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Chapter 10

Preclinical uPAR-targeted multimodal imaging of locoregional head-and-neck cancers

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ABSTRACT

Objectives: Establishing adequate resection margins and lymphatic mapping are crucial for the prognosis of oral cancer patients. Novel targeted imaging modalities are needed, enabling pre- and intraoperative detection of tumour cells, in combination with improved post-surgical examination by the pathologist. The urokinase-receptor (uPAR) is overexpressed in head and neck cancer, where it is associated with tumour progression and metastasis.

Material and methods: To determine suitability of uPAR for molecular imaging of oral cancer surgery, human head and neck tumours were sectioned and stained for uPAR to evaluate the expression pattern compared to normal mucosa. Furthermore, metastatic oral squamous carcinoma cell line OSC-19 was used for targeting uPAR in in vivo mouse models. Using anti-uPAR antibody ATN-658, equipped with a multimodal label, the in vivo specificity was investigated and the optimal dose and time-window were evaluated.

Results: All human oral cancer tissues expressed uPAR in epithelial and stromal cells. Hybrid ATN-658 clearly visualized tongue tumours in mice using either NIRF or SPECT imaging. Mean fluorescent TBRs over time were 4.3 ± 0.7 with the specific tracer versus 1.7 ± 0.1 with a control antibody. A significant difference in TBRs could be seen between 1 nmol (150 μg) and 0.34 nmol (50 μg) dose groups (n = 4, p < 0.05). Co-expression between BLI, GFP and the NIR fluorescent signals were seen in the tongue tumour, whereas human cytokeratin staining confirmed presence of malignant cells in the positive cervical lymph nodes.

Conclusion: This study shows the applicability of an uPAR specific multimodal tracer in an oral cancer model, combining SPECT with intraoperative guidance.
Highlights

– Novel tumour-specific multimodal imaging agent that will aid in surgical procedures.
– Recognizing both neoplastic as well as tumour surrounding stromal cells.
– Antibody-based, providing large imaging windows and high specific signals.
– One administration sufficient for pre-, intra- and postoperative imaging.
– The agent can be applied in low doses and visualizes millimetre sized metastases.

INTRODUCTION

With more than 500,000 new cases diagnosed each year head and neck cancer is the sixth most common cancer worldwide with surgery as the primary curative treatment. An inadequate resection margin in oral squamous cell carcinoma (OSCC) surgery is an important negative prognostic indicator for loco-regional control, distant metastasis, and overall survival. Recent studies suggest that inadequate resection margins (defined as tumour-positive or with a negative margin of <5 mm) in oral cancer surgery range up to 85%, when the complete resection margin is assessed [1]. Nevertheless, time and costs hamper a thorough complete pathological assessment of the surgical specimen as clinical routine. Instead, representative samples are taken, resulting in the adequate analysis of only 0.1–1% of the resection margin [1]. Currently, surgical management of OSCC aims at achieving a macroscopic tumour-free margin of 1 cm, but the conventional approach of intraoperative assessment of tumour borders by visual inspection and palpation is not sufficient [2]. On the other hand, increasing the tumour-free margin often results in impairment of swallowing function and speech disorders [3-5]. Novel imaging modalities are being investigated which could improve the surgical management survival rates of OSCC patients in several ways. Adequate preoperative tumour staging will facilitate treatment selection, whereas intraoperative imaging techniques will improve tumour delineation during surgery. In the post-operative phase, imaging techniques can enhance pathologic examination ex vivo. Tumour specific multimodal imaging, combining preoperative nuclear imaging techniques (SPECT/PET) with intraoperative fluorescence optical guidance in one agent, could improve accuracy in all these phases [6]. The applicability of fluorescence guided imaging during oncologic surgery has recently been shown in clinical practice [7-9]. Fluorescence-guided surgery (FGS) does not only assist the surgeon in distinguishing tumour from normal tissue but can also assist in recognizing vital structures, such as nerves. Combining tumour specific FGS with conventional nuclear techniques in one single agent overthrows partly the limited tissue penetration depth of maximal 10 mm and aids in marking tumour margins.
FGS and multimodal imaging rely on the combination of a tumour targeting tracer, conjugated with a fluorescent and radioactive label, together with validated imaging systems customized for both types of label. After injection the probe will accumulate in the target tissue and enable visualization. Therefore the choice of a good target/tracer combination is crucial.

