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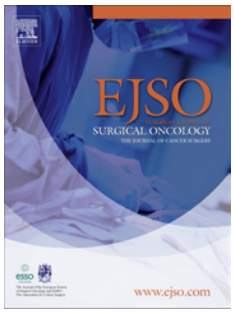

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The prognostic relevance of tumor hypoxia markers in resected carcinoma of the gallbladder

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Abstract

Background

Intratumoral hypoxia has been suggested to drive more aggressive tumor behavior. Our aim was to define whether markers of tumor hypoxia are predictors of outcome in patients with gallbladder carcinoma.

Patients and methods

From 1996 to 2006, 34 patients underwent resection for gallbladder carcinoma. The median follow-up was 12.6 months. Immunohistochemical stains for VEGF, HIF1α, GLUT1, GLUT3, CA9 and EGFR were performed on archival tissue. Immunohistochemical results were correlated with clinical and histopathological parameters. Cumulative overall survival (OS) rates were estimated using the Kaplan-Meier method. Multivariable Cox regression models were used to identify predictors of OS.

Results

The median OS was 11.9 (IQR: 3.4 – 22.0) months. Ubiquitous VEGF staining was observed in all gallbladder carcinomas. High (>50% of tumor cells) EGFR expression was associated with worse OS (p=0.03). CA9 expression was less prevalent in poorly differentiated tumors (p=0.02). GLUT3, GLUT1 and HIF1α expression were not associated with survival, but did correlate with the presence of lymph node metastasis (p=0.02), tumor differentiation (p=0.04) and tumor stage (p=0.03) respectively. High EGFR expression, TNM stage and preoperative serum CA19.9 were retained as independent predictors of OS in multivariable analysis.

Conclusion

In gallbladder cancer high expression of EGFR is an independent predictor of survival.

Introduction

In the United States, gallbladder cancer is the most common biliary tract malignancy and is the fifth most common malignancy of the gastrointestinal tract. SEER data (1) and data from the National Cancer Database (2), have estimated for the United States an annual incidence of approximately 2800 gallbladder cancer related deaths. Despite substantial improvement over the last two decades, the overall prognosis of patients presenting with gallbladder carcinoma remains poor (3). This can primarily be explained by early spread by lymphatic, perineural and hematogenous routes and direct invasion into the liver. Moreover, the lack of effective chemotherapy leaves us impotent to cure many of these patients.

In the search for new therapeutic targets and prognostic markers the concept of tumor hypoxia has been extensively studied. Intratumoral hypoxia has been found to be a potent stimulator of metastasis and is considered an indicator of more aggressive tumor behavior (4). Hypoxia inducible factors (HIF) translate the hypoxia signal and are the key regulators of a large panel of genes that are exploited by tumor cells for survival and angiogenesis, resistance to treatment and important steps in metastasis such as epithelial-mesenchymal transition (EMT). Vascular endothelial growth factor (VEGF) (5), glucose transporter member 1 and 3 (GLUT1 and GLUT3) (6), carbonic anhydrase IX (CA9) (7) and epidermal growth factor receptor (EGFR) (8) have all been implicated as crucial regulators of the tumor response under hypoxia downstream or upstream of HIF1α.

The aim of the current study was to analyze the immunohistochemical expression pattern of a panel of hypoxia markers and their prognostic relevance in patients with gallbladder cancer.

Material and Methods

Prospectively collected data of a single-centre cohort of patients, who underwent surgery for gallbladder carcinoma between December 1996 and December 2006, were reviewed. A total of 34 patients (F/M: 20/14; median age 72 years (range: 44–91) underwent surgical resection with curative intent over this 10-year period.

A curative resection (R0) was defined as negative resection margins by light microscopical examination. For each patient prospectively registered clinicopathological variables were extracted from the electronic clinical records: demographic data (age, gender), presenting symptoms, biochemistry, the tumor markers CA19.9 and CEA and surgical therapy. All the pathology slides were reviewed by 2 pathologists (EL, NE) with special attention for tumor growth pattern and differentiation, the pathologic margin status, the presence of lymphovascular invasion, perineural invasion and the total number and status of regional and distant lymph nodes harvested. The tumor differentiation was graded (G1-3) according to World Health Organization standards and tumor stage classification was applied according to the current AJCC TNM staging system (9).

