Predictive Factors in the Neoadjuvant Chemoradiotherapy of Gastrointestinal Tumors

Ph. D. Thesis

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2012

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1. Introduction

1.1. *Neoadjuvant chemoradiotherapy in gastrointestinal tumors*

Neoadjuvant chemoradiotherapy (CRT) followed by surgery is a widely accepted treatment in gastrointestinal tumors such as loco-regionally advanced esophageal and rectal cancer.

From theoretical perspective neoadjuvant CRT offers several advantages compared to postoperative treatment. Better oxygenation of the tumor area leads to increased radiosensitivity enhancing radiation response. Downsizing of the tumor may facilitate optimal surgical removal, which may enable function preservation. The combination of radiation and systemic agents decreases tumor seeding, consequently may improve local and distant control rates. In clinical practice patients can tolerate preoperative treatment better, due to less acute toxicity and lack of postoperative complications. Moreover, radiotherapy target volume for treatment planning is easier to define because of visible tumor mass. One of the main disadvantages of the neoadjuvant treatment strategy is the potential overtreatment of patients with early disease. In addition, not all tumors respond to neoadjuvant CRT, thus non-responding patients may progress during the preoperative therapy and suffer from unnecessary toxicity.

The ultimate response to CRT is determined by the quality of treatment, as well as by the genetic make-up of the tumor and the various biochemical pathways implicated in chemoradiosensitivity. Understanding the biology of the disease and pretreatment identification of molecular markers predicting therapeutic response would be invaluable in individualizing patient treatments. In the future current standard treatments for large heterogenous patient groups have to be substituted by more individualized therapies based on clinical–pathological features of the tumor and molecular markers.

1.2. *Neoadjuvant chemoradiotherapy of esophageal tumors*

Esophageal cancer is the eighth most frequent cancer in the world, carrying a poor prognosis, with a 3-30% overall 5-year survival rate (1-2). As regards histology, almost all esophageal tumors are represented by scquamosus-cell carcinomas and adenocarcinomas. Although a marked increase of the adenocarcinomas has been observed during the past decades, squamous–cell cancers still account for the majority of cases (3). Squamous-cell cancer is mostly found in the upper two-thirds of the esophagus and is clearly associated with bad socioeconomic environment, nicotine- and alcohol abuse. Consequently, patients with esophageal SCC often present with cardiovascular, pulmonary, nutritional, and even malignant co-morbidities (1, 4). As a result of low socioeconomic status and lack of early symptoms, esophageal tumors are commonly detected in an advanced stage (5-6). Dysphagia, the classical symptom of esophageal cancer, usually appears when the tumor has obstructed over 50% of the lumen, usually meaning that the tumor is irresectable (1). Furthermore, special anatomic features contribute to the early spread of esophageal tumors promoted by the presence of a complex lymphatic plexus and the lack of fibrous serosa within the thorax. Thus at the time of diagnoses, more than 50% of patients have locally advanced disease with lymph node metastases (5, 7). Even though locally advanced disease requires aggressive treatment approaches for tumor control, the adverse medical and social background of the patients rules it out.

The prognosis of a particular cancer patient is very important in the individualization of treatment, in order to plan the patient's follow-up and to inform the patient about the probable outcome. The prognosis of esophageal cancer patients highly depends on the stage of the disease and the completeness of the surgical resection. A microscopically radical (R0) resection is one of the most important prognostic factors of survival and

long term survival can be expected only in case of complete resection (8). The standard therapy of esophageal cancer in the I-II stages is surgery, however local and distant control rates are disappointingly low even after optimal resection (9), moreover, two out of three patients are inoperable at diagnosis (1, 10). For potentially operable tumors, including both initially resectable and irresectable, locally advanced cancers, neoadjuvant chemoradiotherapy (NRCT) has become the accepted modality of treatment (10-11). The potential benefit of preoperative CRT involves the down-staging of the tumor to facilitate complete resection rate as well as the reduction of metastatic potential by eliminating occult micro-metastases (10-12). Several clinical trials comparing surgery with neoadjuvant CRT plus surgery have shown improvement in local control, but studies are contradictory in terms of survival benefit (13-17). Although, until now there has not been a well designed clinical trial providing firm evidence on the effect of preoperative treatment in esophageal cancer, the positive impact of neoadjuvant CRT on local control and on survival has been supported by meta-analyses of individual trials (11, 18-22).

As regards the technique of neoadjuvant CRT, there is still no agreement on the dose and schedule of chemotherapy and radiotherapy. In clinical trials radiotherapy doses vary from 30 Gy to 60 Gy. Regarding chemotherapy, cisplatin-based regimens in combination with 5-fluorouracil seem to be the most generally accepted, however the dose of cytotoxic drugs and the timing during radiotherapy varies widely (13-14, 16-17). A comprehensive analysis of the effect of various treatment schedules on the efficacy of CRT revealed that higher total radiation dose, shorter treatment time, higher cisplatin and 5-fluorouracil dose determine higher rates of pathological complete responses (pCR), however more aggressive protocols are associated with more heamatological and non-haematological toxicity (23). These acute side effect together with the poor general condition characterizing a significant portion of the esophageal cancer patients often circumvents completion of aggressive neoadjuvant treatment (23). Apart

from escalating doses of CRT, introduction of new drugs such as Paclitaxel in neoadjuvant therapy may help to improve treatment results (24).

Besides acute side effects, increased postoperative mortality and morbidity attributable to neoadjuvant chemoradiotherapy are the cause of major concern in clinical practice. Esophagectomy solely is associated with significant mortality, which varies from 5-7% to 14% and highly depends on the skills and the experience of the surgical team (25). Theoretically, the technique of CRT and the time interval between operation and neoadjuvant treatment may influence the rate of postoperative complications including anastomotic leakage, pulmonary and cardiac side effects(22). Keeping in mind this consideration, usually lower doses are used in neoadjuvant setting than applied for definitive therapy. Available literature data, however, are inconsistent in these terms. The few studies that compared postoperative complications rates between patients who had neoadjuvant CRT and those treated with surgery alone usually found no significant difference between postoperative complication rates (26). Only the EORTC study has reported surplus morbidity and mortality rates in combined treatment, which has presumably been caused by hypofractionated radiotherapy (15).

Recent meta-analyses have proved that multimodal treatment improves survival (2, 11, 19-20), but significant improvement in the long-term survival can only be expected, if patients have pathological complete response (pCR), which occurs in 20-30% of the cases (16, 27). In many clinical studies of neoadjuvant CRT, besides microscopically radical resection (R0) the most important prognostic factor for survival is the pathological response to therapy (8). The survival benefit of patients with no evidence of residual tumor on pathological examination implicates that pCR may serve as a biological marker for favorable clinical course of the disease (27-28). To achieve a more detailed differentiation of response after neoadjuvant CRT several studies have used a histomorphological regression

system such as the five degree tumor regression grading (TRG) suggested by Mandard (29). The additional value of these quantitative classification systems is still not completely clear. Some did not find any correlation between tumor regression grade and survival, whereas others have demonstrated that patients with complete (TRG1) and major response (TRG2) have significantly better survival than patients with minor or no response (TRG3-5) (30-31).

Several studies have evaluated the clinical response after CRT by endoscopy, re-biopsy, endoscopic ultrasound (EUS), computed tomography (CT), positron emission tomography (PET). The results of available studies are contradictory and these methods have limited accuracy in response evaluation, since clinical response was correlated with pathological response in only a few series (31-33). FDG-PET imaging, which shows alterations in tissue metabolism, was thought to refine response evaluation and prognosticate response however, results have proved equivocal (34-37). Currently only pathological examination can correctly differentiate responders from non-responders, therefore clinical response evaluation is a major challenge in the neoadjuvant treatment of esophageal cancer.

Not all the tumors are sensitive to neoadjuvant therapy thus, in the group of non-responder patients this may lead to unnecessary overtreatment with cytotoxic drugs. Consequently, the pre-therapeutic identification of those squamous-cell carcinoma cases that would benefit from neoadjuvant treatment has become an important task in order to avoid preventable toxicity, to lengthen survival and to ameliorate life quality (38-39).

