Lighting up cancer aggressiveness: targeting the urokinase plasminogen activator receptor for intraoperative optical imaging
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Chapter 2

Prognostic impact of urokinase plasminogen activator receptor expression in pancreatic cancer: malignant versus stromal cells

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Part I

Introduction
Chapter 1

Introduction and thesis outline
The radical mastectomy for breast cancer, described by William S. Halsted in 1891, represents a milestone in curative oncological surgery [1]. Believing that “cancer was a local disease, growing per continuum to lymph nodes and subsequent metastasis”, he advocated grossly mutilating en-bloc resections of affected regions in order to achieve cure. Throughout the early 20th century the mindset that cancer can be cured if resected radically enough has laid the foundation of surgical care. However, with an increased understanding of cancer biology, the past century’s technological advancements, and the wish to be less mutilating, cancer treatment has altered significantly from the aggressive dissections initially perpetuated for all patients [2].

Cancer treatment is now a multidisciplinary approach within which physicians have an increasing number of therapies at their disposal. Options include, but are not limited to, minimally-invasive/robotic excisions, ablation, irradiation, chemotherapy, targeted therapy and immunotherapy. Nonetheless, the cornerstone of cure for almost all solid tumors is still surgery, where attaining negative tumor margins is fundamental; positive surgical margins (R1/R2, irradical) repeatedly translate into worse overall survival [3]. Unlike what Halsted proclaimed though, organ-sparing surgeries have proven as effective for the majority of patients as extensive anatomical dissections. Another change, compared to Halsted’s era, is that surgeons operate in, sometimes quite literally, different landscapes. First of all, due to neoadjuvant therapy, operating fields are full of fibrosis, inflammation and necrosis, challenging the discernment between malignant and reactive tissue. Secondly, with the advent of laparoscopic and robotic procedures, the visual field is digitalized and the discriminatory nature of tactile feedback is distorted, if not unfeasible.

The emergence of intraoperative imaging
In response to these challenges, intraoperative imaging modalities have entered the operating theatre to aid in navigation between malignant and non-malignant (i.e. normal, benign or reactive tissue) tissue. These modalities, each with their own strengths and weaknesses, can be categorized into conventional, optical, nuclear, and endogenous reflectance [4]. One of the most promising techniques is the optical imaging technique called fluorescence guided surgery (FGS) as it is relatively simple, gives real-time images, results in high contrast and sensitivity, and lacks ionizing radiation. FGS camera systems function by emitting a spectrally
resolved near-infrared (NIR) light that subsequently excites the exogenous contrast agent (i.e. fluorophore) [4-8]. An excited fluorophore emits photons that are then captured by a detector and translated into a digital image and superimposed upon white-light images. As a result anatomical or pathophysiological structures ‘light up’ on screen, enabling clear identification of the desired tissue [9].

Identifying cancerous lesions revolves around creating a large enough contrast between malignant and benign tissue using fluorescent contrast agents [8]. Sole NIR fluorophores, such as indocyanine green (ICG) or IRDye800CW, generally do not sufficiently accumulate in tumours [10]. Therefore a fluorophore needs to be conjugated to a targeting agent that recognizes a tumor and not surrounding healthy tissue. Furthest along in clinical development are the folate receptor targeting OTL38 (pafolacianine; Cytalux) for ovarian cancer, the carcinoembryonic antigen targeting SGM-101 for colorectal cancer, and the cathepsin targeting LUM015 for breast cancer. Their strength, specific targeting, is also their weakness, either due to non-tumoral expression of the target [7] or heterogenous intra- and intertumoral expression of the target [4, 7, 11]. As a result, only a subset of oncological patients benefit from the current class of fluorescence agents. For the remaining cancer patients there is no suitable fluorescence contrast agent while there is a clinical need for tumor margin identification. The current challenge lies in expanding the library of available contrast agents and, with this expansion, a desire to identifying characteristics of the most optimal target-tracer combination for each patient.

