Possible predictive markers of response to therapy in esophageal and rectal cancers

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Possible p redictive markers of response to therapy in esophageal and rectal cancers

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1. Abbreviations and glossary

Akt	Protein kinase B
Akt/NF-KB	Protein kinase B / Nuclear Factor Kappa B
Akt/XIAP	Protein kinase B /X-linked inhibitor of apoptosis protein
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BMI	body mass index
CEA	Carcinoembryonic antigen
CRT	chemoradiotherapy
CT	computed tomography
ESCC	esophageal squamous cell cancer
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRH-R	pituitary-type growth hormone-releasing hormone receptor
GST	glutathione S-transferase
Gy	gray
Hps	heat shock protein
HSP16.2	heat shock protein16.2
Hsp27	heat shock protein 27
Hsp90	heat shock protein 90
IGF-I	insulin-like growth factor I
IgG	immunoglobulin G
KPS	Karnowsky score
MRI	Magnetic Resonance Imaging
NCRT	neoadjuvant chemoradiotherapy
OS	overall survival
p-Akt	Phospho- Protein kinase B

pCR	pathologic complete response
PI3K	phosphatidylinositol 3-kinase
pp23	placental protein 23
pp25	placental protein 25
sHsp	small Hsp
SOUL	heme-binding protein
SPSS	Statistical Package for the Social Sciences software
TNF-alfa	tumor necrosis factor alfa
TRG	tumor regression grade
5-FU	5-Fluorouracil

2. Introduction

Esophageal cancer is one of the most lethal malignancies and ranks as the eighth most common cancer in the world and the sixth most common cause of death from cancer (Ferlay et al., 2015). The incidence of esophageal cancer is on the rise due to an increase in adenocarcinomas located in the lower parts of the oesophagus (Mariette et al., 2007). The localization of esophageal squamous cell cancer (ESCC) is generally in the upper two-thirds of the esophagus. The development of ESCC has been linked to nicotine and drug abuse as well as to poor socioeconomic status (Keighley, 2003). Patients with ESCC often have additional illnesses that are related to alcohol and nicotine consumption, such as liver cirrhosis, chronic obstructive pulmonary disease and concomitant cancers of the lung or head and neck region (Nakajima and Kato, 2013).

Between 2000–2004 and 2005–2009, oesophageal cancer mortality declined by 7% (from 5.34 to 4.99/100 000) in men and by 3% (from 1.12 to 1.09/100 000) in women in the EU. Predictions for 2020 show persistent declines in mortality rates for men and stable rates for women in the EU overall. Although ESCC accounts for ~90% of the cases of oesophageal cancer worldwide, mortality rates associated with adenocarcinomas are rising and have surpassed those of ESCC in several regions in the EU (Castro et al., 2014). Oesophageal carcinoma is rare in young people and increases in incidence with age, peaking in the seventh and eighth decades of life. Adenocarcinomas are three to four times more common in men than in women, whereas the sex distribution is more equal for ESCC (Muro et al., 2019).

All patients with newly developed dysphagia, gastrointestinal bleeding, recurrent aspiration, vomiting, weight loss and/or loss of appetite should undergo an upper intestinal endoscopy. Approximately three-quarters of all adenocarcinomas are found in the distal oesophagus, whereas ESCCs occur more frequently in the proximal to middle oesophagus (Rustgi and El-Serag, 2014). Biopsies should be taken from all suspect areas. The minimal recommended number of biopsies is not defined. The diagnosis should be made from an endoscopic biopsy with the histology classified according to the World Health Organization (WHO) criteria. The differentiation

between SCC and adenocarcinoma is of prognostic and clinical relevance. Immunohistochemical staining is recommended in poorly and undifferentiated cancers (G 3/4) to differentiate between squamous cell carcinoma and adenocarcinoma according, to the WHO. Additionally, small cell carcinomas and other rare histologies (endocrine tumors, lymphoma, mesenchymal tumors, secondary tumors and melanoma) must be identified separately from squamous cell carcinomas and adenocarcinomas and should be treated accordingly (Muro et al., 2019).

At the time of diagnosis, two out of three patients will have tumors that are considered inoperable because of the extent of the tumor or other patient co morbidities. Surgical resection is possible in only 15–20% of the patients, following surgical exploration. Survival at 5 years is below 10% for all patients, independent of having undergone surgery or not (Mariette et al., 2007).

Neoadjuvant radiochemotherapy (NCRT) is the accepted modality of therapy for locally advanced ESCC, since preoperative radiochemotherapy has been shown to increase long-term survival (Mariette et al., 2007, Gebski et al., 2007, Hanna et al., 2016). For submucosal cancer without metastases (T1bN0M0), definitive chemoradiotherapy is a potentially curative treatment option. Adjuvant or neoadjuvant therapy is combined for tumors that invade the muscular propria or adventitia, and/or with lymph node metastases. Patients whose tumors invade an adjacent organ, or patients with distant metastases, undergo chemotherapy, radiotherapy or chemoradiotherapy (Nakajima and Kato, 2013).

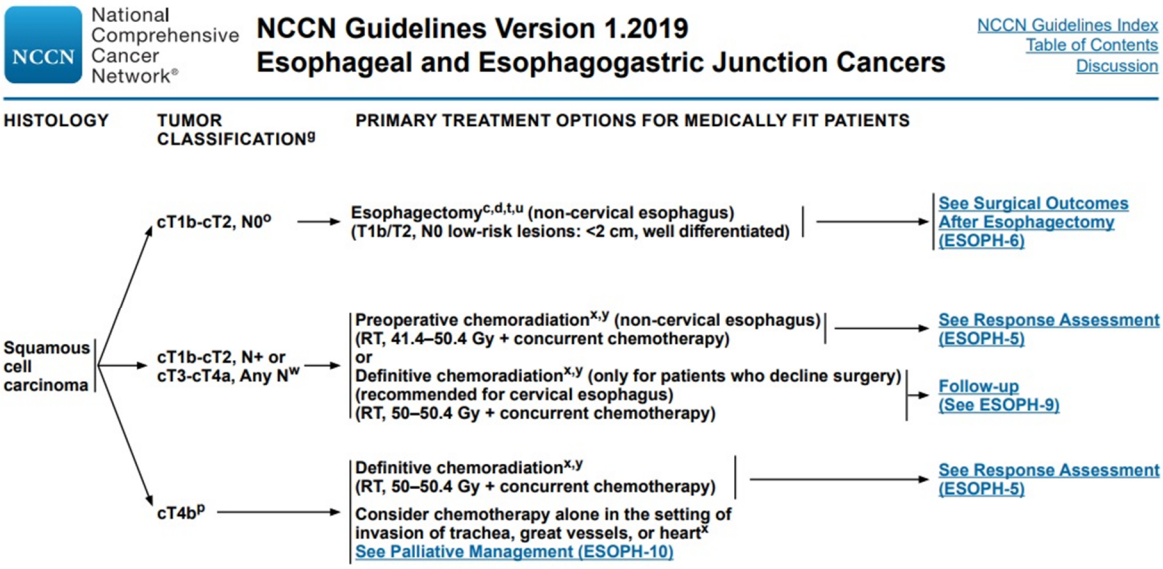

Figure 1. Figure shows the recommended NCCN guidelines for the treatment of locally advanced ESCC.

The goals of applying neoadjuvant chemotherapy and radiotherapy in combination are to utilize the radiosensitising effects of chemotherapy in order to maximize tumor reduction as well as to maximize local control (Gebski et al., 2007).

Cisplatin is a platinum drug that is generally used in the treatment of ESCC. In monotherapy or in combination with other drugs, cisplatin has been demonstrated to improve patient outcomes (Ui et al., 2014).

A number of patients receiving NCRT respond poorly or do not respond at all to therapy. There have been numerous studies examining potential markers of response to treatment in order to avoid unnecessary toxicity to patients and to improve their life-quality and survival (Gillham et al., 2007, Sarbia et al., 2007).

Response to treatment can be optimized by tailoring the dosage of administered cisplatin, 5-FU and irradiation. According to an earlier study, the dose of irradiation, between 30 and 45 Gys, is directly correlated with the complete pathological response of the ESCC at stage II/III ESCC (Ordu et al., 2014). A meta-analysis involving 1335 patients showed that there is a dose-response relationship between increasing protocol prescribed radiotherapy, 5-fluorouracil, cisplatin dose and

pathological complete response to treatment (Geh et al., 2006). Other significant factors influencing response to treatment were found to be radiotherapy treatment time and median age of patients (Geh et al., 2006).

