Effects of Neoadjuvant Therapy on Tumour Target Expression of Oesophageal Cancer Tissue for NIR Fluorescence Imaging

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Abstract

Purpose

Oesophaegal cancer patients with a clinical complete response (CR) after neoadjuvant chemoradiotherapy (nCRT) are candidates for an active surveillance strategy. Regrowth rates of 40% after initial clinical CR indicate that identification of a true complete response to nCRT remains challenging. Near-infrared tumour-specific fluorescence endoscopic imaging might help to discriminate patients with a true complete response from patients with residual disease. This study aims to find potential markers to enable molecular imaging in oesophageal cancer and to assess the effect of nCRT on marker expression.

Procedures

Oesophageal cancer tissue slides of diagnostic biopsies (n=41) (pre-treatment) and paired surgical specimens (n=31) (post-treatment) were collected. Tissue slides of patients with adenocarcinoma (n=29) and squamous cell carcinoma (n=12)) were included. Immunohistochemistry was performed to assess expression of the tumour markers CEA, EpCAM, VEGF-α, EGFR, and c-MET in the tumour and compared to the expression of these markers in surrounding healthy tissue. A total immunostaining score (TIS, range 0–12), which combines the percentage and intensity of stained cells, was calculated. The TIS of pre-treated biopsies were compared with the TIS of the post-treatment surgical specimens to assess the effect of neoadjuvant therapy on the marker expression.

Results

The median TIS of EpCAM in adenocarcinomas was 10, vs. 0 in healthy mucosa ($p < 0.001$). The median TIS of EGFR in squamous cell carcinoma was 12, vs. 4 in healthy mucosa ($p < 0.001$). Neoadjuvant therapy did not affect the expression of the markers.

Conclusion

EpCAM and EGFR appear to be the most suitable targets for tumour-specific NIR fluorescence imaging of oesophageal adenocarcinoma and squamous cell carcinoma, respectively. Unaffected expression of all suitable markers by neoadjuvant therapy implies that the diagnostic biopsy can be used to select a patient-specific target for response evaluation by molecular imaging.

Introduction

Oesophageal cancer is the eighth most common cancer worldwide, with over 500,000 patients diagnosed annually (1). With the introduction of neoadjuvant chemoradiotherapy (nCRT), cure rates and overall survival have drastically improved over the last two decades. In the Netherlands, approximately 30% of all oesophageal cancer patients achieve a pathologically complete response in the resected specimen after neoadjuvant chemoradiotherapy (2, 3). These patients will likely not benefit from surgery. This led to the introduction of active surveillance for these patients in clinical trials. This organ preserving treatment strategy may be offered to patients with a complete clinical response based on imaging modalities including PET-CT and white light endoscopy (i.e., a clinical complete response). (4). However, regrowth in 40% of patients during active surveillance underline the fact that small residual tumour can be missed with conventional diagnostic tools (5, 6). This may for instance be due to the difficulty to discriminate between treatment related fibrosis and residual tumour. New imaging modalities may improve tumour identification after nCRT for oesophageal cancer, either during endoscopic response evaluation, or during surgery.

Tumour-specific fluorescence imaging is a modality that can be used to perform real-time imaging of tumour cells. To perform fluorescence imaging, a tumour-specific tracer comprising of a fluorophore and a targeting agent which binds to a specific ligand or is activated by the tumour-specific environment, is required. The fluorophore can be excited by a specific light source, emitting photons that are detected by a fluorescence camera. In the near-infrared (NIR) spectrum (700 – 900 nm), factors that impair optical tissue properties (absorption, scattering, and tissue autofluorescence) are limited in human tissue, resulting in a higher light penetration depth and enhanced contrast compared to visible light (7, 8).

For a tumour-targeted fluorescence tracer to adequately delineate a tumour, overexpression of a specific tumour marker in cancer cells is vital. Likewise, these biomarkers must be absent or have reduced expression in healthy tissue and fibrosis. A recent study showed that carcinoembryonic antigen-related cell adhesion molecule 5 (CECAM5, from here on to be referred to as CEA), epithelial cell adhesion molecule (EpCAM), vascular endothelial growth factor α (VEGF-α), and epithelial membrane antigen (EMA) were all overexpressed in oesophageal adenocarcinoma tissue, with only minimal expression in healthy tissue (9). However, effects of neoadjuvant treatment on the expression of these markers and marker expression in oesophageal squamous

cell carcinoma are unknown. Oesophageal squamous cell carcinoma is particularly interesting as these tumours tend to show higher rates of pathologically complete responses after chemoradiation (approximately 40% vs. 20% in adenocarcinomas) (10). This study aims to find new molecular markers for tumour-specific imaging of oesophageal adenocarcinoma and squamous cell carcinoma that may be suitable targets for NIR fluorescence imaging during endoscopic response evaluation. The second aim was to explore the effect of neoadjuvant treatment on expression of these markers.

Materials and Methods

This study was reviewed and approved by the Medical Ethical Committee of the Erasmus Medical Centre (METC Erasmus MC, MEC-2021-0339) and was conducted according to the Declaration of Helsinki.

