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Short Communication

Overexpression of Insulin Receptor Substrate-1 Emerges Early in Hepatocarcinogenesis and Elicits Preneoplastic Hepatic Glycogenosis

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Insulin receptor substrate-1 (IRS-1) is a multisite docking protein occupying a central position in signaling cascades stimulated by a number of growth factors including insulin. Using Western blotting and immunohistochemistry, we investigated the expression of IRS-1 in more than 400 preneoplastic foci of altered hepatocytes and in 12 hepatocellular carcinomas induced in rats by oral administration of *N*-nitrosomorpholine. In both *N*-nitrosomorpholine-treated and untreated rat livers, IRS-1 was demonstrable by Western blotting, but with the exception of a few single hepatocytes it was not detectable in the normal parenchyma by immunohistochemistry. In contrast, immunohistochemistry revealed that IRS-1 was strongly expressed in the majority of foci of altered hepatocytes particularly in approximately 97% of the clear/acidophilic and mixed cell foci showing excessive storage of glycogen (glycogenosis). In glycogen-poor basophilic foci of altered hepatocytes and hepatocellular carcinomas, IRS-1 was not detected by immunohistochemistry, but a weak expression was observed in small subpopulations of three hepatocellular carcinomas containing remnants of glycogen. These results indicate that the focal overexpression of IRS-1 is an early event in hepatocarcinogenesis, which is closely correlated with preneoplastic hepatic glycogenosis. During progression from glycogenotic foci to hepatocellular carcinomas, IRS-1-overexpression is gradually down-regulated, and this late event is associated with a fundamental metabolic shift leading to the malignant neoplastic phenotype. *(Am J Pathol 1998, 152:341–345)*

Early changes in energy metabolism including enzymatic alterations associated with an excessive storage of glycogen (glycogenosis) in foci of altered hepatocytes (FAH) are a hallmark of experimental[1] and human[2,3] hepatocarcinogenesis elicited by various oncogenic agents such as chemicals, viruses, and radiation.[4] Progression from preneoplastic hepatocellular glycogen storage foci (GSF) to hepatocellular neoplasms is characterized by additional enzymatic changes indicating a preferential channeling of glucose toward the pentose phosphate pathway and glycolysis.[5] This metabolic shift is accompanied by a gradual decrease of the glycogen initially stored in excess and an increase in (basophilic) ribosomes[1] and cell proliferation[6] leading via mixed cell foci (MCF, composed of glycogenotic and basophilic cells) to the basophilic neoplastic phenotype. Biochemical studies *in situ* and in laser-dissected focal preneoplastic liver lesions revealed a metabolic pattern in the glycogenotic foci that mimics a response to insulin.[7,8] This notion has been corroborated by the observation that a focal hepatic glycogenosis and hepatocellular neoplasms may be induced directly in diabetic rats by intrahepatic transplantation of pancreatic islets hypersecreting insulin under these conditions.[9,10]

Insulin is a pleiotropic hormone exerting metabolic and mitogenic effects on the hepatocytes, which are mediated by a signal transduction cascade engaging insulin receptor substrate-1 (IRS-1) as a principal intracellular substrate of the insulin receptor tyrosine kinase.[11] Overexpression of IRS-1 in hepatocellular carcinoma[12,13] (HCC) and transforming activity of IRS-1-overexpression in hepatic[14] and nonhepatic[15] cell lines have been reported, but the expression of IRS-1 in early stages of hepatocarcinogenesis *in situ* has not been investigated. Using an immunohistochemical approach, we studied IRS-1-expression in preneoplastic GSF, MCF and basophilic cell foci (BCF), and in HCC induced in rat liver by *N*-nitrosomorpholine (NNM).

