Inhibition of Growth and Metastasis of Human Hepatocellular Carcinoma by Antisense Oligonucleotide Targeting Signal Transducer and Activator of Transcription 3

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Abstract

Purpose: Hepatocellular carcinoma (HCC) is an aggressive malignancy and is a devastating clinical complication of chronic liver disease. Therapeutic options are limited mainly because the genetic and biochemical understanding of this disease remains fragmented. We intended to study the role of signal transducer and activator of transcription 3 (STAT3) aberrant signaling in HCC malignancy, and the therapeutic potential of inhibition of STAT3 expression for HCC.

Experimental Design: A 2′-O-methoxymethylribose – modified phosphorothioate antisense oligonucleotide (ASO) was used to knock down STAT3 expression in different human HCC cell lines, including the highly metastatic HCCLM3 derived from orthotopic implantation and subsequent lung metastasis in athymic mice. The effects of STAT3 ASO treatment on HCC cells, metastasis, and animal survival following HCCLM3 orthotopic implantation were evaluated.

Results: Specific suppression of phosphorylated STAT3 reduced its DNA-binding activity, inhibited the expression of vascular endothelial growth factor, survivin, matrix metalloproteinases 2 and 9, reduced cell proliferation and migratory potential, induced apoptosis in vitro, and inhibited intradermal angiogenesis and s.c. tumorigenesis upon injection in mice. In mice bearing orthotopically implanted HCCLM3, STAT3 inhibition following therapeutic treatment with STAT3 ASO reduced circulating vascular endothelial growth factor and basic fibroblast growth factor, decreased intratumor CD34-positive microvessel density, intrahepatic and intraperitoneal transmission, and lung metastasis. HCC tumor volume and weight were reduced and the survival time of mice bearing orthotopically xenografted HCC was approximately doubled in STAT3 ASO – treated mice (P < 0.05).

Conclusions: Constitutively activated STAT3 is essential for the growth, survival, and metastasis of HCC, suggesting that STAT3-targeted therapy may have utility for HCC.

Heptatocellular carcinoma (HCC) is one of the most common and aggressive malignancies, ranking fifth in the world and second in China in cancer-related deaths (1). Although improvement in survival with available treatments has been reported, HCC mostly engenders a poor prognosis, with a >60% recurrence rate within 5 years after resection due to invasion-related spreading (2). Unlike many other tumors, the signaling pathways critical for HCC pathogenesis are still undefined, even though the causes for the disease are largely identified. Recent results from gene profiling analysis indicate that the expression signature of primary HCC is very similar to that of its corresponding metastases, suggesting that transcriptional changes which control metastatic progression are initiated in the primary tumors (3). Thus, it is critical to identify the molecular mechanisms controlling the invasive and metastatic potential of primary HCC.

The transcription factor, signal transducer and activator of transcription 3 (STAT3), a highly studied member of the STAT family, has recently been shown to be a convergent point for a number of oncogenic signaling pathways due to its constitutive activation in a variety of cancers (4). Normally under tight regulation, STAT3 is transiently activated by phosphorylation in response to a number of cytokine and growth factors (5–7); however, it is persistently activated in multiple tumor types at high frequency, including multiple myeloma, head and neck, prostate, pancreatic, and non–small cell lung cancer, among others (8, 9). Activated STAT3 contributes to oncogenesis through the promotion of tumor cell proliferation and angiogenesis coupled with the suppression of apoptosis and host tumor immunity (10). Specific inhibition of STAT3 signaling has been shown to inhibit tumor growth and improve survival (11), suggesting STAT3 as a novel drug target for therapeutic intervention in human cancers.
We have previously established an orthotopic model for HCC by inoculation of human HCC into the livers of athymic mice, HCCLM3, which is more reflective of HCC development in humans with spontaneous lung metastasis in all animals (12–15). Here, we show that HCCLM3 harbors constitutively activated STAT3 and that inhibition of STAT3 using a specific 2′-O-methoxylethyl (2′-MOE)–modified antisense oligonucleotide (ASO) reduced proliferation, induced apoptosis, decreased mobility, and impaired tumor angiogenesis and tumorigenesis of HCCLM3 cells when injected into athymic mice. In support of these findings, the expression of STAT3 target genes, vascular endothelial growth factor (VEGF) and matrix metalloproteinase 2 (MMP2), was reduced following STAT3 suppression. Furthermore, the malignant development of HCCLM3 tumors orthotopically implanted in athymic mice prior to therapeutic treatment with STAT3 ASO was effectively inhibited, including inhibition of tumor growth, local transmission, and lung metastasis, resulting in significantly prolonged survival time. Thus, the present results show that STAT3 signaling plays a key role in HCC growth and metastasis, and suggests that targeting STAT3 may have therapeutic benefit for patients with primary or recurrent HCC.

