Mitogenic insulin receptor-A is overexpressed in human hepatocellular carcinoma due to EGFR-mediated dysregulation of RNA splicing factors

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Running title: Insulin receptor isoform A splicing in liver cancer (51/60 characters)

Key words: insulin receptor, splicing, EGFR, CUGBP1, hnRNP

Financial support: H. Chettouh is a fellow from Ministère de l’Enseignement Supérieur et de la Recherche and Fondation ARC pour la Recherche sur le Cancer. This work was supported by grants from INSERM, GEFLUC, Cancéropôle Ile de France and INCa.

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Conflict of interest: No

Word count: 5,600
Total number of figures and tables: 7 figures
Total number of supplementary figures and tables: 6 figures and 5 tables
Legends to supplementary figures
Abstract (213/250 words)

Insulin receptor (IR) exists as two isoforms resulting from the alternative splicing of IR pre-mRNA. IR-B promotes the metabolic effects of insulin while IR-A rather signals proliferative effects. IR-B is primarily expressed in the adult liver. Here we demonstrate that the alternative splicing of IR pre-mRNA is dysregulated in a panel of 85 human hepatocellular carcinoma (HCC) while being normal in adjacent non-tumour liver tissue. An IR-B to IR-A switch is frequently observed in HCC tumours regardless of tumour etiology. Using pharmacological and siRNA approaches, we show that the autocrine or paracrine activation of the epidermal growth factor receptor (EGFR)/MEK pathway increases the IR-A:IR-B ratio in HCC cell lines, but not in normal hepatocytes, by upregulating the expression of the splicing factors CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF. In HCC tumours, there is a significant correlation between the expression of IR-A and that of splicing factors. Dysregulation of IR pre-mRNA splicing was confirmed in a chemically-induced model of HCC in rat but not in regenerating livers after partial hepatectomy. This study identifies a mechanism responsible for the generation of mitogenic IR-A and provides a novel interplay between IR and EGFR pathways in HCC. Increased expression of IR-A during neoplastic transformation of hepatocytes could mediate some of the adverse effects of hyperinsulinemia on HCC.
Introduction

Recent epidemiological data showing that hyperinsulinemia is a risk factor for a variety of malignancies has renewed the interest for studies examining insulin receptor (IR) signalling in cancer development (1-3). Dysregulations of IR expression and/or downstream signalling have been reported in cancers (4, 5). Hepatocellular carcinoma (HCC), which is the most common primary liver tumour, is no exception. Indeed, elevated insulin levels in serum have been shown to be predictive of HCC recurrence in hepatitis C virus (HCV) and hepatitis B virus (HBV) carriers (6, 7). Furthermore, the use of exogenous insulin or of insulin secretagogues might increase the risk of HCC among diabetics (8, 9). IR expression was found to be slightly higher in a small collection of HCC relative to the adjacent non-tumour liver tissue (10). Moreover, aberrant expressions of IRS-1 and IRS-2, which are IR cytosolic substrates occur in HCC (11, 12).

IR is a heterotetrameric $\alpha_2\beta_2$ receptor with structural and functional homologies with insulin-like growth factor type 1 receptor (IGF-1R). IR is expressed as two isoforms, IR-A and IR-B, which originate from the alternative splicing of exon 11 in the IR pre-mRNA (IR-A$^{\text{Ex11}}$; IR-B$^{\text{+Ex11}}$) and differ from 12 amino acids located at the carboxyl terminus of the extracellular $\alpha$-subunit. Exon 11 skipping is developmentally regulated and IR-A is predominantly expressed in embryo and fetal tissues (13). This isoform becomes less expressed in adult differentiated tissues especially in target tissues of insulin such as the liver, which expresses nearly exclusively IR-B. The structural feature of IR-A isoform confers specific functional properties in terms of ligand affinity. Indeed, IR-A is a high-affinity receptor not only for insulin but also for insulin-like growth factor-II (IGF-II), a fetal growth peptide produced by the liver, which is overexpressed in HCC tumours and cell lines (14). IR-A also displays a faster internalization and recycling kinetics and a higher propensity to signal proliferation and survival compared to IR-B (15). IR-A is overexpressed in tumours from
different tissue origin including breast, colon and lung (4) and in a variety of cancer cell lines including the hepatoblastoma-derived HepG2 cell line (16).

The recognition of exons and introns during pre-mRNA splicing relies on regulatory sequence elements located within exons and flanking introns that are recognized by splicing factors. Oncogenic signals involving receptor tyrosine kinases (RTKs), Ras, phosphatidylinositol 3-kinase (PI3K), AKT and c-Jun N-terminal kinases (JNKs) may promote aberrant mRNA splicing in cancer cells by regulating the expression, the localization and/or the phosphorylation of splicing factors (17, 18). Using IR minigenes transiently transfected in myoblasts, HepG2 or HEK293 cells, splicing factors belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP), SR and CELF/Bruno-like families have been identified as regulators of IR pre-mRNA exon 11 inclusion (16, 19-21). The contribution of these splicing factors in the regulation of endogenous IR pre-mRNA splicing has not been yet explored in cancer cells. In addition, the signal pathways and molecular mechanisms that regulate the function of these splicing factors are currently unknown in the context of IR-A alternative splicing.

