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Early detection of squamous cell carcinoma in carcinogen induced oral cancer rodent model by ratiometric activatable cell penetrating peptides

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ABSTRACT

Objectives: Ratiometric cell-penetrating-peptides (RACPP) are hairpin-shaped molecules that undergo cleavage by tumor-associated proteases resulting in measurable Cy5:Cy7 fluorescence ratiometric change to label cancer *in vivo*. We evaluated an MMP cleavable RACPP for use in the early detection of malignant lesions in a carcinogen-induced rodent tumor model.

Methods: Wild-type immune-competent mice were given 4-nitroquinoline-oxide (4NQO) for 16 weeks. Oral cavities from live mice that had been intravenously administered MMP cleavable PLGC(Me)AG-RACPP were serially imaged from week 11 through week 21 using white-light reflectance and Cy5:Cy7 ratiometric fluorescence.

Results: In an initial study we found that at week 21 nearly all mice (13/14) had oral cavity lesions, of which 90% were high-grade dysplasia or invasive carcinoma. These high-grade lesions were identifiable with white light reflectance and RACPP Cy5:Cy7 ratiometric fluorescence with similar detectability, Area Under Curve (AUC) for RACPP detection was 0.97 (95% Confidence interval (CI) = 0.92–1.02, $p < 0.001$), sensitivity = 89%, specificity = 100%. In a follow up study, oral cavity lesions generated by 4NQO were imaged and histologically analyzed at weeks 16, 18 and 21. In this study we showed that RACPP-fluorescence detection positively identified 15 squamous cell carcinomas (in 6 separate mice) that were poorly visible or undetectable by white light reflectance.

Conclusions: RACPP ratiometric fluorescence can be used to accurately detect carcinogen-induced carcinoma in immunocompetent mice that are poorly visible or undetectable by white light reflectance.

Introduction

Head and neck squamous cell carcinoma (HNSCC), which comprises cancer of the oral cavity, pharynx and larynx, is the 6th leading malignancy worldwide. With a reported annual burden of 633,000 incident cases, 355,000 deaths, and a 5 year overall survival rate ~60%, patient prognosis remains poor as diagnosis often occurs late into disease progression when advanced stage cancer is unresponsive to therapy [1–3]. Established risk factors for HNSCC include tobacco use and alcohol consumption [4]. Human Papilloma Virus (HPV) status has also been identified as a risk factor for HNSCC [5,6]. Initial detection can present as ulcerations or exophytic growths with [7] conclusive diagnosis made by histological examination of biopsied lesions. Poor clinical diagnosis is partly due to the high degree of variability in visual presentation of lesions and the lack of simple reliable non-invasive methods to accurately identify potentially cancerous lesions.

A carcinogen induced mouse model of oral cavity cancer, (the most common type of HNSCC), has been reported using the addition of 4-nitroquinoline oxide (4NQO) to drinking water [10]. This chemical carcinogen promotes formation of DNA adducts similar to those induced by tobacco and other carcinogens. It has been shown that the activation of PIK3-AKT-mTOR pathway, which is a known precursor to MMP2 and 9 expression, occurs in this model. Mutations and change in expression of p53, which are also known precursors for elevated MMPs, were found in a rat model of 4NQO mediated SCCA [11]. At a cellular level, 4NQO induces various stages of carcinogenesis including dysplasia, neoplasia, in situ

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carcinoma, and squamous cell carcinoma (SCC) [12,13]. For these reasons it is thought that such an animal model would better mimic the molecular pathogenesis of human disease compared to more widely used xenograft and transgenic animal models [14].

Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expression have been shown to correlate with cancer grade [15] and decreased patient survival [16,17]. In carcinomas of the tongue, increased MMP2 and 9 expression correlates with an increased incidence of lymph node metastases [18]. Furthermore, the orthology of MMP 2 and 9 in human and mouse have been well established [19], suggesting that a molecular probe designed to detect these MMPs in animal models could be translated for clinical applications. Ratiometric ACPPs (RACPPs) undergo a change in the ratio of Cy5:Cy7 fluorescent emission upon cleavage by proteases. The cationic fragment of the RACPP is retained at the activation site, typically within the tumor. We have previously reported the use of MMP activatable cell penetrating peptides (ACPPs) probes for image guided surgery of tumors in various animal models of breast cancer [20], pancreatic cancer [21], melanoma and head and neck xenografts [22,23]. We have also demonstrated significant correlation of ratiometric fluorescence (Cy5:Cy7) from the MMP cleavable RACPP with tumor burden in a pancreatic tumor mouse model [21]. Concentration of other MMPs including, MMP 1 and 3 in human saliva and oral cancer are strong diagnostic markers of oral SCCA [8]. There is also some evidence to suggest that MMP7 gene expression is associated with early stages of oral cancer [9].

Here we report the use of serial MMP sensitive RACPP imaging to detect oral cavity SCCA induced by 4NQO in drinking water of mice. Additionally, we report the ability of MMP sensitive RACPP to highlight small SCCA lesions that are poorly visible or non-detectable using white light reflectance. The ability to detect SCCA before it becomes apparent on white light inspection suggests that this technology might be leveraged for early detection of SCCA of the head and neck.