The urokinase-type plasminogen activator receptor (uPAR) is associated with many aspects of tumour development and growth, also in head and neck cancer [11-13]. uPARs primary function is to concentrate the extracellular matrix remodelling capacity of its ligand urokinase-type plasminogen activator (uPA) to the cell membrane of migrating cells [14]. Hence, uPAR is known to play an important role in tumour invasion, angiogenesis and (micro-) metastasis, and over-expression is found in the majority of carcinomas [11]. Recently, we described the development and preclinical validation of the first clinically relevant antibody-based uPAR-specific multimodal imaging tracer, combining both preoperative SPECT with NIR fluorescent intraoperative imaging [6]. That in vivo study showed the feasibility to resect colorectal cancer guided by a tumour-specific fluorescent signal, whereas the nuclear component allowed pre-operative non-invasive assessment of SPECT imaging. In oral cancer patients, uPAR is correlated with cancer invasion, secondary regional lymph node metastasis [15] and higher recurrence rates [15-17]. Various prognostic studies have related uPAR expression to worse disease-specific survival, 5-year overall survival and decreased overall survival in patients with early stage disease [18-20]. High endogenous levels of uPAR are found in hypoxic regions and invasive borders of solid tumours, including head-and-neck cancer, on both neoplastic as well as stromal cells, while in normal tissues and dysplastic epithelium little uPAR is present [11, 16, 21, 22].

The aim of this study is to evaluate uPAR expression in H&N tumours and to assess the feasibility of a previously designed multimodal uPAR specific antibody [6] to visualize OSCC in a xenograft model, using a clinically available intraoperative fluorescence imaging system and SPECT system.

**MATERIALS AND METHODS**

**Human samples and immunohistochemical staining**

Randomly chosen paraffin-embedded tumour blocks from 10 oral cancer patients who had surgery between 2013 and 2015 were obtained from the LUMC pathology department and sectioned. Tumour sections were deparaffinised and rehydrated. Endogenous peroxidase was blocked for 20 min in 0.3% hydrogen peroxide in methanol. The slides were treated for antigen retrieval in citrate buffer for 10 min at 95 °C (DAKO PT Link). Sections were incubated overnight with pre-determined optimal antibody dilution (2,4 ug/
ml ATN-615 in PBS). After 30 min of incubation with DAKO envision + HRP anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark) the sections were visualized using diaminobenzidine solution (DAB+; DAKO kit). All sections were counterstained with haematoxylin, dehydrated and finally mounted with pertex. The staining pattern was evaluated as previously described [18, 22]. All samples were handled anonymously according to the national ethical guidelines ('Code for Proper Secondary Use of Human Tissue', Dutch Federation of Medical Scientific Societies) and were approved by the Institutional Ethics Committee of the Leiden University Medical Centre.

Cell lines
For in vitro and in vivo studies, the metastatic oral squamous cell carcinoma cell line OSC-19 was used. The cell line was transfected with Luc2-copGFP and grown as previously described [23].

Antibodies
Mouse monoclonal antibodies ATN-615 and ATN-658 are both of the IgG1 k isotype and bind with high affinity (Kd ≈ 1 nM) to the same epitope on domain D3 of uPAR. ATN-615 is optimized for in vitro experiments and ATN-658 for use in vivo and both are extensively validated [24, 25]. As a control, we used the monoclonal antibody MOPC-21, isotype IgG1k, purchased from BioXCell (West Lebanon, USA) which has no known specificity for human and rodent tissues.

Flow cytometry
Flow cytometry was performed as previously described [6].

Tracer
The development of hybrid ATN-658 and MOPC-21 was previously described by our group [6]. In short, both antibodies were conjugated with the zwitterionic fluorophore ZW800-1 (λex = 773 nm, λem = 790 nm) and radiolabeled with 111In Cl via MSAP (multifunctional single attachment point). Labelling ratios were computed as follows: First, the concentration was calculated by dividing the fluorescent signal over the extinction coefficient (dye 249,000 M−1 cm−1 and antibody 225,000 M−1 cm−1) and multiplied by 10^6. Then, concentrations were divided by each other, resulting in the labelling ratio.

