Translational Oncology
www.transonc.com

Volume 7 Number 2 pp. 213–220 213

Molecular Imaging of Oral Premalignant and Malignant Lesions Using Fluorescently Labeled Lectins

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Abstract

Aberrant glycosylation during carcinogenesis results in altered glycan expression on oral cancer cells. The objective of this study was to detect this atypical glycosylation *via* imaging of fluorophore-conjugated lectins. Paired normal and tumor tissue from seven patients with oral squamous cell carcinoma were investigated for sialic acid expression *via* the legume protein wheat germ agglutinin (WGA). Fluorophore (Alexa Fluor 350 and Alexa Fluor 647) conjugated WGA was topically applied to the tissue samples and imaged using a custom wide-field fluorescence imaging system. All seven patients had histologically confirmed disease with 6/7 exhibiting squamous cell carcinoma and 1/7 exhibiting dysplasia. Fluorescent data collected from all patients demonstrated that fluorophore conjugated WGA could distinguish between pathologically normal and diseased tissue with the average signal-to-noise ratio (SNR) among all patients being 5.88 (P = .00046). This SNR was statistically significantly higher than the SNR from differences in tissue autofluorescence (P = .0049). A lectin inhibitory experiment confirmed that lectin binding is molecularly specific to overexpressed tumor glycans and that fluorescence is not due to tissue optical properties or tissue diffusion differences. These results illustrate that changes in tumor glycan content of oral neoplasms can be detected with optical imaging using topically applied fluorescently labeled WGA. Lectin targeting of oral lesions using optical imaging may provide a new avenue for the early detection of oral cancers.

Translational Oncology (2014) 7, 213–220

Introduction

Oral cancer, which includes cancers of the lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx (throat), is the sixth most common cancer nationally and the third most prevalent cancer in developing countries [1–3]. Oral cancer's five year survival rate has slightly increased over the past four decades to 65% in 2009; however, unfortunately its increase has not improved as much as other cancers over the same period [3]. This is because clinicians face considerable challenges in visually identifying oral neoplasia at an early stage, leading to many diagnoses occurring late in neoplasia progression [3,4].

Currently disease progression, surgical margins, metastasis and extent of invasion are decided based on diagnostic methods such as X-rays, CT scans or PET images carried out prior to surgery [5,6]. These techniques, though clinically useful, have safety concerns, cannot predictably detect tumors less than 1 cm in diameter (equating to greater than 1 million cancerous cells), and cannot be generated in real time to guide the surgeon intra-operatively.

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Received 16 December 2013; Revised 16 December 2013; Accepted 20 December 2013

1936-5233/14 http://dx.doi.org/10.1016/j.tranon.2014.02.006

In recent years, there have been a number of scientific approaches to the problem of oral lesion detection (i.e. ViziLite, VELscope, Trimira, OralCDx, etc.). However, the effectiveness of these technologies is inconsistent [5,7]. The literature suggests that these modalities fail to noticeably improve the detection of oral carcinomas from standard head and neck examinations routinely performed by physicians [7]. A major reason for the inconsistency, poor specificity and inability to detect earlier stage cancer is the oversight of these technologies to target advanced stage anatomical changes instead of early stage molecular level alterations.

Optical molecular imaging provides a non-invasive, *in vivo*, rapid and cost effective method to detect early molecular level changes in neoplastic tissue, based on its ability to specifically analyze molecules of interest. More importantly, optical molecular imaging can be performed with minimum training, increasing its potential to be used in the general physicians' office. Possible targets for optical imaging are the glycoproteins and glycolipids on the cell surface. These cellular glycomolecules are completed during the post-translational event called glycosylation, which is known to be abnormal in human disease progression such as carcinogenesis and metastasis [8–10]. This irregular glycosylation results in varying glycosyl residues on the cell surface during pathological changes, highlighting the clinical importance of this alteration as a potential target by which to detect oral cancer.

A prime example of aberrant glycosylation in carcinogenesis is the overexpression of sialyl Lewis A and sialyl Tn antigen in cancers of the pancreas, colon, stomach and esophagus [11,12]. Moreover, increased sialytransferases and sialic acid content on cell glycoconjugates has long been linked to oral cancer and malignant transformation [13,14]. Increased sialic acid content can reach up to 10e+09 sialic acid residues per tumor cell [15]. Further, Rajpura et al. showed statistically significantly higher levels of sialic acid in oral cancer patients compared to normal patients (63.70mg/dl versus 30.25mg/dl, respectively; $P < .001$, 41 patients) [16]. Silvia et al. and Joshi et al. showed similar significant results for sialic acid overexpression in oral cancer patients [17,18].