Tissue selection

Formalin-fixed paraffin-embedded (FFPE) tissue blocks of oesophageal cancer patients that underwent surgery were collected and reviewed by a dedicated pathologist (MD). Tissue blocks containing both tumour and healthy tissue were selected. Pre-neoadjuvant treatment tissue (diagnostic biopsies) was paired with the corresponding post-neoadjuvant treatment tissue block (resection specimens). Tissue slides from patients with a pathological complete response (i.e. Mandard 1), a near-complete response (i.e. Mandard 2) and minimal response (i.e. Mandard 4 – 5) were selected. Since there were no tumour cells detected in the resected specimen from patients with a pathological complete response, tissue slides were selected based on reactive changes such as fibrosis. All tissue blocks were collected from the tissue bank from the Erasmus University Medical Centre from patients that underwent surgery between 2010 and 2019. Tissue slices of 4 µm were cut and further processed for immunohistochemical (IHC) staining.

Marker selection

The following markers were selected for IHC staining: CEA, EpCAM, VEGF-α, epithelial growth factor receptor (EGFR), and c-mesenchymal-epithelial transition factor (c-MET). These markers were selected based on their known overexpression in most cancer types, their location on the cell membrane, and the (pre-)clinical availability of a fluorescent agent.

Immunohistochemistry staining

Tissue slides were deparaffinized in xylene, and then rehydrated in a series of decreasing concentrations of diluted ethanol (100%, 70%, and 50%). The slides were rinsed with demineralized water and treated with 0.3% hydrogen peroxide (from Merck Millipore, Darmstadt, Germany) for 20 minutes at room temperature to block endogenous peroxidase. The used method for Antigen Retrieval was dependent on the protocol of the antibody (Supplementary table 1). Antigen retrieval for CEA, EpCAM was performed by heat induction at 95°C for 10 minutes using Envision FLEX target retrieval solution low pH (DAKO). For c-MET and EGFR, Envision FLEX target retrieval solution high pH (DAKO) was used. No antigen retrieval was used for VEGF-α. The slides were incubated overnight at room temperature with the primary antibody in predetermined optimal dilutions diluted in 1% BSA/PBS (Supplementary table 1). A negative control was also incubated with 1% BSA/PBS, and tissue sections known to express the marker(s) were used as positive controls. The following day, slides were washed with PBS and then incubated with HRP-labelled secondary antibodies (either anti-mouse or anti-rabbit, both from Dako EnVision+ System) for 30 minutes at room temperature. For staining, 3,3-diaminobenzidine tetrahydrochloride solution (DAB, K3468, DAKO) was applied for 10 minutes, and for counterstaining hematoxylin (VWR international, Amsterdam, the Netherlands) was applied for 15 seconds. Finally, the tissue sections were dehydrated and mounted in Pertex (Histolab, Askim, Sweden).

Scoring methods

All biopsies and surgical specimens were scored for expression of all five tumour markers by a trained pathologist using the Nanozoomer 2.0 HT (Hamamatsu Photonics, Shizuoka, Japan) with a magnification of 80 x. Scoring was done according to the total immunostaining score (TIS), which consists of an intensity score (IS) and proportion score (PS) (11). The IS represents the intensity of staining of the cells and ranges between 0 and 3 (0 = no staining; 1 = weak; 2 = moderate; 3 = strong). The PS represents the proportion of cells that is stained and ranges between 0 and 4 (0 = none; 1 = 1 – 10%; 2 = 11 – 50%; 3 = 51 – 80%; 4 = 81 – 100%). The TIS is calculated by multiplying the IS and the PS. Marker expression was categorized in low expression (TIS 0-5) and high expression (TIS 6-12). Both tumour and adjacent mucosa were scored, and a tumour-to-mucosa ratio was calculated for each tissue slide. A ratio of ≥ 2 was deemed as adequate contrast.

Statistical analysis

Statistical analysis was performed with R-studio software (version 4.1.0, R Foundation for statistical computing, Vienna, Austria). Patient characteristics were presented with descriptive statistics. Marker expression in the tumour was based on the pre-treatment biopsies. Effects of nCRT were assessed by comparing TIS of pre- and post-treatment tissue samples of the same patient (i.e. paired samples). Differences in marker expression between tumour and adjacent mucosa were assessed with the Mann-Whitney U test. Differences in marker expression between the paired pre- and post-treatment tissue samples was assessed with the Wilcoxon signed rank test. A p-value ≤ 0.05 was considered significant.

Results

Tissues of 41 patients were included. In total, 31 paired pre- and post-treatment tissue samples were included and for ten patients, only the pre-treatment biopsies were available. Patient- and tumour characteristics are presented in Table 1.

Marker expression in oesophageal adenocarcinoma biopsies and healthy mucosa

In adenocarcinoma, overexpression in tumour compared to healthy mucosa was observed for CEA (median TIS: 6 vs. 2, $p < 0.001$), EpCAM (median TIS: 8 vs. 0, $p < 0.001$), and VEGF-α (median TIS: 8 vs. 8, $p < 0.01$) (Figure 1). Out of these three markers, EpCAM had the highest tumour-to-mucosa ratio (median: 10) and the highest proportion of samples with a ratio ≥ 2 (97%) (figure 2a). Figure 3a presents representative stained tissue slides for each tumour marker on adenocarcinoma. Supplementary table 2 presents a more detailed overview of the TIS in adenocarcinomas for each tumour marker.

