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Potential imaging targets in primary head and neck squamous cell carcinoma and lymph node metastases

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ABSTRACT

Purpose: To investigate glycoprotein nonmetastatic melanoma protein B (GPNMB) and vascular endothelial growth factor (VEGF) as potential fluorescent imaging markers by comparing their protein expression to epidermal growth factor receptor (EGFR).

Materials and methods: Thirty-eight paired samples of untreated head and neck squamous cell carcinoma (HNSCC) primary tumours (PT) and corresponding synchronous lymph node metastases (LNM) were selected. After immunohistochemical staining, expression was assessed and compared by the percentage of positive tumour cells. Data were analysed using the Mann-Whitney test, effect sizes (ES) and Spearman’s correlation coefficient (r).

Results: GPNMB expression was observed in 100 % of PT, and median 80 % (range 5–100 %) of tumour cells, VEGF in 92 % and 60 % (0–100 %), EGFR in 87 % and 60 % (0–100 %) respectively. In corresponding LNM, GPNMB expression was observed in 100 % of LNM and median 90 % (20–100 %) of tumour cells, VEGF in 87 % and 65 % (0–100 %), and EGFR in 84 % and 35 % (0–100 %). A positive correlation was found between expression in PT and LNM for GPNMB (r = 0.548) and EGFR (r = 0.618) (p < 0.001), but not for VEGF (r = −0.020; p = 0.905). GPNMB expression was present in a higher percentage of tumour cells compared to EGFR in PT (p = 0.015, ESR = −0.320) and in LNM (p < 0.001, ESR = −0.478), while VEGF was not (p = 1.00, ESR = −0.109 and −0.152, respectively).

Conclusion: GPNMB expression is higher than EGFR in untreated HNSCC PT and corresponding LNM, while VEGF expression is comparable to EGFR. GPNMB is a promising target for fluorescent imaging in HNSCC.

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1. Introduction

Intraoperative near-infrared fluorescence molecular imaging (FMI) is a technique that may reduce the positive or inadequate surgical margin rate, which has been reported in 85 % of oral squamous cell carcinomas \([1]\), by better delineation of the tumour and increase the chance for complete tumour removal using a fluorescent agent conjugated to a targeted antibody. For successful use of FMI, the contrast between the tumour and normal background tissue, as quantified by calculating the tumour-to-background ratio (TBR), is essential \([2,3]\). Promising results have been found for intraoperative ex vivo margin assessment of primary HNSCC in patients, by using cetuximab-IRDye800CW or panitumumab-IRDye800CW \([4,5]\). A sensitivity of 100 % and a specificity of 74–91 % was found, with an in vivo TBR of 2.5 and ex vivo TBRs of 3.1–6.5 \([4,5]\). With ex vivo FMI of cervical lymph node metastases, a 92 % sensitivity and 97 % specificity were found with a TBR of 5.8 \([6]\).

In vivo imaging is complicated by several factors, such as ambient light, camera angle, and distance between the camera and the patient \([7]\), resulting in lower TBRs. Higher TBRs could lead to better in vivo distinction between tumour and healthy tissues, allowing for image-guided resections of the primary tumour and lymph node metastases.

Studies have focused on targeting the epidermal growth factor receptor (EGFR), which is expressed in 90 % of HNSCC tumours. However, in these studies, fluorescent EGFR-signal was also present on the cell membrane of normal basal and suprabasal mucosal epithelial cells, and accumulation was noted in salivary gland tissue, which led to lower TBRs \([8]\).

Other FMI targets expressed by HNSCC may better distinguish tumour from surrounding healthy mucosa, resulting in higher TBRs. One potential target is glycoprotein nonmetastatic melanoma protein B (GPNMB), which is commonly expressed in HNSCC \([9,10]\). Another potential target is vascular endothelial growth factor (VEGF), for which the fluorescent tracer bevacizumab-IRDye800CW is already available \([4,5]\). A sensitivity of 100 % and a specificity of 74–91 % was found, with an in vivo TBR of 2.5 and ex vivo TBRs of 3.1–6.5 \([4,5]\). With ex vivo FMI of cervical lymph node metastases, a 92 % sensitivity and 97 % specificity were found with a TBR of 5.8 \([6]\).

