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Variants of CTGF are associated with hepatic fibrosis in Chinese, Sudanese, and Brazilians infected with Schistosomes

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Abnormal fibrosis occurs during chronic hepatic inflammations and is the principal cause of death in hepatitis C virus and schistosome infections. Hepatic fibrosis (HF) may develop either slowly or rapidly in schistosome-infected subjects. This depends, in part, on a major genetic control exerted by genes of chromosome 6q23. A gene (connective tissue growth factor [*CTGF*]) is located in that region that encodes a strongly fibrogenic molecule. We show that the single nucleotide polymorphism (SNP) rs9402373 that lies close to *CTGF* is associated with severe HF ($P = 2 \times 10^{-6}$; odds ratio [OR] = 2.01; confidence interval of OR [CI] = 1.51–2.7) in two Chinese samples, in Sudanese, and in Brazilians infected with either *Schistosoma japonicum* or *S. mansoni*. Furthermore, SNP rs12526196, also located close to *CTGF*, is independently associated with severe fibrosis ($P = 6 \times 10^{-4}$; OR = 1.94; CI = 1.32–2.82) in the Chinese and Sudanese subjects. Both variants affect nuclear factor binding and may alter gene transcription or transcript stability. The identified variants may be valuable markers for the prediction of disease progression, and identify a critical step in the development of HF that could be a target for chemotherapy.

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Abbreviations used: CI, confidence interval of OR; CTGF, connective tissue growth factor; ECMP, extracellular matrix protein; HBV, hepatitis B virus; HF, hepatic fibrosis; OR, odds ratio; SNP, single nucleotide polymorphism.

Hepatointestinal schistosomiasis is caused by two species of helminths: *Schistosoma japonicum*, which is prevalent in Asia, and *S. mansoni*, which is prevalent in Africa and South America. Both worms develop in their host mesenteric system and lay eggs triggering inflammation in the hepatic periportal space in which they are trapped. Worms live for years, and thus, chronic liver inflammation and significant tissue destruction are common in infected subjects. Tissue repair begins with the deposit of extracellular matrix proteins (ECMPs) in the damaged tissues, which are later replaced by normal hepatocytes. In some subjects, ECMPs accumulate in the periportal space, forming fibrosis deposits that reduce blood flow, causing varicose veins. Subjects die from the subsequent effects of hepatic fibrosis (HF). 5–10% of the 350 million infected subjects may develop severe HF. There are no good markers for predicting HF progression in schistosome-infected subjects. HF development is strongly

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influenced by a major locus located at chromosome 6q23 (Dessein et al., 1999). This region contains two major candidate genes: *IFNGR1* encodes a chain of the IFN-γ receptor (IFN-γ being an antifibrogenic cytokine that protects against HF; Henri et al., 2002; Chevillard et al., 2003); and connective tissue growth factor (*CTGF*), which encodes a profibrogenic molecule produced by hepatocytes (Kobayashi et al., 2005; Gressner et al., 2007), hepatic stellate cells, myofibroblasts, and endothelial cells (Gressner and Gressner, 2008). *CTGF* transcripts are overexpressed in livers affected by fibrosis of various etiologies (Rachfal and Brigstock, 2003). In this report, functional single nucleotide polymorphisms (SNPs) implicate *CTGF* as a major actor in severe HF in schistosome-infected Chinese, Sudanese, and Brazilian subjects.

RESULTS AND DISCUSSION

Several independent polymorphisms are associated with severe HF in Chinese fishermen