Marker expression in oesophageal squamous cell carcinoma biopsies and healthy mucosa

In squamous cell carcinoma, overexpression in tumour compared to healthy mucosa was observed for EGFR (median TIS: 12 vs. 4, < 0.001), EpCAM (median TIS: 4 vs. 0, $p < 0.001$), and VEGF-α (median TIS: 8 vs. 8, $p < 0.01$) (Figure 1). Out of these markers, EGFR had a median tumour-to-mucosa ratio of 3, with 100% of the samples having a ratio of ≥ 2 and EpCAM had a median tumour-to-mucosa ratio of 4, with 82% of the samples having a ratio of a ratio ≥ 2 (figure 2b). c-MET and CEA were not overexpressed in oesophageal squamous cell carcinoma compared to healthy mucosa. Figure 3b presents a representative stained tissue slide for each tumour marker.

Supplementary table 2 presents a more detailed overview of the TIS in oesophageal squamous cell carcinoma for each tumour marker.

Marker expression in healthy tissue

Marker expression in healthy mucosa is scored and presented for adenocarcinoma and squamous cell carcinoma combined (figure 1). VEGF-α expression was high in healthy mucosa in 73% (median TIS: 8) of the samples and EGFR expression was high in healthy mucosa in 36% (median TIS: 4) of the samples. c-MET, CEA, and EpCAM had no samples with 'high expression' in the healthy mucosa (Figure 1, Supplementary Table 2). The location of staining in the healthy tissue samples per tumour marker is summarised in Table 2.

Effects of neoadjuvant therapy on marker expression

Differences in marker expression between pre- and post-treatment samples were scored for adenocarcinoma and squamous cell carcinoma combined. Neoadjuvant therapy did not affect expression of any of the markers. Only VEGF-α expression was observed in scar tissue of post-treatment tissue samples of patients with a pathological complete response (median TIS: 3). All other markers had no expression in the scar tissue of post-treatment tissue samples (mean TIS: 0). Figure 4 presents an overview of tumour marker expression in paired pre- and post-treatment samples for all five tumour markers, categorized for response to treatment.

Discussion

In this study, five possible targets for targeted fluorescence imaging of both oesophageal adenocarcinomas and oesophageal squamous cell carcinoma were evaluated using IHC. CEA, EpCAM and VEGF-α were significantly overexpressed in oesophageal adenocarcinomas. In oesophageal squamous cell carcinomas, EGFR, EpCAM, and VEGF-α were significantly overexpressed. Although VEGF-α is overexpressed in both adenocarcinomas and squamous cell carcinoma, our study showed high expression of this marker in the healthy mucosa, stromal cells, scar tissue and muscle layers of all oesophageal cancers, which makes this a suboptimal target for fluorescence imaging. This was also reflected in the median tumour-to-mucosa ratios of VEGF-α of 1.5 for adenocarcinoma and 1.0 for squamous cell carcinoma. Based on the percentage of samples with a tumour-to-mucosa ratio higher than 2, the most suitable targets for oesophageal adenocarcinomas and squamous cell carcinoma are EpCAM and EGFR, respectively.

In resection specimens with a pathologically complete response, no expression of the most promising tumour markers (EpCAM, EGFR and CEA) was observed in the areas with therapy effect or tumour scar tissue, while there was still expression of the markers in resection specimens with a near-complete response. Importantly, expression of all relevant tumour markers was not affected by neoadjuvant therapy in the latter group of patients. This implies that a patient-specific approach may be feasible in which the optimal marker could be selected based on the diagnostic (i.e. pre-treatment) biopsy. Overall, these results provide promising results regarding *in vivo* NIR fluorescence imaging of oesophageal adenocarcinoma and squamous cell carcinoma in future studies.

Our observation of significantly lower expression of all tested markers in patients with a pathologically complete response, along with the absence of c-MET, CEA, EGFR and EpCAM expression in post-treated specimen in these patients raises the question if these markers might be good targets for therapy in oesophageal cancer patients. Some of our tested markers already has been evaluated as potential therapy targets (12). Antibodies targeting c-MET, as rilotumumab and obinutuzumab, have been tested in oesophageal patients and have failed to show any survival benefit (13, 14). The EGFR-targeted therapeutics cetuximab, nimotuzumab, gefitinib and icotinib all have proven to be effective in the treatment of oesophageal squamous cell carcinomas (15-19). Only cetuximab has been tested in oesophageal adenocarcinomas and has also been shown to be effective (15). The anti-VEGF antibodies bevacuzimab and ramucirumab had no survival benefit in patients with oesophageal adenocarcinomas (20, 21). Indeed, the surgical complications such as anastomotic leakage were higher after treatment with bevacuzimab. It is reasonable to hypothesize that these high complications rates might be partly due to the high expression of VEGF-α in healthy mucosa for which an established link with erosive esophagitis exists. The oesophageal condition is induced by chronic acid reflux, which is a well-documented risk factor for oesophageal adenocarcinomas (22, 23).