In the present study, we have examined the status of IR-A in a large panel of human HCC tumours. The observation that IR-A is markedly upregulated to the detriment of IR-B in malignant lesions but not in adjacent non-tumour liver tissues led us to investigate the underlying mechanism. We identified a new mechanism by which activation of the epidermal growth factor receptor (EGFR) pathway triggers aberrant IR alternative splicing and subsequent IR-A overexpression in HCC cells through the up-regulation of the splicing factors CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1 and SF2/ASF. Dysregulation of IR pre-mRNA splicing was confirmed in chemically-induced HCC in rat but not in regenerating livers after partial hepatectomy.
Material and methods

Patients and liver tissue specimens

Two independent collections of HCC tumours and adjacent non-tumoral liver tissues were used in this study. Samples were collected from HCC patients undergoing curative liver resection or liver transplantation at the Saint-Antoine Hospital (tissue collection #1, n = 42; tissue collection #2, n = 43) in accordance with the French laws and regulations. Patient characteristics are recapitulated in Supplementary Table 1. Normal-histologically liver tissue (control liver samples) was obtained from 11 patients undergoing partial hepatectomy for the treatment of metastases or benign tumour. Tissue samples were flash-frozen in liquid nitrogen and stored at −80°C until analyses.

Cell culture and treatments

HepG2, Hep3B, and HuH7 cells were obtained from the American Type Culture Collection. HuH6 and PLC/PRF5 cells were provided by Dr Christine Perret (Institut Cochin, France) and were characterized for β-catenin^G34V (HuH6), axin-1^ΔEx4 (PLC/PRF5) and p53^R249S (PLC/PRF5) mutations. Cell lines were cultured as previously reported (22). Primary cultures of human hepatocytes were established as previously described (23). Cells were incubated with pharmacological inhibitors described in Supplementary Table 2.

Liver carcinogenesis and regeneration in rats

Liver carcinogenesis was induced in rats by i.p injection of diethylnitrosamine (DEN) according to previously established protocol (24). Liver regeneration was induced in rats by two-third liver hepatectomy using a conventional procedure (25). Animals were sacrificed one day, three days or ten days after hepatectomy. Animals were maintained in accordance with the French guidelines for the humane treatment and care of laboratory animals.
**DNA synthesis**

Cells were incubated for 17 h with or without 10 nM insulin or IGF-II and [Methyl-\(^{3}\)H] thymidine (1 $\mu$Ci/well) was added for the last 3 h of the incubation period. The amount of radioactivity incorporated into DNA was determined as previously described (26).

**Real-time cell impedance analysis**

Proliferation was monitored using xCELLigence System (Roche Applied Science), which measures in real time electrical impedance and provides quantitative information on cell adherence. Cells were incubated in 0.3% fetal bovine serum (FBS) overnight and 10 nM insulin or IGF-II was added. Cell Index slope (a parameter calculated as a relative change in measured electrical impedance) was measured using the RTCA Software 1.2.

**Western blotting, antibody array and ELISA**

Antibodies are described in Supplementary Table 3. Blot quantifications were performed by scanning densitometry using ImageJ software (NIH Image). To evaluate splicing factor phosphorylation, cell lysates were incubated with alkaline phosphatase (1 U/$\mu$g protein) for 30 min at 37°C and analysed by Western blot on 4-12% polyacrylamide gradient gels. The phosphorylation status of 42 RTKs was determined by Proteome Profiler array (R&D Systems). Total amounts of IR were quantified in liver tissue extracts by ELISA (R&D Systems).

**RNA interference**

The expression of specific mRNA was down-regulated using a mixture of four siRNAs (ON-TARGETplus SMARTpool; Dharmacon). Control siRNA was a pool from Dharmacon.
(siGENOME non-targeting siRNA pool). Cells were cultured for 24 h to 40% confluency, transfected with 100 nmol/L siRNA using Lipofectamine 2000 (Life Technologies SAS) for 5 h, and harvested 72 h later for mRNA and protein analyses.

**Transient plasmid transfections**

Cells were transfected with 2.5 μg of a plasmid coding for a dominant-negative RAF-1 mutant (RAF-C4) (27) or of a control empty plasmid together with 0.5 μg of pβgal-Control (Clontech Laboratories, Inc.) by using Lipofectamine 2000.

