Folate receptor beta targeting for in vivo optical imaging of head and neck squamous cell carcinoma

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Introduction

In the United States, head and neck cancer has an annual incidence of over 52,000 new cases and causes over 11,000 annual deaths. Squamous cell carcinoma of the head and neck (HNSCC) represents 90% of all head and neck cancers. The surgical resection of head and neck cancers requires high precision to maximize tumor resection without compromising the many complex functions carried out in the oral cavity and neck. Techniques to improve the intraoperative visualization of tumors are needed to increase the efficacy and decrease the morbidity of HNSCC resection.

The folate receptor (FR) is a high-affinity folic acid binding endocytic receptor uncommonly expressed in normal tissues. The α isoform (FR-α) is found in the some epithelial tissues such as the salivary gland and is overexpressed in a variety of epithelial neoplastic cells. In contrast, functional expression of the β isoform (FR-β) is normally limited to only activated macrophages. Importantly, in many malignancies FR serves as a target for the delivery of tumor specific drugs and imaging markers. Folic acid conjugated fluorescent dyes have been used to guide the resection of FR expressing tumors in mouse models and humans.

However, the potential utility of FR in HNSCC is unclear due to an incomplete characterization of the receptor's expression. FR-α mRNA expression has been reported to be low in HNSCC. Nevertheless, in one patient sample, FR expression of an unknown isoform was detected in 45% of primary tumors. Furthermore, FR expression has been inversely correlated with disease-free survival in HNSCC patients in general and with overall survival in those with lymph node metastasis. We hypothesized that tumor infiltrating macrophages expressing FR-β could allow fluorescent visualization of HNSCC tumors using folate conjugated dyes even when FR expression in cancer cells is low.

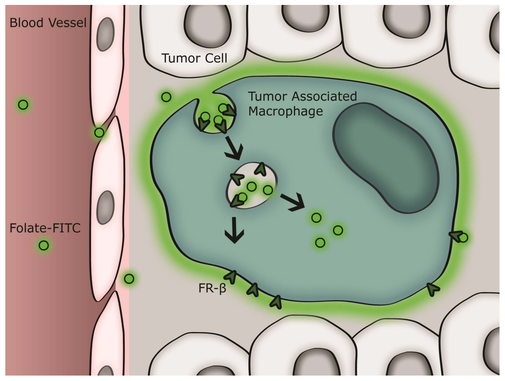

Figure 1. Mechanism of HNSCC tumor targeting by folate-FITC. Folate-FITC is an always on fluorescent small molecule administered intravenously. Upon reaching the inherently leaky tumor vasculature, the probe can exit into the tumor microenvironment. There, tumor associated macrophages expressing FR-β engulf folate-FITC via receptor mediated endocytosis. The probe dissociates from its binding receptor within endocytic vesicles and becomes internalized by the cell. FR-β is finally recycled back to the cell surface.

Subjects and Methods

A tissue microarray (TMA) was constructed with the primary tumor tissue and matched tumor free surgical margins from 22 patients who underwent HNSCC resection (Table 1).

Case#/Age	Stage	Primary Tumor Location
1/84	T1N0M0	tongue
2/60	T1N2aM0	tongue
3/59	T2N1M0	tongue
4/52	T2N2cM0	tongue
5/52	T2N2cM0	tongue
6/43	T3N0M0	tongue
7/50	T3N1Mx	tongue
8/56	T3N2bM0	tongue
9/64	T3N2cM0	tongue
10/62	T4N2bM0	tongue
11/68	T4N2cM0	tongue
12/68	T1N0M0	tonsil
13/59	T1N1M0	tonsil
14/52	T2N0M0	tonsil
15/77	T3N2bM0	tonsil
16/71	T3N1M0	supraglottic larynx
17/62	T3N2M0	supraglottic larynx
18/53	T3N1M0	glottic larynx
19/71	T3N1M0	glottic larynx
20/52	T4aN0Mx	glottic larynx
21/59	T4aN2M0	glottic larynx
22/74	T2N0M0	hypopharynx

Table 1. Characteristics of TMA specimens.

Immunohistochemistry was performed to evaluate the expression of FR-α, FR-β, transforming growth factor-β (TGF-β), the macrophage marker CD68 and the alternatively activated macrophage marker arginase-1 (arg1) using appropriate positive and negative controls for staining. A pathologist (J.T.) scored the FR-α and FR-β staining of the specimens. Staining intensity (0+, 1+, 2+, 3+) and area of specimen stained (0%, <1%, 1-100%) were determined. The overall staining pattern of CD68, TGF-β and arg1 was compared with that of FR-α and FR-β.

To examine the use of folate targeting for image guided surgery, orthotopic xenograft HNSCC tumor models were generated from mice. Injections of HN5 or FaDu HNSCC tumor cell lines were made into the submental triangle of nude and SCID mice respectively. Tumors were allowed to grow for 2-3 weeks. The mice received 0.8 mg/kg intravenous injections of fluorescein isothiocyanate conjugated folate (Folate-FITC) and were imaged for fluorescent emission under 495nm light two hours later. Mouse tissues were then sectioned for examination using fluorescent microscopy.