Different expression rates of the investigated markers were observed between adenocarcinomas and squamous cell carcinomas. Expression rates of EGFR are clearly higher in squamous cell carcinomas, while CEA and EpCAM expression are higher in adenocarcinomas. This could be explained by the different pathogenesis and aetiology between these two subtypes. Adenocarcinomas develop from metaplasia which can be triggered by chronic acid reflux, while squamous cell carcinoma develops from hyperplasia and is associated with chronic tobacco and/or alcohol exposure (23). All tested markers play a role in the development of tumours by promoting tumour

proliferation and/or angiogenesis (24-29). Previous studies also have shown that oesophageal adenocarcinomas and squamous cell carcinomas have different molecular characteristics, which suggests that different mutational patterns and cell signalling pathways drive the tumorigenesis in adenocarcinomas and squamous cell carcinomas (30, 31). Higher expression of CEA and EpCAM in adenocarcinomas can also be partly explained by the fact that adenocarcinomas arise form glandular cells and they retain features of glandular epithelial cells, including the naturally expression of epithelial markers as CEA and EpCAM (32, 33).

The immunohistochemistry results for adenocarcinoma are comparable to the results of de Gouw et al. (9), who also reported overexpression of EpCAM and CEA with only minimal expression of these markers in healthy tissue. In addition, they also found that EGFR and C-met were not overexpressed in adenocarcinomas and that VEGF-α was overexpressed in both the adenocarcinoma and the healthy tissue causing insufficient tumour-to-mucosa ratios. No similar studies were found for the evaluation of tumour-specific targets via IHC in squamous cell carcinoma. Although no clinical trials with endoscopic NIR fluorescence imaging for oesophageal cancer have been performed, three studies report on the ability to identify Barrett's segments (34-36). In two of these studies, in addition to conventional white light imaging, occult lesions were identified using this technique, after intravenously or topically administration of the VEGFα antibody Bevacuzimab conjugated to the fluorophore 800CW and after the topical administration of the EGFR antibody Cetuximab conjugated to 800CW. With the C-met targeting tracer EMI-137, fluorescence endoscopy was able to identify almost all lesions. However, target-to-background ratios were modest. Although our immunohistochemistry results did not show significant overexpression of these three markers in adenocarcinomas, these studies showed that they can identify Barrett's lesions with fluorescence endoscopy. This could be explained by the possibility that the used tracers may bind to different epitopes than the antibodies that were used for the immunohistochemistry staining. This highlights the importance of (pre)clinical validation of immunohistochemistry results. More importantly, the molecular characteristics between Barret lesions and adenocarcinoma can differ.

For oesophageal cancer, endoscopic NIR fluorescence imaging may be used during clinical response evaluation after neoadjuvant therapy. As approximately two thirds of the missed residual tumour lesions are located in the mucosa, endoscopic NIR fluorescence imaging may be a good candidate to improve detection of these currently missed lesions (37). The other one third of the currently missed residual tumour lesions are located in the submucosa. Depending on the depth of these lesions, these could be detected with NIR fluorescence imaging,

possibly improving clinical response evaluation (7). Although earlier detection and resection of residual disease does not appear to improve overall survival, it will result in less invasive response evaluations for these patients (38).

Tumour-specific NIR fluorescence imaging can also be used during oesophagectomy. In general, intraoperative NIR fluorescence imaging can be used for detection of occult metastases and resection margin assessment (39). One such application in oesophageal cancer may be to identify peritoneal metastases, in which case resection should be avoided. NIR fluorescence imaging may also aid in detection of tumour positive lymph nodes. For instance, the identification of enlarged (metastatic) lymph nodes seen on preoperative FDG-PET/CT-scan. Although we did not assess tumour marker expression in metastatic lymph nodes, a previous study showed that expression of tumour markers was highly correlated between the primary tumour and metastatic lymph nodes (9). The application of intraoperative NIR fluorescence imaging for margin assessment appears limited after oesophagectomy, as tumour positive resection margins are only reported in 5 – 7.5% of the cases (40). Besides NIR fluorescence imaging, these identified tumour markers may also be suitable for other purposes such as targeted PET-imaging or targeted therapy.

Performing IHC assessment to assess overexpression of suitable tumour targets is an important first step in studying tumour-specific NIR fluorescence imaging of oesophageal cancer. Despite the promising targets our study has provided, a good correlation between the level of tumour marker expression and the observed in vivo contrast for specific fluorescent tracers is not guaranteed. A variety of pharmacokinetic variables can lead to insufficient binding of the tracer to the target. Moreover, performance of a tumour-specific tracer depends on its affinity and the dose and the interval between administration and imaging. Finally, clinical tracers may bind to different epitopes and with different affinity than the antibodies that were used for the immunohistochemical staining. As such, the next step consists of validating these results by performing feasibility studies in vivo.

Conclusion

Our study demonstrates that EpCAM for oesophageal adenocarcinoma and EGFR for squamous cell carcinoma are the most promising targets for NIR fluorescence imaging. Non-curative neoadjuvant therapy does not negatively affect expression of the assessed markers and no expression of the most promising targets was

observed in tumour scar tissue. These results form an important foundation for subsequent clinical trials with tumour-specific NIR fluorescence imaging to improve tumour detection of residual oesophageal cancer after neoadjuvant therapy.