**RNA isolation and analysis of gene expression**

Total RNA was extracted from cell cultures using RNeasy Mini kits (Qiagen). For tissues, a preliminary RNA extraction step was performed using TRlzol Reagent (Life Technologies). Quantitative measurements of transcripts were performed by real-time PCR on a LightCycler 480 instrument (Roche) using SYBR Green chemistry and specific primers (Supplementary Table 4). For each sample, gene expression was normalized to that of HPRT mRNA content and was expressed relatively to the same calibrator. The relative quantity of each target gene was determined from replicate samples using the formula 2^-ΔΔCt. Qualitative evaluation of IR isoform expression was performed by RT-PCR analysis using primers for the flanking exons 10 and 12 (Supplementary Table 4) and analyzed on 2% agarose gels.

**Immunofluorescence analysis**

Cells seeded on glass coverslips were fixed with 4% paraformaldehyde, blocked with 1% BSA and 10% FBS in PBS followed by overnight incubation with a 1:50 dilution of the primary antibody in PBS at 4°C. Cells were then incubated with a 1:200 dilution of FITC-conjugated goat anti-mouse or mouse anti-goat antibody in PBS for 1 h at room temperature. The slides were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) for nuclei
detection. Fluorescence was visualized using an immunofluorescence microscope (Leica Microsystèmes) with a DFC300 FX digital camera.

**Statistical Analysis**

Results are given as mean ± SEM. Data were analyzed using Prism 5.0 software (GraphPad Software Inc.). Expression data obtained from human and rat liver tissue specimens were compared using Mann-Whitney *U*-test (unpaired data) and Wilcoxon test (paired data). Correlations between IR-A and splicing factor mRNA expression in human liver tissue samples were performed using Spearman’s rank correlation coefficient. Data obtained from *in vitro* experiments in cell lines were compared using the unpaired Student’s *t*-test. Statistical tests were two-sided. *P* value of less than 0.05 was considered significant.
Results

IR is upregulated and aberrantly spliced in human HCC tumours.

We examined the expression level of IR in HCC tumours (T) and paired surrounding non-tumour liver tissues (NT) from 42 patients (tissue collection #1) as well as in healthy livers from 11 subjects (Cont). Real-time quantitative PCR experiments were conducted with a pair of primers that amplifies the two IR isoform mRNA (total IR). Total IR mRNA expression was similar between non-tumour liver tissues and healthy livers while it was significantly higher in HCC tumours compared to matched non-tumour tissues (Figure 1A). Similar data were obtained using an independent collection of 43 paired T/NT samples (tissue collection #2) (Supplementary Figure 1A). In paired T/NT samples available for protein analysis (n = 27, collection #1), Western blotting (Figure 1B) and ELISA (Figure 1C) confirmed IR overexpression at the protein level in HCC tumours.

Then we analysed the expression of IR-B (+Ex11) and IR-A (-Ex11) isoforms by PCR only since there are no discriminative antibodies. Standard PCR experiments using primers for the flanking exons 10 and 12 combined with agarose gel electrophoresis showed that IR-A mRNA was detected in tumours but not in adjacent non-tumour tissues whereas IR-B mRNA expression was decreased in tumours in comparison with adjacent non-tumour tissues (Figure 1D). These findings were supported by quantitative PCR using primer pairs specific for each isoform. As shown in Figure 1E (tissue collection #1) and in Supplementary Figure 1B (tissue collection #2), IR-A mRNA was expressed at significantly higher levels in HCC tumours than in non-tumoral surrounding livers and healthy liver tissues. The relative expression ratio of IR-A to IR-B (IR-A:IR-B) was also significantly higher in tumours compared with adjacent non-tumour tissues indicating increased alternative splicing of IR mRNA (Figure 1F and Supplementary Figure 1C). Supplementary Figure 1D recapitulates the fold inductions of total IR, IR-B, IR-A and IR-A:IR-B ratio in each of the 85
HCC samples compared with the matched adjacent liver tissue. In tissue collection #1, IR-A mRNA transcripts and IR-A:IR-B ratio were increased by 5.21 ± 0.68 and 3.89 ± 0.70 folds in HCC specimens respectively while IR-B and total IR mRNA were increased by only 2.02 ± 0.20 and 2.00 ± 0.27 folds, respectively. Tumour induction of IR-A splicing was observed regardless of the etiology of the liver disease and the presence or absence of significant fibrosis/cirrhosis in the peritumoral liver (Supplementary Figure 1E).

**HCC cell lines express the IR-A isoform and proliferate in response to insulin and IGF-II.**

We tested five human HCC cell lines, all of which overexpressed IR in comparison with normal primary human hepatocytes (Figure 2A). Phospho-RTK array analysis revealed that after treatment with 10% FBS, IR was, together with IGF-1R and EGFR, the most intensively phosphorylated RTK in HCC cells (Figure 2B). Insulin (Figure 2C) and IGF-II (Figure 2D) promoted proliferation in HCC cells but not in hepatocytes as evaluated by the incorporation of [3H]thymidine into DNA and by real-time monitoring of cell index based on impedance variations. Standard PCR experiments clearly evidenced the co-expression of the two IR isoforms in HCC cell lines while normal hepatocytes predominantly expressed IR-B (Figure 2E). Quantitative PCR experiments confirmed that the IR-A:IR-B ratio was significantly increased in HCC cell lines, specially in HuH6 and PLC/PRF5 cells (Figure 2F).