The prognosis for ESCC, especially in advanced stages, remains dismal, despite improvements in multimodal treatment (Nakajima and Kato, 2013). The identification of markers that signal poor response to treatment is essential as these can be targets for individualized, more effective therapy. Clinically, the determination of the survival rate of patients is equally important as evaluating the response to treatment, which is measured by the tumor regression grade (TRG) and the clinical downstaging of the tumor. Therefore, it was the aim of our study to identify possible predictive biomarkers of response to NCRT and to overall survival of patients.

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is considered one of the major anti-apoptotic pathways in cells (Bardeesy and DePinho, 2002, Chung et al., 2008, Mistafa and Stenius, 2009). The pathway is activated by growth factors and is frequently overexpressed in cancer cells (Baba et al., 2010). This antiapoptotic signaling pathway plays an essential role in controlling various processes in cells, which are often characteristic of cancer such as cellsurvival, cellmigration or proliferation (Faes and Dormond, 2015). PI3K is initially activated by tyrosine kinase receptors, then PI3K catalyzes the phosphorylation of PtdIns(4,5) P2 (phosphatidylinositol 4,5-bisphosphate) to PtdIns(3,4,5) P3 (phosphatidylinositol 3,4,5-trisphosphate), which causes the recruitment and activation of PDK1 and Akt to the plasma membrane. Subsequently, Akt affects a number of downstream enzymes, which leads to the survival and proliferation of the cell. Mutations of both these downstream effectors and of Akt family genes are prevalent in different types of human cancers (Carpten et al., 2007). Even one mutation, a substitution of an amino acid , E17K, in the PH domain of Akt results in the constitutive recruitment of Akt to the cell membrane (Carpten et al., 2007, Faes and Dormond, 2015).

A few studies have previously shown that Akt can interact with other kinases besides PI3K to effect cell transformation (Mahajan and Mahajan, 2012). Ack I (activated CDC42-associated kinase 1), a non-receptor tyrosine kinase and Ser/Thr kinase I-κ-B kinase epsilon (Ικκε) are both capable of activating Akt independent of the PI3K

pathway (Xie et al., 2011). The activation of the anti-apoptotic phosphorylated-Akt (p-Akt)-mediated pathways, such as those for PI3K/Akt, Akt/NF-KB and Akt/XIAP, has been shown to correlate with a poor response to NCRT and lower overall survival of ESCC patients (Zhu et al., 2015, Ui et al., 2014, Jin et al., 2016). Based on these numerous reports, it is understandable, that drugs targeting AKT have been in the focus of drug development against cancer (Rodon et al., 2013).

The proteins activating the p-Akt pathways, for example Heat shock protein 90 (Hsp90) and protein Aurora-A, have been identified as possible targets of therapy (Wang et al., 2016, Ui et al., 2014).

Heat shock proteins (Hsp) are a ubiquitous group of proteins found in all living organisms. They are expressed in response to different types of stress including environmental changes such as extreme temperature, anoxia, hypoxia, various chemical agents that can cause stress for cells and induce protein denaturation (Macario and Conway de Macario, 2007).Heat shock proteins function as molecular chaperones aiding the folding and assembly of proteins, thus playing an important role in cytoprotection and cell survival (Hermisson et al., 2000). Their classification is based on the molecular weights of Hsp-s (Ciocca and Calderwood, 2005). Hsp-s are ATP-dependent proteins with ATPase activity, excepting the group of small Hsp-s (Bepperling et al., 2012).

Hsp90 is a chaperone protein, which ensures the stable conformations of a number of client proteins implicated in signaling pathways responsible for the progression of malignant cells (Kabakov et al., 2010). Client proteins include EGFR, IGF-1R, CDK4,Akt, ErbB2, c-Met, BCR–ABL, RET, Fms-like tyrosine kinase 3 (FLT3), BRAF, NF-kB, Raf-1, HER2/Neu, NPM–ALK, neuronal nitric oxide synthase (nNOS) (Kamal et al., 2004). Thus, Hsp90 has become a much studied molecular target in cancer research. The overexpression of Hsp90 has been demonstrated in a number of cancers including oropharyngeal squamous cell carcinoma, multiple myeloma, lung-, breast-, ovarian-, and pancreatic cancers (Shi et al., 2014). Hsp90 overexpression was found to be a biomarker for poor progrnosis in esophageal cancer, lung cancer and bladder cancer as well (Tian et al., 2014).

HSP27 is another key regulator in cellular apoptosis, drug resistance and the development, progression, and metastasis of cancer (Wu et al., 2017). Expression of Hsp27 has been shown to indicate poor prognosis. When Hsp27 was experimentally inhibited, matrix metalloproteinase activity decreased which activity is a characteristic of cancer cell invasion and metastasis formation (Gibert et al., 2013). Furthermore, overexpression of HSP27 was shown to be correlated with peritoneal metastasis of epithelial ovarian cancer (Zhao et al., 2012).

The levels of Heat shock proteins in tumor specimens have also been correlated with response to treatment. A previous investigation indicated that the expression level of HSP27 may be inversely correlated with the metastatic behavior of ESCC, furthermore another working group found that a higher expression of Hsp27 was positively correlated with the grade of differentiation of ESCC (Xue et al., 2014, Chen et al., 2006).

Small Hsps (sHsp) have a molecular weight ranging between 2-43 kD and like other Hsps, they also act as molecular chaperones. They exert their effect on a range of client proteins, and through the stabilization of the cytoskeleton, sHsps indirectly regulate complex intracellular processes, such as apoptosis, cell differentiation, thermotolerance and response to cell stress (Carra et al., 2017). The apparent significant role of sHsps in different types of cancer has made them a target for recent research (Sun et al., 2004, Aoyama et al., 1993, Klemenz et al., 1991).

Hsp 16.2 is a small heat shock, which was found to be expressed in neuroectodermal tumors (Bellyei et al., 2007a, Pozsgai et al., 2007, Bellyei et al., 2007b). The overexpression of Hsp16.2 was shown to protect cells from stress conditions by inhibiting the release of cytochrome c from the mitochondria, the nuclear translocation of AIF and endonuclease G, and caspase 3 activation by protecting the integrity of the mitochondrial membrane system. Hsp16.2 inhibits cell death through the activation of Hsp90 followed by activation of lipid raft formation in the cells and by the activation of PI3K-Akt anti-apoptotic pathway. The overexpression of Hsp 16.2 in solid tumors has been thought to be responsible for resistance to cytostatic treatment (Bellyei et al., 2007a).

According to a previous 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 (Pozsgai et al., 2007).

Earlier, we reported that small heat-shock protein (sHsp)16,2, Hsp90, as well as Bax/Bcl-2 ratio correlated with the efficacy of NCRT and could predict outcome in patients with locally advanced ESCC (Farkas et al., 2011).

A recent study showed that tumor necrosis proved to be an independent prognostic variable concerning progression-free and cancer-specific survival (Pollheimer et al., 2010). SOUL is a member of the BH3-domain-only protein family (Szigeti et al., 2006).

BH3 domain-only proteins have been shown to have a key role in the processes of cell death and survival (Szigeti et al., 2010). Bcl-2 proteins with the BH3 domain have been thought to play a part in enabling mitochondrial-mediated apoptosis (Kelekar and Thompson, 1998). Proteins with proapoptotic BH3 domains are considered latent death factors that need to be activated to exert their cell-death enabling roles. There are a few processes through which Bcl-2 family members, such as Bax, Bid and Bim, are activated. Bid and Bim bind to Bax-type proteins at the outer mitochondrial membrane through their BH3-domain (Epand et al., 2003). This triggers the conformational change of the proteins, leading to intramembranous oligomerization and permeabilization of the outer mitochondrial membrane without affecting the integrity of the inner membrane (Soane and Fiskum, 2005, Baines et al., 2005). The interaction between lipid bilayers and proapoptotic Bcl-2 family members contribute to the apoptotic process . While the Bcl2 homologue, Bax can effect both necrotic and apoptotic cell death on its own (Baines et al., 2005) the BH3-domain protein, SOUL can only facilitate cell death caused by other stimuli and induce necrotic cell death (Szigeti et al., 2010). SOUL permeabilizes the outer mitochondrial membrane like proapoptotic Bcl-2 family members and also facilitates the permeabilization of the inner membrane, leading to mitochondrial swelling, the rupture of the outer membrane, and the release of intermembrane proapoptotic proteins. Thus, SOUL facilitates necrotic cell death in oxidative stress (Szigeti et al., 2010).

The purpose of our investigation was 3-fold. We aimed to correlate possible predictive markers of response to NCRT in ESCC as well as their expression to 3-year overall survival. It was also our goal to determine whether dose of NCRT had any effect on the clinical and histological response to NCRT. Finally, we evaluated the association between the clinical parameters (age, Karnowsky score, tumor localization, weight loss) of the patients' and their 3-year overall survival.