Materials and Methods

Cell culture and oligonucleotide treatment. Human HCC cell lines, HCCLM3 (established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, P.R. China), SNU423 (American Type Culture Collection, Manassas, VA), and Huh7 (a gift from Novartis, Emeryville, CA) were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum in 5% CO2 at 37°C. Oligonucleotides were 20mer in length with a phosphorothioate modification (underlined residues below). The 10 central bases were left unmodified to support RNase H–mediated cleavage of targeted mRNA (16). STAT3 ASO was designed to target the coding region of human STAT3 mRNA (GenBank NM_139276.2; 5′-GCCCGCAGATCTGTCGTCTC-3′; ref. 17). The control oligonucleotides had the same chemical design except for the randomized sequence (18). Oligonucleotides were synthesized as previously described (19). For cell treatment, oligonucleotides were pre-mixed with LipofectAMINE (Invitrogen) at 1.4 μg/mL/100 nmol/L in serum-free DMEM were added to cells (37°C, 6 hours). The oligonucleotide-lipid treatment was then replaced with culture medium and incubated for the indicated times.

RNA isolation and reverse transcription-PCR. Total RNA (2 μg) prepared from cells using TRIzol reagent (Invitrogen) was reverse transcribed (Perkin-Elmer, Norwalk, CT) using oligo(dT) priming and amplified with the primer pairs for a 310-bp fragment of STAT3 (5′-GGAGGACAGATGTCGGGAAT-3′ and 5′-CTTGGTTGTCGGAGAGAAC-3′) and a 366-bp fragment of β-actin (5′-AGCAACACGAGATTACCTG-3′ and 5′-CTTGGGGTGGCTTTTAGGA-3′). Ten microliters of PCR product was analyzed on 2% agarose gels (Sigma, St. Louis, MO).

Immunocytochemistry. Cells cultured on cover slides in six-well plates (1.0 × 105/well) were fixed in acetone and then stained with polyclonal rabbit anti-human STAT3 (Lab Vision, Fremont, CA), phosphorylated STAT3 (tyrosine 705) antibodies (Cell Signaling Technology, Beverly, MA), or a polyclonal rabbit anti-human Ki-67 antibody (Dako, Carpinteria, CA) in a 3% hydrogen peroxide-methanol blocking solution. Following incubation with a horseradish peroxidase–conjugated secondary antibody (Dako), stain was developed using 3,3′-diaminobenzide or AEC chromogen substrate followed by counterstaining with hematoxylin.

Cell proliferation assay. Cells (0.6–1.5 × 104) were plated in 96-well plates 18 hours prior to treatment at the indicated concentrations. After incubation for 1 to 3 days, the cells were assayed for proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Chemicon, Temecula, CA) or Promega’s CellTiter 96 (Promega, Madison, WI) following the manufacturer’s protocol.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay was done as previously described (20). Cells were pelleted and lysed in 1 mL of 0.5% NP40 buffer containing 10 mmol/L of Tris-HCl (pH 7.4), 10 mmol/L of NaCl, and 5 mmol/L of MgCl2 on ice for 5 minutes. Nuclei were pelleted by centrifuge and then lysed in a nuclear extraction buffer (HEPES, pH 7.9, 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L DTT). After sonication (4°C, 30 minutes), 20 μg of proteins from nuclear lysates were incubated with 32P-labeled, double-stranded SIE probe (5′-GATTCATGGTGATATCC-3′) at room temperature for 15 minutes. The bound proteins were analyzed on 10% tris-borate EDTA gels (Sigma) using radiography.

