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

Doctoral (Ph.D.) thesis

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1. Introduction

Esophageal cancer (ESCC) 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. The incidence of esophageal cancer is on the rise due to an increase in adenocarcinomas located in the lower parts of the oesophagus.

Neoadjuvant chemoradiotherapy (NCRT) is the accepted modality of therapy for locally advanced ESCC, since preoperative chemoradiotherapy has been shown to increase long-term survival.

A number of patients receiving NCRT respond poorly or do not respond at all to therapy. Response to treatment can be optimized by tailoring the dosage of administered cisplatin, 5-FU and irradiation. The identification of markers that signal poor response to treatment is essential as these can be targets for individualized, more effective therapy.

The Akt pathway is considered one of the major anti-apoptotic pathways in cells. The activation of the anti-apoptotic phosphorylated-Akt (p-Akt)-mediated pathways has been shown to correlate with a poor response to NCRT and lower overall survival of ESCC patients. The proteins activating pAkt pathways, for example Heat shock protein 90 (Hsp90) and protein Aurora-A, have been identified as possible targets of therapy. A recently characterized small Heat shock protein (sHsp), Hsp16.2, was found to be expressed in neuroectodermal tumors. SOUL, a novel member of the BH3-domain-only protein family, has been found to facilitate necrotic cell death in oxidative stress.

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. 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. It has been demonstrated in several studies, 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.

Growth hormone-releasing hormone (GHRH) is a peptide hormone secreted by the hypothalamus, but is also present in various tissues and tumors. Antagonists of growth hormone-releasing hormone have been tested for the treatment of various types of experimental tumors.

Our objectives were the following:

1. To determine the association between treatment parameters (dose of chemotherapy) and response to NCRT in ESCC.
2. To investigate whether there was a correlation between the expression of proteins, p-AKT, SOUL, sHsp16.2, GHRH-R and response to NCRT treatment in ESCC patients.
3. To investigate whether there was 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. To show whether p-AKT, SOUL, sHsp16.2 and GHRH-R could be possible biomarkers for response to NCRT therapy in ESCC patients.

5. To examine the relationship between the clinical parameters (age, Karnowsky index, tumor localization, weight loss) of ESCC patients and their 3-year overall survival.

6. To investigate how intensively pre-treatment rectal tumor samples stained for proteins p-AKT, SOUL, Hsp16.2, Hsp90 and GHRH-R.

7. 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.

8. To investigate whether a correlation could 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. To show whether p-Akt, SOUL, Hsp16.2, GHRH-R could serve as predictors of tumor regression to NCRT in rectal cancer.

2. Materials and Methods

Patients and Methods (for the study regarding esophageal cancer)

Patients, Pre-treatment and Post-treatment

Ninety-two patients with inoperable, loco-regionally advanced (cT3-4, cN0-1, cM0) squamous-cell esophageal cancer received neoadjuvant CRT. Four weeks after the completion of NCRT, restaging was performed and clinical response to treatment was assessed according to RECIST. The histopathological tumor regression grade (TRG) was evaluated.

Immunohistochemistry

Pre-treatment tumor tissue samples 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.

Statistical Analysis

All statistical analyses were carried out using the SPSS 15.0 statistical program (SPSS, Chicago).

Patients and Methods (for the study regarding rectal cancer)

Patients, Pre-treatment and Post-treatment

Sixty-nine patients with median age of 59 years (range 34-78), were treated for rectal adenocarcinoma with neoadjuvant CRT. Pathological response to neoadjuvant treatment was determined by the histological evaluation of the resected specimens.

Immunohistochemistry

Tumor tissue samples were incubated with the following primary antibodies: self-developed anti-Hsp16.2 and anti-SOUL polyclonal primary antibodies, GHRH-R primary antibody, p-AKT and Hsp90 primary antibodies. Immunohistochemical staining was carried out.

Statistical Analysis

All statistical analyses were carried out using SPSS 16.0 statistical program (SPSS, Chicago).

3. Results

Related to the study on Esophageal cancer

Clinical outcome

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. 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%).