**Activation of EGFR promotes IR-A alternative splicing through the MEK pathway in human HCC cell lines but not in normal hepatocytes.**

The EGFR pathway plays a major role in HCC development and is frequently over-activated in HCC (24, 28). Consistently, EGFR is activated in HCC cell lines (Figure 2B). With the objective to identify whether this pathway promoted IR alternative splicing in HCC cells,
HuH6 and PLC/PRF5 cells were incubated with a panel of specific kinase inhibitors to block EGFR and the downstream signalling molecules MEK, PI3K, AKT, mTOR, JNK and STAT3. As shown in Figure 3A and Supplementary Figure 2A, EGFR and MEK inhibitors significantly decreased the IR-A:IR-B ratio in these cells, without modifying the total levels of IR mRNA (data not shown). The JNK inhibitor induced a significant reduction in the IR-A:IR-B ratio in HuH6 but not in PLC/PRF5 cells. EGFR inhibitor gefitinib induced a time-dependent inhibition of IR-A mRNA splicing (Figure 3B and Supplementary Figure 2B). To confirm the role of EGFR activation in the promotion of IR-A expression, the expression of EGFR and of amphiregulin (AR), an EGFR ligand actively secreted by HCC cells (29), was down-regulated with specific siRNAs. Both EGFR (Figure 3C) and AR (Figure 3D) siRNAs induced a significant decrease in the IR-A:IR-B ratio in HuH6 cells. Similar results were obtained with PLC/PRF5, Hep3B and HuH7 cells (Supplementary Figure 2C). The involvement of the MEK pathway in the regulation of IR mRNA splicing was confirmed by performing a time-dependent kinetics with the MEK inhibitor U0126 (Figure 3E and Supplementary Figure 2D) and by transient overexpression of a plasmid coding for a dominant-negative RAF-1 mutant (RAF-C4) in HuH6 and PLC/PRF5 cells (Figure 3F). We also tested the effect of exogenously added AR, EGF or HB-EGF on IR mRNA splicing. These experiments were conducted in Hep3B and HuH7 cells because the activation of EGFR with an exogenous ligand strongly stimulated the ERK pathway above basal in these cell lines, whereas it was less effective in HuH6 and PLC/PRF5 cells (Supplementary Figure 3). In the latter two cell lines, the basal level of ERK phosphorylation was stimulated mainly through an EGFR-dependent pathway. EGFR ligands induced a significant increase of the IR-A:IR-B ratio in Hep3B and HuH7 cells but not in normal human hepatocytes (Figure 3G). U0126 abolished the effect of AR on the IR-A:IR-B ratio (Figure 3G) indicating that activated EGFR promotes IR-A alternative splicing through MEK activation in HCC cells.
EGFR controls the expression of different splicing factors that promote IR-A alternative splicing in HCC cells

To characterize the mechanisms whereby EGFR promoted the emergence of IR-A in HCC cells, we first examined the expression of splicing factors from CELF (CUG-BP1), hnRNP (H, F, and A1 isoforms) and SR (SF2/ASF, SRp20) families that were previously identified as regulators of IR exon 11 splicing using IR minigene transfection systems (16, 19-21). hnRNPA2B1 was also investigated as it has been recently shown to be overexpressed in HCC (30). As shown in Figure 4A, the protein levels of CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1 and to a lesser extent of SF2/ASF were increased in HCC tumours compared with non-tumoral liver tissues. hnRNPF protein level remained unchanged and SRp20 was undetectable in these samples (data not shown). Analysis of transcript expression by qPCR corroborated these findings (Figure 4B). IR-A mRNA levels in tumours were significantly correlated with those of splicing factors (Supplementary Table 5). With the exception of hnRNPF, splicing factors were overexpressed in HCC cell lines compared with normal human hepatocytes (Figure 4C).