Conflict of Interest: The authors declare that they have no conflict of interest.

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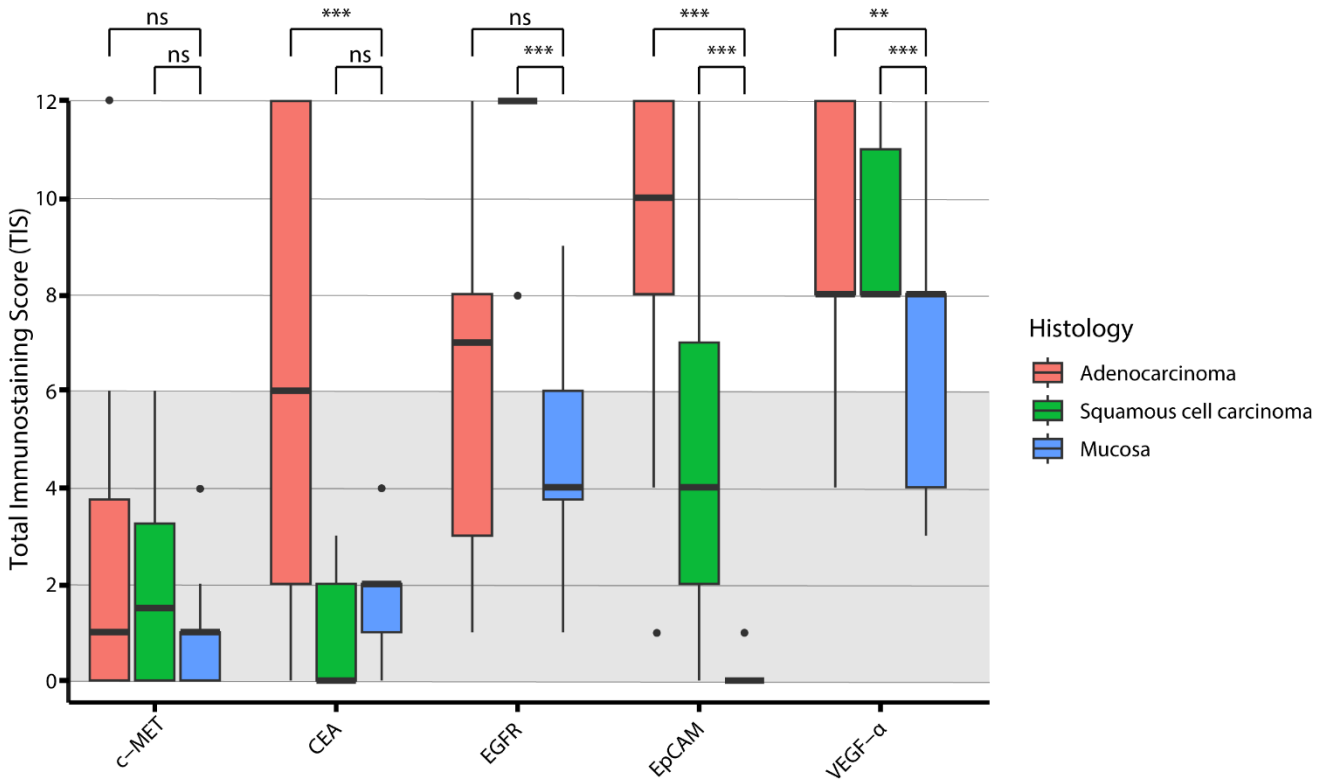

Figure 1: Total immunostaining score (TIS) per tumour marker in oesophageal adenocarcinoma, squamous cell carcinoma, and adjacent mucosa in pre-treated biopsies.

The boxplots represent the medians with q1 and q3 with the error bars representing the range and the dots for outliers. The grey shaded box displays all the TIS < 6, indicating 'low expression'.

