Research Article

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Molecular and metabolic changes in human liver clear cell foci resemble the alterations occurring in rat hepatocarcinogenesis

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Background & Aims: Activation of the AKT/mTOR and Ras/MAPK pathways and the lipogenic phenotype occurs in both a rat model of insulin-induced hepatocarcinogenesis and in human hepatocellular carcinoma (HCC). In the rat model, activation of these pathways is evident within the earliest morphologic detectable alterations, i.e., clear cell foci (CCF) of altered hepatocytes. CCF have also been described in the human liver, but molecular and metabolic alterations within these foci remain to be determined.
Methods: A collection of human liver specimens was examined using electron microscopy, histology, enzyme- and immunohistochemistry, and molecular analysis. Human data were compared to rat preneoplastic CCF and HCC induced by *N*-nitrosomorpholine administration.
Results: CCF occurred in ~33% of extrafocal tissues of human non-cirrhotic livers. Electron microscopy showed massive glycogen storage within CCF, largely due to the reduced activity of the glycogenolytic enzyme glucose-6-phosphatase. Hepatocytes in CCF overexpressed the insulin receptor and glucose transporter proteins. AKT/mTOR and Ras/MAPK pathways as well as enzymes of glycolysis, *de novo* lipogenesis, beta-oxidation, and cholesterol synthesis were upregulated, both in human CCF, and in CCF and HCC of *N*-nitrosomorpholine-treated rats. The Ki-67 proliferation index was 2-fold higher in human CCF than in extrafocal tissue.
Conclusions: The high degree of similarity between human CCF and pre-neoplastic lesions from experimental models of hepatocarcinogenesis in terms of morphologic, molecular and metabolic features suggests a low-grade dysplastic nature of these lesions in human non-cirrhotic livers.

Keywords: Preneoplasia; Insulin receptor; Glycogen storage; Lipogenesis; Proliferation.
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Abbreviations: ACAC, acetyl-coenzyme A carboxylase; ACADM, acyl-CoA dehydrogenase for acyl chains of medium length; ACLY, ATP citrate lyase; AKR1B10, aldo-keto reductase family 1, member B10; AKT, v-akt murine thymoma viral oncogene homolog; AMPK, AMP-activated kinase; chREBP, carbohydrate-responsive element binding protein; CCC, cholangiocellular carcinoma; CCF, clear cell foci; DNA, deoxyribonucleic acid; ERK 1/2, extracellular related kinase; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; FASN, fatty acid synthase; FAH, foci of altered hepatocytes; GLUT, glucose transporter; GSK-3β, glycogen synthase kinase 3β; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma; HNFα, hepatocyte nuclear factor α; HMGCoAR, 3-hydroxy-3-methylglutaryl-CoA-reductase; HUMARA, human androgen receptor gene; IGLK, glucokinase; IRS1, insulin receptor substrate 1; L-FABP, liver fatty acid binding protein; MEK-1, MAP kinase kinase; MKP3, mitogen-activated protein kinase phosphatase 3; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NNM, *N*-nitrosomorpholine; NT, number target; PFKL, phosphofructokinase; PKM2, pyruvate kinase M2; RPS6, ribosomal protein S6; SCD, stearoyl-CoA desaturase; SREBP1, sterol regulatory element binding protein; SQS, squalene synthase; TSC2, tuberous sclerosis protein 2; USP2, ubiquitin-specific protease 2.

Introduction

Human hepatocellular carcinoma (HCC) is one of the most frequent malignant neoplasms and its incidence is rapidly rising in Western Europe and the United States [1]. In particular, it has been shown that 15–20% of HCCs occur in the non-cirrhotic liver [2]. All disorders which might cause liver cirrhosis may also trigger HCC development without liver cirrhosis. Indeed, HCC emerges in non-cirrhotic livers in several congenital disorders of liver cell metabolism, including α_1-antitrypsin deficiency and hemochromatosis. In glycogen storage type I disease, for instance, liver cirrhosis is typically absent and multiple hepatocellular adenomas (HCA) develop and may progress to HCC [3]. Furthermore, mounting epidemiologic evidence indicates that acquired metabolic disorders, such as obesity, hyperinsulinism, and type II diabetes mellitus, are implicated in HCC development as well [4,5]. Many of these cases occur in the non-cirrhotic liver [6,7], in the context of non-alcoholic steatohepatitis, whose potential role in hepatocarcinogenesis has been recently investigated [8,9].

In humans, only high grade dysplastic nodules in liver cirrhosis are accepted as preneoplastic lesions of HCC [10]. The development of HCC in the absence of pre-existing cirrhosis and dysplastic nodules remains poorly understood. Foci of altered hepatocytes (FAH) were initially investigated by Su and Bannasch [11], which were characterized by liver cirrhosis in most cases. The authors classified these foci in glycogen storing foci (i.e., clear

cell foci, CCF), mixed cell foci, and basophilic cell foci, suggesting that FAH represent pre-neoplastic lesions in the cirrhotic liver, since they were detected in almost all resections with HCC and liver cirrhosis.

