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DSG3 as a biomarker for the ultrasensitive detection of occult lymph node metastasis in oral cancer using nanostructured immunoarrays

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SUMMARY

Objectives: The diagnosis of cervical lymph node metastasis in head and neck squamous cell carcinoma (HNSCC) patients constitutes an essential requirement for clinical staging and treatment selection. However, clinical assessment by physical examination and different imaging modalities, as well as by histological examination of routine lymph node cryosections can miss micrometastases, while false positives may lead to unnecessary elective lymph node neck resections. Here, we explored the feasibility of developing a sensitive assay system for desmoglein 3 (DSG3) as a predictive biomarker for lymph node metastasis in HNSCC.
Materials and methods: DSG3 expression was determined in multiple general cancer- and HNSCC-tissue microarrays (TMAs), in negative and positive HNSCC metastatic cervical lymph nodes, and in a variety of HNSCC and control cell lines. A nanostructured immunoarray system was developed for the ultrasensitive detection of DSG3 in lymph node tissue lysates.
Results: We demonstrate that DSG3 is highly expressed in all HNSCC lesions and their metastatic cervical lymph nodes, but absent in non-invaded lymph nodes. We show that DSG3 can be rapidly detected with high sensitivity using a simple microfluidic immunoarray platform, even in human tissue sections including very few HNSCC invading cells, hence distinguishing between positive and negative lymph nodes.
Conclusion: We provide a proof of principle supporting that ultrasensitive nanostructured assay systems for DSG3 can be exploited to detect micrometastatic HNSCC lesions in lymph nodes, which can improve the diagnosis and guide in the selection of appropriate therapeutic intervention modalities for HNSCC patients.

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Introduction

With more than 500,000 new cases annually, squamous cell carcinomas of the head and neck (HNSCC) represent one of the ten most common cancers globally,[1] and result in more than 11,000 deaths each year in the US alone.[2] The 5 year survival of newly diagnosed HNSCC patients is ~50%, and despite new treatment approaches, it has improved only marginally over the past decades.[3] HNSCC has a high propensity to metastasize to loco-regional lymph nodes due to the presence of a rich lymphatic network and the overall high number of lymph nodes in the neck region.[3–8] Even in patients without clinical evidence of lymph node involvement (N0), there is a high incidence of occult lymph node metastasis, ranging from 10% to 50%.[4,5,7] The diagnosis of cervical lymph node metastasis an essential requirement for clinical staging and treatment,[9] and is now widely accepted as the most important factor in HNSCC prognosis.[3,5,6,10] However, due to limitations in the accurate diagnosis of lymph node metastasis, patients with clinically negative nodes often undergo elective neck resection or radiation,[11,12] with the consequent associated morbidity and adverse impact in the quality of life.[12]

Clinical assessments of lymph node metastases include physical examination, imaging modalities such as computed tomography (CT), magnetic resonance imaging (MRI), ultrasonography, and

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[^{18}F]-2-fluorodeoxyglucose positron emission tomography scans (PET).[13,14] However, poor spatial resolution, false positive detection of reactive lymph nodes, and limited sensitivity under 5 mm size,[15–18] can add to potential false negative results. Histopathological, immunohistochemical (IHC), and molecular approaches to evaluate sentinel lymph node biopsies have improved the detection rate of metastatic disease in some cancers,[19,20] but histopathology-based methods can often miss micrometastases, while more sensitive techniques such as IHC and real-time PCR for validated cancer markers are time consuming and require stringent handling procedures and technical expertise.

A recent proteomic analysis of paraffin embedded normal oral mucosa and HNSCC lesions revealed a very high abundance of Desmoglein 3 (DSG3) in both non-neoplastic epithelium and cancer lesions.[21] DSG3 is a transmembrane glycoprotein involved in cell-to-cell adhesion that is exclusively expressed in stratified epithelium.[22] These observations prompted us to explore whether the assessment of DSG3 protein levels could be used to investigate the presence of malignant squamous epithelial cells in cervical lymph nodes, and hence serve as a predictive biomarker for metastasis. In this regard, high sensitivity electrochemical immunoassays have recently gained acceptance in biomedicine.[23] For example, we have developed immunosensors based on nanostructured electrodes coupled to microfluidics and multilabel strategies to achieve highly sensitive detection of protein cancer biomarkers in serum.[24,25] We have combined these strategies into a simple microfluidic immunoarray[26,27] and here explore the suitability of this platform for the rapid and sensitive detection of DSG3 protein. We show that this system can be used to rapidly detect and quantify DSG3 in frozen human tissue sections, distinguishing between clinically positive and negative cervical lymph nodes. Overall, these studies may help develop point-of-care procedures aiding in the diagnosis of invaded lymph nodes in HNSCC patients, thereby facilitating educated decisions regarding appropriate therapeutic intervention modalities, and decreasing the morbidity often associated with HNSCC.