Animal models
Nude Balb/c female mice (Charles River laboratories, l’Arbresle, France), aged 4–6 weeks, were used. Animal experiments were approved by the local animal welfare committee of the Leiden University Medical Centre. Orthotopic tongue tumours were submucosally induced in the tip of the tongue through injection of 40,000 OSC-19-luc2-cGFP cells,
diluted in 20 μL phosphate-buffered saline (PBS). Mice body weight was monitored twice a week and tumour growth was monitored twice a week by bioluminescence (BLI) measurements, using an intraperitoneal injection of 150 μg/g of D-luciferin solution (SynChem, Inc., Elk Grove Village, IL) in PBS, in a total volume of 50 μL, 10 min prior to imaging with the IVIS Spectrum imaging system (Caliper Life Sciences Inc., Hopkinton, MA, USA), and visual inspection of the tongue. BLI signals, being the most sensitive imaging method for the detection of tumour cells [26], served as an in vivo control for the tumour specific fluorescence of the agent. Imaging procedures were performed under isoflurane gas anaesthesia.

Fluorescence and radioactivity measurements

Mice were randomly divided into three groups and received a systemic injection of uPAR-specific tracer (n = 3), a non-specific control tracer (n = 3), or label alone (n = 3). Fluorescent images were acquired at 4, 24, 48, 72 and 96 h post injection. In another experiment mice (n = 6) were sacrificed and overlying skin of the cervical region was removed for gross examination. Whole-body fluorescence imaging was performed and the tongue and cervical lymph nodes were subsequently resected. For the dose finding experiment 4 mice were divided in two groups and received 2/3 or 1/3 nmol of the uPAR specific tracer. The mice were imaged at the same time points to evaluate TBRs. Real-time NIR-fluorescence measurements and fluorescence-guided resections were performed using the FLARE™ NIR imaging system [27]. The Pearl Impulse small animal imaging system (LI-COR) was used as an in vivo preclinical reference system to measure NIR-fluorescence signals and to calculate TBR (tumour-to-background ratios). Fluorescence images of the specific and the control tracer were normalized and regions of interest were marked. Background signals were extracted from normal tongue tissue surrounding the tumour. After measuring the signal intensity from the macroscopic images they were divided by each other using the following formula: TBR = mean signal tumour/ mean signal surrounding tissue.

One mouse was injected with 1 nmol radioactive (35 MBq, 111In) hybrid ATN-658 and evaluated using SPECT and fluorescent optical imaging, to compare the agent/model with the previous study [6].

SPECT scans were conducted with a 3-headed U-SPECT-II gamma camera (MiLabs, Utrecht, the Netherlands). The total body scan was acquired using a 0.6 mm mouse pinhole collimator with energy settings at 171 and 245 keV with a window of 20% and additional background windows of 4.5% [28]. After the last imaging acquisition, the mouse was sacrificed and organs were excised, weighted, and counted for radioactivity with a gamma counter (Wizard2 2470 automatic gamma scintillation counter, Perkin Elmer, USA). The %ID/weight was calculated as followed: (MBq measured in tissue/injected dose *100%) /weight of tissue.
Histological analysis

Tumours and cervical tissue samples were surgically removed and either snap-frozen or paraffin embedded. They were sectioned and scanned on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), using the 800 nm channel, for evaluation of the fluorescent location, or for GFP signals (Nikon eclipse e800, Nikon, Amsterdam, the Netherlands). Subsequently, sections were stained with haematoxylin-eosin and merged images were generated to validate agent distribution and specificity.

Statistical analysis

For statistical analysis GraphPad Prism software (version 5.01, GraphPad Software Inc, La Jolla, California, USA) was used. TBRs are reported in mean and standard deviation. The two-way repeated measurement ANOVA, used to assess the relation between TBRs in the dose groups and time points, was corrected using the Bonferroni correction. P-values equal or lower than 0.05 were considered significant.

