The IGF pathway is activated in insulinomas but downregulated in metastatic disease

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Abstract

Clinical and molecular studies have implicated epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF) and target of rapamycin (mTOR) signaling pathways in the regulation of pancreatic neuroendocrine tumor (PanNET) growth. Interpretation and comparison of these studies is complex due to clinical and molecular tumor heterogeneity. We therefore focused in this study on insulinomas, which we examined for mRNA and protein expression of EGFR, IGF and mTOR signaling pathway components by quantitative real-time PCR (n=48) and immunohistochemistry (n=86). Findings were compared with normal pancreatic islets and correlated with histopathological data and clinical outcome. Insulinomas showed low EGFR and high IGF2 expression. IGFBP2, IGFBP3 and IGFBP6 mRNA levels were 2-4 folds higher than in islets. High protein expression of IGF2, IGF1R and INSR (in 51-92% of the tumors) and low to moderate expression of mTORC1 pathway proteins p-S6k and p-4EBP1 (7-28% of the tumors) were observed. Correlations were found between 1) ERK1 mRNA expression and that of numerous IGF pathway genes, 2) p-ERK and IGF1R protein expression and 3) decrease of IGF pathway components and both metastatic disease and shorter 10 years disease free survival. In conclusion, our observations suggest that high expression of IGF signaling pathway components is a hallmark of insulinomas, but does not necessarily lead to increased mTOR signaling. Reduced expression of IGF pathway components may be an adverse prognostic factor in insulinomas.

Introduction

Over the past years advances have been made in understanding the biology and clinical behavior of gastroenteropancreatic neuroendocrine tumors (GEP-NETs), a heterogeneous group of tumors arising from the diffuse neuroendocrine system (Oberg 2012, Muniraj et al. 2013). The estimated annual incidence of NETs in the USA increased 6.4 fold between
1973 and 2012, with an incidence rate of 3.56/100,000 in gastroenteropancreatic sites (Dasari et al. 2017). GEP-NETs can be divided, based on clinical manifestations, into functioning (syndrome-related) and non-functioning tumors. Today, PanNETs are treated by surgery, biotherapy, chemotherapy and/or molecular targeted therapy using multidisciplinary therapeutic management. Since 2011 the FDA approved the mammalian target of rapamycin (mTOR) inhibitor Everolimus for the treatment of progressive PanNETs and well-differentiated, non-functional GEP-NETs and lung-NETs (RADIANT-3 and -4 studies) (Yao et al. 2011, Yao et al. 2016). mTOR regulates cell survival, proliferation and motility, and also senses cell energy status (Gentzler et al. 2012).

Because of the heterogeneity of PanNETs, analysis of the underlying molecular biology is essential for successful targeted treatment. The basis of positive treatment results for Everolimus is derived from a number of studies implicating mTOR pathway alterations in the proliferation of PanNETs. (Jiao et al. 2011) found mutations in the mTOR pathway genes PTEN and TSC2 in 14% of (non-functioning) PanNETs. Moreover, (Missiaglia et al. 2010) reported downregulation of PTEN and TSC2, inhibitors of the mTOR pathway, in up to 70% of non-functioning and functioning PanNETs, including insulinomas. Whole genome sequencing of insulinomas revealed mutations in the gene YY1, a target of mTOR, in 30% of the tumors (Cao et al. 2013). Finally, several immunohistochemical studies showed positivity for mTOR pathway proteins p-mTOR (range 60-70%), p-S6K (40-80%), and p-4EBP1 (30-90%) in GEP-NETs (Kasajima et al. 2011, Zhan et al. 2012, Qian et al. 2013). However, interpretation and comparison of these studies is complex due to the fact that GEP-NETS/PanNETs comprise heterogeneous tumor sub-types with different clinical and molecular characteristics, and studies have used different diagnostic tools and evaluation criteria to detect alterations in the mTOR pathway.

The mTOR pathway can be activated by various upstream stimuli, including epidermal growth factor receptor (EGFR) and the insulin-like growth factor (IGF) signaling system, which play a pivotal role in cancer development and progression. EGFR activation promotes cell proliferation via MAPK and PI3K signaling routes, and indications for a role of EGFR signaling in PanNETs have been reported. Immunohistochemical analysis of
EGFR expression showed positivity in 30-65% of mixed populations of PanNETs (Srivastava et al. 2001, Papouchado et al. 2005, Bergmann et al. 2009). The IGF signaling system includes the ligands IGF1 and IGF2, the receptors IGF1R, IGF2R and insulin receptor (INSR), and 6 IGF binding proteins (IGFBP1 to 6) (Lodhia et al. 2015).

Deregulation of the IGF signaling system, for example by upregulation of IGF1R, IGF2 and IGFBP2, has been reported in several malignancies, including GEP-NETs/PanNETs and insulinomas (Wulbrand et al. 2000, Dejeux et al. 2009, Ludovini et al. 2009, Livingstone 2013).

In this study we evaluated mRNA and protein expression patterns of EGFR and IGF signaling pathway components that may regulate the mTOR pathway, as well as the mTORC1 pathway downstream effectors p70S6 Kinase (S6K) and 4E-BP1 (4EBP1) in a large series of insulinomas. Quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC) data were correlated with each other, with histopathology and with clinical patient and follow-up data.
**Materials and methods**

A detailed description of materials and methods can be found in Supplementary Materials and Methods.

**Patient samples**

Detailed data on insulinoma patient's age, sex, disease stage and tumor grade and size are provided as Supplementary Table 1 (Jonkers et al. 2007, Marinoni et al. 2014). All insulinoma patients had hyperinsulinism followed by a hypoglycaemia syndrome. The initial treatment consisted of surgical removal of the primary tumor, and if present liver and/or lymph node metastases. Follow-up treatment for patients with metastatic disease included surgery, Transarterial Embolization or Transarterial Chemoembolization. The tumors were all sporadic, not associated with MEN1 syndrome and classified according to the World Health Organization 2010 staging and grading system.