Materials and methods

Reagents, antibodies, and cell culture

All chemicals and reagents were from Sigma–Aldrich (St. Louis, MO), unless indicated. The following antibodies: goat-anti-human DSG3 [AF1720]; mouse-anti-human DSG3 [MAP1720], biotin labeled goat-anti-human DSG3 [BAF1720], recombinant human DSG3 Fc Chimera protein [1720-DM], were from R&D Systems (MN, USA). The mouse anti-human DSG3 antibody [32-6300] from Invitrogen (MA, USA), and rabbit-anti-cytokeratin Wide Spectrum Screening [N1512] from Dako (CA), were used for immunohistochemistry (IHC). The α-tubulin antibody [11H10] was from Cell Signaling Technology (MA, USA). Biotinylated peroxidase and streptavidin coated magnetic beads were from Invitrogen. Anti-rabbit and anti-mouse biotinylated secondary antibodies were from Vector, Burlingame, CA, US. HN12, HN13 and HN30 cells were described previously.[28] Cal27 and Jurkat cells were from ATCC (VA); and primary human cells from Lonza (MD). See Supplemental information for additional information.

Human clinical tissues and tissue microarrays (TMAs), immunohistochemistry and immunofluorescence

Formalin fixed, paraffin-embedded, and freshly frozen HNSCC and lymph node samples were obtained anonymized with Institutional Review Board approval. Five μm sections from all tissues underwent standard H&E staining for histopathological evaluation and immunostaining. Tissue microarrays used include TMA MC2081 US (Biomax, MD) with 208 representative cases of colorectal, breast, prostate and lung cancers, and normal tissue; TMA LC810 (Biomax, MD), consisting of 40 cases of different types of lung cancers with their matched metastatic lymph nodes (total 80 tissue cores); and the Head and Neck Tissue Microarray Initiative, including 317 HNSCC cases.[29] Tissue processing and analysis are described in detail in Supplemental information. All slides were scanned at 400× magnification using an Aperio CS Scanscope (Aperio, CA) and quantified using the available Aperio algorithms. Immunodetection of DSG3 was quantified according to percent of tumor cells stained (1–25%, 26–50%, 51–75%, or 76–100%).[29] For immunofluorescence, 10 μm cryosections were immunostained with goat-anti-human DSG3 (AF1720), mouse-anti-vimentin and DAPI containing. See Supplemental information for additional details.

Western blot analysis of cell and tissue extract, and microfluidic immunoarrays systems for DSG3

A detailed description of the procedures used for tissue lysate preparation, SDS–PAGE gel analysis and Western blotting, and the fabrication of the microfluidic immunoarrays made of gold nanoparticles layered with glutathione are described in detail in the Supplemental information. Briefly, the immunoarrays consisting of eight sensor elements, made of gold nanoparticles layered with glutathione, were first coated with the capture antibody and transferred to a microfluidic chamber. In parallel, biotinylated horseradish peroxidase and a biotinylated secondary antibody were attached to streptavidin-coupled magnetic beads and collected with a magnet. Next, 5 μl of 5–750 fg/mL of recombinant DSG3 protein standards or 4 μl tissue extract were diluted 1:6000 in RIPA buffer and added to the bioconjugates. The bioconjugates with captured proteins were then magnetically separated, washed, resuspended in a final volume of 110 μL, and immediately injected into the microfluidic channel housing the immunoarrays. At this step, the flow was stopped, incubated for 20 min, washed, and hydroquinone solution was passed through the channel. The amperometric signal was developed by injecting 50 μL of 100 μM H_2O_2. Tissue lysates used for Western blot analysis and microfluidic immunoarrays were made from primary tumors ($n = 4$), lymph node (−) ($n = 3$), and lymph node (+) ($n = 3$).

Results

In a previous proteome-wide analysis of HNSCC progression, we noted that DSG3 was highly expressed in normal oral mucosa and HNSCC lesions.[21] To further investigate a possible use for DSG3 as a predictive biomarker, we first assessed DSG3 expression by immunohistochemistry in an independent cohort of human normal and malignant oral squamous tissues. By H&E histological evaluation, normal squamous epithelium shows a defined basement membrane with layers of differentiating keratinocytes, whereas in the malignant counterpart, this organized pattern is lost (Fig. 1A). Normal tissues sections stained for DSG3 show that it is predominantly expressed in the basal and suprabasal layers of the normal squamous epithelium, while in SCC DSG3 expression is restricted to cancer cells. Stromal cells were negative. We next evaluated DSG3 expression in a HNSCC tissue microarray (TMA) containing 317 evaluable cores.[29] DSG3 was readily detected in all HNSCC cores and localized to tumors cells (Fig. 1B). Within these cases, well-differentiated carcinomas ($n = 120$) had the highest percentage of DSG3-positive cells (∼90%). The moderate- ($n = 119$), and poorly-differentiated ($n = 66$) cores showed slightly lower proportion of DSG-reactive cells (80% and 70%, respectively), the remaining 12 cores consisting of corresponding to non-squamous tissues were