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Interleukin-6 and Oncostatin M are elevated in liver disease in conjunction with candidate hepatocellular carcinoma biomarker GP73

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Abstract

The Golgi phosphoprotein GP73 is elevated in the circulation of individuals with a diagnosis of hepatocellular carcinoma. Its usefulness as a biomarker of HCC is questioned, since it has also been reported to be elevated in the circulation of people with liver cirrhosis. Regulation of GP73 by inflammatory cytokines is therefore of interest. The interleukin-6 (IL-6) family cytokines were tested for effects on GP73 mRNA and/or protein levels in human hepatoblastoma HepG2 cells. Levels of GP73 mRNA and protein were up-regulated in HepG2 cells following treatment with either proinflammatory cytokine IL-6 or the related cytokine oncostatin M (OSM). Induction required the shared receptor subunit gp130, and correlated with increased tyrosine phosphorylation of STAT3. Maximal cytokine-mediated induction was not observed in the presence of protein synthesis inhibitor cycloheximide, suggesting additional regulatory factors play an important role. ELISA measurement of GP73 and IL-6 levels in the sera of patients with pre-malignant liver disease revealed a significant correlation between circulating levels of the two proteins. Similarly, a sensitive ELISA assay was developed to measure circulating OSM. OSM levels were elevated 6–7 fold in sera from patients with either cirrhosis or HCC relative to controls without liver disease. Although there was an association between levels of GP73 and OSM in serum from people with liver cirrhosis, there was not a statistically significant correlation in HCC, suggesting that the role of the cytokines in determining circulating levels may be complex. To our knowledge, this is the first report of OSM elevation being associated with liver disease.

Keywords

Golgi phosphoprotein; GOLPH2; GOLM1; cirrhosis

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common, aggressive solid malignancies worldwide, accounting for more than two-thirds of all primary liver cancers. Furthermore,

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the incidence of HCC in the United States is increasing [1–3]. The most important risk factor for the development of HCC is cirrhosis [1–3]. Cirrhosis is a chronic disease of the liver where the normal liver architecture is replaced by fibrotic scar tissue, with an eventual decline of liver function. Distinguishing people with cirrhosis from those with HCC is one of the most sought after and difficult goals to establish who is at risk for liver cancer [4]. Serum proteins that help in risk stratification for HCC or assist in early detection of HCC will be of enormous significance, bringing more patients to proper timely imaging to diagnose HCC. The current, non-invasive marker AFP, and its fucosylated glycoform, AFP-L3, and even the newer markers, such as DCP, are of limited value, detecting cancer in roughly 30–70% in all cases [5].

We have been using a glycoproteomics strategy to identify new serum markers for HCC. One of our lead biomarkers for HCC is the glycoprotein, GP73. GP73 (aka GOLPH2, GOLM1 gene product) is a Golgi resident protein that normally is present at low abundance (nanogram levels) in the circulation of healthy subjects [6–8]. In an initial report, the level of circulating GP73 was higher in individuals with HCC than in those with cirrhosis ($P<0.001$), with an AUROC of 0.79 [7]. In a larger cohort, GP73 had an AUROC of 0.89 for the detection of stage I/II HCC ($p<0.0001$; [8]). Other groups have evaluated this promising biomarker in Asian and European HCC cohorts [9–13]. Serum GP73 is consistently elevated in people with liver disease relative to those without liver disease, although the lower levels seen with Asian cirrhotics might suggest that the marker will prove more useful in certain populations [12]. Thus, the true utility of GP73 as a biomarker of HCC will require additional data.

Normal liver tissue has very low levels of GP73, largely restricted to biliary epithelial cells, whereas overall tissue levels are increased and the protein also is detected in hepatocytes in diseased liver [14, 15]. Thus, it seems likely that the GP73 detected in the circulation arose from the diseased tissue. Although circulating GP73 has not been detected in serum from individuals with other cancers [8, 12], increased expression of the protein has been detected in tumors, including lung adenocarcinoma [16], testicular seminomas [17], renal cell carcinoma [18], and prostate cancer [19–21]. GP73 was detected in the urine, but not the serum, of patients with prostate cancer [20, 22]. Although at this time there is no evidence for a role for GP73 in the carcinogenesis process, the potential importance of the protein as a cancer biomarker suggests a need to study the regulation of GP73 synthesis and/or its secretion into the circulation.

At present, little is known about the regulation of the GOLPH2 gene that codes for GP73. In vivo, GP73 levels increase in hepatocytes in the context of acute hepatitis as well as in autoimmune hepatitis, consistent with regulation by inflammatory mediators [14]. In cultured hepatocytic cells, intracellular GP73 levels are modestly up-regulated by treatment with IFN-gamma and strongly down-regulated by TNF-alpha treatment [15]. Viral infection also appears to increase GP73 expression [23].

Our interest in GP73 as a biomarker for HCC prompted us to investigate additional factors involved in GP73 regulation. As mentioned above, GP73 elevations might be induced by inflammatory cytokines. Members of the interleukin-6 (IL-6) cytokine family have been implicated the liver response to acute injury, specifically as an acute phase response protein and an inducer of liver regeneration [24, 25]. Of specific interest, higher levels of circulating IL-6 in people with chronic HBV infection have been reported to correlate with increased risk for HCC [26]. IL-6 also has been implicated in the development of HCC in an animal model [27]. IL-6 is the protoype for a family of structurally related cytokines that share a common receptor subunit, gp130 [28, 29]. Our present study was therefore aimed at first

determining whether IL-6 family cytokines could affect GP73 levels in cell culture, and then to determine if there is a relationship between cytokine levels and GP73 in people.

We present evidence that GP73 is up-regulated by IL-6 as well oncostatin M (OSM), using the well-differentiated hepatoblastoma HepG2 cell line. Cytokine-induced up-regulation of GP73 involves the receptor gp130 and the downstream transcriptional activator STAT3. In addition, the serum level of IL-6 correlates with elevation of GP73 in the circulation of patients with liver disease. In addition, we report for the first time that circulating OSM levels are elevated in patients with either HCC or cirrhosis.