Rectal cancer is the third most frequent malignancy in males and second in females, accounting for about 1.2 million new cases per year worldwide (Jemal et al., 2011). The incidence of rectal cancer in the European Union is ~125 000 per year, i.e. ~35% of the total colorectal cancer incidence, reflecting 15–25 cases/100 000 population per year and is predicted to increase further in both genders. The mortality is 4–10/100 000 population per year. Median age at diagnosis is ~70 years, but predictions suggest that this figure will rise in the future. Evidence is accumulating that rectal cancer is distinct from colon cancer with different aetiologies and risk factors (Wei et al., 2004), possibly reflecting different environmental exposures. High body mass index, body or abdominal fatness and diabetes type II are seen as risk factors. Longstanding ulcerative colitis and Crohn's disease affecting the rectum, excessive consumption of red or processed meat and tobacco as well as moderate/heavy alcohol use increase the risk (Glynne-Jones et al., 2018).

The treatment mainly depends on the clinical stage of the tumor and, for the majority of the patients, combined multimodal therapy consisting of surgery, chemotherapy and radiotherapy is recommended. Based on data from randomised clinical trials, pre-operative neoadjuvant chemoradiotherapy (NCRT) followed by surgery is established as the standard treatment in locally advanced rectal tumor including cT3/T4 and/or cN+ stage cancers (Bosset et al., 2006, Gerard et al., 2006, Sauer et al., 2004). 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 NCRT- induced tumor response which varies among individual patients (Bouzourene et al., 2002).

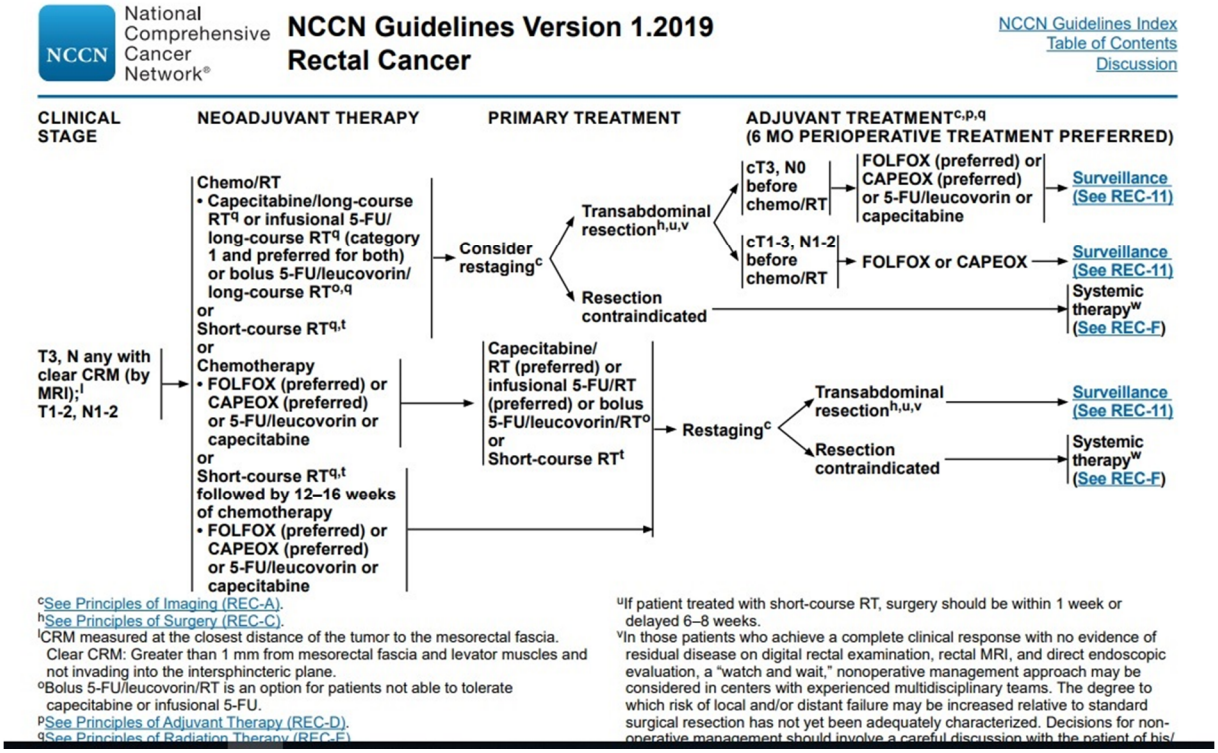

Figure 2. Figure shows the recommended NCCN guidelines for the treatment of locally advanced rectal cancer.

Although some patients do not respond at all and their disease actually shows progression, others undergo surgery and their surgical samples are completely devoid of viable tumor cells, indicating a pathologic complete response (pCR). The incidence of pCR ranges between 10% to 30% and has been associated with decreased local recurrence, longer recurrence-free survival, and increased sphincter-preservation rates (Kalady et al., 2009). 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 (Dhadda et al., 2011, Suarez et al., 2008, Vecchio et al., 2005, Bouzourene et al., 2002). 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. The factors that predict response to therapy in rectal cancer have not been fully explored yet. 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. Several retrospective studies have investigated the

factors associated with tumor response to neoadjuvant chemoradiation including higher radiation doses, different chemotherapeutic drugs, interval to surgery, and molecular markers such as P53 gene mutation and endogenous p21 expression (Kalady et al., 2009).

It was our aim to identify possible predictive biomarkers of response to NCRT. We studied the possible predictive role of Heat shock protein (Hsp90), Hsp16.2, phospho-Akt, SOUL and pituitary-type growth hormone-releasing hormone receptor (GHRH-R) proteins.

Growth hormone-releasing hormone (GHRH) is a peptide hormone secreted by the hypothalamus, but also present in various tissues and tumors. GHRH stimulates the secretion of growth hormone (GH) after binding to pituitary-type GHRH receptors (GHRH-R) on the anterior pituitary (Rekasi et al., 2005, Rekasi et al., 2000, Schally et al., 2008). 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 (Schally and Varga, 1999, Schally et al., 2000, Schally et al., 2001, Schally et al., 2004). The presence of GHRH-R and its splice variants, on different types of cancer cell lines has been demonstrated (Havt et al., 2005, Barabutis and Schally, 2008).

GHRH is considered as a autocrine/paracrine growth factor for tumors, since it was detected in breast, endometrial, ovarian, colorectal, gastric, and lung cancers (Schally et al., 2015). The GHRH/GHRH-R pathway is regarded as a growth factor-signaling pathway in many cancers and modulates the activities of multiple intracellular pathways. Thus, targeting the GHRH/GHRH-R pathway is logical in cancers(Gan et al., 2016). Antagonists of growth hormone-releasing hormone have been tested for the treatment of various types of experimental tumors (Kahan et al., 2000, Kiaris et al., 1999, Kiaris et al., 2003, Chatzistamou et al., 2001a, Busto et al., 2002). 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 (Chatzistamou et al., 2001b, Braczkowski et al., 2002, Zeitler and Siriwardana, 2002).

Antagonists of GHRH, JMR-132 and MIA 602, decreased the expression of the levels of anti-apoptotic proteins, phospho-Akt, phospho-GSK3β and phosho-ERK 1/2. Increased levels of pro-apoptotic proteins, caspase-3 and poly(ADP-ribose) (PARP) were also detected, following treatment. In glioblastoma cells, the nuclear translocation of apoptosis inducing factor (AIF) and Endonuclease G (Endo G) and the mitochondrial release of cytochrome c (cyt c) were found after treatment, which showed that the cells were undergoing apoptosis. In vivo experiments on nude mice showed significantly extended tumor doubling times, after the treatment with the GHRH-antagonist (Pozsgai et al., 2010).

In vitro studies on breast, glioblastoma and ovarian cancer cell lines showed that the GHRH antagonist, MIA-602 decreased tumor cell viability significantly, inhibited cell invasion and suppressed the release ofmatrix metalloproteinases. Wound-healing experiments showed reduced cellular motility in all three cell lines. The downregulation of NF-kappaB and beta-catenin and the upregulation of caveolin-1 and E-cadherin was found. These characteristics of MIA-602 showed that GHRH-antagonists inhibit cancer cell proliferation and metastasis development (Bellyei et al., 2010).