Abbreviations: *ns = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001*

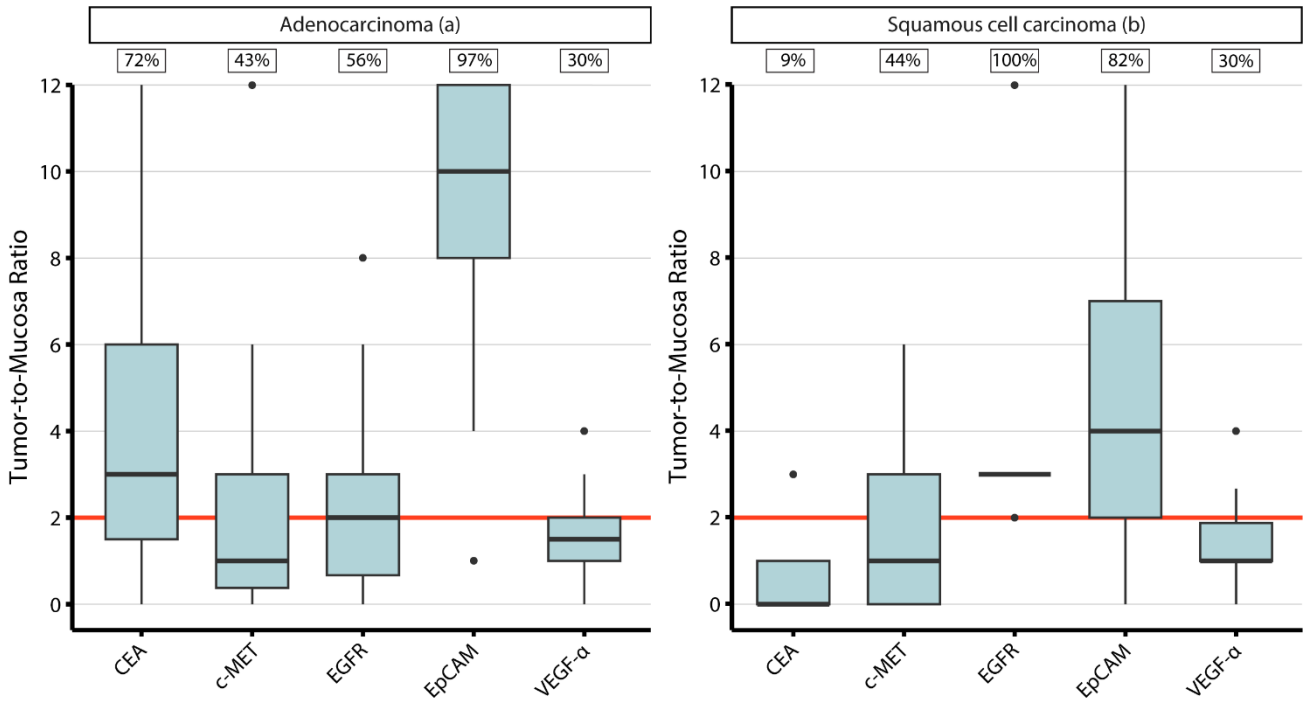

Figure 2: Median tumour-to-mucosa ratios per tumour marker oesophageal adenocarcinoma (a) squamous cell carcinoma (b).

The boxplots represent the medians with q1 and q3 with the error bars representing the range and the dots for outliers. The percentages in the boxes present the proportion of tissue samples with a tumour-to-mucosa ratio of ≥ 2.0. The red line displays a tumour-to-mucosa ratio of 2 or higher, indicating adequate contrast.

