Adiponectin Modulates C-Jun N-Terminal Kinase and Mammalian Target of Rapamycin and Inhibits Hepatocellular Carcinoma

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BACKGROUND & AIMS: Epidemiological studies have shown that obesity is a risk factor for hepatocellular carcinoma (HCC). Lower adiponectin levels are associated with poor prognosis in obese HCC patients, hence it is plausible that adiponectin acts as a negative regulator of HCC. We investigated the effects of adiponectin on HCC development and its molecular mechanisms. **METHODS:** Assays with Huh7 and HepG2 HCC cells were used to examine the signal transduction pathways involved in the protective functions of adiponectin in HCC. These studies were followed by in vivo approaches using HCC xenografts and tumor analysis. Results from in vitro and in vivo findings were corroborated using human HCC tissue microarray and analysis of clinicopathological characteristics. **RESULTS:** Adiponectin increased apoptosis of HCC cells through activation of caspase-3. Adiponectin increased phosphorylation of c-Jun-N-terminal kinase (JNK) and inhibition of c-Jun-N-terminal kinase–phosphorylation inhibited adiponectin-induced apoptosis and caspase-3 activation. Adiponectin increased phosphorylation of 5′-adenosine monophosphate–activated protein kinase and tumor suppressor tuberous sclerosis complex 2 and inhibited mammalian target of rapamycin phosphorylation. Inhibition of 5′-adenosine monophosphate–activated protein kinase phosphorylation not only inhibited adiponectin-induced c-Jun-N-terminal kinase phosphorylation, but also blocked biological effects of adiponectin. Adiponectin substantially reduced liver tumorigenesis in nude mice. Importantly, analysis of adiponectin expression levels in tissue microarray of human HCC patients revealed an inverse correlation of adiponectin expression with tumor size. **CONCLUSIONS: Adiponectin protects against liver tumorigenesis; its reduced expression is associated with poor prognosis in obese patients with HCC.**

Keywords: Hepatocellular Carcinoma; Adiponectin; TSC2; mTOR.

Obesity is an important risk factor for numerous health problems, including carcinogenesis,[1] and its biological effects are mediated through adipocytokines.[2] Adiponectin is an adipocytokine,[3–6] the expression of which is reduced in obese/diabetic murine model *db/db* mice.[7] Plasma adiponectin levels have also been reported to be significantly reduced in obese humans[8] and in various disease states.[9] Adiponectin reduces tissue triglyceride content, upregulates insulin signaling,[10] and has direct antiatherosclerotic effects.[11] More recently, low levels of plasma adiponectin have been associated with many common forms of cancer.[2] Adiponectin receptors exist in 2 isoforms, AdipoR1 and AdipoR2; AdipoR2 has 67% homology (amino acids) with AdiopR1.[12] AdipoR2 is expressed most abundantly in liver.[13] These receptors mediate cellular functions of adiponectin through activation of various intracellular signaling pathways.[14] Several signaling molecules and pathways, such as 5′-adenosine monophosphate–activated protein kinase (AMPK), nuclear factor–κB, peroxisome proliferator–activated receptor-α, and p38 mitogen–activated protein kinase are known to mediate adiponectin induced signaling.[15–17]

Large population prevalence studies have shown that HCC is clearly associated with obesity.[18] A recent US study reported a great impact of obesity on HCC even after multivariate analysis. This study included 404,576 men and 495,477 women aged 30 years with a body mass index (BMI; calculated as kg/m^2) of 18.5 at enrollment and who were observed for 16 years. Stratification of overall and site-specific cancer-related deaths according to BMI showed that HCC was 1.68 times higher among women with high BMI and was 4.52 times higher for men with high BMI. Most notably, among the male group HCC had the highest relative-risk increase as a consequence of obesity compared with all the cancers studied, including prostate, kidney, gallbladder, colon, rectum, esophagus, stomach, and pancreas.[19] At present, a biological explanation for risk associated between obesity and HCC is not known. Therefore, the effects of obesity on human HCC represent a critical intersection between these 2 important health problems. Considering the fundamental role of adipocytokines in cancer progression,

Abbreviations used in this paper: AMPK, adenosine monophosphate–activated protein kinase; BMI, body mass index; HCC, hepatocellular carcinoma; JNK, c-Jun-N-terminal kinase; mTOR, mammalian target of rapamycin; TMA, tissue microarray; TSC2, tuberous sclerosis complex 2.