We investigated the immunohistochemical expressions of Hsp90, sHsp16.2, p-Akt, GHRH-R and SOUL proteins in pre-treatment biopsy samples of rectal carcinoma and compared their expressions with the grade of histopathologic tumor regression after neoadjuvant therapy. We aimed to correlate the response to CRT with the pretreatment expression of the five proteins cited in order to try to identify one or more as a predictive marker. It was also our onjective to determine the association between pre-treatment clinical data (age, sex, distance from anal verge, pre-treatment cT or cN and tumor regression grade, elapsed time interval) and histopathological response to NCRT in patients with rectal tumor.

2.1 Taken together, the goals of my study were to determine the following:

1. Is there an association between treatment parameters (dose of chemotherapy and irradiation) and response to NCRT in ESCC?

2. Can a correlation be found between the expression of proteins, p-AKT, SOUL, sHsp16.2, GHRH-R and response to NCRT treatment in ESCC patients?

3. Is there a correlation between the expression of cellular proteins, p-AKT, SOUL, sHsp16.2, GHRH-R, and 3-year overall survival in tumor samples from ESCC patients?

4. Can these proteins (p-AKT, SOUL, sHsp16.2 and GHRH-R) be possible biomarkers for response to NCRT therapy in ESCC patients?

5. Is there an association between the clinical parameters (age, Karnowsky score, tumor localization, weight loss) of ESCC patients and their 3-year overall survival?

6. How strongly did pretreatment rectal tumor samples stain for proteins p-AKT, SOUL, Hsp16.2, Hsp90 and GHRH-R?

7. Was there an association between pre-treatment clinical data (age, sex, distance from anal verge, pre-treatment cT or cN and tumor regression grade, elapsed time interval) and histopathological response to NCRT in patients with rectal tumor?

8. Can a correlation be found between the expression of proteins p-AKT, SOUL? Hsp16.2, GHRH-R and histopathological response to NCRT in patients with rectal cancer?

9. Can one or all of proteins p-Akt, SOUL, Hsp16.2, GHRH-R serve as predictors of tumor regression to NCRT in rectal cancer?

3. Materials and Methods

3.1 Patients and Methods for the study regarding esophageal cancer

3.1.1 Patients and tumor specimens

Factor	N = 88
Age (years)	
≤ 60	44 (50%)
> 60	44 (50%)
Clinical T stage	
cT3	66 (75%)
cT4	22 (25%)
Clinical N stage	
cN0	32 (36%)
cN1-2	56 (64%)
Dose of radiotherapy	
≤ 40 Gy	50 (57%)
> 40 Gy	38 (43%)
Dose of cisplatin	
≤ 75 mg/m²	50 (57%)
> 75 mg/m²	38 (43%)
Dose of 5-FU	
≤ 750 mg/m²	66 (75%)
> 750 mg/m²	22 (25%)

Table 1. Patients, tumors and treatments specimens

Ninety two consecutive patients with inoperable, loco-regionally advanced (cT3-4, cN0-1, cM0) squamous-cell esophageal cancer received neoadjuvant NCRT from 2006 to 2010. The pre-treatment staging procedures consisted of endoscopy with biopsy, computed tomography (CT) scan of chest and abdomen and bronchoscopy. The patients were treated with external-beam radiotherapy (a total of 36 to 45 Gy, fraction dose: 1.8-2 Gy) and concomitant chemotherapy during the first week of irradiation: cisplatin (60-100 mg/m^2 intravenously on day 1) and 5-fluorouracil

(750-1000 mg/m^2/day, by continuous intravenous infusion through days 1-5).Two patients died during the treatment and two patients refused control examinations. (Table 1).

Four weeks after the completion of NCRT, restaging was performed and clinical response to treatment was assessed according to RECIST (Therasse et al., 2000). Six to nine weeks after neoadjuvant therapy the patients underwent surgical resection, if there was no evidence of disease progression. Pathological response to treatment was determined by histological evaluation of the resected specimen. The histopathological tumor regression grade based on the presence of residual tumor cells and the extent of fibrosis was evaluated. For this purpose the five point tumor regression grading (TRG) system adapted from Mandard et al. was used (Mandard et al., 1994). The system consists of the following grades: TRG1 (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. Based on the results of previous studies, in order to simplify the statistical analysis, the TRG system was combined into two groups: good responders comprising TRG1-2 and poor responders consisting of TRG 3- 5 (Gerard et al., 2006, Sauer et al., 2004, Mandard et al., 1994). All the patients signed informed consent, which was approved by the Local Ethics Committee.

3.1.2 Preparation of polyclonal antibodies against Hsp16.2 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 (Bellyei et al., 2007a, Bellyei et al., 2007b, Szigeti et al., 2006). 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.

3.1.3 Immunohistochemistry

Sections of the pre-treatment tumor tissue samples were fixed in formalin and embedded in paraffin. Subsequently, they were incubated with the following primary antibodies: self-developed anti-Hsp16.2 and anti-SOUL polyclonal primary antibodies, GHRH-R primary antibody purchased from Abcam (Abcam Inc., Cambridge, MA), p-AKT primary antibody purchased from Cell Signaling. GHRH-R antibody detected the presence of both pituitary-type GHRH-R as well as the splice variants of the GHRH-R. Immunohistochemical staining was carried out by 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). The staining intensity was recorded semiquantitatively as mild (+), moderate (++) or strong (+++), following as described before (Somji et al., 2001). 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.

3.1.4 Statistical Analysis

Statistical analyses were carried out using the SPSS 15.0 statistical program (SPSS, Chicago). Univariate chi-square test was used to compare clinical parameters and biological markers for clinical response and tumor regression grade. To increase the number of patients per group, the categories of the various variables were combined for these analyses: age over 60 years vs. 60 years or below, cT2 vs. cT3-cT4, cN0 vs. cN1-2, tumor localization, radiotherapy dose of higher than 40 Gy vs 40 Gy or below, dose of ciplatin over 75 mg/m^2 vs 75 mg/m^2 or below, 5-FU dose over 750 mg/m^2 vs. 750 mg/m^2 or below. For testing statistical intensity, values of immunohistochemistry were dichotomised into low (0, +) and high (++, +++) intensity categories. All parameters were analyzed afterwards in a logistic regression multivariate analysis. A p value of less than 0.05 was considered statistically significant. The effect of the clinical parameters and the biological markers on

overall survival (OS) was demonstrated using Kaplan-Meier curves and the level of significance was determined using the log-rank test. The survival functions were computed from the date of the first symptoms/the start of neoadjuvant CRT by using Kaplan-Meier estimates, and the log-rank test was used to assess the equality of survival functions. The univariate and multivariate Cox regression analyses were performed to test for the independent influence of potential prognostic factors on overall survival (OS). Probability (p) values < 0.05 were considered statistically significant, and statistical tests were based on a two-sided significance level. Statistical analyses were performed with use of Statistical Package for the Social Sciences software (SPSS, Chicago, IL).

3.2 Patients and Methods for the study regarding rectal cancer

3.2.1 Patients, Pre-treatment and Posttreatment

Factor	N = 64
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

Table 2. Patient and tumor characteristics

Sixty four 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. All the patients signed informed consent, which was approved by the Local Ethics Committee. The main clinical characteristics of the patients are given in Table 2.

3.2.2 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. (Mandard et al., 1994). 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: TRG1 (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 (Gerard et al., 2006, Sauer et al., 2004, Mandard et al., 1994).

3.2.3 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 [9,10] 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.

3.2.4 Immunohistochemistry

Sections from the tumor tissue samples were fixed in formalin and embedded in paraffin. Subsequently, they were incubated with the following primary antibodies: self-developed anti-Hsp16.2 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 (Somji et al., 2001). 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.

3.2.5 Statistical Analysis

Statistical analyses were carried out using SPSS 16.0 statistical program (SPSS, Chicago). Univariate chi-squre test with Fishers correction for small samples was used, if necessary, 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 Related to the study on Esophageal cancer

4.1.1 Clinical outcome

Patients underwent restaging, whereas 2 patients died during the treatment and 2 patients refused control examinations. Clinical evaluation found that 36 (39%) tumors showed clinical response to neoadjuvant CRT, 4 (4%) patients had complete remission, 32 (35%) patients had partial remission, 42 patients had stable disease (46%), 14 patients had progressive disease (15%). Resection was performed in 42 (46%) cases, with R0 resection rate of 47%. Histopathological evaluation of response to preoperative CRT in resected oesophageal specimens revealed a complete response (TRG1) in 6 of 42 cases (14%) and significant response (TRG2) in 16 of 42 cases (38%). Hence, good responders accounted for 52% of the patients, while poor responders represented the remaining 48% of the patients.

