Targets in the microenvironment of rectal cancer

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Introduction to the thesis

Introduction

Drug development in oncology was initially focused on inducing DNA damage in proliferating cancer cells, with currently more emphasis on targeted agents and immunotherapy. New approaches are critical as many patients still have recurrence of their disease and develop resistance to their cancer treatment.

More recently it was demonstrated that the environment of cancer cells play a role in cancer progression (1-7). Apart from cancer cells, tumors also contain endothelial cells, bone marrow-derived progenitor cells, immune cells, cancer-associated fibroblasts, and other stromal cells (8). Through cell-cell contact and under the influence of soluble molecules, these cells can interact during tumorigenesis. This cross-talk between tumor and microenvironment cells is increasingly acknowledged as an important characteristic of cancer, hence, a potential target for treatment.

Sustained angiogenesis is a key component of the tumor microenvironment that fosters cancer growth by providing oxygen and nutrients. In turn, the levels of oxygen and nutrients that determine the metabolic and transcriptional profile of cancer cells (8). Low levels of oxygen are related to glycolysis, activated oncogenes that support cancer cell proliferation, and upregulated proinvasive pathways in cancer cells. Furthermore, the oxygen shortage occurring in fast-growing tumors or after antiangiogenic therapy activates the hypoxia-inducible factor 1α (HIF1α)-mediated synthesis of proangiogenic factors. In a vicious cycle, these proangiogenic factors will help to expand the neoplastic vasculature and thus promote cancer growth and dissemination. Apart from tumor vessels, stroma-derived chemokines also foster growth, dissemination, and survival of cancer cells. Chemokines are low-molecular-weight proteins involved in (tumor)cell migration that are produced by stromal cells, and their receptors are expressed by cancer cells. Together, ligands and receptors form an important network supporting cancer cell proliferation and distant spread (9). Furthermore, cancer cells

that reside in a chemokine-rich microenvironment like the bone marrow can be protected from chemotherapy induced damage (10, 11).

Treatment directed at cancer cells elicits a response in the tumor microenvironment. Chemotherapy induces cellular stress and the release of a variety of cytokines, growth factors and chemokines by stromal cell depending on the drug's mechanism of action (6). Radiotherapy causes hypoxia, cellular stress and the production of reactive oxygen species which can also stimulate the stromal cells (12). This way released chemokines and growth factors from the stroma bind to receptors on cancer cells and stimulate cancer cell growth and survival. These events can alter the sensitivity of tumors to chemotherapy and radiotherapy. These events are present in several malignancies. However, little is known about rectal cancer, which is a major contributor to cancer-related morbidity and mortality worldwide (13).

Until recently molecular heterogeneity within a tumor between patients and lesions, within lesions, and in time was neglected. This is however currently drawing more attention (14). Tumor heterogeneity may be missed with only one biopsy. Therefore, standard biopsy analyses for presence of growth factors and chemokines, combined with in vivo whole-body imaging of these factors are an attractive opportunity to get insight into the behavior of these targets in normal and tumor tissues. This can be achieved by labeling compounds directed against these factors with radioactive isotopes or fluorescent dyes. Imaging can then be performed with single-photon emission computed tomography (SPECT) or positron emission tomography (PET) for radionuclides, or optical imaging for fluorescent probes (15, 16). Furthermore, molecular imaging with radiolabeled therapeutic antibodies against soluble growth factors would allow us to assess the biodistribution and tumor uptake of these therapeutics, and could serve as a readout for target saturation (17). This, in turn, may support the rational dosing of these agents in the clinic.

The aim of the thesis

The aim of this thesis was to explore targets in the microenvironment of rectal cancer with a focus on angiogenic growth factors and chemokines.

Outline of the thesis

In **Chapter 2** a literature review was performed about clinical and biological differences and similarities between rectal and colon cancer, and it was analyzed how they influence current standard treatment and might influence the design of future clinical approaches. PubMed and Google Scholar were searched for research and review articles in English, as well as meta-analyses published up to May 2015. We used the following search terms: "rectal cancer", "colon cancer", "epidemiology", "histology", "gene" "(neo)adjuvant treatment", "metastasis", "targeted drugs", "tumor microenvironment", in various combinations. We also consulted current European Society for Medical Oncology, Dutch, and National Comprehensive Cancer Network Clinical Practice Guidelines for rectal and colon cancer, and the registry for clinical studies of the ClinicalTrials.gov site (National Institute of Health, United States of America) .