A number of recent studies have attempted to identify markers that could be used to predict response to neoadjuvant therapy. Serum markers were also examined with the same purpose. Kim *et al.* analyzed serum carcinoembryonic antigen (CEA) levels and found that an increased CEA level predicted relapse and correlated well with visceral involvement, while clinical response correlated with decreased CEA values (40). In another

study, elevated plasma DNA was demonstrated to be a more reliable marker than CEA as an indicator of the presence of recurrent disease (41). Gene expression arrays have been used on the basis that cancer is the consequence of a malfunction of gene expression and in recent clinical studies various gen panel classifiers have been identified as a predictor of response (42-43). Unfortunatelly, no clinically useful predictors of response to neoadjuvant therapy in squamous-cell esophageal cancer have yet been found.

1.3. *Neoadjuvant chemoradiotherapy of rectal cancer*

Colorectal cancer is the third most frequent malignancy in males and second in females, accounting for about 1.2 million new cases per year worldwide (44). About 30% of all colorectal cancers are diagnosed in the rectal region (45). Although 5-10 % of colorectal adenocarcinomas are inherited in autosomal dominant manner, environmental factors have a strong role in the development of rectal cancer. The most important risk factors identified are dietary components, obesity, lack of physical exercise, alcohol abuse, inflammatory bowel disease and some medical therapies (46) (47). Using data of population-based cancer registries the estimated colorectal cancer relative survival has been found to be around 53-54 % in both sexes for Europe (48), however a variability between countries has been found, which may be explained by differences in stage at diagnosis and quality of treatment (49).

The primary treatment of rectal cancer mainly depends on the clinical stage of the tumor. Although, currently the majority of the patients receive combined multimodal therapy consisting of surgery plus radiotherapy with or without chemotherapy, good quality surgery is still thought to be the most important element of the treatment. At present, total mesorectal excision (TME) means the standard surgical technique, which has lead to tremendous changes in local control rates during the past 15-20 years (50-51). TME has reduced local relapse rate from 20-50 % to below 10% in

cancer centres with a large case volume, since the efficacy of this method highly depends on the training of each surgeon, which still represents one of the major prognostic factors in the treatment of rectal cancer (52).

The purpose of pre-operative CRT is to reduce the volume of a rectum tumor, thus to facilitate resection and increase the likelihood of a sphincter-preserving procedure, and to improve local control (53). Meta-analyses of trials comparing surgery versus surgery plus radiotherapy without concurrent chemotherapy revealed significant improvement of the local control rate, cancer specific and overall survival, especially with preoperative radiotherapy at biological effective doses above 30 Gy (54-56). We should keep in mind that all these early trials, which proved the benefit of neoadjuvant radiotherapy compared to surgery alone, were conducted in the era before the introduction of TME. Adding concurrent chemotherapy to radiotherapy in the neoadjuvant setting can further decrease local failure rate compared to radiation alone, but has no effect on survival and more acute side effects are expected in combined treatment (53, 57-58). In a German clinical trial, it has been also demonstrated that in stage II-III rectal cancer preoperative CRT provides better local control with less acute and late toxicity compared to postoperative chemoradiotherapy. Moreover this trial has found that higher rate of sphincter preservation after neoadjuvant chemoradiotherapy can be achieved (53). Regarding sphincter preservation, however, results of other randomised trials are inconsistent and there is no solid evidence supporting the idea, that CRT with delayed surgery can increase the number of preserved sphincters due to downsizing the tumour and by allowing more conservative surgeries in the lower third of the rectum (59-61).

Currently two widely accepted radiotherapy schemes are used as neoadjuvant therapy in rectal cancer: short-course radiotherapy (25 Gy in 5 days) followed by immediate operation and the combined modality treatment with conventional fractionation (45-50 Gy in 5-6 weeks) followed

by delayed surgery. It is difficult to accurately compare the two methods, since in clinical studies they were usually not evaluated in the same patient population. Most trials used short –course preoperative radiation including patients with cT1-T3 disease, whereas available data on conventional combined therapy was limited to patients with cT3-T4 and /or N+ disease. The only trial comparing the two radiotherapy regimens in the treatment of a limited number of rectal cancer patients found no difference in terms of local control and survival rates (61). Both of these therapies can improve local control (53, 57-58, 62) however, after short-course radiotherapy no down-staging can be expected, besides in cases with positive circumferencial resection margin after TME no benefit from this short radiation schedule has been proved (61).

Based on data from randomised clinical trials, pre-operative chemoradiotherapy (CRT) followed by surgery is established as the standard treatment in locally advanced rectal tumor including cT3/T4 and/or cN+ stage cancers (53, 57-58). At present for standard neoadjuvant CRT with conventional fractions (1,8-2 Gy, 5 days a week) doses in range of 45-50,4 Gy are used to treat the whole pelvis. A boost dose of 5,4 Gy can be delivered to the primary tumor, however it is not clear whether higher doses can improve survival. Radiotherapy is usually combined with concurrent, continuous 5-Fluorouracil-based chemotherapy schedules (53, 57-58, 63). During CRT mild to moderate acute side effects such as diarrhoea, acute proctitis, dysuria occur frequently (64). Late complications including small bowel damage, urogenital dysfunction, increased risk of secondary cancer are less common (65-66). Using intensity modulated radiotherapy (IMRT) may reduce the dose of critical structures, thus can prevent the development of radiation-induced complications, however the real clinical value of this method still needs to be proved (67). Intensification of preoperative CRT is endeavoured with the application new cytotoxic drugs such as oxaliplatin and capecitabine as well as with targeted therapies (68-70).

Currently the stage of the disease is considered to be one of the most important prognostic factors for survival in rectal cancer patients (71). It has been demonstrated in several studies however, that clinical outcome depends not only on the initial stage of the tumor, but also on the CRT-induced tumor response which varies among individual patients (72). Response evaluation can be based on clinical restaging or on pathological examination of surgical specimens. In clinical practice widely used MRI and CT have a low accuracy in response evaluation and data from clinical studies suggest that the most promising non-invasive method for identification of responders after neoadjuvant CRT is FDG-PET (73). Significant decrease of standardized uptake (SUV) on post-treatment PET in responders compared to non-responders has been demonstrated in several studies (74-75). Even with the use of advanced imaging tools, it remains a problem to detect small foci of residual tumor cells and to identify complete remissions after CRT. Pathological assessment after preoperative CRT can provide more useful data on treatment-induced tumor response. Down-staging, especially complete destruction of tumor cells known as pathological complete response (pCR) has been found to have a prognostic relevance (76-77). In many studies those who had pCR after neoadjuant CRT could expect improved outcomes in terms of local control rates independent from their initial clinical T and N stage (76-77). Furthermore, a growing body of evidence indicates that pathological response to neoadjuvant treatment can be measured with the histopathological tumor regression grade (TRG), which appears to be an independent predictor of disease-free survival (72, 78-80). The various histopathological responses to the same CRT protocol were not due to differences in stage but rather to the biological features of the tumors. To achieve a more patient-tailored, individualised treatment it would be imperative to understand the biological factors that determine sensitivity or resistance to neoadjuvant CRT, as this would spare poor-responding patients from undergoing ineffective

treatment as well as help to select candidates for new therapeutic approaches.

1.4. *Biological markers*

Several molecular markers have been studied as potential indicators of response to CRT in esophageal and rectal cancer. In the current study we investigated the possible predictive role of Heat shock protein (Hsp) 90, Small Heat hock protein (sHsp) 16.2, phospho-Akt, B-cell-associated leukemia protein 2 (Bcl-2), Heme-binding protein 2 (SOUL) and pituitary-type growth hormone-releasing hormone receptor (GHRH-R).

Heat shock proteins, a ubiquitous group of proteins found in all living organisms, are expressed in response to different types of stress including environmental changes. They function as molecular chaperones aiding the folding and assembly of proteins, and their refolding or –in some cases- elimination, if the damage done to the protein is irreversible. Hence Heat shock proteins play an important role in cytoprotection and cell survival (81)-(82). Hsp90 is an ATP-dependent chaperone which ensures the stable conformations of a number of client proteins implicated in signaling pathways responsible for the progression of malignant cells (83). Thus, Hsp90 has become a much studied molecular target in cancer research.