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the growth regulation of HCC cells by adiponectin might effect their malignant progression. Therefore, in the present study we investigated the effects of adiponectin on apoptosis and proliferation of HCC. We also elucidated the signal transduction pathways regulating adiponectin-induced changes in the cancerous properties of HCC.

Materials and Methods

Antibodies

Antibodies for AdipoR1, AdipoR2, Bcl-2, Bax, cleaved-caspase-3, caspase-3, cyclin D1, proliferating cell nuclear antigen, phospho−c-Jun-N-terminal kinase (JNK), JNK, phospho-AMPK, AMPK, phospho-mammalian target of rapamycin (mTOR), mTOR, phospho- tuberous sclerosis complex 2 (TSC2), TSC2 were purchased from Cell Signaling (Danvers, MA).

Cell Culture and Reagents

HepG2 cells (ATCC, Manassas, VA) derived from a human hepatoblastoma[20] and Huh7 cells derived from a well-differentiated HCC[21] were cultured in minimum essential medium (ATCC) and Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), respectively, supplemented with 10% fetal bovine serum (Fisher Scientific [Hyclone], Pittsburgh, PA). THLE-2 cells (ATCC) derived from primary normal liver cells and human hepatocytes (Lonza, Walkersville, MD) were used as controls. THLE-2 cells were cultured in ATCC complete growth medium (ATCC) containing 5 ng/mL epidermal growth factor, 70 ng/mL phosphoethanolamine, and 10% fetal bovine serum. Human hepatocytes were cultured in HCM hepatocyte culture medium (Lonza). Full-length and globular Adiponectin human-HEK293 was purchased from Biovendor (Candler, NC). For adiponectin treatment, cells were serum-starved for 16 hours followed by adiponectin treatment in fresh media as indicated.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction

Total cellular RNA was extracted and reverse transcription polymerase chain reaction was carried out using specific sense and antisense polymerase chain reaction primers. Please see Supplementary Materials for primer details.

Western Blot

Whole cell lysate was prepared following a previously described method.[22,23] Please see Supplementary Materials for details.

Quantification of DNA/Cell Proliferation Assay by Bromodeoxyuridine Incorporation

Bromodeoxyuridine incorporation assays were performed as described previously.[22,23] For details of bromodeoxyuridine incorporation assay see Supplementary Materials.

Caspase Activity Assay

Caspase-3 like activity was detected in cell lysates after various treatments using an Ac-DEVD-AFC substrate (Calbiochem, San Diego, CA). Measurements were made using a fluorescence microplate reader (Beckman Coulter, Fullerton, CA). Control groups treated with inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone [BD Pharmingen], Alexis Biochemicals, San Diego, CA), were included to ensure specificity.

Cell Viability/Apoptosis Assay

Cell viability assay was performed[22] following our published protocol. For details of XTT reduction assay, please see Supplementary Materials.

Inoculation of HepG2 Cells Into Nude Mice and Treatment With Adiponectin

HepG2-xenografts in nude mice were developed and treated with recombinant adenovirus-adiponectin. All the experimental protocols were approved by the Institutional Animal Care and Use Committee at Emory University. For details, please see Supplementary Materials.

Immunohistochemistry of Human HCC TMA

Adiponectin expression was examined in paraffin-embedded sections of human HCC samples using TMA. In total, 140 cases of HCC diagnosed between 1985 and 2009 were studied. Immunohistochemistry was performed using monoclonal adiponectin (1:80) (Chemicon International, Temecula, CA), Bcl-2 (1:100), Bax (1:100), cleaved-caspase-3 (1:100), and caspase-3 (1:100). For TMA details, please see Supplementary Materials. These studies were approved by the Institutional Review Board at Emory University.

Statistical Analysis

All experiments were independently performed 3 times in triplicates. Data were analyzed using a combination of Fisher's exact test, *t* tests, 2-tailed distribution, analysis of variance, and Pearson's correlation. TMA data were analyzed using the nonparametric Mann-Whitney test, Kruskal-Wallis test, and Spearman correlation coefficient. Specifically, when comparing the expression level of adiponectin between 2 categorical factors, such as node status, Mann-Whitney tests were used. When comparing these expressions between more than 2 categories, Kruskal-Wallis tests were carried out. Correlation of the expression level of adiponectin with a continuous variable such as size was assessed using the Spearman correlation coefficient.