Intraoperative fluorescence imaging in cancer
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SUMMARY

FUTURE PERSPECTIVES

LUCIA M.A. CRANE
SUMMARY

In the last decades, developments in imaging have led to sensitive imaging modalities that are indispensable in cancer diagnosis and follow-up. A relatively new technique is fluorescence imaging, which has the potential to facilitate intraoperative tumor detection in surgical oncology. Several years of preclinical research have led to the recent introduction of this technique into the clinic. This thesis provides an overview of the technical and biological requirements for intraoperative fluorescence imaging, supported by data from the first clinical feasibility studies in this field, with the emphasis on surgical and gynecologic oncology. The thesis is subdivided into two sections; intraoperative imaging using a non-specific fluorescent agent (Part I) and tumor-targeted imaging (Part II).

A general introduction on intraoperative imaging is provided in chapter 1. This chapter highlights the background of non-specific and tumor-targeted fluorescence imaging in oncology and illustrates the conditions for clinical implementation. Furthermore, the outline of the thesis is clarified in the introduction.

The first part of the thesis, Part I, focuses on intraoperative near-infrared fluorescence (NIRF) imaging for the detection of the sentinel lymph node (SLN). The standard SLN procedure is based on dual modality detection using radiocolloid and a blue dye; NIRF imaging using indocyanine green (ICG) is a novel method of which the value yet has to be determined. We discuss three clinical pilot studies that compare NIRF imaging to the standard method.

Chapter 2 describes a technical feasibility study for SLN detection using NIRF imaging in cervical cancer. Lymph node status is a major prognostic factor in cervical cancer, making full pelvic lymphadenectomy an essential part of surgical treatment. Lymph node metastases are reported in approximately 25% of cases, indicating that three quarters of patients will not benefit from either limited or extensive pelvic lymph node dissection. Complications such as lymphedema are reported by one fifth to one third of patients. The role of the SLN procedure in cervical cancer is still a matter of debate because the bilateral drainage pattern from the uterine cervix and the localization of SLNs deep in the pelvis may impede detection. We hypothesized that NIRF imaging may potentially provide two advantages: I) the one-step procedure does not pose stress on patients, as is the case with preoperative radiocolloid injections; and II) the high signal-to-background ratio of ICG may allow detection deep in the pelvis. After conducting a pilot study of 10 patients, we concluded that NIRF imaging is technically feasible. SLNs were found with fluorescence in six out of ten patients, with bilateral SLNs in three of these six patients. Improvements in the flexibility of the camera system are required for optimal fluorescence detection in the pelvis. Furthermore, visualization of the lymph nodes that are located deep in the pelvis, especially in more obese patients was limited by the penetration depth of ICG of ~1 cm, necessitating improvements in fluorescent agents and instrumentation.
In contrast to cervical cancer, the SLN procedure has been proven safe in vulvar cancer, and is now part of standard practice. Again, disadvantages of the technique are the use of radioactivity and the preoperative radiocolloid injection in the vulva. Both issues are avoided when using NIRF imaging, which was the main motivation for the technical feasibility study described in chapter 3. Ten patients with squamous cell cancer of the vulva were included in this pilot study. Transcutaneous lymphatic mapping was possible in five out of ten patients and was limited to lymph nodes located at a depth of <24 mm, moreover, these patients had a lower median BMI than patients in whom transcutaneous fluorescence could not be detected. These findings suggest that NIRF imaging may primarily find its value in lean patients. Larger studies are needed to determine I) the maximum BMI in which the technique is feasible; and II) the maximum distance between lymph nodes and skin in which fluorescence can still be detected transcutaneously.

In chapter 4, we discuss intraoperative NIRF imaging for SLN detection in breast cancer. Preliminary conclusions from this pilot study are that detection rates are comparable to radiocolloid. However, analogous to the above mentioned pilot studies, pitfalls are the limited transcutaneous detection of fluorescence and technical limitations of the camera system. Comparable studies conducted in Japan and the USA indicate that ICG yields good results in SLN detection, however, uniform, sensitive and flexible camera systems are needed. This study is a step-up towards tumor-targeted fluorescence imaging, which may help detect metastases with a tumor-specific agent.