From 48 insulinoma patients snap frozen tumor tissue was available for RNA analysis, and from 26 patients also formalin-fixed, paraffin-embedded material. Two paraffin-embedded tissue micro arrays (TMAs) were available for immunohistochemical analysis, containing 49 insulinomas (TMA1) and a second TMA with 11 additional insulinomas (TMA2). TMA1 furthermore contained 92 additional PanNETs (12 gastrinomas, 11 glucagonomas, 10 vipomas and 59 non-functioning PanNETs), of which data can be found in Supplementary Table 6. Patient material was used according to the Code for Proper Secondary Use of Human Tissue in The Netherlands (https://www.federa.org/, update 2011) and according to the cantonal ethics committee of Bern (KEK-BE 105-2015).

**RNA isolation**

Total RNA was isolated from snap frozen insulinomas using the Qiagen RNeasy Mini Kit, and had a RIN value ≥ 6.5.
Control MPV™ Total RNA from normal human pancreas, liver, lung and adrenal gland (Stratagene) and total RNA from normal, single donor human pancreatic islets (a gift of Dr E. de Koning, Leiden University Medical Center, The Netherlands) were included as controls.

Quantitative Real-Time PCR

Total RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). qRT-PCR reactions were performed using two commercially available SYBR green mixes, iQ™ SYBR® Green Supermix (BioRad) and SensiMix™ SYBR & Fluorescein Kit (BioLine). All primers (Supplementary Table 2) were purchased from Biolegio.

Immunohistochemistry

Immunohistochemical staining on freshly cut 4 µm-thick formalin-fixed, paraffin embedded tissue sections was performed using primary antibodies against EGFR, IGF2, IGF1R, INSR, p-AKT, p-ERK, p-S6K and 4-EBP1. Detailed information on antibodies and staining conditions can be found in Supplementary table 3. Immunohistochemical staining was scored as: 0, absent; 1, weakly positive in ≥10% of cells; 2, moderately positive in ≥10% of cells, 3, strongly positive in ≥10% of cells.

Statistical analysis

Statistical analysis was performed using SPSS version 20 (IBM). Mean relative gene expression levels between groups were compared with the F-test and Student t-test. Associations between relative gene expression levels and immunostaining levels were determined using Pearson’s correlation. All P-values were considered statistically significant if ≤ 0.05 in two-sided tests. Survival curves were created using the Kaplan–Meier method, the log-rank test was used to test for differences between subgroups. Details on assessment of disease free or overall survival rates can be found in the Supplementary Materials and Methods. Cox-regression was used for multivariate analysis.
Results

mRNA expression in insulinomas

Neuroendocrine markers

From 48 insulinomas mRNA was analyzed. To check for the endocrine nature of the
tumors the relative mRNA expression levels of insulin (INS), chromogranin (CGA) and
synaptophysin (SYP) were analyzed by quantitative RT-PCR (qRT-PCR). In addition,
mRNA from normal human tissues (whole pancreas, pancreatic islets, liver, lung and
adrenal gland) was analyzed as controls. Expression levels were normalized to
glucuronidase beta (GUSB), which exhibited the most stable expression level in all
samples after comparing the expression levels of 4 housekeeping genes.
Table 1 shows high mean and median expression levels of INS, CGA and SYP mRNA
(8351, 89 and 2.3 normalized to GUSB, respectively), consistent with the neuroendocrine
character of insulinomas. In normal pancreatic islets the mRNA expression levels of INS,
CGA and SYP were respectively 4151, 15 and 0.5. In normal pancreatic tissue the mRNA
levels for these genes were significantly lower. Normal adrenal mRNA showed a high
expression level of CGA (57) and SYP (1.3), but very low INS expression (0.2). In normal
liver and lung tissue very low mRNA expression levels of CGA, SYP and INS were found
(data not shown; analyses of normal controls were performed three times in duplicate).
When compared to normal pancreatic islets the mean expression levels of INS, CGA and
SYP mRNA in insulinomas showed a 2.0, 6.0 and 4.8 fold increase, respectively (Table 1).

EGFR, ERK and AKT

Since EGFR signaling via MAPK and AKT pathways has been reported to be active in
PanNETs, we examined the mRNA expression levels of EGFR, ERK1, ERK2 and AKT in the
48 insulinomas. EGFR mRNA expression was low (0.10), with a relative expression of
0.15 as compared to normal pancreatic islets (Table 1). ERK1, ERK2 and AKT mRNA
expression levels were 1.1, 1.6 and 2.6, respectively. ERK1 expression was at the same level as in normal pancreatic islets, while the relative expression levels of ERK2 and AKT showed a 0.3 and 0.2 fold decrease.

**IGF pathway**

Table 1 shows the mRNA expression levels of IGF pathway-related genes in insulinomas. In contrast to very low IGF1 levels, the mean mRNA expression level of IGF2 is 1.5, which is a 12.4 fold increase compared to normal pancreatic islets (median: 3.5 fold increase). IGFBP2 has an expression level of 4.8, which is 4.0 fold higher than in normal pancreatic islets. Despite the fact that IGFBP3 and IGFBP6 showed expression levels of 0.21 and 0.23 (normalized to GUSB), their relative expression was 2-3 folds higher than in normal pancreatic islets. The other IGF pathway genes showed low expression levels (0.01-0.2).

**mTOR pathway**

The mean mRNA expression levels of MTOR and RPS6KB1 (coding for S6K protein) (were low (0.01-0.02) in insulinomas, whereas EIF4EBP1 (coding for 4EBP1 protein) is expressed at the level of GUSB (Table 1). In normal pancreatic islets all 3 genes showed a 0.4-0.6 fold decreased mRNA expression level.