1 Although almost all current FGS clinical trials currently use NIR (700 - 900 nm) light, the first clinical trials demonstrating the concept of fluorescence guided surgery used blue (380 - 500 nm) light to visualize brain tumors, colorectal carcinomas and ovarian cancer [5-7]. This change in light source is due to the favourable imaging characteristics of NIR light; absorption by biomolecules (i.e. deoxyhemoglobin, oxyhemoglobin, water, and lipids), scattering and tissue autofluorescence are all favorably low with NIR light compared to blue light. Consequently, background fluorescence is minimized, resulting in greater contrasts with which to discriminate structures. In addition, NIR light emitted by the camera system penetrate deeper into, and out of, tissue, resulting in improved imaging depths of up to several millimetres [8]. A more recent development has been the utilization of the second NIR window (NIR II; 900 - 1450 nm) with even better imaging characteristics, but translation to the clinic is currently limited by the availability of the appropriate camera systems [4].

2 One exception is using ICG for imaging of hepatobiliary tumors. Healthy hepatocytes rapidly excrete ICG into the biliary ducts. Hepatocytes located adjacent to the tumor cannot metabolize ICG in this manner and as a consequence ICG accumulates in these hepatocytes resulting in a fluorescent rim of hepatocytes incapable of metabolizing ICG around the tumor. This phenomenon is subsequently utilized for intraoperative resection of hepatobiliary tumors [10].

3 Due to folate β expression in macrophages, pafolacianine signal also accumulates in lymph nodes with macrophages expressing folate β, resulting in false-positive fluorescence lymph nodes [11].

4 10% of ovarian cancer patients do not have folate receptor expression in their tumors and for solid tumors of other origins folate receptor expression is far more limited [7].
Chapter 1

This thesis addresses this challenge by firstly confirming the urokinase plasminogen activator receptor (uPAR) as a potential target and secondly by evaluating the effect of tracer size on FGS using novel uPAR-targeted tracers. Thereafter, by combining the current literature and the studies described in this thesis the endgoal is to define a clinical framework for uPAR FGS.

The case for using the urokinase plasminogen activator receptor as target

The extracellular matrix (ECM) forms the scaffolding where cells are embedded in, guaranteeing the structural integrity of tissues and organs. Tissue maintenance, differentiation and proliferation, therefore requires a coordinated response between the disintegration and reassembling of the complex network of collagens, proteoglycans and glycoproteins that the ECM consists of. The uninhibited cell growth and eventual invasiveness that defines all solid cancers implicates that pathways involved in ECM remodelling are dysregulated [12]. As such proteins involved in tissue remodelling are a promising source of FGS targets that can be utilized in a universal nature (i.e. for all solid tumors).

An important regulator of ECM proteolysis and cell signalling, involving process like proliferation, differentiation and migration, is the urokinase plasminogen activator receptor (uPAR). Initially, its involvement was regarded as rather simple; as a receptor for urokinase plasminogen activator (uPA; urokinase), uPAR localized the proteolytic cascade initiated by uPA towards the leading edge of cells. The subsequent proteolysis creates room for cell migration into the direction where uPAR was expressed. Over 35 years later the story is much more complex. uPAR narrowly orchestrates cancer aggressiveness managing not only extracellular processes such as proteolysis, but also influencing intracellular pathways involved in tumor progression through its over 42 interacting proteins [13]. Not surprisingly, down-regulating uPAR downregulates signaling pathways involved in eight of the ten Hallmarks of Cancer [14].

For molecular imaging applications, such as FGS, more important than the targets fundamental role in tumor progression is its expression pattern. The ideal target has no expression in non-malignant tissue while being overexpressed in malignant tissue as such an expression pattern allows for a high tumor-normal signal ratio. uPAR expression has been described on neoplastic cells and tumor-associated stromal cells, including neo-angiogenic endothelial cells, cancer-associated fibroblasts, and tumor-associated macrophages [15]. Furthermore, it is completely absent in surrounding non-malignant tissue. This pattern remains an overarching theme for virtually every solid malignancy [16]. With such an expression pattern, targeting uPAR is, not surprisingly, considered particularly suited for FGS [15]. However, up to now, no uPAR targeting tracer has made it to the clinic for FGS and only recently
uPAR-targeted positron emission tomography (PET) trials have reported results while the clinical potential is evident.