We next used a siRNA strategy to examine the contributory role of splicing factors in IR-A mRNA expression in HCC cells (Figure 4D). The siRNA against CUGBP1, hnRNPH, hnRNPA1 or hnRNPA2B1 induced a significant decrease in the IR-A:IR-B ratio in the four HCC cell lines (Figures 4E and 5A). The knockdown of SF2/ASF and of hnRNPF had no effect on the IR-A:IR-B ratio in the PLC/PRF5 cell line while it has a decreasing effect in the three other cell lines (Figures 4E and 5A). In HuH7 cells, each siRNA inhibited the stimulatory effect of exogenously added EGFR ligands on the IR-A:IR-B ratio (Figure 5A) indicating that these splicing factors play a contributory role in the promotion of IR-A expression by EGFR.
We next examined whether EGFR signalling could regulate splicing factor function through modifications of expression, subcellular localization and/or phosphorylation state. The blockade of EGFR signalling with EGFR siRNA or gefitinib decreased CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1 and SF2/ASF at both mRNA (Figure 5B) and protein (Figure 5C) levels in HuH6 cells while hnRNPF levels remained unchanged. With the exception of hnRNPA1, similar results were obtained in HuH7 cells (Supplementary Figure 4A). Reciprocally, in these cells, EGFR ligands increased the expression levels of CUGBP1, hnRNPH, hnRNPA2B1 and SF2/ASF but not of hnRNPA1 and hnRNPF (Supplementary Figure 4B). Immunofluorescence experiments showed that splicing factors were mainly expressed in the nuclear compartment in HCC cells, which is fully compatible with their function as promoter of mRNA alternative splicing (Supplementary Figure 5). The blockade of EGFR signalling with gefitinib or U0126 did not affect their subcellular localization. The phosphorylation status of splicing factors was then analysed by comparing the electrophoretic migration profiles in the presence or absence of alkaline phosphatase. Treatment with alkaline phosphatase alone caused a marked shift in the migration of CUGBP1, hnRNPH and SF2/ASF but not of hnRNPA1, hnRNPA2B1 and hnRNPF in control cells indicating that only CUGBP1, hnRNPH and SF2/ASF were phosphorylated in HCC cells (Figure 5D). The inactivation of the EGFR pathway with EGFR siRNA or gefitinib did not modify the electrophoretic mobility patterns obtained with or without alkaline phosphatase (Figure 5D) indicating that EGFR activation did not modify splicing factor phosphorylation in these cells.

**IR mRNA splicing is altered during liver carcinogenesis but not during liver regeneration in rat models.**

Finally, we took advantage of two experimental models developed in rats to gain further insights into the relationship between IR-A expression and hepatocellular proliferation.
under pathological conditions. First, we investigated the rat model of HCC and cirrhosis induced by weekly *i.p.* injections of DEN during 16 weeks that we previously established (24). Quantitative PCR experiments showed that the IR-A:IR-B ratio was significantly increased in tumours compared with the cirrhotic matched liver tissues, while total IR mRNA levels remained unchanged in this model (Figure 6A). The expression of some splicing factors involved in IR-A splicing was significantly increased in tumours (Figure 6B). To determine whether IR mRNA splicing was altered during non-tumoral hepatocellular proliferation, we analysed IR-A:IR-B ratio and total IR mRNA levels in rat liver 1, 3 and 10 days after a two-third hepatectomy. In this model, maximal hepatocyte proliferation occurs 1 day after hepatectomy. No significant change in IR-A:IR-B ratio and total IR mRNA was observed during rat liver regeneration (Figure 6C). The analysis of splicing factor expression showed that CUGBP1, hnRNPH and hnRNPA2/B1 levels remained constant during the liver regenerative process while a transient induction of hnRNPA1, hnRNPF and SF2/ASF levels was observed (Figure 6D). This might suggest that hnRNPA1, hnRNPF and SF2/ASF have other targets and/or functions during liver regeneration.
Discussion

In the vast majority of HCC tumours that we tested, IR-A expression was strongly increased as compared with healthy liver or adjacent non-tumoral liver tissue suggesting that IR-A is a hallmark of hepatocellular cancer. Experiments conducted in rats support these conclusions since the induction of IR-A expression occurred in DEN-induced HCC tumours but not in proliferating hepatocytes from regenerating livers. Increased IR-A splicing was observed in human HCC tumours irrespective of etiology suggesting that IR-A emergence is a general molecular mechanism of liver carcinogenesis.

Hyperinsulinemia is a major determinant for HCC. Hyperinsulinemia is favoured by chronic liver diseases as a result of liver dysfunction and inflammation and decreased hepatic insulin clearance. Elevated insulin levels are predictive of HCC recurrence in HCV and HBV carriers (6, 7). Hyperinsulinemia is also a common feature of type 2 diabetes and obesity, which are independent risk factors for HCC (31). Recent clinical studies examining the impact of diabetes treatments on HCC incidence show that exogenous insulin and insulin secretagogues tend to increase HCC risk among diabetics (8, 9).

The underlying mechanisms that may account for the development of HCC in the presence of hyperinsulinemia are probably multiple (32). Our findings support the hypothesis that hyperinsulinemia has growth-promoting effects by directly stimulating IR-A activity on liver cancer cells. Due to their structural homology, IR-A may heterodimerize and form hybrids with IR-B and IGF-1R. Heterodimer formation will depend upon the relative expression of each hemireceptor. In accordance with previous studies (33), IGF-1R was expressed in a limited number of HCC tumours (24%). Thus, it is reasonable to assume that HCC tumours express more frequently a mixture of IR-A/IR-A and IR-A/IR-B dimers. Both dimers can bind insulin with high affinity and stimulate cell proliferation and survival (34). Receptor dimers engaging IR-A can also bind the mitogen IGF-II with a high affinity. IGF-II
is overexpressed in HCC notably as a result of loss of promoter specific imprinting and reactivation of fetal promoters (14). Consistently, IGF-II mRNA expression was increased in 17% of IR-A expressing HCC in our collection. Therefore, receptor dimers engaging IR-A can also support HCC lesions to prosper in a high-IGF-II environment.

