Chapter 7

Pilot study to determine the effect of fractionated radiotherapy on expression of pro-angiogenic factors in esophagus carcinoma

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Ongoing
Abstract

Preclinical studies indicate that the anti-tumor effect of radiation therapy (RTx) can be enhanced by angiostatic treatment. Fractionated (FR) RTx can significantly induce the expression of pro-angiogenic factors in vitro and enhances tumor perfusion in vivo. These results suggest that an improved anti-tumor activity may be caused by inhibiting the pro-angiogenic response induced by RTx. To what extent and when this pro-angiogenic tumor response occurs in patients is unknown. To determine when the RTx-induced angiogenic response occurs in patients we designed a non-randomized, interventional explorative clinical trial for adult patients with primary esophageal carcinoma undergoing standard neoadjuvant chemoradiation. The first primary objective in this study is to determine the time point of induction of vascular endothelial growth factor (VEGF) mRNA expression in the tumor tissue during fractionated RTx. The second primary objective is to determine whether the possible induction of VEGF expression can be counteracted by administration of bevacizumab, a monoclonal antibody against VEGF. To assess these two aims a pretreatment and on treatment tumor biopsy is obtained for further analysis. Here, the preliminary results of the first patients included in this study are presented.
Introduction

More than half of all cancer patients undergo radiotherapy (RTx) at some stage during their treatment (1,2). Despite technical advances that continue to improve the efficacy of RTx, normal tissue toxicity is often a limiting factor and a significant number of patients still display local failure (1,3). Consequently, efforts are being made to improve the anti-tumor activity of RTx by enhancing the radiosensitivity of tumor cells. An important factor determining the radiosensitivity is the tumor microenvironment, including the tumor vasculature (4). Cancer cells stimulate the formation of new blood vessels out of pre-existing ones, a process called tumor angiogenesis. Tumor vasculature provides oxygen to the tumor tissue which is prerequisite for effective RTx. Interestingly, several preclinical studies have demonstrated that the combination of RTx with drugs that inhibit the growth of tumor blood vessels, i.e. anti-angiogenic drugs, can induce beneficial effects on tumor growth inhibition (5-8).

This apparent contradiction has been partly explained by the predominantly preclinical observation that anti-angiogenic drugs counteract the pro-angiogenic growth factors thereby causing transient normalization and stabilization of the tumor vessels (7,9). An improvement in tumor perfusion and oxygenation may enhance the efficacy of RTx. However, both treatment modalities have to be precisely dosed and scheduled since prolonged angiostatic treatment causes vascular disruption, which blocks the oxygen supply towards the tumor tissue (6,9-11). In addition, evidence for vessel normalization in patients is lacking and the relatively short window of normalization would require extensive monitoring of tumor perfusion to identify the optimal time-point to apply RTx.

An additional mechanism by which angiostatic drugs might enhance the efficacy of RTx is the induction of pro-angiogenic growth factors by irradiation (12-14). While RTx has been suggested to hamper tumor angiogenesis (15-17), it has also been described that low dose irradiation can enhance vascularization (17-19). Indeed, we have also found that RTx can induce vascular endothelial growth factor (VEGF) expression and that clinically applied fractionated radiation schemes improve tumor perfusion and tissue viability in preclinical models (Chapter 6). Both effects could be counteracted by angiostatic treatment. Similar as for vessel normalization, almost no evidence for a RTx induced pro-angiogenic response in patients is available. While it has been described that VEGF expression is enhanced in rectal carcinoma after fractionated RTx the exact timing remains elusive (14,20).

The aim of this explorative clinical trial is to determine whether a pro-angiogenic response occurs in tumors of patients with primary esophageal cancer that receive fractionated RTx (21). To that end, an exploratory clinical trial was designed to compare the angiogenic response in pre-treatment and on-treatment tumor biopsies from patients treated with neo-adjuvant chemoradiation. The primary endpoint was detection of elevated VEGF mRNA expression level in the tumor tissue during chemoradiation as compared to the pretreatment tumor tissue. Here, the setup of this ongoing study is described and the preliminary results are presented.
Materials and methods

Patients
This study was approved by the institutional medical ethical committee (METC) of the VU University medical center (Amsterdam, The Netherlands). All patients receive oral and written study information and give informed consent according to standard ethical procedures. Patients (>18 years) with previously untreated primary esophagus carcinoma (both adenocarcinoma and squamous carcinoma) are eligible for inclusion when receiving standard neo-adjuvant chemoradiation, provided that a pre-treatment tumor biopsy is collected. Standard neo-adjuvant treatment consists of chemoradiation (5 weeks paclitaxel and carboplatin with concurrent radiotherapy (41.4 Gy in 23 fractions, 5 days per week)). Patients using coumarine derivates are excluded. For the cohort receiving the monoclonal antibody bevacizumab (Avastin, Roche), extra exclusion criteria apply; Inflammation of the gastro-intestinal tract, diastolic/ systolic hypertension (>90/>140 mmHg) not responding to treatment, arterial thrombo-embolism in medical history and surgery within the month prior to start of bevacizumab treatment. Also, pregnant women or women in their fertile period not using anti-conception are excluded.