In conclusion, insulinomas show low expression levels of EGFR mRNA, high expression of IGF2, a 2-4 fold increased expression of IGFBP2, IGFBP3 and IGFBP6, and a twofold reduced expression of MTOR, RPS6KB1 and EIF4EBP1, as compared to pancreatic islets.

**Correlations between mRNA expression patterns**

Pearson correlation analysis between mRNA expression levels of different signaling pathway genes is shown in Supplementary Table 4. Correlations between substantially expressed genes include those 1) between ERK 1, ERK2 and AKT and 2) between ERK1 and IGF pathway genes IGFBP2 (inverse correlation), IGFBP6, and all receptors (detailed
Protein expression in insulinomas

EGFR, ERK and AKT

Immunostaining was performed on 86 insulinomas, including 26 that were also analyzed for mRNA expression levels (see above), and 60 cases present as single or duplicate cores on the TMAs. All tumors were negative for EGFR, which is in agreement with the low assessed EGFR mRNA expression levels. As a positive control a human premalignant laryngeal lesion was used, showing strongly positive membranous staining. In normal human pancreas a low number of acinar cells showed a weakly positive membranous staining, whereas the islet cells were negative for EGFR (Table 2; Fig. 1, A-C).

Table 2 shows the IHC data for p-AKT and p-ERK. A lung carcinoma harboring a K-ras exon 2 mutation served as positive controls (Supplementary Figure 1, A and B respectively). A moderate to strong nuclear p-AKT expression was seen in normal pancreatic islet cells, whereas the acinar cells were negative. In normal pancreas no p-ERK could be detected. Moderately to strongly positive nuclear p-AKT staining was observed in 22% and nuclear p-ERK in 32% of insulinomas (Fig. 1, D-F). Twenty-four % of the insulinomas showed neither nuclear nor cytoplasmic p-ERK staining; 36% were negative for p-AKT. Moderate to strong simultaneous expression of nuclear p-AKT and p-ERK was found in 10% of the tumors, while in 22% no co-expression of nuclear p-AKT and p-ERK could be detected (double-negative).

IGF pathway proteins

IHC results for IGF pathway proteins IGF2 and receptors IGF1R and INSR are shown in Table 2 and Fig. 1, G-O. In 92% of the insulinomas a strong cytoplasmic, diffusely granular pattern of IGF2 was observed. A similar pattern of lower intensity was seen in normal pancreatic islet cells, whereas acinar cells showed a strong, aggregated...
extracellular IGF2 localization, which could be a sign of internalization of IGF1R and/or INSR after ligand binding (Rajapaksha & Forbes 2015).

Tumors indeed exhibited high, diffuse cytoplasmic expression levels of IGF1R and INSR (78% and 83%, respectively), while in 51% of the cases also a membranous IGF1R staining and in 38% a perinuclear localization of INSR was observed. These patterns could also be recognized in the normal pancreas, i.e. the islet cells showed a granular cytoplasmic IGF1R and INSR expression pattern, whereas the acinar compartment showed a membranous IGF1R localization, and a cytoplasmic INSR expression with perinuclear localization. These data indicate that the IGF pathway is active in insulinomas.

\*mTORC1 pathway proteins p-S6K and p-4EBP1*

Table 2 and Figure 2 (A-F) show the IHC results for p-S6K and p-4EBP1. Normal human colon tissue served as positive control (Supplementary Figure 1, C and D).

In 28% of insulinomas a weakly to moderately positive cytoplasmic p-S6K staining pattern was seen. Also 28% of tumors showed nuclear staining. Normal pancreatic islets showed the same staining pattern but with lower intensity. In contrast, in approximately 75% of normal pancreatic acinar cells a moderately to strongly positive perinuclear and/or diffuse nuclear p-S6K staining was detected. Interestingly, in tumor-adjacent pancreatic tissue we observed a stronger p-S6K staining in acinar and islet cells than in normal control pancreatic tissue (data not shown).

Only 37% of insulinomas exhibited a weakly to moderately positive nuclear p-4EBP1 staining, and in 22% also cytoplasmic staining was detected (Table 2). Normal pancreatic islets did not show p-4EBP1 expression, and areas, predominantly at the periphery of lobules, showed a moderately to strongly positive diffuse cytoplasmic, and in 90% also strong nuclear, immunostaining in acinar cells (Fig. 2, D-E). Strikingly, exocrine pancreatic tissue adjacent to the tumor often displayed a stronger nuclear and cytoplasmic immunostaining.
In conclusion, insulinomas show high expression levels of IGF2, IGF1R and INSR, no EGFR expression and low levels of phosphorylated mTORC1 pathway proteins.

Positive correlations between protein expression patterns

Pearson correlation analysis of protein expression levels shows a correlation of IGF2 with cytoplasmic IGF1R (p=0.019). The latter also correlates with nuclear p-ERK (p=0.003), which in turn correlates with nuclear p-AKT (p=0.011) and nuclear p-S6K (p=0.002). In addition cytoplasmic INSR correlates with nuclear p-S6K (p=0.024) (Supplementary table 5).

Correlation of mRNA and protein expression with clinicopathologic parameters in insulinomas

Mean relative gene expression levels of insulinoma subgroups were compared using Student's t-test (Table 3A). Of the 20 genes analyzed, only the relative expression level of IGF2R compared to normal pancreatic islets was significantly lower in grade 2/3 than in grade 1 insulinomas (p=0.039). A decrease in expression in grade 2/3 tumors was also seen for IGF1R, INS and IGF2, although not statistically significant. At the protein level (Table 3B) a significantly lower membranous INSR and cytoplasmic p-4EBP1 staining intensity was found in grade 2/3 as compared to grade 1 insulinomas (p=0.004 and p=0.001, respectively).