Increasing evidence indicates that alternative splicing is subject to regulation by extracellular stimuli such as ligands for RTKs. During the preparation of this manuscript, Zhou and colleagues have reported that massive reprogramming of alternative splicing is induced upon EGF treatment in HEK293T cells (18). In these cells, EGF-induced mRNA splicing was efficiently blocked with PI3K/AKT inhibitors while inhibition of other pathways (such as the MEK pathway) had much lesser effects. Here, we demonstrate that the stimulation of EGFR activity with exogenous ligands or with autocrine amphiregulin loop enhances IR-A mRNA splicing through MEK activation in HCC cells. In full agreement with these results, the two cell lines that exhibit a high basal level of ERK phosphorylation controlled by EGFR, namely HuH6 and PLC/PRF5 cells, have the highest ratio of IR-A:IR-B. The finding that the EGFR/MEK pathway promotes aberrant IR-A splicing in HCC cells makes logical sense in relation to cellular transformation as this pathway is frequently dysregulated in human HCC (28) and participates in liver carcinogenesis in animal models (35, 36). Consistently, high serum levels of EGF are associated with increased HCC risk in patients with cirrhosis (37, 38). EGFR ligands are overproduced by HCC tumours and also by cells from tumour environment such as cirrhotic hepatocytes, hepatic fibroblasts and inflammatory cells (29, 39-41). Taken together, these data suggest that EGFR activation of IR-A splicing in HCC cells may result not only from autocrine but also from paracrine stimulatory loops involving EGFR ligands and that tumour environment could play a prominent role in this process. Of particular note, while normal hepatocytes are known to be responsive to EGFR ligands with respect to proliferation (42), EGFR ligands could not
promote the IR-B to IR-A shift in these cells. These data suggest that the intracellular mechanisms coupling EGFR stimulation to IR-A production are specific of transformed hepatocytes.

The involvement of EGFR in the control of alternative splicing remains largely uninvestigated in cancer in general and in HCC in particular. A recent study has revealed that the activation of EGFR promoted the splicing of the oncogenic variant ΔEx2p73, a dominant-negative inhibitor of p53/p73 (43). Our study identifies IR-A isoform as a novel target of EGFR splicing function in HCC. Our findings may also have broader implications given that the activation of EGFR is increased in other tumours such as colon carcinoma. Consistently, preliminary data from ours show that the blockage of EGFR signalling markedly reduced the IR-A:IR-B ratio in SW480 and LoVo colon cancer cell lines (Supplementary Figure 6).

Studies conducted with IR minigene constructs have shown that CUGBP1, hnRNPH and hnRNPA1 bind to exonic and intronic splicing silencer elements, which affects the selection of splice sites and favours IR exon 11 skipping (19-21). The overexpression of these splicing factors significantly alters the expression of IR splice variants in disease. Thus, in myotonic dystrophy type 1 (DM1), the overexpression of CUGBP1 and hnRNPH is responsible for aberrant IR-A splicing in myoblasts (19, 20). So far, CUGBP1, hnRNPH and hnRNPA1 have been found to be overexpressed in HCC (44-46). Our data support these findings and highlight the contribution of CUGBP1, hnRNPH and hnRNPA1 in the promotion of IR-A splicing in HCC cells. Our data also identify hnRNPA2B1 as a new player promoting the exclusion of exon 11 from the IR pre-mRNA. The correlations we report between the expression of splicing factors and that of IR-A in HCC tumours support the role of abnormally expressed splicing factors in the production of IR-A in HCC.

SRp20 was undetectable in human HCC tumours. Recently, SRp20 has been identified as crucial for hepatocyte differentiation and metabolic function in mice with hepatocyte-
specific deletion (47). SRp20 also antagonizes CUGBP1-mediated repression of exon 11 inclusion in minigene experiments and it seems that the SRp20:CUGBP1 relative ratio dictates the degree of exon inclusion (16). In this context, the loss of expression of SRp20 might be a characteristic of the dysfunction and transformation of hepatocytes and could promote the activity of CUGBP1 which is overexpressed in HCC.