MATERIALS AND METHODS

Human subjects and sera collection

Patient samples were obtained from California Pacific Medical Center or from the University of Michigan. For samples obtained from California Pacific Medical Center, demographic and clinical information was obtained under an Institutional Review Board approved study protocol and written informed consent was obtained from each subject. A blood sample was collected from each subject in a serum separator tube, spun within 2 hours and the serum stored at −80°C until testing. The presence of HCC was excluded by histopathology, including all T1 lesions, or if histopathology was not available by two imaging modalities (ultrasound, magnetic resonance imaging, or computed tomography) showing the absence of a vascular enhancing mass > 2 cm. For some individuals, diagnosis of cirrhosis was based on liver histology or clinical, laboratory and imaging and included evidence of hepatic decompensation or portal hypertension. The 34 serum samples that were analyzed for IL-6 and GP73 levels were derived from a total of 26 individuals, with 2–3 samples drawn from several. The population included 18 males and 8 females, ranging in age from 33–61, with mean of 53.

For samples obtained from the University of Michigan, the University of Michigan's Institutional Review Board approved the study protocol and written informed consent was obtained from each subject. Demographic and clinical information were obtained Institutional Review Board approved the study protocol and written informed consent was obtained from each subject. Demographic and clinical information were obtained. Consecutive patients with HCC and patients with cirrhosis that were age, gender, and race/ethnicity matched to the HCC patients were enrolled from the Liver Clinic during this period. The diagnosis of HCC was made by histopathology, including all T1 lesions, and, if histopathology was not available, by two imaging modalities [ultrasound (US), magnetic resonance imaging (MRI), or computed tomography (CT)] showing a vascular enhancing mass of >2 cm. Diagnosis of cirrhosis was based on liver histology or clinical, laboratory, and imaging evidence of hepatic decompensation or portal hypertension. There were 20 samples each in the cirrhosis and HCC groups. The populations were between 46 and 85 years of age, with mean of 63.7 (HCC), vs. 46–79, mean 60.8 (cirrhosis), with a gender distribution for both groups of 16 males and 4 females.

For comparison, sera from 20 anonymous healthy controls, aged from 42–62 (mean of 53) with an equal number of males and females was obtained from a commercial supplier (Biological Specialty Corp., Colmar, PA).

Cells used and culture conditions

HepG2 cells, a stable tissue culture line derived from a human hepatoblastoma [30], were obtained from American Type Culture Collection. HepG2.2.15 cells are a sub-line of HepG2 cells containing an integrated HBV genome that support continuous virus replication [31]. Both were grown in DMEM containing 10% fetal calf serum. HepG2 or HepG2.2.15 cells

were treated with cytokines or drugs for 48 hrs unless otherwise indicated. Recombinant human OSM (Abcam or Humanzyme), recombinant human IL-6 (R&D systems or Humanzyme), recombinant human IL-11 (Insight Genomics), recombinant human LIF (Insight Genomics), recombinant human CNTF (R&D systems), recombinant human cardiotrophin-1 (R&D systems), mouse monoclonal anti-Human gp130 receptor (Invitrogen or R&D Systems), human interferon-alpha (PBL Biomedical Laboratories) and cycloheximide (Calbiochem) were used as indicated.

Preparation of cell lysates and immunoblotting

Cells were lysed in TNE lysis buffer consisting of 10mM Tris-HCl, pH 8.0, 1% IGEPAL, 0.15M NaCl, 1mM EDTA and 1X protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Lysates were denatured and resolved by SDS-PAGE, using commercially prepared gels in Tris-HEPES buffers (Pierce). Proteins were transferred to PVDF and GP73 was detected by incubation of the membrane with anti-GP73 rabbit polyclonal antibody at a 1:5000 dilution for 1h; total STAT3, serine phosphorylated STAT3(S727), tyrosine phosphorylated STAT3(Y705) were detected using a rabbit anti-STAT3, anti-Phospho-STAT3 (S727) or anti-Phospho-STAT3 (Y705) antibody (Cell Signaling Technology) at a 1:2500 or 1:1000 dilution for 1h. Proteins were detected using IR-dye-conjugated goat anti-rabbit secondary antibody (Licor, Lincoln, NE) and infrared imaging was performed on a Licor Odyssey instrument running Odyssey 2.1 software.

RNA preparation, reverse transcription and real time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carsbad, CA). First strand cDNA synthesis was performed using 1ug of total RNA and SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's protocol. Real time PCR was performed on 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA) using TaqMan pre-developed Assay Reagents for GP73 (Hs00213061_m1) and GAPDH (402869).

Immunofluorescence staining

Cells were fixed in 4% formaldehyde for 5min, permeablized with 0.1% Triton X-100 for 5min and incubated with primary antibodies for 1h and visualized using Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 555 anti-mouse IgG (Invitrogen). Nuclei were visualized by inclusion of Hoechst 33342 (Invitrogen). Primary antibody used was a polyclonal rabbit anti-GP73 diluted 1:500. Competing antibodies were mouse monoclonal anti-gp130 or anti-p21(WAF/CiP1; Santa Cruz) diluted as indicated in the figure legend. Cultures were viewed by wide-field immunofluorescence microscopy on a Nikon Inverted TE2000U, and images captured using NIS Elements AR3.0 software.

ELISA detection of IL-6 and GP73

Human IL-6 ELISA was performed according to manufacturer's protocol (R&D Systems, Minneapolis, MN). 50 μl of each serum sample was used per assay, normal pooled human serum from Sigma (H4522) was used as a negative control. GP73 levels were measured as described previously [8], and are expressed as relative to a commercially obtained pooled normal human serum (Sigma-Aldrich; St. Louis, MO) or as absolute values based on recombinant protein standard.

ELISA assay for oncostatin M

Mouse anti-human OSM antibody, biotinylated goat anti-human OSM antibody, horseradish peroxidase conjugated streptavidin and recombinant human OSM were obtained from R & D Systems (human OSM Duo-Set). All other reagents were obtained from the indicated source or from Sigma/Aldrich.