Apoptosis analysis. Cells were plated (1.5 × 105/cm2 dish) 24 hours before treatment. For flow cytometry analysis, the cells were collected 24 hours after treatment and fixed in 70% ethanol followed by RNase treatment (37°C, 30 minutes). The cells were analyzed for death after adding propidium iodide (50 μg/mL, Sigma) using FACSscan flow (Becton Dickinson, San Jose, CA). For Western immunoblot analysis, 25 μg of cell lysate prepared with radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (EMD Biosciences, San Diego, CA) were separated on NuPAGE 4% to 12% Bis-tris gel (Invitrogen). The blotting membranes were stained with the following antibodies at appropriate dilutions: polyclonal rabbit anti-human STAT3, anti-phosphorylated tyrosine 705 STAT3 (1:1,000), and anti-caspase 3 (1:800) antibody (Cell Signaling Technology), anti-PARP (1:2,000) antibody (BD Pharmingen, San Diego, CA), and anti-β-actin (1:2,000) and anti-α-tubulin (1:5,000) antibodies (Sigma) in 0.05% Tween 20/TBS containing 5% nonfat skimmed milk (4°C, overnight). Proper secondary antibodies coupled with horseradish peroxidase (Bio-Rad, Hercules, CA) were used for the detection of immunospecifc bands using ECL-Plus kit (Amersham Biosciences, Piscataway, NJ).

In vitro migration assay. The upper wells of 24-well transwell chambers with polycarbonate filters (8 μm pore size; Costar, Acton, MA) were coated with 50 μL of Matrigel (0.8 μg/mL, 37°C, 2 hours; BD Biosciences, San Diego, CA). HCCLM3 cells (1.0 × 105 in 100 μL DMEM) collected at 24 hours after treatment were added to precoated wells. The cells were then allowed to migrate toward the lower compartment containing conditioned medium as a chemoattractant (37°C, 40 hours). The cells migrating to the membrane were enumerated with Giemsa staining.

In vivo tumorigenicity and intradural tumor angiogenesis. Male 6- to 8-week-old BALB/c nu/nu mice were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, P.R. China. Animals were maintained in accordance with the recommendations of the NIH Guidelines for Care and Use of Laboratory Animals. HCCLM3 cells (1.2 × 106, viable by trypan blue exclusion) mixed with 0.1 mL of Matrigel (1 μg/mL; BD Biosciences) were s.c. injected into three different regions on the flanks of mice (three sites/mouse, n = 6) under halothane anesthesia. Tumor sizes were measured once a week (tumor volume = ab2/2 in mm3, where a and b are the longest and the shortest perpendicular diameters of the tumor, respectively), and tumor weights were taken at termination on day 35. For observation of tumor angiogenesis, similarly treated cells were injected intradermally in the flanks of mice (n = 4). The inner side of the skin bearing tumor was exposed for photography and luciferase analysis (Chemiluminescence, Promega, Madison, WI) following the manufacturer’s protocol.

Therapeutic treatment of orthotopic HCCLM3 tumors with STAT3 ASO. Tumor fragments (1 mm3 in size) were intrathecally implanted into male BALB/c nu/nu mice under sterile conditions. Mice were then randomly assigned into three groups (n = 15 each) and treated i.p. with oligonucleotide in saline solution. On day 35 after
implantation (i.e., 3 days after the last dose), nine mice randomly selected from each group were sacrificed to examine tumor growth and metastases. The remaining mice were monitored for death (euthanized when appearing moribund). Another 45 mice were treated in a similar fashion, except that the first injection was delayed until 10 days after implantation.

**Determination of intrahepatic HCCLM3 tumor development and metastasis.** Intrahepatic tumor volume was determined as above. Blood, liver tumor, and lung tissues were collected. Plasma VEGF and basic fibroblast growth factor (bFGF) were measured by ELISA (R&D Systems, Minneapolis, MN). Formalin-fixed liver tumors were examined for STAT3 protein levels and microvessel density using immunohistochemistry methods (STAT3 antibody from Lab Vision, rat anti-mouse CD34 antibody from Serotec Ltd., Kidlington, Oxford, United Kingdom, and an alkaline phosphatase-conjugated secondary antibody from Chemicon). The mean microvessel density was determined by counting the three fields that had the highest vessel density in each group (0.708 mm² at 200×). Lung tissue was evaluated for metastases by sequential sections of paraffin-embedded tissue.

**Statistical analysis.** Groups from cell culture and in vivo experiments were compared using two-tailed Student’s t tests and results were presented as means ± SD. Animal treatments for survival studies were compared by log-rank and Kaplan-Meier methods. P < 0.05 indicated significant difference when compared with vehicle control, unless specified in the figure legends.