The association between protein expression and response to NCRT in ESCC

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

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

The association between treatment parameters and response to NCRT in ESCC

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. The administered dose of 5-Fluorouracil (5-FU) did not significantly affect TRG and clinical response .

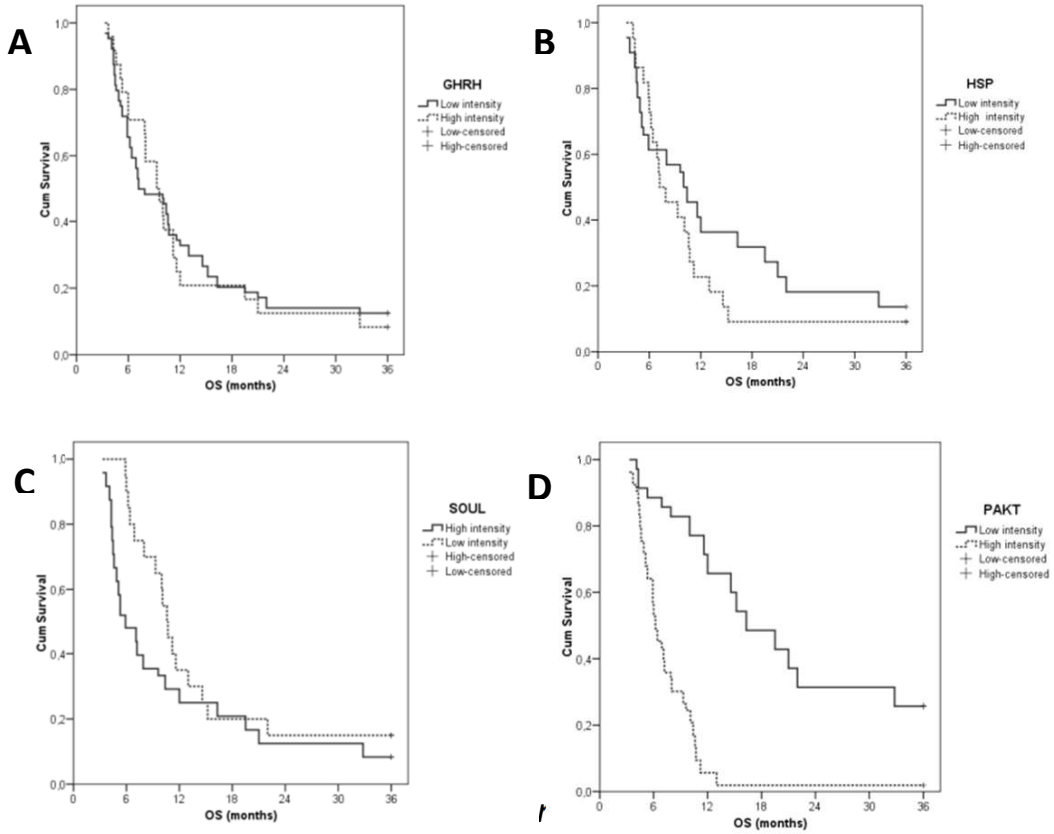
The *association between treatment parameters and response to NCRT in ESCC*

Treatment parameters	**Clinical Downstaging (n=88)**		***p value***
	Responder	**Non-responder**	
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)		
	Responder	**Non-responder**	
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%)	

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

The intensity of GHRH-R (Fig.1A) staining did not affect 3-year OS significantly (p=0.891). Low expression of Hsp16.2 and SOUL (Fig.1B, 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.1D) increased the 3-year OS significantly (p=0.00).



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.