**The case for considering structural variations of tracers**

In 2019, Hernot et al. listed 19 different fluorescent tracers that had been or were being evaluated clinically for FGS [9]. Remarkably, these contrast agents differed not only in their target but also in their structure; ranging from therapeutic monoclonal antibodies repurposed for imaging, to natural occurring ligands conjugated with a fluorophore, and from knotted peptides conjugated with a fluorophore to activatable tracers that only become fluorescent upon enzymatic cleavage. These differences in structure directly translate into differences in distribution, metabolism and excretion; characteristics surgeons will need to contemplate as they will influence uptake by target tissue and clearing organs, tissue penetration, time before image acquisition and imaging contrast [17, 18]. OTL38, for example, consists of a natural ligand (folate analogue) conjugated to a NIR fluorescence dye with a molecular weight of 1.414 Dalton (Da) while SGM-101 is a chimeric antibody conjugated to a NIR fluorescence dye with a molecular weight of approximately 150,000 Da (> 100 times larger). As a result, OTL38 has a relatively rapid clearance from the plasma with imaging windows 2 – 6 hours after administration while SGM-101 has a longer terminal half life and has to be administered 2 – 4 days before surgery [11, 19]. While a more rapid imaging window seems favorable, reducing molecular weight to increase pharmacokinetics can influence imaging outcomes. In a PET study using CD105 targeting antibodies and derivates, the smaller antibody fragments resulted in earlier but lower peak signal [20, 21]. This can potentially reduce contrast. Therefore, when designing a targeted agents, its structural characteristics should be carefully selected as structural variations can ultimately make or break the subsequent clinical usability.

Practically, there exists a whole realm of deconstructed, rebuilt and fused molecules, where the possibilities are endless. However, generally speaking, they can be divided into one of the following categories: antibodies, antibody fragments, recombinant protein scaffolds, peptides or small molecules [9]. Antibodies are an appealing choice due to their naturally high specificity and affinity. In addition, there is extensive experience creating, optimizing and translating antibodies to the clinic [22]. The translation for FGS can be rather rapid by labelling already FDA-approved (therapeutic) antibodies with fluorescent dyes [9]. However, in addition to their extended half-life identified above, their large size limits intra-tumoral penetration and diffusion resulting in heterogeneous intra-tumoral antibody concentrations and consequently effectiveness [23, 24]. As a result, other constructs are being considered. However, what the optimal characteristics of an adjusted construct are have not been clearly defined.
Thesis outline

This thesis aims to expand on the current knowledge of urokinase plasminogen activator receptor (uPAR) fluorescence guided surgery (FGS) with the endgoal of defining a clinical framework for uPAR molecular imaging. While the overarching focus is uPAR, this thesis also attempts to draw more general conclusions for the whole field of FGS. To achieve the before-mentioned goals this thesis is divided into five parts.

Part I. Introduction
The current chapter, Chapter 1, introduces important background concepts about FGS, uPAR, and tracer design so as to set the stage for the work in Parts II – V. The chapter ends with a thesis outline.

Part II. uPAR as a tumor target in various tumortypes
In Chapter 2 the expression pattern in both malignant and stromal cells of is determined uPAR in pancreas adenocarcinoma and correlated with prognosis of patients. In Chapter 3 the immunohistochemical expression of seven promising FGS targets, including uPAR, are compared in patients with high-risk cutaneous and mucosal squamous cell carcinoma of the head and neck. In addition, a novel scoring system is introduced for comparing the appropriateness of targets for FGS.

Part III. uPAR as target: Beyond cancer imaging
Not only does uPAR play a fundamental role in cancer, it also plays a central role in other pathologies. Therefore, in Chapter 4, a side step is made to discuss the potential of uPAR imaging in non-neoplastic diseases in order to identify possible novel avenues for molecular imaging.

Part IV. Development of uPAR targeted tracers
Once uPAR has been identified as a potential target a fluorescent tracer needs to be designed that targets uPAR. The opportunities and pitfalls of fluorescence anti-uPAR tracer design are discussed in Chapter 5. Subsequently, Chapter 6 presents the results of the preclinical development of a humanized anti-uPAR monoclonal antibody. Furthermore the tracers potential for multimodal optical and photoacoustic fluorescence imaging for urothelial cell carcinoma patients is demonstrated. In Chapter 7, this antibody is cleaved into F(ab’)_2 and fab fragments and the fluorescent fragments are compared to the original humanized antibody in three orthotopic tumor models.
Part V. Summary and general discussion
After a summary in chapter 8, the stage is set for the discussion in chapter 9. This discussion includes uPAR as a next-generation target, the optimal structural characteristics of an uPAR FGS tracer, the translation of uPAR FGS to the clinic and the relevance of uPAR molecular imaging in other fields besides oncology. Where relevant, the discussion is generalized to the whole field.
References