### Results

**STAT3 mediates proliferation, survival, and migration of a highly metastatic human HCC cell line.** The constant expression of STAT3 was easily detectable in HCCLM3, an HCC selected for its highly metastatic behavior in vivo. Treatment of HCCLM3 with STAT3 ASO inhibited STAT3 mRNA expression in a dose- and time-dependent manner, whereas the same 2′-MOE–modified, sequence-randomized oligonucleotide had no effect (Fig. 1A), indicating the presence of a sequence-specific, hybridization-based antisense activity. The ASO had an IC₅₀ of 250 nmol/L for the inhibition of STAT3 mRNA at 24 hours posttransfection (Supplementary Fig. S1A). Immunocytochemical staining revealed that both precursory and tyrosine 705–phosphorylated STAT3 proteins were highly expressed in the nuclei of HCCLM3 cells, whereas both were specifically and significantly reduced following STAT3 ASO treatment, but not by control oligonucleotide treatment (Fig. 1B). Electrophoretic mobility shift assay analysis of nuclear extracts from STAT3 ASO–treated HCCLM3 cells showed significantly reduced STAT3 binding to the high-affinity SIE sequence (20), as compared with control oligonucleotide or vehicle lipid–treated cells (Fig. 1C).

We investigated the effects of specific suppression of STAT3 on the proliferation and survival of HCCLM3 cells. Cell proliferation was found to be inhibited in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Supplementary Fig. S1B-C) and nuclear Ki-67 staining (Fig. 2A) by STAT3 ASO treatment. In keeping with disrupted cell proliferation, HCCLM3 cell death was increased as examined by flow cytometry using propidium iodide staining (Fig. 2B). The percentage of HCCLM3 cells in the pre-G₁ fraction was markedly higher in STAT3 ASO than in control oligonucleotide–treated cells. Caspase 3 was found to be specifically activated following the suppression of STAT3 (Fig. 2C), suggesting that apoptosis was induced as a consequence of arrested proliferation following STAT3 suppression.

To understand the role of STAT3 in HCC more broadly, we further examined the effects of STAT3 suppression on two additional popularly used human HCC lines: SNU423 and Huh7. With STAT3 ASO treatment, the cell proliferation of both lines was significantly reduced in a sequence-specific and dose-dependent manner (Fig. 2D). Tyrosine-phosphorylated STAT3 levels were significantly inhibited in both lines treated with STAT3 ASO, however, apoptosis was induced only in SNU423, not in Huh7 (Fig. 2E), as indicated by both caspase 3 and PARP cleavage. These results suggest that STAT3 may control HCC cell proliferation generally, yet the participation of other genes in STAT3–mediated antiapoptosis seems to be specific to individual HCC cell subtypes.

As migratory behavior is a surrogate of invasive potential, we examined HCCLM3 mobility in Matrigel using a transwell assay. STAT3 ASO–treated viable HCCLM3 cells migrated toward their conditioned growth medium in significantly fewer
numbers than their vehicle or control oligonucleotide–treated counterparts (Fig. 2F), suggesting that the invasive potential of HCCLM3 was decreased after the inhibition of STAT3.

Angiogenic, apoptotic, and metastatic gene expression are regulated by STAT3 in HCCLM3 cells. Because STAT3 significantly regulated HCCLM3 growth and migration, we examined the expression of genes involved in tumor development, survival, and/or transmission. HCC is considered to be a hypervascularized tumor and VEGF expression is increased in patients (21). VEGF expression is regulated in part by activated STAT3 (22) as well as by HIF-1α (23). We found that HCCLM3 expressed three variants of alternatively spliced VEGF mRNA, among which VEGF 121 and 165 were dominant (Supplementary Fig. S2A). Following STAT3 ASO treatment, all three VEGF mRNAs were reduced (Supplementary Fig. S2B) and the VEGF protein level in culture supernatant was also diminished (Supplementary Fig. S2C).

