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Title: Near-infrared fluorescence-guided surgery : pre-clinical validation and clinical translation

Issue Date: 2014-05-01

Chapter 15

Summary and Future Perspectives

SUMMARY

Complete surgical resection of a tumor still is the single most important prognostic factor in most types of cancer. Many preoperative imaging modalities are being used to diagnose and stage patients and to finalize the surgical planning. However, during surgery only a limited number of tools can be used to identify tumors, and more importantly to differentiate between normal tissue and tumor tissue. Since it can be extremely challenging to assess the resection margins during surgery, irradical resections unfortunately still occur in a substantial part of patients. Near-infrared (NIR) fluorescence imaging has the potential to be of major impact on surgical treatment and surgical treatment in surgical oncology in particular. Intraoperative real-time visualization of tumors and lymph nodes can hopefully decrease the number of irradical resections and improve surgical outcome parameters such as time of surgery and surgical complications (**chapter 1**).

This thesis focus on preclinical validation of novel fluorescent contrast agents for solid tumor imaging (**Part I**), the clinical introduction of NIR fluorescence sentinel lymph node imaging in several cancer types using indocyanine green (**Part II**) and the clinical translation of NIR fluorescence imaging using clinically available fluorescent contrast agents for solid tumor imaging (**Part III**).

Part 1: Pre-clinical validation of near-infrared image guided surgery

Chapter 2 describes the preclinical optimization of intraoperative visualization of colorectal liver metastases using NIR fluorescence and indocyanine green (ICG). ICG has shown to passively accumulate around liver metastases, however optimal conditions in terms of injection time and dose were never tested. Several different time-points of ICG administration prior to surgery and several different dose groups were tested in an experimental syngeneic liver metastases rat model. All metastases could be identified after ICG injection. The highest tumor-to-liver ratio was obtained when ICG was administered 72 hours prior to surgery.

In head and neck surgery, positive resection margins severely impact patient outcome. Intraoperative assessment of resection margins could be of great value and has the potential to decrease the number of irradical resections. Since most head and neck cancers show high levels of EGFr expression, **chapter 3** describes the successful use of the EGFr –targeting nanobody 7D12 in an experimental orthotopic tongue

cancer mouse model. All tumors could be clearly identified and most importantly, the negative control nanobody showed no fluorescent uptake in the tumor.

Chapter 4 describes the use of the zwitterionic fluorophore ZW800-1 which was conjugated to the tumor specific peptide cRGD (targeting integrins) and to the α -specific peptide cRAD. These probes were injected in mice bearing subcutaneous and orthotopic HT-29 human colorectal tumors. Both subcutaneous and orthotopic tumors could be clearly identified using cRGD-ZW800-1. A significantly higher signal-to-background ratio (SBR) was observed in mice injected with cRGD-ZW800-1 compared to mice injected with cRAD-ZW800-1 or ZW800-1 alone when measured at 24 h after probe administration. The clearance of cRGD-ZW800-1 permitted visualization of the ureters and also generated minimal background fluorescence in the gastrointestinal tract.

Part 2: Clinical translation: Sentinel lymph node imaging

In vulvar cancer patients, SLN mapping is standard of care to obtain nodal staging. Typically, a blue dye and radiocolloids are being used, which have certain disadvantages. In **chapter 5**, the use of NIR fluorescence imaging was tested in vulvar cancer patients using different doses of ICG premixed with human serum albumin (complex ICG:HSA). In this study, a high identification rate of 100% was demonstrated. SLN mapping in vulvar cancer patients is in particular an interesting indication since the lymph nodes are often located superficially which enables NIR fluorescence imaging to percutaneously identify the SLN.

In cervical cancer, sentinel lymph node biopsies are not yet standard of care due to disappointing identification rates and high false-negative rates. In **chapter 6**, NIR fluorescence and ICG:HSA was used for sentinel lymph node mapping in cervical cancer patients. Three dose levels (1.6 ml of 500, 750 and 1000 μ M ICG:HSA) were tested. A high intraoperative identification rate of 100% was observed, no false negative cases were observed and no significant differences between dose groups could be identified. NIR fluorescence therefore has the potential to be used for SLN mapping in cervical cancer patients and can thereby possibly prevent a total lymphadenectomy in a large number of patients. However, this has to be assessed in larger clinical trials.