The mean mRNA expression level of INS relative to normal pancreatic islets was significantly lower in metastatic insulinomas than in non-metastatic (p<0.0001). In contrast, the relative expression of IGFBP3 was higher in metastatic tumors, although the difference was not statistically significant. The protein expression levels of IGF2, cytoplasmic IGF1R and INSR, membranous INSR and cytoplasmic p-S6K were significantly lower in metastatic tumors than in non-metastatic tumors (p=0.001, p=0.026, p=0.035, p=0.004 and p=0.030, respectively).
Comparison of the mean mRNA expression levels between tumors < and ≥ 2 cm revealed INS and 4EBP1 mRNA levels to be significantly lower in tumors ≥2cm (p=0.009 and p=0.003, respectively). This was also observed for IGF2 protein expression (p=0.001). A higher disease stage (stage IV versus stage I+II) correlated with a lower expression of INS, IGF2 and IGF1R mRNA, as well as IGF2, p-ERK (nuclear and cytoplasmic), cytoplasmic IGF1R and in membranous INSR protein.

In conclusion, insulinomas appear to reduce IGF pathway gene expression both at the mRNA and protein level in relation to tumor grade, metastatic potential, size and disease stage.

**Correlation of mRNA and protein expression and clinicopathologic parameters with survival in insulinomas**

Lower mRNA expression levels of INS, IGF1R and INSR-A (p≤0.019 for disease free and p≤0.032 for overall survival) and higher levels of IGFBP3 (p<0.0001 for disease free and p=0.001 for overall survival) correlated with shorter 10 years survival (Figure 3 and suppl. Fig 2).

Lower protein levels of cytoplasmic IGF2, IGF1R and INSR (p≤0.035 for disease free survival; P≤0.033 for overall survival) correlated with shorter 10 years survival rates (Fig 4 and suppl. Fig 3).

Univariate analysis of clinicopathological parameters of tumors showed very strong associations of grade, metastatic disease, tumor size and disease stage with both 10 years disease free and overall survival (p<0.0001) (Fig 3 and Supplementary Fig 2 E-H, and Fig 4 and Suppl Fig 3 D-G).

In Table 4 a summary of parameters that correlate with disease outcome in univariate analysis results is shown.

When comparing high versus low gene expression of either INS, IGF1R, INSR-A or IGFBP3 with grade, metastatic disease and tumor size in multivariate analysis no significant associations were found. In multivariate analysis of moderate versus high IGF2 protein
expression, grade and tumor size, grade (p=0.024, HR 6.81) and tumor size (p=0.022, HR 17.77) were significantly correlated with 10 year survival. When comparing IGF1R (high versus low expression) with grade and tumor size in multivariate analysis only tumor size (p=0.032, HR 7.03) correlated with 10 year survival. This was also found when comparing INSR (high versus low expression) in multivariate analysis with grade and tumor size (tumor size: p=0.007, HR 9.31).

Protein expression analysis in PanNETs other than insulinomas

We also analyzed protein expression in 92 PanNETs other than insulinomas, which were available on the TMA1. Data evaluation and analysis are available as supplementary data (Suppl Results, Suppl Tables 6, 7 and 8).

Discussion

Since PanNETs are a heterogeneous group of neoplasms, understanding the underlying molecular biology of the different subgroups is essential to offer adequate treatment (Cives et al. 2016). Our study focused on mRNA and protein expression of EGFR, IGF and mTOR signaling pathway components in insulinomas. We found that, compared to pancreatic islets, insulinomas show low expression levels of EGFR mRNA, high expression of IGF2, a 2-4 fold increased expression of IGFBP2, IGFBP3 and IGFBP6, and a twofold reduced expression of MTOR, RPS6KB1 and EIF4EBP1. At the protein level, high expression levels of IGF2, IGF1R and INSR were detected, whereas no EGFR and relatively low levels of mTOR pathway proteins were observed. Correlation of expression data with clinicopathological data revealed a decrease of several IGF pathway components in relation to tumor grade, metastatic disease, tumor size and disease stage. Low mRNA expression levels of IGF2, IGF1R and INSR-A but high levels of IGFBP3 correlated with shorter 10 years overall and disease-free survival. Decreased protein
expression of IGF2, cytoplasmic IGF1R and INSR also correlated with shorter survival rates.

Activated EGFR enhances tumor growth, invasion and metastatic spread and promotes cell survival. Abnormal expression of (mutated) EGFR is often found in neoplasms, particularly in breast, non-small-cell lung, head and neck and colorectal cancer, which is utilized in targeted therapy with TKIs or antibodies directed against EGFR (Ciardiello & Tortora 2008, Garraway & Janne 2012). An initial phase II trial using gefitinib treatment of PanNETs, however, did not show much efficacy, i.e. no objective responses and a 6-months progression-free survival of 10% for islet cell carcinomas (Hobday et al. 2006).

In our study we found low levels of EGFR mRNA and neither detectable protein expression in insulinomas and normal pancreatic islets. These data thus may explain the low efficacy of EGFR inhibitors in the treatment of PanNETs. Nevertheless, other studies have reported detectable expression of EGFR in 18-66% of the tumors (Wulbrand et al. 1998, Srivastava et al. 2001, Fjallskog et al. 2003, Papouchado et al. 2005, Gilbert et al. 2013), with low expression in benign PanNETs and high expression rates in both well-differentiated PanNETs and poorly differentiated pancreatic neuroendocrine carcinomas (Bergmann et al. 2009). In these studies, however, mixed groups of PanNETs were subjected to immunohistochemical staining protocols differing in, amongst others pretreatment steps, primary antibodies, and evaluation criteria. We have utilized a commonly used EGFR immunostaining protocol and a primary EGFR-specific antibody, resulting in intense EGFR membrane staining in head and neck premalignancy control specimens as well as in pancreatic ducts adjacent to negative insulinomas. Based on our results we can conclude that EGFR signaling does not play a pivotal role in insulinoma carcinogenesis and progression.