In general, introduction of a new technique into the clinic involves a learning curve, and intraoperative fluorescence imaging is no exception. In order to give an explanation of the technique, and disperse the concept in academic institutions, we produced a video to illustrate the technical and practical implementation of NIRF imaging for SLN. Chapter 5 consists of the textual protocol of the article; the accompanying video is provided on the supplementary CD on the back cover of this thesis.

Part II of this thesis focuses on tumor-targeting and tumor-targeted intraoperative fluorescence imaging. As every tumor has a unique biological profile, there is no ‘universal’ target that applies to all cancers. A possible exception is the vascular endothelial growth factor (VEGF), which is overexpressed when blood supply in the growing tumor falls short. However, VEGF expression depends on the tumor activity. Another example is the glucose metabolism, monitored through FDG-PET, but extensive research has shown limitations in less metabolic active tumors.

Target-finding is an issue that needs to be addressed separately for each individual tumor type. Databanks with prospectively collected patient material are essential for biomarker research. Hypotheses coming forth from such analyses can be tested in a mouse model mimicking a human disease. In chapter 6, an overview is presented of the several animal models that have been developed in cancer, with the emphasis on hypoxia in esophageal cancer as the basis for finding targets and testing concepts in human xenografts.
In chapter 7, we provide an overview of the most promising targets for intraoperative imaging in epithelial ovarian cancer (EOC). In selecting targets, we focused on those that are close to translation into the clinic. Folate receptor alpha (FR-α) is by far the most promising target in EOC, based on its overexpression in 80-90% of tumors. FR-α has already been successfully targeted and a number of clinical trials are currently being conducted on folate-based imaging agents and therapeutics. Vascular Endothelial Growth Factor (VEGF) and Epidermal Growth Factor Receptor (EGFR) are two markers that are upregulated in EOC and have clinically approved therapeutic antibodies. The contrast agents that are based on chemokine receptors, in particular CXCR4, and matrix metalloproteinases (MMPs) are currently being investigated in animal models, and may in the future also be of value in intraoperative imaging as these indicate areas of disseminated disease.

Chapter 8 focuses on the development of a new scoring system for selection of biomarkers for tumor-targeting in colorectal cancer (CRC). A large number of biomarkers have been identified in CRC, but to date no adequate selection system or criteria have been described. We presented the TArget Selection Criteria (TASC), a scoring system that facilitates selection of targets for imaging purposes. The TASC score is based on seven biomarker characteristics that are needed for successful application in imaging: I) extracellular biomarker localization; II) expression pattern; III) tumor-to-healthy ratio; IV) percentage of positive tumors; V) reported successful use of the biomarker in in vivo imaging studies; VI) enzymatic activity; and VII) internalization. When applying TASC, we identified seven targets that are most promising for imaging in CRC: carcinoembryonic antigen (CEA), chemokine receptor 4 (CXCR4), EGFR, epithelial cell adhesion molecule (EpCAM), MMPs, mucin 1 (MUC1) and VEGF-A. All could be candidates for future tumor-specific intraoperative fluorescence imaging in CRC. Cross-validation of the TASC scoring system is necessary to prove its applicability in target selection.

As mentioned above, the folate receptor is one of the most potent targets in ovarian cancer. We conducted a retrospective study on the effect of chemotherapy on the expression of FR-α in ovarian cancer tissue samples, described in chapter 9. Chemotherapy is a vital part of the treatment of ovarian cancer and targeted imaging agents and drugs are therefore only functional if the target is not affected by (neo-)adjuvant chemotherapy. In this study, we focused on the possible effect of chemotherapy on expression rates of FR-α in ovarian cancer. Immunohistochemical staining on 361 patient tissue samples shows expression (weak, moderate or strong) of FR-α on 82% of serous ovarian cancers and on 17% of ovarian cancers of other histologic subtypes (p<0.001). Sub-analysis on 28 matched serous samples showed no difference in expression by vital tumor tissue before and after chemotherapy by (p=0.149). The grade of FR-α expression showed not to be a prognostic marker for disease-free and overall survival. These data illustrate that FR-α is an attractive target in serous ovarian carcinoma, furthermore, expression rates are not significantly altered by chemotherapy.

