Prognostic factors and predictive tests in the treatment of head and neck squamous cell carcinoma

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chapter 1

General introduction

Frank J.P. Hoebers

EPIDEMIOLOGY AND ETIOLOGY OF HEAD AND NECK CANCER

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer, with an incidence of 780,000 new cases a year worldwide [1]. These tumors arise in the different anatomical sites of the head and neck region: nasopharynx, oral cavity, oropharynx, hypopharynx and/or larynx. This introduction and the remaining of this thesis will focus on the latter four of these tumor sites, since the nasopharynx is regarded as a different entity with different etiology and different treatment options.
In developed countries, these non-nasopharyngeal HNSCC are strongly related to tobacco smoking and the use of alcohol [2]. A smaller proportion of tumors is related to chronic viral infection with human papilloma virus (in oropharyngeal carcinoma) [3]. The incidence of HNSCC shows a male to female ratio of approximately 2:1, which is related to gender differences in the frequency of tobacco and alcohol abuse [4]. Cigarette smokers are at an increased risk for multiple HNSCC as well as for lung cancer. Patients with head and neck cancer have an increased risk for developing esophageal and lung cancer [5].

TREATMENT OF HEAD AND NECK CANCER

Locoregional treatment for patients with HNSCC depends mostly on tumor site within the head and neck region and tumor stage and includes several options such as surgery, radiotherapy, chemoradiation, or a combination of these modalities. For early stage disease, single modality treatment is preferred, being either surgery or radiotherapy. With this policy, acceptable treatment results can be achieved without the added toxicity arising from combined treatments. With respect to the treatment with radiotherapy, improvements in outcome have been achieved by the introduction of altered fractionation schedules (using acceleration or hyperfractionation) compared to standard fractionated radiotherapy [6-9], leading to improved locoregional control and survival but also increased acute toxicity.
For advanced stage operable disease, combined modality treatment by surgery and radiotherapy is usually indicated. Although no randomized trials have addressed the efficacy of postoperative adjuvant radiation therapy in the treatment of HNSCC, retrospective series with matched pairs analyses have demonstrated the benefits of postoperative radiotherapy in case of adverse pathological factors [10-13]. Common indications for post-operative radiotherapy include factors like close (less than 5 mm) or positive margins, extracapsular extension, multiple positive nodes, invasion of the soft tissues of the neck, lymph-angio-invasion, perineural spread, infiltrative/spidery growth and/or T3-4 tumors. In high-risk patients (typically with extranodal spread, multiple involved nodes, and/or positive margins) treated with surgery and postoperative radiotherapy, the risk of locoregional disease recurrence is still over 30 percent, with frequent distant metastases

(up to 25 percent) and poor five-year survival rates (40 percent) [14]. Recently, it was shown in 2 randomized trials that in patients at high-risk for disease recurrence, the addition of chemotherapy to postoperative radiotherapy improved locoregional control and survival [15,16].

In patients with tumor extension regarded as inoperable or in patients with advanced disease requiring mutilating surgery (e.g. total glossectomy or total laryngectomy), organ-preservation strategies have demonstrated that combined chemotherapy and radiotherapy may result in long-term disease control and improved outcome compared to radiation alone [17-23]. In a meta-analysis it was shown that the improvement in survival was mainly achieved by the use of concurrent chemotherapy during radiation, rather than by induction or adjuvant chemotherapy [18,23]. The largest effect on survival was observed by the addition of single agent cisplatin concurrently to radiotherapy [18]. Concurrent cisplatin-based chemoradiation is now considered standard of care in advanced stage head and neck cancer.

The introduction of postoperative and definitive chemoradiation has led to more intensified treatment protocols with not only improvements in locoregional control and survival, but also with more treatment related toxicity [15,16,20,21]. The incidence, severity and duration of mucositis is increased after chemoradiation compared to radiation alone, leading to frequent need for tube feeding. Administration of cisplatin is also associated with hematological, renal and ototoxicity. Due to this toxicity profile, compliance to the chemoradiation schedules has been moderate in the order of 70% [21].

These increasingly toxic and potentially more effective therapies for head and neck cancer are administered to a patient population that is characterized by frequent comorbidity [24-26]. This comorbidity is related to the use of tobacco and alcohol and consists mainly of non-malignant cardiovascular disease, pulmonary dysfunction, psychosocial disorders and the occurrence of multiple primary tumors. This serious comorbidity obviously will negatively affect compliance to therapy. Moreover, comorbidity is not only an exclusion criterion for treatment within clinical trials, it is by itself also a factor that leads to both reduced overall survival [25] and reduced cancer specific survival [27].

Improved understanding of tumor processes involved in malignant behavior and their underlying molecular changes, has led to new developments in treatment strategies, which are usually directed against specific molecular properties of cancer cells. These so-called targeted therapies act against a specific receptor or a signal transduction pathway. Recently, a randomized trial was performed in which it was demonstrated that cetuximab, as an inhibitor of the epidermal growth factor receptor (EFGR), improved overall survival when added to radiotherapy [28]. Given the targeted nature of these new types of treatment, it is to be expected that these will only be efficacious when the target is present in the cancer cells of interest.

PREDICTIVE TESTING

Thus, it can be concluded that the treatment of HNSCC has become more effective by altered radiotherapy fractionation schedules and the addition of chemotherapy to definitive or postoperative radiotherapy. However, these treatment approaches are all accompanied by increased toxicities and are given to a possibly vulnerable patient population. This may lead to decreased compliance to therapy and thus worsening of treatment results.

Therefore, accurate prediction of tumor behavior, such as metastatic potential and response to different treatments, would enable a more individualized approach by selecting the optimal treatment. Patients that would be identified as non-responders by appropriate predictive testing could be spared a non-effective yet toxic treatment and be offered an alternative curative treatment or palliative therapy. On the other hand, patients who are predicted to be in a very good-prognostic group, would in theory not need intensification of therapy and could be offered standard, less toxic regimes.

Besides the argument of treatment selection with the best changes of response from the medical point of view, there is also the argument of exposing the patient to the best suited cytotoxic therapy from an economic point of view: combination treatments, especially when new targeted drugs are involved, are much more expensive that the standard, classical therapies.

Thus, there remains an urgent need to find better ways to predict treatment outcome and guide treatment choices for individual patients.

Prognostic factors are usually patient or tumor related factors that are present before therapy is initiated. Strong prognostic factors are indicators of patient outcome for specific therapies and might be used in the selection of therapy modalities. Predictive assays are tests that can be used to predict the response to therapy. A predictive test commonly is a parameter that is based on specific therapy-induced changes and that could be used to discriminate good-responders from poor-responders. A robust predictive assay should ideally be a reliable test with optimal sensitivity and specificity, with proven reproducibility and validated efficacy. In addition, an easy applicable, fast and preferably non-invasive method would further facilitate clinical introduction.

TREATMENT SELECTION AND FACTORS ASSOCIATED WITH OUTCOME

Treatment selection for patients with HNSCC is usually based on classical tumor and patient related criteria. These include the head and neck tumor anatomical site and tumor staging according to the TNM classification system [29], as determined by clinical examination and radiological evaluation. Patient factors include age, performance status, co-morbidity and weight loss.

However, categorization of patients according to tumor site and stage does not accurately and fully predict outcome. E.g. early stage (TNM stage I-II) disease like laryngeal cancer, treated with radiotherapy shows locoregional control rates of 80-90%, but still 10-20% of patients fail this therapy [30]. Similarly, it appears that for the advanced stage HNSCC (Stage III – IV) treated with concurrent chemoradiation TNM-staging criteria may not have enough discriminative properties, since it was shown recently that T-stage did not predict outcome in patients treated with chemoradiation [31,32].
Apparently, other factors are involved determining disease outcome and response to treatment. The list of possible factors is extensive and the number of factors is increasing over time as studies are being performed. It is beyond the scope of this part of the thesis to give an overview of all predictive factors involved in the treatment of HNSCC. However, the following predictive factors were investigated in this thesis and their predictive properties were subsequently tested, as described in the next chapters.

CISPLATIN-DNA ADDUCTS

The chemotherapeutic agent cisplatin is the most active drug used in concurrent chemoradiotherapy for HNSCC [18]. The cytotoxic effect of cisplatin is exhibited through the formation of cisplatin-DNA adducts, which are formed when cisplatin reacts with the cellular DNA by binding to nucleotides. The majority of adducts are either intrastrand adducts with cisplatin bound between two guanine (GG) nucleotides or adenine-guanine (AG) nucleotides [33]. Measurement of cisplatin-DNA adducts can be performed both in tumor and normal tissue. The level of adducts has been shown to correlate with cytotoxicity in vitro [34], and with response to treatment in some clinical series [35-38], but not all [39-41]. In most studies [35-38], adducts were measured in normal tissue, assuming that this could serve as surrogate marker for tumor tissue. The cisplatin-DNA adduct assay may therefore provide a simple and suitable test to select patients for cisplatin-based combined modality treatment and to predict outcome.

TUMOR VOLUME

The volume of a primary head and neck tumor as present on baseline, pre-treatment diagnostic imaging can be computed by delineation of the Gross Tumor Volume on each imaging section and using digital 3-D reconstruction of imaging series [42]. In a number of series on patients with HNSCC treated by radiotherapy alone, tumor volume was associated with locoregional control [43-45]: Larger tumors showed decreased locoregional control compared to smaller tumors. Most of these studies included patients with all tumor stages. In most concurrent chemoradiation schedules however, only patients with T3-4 tumors are included. It has been demonstrated that in this advanced disease population, T-stage does not accurately predict outcome [31]. This may be caused by poor discriminative properties of T-staging due to heterogeneity of tumors within the T3-4 category: E.g. a small (low volume) tumor with bony invasion (staged as T4) may respond better to chemoradiation

than a bulky (high volume) tumor without bone invasion, staged as T3. Pre-treatment primary tumor volume may therefore serve as a better indicator of treatment response and outcome compared to classical TNM-staging.

HYPOXIA

Tumor hypoxia is a frequently occurring phenomenon in HNSCC. In clinical series it was demonstrated that it was an important negative prognostic factor for outcome after treatment of malignant tumors by both radiotherapy and surgery [46-50]. Malignant cells have been shown to be more radioresistant under hypoxic circumstances, leading to less cell kill. Strategies that aimed at modifying the effects of tumor hypoxia, like adding a hypoxic cell sensitizer (nimorazole) to radiotherapy have resulted in improved treatment results [51]. Identification of patients with hypoxic tumors would enable proper selection of patients for appropriate hypoxia modifying or exploiting therapies. Examples of these include the use of hypoxic cell sensitizers [51], hypoxia specific cytotoxic agents like tirapazamine [52] or the addition of nicotinamide and carbogen-breathing to accelerated radiotherapy (ARCON) [53]. For this a reliable and easily applicable method to measure tumor hypoxia before treatment would be needed. Tumor oxygenation is frequently measured invasively with an Eppendorf electrode, limiting its application in clinical practice. Measuring tumor hypoxia by non-invasive methods includes the use of bioreductive markers, such as the 2-nitro-imidazole derivatives. Examples of these include pimonidazole [54] and EF5 [55]. The use of these markers is limited to prospective studies, since they require intravenous administration of the marker, which can be visualized subsequently by immunohistochemistry in tissue sections. It also requires tissue to be examined, either obtained during surgery or as biopsy. The staining pattern of these markers should ideally be hypoxia specific, providing a tool to quantify the extent of tumor hypoxia. In-vivo, non-invasive imaging using radioactive markers of hypoxia therefore represent an attractive tool to visualize hypoxia before and/or during treatment [56-58]. It could be used in patients scheduled both for surgery and radiotherapy, offers the possibility to observe changes in hypoxia during treatment and might be used to guide the use of specific hypoxia-directed therapies.

APOPTOSIS

The mechanism of apoptosis (programmed cell death) is an important factor contributing to cell death in response to treatment with radiation and many chemotherapeutic agents [59]. The relative contribution of apoptosis to the overall cytotoxic effect of an anti-cancer treatment modality has been studied intensely [60-62] and it appears to vary greatly between different tumor types and normal tissues [63].

The process of apoptosis can be assessed indirectly by the presence of various molecular markers (e.g. P53, Bcl-2, Bax and Bcl-X_L), by histological changes indicative of apoptosis (e.g. Apoptotic Index) or by measuring fragmentation of nuclear chromatin (using the TUNEL assay). These parameters have been studied in several cancer sites, including HNSCC, in

an attempt to establish a relation between the degree of apoptotic activity on baseline, pre-treatment tumor specimens and outcome/prognosis [64-72]. Increased levels of some of these markers have been associated with radio- and chemoresistance and poor clinical outcome in various types of cancer, including HNSCC [66,70,73-75]

For the purpose of prediction of therapy response, studies have focused on the value of treatment-induced apoptosis as predictive factor for outcome [76]. With this approach, it would be feasible to document early treatment-induced apoptosis. It was hypothesized that this would enable early prediction of treatment outcome and possibly treatment adaptation and individualization. Recently, in vivo imaging of apoptosis was reported using radiolabeled Annexin-V [76-80]. At an early stage of the apoptotic process, the membrane-bound lipid phosphatidylserine (PS) becomes exposed at the outer leaflet of the cell membrane bi-layer [81,82]. Annexin is an endogenous human protein with a high affinity for PS, which serves as a recognition site for macrophages that digest and remove apoptotic cells. Annexin can therefore be used as a marker to demonstrate apoptosis.

It was demonstrated that ^{99m}Tc-Hynic-rh-Annexin V scintigraphy correlates with radiation-induced cytologically confirmed apoptosis in non-Hodgkin lymphoma [83] and can be used to identify patients that have a favorable prognosis [84,85]. ^{99m}Tc-Hynic-rh-Annexin V scintigraphy might therefore also be of value as a predictive test early during treatment to monitor apoptosis induction in HNSCC.

OBJECTIVES OF THIS THESIS

With the increasing efficacy but also toxicity of combined treatment options for HNSCC patients, there is a growing need for adequate selection of the appropriate treatment for a patient. This is especially true when considering the new targeted drugs, since these will only be active if the target of interest is present.

The objectives of this thesis are therefore to investigate the value of a number of factors that are involved in prognosis and prediction of treatment response in HNSCC. For this we focused specifically on predictive tests that would be simple in application and minimally invasive for the patients, while ideally preserving the predictive capabilities of the test.

We chose to study the formation of cisplatin-DNA adducts and primary tumor volume as possible predictive factors in cisplatin-based chemoradiation. Furthermore, we wanted to test the feasibility of scintigraphy, employing markers for the detection of hypoxia and apoptosis in vivo, as a non-invasive test.

OUTLINE OF THE THESIS

In **chapter 2,** we tested the predictive value of cisplatin-DNA adduct formation in patients with HNSCC, treated by concurrent cisplatin-radiation. We examined whether adduct levels in tumor and/or normal tissue (white blood cells (WBC) and buccal cells) would

be associated with treatment outcome. The adduct levels were also related to the two different cisplatin-dose and administration schedules, which were used in the randomized RADPLAT trial on intravenous (IV) vs. intra-arterial (IA) chemoradiation. The formation of adducts after the standard 100 mg/m^2 IV schedule was compared with that of the high-dose, selective IA administration of cisplatin 150 mg/m^2, both for primary tumor and normal tissue.

In **chapter 3,** we analyzed cisplatin-DNA adduct formation in normal tissue and primary tumor in patients treated with different schedules of intravenous cisplatin-based chemoradiation. We investigated the correlations between the two major forms of cisplatin-DNA adducts (GG- and AG-adducts). We also wanted to explore relationships between adducts in primary tumor and normal tissue. We specifically wanted to test whether the level of adducts in tumors are reflected by those in normal tissues. In studies focusing on the predictive value of cisplatin-DNA adduct levels this would then justify the use of more easily obtained normal tissues as a surrogate for tumor samples.

In **chapter 4,** the results of a prospective study on concurrent chemoradiation with daily low dose cisplatin (RADPLAT daily LD) for advanced stage HNSCC are described. This treatment policy was used as a standard outside the randomized RADPLAT trial for patients that were refusing the trial or for those who were ineligible for the trial.

In **chapter 5,** the impact of primary tumor volume on locoregional control and survival after RADPLAT daily LD was investigated. For this, the tumor volume was calculated from the baseline pre-treatment MRI-scan. Together with tumor volume, other prognostic factors known to be involved in the outcome of HNSCC were tested in uni- and multi-variate analysis.

In **chapter 6,** our initial experience is reported with in vivo imaging of tumor hypoxia using ^{99m}Tc-BRU 59-21 scintigraphy in patients with head and neck cancer. This is a novel radioactive labeled bioreductive marker, a 2-nitro-imidazole agent, that has been shown to selectively localize in tissues with a low oxygen concentration due to ischemia [86,87], in tumor cells incubated under hypoxic conditions and following IV injection in animal models representative of poorly perfused tumors [88]. In this study we tried to validate in vivo hypoxia imaging by comparing the uptake of ^{99m}Tc-BRU 59-21 on SPECT scanning with the staining for pimonidazole, an acknowledged hypoxia marker, on tissue sections obtained after surgery.

In **chapter 7**, a possible artifact of pimonidazole staining was examined: Differentiation-associated staining with anti-pimonidazole antibodies in well differentiated keratinizing head and neck tumors. The bioreductive marker pimonidazole is reduced under hypoxic circumstances and becomes subsequently bound to macromolecules. Pimonidazole antibodies can therefore be used for staining of hypoxic tissues [89,90]. It was reported that pimonidazole staining may also occur in highly differentiated or keratinized tumor tissues [91,92], raising the question of whether this staining is hypoxia-specific. Non-specific oxygen-independent binding of pimonidazole obviously would affect the outcome of

studies focusing on hypoxia quantification by pimonidazole and other bioreductive markers.

In **chapter 8,** ^{99m}Tc-Hynic-rh-Annexin V scintigraphy was applied for in vivo imaging of apoptosis in tumor and normal tissue in patients with advanced HNSCC treated with concurrent cisplatin and radiotherapy. We tested the feasibility of the Annexin-scintigraphy as a non-invasive technique to demonstrate treatment-induced apoptosis in vivo, early during treatment. The purpose was to determine the degree of uptake in normal tissue, primary tumor and lymph node metastases and to evaluate the treatment-induced Annexin-uptake in relation to radiation dose. We questioned whether Annexin uptake would correlate with treatment response.

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chapter 2

Prediction of treatment outcome by cisplatin DNA-adduct formation in patients with stage III/IV head and neck squamous cell carcinoma, treated by concurrent cisplatin-radiation (RADPLAT)

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ABSTRACT

The purpose of our study was to test the predictive value of cisplatin-DNA adduct levels in HNSCC-patients, treated with cisplatin-radiation. Patients with advanced-stage HNSCC were treated within a randomized trial, investigating the optimal route of cisplatin administration, concurrently with radiation. Cisplatin was administered intra-arterially (IA, 150 mg/m^2, with systemic-rescue by sodium-thiosulphate) or intravenously (IV, 100 mg/m^2). In a subgroup, adducts were quantified in normal tissue and tumor. ^{32}P-postlabeling was used to quantify intrastrand GG- and AG-adducts. Adduct levels were correlated with treatment outcome. Thirty-five patients were included (21 IV and 14 IA). At median follow-up of 27 months, Locoregional control was 75% at 1 and 70% at 2-years. Adduct levels in tumor were 4-5 fold higher than in white blood cells (WBC) for both IA and IV treatment ($p=0.01$). Adduct-formation in WBC and buccal cells was higher in IV-treated patients compared with IA infusion ($p=0.049$ and $p=0.005$ for GG-adducts in WBC and buccal cells, respectively). Adducts in tumors after IA infusion were not statistically different from those after IV. A strong correlation was observed between GG- and AG-adduct formation ($r=0.86$, $p<0.001$). Patients with higher GG adduct levels (>median) in primary tumor had significantly better disease free survival (DFS) than patients with lower (<=median) adduct levels ($p=0.02$). For overall survival, a non-significant trend was observed, again in favor of patients with higher adduct levels ($p=0.06$). In conclusion, cisplatin-DNA adduct-formation in primary tumor appears to be predictive for DFS in HNSCC. No differences were observed in intra-tumoral adduct levels between IA or IV-treatments, despite the selective infusion of high-dose cisplatin with the IA procedure. However, systemic adduct levels (WBC and buccals) from IV patients were higher than in IA patients, consistent with less systemic exposure after IA administration.

INTRODUCTION

Outcome for patients with advanced stage head and neck squamous cell carcinoma (HNSCC) have been unsatisfactory for treatment by radiation alone. Several strategies have been pursued to improve results of radiotherapy, including neo-adjuvant chemotherapeutic regimens, adjuvant systemic treatment and the concurrent administration of chemotherapy during the radiation treatment course. In several studies on locally advanced HNSCC, it was shown that cisplatin-based chemotherapy, given concurrently with radiation therapy improved both locoregional control and overall survival [1-4]. This combined treatment is now considered the standard of care not only in advanced stage HNSCC but also in a variety of other solid tumors [5-9]. However, further improvement is warranted since locoregional recurrence rates of up to 30-40% are observed.

In an attempt to improve results of inoperable advanced stage HNSCC treated by concurrent chemoradiotherapy, Robbins et al. developed a treatment protocol using high dose cisplatin (150 mg/m^2) administered selectively to the tumor by intra-arterial (IA) infusion, with systemic rescue by sodium thiosulphate (STS), concurrent with radiotherapy, referred to as the RADPLAT protocol [10,11]. High locoregional control rates were observed. Acute toxicity was considerable, mainly hematological and/or mucosa-related, but appeared to be manageable. Similar favorable results were obtained in our institute in a phase II trial on 79 patients with stage IV HNSCC, treated by the RADPLAT protocol [12]. The 1- and 2-year locoregional control rates for this group of patients with inoperable disease were 82% and 69%, respectively, with a 3-year overall survival probability of 43%.

Based on these favorable results we started a randomized phase III trial in 1999 to investigate whether the IA administration of high dose cisplatin (150 mg/m^2) is superior to standard dose intravenous (IV) (100 mg/m^2) administration of cisplatin, which has been the standard in combined modality treatment.

Many in vitro and animal studies have shown that cisplatin can improve the anti-tumor effect of radiotherapy, especially when both are given concurrently. Moreover, this effect is dependent on the cisplatin dosage [13,14]. Both radiation and cisplatin cause DNA-damage, which is responsible for the cytotoxic effect. The underlying mechanism of both modalities however differs. Radiation causes single strand and double strand DNA breaks and DNA-base damage, whereas cisplatin induces cisplatin-DNA adducts, formed when cisplatin reacts with the cellular DNA by binding to nucleotides. The majority of adducts are either intrastrand adducts with cisplatin bound between two guanine nucleotides (GG) or adenine-guanine nucleotides (AG) [15]. Other types of adducts include mono-adducts of cisplatin to a single nucleotide or interstrand crosslinks. Cisplatin-DNA adducts can be determined both in tumor and normal tissue. The level of these adducts has been shown to correlate with cytotoxicity in vitro [16], and with response to therapy in patients [17-19]. In patients with non-small cell lung cancer (NSCLC) treated with chemoradiotherapy, Van der Vaart et al. [20] found a significant correlation between the level of adducts, measured

in buccal cells, and overall survival. This assay may therefore provide a suitable test to select patients for cisplatin-based combined modality treatment and to predict outcome. The purpose of the present study was to explore relationships between adduct levels in tumor and normal tissue in patients with HNSCC treated with 2 different schedules of cisplatin-based chemoradiation and to investigate relationships with treatment outcome. Furthermore, we studied whether selective IA administration of high dose cisplatin (150 mg/m^2) would result in higher levels of adduct formation compared to standard dose (100 mg/m^2) administered IV.

MATERIAL AND METHODS

RADPLAT TREATMENT

From November 1999 until November 2004, 240 patients were entered in a multicenter randomized phase III trial in advanced stage HNSSC, investigating the optimal route of cisplatin delivery during cisplatin-based chemoradiation (RADPLAT). Randomization was between 2 treatment arms: Arm 1, standard intravenous (IV) administration of cisplatin or arm 2, high-dose selective intra-arterial (IA) delivery of cisplatin. Main eligibility criteria included: squamous cell carcinoma of the oral cavity, oropharynx or hypopharynx, inoperable disease due to extension of the primary tumor or functional inoperable disease due to extension of the primary tumor, Karnofsky performance score at least 60, TNM stage III/IV disease based on T3-4 status of primary tumor with any N-status, no distant metastases, age at least 18 year, no prior chemotherapy, no prior surgery or radiation therapy to the head or neck, WBC at least 4.0, platelets at least 100,000, calculated or 24 hour creatinine clearance over 50 ml/min, no prior cerebro-vascular accident, and signed informed consent form prior to study entry. The randomized trial including the cisplatin-DNA adduct sub-study were approved by the medical ethics committee of the hospital.

RADPLAT treatment consisted of concurrent cisplatin-based chemoradiation. Radiotherapy was given with 4-6 MV photon linear accelerators. Target volume included the primary tumor and the bilateral neck for a dose of 46 Gy in 23 fractions. A boost was given to the known macroscopic tumor extensions at the primary tumor site and lymph node metastases to a dose of 24 Gy in 12 fractions. For bilateral pathological lymph nodes in the posterior part of the neck, the spinal cord was shielded in the photon beams after 40 Gy and treatment was given with electron beams. The total dose delivered was 70 Gy in 35 fractions, 5 fractions per week, with an overall treatment time of 7 weeks. The radiation technique was either a conventional 3-field beam set-up or an IMRT-plan, depending on resources.

Table 1: Patient, tumor and treatment characteristics.

RADPLAT:	**IV**	**IA**	
Number of patients	21	14	
Follow-up (median, months)	26	27	
Sex ratio (male: female)	2:1	2:1	
Mean age (range) (years)	57 (25-82)	54 (32-68)	
Primary tumor site:			
Oral cavity (%)	19	21	
Oropharynx (%)	71	36	p=0.05
Hypopharynx (%)	10	43	
T-stage:			
T3 (%)	38	50	
T4 (%)	62	50	
N-stage:			
N0 (%)	19	29	
N1 (%)	14	21	
N2 (%)	48	36	
N3 (%)	19	14	
TNM stage grouping:			
Stage III (%)	14	36	p=0.22
Stage IV A/B (%)	86	64	
RT treatment complete (%)	91	93	
Chemotherapy as scheduled (%)	86	86	

Randomization between IV and IA cisplatin administration was done at the NKI Trial Bureau, stratifying by center, T-stage, N-stage and tumor site. In arm 1, patients received cisplatin IV at a dose of 100 mg/m^2, given as a 30 min infusion, 1 hour before radiotherapy at day 1, 22 and 43 of treatment. In arm 2, cisplatin was delivered IA selectively by a catheterization procedure using the femoral artery. Cisplatin at a dose of 150 mg/m^2 was given over 3-5 minutes, on days 2, 9, 16 and 23, within 1 hour after radiotherapy delivery. For lesions extending over the midline of the patients an infusion on both sides was recommended. For lesions not extending over the midline a single sided infusion was recommended. Concurrently with IA cisplatin, sodium-thiosulphate (STS, 9 g/m^2 in 200 ml distilled water) was given as an IV push over 15-20 minutes, followed by STS 12 g/m^2 IV continuous infusion over 6 hours. In both arms prehydration was given (2000 ml glucose 2,5%/NaCl 0,45%) starting the day before chemotherapy. Following chemotherapy, posthydration was given (3000 ml glucose 2,5%/ NaCl 0,45% in 24 hours), during 2 days for IV and 1 day for IA treatment. Anti-emetics were applied according to standard protocol, using dexamethasone and 5-HT3-antagonists.

CISPLATIN-DNA ADDUCTS

In a subgroup of 35 patients (21 receiving IV and 14 receiving IA cisplatin) treated according to this RADPLAT protocol, we studied cisplatin-DNA adducts after obtaining written informed consent. Before treatment and 23 hours after the end of the first course of chemotherapy, normal tissue samples (white blood cells (WBC) and buccal cells) were obtained. In patients with an accessible primary tumor localization a biopsy of the tumor was also taken. To prevent harvesting necrotic tumor tissue, the biopsy was obtained from the peripheral vital rim of the tumor, adjacent to the normal mucosa. Then the biopsy was rinsed with saline to remove excess blood and stored frozen until analysis. All patients who underwent biopsy had either oral cavity or oropharyngeal carcinoma, with tumor localization accessible for biopsy by direct trans-oral approach. Cisplatin-DNA adducts are induced rapidly (within hours) after chemotherapy administration. In WBC, repair of adducts is limited, with 70-80% of adducts remaining after 15-20 hours after administration [21]. Because of the late persistence of cisplatin-DNA adducts after chemotherapy, the sampling time of 23 hours after administration was chosen.

WBC were isolated from whole blood samples according to a previously published protocol [21], and stored at -80°C until analysis. Buccal cells were collected in phosphate buffered saline (PBS) by scraping the bilateral buccal mucosa with a cotton-swab. The harvested cells were centrifuged (5 minutes at 4°C, 1000 rpm) and resuspended in 0.2 ml of a Tris-EDTA buffer and stored at -80°C until analysis. The biopsy was taken from the periphery of the primary tumor and immediately frozen at –80° C until analysis. Quantification of GG and AG intrastrand adducts was performed by ^{32}P-postlabeling technique according to Pluim et al. [22]. The cisplatin-DNA adduct levels were expressed as fmol per µg DNA. Actual net adduct levels were calculated by subtraction of the pre-treatment/baseline values from the post-treatment adduct values (for WBC and buccal cells). This was not possible for the primary tumor biopsies since no frozen pre-treatment tissue was available.

EVALUATION OF TREATMENT

Patients were followed during treatment twice weekly for adverse effects and/or complications using physical examination and laboratory testing. Acute toxicity was scored using the CTC criteria version 3.0. Six-eight weeks after the end of treatment, the results of therapy were evaluated by means of radiological investigations (by MRI or CT scan and/or ultrasound) and examination under general anesthesia, with biopsies taken in case of suspicious findings. For residual disease in the neck at the time of evaluation, salvage neck dissection could be performed if judged operable. Follow-up visits were planned every 3 months in the first year after therapy, every 4 months in the second year and less frequent thereafter. A follow-up chest X-ray was performed annually.

STATISTICAL ANALYSIS

In a previous study it was shown that the level of adducts in WBC was linearly correlated with cisplatin plasma levels [18]. Therefore, no cisplatin plasma concentrations were obtained. Adduct levels in WBC were compared with those in buccal cells and tumor tissue, both after IV and IA RADPLAT. Analysis of data was performed in SPSS software (version 11.5, SPSS, Inc.). For quantitative comparison of numerical data Student's t-test was applied. Chi-squared and Fisher's exact test were used for analysis of categorical data. The correlation coefficient r was used to calculate the correlation between GG- and AG-adducts in the tissue samples. Locoregional control and survival data were calculated from the start of treatment using the Kaplan-Meier method and Log Rank testing. For analysis of the effect of adduct formation on outcome, adduct levels were categorized as less than or equal to (<=) or higher than (>) the median value. P-values of <= 0.05 were considered statistical significant.

RESULTS

In total 35 patients treated according to the described RADPLAT protocol (21 receiving IV and 14 receiving IA cisplatin) were included in the cisplatin-DNA adduct study. The imbalance in patient numbers over both treatment arms was a matter of chance. The median follow-up of all patients alive at last follow-up was 27 months (range 5-41), with no differences between 2 treatment groups. Table 1 shows the patient, tumor and treatment characteristics. Mean age of patients at start of treatment was 56 years. Patients included in the IV group had fewer hypopharyngeal tumors and more oropharynx tumors compared to the IA group (p=0.05). Analysis by TNM stage showed a little more advanced stages in the IV treatment group (p=n.s.). Compliance to treatment was high: in 7-9% of cases the total dose of radiation was less than 70 Gy and the overall treatment time was limited to 50 days in all patients. Chemotherapy was administered as planned in 86% of cases.

TREATMENT RELATED TOXICITY

Acute mucositis was a little more severe in patients after IV treatment, compared to IA treatment, although this was not statistically different (p= 0.41). Tube feeding (by nasogastric tube or percutaneous gastrostomy) due to mucositis and dysphagia was needed in 70% of cases independent of chemotherapy schedule. Hematological toxicity (>= grade 3) was observed in approximately 40% of patients, irrespective of treatment arm. Acute renal toxicity (defined as increase in serum creatinine >= CTC grade 2) was observed in 30% of IV and 0% of IA patients (p=0.03). No treatment related deaths were observed. See table 2 for summary of acute toxicities.

Table 2: Acute toxicity per treatment modality.

RADPLAT:	IV	IA	
Acute mucositis:			
Grade 2 (%)	15	29	p=0.41
Grade 3 (%)	85	71	
Tube feeding during treatment (%)	67	71	
Hematological toxicity, ≥ grade 3 (%)	40	43	
Serum creatinine toxicity, ≥ grade 2 (%)	30	0	p=0.03

RESPONSE TO TREATMENT, LOCOREGIONAL CONTROL AND SURVIVAL

Treatment response was evaluated 6-8 weeks after treatment. The complete response (CR) rate at the primary tumor site was 97%. CR rate in the neck in the case of nodal metastases was 84%. In 4 patients, less than a CR in the neck was observed. In 2 of them a salvage neck dissection (removing the residual mass) was done, resulting in an overall CR rate (including salvage surgery) of 94%. The estimated locoregional (LR) tumor control rates including all 35 patients were 75% at 1 year and 70% at 2 years. The rates for disease free survival (DFS) were 65% and 51% at 1 and 2 years respectively. Overall survival (OS) rates at 1 and 2 years were 85% and 62%, respectively. In this small subset of 35 patients from a larger randomized phase III trial, there were no differences in response rates, LR control, DFS or OS between the two treatment arms (IV versus IA), although imbalances in distribution of tumor sites over the treatment arms limits interpretation of these results.