MMPs are involved in the digestion of extracellular matrices, penetration of stroma, and establishment of transmission during malignancy. Recently, activated STAT3 was reported to bind directly to the MMP2 promoter region and transactivate MMP2 transcription in melanoma cells (24), and both MMP2 and MMP9 have been implicated in HCC (25, 26). Both latent and active forms of MMP2 and MMP9 were easily detectable in the culture supernatants of HCCLM3 as indicated by the digestion of gelatin, however, both were inhibited following STAT3 ASO treatment (Supplementary Fig. S2D). These data suggest that in addition to MMP2, STAT3 likely controls MMP9 transcription in HCCLM3 as well. STAT3 ASO treatment also suppressed survivin mRNA expression in HCCLM3 (Supplementary Fig. S2E), in agreement with previous work showing STAT3 regulation of survivin expression in many tumor cell types (27, 28). Thus, STAT3 seems to be a common regulator of VEGF, MMPs, and survivin expression in HCCLM3.

We also did mRNA expression profiling (Sigma Pathway-Finder Gene Array) following STAT3 antisense treatment in HCCLM3 cells. In addition to the inhibition of VEGF, MMP9, MMP2, and survivin, the expression of Akt-1, Bcl-x, c-Myc, and cyclin D1, but not Src, were specifically reduced, supporting the potential involvement of these gene products in Stat3-induced uncontrolled cell growth, desensitization of apoptosis, and prosangiogenic activities of this malignant HCC. Moreover, the expression of Fas and tumor necrosis factor–related apoptosis inducing-ligand receptors, two important cell death–inducing proteins, are interestingly found to be significantly increased after Stat3 antisense treatment (data not shown). In a mouse HCC model of liver-specific transgenic expression of SV40 viral T/t antigen, phosphorylated Akt (serine 473), but not phosphorylated Src (tyrosine 416), was inhibited in tumor tissue following STAT3 suppression by an ASO-targeting mouse STAT3 (data not shown), suggesting that the activation of Akt downstream of activated STAT3 signaling is involved in HCC pathology. Further elucidation of other potentially contributory signal transduction pathways is necessary to more fully define

Fig. 2. Effects of STAT3 suppression on proliferation, survival, and migration of HCC cells. HCC cells were treated with STAT3 ASO, control oligonucleotide (Ctrl ODN), or LipofectAMINE (Lipo). A, immunocytochemical staining of Ki-67 in HCCLM3 (250 nmol/L, 24 hours). B, flow cytometry analysis of dying cells in HCCLM3 following propidium iodide staining (250 nmol/L, 24 hours). C, Western analysis of caspase 3 cleavage following STAT3 suppression in HCCLM3 (250 nmol/L, 24 hours). D, reduction of proliferation in SNU423 (left) and Huh7 (right) treated with STAT3 ASO at the indicated concentration (72 hours). E, Western analysis of caspase 3 and PARP cleavage following STAT3 inhibition in SNU423 (left) and Huh7 (right) after treatment (100 nmol/L, 48 hours). F, HCCLM3 cell migratory ability in transwell assay. Giemsa staining of the cells on transwell membrane (top) and quantitation (bottom) (250 nmol/L, 24 hours). Columns, means; bars, SD; *, P < 0.05.
STAT3-dependent mechanisms of HCCLM3 tumor growth and metastasis.

**STAT3 is essential for HCC in vivo angiogenesis and tumorigenesis.** To show the role of STAT3 in HCC growth and angiogenesis in vivo, in vitro ASO-transfected HCCLM3 cells were injected intradermally in athymic mice. HCCLM3 tumors arising from STAT3 ASO–treated cells showed significantly less neovascularization in number, size and branch of the neovessels than HCCLM3 treated with either control (Fig. 3A). The average count of vessels around tumors was inhibited to ~30% of control groups following STAT3 ASO treatment (Fig. 3B).

HCCLM3 was shown to be 100% tumorigenic after s.c. injection (12), however, tumorigenicity was significantly impeded by STAT3 ASO treatment (Fig. 3C).

Both tumor size and tumor weights at the end of the study were reduced in STAT3 ASO–treated cells (Fig. 3D-F), whereas control oligonucleotide treatment showed a slight effect on tumorigenesis that was not significantly different from LipofectAMINE treatment.