Each well of 96-well Maxisorb plate (Nunc) was coated overnight with 100 ng of mouse anti-human OSM in PBS. The plates were washed with PBS, 0.05% Tween 20 (PBST), blocked overnight at 4°C with 0.2 mm filtered 1% protease and IgG free BSA (Jackson ImmunoResearch) in PBS and washed once more with PBST. One hundred microliters of serum samples diluted 1:3 with filtered 1% BSA in PBS were incubated in the wells overnight at 4°C. Pure human recombinant OSM prepared in filtered 1% BSA in PBS was used as a standard. The plates were washed with PBST and then incubated with 75 ng/ml biotinylated goat anti-human OSM antibody in filtered 1% BSA in PBS for 2 hours at room temperature. The plates were washed again with PBST and incubated for 20 minutes with HRP-streptavidin in filtered 1% BSA in PBS at room temperature. The plates were washed once again with PBST and incubated with 100 ml/well of tetramethylbenzidine peroxidase substrate solution (Rockland) for 30 minutes. Reactions were stopped with 50 ml of 2N H_2SO_4. The optical density of the colorimetric reaction in each well was determined at 450 nm with a Synergy 2 plate reader from BioTek. The optical density of each well at 540 nm was subtracted from the OD_{450} to correct for the contribution of the plate.

Statistical analyses

All data are represented as means +/− standard deviation (SD), unless otherwise indicated. The choice of parametric or non-parametric methods was based on data normality, which determined using the Shapiro-Wilk test and the Kolmogorov-Smirnov test. The relationship between IL-6 and GP73 levels in sera, as well as between GP73 and OSM, was explored by linear regression analysis. Regression diagnostics were performed to ensure validity, with attention given to influential points and outlier points. Statistical significance was defined as P value <0.05. The **R** statistical package (version 2.11) was used for all patient sample analyses (http://www.r-project.org/).

RESULTS

IL-6 and OSM increase GP73mRNA and protein levels in HepG2 cells

To test the influence of pro-inflammatory cytokines on GP73 expression, we chose to use cultured human cells of hepatocyte origin. It was reported previously that well-differentiated human hepatoblastoma HepG2 cells express low amounts of GP73, whereas the HepG2 derived cell line, HepG2.2.15, expresses easily detectable levels of GP73 [15]. We obtained similar results by immunoblotting; GP73 tends to run as a broad band, due at least in part to glycosylation heterogeneity (Fig. 1A). Quantification by reverse transcription of mRNAs from the two cell lines and real time PCR confirmed that the latter cells contain ca. 100 times more GP73 mRNA (Table 1).

To determine whether GP73 expression is affected by IL-6 family cytokines, HepG2 cells were plated and treated for 2 days with various cytokines at 50 ng/ml; fresh medium supplemented with cytokines was added after 24 hours. After 2 days of treatment, RNA was prepared from cells and reverse transcription followed by real time PCR was performed. Values were calculated relative to a standard curve created from linearized plasmid containing GP73 cDNA; GAPD was quantified in parallel to ensure equal loading (data not shown). Both IL-6 and OSM significantly up-regulated GP73 mRNA levels, with increases of >4 and >10 fold, respectively; the cytokines CNTF, CT-1, IL-11 and LIF had little effect (Fig. 1B). OSM treatment was dose responsive, with increasing levels of GP73 observed from at a dose as low as 2 ng/ml (Table 1). The response to IL-6 also increased with dose, although there was a plateau effect.

To confirm that cytokine treatment also increased GP73 at the protein level, cell lysates and supernatants were examined for the presence of GP73. We have previously shown that

supernatants of HepG2.2.15 cells contain readily detectable levels of GP73 after concentration [32]. However, we were unable to detect GP73 in supernatants of OSM treated HepG2 cells, even after concentration (data not shown). Despite our inability to detect secreted GP73, we were able to detect GP73 in cell lysates (Fig. 1C); HepG2.2.15 cell lysate is also shown for comparison. Although after cytokine treatment levels of GP73 in HepG2 cells remain far below those seen in HepG2.2.15 cells, it is also clear that both cytokines result in increases in GP73 gene products at the protein as well as the mRNA level.

Mechanism of GP73 induction by IL-6 family cytokines

The IL-6 family cytokines share a common receptor subunit, gp130, which heterodimerizes with several cytokine-specific subunits to form distinct receptor complexes [28]. To assess whether the induction of GP73 expression was a consequence of cytokine-receptor interaction, an antibody to gp130 that prevents cytokine binding was added to cultures along with OSM. In the absence of OSM, low levels of GP73 were detected in HepG2 cells, but addition of OSM resulted in all cells in the culture strongly expressing GP73 (Fig. 2). Inclusion of increasing amounts of antibody to gp130 with a constant amount of OSM resulted in progressively less GP73 induction; a similar amount of an irrelevant monoclonal antibody did not reduce the OSM-dependent GP73 increase. This reduction was confirmed at the RNA level by real-time PCR, with only a 1.6-fold increase in GP73 mRNA level when 2 μl of antibody was included along with 10 ng/ml OSM, versus a 5.1-fold increase with OSM alone (Table 1). The high basal level of GP73 in HepG2.2.15 cells was not reduced by anti-gp130 treatment (Table 1). The ability of OSM to induce GP73 expression in another human cell line of hepatoma origin, Hep3B, also was examined by real-time PCR. OSM treatment (10ng/ml) resulted in levels of GP73 mRNA that were 1.38-fold (SD=0.52) those of untreated control cells. In parallel, treatment of Hep3B cells with the same level of IL-6 resulted in mRNA levels that were 2.17-fold (SD=0.518) elevated relative to controls. The stronger effect of IL-6 contrasts with the HepG2 cell results, but is consistent with the report that Hep3B cells lack the cytokine-specific chain of the OSM receptor (the non-GP130 component,).

Binding of cytokines to gp130-containing receptor complexes has been found to activate the transcription factor STAT3 via phosphorylation [25, 28]. We evaluated levels of total STAT3 as well as phosphorylated STAT3 in lysates of untreated cells versus cells treated with OSM or IL-6 for 0.5, 1.0 or 3.0 hours (Fig. 3A). Although levels of total STAT3 were unchanged by cytokine treatment, phosphorylation on Tyr-705 was strongly induced by OSM at 0.5–1 hr of treatment, declining by 3 hr. IL-6 treatment also induced tyrosine phosphorylation within 0.5 hr, although the effect was less pronounced. Neither cytokine showed an effect on serine phosphorylation.

The above results do not distinguish whether the *GOLPH2* gene that encodes GP73 is transcriptionally activated by STAT3 directly, or indirectly via up-regulation of one or more other regulatory factors. To begin to address this question, cells were treated with OSM in the presence or absence of cycloheximide (CHX) to inhibit new protein synthesis. In the absence of CHX, GP73 mRNA levels increased steadily from 1 to 3 to 6 hours (Fig. 3B). Note that CHX treatment alone modestly elevated GP73 at all times. The combination of OSM plus CHX resulted in an increase in GP73 mRNA after one hour (approximately an additive effect of the separate OSM plus CHX treatments), but there was no further increase with additional incubation time. Thus, it seems that STAT3 modestly increased transcription of GOLPH2 independent of new protein synthesis, but that additional regulatory factors induced by STAT3 contribute to further increase GP73 mRNA levels.

