ORIGINAL ARTICLE

Expression of IGF-I, IGF-II, and IGF-IR in gallbladder carcinoma. A systematic analysis including primary and corresponding metastatic tumours

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Aims: The insulin-like growth factor (IGF) system has been implicated in tumour development and progression. This study was designed to analyse the expression of the IGF-I receptor (IGF-IR) and its ligands (IGF-I, IGF-II) in gallbladder cancer.

Methods: IGF-I, IGF-II, and IGF-IR immunoreactivity was investigated in 57 gallbladder carcinomas and corresponding lymph node (n = 11) and hepatic (n = 7) metastases using a tissue microarray technique and correlated with tumour stage, grade, and patient outcome.

Results: Cancer tissue allowing a reliable evaluation of IGF-I, IGF-II, and IGF-IR was present in 55 of 57 primary tumours and 17 of 18 metastases. IGF-I and IGF-II immunoreactivity was seen in 25 and 14 of the 55 primary tumours, in addition to six and three of the 17 metastases, respectively. No associations with tumour stage, grade, or prognosis were detected. IGF-IR was expressed in 52 of 55 primary tumours and all 17 metastases. IGF-IR staining intensity decreased with tumour cell dedifferentiation. Moreover, IGF-IR expression in less than 50% of cancer cells was an independent marker of poor prognosis in multivariate analysis (risk ratio, 4.0; 95% confidence interval, 1.4 to 11.2; p = 0.01).

Conclusions: The expression of IGF-IR and its ligands provides evidence for the existence of an auto/paracrine loop of tumour cell stimulation in gallbladder cancer and makes this type of cancer a candidate for therapeutic strategies aimed at interfering with the IGF pathway. The recognition of IGF-IR as a new independent prognostic biomarker may help to identify patients who might benefit from adjuvant treatment.

Carcinoma of the gallbladder is the most common malignant tumour of the biliary tract. Owing to its non-specific symptoms, gallbladder cancer is generally diagnosed late in the disease course.¹ Thus, prognosis is generally poor, with a 32% five year survival rate for lesions confined to the gallbladder mucosa and a 10% one year survival rate for more advanced stages.²

"The regulation of insulin-like growth factor receptor expression is closely associated with the function of several tumour suppressor genes and oncogenes"

The insulin-like growth factor (IGF) system plays an important role in growth and development and has recently been implicated in tumour development and progression.^{3–5} Members of the IGF system are the IGF ligands (IGF-I and IGF-II); cell surface receptors, including the IGF-I receptor (IGF-IR) and the IGF-II receptor (IGF-IIR); and six different types of binding proteins.^{3–5} Both IGF-I and IGF-II interact with IGF-IR, a transmembrane tyrosine kinase that is structurally and functionally related to the insulin receptor. IGF-II also binds to IGF-IIR, which functions as a clearance receptor by endocytosis and intracellular degradation of its ligand.³

The binding of IGF-I and IGF-II to the extracellular subunit of IGF-IR activates the tyrosine kinase activity of IGF-IR and in turn the mitogen activated protein kinase and phosphoinositol-3-kinase cascades, thus mediating mitogenic, antiapoptotic, and differentiation effects.³ In addition, IGF-IR signalling has been implicated in carcinogenesis and tumour progression by modulating cancer cell motility and adhesion,⁶ in addition to angiogenesis.⁷ Mutations of IGF-IR

or chromosomal amplifications are rare.⁵ However, the regulation of IGF-IR expression is closely associated with the function of several tumour suppressor genes and oncogenes. Thus, expression of wild-type p53 inhibits IGF-IR expression, whereas mutant p53 has been shown to upregulate IGF-IR expression.⁸

Overexpression of IGF-IR has been noted in a wide variety of human carcinomas of glandular or transitional cell origin.⁹ Recently, we and other groups demonstrated the simultaneous expression of both ligands (IGF-I and/or IGF-II) and receptor (IGF-IR) within the same tumour, thus providing evidence for an auto/paracrine loop of cancer cell stimulation in colorectal,^{10–11} prostate,¹² and renal¹³ cancer.

Data regarding the involvement of the IGF system in gallbladder cancer are currently lacking. Therefore, we performed a systematic immunohistochemical analysis of a large series of gallbladder carcinomas including synchronous and metachronous metastatic tissues for the expression of IGF-I, IGF-II, and IGF-IR with respect to associations with tumour stage, grade, histological subtype, and prognosis.