Therapeutic approach

The treatment was considered complete when a cholecystectomy with negative resection margins (R0) was performed in case of a tumor limited to the lamina propria (pT1). In case of positive resection margins (R1) or if the depth of invasion reached the muscularis propria (pT>1), the cholecystectomy procedure was extended with both a resection of liver segments 4b and 5 and a lymph node dissection. When the cystic duct resection margin was positive for tumor, the extrahepatic bile duct was

excised to clear margins. Preoperative or postoperative staging consisted of an abdominal ultrasound, a CT-scan and/or MRI of the abdomen, a chest X-ray and biochemistry with tumor markers (CEA, CA 19.9 and occasionally α-fetoprotein).

Immunohistochemistry

Archival paraffin-embedded surgical material was retrieved from the 34 patients. Immunohistochemistry was used on 5 micrometer sections of all tumors to study the expression of VEGF, HIF1α, GLUT1, GLUT3, CA9 and EGFR proteins. Monoclonal antibodies were used for HIF1α (BD Biosciences, Heidelberg, Germany; dilution 1/100), CA9 (Abcam, Cambridge, United Kingdom; dilution 1/25), and EGFR (Zymed Laboratories, Carlsbad, CA92008, USA; dilution 1/50). Polyclonal antibodies were used for VEGF (Santa Cruz Biotechnology Inc, California, USA; dilution 1/100), GLUT1 (Chemicon/Biognost, Belgium; dilution 1/1000), and GLUT3 (Chemicon/Biognost, Belgium; dilution 1/500). For visualisation the Envision (Dako, Glostrup, Denmark) protocol was used. All slides were counterstained with Harris hematoxylin.

All immunohistochemically stained slides were evaluated by one pathologist (EL) who was blinded to the patients' clinical data. In case of staining for CA9, GLUT1, and GLUT3 the percentage of positive tumor cells was semi-quantitatively scored and categorized into 4 groups: < 5%, 5-25%, 25-75% and > 75% of positive tumor cells. HIF1α was semiquantitatively scored as negative, <5% and >5% of tumor cells staining positive, while EGFR was categorized in 4 semi-quantitatively assessed groups: < 10%, 10-25%, 25-75% and > 75% of positive tumor cells. The staining pattern within the tumor was classified as membranous, cytoplasmatic or nuclear. In

case of GLUT1 and GLUT3 stains the staining intensity was also recorded: negative, weak or strong (in comparison to the staining intensity of red blood cells).

Follow-up

Follow-up data were recorded from the patient's medical records and completed by a telephone survey performed on the 1st of August 2009. Postoperative mortality was defined as in-hospital mortality irrespective of the cause. Overall survival (OS) was defined as the time (months) from the day of surgery until death of any cause. Median post-operative follow-up was 12.6 months (range: 4 days – 113.8 months).

Statistics

Statistical calculations were carried out using JMP version 8.0.1 for Mac (SAS). Medians with range were reported for non-binomially distributed data. The Mann-Whitney test was performed to compare continuous data. The Fisher's exact or Pearson's chi-square test was used to compare categorical data whenever appropriate. Survival rates were estimated according to the Kaplan-Meier method using Log-Rank statistics for comparison. Univariable and multivariable Cox regression models were used to identify predictors overall survival. Variables considered were: Age (years), gender (M/F), ASA score (1-5), expression of EGFR, CA9, GLUT3, GLUT1, HIF1α and VEGF, TNM stage, tumor differentiation (G1-3), lymph node status (pN), tumor staging (pT), resection status (R0-2), lymphovascular invasion (LVI), perineural invasion and preoperative serum CA19.9 and CEA levels.

Candidate predictors for the multivariable model were all predictors with $p < 0.1$ in the univariable analyses. A backward selection was applied to reduce the final

multivariable model to 3 variables. Two-sided P-values < 0.05 were considered statistically significant.

Results

Clinical data

Twenty-one patients underwent elective surgery for gallbladder carcinoma, while in 13 cases the diagnosis of gallbladder cancer was an incidental finding after laparoscopic cholecystectomy. All surgical procedures that were performed are summarized in table 1. An R0 resection was obtained in 14 of 34 patients. Concurrent cholecystolithiasis was present in 32 patients. Median preoperative serum CEA levels were 2.7 µg/L (range: 0.5 - 42.5 µg/L). Median preoperative serum CA19.9 levels were 76 kU/L (range: 1 - 13681 kU/L).