**STAT3 ASO treatment decreases circulating VEGF and neovascularization in athymic mice bearing orthotopic HCCLM3 tumors.** An advantage of the HCCLM3 model is intrahepatic and local transmission with concurrent spontaneous metastasis to the lung in all animals after orthotopic implantation of the tumor in livers of athymic mice. This malignancy pattern of HCCLM3 tumor development mirrors that of clinical HCC. We evaluated the therapeutic relevance of STAT3 ASO treatment in this orthotopic HCC model, using two postimplantation regimens chosen to mimic the therapy of primary and recurrent (later stage) disease (Fig. 4A, Materials and Methods). Intraportal dosing of STAT3 ASO following orthotopic implantation inhibited STAT3 protein expression in recovered HCC tumors (Fig. 4B, day 35). Although both HCCLM3 tumor cells and mouse liver endothelial cells can produce VEGF in this model, circulating human VEGF protein was significantly
reduced to ~27% of the control levels following regimen 1 treatment with STAT3 ASO (Fig. 4C, day 35). Circulating human bFGF was similarly inhibited (Fig. 4C), but it is unclear if bFGF is another STAT3 target gene in HCC or if this effect is secondary to STAT3 inhibition. The inhibitory potency of the two treatment regimens on the production of these two angiogenic factors was similar.

With down-regulation of VEGF and bFGF, we were interested to know if the neovascular development in HCCLM3 tumors changes accordingly. Staining of the liver tumor tissues with murine CD34-specific antibody showed that the microvessel density within tumors was significantly reduced in STAT3 ASO–treated mice compared with controls (Fig. 4D and E, day 35), indicating that angiogenesis is promoted through STAT3 activation in this orthotopic tumor model and that VEGF produced from tumor cells rather than liver endothelium likely provides a primary angiogenic signal.

Therapeutic treatment with STAT3 ASO inhibits metastasis and growth of orthotopically transplanted HCCLM3 tumors in athymic mouse. Observation of local metastases of implanted tumors revealed that in the animals treated with regimen 1 STAT3 ASO, intraceliac seeding, bloody ascites, diaphragmatic metastasis, and intrahepatic transmission were all significantly decreased in incidence compared with saline or control oligonucleotide–treated mice (Fig. 5A; Table 1). Liver tumor volume and weight were reduced to 43% and 54% of saline-treated values, respectively (Table 2). In accordance with the local effect, the incidence of mice with lung metastasis dropped to 0% from either 100% in saline (P < 0.001) or 89% in control oligonucleotide (P < 0.001)–treated animals, respectively (Table 2) following STAT3 ASO treatment.

Following regimen 2 delayed treatment, a slight loss in potency of STAT3 ASO was observed, with less inhibition of intraceliac seeding, tumor size and weight, and lung metastases (Tables 1 and 2). Nevertheless, the overall effect of the delayed treatment was significantly different from either control, indicating that STAT3 targeting with an ASO approach is effective even after the establishment of orthotopic HCC.

**STAT3 ASO treatment extends survival time of mice bearing orthotopic HCC tumors.** Animals bearing orthotopic HCCLM3 tumors eventually die from tumor rupture, liver failure, and complications arising from unchecked growth of the tumor, similar to the fate of late-stage patients with advanced HCC. After completion of treatment on day 32, six mice from each group were continually monitored for survival. Regimen 1 STAT3 ASO treatment significantly extended mean survival time.
was persistently activated in several HCC cell lines. This finding supports previous reports implicating STAT3 in HCC, for example, silencing by methylation of SOCS-1, a negative regulator of STAT3, results in constitutive activation of STAT3 in the majority of primary human HCCs examined (29); STAT3 is overexpressed in proteome microarray analysis of primary HCC (30); STAT3 phosphorylation is highly positive in immunohistochemistry analysis of HCC biopsies (31); and STAT3 DNA binding activity is observed in chemically induced HCC (32). These data collectively indicate that HCC is an additional tumor type that harbors constitutively active STAT3.

Promotion of growth, desensitization of apoptosis, augmentation of angiogenesis, and modulation of host tumor immunity are hallmarks of STAT3 activity in tumor cell studies as well as in syngeneic and xenograft models (4). The amelioration of the malignant behaviors of HCCLM3 following orthotopic implantation, including impeded migration, hampered neovascularization, inhibited local spreading, and reduced lung metastasis lead us to the conclusion that STAT3 also mediates the metastatic potential of HCCLM3, a highly malignant HCC. This hypothesis is further supported by the finding that the production of VEGF and MMP2, two well-characterized target genes of STAT3 (21, 22, 25), are down-regulated following the inhibition of STAT3 and that VEGF and bFGF proteins are reduced in the serum of STAT3 ASO–treated tumor-bearing mice. The control of MMP9 by STAT3 is less well-characterized (33) and not previously reported in HCC. It is not yet clear whether MMP9 expression in HCCLM3 cells is regulated by STAT3 at the transcriptional or posttranscriptional level. HCCLM3 features high lung metastatic potential and resembles typical cases of HCC in the clinic, yet it will be important to further examine if constitutive STAT3 signaling also controls the tumorigenicity of other subtypes of HCC, e.g., tumors displaying lower lung metastasis or lymph node transmission, although these are less dominant behaviors in HCC.

