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ORIGINAL ARTICLE

Is there a correlation between molecular markers and response to neoadjuvant chemoradiotherapy in locally advanced squamous cell esophageal cancer?

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Summary

Purpose: *To evaluate the expression of epidermal growth factor receptor (EGFR), p53, p21 and thymidylate synthase (TS) in a pretherapy biopsy specimen of locally advanced squamous cell esophageal cancer and correlate these markers with response to neoadjuvant chemoradiotherapy.*

Methods: *Sixty-two patients with histopathologically proven locally advanced (T3 or greater) squamous cell esophageal cancer were enrolled. The expression of EGRF, p53, p21 and TS markers was assessed with immunohistochemistry. Semiquantitative assessment of expression of these markers was performed based on the percent of the stained cells. Radiotherapy (45-50.4 Gy) was delivered concomitantly with 5-fluorouracil (5-FU)/leucovorin (LV)/cisplatin (CIS) chemotherapy. Five to 6 weeks after chemoradiation, response to treatment was assessed. Medically fit and operable patients were operated. The resected material underwent histopathological evaluation of tumor expansion, histological classification after initial multimodality treatment (yp TNM), residual status and tumor regression grade (TRG).*

Results: *Out of 62 patients enrolled, 41 (66%) were evaluated for molecular markers. Clinical response rate was 43.9%. Out of 41 patients, 12 (29%) underwent surgery. TRG 1 was noted in 58% of the patients. In a pretherapy tumor specimen, positive expression was noted in 80, 90, 80 and 71% for EGFR, p53, p21 and TS, respectively. We noted no statistically significant difference neither between tumor marker expression and clinical response to chemoradiation, nor between tumor marker expression and TRG.*

Conclusion: *We registered no difference in response to treatment between EGFR, TS, p21 and p53 positive and negative staining.*

Key words: chemoradiation, esophageal cancer, molecular markers

Introduction

Despite improvements in diagnostics, surgical resection and (neo) adjuvant therapy, overall survival in esophageal cancer remains poor. Survival rate of patients with locally advanced (T3, T4, N+) squamous cell esophageal cancer is particularly low [1-5]. Surgery alone for locally advanced disease results in 5-year overall survival rate of only 20-25%. The addition of combined modality strategies (namely neoadjuvant chemoradiotherapy) tends to improve survival by increasing resectability and opposing metastatic spread. Nevertheless, results in 5-year overall survival rates are only 30-35%. The highest percentage (50-60%) in 5-year overall survival rate is noted in patients with pathological complete response (pCR) to chemoradiotherapy [5-8]. Multivariate analysis of TRG according to Mandard criteria revealed that it is probably the most significant prognostic factor of disease-free survival [9,10]. In patients with TRG 1-2, 3-year overall survival rate is over 60% [9,10]. Unfortunately, preoperative chemoradiation produces pCR in no more than

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20-30%, meaning that many patients fail to achieve adequate response. On the other hand, conventional chemotherapeutic agents are unspecific for tumor cells, so they are frequently accompanied with considerable toxicity which leads to increased treatment morbidity and even mortality. This means that some patients receive toxic treatment from which they will have very little or no benefit whatsoever. Therefore, predictive markers are needed to allow tailored therapy which will result in increased efficacy and decreased toxicity. The attention is focused today on the molecular markers that distinguish biological tumor behavior and potential response to the applied therapy [11-15].

The aggressiveness of squamous cell esophageal cancer, its unfavorable prognosis, as well as individual response to multimodal therapy is largely explained by the biological characteristics of the tumor. The malignant phenotype of the tumor is determined by disorders in the expression of molecular factors essential for tumor cell growth and proliferative activity such as EGFR, VEGF, p53, p21, TS, HER-2, MIB-1, CD34, NF-kB, GSTP1 etc. [16-19]. Some of these biomarkers are proven to be significant predictive markers in other tumors such as HER-2 in breast cancer, BCR-ABL in chronic myeloid leukemia, EGFR in lung cancer, mutations in KRAS in colonic cancer etc. Despite extensive investigation in this field the current evidence for the role of biomarkers in predicting response to therapy in esophageal cancer is still evolving. Up to now, there are no established predictive biomarkers in esophageal cancer to individualize patient treatment [20].