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

There was a significant decrease in 3-year OS in patients whose pre-treatment weight-loss (Fig.2C) exceeded 10% of their body mass (p=0.045). Patients with upper-third ESCC had a significantly higher 3-year OS, than patients with middle and lower third tumors (p=0.002). No significant correlation was found between the other clinical parameters and 3-year OS. (Figure 2)

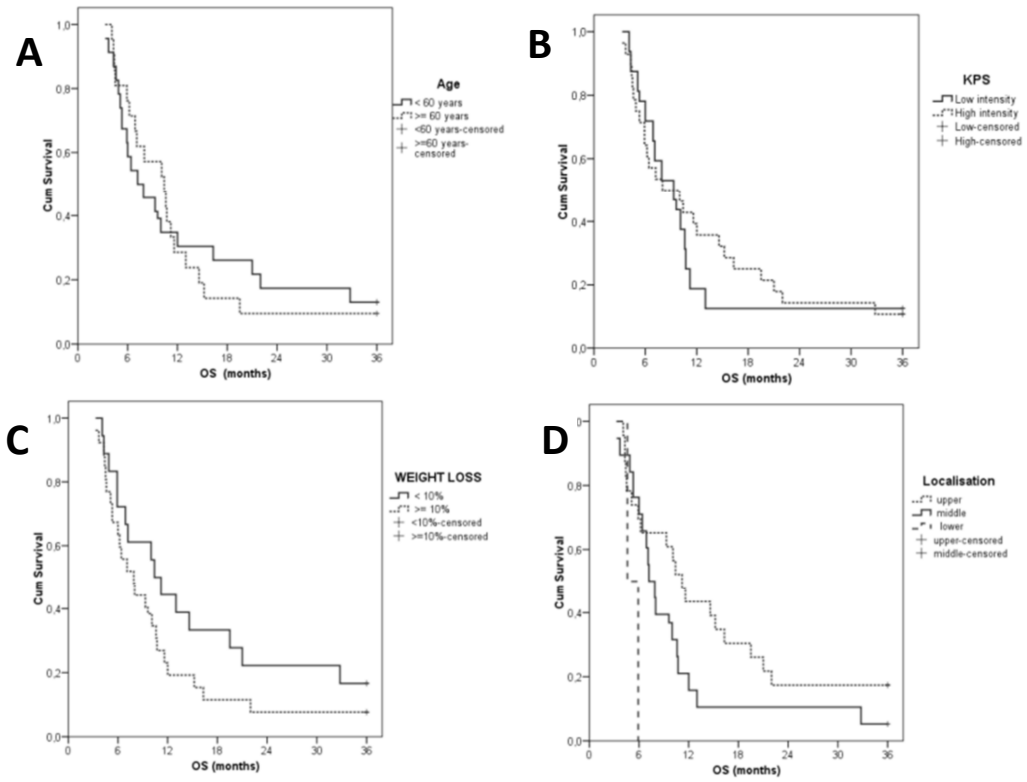


Figure 2.

The relationship between clinical parameters age (A) p=0.875, Karnowsky score (B) p=0.6, pre-treatment weight-loss (C) p=0.045, tumor localization (D) p=0.002 and 3-year OS.

Related to the study on Rectal Cancer

Histopathological Response to Neoadjuvant CRT

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%).

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 2.)

Table 2
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%)
Hsp16.2	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.

Association between Protein Expression and Histopathological Response to CRT

SOUL, Hsp16.2 and p-Akt staining did not show a significant association with tumor regression grade. 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). (Table 3)**.**

Table 3. Relationship between protein expression and histopathological response to neoadjuvant CRT (n=64)

Markers	**Case no.** (n=64)	**Good response** (n=31)	**Poor response** (n=33)	***p***
pp23				
low intensity	20 (31%)	8 (12%)	12 (18%)	
high intensity	44 (69%)	23 (36%)	21 (33%)	0.43
pp25				
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-square test, level of significance $p<0.05$

4. Discussion

In accordance with previous studies, we found that higher radiation doses (over 40Gy) led to an increased number of clinical responders in patients with ESCC, and that application of higher cisplatin doses (over 75mg/m^2) resulted in more histopathological responders.

Due to the poor prognosis of ESCC, it is of great importance, that responders be identified before initiating treatment. 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, which indicated the potential of these proteins as markers of response. Besides 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. 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. 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.