Tumor biopsies and blood sampling
Six pretreatment and six on-treatment tumor biopsies are collected during ultrasound endoscopy. The time point at which the on-treatment tumor biopsy is obtained depends on the patient cohort. Tumor tissue from the surgical resection is also collected. All tissue samples are processed as FFPE or snap-frozen in liquid nitrogen and stored at -80°C until further processing. Blood samples (2x 6 mL) are collected at 4 different time points: i) Before start of chemoradiation, ii) in the week of the on-treatment biopsy, iii) after completion of the chemoradiation and iv) before surgery.

Study design
The study design encompasses consecutive cohorts of patients that receive chemoradiation for 1 up to 4 weeks, starting with the 1-week cohort. A ≥ 2-fold increase in VEGF mRNA expression in on-treatment vs. pre-treatment tumor tissue is considered as enhanced expression. The lower limit of the confidence interval was set at 60% and the initial number of patients included in each cohort is 6. In case all 6 patients in a cohort show enhanced VEGF mRNA expression this is considered as the time point of induction (6 out of 6; 95% confidence interval for the probability of enhanced expression: 61%-100%). If 1 out of 6 patients does not show enhanced expression, an additional 4 patients are included. If these additional patients all show elevated expression, this cohort is considered as the time point of induction (9 out of 10; confidence interval: 60%-99%). As soon as 2 patients in a cohort do not demonstrate elevated VEGF expression, the lower limit of the confidence interval is below 60% (<40% for 6 patients or <50% for 10 patients), and the study proceeds to the next cohort (Figure 1).

Once the induction time point has been identified, a final cohort will be added in which patients will receive bevacizumab, starting at the identified time point of induction. The number
Pilot study: FR RTx and pro-angiogenic factor expression

of patients included in this cohort will equal the number of patients that was needed to identify the induction time point. If bevacizumab is not well tolerated, it will be discontinued for this patient. If in one patient any grade 3-4 side effects are observed, related to bevacizumab treatment, the whole bevacizumab cohort will be discontinued. Bevacizumab will be stopped at least 4 weeks before the surgical resection of the tumor tissue to assure complete metabolism of the drug, so that it will not interfere with the wound healing after surgery.

Figure 1. Study flowchart.

Quantification of VEGF mRNA expression
The mRNA VEGF expression in the tumor tissue is determined using qPCR. RNA isolation from snap-frozen biopsies is performed using Trizol (Life Technologies). The final RNA concentration is determined with the Nanodrop ND-1000. For subsequent reverse transcription we use 500 ng RNA, with the iScript kit (Biorad) following the manufacturers protocol. The resulting cDNA is used for the qPCR reaction, with the SYBR green supermix (Biorad) and a total samples volume of 25 µL. For primers sequences, see Supplementary table 1. PCR is performed on the CFX96 (biorad) and the following cycling conditions are used: 95°C for 5 minutes, followed by 95°C for 10 seconds and 60°C for 30 seconds for 40 cycles. Expression levels are normalized to reference genes.

Quantification of viable tissue in tumor biopsies
Quantification of viable and necrotic tissue is performed on frozen sections or paraffin embedded tumor sections by standard haematoxylin and eosin (H/E) staining. Frozen sections
of 6 µm are first fixed in ice-cold 60% ethanol/40% isopropanol for 20 minutes. After washing with PBS, slides are emerged in haematoxylin for 2 minutes, rinsed in tap water for 5 minutes, and emerged in eosin for 1 minute. Paraffin embedded sections (5µm) are dewaxed in neo-clear and then rehydrated through a graded series of alcohol. After washing with PBS, all slides are emerged in haematoxylin for 4 minutes, rinsed in tap water for 5 minutes, and emerged in eosin for 10 seconds. All slides are then dehydrated through a graded series of alcohol, fixed with neo-clear (Millipore) and mounted with neo-mount (Millipore). Percentage of viable tissue is scored by 2 independent observers. With a 10x10 grid, the number of cross-sections is counted, covering viable tumor tissue. Cross sections not covering tumor tissue are subtracted from the total number of cross sections and a percentage of viable and necrotic tissue is then calculated.

Results

The aim of the current study is to determine whether and when fractionated RTx will induce a proangiogenic response in cancer patients. To that end, a clinical pilot study was designed including patients with previously untreated primary esophagus carcinoma (Figure 1). From January until August 2015 a total of 5 patients signed informed consent. One patient refrained from the on-treatment biopsy, and 1 patient did not receive neo-adjuvant chemoradiation because of metastatic disease. Both patients were therefore excluded from the study. All remaining patients were included in the first cohort, i.e undergoing the on-treatment tumor biopsy at the end of the first chemoradiation treatment week. All included patients were male, with age ranging between 57 and 67 years. Adenocarcinoma was diagnosed in all 5 patients (Table 1).