Correlation of IL-6 and GP73 levels in patient sera

Based on the above cell culture experiments, we hypothesized that GP73 levels, in people, would be elevated under conditions where IL-6 and / or OSM levels in the circulation were also elevated. Since assays to measure very low levels of IL-6 are commercially available, the possibility that IL-6 and GP73 levels correlate in vivo was tested first. Levels of GP73 and IL-6 were measured in sera collected from 34 patients with chronic liver disease, mostly in the context of chronic infection with HBV or HCV, who were at elevated risk for HCC development. In previous work, a similar patient cohort exhibited moderate elevations of GP73, while those with HCC had significantly higher levels [7]. Consistent with that study, elevations of GP73 were observed in the present patient population relative to an aged-matched control population (Fig. 4B). In addition, IL-6 elevations were observed in the patient samples, in comparison with controls (Fig. 4A). Linear regression analysis of the data obtained from the individuals with liver disease revealed a significant relationship between the levels of GP73 (normalized to alpha-1-acid glycoprotein) and the levels of IL-6 ($p=0.040$) (Fig. 4D), after removal of one sample based on linear regression diagnostics (this individual was diagnosed with HCC one year after the serum sample was collected). There was no such correlation in the population of normals (Fig. 4C). Thus, the data support the hypothesis that IL-6 is a likely contributor to the over-expression of GP73 observed in patients with liver disease.

OSM levels are elevated in individuals with liver disease

Since a commercial ELISA assay for OSM was not available, a sensitive assay for the cytokine was developed. A standard curve for the assay using purified OSM is shown in Fig. 5A, illustrating that the linear range extends from ca. 10 pg/ml to >100 pg. The assay was then used to measure OSM levels in sera from three groups, with 20 individuals per group – normals with no known liver disease, cirrhotics, and those with a diagnosis of HCC. As shown in Fig. 5B, both cirrhotic and HCC groups significantly elevated levels of circulating OSM relative to the control group (means of 75.08 and 62.85 vs. 10.12 pg/ml). However, when we examined GP73 levels in these groups, we also saw a significant elevation in the two disease groups relative to controls (means of 280.38 ng/ml in HCC and 295.44 in cirrhosis, vs. 7.86 in the normals. When the cirrhotic group was evaluated for possible correlation between GP73 and OSM, only a weak correlation was seen after removal of an outlier with very high levels for both parameters that was biasing the analysis ($R^2=0.1567$, $P=0.05$; data not shown). In the HCC group, the association between GP73 and OSM did not meet statistical significance ($R^2=0.116$, $P=0.339$; data not shown). However, when the two disease groups were combined, a highly significant correlation between GP73 and OSM levels was revealed (Fig. 5C; $R^2=0.16$, $P=0.01$). Thus, it is likely that both IL-6 and OSM contribute to the elevation of GP73 seen with liver disease.

DISCUSSION

There is a growing body of evidence that GP73 serum levels correlate with the presence of HCC [7–10, 33–35]. However, although GP73 levels in the circulation do not appear to be elevated in healthy subjects, these and other reports suggest elevation of the marker in people with inflammatory liver diseases, in the absence of cancer [14]. Thus, its usefulness in distinguishing cancer from cirrhosis or other liver conditions associated with inflammation is still being evaluated. Work by others has shown that GP73 levels are affected by TNF-α and interferon-γ in tissue culture systems [15]. We sought to extend these studies by evaluating the effects of cytokines of the IL-6 family on GP73. In cultured HepG2 cells, IL-6 and OSM increased protein and mRNA by as much as 16-fold. This cytokine-induced increase in expression required binding to the common IL-6 family receptor subunit gp130, as antibody to gp130 that blocks cytokine binding abrogates the

induction. It is of interest to note that the antibody did not affect the constitutively high level of GP73 expressed in the HepG2.2.15 cells (Table 1). Although at this time we do not know the molecular basis for the high expression of GP73 in these cells, it seems unlikely to be due to an auto-endocrine effect as a result of secretion of one of the cytokines.

We next tested whether a known downstream effector of gp130, STAT3, is involved in GP73 modulation. Comparison of the dose response to IL-6 versus OSM (Table 1) suggests that OSM induces a sharper increase in GP73, whereas IL-6 produced a more modest response. It is unclear at this time whether the difference in the response of GP73 to the two cytokines is a consequence of the differential phosphorylation of STAT3, or to other cytokine-specific changes. However, the relative increase in GP73 mRNA in the presence versus absence of cycloheximide suggests a model in which maximal induction requires the production of additional STAT3-induced regulatory factors. The promoter of the GOLPH2/GOLM1 gene is uncharacterized, with two transcription start sites that produce transcripts with different 5' untranslated regions (Genbank reference sequences NM_016548 and NM_177937.2). Both transcripts produce the same protein. Further work will be needed to establish the specific factors that activate the GOLPH2 promoter.

The function of GP73 is not known although it does not appear to be essential, as mice that produce only a severely truncated form of the protein survive without apparent hepatic dysfunction, despite morphologic changes in the tissue [36]. GP73 is normally anchored in the membrane by a single N-terminal membrane-spanning domain. GP73 therefore represents a low abundance serum protein that is normally intracellular, and is probably shed from cells following proteolytic cleavage. Thus, the elevation of GP73 in the serum of individuals with HCC may require both increased synthesis and secretion. We tested whether OSM treatment of HepG2 cells resulted in GP73 secretion, but levels of the glycoprotein remained undetectable in the culture supernatant (data not shown). Given that cytokine-induced intracellular levels of GP73 remain well below the levels observed in the HepG2.2.15 cell line (Fig. 1), where secreted GP73 can be detected, it is possible that overall expression is still too low to detect the secreted fraction.

Elevation of IL-6 has been implicated in HCC development [26, 27]. We were interested in whether GP73 might reflect an elevation of the inflammatory cytokine and thus represent a risk factor for HCC development. Using serum from people with non-malignant liver disease, there was a clear and statistically significant correlation between levels of GP73 and IL-6. Although the number of patients represented in this study was small (n = 34), we hypothesize that IL-6 may play a role elevating circulating levels of GP73.