Our most striking finding was that 92% of insulinomas stained moderately to strongly positive for the IGF2 protein, which implicates autocrine activation of the IGF pathway in tumorigenesis, also reported by others (Samani et al. 2007, Weroha & Haluska 2012, Denduluri et al. 2015). This corresponded well with the 12.4 fold higher IGF2 mRNA level in insulinomas compared to normal pancreatic islets. IGF2 is an imprinted gene,
expressed primarily from the paternal allele. Methylation of the IGF2 regulatory regions has been reported in many cancers, resulting in loss of imprinting and protein overexpression (Murphy et al. 2006, Cerrato et al. 2008, Dejeux et al. 2009, Livingstone 2013, Creemers et al. 2016). Dejeux et al. 2009 reported hypermethylation of the differentially methylated region 2 (DMR2) as a specific event in insulinomas, leading, in a subset of the samples, to IGF2 mRNA overexpression compared to normal pancreatic tissue, other PanNETs (gastrinomas and non-functioning) and small intestinal endocrine tumors. At the protein level they found moderate to high expression levels in 14/28 insulinomas. The use of a different primary antibody and unspecified immunostaining procedure might explain the lower frequency of IGF2 positive insulinomas. Hoog et al. 2001 detected higher levels of IGF2 protein in 16 out of 18 insulinomas, which is in accordance with our findings. In contrast to IGF2, the mRNA expression of IGF1 was very low in insulinomas and not detectable in normal pancreatic islets.

The IGF signaling pathway is activated by binding of the ligands IGF1, IGF2 or insulin to their respective receptors, IGF1R and INSR. Posttranscriptional alternative splicing of INSR results in two isoforms, INSR-A (which lacks exon 11) and INSR-B. IGF2 binds with similar affinity both to IGF1R and INSR-A, which promotes cell growth, proliferation and survival (Chao & D’Amore 2008). IGF1R and INSR are overexpressed in a variety of cancers, including breast, prostate, osteosarcoma and thyroid carcinomas (Lodhia et al. 2015). In our study we found moderate to strong cytoplasmic and membranous IGF1R and cytoplasmic INSR protein expression in 51%, 78% and 83% of the insulinomas respectively, which was at least similar, or stronger in staining intensity than observed in pancreatic islets. Since we found a correlation between IGF1R and high IGF2 protein expression levels, these data suggest the presence of an autocrine proliferative loop in insulinomas, as described for other cancer types (Bergman et al. 2013, Livingstone 2013).

Binding of IGF1 or IGF2 to IGF1R or INSR leads to autophosphorylation of the β subunit tyrosine kinase and recruitment of INSR substrates (IRS), inducing activation of the MAPK/ERK and PI3K/AKT signaling pathways (Alvino et al. 2011). Phosphorylation of AKT
leads to activation of mTOR and downstream effectors of mTORC1 S6K and 4EBP1, both regulators of mRNA translation and involved in cell proliferation and survival (Robbins & Hague 2015). Although 51 and 83% respectively, of the insulinomas in our study express IGF1R and INSR protein, only 22% and 32% respectively, strongly express nuclear p-AKT and p-ERK, and 4 and 19% respectively, express p-S6K and p-4EBP1. Interestingly, a positive correlation was found between cytoplasmic IGF1R expression on the one hand and IGF2 and nuclear p-ERK on the other. Also at the mRNA level a correlation was observed between AKT, ERK1, RPS6KB1 and MTOR. The fact that IGF1R signaling does not necessarily results in AKT/mTOR signaling might be the result of an intact PTEN expression in about two third of the tumors, inhibiting PI3K and subsequent downstream signaling. Reduced PTEN expression or altered subcellular localization has been reported to activate PI3K/AKT/mTOR signaling in PanNETs (Perren et al. 2000, Missiaglia et al. 2010), which might have occurred in the remaining one third of the insulinomas in our study. In a pilot study of 10 cases we indeed observed loss of nuclear PTEN expression, as compared to normal islets, in 4 cases with p-AKT and p-S6K expression (Data not shown). (Komori et al. 2014) also found active mTOR signaling in 22-35% of a group of 14 insulinomas tested for amongst others p-mTOR, p-S6K and p-4EBP1. The percentage of tumors exhibiting active AKT/mTOR signaling varies significantly in different studies due to different PanNET subgroups tested, as well as different antibodies and cut-off criteria for positivity used (Ghayouri et al. 2010, Kasajima et al. 2011, Qian et al. 2013). It is tempting to speculate that p-AKT and/or p-S6K are putative predictive markers of response to Everolimus. A recent study on PanNETs indeed showed anecdotal evidence of p-AKT for this role in a primary cell culture model for response to Everolimus (Falletta et al. 2016).

We observed a decreased level of several IGF pathway components in relation to tumor grade, metastatic disease, tumor size and and disease stage. The protein expression levels of IGF2, cytoplasmic IGF1R, cytoplasmic and membranous INSR, and cytoplasmic p-S6K were significantly lower in tumors from patients with metastatic disease than from those with non-metastatic disease. Also the mean mRNA expression levels of INS was
significantly lower in patients with metastatic insulinomas as compared to non-metastatic
insulinomas. This finding is furthermore reflected in the lower levels of these IGF
pathway proteins and their mRNAs in association with shorter 10 years disease-free
survival and overall rates. A decrease in both IGF1R protein and gene expression levels
has been described before in esophageal adenocarcinoma, colorectal and breast cancer
(Schnarr et al. 2000, Allison et al. 2007, Kuklinski et al. 2011, De Bruijn et al. 2015) and
might reflect a dedifferentiation process. Indeed, two recent genomic studies have
identified distinctive m(i)RNA expression profiles, separating PanNET with liver
metastases (metastasis-like primary tumor subtype) from well-differentiated PanNETs
(well-differentiated islet/insulinoma tumor subtype), further underscoring
dedifferentiation to be reflected in gene expression signatures (Sadanandam et al. 2015,
Scarpa et al. 2017). Decreased insulin signaling was one described hallmark of mouse
metastasis like primary PanNET (Sadanandam et al. 2015). It remains to be studied
whether the changes in gene expression seen in insulinomas reflect a different entity or
are the result of tumor progression.