In our study, we evaluated the expression of EGFR, p53, p21 and TS in biopsy specimens of locally advanced squamous cell esophageal cancer and correlated these markers with response to neoadjuvant chemoradiotherapy.

Methods

Inclusion criteria

Sixty-two patients older than 18 years with histopathologically proven locally advanced (T3 or greater) squamous cell esophageal cancer were enrolled in this study. Patients with poor performance status, acute and chronic uncontrolled severe physical and mental disorders were excluded, as well as patients previously treated for esophageal cancer with any antitumor agent.

Diagnostic workout

After receiving written informed consent, a full clinical examination was conducted as well as endoscopy with tumor biopsy and histopathological exploration, complete blood count and serum biochemistry. Also performed were barium esophagography and computed tomography (CT) of the chest and upper abdomen.

Treatment plan

Radiotherapy was delivered as a single daily dose of 1.8 Gy per fraction in 24-28 fractions up to a total dose of 45-50.4 Gy to a reference point according to ICRU 50/62, through 3 or 4 fields. Radiotherapy lasted 5-6 weeks and was delivered with linear accelerators using high energy photons (more than 8 MeV). Chemotherapy with CIS/5-FU/LV was administered concomitantly in 4 cycles every 14 days. Each chemotherapy cycle was administered in two days: 50 mg/m^2 CIS on days 1, 15, 29 and 43 of treatment with adequate hydration, 20 mg/m^2 LV i.v. infusion over 2h, 5-FU 400 mg/m^2 short i.v. infusion and 600 mg/m^2 5-FU as 22h i.v. infusion on days 1, 2, 15, 16, 29, 30, 43 and 44.

During treatment, toxicity was evaluated according to NCI-CTC criteria [21].

Immunohistochemistry

Immunohistochemical study was conducted on 4 μm thick slices of paraffin blocks previously fixed in 10% formaldehyde in phosphate buffered saline. The list of antibodies and immunohistochemical staining in order to prove expression of EGRF, p53, p21 and TS markers is presented on Table 1. The sensitive and specific immunohistochemical method of LSAB+/HRP was used. After de-

Table 1. Primary antisera and visualizing immunohistochemical methods

Primary antiserum and clone (mo-monoclonal, po-polyclonal)	*Immunogen*	*Manufacturer/Catalogue number*	*Antibody dilution/ antigen demasking*	*IHH method*
EGFR (po goat anti-human EGFR)	c-terminus protein EGFR of human origin	Santa Cruz Biotechnology, USA, SC-03	1:200 / MW, proteolytic digestion, proteinase K 370C, 21 min.	LSAB+/HRP
TS (mo mouse anti-thymidylate synthase) (106/4H4B1)	Recombinant human thymidylate synthase	Zymed Laboratories Int, USA, 18-0405	1:100 / MW, citrate buffer pH6, 21 min.	LSAB+/HRP
p53 (mo mouse anti-human) (DO-7)	Recombinant human protein p53 wild type isolated from E. Coli	DAKO A/S, Denmark, M7001	1:50-1:100 / MW, citrate buffer pH6, 21 min.	LSAB+/HRP
p21RAS (mo mouse anti-human) (NCC-RAS-001)	recombinant c-H-ras protein p21	DAKO A/S, Denmark, M0637	1:50 / MW, citrate buffer pH6, 21 min.	LSAB+

IHH: immunohistochemical, LSAB$^+$/HRP: Labelled StreptAvidin-Biotin/Horse Radish peroxidase, MW: microwave

paraffinisation of the sections, demasking was performed by exposing tissue slices immerged in solution for demasking (0.01 M citrate buffer pH 6.0) to microwaves (800 W) for 7-21 min. Once the deparaffinisation and demascation was obtained, endogenous peroxidase was blocked by immersing slices in water for 5 min at room temperature. The incubation with primary antibody lasted for 60 min at room temperature and then incubation followed with biotinized anti-rabbit, anti-mouse and anti-goat immunoglobulins for 30 min at room temperature. The next step was incubation of the slices with streptavidin conjugate for 30 min at room temperature. Finally, the slices were incubated for 5 min at room temperature in a substrate-chromogen mixture (H_2O_2 and 3-amino-9-ethyl-carbasol in N, N-dimethylphormamide [AEC+ Substrate-Chromogen kit, Cat No K 3469, DAKO-Denmark]). As general dilution of antiserum and as a tool for rinsing between these steps, 0.1M phosphate buffer pH 7.4 was used. Cell nuclei were stained with Mayer haematoxylin.

