, Roth, Germany) and a glass coverslip.

2.5 | Therapeutic efficacy study

Tumor resection was performed 19 days after tumor implantation under visual guidance. General anesthesia was performed before surgery (intraperitoneal injection of medetomidin [0.2 µg/g]/ketamine [0.1 mg/g] associated with a subcutaneous injection of buprenorphine [0.1 mg/kg]). Surgery consisted in a direct approach through an incision in regard to the tumor followed by the tumor dissection and resection. At the end, the skin was closed using PDS\(^{\text{II}}\) (polydioxanone 5.0 monofilament synthetic absorbable suture). Surgery was performed on extensive tumors with macroscopically positive surgical margins. After surgery, mice were weighed three times a week and bioluminescence imaging was performed once a week.

Twenty-three days after surgery, a total of 36 mice were randomized into three groups: control (n = 10), irradiation only (n = 13), and IV injection of AGuIX (200 µL at [Gd\(^{3+}\)] approximately 50 mmol/L) 1 hour before the irradiation (n = 13). Irradiation consisted of a single 10 Gy dose delivered with a radiation source operating at 200 keV with a 2 mm Al-filter. It was focused on the upper aero-digestive tract and performed at day 42. The mice were followed-up after irradiation as described before. Mice were sacrificed in case of advanced tumor stage (clinical signs: deterioration of the general status, isolation, cervical wounds or hematoma, tumor volume >600 mm\(^3\), and/or 20% weight loss).

2.6 | Statistical analysis

Statistical analyses were performed using the two-tail non-parametric Mann-Whitney t test. The results are expressed as the means ± SD for the biodistribution study and as the mean ± SE of the mean for the follow-up of tumor growth. To compare survival among different treatment groups, the Kaplan-Meier survival data were plotted versus time after tumor implantation. These data were subsequently analyzed using a log-rank test. In both cases, statistical significance was considered when P < .05.

3 | RESULTS

3.1 | Biodistribution study

The in vivo biodistribution of AGuIX labeled with cyanine 5.5 (AGuIX-Cy5.5) and their ability to accumulate in head and neck tumors were assessed following their IV injection in the tail vein of nude mice bearing orthotopic CAL33-Luc xenografts. After IV injection, AGuIX-Cy5.5 nanoparticles were distributed in the whole-body and were rapidly filtered by the kidneys before a final elimination through the bladder (Figure 1A,B). A weak hepatic accumulation was also observed. A fluorescent signal was detected in the CAL33-Luc orthotopic tumors from the first measurement point (30 minutes after injection) and up to 24 hours after injection. The maximum fluorescence tumor/skin ratio was observed 5 hours after IV administration of AGuIX-Cy5.5 and persisted 24 hours after (Figure 1C).

The tumor accumulation of AGuIX-Cy5.5 was then evaluated in another human head and neck orthotopic tumor model (SQ20B) using the same protocol. The similar biodistribution was observed with a rapid renal clearance followed by the excretion of the nanoparticles in the urine (Figure 2A, B). Passive accumulation into the tumors was detected in this model after 30 minutes with a maximum fluorescence tumor/skin ratio at 24 hours after IV injection (Figure 2C).

Twenty-four hours after IV injection, the amount of AGuIX-Cy5.5 semi-quantified in CAL33-Luc and SQ20B orthotopic tumors were comparable (approximately 2200 photons/pixel/500 ms).

Fluorescence confocal microscopy was performed on CAL33-Luc and SQ20B frozen tumor sections to confirm...
the presence of AGuIX-Cy5.5 in the tumors. For the two tumor models, the fluorescent nanoparticles were mainly found at the periphery of the tumors. At early time after injection (1 hour 30 minutes and 5 hours), punctuate fluorescent signals were detected on CAL33-Luc tumor slices corresponding to the presence of AGuIX-Cy5.5. The fluorescent signal was more diffuse at 24 hours and seemed to be located in the cytoplasm of the cells for the two tumor models (Figure 3).

3.2 | Radiosensitizing study

Tumor resection under visual guidance was performed 19 days after tumor implantation. Twenty-three days after resection, mice were exposed to conventional radiotherapy after IV injection of AGuIX (Figure 4).