In-vitro and preclinical studies showed that premixing ICG with HSA increased fluorescent yield and increased hydrodynamic diameter, which possibly results in better retention in the SLN.¹ However, a randomized clinical study in breast cancer patients showed no differences between using ICG alone and

ICG:HSA.² This can possibly be explained by the physiologically available proteins in lymph fluid, which eliminate the need of premixing with HSA. In **chapter 7**, a randomized study is described, which tested the need of premixing ICG with HSA in cervical cancer patients. Based on **chapter 6**, a dose level of 500 μ M ICG (1.6 ml) alone or ICG:HSA was used. No significant differences could be observed between the ICG alone group and the ICG:HSA group. These results show that ICG alone can be used for SLN mapping in cervical cancer patients.

In **chapter 8**, the use of NIR fluorescence and ICG:HSA at different dose levels was tested in a clinical trial in melanoma patients. In all patients (N = 15), the SLN could be identified using NIR fluorescence. Thereby, SLNs could be percutaneously identified using NIR fluorescence in several cases. Using NIR fluorescence in melanoma patients, time of surgery could possibly be decreased. Furthermore, NIR fluorescence outperformed blue dye staining and the benefit of using patent blue needs to be assessed in future larger clinical trials.

The SLN procedure is not standard of care in oral cavity and oropharynx cancer patients. NIR fluorescence imaging using ICG:HSA was tested in patients which were planned for surgery for oral cavity or oropharynx carcinoma in **chapter 9**. Since standard of care in our center includes a complete neck dissection, a perfect platform is created to test the flow to higher tier lymph nodes using ICG:HSA. In all patients, a SLN could be identified during surgery and one false-negative case was observed. This can be explained by multiple drainage patterns, resulting in a subset of SLNs becoming fluorescent later, being incorrectly identified as higher tier lymph nodes. Future research should therefore be focused on fluorescent tracers that are retained in the first draining node(s), which makes it possible to perform imaging later after injection of the dye.

Previous clinical studies showed that SLN mapping in breast cancer patients can be performed using NIR fluorescence and that 1.6 ml 500 μ M of ICG alone is most optimal to use. In chapter 10, the need to use patent blue when using NIR fluorescence and radiocolloids has been assessed in a randomized trial. The results of this study show that patent blue can be omitted when NIR fluorescence and radiocolloids are used. In all cases, the SLN was identified with NIR fluorescence earlier than with patent blue. Furthermore, to preliminarily assess the needs of radiocolloids for SLN mapping in breast cancer patients the gamma probe was not used during the first 15 minutes of the operation. To identify the SLN, the gamma probe was still needed in 25% of patients. However, the body mass index of patients in whom the gamma probe was needed to identify the SLN

was significantly higher compared to the body mass index of patients in whom the SLN could be identified using only NIR fluorescence (33.1 ± 9.9 vs. 24.4 ± 5.4). These results suggest that BMI might be used eventually to select those patients in whom SLN mapping can be performed without the use of radiocolloids.

In summary, this thesis shows that a dose of $500\mu\text{M}$ of ICG administered as a volume of 1.6 ml without the need of premixing with HSA is optimal to use for the sentinel lymph node procedure in various cancer types that were studied in this thesis. Another recently developed approach is combining radioactive colloids with indocyanine green in one multimodal tracer.^{3,4} The advantage of this approach is the necessity of only one injection. Moreover, rather than visualizing lymph flow with fluorescence imaging, the hybrid approach individually illuminates the very nodes identified on lymphoscintigrams and SPECT/CT and thus combines preoperative results with intraoperative findings.

Part 3: Clinical translation: Solid tumor imaging

Chapter 11 was performed to clinically translate the results from **chapter 2**. In this study, ICG was injected prior to surgery to intraoperatively identify colorectal liver metastases using NIR fluorescence imaging. In 40 patients, median tumor-to-liver ratios of 7.0 (range 1.9-18.7) were observed. No differences in tumor-to-liver ratios were observed between dose and time groups. Therefore, the lowest tested dose of 10 mg is recommended. In 12.5 % (95 % CI: 5.0 -26.6) of patients, additional metastases, which would otherwise be missed, could be identified and resected using NIR fluorescence. Furthermore, it was shown that ICG accumulated in CK7-positive immature hepatocytes. It is known that immature hepatocytes often have impaired expression of their organic anion transporters, which are essential for the transport of many organic anions, including ICG. Consequently, the biliary excretion of ICG in immature hepatocytes can be reduced by 90%. Therefore, the pattern of rim fluorescence could be explained by the presence of immature hepatocytes in the liver tissue surrounding the tumor that have taken up ICG, but exhibit impaired biliary clearance.