CISPLATIN-DNA ADDUCTS

In table 3 and figures 1 and 2 the results are given for the cisplatin-DNA adduct levels in WBC, primary tumor and buccal cells for both treatment modalities: standard dose IV or high dose IA administered cisplatin. Adduct levels in primary tumor were 4-5 fold higher than in

Table 3: Cisplatin-DNA adduct levels, 23 hours after infusion (in fmol/µg DNA).

		WBC		Tumor		Buccal cells	
RADPLAT modality		**GG**	**AG**	**GG**	**AG**	**GG**	**AG**
IV 100 mg/m2	*N*	*21*	*21*	*7*	*7*	*6*	*6*
	Mean	1.01	0.10	3.87	0.41	1.70	0.36
	SD	0.39	0.05	1.10	0.09	0.26	0.08
IA 150 mg/m2	*N*	*14*	*14*	*5*	*5*	*5*	*5*
	Mean	0.76	0.08	4.55	0.43	0.92	0.16
	SD	0.30	0.04	1.16	0.18	0.43	0.13

WBC or buccal cells, after both IV and IA treatment (p=0.01). The cisplatin-DNA adducts in WBC (a measure of systemic exposure) were higher in IV-treated patients compared to selective IA infusion (p=0.049 for GG-adducts, p=0.054 for AG-adducts). Similar results were found for buccal cells: Significantly more adducts in buccal cells after IV treatment than after IA treatment were found (p=0.005 for GG-adducts, p=0.01 for AG-adducts), indicative of more systemic exposure to cisplatin after standard IV compared to selective high-dose IA cisplatin administration. The ratio between GG- and AG-adducts was similar in all tissue samples studied, both after IV and IA RADPLAT treatment.

Figure 1. GG-adduct levels in fmol/µg DNA in WBC (circles), primary tumor biopsies (squares) and in buccal cells (triangles), 23 hours after administration of cisplatin. Open symbols represent individual data points, closed symbols represent means. Errors are SD.

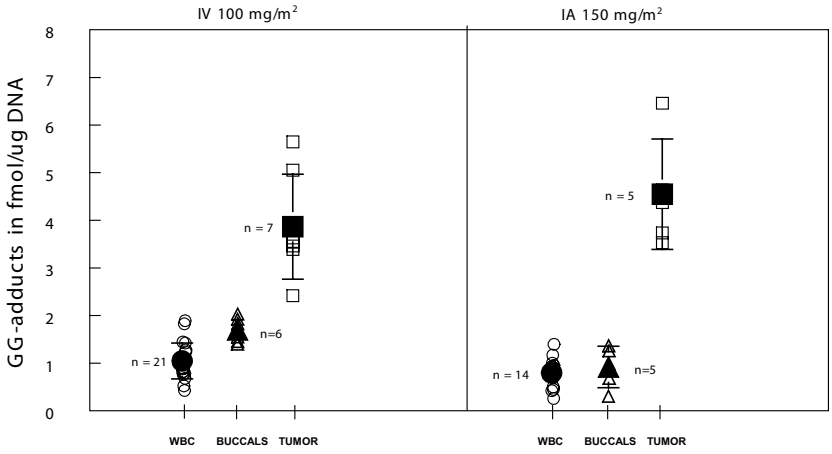


Figure 2: AG-adduct levels in fmol/µg DNA in WBC (circles), primary tumor biopsies (squares) and in buccal cells (triangles), 23 hours after administration of cisplatin. Open symbols represent individual data points, closed symbols represent means. Errors are SD.

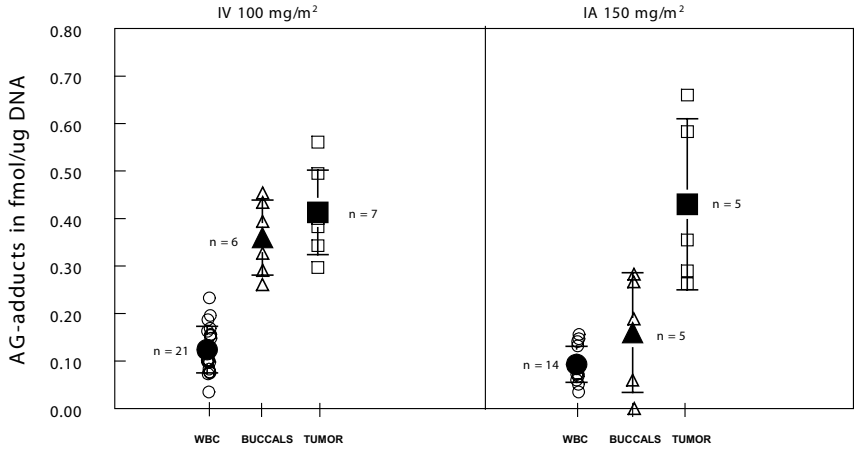
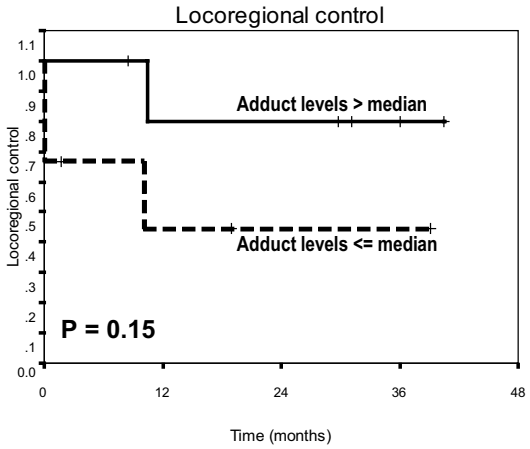
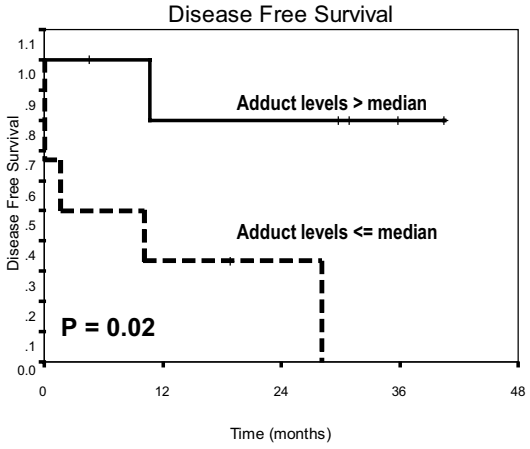


Figure 3: Treatment outcome by GG-adduct levels in primary tumors. Panel A represents locoregional control, panel B disease free survival, panel C overall survival.

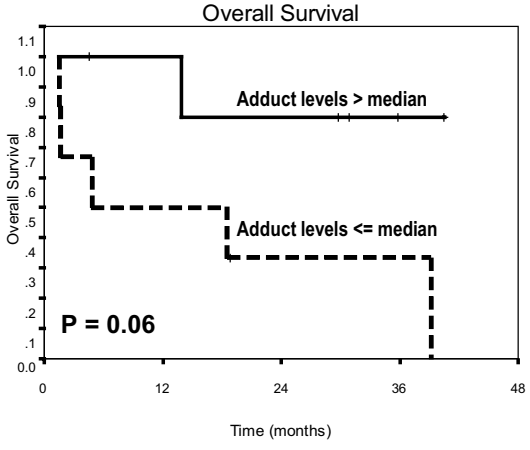
A



B



C



Adduct levels in the primary tumors after IA infusion were only slightly higher, but not significantly, compared to levels after IV treatment, despite the use of higher cisplatin doses and selective tumor-directed infusion. The inter-individual variation in adduct formation was limited in normal tissue, but more pronounced in primary tumor. Intrastrand GG-adduct levels were 5-10-fold higher than AG-adducts, both in normal tissue and in primary tumor. The levels of GG-adducts and AG-adducts in the normal tissue and primary tumor samples were statistically significantly correlated ($r=0.86$, $p<0.001$). Therefore, the analysis of treatment outcome in relation to the adduct levels was calculated for the most predominant adduct-form: GG-adducts. No significant correlations were found between the levels of adducts in normal tissue (WBC) and in primary tumor.

Analysis of 2-year LR-control rates of all patients by the level of adduct formation in primary tumor (<= median versus > median) showed non-significant differences ($p=0.15$), although patients with higher GG-adduct levels had a better LR control, compared to patients with lower adduct levels: 80% and 44%, respectively (figure 3A). When analyzing the results for DFS by adduct levels in primary tumor, a significantly better DFS for patients with higher adduct levels was observed compared to those with lower adduct levels (figure 3B): The 2 year DFS rates were 80% and 33%, respectively ($p=0.02$). For OS there was a trend towards better OS for patients with higher adduct levels ($p=0.06$) (figure 3C). Adduct levels within WBC had no predictive value for either LR control, DFS or OS. In table 4 the patient distribution is given according to the classification by adduct levels in the primary tumor (<= median versus > median).

Analysis of GG-cisplatin-DNA adducts in tumor per either IA or IV treatment resulted again in better control rates and survival for patients with adduct levels > median compared to levels <= median, although differences were not statistically significant. No relationship was found between the level of cisplatin-DNA adducts and the occurrence and/or severity of acute hematological, mucosal or renal toxicity (data not shown).

DISCUSSION

The hypothesis of the present randomized trial was that in patients with advanced stage HNSCC, selective IA infusion of high dose cisplatin with systemic rescue by sodiumthiosulphate (STS) would result in improved locoregional control as compared to standard dose IV cisplatin-chemotherapy, without increased treatment-related toxicity. In a subset of patients from the trial, adduct levels in tumor and normal tissues were evaluated. Imbalances in patient numbers and tumor sites over both treatment arms have occurred, which limits the interpretation of outcome by treatment arm. This however, does not influence the analysis of adduct levels and their predictive value as such. In contrast to our expectations, the adduct levels in the primary tumor were not significantly different between patients with IA and IV administration of cisplatin, although levels after high dose IA treatment were slightly higher compared to IV treatment. Due to the relatively low number of patients who had biopsies taken, a true

difference between adduct levels in primary tumor after IV vs. IA treatment might have been missed. As hypothesized, the opposite was true for adduct formation at the systemic level: statistically significant higher adduct levels were observed in WBC and in buccal cells after IV cisplatin, compared to selectively delivered cisplatin during IA infusion. These results indicate that selective IA infusion with systemic rescue by STS results in lower systemic cisplatin-exposure but does not lead to higher local cisplatin-adduct levels at the primary tumor site.

Table 4: Characteristics of patients according to primary tumor adduct level status.

Primary Tumor Adduct level:	**<= median**	**> median**
Tumor site		
Oral cavity	3	2
Oropharynx	3	4
T-stage		
T3	1	2
T4	5	4
N-Stage		
N0	0	2
N1	1	0
N2	3	3
N3	2	1
TNM-stage group		
Stage III	0	2
Stage IV	6	4
Treatment		
RADPLAT IV	5	2
RADPLAT IA	1	4

Cisplatin-DNA adduct formation appears to be predictive for DFS: Higher GG-adduct levels in the primary tumor resulted in improved DFS compared to lower adduct levels. The predictive value of adduct formation in the primary tumor appears to be independent of treatment modality, being IV or IA. Similar but not significant results were found for LR control and OS: Patients with higher adduct levels had better outcome compared to those with lower levels. Levels of adduct formation in WBC were not predictive for outcome. In previous studies in which adduct levels were investigated in normal tissue, a positive correlation was found between adduct formation and outcome [18-20]. This would suggest that normal tissue (i.e. WBC) might be used as a surrogate marker for tumor tissue with respect to the predictive value of cisplatin-DNA adduct formation. In our present study however, we did not find a correlation between adducts in WBC and in tumor.

This lack of correlation between adducts in tumor and normal tissue was confirmed in our ongoing studies in chemoradiation protocols (unpublished data). Similar results were found in a study by Moses et al. [23] in an animal model, treated with IV or IA cisplatin, demonstrating lack of correlation between tumor platinum content and serum platinum concentration. This would suggest that different mechanisms in tumor and normal tissue play a role. For normal tissue, the adduct formation in WBC probably reflects total dose and system exposure to cisplatin. In primary tumors many other factors play a role: tumors are very heterogeneous in terms of perfusion, in differences in pH, presence of necrosis and capacities to repair damage from cytotoxic agents. All these factors probably contribute to the lack of correlation between systemic cisplatin exposure and intra-tumoral cisplatin-adduct formation. From our and other data it appears that the predictive value of adduct levels in the primary tumor is higher than of those in normal tissue and that for purposes of individualization, primary tumor adduct levels should be included.

To the best of our knowledge, this is the first direct comparative study in patients investigating the formation of cisplatin-DNA adducts in primary tumor tissue after IV vs. IA cisplatin-based chemoradiotherapy. Most studies measured intra-tumoral platinum content instead of cisplatin-DNA adducts in HNSCC patients [24-27] or cervical cancer patients [28-30].

Gouyette et al. reported on intra-tumoral platinum-content in HNSCC patients after cisplatin administration IV or IA [24]. Platinum concentrations in biopsies taken after IA infusion were slightly increased relative to IV treatment, but not statistically different. In this study no STS was given systemically in IA patients. In a publication of Los et al., intra-tumoral platinum-content and cisplatin-DNA adducts were measured in biopsies after IA RADPLAT treatment (150 mg/m^2, with systemic STS neutralization) in HNSCC patients [25]. The observed intra-tumoral platinum content correlated with adduct formation and was compared with data from Gouyette's paper [24] and it was concluded that high dose IA cisplatin yielded statistically higher platinum-concentrations than conventional dose IV. However, this comparison with historical controls from another institute is inherently difficult, in particular because the timing of the biopsy was different and because the IV schedule used in Gouyette's paper (cisplatin, 2 x 50 mg/m^2 over 2 days) was different from the commonly applied schedule of a single infusion of 100 mg/m^2. In a study from Sileni et al. in HNSCC patients, cisplatin was administered IA (without STS) and IV [26]. In primary tumor biopsies taken after the first infusion no increase in platinum content in IA patients compared to IV patients was shown.

In animal studies, there are again conflicting results. Moses et al. observed no benefit from IA treatment in terms of intra-tumoral platinum concentrations compared to IV administration [23]. On the other hand, Jakowatz et al. reported on increased intra-tumoral platinum levels in animals, treated with prolonged IA infusion times (3-48 hours), but not in rapid, short infusions (30 min), when compared to IV infusion [31].

Possible reasons for the non-significant differences in adduct formation between IA and IV treatment in our study include saturation of cisplatin in the tumor as suggested by Moses et al. [23]. In addition, the total exposure of the tumor to cisplatin (i.e. total dose of cisplatin x time of infusion) after a short (3-5 min) IA infusion may be limited and could be more or less comparable to an IV administration as observed in animal studies in which increased intra-tumoral platinum levels were seen at longer IA infusions [31]. Since the proportion of cisplatin reaching the systemic circulation after the IA infusion is neutralized by STS, there is only a first-passage effect in the tumor during the IA infusion. For IV treatments, no neutralization was done and exposure to cisplatin may therefore be prolonged, resulting in adduct levels being comparable to those after IA administration. Finally, the number of patients from whom biopsies were taken, was relatively small and this might have been responsible for the lack of a statistically significant result. Since only a single biopsy per patient was taken, no information is obtained regarding possible intra-tumoral variation in adduct levels.

Levels of adduct formation in our study in normal tissues were significantly higher in IV than in IA treated patients (table 3), indicating that IA administration with STS indeed selectively exposes tumor with reduced systemic exposure to cisplatin. Consistent with this finding is the observation that acute renal toxicity was less frequent in IA patients (table 2). These observations are in agreement with data from other studies where the area under the curve (AUC) of cisplatin was lower after IA treatment compared to IV cisplatin [26,28].

Adduct levels in primary tumors were 4-5 fold higher than in WBC both after IV and IA cisplatin, as shown previously by others [32]. The same relative increase in levels in tumor over normal tissue was shown for cisplatin concentration measurements in animal experiments using IA and IV delivery [31]. The levels of adducts within WBC after IV chemotherapy and their relative occurrence (GG- versus AG-adducts) were within the same range as previously reported in patients with advanced stage NSCLC treated by cisplatin and gemcitabine [33]. It was also observed that adducts in tumor were higher than in buccal cells, both tissues being derived from the same mucosal squamous epithelium. The explanation for the observed increase in adduct levels in tumor over buccal cells is unknown. It might be explained by tissue specific differences in one or more of the following factors. These include the uptake of cisplatin from the circulation, efflux of cisplatin from the cells by active transport, cisplatin-binding capacity to the DNA or repair of cisplatin-DNA adducts. Buccal cells and squamous cell tumors may also differ in the degree of cell viability and in the proximity to blood vessels. Furthermore, it is a consistent finding in previous studies that tumor adduct levels are higher than in surrogate tissues [19,32].

Whether IA administration of cisplatin during radiation is superior to IV cisplatin in terms of improved locoregional control and/or survival in locally advanced HNSCC is as yet unknown and will be determined when the final results of the RADPLAT trial become

available. Analysis of the present study is limited by the number of patients and the imbalances in distribution in tumor-sites. Although the single measurement of adduct formation in primary tumor after the first course of chemotherapy revealed no differences in adduct levels between IA and IV treatment, it should be noted that not only the dose of cisplatin administered per course (150 mg/m^2 vs. 100 mg/m^2), but also the number of courses (4 vs. 3) was higher in IA patients. Furthermore, the IV treatment is given every 3 weeks, whereas in the IA treatment cisplatin is delivered during the first 4 weeks of treatment. All these factors may contribute to differences in sustained adduct levels throughout the course of treatment.

In conclusion, intra-tumoral cisplatin-DNA adduct formation seems to be predictive for DFS, independently of treatment modality (IV vs. IA cisplatin). High-dose IA cisplatin during concurrent chemoradiotherapy for advanced stage HNSCC did not result in statistically significant higher cisplatin-DNA adduct levels compared to standard dose IV cisplatin administration in the primary tumor, despite the selective mode of drug delivery during IA catheterization. However, adduct formation in WBC and buccal cells was significantly increased after IV treatment, when compared to IA administration, indicating increased systemic cisplatin exposure.

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chapter 3

Cisplatin-DNA adduct formation in patients treated with cisplatin-based chemoradiation: lack of correlation between normal tissues and primary tumor

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ABSTRACT

Purpose

In this study, the formation of cisplatin-DNA adducts after concurrent cisplatin-radiation and the relationship between adduct-formation in primary tumor tissue and normal tissue were investigated.

Methods

Three intravenous cisplatin-regimens, given concurrently with radiation, were studied: daily low-dose (6 mg/m^2) cisplatin, weekly 40 mg/m^2, three-weekly 100 mg/m^2. A ^{32}P-postlabeling technique was used to quantify adducts in normal tissue (white blood cells (WBC) and buccal cells) and tumor.

Results

Normal tissue samples for adduct-determination were obtained from 63 patients, and tumor-biopsies from 23 of these patients. Linear relationships and high correlations were observed between the levels of GG- and AG-adducts in normal and tumor tissue. Adduct levels in tumors were 2-5 times higher than those in WBC ($p<0.001$). No significant correlations were found between adduct levels in normal tissues and primary tumor biopsies, nor between WBC and buccal cells.

Conclusions

In concurrent chemoradiotherapy schedules, cisplatin adduct levels in tumors were significantly higher than in normal tissues (white blood cells). No evidence of a correlation was found between adduct levels in normal tissues and primary tumor biopsies. This lack of correlation may, to some extent, explain the inconsistencies in the literature regarding whether or not cisplatin-DNA adducts can be used as a predictive test in anticancer platinum therapy.

INTRODUCTION

Concurrent chemoradiotherapy is more effective than radiotherapy alone, both in *in vitro* studies [1,2] as well as in clinical studies in many different tumor types, including advanced head and neck squamous cell carcinoma (HNSCC) and cervical cancer, leading to improvements in locoregional control and/or survival [3-6]. In a meta-analysis on concurrent chemoradiation in HNSCC, the addition of concurrent single agent cisplatin to radiotherapy was the most effective treatment regime with the largest improvement on overall survival [7]. Concurrent cisplatin-based chemoradiation is now considered standard of care in advanced stage HNSCC and cervical cancer.

In addition to the increased efficacy of the combined treatment, it was shown that the concurrent regimens are accompanied by higher acute toxicity rates compared to radiation alone [3,5], with more severe mucositis and gastro-intestinal toxicity.

Since a substantial number of patients treated with concurrent chemoradiation still fail to respond to this toxic treatment, there is a need for an accurate predictive assay, based on which patients can be selected that are likely to respond to the therapy. This strategy may also provide a tool to individualize and tailor treatment, based on evaluation of the predictive assay, early during therapy.

One potential predictive marker is the formation of cisplatin-DNA adducts, which are formed when cisplatin reacts with the cellular DNA by binding to nucleotides. The majority of adducts are either intrastrand adducts with cisplatin bound between two guanine (GG) nucleotides or adenine-guanine (AG) nucleotides [8]. Cisplatin-DNA adducts can be measured in tumor and normal tissue. The level of adducts has been shown to correlate with cytotoxicity *in vitro* [9], and with response to therapy in patients [10-13]. In most of these studies [10-12] adduct measurements were performed in normal tissue, with the assumption that normal tissue can be used as surrogate marker for tumor.

In our institute, study protocols with cisplatin-DNA adduct measurements are ongoing in patients with HNSCC and cervical cancer, all treated with concurrent cisplatin-based chemoradiation. The objectives of the current study are 1) to investigate the two major forms of cisplatin-DNA adducts (GG- and AG-adducts) after different schedules of cisplatin given concurrently with radiation and 2) to explore relationships between adducts in primary tumor and normal tissue. We specifically wanted to investigate whether the level of adducts in tumors are reflected by those in normal tissues. In studies focused on the predictive value of cisplatin-DNA adduct levels this would then justify the use of more easily obtained normal tissues as a surrogate for tumor samples.

PATIENTS AND METHODS

CONCURRENT CHEMORADIATION PROTOCOLS

This study on adduct-formation was approved by the medical ethical committee of the participating hospitals. Main eligibility criteria were: patients scheduled for cisplatin-chemoradiation; no previous treatment with cisplatin; and informed consent. Eligible patients were informed about the nature of the protocol and after written informed consent, were entered in the study. Patients were recruited from one of the following regimens.

In advanced stage HNSCC patients, two different cisplatin-based concurrent chemoradiation protocols (RADPLAT) were used. The **RADPLAT 100** schedule, which is the most commonly administered schedule in HNSCC [3], consisted of cisplatin intravenously (IV) at a dose of 100 mg/m^2, given as a 30 min infusion, 1-2 hours before radiotherapy (RT) at day 1, 22 and 43 of treatment. This treatment was part of a randomized trial on IV versus intra-arterial chemoradiation. In **RADPLAT daily LD**, low dose (LD) cisplatin was given as a 1-2 min IV infusion at a dose of 6 mg/m^2 daily, for a total number of 20 doses, 1-2 hours prior to RT. This treatment was shown to be an effective alternative in HNSCC [14,15]. Patients ineligible for or refusing the randomized trial on intra-arterial chemoradiation were treated with RADPLAT daily LD since this treatment could be given on an outpatient basis. The RT target volumes for all schedules included the primary tumor and the bilateral neck at a dose of 46 Gy in 23 fractions. A boost was given to the macroscopic tumor extensions at the primary tumor site and lymph node metastases at a dose of 24 Gy in 12 fractions, resulting in a total dose of 70 Gy in 35 fractions.

In patients with advanced stage squamous cell cervical cancer, concurrent chemoradiation (**CERVIX 40**) consisted of weekly administration of cisplatin IV as a 4-hour infusion at a dose of 40 mg/m^2 followed by RT within 1-2 hours. The total number of doses was 5-6, depending on external beam RT schedule and the number of intracavitary brachytherapy applications. The total radiation dose was usually 46 Gy to the cervical tumor, uterus and pelvic lymph nodes, with a boost to the cervix tumor and other involved regions, to a total dose of 60-74 Gy, depending on treated volume and whether or not intracavitary brachytherapy was given.

CISPLATIN-DNA ADDUCTS

Before and after chemotherapy, normal tissue samples (white blood cells (WBC) and buccal cells) were collected. In patients with an accessible primary tumor, a biopsy of the tumor was also taken. To avoid harvesting necrotic tissue, the biopsy was taken at the viable peripheral rim of the tumor. Samples were obtained at different times, due to logistic reasons: For the patients in the RADPLAT 100 study, this was done 23 hours after the end of administration of the first cisplatin infusion (given on day 1 of treatment). In the patients in the CERVIX 40 study, samples were taken 20 hours after the end of administration of the first weekly cisplatin infusion (given on day 1 of treatment). For the patients in the RADPLAT

daily LD group, samples were taken 1 hour after the 5th dose on day 5 of treatment. WBC were isolated from whole blood samples according to a previously published protocol [16]. Buccal cells were collected in phosphate buffered saline by scraping the bilateral buccal mucosa using a cotton-swab. In HNSCC patients, the buccal mucosa could be located within the RT treatment fields, depending on tumor site. The harvested cells were centrifuged (5 minutes at 4°C, 1000 rpm) and resuspended in a Tris-EDTA buffer and stored at -80°C until analysis. Tumor biopsies were taken and immediately frozen at –80°C until analysis. Quantification of GG- and AG-intrastrand adducts was performed by a ^{32}P-postlabeling technique as previously described [17]. Internal standardization was incorporated in the present analysis method, by adding 300 fmol of TT nucleotides to each sample. From previous work, the reproducibility of the assay is known by analysis of duplicate specimens within the same experiment (within-run reproducibility) and by analysis of duplicate specimens in separate experiments (between-run reproducibility) [17]. The reproducibility was described for WBC and tumor samples and was within 10% for the within-run precision and between 2-20% for the between-run precision. It was also determined for buccal cells in the same way and similar reproducibility was obtained. The concentration of DNA present in the samples was measured spectrophotometrically at 260nm with the Nanodrop ND-1000 (Nanodrop Technologies Inc, Wilmington, DE, USA). The cisplatin-DNA adduct levels were expressed as fmol per µg DNA. The lower limit of quantification for the Pt-GG and Pt-AG adducts was 0.087 fmol/µg DNA and 0.053 fmol/µg DNA, respectively.

STATISTICAL ANALYSIS

Analysis of data was performed in SPSS software (version 11.5, SPSS, Inc.). For quantitative comparison of numerical data between groups, the Student's *t*-test was applied. The Pearson correlation coefficient and Spearman's rank correlation coefficient were calculated for analysis of correlations between different samples (WBC, buccal cells and primary tumor) on an intra-patient level.

RESULTS

Samples for cisplatin-DNA adduct determination were obtained from 63 patients: 27 from RADPLAT daily LD, 15 from CERVIX 40, and 21 from RADPLAT 100. WBC samples were taken from 61 patients, buccal cells from 25, and tumor biopsies from 23 of these patients. The reasons for the missing data for the normal tissue samples were: no collection of samples due to logistics or (in minority of cases) not sufficient volume for analysis. The reason for missing primary tumor biopsy data were: tumor not accessible for direct out-patient-based biopsy (in HNSCC patients) or refusal (in cervix cancer patients).

In WBC, all but 3 of the 60 available baseline samples were below the LLQ for the GG-adducts, and all but 2 below the LLQ for the AG-adducts. This was probably due to some

background signal inherent in the post-labeling method, since all patients had not been treated before with platinum-chemotherapy. The yield of DNA, obtained from buccal cell samples was rather low, ranging from 1 – 10 µg. Baseline samples of buccal cells were available from 16 of 25 patients, of whom post-treatment samples were also available. The baseline values of GG-adducts in buccal cells ranged from 0.067 to 0.745 fmol/µg DNA (mean 0.282, SD 0.19) and baseline values of AG-adducts in buccal cells ranged from 0.087 to 1.538 fmol/µg DNA (mean 0.398, SD 0.38). All but 2 of the baseline GG-adduct values were above the LLQ and all the baseline AG-adduct values were above the LLQ. This was probably due to the low DNA quantities obtained from buccal cell samples. The difference in adduct levels from baseline to post-infusion values was significant for the GG-adducts, but not for the AG-adducts. This implies that for measuring low quantities of AG-adducts in the low amounts of buccal cell DNA available, we reached the limits of quantification with this postlabeling method.

In table 1 the results are presented for the GG- and AG-adduct levels in normal tissue and primary tumor for the 3 different cisplatin-chemoradiation regimes. Adduct levels in primary tumor were 2-5 times higher than those in WBC for all 3 treatment regimes for both GG- and AG-adduct formation (Student *t*-test, $p<0.001$ for both adduct types). For the comparison of the adduct levels from the 3 different treatment protocols, the data from the RADPLAT daily LD were omitted, since the daily administration schedule and sampling time (1 hour after the infusion) were different from the other 2 regimens. The adduct levels in the RADPLAT 100 schedule were statistically significantly higher than those after the CERVIX 40 schedule for both tumor (Student *t*-test, $p= 0.01$) and normal tissues (Student *t*-test, $p<0.01$).

Table 1. Cisplatin-DNA adducts (in fmol/µg DNA) in normal tissue and primary tumor after different schedules of cisplatin-based chemoradiation. See text for explanation of treatment schedules.

Treatment schedule		**WBC**		**Buccal cells**		**Tumor**	
		GG	**AG**	**GG**	**AG**	**GG**	**AG**
RADPLAT daily LD	*N*	*26*	*26*	*11*	*11*	*6*	*6*
	Mean	0.34	0.05	0.84	0.21	0.66	0.10
	SD	0.10	0.19	0.39	0.14	0.37	0.05
CERVIX 40	N	14	14	7	7	10	10
	Mean	0.44	0.06	0.87	0.22	1.94	0.26
	SD	0.17	0.03	0.26	0.06	1.47	0.26
RADPLAT 100	*N*	*21*	*21*	*7*	*7*	*7*	*7*
	Mean	1.05	0.12	1.56	0.34	3.87	0.41
	SD	0.38	0.05	0.43	0.09	1.10	0.09

Abbreviations: N, number of patients. SD, standard deviation. WBC, white blood cells. GG, GG-adducts. AG, AG-adducts.

A highly significant linear correlation (Pearson correlation, r=0.93, p<0.001, n=61) was observed between the level of GG- and AG-adducts in WBC (see figure 1a), with a mean ratio of GG/AG adducts of 7.6 +/- 2.1 SD. Similar linear relationships and ratios were found for GG- and AG-adducts in primary tumor (r=0.89, p<0.001, n=23, and ratio 8.5 +/- 2.8, figure 1b) and buccal cells (r=0.85, p<0.001, n=23, and ratio 3.9 +/- 1.3, figure 1c).

A trend was observed between GG-adduct levels in WBC and buccal cells, although not significant (r=0.38, p=0.07, n=24). No significant correlations were found between tumor and normal tissue: tumor vs. WBC (r=0.35, p=0.13, n=21,) and tumor vs. buccal cells (r=-0.003, p= 0.99, n=9). See figure 2 for scatter plots. Similar results were found for the AG-adducts: no significant correlations were found between adducts in tumor vs. WBC (r=0.14, p=0.55, n=21), tumor vs. buccal cells (r=0.25, p=0.58, n=7) or WBC vs. buccal cells (r=0.26, p=0.24, n=22).

DISCUSSION

There are two main conclusions from the 63 patients included in these analyses. Firstly, intra-tumoral adduct levels were substantially higher than those in normal tissue (WBC) at all cisplatin-dose levels examined, and secondly, no positive correlations were evident between adducts in tumors and normal tissues. It should be noted that the various schedules, the cisplatin doses and the duration of infusions differed, as well as the sampling times. However, all analyses on adducts were performed on paired samples, within the same patient. This eliminates variance of these factors since the normal tissue and tumor samples all were obtained at similar time points after the cisplatin infusion. In the RADPLAT daily LD, some accumulation from the previous four daily 6 mg cisplatin infusions will have occurred and affect the day 5 measurement after the 5th infusion. In the RADPLAT 100 and CERVIX 40 patients, no such accumulation will have occurred since the sampling was done 20-23 hours after the first infusion of cisplatin.

Relatively little information is available regarding the in vivo formation of intra-tumoral cisplatin-DNA adducts in clinical series [13,18]. Most studies focused on intra-tumoral platinum concentrations, both in HNSCC [19,20] and cervical cancer patients [21,22]. These studies are mostly characterized by relatively low numbers of patients, probably due to the invasive nature of the procedure. The data on correlations between adducts and platinum content are contradictory: In an experimental study [23] no relationship could be established between the intra-tumoral adduct levels and platinum content, although in one clinical study, a significant correlation was found [18].

Adduct levels in primary tumors were consistently 2-5 fold higher than in WBC. This was true for both GG and AG adducts. This finding was previously described in anecdotal clinical cases [24,25]. Similar observations were made in platinum content studies in an experimental tumor model [26] and in HNSCC patients [20]. Adducts in tumor were

Figure 1. Correlation-plots of GG- and AG-adduct levels in white blood cells (WBC) (panel A), primary tumor biopsy (panel B), buccal cells (panel C). In each panel, the 3 different treatment groups are depicted: RADPLAT daily LD 5x6 mg (squares), CERVIX 40 mg (circles), and RADPLAT 100 mg (triangles).