Small Hsps (sHsp) have a molecular weight ranging between 2-43 kD and like other Hsp, they also act as molecular chaperones. They increase the cells' resistance to stress by suppressing the aggregation of denatured proteins or by storing aggregation prone proteins. The amount and location of sHsp in a cell can be different according to the lack or presence of various physiological stressors, such as heat, hypoxia or cell development (84). As consequence of the central role of sHsps in the cytoprotection, changes in their structure or function – due to mutation in their DNA- is likely to lead to the damage of the cell and finally, to the development of a disease. sHsps have the potential to guard cells from damage and disease

but when they are disturbed or are present in tumorous cells, they can foster disease. Thus, sHsps have been linked to various human illnesses (84). The apparent significant role of sHsps in different types of cancer has made them a target for recent research (85-87). It is particularly worthwhile to examine the role of sHsps in cancer. The discovery that a positive correlation exists between the levels of alphaB-crystallin-one of the most studied sHsp- and lymph node involvement in breast cancer turned the attention of researchers towards the possibility of sHsps being used as tumor markers (84). A recently characterized sHsp, Hsp16.2, was found to be expressed in neuroectodermal tumors (88-90). In a consequent study, Hsp16.2 expression was shown to be directly correlated with the histological grade of different types of brain tumors, indicating its potential relevance as a tumor marker in brain cancers (89). Hsp16.2 is a novel small heat shock protein that synthesis is induced by heat stress and it has an ATP-independent chaperone activity similarly to other small heat shock proteins. Suppression of Hsp16.2 sensitized cells to apoptotic stimuli, while over-expressing of Hsp16.2 protected cells against H_2O_2 and taxol induced cell death (90). Under stress conditions, Hsp16.2 inhibited the release of cytochrome c from the mitochondria, nuclear translocation of AIF and endonuclease G, and caspase3 activation by protecting the integrity of mitochondrial membrane system. Furthermore, Hsp16.2 was found to bind to Hsp90, thus Hsp16.2 mediated cytoprotection requires Hsp90 activation. Hsp16.2 over-expression facilitated lipid rafts formation, and increased Akt phosphorylation supporting the idea that stabilization of lipid rafts is essential to Akt activation (90). The inhibition of PI-3-kinase-Akt pathway by LY-294002, or wortmannin, significantly decreased its protective effect. Taken together, these data indicated that one of the main mechanisms by which Hsp16.2 inhibits cell death is the activation of Hsp90 followed by activation of lipid raft formation and by the activation of PI-3-kinase - Akt cytoprotective pathway.

Another attractive target for anticancer therapy is the Akt signaling pathway, which is activated by growth factors and is frequently overexpressed in cancer cells (91). The Akt pathway is considered as one of the major anti-apoptotic pathways in cells (92-94). Ability to trigger cell apoptosis is responsible for therapeutic effect of chemotherapeutic drugs. Deregulation of apoptotic cell death renders tumor cells refractory to cancer therapeutics.

B-cell-associated leukemia protein 2 (Bcl-2) and its family member proteins regulate apoptosis though the intrinsic mitochondrial apoptosis pathway that is activated in response to genotoxic stress stimuli (95). Bcl-2 family proteins consist of conserved regions of amino acid sequences, known as Bcl-2 homology (BH) domains, which play a role in apoptosis regulation. Based on their function the members of Bcl-2 family can be divided into pro-apoptotic and pro-survival proteins (96). Pro-survival protein Bcl-2 contains up to four BH domains and inhibits apoptosis by regulating the release of certain proteins such as cytochrome c from the mitochondria. One part of pro-apoptotic proteins have a multi-domain structure, like BCL2-associated X protein (Bax), that shows proapoptotic activity by releasing apoptogenic proteins from mitochondrial inter-membrane space and permeabilizing the outer mitochondrial membrane (97-98). Other group of pro-apoptotic proteins are BH3-only- domain proteins, which are known to be essential initiators of apoptosis through binding to other Bcl-2 family members (96). Deregulation of Bcl-2 family members often contribute to malignant tumor pathogenesis and therapeutic resistance to anticancer treatments.

A recent study showed that tumor necrosis proved to be an independent prognostic variable concerning progression-free and cancer-specific survival (99). Heme-binding protein 2 (SOUL) has a sequence homologous to Bcl-2 homology 3 (BH3) domain of Bcl-2 proteins, so it is thought to be a novel member of the BH3-domain-only protein family

(100). SOUL is expressed in various tissues to different extents, for instance elevated SOUL expression has been found in pancreatic adenocarcinomas compared to normal pancreas tissue (100). SOUL is located mostly in the cytoplasm, although a smaller fraction of SOUL is associated with the mitochondrium (100) . Besides binding hem it may play a role in the complex process of cell death. Recombinant SOUL protein has facilitated the mitochondrial permeability transition, collapse of mitochondrial membrane potential and the release of pro-apoptotic mitochondrial intermembrane proteins in vitro experiments (101). Although these mitochondrial proteins like cytochome c are implicated in the process of apoptotic cell death, available data suggest that SOUL predominantly mediates cell death through the induction of mitochondrial permeability transition resulting in swelling of the matrix, and ultimately in membrane disruption and necrotic cell death (101). SOUL cannot induce cell death alone, but has been found to facilitate necrotic cell death in oxidative stress by aiding the permeabilization of the inner and outer mitochondrial membranes (101).

Growth hormone-releasing hormone (GHRH) is a peptide hormone secreted by the hypothalamus, but also present in various tissues and tumors, stimulates the secretion of growth hormone (GH) after binding to pituitary-type GHRH receptors (GHRH-R) on the anterior pituitary (102-104). GH stimulates the production of the insulin-like growth factor I (IGF-I), which plays a major role in malignant transformation, metastasis and tumorigenesis in various cancers (105-108). The presence of GHRH-R and its splice variants, on different types of cancer cell lines has been demonstrated (109-110). Antagonists of growth hormone-releasing hormone have been tested for the treatment of various types of experimental tumors (111-115). Antagonists of GHRH inhibit the secretion of GH and block the binding of autocrine GHRH to receptors on tumor cells, and thus suppress its action and the tumoral production of IGF-I (116-118).

2. Thesis

The aim of this study was to investigate certain molecular-biological markers which characterize the two major cell death pathways as possible clinically useful predictors of response to neoadjuvant CRT for esophageal and rectal cancer. The main objectives of my research were the following:

1. to assess the efficacy and tolerability of the neoadjuvant chemoradiotherapy regimen used in treatment of loco-regionally advanced squamous cell esophageal carcinoma

2. to determine a correlation between the expression of heat shock proteins (HSP90 and HSP16.2) and the clinical or pathological response to neoadjuvant CRT in esophageal cancer

3. to determine a correlation between the Bax/Bcl2 ratio, representing the apoptotic route of cell death, and the clinical or pathological response to neoadjuvant CRT in esophageal cancer

4. to determine a correlation between SOUL implicated in necrotic cell death and the clinical or pathological response to neoadjuvant CRT in esophageal cancer

5. to identify, whether there is any difference in tumor-related protein expression of squamous cell carcinomas arising in the middle or in the upper third of esophagus

6. to evaluate efficacy of neoadjuvant CRT regarding pathological response in loco-regionally advanced rectal cancer and to assess the impact of patient/therapy-related clinical factors on tumor response

7. to identify, whether the expression of heat shock proteins (HSP90 and Hsp16.2) are correlated with histopathological response after neoadjuvant therapy of rectal cancer

8. to identify, whether expression of p-AKt has any influence on histopathological response after neoadjuvant therapy of rectal cancer

9. to evaluate the impact of GHRH-R expression on histopathological response after neoadjuvant therapy of rectal cancer

10. to evaluate the impact of necrosis-inducing heme-binding protein 2 (SOUL) expression on histopathological response after neoadjuvant therapy of rectal cancer

11. to identify one or more tumor-associated proteins as independent predictive markers of the response of individual rectal tumors to neoadjuvant CRT

3. MATERIALS AND METHODS

3.1. Materials and Methods in Esophageal Study

Patients and tumor specimens.

Twenty patients with esophageal cancer, candidates for NRCT, were enrolled in the study between 2005 and 2006. All the patients had squamous-cell cancer, with stages cT3-4, cN0-1, cM0, located in the upper two-thirds of the esophagus (Table I). All the patients signed informed consent, which was approved by the Local Ethics Committee.

Table I. Esophageal patient characteristics and clinical outcome.