We observed that the siRNA-mediated knockdown of SF2/ASF and hnRNPF led to different effects on endogenous IR mRNA splicing, depending on the HCC cell lines. Both siRNA were without effect in PLC/PRF5 cells while they induced a marked decrease of the IR-A:IR-B ratio in the three other cell lines. In apparent contradiction with these data, siRNA against SF2/ASF or hnRNPF increased the IR-A:IR-B ratio in HepG2 cells transfected with a IR minigene (16, 21). These discrepancies may reflect differences in the experimental approaches (endogenous transcript versus minigene). Indeed, in the same study, the down-regulation of hnRNPF was without effect on endogenous IR-A:IR-B ratio in HepG2 cells (21). In other cell contexts, SF2/ASF has been identified as an oncoprotein (48) and as a promoter of exon skipping (49-51). Therefore, it is not excluded that SF2/ASF may favour exclusion of IR exon 11 and thus promote the expression of IR-A in HCC.

To date, knowledge of the mechanisms leading to changes in splicing factor concentrations is limited and the contribution of EGFR-dependent pathways in these processes remains unexplored. Our data reveal that, except hnRNPF, EGFR activation mediates CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1 and SF2/ASF up-regulation at mRNA and protein levels in HCC cells. The blockade of EGFR signalling affected neither their phosphorylation profile nor their subcellular localization indicating that EGFR predominantly regulates splicing factor expression at a transcriptional level. The detailed mechanisms through which EGFR stimulates splicing factor expression remain to be studied.
In conclusion, the present study shows that the dysregulation of the alternative splicing of IR toward a proliferative phenotype is a frequent event in HCC. The underlying mechanism has been elucidated and connects the activation of EGFR to the regulation of a variety of splicing factors (Figure 7). These findings expand our knowledge of the oncogenic mechanisms activated by EGFR in HCC and provide mechanistic support to the hypothesis that hyperinsulinemia participates to the development of HCC. They also provide a strong molecular rationale to ongoing clinical trials that target IR/IGF-1R RTK in HCC and open new strategies in modulating IR-A splicing therapeutically.
Acknowledgments

The authors thank Cancer-Est Tumour Bank for providing human liver tissues.
References


Legends to figures

Figure 1. IR status in human HCC tumours.

(A) qPCR analysis of total IR mRNA expression in 42 human paired HCC (T) / non-tumour (NT) liver tissue samples (tissue collection #1) and 11 healthy liver tissue samples (Cont). (B) Representative Western blot showing IR overexpression in HCC. (C) Total IR protein levels measured by ELISA in 27 paired T/NT liver tissue samples. (D) Representative semi-quantitative PCR analysis showing IR-A induction in HCC. (E) qPCR evaluation of IR-A mRNA expression in T/NT liver tissue collection. (F) Relative IR-A:IR-B ratio in T/NT liver tissue collection. Statistical analysis: NT versus Cont, Mann-Whitney test for unpaired values, $p$ is not significant (NS); T versus NT: Wilcoxon test for paired values, $^* p < 0.05$, $^{***} p < 0.001$.

Figure 2. IR status in human HCC cell lines.

(A) Representative blot of three independent experiments showing IR expression in normal human hepatocytes and HCC cell lines. (B) RTK Profiler array showing IR, IGF-1R and EGFR tyrosine phosphorylation in Hep3B and HuH6 cells cultured in complete medium for 24 h. Green boxes delineate control spots. (C) Proliferation of hepatocytes and HCC cells in response to insulin evaluated by [³H]thymidine incorporation into DNA (left) and by real-time impedance measurements (right). (D) Proliferation of hepatocytes and HCC cells in response to IGF-II using the same techniques. (E) Representative image of three independent experiments showing IR-A and IR-B mRNA isoforms in normal human hepatocytes and HCC cell lines by semi-quantitative PCR and agarose gel electrophoresis. (F) IR-A:IR-B ratio in HCC cell lines relative to normal hepatocytes. Statistical analysis: n = 3-6 in duplicate; Student’s $t$-test; $^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$. 
Figure 3. EGFR-dependent signalling increases IR-A mRNA splicing in HCC cells.

(A) HuH6 cells were treated for 24 h with inhibitors against EGFR (gefitinib, 2.5 μM), MEK (U0126, 5 μM), PI3K (LY294002, 10 μM), AKT (Inh AKT VIII, 5 μM), mTOR (rapamycin, 1 μM), JNK (SP600125, 5 μM) or STAT3 (WP1066, 1 μM). The relative IR-A:IR-B ratio was evaluated by quantitative (left) and semi-quantitative (right) PCR. (B) Effect of treatment duration with gefitinib (2.5 μM) on the IR-A:IR-B ratio in HuH6 cells. (C) HuH6 cells were transiently transfected with 100 nM of control siRNA (siCont) or EGFR siRNA (siEGFR). After 72 h, the IR-A:IR-B ratio was analysed by quantitative (left) and semi-quantitative (right) PCR. (D) HuH6 cells were transiently transfected with 100 nM of control siRNA (siCont) or amphiregulin siRNA (siAR) and the IR-A:IR-B ratio were analysed by qPCR. (E) Effect of treatment duration with U0126 (5 μM) on the IR-A:IR-B ratio in HuH6 cells. (F) HuH6 and PLC/PRF5 cells were transiently transfected with 2.5 μg of a plasmid coding a dominant-negative RAF-1 mutant (RAF-C4) or of a control empty plasmid for 72 h. The IR-A:IR-B ratio was analysed using qPCR. (G) Hep3B, HuH7 and hepatocytes were treated for 24 h with EGFR ligands (AR, EGF or HB-EGF at 50 ng/ml) in the presence or absence of the MEK inhibitor U0126 (5 μM) and the IR-A:IR-B ratio was analysed by quantitative (upper) and semi-quantitative (lower) PCR. Statistical analysis: n = 3 in duplicate; Student’s t-test; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4. Splicing factors from CELF, hnRNP and SR families are overexpressed in HCC cells and favor IR-A mRNA expression.