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Table 1. Expression of IRS-1 in Different Types of Preneoplastic and Neoplastic Liver Lesions Induced in Rats By *N*-Nitrosomorpholine

Type of liver lesion	Incidence of liver lesions	Number of liver lesions	Glycogen content (periodic acid-Schiff reaction)	IRS-1 positive lesions	
				Number	Immunoreaction
Glycogen storage focus	9/9	93	+++	89	+++
Mixed cell focus	9/9	330	++ to +	321	++ to +
Basophilic cell focus	3/9	6	−	0	−
Hepatocellular carcinoma	10/13	12	++ to −	3	+ to −

Intensity of IRS-1 and periodic acid-Schiff reaction: +++, strong; ++, moderate; +, weak; −, negative.
Animals with hepatocellular carcinomas were sacrificed between 18 and 26 weeks after withdrawal of *N*-nitrosomorpholine.

Materials and Methods

Animals and Tissue Sampling

Preneoplastic hepatocellular lesions were induced in adult, male Sprague-Dawley rats by limited oral exposure (stop model) to 12 mg of NNM per kg of body weight for 7 weeks as described previously. Tissue of 9 NNM-treated and 9 untreated animals was obtained 15 and 20 weeks after withdrawal of NNM. Detailed studies on the dose and time dependence of the cellular phenotype in hepatic preneoplasia and neoplasia were performed on the same tissue previously.[16] HCCs were produced by oral administration of the same dose of NNM for 10 weeks. Between 18 and 26 weeks after withdrawal of NNM, 10 of 13 animals developed HCC compared with 0 of 13 controls. Two of the HCC-bearing rats contained two separate neoplasms each so that a total of 12 HCC were available. Liver tissue was snap-frozen at −150°C and stored at −80°C. Six-μm serial cryostat sections were stained with hematoxylin and eosin or treated with periodic acid-Schiff reaction for the demonstration of glycogen and counterstained with toluidine blue. More than 400 FAH and 12 HCCs were investigated and were classified according to published criteria. FAH were subclassified into GSF, MCF, and BCF.[4] With the exception of one small GSF in one animal, no FAH were found in untreated controls. Additional details of the incidence, number, and classification of the liver lesions are given in Table 1.

Western Blot Analysis

Freeze-dried liver tissue was lysed in a buffer containing 50 mmol/L Tris-buffered saline (pH 7.4), 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium desoxycholate, 1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 2.5% sodium dodecyl sulfate, and 5% 2-mercaptoethanol. To remove cell debris, two centrifugations at 15,000 × *g* for 10 minutes at 4°C followed. After 5 minutes of boiling, a polyacrylamide gel electrophoresis was performed using 4 to 15% gradient gels. Protein separation and transfer to a nitrocellulose membrane (Hybond C©, 0.45 μm, Amersham, Little Chaltfont, England) were achieved by means of the PhastSystem© (Pharmacia, Uppsala, Sweden). Subsequently, the membrane was treated with 3% nonfat dried milk in phosphate-buffered saline for 20 minutes, incubated with 3 μg/ml anti-IRS-1 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) for 30 minutes at room temperature (RT) and an additional 24 hours at 4°C, and followed by 1.2 μg/ml alkaline phosphatase-labeled goat anti-rabbit IgG (90 minutes, RT; Jackson ImmunoResearch, West Grove, PA). After treatment with 0.05% Tween 20 in phosphate-buffered saline for 15 minutes, bands were visualized with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Bachem, Heidelberg, Germany). The antibody used specifically binds a protein from rat liver, which has a molecular mass of approximately 165 kd (Figure 1, lanes A to D). This is within the range of 165–180 kd reported for IRS-1 by several groups in different tissues.[11] For positive control, 3T3 cell lysate (Upstate Biotechnology) was used (Figure 1, lane A). Lysate of preneoplastic liver tissue (Figure 1, lane C) was obtained from NNM-treated rats containing FAH in a parenchymal fraction of less than 5%.[16] In this cell lysate derivatives of the extrafocal tissue by far exceeded those of the preneoplastic FAH. In contrast, lysate of HCC (Figure 1, lane D) was predominantly derived from neoplastic cells, which were intermingled with stromal but not with parenchymal liver cells.