	Median
Age (years)	60 (41-69)
Distance from teeth (cm)	25 (17-31)
Male/Female	16/4
T3/T4	11/9
N0/N1	6/14
Resection	19/20
Complete resection (R0)	9

The staging procedures included endoscopy with biopsy, endoscopic ultrasound, computed tomography (CT) scan of chest and abdomen and bronchoscopy. From each patient one biopsy was taken from the tumor and one biopsy from the intact part of the esophagus to serve as control. The biopsy from the tumor was divided into two parts. One tumor sample and the normal tissue sample were immediately frozen in liquid nitrogen and the other tumor sample was formalin-fixed for pathological examination. The biochemical examinations were carried out on fresh frozen samples. The patients then received external-beam radiotherapy (total of 36 Gy, fraction dose: 1.8 Gy) and concomitant chemotherapy during the first week of irradiation: cisplatin (100 mg/m^2 intravenously on day 1) and 5-fluorouracil (1000 mg/m^2/day, continuous intravenous infusion through days 1-5). Four weeks after the completion of RCT, the clinical response to treatment was assessed according to the Response Evaluation Criteria In Solid Tumors (RECIST), (control CT scan and endoscopy with biopsy) (119). Six to nine

weeks after the neoadjuvant therapy if there was no evidence of disease progression, the patients underwent definitive surgical resection. Pathological response to treatment was determined by the histological evaluation of the resected specimen. Side-effects were documented in conformity with the Common Terminology Criteria for Adverse Events, Version 3.0. (http://ctep.cancer.gov).

Preparation of polyclonal antibodies against Hsp 16.2 and SOUL.

Rabbits were immunized subcutaneously at multiple sites with 100 pg of recombinant Hsp16.2/ Glutathione S-transferase (GST) or SOUL/GST fusion proteins dissolved in Freund's complete adjuvant, as described before (88, 90). Then four subsequent booster injections of 50 pg doses at 4-week intervals were given. Blood was collected 10 days after the last boosting, and the antisera were stored at -20 C. IgGs were affinity purified from the sera by protein G-Sepharose chromatography (Sigma-Aldrich, Munich, Germany) according to the manufacturer's protocol.

Immunoblot analysis.

The tissue specimens were homogenized in chilled lysis buffer of 0.5 mM sodium metavanadate, 1 mM EDTA and protease inhibitor mixture in phosphate-buffered saline in a Teflon/glass homogenizer, then centrifuged for 10 minutes. Isolation of the cytosol and nuclear fractions was carried out by standard laboratory protocols described previously (120). The samples were equalized to 1 mg/ml total protein concentration using Biuret's method and subjected to SDS-PAGE. The proteins (20 μg/lane) were separated on 15% gels and then transferred to nitrocellulose membranes. The membranes were blocked in 5% low fat milk for 1 h at room temperature, then exposed to the primary antibodies at a dilution of 1:2,000 at 4°C overnight in blocking solution. The primary antibodies used were: anti-Hsp 16.2 , anti-SOUL, anti-Hsp 90 (Cell Signaling, Danvers, MA, USA), anti-Bax (Cell Signaling, Danvers, MA, USA) and anti-Bcl-2 (Cell Signaling, Danvers,

MA, USA) antibodies. Appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, Munich, Germany) were used for 2 h at room temperature and at 1:5,000 dilution. Peroxidase labeling was visualized with enhanced chemiluminescence (ECL) using an ECL Western blotting detection system (GE Healthcare, Uppsala, Sweden). The developed films were scanned, and the pixel volumes of the bands were determined using NIH Image J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). All the experiments were repeated four times.

Statistical analysis.

Statistical analysis was performed by analysis of variance followed by Student's *t*-test and the Mann-Whitney *U*-test. Statistical significance was set at $p<0.05$. The analyses were performed using the statistical software SPSS for Windows. (version 11.5; SPSS Inc., Chicago, IL, USA)

3.2. Materials and Methods in Rectal Study

Patients, Pre-treatment and Posttreatment

Sixty nine consecutive patients with median age of 59 years (range 34-78), were treated for rectal adenocarcinoma with neoadjuvant CRT between January 2005 and December 2006. All the patients had locally advanced tumors (cT3/T4 and /or cN+ and cM0). Preteatment workup consisted of digital rectal examination, sigmoidoscopy, biopsy, abdomino-pelvic CT, pelvic MRI, chest x-ray or CT. In all cases 3D planned conformal radiotherapy was carried out with belly board in prone position, with 18 MV photons. Primary tumor as well as lymph nodes at risk were covered with 3 irradiation fields and received 45 Gy in 25 fractions over a period of 5 weeks. As a concomitant chemotherapy, 500 mg/m^2 of 5-Fluorouracil

continuous infusion and 30 mg/m^2 Folic acid bolus on days 1-5 of 1st and 5th weeks of radiotherapy was administered. Four weeks after the completion of CRT, patients were re-staged and definitive surgical resection was performed six to nine weeks after neoadjuvant therapy in 64 cases. All the patients signed informed consent, which was approved by the Local Ethics Committee. The main clinical characteristics of the patients had underwent operation are given in Table II.

Table II. Rectal patient and tumor characteristics

Factor	N
Age (years)	
≤ 60	32 (52%)
> 60	32 (48%)
Sex	
Male	35 (55%)
Female	29 (45%)
Clinical T stage	
cT2	2 (3%)
cT3	55 (86%)
cT4	7 (11%)
Clinical N stage	
cN0	25 (39%)
cN1-2	39 (61 %)
Distance from AV (cm)	
<5	22 (35%)
5-10	26 (40%)
>10	16 (25%)
Time to surgery (weeks)	
≤ 7	37 (58%)
>7	27 (42%)

AV: anal verge

Histopathological Evaluation

Pathological response to neoadjuvant treatment was determined by the histological evaluation of the resected specimens using rectal radiotherapy grading system adapted from Mandard et al. (29). This five point tumor

regression grading (TRG) is based on the presence of residual tumor cells and the extent of fibrosis and consists of the following: TRG 1 (complete regression) is defined as the absence of residual tumor and fibrosis extending through the different layers of the rectal wall, TRG2 is characterized by the presence of rare residual tumor cells scattered throughout the fibrosis, TRG3 shows an increase in the number of residual tumor cells, but the fibrosis still predominates, TRG4 demonstrates residual tumor outgrowing the fibrosis and TRG5 is characterized by the absence of any tumor regression. In accordance with previous studies in order to simplify the statistical analysis, the TRG was combined into two groups: good responders comprising TRG1-2 and poor responders consisting of TRG 3- 5 (29, 53, 58).

Preparation of Polyclonal Antibodies against Hsp16.2 and SOUL

Rabbits were immunized subcutaneously at multiple sites with 100 pg of recombinant Hsp16.2/GST and SOUL/GST fusion proteins, which was expressed as described previously (88,90) in Freund's complete adjuvant. Four subsequent booster injections at 4-week intervals were given with 50 pg of protein in Freund's incomplete adjuvant. Blood was collected 10 days after boosting, and the antiserums were stored at -20 C. IgGs were affinity purified from sera by protein G-Sepharose chromatography (Sigma) according to the manufacturer's protocol.

Immunohistochemistry

Sections from the pretreatment tumor tissue samples were fixed in formalin and embedded in paraffin. Subsequently, they were incubated with the following primary antibodies: self-developed anti-Hsp 16.2 and anti-SOUL polyclonal primary antibodies, GHRH-R primary antibody purchased from Abcam (Abcam Inc., Cambridge, MA), p-AKT and Hsp90 primary antibodies purchased from Cell Signaling (GHRH-R antibody detected the presence of both GHRH-R as well as the splice variants of the

GHRH-R). Immunohistochemical staining was carried out according to the streptavidin-biotin-peroxidase method with hydrogen peroxide/3-amino-9-ethylcarbazole development using the Universal kit. Only secondary IgG was incubated with the control sections. The evaluation of the slides was done with the help of an Olympus BX50 light microscope with incorporated photography system (Olympus Optical Co., Hamburg, Germany). Staining intensity was recorded semiquantitatively as mild (+), moderate (++) or strong (+++), following as described before (121). For internal positive control, the normal cellular and vascular structures of the samples were used. Positive areas around necrotic fields were excluded due to their probable stress related up-regulation. All slides were assessed by the same experienced pathologist blinded to clinico-pathological data.