Median length of hospital stay was 9 days (range: 1 - 29 days). Nine patients developed post-operative complications. Three patients died in-hospital of postoperative complications.

Twenty-two patients had stage IV disease at the time of diagnosis. Eight patients with stage IV disease received adjuvant gemcitabine-based chemotherapy post-operatively. The median OS for the entire study cohort was 11.9 months (IQR: 3.4 – 22.0). At the end of follow-up, 5 patients remained alive.

Histopathological data

Histological data are summarized in table 2. All carcinomas showed a glandular growth pattern. In addition to this pattern a papillar growth pattern was demonstrated in 15 patients. Carcinomas were composed mostly of clear cells in 12 patients.

Seventeen tumours were poorly differentiated. Lymph node status was evaluable in 22 patients. Positive lymph nodes were found in 16 patients.

Immunohistochemistry (Figure 1a-f)

CA9 expression (Figure 1a)

CA9 staining was found on the membrane of tumor cells in all but one patient. This expression pattern was found in less than 5% of tumor cells in 7 tumors. A more extensive expression pattern ranging from 5 to 25% of tumor cells was found in 9 tumors. In 6 tumors membranous CA9 expression was found in more than 75% of tumor cells. CA9 expression did not correlate with R status, pT, pN or pM. After pooling all CA9 positive cases, CA9 expression was found to inversely correlate with tumor grade (p=0.017). Median OS was 17.0 (IQR: 11.9 – 41.1) and 6.8 (IQR: 3.0 – 19.3) months for patients with more than 50% or less than 50% of CA9 expression in their tumor respectively (p=0.21, Log Rank).

EGFR expression (Figure 1b)

EGFR positive gallbladder carcinomas predominantly expressed the protein on the membrane of tumor cells and to a lesser degree also in the cytoplasm. Nevertheless, large differences in the percentages of stained cells were noted with percentage values ranging between less than 10% and more than 95%. In 17 cases more than 50% of tumor cells stained for EGFR. Patients with EGFR expression in more than 50% tumor cells had a median OS of 3.7 months (IQR: 2.4 – 14.0) compared to 15.8 months (IQR: 11.4 – 62.4) in patients with EGFR expression in < 50% tumor cells. (p=0.03, Log Rank; Figure 2). EGFR expression did not correlate with the R status, pT, pN, pM and tumor grade.

GLUT3 expression (Figure 1c)

GLUT3 expression on the other hand was found to be expressed ubiquitously (>95%) in the cytoplasm of tumor cells and to a much lesser extent also on the membrane. In

2 tumors this pattern was not present with GLUT3 expression that was higher on the tumor cell membrane than in the cytoplasm. A weak GLUT3 staining intensity was seen in 8 tumors. The remaining 26 tumors clearly showed moderate to strong GLUT3 staining intensity. The presence of positive lymph nodes (pN1) was correlated with more extensive GLUT3 expression (p=0.015). On the other hand, the extent of GLUT3 expression did not correlate with survival, R status, pT, pN, pM or tumor grade.

GLUT1 expression (Figure 1d)

GLUT1 expression was generally found on the tumor cell membrane and to a lesser extent in the cytoplasm. Strong GLUT1 staining was seen in 24 tumors. The extent of GLUT1 expression correlated with higher tumor grade (p=0.04). The extent of GLUT1 expression did not correlate with survival, R status, tumor stage, the presence of positive lymph nodes or distant metastases. Similarly, GLUT1 staining intensity did not correlate with survival, R status, pT, pN, pM or tumor grade.

HIF1α expression (Figure 1e)

Nuclear HIF1α staining was identified in the tumors of 15 patients. Staining intensity was generally weak and present in less than 5% of tumor cells. Two patients had a more diffuse and more intense staining pattern with HIF1α. HIF1α expression did not correlate with survival, R status, the presence of positive lymph nodes or the presence of distant metastasis. When grouping all HIF1α positive cases, HIF1α positivity was correlated with higher pT scores (p=0.029). The correlation between HIF1α positivity and tumor grade did not reach significance (p=0.076). Finally, no correlation was

found between HIF1α staining and the expression of CA9 (p=0.495), EGFR (p=1.000), GLUT1 (p=0.724), GLUT3 (p=0.311) and VEGF (p=0.113).