We also evaluated whether OSM was elevated in association with GP73 in sera of patients with liver disease. This required the development of a sensitive ELISA assay for OSM. Unfortunately, insufficient material remained from the samples analyzed for IL-6 expression, so a separate set of serum samples (20 HCC and 20 cirrhotic) were analyzed for OSM and GP73 levels. Although OSM was clearly elevated in individuals with both liver diseases, the correlation with GP73 was weak when the diseases were evaluated separately. However, combining the two disease groups revealed the correlation. It is possible that may OSM elevate the expression of GP73, but have a variable effect on the secretion of the protein into the circulation.

Previously, the cytokine OSM has been implicated in promoting a differentiated hepatocytic phenotype (for instance, see [37]). To our knowledge, this is the first report linking elevated OSM levels with liver cirrhosis or HCC. Involvement of IL-6 also establishes a potential link between GP73 and both liver regeneration and the hepatic acute phase response [24, 25]. Our findings raise the interesting possibility that GP73 over-expression is induced

initially by inflammation, specifically by inflammatory cytokines such as IL-6 OSM, possibly in response to chronic active viral infection. It is thus possible that the cytokine milieu present in an individual may contribute to the phenotypic heterogeneity observed for HCCs. The ability of circulating cytokines and growth factors such as IL-6 and OSM to influence the level of other circulating factors that are used as biomarkers of disease demonstrates, at a minimum, the complexity in interpreting biomarker values. Additional studies to explore the effect of other inflammatory processes would be warranted. More profoundly, it may suggest cause and affect relationships that offer clues as to GP73's biological functions and roles in the host response to inflammation and growth signaling.

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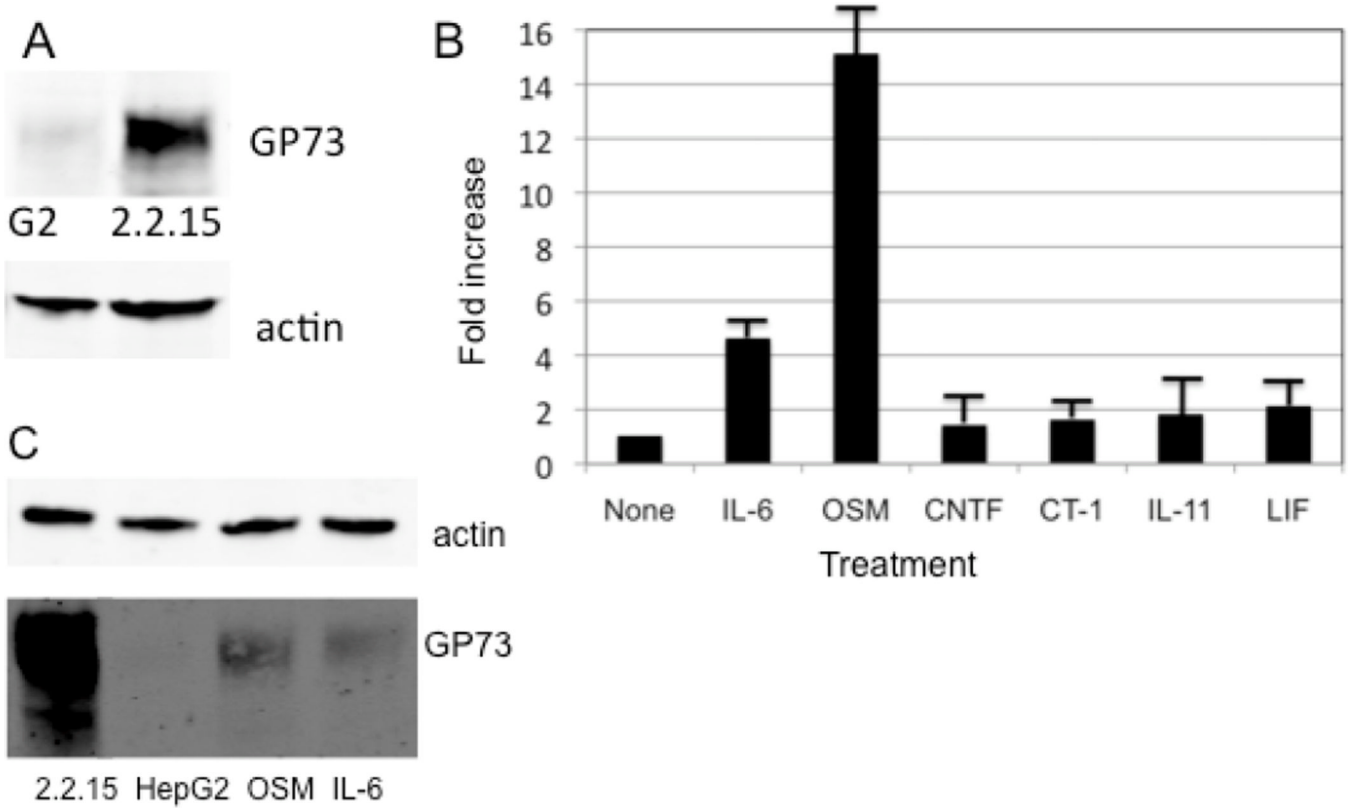

Fig. 1. GP73 mRNA and protein before and after treatment with IL-6 family cytokines

A. Equal amounts of lysates from HepG2 cells (left) or HepG2.2.15 cells (right) were analyzed by immunoblotting with polyclonal anti-GP73 (top) or actin (bottom). Only the relevant portion of each blot is shown.

B. HepG2 cells were treated with the indicated cytokine at 50 ng/ml for 48 hours, at which point total RNA was isolated and used to measure GP73 by real time PCR. Values are given as fold increase over untreated cells.

C. Total cell lysates were prepared from HepG2.2.15 cells, untreated HepG2 cells, and HepG2 cells treated with IL-6 or OSM for two days, and these were compared by immunoblotting. Blots were probed for GP73 or actin as indicated, with only the relevant portion of each blot shown.