We also found that low SOUL staining in tumor samples was associated with significantly improved clinical and histopathological response. In recent studies, we found evidence that tumor necrosis factor alfa (TNF-alfa) could be implicated in increased resistance to chemotherapy in prostate cancer. Therefore, we hypothesized that by generating a higher

grade of necrosis inside the tumor, SOUL could make tumor cells less sensitive to chemotherapy.

Nutritional status has been proven to be predictive of OS. 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%.

We showed, 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. This is in line with an earlier study, in which we reported that a higher rate of response could be observed in patients with upper-third ESCC, compared to patients middle third ESCC.

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. In the present study we found that 48% of the patients showed a good response (TRG1 and TRG2).

It was demonstrated that besides radiation dose, the time between surgery and neoadjuvant treatment has a significant impact on tumor regression. In our study, 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.

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.

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. 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.

Antagonists of growth hormone-releasing hormone (GHRH) have been tested for the treatment of various types of experimental tumors, including malignant gliomas, breast cancer, ovarian cancer (98), prostate and lung cancers. 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.

5. Conclusions

1. A higher dose of irradiation resulted in a significantly higher number of clinical responders among ESCC patients. A higher dose of Cisplatin (above 75 mg/m^2), significantly increased the number of TRG responders.

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 Karnowsky 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.

6. 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

Bognár L, Hegedűs I, Bellyei S, Pozsgai É, ***Zoltán L***, Gombos K, Horváth ÖP, Vereczkei A, Papp A.: ***Prognostic role of HPV infection in esophageal squamous cell carcinoma.*** Infect Agent Cancer. 2018 Nov 29;13:38. doi: 10.1186/s13027-018-0210-9. eCollection 2018.PMID:30519280

IF: 2,123

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László Z., Boronkai Á., Szappanos Sz., Lőcsei Z., Kalincsák J., Farkas R., Al Farhat Y., Sebestyén Zs., Sebestyén K., Kovács P., Csapó L., Mangel L.:
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IF: 0.291

Szappanos, Sz., Farkas, R., Lőcsei, Z., ***László, Z.***, Kalincsák, J., Bellyei, Sz., Sebestyén, Zs., Csapó, L., Sebestyén, K., Halász, J., Musch, Z., Beöthe, T.,

Farkas, L., Mangel, L., [New methods in the treatment of localised prostate cancer:usage of dynamic arc therapy and kV cone-beam CT positioning] ***Új módszerek a prosztatadaganatok sugárkezelésében: dinamikus ívbesugárzás és kilovoltos „cone-beam" komputertomográfiás ellenőrzés***, Orvosi Hetilap, 2014, 155(32), 1265-1272.

Kalincsák J., ***László Z.***, Sebestyén Zs., Kovács P., Horváth Zs., Dóczi T., Mangel L.: ***Új lehetőség az agyi áttétek sugárkezelésében: együttesen alkalmazott teljes koponya besugárzás és integrált sztereotaxiás sugársebészet***, Ideggyógyászati Szemle, 2015 Nov 30; 68 (11-12):391-7.

Mangel L., ***László Z.***, Varga Zs., Sebestyén Zs., Szappanos Sz., Lőcsei Z., Mezősi E., Horváth Ö.P., Battyáni I., Zemplényi A., Földi I., Kollár L.: ***Hasüregi daganatáttétek stereotaxiás sugárkezelése egy ülésben. Beszámoló az első hazai, koponyán kívüli sugársebészeti beavatkozásról***, Orvosi Hetilap, 2015, 156(39), 1593–1599.
IF: 0.291

Cumulative IF: 7.515

Abstracts in the topic:

László Z, Farkas R, Kalincsák J, Mangel. L ***Neoadjuváns radiokemoterápiás kezelés hatékonyságának vizsgálata és prediktív markerek keresése lokoregionálisan előrehaladott nyelőcsőtumorok esetében*** Magyar Onkológia 55:129–146, 2011

Farkas R, Pozsgai É, Bellyei Sz, ***László Z***, Szigeti A, Sebestyén K, Rápolti E, Gömöri É, Papp A, Mangel L. ***Klinikai és molekuláris prediktív markerek vizsgálata rectumtumoros betegeknél*** Magyar Onkológia 55:129–146, 2011