We first studied HF in Chinese fishermen infected with *S. japonicum*. Covariates influencing the development of HF were gender, exposure, and antischistosome treatments. We used these covariates to test, in 99 cases and 201 controls, whether SNPs (three SNPs per gene) in *CTGF* and *IFNGR1* were associated with HF. SNP rs9399005 in close proximity to *CTGF* was associated with HF (P = 0.02). Sequencing *CTGF* (3.2 kb), 15.3 kb of sequence upstream from the starting codon and 14.1 kb of sequence in the 3′ untranslated region, revealed 61 SNPs, of which 50 had been previously described. The 33 SNPs with a minor allele frequency > 20% were grouped into seven correlation ($r^2 = 0.8$) bins (I–VII; Fig. 1). We selected 22 SNPs for further genotyping: at least two SNPs per bin and five SNPs not included in bins but with potential functional effects, as assessed by in silico analysis. SNPs associated with HF (Table I) were SNPs rs1257705 (P = 0.02; odds ratio [OR] = 2.3) and rs12526196 (P = 0.02; OR = 2.2) in bin II, SNPs rs6918698 (P = 0.02; OR = 2) and rs3037970 (P = 0.003; OR = 2.6) in bin III, SNPs rs1931002 (P = 0.004; OR = 2.3) and rs2151532 (P = 0.02; OR = 2) in bin IV, SNP rs9402373 (P = 0.015; OR = 2) in bin VI, and SNP rs9399005 (P = 0.02; OR = 2.2). The covariates were gender (P < 0.001), exposure (fishing years: P < 0.001; born on boat: P < 0.01), and number of antischistosome treatments (P < 0.01).

SNP rs3037970 excluded rs6918698 in a multivariate analysis testing both SNPs simultaneously. Similarly, SNP rs1931002 excluded SNP rs2151532, and SNP rs1256196 excluded SNPs rs1257705 and rs9399005. This indicated that SNPs rs12526196, rs30337970, rs1931002, and rs9402373 had the strongest association with HF. When all four SNPs were tested in the same regression model (Table I, bottom), SNPs rs12526196 (P = 0.007; OR = 3), rs9402373 (P = 0.002; OR = 2.8), and rs1931002 (P = 0.002; OR = 2.8) were independently associated with HF. We also tested a more severe phenotype, which included advanced HF only if patients displayed evidence of portal hypertension. We found that SNPs rs9402373 (P = 0.005; OR = 2.6) and rs3037970 (P = 0.05; OR = 2) were associated with this phenotype; SNP rs1256196 also showed a trend for an association with this more severe disease phenotype (P = 0.12). Gender (P = 0.001) was a significant covariate.

rs1257705, rs12526196, and rs9402373 polymorphisms are also associated with severe HF in Chinese farmers

We sought the replication of these results in an additional independent sample of Chinese farmers infected with *S. japonicum*. The SNPs that showed evidence of an association with HF in fishermen were genotyped in farmers (113 controls and 181 cases). The analysis of the SNPs separately showed associations between severe HF and SNPs rs9402373 (P = 0.003; OR = 2.2) and rs1256196 (P = 0.02; OR = 1.9; Table I). Other SNPs were not associated (P > 0.1). Multivariate analysis of these SNPs simultaneously indicated that SNPs rs9402373 (P = 0.03; OR = 2.3) and rs1256196 (P = 0.02; OR = 1.9) were independently associated with severe disease. Covariates were birthplace (endemic or not endemic: P = 0.03) and infection with hepatitis B virus (HBV; P = 0.05).

We next investigated whether HF caused by *S. mansoni* in endemic subjects in Sudan and Brazil was also affected by *CTGF* allelic variants. *CTGF* polymorphisms associated with HF in Chinese fishermen were genotyped in both samples.

rs1257705, rs12526196, and rs9402373 polymorphisms are also associated with severe HF in Sudanese patients

In Sudanese and Brazilian samples, the minor SNP 1256196 allele frequencies (4.4 and 6.6%, respectively) were lower than in Chinese fishermen and farmers (17.03 and 16.52%, respectively); this and smaller sample sizes reduced (from >74% in Chinese to <20% in Sudanese and Brazilians) the power to detect an association with the SNP rs12526196. Nevertheless, the analysis (Table II, left) in Sudanese patients (152 controls and 62 cases) suggested the association of rs1256196 (P = 0.066; OR = 6.8) with HF and indicated the association of rs9402373 (P = 0.008; OR = 5.2) with HF. An additional association was found for rs12527705 (P = 0.069; OR = 6.7). Multivariate analysis of these SNPs simultaneously indicated that SNPs rs9402373 (P = 0.008; OR = 5.2) and rs1256196 (P = 0.059; OR = 7.3) were independently associated with HF in the presence of age as a covariate (P = 0.05).

rs6918698 and rs9402373 polymorphisms are associated with severe HF in Brazilian patients

Genotyping data analysis of the Brazilian patients (61 cases and 75 controls) showed (Table II, right) that SNPs rs9402373 (P = 0.02; OR = 2.6) and rs6918698 (P = 0.008; OR = 3) were associated with HF without additional covariates. The genotypes associated with disease for both SNPs were similar to those observed in Chinese and Sudanese subjects. Multivariate analysis indicated that SNPs rs9402373 ($P = 9 \times 10^{-4}$; OR = 4.3) and rs6918698 ($P = 5 \times 10^{-4}$; OR = 4.9) were independently associated with HF in Brazilians.