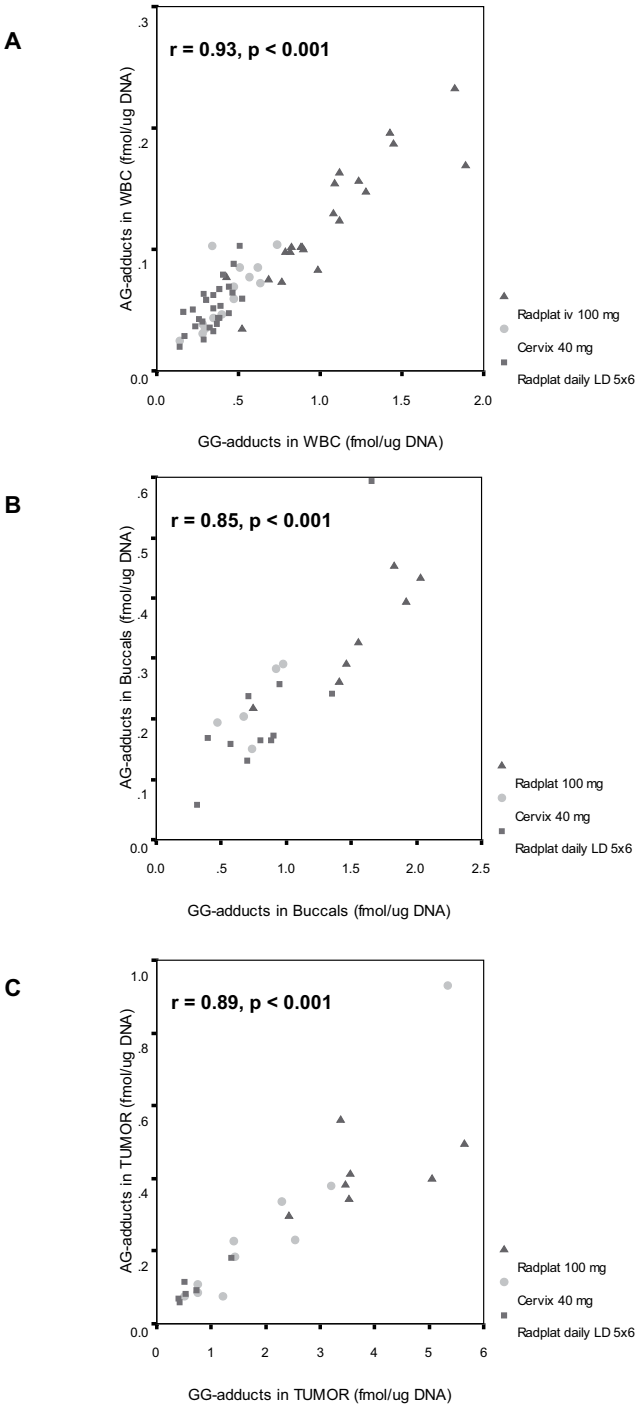
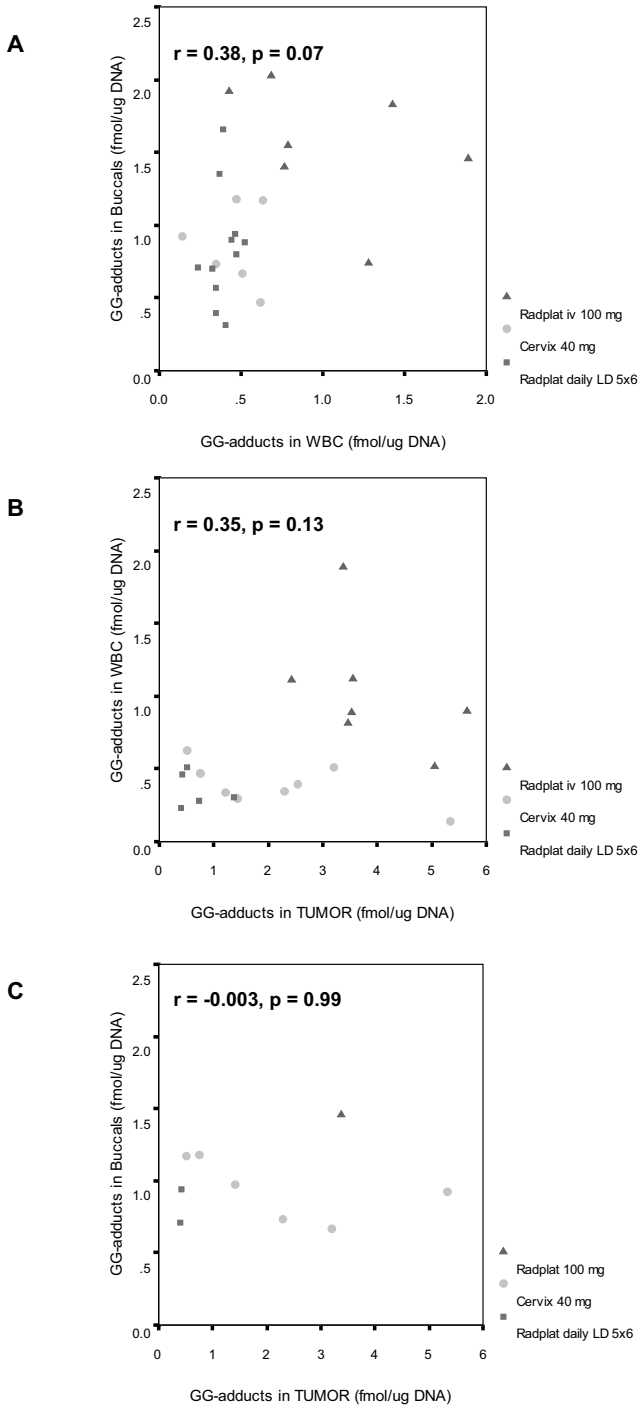


Figure 2. Correlation-plots of GG-adducts in white blood cells (WBC) versus buccal cells (panel A), and normal tissue versus tumor (panel B and C). In each panel, the 3 different treatment groups are depicted: RADPLAT daily LD 5x6 mg (squares), CERVIX 40 mg (circles), and RADPLAT 100 mg (triangles).



also higher than in buccal cells in the CERVIX 40 and RADPLAT 100 group, but not in the RADPLAT daily LD group. We observed a linear relationship between the two major adduct forms (AG and GG) for both normal tissues and tumor (figures 1A-C), although GG adduct formation was 5-12 times increased relative to the AG adducts, as reported earlier [8,27]. The linear relationships between GG and AG adducts serve as a validation of the assay. Apparently within a sample, both types of adducts are present in an equal proportion, although the absolute amounts differ greatly. In previous studies, the type of adduct responsible for the cytotoxic effect of platinum compounds has been investigated. From two of these studies [27,28] it was concluded that the AG-adduct was responsible for the platinum cytotoxicity. From our study, such a conclusion cannot be made, since both types of adducts were present in equal proportions.

Adduct formation in different tissue samples showed a lack of correlation between tumor and normal tissue. One might have expected that higher adduct levels in normal tissue would be accompanied by more adducts in tumor, although this was not the case. Similar results were found in an animal study [29] and in a clinical series of uterine cervix cancer patients [22], both demonstrating lack of correlation between tumor platinum and serum platinum concentrations. If adduct formation were merely a matter of cisplatin exposure, then a positive correlation would be expected. The reasons for the higher levels of adducts in tumor versus normal tissue and the lack of correlation between them, may be explained by differences between tumor and normal tissue in one of the following factors: Tumors are heterogeneous in terms of blood supply and perfusion, resulting in differences in cisplatin uptake and diffusion, drug-pumps may diminish intra-cellular cisplatin concentrations by active trans-membrane transport of cisplatin, prohibiting adducts to be formed, and tumor cells may have less effective capacities to repair damage from cytotoxic agents.

Adducts are formed rapidly and in a dose-dependent fashion within 1-2 hours after cisplatin exposure, with a gradual decrease (repair) within the next 20-24 hours [11,27,30]. The persistence of cisplatin-DNA adducts may therefore be regarded as a measure of repair and possibly be used as predictive assay. We therefore chose 20-23 hours after the end of chemotherapy infusion to measure adducts. Ideally, more frequent measurements would have generated more information on the rate of adduct formation, repair, and total exposure to adducts (like an AUC analysis) [16]. However, obtaining repeated biopsies is not feasible in clinical practice.

The results from the buccal cell samples need to be interpreted with caution, especially the AG-adduct levels, since uncertainties remain. With the low quantities of AG-adducts in the low amounts of buccal cell DNA we could extract, we reached the limits of quantification of the postlabeling method.

The rationale for measuring cisplatin-DNA adducts is that it could be used as a predictive assay: higher levels of adducts would predict favorable treatment outcome. Many studies have been performed for this purpose, investigating adducts in normal tissue (WBC and buccal cells) [10-12,31-35]. These study designs are based on the assumption that normal

tissue can be used as a surrogate marker for tumor tissue with respect to cisplatin-DNA adduct formation. In our present study, however, we did not find such a correlation. This might explain why the results on the role of adduct formation to predictive outcome are heterogeneous and contradictory, as illustrated below.

Several studies on adduct formation in WBC showed that the level of adducts was positively correlated with response to chemotherapy in patients with advanced disease in a variety of tumor sites [11,32], while others showed no correlation [34,35]. One study showed a positive correlation in one tumor site (ovarian cancer), but not in the other (breast cancer) [31], and in another study the level of adduct formation showed a negative association with survival for day-5 adducts, while there was no difference for day-1 adducts [33]. In studies on adduct levels in buccal cells, a positive correlation was found between adducts and either disease response [10] or better survival in non-small cell lung cancer (NSCLC) [12]. We recently showed that adduct formation in primary tumor appeared to be associated with better progression-free survival in HNSCC [13].

Differences were observed between the levels of intra-tumoral adducts for the 3 chemoradiation schedules, with lower adducts after lower dosages of cisplatin. Based on this, however, one cannot predict that the schedules with less adducts will result in less cytotoxicity, since not only the cisplatin dose but also timing and schedule of cisplatin administration are crucial determinants of efficacy [1]. These factors may contribute to differences in the formation and rate of repair of adducts, resulting in different exposures to cisplatin-DNA adducts. These differences make it difficult to extrapolate from the observed adduct values in tumor and normal tissues to a prediction of superiority of one schedule over another.

In future studies, immunohistochemistry on repair proteins like e.g. ERCC1 [36] or gene expression profiling studies on platinum resistance [37], could be used to improve the prediction of cisplatin sensitivity and prediction of therapy response.

In conclusion, we have demonstrated that in concurrent chemoradiotherapy schedules, cisplatin adduct levels in tumors were significantly higher than in normal tissues (white blood cells). No evidence of a correlation was found between adduct levels in normal tissues and primary tumor biopsies. This lack of correlation may, to some extent, explain the inconsistencies in the literature regarding whether or not cisplatin-DNA adducts can be used as predictive test in anticancer therapy.

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chapter 4

Concurrent chemoradiation with daily low dose cisplatin for advanced stage head and neck carcinoma

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ABSTRACT

Background and purpose
To evaluate treatment results of concurrent chemoradiation with daily low dose cisplatin.

Material and methods
121 patients with advanced stage HNSCC were treated with RT (35x2 Gy) and cisplatin (6 mg/m^2 i.v.x20, daily before RT). After 47 patients, the treatment protocol (*Standard Group*) was changed: Daily i.v. prehydration and accelerated RT were given to the subsequent 74 patients (*Hydr-Ac-RT Group*).

Results
Mean follow-up was 29 months (range 7–62). More chemotherapy could be administered in the Hydr-Ac-RT Group (maximum no. of 20 cisplatin-infusions increased from 59 to 91% of patients, p=0.008), with less renal toxicity (p<0.001) and less hospital admissions (p<0.02). However, mucositis was more pronounced and tubefeeding more frequent in the Hydr-Ac-RT Group. The CR rate of the primary tumor increased from 74% (Standard Group) to 90% (Hydr-Ac-RT Group) (p=0.06), although this did not lead to an improvement in loco-regional control.

Conclusions
Concurrent chemoradiation with daily low dose cisplatin is feasible and effective for selected patients with advanced HNSCC. Although the addition of accelerated RT resulted in more mucositis and tubefeeding, the introduction of prehydration led to better compliance to therapy with more chemotherapy administered and less hospital admissions.

INTRODUCTION

Concurrent radiotherapy (RT) and chemotherapy has led to important improvements in locoregional control and survival for patients with advanced stage head and neck squamous cell carcinoma (HNSCC) compared to treatment with radiation alone [1,2]. The largest effect on survival was observed with concurrent single agent cisplatin added to radiotherapy [3].

Several cisplatin-dose regimens are used in clinical practice. Cisplatin-schedules using three-weekly intravenous (IV) infusions are widely applied in primary chemoradiation [2], but chemoradiation with a daily low dose of cisplatin has also been seen studied [4-6]. An alternative to IV administration of chemotherapy is the intra-arterial (IA) approach [7]. From early 2000 to the end of 2004 all patients with advanced stage HNSCC were offered participation in a randomized trial on IA versus IV cisplatin with concomitant radiation. Main eligibility criteria of this trial included: squamous cell carcinoma of the oral cavity, oropharynx or hypopharynx, inoperable disease, TNM-stage T3-4, any N-status, M0, Karnofsky score >=60, age >=18, no prior chemotherapy or prior surgery or radiation therapy to the head or neck, creatinine clearance > 50 ml/min, no prior or concurrent malignancies except basal cell carcinoma of the skin or carcinoma in situ of the cervix and no prior cerebro-vascular accident.

Due to the abuse of nicotine and alcohol, the HNSCC patient-population is characterized by frequent co-morbidity (non-malignant cardio-vascular and/or pulmonary disease) [8], alcohol-abuse related psychosocial problems, and the occurrence of 2nd primary tumors [9]. Therefore, a significant proportion of patients may be ineligible for clinical trials. During the accrual period of the randomized trial, patients ineligible for or refusing the trial were treated with concurrent chemoradiation with daily low dose cisplatin (RADPLAT daily LD).

The purpose of this study was to evaluate prospectively a cohort of patients, which during the accrual of the randomized trial were treated with RADPLAT daily LD, either because of ineligibility for or refusal of the randomized trial.

MATERIAL AND METHODS

The total number of patients with advanced stage HNSCC who were treated with concurrent cisplatin-based chemoradiation between January 2000 and April 2005 was 390. Of these, 248 were treated within the protocol on intra-arterial and intravenous chemoradiation (RADPLAT trial). The remaining 142 patients were treated according to standard hospital practice with the RADPLAT daily LD protocol and were prospectively registered in a database. After exclusion of 21 patients, who received this treatment as a re-irradiation, 121 patients were left for this analysis.

Patient and tumor characteristics are given in table 1. The mean age of patients was 61 years (range 36-85). A synchronous tumor was present in 10 cases (8%): a second HNSCC in 7 patients,

NSCLC in 2 and trachea carcinoma in 1 patient. The reason for treatment with RADPLAT daily LD was refusal of the randomized RADPLAT trial in 21%, in 79% ineligibility for the trial. In these latter patients, ineligibility was mainly due to odd head and neck tumor site (20%), previous vascular history (cardiac ischemia, occlusive vascular disease, CVA) (19%), recurrent tumors (11%), synchronous tumors (8%), 2nd primary tumors (11%), poor mental and general condition (8%), unknown primary with extensive neck involvement (5%), and others (18%).

Table 1. Patient and tumor characteristics.

	Frequency (%)
Primary Tumor site	
oral cavity	18
oropharynx	41
hypopharynx	20
larynx	8
unknown primary	4
other	8
T-stage	
Tx (UPT)	4
T1	4
T2	8
T3	33
T4	44
recurrent primary	7
N-stage	
N0	26
N1	17
N2	39
N3	15
recurrence in neck	3
TNM stage	
Stage II	2
Stage III	17
Stage IV	73
recurrence	8
Gender	
Male	65
Female	35

Abbreviations: UPT, unknown primary tumor. FU, follow-up. Other primary tumor sites: cervical esophagus (3 cases), nasal cavity/sinus (4), auditory canal (1) and trachea (1). TNM stage is given according to the AJCC criteria [15].

Radiotherapy was usually given with 4-6 MV photon linear accelerator, with a dose of 46 Gy in 23 fractions to the primary tumor and the bilateral neck, followed by a boost to the primary tumor and lymph node metastases to a dose of 24 Gy in 12 fractions, resulting in a total dose of 70 Gy in 35 fractions. Treatment was given by a standard 3-field technique or by an IMRT-technique depending on resources. Cisplatin was given as a 1-2 min IV infusion at a dose of 6 mg/m^2 daily, for a total number of 20 doses, 1-2 hours prior to irradiation. Treatment was given on an outpatient-basis.

After the first 47 patients (June 2002) the original treatment protocol with the instruction to patients of an oral fluid intake of at least 1.5 liter was adapted (*Standard Group*), since we observed frequent premature cessation of cisplatin-administration due to toxicity. It was decided to include 1 liter of saline IV prehydration at each cisplatin-infusion. In addition, the overall treatment time (OTT) of radiotherapy was changed from 7 to 6 weeks, since the importance of acceleration of RT was recognized [10]. The 1-week shortening of OTT was accomplished by giving a second fraction of 2 Gy once weekly, 6-8 hours after the first one, during week 2 to 6. This adapted treatment was given to the subsequent 74 patients (*Hydr-Ac-RT Group*).

EVALUATION OF TREATMENT

Patients were followed during treatment twice weekly for adverse effects and/or complications using physical examination and laboratory testing. The worst acute toxicity was scored using CTC-criteria version 3.0. Therapy results were evaluated 6-8 weeks after the end of treatment by means of radiological investigations (MRI or CT scan and/or ultrasound) and/or examination under general anesthesia. For residual disease in the neck at the time of evaluation, salvage neck dissection was performed if judged operable.

STATISTICAL ANALYSIS

For quantitative comparison of numerical data, the Student's *t*-test was applied. Chi-squared and Fisher's exact test were used for analysis of categorical data. Locoregional control and survival data were calculated from the start of treatment using the Kaplan-Meier method. A Cox Univariate and Multivariate Proportional Hazard Regression analysis was done to establish factors that independently contributed to treatment-outcome. Two-sided P-values of <0.05 were considered statistically significant.

RESULTS

TREATMENT COMPLIANCE AND ACUTE TOXICITY

The mean follow up was 29 months (range 7 – 62) for patients alive at last follow-up. Main results of treatment compliance, number of cisplatin shots and acute toxicity are given in table 2 for the total patient group and the *Standard Group* and *Hydr-Ac-RT Group*. In 12% of all patients, hospital admission from the start of treatment was needed, due to reasons of co-morbidity. During treatment, 36% of all patients were admitted to the hospital because

of toxicity (e.g. dehydration, renal function impairment, and severe mucositis). Other toxicity and complications during treatment included bleeding from the external carotid artery (1 patient), unexplained lung bleeding (1 patient), and need for tracheotomy (6 patients).

Table 2. Treatment compliance, acute treatment related toxicity and treatment results according to the RADPLAT daily LD treatment protocol, for total group, Standard group and Hydr-Ac-RT group. See text for treatment details.

		Total group	Standard Group	Hydr-Ac-RT Group	P-value
		n=121	n=47	n=74	(Standard vs. Hydr-Ac-RT Group)
Mean follow-up (months)		29	49	20	p<0.001
RT completed (%)		94	96	93	p= n.s.
Mean OTT (days)		43	46	41	p<0.001
Max. no. of 20 shots (%)		78	59	91	p=0.008
Mean no. cisplatin shots		18.7	17.3	19.6	p<0.001
Mucositis	Grade 1-2 (%)	20	35	11	
	Grade 3 (%)	79	65	87	p=0.009
	Grade 4 (%)	1	0	1	
Tube feeding (%)		81	70	88	p=0.03
Hemoglobin	no - Grade 1 (%)	66	63	69	
	Grade 2 (%)	32	35	30	p= n.s.
	Grade 3 (%)	2	2	1	
WBC	no - Grade 1 (%)	44	39	47	
	Grade 2 (%)	24	28	21	
	Grade 3 (%)	23	22	24	p= n.s.
	Grade 4 (%)	9	11	7	
Platelets	no - Grade 1 (%)	88	82	91	
	Grade 2 (%)	6	9	5	p= n.s.
	Grade 3-4 (%)	6	9	4	
Renal function	no (%)	65	37	83	
	Grade 1 (%)	22	39	11	p<0.001
	Grade 2 (%)	12	22	6	
	Grade 3 (%)	1	2	0	
Hospital admission due to toxicity (%)		36	50	27	p=0.02
CR rate primary tumor		84	74	90	p=0.06
CR rate neck (%)		75	77	74	p= n.s.
LR control at 3 years (%)		55	51	57	p= n.s.
DFS at 3 years (%)		33	34	28	p= n.s.
OS at 3 years (%)		36	38	33	p= n.s.

Abbreviations: Hydr-Ac-RT, accelerated chemoradiotherapy with hydration. vs., versus. RT, radiotherapy. OTT, overall treatment time. Max, maximum. No., number. WBC, white blood cells. CR, complete remission. LR, loco-regional. DFS, disease free survival. OS, overall survival. n.s., not statistically significant.

Addition of hydration to cisplatin infusion and acceleration of RT improved compliance to treatment as illustrated in table 2: more chemotherapy could be administered (mean no. of cisplatin infusions increased from 17.3 to 19.6, $p<0.001$ and the maximum no. of 20 infusions increased from 59 to 91%, $p=0.008$), with less renal toxicity (Grade 2 incidence decreased from 24% to 6%, $p<0.001$) and less hospital admissions (frequency decreased from 50% to 27%, $p<0.02$). However, mucositis was more pronounced (increase in grade III mucositis from 65% to 87%, $p<0.02$) and tube feeding was more frequent (increase from 70% to 88%, $p=0.03$). Hematological toxicity was mild, and consisted of mainly leucopenia (Grade 3 in 23%, grade 4 in 9%), with no differences in hematological values between the 2 treatment protocols.

TREATMENT OUTCOME

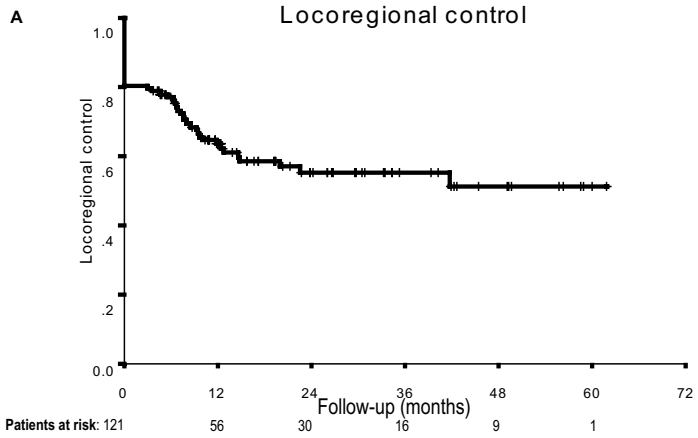
A complete response (CR) of the primary tumor was observed in 84% of cases. The initial CR-rate in the neck was 75%, resulting in an overall CR-rate of 69% (table 2). The CR-rate for the smaller synchronous 2nd primary tumor was 100% (10 out of 10 patients). Salvage surgery for residual disease was performed within 2 months in 18 of 34 patients (53%), who had a partial remission. The CR-rate, including salvage surgery was 85%. In the remaining 15% of patients only a partial remission was achieved.

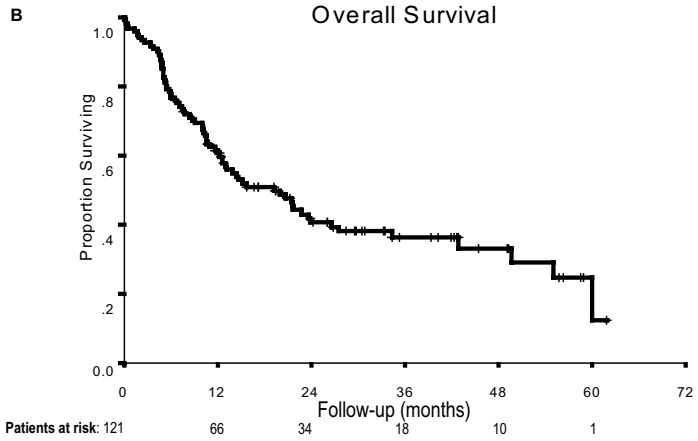
The loco-regional (LR) control rates for all 121 patients were 55% and 55% at 2 and 3 years, respectively (figure 1A). Disease free survival (DFS) was 36% and 33% at 2 and 3 years, respectively. Overall survival (OS) was 41% and 36% at 2 and 3 years, respectively (figure 1B).

In uni- and multivariate analysis, the following factors were tested: primary tumor site, degree of (co-) morbidity (using the ASA classification) [11], TNM-stage, whether or not patient was ineligible for the RADPLAT trial, age, gender, acceleration of RT, no. of cisplatin infusions (table 3). Significantly associated with improved DFS were: Hypopharynx/oropharynx site (compared to other sites, mainly oral cavity), less advanced TNM stage and lower ASA-comorbidity classification. Tumor site and TNM staging were significant factors for LR control in univariate analysis, but did not reach statistical significance in multivariate analysis. Although the CR rates in the Hydr-Ac-RT Group appeared to be improved compared to the Standard Group (90% vs 74%, $p=0.06$, see table 2), loco-regional control and survival were not statistically different for the 2 different treatment protocols (table 2).

At last follow-up, 34% of cases were alive with no evidence of disease, 6% were alive with disease, and 73 patients (60%) deceased. The cause of death was disease-related in 72% of cases (38% LR tumor, 27% distant metastases, both LR and distant in 7%). In the other 28% causes of death were related to treatment induced toxicity (n=2 patients: respiratory failure due to aspiration pneumonia and septicemia), 2nd primary tumor, or pulmonary non-malignant or cardiovascular disease.

Figure 1. Panel A represents locoregional control, panel B overall survival for all patients treated with concurrent chemoradiation with daily low dose cisplatin.





LATE TOXICITY

No differences in late toxicity were seen between the Standard treatment group and the Hydration-acceleration treatment group. Prolonged tubefeeding for at least part of the dietary intake was reported in 29% of cases with a mean duration of 8 months (range 4 -24 months). Fibrosis of subcutaneous tissue was documented in 9%. Multiple episodes of aspiration pneumonia were recorded in 4 patients, stenosing of the pharynx/esophagus (requiring dilatation) in 3. Osteoradionecreosis was observed in 2 patients, late renal toxicity in 1 patient.

DISCUSSION

In this series of 121 patients with advanced stage HNSCC, the chemoradiation protocol with daily administration of low dose cisplatin concurrently with radiotherapy (RADPLAT daily LD) appeared to be feasible and effective. The schedule was chosen as a standard treatment outside the context of the randomized RADPLAT IA trial based on the fact that IV cisplatin concurrently with radiation is acknowledged as standard therapy for advanced HNSCC [3]. The advantage of the RADPLAT daily LD schedule is that it can be given on an outpatient basis and that, in patients unfit for high dose cisplatin, adaptation or cessation of chemotherapy administration can be done early before irreversible toxicity may occur.

The majority of patients (79%) were included in this protocol because of ineligibility for the randomized trial and therefore it represents a negative selection. This negative selection bias is also reflected by the fact that 12% of patients were treated clinically during the whole course, although this regimen was designed as an outpatient treatment.

In addition, the mean age of our patients was 7 years older than in the RADPLAT phase II trial [7], also negatively influencing prognosis. The results of treatment appear comparable to results obtained in other series [4,5]. However, comparison of these series should be performed with care due to the negative selection of patients in this series.

In our series we demonstrated that prehydration improved compliance to the protocol: more chemotherapy could be administered, with less acute nefrotoxicity. This is in agreement with the routine use of forced hydration in high dose cisplatin-based chemotherapy [12]. However, in the daily low dose cisplatin schedules reported in literature prehydration is not incorporated [4,5]. In the current series, which obviously was not a controlled trial, acceleration of RT was not associated with improved LR control, although the response rates were higher in the group that was treated with accelerated RT, with a borderline significant p-value. Acute toxicity (need for tube feeding and mucositis) after accelerated RT was increased as previously reported [10]. These acute reactions however, were manageable in clinical practice and late toxicity was not altered.

In multivariate analysis, the ASA classification, which is a recording of co-morbidity, was a strong predictor for survival, not for LR control, as reported earlier in our series of intra-arterial chemoradiation [13]. Other prognostic factors that affected outcome included TNM stage and tumor site as reported previously [14]. The factor whether a patient was ineligible or refused the randomized trial was not associated with differences in LR control or DFS. This may be explained by the fact that ineligibility for the randomized trial in part may be due to negative prognostic factors (e.g. recurrence or extensive co-morbidity), but also due to factors that do not necessarily imply a worse prognosis (e.g. odd tumor sites, stage III disease based on early nodal disease).

In conclusion, the concurrent chemoradiation with daily low dose cisplatin for advanced head and neck cancer patients is feasible and effective. Although the addition of acceleration of RT resulted in more mucositis and tubefeeding, better compliance to

Table 3. Results of Cox Proportional Hazard Regression analysis for locoregional control and disease free survival.

Endpoint	Variable	UV analysis		MV analysis	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Locoregional control	Site (hypopharynx/oropharynx vs. other)	1.8 (1.0-3.2)	0.04	1.7(1.0-3.0)	0.06
	ASA score (1-3)	1.4 (0.8-2.2)	0.2	-	-
	TNM stage (I, II, III, IV/recurrence)	2.4 (1.0-5.8)	0.05	2.2(0.9-5.5)	0.08
	Reason protocol (ineligible/refusal)	1.6 (0.9-3.1)	0.1	-	-
	Age	1.0 (0.97-1.02)	0.7	-	-
	Gender (male/female)	0.8 (0.4-1.4)	0.4	-	-
	Accelerated RT (yes/no)	0.8 (0.5-1.5)	0.5	-	-
	No of courses cisplatin	0.96 (0.90-1.02)	0.2	-	-
Disease free survival	Site (hypopharynx/oropharynx vs. other)	1.8 (1.2-2.9)	0.007	1.7 (1.2-2.7)	0.03
	ASA score (1-3)	1.7 (1.2-2.5)	0.005	1.7 (1.2-2.5)	0.005
	TNM stage (I, II, III, IV/recurrence)	2.3 (1.2-4.5)	0.01	2.1 (1.0-4.5)	0.05
	Reason protocol (ineligible /refusal)	1.2 (0.7-1.2)	0.4	-	-
	Age	1.00 (0.98-1.02)	0.6	-	-
	Gender (male/female)	1.1 (0.7-1.7)	0.6	-	-
	Accelerated RT (yes/no)	0.9 (0.6-1.4)	0.6	-	-
	No of courses cisplatin	0.95 (0.90-1.00)	0.06	0.95 (0.90-1.01)	0.1

Note: The variable "Reason protocol (ineligible/refusal)" applies to the reason why the patient was treated with the RADPLAT daily LD protocol: Due to either ineligibility for or refusal of the randomized RADPLAT trial on intra-arterial versus intravenous chemoradiation.
Abbreviations: UV, univariate. MV, multivariate. CI, confidence interval. RT, radiotherapy. No, number.

therapy with more chemotherapy administered and less hospital admissions was achieved due to the addition of hydration. The locoregional control and survival rates are a reflection of the selected patient population. Comparison of the obtained results with other series on concurrent chemoradiation should be performed with care due to the negative selection of patients in this series.

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chapter 5

Primary tumor volume predicts locoregional control and survival after concurrent chemoradiation with daily low dose cisplatin for advanced stage head and neck carcinoma

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Submitted

ABSTRACT

Background and Purpose

To evaluate the prognostic value of primary tumor-volume in patients with advanced stage HNSCC treated with concurrent cisplatin-chemoradiation.

Material and methods

Forty-six patients were treated with radiotherapy and cisplatin (6 mg/m^2 i.v.x20, daily). Tumor sites were: oropharynx (n=33), oral cavity (n=10), hypopharynx (n=2), and larynx (n=1). Baseline primary tumor-volume was recorded from diagnostic MRI-scans. In univariate and multivariate analysis, the prognostic impact of primary tumor-volume and other prognostic factors for locoregional control and disease free survival was tested.

Results

Mean tumor-volume was 28 cm^3 (median 23 cm^3, range 3-112). Tumor-volume and T-stage were positively correlated: T3-tumors had a mean tumor-volume of 19 cm^3, whereas the volume of T4-tumors was 40 cm^3 (p=0.003). The LR control-rate at 3-years was 81% for patients with tumor-volumes <median compared to 48% for tumor-volumes >=median (p=0.036). At multivariate analysis, tumor volume remained a significant determinant of locoregional control and disease free survival when adjusted for other prognostic factors.

Conclusions

In advanced stage HNSCC treated with concurrent chemoradiation, primary tumor-volume is associated with LR control and DFS. Larger studies are needed to confirm whether incorporation of tumor volume in the staging system improves the prediction of treatment outcome and can serve as a tool to guide treatment options.

INTRODUCTION

For patients with advanced stage head and neck squamous cell carcinoma (HNSCC) the treatment of choice is concurrent cisplatin-based chemoradiation [1]. This treatment is effective, resulting in locoregional control rates of 50-70 % [2-4]. However, improvement of these results is still warranted, since salvage treatment of recurrences is often unsuccessful [5]. Compared to treatment with radiation alone, the toxicity of concurrent chemoradiation is substantial [2].

Given the limitations in cure rates and the increased toxicity after concurrent chemoradiation for advanced stage HNSCC, selection of patients who are likely to respond favorably to this therapy is increasingly important. Selection for treatment is usually based on TNM-staging criteria [6]. It appears that for the advanced stage HNSCC (Stage III–IV) treated with concurrent chemoradiation these criteria may not have enough discriminative properties, since it was shown recently that T-stage did not predict outcome in patients treated with intra-arterial chemoradiation [7,8]. Therefore, other factors that are able to predict treatment response are needed. One such factor might be the pre-treatment volume of the primary tumor, as derived from CT or MRI. In a number of studies on patients treated with radiation alone, tumor volume was associated with local control [9-11]. Similar associations have been found between tumor volume and locoregional control after primary surgery in supraglottic laryngeal carcinoma [12] and hypopharyngeal carcinoma [13], again an indication that tumor volume is of relevance in outcome after HNSCC.