MATERIAL AND METHODS

Case selection

Formalin fixed, paraffin wax embedded specimens of 57 primary gallbladder carcinomas from consecutive patients (39 women, 18 men) undergoing surgery between April 1984 and December 2002 were chosen for analysis. The mean and median ages of the patients were 71 and 72 years (range, 35–91), respectively. Resection categories of primary tumours

Abbreviations: CI, confidence interval; IGF-I/II, insulin-like growth factor type I/II; IGF-IR, insulin-like growth factor type I receptor; RR, relative risk; TMA, tissue microarray

Table 1 Histological subtypes of primary gallbladder carcinoma		
Histological subtype	N	%
Tubular adenocarcinoma	43	75
Papillary adenocarcinoma	5	9
Mucinous adenocarcinoma	2	4
Clear cell adenocarcinoma	2	4
Signet ring cell adenocarcinoma	1	2
Adenosquamous carcinoma	4	7
Total	57	100

were R0 in 28, R1 in 16, and R2 in 13 cases. Eleven corresponding synchronous lymph node metastases, in addition to four synchronous and three metachronous corresponding hepatic metastases were included in the investigation. pT categories were adjusted according to the recent update of the TNM system:¹⁴ Stage pT1a was present in one, pT1b in four, pT2 in 23, pT3 in 27, and pT4 in two cases. Tumour grades and histological subtypes were evaluated according to the World Health Organisation guidelines.¹⁵ Neither ethical committee approval nor informed consent is required for retrospective studies dealing with archival material at our institution.

Immunohistochemistry

A tissue microarray (TMA) technique was used for immunohistochemical analysis. TMAs were prepared using a manual tissue arraying instrument (Beecher, Silver Spring, Maryland, USA), as has been described previously.¹⁶ To account for cancer tissue heterogeneity, between three and five cylindrical core biopsies, 0.6 mm in diameter, were taken from different sites in each tumour that had been selected on the original tumour slides to include all patterns of differentiation. TMA sections (4 µm thick) were mounted on Superfrost^{TM} slides for immunohistochemical analysis using an automated immunostainer (Dako-Autostainer, Universal Staining System; Dako, Glostrup, Denmark). Briefly, TMA sections were dewaxed, rehydrated in graded

alcohols, and treated for five minutes with 1% H₂O₂. To detect IGF-I and IGF-II, sections were then treated for antigen retrieval (1% trypsin for 20 minutes at room temperature) and subsequently incubated with the following monoclonal antibodies: anti-IGF-I (clone I-5C9; Linaris, Wertheim, Germany; 1/500 dilution) and anti-IGF-II (clone W2-H1; Linaris; 1/500 dilution). Binding of the primary antibodies was assessed by the Dako EnVision+^{TM} System detection kit. For the detection of IGF-IR, sections were submitted to microwave antigen retrieval (30 minutes at 160 W, EDTA, pH 8.0) and were subsequently incubated for 30 minutes with a mouse monoclonal antibody directed against the α subunit of IGF-IR (clone 24–31; NeoMarkers, Fremont, California, USA; 1/50 dilution). Binding of the IGF-IR primary antibody was assessed by the Dako ChemMate^{TM} detection kit.

Immunohistochemical evaluation and controls

Distinct membranous and/or cytoplasmic staining was considered positive, and immunoreactivity was documented in categories as follows, assessing the average positivity of the core biopsies: focal (+; < 10% of tumour cells positive), moderate (++; 10–50%), or extensive (+++; > 50%). In addition, the intensity of IGF-IR immunostaining was arbitrarily categorised as either weak (1+), moderate (2+), or strong (3+). Sections of a colon carcinoma known to be

Table 2 IGF-I, IGF-II, and IGF-IR immunoreactivity in primary gallbladder carcinomas (n = 55)						
Degree of staining	IGF-I		IGF-II		IGF-IR	
	N	%	N	%	N	%
Negative	30	55	41	75	3	5
<10% positive	15	27	4	7	1	2
10–50% positive	7	13	6	11	1	2
>50% positive	3	5	4	7	50	91
Overall positive	25	45	14	25	52	95

IGF-I/II, insulin-like growth factor type I/II; IGF-IR, insulin-like growth factor type I receptor.

Figure 1 Well differentiated tubular adenocarcinoma of the gallbladder showing distinct granular cytoplasmic (A) IGF-I and (B) IGF-II immunostaining; original magnification, ×100. IGF-IR expression in gallbladder cancer: (C) strong and predominantly membranous immunostaining in well differentiated adenocarcinoma, but (D) only weak staining of tumour cells in poorly differentiated adenocarcinoma; original magnification, ×150. IGF-I/II, insulin-like growth factor type I/II; IGF-IR, insulin-like growth factor type I receptor.

positive for IGF-I, IGF-II, and IGF-IR served as positive controls. Negative controls included omission of the primary antibody and incubation with Dako ChemMate antibody diluent.

Statistical analysis

Subgroups according to pT stage and tumour grade were compared with respect to possible differences in immunoreactivity using Fisher's exact test. Patient outcome was assessed using the Kaplan–Meier method and compared by means of the log rank test. A Cox's proportional hazards regression model was used for multivariate testing.