VEGF expression (Figure 1f)

VEGF was found to be strongly expressed in the cytoplasm of >75% of tumor cells in all tumors. Therefore no correlations between VEGF expression and survival, R status, pT, pN, pM or tumor grade could be found.

Univariable analysis

Univariable Cox regression identified high EGFR expression (p=0.04), TNM stage (p=0.001), Tumor differentation (p=0.006), pT stage (p=0.05), resection status (p=0.1), perineural invasion (p=0.01) and increasing preoperative serum CA19.9 levels (p=0.01) as candidate predictors for the multivariable model.

Multivariable analysis

In multivariable Cox regression analysis high (>50%) EGFR expression, higher TNM staging and high preoperative serum CA19.9 were retained as independent prognostic variables associated with worse OS (see table 3).

Discussion

Since complete surgical resection is the only potentially curative treatment for gallbladder carcinoma many centers have adopted a more aggressive surgical approach, often combining liver and extrahepatic biliary resections. (10, 11) More effective adjuvant chemotherapeutic regimens need to be found in order to improve the overall prognosis of this cancer. Indeed, current gemcitabine-based chemotherapeutic regimens are impotent against this aggressive tumor. (12)

A plea for tumor hypoxia as a surrogate marker of more aggressive tumor behavior.

In the search for new therapeutic targets and prognostic markers the concept of tumor hypoxia has been extensively studied. Intratumoral hypoxia has been found to be a potent stimulator of metastasis and is considered an indicator of more aggressive tumor behavior. (4) Also, tumor hypoxia stimulates angiogenesis, which has been found to play a crucial role in the outgrowth and progression of malignant tumors. In this retrospective unicentric study weak immunohistochemical nuclear staining for HIF1α was found only in a portion (44.1%) of the tumor. We found the expression of HIF1α to be correlated with advanced tumor stages. HIF1α levels are known to be low under normoxic conditions. Intratumoral hypoxia, as well as genetic abnormalities stabilize HIF1α which binds to the hypoxia responsive element (HRE) in the promoter regions of HIF-downstream gene products such as VEGF, GLUT1 and CA9. A significant correlation between HIF1α and VEGF, GLUT1 and CA9 was not confirmed in our small series.

VEGF is ubiquitously present in gallbladder carcinoma.

Currently available monoclonal antibodies such as bevacuzimab (avastin®) efficiently inhibit the actions of VEGF. As VEGF was strongly expressed in all gallbladder carcinoma samples examined, anti-VEGF therapy could well be an important adjunct to current chemotherapeutic regimens. Interestingly, therapeutic strategies where anti-VEGFR and anti-VEGF therapy are combined are being introduced into clinical practice and seem likely to form a powerful tool against a set of highly resistant solid tumors. Indeed, the results of a NCI-sponsored phase I trial (NCT00350753) evaluating the combined targeted therapy with the tyrosine-kinase inhibitor sorafenib (i.e. a Raf kinase and VEGFR inhibitor) and bevacuzimab confirmed promising clinical activity. (13) Nevertheless, a long way to go remains before such therapies will be used in routine practice as issues occurred with sorafenib dosing, resulting in the need for dose reduction in 74% of included patients.

Expression of glucose transporters 1 and 2 was not associated with worse oncological outcome.

Glucose metabolism is increased in most cancers. Hypoxic adaptations promote aerobic glycolysis. Hence, members of the glucose transporter family and in particular GLUT1 may play an important role in tumor progression. The expression of GLUT1 can be induced in cancer cells by oncogenes, growth factors, interleukin-1, local hypoxia and inflammatory changes. Importantly, variable intratumoral expression of GLUT1 has been noted with an intense immunoreactivity in aggressive regions of the tumor, such as the poorly differentiated and central hypoxic areas. (14) In our study we did not specifically study this association. GLUT1 is thought to enhance the activity of the matrix metalloproteinases, whose activities have been directly related to tumor invasiveness and metastasis. (15) In contrast to our study, a larger study

found a strong association between GLUT1 expression and neoplastic progression in gallbladder carcinomas. In their study GLUT1 expression identified a subgroup of gallbladder carcinomas with worse survival. (16) However, this association might be reflected in the correlation we found between the extent of GLUT1 expression and higher tumor grades. GLUT3 on the other hand, is also expressed in human malignant tissue in response to hypoxia (17), but clinical studies have shown inconsistent results. (18, 19) We found that extended GLUT3 expression was correlated with the presence of lymph node metastases. It therefore seems useful to include GLUT3 in the panel of immunohistochemical markers that define gallbladder carcinomas with worse outcome.