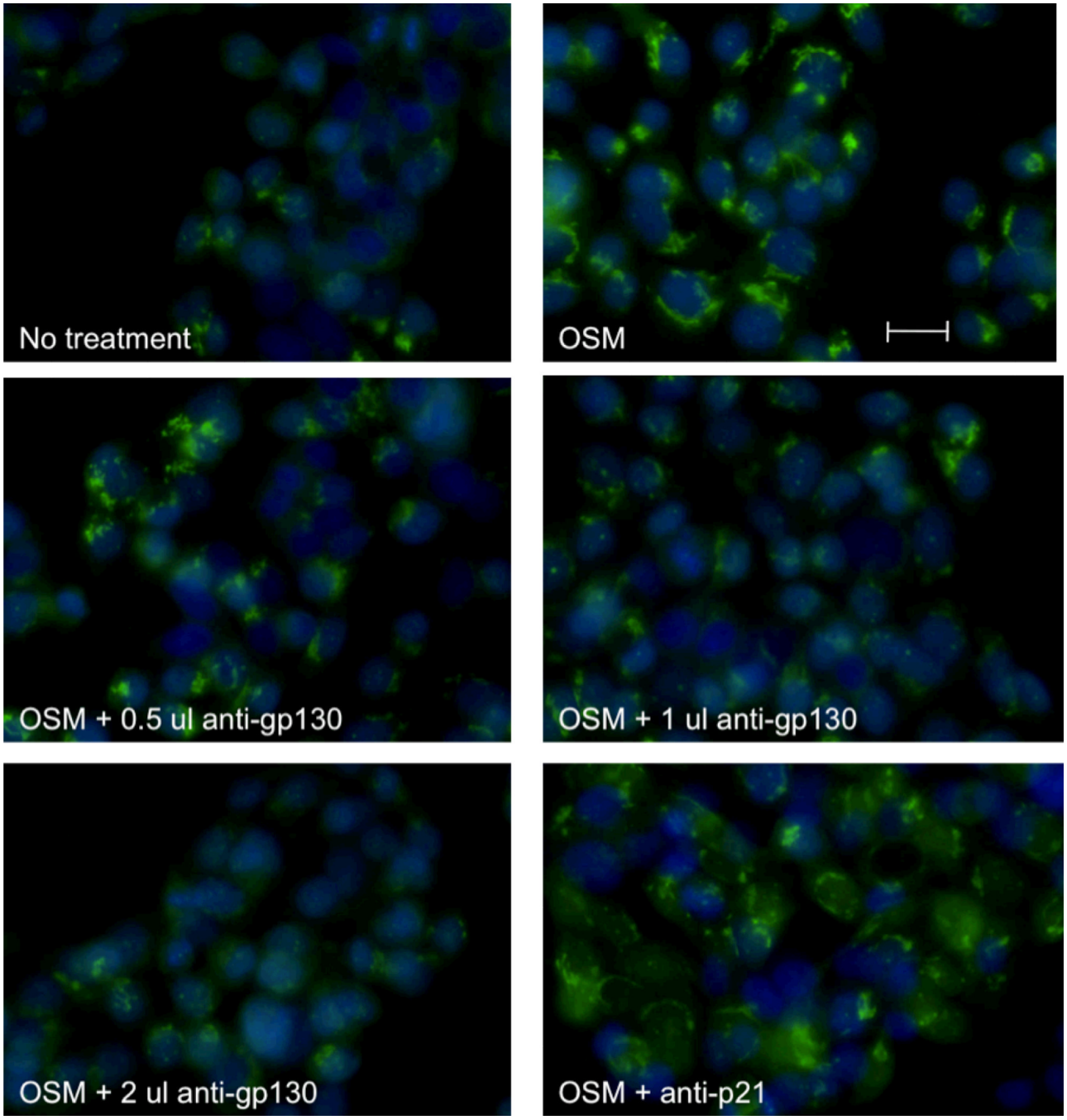

Fig. 2. Inhibition of OSM-induced GP73 by addition of anti-gp130 to cultures

HepG2 cells were treated with OSM either in the absence or presence of an increasing amount of anti-gp130 antibody (concentration of 1 μg/ml), or a control antibody to p21 (5 μg) as indicated in the figure. After two days, cells were stained with anti-GP73 (green). Cells were also stained with Hoechst 33342 to visualize nuclei. All exposure settings were the same for each field shown. Scale bar, 100 μm.