RESULTS

Histopathology

Forty three of 57 cases were tubular adenocarcinomas, whereas the remaining tumours displayed either papillary, mucinous, clear cell, signet ring cell, or adenosquamous differentiation (table 1). The series included 16 well differentiated (G1) carcinomas, 19 moderately differentiated (G2) carcinomas, 21 poorly differentiated (G3) carcinomas, and one undifferentiated (G4) carcinoma. Lymphatic vessel and/or venous invasion was seen in 41 and 16 cases, respectively.

Immunohistochemistry

Cancer tissue allowing a reliable evaluation of IGF-I, IGF-II, and IGF-IR immunoreactivity was present in 55 of the 57 primary tumours and 17 of the 18 metastases.

Distinct granular cytoplasmic IGF-I and IGF-II immunoreactivity of cancer cells was seen in 25 and 14 of the 55 primary tumours, respectively, with only a few cases showing extensive immunostaining (fig 1A, B; tables 2 and 3). IGF-I and IGF-II immunoreactivity was seen in six and three of the 17 metastases, respectively. No associations with tumour stage, tumour grade, histological subtype, or presence of vascular invasion were found for either IGF-I or IGF-II. However, both proteins were related to one another, because 12 of 25 IGF-I positive tumours expressed IGF-II compared with only two of 30 IGF-I negative tumours ($p < 0.001$).

With regard to IGF-IR, predominantly membranous staining of tumour cells was noted in 52 of 55 primary and all 17 metastatic carcinoma tissues. Immunoreactivity of more than 90% of cancer cells occurred in 38 of 55 primary tumours. With respect to intensity of immunoreactivity, weak staining was found in three, moderate staining in 16, and strong staining in 33 of the 55 primary cancer cases (fig 1C, D). With regard to overall IGF-IR immunoreactivity, no associations with IGF-I or IGF-II expression, tumour stage, tumour grade, histological subtype, or presence of vascular invasion were

Figure 2 Disease free survival (weeks) in patients with gallbladder cancer related to insulin-like growth factor type I receptor (IGF-IR) expression. There was a favourable outcome of patients with tumours showing extensive IGF-IR immunoreactivity ($> 50\%$ of tumour cells) compared with those with tumours showing only focal or moderate IGF-IR staining ($\leq 50\%$ of tumour cells; $p = 0.001$, log rank test).

found. However, IGF-IR staining intensity was pronounced in low grade tumours: 12 of 14 well differentiated (G1) carcinomas showed strong staining compared with 21 of 51 moderately and/or poorly differentiated (G–G4) tumours ($p = 0.005$).

Survival analysis

Follow up data were available for all patients. The overall median survival time was 45 weeks (range, 0.4–604), with a one year survival rate of 34% and a five year survival rate of 6%. There were four procedure related deaths (within 28 days). Forty four patients died (range, 10–175 weeks; median, 31) owing to tumour progression, eight died (range, 12–604 weeks; median, 44) from causes unrelated to gallbladder cancer without evidence of residual tumour, and one is alive with metastatic disease. Patients with complete tumour resection (R0) had a longer overall median survival (78 weeks; range, 14–604) compared with those with either R1 (21 weeks; range, 5–90) or R2 resections (18 weeks; range, 0.4–42) ($p < 0.001$; log rank test).

In patients with complete tumour resection (R0 status) survival was influenced by tumour grade: only 12 of 19 patients with low grade (G1–2) tumours died as a result of tumour progression, compared with eight of nine patients with high grade (G3–4) tumours ($p = 0.004$; log rank test). However, outcome was independent of tumour stage

Table 3 IGF-I, IGF-II, and IGF-IR immunoreactivity in primary gallbladder carcinomas ($n = 55$) related to tumour stage and tumour grade

Tumour characteristic	IGF-I positive		IGF-II positive		IGF-IR positive	
	N	%	N	%	N	%
Stage						
pT1a ($n = 1$)	1	100	1	100	1	100
pT1b ($n = 4$)	2	50	0	0	4	100
pT2 ($n = 21$)	6	29	5	24	19	90
pT3 ($n = 27$)	14	52	6	22	26	96
pT4 ($n = 2$)	2	100	2	100	2	100
Grade						
G1 ($n = 14$)	5	36	3	21	14	100
G2 ($n = 19$)	10	53	7	37	19	100
G3 ($n = 21$)	10	48	4	19	19	90
G4 ($n = 1$)	0	0	0	0	0	0

None of the differences was significant.

IGF-I/II, insulin-like growth factor type I/II; IGF-IR, insulin-like growth factor type I receptor.