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2. Methods

Consecutive patients who had undergone resection of a primary HNSCC with a neck dissection as an initial treatment between March 2014 and August 2020 in the University Medical Centre Groningen (UMCG) were retrospectively selected for this study. Only patients with histopathologically confirmed positive lymph node metastasis in the neck were selected. This led to 41 patients of whom tissue of both the primary tumour and the paired synchronous lymph node metastasis was available. Demographic information, as well as relevant medical and treatment information, was collected. Formalin-fixed paraffin-embedded blocks were retrieved from the archives of the Department of Pathology of the UMCG, and 3 μm thick sections were cut and stained for haematoxylin & eosin (H&E), GPNMB, VEGF-A, and EGFR. Immunohistochemical staining was performed as described previously \([16]\).

Tumour tissue of three patients had to be excluded due to the absence of tumour after re-sectioning the archival paraffin-embedded tissue, resulting in data from 38 patients for analysis.

Two investigators evaluated immunohistochemistry results using H-scores (JvS, a PhD candidate trained in scoring immunohistochemistry, and BvdV, a dedicated head and neck pathologist). H-scores were calculated as the product of staining intensity (0–2; 0 = absent, 1 = weak, 2 = strong) multiplied by the percentage of all viable tumour cells showing staining (0–100 %). H-scores, therefore, range from 0 to 200.

Primary tumours and lymph nodes with at least 5 % of viable tumours showed staining, with at least 5 % of viable tumours showing expression were considered positive. Hence, an H-score of ≥5 was deemed positive, and an H-score < 5 was deemed negative. Normal tissue surrounding viable tumour cells can be viewed as a negative control. However, since H-scores assess the percentage of viable tumour cells, normal tissue could not be evaluated by H-scores.

2.1. Statistical analysis

Groups of three or more were compared using the Kruskal-Wallis test for non-parametric data. Mann-Whitney U test was used for comparing two groups with posthoc Bonferroni corrections. In the case of a significant Kruskal-Wallis test, all three targets were compared, resulting in six Mann-Whitney U tests. Spearman’s correlation coefficients were used to compare two continuous variables. Effect sizes were calculated (ES r) \([17]\). A Bonferroni-corrected P-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS (version 23 for Windows; IBM Corp., Armonk, New York).

3. Results

3.1. Patients

Tumour tissue of 38 patients, 31 males (82 %), and seven females (18 %) was used (Table 1). The median age was 65 years (range 41–81). The median follow-up time was 20.9 months (range 1.8–87.3). The predominant site was the larynx (45 %), followed by the hypopharynx (29 %) and the oral cavity (24 %). Most patients had a T4 tumour (53 %) and N2 nodal (58 %) status.

3.2. Expression in primary HNSCC

In primary HNSCC, GPNMB expression with an H-score of ≥5 was

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31 (82)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (18)</td>
</tr>
<tr>
<td>Age, in years</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>65</td>
</tr>
<tr>
<td>Range</td>
<td>41–81</td>
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<tr>
<td>Primary tumour site</td>
<td></td>
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<tr>
<td>Oral cavity</td>
<td>9 (24)</td>
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<tr>
<td>Oropharynx</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>11 (29)</td>
</tr>
<tr>
<td>Larynx</td>
<td>17 (45)</td>
</tr>
<tr>
<td>Differentiation grade</td>
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<tr>
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</tr>
<tr>
<td>Moderate</td>
<td>27 (71)</td>
</tr>
<tr>
<td>Poor</td>
<td>10 (26)</td>
</tr>
<tr>
<td>T-classification</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5 (13)</td>
</tr>
<tr>
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<td>5 (13)</td>
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<td>8 (21)</td>
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<tr>
<td>N-classification</td>
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<tr>
<td>2</td>
<td>22 (58)</td>
</tr>
<tr>
<td>3</td>
<td>12 (32)</td>
</tr>
</tbody>
</table>
observed in 100%, VEGF in 92%, and EGFR in 87% of the tumours (Table 2). The median H-score was 100 for GPNMB (range 5–200), 60 for VEGF (0–180), and 60 for EGFR (0–200) (Fig. 1A). The H-scores across the HNSCC sites for each target can be seen in Table 3. GPNMB was expressed in a higher percentage of tumour cells than EGFR (p = 0.015, ESr = 0.320), while VEGF was not (p = 1.00, ESr = 0.109). No significant differences in H-scores of GPNMB, VEGF, or EGFR were found between different HNSCC sites (range ESr 0.006 to 0.386), T-stages (range ESr 0.0 to 0.470), or differentiation grades (range ESr – 0.006 to –0.010). In primary tumours, GPNMB showed higher expression compared to EGFR, and VEGF did not.