Statistical Analysis

All statistical analyses were carried out using SPSS 16.0 statistical program (SPSS, Chicago). Univariate chi-square test was used to compare clinical parameters and biological markers for tumor regression grade. To increase the number of patient per group, the categories of the various variables were combined for these analyses: age over 60 years vs. below 60 years, cT2 vs. cT3 vs. cT4, cN0 vs. cN1-2, distance from the anal verge less than 5 cm vs. between 5 and 10 cm vs more than 10 cm, time to surgery within 7 weeks vs. over 7 weeks. For statistical testing intensity values of immunohistochemistry were dichotomised into low (0, +) and high (++, +++) intensity categories. All parameters were analysed afterwards in a logistic regression multivariate analysis. A *p* value of less than 0.05 was considered statistically significant.

4. Results

4.1. Results in Esophageal study

Clinical outcome.

A 65% clinical response rate was found. One patient had complete remission (5%), 12 patients had partial remission (60%), 5 patients had stable disease (25%), 1 patient had progressive disease (5%) and 1 patient died during the treatment (5%) (Table III).

Table III. Response evaluation regarding esophageal cancer.

	Clinical response	Histological response
Complete remission	1	2
Partial remission	12	11
Stable disease	5	5
Progressive disease	1	1
Death	1	-

The patients with complete or partial remission underwent definitive surgery. The following histological response was observed: no residual tumor tissue in 2 patients (10%), down-staging of the tumor size (T) or lymph node involvement (N) in 6 (30%) and 5 (25%) cases, respectively. Complete (R0) resection was possible in 9 cases (70%) and no perioperative mortality occurred. Grade 3 or 4 gastrointestinal, hematological and pulmonary side-effects occurred, one patient died due to severe sepsis (Table IV).

Table IV. Adverse events detected during the treatment of esophageal cancer.

Grade 3-4 gastrointestinal side-effects	6/20 (30%)
Grade 3-4 hematological side-effects	3/20 (15%)
Grade 3-4 pulmonary side-effects	3/20 (15%)
Treatment related death	1/20 (5%)

Detection of possible new markers by Western-blot.

All twenty squamous-cell esophageal cancer and corresponding normal esophageal tissue samples were examined by Western-blot. The tumor samples from the patients with no clinical response contained approximately double the level of Hsp 90 and Hsp 16.2, significantly higher than responding tumors (p=0.049 and p=0.019 respectively). They also expressed SOUL at a higher level and had a lower Bax/Bcl-2 ratio than those with good clinical response, but these results were not significant (p=0.247 and p=0.883) (Figure 1).

Figure 1: Tumor-associated proteins and clinical response (statistically significant difference between responder and non-responder tumor tissue)*

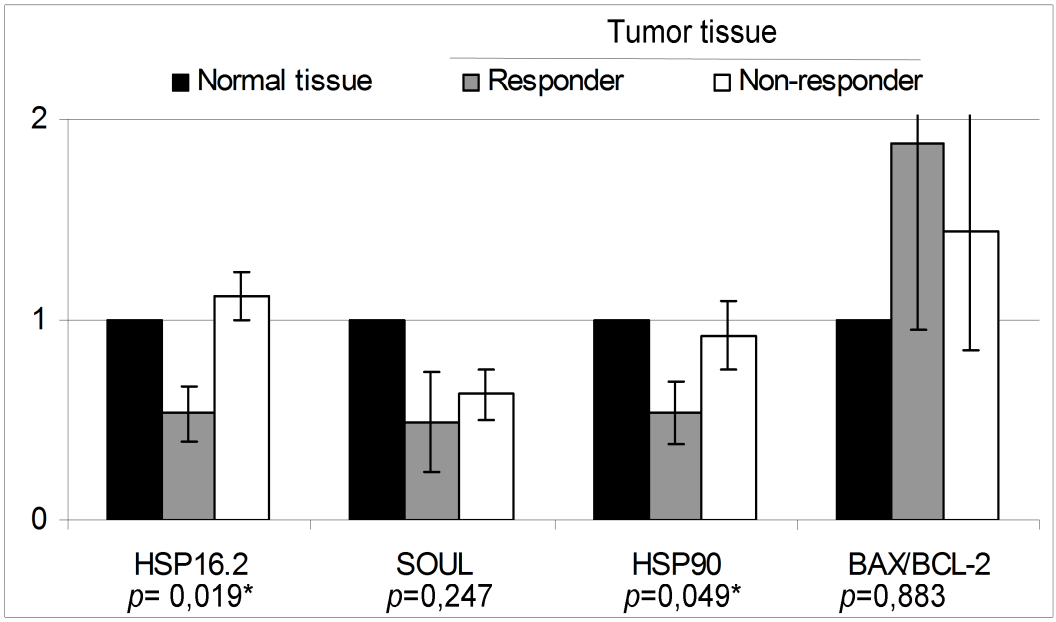


Figure 1

The results of the pathological examination were similar to the clinical results. The tumors with no histological response expressed twice as much Hsp90 (p=0.0005) and Hsp16.2 (p=0.002) and 1.5 times more SOUL (p=0.218) than the responders. On the other hand, a lower Bax/Bcl-2 ratio was seen in the non-responders compared to the responders, but as SOUL this result was not significant (p=0.499) (Figure 2).

Figure 2: Tumor-associated proteins and pathological response (statistically significant difference between responder and non-responder tumor tissue)*

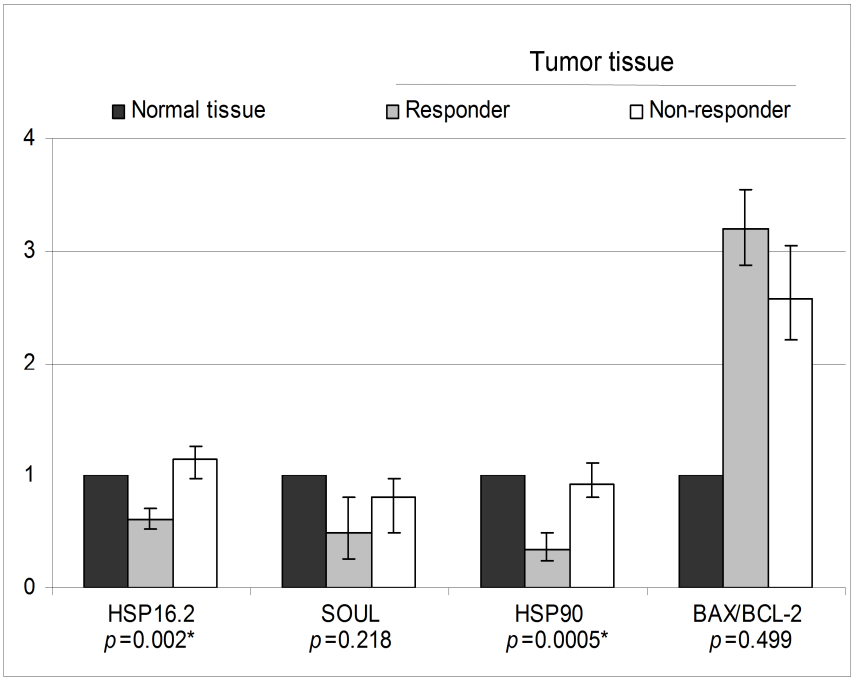


Figure 2

Particularly interesting results were observed when the samples were analyzed according to the tumor location. The upper tract tumors expressed the Hsp proteins in significantly lower quantities than the tumors located in the lower-third of the esophagus (Hsp90 upper *vs*. middle-third p=0.006 and Hsp16.2 upper *vs*. middle-third p=0.012). The SOUL protein was also expressed in significantly smaller quantities in the upper-third of the esophagus (p=0.047). Although the Bax/Bcl-2 ratio seemed to be lower in the middle-third tumors, the difference was not significant (p>0.05) (Figure 3 and Figure 4).