($p = 0.13$; log rank test). With regard to the investigated biomarkers, patients with tumours showing extensive IGF-IR immunoreactivity had a longer median survival (78 weeks; range 6–604) compared with those who had tumours showing no or only focal/moderate IGF-IR immunoreactivity (50 weeks; range, 29–66). Fourteen of 22 patients with tumours showing extensive IGF-IR immunoreactivity died as a result of tumour progression, compared with all six patients showing no or only focal/moderate IGF-IR immunoreactivity ($p = 0.001$; log rank test; fig 2). In contrast, expression of both receptor ligands, IGF-I and IGF-II, had no influence on patient outcome. Multivariate analysis showed incomplete tumour resection (R1 and R2) versus complete tumour resection (risk ratio (RR), 28.0; 95% confidence interval (CI), 9.1 to 86.2; $p < 0.001$), IGF-IR immunoreactivity of less than 50% of cancer cells versus immunostaining of more than 50% of cancer cells (RR, 4.0; 95% CI, 1.4 to 11.2; $p = 0.01$), and tumour grades 3–4 versus 1–2 (RR, 2.3; 95% CI, 1.1 to 4.7; $p = 0.02$) as independent prognostic factors with regard to tumour specific survival.

DISCUSSION

The expression of IGF-IR has been documented in human carcinomas of different origin.⁹ Moreover, the simultaneous expression of both IGF-IR and its ligands (IGF-I and/or IGF-II) within the same tumour has provided evidence for an auto/paracrine loop of cancer cell stimulation.^{10–13}

Our investigation is the first to analyse the expression of the IGF system in gallbladder cancer. According to our results, IGF-IR is expressed in almost all primary tumours and in corresponding metastases, whereas IGF-I and IGF-II are found only in a few cases. In agreement with recent data obtained from colorectal cancer,¹⁷ the IGF system seems to be involved at an early stage during carcinogenesis, because distinct immunostaining was already present in low stage and low grade tumours and the IGF-IR staining intensity decreased significantly with tumour cell dedifferentiation. The lack of association between overall IGF-I, IGF-II, and IGF-IR expression and both tumour stage and grade may be explained by the small sample size.

“The insulin-like growth factor (IGF) system seems to be involved at an early stage during carcinogenesis, because distinct immunostaining was already present in low stage and low grade tumours and the IGF receptor staining intensity decreased significantly with tumour cell dedifferentiation”

The main prognostic factors for gallbladder cancer are resection status and TNM stage,^{18–19} and there is a correlation between the level of tumour invasion and the presence of lymph node metastases.² Because the prognosis of gallbladder cancer is generally poor and it is difficult to cure by surgery alone, identification of new prognostic biomarkers would help identify patients who might benefit from additional treatment. According to our data, IGF-IR immunoreactivity might be useful as a new independent prognostic marker, because tumours with staining of less than 50% of cancer cells showed a significantly worse outcome in multivariate analysis. Similar results have been demonstrated for colorectal²⁰ and breast²¹ cancer, whereas in endometrial,²² oesophageal,²³ and a subset of renal clear cell cancers²⁴ high IGF-IR expression was associated with an unfavourable prognosis. High IGF-IR immunoreactivity may simply identify a relatively well differentiated tumour that requires IGF-IR for proliferation.²⁵ In addition, tumour microenvironmental stress has recently been shown to induce IGF-IR expression.²⁶ Cancer cells with low IGF-IR values may thus

Take home messages

- This is the first study of the insulin-like growth factor (IGF) signalling pathway in gallbladder cancer
- The expression of the IGF type I receptor (IGF-IR) and its ligands suggests that an auto/paracrine loop of tumour cell stimulation exists in gallbladder cancer
- IGF-IR expression in less than 50% of cancer cells was an independent marker of poor prognosis in multivariate analysis so that therapeutic strategies aimed at interfering with the IGF pathway might be useful in this disease
- The recognition of IGF-IR as a new independent prognostic biomarker may help to identify patients who might benefit from adjuvant treatment

represent a subpopulation that is able to adapt to this stress and develop a more metastatic phenotype.²⁰

Most patients with gallbladder cancer present at an advanced stage. However, the role of adjuvant chemotherapy and radiotherapy has not been fully defined.²⁷ Moreover, cancers overexpressing IGF-IR have been shown to be more resistant to drug and radiation induced apoptosis.⁹ The concept of receptor targeting, however, is now well established in anticancer treatment and, according to recent experimental data, the growth of many established cancers can be inhibited by pharmacological strategies aimed at reducing IGF-IR signalling.^{4–5, 28–31} These observations will lead to clinical trials with new drug candidates, such as anti-IGF-IR antibodies, IGF-IR tyrosine kinase inhibitors, and IGF-IR gene silencing by antisense RNA interference in the future and might help to improve the poor prognosis of patients with gallbladder cancer.

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