We performed a meta-analysis with SNP rs9402373 on the data obtained in the four samples. No covariates were included in the analysis. The meta-analysis confirmed that the

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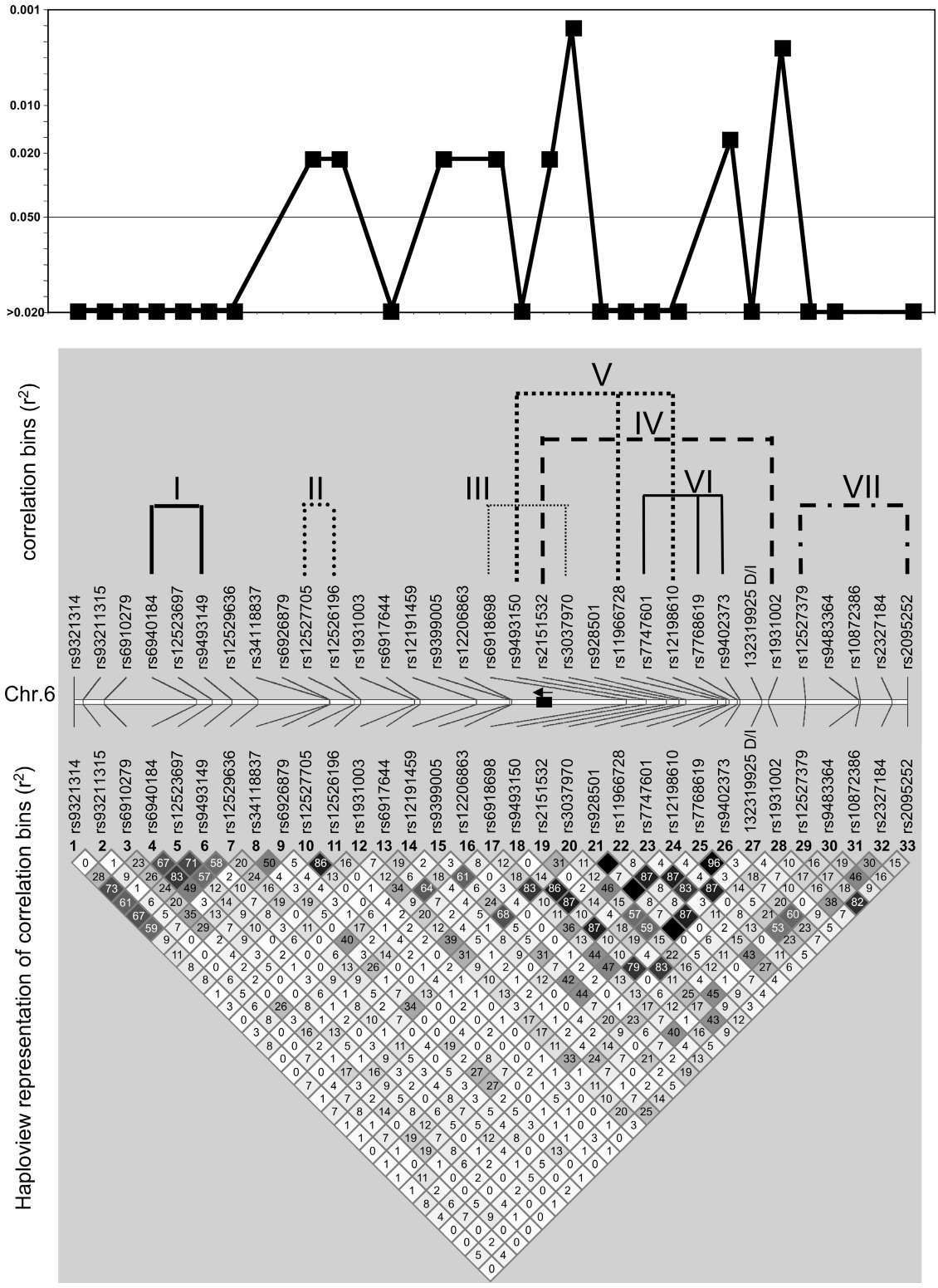