During the follow-up, the control group exhibited a constant and progressive tumor growth. In contrast, in both irradiated groups, with or without AGuIX administration, a stabilization of tumor progression was observed after the radiotherapy. Then, around 10 days after radiotherapy, a constant progression of the tumor volume was observed in the irradiated only group, whereas in the irradiated group with AGuIX, tumor growth stayed stable (Figure 4).

The median survival time exhibited by the control group was 54 days and it was extended to 75 days in the irradiated only group and to 196 days in the irradiated group with nanoparticle injection (Figure 4). Combining AGuIX nanoparticles and irradiation induced an increase of survival of around 261% ($P < .03$) compared to the irradiated only group.

4 | DISCUSSION

In the last decade, the theranostic properties of the gadolinium-based nanoparticles AGuIX were evaluated on different tumor models. They were validated as a powerful contrast agent for multimodal imaging (MRI, SPECT, PET, and fluorescence imaging)\(^8\) and as radiosensitizer in vitro and in vivo, in many tumor models (melanoma, lung and brain tumors, etc), using various routes of administration and different irradiation protocols.\(^5,15,17\) AGuIX were also used for the therapeutic management of the orthotopic HNC model.\(^22\)
FIGURE 2  In vivo biodistribution of AGuIX-Cy5.5 in nude mice carrying orthotopic head and neck SQ20B tumors. A, two-dimensional-fluorescence images (200 ms integration time, contrast fixed between 1780 and 7240) obtained 30 minutes, 1 hour 30 minutes, 3 hours, 5 hours, and 24 hours after IV AGuIX-Cy5.5 nanoparticles injection (200 μL at [Gd³⁺] approximately 20 mmol/L). the red arrows indicate the presence of the nanoparticles in the left cheek tumor. B, biodistribution in organs 24 hours after administration of AGuIX-Cy5.5 nanoparticles. ROIs are defined on the extracted organs to semiquantify the amount of photons detected per pixel after a 500 ms time exposure. C, ratio of the tumor signal under the skin signal at different time points after a 500 ms time exposure on ventral position [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3  Distribution of fluorescent nanoparticles on the frozen sections of CAL33-Luc and SQ20B tumors at different time points after IV administration of AGuIX-Cy5.5 (200 μL at [Gd³⁺] approximately 20 mmol/L). tumors were observed by fluorescence microscopy (in blue: DAPI staining of the nuclei; in red: Cy5.5 signals). The white arrows show AGuIX-Cy5.5 signals [Color figure can be viewed at wileyonlinelibrary.com]
The advantage of orthotopic head and neck carcinoma animal models is that they take into account the cancer microenvironment, which can influence pathological and clinical features as well as the response to treatments, rendering them more representative of human HNCs. Several methods have been developed for orthotopic head and neck tumors setup. One of the most important consisted in cancer cells injection directly into the tongue of the mice. The tumors expanded quickly with this model and the mice had to be sacrificed 10 to 12 days after cells injection. Moreover, vital structures of the oral cavity were damaged by the hydrostatic pressure of the syringe during cells injection, and the mice had to be sacrificed after surgery. This short period of time did not allow long-term efficacy evaluation of new treatments. In our study, our previously developed HNC orthotopic animal model was used to evaluate the benefits of AGuIX. In this model, tumor fragment of human head and neck CAL33-Luc or SQ20B subcutaneous tumors were implanted in the oral cavity of mice. This tumor model is relevant and representative of human HNC. It presents slow tumor growth and allows studying of novel surgical or medical treatments.21

FIGURE 4  Orthotopic CAL33-Luc head and neck tumor model. A, scheme of the experimental irradiation protocol. B, tumors growth evaluated by bioluminescence imaging obtained from CAL33-Luc head and neck tumors bearing mice after tumor removal surgery without treatment (n = 10), treated by irradiation only (n = 13), treated by irradiation 1 hour after IV injection of AGuIX (n = 13). Irradiation (10 Gy) was performed at day 23 after resection. C, survival curves from CAL33-Luc head and neck tumors bearing mice after resection without treatment (blue, n = 10), treated by irradiation only (orange, n = 13), treated by irradiation 1 hour after IV injection of AGuIX (green, n = 13). Irradiation (10 Gy) was performed at day 23 after resection [Color figure can be viewed at wileyonlinelibrary.com]
fluorescent, and we assumed that AGuIX and AGuIX-Cy5.5 have the same biodistribution and that AGuIX are also present in tumors.8,18

