Response to chemoradiatiotherapy in squamous cell carcinoma of the esophagus: evaluation of some prognostic factors

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Objective: To evaluate the predictive values of the expression of factor VIII, CD-34, p53, bcl-2, and DNA ploidy regarding the response to chemoradiation of squamous cell carcinoma of the esophagus.

Design: Retrospective analysis of pretreatment biopsies with immunohistochemistry and flow cytometry. The results were correlated to tumor response (complete vs. noncomplete) following chemoradiation with three cycles of 5-FU and cisplatin combined with 40–64 Gy of radiation.

Subjects: 44 consecutive patients with squamous cell carcinoma of the esophagus treated with chemoradiation with a curative intent from 1992–2000.

Main outcome measures: Treatment response.

Results: No correlations were found between the expressions of p53, bcl-2, or DNA ploidy and tumor response to chemoradiation. A positive correlation was found between factor VIII expression and a complete tumor response ($p = 0.0357$). However the other marker for angiogenesis, CD-34, showed a negative correlation ($p = 0.0493$). Both markers indicate blood vessel density meaning that, in this study, many vessels indicated a favorable response if measured with factor VIII, but a poor response if measured with CD-34.

Conclusion: It is not possible to predict tumor response to our chemoradiation protocol through the analysis of pretreatment expression of p53, bcl-2 or DNA ploidy in biopsy specimens. In spite of significant correlations between complete tumor responses and the expressions of the markers for angiogenesis this significance may be questionable since one of the two markers, factor VIII had a positive and the other,CD-34, a negative correlation to tumor response.

Keywords: chemoradiation, response, prognostic factor, apoptosis, p-53, angiogenesis, DNA ploidy

Introduction

Esophageal cancer has a well known poor prognosis.[1–3] The 5-year survival has been in the order of 5%–10% following surgery or radiotherapy. These poor results have stimulated the development of new treatment techniques including more radical surgical procedures.[4,5]

However, at least in the Western countries, esophageal cancer is mostly diagnosed at a late disseminated stage. Then locoregional treatments such as surgery and/or radiotherapy are insufficient to cure the patients. In this situation it is rational to include a systemic treatment, ie, chemotherapy into the protocols.

Results of chemotherapy have been poor until the last decade when a number of different series of concomitant chemoradiotherapy, mostly including cisplatinum, have shown promising results with complete response rates above 40%.[6–10.]

However these chemoradiotherapy protocols, often in neoadjuvant settings, are followed by rather high morbidity and treatment-related mortality.[11] It would therefore

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be of interest to be able to predict which patient is a responder and which is not before the administration of the treatment. It would then be possible to exclude nonresponders from a potentially harmful treatment.

In this study microbiologic markers have been evaluated in two groups of patients, responders and nonresponders, treated according to the same chemoradiotherapy protocol.

Angioneogenesis in tumor tissue has been studied, since it is known that good oxygenation of tumor cells enhances the radiosensitivity and improves the delivery of cytotoxic agents.[12,13] A high microvessel density with an improved circulation enhances this crucial oxygenation. It is however also known that angioneogenesis may be a limiting factor regarding tumor growth and possibly also regarding the tendency to metastasize.[14,15] Accordingly a pronounced angioneogenesis may both act as an adjunct to chemoradiotherapy and at the same time be a marker of poor prognosis regarding tumor biology. We have investigated pretreatment microvessel density within tumors expressed through factor VIII and CD-34 and correlated the results to tumor response after chemoradiation.

Apoptosis, or programmed cell death, is a physiological mechanism regulating homeostasis in both normal and tumor tissue by the elimination of unnecessary cells.[16] Failure of tumor cells to undergo apoptosis can result in uncontrolled accumulation of cells. It is known that apoptosis can be induced by chemotherapuetic agents and radiation[17] and by oncogenes such as bcl-2 and p53.[18,19] Like a neoplasm with fully established tumor cell necrosis, a carcinoma with a high incidence of apoptotic nuclei of the neoplastic parenchymal cell carcinoma is looked upon as a neoplasm with a bad prognosis for the patient, requiring particular types of therapy. P53 expression has been correlated to survival in carcinoma of the breast,[20] colon,[21] stomach,[22] and lung,[23] while the prognostic value of p53 in esophageal cancer remains more uncertain.[24,25] Mutations of the tumor suppression antigen p53 are among the most commonly detected genetic abnormalities in human neoplasia in general and in squamous cell carcinomas in particular. The results of an immunohistochemistry (IHC) analysis of p53 can govern both the therapy and the prognosis of the patient´s carcinoma. It is however a rather well established fact that the simple finding of an IHC overexpression of some kind, does not yet in itself become a good predictor of the subsequent course of the patient's neoplastic disease. It is neither decisive for the choice of therapy. We have studied the correlation between tumor response and bcl-2 and p53.