Immunohistochemistry

Six-μm cryostat sections were mounted on poly-L-lysine-coated slides (Sigma, St. Louis, MO), fixed in 100% methanol (15 minutes, −20°C), blocked with goat serum 1:30 in Tris-buffered saline, and incubated with 10 μg/ml anti-IRS-1 antibody for 30 minutes at RT followed by an additional 24-hour incubation at 4°C. After each following step, sections were rinsed with 0.05 mol/L Tris buffer (pH 7.4) twice for 3 minutes. Eighteen μg/ml goat anti-rabbit IgG (Jackson ImmunoResearch) followed by a 1:10-diluted rabbit alkaline phosphatase-anti-alkaline phosphatase complex (Sigma) were applied in two cycles. After washing with distilled

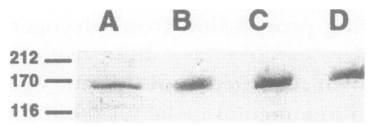

Figure 1. Lanes A to **D**, Western immunoblot analysis demonstrating 165-kd insulin receptor substrate-1 in untreated (**lane B**) and *N*-nitrosomorpholine-treated (**lanes C** and **D**) rat liver tissue homogenates. Aliquots (10 μg) of total cellular protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and probed with anti-IRS-1 polyclonal antibody; bound antibody was detected with alkaline phosphatase-labeled goat anti-rabbit IgG. **lane A**, 3T3 cells (positive control); **lane B**, untreated rat liver; **lane C**, preneoplastic rat liver; **lane D**, hepatocellular carcinoma; **left**, molecular weight (kd).

water for 5 minutes, sections were stained with new fuchsin. Blocking of the endogenous alkaline phosphatase was achieved by adding 1.73 mmol/L levamisole (Sigma). Some of the sections were counterstained with 1% cuprolinic blue (BDH, Poole, England). Finally, sections were covered with glycerol gelatin.

Controls

To verify the specificity of the immunostaining, the primary antibody was substituted in serial sections with 10 μg/ml affinity-purified rabbit IgG (Jackson ImmunoResearch). In addition to this substitution control, a liquid adsorption control was conducted in serial sections after preincubation of anti-IRS-1 antibody with its corresponding immunizing peptide (rat IRS-1 peptide, Upstate Biotechnology) at a mass ratio of 1:10 g/g for 3 hours at RT. Both controls were negative (Figure 2).

Results

IRS-1 protein was clearly demonstrable in both the untreated (Figure 1, lane B) and the NNM-treated rat livers (Figure 1, lanes C and D) by Western blot analysis. With the exception of a few single hepatocytes, IRS-1 was not detected in the normal liver parenchyma of treated and untreated animals by immunohistochemistry. This was apparently due to the very low level of IRS-1 in normal hepatocytes.[17] In contrast, the vast majority of preneoplastic foci of altered hepatocytes including the one small GSF found in one control animal showed a more or less pronounced immunoreactivity for IRS-1 (Table 1). The immunoreaction was limited to the cytoplasm sometimes showing a striking perinuclear localization. IRS-1-expression was particularly pronounced in the early appearing GSF (Figure 2, A and B). From the 93 GSF observed in NNM-treated rats, 96% were IRS-1-positive. In the somewhat later developing MCF, IRS-1 was also clearly demonstrable by immunohistochemistry (Figure 2, E and F). Ninety-seven percent of the 330 MCF observed were positive. However, expression of IRS-1 was less pronounced in MCF compared with GSF. The extent of IRS-1-immunoreactivity closely correlated with the number of glycogen-storing cells within the FAH. The immunoreactivity decreased with increasing replacement of glycogenotic by glycogen-poor basophilic cells. FAH composed exclusively of basophilic cells were rare, but all of the 6 BCF found were negative for IRS-1 (Figure 2, G and H). The same applies to the majority of the 12 HCCs investigated in which glycogen-poor basophilic cells prevailed (Table 1; Figure 2, I and J). Only in 3 HCCs, a weak staining for IRS-1 was observed, but immunoreactivity was limited to highly differentiated cellular subpopulations still containing some glycogen (Figure 2, K and L).