In **chapter 12**, an attempt was made to use ICG to explore the enhanced permeability and retention (EPR) effect to intraoperatively identify pancreatic tumors. Even after a macroscopically curative resection of pancreatic tumors, tumor cells might be observed by microscopy at one or more edges of the resected specimen in 17–74%.⁵⁻⁷ Better visualization of pancreatic tumors during

surgery could therefore decrease the number of irradical resections. The EPR effect, which is based on leaky vessels and hampered lymphatic drainage and results in passive accumulation of agents in tumors has been described extensively. Since no tumor-specific contrast agents are yet available clinically, ICG was injected intravenously with the objective to explore the EPR effect in pancreatic cancer patients. However, no adequate contrast between tumor and normal pancreatic tissue could be observed in all but one patient, unfortunately. This is possibly the result of different tumor biology of pancreatic cancer when compared to other cancer types. Several earlier studies reported a lower perfusion of tumor tissue in comparison with healthy pancreas tissue, which might decrease availability of ICG for a potential EPR effect of the tumor.^{8,9} Concluding, pancreatic cancer is not a suitable tumor type to exploit the EPR effect. On the other hand, results will not be distorted by the EPR effect when using tumor-targeting fluorescent agents.

The intraoperative identification of parathyroid adenomas can be challenging, which results in surgical complications and reoperations. In the past, methylene blue has been used to macroscopically stain parathyroid glands during surgery. However, since high doses were necessary for visual identification and toxicity could occur, this was stopped. As methylene blue is a medium-strength fluorophore when diluted to doses invisible to the human eye, **chapter 13** describes the use of NIR fluorescence and low-dose methylene blue in parathyroid surgery. In 10 of 12 patients, histology confirmed a parathyroid adenoma and in 9 of these patients, NIRF could clearly identify the parathyroid adenoma during surgery. Seven of these 9 patients had a positive preoperative ^{99m}Tc-sestamibi SPECT scan. Importantly, in two patients, parathyroid adenomas could be identified only using NIRF. This is the first study to show that low-dose MB can be used as NIRF tracer for identification of parathyroid adenomas, and suggests a correlation with preoperative ^{99m}Tcsestamibi SPECT scanning.

As discussed in the **chapter 13**, based on an unknown mechanism, methylene blue accumulates in neuro-endocrine tissue. This mechanism could be used to identify neuro-endocrine tumors of the pancreas by injecting methylene blue during surgery. **Chapter 14** describes a case study in which methylene blue was intravenously injected in a patient suffering from an extremely rare solitary fibrous tumor of the pancreas. In this case, the pancreatic tumor could be clearly identified during surgery and maximum tumor-to-background ratio of approximately 3 was observed.

FUTURE PERSPECTIVES

NIR fluorescent contrast agents

The clinical availability of the fluorescent contrast agents indocyanine green and methylene blue, which were used in part II and III of this thesis resulted in a fast introduction of NIR fluorescence imaging in cancer surgery. Thereby, it significantly contributed to the practical knowledge of using NIR fluorescence imaging during surgery. However, both these agents cannot be conjugated to other molecules without changing their chemical structure, which will result in the necessity to conduct phase 0 and 1 clinical trials. Moreover, fluorescent brightness of both ICG and MB are by far not optimal. In order to determine the true clinical benefit of this technique, development and clinical assessment of contrast agents tailored to specific applications are essential. In this perspective, the development of novel targeted probes starts with clinically-compatible fluorophores. Although several fluorophores have been studied and tested preclinically, to date only two fluorophores made it towards the process of clinical translation. IRDye 800CW (LI-COR Biosciences, Lincoln, NE) and ZW800-1 (The FLARE Foundation, Wayland, MA) are both small molecules which are easily conjugatable to targeting ligands. Initial toxicity studies of both compounds showed no toxicity and the purely renal clearance of ZW800-1, and the combined renal and hepatic clearance of IRDye 800CW, enable these agents to be used for imaging of ureters and bile ducts.