4.1.2 The association between protein expression and response to NCRT in ESCC

Tumor samples taken before initiation of treatment were stained for four molecular markers (SOUL, Hsp16.2, GHRH-R) and p-Akt), then the intensity of staining was evaluated in both the responding and non-responding groups. Responsiveness to NCRT was also determined according to clinical downstaging and TRG classification. Among the markers evaluated, expression of GHRH was low in 90% of the tumor specimens and GHRH-R staining did not show a significant association with tumor response to CRT. However, high expression levels of Hsp16.2 in the pre-treatment tumor biopsies were significantly correlated with poor clinical and histopathological response ($p = 0.001$, $p = 0.000$ respectively). High intensity staining for p-AKT was also associated with significantly lower rate of good clinical and histopathological response ($p = 0.02$, $p = 0.032$ respectively). Low expression of SOUL resulted in twice as many clinically responding patients ($p = 0.037$) and four times as many histopathologically responding patients ($p = 0.001$). The relationship between the expression of the proteins and response to NCRT is shown in (Table 3).

Clinical Downstaging (n = 88)				
Molecular Markers		**Responder**	**Non-responder**	***p value***
SOUL	Low intensity	25 (28%)	23 (26%)	***p = 0.037***
	High intensity	12 (14%)	28 (32%)	
Hsp16.2	Low intensity	26 (30%)	18 (20%)	***p = 0.001***
	High intensity	11 (12%)	33 (38%)	
GHRH-R	Low intensity	24 (27%)	40 (46%)	*p = 0.158*
	High intensity	13 (15%)	11 (12%)	
p-Akt	Low intensity	20 (23%)	15 (17%)	***p = 0.020***
	High intensity	17 (19%)	36 (41%)	
TRG (n = 42)				
Molecular Markers		**Responder**	**Non-responder**	***p value***
SOUL	Low intensity	18 (42%)	6 (15%)	***p = 0.001***
	High intensity	4 (10%)	14 (33%)	
Hsp16.2	Low intensity	22 (52%)	2 (5%)	***p = 0.000***
	High intensity	0 (0%)	18 (43%)	
GHRH-R	Low intensity	17 (41%)	14 (33%)	*p = 0.592*
	High intensity	5 (12%)	6 (14%)	
p-Akt	Low intensity	15 (36%)	7 (17%)	***p = 0.032***
	High intensity	7 (17%)	13 (30%)	

Table 3. The relationship between protein (SOUL, Hsp16.2, GHRH-R and p-Akt) expression and clinical and histopathological response to NCRT

4.1.3 *The association between treatment parameters and response to NCRT in ESCC*

We investigated whether the dosage of chemotherapy (Cisplatin and 5-Fluorouracil) and irradiation affected the clinical downstaging and TRG of the tumors. A higher dose of irradiation (41-45 Gy) resulted in a significantly higher number of clinical responders ($p = 0.009$), while the dosage didn't significantly affect TRG. A higher dose of Cisplatin (above 75 mg/m^2), on the other hand, significantly increased the number of TRG responders ($p = 0.004$) but did not significantly affect clinical response. In our study the administered dose 5-Fluorouracil (5-FU) did not significantly affect TRG and clinical response (Table 4).

Clinical Downstaging (n = 88)				
Treatment parameters		**Responder**	**Non-responder**	***p value***
Dose of radiation	**36-40Gy**	15 (17%)	35 (40%)	***p = 0.009***
	41-45 Gy	22 (25%)	16 (18%)	
Dose of Cisplatin	**Below 75mg/m^2**	18 (20%)	32 (36%)	*p = 0.188*
	Above 75mg/m^2	19 (22%)	19 (22%)	
Dose of 5-FU	**Below 750mg/m^2**	26 (30%)	40 (46%)	*p = 0.383*
	Above 750mg/m^2	11 (12%)	11 (12%)	
TRG (n = 42)				
Treatment parameters		**Responder**	**Non-responder**	***p value***
Dose of radiation	**36-40Gy**	8 (19%)	10 (24%)	*p = 0.372*
	41-45 Gy	14 (33%)	10 (24%)	
Dose of Cisplatin	**Below 75mg/m^2**	8 (19%)	16 (38%)	***p = 0.004***
	Above 75mg/m^2	14 (33%)	4 (10%)	
Dose of 5-FU	**Below 750mg/m^2**	14 (33%)	16 (38%)	*p = 0.241*
	Above 750mg/m^2	8 (19%)	4 (10%)	

Table 4. The association between treatment parameters and response to NCRT in ESCC

4.1.4 The relationship between expression of pre-treatment proteins (SOUL, Hsp16.2, GHRH-R and p-Akt) and 3-year overall survival (OS)

It was our aim to examine whether there was a correlation between pre-treatment protein expression and 3-year OS. The intensity of GHRH-R (Fig.3A) staining did not affect 3-year OS significantly (p = 0,891). Low expression of Hsp16.2 and SOUL (Fig.3B,C) did not significantly increase 3-year OS (p = 0.19 and p = 0.63 respectively), however, a non-significant improvement after about 8 months in the 3-year OS was apparent. Interestingly, low intensity staining for p-Akt (Fig.3D) increased the 3-year OS significantly (p = 0.00) (Table 5.).

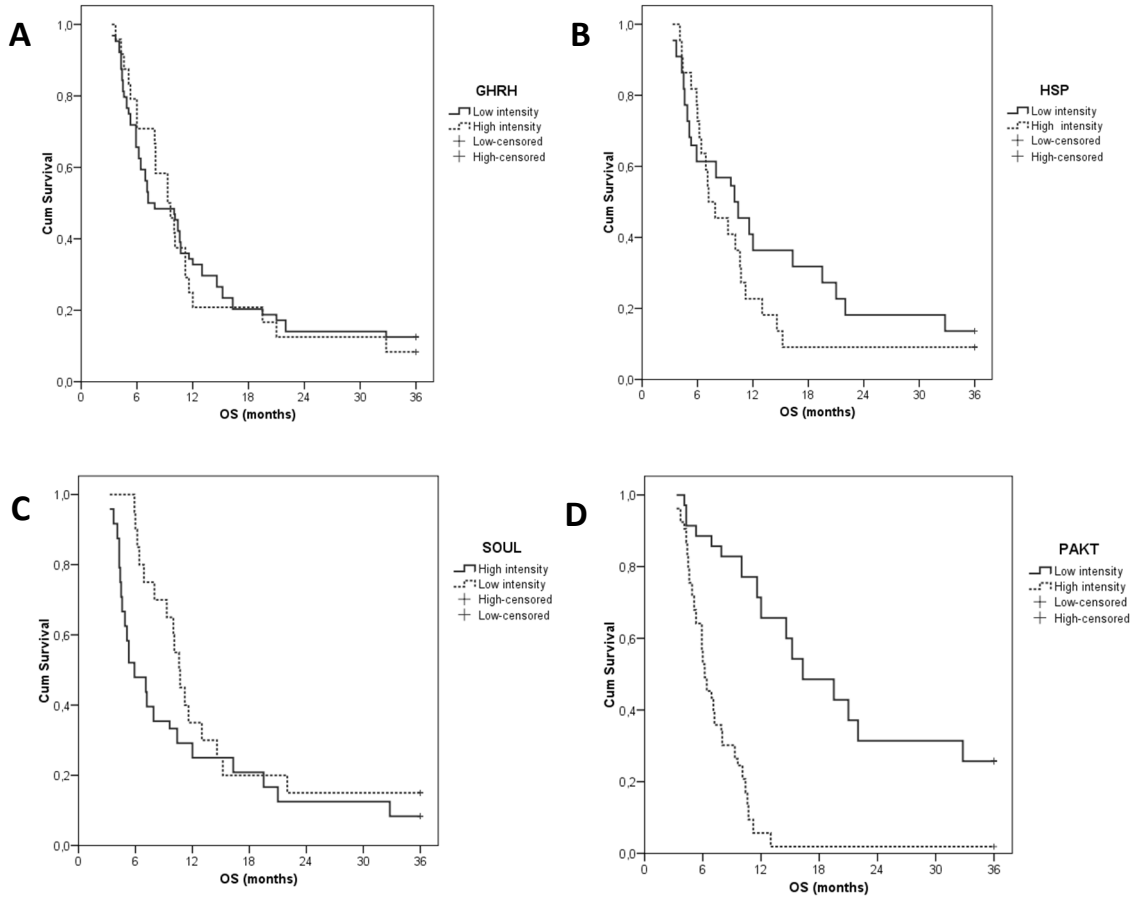

Figure 3. The relationship between expression of pre-treatment proteins and 3-year overall survival (OS)

The relationship between pre-treatment proteins GHRH-R (A) p = 0.891, Hsp16.2 (B) p = 0.19, SOUL (C) p = 0.63, and p-Akt (D) p = 0.00 staining and 3-year OS. The effect of biological markers on overall survival was demonstrated using Kaplan-Meier curves and the level of significance was determined using the log-rank test. Probability (p) values < 0.05 were considered statistically significant.