Figure 1. Correlation bins for *CTGF* and flanking regions in the fisherman sample. 33 markers were genotyped in 70 unrelated subjects, as described in Materials and methods. Correlations (r^2 values) between SNPs were determined using Haploview software (available at http://www.broadinstitute.org/mpg/haploview). The darkest colors indicate the strongest correlations (bottom). Correlation bins ($r^2 > 0.8$; middle) were as follows: bin I, SNPs rs6940184 and rs9493149; bin II, SNPs rs12527705 and rs12526196; bin III, SNPs rs6918698 and 3037970; bin IV, SNPs rs2151532 and rs1931002; bin V, SNPs rs9493150, rs11966728, and rs12198610; bin VI, SNPs rs7747601, rs7768619, and rs9402373; and bin VII, SNPs rs12527379 and rs2095252. p-values obtained in the association studies with the 25 selected SNPs in Table I are shown (top).

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CC genotype was associated with severe fibrosis ($P = 2 \times 10^{-6}$; OR = 2.01; confidence interval of OR [CI] = 1.51–2.68). We also performed a meta-analysis with SNP rs12526196 on the data obtained in both the fisherman and farmer Chinese samples and in the Sudanese sample. This indicated that the TT genotype is associated with HF in these populations ($P = 6 \times 10^{-4}$; OR = 1.93; CI = 1.3–2.8).

To further support our findings, we tested, in Chinese fishermen, whether any SNPs within 7 Mb around *CTGF* were correlated ($r^2 > 0.6$) with the SNPs associated with HF (Fig. 2), and could account for the association. We found no correlation.

rs9402373 and rs12526196 polymorphisms affect nuclear factor binding and may alter gene transcription or transcript stability

The associated polymorphisms may create or alter DNA–protein interactions. We performed an in silico analysis (http://jaspar.cgb.ki.se) using an 85% threshold cut-off score (similarity matrix). DNA carrying the rs9402373C allele may bind specifically to BRAC1 (86.7% homology) and Broad-Complex 3 (89.4%). The rs9402373G allele appears to be able to bind to RUSH1-alfa (94.3%), FOXC1 (90.2%), Dof2 (88.8%), Dof3 (96.6%), MNB1A (93.2%), PBF (96%), and Mafb (85.3%). The rs12526196C allele may not specifically bind one transcription factor whereas the rs12526196T allele may specifically bind Foxq1 (87.6%), SRY (91%), FOXL1 (86.8 and 85.8%), SOX9 (85.6%), Sox5 (87.5%), and Sox17 (88%). To confirm these findings, we performed EMSA experiments; EMSA also showed a greater binding affinity of nuclear factors for the rs12526196T allele (Fig. 3 A). The rs9402373 C allele also bound nuclear factors that were not bound by the G allele (Fig. 3 B). This binding was competed with a specific unbiotinylated probe (Fig. 3 B). EMSA did not reveal allele-specific binding for the rs12527705 and rs1931002 polymorphisms (Fig. 3 A).

We show in this report that two SNPs in the *CTGF* gene are associated with severe HF across various populations. This result is remarkable given the fact that these populations are ethnically different and are infected by two different schistosome species. However, this result was not completely unexpected, as this gene belongs to a chromosomal region associated by us (Dessein et al., 1999) and others (Blanton et al., 2005), with aggravated fibrosis in a Sudanese population

Table I. SNPs in the region flanking *CTGF* are associated with severe HF in two Chinese samples