An interesting finding was that the shorter 10 years overall and disease-free survival
rates also correlated with higher levels of IGFBP3 mRNA in insulinomas. This gene
belongs to a family of 6 IGF binding proteins, which function as transport proteins for
IGF1 and IGF2 in the peripheral circulation, and in this way limit the bioavailability of
IGFs, as well as modulators of cell function via amongst others IGF1R-dependent
mechanisms (Baxter 2014). IGFBP3 has been proposed to function as either tumor
promotor or suppressor (Baxter 2014). On the one hand overexpression is detected in
association with tumor progression in many tumor types, such as head and neck
carcinoma (Marimuthu et al. 2013), melanoma (Xi et al. 2006) or renal clear cell
carcinoma (Takahash et al. 2005), and on the other hand (Ren et al. 2007) found a
higher IGFBP3 mRNA expression in benign as compared to malignant breast tumors. In
agreement with our study, (Hansel et al. 2004) found higher expression levels of IGFBP3
in well differentiated PanNETs of patients with metastatic disease, although this
particularly comprised non-functioning tumors and no metastatic insulinomas were
A high IGFBP3 mRNA expression was also identified in PanNETs of the metastasis-like primary PanNET subtype in a recent genomic analysis (Scarpa et al. 2017).

In conclusion, our observations suggest that insulinomas are characterized by high expression levels of IGF signaling pathway components, with a possibility of a strong autocrine loop especially in benign well-differentiated insulinomas. This IGF-signaling pathway appears to be downregulated during tumor progression, coinciding with a shorter 10 years disease free survival.

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**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Legends to the figures

Figure 1. Representative examples of immunohistochemical EGFR, pAKT, pERK, IGF2, IGF1R and INSR protein expression in human tissue: normal pancreas (B, D, G, J, M), a premalignant laryngeal lesion (A) and insulinomas (C, E, F, H, I, K, L, N, O). Strong membranous EGFR expression in a premalignant laryngeal lesion (A), weakly positive expression in normal acinar cells, and no detectable expression in the islets (B) and in insulinomas (C). Moderate to strong nuclear p-AKT expression in normal pancreatic islets (D), and moderate to strong nuclear and moderate cytoplasmic expression in an insulinoma (E). Strong nuclear p-ERK immunostaining in an insulinoma (F). Strong aggregate like, extracellular IGF2 localization in normal acinar cells, and a moderate, diffuse granular cytoplasmic staining pattern in pancreatic islets (G). Strong, granular cytoplasmic IGF2 expression in a non-metastatic insulinoma and weakly positive, diffuse cytoplasmic expression in a metastatic insulinoma (H and I respectively). Strongly positive, membranous IGF1R expression in normal acinar cells and moderately positive, granular cytoplasmic expression pattern in islet cells (J). In insulinomas a strong, cytoplasmic expression (K) or combined cytoplasmic and membranous IGF1R expression pattern (L) is seen. Moderate to strong cytoplasmic, perinuclear INSR expression in normal acinar cells with a granular, cytoplasmic pattern in islet cells (M). Moderate, diffuse granular and strong perinuclear INSR expression (N) or weak to moderate, diffuse expression (O) in insulinomas. Original magnifications 200X.

Figure 2. Representative examples of immunohistochemical p-S6K and p-4EBP1 protein expression in normal human pancreatic tissue and insulinomas. Strong, perinuclear expression pattern of p-S6K in normal acinar cells, while the islets cells show a very weak, diffuse cytoplasmic expression (A). Weak cytoplasmic and moderate to strong nuclear expression of p-S6K in insulinoma (B). Moderately to strongly positive diffuse cytoplasmic and nuclear p-4EBP1 immunostaining in normal exocrine pancreas cells at the periphery of a lobule (C). No detectable p-4EBP1 expression in normal pancreatic islet (D). Strong nuclear and weak to moderate cytoplasmic p-4EBP1 expression in tumor
adjacent exocrine tissue (E). Moderate to strong nuclear and weak cytoplasmic p-4EBP1 expression in insulinoma (F).

Figure 3. Kaplan-Meier analysis showing 10 year disease-free survival rates of insulinoma patients with regard to mRNA expression (A-D) and clinicopathological parameters (E-H). Correlation between survival and mRNA expression, A: INS (cut off 25th percentile), B: IGF1R (cut off 25th percentile), C: INSR-A (cut off 25th percentile), D: IGFBP3 (cut off 25th percentile), E: Grade (Grade1 versus Grade 2+3), F: Disease (non-metastatic versus metastatic), G: Tumor size (<2 cm versus ≥2 cm) and H: Disease stage (Stage I+IIa versus IV).

Dotted lines in plot A-D refer to low expression.

Figure 4. Kaplan-Meier analysis showing 10 year disease-free survival rates of insulinoma patients with regard to protein expression (A-C) and clinicopathological parameters (D-G). Correlation between survival and protein expression, A: IGF2 (moderate (2) vs high (3) expression), B: cytoplasmic IGF1R (low vs high expression), C: cytoplasmic INSR (low vs high expression), D: Grade (Grade1 versus Grade 2+3), E: Disease (non-metastatic versus metastatic), F: Tumor size (<2 cm versus ≥2 cm) and G: Disease stage.