DNA ploidy is known to indicate prognosis in squamous cell carcinoma of the esophagus through the detection of aneuploid peaks in flow cytometric analysis.[26] We have investigated the pretreatment DNA ploidy and then correlated this analysis to the response to chemoradiation therapy.

Patients and methods

Patients

Forty-four patients were recruited from a consecutive group of patients, who had received planned preoperative or fulldose chemoradiation for squamous cell carcinoma of the esophagus during 1992–2000. The patients were separated into two groups depending on response to oncological therapy (complete response [CR] vs non-CR). See Table 1 for details.

Eligibility criteria for the study included histologically confirmed squamous cell carcinoma of the esophagus without signs of distant metastases, ie tumor stage T1-4, N0-1, M0 according to the International Union against Cancer.[27] The patients had to have a functional performance status of ≤2 according to the World Health Organization (WHO) classification and they should not suffer from any other condition that could be worsened by the planned treatment such as

Table 1 Patient and tumor characteristics

	Total	CR	Non-CR	p-value
No. of patients entered	44	23	21	
No. of women	23 (52)	11 (48)	12 (57)	0.53 n.s.
Age mean, range	62, 44–82	64, 44–74	60, 45–82	0.23 n.s.
Grade of differentiation				
High	1 (2)	1 (4)	–	1.0 n.s.
Moderate	20 (45)	9 (39)	11 (52)	0.36 n.s.
Low	23 (53)	13 (57)	10 (48)	0.66 n.s.
Tumor location in the esophagus				
Upper	12 (27)	6 (26)	6 (29)	0.85 n.s.
Middle	19 (43)	9 (39)	10 (48)	0.57 n.s.
Lower	13 (30)	8 (35)	5 (24)	0.51 n.s.
Tumor length (cm)				
<5	14 (32)	8 (35)	6 (29)	0.75 n.s.
≥5 to ≤10	29 (66)	15 (65)	14 (67)	1.0 n.s.
>10	1 (2)	–	1 (5)	1.0 n.s.
Tumor stage (UICC)				
IIA	10 (23)	8 (35)	2 (10)	0.07 n.s.
IIB	1 (2)	1 (4)	–	1.0 n.s.
III	31 (70)	14 (61)	17 (81)	0.12 n.s.
IV	2 (5)	–	2 (10)	0.22 n.s.

Notes: Figures in brackets show percent of group (Total, CR, or non-CR).
Abbreviation: CR, complete response.

serious heart (ie, heart failure or angina) or kidney disorders. Hematological and renal function test parameters had to be normal (leucocytes $\geq 3.0 \times 10^9$/L, platelets $\geq 100 \times 10^9$/L, cr-EDTA clearence >65 mL/min). There should not be a history of other squamous cell malignancy prior to entry nor should there be any history of any type of malignant tumor during the last five years before inclusion. Before entry into the study the patients were investigated with spirometry, electrocardiogram, barium esophagogram, chest X-ray, computed tomography (CT) scan of the chest and upper abdomen, abdominal and endoscopic ultrasound (EUS), esophagoscopy with biopsies, and finally bronchoscopy, if the tumor was located at or above the carina. Patients with intraluminal airway growth were excluded. The results of these pretreatment investigations were evaluated by a surgeon and an oncologist to find out if the patients were fit for the planned treatment.

Surgical treatment

Before starting the treatment the patients were stratified as primarily resectable or unresectable by the surgeon and the oncologist. This stratification was repeated after the primary chemoradiation treatment, when a new CT scan and EUS (if available) were performed. The standard surgical technique was an Ivor Lewis procedure with a gastric pull-up or with a colon interponate as the esophageal substitute.