Figure 3:Tumor –associated proteins and tumor location. (p-values, upper vs. middle-third tumors)

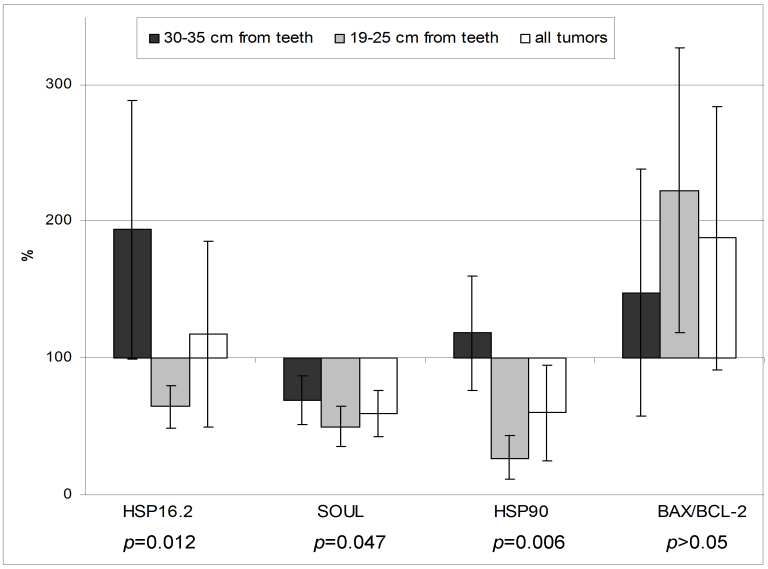
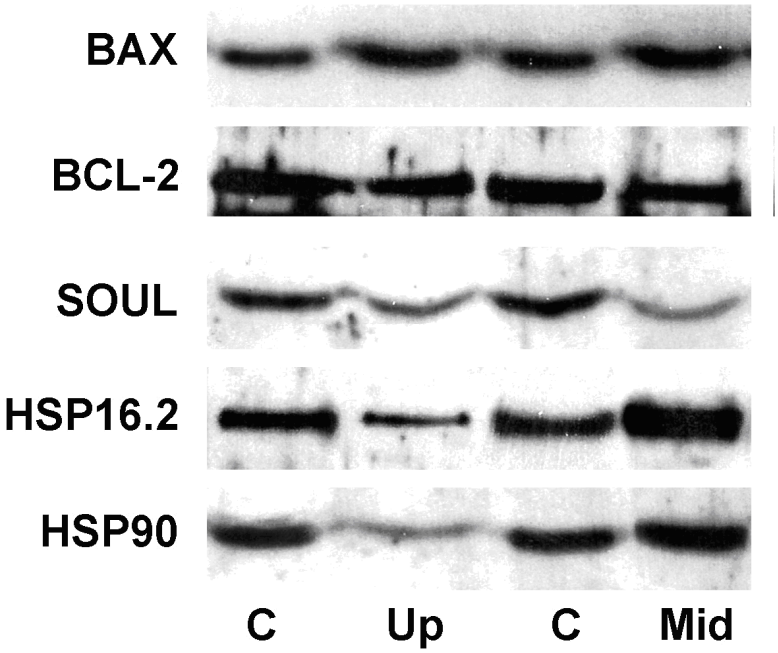


Figure 3

Figure 4:Western blot: Upper part tumors expressed chaperone proteins in significantly lower amounts than tumors located in the lower part of the esophagus



4.2. *Results in Rectal Study*

Histopathological Response to Neoadjuvant CRT

Curative resection was performed in 64 (92 %) cases. The surgical intervention was a low anterior resection in 49 cases (70%) or abdomino-perineal resection in 15 cases (21%), with R0 resection rate of 90%. Pathological evaluation of response to preoperative CRT in resected rectum specimens revealed complete response (TRG1) in 11 of 64 cases (17%) and significant response (TRG2) in 20 of 64 cases (31%). Hence good responders encompassing TRG1 and TRG2 categories account for 48% of patients, while poor responders including TRG3 for 19 cases (30%), TRG4 for 12 cases (19%) and TRG5 for 2 cases (3%) represented the remaining 52% of the patients.

Protein Expression in Pre-treatment Biopsy Specimens

Immunohistochemical evaluation of the pre-treatment biopsy specimens showed high intensity staining (++, +++) for SOUL, Hsp 16.2, Hsp90 and for GHRH-R in 67%, 61%, 58% and 25% of the cases, respectively. High intensity p-Akt staining was found in all the rectum biopsy specimens (Table V.). Typical staining of the examined markers is shown in FIGURE1

Table V. Immunhistochemical expression of proteins in pre-treatment biopsy specimens

Markers	Immunhistochemical expression			
	low intensity		high intensity	
	0	+	++	+++
SOUL	0 (0%)	23 (33%)	38 (55%)	8 (12%)
Hsp 16.2	0 (0%)	27 (39%)	33 (48%)	9 (13%)
Hsp 90	1 (1.5%)	28 (40.5%)	28 (40.5%)	12 (17.5%)
p-Akt	0 (0%)	0 (0%)	6 (9%)	63 (91%)
pGHRH-R	0 (0%)	44 (64%)	25 (36%)	0 (0%)

Association Between Pre-treatment Clinical Data and Histopathological Response to CRT

None of the pre-treatment clinical characteristics except the elapsed time interval between the end of neoadjuvant therapy and surgery was found to be statistically related to histopathological response. The patients who were operated on 7 weeks or more after CRT ended, had a significantly higher chance of showing a good response to neoadjuvant treatment, than those who underwent surgery within 7 weeks (63% versus 37%, p=0.041) following CRT. Univariate analysis of the correlation between other clinical parameters including age, sex, distance from anal verge, pre-treatment cT or cN and tumor regression grade revealed no statistically significant association (Table VI).

FIGURE 5. Immunohistochemistry for GHRH-R and Hsp90 in pre-treatment rectal carcinoma biopsies. Low intensity staining: GHRH-R (A) and Hsp90 (C), high intensity staining: GHRH-R (B) and Hsp90(D). The magnification is 100x (A,C,D) and 200x (B).

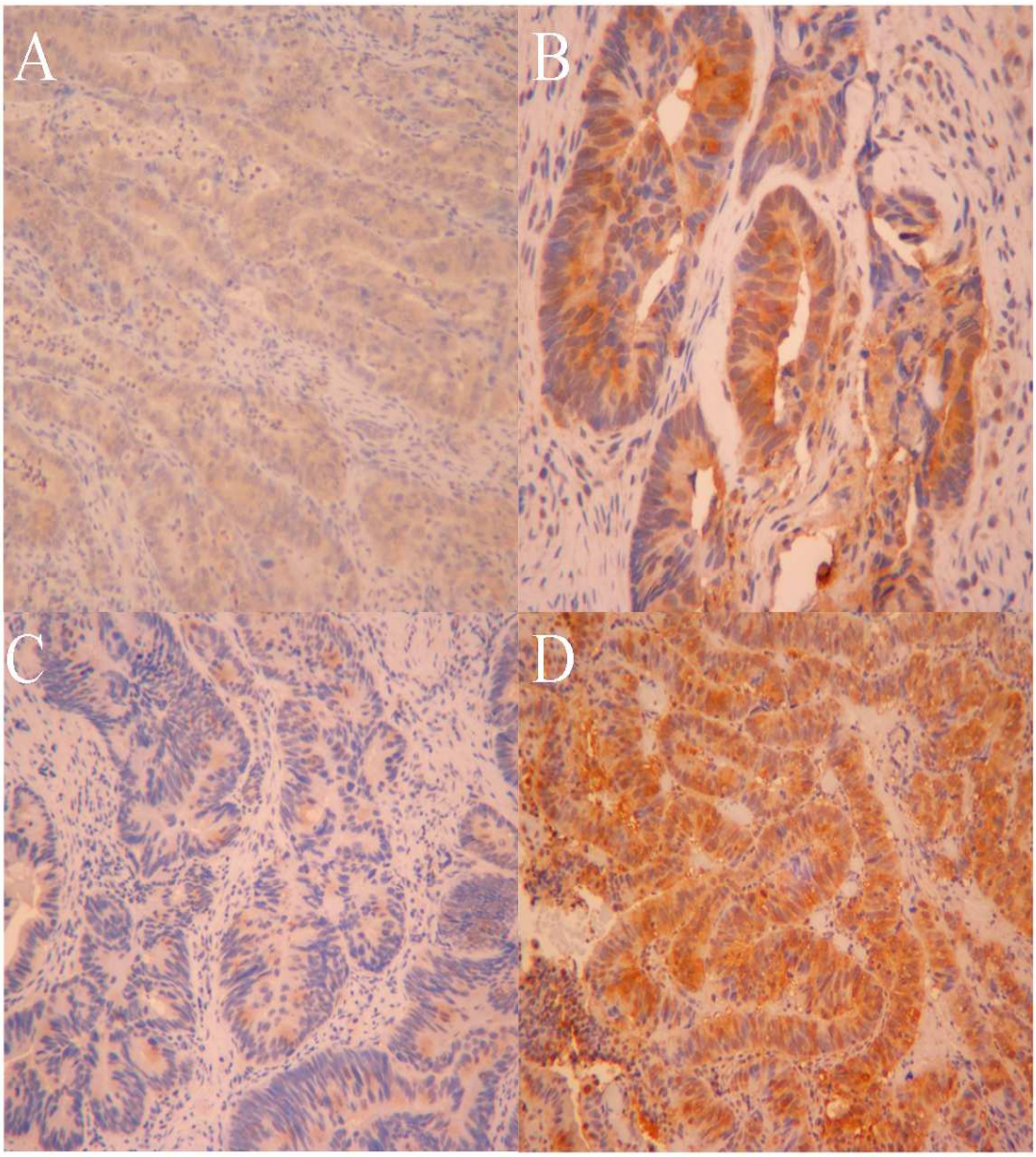


Table VI. Relationship between clinical factors and histopathological response to neoadjuvant RCT (n=64)