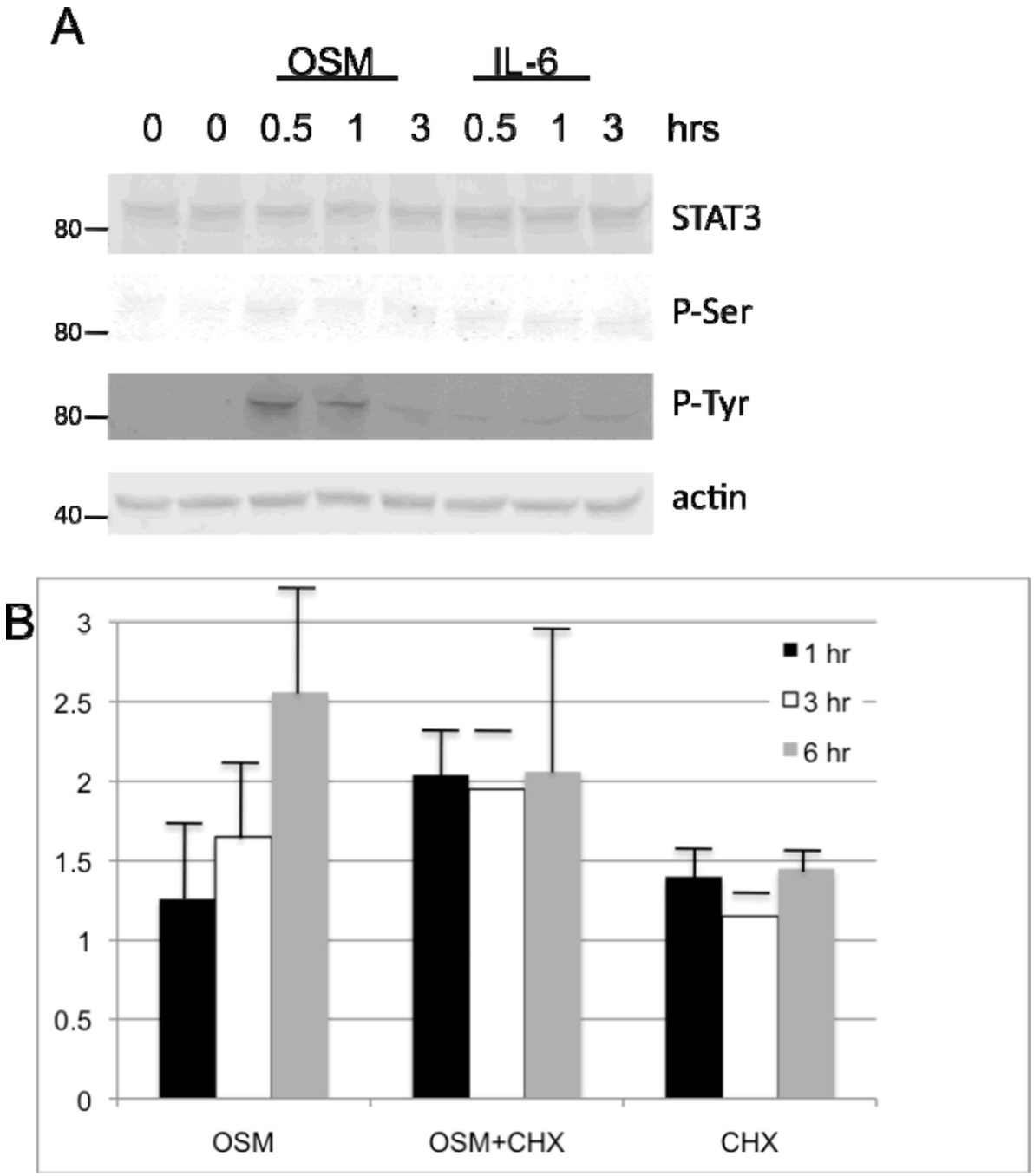

Fig. 3. Influence of IL-6 and OSM on STAT3 and GP73

Total cell lysates were prepared from HepG2.2.15 cells, untreated HepG2 cells, and IL-6 or OSM-treated HepG2 cells, and these were compared by immunoblotting. Only the relevant portions of each blot are shown.

A. Cells were treated for the indicated times. Blots were probed for total STAT3, serine phosphorylated STAT3(S727), tyrosine phosphorylated STAT3(Y) and actin, as indicated.

B. Cells were treated for the indicated times with OSM (10 ng/ml), cyclohexamide (100 μg/ml) or both for the times indicated. Induction of GP73 mRNA was measured by real-time PCR assay as in Fig. 2. Values shown are relative to untreated HepG2 cells, which have

been set to unity. Each bar represents the mean of three separate experiments, with SEM indicated.

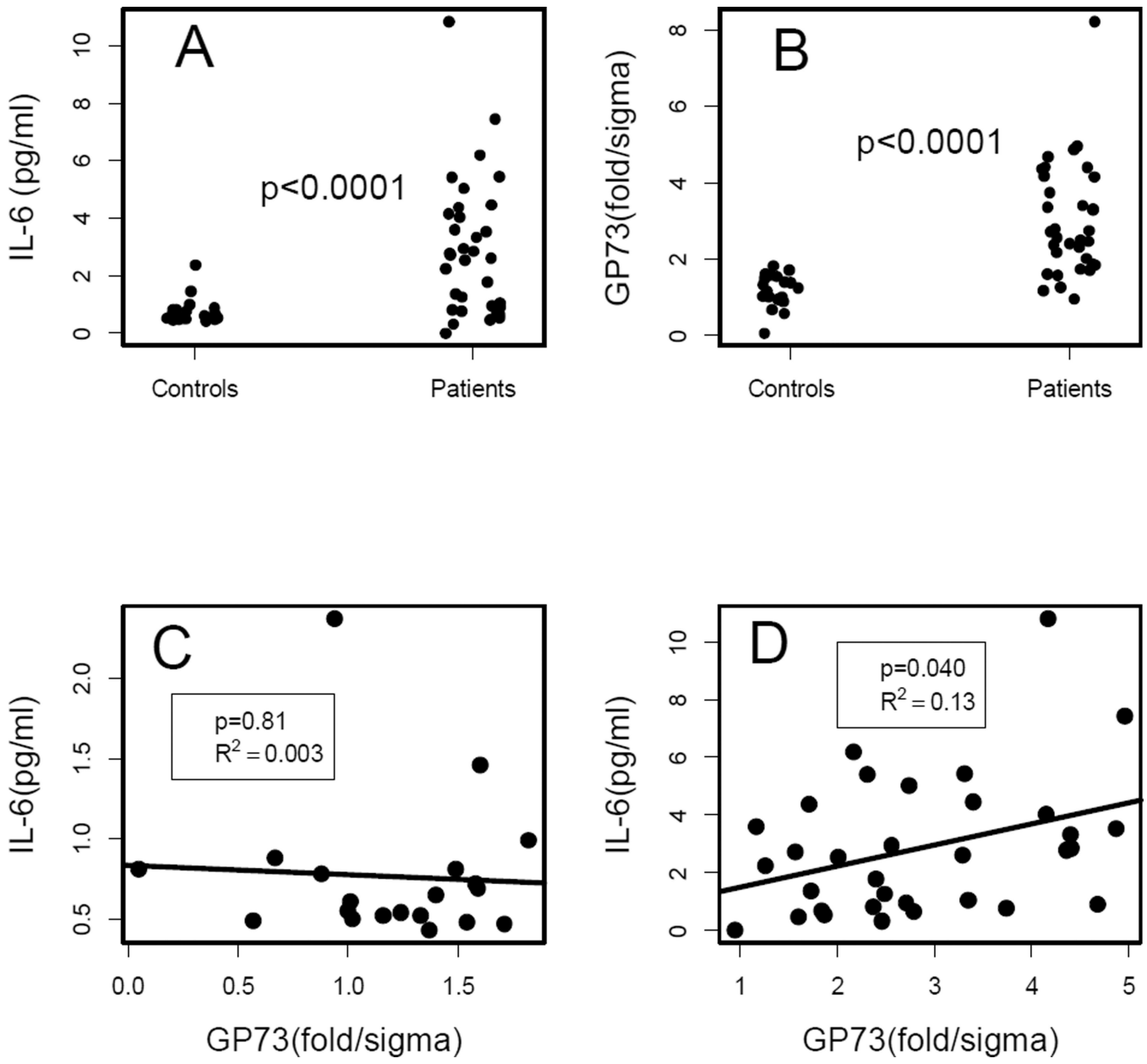

Fig. 4. Correlation of GP73 levels and IL-6 levels in sera of patients with chronic liver disease but not healthy controls

Both GP73 and IL-6 levels were measured in each serum sample by ELISA

A. Comparison of serum IL-6 levels in healthy controls versus patients with cirrhosis. IL-6 levels were determined using a standard curve and are expressed as pg/ml.

