Theses

Predictive factors for response to neoadjuvant therapy in patients with oesophageal cancer

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1. Introduction

The prognosis for patients with oesophageal cancer (EC) remains poor with 5-year survival rates of 10—20% only. In some selected patients, 5-year survival rates of 40% have been reported after extended lymph node dissection. Introduction of preoperative radio-chemotherapy (RCX) may improve the overall survival rate, in patients in whom RCX led to a complete tumour response, defined as the absence of vital tumour cells within the resected specimen. 5-year survival rates of >60% have been reported in these patients. According to many phase II - and some randomized phase III studies, a complete tumour response can be expected in 20-30% regardless of the applied protocol, type of histology and tumour stage. On the other hand, perioperative morbidity and mortality are increased after RCX. This affects all patients including those who show only a partial or no response at all after preoperative RCX. Therefore, identification of factors which predict a response to RCX prior to the onset of this therapy are urgentiy required.

Linear correlation between response and proliferating activity of the tumour cells in limited patient cohors was published. A multivariant analysis of proliferation factor (defined as rate of MIB-1 positive /all tumor cells) and tumour vascularisation (expressed by VEGF, vascular endothelial growth factor and CD34) was not yet published. A vascular network serves as a prerequisite for nutritional supply for a growing tumour, otherwise the tumour becomes necrotic or

remains in a state of dormancy. VEGF-mRNA is markedly upregulated in many human tumours including gastrointestinal tract carcinomas. In all these tumours, VEGF- mRNA is expressed by tumour cells but not by endothelial cells. On the other hand, the VEGF protein is detectable not only in the tumour cells but also in blood vessels indicating an accumulation of the tumour-secreted VEGF within the target cells. Correlation of VEGF expression with T-stage was reported in patients with EC. Expression of VEGF was inversely correlated with 5-year survival rate in squamous cell carcinoma of the esophagus. The carcinogenetic role of apoptosis factor bcl-2 is well-known, but its predictive effect has not been investigated.

It is a well-known fact that human papilloma viruses (HPV) participate in the pathogenesis of several types of planocellular carcinoma, however, these were detectable in other tissue type cancers. Members of 55 nm sized papovirus family can be divided into two groups. „High risk" types: 16, 18, 31, 33, 35, 39 and 52, can be found mainly in malignant tumors. „Low risk" types: 6 and 11, detectable in benign lesions (condyloma acuminatum). The viral DNA of „high risk" types is built in the chromosomes of the host cell triggering productions of two proteins. E6 and E7 proteins are products of proto-oncogenes. E6 binds to p53, and E7 to pRB which are well-known tumor-suppressor genes and these bindings inhibit their expression. HPV prefers epithelial structures. In 1989, Syrjanen isolated this pathogen from esophageal cancer. It is estimated that

infection of esophageal cancers is between 0% to 70% depending on geographic variances and different methods. In average, 20-30% of esophageal cancer patients are infected with HPV.

It is well-known that certain groups of tumors showing HPV-infection above 80% such as anal carcinoma or cervical cancer demonstrate complete response after RCX in 80-95% of cases. This finding has raised the hypothesis of a relationship between 20-30% response rate in esophageal cancer patients and the similar ratio of HVP infection. It has not yet been established however, that patients with esophageal carcinoma showing response are HPV carriers.

2. Aims

1. HPV-infection and prediction (using polymerase chain reaction and Southern blot hybridization)
Is there a correlation between HPV-infection and response for neoadjuvant radio-chemotherapy?
Are all patients showing complete response infected with HPV?

2. Immunohystochemistry and prediction
Is there a correlation among investigated factors and response for RCX? (Investigated factors: angiogenesis – VEGF, tumour vascularisation – CD34, proliferation – MIB-1, apoptosis – bcl-2)
Has the rate of expression of the factors predictive value?

3 Survival and prognosis

Is there a prognostic correlation among investigated factors according to the clinical and pathological parameters?