					Chinese fishermen					Chinese farmers				
	SNP	Position	Bins	Genotype	Controls	Cases	OR	95% CI	P	Controls	Cases	OR	95% CI	P
					%	%				%	%			
Univariate analysis	rs1257705	132304944	II	AA	69.1	83.7	2.26	1.13–4.5	0.02	65.1	72	1.67	0.98–2.84	0.06
	rs12526196	132305169	II	TT	67.9	82.5	2.21	1.13–4.4	0.02	61.6	71.8	1.85	1.1–3.1	0.02
	rs9399005	132310657		CC	17.2	26.3	2.2	1.1–4.29	0.02					NS
	rs6918698	132314950	III	CC	26.6	35.1	2	1.1–3.7	0.02					NS
	rs3037970	132316891	III	−/−	27.3	38.8	2.59	1.4–4.8	0.003					NS
	rs1931002	132320175	IV	CC	52.5	66.7	2.32	1.3–4.13	0.004					NS
	rs2151532	132316423	IV	AA	55.4	65	1.98	1.12–3.5	0.02					NS
	rs9402373	132319124	VI	CC	50.3	60.6	2.04	1.15–3.54	0.015	25	43.1	2.23	1.32–3.76	0.003
Multivariate analysis	rs12526196		II	TT			3	1.4–6.6	0.007			1.88	1.13–3.19	0.018
	rs9402373		VI	CC			2.8	1.5–5.4	0.002			2.26	1.33–3.83	0.03
	rs1931002		IV	CC			2.81	1.47–5.37	0.002					

Data are provided for two independent Chinese samples (fishermen and farmers living in a region endemic for *S. japonicum*). Cases and controls were defined as indicated in Materials and methods and in Table S1. The association between genotypes and HF phenotypes were first tested separately (univariate analysis) and then simultaneously (multivariate analysis that included SNPS rs12526196, rs1931002, rs3037970, and rs9402373, which showed the strongest associations when tested against SNPs from the same bin). Bins represented correlation ($r^2 > 0.8$) groups, and genotype was the aggravating genotype. In the Chinese fisherman sample ($n = 300$, 99 cases and 201 controls), the covariates were the number of years of fishing, being born on a boat, sex, and the number of Praziquantel treatments. In the Chinese farmer sample ($n = 294$, 113 controls and 181 cases), the covariates were age, whether HF was endemic or not endemic, and whether they had been infected with HBV ($P = 0.05$; OR = 2.38). P, p-value.

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originating from West Africa and in an Arabic population in Egypt. Nevertheless, minor SNP alleles, such as the T allele of SNP rs12526196, were present at a low frequency in Sudanese and Brazilians, and our study may have lacked the statistical power to detect clear associations between these SNPs and fibrosis in these samples.

In cases in which covariates could be evaluated accurately, our analysis included those potentially affecting the fibrosis phenotype in the analysis. Accounting for nongenetic covariates slightly improved the association in all cases. Nevertheless, the effects of all covariates (except for HBV infections) on the genetic associations reported in this paper were modest.

This is in part caused by the fact that we selected populations with high exposure (males and females) to infection. This is illustrated in the results of the meta-analysis that was performed without including the covariates, and confirmed the associations of SNPs rs12526196 and rs9402373 with HF. Nevertheless, past or present HBV infections had a significant effect on the genetic associations (HBV and HCV infections were tested if a suitable treatment of the affected subjects could be performed by the local health authority). Such a significant effect is possibly caused by the fact that HBV infections aggravate HF and portal hypertension, thus confounding the genetic effect: a subject with a protective allele may have a more severe case of disease if he had been infected by both schistosome and HBV.

In this regard, HBV or HCV infections do not cause periportal fibrosis detectable by ultrasound. Thus, the genetic control described in this report relates to HF caused by schistosomes. Our results do not allow concluding that SNPs rs1256196 and rs9402373 also modulate HF caused by HBV and/or HCV, even though Hepatitis virus infections have likely aggravated HF in certain study subjects.

SNP rs9402373 was associated with HF in all four samples tested. This association was obtained in Chinese and Sudanese subjects by using a strict fibrosis phenotype or a more severe phenotype (HF plus evidence of portal blood hypertension). SNP rs1256196 was also independently associated with HF in Chinese and Sudanese subjects. Our EMSA data indicated that allelic variants of SNPs rs1256196 and rs9402373 bind differently to nuclear factors, suggesting that they may affect the regulation of gene transcription or the stability of transcripts.

During the course of our study, SNP rs6918698 has been shown to be associated with sclerodermia in Caucasians (Fonseca et al., 2007). Two other groups have failed to replicate this finding (Gourh et al., 2008; Morita et al., 2008). Our findings of an association of HF with rs6918698CC in Brazilians and in Chinese fishermen were not replicated in the two other samples, and the rs6918698CC genotype is not the aggravating phenotype in sclerodermia. Thus, SNP rs6918698 may be in linkage disequilibrium with another causal SNP.