Suppl. Figure 1. Control immunoperoxidase stainings for p-AKT, p-ERK, p-S6K and p4-EBP1. Moderate to strong nuclear and weak cytoplasmic expression of p-AKT (A) and moderate nuclear expression of p-ERK (B) in a lung tumor harboring a K-ras exon 2 mutation. Moderate nuclear p-S6K expression (C) and moderate cytoplasmic p-4EBP1 expression (D) in glandular normal colon cells. A low percentage of cells also shows a moderate to strong nuclear p-4EBP1 staining pattern (D). Original magnifications 200X.
**Suppl. Figure 2.** Kaplan-Meier analysis showing 10 year overall survival rates of insulinoma patients with regard to mRNA expression (A-D) and clinicopathological parameters (E-H). Correlation between survival and mRNA expression, A: INS (cut off 25th percentile), B: IGF1R (cut off 25th percentile), C: INSRL-A (cut off 25th percentile), D: IGFBP3 (cut off 25th percentile), E: Grade (Grade1 versus Grade 2+3), F: Disease (non-metastatic versus metastatic) G: Tumor size (<2 cm versus ≥2 cm) and H: Disease stage (Stage I+IIa versus IV). Dotted lines in plots A-D refer to low expression.

**Suppl. Figure 3.** Kaplan-Meier analysis showing 10 year overall survival rates of insulinoma patients with regard to protein expression (A-C) and clinicopathological parameters (D-G). Correlation between survival and protein expression, A: IGF2 (moderate (2) vs high (3) expression), B: cytoplasmic IGF1R (low vs high expression), C: cytoplasmic INSR (low vs high expression), D: Grade (Grade1 versus Grade 2+3), E: Disease (non-metastatic versus metastatic), F: Tumor size (<2 cm versus ≥2 cm) and G: Disease stage. Dotted lines in plots A-C refer to low expression.
Table 1  Mean mRNA expression levels of neuroendocrine related genes and genes in the MAPK/ AKT and IGF pathway in 48 insulinomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean (± SD)</th>
<th>Median</th>
<th>Mean (± SD)</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGA</td>
<td>88.91 ± 95.94</td>
<td>59.94</td>
<td>6.04 ± 6.61</td>
<td>3.92</td>
</tr>
<tr>
<td>SYNAPT</td>
<td>2.28 ± 1.92</td>
<td>1.83</td>
<td>4.83 ± 4.31</td>
<td>3.61</td>
</tr>
<tr>
<td>INS</td>
<td>8351.20 ± 8507.11</td>
<td>6269.65</td>
<td>2.01 ± 2.03</td>
<td>1.48</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.10 ± 0.10</td>
<td>0.07</td>
<td>0.15 ± 0.17</td>
<td>0.11</td>
</tr>
<tr>
<td>AKT</td>
<td>2.58 ± 1.45</td>
<td>2.28</td>
<td>0.75 ± 0.45</td>
<td>0.63</td>
</tr>
<tr>
<td>ERK1</td>
<td>1.10 ± 0.73</td>
<td>0.93</td>
<td>1.03 ± 0.64</td>
<td>0.95</td>
</tr>
<tr>
<td>ERK2</td>
<td>1.56 ± 0.89</td>
<td>1.45</td>
<td>0.68 ± 0.49</td>
<td>0.55</td>
</tr>
<tr>
<td>IGF1</td>
<td>0.04 ± 0.13</td>
<td>0.01</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>IGF1R</td>
<td>0.11 ± 0.12</td>
<td>0.06</td>
<td>0.82 ± 0.92</td>
<td>0.49</td>
</tr>
<tr>
<td>IGF2</td>
<td>1.46 ± 3.12</td>
<td>0.43</td>
<td>12.44 ± 22.64</td>
<td>3.54</td>
</tr>
<tr>
<td>IGF2R</td>
<td>0.13 ± 0.09</td>
<td>0.11</td>
<td>0.67 ± 0.42</td>
<td>0.58</td>
</tr>
<tr>
<td>IGF bp1</td>
<td>0.03 ± 0.07</td>
<td>0.00</td>
<td>1.63 ± 4.13</td>
<td>0.16</td>
</tr>
<tr>
<td>IGF bp2</td>
<td>4.81 ± 5.69</td>
<td>3.44</td>
<td>4.04 ± 4.41</td>
<td>3.18</td>
</tr>
<tr>
<td>IGF bp3</td>
<td>0.21 ± 0.32</td>
<td>0.09</td>
<td>2.86 ± 4.74</td>
<td>1.24</td>
</tr>
<tr>
<td>IGF bp6</td>
<td>0.23 ± 0.32</td>
<td>0.12</td>
<td>2.03 ± 1.99</td>
<td>1.37</td>
</tr>
<tr>
<td>INSR-A</td>
<td>0.22 ± 0.20</td>
<td>0.19</td>
<td>1.30 ± 0.70</td>
<td>1.27</td>
</tr>
<tr>
<td>INSR-B</td>
<td>0.10 ± 0.09</td>
<td>0.08</td>
<td>0.75 ± 0.54</td>
<td>0.70</td>
</tr>
<tr>
<td>mTOR</td>
<td>0.01 ± 0.01</td>
<td>0.01</td>
<td>0.35 ± 0.44</td>
<td>0.22</td>
</tr>
<tr>
<td>RPS6KB1</td>
<td>0.02 ± 0.02</td>
<td>0.01</td>
<td>0.58 ± 0.61</td>
<td>0.39</td>
</tr>
<tr>
<td>EIF4EBP1</td>
<td>0.84 ± 0.87</td>
<td>0.58</td>
<td>0.45 ± 0.46</td>
<td>0.30</td>
</tr>
</tbody>
</table>

IGF1 relative to pancreatic islets cannot be calculated since IGF1 was not detectable in normal pancreatic islets
Table 2 Immunohistochemical expression of proteins in the EGFR/MAPK/AKT/IGF/mTOR pathways in insulinomas, presented as percentage of the samples with a specified staining intensity.