(A) Representative blots (left) and quantifications (right) showing splicing factor protein levels in HCC (T) and surrounding non-tumour tissue (NT) from 27 paired liver specimens (collection #1). (B) qPCR analysis of splicing factor mRNA levels in 42 paired T/NT liver tissues (collection #1). (C) Representative analysis (n = 2) of splicing factor protein levels in
normal human hepatocytes and HCC cell lines by Western blotting. (D) HuH6, PLC/PRF5 and Hep3B cells were transiently transfected with 100 nM of specific siRNA against CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, hnRNPF or SF2/ASF or with a control siRNA (siCont) for 72 h and protein levels were examined by Western blotting. (E) Using qPCR, the IR-A:IR-B ratio was analysed in cells transiently transfected with control siRNA or with siRNA targeting splicing factor mRNA. Statistical analysis: n = 3; Student’s t-test; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. Splicing factors are involved in the stimulatory effect of EGFR ligands on IR-A expression and are under the control of EGFR signaling.

(A) HuH7 cells were transiently transfected with 100 nM of specific siRNA against CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, hnRNPF or SF2/ASF or with a control siRNA (siCont) for 72 h. For the last 24 h, cells were incubated with or without EGFR ligands (50 ng/ml) and the IR-A:IR-B ratio was analysed by qPCR. (B) HuH6 cells were transiently transfected with 100 nM of EGFR siRNA or control siRNA for 72 h or treated with or without gefitinib (2.5 μM). The expression of splicing factors was analysed by qPCR. (C) Representative blots of three independent experiments showing down-regulation of CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1, and SF2/ASF protein levels upon inhibition of EGFR signalling with EGFR siRNA or gefitinib in HuH6 cells (left) and blot quantifications (right). (D) HuH6 cells were transiently transfected with 100 nM of EGFR or control siRNA for 72 h or treated with or without gefitinib (2.5 μM). Cell extracts were then treated or not with alkaline phosphatase and analysed by Western blotting. Statistical analysis: n = 3; Student’s t-test; *p < 0.05, **p < 0.01 compared to controls; ###p < 0.001 compared to cells stimulated with an EGFR ligand.
Figure 6. Alteration of IR mRNA splicing during liver carcinogenesis but not during liver regeneration in rat.

(A) The IR-A:IR-B ratio (left) and the total IR mRNA content (right) were evaluated by qPCR in 5 paired HCC (T) / non-tumour (NT) liver tissue samples obtained from rats weekly *i.p* injected with DEN during 12 weeks. (B) qPCR analysis of splicing factor mRNA levels in rat T/NT liver tissues. (C) The relative IR-A:IR-B ratio (left) and the total IR mRNA (right) content were evaluated by qPCR in liver tissue samples from rats 1 day (n = 5), 3 days (n = 4) and 10 days (n = 6) after partial hepatectomy (PH) and compared to baselines (Day 0). (D) qPCR analysis of splicing factor mRNA levels in rat regenerating liver tissues. Statistical analysis: Wilcoxon test for paired values, *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 7. Model for the regulation of IR pre-mRNA alternative splicing by EGFR in HCC cells.

Upon ligand binding, EGFR activation triggers an intracellular signalling cascade implicating MEK activation. This results in the stimulation of transcription of genes coding for different splicing factors namely CUGBP1, hnRNPH, hnRNPA1, hnRNPA2B1 and SF2/ASF. hnRNPF expression is not controlled by EGFR-dependent pathway. The interaction of splicing factors with IR pre-mRNA promotes IR exon 11 alternative splicing. As a consequence, the expression of IR-A isoform is increased to the detriment of IR-B, which allows the transmission of proliferative signals in response to insulin and IGF-II.
Mitogenic insulin receptor-A is overexpressed in human hepatocellular carcinoma due to EGFR-mediated dysregulation of RNA splicing factors

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Cancer Res Published OnlineFirst April 30, 2013.

Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-3824

Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/04/29/0008-5472.CAN-12-3824.DC1

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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