The purpose of this study was to examine the prognostic value of pretreatment primary tumor volume in addition to other clinical factors in patients with advanced stage HNSCC treated with concurrent chemoradiation with daily low dose cisplatin (RADPLAT daily LD). This tumor-volume study represents a subgroup of patients from a larger series, on whom we have reported the clinical results recently [14]. The association between clinical factors (including tumor volume) and outcome measures (locoregional control and survival) were investigated.

MATERIAL AND METHODS

Between 2000 and 2005, 142 patients with HNSCC were treated with the RADPLAT daily LD protocol and were prospectively registered in a database. Of these, 21 patients received this treatment as a re-irradiation and were excluded from this analysis. Of the remaining 121 patients, those with primary tumors in the oral cavity, oropharynx, hypopharynx or larynx and who had a pre-treatment MRI-scan available were selected. The reason for exclusion of patients with diagnostic CT-scans was that volumes derived from CT and MRI are not comparable, since the volumes from CT are generally larger than those generated from MRI [15]. The standard hospital policy on diagnostic radiological evaluation was to perform MRI-scan in patients with disease located cranially from the hyoid bone (typically

in the oral cavity or oropharynx) and CT-scan in case of disease caudally from the hyoid (in the larynx or hypopharynx). Ten patients undergoing this treatment because of recurrent disease (locally or regionally) were also excluded. In total, 46 patients with previously untreated primary advanced HNSCC were left for the current analysis.

Patient and tumor characteristics are given in table 1. The mean age of patients was 61 years (range 40 - 85). The treatment of RADPLAT daily LD consisted of radiotherapy, usually given with 4-6 MV photon linear accelerators. Target volume included the primary tumor and the bilateral neck to a dose of 46 Gy in 23 fractions. A boost was given to the macroscopic tumor extensions at the primary tumor site and lymph node metastases to a dose of 24 Gy in 12 fractions, resulting in a total dose of 70 Gy in 35 fractions. Treatment was given by a standard 3-field technique (2 opposing laterals for the upper neck region, with an adjacent supraclavicular field for the lower neck) or by CT-planning (using an intensity modulated radiotherapy technique) depending on resources. Cisplatin was given as a 1-2 min IV infusion at a dose of 6 mg/m^2 daily, for a total number of 20 doses, 1-2 hours prior to irradiation. Treatment was given on an outpatient-basis. In June 2002, the original treatment protocol with the instruction to patients of an oral fluid intake of at least 1.5 liter was adapted, since we observed frequent premature cessation of cisplatin-administration due to toxicity. Sixteen patients were treated within this original protocol. It was decided to include 1 liter of saline IV prehydration at each cisplatin infusion. In addition, the overall treatment time (OTT) of radiotherapy was changed from 7 to 6 weeks, since the importance of acceleration of RT was recognized [16]. The 1 week shortening of OTT was accomplished by giving a second fraction of 2 Gy once weekly, 6-8 hours after the first one, during week 2 to 6. This adapted treatment was given to the subsequent 30 patients.

Primary tumor volume assessment was performed by delineation of the Gross Target Volume (GTV), which included all visible tumor on pretreatment MRI scans. MRI scans were performed on a 1.5-Tesla scanner (Siemens Magnetom 63 SP4000; Siemens, Erlangen, Germany). Slice thickness was <=4 mm, with an inter-slice gap of <=1 mm. The field of view for the axial views was 16-18 cm for T1-weighted sequences and 18-20 cm for T2-weighted sequences. T1-weighted images were obtained before and after injection of intravenous gadolinium. Post-contrast images were acquired using fat saturation. An experienced head and neck radiologist (F.A.P.), who was blinded to the patients' outcome, performed the primary tumor volume delineations, using multiple planes for optimal assessment of tumor extension. All images were transferred to our in-house developed workstation (Worldmatch Workstation) in DICOM format [17] for 3-D computation of tumor volume. No volume measurement of lymph nodes was performed.

Table 1. Patient, tumor and treatment characteristics.

		Frequency (%)
Gender		
	Male	34 (74%)
	Female	12 (26%)
ASA comorbidity classification		
	1	10 (23%)
	2	23 (54%)
	3	10 (23%)
	missing	3
Pre-treatment weight loss		
	< 10%	31 (67%)
	>= 10%	15 (33%)
Primary Tumor site		
	oral cavity	10 (22%)
	oropharynx	33 (72%)
	hypopharynx	2 (4%)
	larynx	1 (2%)
T-stage		
	T1	2 (4%)
	T2	2 (4%)
	T3	20 (44%)
	T4	22 (48%)
N-stage		
	N0	11 (24%)
	N1	8 (17%)
	N2	18 (39%)
	N3	9 (20%)
TNM stage		
	Stage III	7 (15%)
	Stage IV	39 (85%)
Neck level involvement		
	No, level I-II-III	39 (85%)
	Level IV	7 (15%)
No. of cisplatin infusions		
	Mean (range)	18.9 (8 - 20)
Accelerated RT		
	no	16 (35%)
	yes	30 (65%)

Abbreviations: No., number. RT, radiotherapy.
ASA-classification according to Wolters et al. [18], TNM stage according to the AJCC criteria [6].

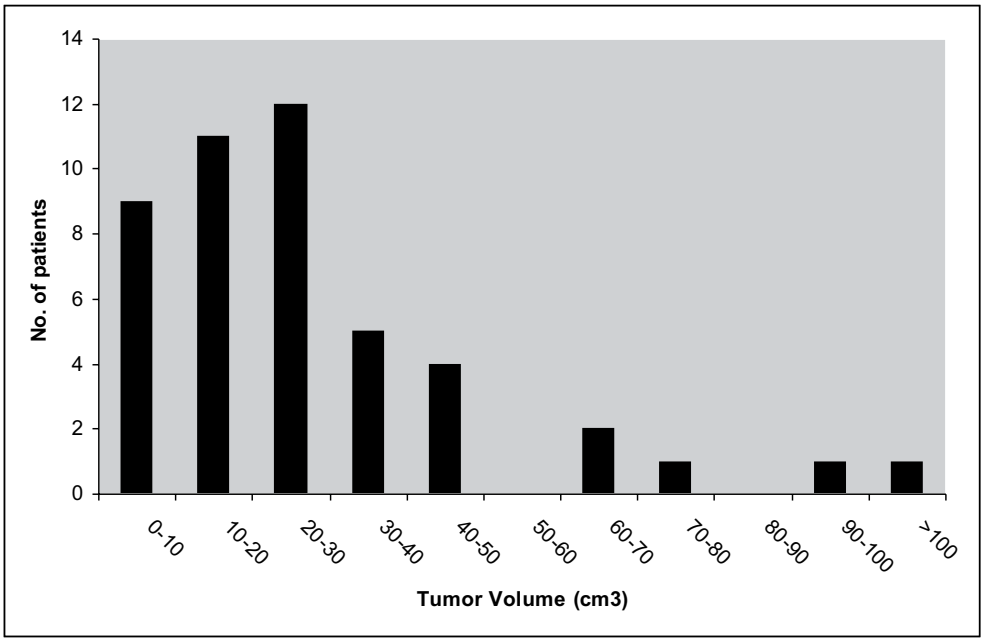
EVALUATION OF TREATMENT

The results of therapy were evaluated 6-8 weeks after the end of treatment by means of radiological investigations (by MRI or CT scan and/or ultrasound) and/or examination under general anesthesia, with biopsies taken in case of suspicious findings. For residual disease in the neck at the time of evaluation, salvage neck dissection was performed if judged operable.

STATISTICAL ANALYSIS

For quantitative comparison of numerical data, the Student's *t*-test and Wilcoxon Mann Whitney test were applied. Chi-squared and Fisher's exact test were used for analysis of categorical data. For analysis of Locoregional (LR) control, subjects were at risk from start of treatment until LR recurrence (event) or death (censored), whichever came first. For analysis of disease free survival (DFS), subjects were at risk from start of treatment until LR recurrence (event), distant metastases (event) or death (event), whichever came first. For overall survival (OS) analysis, subjects were at risk from start of treatment until death (event). Kaplan-Meier plots and Log Rank testing were used to describe survival. A Cox Univariate Proportional Hazard Regression analysis was done to establish factors that were associated with LR control and survival. To evaluate whether tumor volume was a significant predictor for LR control and DFS when corrected for other factors, a multivariate analysis was performed.

Figure 1. Distribution of primary tumor volumes in cm^3 of all 46 patients, treated with concurrent chemoradiation with daily low dose cisplatin.



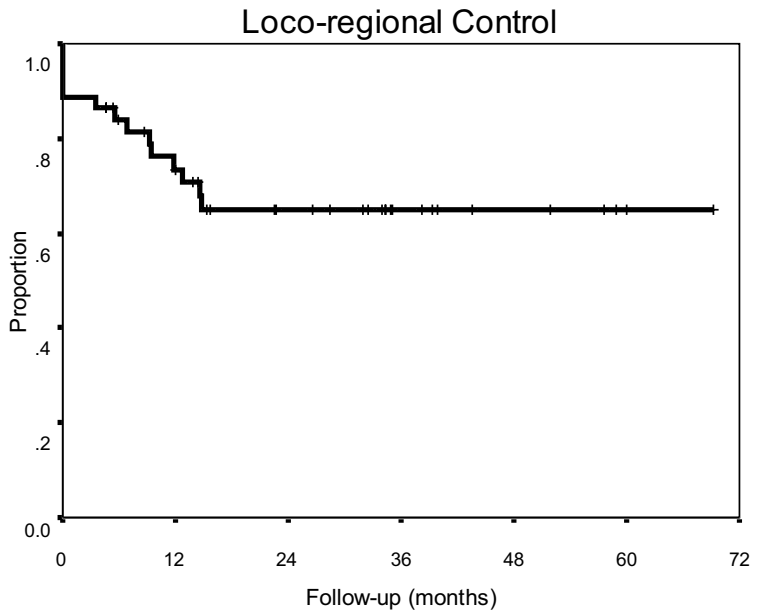
Because of the small population, we did not include all univariately significant factors in one model but added them, one at a time, to a model with tumor volume and focused on the (change in) hazard ratios, rather than p-values. A small (<=10%) change in the hazard ratio for tumor volume from univariate to multivariate analysis would indicate in independent effect of tumor volume. Patient- , tumor- and treatment-related factors included: gender, age, pretreatment weight loss (percentage of body weight), comorbidity according to the ASA-classification [18], tumor site, T-classification, N-classification, primary tumor-volume, and neck-level involvement. The treatment-related factors included the number of cisplatin-infusions and fractionation-schedule (standard vs. acceleration). Two-sided P-values of <=0.05 were considered statistically significant.

RESULTS

PRIMARY TUMOR VOLUME

The mean tumor volume for all patients was 28 cm^3 (median 23, range of 3 – 112 cm^3). See figure 1 for tumor volume distribution. The mean tumor volume by site was: oral cavity (n= 10) 41 cm^3, oropharynx (n = 33) 24 cm^3, hypopharynx (n= 2) 32 cm^3, larynx (n=1) 25 cm^3. The volume difference between oral cavity and oropharynx carcinoma was of borderline significance (Wilcoxon Mann Whitney test, p=0.079). T3-tumors had a mean tumor volume of 19 cm^3, and T4-tumors a mean of 40 cm^3 (Wilcoxon Mann Whitney test, p=0.002). No correlation was observed between tumor volume and N-stage.

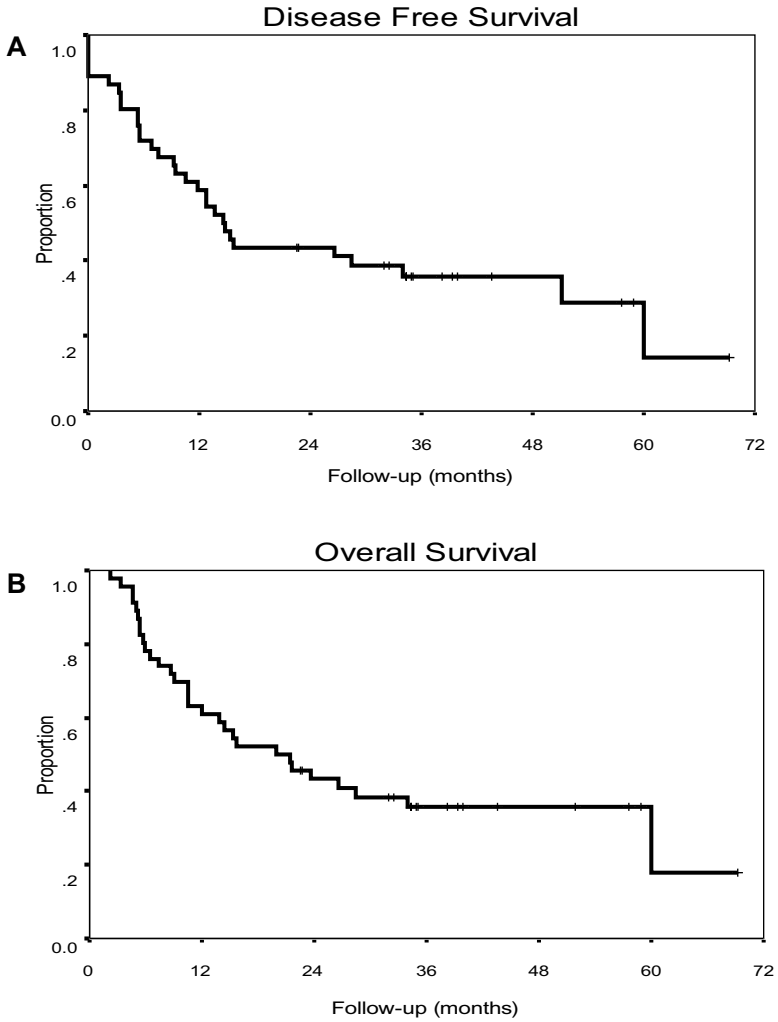
Figure 2. Locoregional control for all 46 patients, treated with concurrent chemoradiation with daily low dose cisplatin.



TREATMENT OUTCOME

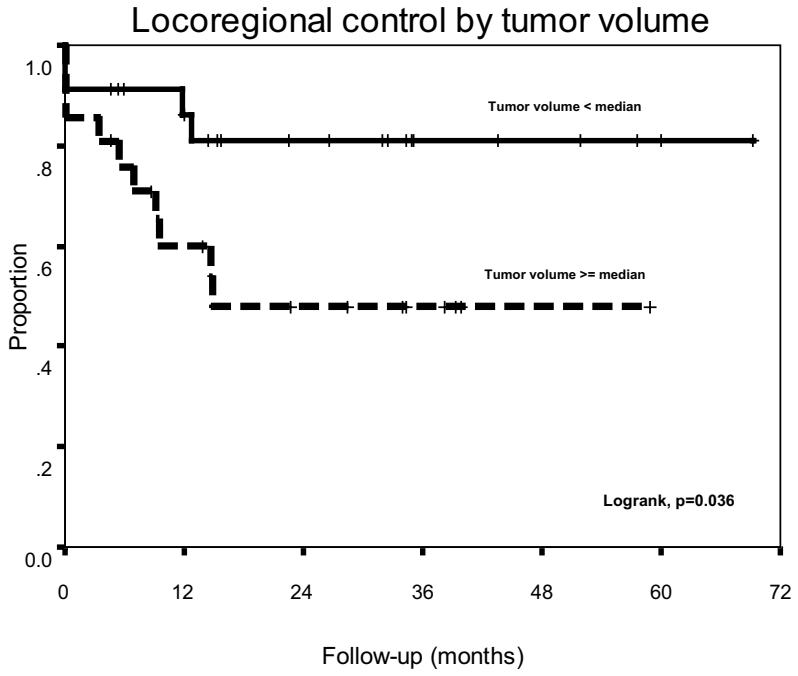
All patients completed the treatment. The mean follow-up of patients alive at last follow-up was 40 months (range 23 – 69). At the time of treatment evaluation (6-8 weeks after the end of therapy) a complete response (CR) of the primary tumor was observed in 89% of cases. The initial CR rate in the neck was 68%, resulting in an overall CR rate of 66%. Salvage surgery for residual disease was performed within 2 months in 9 of 15 patients (60%), who had a partial remission. The CR rate, including salvage surgery was 86%. In the remaining 14% of patients only a partial remission was achieved.

Figure 3. Disease free (Panel A) and overall survival (Panel B) for all 46 patients, treated with concurrent chemoradiation with daily low dose cisplatin.



The LR control rates were 65% at 2 and 3 years (figure 2). DFS was 43% and 36% at 2 and 3 years, respectively (figure 3A). OS was 43% and 36% at 2 and 3 years respectively (figure 3B). Tumor volume was associated with LR control: 3 year rates of LR control were 81% and 48% for tumor volume < 23 cm^3 (median) and tumor volume >= 23 cm^3, respectively (Logrank, p=0.036, figure 4). Oropharyngeal tumors (mean volume 24 cm^3) had significantly better 3 year LR control rates compared to oral cavity tumors (mean volume 41 cm^3): 75% vs. 44% (Logrank, p=0.013). T3-tumors (mean volume 19 cm^3) had significantly better 3-year LR control rates compared to T4-tumors (mean volume 40 cm^3): 78% vs. 44% (Logrank, p=0.033).

Figure 4. Locoregional control by primary tumor volume. Tumors were classified as smaller than (solid line) or equal to/larger than (broken line) median (i.e. 23 cm^3). The difference in locoregional control between the 2 groups was statistically significant (p=0.036).



The results of the univariate Cox regression for the endpoints locoregional control and disease free survival are given in table 2. Oral cavity tumors (as compared to other sites, mostly oropharynx), advanced T-stage, worse ASA-score and larger tumor volumes were significantly associated with worse locoregional control. For each 10 cm^3 increase in tumor volume, the relative risk of locoregional failure increases with about 30% (i.e. a 3% relative decrease in locoregional control per cm^3). Tumor site and volume were also significantly associated with DFS. In the multivariate analysis (table 3), we tested whether tumor volume remained a predictive factor when adjusted for other predictive factors

(as mentioned in the Material and Methods section, the MV analysis was limited due to the small patient group). For this, we included tumor volume and one other factor at a time and repeated this for the 4 significant factors from the UV analysis. It appeared that the hazard ratio for tumor volume from univariate analysis (hazard ratio 1.29) changed only a little when other factors were introduced in the model (table 3). The change in hazard ratio ranged from 1 to 8%, indicating that the effect of tumor volume in univariate analysis remained in multivariate analysis. The same analysis was performed for DFS and similar results were obtained (table 3), with only little changes (0-3 %) in hazard ratio for tumor volume from univariate to multivariate analysis, again indicative of an independent effect of tumor volume on DFS.

Table 2. Results of Cox Univariate Hazard Regression analysis for locoregional control and disease free survival. For each tested dichotomous variable, the former category is the reference category.

Variable	UV analysis HR (95% CI)	p-value	UV analysis HR (95% CI)	p-value
	Locoregional control		*Disease Free Survival*	
Site (other vs oral cavity)	3.2 (1.0 – 9.5)	0.04	2.2 (1.0 – 4.8)	0.05
T-stage (T1-3 vs T4)	4.0 (1.2 – 12.8)	0.02	2.0 (0.9 – 4.0)	0.07
N-stage (N2+ vs N0-1)	1.4 (0.5 – 3.9)	0.6	0.9 (0.4 – 1.9)	0.8
Tumor volume (per 10 cm3)	1.3 (1.1 – 1.5)	0.005	1.2 (1.0 – 1.4)	0.01
Level IV involvement (no vs yes)	3.1 (1.0 – 10.0)	0.06	1.6 (0.6 – 4.1)	0.4
ASA score (1-3, continuous)	2.1 (0.9 – 6.4)	0.02	1.4 (0.8 – 2.5)	0.2
Weight loss > 10 % (no vs yes)	2.2 (0.8 – 5.1)	0.1	1.8 (0.8 – 3.7)	0.1
Age (<61y vs > 61y)	1.5 (0.5 – 4.3)	0.5	1.1 (0.5 – 2.2)	0.8
Gender (male vs female)	0.2 (0.0 – 1.5)	0.1	0.6 (0.2 – 1.4)	0.2
Accelerated RT (yes vs no)	2.2 (0.8 – 6.2)	0.2	1.4 (0.7 – 3.0)	0.4
Completed 20 cisplatin infusions (yes vs no)	0.6 (0.1 – 4.9)	0.7	1.5 (0.6 – 3.8)	0.4

Abbreviations: UV, univariate. MV, multivariate. HR, hazard ratio. CI, confidence interval. RT, radiotherapy. No., number.
ASA-classification according to Wolters et al. [18]

At last follow-up, 35% of cases were alive with no evidence of disease and 65% of patients were deceased. The cause of death was disease-related in 76% of cases (35% LR tumor, 38% distant metastases, both LR and distant in 3%). In the remaining 24% of patients, the cause of death was cardio-vascular disease (7%), pulmonary disease (7%), second primary tumors (7%) and others (3%).

DISCUSSION

In this study, the prognostic value of primary tumor volume on treatment outcome was demonstrated in patients with advanced stage HNSCC after intravenous concurrent chemoradiation. Larger tumor volume was associated with worse locoregional control and disease free survival. In the limited multivariate analysis, tumor volume remained a prognostic factor for both endpoints.

In general, concurrent chemoradiation is indicated in patients with advanced stage HNSCC. This is mainly based on advanced TNM-staging, i.e. T3-T4 stage. Although T-stage is an important prognostic factor for locoregional control across the range of T-stages from T1 to T4 treated with radiotherapy [19], it appears that for advanced stages (T3-4) treated with concurrent chemoradiation the TNM-staging criteria may not have enough discriminative properties. This was illustrated recently in two series on targeted intra-arterial chemoradiation by the finding that T-stage did not predict outcome [7,8]. In both studies, primary tumor volume was an independent factor contributing to locoregional control. The increase in relative risk of local failure per cm^3 tumor volume in our previous study [7] on intra-arterial chemoradiation was comparable (2.6%) to the relative increase in the present study. Therefore, primary tumor volume may be used as a more appropriate factor to predict outcome after concurrent chemoradiation.

Table 3. Results of Multivariate analysis for locoregional control and disease free survival. The analysis was performed with the strongest factor in univariate analysis (tumor volume) and 1 other factor at a time, derived from the univariate analysis.

Variable	HR	p-value	HR	p-value
	Locoregional control		*Disease Free Survival*	
Tumor volume	1.19	0.08	1.16	0.06
T-stage	2.84	0.11	1.47	0.35
Tumor volume	1.3	0.007	1.19	0.019
Site	3.07	0.052	2	0.09
Tumor volume	1.31	0.004	1.19	0.016
ASA-score	2.01	0.1	1.59	0.13
Tumor volume	1.23	0.04	1.2	0.02
Level IV involvement	1.86	0.38	1.06	0.92

Abbreviation: HR, hazard ratio.
ASA-classification according to Wolters et al. [18]

To our knowledge, the current series is one of the first to establish a significant role for primary tumor volume in predicting outcome after intravenous chemoradiation for advanced stage HNSCC. Chufal et al. [20] also reported on the impact of tumor volume on outcome after chemoradiation, although they did not use full 3-D reconstruction for calculation of tumor volume, but estimated volume based on the largest diameter in 3 dimensions. For analysis, they used the total tumor volume as the sum of primary and nodal tumor volume. In their multivariate analysis, tumor volume was associated with survival but not locoregional control.

In studies on treatment with definitive RT alone, primary tumor volume was evaluated in several series in different tumor sites. In glottic and supraglottic laryngeal cancer, tumor volume was strongly associated with local control [10,21-25]. Tumor volume also was an independent factor for local control in hypopharyngeal carcinoma [11,26]. In oropharyngeal carcinoma, the available literature shows conflicting results. For T1-T4 tonsillar carcinoma, tumor volume was associated with local control in univariate analysis, but lost its predictive value to T-stage after multivariate analysis [9]. This was confirmed by Nathu et al. [27] and by Mendenhall et al. [28], who demonstrated that T-stage was more important than tumor volume in series on all oropharyngeal carcinoma subsites. Chao et al. reported on a series of mostly advanced stage oropharyngeal carcinoma from different subsites, treated with IMRT and demonstrated that tumor volume was associated with outcome [29]. However, in their series patients were treated with definitive RT, with or without concurrent chemotherapy. Data for the role of tumor volume in oral cavity tumors are lacking, since they are usually not treated with radiotherapy alone.

So in summary, it appears that tumor volume is a significant factor associated with outcome after radiotherapy in laryngeal and hypopharyngeal carcinoma. For oropharyngeal carcinoma, it appears that the effect of tumor volume is weak if all T-stages are considered, probably due to the fact that within the early T-stages the exact calculation of tumor diameter (which is related to volume) is taken into account. In the T3-T4 category, staging is not based on size/volume criteria but on less accurate criteria like invasion of adjacent structures. The latter may have prognostic impact for surgical treatment, but this may not be the case for (chemo-) radiotherapy, since from a radiobiological point of view deep invasion may not to be associated with poorer outcome.

In our series on concurrent chemoradiation, larger tumor volume was associated with worse locoregional control, when the subsets of patients with oropharyngeal carcinoma and oral cavity carcinoma were analyzed separately. However, this did not reach statistical significance, possibly due to the limited number of patients within each group (data not shown).

In most series reported so far [9-11,21-29], tumor volume was based on calculation performed on CT-scans. In our series reported here and others [7,24,30], MRI scans were used to determine tumor volumes. From comparative studies it is known that volumes derived from MRI are significantly smaller than those delineated on CT-scans [15,31,32]. This finding and

the fact that many studies used different cut-off points to define larger and smaller tumors, limit the interpretation possibilities of these studies for decision making in clinical practice.
The locoregional control rates in this study are comparable to those reported in other series of concurrent chemoradiation with daily low dose, including our recently reported data [3,14,33].
In conclusion, in advanced stage HNSCC treated with concurrent chemoradiation, primary tumor volume is significantly associated with locoregional control and disease free survival. In this limited consecutive series of patients the prognostic value of primary tumor volume remained in multivariate analysis. Larger studies using uniform volume measurements are needed to establish tumor volume as a predictive factor in the staging system and to investigate whether it could serve as a tool to guide treatment options.

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chapter 6

Phase 1 study to identify tumor hypoxia in patients with head and neck cancer using ^{99m}Tc-BRU 59-21

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ABSTRACT

The aim of this study was to assess the safety and biodistribution of ^{99m}Tc-BRU 59-21, a novel radioactively labeled 2-nitro-imidazole hypoxic marker, in head and neck cancer patients and to correlate uptake with pimonidazole staining.

^{99m}Tc-BRU 59-21 was administered intravenously (mean dose 824 MBq, range 780-857 MBq) to ten head and neck cancer patients scheduled for primary surgery, and whole-body images and SPET scans were then obtained. Uptake of radioactivity in the regions of interest was determined and tumor to normal tissue ratios were calculated after correlative evaluation with MRI/CT. Twelve to 16 h before surgery (up to 2 weeks after the scan), patients received pimonidazole intravenously. Tumor sections were stained immunohistochemically for pimonidazole binding.

No serious adverse events were reported. In five patients there were ten adverse events, which were mild in intensity and resolved completely without intervention. Uptake of ^{99m}Tc-BRU 59-21 was observed in eight of the ten primary tumors. Tumor to normal tissue ratios on the SPET scans for primary tumor and lymph nodes increased from 1.8 (range 0.9-2.7) to 2.1 (range 0.8-3.7) between 30 min and 3 h post injection. Tumor to normal tissue ratios in the primary tumor were significantly correlated with pimonidazole staining for SPET scans performed 30 min and 3 h post injection ($p=0.016$ and $p=0.037$, respectively). When primary tumor and involved lymph nodes were considered in conjunction, correlation between the tumor to normal tissue ratio and pimonidazole staining was observed for early ($p<0.001$) but not for late SPET scans ($p=0.076$). However, late scans showed better tumor delineation than early scans. Administration of ^{99m}Tc-BRU 59-21 in head and neck cancer patients appears to be safe and feasible. Uptake and retention in tumor tissue was observed, suggestive of tumor hypoxia, and this was supported by correlations with staining for the hypoxic marker pimonidazole.

INTRODUCTION

Tumor hypoxia is an important negative prognostic factor for outcome after treatment of malignant tumors by both radiotherapy and surgery [1-5]. If a reliable and easily applicable method to measure tumor hypoxia before treatment were available, patients could be selected for additional treatment aimed at modifying or exploiting hypoxia.

Tumor oxygenation is frequently measured with an Eppendorf electrode. This is an invasive procedure, limiting its application in clinical practice. Measuring tumor hypoxia by non-invasive methods includes the use of bioreductive markers, such as the 2-nitro-imidazole derivatives. Clinically approved hypoxic markers include pimonidazole [6] and EF5 [7] and can be visualized by immunohistochemical staining in tissue sections. In a previous study, we have shown that pimonidazole staining correlates significantly with diffusion-limited hypoxia [8].

^{99m}Tc-BRU 59-21, a novel 2-nitro-imidazole agent, has been shown to selectively localize in tissues with a low oxygen concentration due to ischemia [9,10], in tumor cells incubated under hypoxic conditions and following intravenous injection in animal models representative of poorly perfused tumors [11]. Preliminary results from a phase I study indicated that ^{99m}Tc-BRU 59-21 has an acceptable safety profile in healthy volunteers [unpublished data from Bracco Research]. Given the selective uptake of ^{99m}Tc-BRU 59-21 in hypoxic tissues and the non-invasive method of assessment by external nuclear scanning, ^{99m}Tc-BRU 59-21 could be an attractive agent for determination of the oxygen status of malignant tumors in vivo. The objective of this phase I study was therefore to assess the safety and biodistribution of ^{99m}Tc-BRU 59-21 in head and neck cancer patients and to correlate uptake of the agent in vivo with staining by pimonidazole, a recognized hypoxic marker.

MATERIALS AND METHODS

This was a single-centre phase I study, approved by the ethics committee of the Netherlands Cancer Institute. It involved ten patients with primary squamous cell carcinoma of the head and neck, classified as stage T2-4NxM0 according to the TNM system [12] and scheduled for primary surgery. After written informed consent had been obtained, a single intravenous (IV) injection of ^{99m}Tc-BRU 59-21 was administered and imaging was performed in order to study the biodistribution of the agent. Safety was monitored by pre- and post-dose evaluation by means of medical history, clinical examination, laboratory evaluations and ECG. Before surgery (performed within 2 weeks after the scans), pimonidazole was administered by i.v. infusion. After resection, tumor sections were made for immunohistochemical staining. Three female patients and seven males were included. The mean age of patients at the time of scintigraphy was 63 years (range 51-78). Further patient data are given in Table 1.

Table 1. Summary of patient data on tumors and treatment.

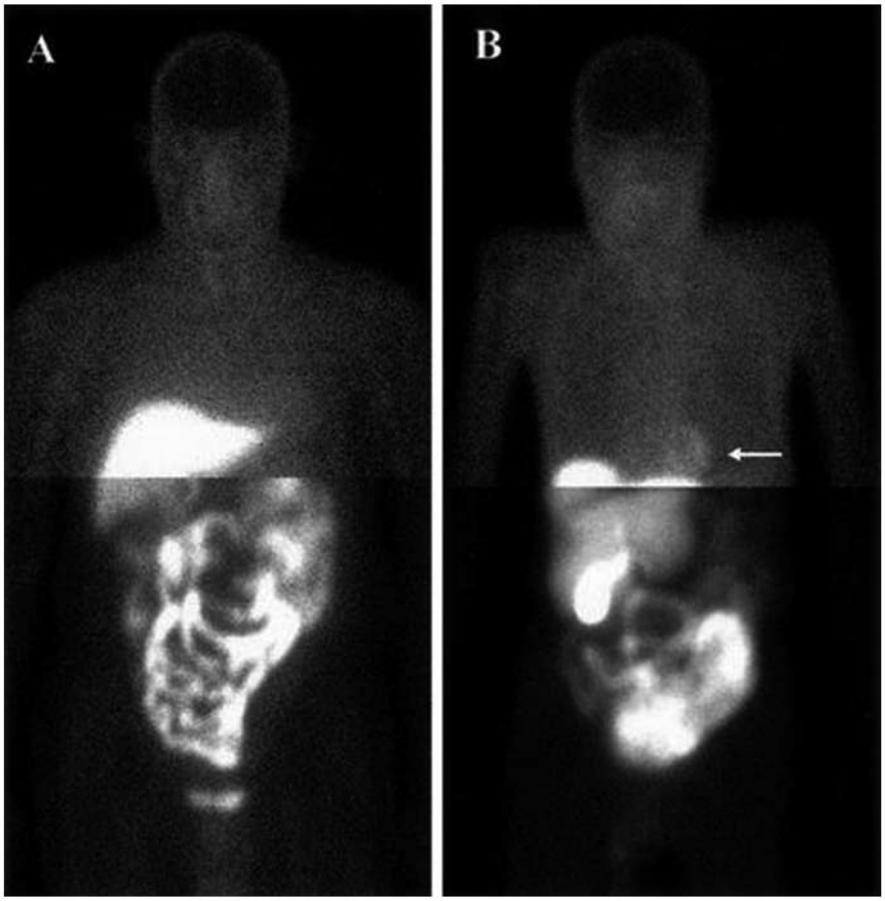
Patient	Tumor site	Clinical TNM stage	Surgery	Pathological TNM stage	Postoperative radiotherapy
101	Left mobile tongue	T2N0	Partial glossectomy	T2N0	No
102	Left mobile tongue	T2N2c	Partial glossectomy and bilateral neck dissection	T2N2c	Refused
103	Larynx	T4N0	Laryngectomy	T4N1	Yes
104	Left tonsillar region	T3N1	Commando procedure	T3N2b	Yes
106	Right retromolar region	T3N0	Commando procedure	T2N1	Yes
107	Right mobile tongue	T2-3N0	Partial glossectomy and supra-omohyoidal neck dissection	T2N2b	Yes
108	Left base of tongue	T2N1	Commando procedure	T2N2b	Yes
109	Left mobile tongue	T2N0	Partial glossectomy and supra-omohyoidal neck dissection	T2N0	No
110	Left base of tongue	T3-4N0	Commando procedure	T3N0	Yes
112	Left floor of mouth and tongue	T3N0	Commando procedure	T2N0	Yes

The ^{99m}Tc-BRU 59-21 used in this study was prepared using a kit containing two vials [13]. Reconstitution of vial A, containing 2 mg BRU59-21, the 2-nitro-imidazole derivative, was performed by adding ^{99m}Tc-pertechnetate solution and saline. Vial B, containing pentetate pentasodium and stannous chloride, was reconstituted with saline to form stannous pentetate solution. The stannous pentetate solution of vial B was added to vial A to form ^{99m}Tc BRU 59-21. Before administration, the radiochemical purity (RCP) of the agent was calculated using a combined high-performance liquid chromatography (HPLC) and paper-liquid chromatography method for each patient. The RCP obtained in all ten injected patients had a mean value of 93.5% (range 90.5%-95.5%), well above the required 90% level. A mean dose of 824 MBq (range 780-857 MBq) was administered IV.