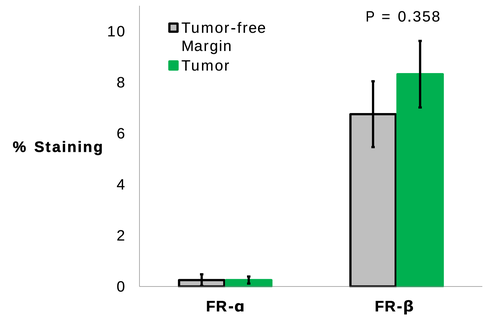

Figure 2. FR-α and FR-β staining area in TMA specimens. Mean percentage of specimen area stained ± SE. Tumor samples were compared with specimens from tumor-free surgical margins. Paired comparisons made with the Wilcoxon signed-rank test.

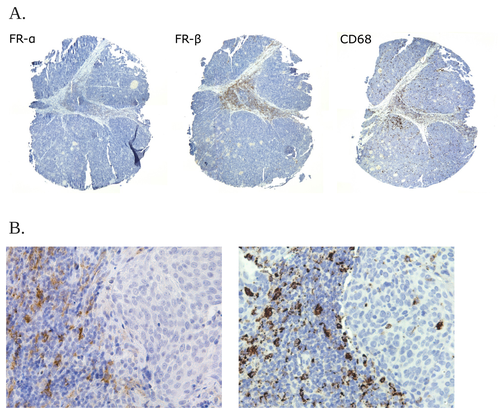

Figure 3. (A) TMA staining of tumor specimen for FR-α, FR-β and CD68. No FR-α expression seen. FR-β staining occurs only in inflammatory regions and overlaps with a subset of CD68$^+$ macrophages. (B) Higher magnification view of FR-β and CD68 staining. FR-β$^+$ cells do no occur within nests of neoplastic cells but rather in the adjacent inflammatory stroma.

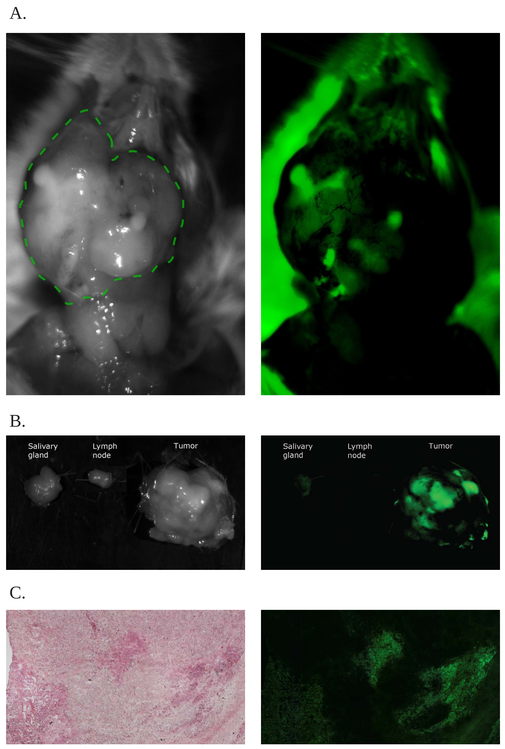

Figure 4. Folate-FITC targeting in tumor xenograft model. (A) Ventral *in vivo* view of mouse head and neck with orthotopic tumor xenograft after folate-FITC injection. White light image on left with tumor outlined in green. Same view under fluorescent light on right. Fur in the background exhibits auto-fluorescence unrelated to folate-FITC uptake. (B) *Ex vivo* view of resected tumor, salivary gland and normal lymph node. Minimal fluorescent signal is detected in the salivary gland and no signal is present in the lymph node. (C) Scanning view of tumor section with H&E staining (left) and fluorescent microscopy (right). Folate-FITC uptake is seen in regions of inflammation and necrosis.

Results

TMA:

- FR-α expression was absent or minimal in tumor specimens (mean staining area <1%, intensity 0+).
- All 22 tumors sampled were positive for FR-β (mean staining area 8.3%, intensity 3+).
- FR-β$^+$ cells in HNSCC are non-neoplastic CD68$^+$ tumor associated macrophages located in the tumor stroma
- No association was observed between FR-β staining and either TGF-β or arg1 staining.

Tumor xenografts:

- Tumors showed strong fluorescence in vivo after folate-FITC injection.
- Mean area of folate-FITC uptake was 25% of visible tumor area. Mean signal amplification over normal tissue was 12-fold.
- Normal tissues did not have significant folate-FITC uptake
- Consistent with our TMA data, fluorescence within the tumors was confined to areas of inflammatory cell infiltration.

Conclusion

HNSCC tumors contain a significant population of FR-β expressing macrophages. The previously reported prognostic value of FR expression in HNSCC may be due to the receptor's ability to act as a marker for tumor associated macrophages, which have been linked to the promotion of tumor progression. In contrast to many other carcinomas, the HNSCC tumor cells in our TMA did not express FR-α. Despite this, folate conjugated FITC dye was able to target and specifically label tumor xenografts in mice, allowing for macroscopic fluorescence imaging. The folate linked delivery of fluorescent dye into the tumor microenvironment can facilitate image guided surgery even when HNSCC tumor cells themselves do not express FR. Further refinement of a folate probe through its use in combination with other tumor microenvironment targeting strategies can increase tumor imaging efficacy.

References

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Acknowledgments

This study was supported by the Cancer Prevention and Research Institute of Texas (RP120094) and the UT Southwestern Summer Medical Student Research Program.