Semiquantitative assessment of expression of all investigated markers was performed on tissue samples stained by immunohistochemical methods based on intensity of immunohistochemical staining, taking into account the percent of stained cells as presented on Table 2.

Response evaluation

Five to 6 weeks after chemoradiation, tumor response to treatment was assessed using the RECIST criteria [22] and operability was estimated again for each patient. Medically fit patients converted to operable stage were operated. Surgical approach was individually tailored and included resection of the esophagus and proximal stomach with regional lymph nodes. The resected material underwent histopathological evaluation of yp TNM (histological classification after initial multimodality treatment), residual status and TRG according to Mandard criteria [10].

Patients not eligible for surgery continued with chemotherapy and/or best supportive care.

Regular follow-up was performed every 3 months during first 2 years, and then every 6 months. Follow-up consisted of physical examination, tumor assessment (esophagoscopy, CT of chest and upper abdomen every 6 months) and evaluation of treatment toxicity according to NCI-CTC criteria.

Statistics

In statistical analysis the R package was used (version 2.8.1; 2008-12-22; Copyright (C) 2008; The R Foundation for Statistical Computing; ISBN 3-900051-07-0). Basic patient characteristics were summarized. Frequency tables were formed for categorical variables, and for continuous variables descriptive statistics was used (median, range and frequency distribution). The overall rate of clinical and histopathological complete response was presented with 95% confidence interval. For statistical significance testing between EGRF, TS, p21 and p53 positive and negative tissue samples regarding clinical and histopathological response, Fisher's exact test was used. The results were presented in Tables.

Table 2. Immunoreactivity assessment

EGFR, TS (cytoplasmic and/or membrane reactivity)	
0	No immunoreactivity
+	Low or focal immunoreactivity in < 10% tumor cells
1	Low or focal immunoreactivity in 10-50% tumor cells
2	Clear immunoreactivity in 10-50% tumor cells
3	Clear immunoreactivity in ≥ 50% tumor cells
p53, p21 (nuclear reactivity)	
0	No immunoreactivity
+	Clear immunoreactivity in < 1% tumor cells
1	Clear immunoreactivity in 1-9.9% tumor cells
2	Clear immunoreactivity in 10-49% tumor cells
3	Clear immunoreactivity in ≥ 50% tumor cells

Results

Forty-one (66%) out of 62 patients were evaluated for molecular markers. Their clinical characteristics are presented in Table 3.

Chemoradiotherapy was applied to all patients, and they all finished radiotherapy with the total prescribed dose. All 4 cycles of chemotherapy were administered to 25 (61%) patients. In the remaining 16 (39%) chemotherapy was interrupted due to high grade toxicity: in 10 patients after 2 cycles and in 6 patients after 3 cycles.

Five to 6 weeks after chemoradiation patients underwent evaluation of response to treatment. The results are presented on Table 4. Objective response rate (complete /CR and partial response /PR) was 43.9% (95% confidence interval 29.89-58.96) and these patients were judged as sensitive to chemoradiation. Patients with stable disease (SD) or disease progression (PD) were judged as insensitive. Out of 8 patients with

Table 3. Patient characteristics

Characteristics	*N=41*	*%*
Gender		
Male	34	83
Female	7	17
Age (years), median (range)	58 (34-74)	
Tumor localization		
Upper third	23	56
Middle third	15	37
Lower third	3	7
Histopathological grade		
I	15	37
II	17	41
III	9	12
T stage		
T3	19	46
T4	22	54
N stage		
N0	18	44
N+	23	56
M stage		
M0	38	93
M+	3	7

Table 4. Clinical response to chemoradiation in 41 patients

	N	*%*	*95% confidence interval*
Complete response	3	7	2.52-19.43
Partial response	15	36.5	23.59-51.88
Stable disease	15	36.5	23.59-51.88
Progressive disease	8	20	10.23-34.01

Table 5. Pathological response rate (12 patients)

	N	*%*	*95% confidence interval*
TRG1	7	58	31.95-80.67
TRG2	2	17	4.70-44.80
TRG3	3	25	8.89-53.23

TRG: tumor regression grade

therapeutic failure, local progression was observed in 3 patients, while 5 developed distant metastases (lungs, liver and bones).