The availability of a clinical grade folate-based contrast agent (folate-FITC; Endocyte...
Inc.) offered us the opportunity to investigate the value of tumor-targeted intraoperative fluorescence imaging in ovarian cancer patients. To our knowledge, the study described in chapter 10 is the first to apply tumor-targeted intraoperative imaging in a clinical setting. In a patient with extensive peritoneal disseminated disease, we were able to visualize tumor nodules as small as 1 mm in vivo, and tumor deposits <1 mm ex vivo. A moderate signal was seen in serous tumors without dissemination, whereas fluorescence was neither observed in benign tumors nor in an inflammatory process of the ovary. Postoperative determination of FR-α expression showed excellent correlation of fluorescence intensity in vivo and FR-α expression by the tumor. Additionally, fluorescence microscopy for FITC in excised tissue correlated well with both FR-α expression and in vivo fluorescence. This study shows that tumor-targeted intraoperative fluorescence imaging is feasible. Future clinical studies are needed to establish the detection accuracy and the definitive clinical value for staging and intraoperative decision making.

CONCLUSIONS AND FUTURE PERSPECTIVES
In this thesis, we illustrate several aspects of intraoperative fluorescence imaging in surgical and gynecologic oncology. The clinical pilot studies indicate that this technique is technically feasible, both for detection of the sentinel lymph node (SLN) and tumor-targeted imaging. In the following paragraphs, the most important conclusions of this thesis are expanded and suggestions for future developments are discussed.

TECHNICAL IMPROVEMENTS TO THE FLUORESCENCE CAMERA SYSTEM
When working with a prototype such as the intraoperative camera that was used in all the clinical studies in this thesis, it is inevitable that points for improvement come to light. Second-generation systems will undoubtedly be more refined and customized. The most important technical improvements concern the flexibility of the camera system. For intraoperative imaging of superficially located targets such as inguinal or axillary lymph nodes, the current system is reasonably adequate. However, when using the camera system in cervical cancer (chapter 2), the localization of lymph nodes deep in the pelvis hampered optimal detection. A more flexible and smaller camera head, in particular one with more rotation freedom, is needed for fast and smooth imaging in the pelvis and/or abdomen.

An advantage of the current system is its working distance from the surgical field. The free-standing camera system is positioned approximately 25 cm above the patient, allowing for visualization of a relatively large field-of-view (max. 15x15 cm) and synchronous, real-time surgical actions. Other groups have worked with the Photodynamic Eye (PDE; Hamamatsu Photonics K.K.), a hand-held fluorescence detector. To our opinion, a hand-held system is not preferable, as it hinders the surgeon in simultaneous dissection of fluorescent tissue. Furthermore, neither the PDE nor other commercially available fluorescence detectors such as IC-view (Pulsion Medical Systems) are equipped for si-
multaneous detection of color and fluorescence. This feature is useful, if not indispens-
able, in anatomical positioning, especially in the pelvis and abdomen.

The number of laparoscopic and endoscopic surgeries is increasing and the first laparo-
scopic fluorescence camera systems have now been tested in clinical studies. It is expected
that future developments in the field of intraoperative imaging will also be focused on
multispectral laparoscopic cameras.

FLUORESCENT AGENTS
Currently, the only two FDA-approved fluorescent agents are indocyanine green (ICG)
and fluorescein isothiocyanate (FITC). Both have been used for several decades as diag-
nostic agents and have a well-known pharmacologic profile. The emission peak of FITC
lies in the visible spectrum, resulting in a higher degree of autofluorescence by surround-
ing tissue and absorption by hemoglobin. Although we showed that tumor-targeted im-
aging is possible with FITC, we assume that these results could only be obtained due to
the superficial location of tumor tissue. The wavelength of FITC is most likely too weak
to penetrate tissue beyond 5 mm.