Methods

All animal studies were approved by the UCSD Institutional Animal Care and Use Committee protocol #S04011.

Reagents

4NQO

Stock solution: A concentrated stock solution was made by dissolving 4NQO (Sigma Aldrich) in propylene glycol or DMSO (Sigma Aldrich) at a concentration of 4 mg/mL as previously described [10]. The stock solution was stored at 4 °C and diluted into the drinking water at a final concentration of 50 μg/ml.

Vehicle: Initial experiments (Cohort 1) were performed with 4NQO dissolved in propylene glycol. During the course of the experiment, we noted that the use of propylene glycol as a vehicle for 4NQO delivery resulted in non-specific fluorescent signal in the dentition following injection of RACPP. To minimize this nonspecific signal, we tested stock solutions of 4NQO made in other solvents including ethanol (2 mg/ml), PEG 300 (4 mg/ml), DMSO (4 mg/ml). The intensity of non-specific fluorescence signal of the dentition was greatest in mice given propylene glycol and least in mice given DMSO (Supplemental Fig. 1). Subsequent experimental animals (part of cohort 2) were administered with 4NQO using DMSO as vehicle.

RACPP: The peptide sequence H_2N-e_9-c(SS*t*Bu)-o-PLGC(Me)AG-r_9-c-amide was synthesized using standard FMOC chemistry. Peptide was purified by HPLC and reacted with Cy5-maleimide in presence of N-methylmorpholine (NMM) in dry DMSO. Following this, triethylphosphine (TEP) was added in excess for deprotection of the d-cysteine (i.e. removal of –StBu to generate the thiol). This compound was precipitated in 80:20 hexane:EtOAc and purified by HPLC using a C18 column. Thereafter, the peptide was reacted with peg_{12}-maleimide (Quanta Biodesign) in presence of NMM followed by purification with HPLC. Peptide was then reacted with Cy7-NHS ester in dry DMSO with NMM for 24 h to obtain MMP cleavable RACPP: (Cy7)-e_9-c(peg_{12})-o-PLGC(Me)AG-r_9-c(Cy5) [24]. The selectivity of PLGC(Me)AG-RACPP for MMP2 and MMP9 and other related enzymes is highlighted in Supplemental Fig. 3.

Experimental model

In experimental cohort 1, 15 female C57Bl/6 mice, 4–6-weeks of age, weighing 18–20 g, and 5 age, gender and species matched control mice were used. The 15 experimental mice were given 4NQO in their drinking water for 16 weeks while control mice were given vehicle only (Fig. 1). At the end of 16 weeks, all mice were converted to drinking water without 4NQO and vehicle until the study endpoint at 21 weeks. One mouse died at week 7 for reasons unrelated to oral cavity tumor development and was excluded from analysis. Mice were evaluated for tumor growth every 2 weeks from week 11 to week 21. In each case the mice were anesthetized with isoflurane (2% in O_2) and the oral cavity was imaged using both white light reflectance and Cy5:Cy7 ratiometric fluorescence. Oral cavity examinations were performed ninety minutes after intravenous injection of 10 nmoles of MMP sensitive RACPP

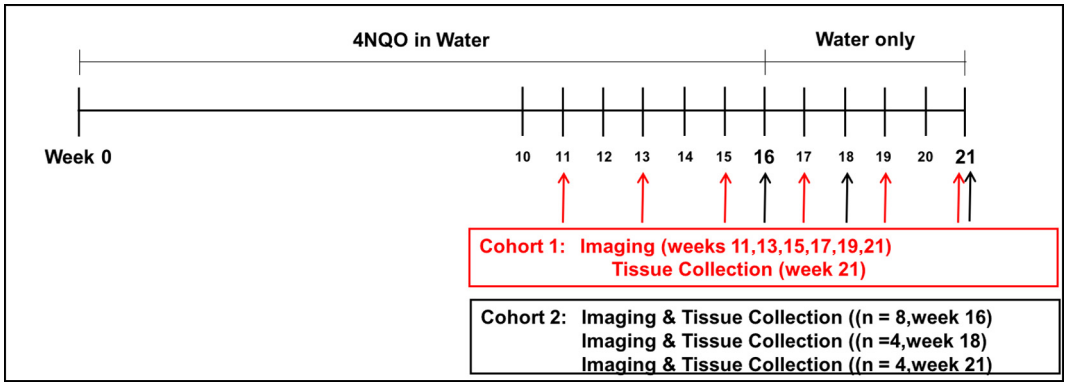

Fig. 1. Experimental scheme. Cohort 1: A cohort of 14 mice were given 4NQO in their drinking water for 16 weeks at which time they were converted to regular drinking water until week 21. White light and ratiometric fluorescent (RF) imaging was done biweekly starting at 11 weeks, continuing until week 21 at which time tissue was collected for histological analysis. Cohort 2: A cohort of 16 mice were given 4NQO in their drinking water for 16 weeks followed by single time point imaging and tissue collection at week 16 (n = 8), week 18 (n = 4) and week 21 (n = 4).