Oncological treatment

The protocol[6] included three five-day courses of cisplatinum (100 mg/m^2 on the first day of each course) and 5-fluorouracil (a continuous 120-hour infusion on day 1 through 5 of each course with 750 mg/m^2 administered on each day of treatment) with a two-week rest between the treatments. Radiation was administered daily on weekdays from the first day of the second course of chemotherapy up to a preoperative dose of 40 Gy or through a full dose treatment of 64 Gy. The radiation treatment (preoperative or full dose) was decided on before the treatment started. The daily fractionation was 2 Gy (see Figure 1).

When no tumors were found postoperatively after histopathological examination the responses were classified as pathological CR. Patients not operated on were evaluated with clinical methods (X-ray of the esophagus, endoscopy, CT scans and, when available, EUS). Responses were classified as clinically CR, when no manifestations of malignancy could be found with clinical techniques. All other tumors, not classified as complete responses, were grouped together as incomplete responses (non-CR) regardless of degree of response.

Biopsy procedure

The biopsies obtained during endoscopies were paraffin-embedded and formaldehyde-fixed. These procedures were performed at the time of diagnosis. Hence no patients had received any anticancer treatment prior to the biopsy procedure.

A minimum of three macroscopically representative biopsies were sampled from gross tumor tissue through an endoscope (various Olympus video gastroscopes) with biopsy graspers (Olympus FB-24K®).

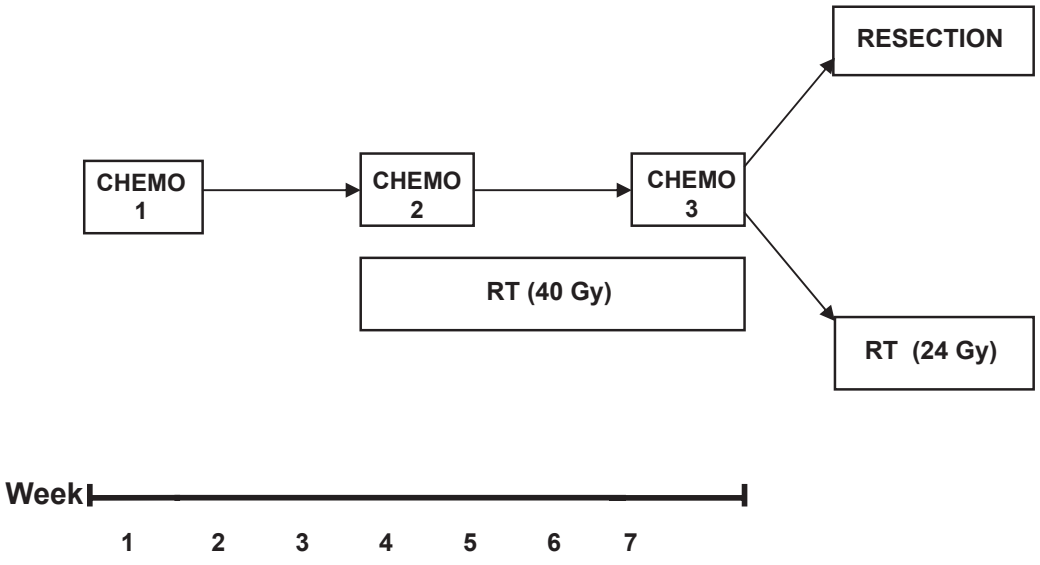

Figure 1 Oncological treatment protocol.
Abbreviations: Chemo, chemotherapy; RT, radiotherapy.

Laboratory methods

Factor VIII, CD-34, p53 and bcl-2 were all analyzed according to standard immunohisto-chemistry methods utilizing the avidin biotinylated immunoperoxidase-staining technique (Hsu 28). The reason why factor VIII and CD34 were chosen as IHC markers for angiogenesis was the fact that among the antisera raised against endothelial cells available in our laboratory, these were found to be those which in pilot studies showed themselves to give the histotechnically most optimal results.

A standardized procedure for cutting out specimens from the large resection material for subsequent light microscopy was applied. Particular attention was paid to visualize the free borders of the specimen. This was performed by marking them with India ink.

The sections were deparaffinated in xylene and rehydrated in ethanol and distilled water. Antigen retrieval was performed through pressure boiling for two minutes in 10 mM citrate buffer (factor VIII, CD-34, p-53, and bcl-2) and through digestion with proteinase K (DAKO, code 2019) (factor VIII). They were then immunostained (see Table 2 for primary antibodies) and endogenous peroxidase activity was blocked with hydrogen peroxide before exposure to biotinylated secondary linking antibody, avidin-biotinylated enzyme complex, diaminobenzidine (DAB) as chromogen and hematoxylin as counterstain.