Clinical factor	Case No (n=64)	Good response (n=31)	Poor response (n=33)	*P*
Age (years) ≤ 60 > 60	 32(50%) 32(50%)	 13(20%) 18(28%)	 19(30%) 14(22%)	0,21, *0,15
Sex Male Female	 35(55%) 29 (45%)	 17(26%) 14(22%)	 18(28%) 15(24%)	0,98 *0,59
Clinical T stage cT2 cT3 cT4	 2 (3%) 55 (86%) 7 (11%)	 2(3%) 26(41%) 3(5%)	 0(0%) 29(45%) 4(6%)	0,28
Clinical N stage cN0 cN1-2	 25 (39%) 39 (61%)	 12(19%) 19(30%)	 13(20%) 20(31%)	0,95 *0,57
Distance from AV (cm) <5 5-10 >10	22(35%) 26(40%) 16(25%)	11(17%) 13(20%) 7(11%)	11(17%) 13(20%) 9(14%)	0,91
Time to surgery (weeks) ≤ 7 >7	37(58%) 27(42%)	14(22%) 17(27%)	23(36%) 10(16%)	**0,047** *0,041

Statistical analysis with chi-squere test, * Fishers correction for small samples, level of significance $p<0,05$

Association between Protein Expression and Histopathological Response to CRT

Among the markers evaluated in pre-treatment biopsy specimens, SOUL, Hsp16.2 and p-Akt staining did not show a significant association with tumor regression grade. However, high levels of Hsp90 and GHRH-R

expression in the pre-treatment tumor biopsies were significantly correlated with poor histopathological response (p=0.00002, p=0.00006 respectively). The relationship of immunohistochemical factors with tumor regression grade are shown in Table VII**.**

Table VII. Relationship between protein expression and histopathological response to neoadjuvant RCT (n=64)

Markers	**Case no. (n=64)**	**Good response (n=31)**	**Poor response (n=33)**	***P***
SOUL				
low intensity	20 (31%)	8(12%)	12(18%)	
high intensity	44 (69%)	23(36%)	21(33%)	0,43
Hsp16.2				
low intensity	25(39%)	15(23%)	10(16%)	
high intensity	39(61%)	16(25%)	23(36%)	0,29
HSP90				
low intensity	28(44%)	23(36%)	5(8%)	
high intensity	35(55%)	8(13%)	27(42%)	**0,00002**
P-AKT				
low intensity	6(9%)	4(6%)	2(3%)	0,75
high intensity	58(91%)	27(42%)	31(49%)	
GHRH				
low intensity	42(66%)	28(44%)	14(22%)	**0,00006**
high intensity	22(34%)	3(5%)	19(29%)	

Statistical analysis with chi-squere test, level of significance $p<0,05$

Multivariate analyses confirmed that the association of GHRH-R and Hsp90 expression with the therapeutic response was significant (for pGHRH odds ratio, 0.198; 95% confidence interval, 0.042-0.941; $p<0.05$ and for Hsp90 odds ratio, 0.218; 95% confidence interval, 0.074-0.647; $p<0.001$) after data was adjusted to account for the clinicopathological parameters and expression of the other markers.

5. DISCUSSION

5.1. Discussion regarding esophageal cancer

The efficacy and toxicity of neoadjuvant chemoradiotherapy depends on the treatment protocol (23). Our preoperative CRT scheme resulted a in pCR rate of 5%, which is lower than response rates reported in previous trials (16, 27). These results can be explained by the relative low radiation dose applied in our study. The intensification of neoadjuvant therapy, however, requires better patient selection, since more aggressive treatment could lead to serius or even fatal sideeffects. There is a strong need to individualize the therapeutic approach of esophageal cancer in order to reflect performance state, co- morbidity, estimated prognosis and chemoradiosensitivity of tumors.

Sensitivity to oncological therapy is determined by several molecular factors. Bcl-2 and its family members influence cell behavior in response to genotoxic stress (95). While Bcl-2 inhibits apoptosis by regulating the release of certain proteins such as cytochrome c from the mitochondria, Bax shows proapoptotic activity by permeabilizing the outer mitochondrial membrane (97-98). In the present study the patients with clinical and histological response to neoadjuvant therapy seemed to have a higher Bax/Bcl-2 ratio, whereas seemingly lower Bax/Bcl-2 ratios were found in the non-responders, although this trend was statistically not significant. Similarly better survival of patients with proapoptotic p21 positive esophageal tumors treated with RCT compared to those with no p21 expression has also been demonstrated (122), while elevated levels of survivin, an apoptosis inhibitor and key factor in resistance to RCT, predicted a significantly reduced median survival in patients receiving preoperative therapy (123-124).

Hsps are a group of proteins that are present in all cells in all life forms. Their production is induced when a cell undergoes various types of

environmental stress such as heat, cold and hypoxia. The anti-apoptotic activity of Hsps including small Hsps has been reported previously (125-126). These proteins are molecular chaperones helping to preserve original protein function and activity by protecting cells against various stress stimuli (*e.g.* hydrogen peroxide, taxol) (88, 127). Hsps are highly expressed in cancer cells and are essential to their survival. Hsp90 plays a particularly versatile role in cell regulation, forming complexes with a large number of cellular kinases, transcription factors and other molecules. Wu and coworkers demonstrated that Hsp90 was selectively expressed in esophageal cancer tissue compared to the corresponding normal tissue, and the inhibition of Hsp90 resulted in decreased proliferation and viability as well as radiosensitisation of esophageal cancer cells (128). Hsp16.2 forms self-aggregates and binds to Hsp90, thus promoting the effect of the latter protein. The over-expression of Hsp16.2 inhibits cell death *via* the stabilization of the mitochondrial membrane, activation of Hsp90, stabilization of lipid rafts and by the activation of the Phosphatidylinositol-3-kinases-Akt cytoprotective pathway (88). Both Hsps and small Hsps were confirmed as playing a role in the development of tumors, for example malignant brain tumors (84, 89). Furthermore, their overexpression in cancer cells has been linked to increased tumor growth and resistance to RCT (125-126). Elevated Hsp expression in malignant cells plays a key role in protection against spontaneous apoptosis associated with malignancy, as well as against apoptosis generated by therapy. These are mechanisms which may underlie the role of Hsp in tumor progression and resistance to treatment (129). Hence, the observation that the upper esophageal tumors expressed the Hsps at significantly lower levels than the middle-third tumors, is of particular importance, since it may be the possible explanation of the widely known fact, that cervical esophageal cancer has a superior sensitivity to multimodal therapy. As expected, the samples from the non-responding esophageal tumors expressed Hsp90 and Hsp16.2 at twofold compared to the levels of the responding tumors.

Response to stress may not only result in apoptosis, but also in necrotic cell death. The recently identified heme-binding protein SOUL sensitizes cells to necrosis by promoting the opening of mitochondrial permeability transition pores under stress (100). SOUL was also observed at a higher level in non-responders compared to the responding tumors. Overall the results were almost identical for both the clinical and pathological response. The results also suggested that the response to preoperative RCT may be related to the activation of stress mechanisms which act through different signal transduction pathways.