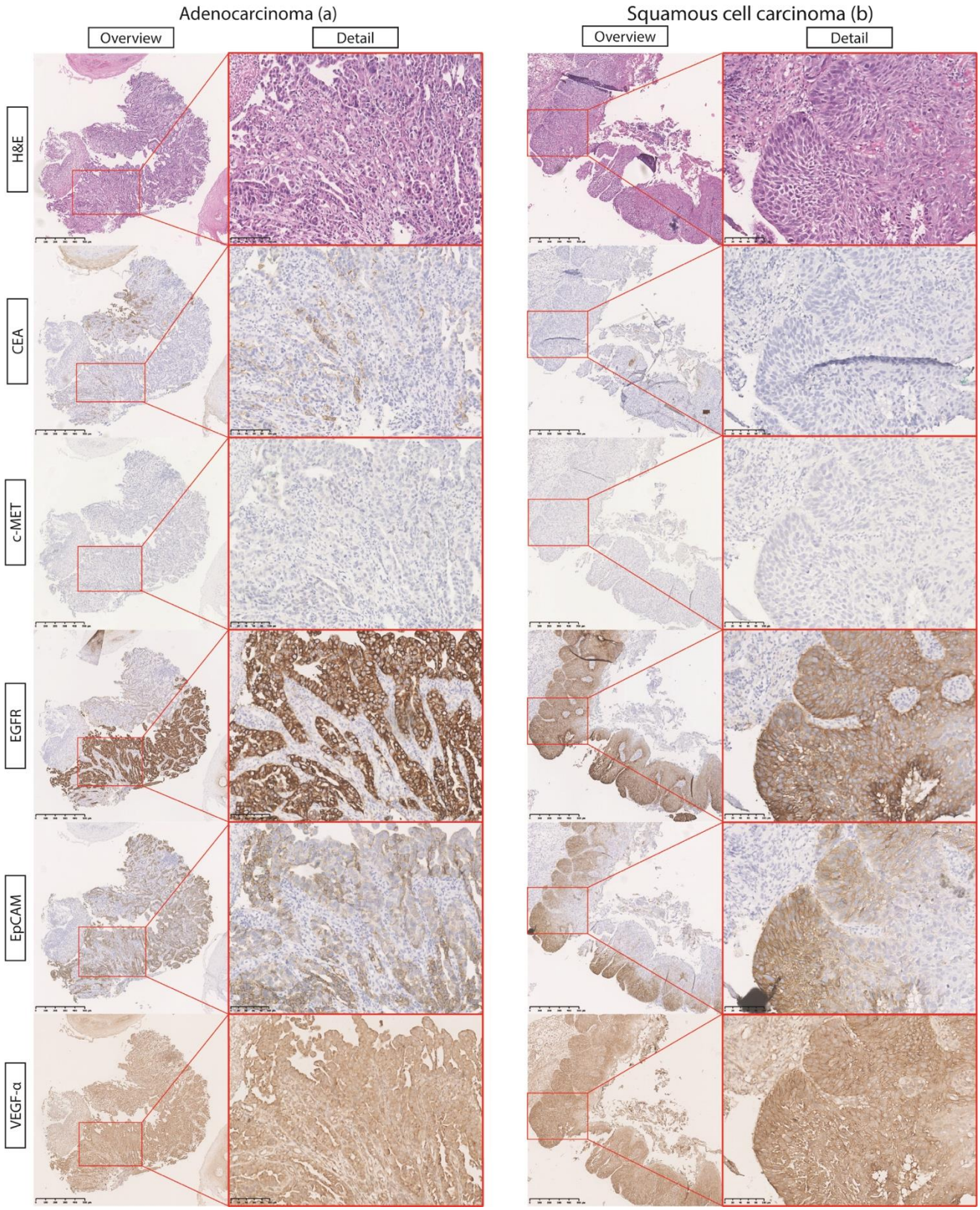

Figure 3: A representative tissue slide of an adenocarcinoma and a squamous cell carcinoma with the H&E slide and the five tumour markers

These images show representative slides of an adenocarcinoma and squamous cell carcinoma case stained with the five tumour markers and the corresponding H&E slide. The first row shows an overview of the tumour sample, and the second row shows a detailed tumour sample. The area of the detailed tumour staining is indicated with a rectangle in the tumour overview. The whole black bar represents 500 µm in both the overview as the detailed images.

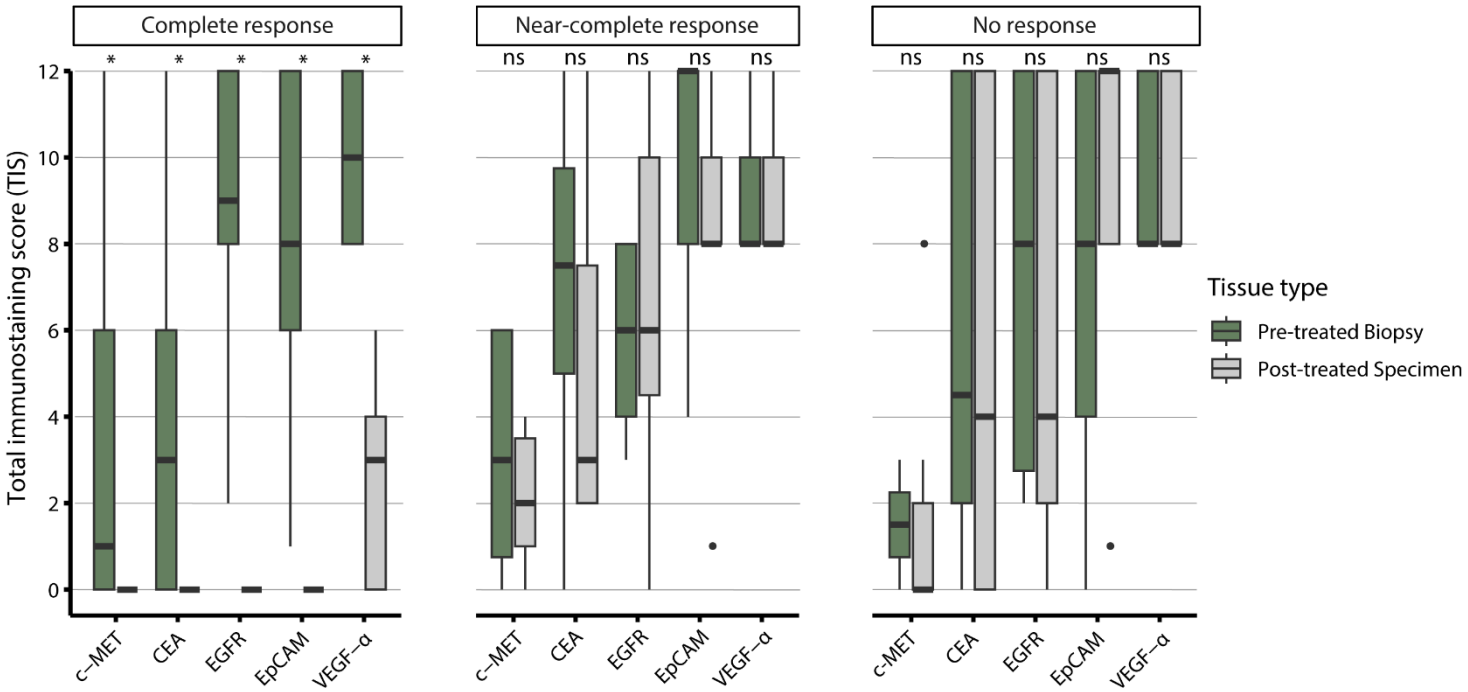

Figure 4: Comparison of total immunostaining score (TIS) per tumour marker between paired pre-treated biopsies and post-treated specimen (adenocarcinoma and squamous cell carcinoma combined).