Low CA9 expression inversely correlates with tumor differentiation.

CA9 is a member of the carbonic anhydrase family and is thought to regulate the extracellular pH in the tumor stroma and increase the intracellular pH. (20). Its membranous expression is induced by HIF in response to tumor hypoxia allowing enlarging tumors to adapt to an oxygen-poor microenvironment. Indeed, rapidly dividing tumor cells display increased glycolysis resulting in the accumulation of lactic acid, acidifying the tumor microenvironment. In our series, membranous expression of CA9 was found in 97% of gallbladder carcinomas. In multivariable Cox regression analysis for OS, CA9 was not an independent prognostic variable. The relationship between low CA9 expression and poor prognosis has been shown in studies with cervical carcinoma (21), colorectal carcinoma (22), esophageal cancer (23) and renal cell carcinoma (7). It is unclear whether CA9 expression is just a surrogate of tumor progression or directly influences tumor behavior. Decreased expression occurs in tumors with the highest malignant potential. Indeed, in advanced

tumors low CA9 expression is often noted. Similarly, in our study an inverse correlation was found between CA9 expression and tumor differentiation. As the tumor progresses continued CA9 expression is probably no longer a requirement to confer proliferation advantage. An alternative hypothesis suggests that cumulative genetic lesions involved in cancer progression could alter the pathways of hypoxia response leading to consistantly low CA9 expression (24).

High EGFR expression is an independent prognostic variable associated with worse overall survival.

In our study, high (>50% of tumor cells) EGFR expression identified a subgroup with worse cancer-related survival. This finding is in accordance with the common feature in a variety of human cancers that overexpression of EGFR leads to more aggressive tumor phenotypes and resistance to standard therapy. EGFR is a transmembrane receptor tyrosine kinase that promotes tumor cell proliferation, angiogenesis and inhibits apoptosis. The precise mechanisms that lead to EGFR overexpression are not fully elucidated. Besides genetic alterations (e.g. receptor-activating mutations), translational upregulation of EGFR provides a non-mutational explanation for its overexpression. (25) Indeed, recent evidence suggests that tumor hypoxia initiates an oncogenic program that is HIF2α-dependent and results in aberrant EGFR expression. EGFR inhibitors such as cetuximab (erbitux ®), erlotinib (tarceva ®), gefitinib, or lapatinib are currently being used in clinical practice for a multitude of malignant tumors. EGFR inhibitors can decrease VEGF expression and tumor angiogenesis by both HIF1-independent and -dependent mechanisms. (26) Cetuximab has already shown promising results in combination with other agents (e.g. irinotecan, oxaliplatin) in a selected group of metastatic colorectal cancer patients. (27, 28) Also, a non-

randomized open label phase II trial is currently recruiting patients with metastatic gallbladder cancer refractory or intolerant to standard systemic chemotherapy in order to evaluate the combined effect of anti-EGFR (erlotinib) and anti-VEGF (bevacuzimab) (NCT00350753).

We are well aware that the small sample size and retrospective nature of this study calls for caution when interpreting the results. Therefore, our conclusions need confirmation in a large multivariable analysis.

In conclusion, our study showed that the expression of EGFR, CA9, GLUT1, GLUT3, and HIF1α in resected gallbladder carcinoma correlates with survival, tumor grade, tumor stage or the presence of lymph node metastases. As VEGF expression was prominent in all gallbladder carcinomas, anti-VEGF targeting may be a good therapeutic strategy in the future. Use of this panel of immunohistochemical markers could be introduced into routine clinopathological practice to predict oncological outcome of gallbladder cancer and identify high-risk patients in need for targeted therapies.

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Statement of Interest

None of the authors have any actual or potential conflict of interest to disclose including any financial, personal or other relationships with other people or organizations within that could inappropriately influence (bias) this work.

Tables

Table 1: Operative procedures of included patients (N=34).

	N
Laparoscopic CCE	13
Open CCE	5
Open CCE, HJS, GES	1
Open CCE, EHBD	3
Open CCE, EHBD, segmentectomy 4B and 5	5
Open CCE, trisectionectomy	1
Open CCE, left hemihepatecomy	2
Open CCE, Whipple procedure	2
Open CCE, distal gastrectomy	2

CCE, cholecystecomy; HJS, hepaticojejunostomy; GES, gastroenterostomy; EHBD, extrahepatic bile duct resection

Table 2: Clinicopathological characteristics of the included patients (N=34).