The possibility that the strong association between IRS-1-expression and glycogen accumulation might be due to an unspecific binding of the antibody to the glycogen molecule has been excluded by the following observations. Although considerable amounts of glycogen were also stored in the extrafocal hepatocytes of NNM-treated animals (Figure 2A) and in the parenchyma of untreated control animals, the immunoreaction for IRS-1 was invariably negative in these parenchymal portions (Figure 2B). Moreover, glycogen was eluted during the immunohistochemical procedure, particularly during the large number of washing steps. This was verified by the application of both the immunoreaction and the periodic acid-Schiff reaction in the same sections. When the periodic acid-Schiff reaction was conducted after the immunoreaction, all IRS-1 positive foci were completely devoid of glycogen.

Discussion

The increased immunoreactivity for IRS-1 in the majority of FAH and some HCC may in principle result from an enhanced expression of the mRNA of IRS-1 or from a reduced degradation of the protein, but it has been reported by several authors that a high protein level of IRS-1 is associated with an abundance of the respective mRNA.[17,18]

Our results show that overexpression of IRS-1 is an early event in hepatocarcinogenesis, which closely correlates with the accumulation of glycogen in GSF and MCF. This correlation applies not only to the increased expression of IRS-1 in glycogenotic cells of GSF and MCF but also to the largely concomitant reduction of both IRS-1-overexpression and glycogen accumulation during the appearance of dedifferentiated basophilic cells in MCF, BCF, and hepatocellular neoplasms. In the few HCC that showed an inconsistent weak IRS-1-positivity there were always some remaining glycogenotic cells in the same cellular subpopulations. The gradual decrease of immunoreactivity with increasing replacement of glycogenotic by glycogen-poor basophilic cells indicates a fundamental metabolic shift during progression from early to late FAH in line with previous findings.[4,5]

The results reported strongly suggest that the phenotype of the preneoplastic hepatic glycogenosis is elicited by an overexpression of IRS-1 that represents a major component in insulin signaling, mediating pleiotropic effects in cell metabolism, proliferation, and differentiation. IRS-1 initiates a signal transduction cascade leading to glycogen synthesis via the mitogen-activated protein kinase pathway. Glycogenesis is achieved by several phosphorylation steps resulting in the activation of protein phosphatase 1, which in turn activates glycogen synthase but inactivates glycogen phosphorylase by dephosphorylation.[19] These effects of IRS-1-signaling are in accordance with the maintenance or increase of glycogen synthase activity and the marked reduction in glycogen phosphorylase activity previously demonstrated in GSF by enzyme histochemistry.[5,9] Cytochemical and/or microbiochemical approaches also revealed a number of other enzymatic changes, such as a reduction in the activities of glucose-6-phosphatase and adenylate cyclase, and an increase in the activities of pyruvate kinase and glucose-6-phosphate dehydrogenase in GSF,[4,5,9] which indicates a metabolic pattern resembling the response of hepatocytes to insulin.[8] In addition to these metabolic changes, a slight but significant increase in