RESULTS

Human tissue samples

Immunohistochemical analysis of 10 human OSCC samples revealed a heterogenic uPAR staining pattern in all tumours. Five tumours showed low uPAR expression (<50% positive cells) and 5 high (≥50% positive cells). All tumours showed comparable or enhanced signal intensities at the tumour margin (Fig. 1A). Generally, tumour and stromal cells showed a moderate staining intensity with varying membranous or cytoplasmic staining. In 5 tumours stromal uPAR expression predominated over tumour expression with highest intensities on (myo-)fibroblast, neutrophils and granulocytes (Fig. 1B). Sporadically, some weak staining was seen in normal buccal mucosa tissue closely to the tumour (Fig. 1C), mostly parabasal (stratum spinosum) while the basal cells itself were uPAR negative. Keratinized cells (squamous eddies) showed high uPAR expression (Fig. 1D). uPAR expression was also found on (neo-)angiogenic endothelial cells (Fig. 1E) surrounding the tumour, whereas no expression was found in muscle tissue, lymphocytes and in normal salivary gland cells or ducts.
Study design

uPAR specific antibody ATN-658 and isotype antibody control MOPC-21 were conjugated to the hybrid label (DTPA-Lys(ZW800)-Cys-NHS) in mean ratios (dye:antibody) of 1.7:1 and 2.2:1 respectively (Fig. 2A). Flow cytometry analyses showed moderate uPAR expression on OSC-19 cells and no specific binding was detected using the control antibody (Fig. 2B). Mice were inoculated with OSC-19 cells and developed a primary tongue tumour plus adjacent cervical lymph node metastases (Fig. 2C) within three weeks [29]. First, mice were injected with non-radioactive hybrid ATN-658 specific tracer (n = 4), control IgG hybrid tracer (n = 4) or NIRF label alone (n = 3). Fluorescence in vivo images were taken both with the PEARL small animal imaging system and the clinical FLARE intraoperative imaging system. Mice were sacrificed and tumours and metastases were resected for ex vivo evaluation using the Odyssey infrared imaging system and immunohistochemistry. Eventually, one mouse was injected with the radiolabeled hybrid ATN-658 for SPECT imaging and biodistribution of the tracer.
In vivo specificity

All primary tumours were clearly identified by the uPAR-specific hybrid tracer using both the PEARL and the FLARE imaging systems, but the IgG control tracer did not discriminate tumour from the surrounding tissue, as indicated for the FLARE system in Fig. 3A–C. Extra-tumour fluorescent signals were detected through the skin with the uPAR-tracer until 72 h after injection, suggesting the presence of cervical metastases. Determined with the standardized preclinical PEARL system, the mean TBR of the uPAR specific tracer increased from 1.8 to 2.5 between 4 h and 24 h after injection, after which it remained constant at 2.5 for at least 96 h. (Fig. 3D). Differences in TBR between the tumour-specific and both control tracers, i.e. hybrid labeled MOPC-21 and ZW800 dye alone, were statistically significant at all time points (p < 0.05) (Fig. 3D). TBRs determined with the FLARE system showed a similar difference between the uPAR specific (3.0 ± 0.1 at 4 h, 4.4 ± 0.4 at 24 h, 4.7 ± 0.5 at 48 h and 4.9 ± 0.6 at 72 h) and control tracer (1.9 ± 0.1 at 4 h, dropping to 1.6 ± 0.1 at 72 h).
The estimated half-life time of the hybrid ATN-658 and hybrid MOPC21 was 15–20 h, which is comparable with our previous findings. In a dose finding experiment we studied three different doses of hybrid ATN-658 (1, 0.67 and 0.34 nmol). The TBRs of the middle and the highest dose groups are comparable between 4 h and 72 h, whereas the lowest dose (50 μg; 0.34 nmol) showed decreasing TBRs over time (Fig. 3E). Significant differences were seen between the two highest and the lowest dose group (p < 0.05).
**Resection and histology**

After 48 h, the mice were sacrificed and the cervical skin was removed. Substantially higher fluorescent signals were detected from metastatic cells within the cervical lymph nodes, as confirmed by BLI. Fig. 4 shows a typical example of BLI and NIR fluorescent signals from the cervical lymph nodes. The head of the mouse is shielded to prevent overexposure of the BLI signal originating from the primary tongue tumour. Unfortunately no GFP fluorescence could be detected in the paraffin embedded sections of the cervical lymph nodes, probably due to required but too extensive wash steps. Therefore, immunohistochemistry with a wide spectrum anti-cytokeratin antibody was used to confirm the presence of tumour cells (Fig. 4B). Ex vivo images of the tongue clearly showed tumour specific accumulation of the fluorescent tracer, whereas no signals were observed in the normal tongue musculature. Furthermore, despite the relatively large size of the antibody-based tracer, it was homogenously distributed through the tongue.