		Mean		Median	
		Estimate	**Std. Error**	**Estimate**	**Std. Error**
GHRH-R	Low intensity	12.641	1.301	7.200	1.556
	High intensity	12.438	1.929	9.300	1.225
	Log Rank (Mantel-Cox) Sig.: 0.891				
Hsp16.2	Low intensity	14.334	1.699	10.000	1.706
	High intensity	10.836	1.289	7.200	0.995
	Log Rank (Mantel-Cox) Sig.: 0.191				
SOUL	Low intensity	14.285	1.558	10.600	0.474
	High intensity	11.169	1.470	5.900	0.989
	Log Rank (Mantel-Cox) Sig.: 0.63				
p-Akt	Low intensity	20.194	1.921	16.300	2.415
	High intensity	7.560	0.650	6.200	0.520
	Log Rank (Mantel-Cox) Sig.: **0.0000**				
Age	< 60 years	13.193	1.658	7.200	1.399
	>= 60 years	11.919	1.351	10.400	1.403
	Log Rank (Mantel-Cox) Sig.: 0.875				
Karnofsky score	Low intensity	11.609	1.691	9.300	1.411
	High intensity	13.143	1.395	8.000	1.871
	Log Rank (Mantel-Cox) Sig.: 0.600				
Pre-treatment weight-loss	< 10%	15.539	1.918	10.400	0.900
	>= 10%	10.540	1.182	7.900	0.961
	Log Rank (Mantel-Cox) Sig.: **0.045**				
Tumor localization	upper	14.957	1.628	11.200	0.847
	middle	10.487	1.399	7.200	0.616
	lower	5.260	0.375	4.600	-
	Log Rank (Mantel-Cox) Sig.: **0.002**				

Table 5. The relationship between pre-treatment proteins staining and clinical parameters and between OS (months).

4.1.5 The relationship between clinical parameters (age, Karnofsky score, pre-treatment weight-loss, tumor localization,) and 3-year OS

We evaluated the effect of the individual clinical parameters on 3-year OS. The cutoff value for age was 60 years, for weight-loss (between initial symptoms and beginning of NCRT) was 10% of original body mass, and for Karnofsky score 80%. We could not detect a significant difference in 3-year OS among our patients in the two age groups (Fig. 4A) or in the groups assigned according to their Karnofsky score (Fig. 4B), although there was a non-significant improvement in the OS of younger patients after 12 months. However, there was a significant decrease in 3-year OS in patients whose pre-treatment weight-loss (Fig. 4C) exceeded 10% of their body mass ($p = 0.045$). The localization of the tumor affected 3-year OS greatly (Fig. 4D). Patients with upper-third ESCC had a significantly higher 3-year OS, than patients with middle and lower third tumors ($p = 0.002$) (Table 5.).

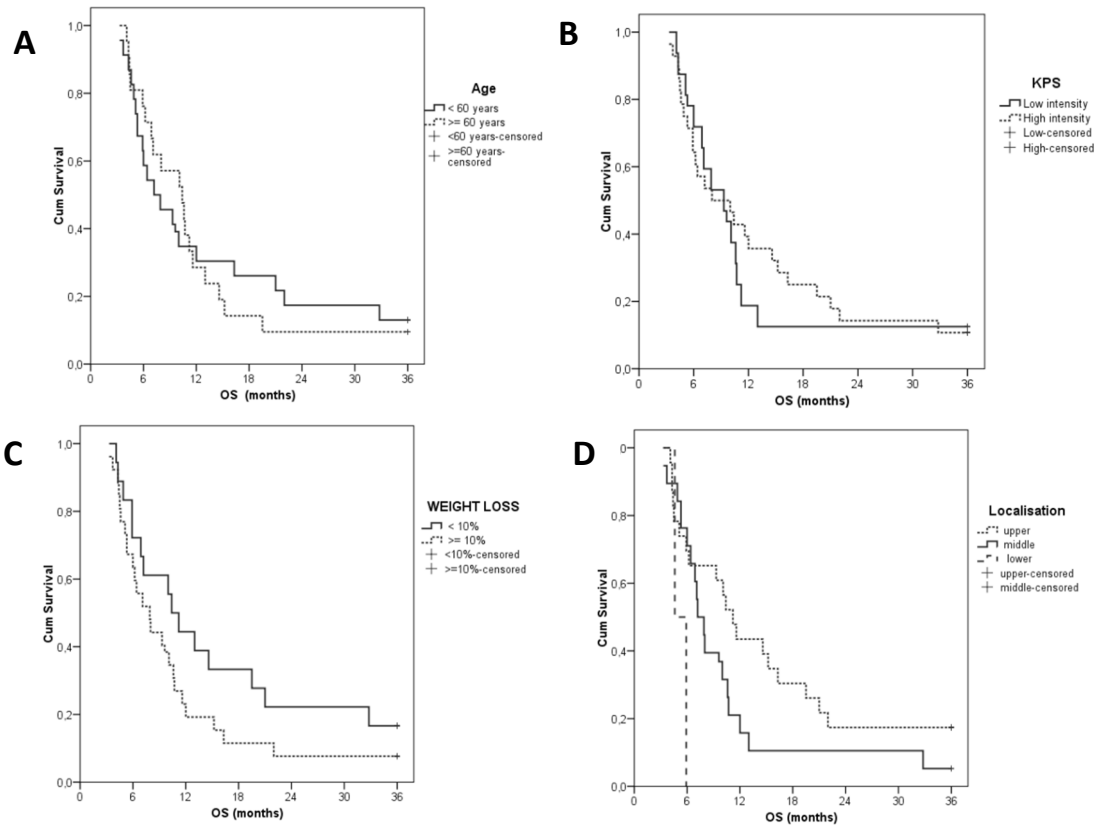

Figure 4. The relationship between clinical parameters and 3-year OS

The relationship between clinical parameters age (A) p = 0.875, Karnofsky score (B) p = 0.6, pre-treatment weight-loss (C) p = 0.045, tumor localization (D) p = 0.002 and 3-year OS. The effect of the clinical parameters on overall survival was

demonstrated using Kaplan-Meier curves and the level of significance was determined using the log-rank test. Probability (p) values < 0.05 were considered statistically significant.

4.2 Related to the study on Rectal Cancer

4.2.1 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 20 of 64 cases (31%) and significant response (TRG2) in 11 of 64 cases (17%). 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.

4.2.2 Protein Expression in Pre-treatment Biopsy Specimens

Immunohistochemical evaluation of the pre-treatment biopsy specimens showed high intensity staining (++, +++) for SOUL, Hsp16.2, 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 6). Typical staining for the examined markers is shown in Fig 5**.**

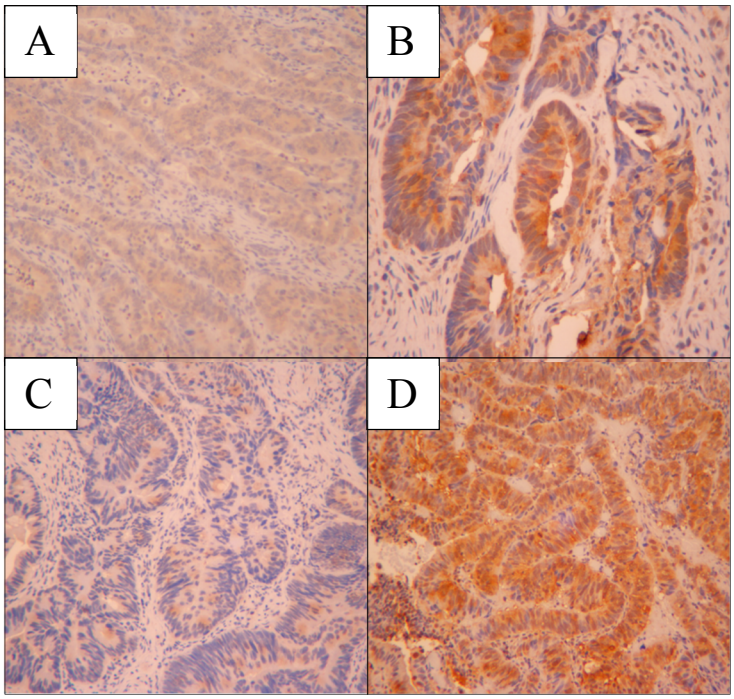

Figure 5. Immunohustochemistry

Immunohustochemistry for GHRH-R and Hsp90 in pretherapy 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).