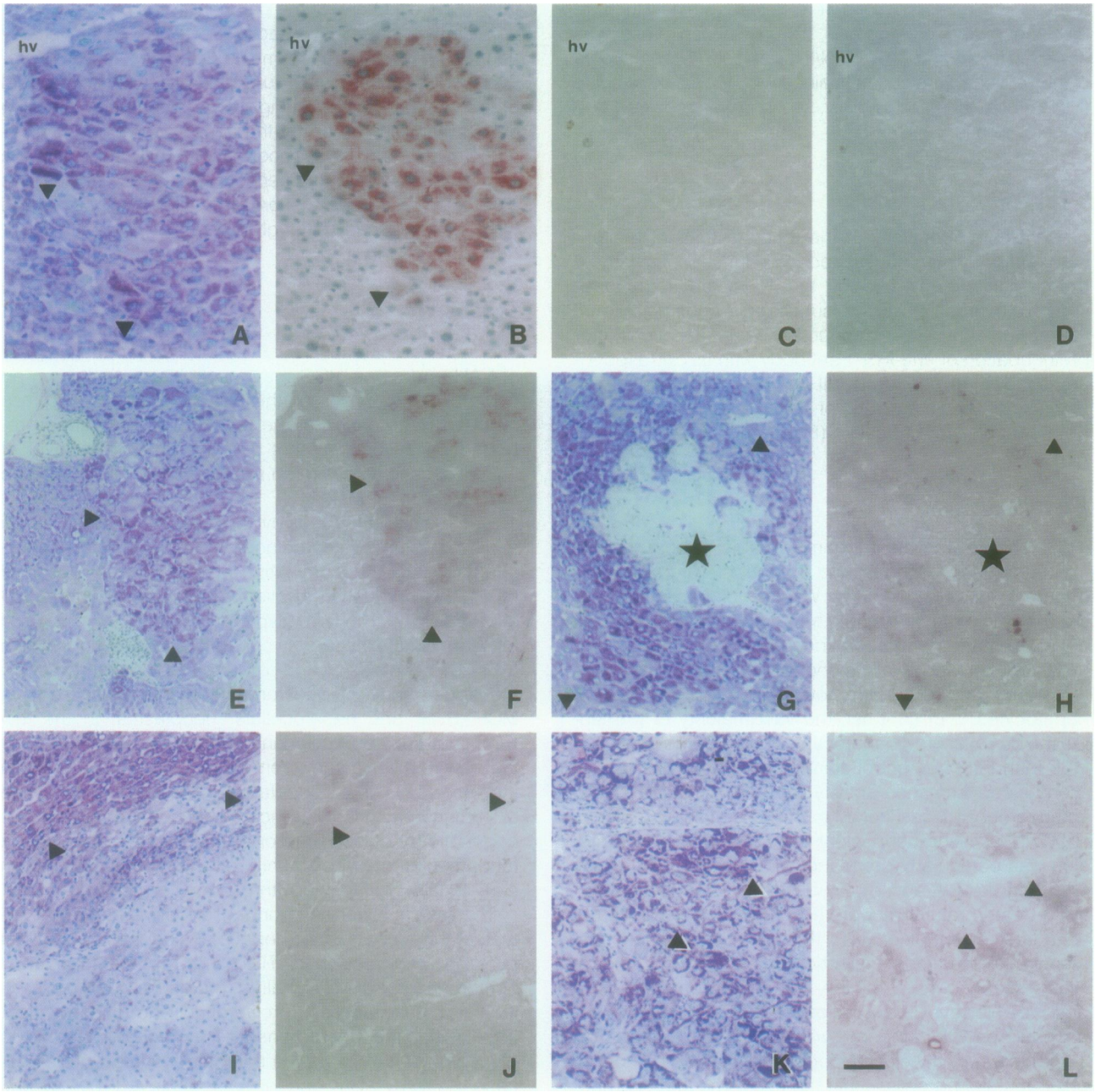

Figure 2. A to **L**: Correlation of glycogen content as demonstrated by the periodic acid-Schiff reaction (**A, E, G, I,** and **K**) with the expression of insulin receptor substrate-1 (IRS-1) detected *in situ* with anti-IRS-1 rabbit polyclonal antibody (**B, F, H, J,** and **L**). The specificity of the immunoreaction (alkaline phosphatase-anti-alkaline phosphatase method) was verified by substitution (**C**) and liquid adsorption control (**D**) in serial cryostat sections of preneoplastic (**A** to **H**) and neoplastic (**I** to **L**) hepatocellular lesions induced in rats with *N*-nitrosomorpholine. **A:** Preneoplastic glycogenotic focus (▼) showing pronounced positive immunoreactivity for IRS-1 in contrast to the negative reaction in the surrounding tissue (**B**, counterstained with cuprolinic blue), in the substitution (**C**), and in the liquid adsorption controls (**D**). **E:** Mixed cell focus (▼) composed of glycogenotic and glycogen-poor cells showing immunoreaction for IRS-1 (**F**). **G:** Glycogen-poor, basophilic cell focus (★) within a glycogenotic focus (▼) associated with positive immunoreaction for IRS-1 in glycogenotic (▼) but negative reaction in glycogen-poor (★) cell population. **I:** Portion of a glycogen-poor, basophilic hepatocellular carcinoma adjacent to preneoplastic mixed cell focus (▼) associated with negative immunoreaction for IRS-1 in the HCC (**J**) but weak immunoreaction in the preneoplastic cell population (▼). **K:** Portion of hepatocellular carcinoma containing relatively large amounts of glycogen including some prominent glycogenotic (clear) cells (▼), showing immunoreaction for IRS-1 (**L**), particularly in glycogenotic cells (▼). Magnification, ×64; 100-μm bar; **hv**, hepatic venule.

cell proliferation occurs in GSF compared with the surrounding parenchyma,[6] which is also compatible with a response to an insulinomimetic effect.[8,20]

Recently, Dombrowski et al[9,10] provided evidence for a direct induction of preneoplastic hepatic glycogenosis and hepatic neoplasms by low-number intrahepatic transplantation of pancreatic islets hypersecreting insulin in diabetic rats. It is noteworthy in this context that patients suffering from diabetes mellitus have a significantly increased risk of developing HCC.[21] A hepatic glycogenosis associated with poorly controlled diabetes mellitus has been known for decades[22] but has not been related to human hepatocarcino-