Parameter		%
Age (range)	72 (44 – 91) years	
Gender (M:F)	14/20	41.2/58.8
ASA-score		
1	3	8.8
2	22	64.7
3	9	26.5
pT		
1	3	8.8
2	17	50.0
>2	14	41.2
Tumor grade		
1	5	14.7
2	12	35.3
3	17	50.0
pN		
0	6	17.6
1	16	47.1
x	12	35.3
pM		
0	9	26.5
1	22	64.7
x	3	8.8
Tumor stage		
I	8	23.6
II	3	8.8
III	1	2.9
IV	22	64.7
Perineural invasion		
absent	13	38.2
present	21	61.8
Lymphatic invasion		
absent	13	38.2
present	21	61.8
Vascular invasion		
absent	13	38.2
present	21	61.8
R-status		
negative	14	41.2
positive	20	58.8

ASA-score: American Society of Anesthesiologists; R-status: resection margin status.

Table 3: Multivariable Cox regression model

	p-value	HR (95% CI) per unit change
OS		
EGFR	0.04	2.50 (1.04 – 6.07)
TNM staging	0.003	1.78 (1.21 – 2.84)
Preoperative serum CA 19.9	0.008	1.03 (1.01 – 1.05) *

HR (95% CI), hazard ratio with 95% confidence interval; OS, overall survival; CSS, cancer-specific survival; * 100 unit HR (95% CI)

Figure legends

Figure 1:

1a. Strong and diffuse membranous CA9 expression. (original magnification 50x)

1b. EGFR showed a predominantly membranous expression pattern within the tumors. (original magnification 100x)

1c. Positive lymph nodes were more prevalent in case of extensive cytoplasmic GLUT3 expression (p=0.015). (original magnification 200x)

1d. A diffuse membranous staining pattern for GLUT1 correlated with higher tumor grades (p=0.04). (original magnification 100x)

1e. Nuclear HIF1α positivity was associated with higher pT scores (p=0.029). (original magnification 100x)

1f. Strong and diffuse cytoplasmic VEGF expression was seen in all tumors. (original magnification 100x)

Figure 2: Overall survival for tumors with low and high EGFR expression.

Estimated overall survival for EGFR low (15.8 months (IQR: 11.4 - 62.4) (red) and EGFR high (3.7 months (IQR: 2.4 – 14.0) (blue) groups.

The difference is statistically significant (p=0.03; Log-Rank)