Planar and single-photon emission tomography (SPET) images were obtained over a 3- to 4-h period following intravenous injection. At 30 min and 3-3.5 h post-injection, SPET images were obtained. Whole-body planar images were obtained at 1, 2 and 3 h post injection.

The presence of localized ^{99m}Tc-BRU 59-21 radioactivity on the dynamic, SPET and static nuclear images was determined by visual analysis and analysis of regions of interest (ROI's) on the SPET images. ROI's included the primary tumor, clinically involved lymph nodes (>1 cm) and normal tissue (deep neck musculature with low-intensity uptake). For each ROI, the number of pixels, the number of counts and the counts/pixel were calculated. The data were expressed as counts/pixel in the ROI, and as the ratio between counts/pixel in tumor and normal tissue.

Figure 1. Anterior ^{99m}Tc-BRU 59-21 planar images displayed with dual intensity showing a normal distribution of the tracer (**A**) and abnormal myocardial uptake (*arrow* on **B**).



Twelve to 16 h before surgery, 0.5 mg/m^2 pimonidazole (Hypoxyprobe-1; Natural Pharmacia International, Belmont USA) was administered by i.v. infusion. After surgery, sections from primary tumor and/or lymph node metastases were obtained from the Department of Pathology and stained with a biotinylated antibody against pimonidazole (kindly supplied by Dr. J.A. Raleigh, University of North Carolina, Chapel Hill, USA), according to our previously reported protocol [8]. Image analysis was done on digital microscopic images using image analysis software (IPLab 2.5 and SCIL-Image); pimonidazole-positive areas were measured by thresholding on the pimonidazole stain in the total tissue section.

To test for correlations between the uptake of ^{99m}Tc-BRU 59-21 in the tumor and pimonidazole staining on tumor sections, linear regression analysis was performed.

RESULTS

No serious adverse events were reported during the study period. In five of the ten patients a total of ten adverse events were reported. Side-effects were of mild intensity, and patients recovered completely without medical intervention. Effects included: pain at the primary tumor site and pain in the ear, dizziness, metal taste, dry mouth, erythema on the wrist and headache, all of which occurred after injection. The other three adverse events occurred in one patient and included an increase in white blood count and creatinine and a decrease in hemoglobin. On re-testing, laboratory results improved and the patient recovered without any sequelae. Four of these adverse events were assessed as possibly causally related, two as being of unknown cause and four as unrelated.

After injection of ^{99m}Tc-BRU 59-21, whole-body images showed activity in the liver, gallbladder, intestines and urinary bladder. Unexpected cardiac uptake was seen in four patients, presumably due to subclinical cardiac disease (Fig. 1). Early SPET showed activity in the salivary glands, in the anterior part of the oral cavity (probably saliva), at the site of the soft palate and in the thyroid.

Figure 2. A CT scan showing a T4 laryngeal carcinoma (*arrow*). Early (**B**) and late (**C**) transaxial ^{99m}Tc-BRU 59-21 SPET images show intense uptake at the tumor site.

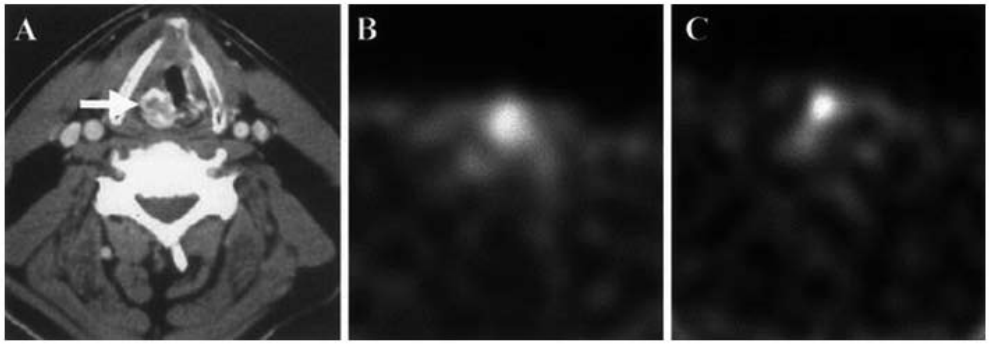


Figure 3. A Coronal MRI STIR sequence showing a T3 oropharyngeal carcinoma (*arrow*). **B** Early coronal ^{99m}Tc-BRU 59-21 SPET showing slightly increased uptake at the tumor site. By contrast, late coronal ^{99m}Tc-BRU 59-21 SPET (**C**) shows more intense tumor uptake.

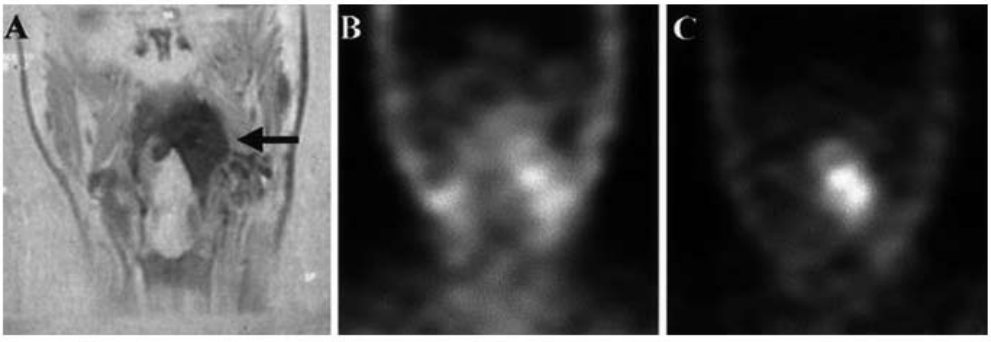
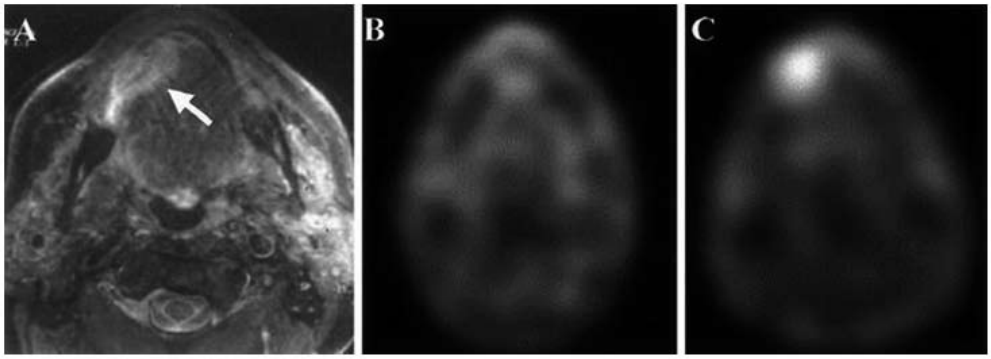


Figure 4. A Transaxial MRI showing a T2 tumor in the right side of the tongue (*arrow*). Tumor uptake of ^{99m}Tc-BRU 59-21, not observed on early transaxial SPET (**B**), is intense on late SPET (**C**).



In three patients, the nuclear scan was visually assessed as negative at the primary tumor site. However, in one of those patients (no. 102) the tumor to normal tissue ratio was greater than 1. In the remaining seven patients, uptake at the primary site was observed (Figs. 2, 3, 4). In all patients, tumor was better delineated on the late SPET scans. On static whole-body images, the primary tumor could not be identified in any of the patients.

In three patients, four clinically involved lymph nodes were present. Only two of these showed increased uptake on the SPET scan. In one patient, a 2-cm neck node showed no increased uptake, while the other patient had a 1-cm neck node only, which was too small for detection by SPET. The tumor to normal tissue ratios for primary tumor and lymph nodes on the SPET scans increased from 1.8 (range 0.9-2.7) for the early scan to 2.1 (range 0.8-3.7) for the late scan (Table 2).

No adverse events were reported after pimonidazole infusion. Pimonidazole positive staining in the histological tumor sections was observed in all ten primary tumors and seven tumor-positive lymph nodes (Table 2).

Correlations between SPET scan data and the pimonidazole-positive fraction are presented graphically in Fig. 5. For the early (30 min) SPET scans, considering either primary tumor alone or primary tumor in conjunction with lymph nodes, there were statistically significant correlations between the tumor to normal tissue ratio for counts/pixel and the pimonidazole fraction (upper 2 panels, with $r=0.73$, $p=0.016$ and $r=0.86$, $p<0.001$, respectively). The same trends were seen with the 3-h SPET scan data (lower 2 panels), although only data for primary tumor ($r=0.67$, $p=0.037$) showed a significant correlation with the pimonidazole-positive fraction.

Table 2. Patient data regarding tumor volume (from histology), ^{99m}Tc-BRU 59-21 scan outcome, SPET analysis and pimonidazole staining.

Patient	Site	Volume (cm3)	Tumor (0.5 h) (counts/ pixel)	Normal tissue (0.5 h) (counts/ pixel)	Tumor (3 h) (counts/ pixel)	Normal tissue (3 h) (counts/ pixel)	Pimonida-zole (% tissue)
101	PT	6	42.4	41.9	18.5	23.6	0.4
102	PT	4.5	114.2	41.8	51.5	24.1	6.3
	LN left		125.1	41.8	53.7	24.1	8.6
	LN right		124	41.8	60	24.1	11.3
103	PT	3	83.1	38.1	31.5	13.8	3.7
	LN right	1		38.1		13.8	0.3
104	PT	33	89.3	49.3	54.1	14.6	7.7
	LN left		70.7	49.3	26	14.6	3.5
106	PT	23.7	84.6	61.1	47.1	26.6	0.8
	LN right			61.1		26.6	0
107	PT	11.4	83.7	58.9	61.6	20.2	1.5
	LN right			58.9		20.2	2.7
108	PT	6	110.9	69.4	68.7	33	5
	LN left			69.4		33	3.9
109	PT	3.8	58	66.9	26.1	29.9	0.2
110	PT	81	95.2	63.6	59.9	29.6	4.2
112	PT	14.7	130.8	79.2	66.3	29.7	4.8

Abbreviations: PT, Primary tumor; LN, lymph node.

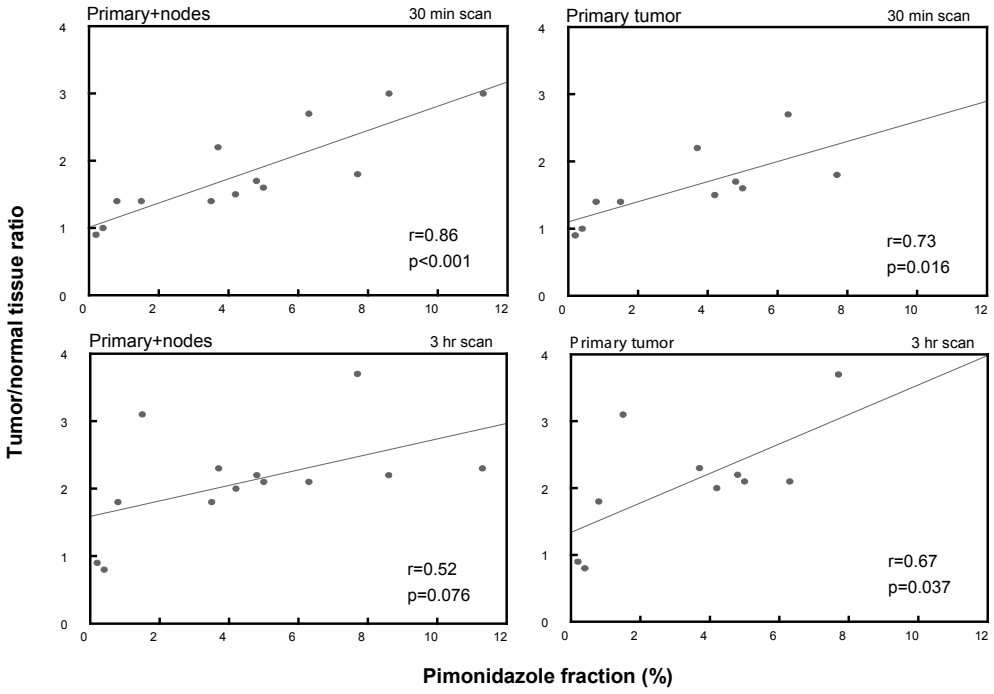
DISCUSSION

Nuclear imaging with intravenously administered ^{99m}Tc-BRU 59-21 appeared to be safe and feasible, without significant adverse effects. The reported events were similar to results from a volunteer study [unpublished data from Bracco Research]. No adverse events were reported for pimonidazole at the dose level used. Uptake on the SPET scan was observed in eight of ten primary tumors; the uptake was especially evident on the 3-h scan, since the delineation of tumor from normal tissue was facilitated by decreased background activity.

To our knowledge, this is the first comparison of tumor hypoxia in cancer patients using in vivo uptake of a radioactively labeled 2-nitro-imidazole and immunohistochemical staining on tumor sections with a similar 2-nitro-imidazole bioreductive marker. There are inherent problems in comparing the two methods (external scanning and immunohistochemistry). One is an intensity measurement of radioactivity (SPET) while

the other is an area measurement (pimonidazole staining). The effective volume being measured is also different: slice thickness is 5 mm for SPET and only 5 µm for tissue sections. In addition, the immunohistochemistry results are obtained from only a small fraction of the total tumor. Whether pimonidazole staining data on small areas is representative for the whole tumor is the subject of ongoing study.

Figure 5. Relationship between pimonidazole staining and ^{99m}Tc-BRU 59-21 uptake in tumor, represented as the tumor to normal tissue ratio of counts/pixel. In the *left-hand panels*, data for the primary tumor and lymph nodes are shown, and in the *right-hand panels*, data for primary tumor only. The *upper two panels* show data for the early SPET scans (0.5 h) while the *lower two panels* show data for the late SPET scans (3 h). In each panel the correlation coefficent (r) and the level of significance (p-value) are indicated.



To improve results in future studies with ^{99m}Tc-BRU 59-21, image fusion between SPET data and MRI/CT scan [14] should be considered in order to locate tumors on the SPET scan more accurately. In addition, the optimum interval between administration and SPET scanning must be determined. Scans at least 3 h after injection appear to be necessary in order to obtain better tumor delineation and decreased non-specific background activity. Future studies using ^{99m}Tc-BRU 59-21 should include a larger number of patients to determine its prognostic value by correlating uptake with treatment outcome.

In conclusion, in vivo evaluation of tumor hypoxia with ^{99m}Tc-BRU 59-21 appears to be safe and feasible. Uptake and retention in tumor tissue can be assessed with SPET, and the observed correlations with pimonidazole staining indicate that it has promise as a method to assess tumor hypoxia.

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chapter 7

Differentiation-associated staining with anti-pimonidazole antibodies in head and neck tumors

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ABSTRACT

BACKGROUND AND PURPOSE

Hypoxia is a strong negative prognostic factor for all three major treatment modalities for cancer. The bioreductive drug pimonidazole is currently under clinical investigation as a hypoxia marker. In human head and neck tumors, in addition to staining patterns typical of chronic hypoxia, staining was seen specifically around areas of keratinization, raising the question of whether this is hypoxia-related. This could influence quantitative hypoxia estimates using this marker. We investigated here whether the differentiation-related staining was caused by locally high reductive enzyme levels.

PATIENTS AND METHODS

The nitrotetrazolium compound NBT was used, which is reduced by nitroreductases to yield a blue color. The assay was validated on three genetically related MDA231 human mammary carcinoma cell lines: wildtype, overexpressing DT-diaphorase (DT1), and overexpressing cytochrome p450 reductase (R4). Increased NBT staining under normoxia was indeed seen for both R4 and DT1 lines. Pimonidazole staining under normoxia was only seen in the R4 line.

RESULTS

Frozen tumor sections from 20 patients with head and neck cancer injected with pimonidazole were incubated with NBT. Parallel sections were stained for pimonidazole. Staining patterns were then compared on matched images, and areas of keratinization scored for the presence or absence of pimonidazole and NBT. Pimonidazole staining was seen in 56% of keratinized areas, and of these, 78% showed increased NBT staining, indicating that high reductase levels are not a necessary requirement for differentiation-associated pimonidazole staining. In a second series, frozen sections of tumors from 15 patients not receiving pimonidazole were incubated with NBT and compared with staining after incubation with pimonidazole under both oxic and hypoxic conditions. Pimonidazole staining of some keratinizing areas under oxic conditions was seen. Of these areas, only a proportion (70%) showed increased NBT staining, confirming the lack of correspondence between keratin-associated pimonidazole staining and reductase levels.

CONCLUSION

Hypoxia-independent pimonidazole staining can occur in more differentiated head and neck tumors, necessitating caution in hypoxia quantification. These data argue against a causative role for locally high reductase levels in differentiation-associated staining. DT-diaphorase appears to play no role in pimonidazole reduction.

INTRODUCTION

2-Nitroimidazoles become reductively activated and bind to macromolecules in the presence of low oxygen levels and therefore are widely used as markers of hypoxia. Pimonidazole has been validated as a hypoxia marker in preclinical studies [1,2] and is under current investigation in the clinic [3-6]. In addition to the presence of staining in areas in the tumor suspected for hypoxia, i.e. far from blood vessels, and in peri-necrotic tissue, another general finding in most of these studies [7,8] is the presence of pimonidazole staining in highly differentiated or keratinized tumor tissue, raising the question of whether this staining is hypoxia-specific. This could have obvious implications for selecting patients with tumors having high or low hypoxic fractions for further hypoxia-modulating therapies.

The reduction of pimonidazole requires nitro-reductases. One electron (1-e) reductases such as NADPH:cytochrome p450 reductase, xanthine oxidase, and aldehyde dehydrogenase are able to reductively activate 2-nitroimidazoles in two separate 1-e reaction steps. The addition of the first electron to form a nitro radical anion is reversible in the presence of molecular oxygen. This step is believed to be the basis of the oxygen dependence of 2-nitroimidazole bioreductive activation and binding. Only in the presence of low oxygen levels can the second reduction step occur allowing a cascade of reductive events to proceed giving products which have the potential to bind to cellular macromolecules. In contrast to 1-e reductases, 2-e reductases like DT-diaphorase are able to bypass the first oxygen-dependent reduction and thus reduce 2-nitroimidazoles independently of the oxygenation status of the cell.

Chapman et al. [9] were the first to report the binding of misonidazole (a closely related analogue of pimonidazole) in normal, presumably normoxic, tissues. Cobb et al. [10,11] described misonidazole retention in stratified squamous epithelia. He also found a correlation between misonidazole retention in normal tissue and elevated oxygen-independent reductase levels and concluded that the retention was more likely to be due to high levels of these reductases rather than to the presence of hypoxia in these normal tissues. In contrast, Parliament et al. [12] studied the high binding of misonidazole to esophageal mucosa in more detail and concluded that an oxygen-insensitive process was not a major cause. They suggested that this high binding resulted primarily from a high level of oxygen-sensitive bioreductive activity.

In the present study, we therefore investigated whether differentiation-related staining is due to high levels of 1- or 2-e reductases. Clarification of the hypoxia specificity of this staining is of importance for the quantification of hypoxia by pimonidazole, and possibly other bioreductive markers, especially in tumor sites that are known to contain considerable amount of keratinization, such as in head and neck tumors. Non-specific oxygen-independent reductase levels using a color reaction on frozen sections were therefore compared with pimonidazole binding in areas of keratinization.

MATERIALS AND METHODS

PATIENTS

Twenty patients with squamous cell carcinoma of the head and neck, treated with primary surgery, were injected with pimonidazole (0.5 g/m^2, IV) the evening before the operation to remove the tumor. Pimonidazole hydrochloride (Hypoxyprobe™-1; Natural Pharmacia International, Belmont, USA) was diluted in sterile 0.9% saline for intravenous injection. The study was approved by our Institute Review Board and written informed consent was obtained from all patients. A further 15 patients, also with squamous cell carcinomas of the head and neck but who were not given pimonidazole, were used in a second series. For both series, a biopsy was taken from the resection specimen and frozen in liquid nitrogen. For sectioning, the tissues were mounted on the freeze microtome head with OCT embedding medium and 7 µm sections were cut at –20 °C.

IMMUNOHISTOCHEMISTRY

Sections were fixed in 4% formaldehyde for 10 min. After blocking with 0.3% hydrogen peroxide in methanol, the slides were incubated with serum free protein block, followed by incubation with a biotinylated antibody against pimonidazole (supplied by Dr J.A. Raleigh, 1/1000, overnight, 4 °C). Sections were subsequently incubated with streptavidin-peroxidase (UltraVision Detection System, Lab Vision, USA) for 10 min at room temperature. Finally, peroxidase activity was detected with DAB substrate in distilled water.

REDUCTASE ACTIVITY ASSAY

Frozen sections were fixed in formaldehyde vapor for 30 s to minimize diffusion of enzyme. They were then incubated at 37 °C for 5 min in phosphate buffer (pH 7.2), NBT (3,3'-(3,3'-dimethoxy-4,4'-biphenylene)bis[2-4-nitrophenyl-5-phenyl-2H-tetrazolium chloride]) 1.2 mM; MgCl 0.05 M; NADPH 2.4 mM and menadione (vitamin K). Sections were terminally fixed in 10% formaldehyde and washed in water. Finally, they were rinsed in acetone, dehydrated in alcohol, cleared in xylene and mounted in Depex.

NBT staining density was estimated semi-quantitatively. For the cell lines, we used an arbitrary scale of 0 to +++. The absence of any except minimal blue staining was recorded as 0, a clearly positive blue stain +, a strong blue ++ and very strong blue verging on black +++. On the tumor sections, areas of keratinization were scored for the presence or absence (+ or –) of increased NBT staining (strong blue staining). Scoring was done independently by two or more different observers.

TISSUE CULTURE

The human mammary carcinoma cell line MDA 231 (wild type) and MDA 231 overexpressing cytochrome p450 reductase (clone R4) or DT-diaphorase (clone DT1) have been described [13,14]. DT diaphorase activity was 10,000 nmol cyt.c reduced/min per mg protein for the

DT-1 clone and 12 for the wild type. Cytochrome p450 reductase activity was determined to be 300 nmol cyt.c reduced/min per mg protein for the R4 clone and 6 for the wild type. Overexpression of DT-diaphorase in comparison with wildtype cells was confirmed by western blotting. All cell lines were maintained in exponential growth phase in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% fetal calf serum and puromycin (5 µg/ml). They were cultured at 37 °C in water-saturated atmosphere of 95% air and 5% CO_2.
After trypsinization, wildtype and transfected cells were grown on coverslips for 2 days and washed with PBS before incubation with pimonidazole or fixation in formaldehyde vapor for the NBT assay.

INCUBATION WITH PIMONIDAZOLE UNDER OXIC VERSUS HYPOXIC CONDITIONS

Fresh frozen tissue sections or cells grown on coverslips were incubated for 4 h at 37 °C in 95% air and 5% CO_2 or under hypoxic conditions in a sealed chamber. Incubation medium contained DMEM, 10% FCS, Hepes (25 mM), NADHP (2.4 mM) and 0 or 3 mM pimonidazole. Tissue sections from 15 different tumors were studied. For each tumor, parallel sections incubated without pimonidazole were used as a negative control. To create a hypoxic environment, a GasPak 100™ anaerobic system (Becton Dickinson Microbiology Systems, Sparks, USA) was used. In this system, hydrogen generated from a sodium borohydride tablet, combines in the presence of a palladium catalyst with the oxygen in the jar to form water, thus exhausting the oxygen. Evans [15] previously reported that the oxygen concentration in these jars was reduced to less than 0.4% in 100 min. A clonogenic assay for radiosensitivity was used to evaluate the level of oxygen achieved by this system after 3 h, showing an oxygen enhancement ratio of approximately 2.9 for Chinese Hamster CHO cells (data not shown), indicating a high degree of hypoxia. After incubation, slides were immediately fixed in 4% formaldehyde for 10 min followed by washes with PBS.
To assess pimonidazole staining intensity, we scored keratinized and non-keratinized areas according to an arbitrary five-point scale (0, no staining; 5, maximum staining). Non-keratinized areas were chosen next to keratinized areas in differentiated tumors, whereas in tumors showing little or no keratinization, the tumor as a whole was scored.

Table 1. Pimonidazole and NBT staining of wildtype and transfected MDA231 cells.

	NBT staining, normoxia	**Pimonidazole staining**	
		Hypoxia	*Normoxia*
WT	+	+++	0
R4	+++	++++	++
DT1	+++	+++	0

Explanation: WT, wildtype; R4, overexpressing cytochrome p450 reductase; DT1, overexpressing DT-diaphorase; visual score 0 to +++, intensity of NBT staining; visual score 0 to ++++, intensity of pimonidazole binding.

FLOW CYTOMETRY

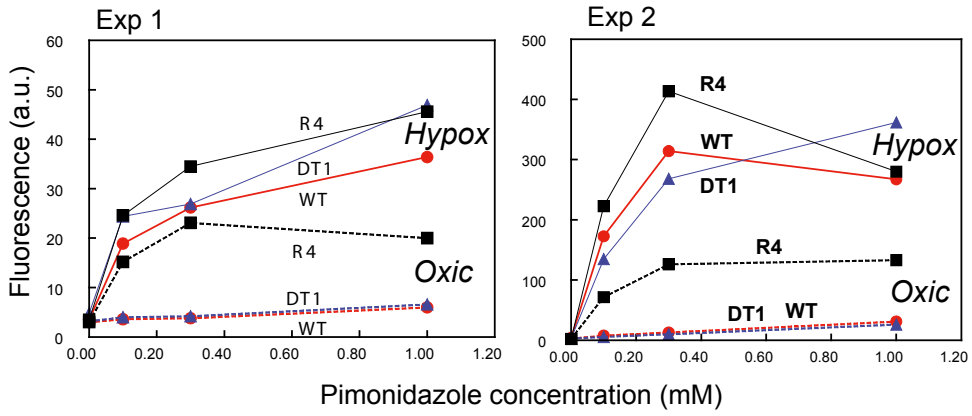
Cells were trypsinized and fixed in 70% ethanol. Fixed cells were stained with pimonidazole using a non-conjugated mouse anti-pimonidazole antibody (supplied by Dr J.A. Raleigh, dilution 1/1000, 1 h, RT) followed by an FITC-conjugated goat anti-mouse antibody (Sigma, USA), according to standard procedures. Cells were assessed for fluorescence using a FACScan flow cytometer (Becton Dickinson, USA) calibrated with fluorescent beads, again according to standard procedures. Debris was gated out from forward versus side scatter plots and the mean green fluorescence of the gated cells measured using WinMDI software.

RESULTS

VALIDATION OF NBT ASSAY

MDA 231 human breast carcinoma cell lines overexpressing either p450 reductase (R4) or DT-diaphorase (DT1) were used to validate the reductase assay. The intensity of the blue staining was scored visually. Both DT and p450 overexpressing cell lines showed more intense NBT staining compared with wildtype cells, indicating that NBT detected increased activity of both reductase types (Table 1). No significant staining difference could be detected between these 1 or 2 electron reductases. In the majority of the R4 cells, NBT staining was predominantly located in the cytoplasm, while in the DT-1 cells, staining was mainly localized in the nucleus.

Figure 1. Pimonidazole staining in MDA231 human mammary carcinoma cell lines detected by flow cytometry. WT, wildtype (circles); DT1, overexpressing DT-diaphorase (triangles); R4, overexpressing cytochrome p450 reductase (squares). Oxic incubations, dashed curves; hypoxic incubations, full curves.



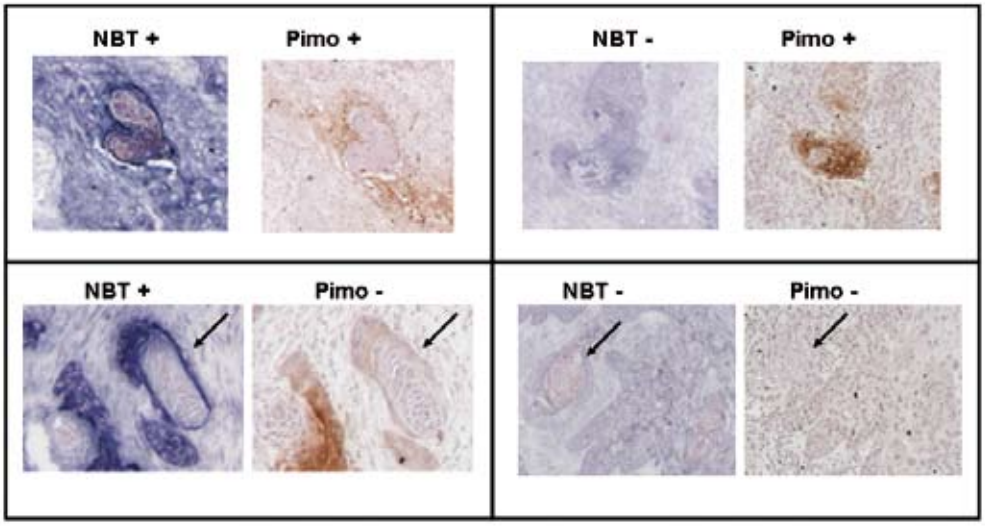
In addition, we incubated the same cell lines with pimonidazole under either oxic or hypoxic conditions. Pimonidazole reduction and binding was quantified by flow cytometry using an anti-pimonidazole antibody and a fluorescent second antibody.

Two separate experiments showed essentially comparable results (Fig. 1). All three lines showed high staining after hypoxic incubation, which was pimonidazole concentration-dependent. Two of the cell lines showed little staining after oxic incubation. In the R4 cell line, however, staining was considerably higher than in the other two, although not as high as after hypoxic incubation. These data indicate that p450 reductase is capable of reducing pimonidazole past the 1-e reduction step under oxic conditions, if expressed at high levels. They also indicate that DT-diaphorase is incapable of reducing this nitroimidazoles under normoxia, despite its capability of 2-e reduction. Moreover, in neither experiment was the DT1 curve significantly higher than for wildtype cells under hypoxia, indicating that DT-diaphorase plays a negligible role in pimonidazole reduction under either condition of oxygenation.

PIMONIDAZOLE STAINING VERSUS REDUCTASE ACTIVITY

Reductase activity was measured on frozen tumor sections of patients previously injected with pimonidazole. A mouse liver was used as a positive control while NADPH was omitted as a negative control. Blue staining varied from almost none to very high and was often heterogeneous throughout the tumor. Areas of keratinization were scored for the presence of increased reductase activity. Corresponding areas in adjacent sections were scored for the presence of pimonidazole. Examples of the four possible outcomes are shown in Fig. 2, illustrating areas positive for both markers, one marker or neither marker.

Figure 2. Staining in frozen sections of head and neck tumors from patients given pimonidazole. Adjacent sections were incubated with NBT. Photos show four matched keratinizing tumor areas stained for pimonidazole and NBT (right and left in each pair), illustrating the four possible outcomes: +/+, –/+, +/– and –/– (first/second symbols representing pimonidazole and NBT staining scores, respectively). Arrows indicate keratinizing areas.



Scoring results are shown in Table 2. Areas of keratinization were present in 10 of the 20 tumors and a total of 64 areas were scored after matching pimonidazole and NBT sections. Pimonidazole staining was seen in 36 of the 64 areas (56%; Table 2, second and fourth data columns). In addition, in these 64 differentiated areas, reductase levels correlated with pimonidazole staining in only 56% (43% both positive, 13% both negative; Table 2, first and fourth data columns). The remaining 44% of the areas showed a mismatch: 13% pimonidazole positive/NBT negative, 31% pimonidazole negative/NBT positive. These data show that keratinizing areas are sometimes but not always associated with pimonidazole staining, and that high reductase levels are not correlated with this staining.

Table 2. Pimonidazole and NBT staining of frozen sections of tumors from patients given pimonidazole. Areas of keratinization were scored for the presence or absence of pimonidazole staining (+ or –). The same areas on consecutive sections were scored for the presence or absence of increased NBT staining (+ or –).