	Immunhistochemical expression			
Markers	**low intensity**		**high intensity**	
	0	+	++	+++
SOUL	0 (0%)	23 (33%)	38 (55%)	8 (12%)
Hsp16.2	0 (0%)	27 (39%)	33 (48%)	9 (13%)
Hsp90	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%)

Table 6. Immunhistochemical expression of proteins in pre-treatment biopsy specimens

4.2.3 *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%, $p = 0.047$) 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 7).

Clinical factor	Case No	Good response	Poor response	*p*
Age (years)				
≤ 60	32 (50%)	13 (20%)	19 (30%)	0.21
> 60	32 (50%)	18 (28%)	14 (22%)	
Sex				
Male	35 (55%)	17 (26%)	18 (28%)	0.98
Female	29 (45%)	14 (22%)	15 (24%)	
Clinical T stage				
cT2	2 (3%)	2 (3%)	0 (0%)	
cT3	55 (86%)	26 (41%)	29 (45%)	0.28
cT4	7 (11%)	3 (5%)	4 (6%)	
Clinical N stage				
cN0	25 (39%)	12 (19%)	13 (20%)	0.95
cN1-2	39 (61%)	19 (30%)	20 (31%)	
Distance from AV (cm)				
< 5	22(35%)	11 (17%)	11 (17%)	
5-10	26(40%)	13 (20%)	13 (20%)	0.91
> 10	16(25%)	7 (11%)	9 (14%)	
Time to surgery weeks				
≤ 7	37 (58%)	14 (22%)	23 (36%)	0.047
>7	27 (42%)	17 (27%)	10 (16%)	

Table 7. Relationship between clinical factors and histopathological response to neoadjuvant CRT (n = 64) (Case No n =64, Good response n = 31, Poor response n = 33)

4.2.4 *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 8**.**

Markers	Case no	Good respone	Poor response	*p*
pp23				
low intensity	20 (31%)	8 (12%)	12 (18%)	0.43
high intensity	44 (69%)	23 (36%)	21 (33%)	
pp25				
low intensity	25 (39%)	15 (23%)	10 (16%)	0.29
high intensity	39 (61%)	16 (25%)	23 (36%)	
HSP90				
low intensity	28 (44%)	23 (36%)	5 (8%)	**0.00002**
high intensity	35 (55%)	8 (13%)	27 (42%)	
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-square test, level of significance $p < 0.05$				

Table 8. Relationship between protein expression and histopathological response to neoadjuvant CRT (Case No n =64, Good response n = 31, Poor response n = 33)

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

The accepted standard treatment modality for locally advanced ESCC is NCRT followed by surgery. NCRT consists of irradiation and concomitant chemotherapy, based on the administration of cisplatin and 5-FU (Mariette et al., 2007, Gebski et al., 2007). An earlier study showed that there was a positive correlation between the administration of higher doses of radiotherapy, 5-FU, cisplatin and complete pathological remission (Geh et al., 2006). Later, a number of other studies found evidence that application of higher cisplatin and irradiation doses resulted in a significantly increased rate of complete responses and improved 5-year OS (de Manzoni et al., 2005, Pasini et al., 2005, Ordu et al., 2014). In accordance with these studies we found that higher radiation doses (over 40 Gy) led to an increased number of clinical responders, and that application of higher cisplatin doses (over 75 mg/m^2) resulted in more histopathological responders. Although advances have been made in therapy, due to the poor prognosis of ESCC, it is of great importance, that responders be identified before initiating treatment (Hanna et al., 2015). In our previous investigation we identified possible novel biomarkers of response to NCRT. We showed that overexpression of Hsp16.2 and Hsp90 in tumor samples was associated with poor response to CRT. Hsp-s are chaperones, that have a major role in cytoprotection through the prevention of the aggregation of stress-accumulated misfolded proteins (Parcellier et al., 2005). Hsp-s have also been implicated in the increased survival of tumor cells (Ui et al., 2014, Xu et al., 2016). A study by Ui et al. showed that the inhibitor of Hsp90 (17-AAG) synergized with cisplatin and helped induce apoptosis in cisplatin –resistant ESCC (Ui et al., 2014). This effect was shown to be modulated through the the Akt/Xiap pathway (Ui et al., 2014). Hsp16.2 is a member of the small heat shock family (Bellyei et al., 2007a, Bellyei et al., 2007b). In accordance with our earlier findings, we found that tumors that stained high for Hsp16.2 had a significantly lower rate of clinical and histopathological response than those that expressed Hsp16.2 at lower levels. We were curious to determine how the levels of p-Akt were associated with the response

to CRT. As expected, mostly, tumors expressing a higher amount of p-Akt proved to be poor responders to CRT. This finding could be in part explained by a previous report, that Hsp16.2 inhibits cell death by binding to Hsp90 and through the activation of the PI-3kinase/Akt cytoprotective pathway (Bellyei et al., 2007a). Our detection of a significant correlation between high staining of proteins p-Akt, Hsp16.2 and poor response could be observed in both clinical and histopathological (TRG) responsiveness. This indicates the potential of these proteins as markers of response. Besides the response to therapy, the length of survival is also important when assessing the efficacy of treatment. It was of particular interest that we found that patients whose tumors showed high staining for Hsp16.2 and p-Akt had a worse 3-year OS than patients whose tumors stained low. The inverse correlation between length of OS and intensity of staining for the protein, however, was found to be significant only for p-Akt but not for Hsp16.2. This latter result could be explained by the relatively small number of patients in our group. Since the role of the activation of the p-Akt pathways in ESCC has been reported by a number of studies, the possibility of using p-Akt pathway as a target in the treatment of cancer has emerged (Wang et al., 2016, Jin et al., 2016, Chang et al., 2016). Our evidence suggests that the selective targeting of Hsp16.2, and by thus, inhibiting the PI-3kinase/Akt pathway, could be a promising tool in the treatment of ESCC. Unlike in our previous study, where the inverse correlation existed, but was not significant, we now found that low SOUL staining in tumor samples was associated with significantly improved clinical and histopathological response. SOUL is a heme-binding protein which has been shown to promote necrotic cell death by inducing mitochondrial permeability (Szigeti et al., 2006). It could be expected, that a higher intensity of necrosis would allow the decrease of the tumor. Surprisingly, a non-significant but negative correlation between the intensity of staining for SOUL and 3-year OS could be detected. In recent studies, we found evidence that tumor necrosis factor alfa (TNF-alfa) could be implicated in increased resistance to chemotherapy in prostate cancer (Sha et al., 2015). Another investigation showed that patients with elevated transmembrane TNF-α expression were more likely to

have a worse prognosis than patients with low tmTNF-α expression in colorectal cancer (Li et al., 2016). Therefore, we hypothesized that by generating a higher grade of necrosis inside the tumor, SOUL could make tumor cells less sensitive to chemotherapy. However, the precise mechanism behind the negative effect of SOUL on response and OS needs to be further elucidated. Various cancers have been found to express GHRH-R and/or its splice variants and GHRH has been shown to act as an autocrine growth factor for many malignancies (Plonowski et al., 2002, Kahan et al., 1999, Garcia-Fernandez et al., 2003, Rekasi et al., 2000, Halmos et al., 2000, Szereday et al., 2003). From the four proteins that were examined, staining for GHRH-R showed no significant correlation with the response to therapy, neither could a difference be detected between patients whose tumors expressed GHRH-R at different levels in the 3-year OS. Moreover, 90% of tumor specimens stained low for GHRH-R. These results are supported by the previous finding that squamous cell carcinoma of the oesophagus was negative for GHRH-R and SV-1, while adenocarcinomas of the oesophagus showed a strong expression of both receptors (Hohla et al., 2008). Numerous studies have examined the effect of individual clinical parameters on the OS of patients. Old age, male gender, low hemoglobin content, low Karnofsky score and low socioeconomic staus have all been correlated to poor OS (Wu et al., 2016, Koppert et al., 2012, Kandaz et al., 2012, Neuhof et al., 2005). In our study, we found no significant correlation between patients over or below 60 years and 3-year OS, but an improvement it the younger age group was apparent after 12 months. Similarly, the Karnofsky score of the patients did not significantly affect the 3-year OS. Since these parameters have been investigated by a number of studies earlier on a large number of patients, it is rational to assume that our results failed to show the expected significant correlation due to the smaller number of patients in our study. Nutritional status has been proven to be predictive of OS. Di Fiore et al. found that a BMI over 18 kg/m^2 was an independent prognostic factor of survival in patients with locally advanced esophageal cancer (Di Fiore et al., 2007) and another investigation showed that a weight-loss above 9.7% from the onset of the disease until the start of the therapy had a significantly unfavorable

impact on survival (Zemanova et al., 2012). Accordingly, we found that those patients who lost more than 10% of their body weight between the appearance of the first symptoms of the illness and the start of NCRT, had a significantly shorter 3-year OS, than those patients who lost less than 10%. Before the introduction of NCRT, tumors in the upper-third of the esophagus were considered to have a worse prognosis, than middle and lower-third ESCC. In an earlier study, we reported that a higher rate of response could be observed in patients with upper-third ESCC, compared to patients middle third ESCC (Papp et al., 2010, Papp et al., 2007). As a continuation of our previous investigation, the degree of 3-year OS of patients with differing localization of tumors was evaluated. We found, that not only the response to NCRT-but the 3-year OS was also significantly better in patients with upper-third tumors than patients with middle or lower third tumors.