ICG is preferable in fluorescence imaging, as its near-infrared emission wavelength yields
superior penetration and a higher signal-to-background ratio. Most studies on intraop-
erative imaging report acceptable results when using ICG for superficial lymph node
mapping; however, we encountered difficulties in visualizing fluorescence from deeper-
seated lymph nodes (chapter 2).

Future progress in fluorescence imaging should certainly include the development of new
fluorescent agents for clinical use, taking into account a number of characteristics. First,
the agent must be non-toxic. Second, the molecule needs to be small enough to be trans-
ported through capillaries and lymph vessels in order to reach tumor cells. Third, a wave-
length in the near-infrared range (750-1000 nm) is preferable to minimize autofluores-
cence and scattering in surrounding tissue. Fourth, the quantum efficiency (brightness) of
the agent must be sufficient to penetrate through tissue. Currently, the novel agent IRDye
800CW (Li-COR Biosciences), which is >50 times brighter than ICG, is being tested for
clinical application.

TUMOR-TARGETED FLUORESCENCE IMAGING
In the last years, a number of studies have reported fluorescence imaging as a tool for SLN
detection. This technique bears several advantages; however, with the current fluorescent
agents it is doubtful whether the detection rate will be high enough. A more interesting
approach would be the intraoperative detection of lymph node metastases by tumor-spe-
cific imaging. Ideally, integrating highly sensitive tumor-targeted imaging with the SLN
procedure would lead to direct feedback on lymph node status. In case of metastatic
involvement, full lymphadenectomy could then be performed during the same surgery.
This approach requires the identification of tumor-specific targets in lymph node meta-
stases. A number of studies illustrate corresponding expression of biomarkers in both pri-
mary tumors and lymph node metastases. In breast cancer, HER2-amplification in both the primary tumor and metastatic lymph nodes was seen in 71% of cases. HER2 can be targeted with trastuzumab, offering the possibility of targeted imaging in the future, not only for breast cancer but potentially also for detection of the circumferential resection margin in esophageal cancer or for disseminated gastric cancer during laparoscopic surgery. Carbonic anhydrase IX (CA-IX), a hypoxia-related transmembrane protein present in renal, vulvar, cervical and colorectal carcinoma, was identified as a possible marker for lymph node metastases in vulvar cancer. Recently, a number of CA-IX inhibitors were developed, including introduction of such a compound as PET imaging agent. A phase I study on pre-operative PET-scanning using a radiolabeled CA-IX-antibody showed accurate detection of clear-cell renal carcinoma. Furthermore, successful targeting of CA-IX with IgG-fragments was recently reported in mice. Theoretically, these findings could be expanded towards tumor-specific intraoperative imaging in lymph nodes through conjugation of a CA-IX-antibody to a fluorescent agent. However, this hypothesis requires extensive scientific investigation.

As is demonstrated in this thesis, thorough research is needed to select the best targets for each tumor type. As not every biomarker is expressed to the same extent, it is possible that targeting a panel of markers will yield better results than focusing on a single factor. Although tumor-targeting is still a developing field of research, swift progress is expected as suitable targets can be of value in diagnostics as well as in intraoperative imaging and therapy. Bevacizumab, a VEGF-A therapeutic antibody, is used as an antitumor drug in several types of cancer such as ovarian, colorectal and renal cell carcinoma. Additionally, PET-scanning using a bevacizumab-based radionuclide can be used for pre-operative visualization of tumors. Conjugation of bevacizumab and trastuzumab to a fluorescent agent for intraoperative imaging is now being investigated (preliminary data). These results illustrate the possible versatility of one single biomarker. The time-consuming and expensive process of introducing a fluorescent compound for targeted imaging to the clinic can be drastically shortened for biomarkers that are already in use for a different purpose, like the ones mentioned above.

In conclusion, tumor-targeted intraoperative imaging bears great potential for detection and treatment of cancer. Research in this field can only be successful in a multidisciplinary collaborative approach, involving molecular biology, chemistry, physics, pharmacy, and clinicians with different backgrounds such as medical oncology, surgery, gynecology, radiology and nuclear medicine. In the near future, both preclinical and clinical studies will undoubtedly shed more light on the possibilities and pitfalls of intraoperative fluorescence imaging in cancer.
REFERENCES