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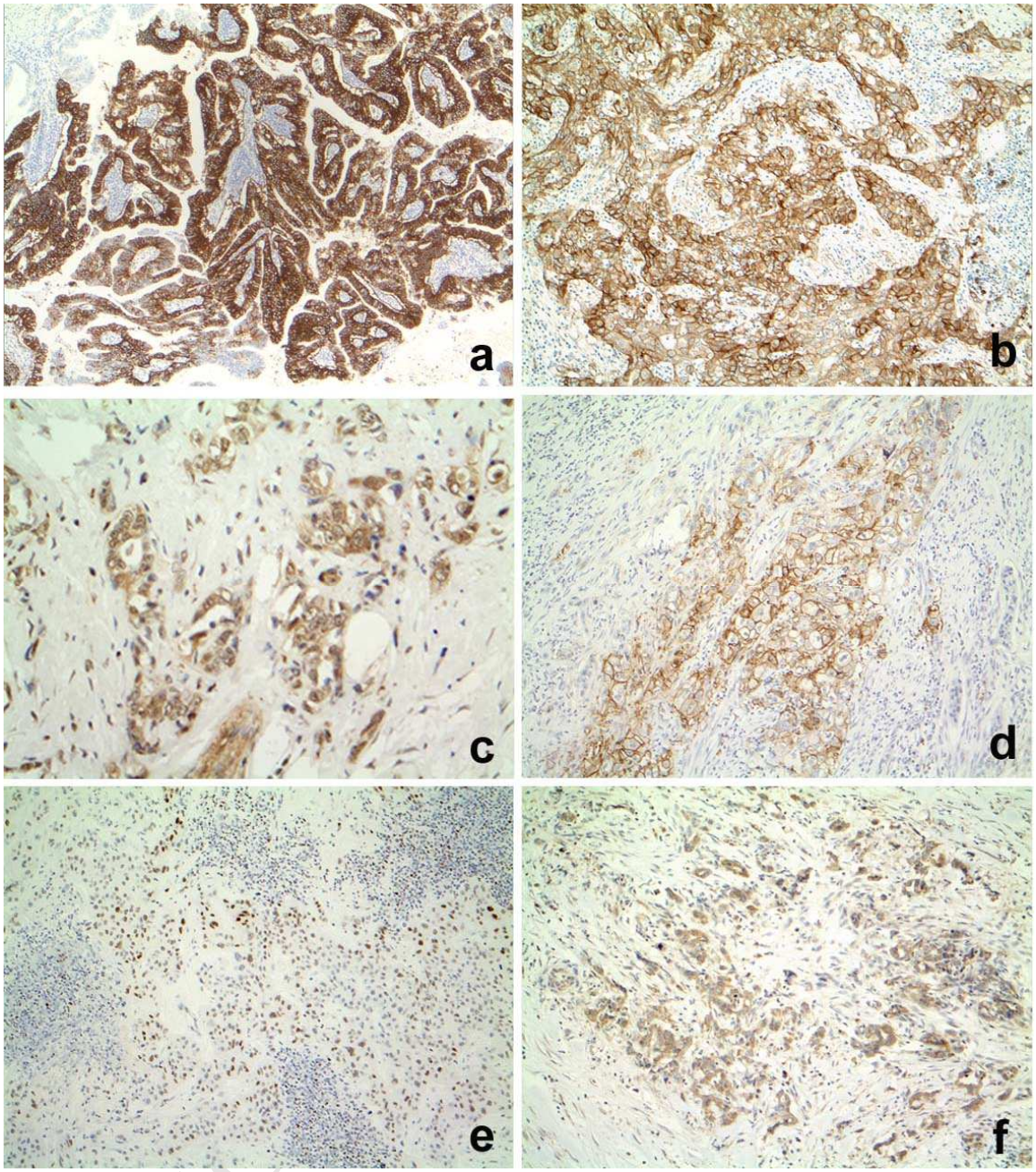

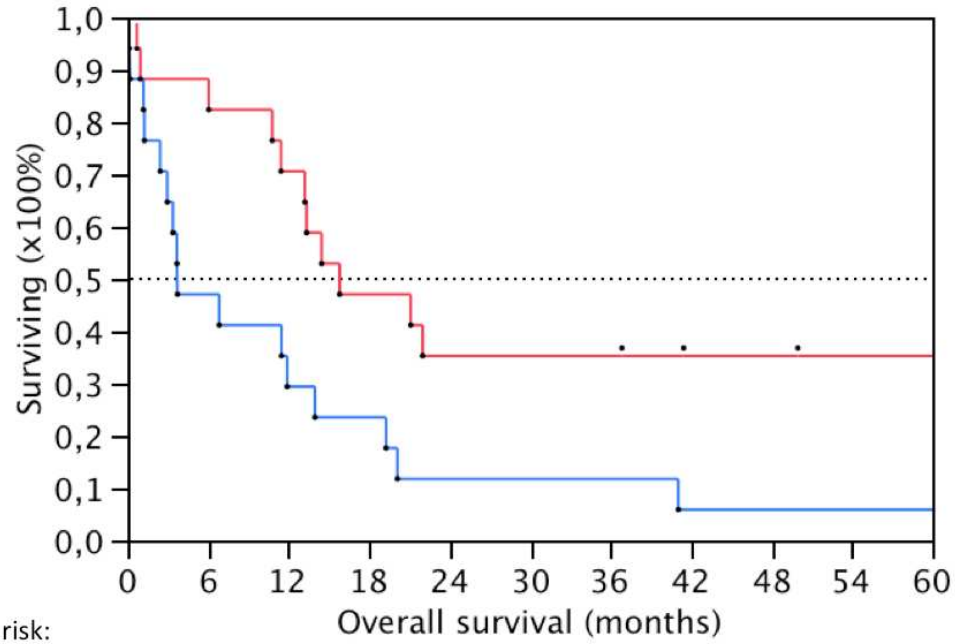

N at risk:						
Low EGFR	17	13	7	7	5	4
High EFGR	17	9	3	3	2	2