NBT/pimonidazole	- / -	- / +	+ / -	+ / +
Number of areas	8	8	20	28
% of total	12.5	12.5	31	43

PIMONIDAZOLE BINDING UNDER OXIC VERSUS HYPOXIC CONDITIONS

To try and clarify whether the pimonidazole staining could occur under oxic conditions, we studied a second series of patients not given pimonidazole. This allowed us to study pimonidazole staining under different conditions of oxygenation by incubating frozen sections with the hypoxic marker compound. Adjacent sections were again incubated with NBT (under normoxia) to measure reductase activity. Sections of head and neck tumors with different degrees of differentiation and keratinization were studied. Staining intensity was scored on an arbitrary five-point scale. When scoring individual keratinized and adjacent non-keratinized areas, pimonidazole staining was more intense under hypoxia than under oxic conditions for both states of differentiation (Table 3). It was also clear that staining was more intense in keratinized areas (columns 1 versus 3, 2 versus 4). However, although both hypoxia and differentiation state affected staining intensity, the influence of hypoxia was the more dominant, such that hypoxia-non-keratinized areas still showed more intense staining than oxic-keratinized areas (columns 3 versus 2).

It was apparent that strongly keratinizing tumors showed a more heterogeneous pimonidazole staining than non-keratinizing tumors (see Fig. 3). Not all tumors showed pimonidazole staining around areas of keratinization under oxic conditions (Table 3, column 2). Of 86 areas that stained positively in air, 70% were associated with increased reductase activity (high NBT staining; data not shown). The remaining 30% had basal NBT staining, again indicating a lack of correlation between keratin-associated staining and reductase levels.

Table 3. Intensity of staining after pimonidazole incubation of frozen tumor sections under hypoxic and oxic conditions. A five-point intensity scale was used (visual scoring), and both differentiated (keratinized) and adjacent non-differentiated areas were scored separately.

Tumor	**Keratin areas**		**Next to keratin areas**	
	Hypoxic	*Oxic*	*Hypoxic*	*Oxic*
SC 219	5	1	2	0
	5	1	2	0
	4	2	4	0
	4	2	4	1
	4	1	3	0
	5	2	2	0
	4	3	2	0
	4	1	1	0
	4	1	2	0
SC 202	4	2	2	0
	4	2	2	0
	3	1.5	2	0
	4	1.5	2	0
	3	1.5	2	0
	3	1.5	2	0
SC 183	4	0	3	0
	3	0	2	0
	4	0	2.5	0
	4	0	3	0
	3.5	0	2.5	0
	1	0	1	0
	2.5	1	2.5	0
	4	0.5	3	0
	2.5	0.5	2.5	0
	4	0.5	3	0
Mean	**3.7**	**1.1**	**2.4**	**0**
SD	**0.9**	**0.8**	**0.7**	**0.2**
N	*25*	*25*	*25*	*25*

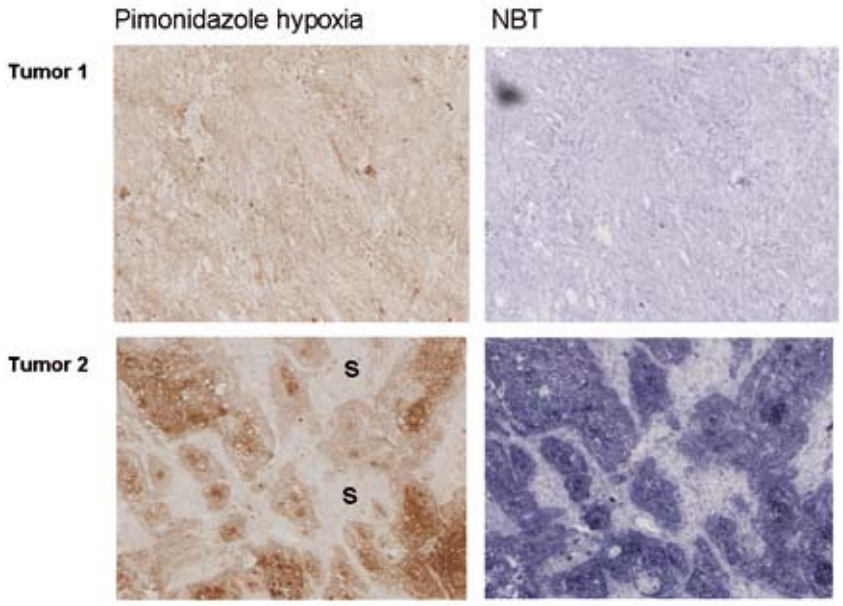
Abbreviations: SD, standard deviation. *N*, number of areas scored.

DISCUSSION

This study tested the hypothesis that the differentiation-associated staining seen with anti-pimonidazole antibodies is, at least in part, not hypoxia-related but due to other factors, specifically high levels of non-specific oxygen-independent nitroreductases. These areas are visually different from the classic chronic hypoxia patterns seen with pimonidazole in many tumors such as those less well differentiated. The classic pattern shows a gradual increase of staining intensity and proportion of cells stained with increasing distance from blood vessels. In contrast, areas of differentiation-associated staining often have very sharp boundaries and exist at variable distances from blood vessels. This raises the obvious question of whether this is indeed hypoxia-related staining.

The fact that pimonidazole staining can occur in these areas was illustrated in both patient series. The finding that pimonidazole incubation of frozen sections under normoxia showed staining in some of these areas was particularly informative, demonstrating that non-hypoxia-related pimonidazole staining can occur. This only occurred in keratinizing areas. There are therefore clearly other factors than oxygen concentration that will influence pimonidazole staining in histological sections. However, the lack of correlation with NBT staining did not support our hypothesis that a major factor could be high reductase levels.

Figure 3. Staining in adjacent frozen sections of head and neck tumors incubated in vitro with pimonidazole or NBT. Left panels, pimonidazole staining after hypoxic incubation; right panels, NBT staining of matched areas in adjacent sections after oxic incubation to monitor reductase levels. Top panels show a poorly differentiated tumor; lower panels show a well differentiated keratinizing tumor, showing heterogeneous pimonidazole staining and high NBT staining in tumor areas. Stromal areas (s) are lighter stained and separate the darker staining tumor areas.



Pimonidazole concentrations used in the in vitro incubation experiments were higher than those reached in human tumors and this could possibly have influenced the non-hypoxic binding. However, we had previously tested a range of concentrations of pimonidazole (data not shown) and found that a high concentration (3 mM) was necessary to obtain a good hypoxia-specific signal. These high concentrations may have been necessitated by non-optimal incubation conditions, or loss of enzyme or co-factors resulting from freezing and cutting.

The assay chosen, reduction of the nitrotetrazolium NBT to a blue product, was tested by staining-related cell lines with different levels of reductase expression. The results showed that both reductases investigated, cytochrome p450 reductase and DT-diaphorase, were capable of reducing NBT under oxic conditions. Both enzymes have been shown to reduce nitroimidazoles. It therefore appeared to be a satisfactory assay to test for variable enzyme levels in tumors as potential mediators of non-hypoxic reduction. The pimonidazole data on these cell lines showed additionally that DT-diaphorase appeared to be incapable of reducing pimonidazole under air, and also possibly under hypoxia. Increased NBT staining in tissue sections therefore could reflect either higher p450 reductase or DT-diaphorase concentrations, but only p450 reductase levels will be relevant to pimonidazole staining. Whether there are other reductases occurring in human tumors which can be detected by NBT is not known.

If high reductase levels are not the cause of the differentiation-associated pimonidazole staining, what then is? One possibility is that the antibody cross-reacts with another protein or cellular constituent. This is unlikely, since staining of control sections not containing pimonidazole (from patients not given pimonidazole and not incubated with pimonidazole in vitro) show low background staining and no specific differentiation-associated staining. It is possible that unreduced pimonidazole binds or is trapped in keratinizing areas and is subsequently detected by the antibody. We have nothing to prove or disprove this possibility. Interestingly, oxygen-independent binding of pimonidazole to melanin in melanoma cell lines has been reported [16] and so by analogy, it is possible that pimonidazole may bind to other cellular constituents such as keratin.

Raleigh and colleagues [7] showed a large overlap between pimonidazole and the differentiation marker involucrin in human cervix tumors. This co-localization supports a relationship between differentiation and pimonidazole staining, and is not inconsistent with the idea that the differentiating cells are also hypoxic. They are also not inconsistent with the notion that some of these areas may not be hypoxic. This still needs further investigation.

Another perhaps more important question is whether the cells in well differentiated areas and which stain with pimonidazole are also clonogenic. For patients treated with radiotherapy, the radioresistance of hypoxic cells is likely to be a major problem, and was the primary reason for originally stimulating the development of bioreductive hypoxia markers. For chemotherapy, a similar reasoning applies, although the chemoresistance

of hypoxic cells is likely to be caused by lower drug concentrations reaching these cells, and the fact that a higher proportion will be out of cycle. The requirement for clonogenicity remains, however. It would therefore be very useful to know what fraction of pimonidazole-stained cells are clonogenic. At present there are no methods to measure this in human tumors, although one could speculate that it could be addressed by studies correlating gene expression patterns under progressive hypoxia and nutrient deprivation with loss of clonogenicity. If an expression pattern inconsistent with clonogenic ability (e.g. reflecting a particular differentiation and energy status) could be found, this could be exploited to investigate the status of pimonidazole stained cells in vivo.

If the differentiation-associated staining is non-hypoxia-related, the 'hypoxic fraction' measured by pimonidazole will be overestimated. How big is this problem? In the present study, differentiation-associated staining was seen in six of the 20 tumors from patients given pimonidazole. In these five patients, we estimated that 30% of the total pimonidazole staining appeared to be differentiation-associated (judgment based on proximity to keratinization and sharpness of borders). This means that in 25% of patients (5/20), the hypoxic fraction would be overestimated by 30%. This is a worst-case scenario since it assumes that the cells showing differentiation-associated staining are not hypoxic, and some of them are likely to be both differentiated and hypoxic.

In conclusion, pimonidazole may give an overestimate of the true hypoxic fraction in some head and neck tumors due to differentiation-associated staining. High reductase levels do not seem to be the cause of this staining, at least not in all keratinizing areas, and therefore it cannot be corrected for by the NBT. The problem addressed here could also be regarded as a question of setting an appropriate intensity threshold, which should ideally exclude the light oxic staining seen in some differentiated areas. This is difficult to realize in practice. It is possible that any overestimate is further compounded by the fact that a proportion of the pimonidazole-stained cells may no longer be clonogenic. This remains an important question. Correlations of the extent of pimonidazole staining with outcome after radiotherapy should help clarify some of these issues.

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chapter 8

^{99m}Tc-Hynic-rh-Annexin V scintigraphy for in vivo imaging of apoptosis in patients with head and neck cancer treated with chemoradiotherapy

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ABSTRACT

Purpose

To determine the value of ^{99m}Tc-Hynic-rh-Annexin-V-Scintigraphy (TAVS), a non-invasive in-vivo technique to demonstrate apoptosis, in patients with head and neck squamous cell carcinoma.

Methods

TAVS were performed before and within 48 hours after the 1st course of cisplatin-based chemoradiation. Radiation-dose given to the tumor at the time of post-treatment TAVS was 6-8 Gy. SPECT-data were co-registered to planning CT-scan. Complete sets of these data were available for 13 patients. The radiation dose at post-treatment TAVS was calculated for several regions of interest (ROI): primary tumor, involved lymph nodes and salivary glands. Annexin-uptake was determined in each ROI, and the difference between post-treatment and baseline TAVS represented the absolute Annexin-uptake: Delta-uptake (ΔU).

Results

In 24 of 26 parotid glands, treatment-induced Annexin-uptake was observed. Mean ΔU was significantly correlated with the mean radiation-dose given to the parotid glands ($r=0.59$, $p=0.004$): Glands that received higher doses showed more Annexin-uptake. ΔU in primary tumor and pathological lymph nodes showed large inter-patient differences. A high correlation was observed on an inter-patient level ($r=0.71$, $p=0.006$) between the maximum ΔU in primary tumor and in the lymph nodes.

Conclusions

Within the dose range of 0–8 Gy, Annexin-V-scintigraphy showed a radiation-dose-dependent uptake in parotid glands, indicative of early apoptosis during treatment. The inter-individual spread in Annexin-uptake in primary tumors could not be related to differences in dose or tumor-volume, but the Annexin-uptake in tumor and lymph nodes were closely correlated. This effect might represent a tumor-specific apoptotic response.

INTRODUCTION

Apoptosis is an important mechanism of cell death in response to treatment with radiation and many chemotherapeutic agents [1]. To what extent apoptosis contributes to the overall cytotoxic effect of an anti-cancer treatment modality has been the topic of intense research during the last decade [2-4]. The relative contribution of apoptosis to the occurrence of cell death varies greatly both between different tumor types and normal tissues [5].

Recently, *in vivo* imaging of apoptosis has proven to be feasible by using radiolabeled Annexin V [6-10]. This endogenous human protein has a high affinity for membrane-bound lipid phosphatidylserine (PS), which becomes exposed at the outer leaflet of the cell membrane bilayer at an early stage of the apoptotic process [11,12]. PS then serves as a recognition site for macrophages that digest and remove apoptotic cells. The reproducibility of the ^{99m}Tc-Hynic-Annexin-V scintigraphy has been demonstrated in head and neck squamous cell carcinoma (HNSCC) patients by serial imaging in untreated patients [13], in which the mean difference in uptake was 6%. We have recently demonstrated that ^{99m}Tc-Hynic-rh-Annexin V scintigraphy (TAVS) correlates with radiation-induced cytologically confirmed apoptosis in non-Hodgkin lymphoma [14] and can be used to identify patients that have a favorable prognosis [15].

The current standard of practice for patients with advanced stage HNSCC is treatment with concurrent cisplatin-based chemoradiation [16,17]. Although this is an effective treatment, it also is accompanied by more severe toxicity than radiation alone. We reasoned that TAVS early during treatment might be used to monitor apoptosis induction in tumor and normal tissue and to give an indication of the radiosensitivity of these structures. In future, this would also offer the possibility to adapt the treatment strategy at an early stage on a patient-by-patient basis.

In the present study we therefore aimed at assessing the feasibility of TAVS as a non-invasive technique to demonstrate treatment-induced apoptosis in vivo, in patients with HNSCC, early during treatment with concurrent chemoradiation. The purpose was to determine the degree of uptake on TAVS in normal tissue, primary tumor and lymph node metastases and to evaluate the treatment-induced Annexin-uptake in relation to radiation dose. Furthermore, we questioned whether the differences in uptake would correlate with treatment response.

MATERIAL AND METHODS

Patients included in this study were recruited between January 2004 and March 2005 from a randomized phase III trial in advanced stage HNSCC, investigating the optimal route of cisplatin delivery during cisplatin-based chemoradiation (RADPLAT). Randomization was between 2 treatment arms: Arm 1, standard intravenous (IV) administration of cisplatin 100 mg/m^2 (1 hour before radiotherapy at day 1, 22 and 43) or arm 2, high-dose selective intra-

Table 1. Patient, tumor and treatment characteristics.

Patient Number	Gender	Age (years)	Tumor site	TNM-Stage	Tumor volume (cm3)	Max ΔU in primary tumor	Interval cisplatin infusion and Annexin scan (hours)	RT technique	Mode of cisplatin administration
1	M	64	oral cavity	III	21	62	52.2	Conventional 3 field	intra-arterially
2	M	58	oropharynx	III	47	203	50.3	IMRT	intra-arterially
3	F	65	oropharynx	IV	25	112	53.1	IMRT	intra-arterially
4	M	55	oropharynx	III	26	36	53.5	IMRT	intra-arterially
5	M	49	hypopharynx	II	13	43	53.3	IMRT	intra-arterially
6	F	66	oral cavity	IV	32	135	53.6	Conventional 3 field	intra-arterially
7	M	60	oropharynx	IV	147	191	54.3	Conventional 3 field	intra-arterially
8	M	46	oropharynx	IV	28	170	52.7	Conventional 3 field	intra-arterially
9	M	55	oropharynx	IV	41	42	49.9	IMRT	intravenously
10	M	57	oropharynx	IV	25	51	51.7	IMRT	intravenously
11	M	48	oropharynx	IV	91	117	51.5	Conventional 3 field	intravenously
12	M	50	hypopharynx	IV	100	132	50.3	Conventional 3 field	intravenously
13	M	26	oropharynx	IV	105	52	50.1	Conventional 3 field	intravenously

Abbreviations: *M*, male. *F*, female. *IMRT*, intensity modulated radiotherapy

arterial (IA) delivery of cisplatin at a dose of 150 mg/m^2 (on day 2, 9, 16 and 23, within 1 hour after radiotherapy delivery). Main eligibility criteria included: inoperable squamous cell carcinoma of the oral cavity, oropharynx or hypopharynx, TNM stage T3-4 status of primary oral cavity or oropharyngeal tumors and T2-3-4 for hypopharyngeal tumors, with any N-status, (functionally) inoperable disease, no distant metastases, age at least 18 year, ability to give informed consent and no prior cerebro-vascular accident. Both the randomized trial and the TAVS study were approved by the medical ethics committee of the hospital. Patients were informed about the nature of the study protocol and signed informed consent before enrolment, separately for both the randomized trial and the TAVS protocol.

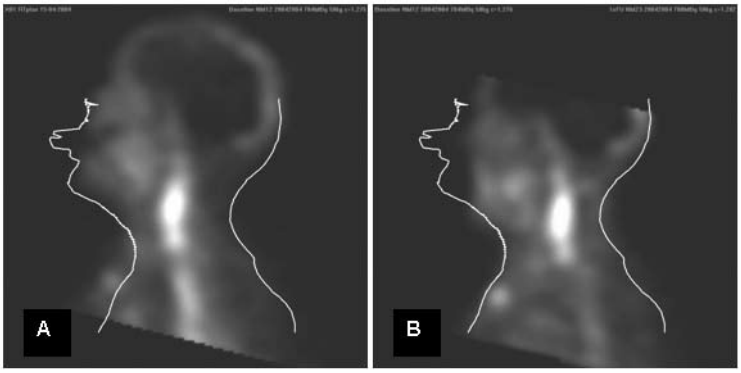
Radiotherapy was given with 4-6 MV photon linear accelerators. Target volume included the primary tumor and the bilateral neck for a dose of 46 Gy in 23 fractions. A boost was given to the known macroscopic tumor extensions at the primary tumor site and lymph node metastases to a dose of 24 Gy in 12 fractions. The total dose delivered was 70 Gy in 35 fractions, 5 fractions per week, with an overall treatment time of 7 weeks. The radiation technique was either a conventional 3-field beam set-up (using conventional simulation or virtual simulation with CT-scan) or an Intensity Modulated Radiotherapy (IMRT-) plan, depending on resources and tumor extent. The IA cisplatin delivery was accomplished by a selective catheterization procedure using the femoral artery, according to the earlier described RADPLAT protocol [18,19]. Concurrently with IA cisplatin sodium-thiosulphate (STS) was administered, to neutralize systemic cisplatin. In both arms prehydration and posthydration were given.

^{99M}TC-HYNIC-RH-ANNEXIN V SCINTIGRAPHY, DIAGNOSTIC IMAGING, RADIATION TREATMENT PLANNING AND IMAGE FUSION

Baseline TAVS was performed within two weeks before the start of concurrent chemoradiation (average interval 5 days, range 1 – 8). Post-treatment TAVS was done within 48 hours after the start of cisplatin chemotherapy. At the time of post-treatment TAVS, patients had received 6 Gy of radiation in case of IV cisplatin, 8 Gy in case of IA cisplatin. Each patient received an average of 847 MBq (range 714- 1032 MBq) of ^{99m}Tc-Hynic-rh-Annexin V (Theseus Imaging Corporation, Boston, USA) by slow intravenous injection 4 hours prior to the planar imaging and single photon emission tomography (SPECT) imaging. Planar images were used to assess the biodistribution. SPECT of the head and neck region was acquired by the step-and-shoot mode, one step per 3 degrees, 30 seconds per frame, matrix size 128x128, using a dual-head gamma camera (Genesis, Philips, Best, The Netherlands) equipped with low-energy, high-resolution collimators. For SPECT reconstructions an iterative algorithm was used and the images were postfiltered using a Butterworth filter (cut off frequency 0.35, order 5). The use of absolute quantitative analysis of SPECT data was validated by comparison of iterative and FBP reconstruction methods. Transaxial, coronal and sagittal slices were visually examined to evaluate tracer uptake at the tumor sites and in normal tissues. The intensity of the obtained images was

corrected by normalization for the injected radioactive dose and body weight.
Baseline diagnostic imaging with MRI (1.5-T system; Somatom; Siemens Medical Systems, Erlangen, Germany) or spiral CT (Tomoscan AVE1, Philips, Best, The Netherlands or HiSpeed CT, GE Medical Systems, USA) was performed within 3 weeks before the start of treatment and repeated 6-8 weeks after the end of the treatment for the evaluation of treatment response.

Figure 1. Sagittal projection of TAVS imaging co-registered with planning CT-scan. The solid line represents the contour of the patient during RT planning-CT scan, including tongue depressor. The projected image is the TAVS. Panel A represents the baseline TAVS; Panel B the post-treatment TAVS. Note the mismatch of contours of the head and neck, mostly due to different position of the chin in Panel B. The misalignment of the lower neck in panel A is due to the flexion of the neck. For the analysis, the matching-procedure was focused on the mandibular region. This patient was excluded from analysis because of misalignment in panel B.



Radiation treatment planning was done with our clinical treatment planning system (TPS) (U-MPlan, University of Michigan, Ann Arbor, USA). Six patients were treated by intensity modulated radiotherapy (IMRT) and the treatment plans were recalculated for the dose at the time of 1st post-treatment TAVS. The regions of interest (ROI) were delineated manually for each patient on the CT-scan. These included the Gross Tumor Volume (GTV) of the primary tumor and/or lymph nodes, the parotid glands, and the submandibular glands. In the other 7 patients, a standard 3-field technique was used by virtual simulation with a CT-scan. In these latter patients, the CT-scan was imported into the TPS in which the delineations were done. The treatment fields were reconstructed and the clinically applied dose distribution at the time of TAVS was recalculated in the TPS. The primary tumor and lymph node volumes were calculated by 3-D reconstruction of the delineated GTV.
SPECT, MRI and CT were performed separately. For the SPECT scan, we obtained reproducibility of the positioning of the head and neck as during RT by the use of immobilization mask, fixed to the table by adhesive tape. All images and the radiation dose data were transferred for image fusion to our in-house developed workstation for co-registration (Worldmatch Workstation) in DICOM format [20]. Keeping the limitations of the mask fixation in mind, it was decided that for more accuracy, matching

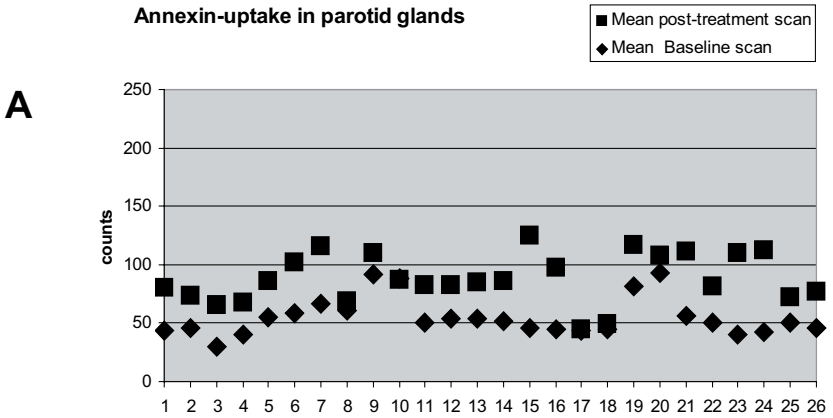
was performed on different anatomical regions for different ROI's. E.g. for the analysis of parotid glands or oropharyngeal tumors, the co-registration was done in such a way that it ensured adequate matching for that area only. For co-registration of ROI's in the neck (e.g. lymph nodes or hypopharyngeal tumors) only the neck and vertebral column was used in the matching procedure. Accurate matching of body contours and bone structures was visually verified. SPECT, composite SPECT/SPECT, CT and SPECT/CT images, obtained using a colourwash technique, were simultaneously examined using linked cursor to evaluate Annexin V uptake in tumor and normal tissues.

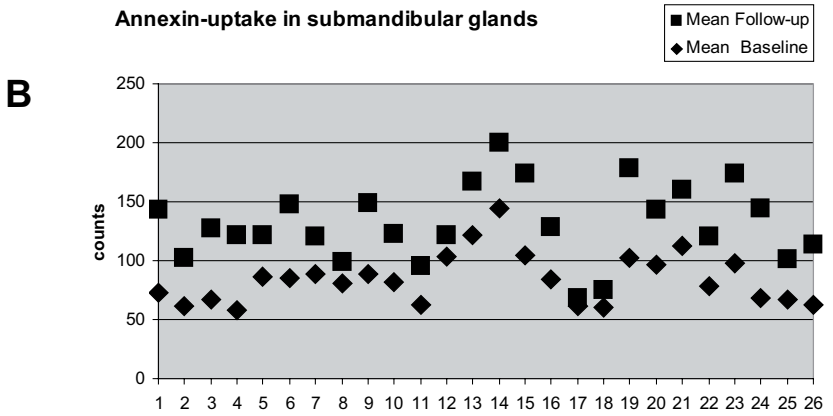
The tumor and normal tissue uptake at baseline and post-treatment were calculated as follows. ROI's were delineated in the planning CT. Each ROI was projected using the registration transformation onto the baseline and post-treatment SPECT scans. The area was next sampled by 10.000 random points and for each point the Annexin uptake was determined in the SPECT scans by trilinear interpolation. Of these 10.000 samples the maximum and mean values were computed. In this way the uptake in the ROI was accurately sampled even though the pixel size in the SPECT scans is relatively large (about 5 mm). For the quantification of Annexin uptake no attenuation correction was performed, because the images were obtained on a conventional gamma camera, without a hybrid system. Subsequently, the difference (ΔU) between the post-treatment and baseline uptake was determined by subtraction of the baseline scan from the post-treatment scan. The subtraction was performed on a point by point basis for all 10.000 points. Then, the mean or maximum value of the difference of both scans ("subtraction scan") was computed and expressed as ΔU. No correction for background activity was made, since this was automatically eliminated by subtraction of the baseline activity from the post-treatment activity, assuming that the background activity is equal in the baseline and post-treatment scan. Radiation dose parameters (mean and maximum dose in cGy) within each ROI were calculated and correlated with corresponding ΔU parameters. It was observed that the maximum ΔU and the mean ΔU were closely related: a linear relationship between both 2 parameters was found for each ROI (tumor: $r=0.93$, $p<0.0001$; parotid gland: $r=0.94$, $p<0.0001$; submandibular gland: $r= 0.90$, $p<0.0001$; lymph node $r=0.94$, $p<0.0001$), indicating that both values would have been representative for the whole ROI.

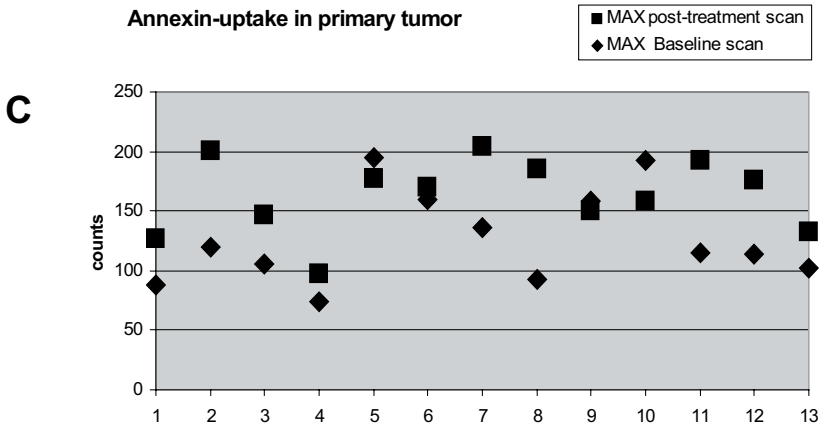
EVALUATION OF TREATMENT

Six-eight weeks after the end of treatment, the results of therapy were evaluated by means of radiological investigations (by MRI or CT scan and/or ultrasound) and examination under general anaesthesia, with biopsies taken in case of suspicious findings. For residual disease in the neck at the time of evaluation, salvage neck dissection was performed if the patient was judged operable. Follow-up visits were planned every 3 months in the first year after therapy, every 4 months in the second year and less frequent thereafter. A follow-up chest X-ray was performed annually.

Figure 2. Baseline (diamonds) and post-treatment (squares) Annexin activity in parotid glands (Panel A), submandibular glands (Panel B) and tumor (Panel C) for all 13 patients. The data for 26 parotid and submandibular glands represent the right and left gland within each patient.



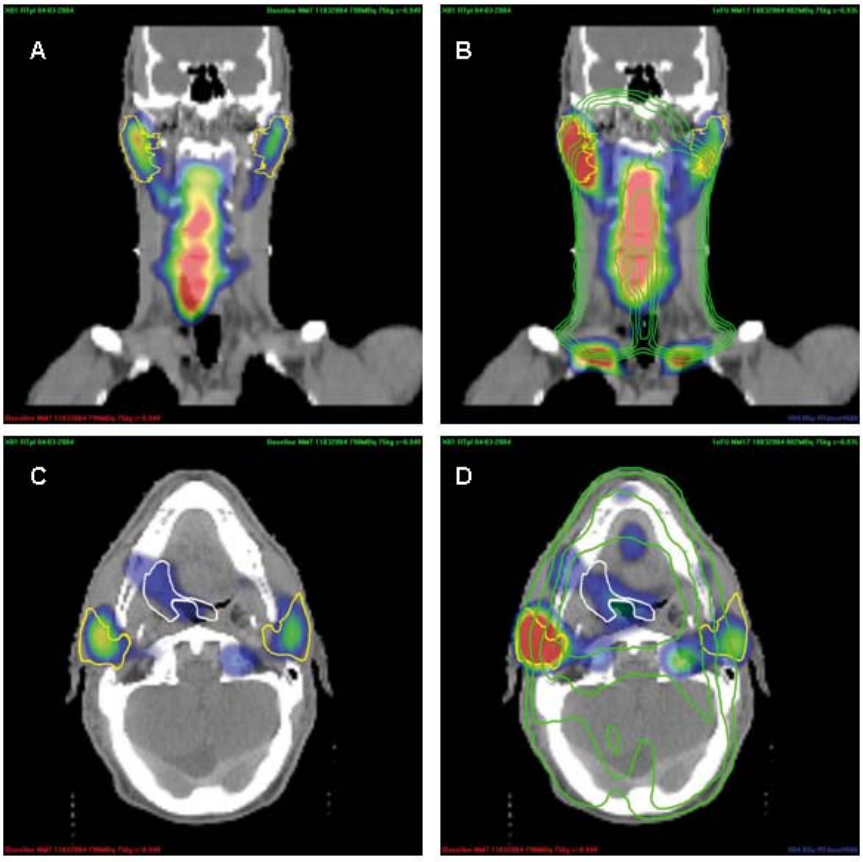




STATISTICAL ANALYSIS

For quantitative comparison of continuous data Student's *t*-test was applied. Chi-squared and Fisher's exact test were used for analysis of categorical data. The Pearson and Spearman rank correlation coefficient *r* were used to calculate correlations between Annexin-uptake and treatment parameters. Locoregional control and survival data were calculated from the start of treatment using the Kaplan-Meier method and Log Rank testing. Two-sided p-values of <0.05 were considered statistically significant.

Figure 3. TAVS imaging co-registered with planning CT-scan in frontal plane and axial plane, A and C at baseline, B and D after treatment, from patient number 2. The Annexin-uptake is represented by color-wash. In panel B and D, the isodose lines show the dose-distribution in relation to the parotid gland and primary tumor (Isodose lines shown: 40%, 60%, 80%, and 95%, from outer to inner side of patient). Note the increased treatment-induced Annexin-uptake in the right parotid gland, in correspondence to the higher radiation dose-distribution, when compared to the left parotid gland. Also note the weak increase in primary tumor Annexin-uptake in the right oropharynx after treatment, and in the anterior floor of mouth (panel D).



RESULTS

Sixteen patients gave their consent for participating in the TAVS study. However, one patient refused post-treatment TAVS scans, in one patient no CT-scan in radiation treatment position was available and in one patient co-registration with SPECT was unsuccessful due to miss-alignment because no immobilization mask and no tongue depressor could be used during SPECT due to pain (Figure 1). The number of patients included in this analysis on ^{99m}Tc-Hynic-rh-Annexin V scintigraphy in advanced stage HNSCC is therefore 13. The mean age of patients was 53 years (range 26-66 years). See table 1 for the patient, tumor and treatment characteristics.