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<tr>
<th>Study subject</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Tumor type</th>
<th>Tumor staging (TNM)</th>
<th>Study cohort</th>
<th>On-treatment tumor biopsy</th>
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Table 1. Patient characteristics

VEGF mRNA expression

The expression of VEGF mRNA was determined for 3 patients in the pre-treatment and on-treatment tumor biopsies. Basal expression of VEGF in the pretreatment biopsies varied considerably between the patients (Figure 2A). Enhanced expression of VEGF in the on-treatment biopsies was observed in 2 out of 3 patients. One patient demonstrated a decrease of VEGF expression (Figure 2B).
Viable tissue in tumor biopsies
During the on-treatment endoscopy, no effect of the first treatment week on the tumor was observed by the gastro-enterologist (MAJ). Percentage of the viable tissue in the pre-treatment tumor biopsies varied between the patients from 48-85%. The percentage of viable tissue in the on-treatment biopsies was higher for each patients compared to the pre-treatment biopsies (differences of 6-24%) (Figure 3A and 3B).

mRNA expression of other growth factors
While VEGF mRNA expression is the primary endpoint of this pilot study, also the expression of other pro-angiogenic growth factors was determined. The expression of placental growth factor (PIGF) (Figure 4A) and platelet derived growth factor (PDGF) (Figure 4B) was enhanced in 1 patient, which also demonstrated enhanced VEGF expression. PIGF and PDGF expression were not enhanced in the other two study patients (Figure 4).
Discussion and future perspectives

The aim of this explorative clinical trial is to determine whether a pro-angiogenic response occurs in tumors of patients with primary esophageal cancer that receive fractionated RTx (21). While the chemotherapy may influence the effect of RTx on VEGF expression, fractionated RTx with curative intent is usually combined with chemotherapy. The relatively easy accessible tumor site and the intended tumor resection after the chemoradiation course were also decisive reasons to choose for this specific patient group. Initial inclusion of patients has been difficult since many eligible patients considered the on-treatment tumor biopsy as too much burden during their intensive treatment schedule. However, providing more information regarding the potential side effects and explanation of the value of this study for future research have improved the inclusion rate.

So far, all patients have been included in the first study cohort undergoing the on-treatment tumor biopsy after 1 week of chemoradiation. Our preliminary results demonstrate that of the 3 patients, 1 failed to show enhanced VEGF expression in the on-treatment tumor biopsies. Therefore, a total of 10 patients will be included in the first cohort. If 1 more patient fails to demonstrate enhanced VEGF expression, this cohort will be closed, and the study will continue with the second study cohort, i.e. patients undergoing the on-treatment tumor biopsy after 2 weeks of chemoradiation. Based on our pre-clinical data, we do not expect to observe VEGF induction within the first week of chemoradiation. However, an induction of VEGF expression with a relative increase of at least 2 times should become apparent within the first 4 weeks of chemoradiation. If we do not observe an induction of VEGF after 4 weeks the study will be closed, since we aim to investigate whether enhanced VEGF expression occurs during chemoradiation.

The exact timing of the enhanced VEGF expression is important for the scheduling of the anti-angiogenic drug. The aim is to restore the balance between anti and pro-angiogenic
factors. Starting too early with the anti-angiogenic drug would result in an overload of anti-
angiogenic factors, which results in tumor hypoxia (7;22). This would make the cancer cells
less sensitive to the RTx (23;24). Starting too late with the anti-angiogenic therapy would allow
accumulation of the pro-angiogenic factors. Previous observations suggest that this could
result in enhanced tumor perfusion and increased radioresistance of the cancer cells (chapter
6).

The final patient cohort will receive bevacizumab (3mg/kg/wk) treatment, starting at the
time point of VEGF induction. Bevacizumab is a monoclonal antibody against VEGF-A (hereafter
VEGF). It binds to VEGF and thereby inhibits the binding of VEGF to its receptor VEGFR2. The
indicated dose has been proven to be clinically well tolerable with low grade toxicities when
given together with paclitaxel and carboplatin in patients with esophageal cancer (25;26).
However, treatment of bevacizumab concurrent with chemoradiation is known to increase
the risk of gastro-intestinal perforations (27;28). This is regardless of the site of the primary
tumor. Although no data is available about the risk in esophageal cancer patients, the overall
incidence is about 0.9% with a relative risk of 2.14 (28).

The aim of adding bevacizumab to the standard chemoradiation regime is to determine
if it counteracts the pro-angiogenic tumor micro-environment. Therefore phosphorylated
VEGFR2 expression will be measured by IHC staining of tumor sections. In addition the
microvessel density of the tumor tissue will be determined, by staining for CD31/CD34 in the
tumor tissue. The results will be compared to the tumor tissue of patients that have not received
bevacizumab and to the pre-treatment tumor tissue of the same patient. These analyses will
give insight in the feasibility of counteracting the pro-angiogenic response with a low dose
anti-angiogenic drug.

Taken together, the knowledge gained in this exploratory clinical trial is relevant for the
design of a following phase II clinical trial. A precisely defined dose-schedule regime should
then be tested for the combination treatment of angiostatic drugs and radiotherapy. The
ultimate aim is to determine whether the addition of anti-angiogenic treatment to RTx is safe
and effective for cancer patients.
References


### Supplementary Table 1. Primer sequences for qPCR.

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