Conversely, it is expected that this passive targeting combined with an active targeting should provide a more efficient accumulation of AGuIX in the tumors. The Arg-Gly-Asp (RGD) sequence, an arginine-glycine-aspartic acid tripeptide, is well known to be highly selective for αvβ3 integrins. The αvβ3 integrins are particularly interesting because they are expressed both on the surface of sprouting vessels and in 25% of human cancers of different types including HNC.20 Many studies have demonstrated that a multivalent presentation of RGD peptides can significantly improve the binding affinity toward αvβ3 integrins and enhance tumor-targeting capabilities. The presence of RGD onto the nanoparticles AGuIX could also increase their active tumor accumulation.20

In this study, tumor resection was performed 19 days after tumor implantation on extensive and invasive tumors located in the cheek of mice explaining limited surgery with incomplete resection and positive surgical margins. Although surgery remains the standard treatment of HNC, tumor volume at this period was important, thus not allowing complete tumor resection. A postoperative radiotherapy was therefore recommended. The delay between surgery and radiotherapy was of 23 days to permit a correct healing process while avoiding tumor growth. During this period, massive inflammatory phenomena with increased vascularity are involved to permit a complete healing before irradiation.

The CAL33-Luc cell line was described as a radiosensitive cell line and therefore promising to evaluate the radiosensitizing effect of the nanoparticles. In this study, after literature review, the radiotherapy was performed as at a single dose of 10 Gy.22–26 We assumed that after resection, AGuIX nanoparticles could accumulate in the postoperative remaining tumor cells aided also by postoperative inflammation. The demonstrated passive accumulation of AGuIX within tumors suggested that a radiosensitizing effect could be expected even after incomplete tumor resection. In our study, statistically significant improvement of the radiotherapy efficiency was shown using gadolinium-based nanoparticles AGuIX. All the irradiated groups presented a significant slower tumor growth compared to the control group. Nevertheless, 10 days after the radiotherapy, tumor volume started to progress in the irradiated only group. There was a statistically significant difference between the irradiated only group and the irradiated group with AGuIX nanoparticles injection. Median survival time was prolonged more than two times in irradiated group with nanoparticles injection compared to irradiated only group.

In this study, we observed a potential complication of irradiation in only one mouse which belongs to irradiated only group. No additional complication of the radiotherapy was observed for the mice irradiated in presence of nanoparticles.

Obviously, other irradiation protocols could now be evaluated, either by changing the radiation dose or by increasing the amount of injected nanoparticles, or by modifying the delay between the injection of nanoparticles and the irradiation, or by using fractionated irradiation protocols.

In the literature, Bozec et al. applied on orthotopic CAL33 tumor models, fractionated doses of radiotherapy, to optimize its efficacy and reduce side effects. Mice were exposed to conventional radiotherapy 3 days a week at 6 Gy. In total, four doses of radiotherapy were delivered.23,24

This technique conventionally used in clinical practice could be tested in our study. It allows delivering over a longer time low doses of radiation per session to avoid radiotherapy toxicity.

5 CONCLUSION

AGuIX gadolinium-based nanoparticles show real theranostic properties: a safe behavior after IV injection with a rapid renal clearance, an adequate tumor accumulation, a good contrast enhancement for medical imaging (MRI and fluorescence imaging), and a therapeutic activity (near-infrared fluorescent imaging-guided surgery and radiotherapy potentiation). In this first trial, the enhancement of radiotherapy by the nanoparticles after incomplete tumor resection was demonstrated in a HNC orthotopic animal model. The use of these nanoparticles could be the first step toward a personalized therapy, combining tumor diagnosis by MRI, fluorescence imaging-guided surgery, and radiotherapy potentiation.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

REFERENCES