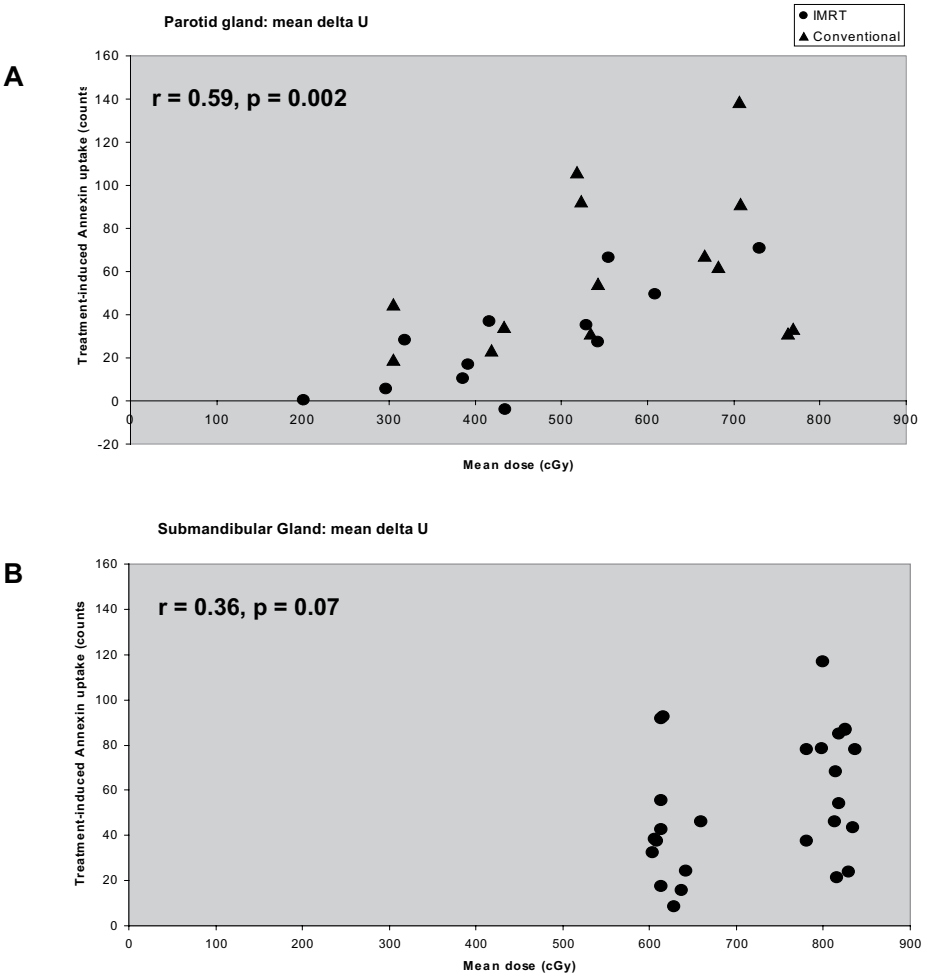
ANNEXIN-UPTAKE IN NORMAL TISSUE AND TUMOR

At baseline scintigraphy, reproducible physiologic Annexin-uptake was detected on the planar images in liver, kidneys, spleen, gall bladder, bone marrow, colon, and urinary bladder, as described earlier [7,14,21].

In the parotid glands, weak baseline uptake was present in all cases. The post-treatment TAVS showed moderate to strong increase in uptake in 24 of 26 parotid glands (figure 2A). The average of the mean number of counts increased from 55 to 88 ($p<0.001$). Visual analysis of the increase in uptake showed that these changes were related to the radiation portals and the dose given to the parotid glands (figure 3). The difference in Annexin-uptake between the post-treatment and baseline TAVS (ΔU) in each parotid gland was correlated with the radiation dose at the time of post-treatment scintigraphy (n=13 patients, n=26 parotids). The mean ΔU showed a positive correlation with the mean radiation dose (Pearson coefficient $r=0.59$, $p=0.002$): parotid glands that received a higher dose of radiation showed a higher Annexin-uptake (figure 4A). The increase in Annexin-uptake in parotid glands that were treated with parotid-sparing IMRT was less than in parotid glands that were treated with a conventional 3-field technique (IMRT 29 counts, 3-field technique 59 counts, $p=0.02$). The given radiation dose at the time of post-treatment TAVS (6 or 8 Gy) and the mode of cisplatin administration (IA or IV) did not affect the uptake in parotid glands. Since xerostomia scoring was not documented sufficiently detailed in the files, we interviewed patients alive at last follow-up for xerostomia grading for the purpose of this study. In 9 patients alive at last follow-up, the treatment-induced Annexin uptake in parotid glands was related to the subjective xerostomia rating using the EORTC QLQ HN-35 questionnaire [22]. No relation could be established in this small set of patients (Data not shown).

For the submandibular glands a similar pattern as with the parotids was observed: absent/weak baseline uptake and moderate to strong increase after the start of chemoradiation (figure 2B). The average of the mean number of counts increased from 85 to 132 ($p<0.001$). No correlation between the ΔU and radiation dose was noted (figure 4B), probably since all submandibular glands were located within the high dose region.

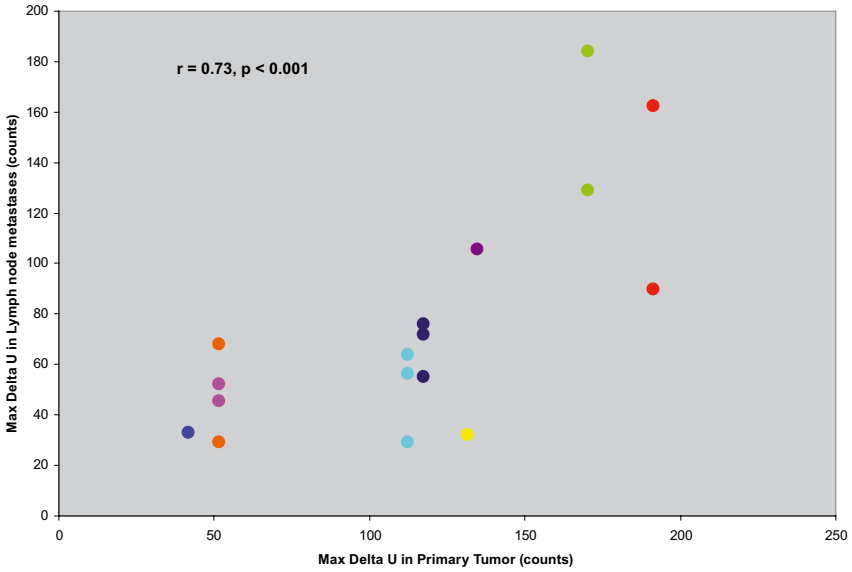
Figure 4. Correlation plot of mean radiation dose (cGy) and the post-treatment increase in Annexin uptake, mean ΔU, in parotid glands (Panel A), normalized for tracer dose and body weight. Circles represent parotids glands treated with IMRT, triangles parotids treated with conventional technique. In Panel B, the correlation between mean radiation dose (cGy) and the post-treatment increase in Annexin uptake, mean ΔU, in submandibular glands is given.



The baseline TAVS showed moderate to strong Annexin-uptake in the primary tumor in all patients, indicative of spontaneous apoptosis or necrosis. On the post-treatment TAVS, the uptake in tumor clearly increased in 9 cases; in the other 4 little or no changes occurred (figure 2C). The average of the maximum number of counts increased from 127 to 163 (p=0.007). The difference between post-treatment and baseline TAVS (ΔU) in primary tumor was not correlated with primary tumor volume (Pearson coefficient r=0.39, p=0.21) nor with the mode of chemotherapy administration (p=0.21). In 9 patients, 17 lymph node metastases were present in the neck (mean lymph node volume 5 cm^3, range 1–19).

The treatment-induced Annexin-uptake (maximum ΔU) in primary tumor was positively correlated with the uptake in corresponding lymph node metastases (r=0.73, p=0.004) as shown in figure 5. To verify that the observed effects of Annexin-uptake in different structures could not be attributed solely to the administered radioactive dose per patient but was tissue specific, we calculated the correlation between the ΔU in tumor and parotid glands. No evidence of such an effect was found (r=0.28, p=0.18).

Figure 5. Correlation plot of the treatment-induced increase in Annexin-uptake, maximum ΔU, in primary tumor and lymph node metastases. Each colour represents an individual patient. Some patients had multiple lymph node metastases originating from the same primary tumor. In these cases, multiple values of treatment induced Annexin uptake in lymph nodes (on the y-axis) correspond to a single value of the primary tumor (on the x-axis).



RESPONSE TO TREATMENT, LOCOREGIONAL CONTROL AND SURVIVAL

The median follow-up of all patients alive at last follow-up was 30 months (range 24–38). The complete response (CR) rate at the primary tumor site was 85%. CR rate in the neck in the case of nodal metastases was 100%. The overall CR rate was 85%. The estimated locoregional (LR) tumor control rates were 68% at 2 and 3 years. The rates for disease free survival (DFS) were 54% at 2 and 3 years. Overall survival (OS) rates at 2 and 3 years were 62% and 40%, respectively. In this small subset of 13 patients from a larger randomized phase III trial, there were no differences in response rates, LR control, DFS or OS between the two treatment arms (IV versus IA). No correlation could be established between baseline or treatment-induced Annexin-uptake (ΔU) and any outcome parameter (response rate, recurrences and/or survival).

DISCUSSION

This study represents our first clinical experience on *in vivo* imaging of apoptosis with TAVS in HNSCC patients, in which we applied co-registration of multiple imaging modalities. By this, we were able to analyze treatment-induced changes in normal and tumor tissue, early during therapy, after 1 cycle of cisplatin chemotherapy and 6-8 Gy of radiotherapy. Treatment-induced Annexin-uptake in the parotid glands could be visualized, indicative of early treatment related apoptosis at mean radiation doses as low as 3-8 Gy (see figure 4A) and one course of cisplatin. The Annexin-uptake in the parotids showed a radiation dose-response relationship: Glands that had received higher doses of radiation demonstrated increased Annexin-uptake.

Loss of parotid gland function, leading to xerostomia is an important long-term side effect of radiotherapy, affecting quality of life of patients. It has well been shown by scintigraphy studies [23] and by salivary flow rate studies [24] that reduction of radiation dose to the parotid glands can maintain parotid function, decrease xerostomia and improve quality of life. Most studies on salivary gland function are performed after a full course of RT (typically 60-70 Gy in 6-7 weeks) [23-26]. Our study design with early in vivo imaging indicated that already after low dose of RT (6-8 Gy) parotid glands may be affected and that the physiologic process leading to loss of parotid gland function already starts early. This is in agreement with observations in experimental rodent studies, in which apoptosis was induced early, after doses of up to 5 Gy [27]. Similar results were obtained in a monkey model, with early radiation induced apoptosis of serous acinar cells of salivary glands [28]. It was suggested that these early effects are mainly responsible for the acute sialoadenitis, which at higher RT doses might progress in chronic xerostomia by damage to and depletion of ductal stem cells within the gland. In the 9 patients assessed for xerostomia, no relation could be established with subjective salivary gland function after radiotherapy and parotid gland Annexin uptake. However, the significance of this analysis is limited since we assessed both parotid glands separately by TAVS and xerostomia questionnaires give an overall impression of function. We did not include follow-up salivary flow studies. In short, our results indicate indirect evidence of early radiation-induced salivary gland damage and, in future studies, this needs to be correlated with functional outcome parameters.

Due to the large interpatient variability in the baseline TAVS (figure 2C), the subtraction method for assessing treatment induced changes was chosen over a relative method (e.g. percentage change). In this figure, it is important to notice that the maximum uptake value of the baseline and post-treatment scan might be located in different points within the tumor ROI. Therefore, these data can not be simply subtracted, but for the calculation of the maximum ΔU in primary tumor and lymph nodes, the baseline scan was first subtracted from the post-treatment scan and subsequently these parameters were calculated from the subtraction scan. The treatment-induced Annexin-uptake in

primary tumor and pathological lymph nodes showed a positive correlation: Patients with primary tumors with a high Annexin-uptake also appeared to have lymph node metastases with high Annexin-uptakes. The ΔU in primary tumor and lymph node metastases showed large inter-patient differences (figure 5). This variation could not be attributed to differences in the route of cisplatin administration (intravenous or intra-arterial chemoradiation), nor to the radiation doses given (6 or 8 Gy), nor to the primary tumor volume. This positive correlation for ΔU in primary tumor and lymph nodes as well as the large inter-patient differences might represent a tumor-specific apoptotic response.

In our series, repositioning of the patient during SPECT scan in RT position was standard procedure, but due to the limitations in fixation not optimal. The repositioning facilitated the co-registration of SPECT and CT-scan. In one patient, misalignment occurred because the positioning was not accurate due to the fact that the RT position could not be reproduced (figure 1). So, for co-registration of (functional) imaging to RT-planning CT scan data in HNSCC, we recommend improvements from our protocol to obtain a reproducible positioning, like the use of an immobilization mask fixed to the SPECT table, the use of the same mattress under the patient, and a laser alignment system. In ongoing studies we have now incorporated these. In the current series, no attenuation correction was applied because the images were obtained on a conventional gamma camera, without a hybrid system. For the purpose of this study, the quantification of Annexin-uptake remains valid, since we used the relative increase from baseline to post-treatment uptake and the degree of attenuation will be equal on both scans.

In this small series of HNSCC, the treatment-induced Annexin-uptake (ΔU) in primary tumor did not predict outcome: no correlation between Annexin-uptake and early response was observed, probably partly due to the high initial response rates (85%). In addition, TAVS also did not predict the locoregional control rates within the first 2 years of follow-up. In our previous experience on TAVS in other tumor sites (mainly lymphoma), a strong correlation has been found between early response (within 3 months) and Annexin-uptake [15]. One explanation for the lack of correlation might be that in the current series these advanced stages HNSCC with large tumors harbor more necrosis, which is also detected by TAVS because of accessibility of PS at the necrotic cell membrane [29]. Van de Wiele et al. showed that in HNSCC, Annexin-scintigraphy correlated with histopathological apoptosis (using TUNEL assay) only in the absence of necrosis [8]. Indeed, in this series, baseline Annexin-uptake in the HNSCC patients was clearly demonstrated, whereas it was not present in our previous study in lymphoma patients [15]. This may suggest the presence of necrosis in large tumors from solid origin. Other factors that may affect Annexin-uptake are the intra- and peri-tumoral lymphocyte infiltration [30]. For the future, we plan to increase the number of HNSCC patients to undergo the TAVS imaging, in order to prove whether or not it can be used for outcome prediction as it was shown in patients with malignant lymphoma and non-small cell lung cancer [14,15].

In conclusion, co-registration of Annexin V scintigraphy with radiotherapy-planning CT-scan showed a radiation-dose-dependent uptake in parotid glands, indicative of early apoptosis during treatment. The interindividual spread in Annexin-uptake in primary tumors could not be related to differences in treatment schedule or tumor volume, but the Annexin-uptake in tumor and lymph nodes were closely correlated. This effect might represent a tumor-specific apoptotic response.

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chapter 9

General discussion and conclusions

Frank J.P. Hoebers

GENERAL DISCUSSION AND CONCLUSIONS

The purpose of the work described in this thesis was to investigate possible prognostic and predictive factors in patients with HNSCC. The identification of these factors is important since they may lead to individualized therapy, tailored to the individual patient. The need for prediction of outcome and prediction of response to therapy is increasing because of a number of factors.

Firstly, treatment efficacy has been improved by more intense treatments (like concurrent chemoradiation) but also the toxicity is increased in these regimens. Therefore, identification of patients who are predicted to benefit or not from this therapy is warranted. Although the results generally have improved, still a large proportion of patients fail to respond to the intensified therapy. A toxic, yet non-effective therapy may be withheld from a patient if a favorable outcome after treatment is unlikely based on the result of a predictive assay. Consequently, the therapy may be adapted according to the assay: This can either be a further increase in the intensity of therapy (e.g. an increase in dose or altered fractionation scheme of radiotherapy or a higher dose or other type of chemotherapy) or another type of treatment might be chosen. So, with this approach, prescribing a non-effective treatment to the patient may be prevented.

Secondly, intensified therapies may be regarded as "over-treatment" in a subset of patients that are selected for this treatment, based on "classical" or "standard" criteria like the TNM-staging system. For example, within the subset of patients with T4 tumors, the chances of cure with standard therapy might be very high for some patients and intensified toxic treatments might not be necessary. Better and more detailed information of (biological or other) tumor characteristics may be helpful in this.

Thirdly, the development of so-called biological or targeted therapies has generated increased interest in prediction. The rationale of these therapies is to target a (tumor-) specific pathway or biological process that is of interest in the tumor under investigation/ treatment. This approach requires that the pathway c.q. process is present at least to some extend in the tumor if activity is anticipated. E.g. a targeted therapy that inhibits a growth factor receptor like EGFR or that promotes apoptosis will only be active if this target or process is present within the tumor. Therefore, identification of the presence of the target may be regarded as a predictive factor.

CISPLATIN-DNA ADDUCTS

In studies on the predictive value of cisplatin-DNA adducts varying results have been reported: In some series, a positive association was observed between the amount of adducts formed and response [1-3]. Other series however found a negative [4] or no association [5,6]. In almost all of these studies, adducts were measured in normal tissue.

In **chapter 2**, we measured adducts both in normal tissue and in tumor in patients with advanced stage HNSCC treated within the frame work of a randomized trial on different routes of administration of cisplatin-based chemotherapy, concurrently with radiation. The clinical hypothesis of this trial was that selective intra-arterial (IA) infusion of high dose cisplatin (150 mg/m^2) with systemic rescue by sodium-thiosulphate (STS) would result in improved locoregional control as compared to standard dose IV (100 mg/m^2) cisplatin-chemotherapy, without increased treatment-related toxicity. In a subset of patients from that trial, adduct levels were evaluated.

The main findings of this study were that the adduct levels in the primary tumor were not significantly different between patients with IA and IV administration of cisplatin, despite the targeted delivery of high dose cisplatin during IA administration. In addition, higher adduct levels were observed in white blood cells (WBC) and in buccal cells after IV cisplatin, compared to those after IA infusion. These observations on differences in adduct formation between the 2 treatment-arms are now confirmed by the clinical outcome of the trial: The IA treatment did not result in improved locoregional control or survival, but the systemic (mainly renal) toxicity after selective IA cisplatin administration with rescue by STS was decreased [7].

With regard to the predictive value of cisplatin-DNA adducts: Levels of adducts in WBC were not predictive for outcome. However, Cisplatin-DNA adduct formation in primary tumor appeared to be predictive for outcome: Higher adduct levels in tumor resulted in improved disease free survival compared to lower adduct levels.

The rationale for determining adducts in normal tissue is that it might be useful as a surrogate marker for tumor tissue with respect to its predictive value. If so, cisplatin-DNA adduct assessment in easily obtained normal tissue samples like WBC or buccal cells would be preferable over adduct assessment in primary tumor, since these might be poorly accessible for direct biopsy. Furthermore, the invasive nature of obtaining biopsies during treatment will limit the clinical applicability in larger patient series.

However, in our experience described both in **chapter 2** and **chapter 3**, we could not establish a positive association between adducts in primary tumor and in normal tissue. This lack of correlation between adducts in tumor and normal tissue was also found in an experimental animal study [8], demonstrating no association between tumor platinum content and serum platinum concentration. The explanation of this is unknown, but we hypothesized that in the process of adduct formation in tumor and normal tissue different mechanisms would play a role. The formation of adducts will in part be determined by the total administered dose and exposure to cisplatin. However, between tumors and normal tissues differences will exist in the capability of cisplatin-uptake within the cell and nucleus, in the rate of adduct formation and in the capacities to repair damage from cytotoxic agents. The observed adduct levels as based on a single measurement in time, is the net resultant of total adduct formation and adduct repair. The reported lack of correlation between tumor and normal tissue may therefore be the explanation why

some [1-3,9] but not all studies [5,6] on the predictive value of adduct formation in normal tissue were positive. A limitation of our study is that we sampled normal tissue and tumor on a single time point only. Therefore, no information is available on the total exposure to adducts over time (analysis based on the "area under the curve").
From our work it is concluded that intra-tumoral adduct levels appear to be predictive for outcome after concurrent chemoradiation for advanced stage HNSCC. Previous work has demonstrated that adducts in normal tissue may be associated with outcome. However, we could not demonstrate a correlation between the adduct levels in tumor and normal tissue. Therefore, at present, the data concerning the use of cisplatin-DNA adducts as predictive assay can not be regarded as conclusive. Application of this test can not be performed routinely in clinical practice and further exploratory adduct studies would be needed.

CONCURRENT CHEMORADIATION WITH DAILY LOW DOSE CISPLATIN

In **chapter 4**, the results of treatment with concurrent chemoradiation with daily low dose cisplatin are described in a series of 121 consecutive patients with advanced stage HNSCC. This treatment was given outside the context of the randomized phase III RADPLAT trial on intravenous vs. intra-arterial chemoradiation. Patients who were ineligible for this trial or refused participation in the trial were treated with the daily low dose cisplatin regimen. The treatment protocol with the instruction to patients for adequate oral fluid intake was changed into the administration of IV prehydration at each cisplatin-infusion, since we observed frequent premature cessation of cisplatin-administration due to toxicity. In addition, the overall treatment time of radiotherapy was changed from 7 to 6 weeks, since the importance of acceleration of RT was recognized [10].
From this series we concluded that addition of accelerated RT resulted in more mucositis and tube feeding, but the introduction of prehydration led to better compliance to therapy with more chemotherapy administered and less hospital admissions.
The locoregional control rates were 55% and 55% at 2 and 3 years, respectively. Disease free survival was 36% and 33% at 2 and 3 years, respectively. Overall survival was 41% and 36% at 2 and 3 years, respectively. In uni- and multivariate analysis, the following factors were significantly associated with improved disease free survival: Hypopharynx/oropharynx site (compared to other sites, mainly oral cavity), less advanced TNM stage and lower ASA-comorbidity classification. Although the complete remission rates in the group treated with hydration and acceleration appeared to be improved compared to the standard group (90% vs. 74%, p=0.06), loco-regional control and survival were not statistically different for the 2 different treatment protocols.
The major cause of death remained cancer-related (in 72% of cases), mainly due to locoregional tumor recurrence (in 38% of patients), due to distant metastases (in 27% of

patients), or both (7%), emphasizing the need for improving treatment results by better selection of patients and developing more efficacious treatment protocols.
The results of our treatment appear comparable with the results obtained in other series on daily low dose cisplatin concurrently with radiation [11,12] or with the preliminary results from the randomized intra-arterial vs intravenous chemoradiation trial [7]. However, comparison of these series should be performed with care due to the selection of different groups of patients for each series. The patients described in chapter 4, treated with the daily low dose cisplatin chemoradiation protocol obviously represented a negative selection of patients, since more than three quarters were ineligible for the randomized trial, because of odd head and neck tumor site, previous vascular history, recurrent tumors, synchronous tumors, 2nd primary tumors, or poor mental and general condition. Moreover, the patients in our series were 7 years older than in the previous RADPLAT phase II trial [13]. The differences in trial participants and non-participants have been investigated by others, showing that the distribution of prognostic factors were very heterogeneous: trial participants were younger and had better performance status and less comorbidity compared with non-participants [14]. However, in 2 large overview studies on the outcomes of trial-participants and non-participants in cancer treatments, it could not be shown that enrolment in clinical trials would lead to improved outcome [15,16].

TUMOR VOLUME

The indications for concurrent chemoradiation are usually based on TNM-staging criteria. These mainly include advanced TNM-staging, i.e. T3-4 stage. However, within these categories of patients, it appears that the discriminative properties of the TNM-system may not be optimal, since it was demonstrated in patients treated with intra-arterial chemoradiation that T-stage did not predict outcome [17,18]. Therefore in **chapter 5**, we tested the prognostic value of pre-treatment tumor volume, as derived from MRI in a subgroup of 46 patients treated with daily low dose cisplatin-based chemoradiation. In a number of previous studies in HNSCC treated by radiation only, tumor volume was associated with local control [19-21].
It was shown that tumor volume was an important factor, determining locoregional control and disease free survival: Larger tumors were associated with worse locoregional control and survival. In multivariate analysis, the predictive value of tumor volume on locoregional control and disease free survival remained present.
The association between tumor volume and disease outcome has now been established for several tumor sites of HNSCC treated by radiation only, including larynx [20,22-26] and hypopharyngeal carcinoma [21,27]. In oropharyngeal carcinoma treated by radiation, some studies showed that tumor volume was not associated with local control [19,28,29] and others did show such association [30]. In addition, tumor volume also predicted

outcome in HNSCC patients treated with intra-arterial chemoradiation [19-21], whereas T-stage could not. Furthermore, in other tumor sites like esophageal cancer [31] and NSCLC [32], tumor volume also had an impact on outcome.

Implementation of tumor volume as prognostic factor is hampered by difficulties in extrapolation of the results from mainly single institution reported series towards broad applicable daily practice, since in all series different cut-points were used to distinguish large and small tumors. Another limitation is the fact that volumes derived from MRI are significantly smaller than those delineated on CT-scans [33,34]. However, these challenges can be overcome by prospective studies that include tumor volume and by adding a correction factor when comparing CT and MRI volumes.

In summary, it appears that tumor volume is an important prognostic factor in HNSCC, treated by chemoradiation. Therefore, this parameter should be incorporated in the staging system and could be used to guide treatment in future treatment protocols. In patients with T3-4 stage disease scheduled for chemoradiation, incorporation of tumor volume may direct the necessity for intensified treatment. In case of a small T3-4 tumor, standard concurrent chemoradiation may be sufficient effective to yield disease control. In patients with large tumors on the other hand, treatment intensification could be considered, e.g. by adding another cytotoxic agent or biological to chemoradiation or by the use of induction chemotherapy before concurrent chemoradiation [35]. The effects of tumor volume on outcome are not limited to primary tumor volume only, since it was also shown that nodal volume was associated with regional control in patients treated with radiotherapy alone or with chemoradiation [36].

TUMOR HYPOXIA

Tumor hypoxia has been identified as an adverse prognostic factor since it can promote resistance to therapy and lead to tumor progression. The presence of hypoxia has been associated with worse locoregional control, disease free or overall survival in several tumor types including HNSCC [37-39], cervical uterine carcinoma [40,41] and sarcoma [42,43]. The negative effects of hypoxia on outcome have been observed in series on radiotherapy [37,38], but also chemotherapy [44] or surgery [45]. Two types of hypoxia are recognized: acute and chronic hypoxia. Acute hypoxia occurs due to the temporary (opening and) closing of blood vessels leading to decreased perfusion and acute changes in oxygenation. The chronic type of hypoxia is related to deprivation of oxygen in tumor areas, due to poorer diffusion to cells at a larger distance from the nearest blood vessels.

Tumor hypoxia is not only identified as an adverse prognostic factor, it also represents a target for new treatment concepts. In a randomized trial from the Danish Head and Neck Cancer Group, the hypoxia radiosensitizer nimorazole significantly improved the outcome of radiotherapy compared with placebo [46]. Another example is the use of ARCON, an acronym of Accelerated Radiotherapy and Carbogen breathing with Nicotinamide

[47]. Carbogen will affect the chronic hypoxia by increasing the blood oxygenation and nicotinamide will diminish acute hypoxia by its vasodilatatory properties. In a meta-analysis on the results of 83 randomized trials [48], investigating several oxygen-modifying therapies in more than 10,000 patients with several tumor sites, it was shown that modification of tumor hypoxia significantly improved the locoregional tumor control after radiotherapy, most pronounced in head and neck cancer. Overall survival was also significantly improved by hypoxic modification.

Tumor oxygenation can be assessed by different methods. It is frequently measured with an Eppendorf electrode, which is an invasive procedure, limiting its application in routine clinical practice. However, the data on the negative impact of hypoxia on patient outcome have been greatly established on series using the Eppendorf electrode method [37-43].

Hypoxia can also be examined by analysis of endogenous hypoxic markers using immunohistochemical staining on tumor sections. Expression of HIF-1α was associated with poorer survival in HNSCC [49]. Other markers that have been used include GLUT-1 [50,51] and CA IX [52].

Measuring tumor hypoxia by non-invasive methods can also be performed by the use of exogenous bioreductive markers, such as the 2-nitro-imidazole derivatives like pimonidazole [53,54] and EF5 [55]. These markers are administered to the patient, taken up in hypoxic areas of the tumor and can be visualized by immunohistochemical staining in tissue sections. Prognostic significant associations between 2-nitro-imidazole markers and clinical outcome have been established for soft tissue sarcoma [56], brain tumors [57] and HNSCC [58]. In contrast, no correlation between pimonidazole staining and outcome was observed for cervical cancer [59]. In the Netherlands Cancer Institute, a study on the prognostic value of pimonidazole staining in HNSCC patients treated with surgery has recently been completed and the results are awaited as follow-up will mature. One disadvantage of the use of endogenous and exogenous hypoxic markers is that it requires tumor tissue for analysis as obtained by surgery or by biopsy. A recent study discovered an indirect marker of tumor hypoxia in osteopontin plasma level, a factor associated with a worse prognosis in patients treated with radiotherapy [60].

Given the large number of markers or assays that can be used to identify patients with hypoxic tumors from those with oxic tumors, it is important to know whether these tests show inter-assay correlation. Previously, we have shown that different measures of hypoxia (pimonidazole staining, HIF-1α expression and the so-called diffusion-limited fraction (i.e. the fraction of cells farther than a certain distances from the nearest blood vessel, a measure of chronic hypoxia) did not show correlation within individual patients [61]. In a recent study by Nordsmark et al. [62], five hypoxic markers predictive for outcome were compared and correlated with outcome after radiotherapy in 67 HNSCC patients. These included tumor oxygenation using pO_2 measurements by Eppendorf-electrode, HIF-1α expression, CA IX expression, plasma osteopontin levels and tumor osteopontin expression. Diversity and lack of correlation among the 5 hypoxic markers was observed

within individual tumors. An association with poorer locoregional control was seen for low pO_2, high HIF-1α expression and high plasma osteopontin, whereas CA IX expression and tumor osteopontin failed to predict outcome. These data indicate that accurate assessment of hypoxia is still a challenge. Although individual markers have been associated with outcome, apparently these markers do measure different biological phenomena related to hypoxia that all affect outcome. These differences may e.g. be related to the presence or absence of acute vs. chronic hypoxia.

Non invasive hypoxia imaging using radioactive labeled hypoxic markers allows determination of the oxygen status of malignant tumors in vivo and represent an attractive tool to visualize hypoxia pre- and/or during treatment [63-65]. It can be applied in patients scheduled both for surgery and radiotherapy and it offers the possibility to observe changes in hypoxia during treatment. In future it might be used to guide the use specific hypoxia-directed therapies. Examples of tracers used for in vivo imaging of hypoxia include ^{18}F-misonidazole PET [63,64] and Cu-ATSM [66,67]. In a number or series on HNSCC, it was shown that hypoxic tumors defined by increased ^{18}F-misonidazole uptake had worse survival and/or higher locoregional failure rates [63,64]. In the study by Rischin et al. [64], patients that had received tirapazamine-chemotherapy (a cytotoxic compound that is activated specifically under hypoxic circumstances) had significantly lower locoregional failure rates, indicating that hypoxic modification can alter sensitivity to therapy. So, non-invasive in-vivo imaging of tumor hypoxia represents an attractive and promising strategy to identify patients who have a worse prognosis and might need additional therapy to overcome hypoxia-related resistance to radiation or chemotherapy.

In **chapter 6**, we investigated the use of ^{99m}Tc-BRU 59-21, a novel 2-nitro-imidazole agent, as a marker for hypoxia and compared this with pimonidazole staining (another 2-nitro-imidazole derivative). It was shown that in vivo uptake of ^{99m}Tc-BRU 59-21 was observed on SPECT scanning in patients with HNSCC scheduled for surgery. The in vivo uptake of the radioactive labeled 2-nitro-imidazole agent was significantly correlated with staining for the other hypoxic marker pimonidazole on stained histological tumor sections, obtained after surgery. Thus, it appears that in-vivo hypoxia imaging using ^{99m}Tc-BRU 59-21 can identify hypoxic tumors as it was previously shown that it also localizes in tissues with a low oxygen concentration due to ischemia [68,69] or in animal models representative of poorly perfused tumors [70]. Although this was only a pilot study, designed for proof of principal, a larger study would be needed to investigate the prognostic value of this in vivo hypoxic marker.

A limitation in the use of hypoxia markers is the fact that the acquired images only provide a snap-shot on the presence of hypoxia within the tumor on a single point in time. It has been shown however by repeated imaging that the hypoxic sub-volumes within tumors can both change in location [71] and decrease in intensity [72] during the course of therapy, reducing the potential utility of pre-therapy planning of hypoxia-driven dose painting to boost the more radio-resistant hypoxic tumor areas [73].

In **chapter 7**, we investigated staining patterns of the hypoxia marker and bioreductive drug pimonidazole. This is important since 2-nitro-imidazole staining of misonidazole (a closely related analogue of pimonidazole) was also observed in normal, presumably normoxic tissues [74] and in keratinizing areas of stratified squamous epithelia [61,75,76], raising the question whether this is hypoxia-related and specific. This could influence quantitative hypoxia estimates using pimonidazole. One possible explanation for the staining in keratinizing areas was the presence of elevated oxygen-independent reductase levels, which might cause non-specific hypoxia-unrelated staining of pimonidazole [75,76]. We therefore investigated whether the differentiation-related staining was caused by locally high reductive enzyme levels. We did indeed observe hypoxia-independent pimonidazole staining in more differentiated, keratinizing head and neck tumors, but could not prove that this was related to locally high reductase levels. However, it does show that pimonidazole may give an overestimate of the true hypoxic fraction in some head and neck tumors due to differentiation-associated staining. The extent of this artifact remains unclear. Therefore, future studies on correlations of pimonidazole staining with outcome after therapy could help clarify this.

ANNEXIN-V SCINTIGRAPHY AND APOPTOSIS

Apoptosis is an important mechanism, by which malignant and normal cells can respond to radiation therapy and chemotherapy. Several studies have tried to investigate whether apoptosis is associated with treatment outcome or prognosis. One way of apoptosis detection is by the use of radiolabeled Annexin V [77-80], an endogenous human protein with a high affinity for membrane-bound lipid phosphatidylserine (PS). This lipid is exposed at the outer leaflet of the plasma membrane bi-layer at an early stage of the apoptotic process [81,82]. Early detection of apoptosis during treatment might be indicative of the effectiveness of therapy. In previous studies we have demonstrated that ^{99m}Tc-Hynic-rh-Annexin V scintigraphy correlates with radiation-induced cytologically confirmed apoptosis in non-Hodgkin lymphoma [83] and can be used to identify patients that have a favorable prognosis in patients with lymphoma [84] and NSCLC [85].
In **chapter 8**, we have applied ^{99m}Tc-Hynic-rh-Annexin V scintigraphy in 13 patients with advanced stage HNSCC treated by concurrent chemoradiation. Baseline Annexin uptake was compared with Annexin uptake after 1 course of cisplatin and 6-8 Gy of radiotherapy for primary tumor, lymph node metastases, and normal tissues (salivary glands). By co-registration of the Annexin-V SPECT-scan and radiation-planning CT-scan we were able to analyze patterns of Annexin uptake in relation to radiation dose at the time of follow-up Annexin scanning. Treatment-induced Annexin-uptake in the parotid glands could be visualized, indicative of early treatment related apoptosis at doses as low as 3-8 Gy and one course of cisplatin. The Annexin-uptake in the parotids showed a radiation dose-response relationship: Glands that had received higher doses of radiation demonstrated

increased Annexin-uptake. Unfortunately, no correlation could be made between treatment-induced salivary gland apoptosis (as expressed by Annexin-uptake) and loss of parotid function (as expressed by patient-reported xerostomia-grading, scintigraphy parotid-functional studies or salivary flow rate methods), since this was not included in the study design. However, our study indicated that already after low doses of RT (6-8 Gy) parotid glands may be affected and that the physiologic process leading to loss of parotid gland function already starts early.