3. Materials and methods

3.1. RCX and patients

We analyzed preparations and clinical data of 26 patients undergoing resection surgery 4 weeks after preoperative CRX due to esophageal carcinoma according to Nauheim protocol in Department of Surgery, Albert Ludwig University, Freiburg between 1999 and 2000. Radiotherapy was administered concomitantly with chemotherapy for 4 weeks at 5 days/week. A total dose of 36 Gy was applied at daily fractions of 1.8 Gy on days 1—5 for 4 weeks. In addition, patients received 500 mg/m2 5- fluorouracil on days 1—5 for 4 weeks and 20 mg/m2 cisplatin on days 1—5 in the first and fourth week. After a break of at least 4 weeks, restaging and resection were performed. PCR was performed in 26 and imunnohistochemistry (IHC) in 21 cases. The clinical parameters and results of IHC were analyzed in a cohort of 56 cases (involving early results). Based on the criterion of complete response presence of tumour cells in surgical preparations could not be confirmed even microscopically. Partial response (PR) was measured by downstaging confirmed with staging before and after CRX and surgical findings. Patients not responding or not showing progression to the treatment are classified

as non-responder (NR) and progression (P) groups, respectively. Esophagogastroscopy, endoluminal esophageal ultrasound, CT and histological examination of biopsy were performed for preoperative staging. Lymph nodes larger than one cm were considered abnormal. Reductions in tumor size and/or in number and/or size of lymph nodes were considered as requirements for downstaging.

3.2. Methods

3.2.1. Immunohystochemistry

Usually 4—6 samples/patient were obtained endoscopcaily to prove malignancy. The specimens were immediately immersed in 4% unbuffered formalin and then prepared according to standard methods. After quenching of endogenous peroxidase with 1% H202. For immunostaining all slides were unmasked by pressure cooking in 10 mmol citric acid, pH 6, for 5 min. Sections were incubated with 0.1 % normal bovine serum to reduce non-specific background staining. Thereafter, the slides were incubated with monoclonal antibodies directed against MIB-1 (dilution 1:600; DIANOVA, Hamburg, Germany), CD34 (dilution 1:200, DAKO, Harnburg, Germany), bcl-2 (1:200, DAKO, Hamburg, Germany) and polyclonal antibodies against VEGF (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. All slides were then incubated with biotinylated secondary antibody at room temperature, followed by incubation with avidin and biotinylated horseradish

peroxidase complex (ABC-method, Vector, Burlingame, CA). Peroxidase activity was visualized by 3-amino-9-ethylcarbazole (AEC; Sigma, München, Germany). The nuclei were slightly counterstained with hematoxylin, and slides fixed with glycerin. Negative control experiments were performed by replacing the primary antibodies with preimmune serum of the corresponding species (mouse, rabbit). For control experiments, sections from a follicular hyperplasia of a tonsil served as positive control.

Five high-power fields (X *400) / slide* (three slides/ patient) from each tumour specimen were analyzed by light microscopy using a morphometric software (Analysis, Softimaging Software GmbH, Münster, Germany). On average, 230 tumour cells/slide were counted. Tumour cells were scored as positive or negative. Proliferating cells were detected by positive staining for MIB-1. Calculation of the proliferation index (P1) was performed as a percentage of all tumour cells (Index = positive turnour cells X 100/positive + negative tumour cells). All results were reported as means ± SD. Calculation of VEGF index and bcl-2 index was performed accordingly.
Microvessel density was assessed in tumour areas showing the strongest density of staining as determined by an initial scan with low magnification. For determination of vessel density, five vascular areas/slide were counted (X 200) using the SIS-computer-assisted analyzing software package (Soft-Imaging Software GmbH, Münster, Germany). The average counts were recorded.

3.2.2. Polymerase chain reaction (PCR)

Five sections (5 µm) were cut from each paraffin block (35mg) and placed in a 500 µl Eppendorf tube. One section was stained with hematoxylin and eosin for histological observation, presented by the same pathogist. DNA was extracted with DNA Mini Kit (Quiagen, Foster City, USA) using modified protocol with digestion with 180 µl ALT Buffer and 30 µl Proteinase K for 16 hours at 56 °C. All procedures were performed carefully to avoid contamination. For detection of HPV DNA, broad-spectrum consensus and highly specific (-16, -18) primers and probes were used. Briefly, the "master mix" contained 78 µl distilled water, 6 µl dNTP nucleotide mix (Boehringer, Mannheim, Germany), 3-3 µl primer-1 and -2, 30 µl PCR incubating buffer, 60 µl Solution-Q and 36 µl $MgCl_2$ (25 mM). To 18 µl of this mixture 2 µl of eluated DNA was added (Table 3.). Samples were then denatured for 10 minutes at 95 °C. After adding of 5 µl Taq-polymerase solution (PCR-Kit, Quiagen, Foster City, USA), 40 cycles were performed using beta-globine and consensus primers, consisting of annealing for 60 sec at 55 °C, extension for 60 sec at 72 °C and denaturation for 60 sec at 94 °C. For HPV-16 and -18 primers 45 cycles were performed with annealing for 40 sec at 57 °C, extension for 40 sec at 72 °C and denaturation for 40 sec at 94 °C. As controls we used beta-globine and standard HPV-DNA (prof. de Villier, ZfKF, Heidelberg). Amplified DNA (14 ul) was electrophoresed with 2 µl bromophenolblue on 2% TEA-agarose gel, using 80 V for 2 hours,

and made visible by ultraviolet light after staining with ethidium bromide.