B. Comparison of serum GP73 levels in healthy controls versus patients with cirrhosis (CPMC samples). GP73 levels were normalized to levels of a reference sample (pooled normal human serum) and are expressed as fold over pooled normal sera obtained from Sigma (see Materials and Methods).

C. Linear regression of GP73 and IL-6 levels in normal controls.

D. Linear regression of GP73 and IL-6 levels in patients with cirrhosis.

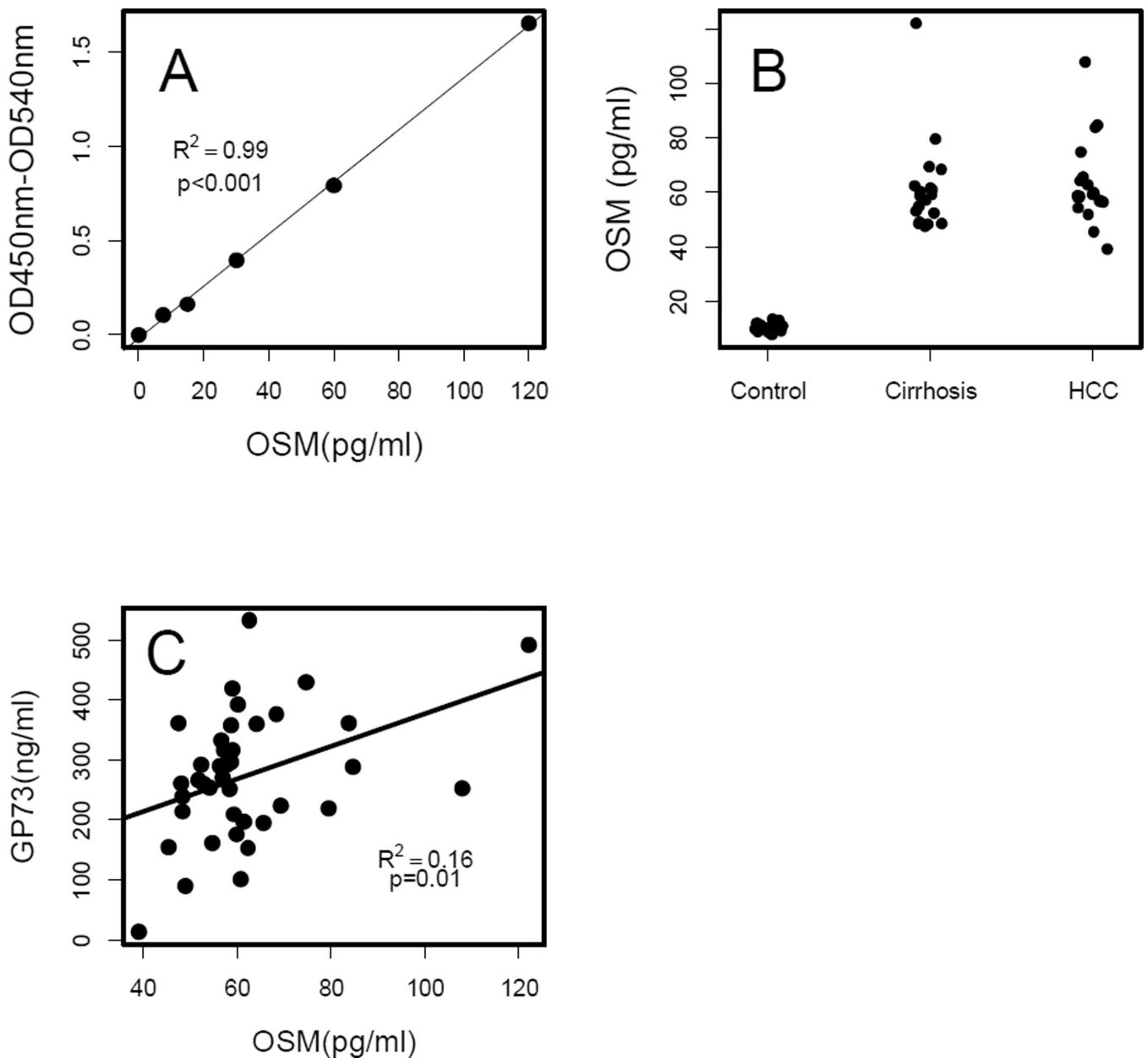

Fig. 5. Measurement of OSM levels in human serum samples and comparison with GP73 levels

A. Development of a sensitive ELISA to measure OSM levels in serum. The standard curve was derived using purified OSM added into commercially obtained normal human serum, and shows that the linear range of the assay extends into the low pg/ml range.

B. The OSM ELISA was used to measure cytokine levels in three groups of twenty individuals with diagnoses of HCC, cirrhosis (both from UM samples) or no known liver disease (controls).

C. Linear regression of GP73 and OSM in the combined cirrhosis and HCC groups, after removal of one sample highly elevated for both parameters, which was over-biasing the analysis.

Table 1

Effects of treatments on GP73 mRNA levels

Treatment	Norm*	SD&
HepG2	1	NA&
HepG2+OSM(2)#	2.39	0.28
HepG2+OSM(10)	5.10	0.33
HepG2+OSM(50)	14.62	1.44
HepG2+OSM(10)+αgp130	1.61	0.20
HepG2+IL-6(2)	3.08	0.38
HepG2+IL-6(10)	3.64	0.90
HepG2+IL-6(50)	4.98	2.45
HepG2.2.15	149.63	13.47
HepG2.2.15+αgp130	137.47	7.91
Hep3B	1	NA
Hep3B+OSM(10)	1.38	0.52
Hep3B+IL-6(10)	2.17	0.52

*Norm, values adjusted relative to untreated HepG2 cells or Hep3B cells (last three rows)

&SD, standard deviation of RT-PCR replicates; NA, Not applicable

#cytokine concentration (ng/ml)