Two independent experienced histopathologists (SF, UF) then evaluated the specimens. They did not know the clinical outcome of the individual patients.

The evaluations were performed in light microscopy in 400 × magnification fields. All tumor tissue was evaluated, excluding the stroma. An average figure from each section was calculated.

Factor VIII

The number of microvessels in each field was calculated. Stains were counted as microvessels when they were not associated with the muscular bundles of arteries or arteriole. A lumen was not necessary for a positive count. Vessels in and along the borders of tumor tissue were counted.

Table 2 Primary antibodies used during immunohistochemistry

Investigated marker	Antibody	Manufacturer
Factor VIII	A082	DAKO
CD-34	NCL-END	Novo Castra
Bcl-2	M887	DAKO
P-53	NCL-CM1	Novo Castra

CD-34

CD-34 was calculated in the same way as factor VIII.

Bcl-2

One or more stained cells in a biopsy were regarded as a positive case. The evaluation was performed at both 100 × and 400 × magnification.

P-53

More than 50% positively stained cells out of the total were regarded as a positive case. The evaluation was performed at both 100 × and 400 × magnification.

DNA

The utilized procedure in the analysis of DNA-ploidy was flow cytometry. This analysis was only performed with specimens from the first consecutive 28 patients due to technical difficulties.

Statistical considerations

The continuing variables have been analyzed with logistic regression and divided into different categories according to different value cut-offs. The category variables have been analyzed in cross tables with Fisher's test and the Chi-squared test.

Ethical considerations

The study was approved by the ethics committee at the Karolinska Institute in Stockholm. All living patients gave their informed consent to take part in the study.

Results

Oncological therapy

Twenty-three patients had complete responses (the CR group), while the other 21 patients (the non-CR group) showed poorer responses (partial responses or less). See Table 3 for details. In accordance with other studies[6] response to chemoradiation was correlated to initial tumor stage ($p < 0.05$).

All 44 patients received ≥40 Gy of radiation and three courses of chemotherapy. Four patients, two from each group, received full dose radiation.

Surgery

Thirty-five of the 44 patients were operated on (80%). Five patients did not receive any operation due to a high location in the esophagus. High tumors were not resected to avoid mutilating laryngectomies. Two patients were not

Table 3 Responses and survival following therapy

	Total	CR group	Non-CR group
Response			
Complete, clinical	5 (11)	5 (22)	–
Complete, pathological	18 (41)	18 (78)	–
Partial, clinical	2 (5)	–	2 (10)
Partial, pathological	11 (25)	–	11 (52)
Stable disease	3 (7)	–	3 (14)
Progressive disease	5 (11)	–	5 (24)
Median survival (months)	24.3	42.3	16.8

Notes: Figures in brackets show percent of group (Total, CR, and nonCR).
Abbreviation: CR, complete response.

Table 4 Delivered treatment

	Gy	Courses of CT	Total	CR	Non-CR
Oncologic treatment					
	40	3	40 (91)	21 (91)	19 (90)
	>40	3	4 (9)	2 (9)	2 (10)
Surgical treatment					
Operations			35 (80)	18 (78)	17 (81)
Resections			32 (73)	18 (78)	14 (67)
Explorations			3 (7)	–	3 (14)

Notes: Figures in brackets show percent of group (Total, CR, and non-CR).
Abbreviations: CR, complete response; CT, computed tomography.

operated on because of local overgrowth on adjacent organs. Finally two patients, initially planned for surgery, were not operated due to poor health following oncologic treatment. In the non-CR group of patients three cases of unexpected advanced tumor growth was found during operation and therefore only explorations were performed. Eighteen of the 23 patients (78%) in the CR group had resections compared to 14 of 21 patients (67%) in the non-CR group. See Table 4 for details.

Angiogenetic markers in CR vs non-CR groups

Factor VIII

A statistically significant difference was found between responders and nonresponders regarding vessel density in tumor tissue expressed as mean numbers of vessels stained for factor VIII/field of vision ($p = 0.0357$).