![Figure 4](image-url)

**Figure 4** Resection and histology. A) Removing the cervical skin resulted in more clear demarcation of the cervical lymph nodes. Lower fluorescent signals are observed in the muscle compared to the skin resulting in a higher TBR. Ex-vivo, the tumour could be clearly recognized at the back table using NIR fluorescence. White arrows locate the tumour and the red circles represent ROIs used to calculate TBRs. B) BLI images show presence of luciferase transfected OSC-19 cells in the cervical lymph nodes and the adjacent fluorescent signals. Immunohistochemistry against human cytokeratin validate the presence of human epithelial cells. C) The presence of the NIR fluorescent signals in the tongue showed co-localization with the GFP signals and could be confirmed to be tumour specific using HE staining. Red arrows show tumour free regions with low fluorescence intensity adjacent to the primary tumour. Left insert show an island of tumour cells next to the primary tumour that is highly fluorescent, underscoring the ability to discriminate between malignant and normal cells at the microscopic level. Right insert shows area with muscle cells that show no fluorescence.
tumour as shown both macroscopically (Fig. 4A) and microscopically (Fig. 4C). GFP signals from quick-frozen tissues confirmed the presence of human neoplastic OSC-19 cells in the tissue specimen and could be co-localized with the NIR fluorescence signal. After fluorescence imaging (GFP and NIR fluorescence), sections were stained with HE. Even sub-millimetre islands of tumour cells located closely to the primary tumour co-localized with the fluorescence, underscoring the ability to discriminate between malignant and normal cells at the microscopic level (red arrows in Fig. 4C).

**SPECT imaging and biodistribution**

SPECT was performed at 24, 48 and 72 h after injection of the tracer, and the biodistribution was analysed after the latest time-point. The tumour could be clearly recognized at all three different time-points. The SPECT images were interpreted semi-quantitatively with mean TBRs from the transversal, coronal and sagittal views of respectively 4.1 ± 1.1, 2.9 ± 0.2 and 4.0 ± 0.9 (Fig. 5A). Tumour specificity was further confirmed by simultaneously acquired NIR fluorescent images (Fig. 5B). Some (non-specific) radioactive signals could be recognized around the lacrimal glands using SPECT, but this could not be verified using fluorescence imaging, probably due to the limited depth penetration of NIR fluorescence. The biodistribution showed similar pattern and kinetics as obtained in the previous study: relatively high percentages (values in %ID/g) of signal in excreting

**Figure 5** Validating uPAR imaging agent and animal model using SPECT. A) SPECT images of the mouse injected with radioactive uPAR-specific hybrid agent at various time-points, including TBRs calculated from the images. Especially at 24h cervical lymph nodes could be clearly recognized (white arrows) and, interestingly, also the lacrimal glands could be seen at this time point (dashed arrows). White circles represent ROIs used to calculate TBRs. B) NIR Fluorescent images taken at 24h and 48h of the same mouse. Tumours (dashed arrows) are clearly visible as are the cervical lymph nodes (white arrows), both at 24h and 72h. Black circles represent ROIs used to calculate TBRs.
organs (liver 4.01 & kidneys 19.13), urine (13.19), spleen (8.77), blood (4.01) and tumour (6.16). Other organs showed moderate to low percentages (values in %ID/g): Heart 2.67, lungs 3.80, sternum 2.58, muscle 1.25, bone 3.46, brain 0.19, stomach 1.88, gall bladder 0.17, intestine 3.10, cecum + colon 3.07 and defecation 2.93.