The results of our in vitro microarray and in vivo orthotopic HCC models implicate multiple known STAT3-regulated genes in the pathology of metastatic human HCC. STAT3-dependent modulation of Akt-1 expression and phosphorylation in our HCC models is supported by previous studies which show transcriptional regulation of the Akt-1 promoter by the v-src/STAT3 pathway (34) and link Akt-1 regulation to the induction

Table 1. STAT3 ASO treatment inhibits local spread of intrahepatic HCCLM3 tumor

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Peritoneal seeding (%)</th>
<th>Bloody ascites (%)</th>
<th>Diaphragm seeding (%)</th>
<th>Intrahepatic transmission (%)</th>
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<td>7 of 9 (77.8)</td>
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<td>3 of 9 (33.3)</td>
<td>5 of 9 (55.6)</td>
<td>5 of 9 (55.6)</td>
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<tr>
<td>STAT3 ASO</td>
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<td>0 of 9 (0)*</td>
<td>0 of 9 (0)*</td>
<td>0 of 9 (0)*</td>
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<tr>
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<td>8 of 9 (88.8)</td>
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<tr>
<td>Control oligonucleotide</td>
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<td>STAT3 ASO</td>
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<td>0 of 9 (0)*</td>
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NOTE: Intraperitoneal tumor invasiveness was scored on day 35 following orthotopic implantation of HCCLM3 tumor fragments and subsequent therapeutic treatment with STAT3 ASO or controls (n = 9 per group). The number of mice with positively invasive tumors or bloody ascites in each group is presented. The percentages of the total group number are indicated in parentheses. Bloody ascites represented >10,000 RBC/mm³ in the peritoneal fluid.

*P < 0.05.

Discussion

Aberrant STAT3 activation has been linked to oncogenesis in a variety of human tumors. In this report, we found that STAT3...
of antiapoptosis in hepatoma cells (35, 36). Although we did not specifically evaluate STAT3-dependent mechanisms of suppression of host tumor immunity in our human in vivo model, we did observe direct effects of STAT3 inhibition on HCCLM3 cells that are consistent with previous reports of evasion of immune surveillance, e.g., STAT3-dependent down-regulation of Fas mediated via c-Jun cooperation (37) and tumor necrosis factor–related apoptosis-inducing ligand expression in tumor cells (38), and increased production of VEGF in animals bearing tumors with constitutively activated STAT3 (4, 9). Recent investigations have shown the suppression of host antitumor immunity mediated by STAT3 expression in tumors (10) and in hematopoietic cells (39, 40). Although we used athymic mice in our HCC model, we cannot rule out the possible effect of STAT3 antisense treatment on host dendritic cells, natural killer cells, or neutrophils. However, this mechanism seems less likely because antitumor effects were largely absent in mice lacking STAT3 expression in hematopoietic cells that were devoid of CD4+ and CD8+ T lymphocytes (40).

Hepatitis B virus (HBV) and hepatitis C virus chronic infection are two known causative factors of HCC and viral proteins are reported to be able to activate STAT3 (41, 42). HBV X protein constitutively enhances tyrosine phosphorylation of STAT3 by specifically activating Jak1 (43). Activated STAT3 has also been shown to directly bind to hepatocyte nuclear factor-3, resulting in cooperative activation of HBV enhancer 1 function, a cytokine and growth factor–responsive element that positively regulates the activity of HBV promoter elements in a liver-specific manner (44). Hepatitis C virus core and NS5A proteins are shown to either directly or indirectly lead to phosphorylation of STAT3 (42, 43, 45). These data suggest that hepatitis B and C viruses subvert STAT3 signaling pathways in hepatocytes to promote their transformation. Interestingly, in our results, whereas proliferation of all three HCC cell lines was critically regulated by STAT3 (4, 9), recent investigations have shown the suppression of STAT3 expression in HCC cells in culture and in rats (49, 50). In the present study, the ASO-mediated specific suppression of STAT3 expression in HCCLM3 has already been noted when STAT3 was suppressed by 50% of its up-regulated levels. Thus, our data strongly suggest that a limited reduction of activated STAT3 in HCC would likely be of benefit although not likely to produce unmanageable toxicity.