Specific glycan changes can be targeted using lectins. Lectins are proteins or glycoproteins of non-immune origin that bind non-covalently to specific oligosaccharide chains extending extracellularly from glycoproteins or glycolipids [19]. Lectins exhibit high specificity in recognizing their specific sugar moieties, and thus are useful analytical tools to study the alterations in cell surface carbohydrates in diseased stages [15,19–21]. The other advantages of using lectin probes are the ease of production due to their abundance, inexpensiveness, ease of labeling with fluorescent probes, heat stability, stability at low pH, and low toxicities as many are part of the normal human diet [22].

As sialic acid residues are overexpressed during carcinogenesis, an appropriate lectin probe specific to sialic acid could provide an advantageous biomarker for oral cancer detection. One particular lectin of interest is the legume wheat germ agglutinin (WGA), which is a carbohydrate-binding lectin of approximately 36 kDa that selectively recognizes sialic acid and N-acetylglucosaminyl sugar residues [11,14,22]. Furthermore, conjugation of this lectin with a fluorophore could provide an effective non-invasive *in vivo* screening method to visualize premalignant and malignant oral lesions in real time.

The objective of our study was to establish a preclinical screening technique that targets an intrinsic fluorophore, nicotinamide adenine dinucleotide (NAD^+/NADH), and sialic acid expression, using fluorescent conjugated WGA, to screen for oral cancers. This proof-of-concept preclinical study will be used to guide later clinical evaluation studies.

Materials & Methods

Clinical Sample Set

Freshly extracted tissue samples were obtained either from patients diagnosed with oral cancer or from scalpel biopsies acquired from patients suspected of having oral cancer. In addition, punch biopsies were acquired from patients suspected to have oral cancer, which entered the study *via* the walk-in clinic. All seven patients gave their written informed consent to participate, and the study was reviewed and approved by the Institutional Review Boards at the University of Minnesota and the Mazumdar Shaw Cancer Center in Bangalore, India. Paired biopsies of clinically normal and abnormal oral mucosa were acquired with patient morbidity in mind, and did not deviate from normal clinical practice (Figure 1). Normal tissue biopsies either came from tissue adjacent to the surgical margin or from a slight extension of suspicious lesion margin (Figure 1). Upon extraction, tissue samples were placed in 1× phosphate buffered saline (PBS) (Sigma Aldrich, Milwaukee, WI) to prevent dehydration and then were immediately used for testing. All materials were used as received, unless noted otherwise.

Topical Application of Lectin Probe

To initially demonstrate the efficacy of fluorescently labeled lectins, Alexa Fluor 647 conjugated WGA (AF647-WGA) (Invitrogen, Carlsbad, CA) was used to target sialic acid residues on the cells' surfaces. This specific fluorophore was used since tissue autofluorescence is minimal in the far red and near-infrared spectrums [23]. Specifically, AF647-WGA (5μM titration in 1×PBS, pH 7.4) was topically applied to the tissue samples in the presence of 10 v/v% dimethylsulfoxide (DMSO) (Sigma Aldrich, Milwaukee, WI). DMSO was used as a permeation enhancer to improve delivery of the lectin conjugate through the epithelium of the tissue samples.

Alexa Fluor 350 conjugated WGA (AF350-WGA) (Invitrogen, Carlsbad, CA) (20μM titration in 1×PBS, pH 7.4, and 10 v/v% DMSO) was then used to demonstrate that analogous results could be obtained in the UV spectrum. The molar concentration of AF350-WGA was increased compared to AF647-WGA to overcome possible autofluorescence background signals. Furthermore, the use of a UV fluorophore allowed for the direct comparison of tissue autofluorescence to the fluorescence of AF350-WGA binding. Briefly, tissue autofluorescence in the UV spectrum at 365nm is largely due to an endogenous fluorophore called nicotinamide adenine dinucleotide (NAD^+/NADH) [24,25]. This physiologically important coenzyme is interesting in the fact that its reduced form (NADH) is fluorescent at 365nm whereas its oxidized form (NAD^+) is not. Due to changes in metabolism during oral carcinogenesis, oral cancer cells have lower levels of NADH [24,25]. To establish an autofluorescence background value at 365nm, epi-illumination (reflectance) images were acquired from the tissue samples under narrow band illumination of UV light using a 365nm ± 7.25nm LED (Opto Technology Inc., Wheeling, IL).

Both Alexa Fluor conjugated WGA molecules were subjected to the following protocols which were slightly modified according to the tissue type of the patients. Pilot experiments were conducted to