Combining these NIR fluorophores with clinically available antibodies could usher in a new generation of NIR fluorescent contrast agents. As recently reviewed by Scheuer and colleagues¹⁰ clinically approved targeted antibodies are available for various tumors and tumor markers, for example bevacizumab (Genentech, San Francisco, CA) against the vascular endothelial growth factor A, cetuximab against the epidermal growth factor receptor (Bristol-Myers Squibb, New York City, NY, and Eli Lilly, Indianapolis, IN), and trastuzumab against the human epidermal growth factor receptor 2 (Genentech, San Francisco, CA). Indeed, van Dam and colleagues are currently accruing patients in the first clinical trial using bevacizumab conjugated to IRDye 800CW.¹¹ Next to antibodies, which have several disadvantages such as their large size and long circulation time, other smaller targeting molecules have been advocated and even successfully translated into the clinic. Cyclic arginine-glycine-aspartic acid (cRGD) has been extensively used clinically in positron emission tomography studies mostly in glioblastoma patients.¹²⁻¹⁴ cRGD conjugated to ZW800-1 (cRGD-ZW800-1) was first described by Choi et al^{15,16} and was preclinically

used to clearly visualize orthotopic colon tumors in mice (*unpublished data*). Another approach to overcome the drawbacks of intact antibodies is the use of smaller antigen binding fragments such as nanobodies (chapter 3 of this thesis).¹⁷

Imaging system development and optimization

During the last decade, many different imaging systems, designed and developed by both academic and industry groups, for open, laparoscopic, thoracoscopic, and robotic surgery were used for NIR fluorescence imaging studies.^{4,18-24} The optics, usability, fluorescence excitation source and power and cost are key issues in imaging system design. There should be a balance between maximizing the fluence rate of the excitation light, which increases tissue penetration depth and minimizing undesirable effects such as skin/eye exposure, irreversible photochemical bleaching of the NIR fluorophore, and tissue heating. To avoid these issues and to avoid the need to wear laser goggles, fluence rates are mostly limited to the 10-25 mW/cm² range. A few imaging systems are able to acquire multiple wavelengths of light at the same time.^{19,23} This enables the visualization of the targeted tissue in relation to the surrounding tissue anatomy. For example, tumor identification and nerve visualization in pelvic surgery for rectal cancer would be extremely beneficial. Furthermore, using software based correction techniques, it is also possible to discriminate between multiple contrast agents emitting at a nearly similar wavelength.^{25,26} NIR fluorescence imaging can also be of particular benefit in laparoscopic and robot assisted surgery, in which there exists diminished tactile feedback. NIR fluorescence-capable fiberscopic systems are now readily available for these applications.^{4,27,28} Further technical developments of these laparoscopic NIR imaging systems are in progress aiming to improve the real-time intraoperative display of NIR fluorophores.

Limitations and leverage

The major disadvantage of using NIR fluorescence and reason for it being an adjunct rather than a substitute of other imaging modalities is the limited penetration depth. NIR fluorescence imaging (which uses reflectance-based systems) is not able to visualize structures that are located more than 5 mm to 1 cm beneath the tissue surface. Therefore, the field of intraoperative imaging is moving towards combining imaging modalities. Imaging modalities based on radioactive tracers, such as preoperative PET and SPECT, and intraoperative gamma probes and gamma cameras, have depth sensitivity to several cm but cannot provide real-time and precise visualization. Intraoperative ultrasound imaging also has superior depth penetration when compared with NIR fluorescence, but requires tissue contact and has problems visualizing smaller and superficial lesions. Combining NIR fluorescence imaging with these modalities leverages the key benefits, whilst overcoming the limited penetration depth of NIR light.

Conclusion and path to routine patient care

NIR fluorescence image-guidance during cancer surgery has the potential to change patient management by visualizing tissue demarcation in real-time, thereby increasing the completeness of surgery and decreasing the morbidity associated with damage to normal structures. As stated before, the limited penetration depth will make NIR fluorescence imaging complementary to existing techniques rather than replacing them. However, results of studies using the first generation imaging systems and clinically available contrast agents are extremely promising and feasibility has been proven in many clinical studies. That leaves the main question, when will it become part of routine patient care? As we know from other promising imaging techniques that made it to wide clinical implementation, such as magnetic resonance imaging and computed tomography, adaptation had to satisfy two major criteria; changing patient management and being clinically realistic. Thus, it needs to have a significant impact on patient care and it cannot disrupt normal workflow. Thereby, since insurance companies and governments try to streamline (reign in) health care, a novel imaging modality also needs to make patient care faster, better, and cheaper. NIR fluorescence imaging has the ability to meet all criteria as the surgeon can possibly identify the targeted tissue faster and it will not disrupt normal workflow, as the surgical field will not be hampered. If NIR fluorescence imaging will increase the number of radical resections and decrease complication rates, then healthcare costs can and will be significantly reduced, but most importantly, patient care and prognosis will be improved.

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