5.2. Discussion for rectal cancer

Neoadjuvant CRT followed by surgery is the widely accepted treatment for locally advanced rectal cancer. The outcome of rectal cancer appears to be correlated with the response to CRT, which is typically quite variable, with significant downstaging occurring in 30-64% of the cases and complete pathological response (pCR) rates ranging from 7 % to 30 % of the cases (72, 80, 130-132). Histopathological regression grading systems have been developed for the quantification of tumor response besides clinico-pathological downstaging. These grading systems are based on the biological effect of radiation on tumors, such as changes in tumor cell density and the extent of fibrosis (29, 130) . According to some authors, tumor regression grade should be regarded as a better marker of chemo-radiosensitivity than downstaging, since tumors often remain at the same stage following neoadjuvant chemoradiotherapy, even if the tumor shows significant histopathological changes (72, 80).

The value of TRG as an independent prognostic factor for disease-free survival has been demonstrated in several studies (78-80). Some studies report that pCR denotes better long term outcome, therefore they evaluate pCR separately (78-79). The results of retrospective analyses suggest however, that it may be possible to combine tumors into a group of good responders (TRG1 and TRG2) and a group of poor responders (TRG3,

TRG4 and TRG5), since those who show significant histopathological regression and complete pathologic regression have a similarly better prognosis than the remaining poorly responding patients (78-79). As with the pathological complete response, the rate of good responders varies highly in published studies ranging from 20 to 60 % of the cases (72, 78-80). In the present study we found that 48% of the patients showed a good response (TRG1 and TRG2). The observed difference in the number of good responders in previous reports might be explained with various treatment protocols including different radiation doses and dissimilar types of chemotherapy, and the diverse intervals between CRT and surgery. It was demonstrated that besides radiation dose the time between surgery and neoadjuvant treatment has a significant impact on tumor regression (133). In accord with this finding, a recent study found that an interval over 8 weeks between the completion of CRT and surgical resection was associated with a significantly higher rate of pCR (134). Similarly, in the present work, an interval longer than 7 weeks between CRT and surgery proved to be associated with a significantly higher rate of good tumor response, supporting the concept that radiation- induced biological changes develop over a longer period of time.

Regarding pre-treatment clinical parameters, in line with other investigations, we did not find any correlation between age, gender, clinical T stage, clinical N stage and tumor regression (79, 135-136). However, in an other study, preoperative CEA level, circumferential extent of tumor, and distance from the anal verge were found to be predictors of histopathological downstaging (135). In the present study we could not confirm the predictive value of distance from the anal verge, as it did not significantly influence the rate of tumor response.

TRG appears to be a good surrogate marker of tumor chemoradiosensitivity, because it mainly depends on biological factors representing the molecular pathways of tumor response rather than on pre-treatment clinical parameters. Accordingly, analyses of pre-treatment

biopsies using various molecular markers have been performed, some with equivocal results. Among the number of potential markers studied, the expressions of Bax, p53 and p27 as well as spontaneous apoptosis and tumor necrosis have been correlated with tumor regression (99, 137-138). Studies by Losi et al. and Lin et al. demonstrated that overexpression of p53 was significantly correlated with a poor clinical outcome (138-139), while others showed that higher levels of Bax and p27 were associated with a favourable outcome (137). On the other hand, another investigation found, that p27 does not predict histopathological response to RCT in rectal cancer (140).The apparent ambiguities in the literature warrant the investigation of novel molecular predictors of response. In our study we showed that the levels of immunohistochemical staining of anti-apoptotic p-Akt, necrosis-facilitating SOUL and Hsp 16.2 involved in cytoprotection, were not related to tumor regression. However, we found a significant correlation between the expressions of GHRH-R and Hsp90 and poor histopathological response. According to our data, rectal cancers that express GHRH-R and/or Hsp90 at high levels responded poorly to neoadjuvant RCT. These findings are important since it is vital, that patients who would not benefit from neoadjuvant CRT do not undergo treatment and lose time until surgery, which is approximately 3 months after the diagnosis is set up. Moreover, for the non-responding patients a tailored therapy is essential. Hsp90 inhibiting compounds are currently being tested in preclinical or phase I-III clinical trials as anticancer agents (83, 141-142). Hsp90 inhibitors have been shown to sensitize human tumors to irradiation, furthermore, some Hsp90 inhibitors bind Hsp90 in malignant cells with much higher affinity than in normal cells (143). For patients with Hsp90-positive rectal cancer, the application of suitable Hsp90 inhibitors would be highly beneficial. Antagonists of growth hormone-releasing hormone (GHRH) have been tested for the treatment of various types of experimental tumors, including malignant gliomas (144), breast cancer (111), ovarian cancer (145), prostate and lung cancers (109-110). GHRH antagonists block

the binding of autocrine as well as paracrine GHRH produced by the cancer cells to GHRH receptors (105, 111). GHRH antagonists have also been demonstrated to induce apoptosis through the key apoptotic signaling pathways in glioblastoma cells (89) as well as to cause DNA damage in colon cancer cells (146). In the present study we found, that rectal tumors expressing GHRH-R at a high level showed little or no tumor regression. Thus, GHRH-R, besides acting as a possible predictive marker could become a target of therapy, similarly to Hsp90, if GHRH antagonists could be introduced into the clinical practice.

6. Conclusion

In conclusion, our results suggest, in line with previous studies, that response to neoadjuvant therapy depends on the treatment protocol including the radiation dose and time between CRT and operation as well as on the biological features of tumor. The identification of biomarkers predicting the responses would allow more effective and individualized treatment.

1. In the present retrospective study of esophageal cancer the rate of complete responses achieved with neoadjuvant chemoradiotherapy was lower than pCR rates reported in the literature, which implies a need for escalation of radiation dose. However, the serious and fatal side effects related to CRT scheme indicate that any intensification of therapy requires thorough selection of patients.

2. In tumor samples from esophageal cancer patients with no clinical or pathological response to neoadjuvant CRT significantly higher levels of HSP90 and HSP16. 2 expression were detectable than in responding tumors, indicating the role of heat shock proteins in resistance against chemo-radiotherapy.

3. Our data demonstrate no significant association between the response of esophageal tumors to CRT and BAX/Bcl2 ratio representing the apoptotic form of cell death.

4. The present study did not find a significant association between the response of esophageal tumors to CRT and the expression of SOUL, a protein implicated in necrotic cell death.

5. Upper tract esophageal tumors expressed HSP proteins and SOUL protein in significantly lower quantities than middle-third tumors, which may contribute to their different sensitivity to CRT.

6. The current investigation indicates that pre-treatment clinical parameters do not influence the chemo-radiosensitivity of rectal cancer, whereas the time interval longer than 7 weeks between neoadjuvant CRT and operation is associated with better tumor response.

7. As regards cytoprotective heat shock proteins, the level of immunhistochemical staining of Hsp 16.2 was not related to tumor regression, whereas the expression of HSP90 was significantly correlated with poor histopathological response to CRT for rectal cancer.

8. The results of our study do not support the view that the expression of anti-apoptotic p-AKT has any influence on histopathological response to CRT for rectal adenocarcinomas.

9. A significant correlation between the expression of GHRH-R and poor histopathological response to neoadjuvant CRT for rectal cancer was demonstrated.

10. In the present study the level of immunhistochemical staining of necrosis-facilitating SOUL was not related to histopathological regression of rectal cancer after neoadjuvant CRT.

11. Our data indicate that GHRH-R and Hsp90 may serve as pretreatment predictors of tumor regression to neoadjuvant CRT in rectal cancer. Moreover GHRH-R and Hsp90 hold promise of providing novel therapeutic options for poor responder patients. Nevertheless, before any clinical implications can be drawn, further studies are warranted to confirm our results.

7. Acknowledgements

This work was performed under the inspiring guidance of Prof. Örs Péter Horváth MD, PhD, DSc. and thorough supervision of László Mangel MD. PhD. I am also very thankful for the invaluable help of Szabolcs Bellyei MD, PhD, who contributed to my work as a second consultant. Special thanks to Éva Pozsgai MD, PhD, Éva Gömöri MD, PhD, Katalin Hideghéty MD, PhD and András Papp MD, PhD for their theoretical and technical help. This work was supported by a grant from the Hungarian Science Foundation 68469, by Research Grants from the Ministry of Health (2006-2008; 01/270), by the Janos Bolyai Research Scholarship of the Hungarian Academy of Sciences and Research Grants from AOKKA-34039-1004/2010.

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