3.2.3. Southern blot hybridization

After staining with ethidium bromide, the DNA was transferred to nitrocellulose by Southern's method. The filters were hybridized with ^{32}P-5' end labeled oligonucleotide probes under stringent conditions using the Oligonucleotide Tailing Kit (Boehringer). After hybridization with 60 µl oligonucleotide for 16 hours at 56 °C, the filters were washed under low stringent condition at room temperature. Anti-digoxigenin-AP-conjugate was used for visualization (DIG-Nucleotide Acid Detection Kit, Boehringer)

3.2.4. Statistics

Survival rates were calculated by Kaplan—Meier's procedure, statistical differences were calculated by Wilcoxon's test, respectively; $P < 0.05$ was regarded as significant. Differences in Chi square test of numbers of stained cells were evaluated by the Student's t-test for independent samples. Multivariate analysis (Cox procedure) was performed to prove independence of investigated variables. Sixteen variables were inciuded. Stepwise forward and backward procedures were used to strengthen the used models.

4. Results

4.1. Clinical-pathological data

Fourteen patients (25%) showed complete pathological response (3/14 adenocarcinoma, *11/42* squamous cell carcinoma). In 23 of 56 patients (41%), NR or even tumour progression was observed (8/14 adenocarcinoma, 15/41 squamous cell). There was no significant correlation between clinical staging and response. The calculated 5-year survival rate of all 56 patients was 25% (median, 26 months). At the last survey 26.8% of patients with squamous cell cancer were still alive (median, 18 months) and 50% of those with adenocarcinoma (median, 17 months; $P = 0.5$).

4.2. HPV-infection (n=26)

4.2.1. Clinical data

More than half of patients (n=17, 65.4%) with CRX treatment responded well to the treatment and 7 patients demonstrated complete response. In these cases lymph nodes were negative as well. However, more than one-third of patients had not shown response, and even progression was observed in one-tenth of them.
It is to be noted that 3 out of 7 patients with adenocarcinoma had responded to CRX. In 24 patients we performed esophageal resection from thoracotomy and reconstruction by Akiyama (in one case by

colonic interposition) and extended lymphadenectomy. In two cases transhiatal approach was performed. Postoperative phase was uneventful in 15 cases. Re-operation was performed in 3 patients (11%) due to the following reasons: suture leakage, empyematic chylothorax and ischemic necrosis of the gastric tube. Pulmonary complications (pneumonia, hydrothorax, respiratory insufficiency) occurred most frequently amongst all complications (7 patients, 27%). Tracheostomy was necessary in five patients (due to permanent artificial respiration). One patient died within 30 days (3.8%). Additional 3 patients died within 2 months (2 patients died due to respiratory insufficiency and septicemia, and one patient due to symptoms of MOF).

4.2.2. PCR and Southern Blot results

HPV-infection has been confirmed in 6 cases (23%). Consensus-primer (the general primer in the identification of HPV) was positive in each of these cases. We observed HPV-16 infection in 5 cases, and HPV-18 infection in one case. Mixed infection has not occurred. Control betaglobin test was positive in each case. HPV-16 infection was identified in all cases with planocellular carcinoma. The only HPV-18 positivity was observed in an adenocarcinoma. Seventy-seven percent of patients (n=20) was virus carrier. Average age of carriers was 57.5 (48.2-73.6) years. Out of the 6 carriers there were 3 complete and 3 partial responders. There was no carrier among non-

responders. From HPV positive tumors 2 were found in the middle third and 4 in the lower third of the esophagus.

4.2.3. Results of survival analyses

There was no significant difference in survival among responders ($p=0.069$), but $p<0.01$ when responder groups were compared to the NR group. Median values were as follows: CR: 42 months, PR: 36 months and NR: 12 months. HPV-positive patients (all in responder group) showed significantly better prognosis ($p=0.032$).

4.3. Immunohystochemistry (n=21 / MIB-1: n=45 / 56)

4.3.1. Clinical data

Significant difference ($P = 0.0026$) in survival was observed regarding tumour response (including hospital death), 53.3% of patients were still alive after complete pathological response (median, 54 months) compared with 36.8% of patients with a PR (median, 40 months) and with 9.5% of those with no change/tumour progression (median, 11 months).

