Abstract. Background: Loss of heterozygosity (LOH) may be a valuable tool for detection of malignant proceedings. The aim of our study was to investigate LOH in the serum of patients with adenocarcinoma of the distal oesophagus and the cardia for diagnostic and prognostic utility. Patients and Methods: Matched tumour and serum samples from 46 surgically treated patients with oesophageal adenocarcinoma and cardia carcinoma divided in two groups were analysed. Twelve markers were examined with a PCR-based microsatellite analysis. Results: A similar high frequency of LOH (range from 77% to 96%) was detected in the tumour and serum of both groups, whereas no LOH was detected in 20 healthy individuals. However, no significant correlation between LOH incidence and clinicopathological characteristics and survival was found. Conclusion: The results indicate that DNA alterations in tumours of the oesophagus and cardia are uniform. The high frequency of LOH in tumour patients underlines the utility of this molecular approach as a diagnostic tool.

The incidence of adenocarcinoma in the gastroesophageal junction has risen significantly over the last decades (1). Often patients with this disease suffer from clinical symptoms only at an advanced stage of this tumour type, with consequent difficulty in achieving a curative treatment.

Therefore, detection of genetic alterations, especially the loss of heterozygosity (LOH) in cancer cell DNA has become established as a promising field in tumour diagnostics (2, 3).

LOH analysis is not only limited to the detection of alterations in cancer cell DNA from tumour specimens alone. There are many reports of free circulating tumour DNA in body fluids such as serum and plasma of cancer patients (4-10). Recent studies have suggested that especially in plasma or serum these genetic markers can be used for diagnosis or prognosis of progression in patients suffering from different types of tumours (11-15).

Tumors located at the gastroesophageal junction, that are diagnosed at an advanced stage at tumour growth, are mostly associated with radical therapies, such as extended surgical interventions and a poor prognosis (16).

Adenocarcinomas of the gastroesophageal junction have been found to have high frequencies of genetic alterations on the chromosomal subregions 3p, 4p, 5q, 9q, 18p and 21. Importantly it has been noted that loss of heterozygosity near the tumour suppressor genes, such as APC on 5q, p16 on 9q, and p53 on 17p, were found in the tumour DNA of patients with adenocarcinoma of the oesophagogastric junction (17). This implies that similar genetic alterations have an influence on the tumourigenesis of these types of carcinomas. Moreover, the detection of these allelic losses in free circulating cancer cell DNA in the serum, could be clinically useful for the detection of disease or the prediction of outcome for patients with carcinomas located at the gastroesophageal junction.

Therefore the purpose of this study was to detect LOH in the DNA of different chromosomal regions of serum and tumour tissue samples from patients who had undergone surgical resection in order to investigate whether this molecular approach has a role in the detection and prognosis of patients with adenocarcinoma of oesophagus and the proximal stomach. The patients were separated into a group with adenocarcinoma of the distal oesophagus and another group with adenocarcinoma of the cardia.

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Patients and Methods

Patients and tissue collection. The study was approved by the ethics committee of the chamber of physicians in Hamburg, Germany. Written informed consent was obtained from all patients for the use of tissue and blood samples, which were collected at the time of surgery in our hospital from patients suffering from adenocarcinoma of the distal oesophagus (n=26), of the cardia (n=20). Control blood samples were collected from healthy individuals (n=20). An experienced pathologist defined cardia or oesophageal adenocarcinoma based on the histopathological observations. Ten ml of blood as a source of genomic DNA were collected in tubes containing EDTA (ethylenediaminetetraacetic acid). Blood for serum DNA was collected in separate serum tubes without EDTA. For extraction of serum after centrifugation, a minimum of 2 ml was collected.

All of the tissue samples and blood were collected from each patient just before the surgical resection. Experienced surgeons performed these procedures, which were mainly distal oesophageal resection with total gastrectomy or distal oesophageal resection with proximal gastric resection in combination with different methods of reconstruction. Representative thin tumour sections were stained with H&E (Hämatoxin and Eosin). Based on the microscopic evaluation of the H&E stained section by an experienced pathologist, tumour cells were collected by microdissection with a sterile razor blade. Non-neoplastic tissue was removed to achieve at least a 70% purity of the neoplastic cell population. Twelve 12 µm tissue sections were placed in 1% SDS (sodium doceyl-sulphate)/proteinase K (0.5 mg/ml/Qiagen, Hidden, Germany) and incubated at 58°C for 24 h. Lymphocytes were collected from the EDTA-blood samples by centrifugation at 4000 rev. per minute for five min. DNA was extracted from the serum and tumour tissue using a QIAamp midi kit and a QIAamp mini kit respectively. After extraction the DNA concentrations were measured photometrically.