DISCUSSION

Establishing resection margins and adequate lymphatic mapping are crucial for the prognosis of oral cancer patients. Both disciplines would be served with a multimodal tracer able to distinguish tumour from normal tissue. We investigated an antibody-based uPAR-targeting multimodal tracer that proved suitable to detect and accurately delineate locoregional H&N tumours in a xenograft mouse tongue model using radioactive as well as NIR fluorescent imaging. Presently, nuclear imaging for H&N cancer is not primarily based on targeting specific tumour-associated proteins [30, 31], but many biomarkers are under investigation for this purpose, including matrix metalloproteinases, and the epidermal growth factor receptor EGFR [32]. Most tumour-associated biomarkers are over-expressed on epithelial cells but some, like vascular endothelial growth factor receptor (VEGFR), are present on tumour surrounding stromal cells. uPAR, the target used in this study, offers the best of both worlds, as shown by the immunohistochemistry data in this study. Targeting neoplastic cells as well as tumour stromal cells is believed to be advantageous for imaging, as it increases the amount of targetable tumour mass, especially at the tumour border, thereby increasing signal intensity and the ability to recognize small tumour nodules or even cells with low specific tumour marker expression levels [33]. Recently, uPAR was targeted with an indocyanine green (ICG) labelled small peptide showing appropriate signals between 6 and 24 h after injection [34]. The same peptide was used in a first-in-human clinical trial for PET imaging of breast, prostate and bladder cancer [35]. High uptake in primary tumours and lymph node metastases was reported. The biodistribution analysis revealed a relatively high accumulation of signal in the liver, which would make this particular tracer less suitable for detection of liver metastases. Because this enhanced liver signal is not easily explained by uPAR affinity, it probably resulted from instability of the peptide/chelator complex. In the present preclinical study for H&N cancer, and in a previous study using other cancer models, we did not notice enhanced levels of our antibody-based uPAR tracer in the liver [6].

Not all uPAR binding antibodies are equally fit to target both neoplastic and stromal cells, as recently shown [36]. Significant differences exist between different uPAR recognizing antibodies in recognizing epithelial expressed uPAR, stromal expressed uPAR or both. Our IHC data indicate clearly that the antibodies used in this study recognize both
neoplastic and stromal cells, showing a millimetre-wide physiological rim around the tumour (as also visible in Fig. 1A and B), creating an ideal target for intraoperative tumour delineation [33]. Due to the species specificity of the uPAR antibodies, this phenomenon is not recognized in the \textit{in vivo} experiments, which is a clear limitation of this and many other studies about targeting of stroma in a pre-clinical animal model [33, 37]. For this reason, even higher signals and better TBRs are expected in primary tumours in human studies.

However, contrary to oral squamous cell carcinomas, cervical lymph nodes (and metastases) are covered with skin and subcutaneous adipose tissue. Therefore, optical tissue properties will have a profound influence on the NIRF signals (10), resulting in the limited depth penetration of fluorescence signals, as has previously been described [29, 38]. This would emphasize the importance of the multimodal nature of the tracer. However, because uPAR is known to be expressed on activated monocytes and macrophages, which are frequently present in first echelon located lymph nodes (sentinel nodes), the actual specificity in recognizing lymph node metastasis could be hampered by the presence of macrophages. But, due to species specificity, this will not be detected within this model.

Image-guidance is already used in sentinel lymph node procedures in the H&N region and combinations of various techniques are currently being evaluated [39]. Multimodal imaging of H&N cancer patients has been evaluated for sentinel lymph node detection using the tracer ICG-99mTc-nanocolloid [40, 41]. Both studies clearly showed additional value in finding additional sentinel lymph nodes. However, these studies used non-specific tracers that do not allow tumour-specific imaging and cannot be used to recognize resection margins and metastasis specifically. As specified in the previous paragraph, an uPAR based tracer will probably detect sentinel lymph nodes, either with metastatic cells or without, because of the presence of macrophages.

Several tumour-specific multimodal agents are under investigating using targeting vehicles of different size. Smaller agents, like peptides and nanobodies possess the advantage of fast tumour accumulation and blood clearance (hours), while larger agents, like the antibody used in this study, offer advantages in prolonged circulation times and adjacent long imaging windows (days) enabling preoperative SPECT/PET scanning and intraoperative fluorescent imaging with a single injection [42]. For multimodal applications IgG-based conjugates might exhibit the most favourable balance between systemic clearance and vascular extravasation, resulting in maximal tumour uptake and subsequent optimal specific signals [42].

In conclusion, although preoperative nuclear imaging techniques are not widely applied for surgical planning in H&N cancer surgery, this pre-clinical study paves the way for a future multimodal tracer combining NIRF imaging of tumour margins with nuclear intraoperative imaging techniques such as free-hand SPECT for the detection of lymph
node metastases [43]. Furthermore, postoperative this technique can aid in a more efficient thorough complete pathological assessment of the surgical specimen.
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