CD-34

A weak negative correlation ($p = 0.0493$) was found when the vessel density was calculated with the CD-34 method as opposed to the results of the factor VIII evaluations.

Apoptosis

Bcl-2

Seven patients in the CR group had positive stains for bcl-2 as did five patients in the non-CR group. All other patients were negative. As a consequence no significant difference was found between the two groups regarding bcl expression.

P-53

No significant differences were found between responders and nonresponders regarding p-53 expression ($p = 0.895$).

DNA-ploidy

DNA

Most of the 28 analyzed tumors were aneuploid ($n = 25$). Two tumors in the CR group ($n = 14$) of patients were tetraploid, as was one of the tumors in the non-CR group ($n = 14$). Hence no difference in DNA-ploidy was found between the groups.

Discussion

Tumor angiogenesis has been shown to relate to survival in a number of different tumors including squamous cell carcinoma of the esophagus.[29] The same relation has also been shown regarding the expression of p-53[25] and apoptosis through bcl-2.[30]

A few investigators have adressed the same issue as in our study, ie, to find predictors of tumor response to demanding oncological therapy. The rationale is to make it possible to avoid the often serious side effects of a demanding and, in the case of the non responders, ineffective treatment.

Imdahl and colleagues[31] found a better response to chemoradiation in tumors with a high proliferation rate determined by MIB-1 immunohistology, but no such correlation was found in their series investigating tumor response and apoptosis. Seitz and colleagues[32] found correlations between clinical response (endoscopy with biopsies two to three weeks after chemoradiation) and lack of p53 overexpression. DNA ploidy (flow cytometry) and cell proliferation (Ki67) was not correlated to tumor response. Ressiot and colleagues, on the other hand, found overexpression of Ki67 to be an independent factor for complete response in esophageal cancer.[33] Sarbia and colleagues[34] found that the expression of cyklin D1 was correlated to a poor response to radiochemotherapy but this correlation was not found in overall survival. The same authors have also investigated bcl-2 and p53 in radiochemotherapy treated patients without finding a significant correlation to tumor response.[35]

Shimada and colleagues found a correlation between low angiogenetic activity, expressed through low serum vascular endothelial growth factor levels, and a good tumor response in squamous cell carcinoma of the esophagus.[36] This finding was supported by the study by Imdahl.[37]

COX-2 displays antiapoptotic functions related to angiogenesis or blocking of bcl-2 action. Takatori and colleagues found that COX-2 mRNA expression in tumor biopsies was closely related to chemoradiotherapy effectiveness in esophageal squamous cell carcinoma.[38]

In our study correlations between angiogenesis and tumor response were found. There were no correlations between apoptosis (bcl-2), p-53 expression or DNA ploidy and the response to chemoradiation. Shimada did not find any correlation between p-53 and tumor response either,[39] but these results were challenged by earlier studies by Ribiero[40] and Yang.[41] One major problem with the results of our study was that the two markers of angiogenesis, factor VIII and CD-34 were correlated to response, but not in the same way. Factor VIII had a significant positive correlation at the 5% level with a p-value of 0.0357 whereas CD-34 had a slight significant negative correlation with a p-value of 0.0493. One explanation for this lack of correlation may be the fact that VIII also is known to be a marker of lymphangiogenesis.

Squamous cell carcinomas of the esophagus have an intratumoral heterogeneity that may influence the representativeness of the biopsies harvested. It has been shown that heterogeneity is less pronounced in different parts of the same tumor compared with samples from different tumors.[42] It is therefore unlikely that our method of sampling can explain the results.

The method of pressure cooking to achieve antigen retrieval during the IHC process is sensible and it has to be performed with caution. However the fact that both positive and negative stains were found after pressure cooking and that the controls utilized were colored as expected supports the accuracy of the method.

Finally it has to be noted that the group of patients was relatively small which may influence the results. However it must be concluded that no major differences can be found regarding apoptosis, p-53 or DNA ploidy if a CR group is compared with a non-CR group in chemoradiation of squamous cell carcinoma of the esophagus. Neither can it be concluded that angiogenesis is a predictor of therapeutic success with the chemoradiation protocol used, since the two markers of angiogenesis show diverse correlations. An unknown procedure related, or biologically determined factor may be responsible for this. It has however to be noted that other studies, as demonstrated above, do not show unequivocal results.

Disclosure

The authors report no conflicts of interest.

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