Neoadjuvant CRT followed by surgery is the widely accepted treatment for locally advanced rectal cancer. The purpose of pre-operative CRT is to reduce tumor volume, thus to facilitate resection and increase the likelihood of a sphincter-preserving procedure, and to improve local control (Sauer et al., 2004). 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 (Vecchio et al., 2005, Bouzourene et al., 2002, Rodel et al., 2005, Carlomagno et al., 2009, Crane et al., 2010). 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 (Mandard et al., 1994, Rodel et al., 2005) . 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 (Bouzourene et al., 2002, Vecchio et al., 2005).

The value of TRG as an independent prognostic factor for disease-free survival has been demonstrated in several studies (Dhadda et al., 2011, Suarez et al., 2008, Vecchio et al., 2005). Some studies report that pCR denotes better long term outcome, therefore they evaluate pCR separately (Dhadda et al., 2011, Suarez et al., 2008). 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 (Dhadda et al., 2011, Suarez et al., 2008). As with the pathological complete response, the rate of good responders varies highly in published studies ranging from 20 to 60% of the cases (Bouzourene et al., 2002, Dhadda et al., 2011, Suarez et al., 2008, Vecchio et al., 2005). 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 (Berger et al., 1997). 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 (Kalady et al., 2009). 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 (Suarez et al., 2008, Das et al., 2007, Rebischung et al., 2002). However, in an other (Das et al., 2007) study, preoperative CEA level,

circumferential extent of tumor, and distance from the anal verge were found to be predictors of histopathological downstaging. 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 a 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 (Pollheimer et al., 2010, Chang et al., 2005, Lin et al., 2006). Studies by Losi et al. and Lin et al. demonstrated that overexpression of p53 was significantly correlated with a poor clinical outcome (Losi et al., 2006, Lin et al., 2006), while others showed that higher levels of Bax and p27 were associated with a favourable outcome (Chang et al., 2005). On the other hand, another investigation found, that p27 does not predict histopathological response to CRT in rectal cancer (Gunther et al., 2003).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 Hsp16.2 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 CRT. 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 (Bohonowych et al., 2010, Holzbeierlein et al.,

2010, Kabakov et al., 2010). 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 (Hwang et al., 2009). 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 (Kiaris et al., 2000), breast cancer (Kahan et al., 2000), ovarian cancer (Engel et al., 2005), prostate and lung cancers (Havt et al., 2005, Barabutis and Schally, 2008). GHRH antagonists block the binding of autocrine as well as paracrine GHRH produced by the cancer cells to GHRH receptors (Kahan et al., 2000, Schally and Varga, 1999). GHRH antagonists have also been demonstrated to induce apoptosis through the key apoptotic signaling pathways in glioblastoma cells (Pozsgai et al., 2007) as well as to cause DNA damage in colon cancer cells (Hohla et al., 2009). 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. Conclusions

1. A higher dose of irradiation resulted in a significantly higher number of clinical responders among ESCC patients, while the dosage didn't significantly affect TRG. A higher dose of Cisplatin (above 75 mg/m^2), on the other hand, significantly increased the number of TRG responders but did not significantly affect clinical response.

2. High expression levels of Hsp16.2 and p-AKT in the pre-treatment tumor biopsies were significantly correlated with poor clinical and histopathological response to NCRT in ESCC patients. Low expression levels of SOUL resulted in twice as many clinically responding patients and four times as many histopathologically responding patients. GHRH-R staining did not show a significant association with tumor response to NCRT.

3. Low intensity staining for p-Akt increased the 3-year OS significantly in ESCC patients. Low expression of Hsp16.2 and SOUL did not significantly increase 3 year OS, however, a non-significant improvement after 8 months in the 3-year OS was shown. The intensity of GHRH-R staining did not affect 3-year OS significantly.

4. Since high levels of Hsp16.2, p-Akt and SOUL were negative prognostic factors in response to NCRT in ESCC patients and were correlated with decreased 3-year overall survival, these biomarkers are potential predictors of response which have implications for clinical practice.

5. There was a significant decrease in 3-year overall survival in ESCC patients whose pre-treatment weight-loss exceeded 10% of their body mass. Patients with upper-third ESCC had a significantly higher 3-year OS, than patients with middle and lower third tumors. There was no significant difference in 3-year OS among patients in the different age groups or in the groups assigned according to their Karnofsky score, although there was a non-significant improvement in the OS of younger patients after 12 months.

6. Immunohistochemical staining showed high intensity staining for p-Akt in all the pre-treatment rectal cancer biopsy specimens. Biopsy specimens varied regarding high intensity staining for SOUL, Hsp16.2, Hsp90 and for GHRH-R.

7. None of the pre-treatment clinical characteristics (age, sex, distance from anal verge, pre-treatment cT or cN and tumor regression grade, elapsed time interval) except the elapsed time interval between the end of NCRT and surgery was found to be statistically related to histopathological response in patient with rectal cancer. The patients who were operated on 7 weeks or more after NCRT ended, had a significantly higher chance of showing a good response to neoadjuvant treatment, than those who underwent surgery within 7 weeks following NCRT.

8. High levels of Hsp90 and GHRH-R expression in the pre-treatment rectal tumor biopsies were significantly correlated with poor histopathological response to NCRT. SOUL, Hsp16.2 and p-Akt staining did not show a significant association with tumor regression grade.

9. Our data indicated that GHRH-R and Hsp90 may serve as predictors of tumor regression to NCRT in rectal cancer. Furthermore, GHRH-R and Hsp90 hold promise of providing novel therapeutic options for poor responder patients.

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8. Publications related to the Thesis

Zoltan L, Farkas R, Schally AV, Pozsgai E, Papp A, Bognár L, Tornoczki T, Mangel L, Bellyei S.

Possible Predictive Markers of Response to Therapy in Esophageal Squamous Cell Cancer.

Pathol Oncol Res. 2017 Nov 4. doi: 10.1007/s12253-017-0342-z.PMID:29103201

IF: 1,9

Farkas R, Pozsgai E, Schally AV, Szigeti A, Szigeti E, ***Laszlo Z***, Papp A, Gomori E, Mangel L, Horvath PO, Bellyei S:

Possible predictors of histopathological response to neoadjuvant chemoradiotherapy for rectal cancer, Journal of cancer research and clinical oncology 2012, 138: (3) pp. 387-395.

IF: 2.91

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Prognostic role of HPV infection in esophageal squamous cell carcinoma.

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7. sz. melléklet

DOKTORI ÉRTEKEZÉS BENYÚJTÁSA ÉS NYILATKOZAT A DOLGOZAT EREDETISÉGÉRŐL

Alulírott

név: **Dr. László Zoltán**

születési név: **László Zoltán**

anyja neve: **Nagy Ilona Rózsa**

születési hely, idő: **Komló, 1981.11.13.**

„Possible predictive markers of response to therapy in esophageal and rectal cancers"

című doktori értekezésemet a mai napon benyújtom a

Pécsi Tudományegyetem Egészségtudományi Kar Egészségtudományi Doktori Iskola **„Tumormarkerek szerepe különféle daganatokban"**

Programjához/témacsoportjához

Témavezető neve: **Dr. Bellyei Szabolcs egyetemi docens**

Egyúttal nyilatkozom, hogy jelen eljárás során benyújtott doktori értekezésemet
- korábban más doktori iskolába (sem hazai, sem külföldi egyetemen) nem nyújtottam be,
- fokozatszerzési eljárásra jelentkezésemet két éven belül nem utasították el,
- az elmúlt két esztendőben nem volt sikertelen doktori eljárásom,
- öt éven belül doktori fokozatom visszavonására nem került sor,
- értekezésem önálló munka, más szellemi alkotását sajátomként nem mutattam be, az irodalmi hivatkozások egyértelműek és teljesek, az értekezés elkészítésénél hamis vagy hamisított adatokat nem használtam.

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