Patients with increased proliferation activity showed significantly better survival. Patients with PI higher than 40% had better prognosis (5 year survival four times better; $p=0.021$). More than half (57.1%) of the patients with adenocarcinoma had no benefit from CRX, including 35.7% of those with squamous cell carcinoma.

4.3.2. Factors investigated for prediction

MIB-1

In 46 of 56 patients who received preoperative RCX, prolifcrative activity of tumour samples obtained prior to RCX could be related to the response after resection.

Tumour samples of 13 patients with a complete pathological response showed a mean PI of 58.81 compared with 53.98 and 45.9 of tumours with PR or NR *(P* = 0.012). None of the tumours with a CR had an index of less than 45. Correlation with survival was statistically significant: an increased Pl led to a better long-term survival *(P* = 0.0114).

VEGF

VEGF expression could be determined in 21 biopsies prior to RCX. Tumour sampies of five patients with a complete pathological response showed a VEGF expression index of 10.7 compared with 18.34 (n=8) and 36.58 (n=8) of tumours with PR or NR *(P* = 0.035). Correlation with survival was statistically significant: a decreased VEGF expression led to a better long term survival (P = 0.0205).

CD34

Tumour samples of five patients with a complete pathological

response showed **a** CD34 index of 10.92 compared with 18.97 *(n* = 8) and 18.16 *(n* = 8) of tumours with PR or NR *(P* = 0.036). None of the tumours with a CR had an index of more than 20. CD34 expression showed a correlation with VEGF expression *(r* = 0.4). Both vascular density and VEGF expression were higher in tumour samples which showed only PR or NR to preoperative RCX compared with tumours of complete responders. There was no correlation of CD34 expression with longterm survival *(P* = 0.63).

bcl-2

Tumour samples of five patients with complete pathological response showed a bcl-2 expression index of 18.4 compared with 22.4 and 16.1 of tumours with PR or NR. Predictive value of bcl-2 expression was not proved.

4.3.3. *Correlation of proliferative activity and VEGF expression*

In tumours which showed a CR to chemoradiation, the relation of mean indices between VEGE expression and proliferative activity was 10.7—58.8. The relation of mean indices of VEGF expression and proliferative activity in tumours with PR or NR was 18.3—53.8 and 36.6—43.5, respectively. *According to these results, it may be expected that tumours with a VEGF/MIB-l quotient of **1:5** or less prior to RCX will respond to this therapy.*

4.3.4. Multivariant analysis of survival and response

Multivariate analysis revealed Pl *(P* = 0.0203), response *(P* = 0.0017) and tumour free resection (R0; *P* = 0.023) as independent variables for survival in patients who received preoperative RCX. All other tested variables (sex, pT, pN, histology, grading) were not significant. Logistic regression defined PI *(P* = 0.0193) and pN *(P* = 0.0002) as independent variables for tumour response in these patients.

4.3.5. Correlation of the HPV infection and IHC-results

HPV-infected patients have showen response for CRX. VEGF-Index and CD34 expression were lower in these cases, than in non-infected cases. Pre- and posttherapeutic expressions of angiogenetic factor demonstrated differrencies. MIB-1/VEGF rates were elevated in HPV- positive cases, potentially leading to better prognosis. It is hypothesized that HPV-infection potentially leads to better prognosis.

4.3.6. Incidental local results

We invetigated 10 patients who underwent non-standardised CRX and surgical resection. The investigated factors were: CD34, MIB-1 and HPV-16 and -18 infection. Three patients showed complete

response. Two of them were HPV-carriers. The mean PI was 56.2 and 47.8 in CR and NR groups, respectivelly. CD34 expression was 23.4 and 46.3 in CR and NR groups repsectivelly, which is a statistically significant difference in spite of the small number of cases. Postoperative complication occoured in 5 of 10 cases.

5. Discussion

The positive effect of praeoperative chemo-radiotherapy (CRX) on survival (rate) is unequivocal. Total pathological response could be observed in 20-30% of the cases. Surgical complications were more severe and more difficult to be managed after CRX. It is suggested that cellular immunity and peri-esophageal inflammatory reactions are responsible for this phenomenon. A higher rate of complications affected all patient groups which underwent CRX, and was also observed in patients without beneficial effect of the therapy. Nauheim protocol was applied and 26.9% complete response and 38.5% partial response were observed. 34.6% of patients did not respond to the therapy. The rate of adenocarcinoma in patient groups in which CRX was effective was 41% and the stage did not significantly affect the response. There was no postoperative complication in 58% of the cases. Fourty percent of the patients showed not response.