Microsatellite analysis. A panel of 12 microsatellite markers was chosen: D17S520 (17p), D17S796 (17p), D17S804 (17p), D9S162 (9p), D9S171 (9p), D9S1746 (9p), D9S126 (9p), D18S51 (18q), D18S70 (18q), ACTBP2 (15q), CSF1R (15q) and D18S479 (18q). The microsatellite markers were tested in the matched tumour DNA, lymphocyte DNA and serum DNA. DNA sequences for the microsatellite markers were obtained from the genome database (http://www.gdp.org/). A 10 µl aliquot of reaction mixture containing 2 µl of DNA of the probes, 1 µl of each primer marker set 0.05 µl of Ampli Taq Gold (Applied Biosystems, Foster City USA) and 5 µl-distilled water, were used. PCR conditions were as follows: 94°C for 16 minutes followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for one minute with a final elongation step at 72°C for 10 minutes. Then 0.2 µl of Rock Size (Applied Biosystems, Foster city USA) and 40 µl of deionised formamide were added to 0.5 µl of the mixture. Then the mixture was heated at 96°C, immediately cooled on ice and analysed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

A reduction in the intensity of one allele in the target sample of more than 50% was considered to represent LOH (example given in Figure 1). Results indicating LOH were repeated at least twice. If the peaks of alleles were too high no exact calculation of LOH was possible. In these cases the PCR product was diluted mainly by HPLC-buffer of a ratio of 1:10.

Statistics. For statistical analysis SPSS for Windows (SPSS®, Chicago, USA) was used. Clinical data were taken from the patient’s charts and hospital records. Correlation between LOH and clinicopathological features was calculated using cross tables chi-square test and Fishers exact probability test. Patient survival was analysed according to the Kaplan-Meier method and compared by a log-rank test.

Results

Patient characteristics. Twenty-six patients with oesophageal adenocarcinoma and twenty patients with cardia carcinoma were recruited for this study. Blood samples from 20 healthy age matched individuals (average age 67 years) were also analysed as control.

Of the 20 patients with cardia adenocarcinoma (Table I), two patients (10%) had T1 tumours, twelve (60%) T2 tumours, five (29%) T3 tumours and one patient (5%) had a T4 tumour. 13 (65%) Patients had G2 tumours and 7 (35%) had G3 tumours. Distant metastases were found in nine (45%) patients and 19 patients (95%) had lymph node involvement in the preoperative staging. Of the patients
with oesophageal adenocarcinoma (Table II), five (19%) had T1, seven (27%) T2 and 14 (54%) T3. In this group 14 patients (54%) had G3 and 12 patients (46%) had G2 tumours. 73% (19 out of 26) of these patients had lymph node involvement. Further details of patient characteristics are shown in Tables I and II. The mean DNA concentration from the serum of patients with adenocarcinoma of the oesophagus was 9.2 µg/ml, from patients with cardia carcinoma 8.9 µg/ml and from 20 healthy individuals 7.9 µg/ml.

LOH tumour and serum DNA. In the DNA of tumour tissue of the patients with oesophageal adenocarcinoma LOH was detected in 20 (77%) whereas in serum DNA the LOH was found in 25 (96%) patients. Average LOH incidence was 2.8 alleles in tumour tissue and 3.0 in serum DNA. In the patients with cardia carcinoma LOH was detected in the tumour DNA in 17 patients (85%) and in 19 patients (95%) in the serum DNA. In this group average LOH was 2.8 alleles in tumour tissue and 3.0 in serum. In serum of 20 healthy individuals no LOH was detected.

In the patients with cardia carcinoma LOH was detected in the tumour DNA in 17 patients (85%) and in 19 patients (95%) in the serum DNA. In this group average LOH was 2.8 alleles in tumour tissue and 3.0 in serum. In serum of 20 healthy individuals no LOH was detected.