*The boxplots represent the median with q1 and q3 with the error bars representing the range and the dots for outliers. Abbreviations: ns = not significant; * = p < 0.05*

Patients, n (%)		41 (100)
Age in years, mean (sd)		67.1 (8)
Gender, n (%)	*Male*	33 (80)
	Female	8 (20)
Tumour histology, n (%)	*Adenocarcinoma*	29 (71)
	Squamous cell carcinoma	12 (29)
Tissue available, n (%)	*Pair*	31 (76)
	Biopsy only	10 (24)
Differentiation grade, n (%)	*Good*	2 (5)
	Good / moderate	5 (12)
	Moderate	17 (42)
	Moderate / poor	5 (12)
	Poor	12 (29)
Neoadjuvant regimen, n (%)	*CROSS*	40 (98)
	MAGIC	1 (2)
Response, n (%)	Complete response	14 (34)
	Near-complete response	17 (42)
	Minimal response	10 (24)
ypT-stage, n (%)	*ypT-0*	14 (34)
	ypT-1	8 (19)
	ypT-2	6 (15)
	ypT-3	13 (32)

Table 1: Patient and tissue baseline characteristics

Tumour marker	Localisation expression in tumour	Localisation expression in healthy adjacent mucosa	Expression in other healthy tissue
c-MET	Membranous staining with cytoplasmic background staining	No / Minimal expression	No expression
CEA	Membranous staining with cytoplasmic background staining	Weak – moderate expression in the upper 33% of the luminal side of the epithelial cell layer.	No expression
EGFR	Membranous and cytoplasmic	Moderate expression in the first 33% – 67% of the basal and para-basal cell layer of the squamous cell epithelium.	Weak expression of 100% of the muscular layers
EpCAM	Membranous and cytoplasmic	No / Minimal expression	No expression
VEGF-α	Membranous and cytoplasmic	Moderate – strong expression in the basal cell layer, gradient weakening towards luminal side	Weak – moderate expression in 100% of the muscular layers Weak – moderate expression in 100% of the stromal cells

Table 2: Location of staining on tumour tissue and adjacent healthy mucosa

This table provides an overview of the more precise localisation of expression of the tumour markers in both tumour tissue and adjacent healthy mucosa.

Protein abbreviation	Antibody	Company	Antigen retrieval	Secondary antibody	Stock concentration
CEA	CI-P83-1	Santa Cruz	EnVision FLEX Target Retrieval Solution Low pH (DAKO)	Anti-mouse	0.2 mg/ml
c-MET	EP1454Y	Abcam	EnVision FLEX Target Retrieval Solution High pH (DAKO)	Anti-rabbit	0.479 mg/ml
EGFR	D38B1XP	Cell signaling technology	Dako PT link Target Retrieval Solution, pH 9.0	Anti-rabbit	0.1 mg/ml
EpCAM	MOC31	Acris antibodies	Dako PT link Target Retrieval Solution, pH 6.0	Anti-mouse	0.64 mg/ml
VEGF-α	G153-694	BD Pharmingen	No antigen retrieval	Anti-mouse	0.5 mg/ml

Supplementary table 1: An overview of the immunohistochemistry methods

This table shows an overview of the used antibodies and methods for the immunohistochemistry staining.

		Low expression		High expression	
Tumour marker	**Histology**	**% TIS 0 (n)**	**% TIS 1 - 5 (n)**	**% TIS 6 - 8 (n)**	**% TIS 9 - 12 (n)**
c-MET	*Adenocarcinoma*	43% (12)	32% (9)	18% (5)	7% (2)
	SSC	38% (3)	50% (4)	13% (1)	0% (0)
	Mucosa	38% (15)	63% (25)	0% (0)	0% (0)
CEA	*Adenocarcinoma*	7% (2)	32% (9)	21% (6)	39% (11)
	SSC	64% (7)	36% (4)	0% (0)	0% (0)
	Mucosa	16% (6)	84% (31)	0% (0)	0% (0)
EGFR	*Adenocarcinoma*	0% (0)	38% (10)	42% (11)	19% (5)
	SSC	0% (0)	0% (0)	11% (1)	89% (8)
	Mucosa	8% (3)	56% (20)	25% (9)	11% (4)
EpCAM	*Adenocarcinoma*	0% (0)	10% (3)	40% (12)	50% (15)
	SSC	9% (1)	45% (5)	27% (3)	18% (2)
	Mucosa	95% (35)	5% (2)	0% (0)	0% (0)
VEGF-α	*Adenocarcinoma*	0% (0)	4% (1)	52% (14)	44% (12)
	SSC	0% (0)	0% (0)	70% (7)	30% (3)
	Mucosa	0% (0)	27% (10)	68% (25)	5% (2)
SCC = Squamous cell carcinoma					

Supplementary table 2: A detailed overview of the TIS scores per tumour marker in pre-treated tissues, stratified for tissue histology

This table shows an overview of the TIS scores per tumour marker in in pre-treated tissue of both adenocarcinomas, squamous cell carcinomas (SCC) and healthy mucosa. The TIS score is a product of a proportion score (0= none, 1=<10%, 2= 10-50%, 3= 51-80%, 4= >80%) and intensity score (0= no staining, 1= weak, 2= moderate, 3= strong).