### 3.3. Expression in lymph node metastasis

In the lymph node metastases, GPNMB expression was observed in 100%, VEGF in 92%, and EGFR in 87% of the cases (Table 2). The median H-score was 118 for GPNMB (range 30–180), 65 for VEGF (0–100), and 35 for EGFR (0–200) (Fig. 1B). GPNMB was expressed in a significantly higher percentage of tumour cells than EGFR (p < 0.001, ES r = –0.478), while VEGF was not (p = 1.00, ES r = –0.152). No significant differences were found between different N-stages for all three targets (range ES r = –0.021 to –0.214).

Representative examples of the immunohistochemical results are shown in Fig. 3. Normal tissue was present surrounding the HNSCC in tissue slides of both the primary tumours and lymph node metastases, and this surrounding tissue did not express any staining. Although H-scores cannot be used to score normal tissue, it can be concluded that expression was absent in normal tissues.

### 3.4. Correlation of expression in primary HNSCC and paired lymph node metastasis

Correlation coefficients between H-scores of primary tumours and corresponding lymph node metastases within the same patients were 0.548 (p < 0.001) for GPNMB, –0.020 (p = 0.905) for VEGF, and 0.618 (p < 0.001) for EGFR (Fig. 2). Correlation coefficients for expression in percentage of tumour cells were 0.432 (p = 0.007) for GPNMB, 0.014 (p = 0.934) for VEGF, and 0.676 (p < 0.001) for EGFR. The percentage of patients with simultaneous expression in the primary tumour and the lymph node metastasis (i.e., H-score ≥ 5), was 100% for GPNMB, 79% for VEGF, and 79% for EGFR. GPNMB, therefore, appears to be the most promising target for simultaneous FMI in both primary tumours and corresponding lymph node metastases.

### 4. Discussion

In this study, we evaluated the expression of GPNMB and VEGF as potential targets for FMI, compared to EGFR as a traditional marker, in primary tumours and paired lymph node metastases by immunohistochemistry. Only GPNMB was expressed in all HNSCC (100%) and in the highest percentage of tumour cells in primary tumours and lymph node metastases. Moreover, all primary tumours and corresponding lymph node metastases showed GPNMB expression.
GPNMB expression was tumour cell specific, with absence of staining in normal epithelium and stromal cells, which indicates that high TBRs might be expected in FMI. In contrast, VEGF and EGFR expression was seen in both the primary tumour and lymph node metastases in only 79% of patients. Our observed expression of VEGF in 92% and EGFR in 87% of primary HNSCC is concordant with the literature where expression percentages of 87.5–95.0% for VEGF and 87.5–92.5% for EGFR have been described [18–20].

In our previous study investigating expression in HNSCC lymph node metastases after initial (chemo)radiotherapy, we found that 100% of the cases showed GPNMB expression and 87% EGFR expression [16]. The results of the current study are comparable to that. We could not score VEGF expression in the previous study due to confounding background staining in all tissue slides. We hypothesised that this was the result of the previous treatment with radiotherapy. A recent study using bevacizumab-800CW in soft-tissue sarcomas reported a false-positive fluorescence signal after neoadjuvant radiotherapy [11]. However, VEGFA targeted fluorescence endoscopy in rectal cancer did show that this technique is feasible after radiotherapy [15]. In our current cohort with previously untreated primary tumours and corresponding lymph nod metastases, no such background staining was seen for VEGF.

Interestingly, EGFR seems to have been upregulated after (chemo)radiotherapy, where the median H-score was 95 in lymph node metastases [16]. This could mean that EGFR-targeted FMI is more sensitive in previously treated patients than untreated patients. The GPNMB expression in lymph nodes after initial (chemo)radiotherapy with a median H-score of 100 found in an earlier study is consistent with our current findings [16].

Although protein expression is not the same as fluorescence uptake and no clear relation between the level of EGFR expression and fluorescence uptake has been found, EGFR expression has been associated with fluorescence intensity in primary tumours and lymph node metastases [6,21]. The positive correlations of GPNMB and EGFR expression between primary tumours and lymph node metastases could, therefore, be interesting for the use of FMI. This finding aligns with earlier results in the literature for EGFR [22]. Clinically, this could mean that when a biopsy of the primary tumour is positive for GPNMB, it can be assumed that the lymph node metastases are positive and could show fluorescence uptake during a subsequent neck dissection. Conversely, in cases of GPNMB positive lymph node metastasis with a (yet) unknown primary tumour, FMI could also be applied in the search for the unknown primary tumour since there is a significant positive correlation and, therefore, the primary tumour is also likely to be positive. This may not apply to EGFR, where expression was seen in both primary tumours and corresponding lymph node metastases in only 79%.