There are increasing attempts to treat primary metastasized rectal cancer with curative intent. The antiangiogenic agent bevacizumab – a humanized antibody against vascular endothelial growth factor A (VEGFA) – administered in combination with standard treatment has improved survival of metastatic (colo)rectal cancer patients. These data provided the rationale for a phase 2 study in patients with primary metastatic rectal cancer. **Chapter 3** describes this study. Fifty therapy-naive patients presenting with a primary rectal tumor and simultaneous metastases to the liver or lungs received short-course radiotherapy followed by preoperative bevacizumab, capecitabine and oxaliplatin, and subsequent radical surgical treatment. The primary end point was the percentage of patients receiving radical curative

treatment at all tumor sites. Secondary end points were 2-year survival, 2-year recurrence rate, and treatment-related toxicity.

In **Chapter 4**, we evaluated the expression of chemokine receptor CXCR4 and its ligand CXCL12 which are both involved in the microenvironment in rectal tumors. Tumors of 46 stage IV patients participating in the phase 2 trial as described in Chapter 3 were studied before and after local radiotherapy and systemic treatment with bevacizumab, capecitabine and oxaliplatin, and subsequent radical surgery. The protein expression of CXCR4 and CXCL12 was analyzed immunohistochemically in paraffin-embedded primary rectal cancer diagnostic biopsies collected before and in surgical rectal specimens collected after radiochemotherapy and bevacizumab. Expression of both factors was assessed in the cytoplasm and nucleus of tumor cells, adjacent stromal cells and normal rectal crypts. In addition, baseline expression of CXCR4 and CXCL12 was correlated with patients' pathologic response to treatment.

Expression of placental growth factor (PlGF) – a VEGFA homolog – is related preclinically to tumor angiogenesis and survival of cancer cells, and correlated with poor survival of colorectal cancer patients (18-20). Moreover, bevacizumab alone or in combination with radiotherapy or chemotherapy increased circulating PlGF levels in (colo)rectal patients (21, 22). In **Chapter 5** we examined immunohistochemically the expression of VEGFA and PlGF, and the mean vessel density (MVD) in the paraffin-embedded rectal tumors of 46 patients enrolled in the clinical study reported in Chapter 3. The protein expression of VEGFA and PlGF was assessed in tumor and stromal cells, and in the epithelial cells of tumor-neighboring rectal crypts before and after radiochemotherapy and bevacizumab. Additionally, VEGFA and PlGF protein expressions at diagnosis were correlated with pathologic response to treatment. The pathologic response was chosen as it

provides early and accurate information on the local effect of pelvic radiotherapy and systemic bevacizumab, capecitabine and oxaliplatin.

Preclinical PlGF inhibition restricts growth and metastasis of various tumors, including those resistant to VEGF receptor (VEGFR) inhibitors, and enhances the efficacy of chemotherapy and VEGFR inhibitors (23). Early clinical trials of humanized monoclonal anti-PlGF antibody RO5323441 showed that anti-PlGF therapy was well tolerated (24, 25). In a randomized trial in patients with recurrent glioblastoma (n=22; ClinicalTrials.gov identifier, NCT01308684), RO5323441 combined with bevacizumab did not improve the response rate compared to single agent bevacizumab (26). However, in the initial study its administration did not coincide with dose-limiting toxicity, therefore, no optimal therapeutic dose is yet defined. Rational dosing might be achieved when tumor and normal body tissues uptake of the antibody is defined by ^{89}Zr-RO5323441 PET. Therefore, as described in **Chapter 6**, we labeled RO5323441 with radioactive Zirconium-89 (^{89}Zr) in order to develop a PlGF-specific PET tracer. The radiochemical purity of the tracer was tested by size-exclusion high-performance liquid chromatography. The stability of the tracer was assessed at 4 °C in solvent (0.9% NaCl), and at 37 °C in buffer (phosphate-buffered saline) and in human serum by trichloroacetic acid precipitation. In vitro immunoreactivity of ^{89}Zr-RO5323441 was tested in a competitive radio-immuno assay with PlGF coated ELISA plates. Ex vivo biodistibution of the tracer was studied in tumor-bearing and in healthy mice. The tumor uptake was determined by small-animal PET imaging in athymic nude mice xenografted with human PlGF-expressing hepatocellular carcinoma or human renal cell carcinoma without detectable human PlGF expression. Indium-111 labeled immunoglobulin G (^{111}In-IgG) served as a control for non-specific tumor uptake and organ distribution assessment. Ex vivo immunofluorescent staining of tumor slides with anti-CD68 antibody labeled with Alexa 488

and RO5323441 labeled with cyanine 5 (Cy5) were used to detect tumor-associated macrophages, and to study the molecular mechanisms behind the specific tracer tumor uptake.

Chapter 7 contains the English summary of this thesis, followed in **Chapter 8** by a discussion and future perspectives. **Chapter 9** contains the Dutch summary and **Chapter 10** the Hungarian summary.

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