<table>
<thead>
<tr>
<th>Insulinomas</th>
<th>negative</th>
<th>weakly positive</th>
<th>positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p-AKT nuclear</td>
<td>60</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>p-AKT cytoplasmic</td>
<td>51</td>
<td>41</td>
<td>8</td>
</tr>
<tr>
<td>p-ERK nuclear</td>
<td>30</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>p-ERK cytoplasmic</td>
<td>38</td>
<td>38</td>
<td>24</td>
</tr>
<tr>
<td>IGF2</td>
<td>1</td>
<td>7</td>
<td>92</td>
</tr>
<tr>
<td>IGF1R cytoplasmic</td>
<td>4</td>
<td>18</td>
<td>78</td>
</tr>
<tr>
<td>IGF1R membranous</td>
<td>38</td>
<td>11</td>
<td>51</td>
</tr>
<tr>
<td>INSR cytoplasmic</td>
<td>4</td>
<td>13</td>
<td>83</td>
</tr>
<tr>
<td>p-S6K nuclear</td>
<td>72</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>p-S6K cytoplasmic</td>
<td>72</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>p-4EBP1 nuclear</td>
<td>93</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>p-4EBP1 cytoplasmic</td>
<td>78</td>
<td>13</td>
<td>9</td>
</tr>
</tbody>
</table>

Staining intensity was defined as negative, weakly positive in >10 % of cells and moderately or strongly positive in >10 % of cells. Abbreviation used: p=phosphorylated
Table 3A  Correlation of mRNA expression levels in insulinomas, relative to normal single donor pancreatic islet mRNA, with grade, tumor size, metastatic disease and disease stage

*p-values <0.05 correspond to lower specific gene expression in tumors with higher grade, metastatic progression, larger size and more advanced disease stage*

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Grade 1 vs Grade 2+3 p-Value</th>
<th>Non-metastatic vs metastatic p-Value</th>
<th>Tumor size &lt;2cm vs ≤2 cm p-Value</th>
<th>Disease stage I+II vs IV p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>INS</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>0.009</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AKT</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ERK1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ERK2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IGF2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.004</td>
</tr>
<tr>
<td>IGF1R</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.024</td>
</tr>
<tr>
<td>IGF2R</td>
<td>0.039</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IGFBP6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>INSR-A</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>INSR-B</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>EIF4EBP1</td>
<td>NS</td>
<td>NS</td>
<td>0.003</td>
<td>NS</td>
</tr>
<tr>
<td>RPS6KB1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant
**Table 3B** Correlation of protein expression levels with grade, tumor size, metastatic disease and disease stage

*p*-values *<0.05 correspond to lower specific protein expression in tumors with higher grade, metastatic progression, larger size and more advanced disease stage*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Grade 1 vs Grade 2+3 p-Value</th>
<th>Non-metastatic vs metastatic p-Value</th>
<th>Tumor size &lt;2cm vs &gt;2 cm p-Value</th>
<th>Disease stage I+II vs IV p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF2</td>
<td>NS</td>
<td>0.001</td>
<td>0.001</td>
<td>0.009</td>
</tr>
<tr>
<td>p-AKT nuclear</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>p-AKT cytoplasmic</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>p-ERK nuclear</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.004</td>
</tr>
<tr>
<td>p-ERK cytoplasmic</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.006</td>
</tr>
<tr>
<td>IGF1R cytoplasmic</td>
<td>NS</td>
<td>0.026</td>
<td>NS</td>
<td>0.021</td>
</tr>
<tr>
<td>IGF1R membranous</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>INSR cytoplasmic</td>
<td>NS</td>
<td>0.035</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>INSR membranous</td>
<td>0.004</td>
<td>0.004</td>
<td>NS</td>
<td>0.004</td>
</tr>
<tr>
<td>p-4EBP1 nuclear</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>p-4EBP1 cytoplasmic</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>p-PS6K cytoplasmic</td>
<td>NS</td>
<td>0.030</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant

Abbreviation used: p = phosphorylated
Table 4  Significance levels (p-values) for univariate analysis of insulinomas, indicating the relation of clinical and molecular parameters with disease outcome for tumors subjected to A mRNA expression analysis and B protein expression analysis

<table>
<thead>
<tr>
<th></th>
<th>OS p-value</th>
<th>DFS p-value</th>
<th></th>
<th>OS p-value</th>
<th>DFS p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade¹</td>
<td>0.007</td>
<td>0.002</td>
<td>Grade¹</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Disease²</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>Disease²</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tumor size³</td>
<td>0.003</td>
<td>&lt;0.0001</td>
<td>Tumor size³</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Disease stage⁴</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>Disease stage⁴</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>INS⁵</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IGF2&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.027&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.035&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF1R&lt;sup&gt;5&lt;/sup&gt;</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IGF1R&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.033&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.012&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>INSR-A&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.032&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0019&lt;sup&gt;a&lt;/sup&gt;</td>
<td>INSR&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.016&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.010&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGFBP3&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations used: OS, overall survival; DFS, disease free survival

¹ Grade 1 vs Grade 2+3; ² non-metastatic vs metastatic disease; ³ tumor size <2 cm vs ≥2 cm; ⁴ disease stage I+II vs IV; ⁵ high vs low mRNA expression (see Figure 2 A-D legend); ⁶ high vs low protein expression (see Figure 3 A-C legend).

<sup>a</sup> p-values refer to shorter 10 years survival for lower expression; <sup>b</sup> p-values refer to shorter 10 years survival for higher expression
Figure 1

199x280mm (300 x 300 DPI)
Figure 2

200x119mm (300 x 300 DPI)
Figure 3

281x461mm (300 x 300 DPI)