In numerous animal models of hepatocarcinogenesis, several types of FAH have been described [12]. Their progression into hepatocellular neoplasms is well documented [13–15]. In the islet transplantation model of hepatocarcinogenesis [16–19], we recently found that both the v-akt murine thymoma viral oncogene homolog (AKT)/mammalian target of rapamycin (mTOR) and the Ras/mitogen activated protein kinase (MAPK) pathways are continuously activated from CCF – representing the earliest lesions – to HCC and play a major oncogenic role as downstream effectors of the insulin signal cascade [20–22]. Simultaneous increased lipogenesis [22] is known as the lipogenic phenotype. Of note, induction of AKT/mTOR and Ras/MAPK pathways and the lipogenic phenotype similarly occur in human HCC and are associated with a dismal patient's prognosis [23,24].

Here, we investigated CCF in human non-cirrhotic liver tissue to unravel their oncogenic potential in a non-cirrhotic background. We compared their proliferation activity, protein expression patterns and enzyme activities to CCF from a chemical model of hepatocarcinogenesis in the rat, i.e., the *N*-nitrosomorpholine (NNM) model [12,13,25]. Our results indicate a remarkable similarity in terms of molecular and metabolic properties between human CCF and CCF and HCC from NNM-treated rats.

Materials and methods

Human samples

Liver specimens were obtained from a total of 262 resected, non-cirrhotic livers. Liver resection specimens showed cancer metastases (n = 173) or advanced primary liver tumors and tumor- like lesions (n = 77, Table 1). The presence of underlying viral hepatitis, hemochromatosis, alpha-1-antitrypsin deficiency, glycogenosis and severe alcoholism was excluded. Institutional Review Board was obtained at the local Ethical Committee of the Universitätsmedizin Greifswald.

Tissue processing, enzyme and immunohistochemistry

Human liver samples were processed for histology, enzyme and immunohistochemistry (Supplementary Table 2) and electron microscopy as previously reported [22,24–28].

Morphometric analysis

CCF were identified in sections stained with H&E and PAS as lesions of enlarged hepatocytes with pale cytoplasm in H&E staining [25], due to extensive glycogen storage, observed in the PAS. Corresponding lesions in enzyme- and immunostained sections were detected by comparison with H&E staining. Other types of FAH [12] were not further investigated. Volume fraction determination [29] and proliferative activity of CCF were evaluated in representative sections, randomly selected from 88 cases containing CCF. The degree of steatosis, steatohepatitis and fibrosis was determined for all liver specimens [30].

Rat liver tissues, treatment, and tissue processing

Three-month-old male Lewis rats (n = 30; 250–300 g body weight) were used. Animals received NNM 5 mg/kg body weight daily by oral application for three (n = 15) and six (n = 15) months, respectively, as previously reported [31]. Rats were housed, fed and treated according to the German Animal Protection Law, as described [17].

Table 1. Non-cirrhotic liver specimens (n = 262) and events of resection.

Liver metastases of	173
Colorectal cancer	98
Mammarian carcinoma	9
Neuroendocrine tumors	11
Pancreas carcinoma	10
Gallbladder carcinoma	6
Stomach carcinoma	8
Cholangiocellular carcinoma (extrahepatic)	4
Esophageal carcinoma	3
Ovarian carcinoma	5
Urothelial carcinoma	3
Renal cell carcinoma	2
Melanoma	2
Gastrointestinal stromal tumor	2
Lung carcinoma	2
Parotis carcinoma	1
Neuroblastoma	1
Carcinosarcoma of the uterus	1
Carcinoma of the thyroid gland	1
Cancer of unknown primary origin	4
Primary liver tumors/tumor-like lesions	77
Hepatocellular carcinoma	15
Cholangiocellular carcinoma	22
Hepatocellular adenoma	4
Bile duct adenoma	1
Hepatoblastoma	1
Angiosarcoma	1
Hemangioma	8
Focal nodular hyperplasia	9
Liver cyst	8
Liver abscess/necrosis	7
Scar	1
Other conditions	12

Quantitative RT-PCR (QRT-PCR)

Primers for human and rat *FASN*, *SREBP1*, *chREBP*, *SQS*, *PKM2*, and ribonucleic acid ribosomal 18S (*RNR-18*) genes were purchased from Applied Biosystems (Foster City, CA).

Kinase assays, assessment of fatty acid synthesis

AKT, MAPK, and RalA kinase activities were assessed with the AKT, p44/42 MAPK kinase (Cell Signaling Technology, Danvers, MA) and RalA G-LISA Activation Assay (Cytoskeleton Inc., Denver, CO) kits, respectively, following the manufacturer's protocols. Fatty acid synthesis was assessed as previously reported [24].

Analysis of clonality by use of the androgen receptor gene assay (HUMARA)

Laser microdissected CCF were analyzed for monoclonality at the *HUMARA* locus by PCR amplification as previously described [25,26].

Statistical analysis

Differences in volume fraction, proliferative activity of CCF, values of QRT-PCR, kinase assays, fatty acid synthesis assessment (Student's *t*, Tukey–Kramer's tests) and differences regarding clinical data (Student's *t*, Fisher's exact test) were considered significant if $p < 0.05$.