Differentiation grade, T-stage, and primary tumour site did not significantly influence expression in GPNMB, VEGF, and EGFR. For GPNMB, this is contrary to the findings of Li et al., who found that TNM-stage correlated with GPNMB expression [10]. Since we did find a medium effect of T-stage on GPNMB H-scores, our non-significant findings may result from a relatively small sample size. However, our current results suggest that any HNSCC tumour is positive for GPNMB expression, regardless of its site, stage, or differentiation grade.

In clinical fluorescence imaging studies, a sensitivity of 100% has been reported for the detection of primary HNSCC tumours and 85–95% for lymph node metastases using EGFR-targeted agents [4–6]. In comparison, we found EGFR expression in only 87% of primary tumours and 84% of lymph node metastases. This discrepancy may be related to the in vivo activity of EGFR receptors, which cannot be measured by immunohistochemistry. The next step towards implementation of GPNMB is therefore to produce a fluorescently labelled GPNMB probe and evaluate its performance in a xenograft mouse model [23]. It can also be topically applied ex vivo on a freshly excised HNSCC specimen and evaluate whether current immunohistochemical results correlate with xenograft or ex vivo findings [24].

### Table 3

<table>
<thead>
<tr>
<th>Target</th>
<th>H-score (median, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>80 (50–150)</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>70 (0–100)</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>100 (30–180)</td>
</tr>
<tr>
<td>Larynx</td>
<td>110 (5–200)</td>
</tr>
</tbody>
</table>

Fig. 2. Expression of corresponding primary tumours and lymph node metastases.
GPNMB: glycoprotein nonmetastatic melanoma protein B; VEGF: vascular endothelial growth factor; EGFR: epidermal growth factor receptor.

Fig. 3. Immunohistochemical staining in primary tumour and lymph node metastasis of the same patient.
GPNMB: glycoprotein nonmetastatic melanoma protein B; VEGF: vascular endothelial growth factor; EGFR: epidermal growth factor receptor.
5. Conclusion

Immunohistochemically, GPNbMB was expressed in a higher percentage of untreated HNSCC primary tumours and corresponding lymph node metastases than EGFR, while VEGF was not. GPNbMB and EGFR expression in untreated primary tumours showed a moderate positive correlation with expression in corresponding lymph nodes. This makes GPNbMB a promising target for optical imaging.

Research funding

No funding to declare.

Ethical statement

Based on the Dutch Medical Research Law (Wet medisch-wetenschappelijk onderzoek met mensen [WMO]), assessment by the hospital's institutional review board (METC/UMCG) was not required.

CRediT authorship contribution statement

Jeroen E. van Schaik: Conceptualization, Data curation, Formal analysis, Writing – original draft. Bert van der Vegt: Investigation, Methodology, Writing – review & editing, Supervision. Lorian Slagter-Menkema: Investigation, Writing – review & editing. Saskia H. Hanemaaijer: Writing – review & editing. Gyorgi B. Halmo: Writing – review & editing. Max J.H. Witjes: Writing – review & editing. Bernard F.A.M. van der Laan: Conceptualization, Writing – review & editing. Rudolf S.N. Fehrmann: Writing – review & editing. Sjoukje F. Oosting: Writing – review & editing. Boudewijn E.C. Plaat: Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

Boudewijn E.C. Plaat has a consultancy role for and has received unrestricted research grants from UMC. Bert van der Vegt reports honoraria received by UMC for expertise or scientific advisory board/consultancy (on request): Visiopharm, Philips, MSD/Merck, Daichi-Sankyo/AstraZeneca; Speaker's fee from Visiopharm, Diaceutics, MSD/Merck. All unrelated to this publication. Sjoukje F. Oosting reports research grants from Novartis (2009), Pfizer (2011) and Celldex Therapeutics (2015); a speakers fee from Merck, and consultancy fees from Genmab, Merck, and Bristol Myers Squibb (all paid to the institution). The other authors have no other funding, financial relationships, or conflicts of interest to disclose.

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