genesis, although the exceedingly high risk of patients with inborn hepatic glycogenosis to develop hepatocellular neoplasms is well established.[4,5]

We have previously shown in different experimental models that the progression from GSF to MCF and hepatocellular neoplasms is regularly accompanied by additional metabolic changes such as an increase in glycolytic enzymes, a reduction of the glycogen initially stored in excess, and a further increase in the activity of glucose-6-phosphate dehydrogenase.[4,5,7] At the same time there is an increasing cell proliferation, which is inversely correlated with the gradual decrease of glycogen.[6] In this report we demonstrate that the initial overexpression of IRS-1 is down-regulated during the progression from the preneoplastic glycogenotic to the malignant neoplastic phenotype. This finding is in accordance with the reduction in glycogen initially stored in excess, but it does not explain the ever increasing cell proliferation. It has been shown by others, however, that insulin-like growth factor II, which is produced in large amounts in the liver during embryogenesis but only at very low levels in mature hepatocytes, is re-expressed during cellular dedifferentiation in late stages of chemical and viral hepatocarcinogenesis.[23] This is the very time point at which a marked expression of transforming growth factor-α has been observed in focal lesions developing in chemical[24] and hormonal[10] hepatocarcinogenesis. Growth stimulation by such growth factors is preferentially or exclusively mediated by IRS-1-independent pathways that have not been completely clarified. It is, thus, conceivable that an autocrine or paracrine activation of alternative signaling pathways by growth factors such as insulin-like growth factor II or transforming growth factor-α replaces IRS-1-signaling at this stage of hepatocarcinogenesis without exerting insulinomimetic effects on glycogen metabolism. The cause of the IRS-1 overexpression in preneoplastic hepatic foci and some highly differentiated subpopulations of HCCs remain to be clarified. The elucidation of this early event may provide a key for the further understanding of the mechanism of hepatocarcinogenesis and for the development of preventive measures. This notion is underlined by the recent finding that the transfection of a human hepatocellular carcinoma cell line with a dominantly expressed truncated type of IRS-1 reversed the malignant neoplastic phenotype to normal.[25]

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