The baseline Annexin-scintigraphy showed moderate to strong Annexin-uptake in the primary tumor in all patients, indicative of spontaneous apoptosis or necrosis. On the follow-up Annexin scan, the uptake in tumor increased in 9 cases; in the other 4 little or no changes occurred. The treatment-induced Annexin-uptake in primary tumor and pathological lymph nodes showed a positive correlation: Patients with primary tumors with a high Annexin-uptake also appeared to have lymph node metastases with high Annexin-uptakes. This positive correlation as well as the large inter-patient differences might represent a tumor-specific apoptotic response.

The treatment-induced Annexin-uptake in primary tumor did not predict outcome: no correlation between Annexin-uptake and early response was observed nor with 2-year locoregional control rates. One explanation for the lack of correlation in our present study might be the presence of necrosis in these large and advanced tumors, which is also detected by Annexin-scintigraphy [86]. In a study by Van de Wiele et al. in HNSCC, it was demonstrated that Annexin-scintigraphy correlated with histopathological apoptosis (using TUNEL assay) only in the absence of necrosis [80]. In our series we indeed observed substantial baseline Annexin-uptake, whereas this was not present in our previous study in lymphoma patients [84].

In our previous experience on Annexin-scintigraphy in lymphoma and NSCLC patients treated with radiotherapy or chemotherapy, a strong correlation has been found between early response (within 3 months) and treatment-induced Annexin-uptake [84,85]. Similar results were obtained by Belhocine et al. [87], who investigated treatment-induced Annexin-uptake in patients with breast cancer, lymphoma and lung cancer treated with chemotherapy and showed that increased uptake after the first course of chemotherapy was associated with better survival. Rottey et al. evaluated pre-treatment Annexin-scintigraphy in patients with several different malignancies, treated with radiotherapy or chemotherapy and observed a higher tumor-to-background ratio in early responders compared to non-responders [88].

In summary, ^{99m}Tc-Hynic-rh-Annexin V scintigraphy represents a non-invasive test to visualize in-vivo treatment-induced apoptosis during anti-cancer therapy, both in tumor and normal tissue. Annexin uptake may be a marker of early treatment-induced salivary gland damage, leading to salivary gland dysfunction and xerostomia. No association was found between Annexin uptake in tumor and outcome, but a positive correlation was observed for Annexin uptake in primary tumor and lymph nodes. For the future, we plan

to incorporate Annexin scintigraphy in new treatment strategies that include agents that have pro-apoptotic properties. An example of this is a drug called Gossypol [89], which is a specific and potent inhibitor of Bcl-X_L and Bcl-2. Expression of these 2 proteins lead to inhibition of apoptosis and over-expression has been reported in many malignant tumors, including HNSCC [90-95] and may lead to resistance to anti-cancer therapy [96]. By inhibition of the anti-apoptotic properties of Bcl-X_L and Bcl-2, Gossypol was identified as an agent that selectively induces apoptosis in cancer cells. In animal studies, Gossypol showed anticancer activity, which was enhanced by the combination with cytotoxic agents like cisplatin [97] or radiation [98]. Therefore, Gossypol appears as an attractive drug for concomitant use with concurrent cisplatin-based chemoradiotherapy.

CONCLUSIONS

The treatment of head and neck cancer patients has been changed greatly during the last decades. From surgery (with or without postoperative radiotherapy) or radiotherapy, the treatment has evolved into combined modality treatment for a large proportion of patients. This includes surgery with postoperative chemoradiation and to a larger extend organ-preserving strategies combining chemotherapy and radiotherapy, usually with concurrent cisplatin-based chemoradiation. The results of treatment have improved by these intensifications, but also the side-effects have increased considerably. Therefore, the selection of patients for the available treatment options is increasingly important. In this thesis, a number of studies are presented that aimed at applying or developing prognostic and predictive tests in order to be able to improve the (future) selection of patients for the appropriate treatment.

FROM THE WORK DESCRIBED IN THIS THESIS THE FOLLOWING CAN BE CONCLUDED.
The predictive value of the formation of cisplatin-DNA adducts was studied in patients treated with concurrent chemoradiation. Adducts in tumor were significantly higher than in normal tissue (white blood cells and buccal cells). In patients treated within the randomized trial on intra-arterial vs. intravenous chemoradiation, lower adduct levels were observed in normal tissue after selective high-dose intra-arterial cisplatin with systemic rescue by sodium-thiosulphate compared to standard dose intravenous chemoradiation, indicating that the intra-arterial administration of cisplatin indeed diminished the systemic exposure to cisplatin. Adduct levels in primary tumor were not statistically different after high-dose selective intra-arterial administration compared to standard intravenous administration. Analysis of the correlation between adducts and outcome showed that higher intra-tumoral cisplatin-DNA adducts appeared to be associated with improved disease free survival. In patients treated with intravenous schedules of cisplatin-based chemoradiation, no correlation was observed between adducts in normal tissue and tumor, suggesting that for this purpose normal tissue samples (like white blood cells) can

not be used as surrogate markers for tumor. Therefore, at present, the data concerning the use of cisplatin-DNA adducts as predictive assay can not be regarded as conclusive. Application of this test can not be performed routinely in clinical practice and further exploratory adduct studies would be needed.

In patients treated with concurrent chemoradiation with daily low dose cisplatin administration, we have demonstrated that the addition of intravenous hydration improves compliance to therapy with more chemotherapy administered, decreased frequencies of nephrotoxicity and less hospital admissions due to toxicity. The introduction of accelerated radiotherapy however resulted in more mucositis and more frequent tube feeding. In this patient population, it was demonstrated that primary tumor volume derived from pre-treatment MRI-scan was an important prognostic factor for both locoregional control and disease free survival.

The in-vivo detection of intra-tumoral hypoxia in patients scheduled for surgery by the use of the radioactive labeled hypoxia marker BRU 59-21 showed an association with another established hypoxia marker, pimonidazole, on tumor tissue sections obtained after surgery, suggesting that this in-vivo marker could be used for non-invasive detection of hypoxia.

In the staining for pimonidazole, non-specific non-hypoxia related staining may occur in keratinizing areas of squamous cell carcinoma, which may lead to an overestimation of the true hypoxia, but we could not prove that this non-specific staining was related to the presence of high reductase levels.

Non-invasive, in-vivo imaging of treatment-induced apoptosis by Annexin-scintigraphy during concurrent chemoradiation showed a radiation-dose dependent Annexin-uptake in parotid glands, indicative of early treatment-induced salivary gland damage. The degree of Annexin-uptake in primary tumor and lymph node metastases was positively correlated, which may imply that the propensity for apoptosis is tumor-specific. No association could be established between Annexin-uptake and outcome, probably due to the presence of necrosis in these advanced head and neck cancers. At present, Annexin-scintigraphy for HNSCC can not be incorporated into treatment protocols outside the context of clinical trials.

FUTURE DIRECTIONS

CISPLATIN-SENSITIVITY

Conflicting results have been obtained in cisplatin-DNA adducts studies: some studies showed a positive association between adducts in normal tissue and outcome, others showed no or even a negative association. We showed that intra-tumoral adducts were associated with outcome, but we could not establish correlations between adducts in tumor and normal tissue. Due to the limitations in the use of cisplatin-adducts, other assays are needed to predict response to cisplatin-based treatment regimens. In the search for predictive tests concerning cisplatin-sensitivity, recently other types of tests have become available besides adduct measurements. These include the assessment of the expression of the excision repair cross-complementation group 1 (ERCC1) protein in tumor, by immunohistochemical methods. This protein is involved in the repair of cisplatin-DNA adducts. It has been shown in surgically treated lung cancer patients that patients with tumors with low expression of ERCC1 benefited from adjuvant chemotherapy, in contrast to patients with high ERCC1 expression [99]. In future studies, sensitivity to cisplatin may be determined not only by assessment of cisplatin-DNA adducts, but also by analysis of the expression levels of ERCC1 and other involved proteins, e.g. using microarray techniques.

TUMOR VOLUME

Primary tumor volume is significantly associated with locoregional control and disease free survival after both radiotherapy alone and concurrent chemoradiation as shown in several studies. However, due to variations in assessment of tumor volume and differences arising from different imaging modalities (CT vs. MRI), studies using uniform volume measurements are needed to establish tumor volume as an independent predictive factor in the staging system. By this, the role of tumor volume might be investigated prospectively to show whether it could serve as a tool to guide treatment options.

IN VIVO FUNCTIONAL IMAGING STUDIES

The results from the pilot study with the radioactive labeled hypoxia marker ^{99m}Tc-BRU 59-21 showed that non-invasive imaging of hypoxia is feasible, using this marker. However, larger studies would be needed to confirm the prognostic value of this non invasive assessment of tumor hypoxia on treatment outcome. For this, the ^{99m}Tc-BRU 59-21 agent could be used. Another promising marker that uses SPECT is HL91 [100], which showed a correlation with response in NSCLC. More recently, positron emission computed tomography (PET) based imaging has been used. Examples of these include the use of nitro-imidazole derivatives like ^{18}F-MISO [64] and ^{18}F-labeled EF5 [101] or others like Cu-ATSM [67]. Comparison of the hypoxic volume as derived from ^{18}F-MISO-PET with the glucose-metabolism by^{18}F-FDG-PET was performed in patients with soft-tissue sarcoma [102], but no correlation between hypoxia and glucose metabolism could be shown.

Future studies on hypoxia imaging should focus on limitations and uncertainties that remain from previous studies: What is the optimal time frame for imaging of hypoxia? Should pre-treatment hypoxia assessment be used or (early) changes in hypoxia during treatment? How should changes during repeat scans during treatment be interpreted?
In our ^{99m}Tc-Annexin-V study, we showed that treatment-induced uptake suggestive of apoptosis was present in both tumor and normal tissue (salivary gland). No association was found between Annexin uptake in tumor and clinical outcome, probably due to the presence of necrosis within these large tumors and the high initial response rates after chemoradiation. For the future, we plan to incorporate Annexin scintigraphy in new treatment strategies that include agents that have pro-apoptotic properties. An example of this is a drug called Gossypol [89], which is a specific and potent inhibitor of Bcl-X_L and Bcl-2. By this, we will be able to study the effects of agents with apoptosis modulating properties. Therefore, we plan to investigate correlations between Annexin-scintigraphy and other biological endpoints for apoptosis like upregulation or downregulation of involved proteins on biopsy samples.

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chapter 10

Summary / Nederlandse samenvatting

Frank J.P. Hoebers

SUMMARY

CHAPTER 1

Chapter 1 contains a general introduction regarding the treatment of head and neck squamous cell carcinoma (HNSCC). This treatment has not only become more effective by intensified treatment schedules, but has also become more toxic in terms of increased side effects. This may lead to decreased compliance to therapy and thus worsening of treatment results.

Therefore, accurate prediction of tumor behavior would enable a more individualized approach by selecting the optimal treatment for the patient. Patients that would be identified as non-responders by a predictive test could be spared a non-effective yet toxic treatment and be offered an alternative treatment. On the other hand, patients who are predicted to be in a very good-prognostic group, would in theory not need intensification of therapy and could be offered standard, less toxic regimes.

Treatment selection for patients with HNSCC is usually based on classical tumor and patient related criteria, including anatomical site and tumor staging according to the TNM classification system. However, categorization of patients according to tumor site and stage does not accurately and fully predict outcome.

The objectives of this thesis were to explore prognostic factors and to investigate predictive tests that might be used to improve prediction of treatment outcome for patients with HNSCC. For this we focused specifically on predictive tests that would be simple in application and minimally invasive for the patients, while ideally preserving the predictive capabilities of the test. We chose to study the formation of cisplatin-DNA adducts and primary tumor volume as possible predictive factors in cisplatin-based chemoradiation. Furthermore, we wanted to test the feasibility of scintigraphy, employing markers for the detection of hypoxia and apoptosis in vivo, as a non-invasive test.

CHAPTER 2

The predictive value of cisplatin-DNA-adduct levels was investigated in 35 patients with HNSCC, treated with concurrent cisplatin-based chemoradiation. Cisplatin was administered intra-arterially (IA, 150 mg/m^2, with systemic-rescue by sodium-thiosulphate) or intravenously (IV, 100 mg/m^2). The rationale for the IA chemoradiation procedure was to increase cisplatin delivery to the primary tumor and to decrease systemic exposure, thereby improving the therapeutic ratio. Adducts were quantified in normal-tissue and tumor. Adduct levels in tumor were 4-5 fold higher than in white blood cells (WBC) for both the IA and IV treatment schedule. No differences were observed in intra-tumoral adduct levels between IA or IV-treatments, despite the selective infusion of high-dose cisplatin with the IA-procedure. However, systemic adduct levels (WBC and buccals) from IV patients were higher than in IA patients, consistent with less systemic exposure after IA-administration. Patients with higher adduct levels (>median) in primary tumor had

significantly better disease free survival than patients with lower (<=median) adduct levels, indicating that cisplatin-DNA adduct-formation in primary tumor may be used for treatment prediction in HNSCC.

CHAPTER 3

The formation of cisplatin-DNA adducts after different schedules of concurrent cisplatin-radiation was investigated and correlations between adducts in tumor and normal tissue were analyzed in 63 patients. Three intravenous cisplatin-regimens, given concurrently with radiation, were studied: daily low-dose (6 mg/m^2) cisplatin, weekly 40 mg/m^2, and three-weekly 100 mg/m^2. Normal tissue samples for adduct-determination were obtained from all 63 patients, and tumor-biopsies from 23 of these patients. Linear relationships and high correlations were observed between the levels of GG- and AG-adducts both in normal and tumor tissue. Adduct levels in tumors were 2-5 times higher than those in WBC. Interestingly, no significant correlations were found between adduct levels in normal tissues and primary tumor biopsies, nor between WBC and buccal cells. This lack of correlation may, to some extent, explain the inconsistencies in the literature regarding whether or not cisplatin-DNA adducts can be used as a predictive test in anticancer platinum therapy.

CHAPTER 4

The treatment results of concurrent radiation (70 Gy in 35 fractions) with daily low dose cisplatin (6 mg/m^2 i.v.x20, daily before RT) were evaluated in 121 patients with advanced stage HNSCC. After the first 47 patients, the original treatment protocol (*Standard Group*) was changed: Daily IV prehydration and accelerated RT were given to the subsequent 74 patients (*Hydr-Ac-RT Group*). It was shown that more chemotherapy could be administered in the *Hydr-Ac-RT Group*, with less renal toxicity and less hospital admissions. However, mucositis was more pronounced and tubefeeding more frequent in the *Hydr-Ac-RT Group*. The complete response rate of the primary tumor increased from 74% (*Standard Group*) to 90% (*Hydr-Ac-RT Group*), although this did not lead to an improvement in loco-regional control. Therefore, we concluded that concurrent chemoradiation with daily low dose cisplatin is feasible and effective for selected patients with advanced HNSCC. Although the addition of accelerated RT resulted in more mucositis and tubefeeding, the introduction of prehydration led to better compliance to therapy with more chemotherapy administered and less hospital admissions.

CHAPTER 5

In 46 HNSCC patients treated with concurrent chemoradiation with daily low dose cisplatin, we studied the prognostic value of pre-treatment primary tumor-volume as derived from diagnostic MRI scans. The mean tumor-volume was 28 cm^3 (median 23 cm^3, range 3-112). Tumor-volume and T-stage were positively correlated: T3-tumors had a mean

tumor-volume of 19 cm^3, whereas the volume of T4-tumors was 40 cm^3. The locoregional control-rate at 3-years was 81% for patients with tumor-volumes <median compared to 48% for tumor-volumes >=median. At multivariate analysis, tumor volume remained a significant determinant of locoregional control and disease free survival when adjusted for other prognostic factors. Larger studies are needed to confirm whether incorporation of tumor volume in the staging system improves the prediction of treatment outcome and can serve as a tool to guide treatment options.

CHAPTER 6

In this phase 1 study we investigated ^{99m}Tc-BRU 59-21 as a novel radioactively labeled 2-nitro-imidazole hypoxic marker, in 10 HNSCC patients, scheduled for surgery. The in vivo uptake of this hypoxic marker on SPECT was correlated with an established hypoxic marker, pimonidazole, for which was stained immunohistochemically on tissue sections. Uptake of ^{99m}Tc-BRU 59-21 was observed in eight of the ten primary tumors. Tumor to normal tissue ratios in the primary tumor were significantly correlated with pimonidazole staining. When primary tumor and involved lymph nodes were considered in conjunction, correlation between the tumor to normal tissue ratio and pimonidazole staining was observed for early (30 min post-injection) but not for late (3 h post-injection) scans. However, late scans showed better tumor delineation than early scans. We concluded that uptake and retention of ^{99m}Tc-BRU 59-21 in tumor tissue was observed, suggestive of tumor hypoxia, and this was supported by correlations with staining for the hypoxic marker pimonidazole.

CHAPTER 7

The bioreductive drug pimonidazole is used as a hypoxia marker. In human head and neck tumors, in addition to staining patterns typical of chronic hypoxia, staining was seen specifically around areas of keratinization, raising the question of whether this is hypoxia-related. We investigated whether the differentiation-related staining was caused by locally high reductive enzyme levels. For this, the nitrotetrazolium compound NBT was used, which is reduced by nitroreductases. Frozen tumor sections from patients with HNSCC injected with pimonidazole were incubated with NBT. Parallel sections were stained for pimonidazole. Pimonidazole staining seen in keratinized areas was not always accompanied by increased NBT staining, indicating that high reductase levels are not a necessary requirement for differentiation-associated pimonidazole staining. In a second series, frozen sections of tumors from patients not receiving pimonidazole were incubated with NBT and compared with staining after incubation with pimonidazole under both oxic and hypoxic conditions. Pimonidazole staining of some keratinizing areas under oxic conditions was seen. Of these areas, only a proportion showed increased NBT staining, confirming the lack of correspondence between keratin-associated pimonidazole staining and reductase levels. We concluded that hypoxia-independent pimonidazole staining

can occur in more differentiated head and neck tumors, necessitating caution in hypoxia quantification. These data argue against a causative role for locally high reductase levels in differentiation-associated staining.

CHAPTER 8

The value of ^{99m}Tc-Hynic-rh-Annexin-V-Scintigraphy (TAVS), a non-invasive in-vivo technique to demonstrate apoptosis was evaluated in 13 HNSCC patients treated with concurrent chemoradiation. TAVS were performed before and within 48 hours after the 1st course of cisplatin. Radiation-dose given to the tumor at the time of post-treatment TAVS was 6- 8 Gy. The radiation dose at post-treatment TAVS was calculated for several Regions of interest (ROI): primary tumor, involved lymph nodes and salivary glands. Annexin-uptake was determined in each ROI, and the difference between baseline and post-treatment TAVS represented the absolute Annexin-uptake: Delta-uptake (ΔU). Treatment-induced Annexin-uptake was observed in parotid glands and mean ΔU was significantly correlated with the mean radiation-dose given to the parotid glands: Glands that received higher doses showed more Annexin-uptake. ΔU in primary tumor and pathological lymph nodes showed large inter-patient differences. A high correlation was observed between the maximum ΔU in primary tumor and in the lymph nodes. No correlation was found between Annexin-uptake and outcome. We concluded that Annexin-V-scintigraphy showed a radiation-dose-dependent uptake in parotid glands, indicative of early apoptosis during treatment. Annexin-uptake in tumor and lymph nodes were closely correlated. This effect might represent a tumor-specific apoptotic response.

CHAPTER 9

This chapter contains a general discussion of the results of the studies described in this thesis. Some conclusions and future directions are given.

NEDERLANDSE SAMENVATTING

HOOFDSTUK 1

In hoofdstuk 1 wordt een algemene introductie gegeven betreffende de behandeling van het plaveiselcel carcinoom van het hoofd-hals gebied (hoofd-hals carcinoom). Deze behandeling is in de laatste jaren niet alleen effectiever geworden door meer intensieve behandelingschema's, maar is ook zwaarder geworden door meer bijwerkingen. Hierdoor bestaat het risico dat patiënten de behandeling niet kunnen afmaken en daardoor kunnen de resultaten van de behandeling als geheel verslechteren. Bovendien blijkt het dat sommige patiënten de intensievere behandeling niet nodig hebben, omdat zij ook met de standaard behandeling al kunnen genezen. Aan de andere kant zijn er patiënten die zelfs met de zwaardere, intensieve behandeling niet een genezing van de kanker bereiken. In beide situaties hebben patiënten wel de nadelen van een zware intensieve behandeling (bijwerkingen), maar hebben zij geen profijt van de voordelen (betere genezingskansen).
Daarom zou een adequate voorspelling van de reactie van een tumor op de behandeling de mogelijkheid bieden tot een geïndividualiseerde aanpak om de optimale behandeling voor een patiënt te kunnen selecteren. De keuze van behandeling voor patiënten met hoofd-hals carcinoom wordt met name gebaseerd op klassieke tumor- en patiënt-gerelateerde criteria, waaronder de lokatie van de tumor en het tumor stadium volgens het TNM classificatie systeem. Echter, het blijkt dat indeling van patiënten volgens deze criteria niet tot een goede voorspelling van de behandelingsuitkomsten leidt.
Het doel van dit proefschrift was om een aantal voorspellende factoren te onderzoeken, die gebruikt zouden kunnen gaan worden om de behandelingsuitkomsten van patiënten met hoofd-hals carcinoom te voorspellen. Hiervoor waren we met name geïnteresseerd in voorspellende testen die simpel in uitvoering waren en slechts weinig belastend zouden zijn voor de patiënt, terwijl de test wel een goede voorspelling moest opleveren. We hebben hiervoor de vorming van cisplatin-DNA adducten en het primaire tumor volume bestudeerd als mogelijke voorspellende factoren bij de behandeling door middel van gelijktijdige chemoradiotherapie. Bovendien hebben we de uitvoerbaarheid onderzocht van scintigrafie, gebruik makend van markers voor hypoxie en apoptose in vivo, als een niet-invasieve test.

HOOFDSTUK 2

De voorspellende waarde van cisplatin-DNA adducten werd onderzocht in 35 patienten met hoofd-hals carcinoom, die behandeld werden met gelijktijdige cisplatin-radio-therapie. Cisplatin werd intra-arterieel (IA, 150 mg/m^2, met systemische toediening van Na-thiosulfaat) of intraveneus (IV, 100 mg/m^2) toegediend. De rationale voor de IA toediening was om de cisplatin afgifte aan de tumor te vergroten en tegelijkertijd de systemische blootstelling te verminderen, waardoor de therapeutische ratio zou worden vergroot. Adducten werden gekwantificeerd in tumor en in normaal weefsel. Adduct

levels in tumor waren 4-5 maal hoger dan in witte bloedcellen (WBC) zowel voor de IA als de IV behandeling. Er werd geen verschil gevonden in tumor adduct levels tussen de IA en IV behandeling, ondanks de selectieve toediening van hoge dosis cisplatin tijdens de IA procedure. Echter, systemische adduct levels (in WBC en wangslijmvliescellen) van de IV patiënten waren hoger dan die van de IA patiënten, samenhangend met een verminderde systemische blootstelling tijdens de IA procedure. Patiënten met hoge levels van GG-adducten in primaire tumor hadden een betere ziekte-vrije overleving dan patiënten met lage levels van adducten. De vorming van cisplatin-DNA adducten in primaire tumor zou daarom gebruikt kunnen worden als een voorspellende factor bij de behandeling van hoofd-hals carcinoom.

HOOFDSTUK 3

De vorming van cisplatin-DNA adducten na verschillende schema's gelijktijdige chemoradiotherapie werd onderzocht en correlaties tussen adducten in tumor en normaal weefsel werden geanalyseerd in 63 patiënten. Het ging om 3 schema's van IV toediening van cisplatin: 6 mg/m^2 dagelijks, 40 mg/m^2 wekelijks and 100 mg/m^2 per 3 weken. Normale weefsel samples voor adduct bepaling werden verkregen van alle 63 patiënten; een tumor biopt van 23 patiënten. Lineaire relaties en hoge correlaties werden gevonden tussen de GG- en AG-adduct levels, zowel in tumor als in normaal weefsel. Adduct nivo's waren 2-5 maal hoger in tumor dan in WBC. Er konden geen correlaties worden aangetoond tussen adduct levels in normaal weefsel en in tumor, noch tussen WBC en wangslijmvliescellen. Dit gebrek aan correlatie zou tot op zekere hoogte kunnen verklaren waarom de gegevens in de literatuur of adducten gebruikt kunnen worden als een voorspellende test niet eenduidig zijn. Bovendien betekent het dat adducten in normaal weefsel niet als surrogaat gebruikt kunnen worden als maat voor de adduct vorming in de primaire tumor.

HOOFDSTUK 4

De behandelingsresultaten van gelijktijdige bestraling en dagelijks lage dosis IV cisplatin (6 mg/m^2) werden geëvalueerd in 121 patiënten met gevorderd hoofd-hals carcinoom. Na de eerste 47 patiënten werd het originele behandel-protocol (*Standaard groep*) aangepast: dagelijkse IV prehydratie werd toegevoegd en de bestraling werd geaccelereerd in de volgende 74 patiënten (*Hydratie-acceleratie groep*). Het bleek dat in de *Hydratie-acceleratie groep* meer chemotherapie kon worden toegediend met minder nier-toxiciteit en minder ziekenhuis opnames. Wel was de mucositis ernstiger en was sondevoeding vaker noodzakelijk. Het percentage complete remissies steeg van 74% in de *Standaard groep* naar 90% in de *Hydratie-acceleratie groep*, hoewel dit niet leidde tot een verbetering in locoregionale controle. We concludeerden dat de introductie van acceleratie in de behandeling met gelijktijdige chemoradiotherapie met dagelijks lage dosis cisplatin leidde tot meer mucositis en sondevoeding, maar dat de toediening van prehydratie leidde tot betere behandeling met meer chemotherapie toediening en minder ziekenhuis opnames.

HOOFDSTUK 5

In 47 patiënten met hoofd-hals carcinoom, die behandeld werden met gelijktijdige chemoradiotherapie met dagelijks lage dosis cisplatin, werd de prognostische waarde onderzocht van het primaire tumor volume, zoals bepaald aan de hand van de diagnostische MRI-scan. Het gemiddelde tumor volume was 28 cm^3 (mediaan 23 cm^3, range 3–112). Tumor volume en T-stadium waren gecorreleerd: T3-tumoren hadden een gemiddeld tumor volume van 19 cm^3, terwijl T4-tumoren een volume hadden van 40 cm^3. De locoregionale controle op 3 jaar was 81% voor patiënten met een tumor volume < mediaan, vergeleken met 48% voor patiënten met een tumor volume >= mediaan. In een multivariate analyse bleef tumor volume een significante factor voor locoregionale controle en ziekte vrije overleving ook na correctie voor andere prognostische factoren. Grotere studies zijn nodig om te bepalen of de invoering van tumor volume in het stageringssysteem de voorspelling van de behandelingsuitkomsten kan verbeteren en of het gebruikt kan worden om een keuze te maken uit de verschillende behandelingsopties.

HOOFDSTUK 6

In deze fase 1 studie hebben we ^{99m}Tc-BRU 59-21 onderzocht als een nieuwe radioactief-gelabelde 2-nitro-imidazole hypoxie-marker in 10 patiënten met hoofd-hals carcinoom die gepland stonden voor chirurgie. Hypoxie is een erkende negatieve factor voor genezing van hoofd-hals carcinoom patiënten. De in-vivo opname van deze hypoxie-marker op SPECT scans werd gecorreleerd met een erkende hypoxie-marker, pimonidazole, waarvoor een immunohistochemische kleuring werd verricht op weefsel coupes. Opname van ^{99m}Tc-BRU 59-21 werd waargenomen in 8 van de tien primaire tumoren. Ratio's tussen opname van ^{99m}Tc-BRU 59-21 in primaire tumor en normaal weefsel waren significant gecorreleerd met pimonidazole kleuring. Analyse van primaire tumor en lymfklier metastasen samen toonde wel correlatie tussen tumor-normaal weefsel ratio en de pimonidazole kleuring voor de vroege scan (30 min na injectie), maar niet voor de late scan (3 uur na injectie). Echter, op de late scans was de afgrenzing van tumor beter dan op de vroege scans. We concludeerden dat de opname en retentie van ^{99m}Tc-BRU 59-21 die in tumor werd geobserveerd een aanduiding was van tumor-hypoxie, omdat deze correleerde met immunohistochemische kleuring voor de hypoxie-marker pimonidazole. De methode van hypoxie-detectie zou in een groter onderzoek toegepast moeten worden om te beoordelen of deze ook prognostische waarde heeft.

HOOFDSTUK 7

Het bioreductieve middel pimonidazole wordt gebruikt als een hypoxie-marker. In hoofd-hals tumoren wordt naast de typische kleuringspatronen passend bij chronische hypoxie ook kleuring gezien rondom gebieden van verhoorning, zodat de vraag werd gesteld of dit wel hypoxie-gerelateerde kleuring betrof. We onderzochten of de differentiatie gerelateerde kleuring in gebieden van verhoorning werd veroorzaakt door plaatselijke

hoge concentraties van reductieve enzymen. Hiervoor werd de nitrotetrazolium stof NBT gebruikt, die wordt gereduceerd door nitroreductasen. Vriescoupes van patiënten met hoofd-hals carcinoom, die pimonidazole intraveneus toegediend hadden gekregen werden geïncubeerd met NBT. Parrelle coupes werden gekleurd voor pimonidazole. Het bleek dat pimonidazole kleuring in gebieden van verhoorning niet altijd vergezeld ging van verhoogde NBT kleuring: hoge reductase levels zijn blijkbaar geen vereiste voor de differentiatie gerelateerde pimonidazole kleuring. In een tweede serie experimenten werden vriescoupes van tumoren van patiënten die geen pimonidazole hadden gekregen, geïncubeerd met NBT en vergeleken met kleuring na incubatie met pimonidazole onder oxische en hypoxische omstandigheden. Pimonidazole kleuring in gebieden van verhoorning werd soms gezien onder oxische condities. Van deze gebieden vertoonde slechts een gedeelte verhoogde NBT kleuring, zodat het gebrek aan overeenkomst tussen verhoorning-geassocieerde pimonidazole kleuring en reductase levels bevestigd werd. We concludeerden dat hypoxie-onafhankelijke pimonidazole kleuring kan optreden in meer gedifferentieerde hoofd-hals carcinomen met gebieden van verhoorning. Het kwantificeren van hypoxie door middel van pimonidazole kleuring kan hierdoor beïnvloed worden. Een causale rol voor hoge concentraties van reductasen kon niet bewezen worden als verklaring voor de differentiatie geassocieerde pimonidazole kleuring.

HOOFDSTUK 8

De waarde van ^{99m}Tc-Hynic-rh-Annexine-V-Scintigrafie (TAVS), een niet-invasieve in-vivo techniek om apoptose aan te tonen werd onderzocht in 13 patiënten met hoofd-hals carcinoom, die behandeld werden met gelijktijdige chemoradiotherapie. TAVS werd verricht voor en binnen 48 uur na de eerste kuur cisplatin. De gegeven bestralingsdosis ten tijde van TAVS was 6-8 Gy. De bestralingsdosis op het moment van TAVS werd berekend voor veschillende structuren: primaire tumor, lymfeklier metastasen en speekselklieren. Opname van Annexine werd bepaald in iedere structuur en het verschil tussen de baseline TAVS en die van na de start van de behandeling vertegenwoordigde de absolute Annexine opname (ΔU). Door de behandeling geïnduceerde Annexine opname werd gezien in de parotis speekselklieren. De gemiddelde ΔU vertoonde een significante correlatie met de gemiddelde bestralingsdosis op de parotiden: klieren die een hogere bestralingsdosis hadden gekregen hadden een hogere Annexine opname. ΔU in primaire tumor en lymfklier metastasen vertoonden grote interindividuele verschillen. Een hoge correlatie werd echter gezien tussen de maximum ΔU in de primaire tumor en de bijbehorende lymfklieren. In tegenstelling tot de gegevens van patiënten met maligne lymfomen, vertoonde de mate van Annexine-opname bij deze hoofd-hals tumoren geen correlatie met de respons op de behandeling. We concludeerden dat Annexine-V-Scintigrafie in parotiden bestralingsdosis afhankelijk was en dat dit een aanduiding was van apoptose vroeg tijdens de behandeling. Bovendien waren

de Annexine opname in primaire tumor en lymfklieren nauw gecorreleerd. Dit effect vertegenwoordigt mogelijk een tumor-specifieke apoptotische respons.

HOOFDSTUK 9

Dit hoofdstuk bevat een algemene discussie over resultaten van de onderzoeken beschreven in dit proefschrift en plaatst deze in perspectief van andere resultaten. Tenslotte worden enkele conclusies getrokken en een aantal toekomstige ontwikkelingen genoemd.

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