It has been shown in many reports that 2′-MOE phosphorothioate-modified oligonucleotides accumulate in the liver following systemic administration in mice and that the ASO pharmacokinetics and pharmacodynamics are closely correlated (49, 50). In the present study, the ASO-mediated specific suppression of STAT3 expression in HCC cells in culture and in tumors is shown by oligonucleotide concentration– and treatment–time–dependency, as well as sequence-specificity. The two therapeutic systemic dosing regimens used confirmed the observed pharmacologic effects and showed the onset-of-action of antiapoptosis and growth inhibition and suppression of metastasis. Conditional ablation of STAT3 signaling partially impairs liver regeneration (46) and tissue-specific ablation of hepatic STAT3 is shown to affect glucose homeostasis and induction of insulin resistance (47). To clarify the potential hurdles for STAT3-targeted therapy, we also designed potent murine and murine/human STAT3 ASOs with the same chemistry and tested them in normal mice. When mouse STAT3 mRNA was inhibited by 85% and 50%, respectively, in liver following treatment for 14 and 21 days, plasma glucose, triglycerides, and cholesterol levels of mice were unchanged; neither liver enzymes nor caspase 3 activation was induced in the liver. Furthermore, these mice displayed no effects on hematologic variables, similar to the normal mice or HCC-bearing mice treated with the human STAT3 ASO. Similar results have been recently reported in an anaplastic large cell lymphoma study in mice (48), supporting the observations that non–tumor cells largely tolerate the lack of STAT3 expression (4). Therefore, the excessive or deregulated expression of activated STAT3 may be required for genesis and malignant growth of HCC (30), whereas normal cells including liver cells may require less STAT3 for proper function. In comparison, a significant antitumor effect on HCCLM3 has already been shown.

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### Table 2. STAT3 ASO treatment reduces HCCLM3 tumor size, weight, and metastasis

<table>
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<th>Treatment groups</th>
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<th>Lung metastasis</th>
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<td>Volume (mm³)</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>Regimen 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>1,471 ± 359</td>
<td>1.73 ± 0.17</td>
</tr>
<tr>
<td>Control oligonucleotide</td>
<td>1,311 ± 131</td>
<td>1.67 ± 0.27</td>
</tr>
<tr>
<td>STAT3 ASO</td>
<td>634 ± 117*</td>
<td>0.93 ± 0.12*</td>
</tr>
<tr>
<td>Regimen 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>1,564 ± 269</td>
<td>1.89 ± 0.24</td>
</tr>
<tr>
<td>Control oligonucleotide</td>
<td>1,372 ± 152</td>
<td>1.77 ± 0.39</td>
</tr>
<tr>
<td>STAT3 ASO</td>
<td>802 ± 158*</td>
<td>1.18 ± 0.11*</td>
</tr>
</tbody>
</table>

*NOTE: Dissection of intrahepatic tumors and evaluation of lung metastasis in mice was done on day 35 following orthotopic implantation of HCCLM3 tumor fragments and subsequent therapeutic treatment with STAT3 ASO or controls (*n* = 9 per group). Tumor sizes are presented by both volume in cubic millimeters and weight in grams (mean ± SD). Lung metastasis is denoted as both the positive metastases in the lungs (the percentages are indicated in parentheses) and the average number of metastases (no.) in lungs per positive case (mean ± SD). *p* < 0.05.
of antisense therapy, further supporting antisense-mediated inhibition of STAT3. Because 70% to 90% of liver cancers exhibit preexisting liver disease (viral hepatitis, inflammation, cirrhosis, etc.), it is preferable to use a highly target-specific drug approach to treat HCC to avoid the possible liver toxicity that can further endanger patients to the risk of liver failure. Our data suggest that the ASO approach offers promise as a safe and effective therapy for HCC, and that targeting STAT3 may have a therapeutic benefit for patients with HCC.

References

Inhibition of Growth and Metastasis of Human Hepatocellular Carcinoma by Antisense Oligonucleotide Targeting Signal Transducer and Activator of Transcription 3

Wen-Chang Li, Sheng-Long Ye, Rui-Xia Sun, et al.


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