5.1. Predictive role of HPV infection

HPV infection of esophageal cancers is between 0% to 60% depending on geographic variances and different detection methods. In average, 20-30% of esophageal cancer patients are infected with HPV. The potential correlation between HPV infection and response to CRT was not yet investigated. HPV infection was detected in 23% of our patients with esophageal cancer and in 35% of responders to CRT. Results of PCR were confirmed by Southern Blot hybridization. Fifty percent of HPV infected patients showed complete pathological remission while the remaining half were partial responders. HPV infection was only detected amongst responder patients. In spite of the small patient cohort, a correlation may be hypothesized between HPV infection and response to CRT, thus HPV infection is assumed as a predictive factor.

5.2. Predictive role of immunohystochemical factors

Correlation between tumour proliferation and vascularissation in cases of esophageal cancer was not yet analysed. Our data revealed that tumours with low vascularisation and angiogenetic activity and those with high proliferative activity respond better to CRT. These findings suggest that proliferation prevails at a cellular basis, while angiogenesis and/or tumour vascularisation is a more complex mulifactorial procedure indepent of the grade of proliferation. PI (besides N status) determinded by regression analysis proved to be

an *independent predictive factor alluding to remission*. The 5:1 or higher rate of PI:VEGF-expression in our patients turned out to be predictive to complete tumour.-remission. The analysed factor of apoptosis (bcl-2) proved not to be predictive.

5.3. Correlation of the investigated factors and survival

Multivariant analysis of all patients undergoing CRT proved proliferation activity (besides remission and R0- resection) as an *independent prognostic factor signifcant to survival.* Overall survival was significantly better in cases with high proliferation activity. Survival of patients with PI higher than 40% was nearly four times higher. There was a significant difference in survival among responders, non-responders and the controll group not receiving CRT. Patients with partial remission also had significantly better survival in comparison to non-responders and the controll group. Thus, *predictive factors referring to remission proved to be progressive factors as well.*

6. Summary

Neoadjuvant chemoradiotherapy (CRT) introduced in the treatment of resectable esophageal cancer produced encouraging results,

however overall prognosis remained poor. Twenty-five to thirty percent of the patients achieve a complete pathological response, following neoadjuvant CRT with higher rate of R0-resections and significamtly higher survival rates, in comparison to patients without neoadjuvant CRT. On the other hand, patient undergoing neoadjuvant CRT show an increased rate of postoperative complications and mortality. In our study, complete pathological response was achieved in 26.9% of the cases, while 34.6% of the patients proved to be partial responders. Predictive factors for response to neoadjuvant CRT was examined in various pathways and methods (CD34, VEGF, MIB-1, bcl-2: immunohistochemistry and HPv-16, -18 detection with PCR and Southern Blot Hybidization). It is well known that tumours with high prolifartive activity respond more to neoadjuvant CRT than those with greater vascularisation (angiogenesis). These two oncological factors were not yet examined in patients with esophageal cancer undergoing neoadjuvant CRT. *In this analysis, a relation of 1:5 or less of VEGF expression playing a key role in angiogenesis and MIB-1 indicating proliferative activity was predictive for response to neoadjuvant CRT.* In addition, multivariate analysis revealed that a proliferative activity higher than 40% resultsin significantly higher survival. The bcl-2 apoptosis-factor was not found to be predictive.

The role of certain types of human papilloma virus (HPV) in the pathogenesis of esophageal cancer is known, with an average infectaion rate of 20-30%. The E6 protein of HPV-16 and 18 causes degradation of p53 tumorsupression-protein resulting inhibition of

apoptosis. HPV is responsible for inhibition, but not for mutation of p53 leading to hyperexpression of VEGF amongst other unfavourable prognosttic factors. This is one of the paradox enigmas of the oncopreventive and oncogenetic impacts of HPV. It was reported that certain highly HPV- infected cancers show more than 90% complete pathological response to neoadjuvant CRT but the potential correlation between HPV infection and response to CRT was not yet investigated. We identified the two most common malignant type of HPV (HPV-16,-18) by PCR and Southern Blot Technique. Results were compared with immunohistochemical findings and clinico-pathologic parameters. *HPV-infection was only detected in responder cases showing remission to neoadjuvant CRT. We presume that HPV- infection contribute via indirect mechanisms (inhibiton of VEGF hyperexpression) to a better prognosis. Our hypothesis was confirmed by survival statistics. Multivariate analysis revealed MIB-1 expression, remission to neoadjuvant CRT and R0 resection as indipendent, significant prognostic factors. In terms of remission to neoadjuvant CRT, MIB-1 and pN proved to be independent prognostic factors.*