After clinical evaluation, 12 (29%) patients out of 41 underwent surgery. All of them had complete resection (R0). Assessing the resected material according to Mandard criteria the histopathological response rate is shown in Table 5. In patients with TRG 1 and 2, chemoradiotherapy was considered to be effective, while in those with TRG 3 or more it was considered ineffective.

Twenty-nine patients were judged as not fit for surgery and, due to poor performance status, received best supportive care only. Eight of them had gastrostomy performed and in one esophageal stent was placed.

Expression of molecular markers

In pretherapy tumor specimens, positive EGFR expression was noted in 80%, and p53 was positive in 90%. Positive staining for p21 was noted in 80% of the evaluated specimens and 71% of tumor specimens were TS positive (Figure 1).

Relationship between EGFR, p53, p21 and TS expression and response to chemoradiotherapy

Three patients clinically assessed as CR were positive for all evaluated markers, as well as most of the PR patients, SD and PD patients. There was no statistically significant difference between tumor marker expression and clinical response. The results of comparison of EGFR, p53, p21 and TS expression and response to chemoradiotherapy are presented in Table 6.

Comparison between EGFR, p53, p21 and TS expression and histopathological response to chemoradiation, is presented in Table 7. Six out of 7 patients with TRG 1 were EGFR and TS positive and all patients with TRG 1 were p53 and p21 positive. Almost all patients with TRG 3 also had all 4 tumor markers positive. We noted no statistically significant difference between tumor marker expression and tumor regression grade.

Table 6. Relationship between EGFR, p53, p21 and TS expression and clinical response to chemoradiotherapy in 41 patients

	Clinical response to chemoradiotherapy					
	CR	*PR*	*SD*	*PD*	*Total*	*Fisher exact test, p-value*
EGFR						
+	3	12	13	6	34	0.928
–	0	3	2	2	7	
P53						
+	3	13	12	7	35	0.298
–	0	2	3	1	6	
P21						
+	3	13	12	7	35	1.00
–	0	2	3	1	6	
TS						
+	3	12	10	5	30	0.644
–	0	3	5	3	11	

CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease

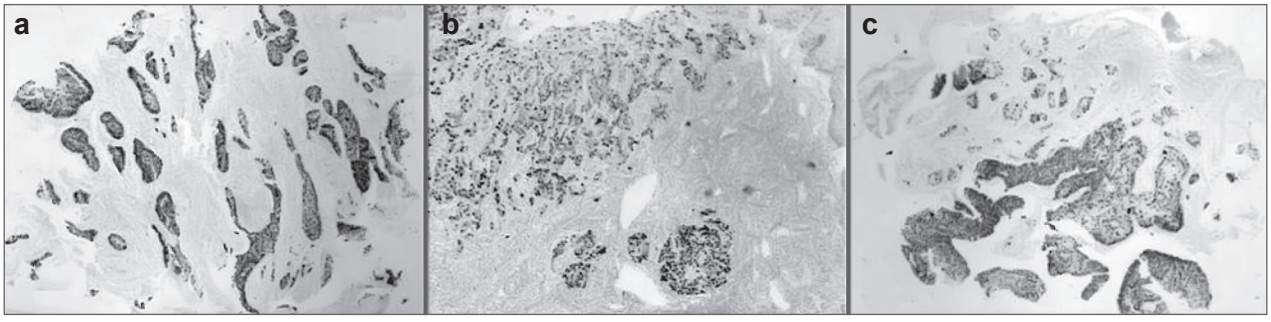

Figure 1. Immunohistochemical stainings in biopsies of esophageal squamous cell carcinomas: **(a)** strong nuclear immunoexpression of p21, **(b)** nuclear immunostaining of p53 and **(c)** nuclear and cytoplasmic immunostaining of TS.