The frequencies of LOH detection in the tumour and serum DNA of both groups for the single primers are listed in Table III. No significant difference was found for the frequency of LOH detection on chromosomal regions 9q, 17p and 18q between cardia and oesophageal adenocarcinoma. LOH number in both tumour and serum DNA on 17p was slightly higher than on the other examined chromosomal regions (Figure 2).

LOH and survival. The median follow-up time after surgery was 24.6 months for all patients. The LOH in the tumours and in serum sample did not correlate significantly with survival in patients with cardia carcinoma (tumour LOH: \( p=0.4 \); serum LOH: \( p=0.82 \)) or in patients with adenocarcinoma of the...
oesophagus (tumour LOH: \( p = 0.46 \); Serum LOH: \( p = 0.11 \)); see Figure 3.

Survivals was not significantly correlated of LOH number either in tumour or in serum of all patients with adenocarcinoma of the gastroesophageal junction (\( n = 46 \)), (Figure 3).

**Association between LOH and pTNM stage.** Here was no association between presence of LOH in the tumour /serum DNA and the T-stage of the tumour in patients with cardia carcinoma (\( p = 0.36 \) tumour/\( p = 0.98 \) serum). In patients with oesophageal adenocarcinoma \( p = 0.4 \) tumour/\( p = 0.62 \) serum). Also no significant correlation between histopathological lymph node involvement and tumour grading and tumour LOH (\( p > 0.05 \)) or serum LOH (\( p > 0.05 \)) could be found in either tumour group.

**Discussion**

The markers chosen in this study were detecting LOH in chromosomal regions located near genes, which play an important role in the development of tumours of the oesophagogastric junction. These genes were p53 (17p), p16 (9p), SMAD 4 (18q) and APC serum (5q).

The main finding of our study was the high frequency of LOH in tumour and serum DNA in patients of both groups, whereas no samples from the control group showed any LOH. These results imply that there was a high degree of genetic instability in these adenocarcinomas on chromosomes 18q, 17p and 9p in accordance with other studies (18-20). Remarkably, the recorded incidence of LOH incidence was higher in serum DNA than in tumour DNA. Hypothetically a more precise dissection technique such as by Laser microdissection of the tumour tissue could provide purer tumour cell samples, which might be expected to give results in line with the serum LOH values.

The high incidence of LOH in both tumour and serum DNA was similar for patients with adenocarcinoma of the distal oesophagus and the cardia. Corresponding to other reports (11, 21-23), this result indicates that genetic changes in these regions in the vicinity of the APC, p16 and p53 genes reflect a similar pattern of carcinogenesis of both tumour types. Thus the same marker panel might be used in a clinical setting to diagnose both tumour types at an early stage of development.

The lack of association between LOH incidence and both tumour grade/stage and survival limits this molecular analysis approach to diagnostic purposes, since a prognostic impact for this type of analysis in adenocarcinoma of the gastroesophageal junction has not been demonstrated. Larger patient numbers and longer follow up data are required to clarify this issue.

The lack of correlation between incidence of LOH and pTNM-Stage in this study may be explained by the early occurrence of multiple genetic changes at the onset of tumourigenesis in adenocarcinoma of the gastroesophageal junction. However, the presence of these multiple alterations at an early stage of tumour growth, as detected in the serum of pT1N0 oesophageal adenocarcinoma patients, could be used as an early diagnostic tool, which may lead to early and more effective treatment of these patients (23). Markers on other relevant chromosomal arms could potentially improve this molecular approach.

Remarkably the frequency of LOH in the serum DNA of cancer patients was similar to that detected in tumour DNA. The discrepancy between chromosomal regions of the LOH in tumour DNA, and the LOH in serum DNA, could be explained by heterogeneity of tumour (24).

In conclusion a high frequency of LOH was detected in the tumour and serum DNA of patients with adenocarcinoma of the gastroesophageal junction, whereas in the serum of 20 age matched healthy individuals no LOH was observed. On the basis of the microsatellite markers used in this study similar frequency of the LOH in the different chromosomal
regions between gastric and oesophageal adenocarcinoma was detected. This could reflect a similarity of the same genetic events in the carcinogenesis between these tumours.

Furthermore a significant association between the LOH appearance and clinicopathological features, such as grade or stage, of these two tumour types was lacking. This result could imply the occurrence of a wide range of genetic alterations at the beginning of tumour growth. Moreover this molecular approach could facilitate the detection of adenocarcinoma of the gastroesophageal junction at an early stage.

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References


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