Table 7. Relationship between EGFR, p53, p21 and TS expression and tumor regression grade in 12 patients

	Tumor regression grade				
	TRG 1	*TRG 2*	*TRG 3*	*Total*	*Fisher exact test, p-value*
EGFR					
+	6	1	2	9	0.138
–	1	1	1	3	
P53					
+	7	2	3	12	0.25
–	0	0	0	0	
P21					
+	7	2	3	12	0.25
–	0	0	0	0	
TS					
+	6	2	3	11	0.533
–	1	0	0	1	

TRG: tumor regression grade

Discussion

EGFR is a member of ERBB transmembrane growth factor receptor family which initiates signal transduction by activation of a receptor-associated tyrosine kinase (TK). Activated TK starts a cascade of downstream phosphorylation and activation of other signal effectors which are potent regulators of important intracellular processes such as cycle progression, apoptosis, cell survival, proliferation, angiogenesis and metastases [23]. Results of various studies on the prognostic significance of EGFR overexpression have led to a hypothesis that this overexpression in tumors correlates with unfavorable prognosis and disease course. EGFR overexpression correlates also with deeper invasion of the tumor, intravascular invasion and risk of local relapses [16,11]. No predictive potential of EGFR overexpression is yet clear but it seems that it may be associated with resistance to apoptosis. In a study of Miyazono et al., quantitative expression levels of EGFR in pretreatment biopsies did not predict the degree of histopathologic response to neoadjuvant radiochemotherapy with cisplatin and 5-FU [11]. In our study, as well, there was no statistically significant difference neither in response to treatment nor in TRG regarding EGFR expression.

5-FU belongs to a family of drugs named antimetabolites and principally acts via inhibition of DNA and RNA synthesis. The key step in 5-FU activity is its binding to TS that depletes the thymidine nucleotide pool and hence DNA synthesis. Overexpression of TS may lead to relative resistance as it may reduce 5-FdUMP binding. In gastric cancer, high TS protein expression predicts resistance to high-dose 5-FU and LV chemotherapy and correlates with poor survival. Low TS levels on the other hand were associated with tumor responses [20]. There is much less data on TS overexpression in esophageal cancer. Our study did not detect statistically significant difference either in response to treatment or in TRG regarding TS expression.

p53 gene is a tumor suppressor gene, involved in the regulation of the cell cycle, apoptosis and DNA repair [11]. It also plays a critical role in tumor development and growth and is also correlated with aggressive tumor behavior. Mutation in p53 has a proven role in squamous cell carcinogenesis and recently it has been shown that mutant p53 plays a role in controlling angiogenesis and has been related to lymphatic spread and distant metastases [24]. Mutant p53 may also correlate with tumor resistance. In gastric cancer p53 negativity has been correlated with good tumor response, but in esophageal cancer the results are conflicting. Whilst one study suggests that p53 negativity may correlate positively to tumor response, other studies did not find any correlation between p53 expression and response to cytotoxic therapy [11,20]. Although p53 may play a critical role in radiation-induced apoptosis, some patients with p53 negative tumors do not respond well to chemoradiation [25]. In our study we found no statistically significant difference in response to treatment and TRG regarding p53 expression.

p21 gene encodes a cyclin-dependent kinase inhibitor which plays a role in terminal differentiation and tumor aging. It could work both as p53-dependent and p53-independent. Its protein also regulates cell cycle and determines pathological lymph node metastases [24]. In a study of Okumura et al. [25] there was no correlation between p21 positive expression and clinical and histological effect of chemoradiation. On the other hand, Nakashima et al. [11] found that strong positive staining of p21 in the absence of p53 is associated with detectable histological response to preoperative chemoradiotherapy. Our study correlates with the study of Okumura. We also did not find statistically significant difference in clinical and histopathological response to treatment regarding p21 expression.

Conclusion

Prediction of tumor response in esophageal cancer is difficult and under intense investigation. Results are still inconclusive. Based on the results of our study, there is no difference in response to treatment between EGFR, TS, p21 and p53 positive and negative staining. Further studies are needed.

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