CTGF is a major profibrogenic growth factor, playing a major role in chondrogenesis and angiogenesis; indeed, $CTGF^{-/-}$ newborn mice die of skeletal defects (Ivkovic et al., 2003; Gressner and Gressner, 2008). CTGF contributes to fibrosis by acting in synergy with various profibrogenic growth factors (Leask and Abraham, 2006), including platelet-derived growth factor, vascular endothelial growth factor, and the master fibrogenic molecule, TGF-β. CTGF is a

Table II. SNPs in the region flanking *CTGF* are associated with severe HF in Sudanese and Brazilian subjects infected with *S. mansoni*

	Sudanese sample							Brazilian sample						
	SNP	Genotype	Controls	Cases	OR	95% CI	P	SNP	Genotype	Controls	Cases	OR	95% CI	P
			%	%						%	%			
Univariate analysis	rs1257705	AA	90.1	98.4	6.68	0.86–51.7	0.069	rs1257705	AA					NS
	rs12526196	TT	90.2	98.4	6.8	0.89–52.8	0.066	rs12526196	TT					NS
	rs3037970	−/−					NS	rs3037970	−/−	80.5	90.2			0.12
	rs6918698	CC					NS	rs6918698	CC + CG	63	83.9	3.05	1.33–6.98	0.008
	rs9402373	CC	78.1	94.9	5.22	1.53–17.7	0.008	rs9402373	CC	61.3	80.3	2.57	1.18–5.64	0.018
Multivariate analysis	rs12526196	TT			7.26	0.93–56.8	0.059	rs6918698	CC			4.92	2–12	5×10^{-4}
	rs9402373	CC			5.23	1.5–18	0.008	rs9402373	CC			4.25	1.79–10.08	9×10^{-4}

The two principal schistosome strains that cause HF are *S. japonicum* in Asia and *S. mansoni* in Africa and South America. We investigated whether HF in an *S. mansoni*–endemic region in Sudan and Brazil was also affected by *CTGF* allelic variants. *CTGF* polymorphisms that were associated with HF in Chinese fishermen were genotyped in both samples. Case and control phenotypes are described in Materials and methods and in Table S1. We first tested for associations between the SNPs and HF phenotypes separately (univariate analysis); the SNPs were then tested simultaneously (multivariate analysis), including SNPs rs12526196, rs1257705, and rs9402373. Genotype is the aggravating genotype. In Sudanese farmers (n = 214, 62 cases and 152 controls), the covariate was age only. In Brazilians (n = 136, 61 cases and 75 controls), there were no covariate. P, p-value.

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TGF-β downstream modulator (Leask and Abraham, 2003; Leask and Abraham, 2006). It increases TGF-β binding to its receptor, interferes with the negative Smad-7 feedback loop affecting TGF-β (Wahab et al., 2005), and inhibits receptor binding of the principal TGF-β antagonist BPM-7 (Abreu et al., 2002). An important consequence of CTGF action on TGF-β is the stimulation of transdifferentiation of hepatic stellate cells and other parenchymal cells into ECMP-producing myofibroblasts, all of which are involved in fibrosis (Kalluri and Neilson, 2003; Neilson, 2005). CTGF also increases ECMP networking through its binding to fibronectin domains on ECMP (Yoshida and Munakata, 2007; Gressner and Gressner, 2008). Inhibiting CTGF with siRNAs prevents or reduces rat tissue fibrosis (Li et al., 2006; George and Tsutsumi, 2007). Thus, CTGF is a major actor in fibrosis, and polymorphisms in *CTGF* could certainly affect HF in schistosome infections. In addition to profibrogenic effects, other CTGF properties, such as stimulation of angiogenesis (Shimo et al., 2001; Brigstock, 2002), stimulation of cell proliferation (Gao et al., 2004), and survival (Croci et al., 2004), partly mediated through its effect on *TGF-β* and BPM-7, may also be directly relevant to other aspects of schistosome-induced pathology. CTGF may contribute to the massive hepatic angiogenesis observed in schistosome-infected subjects and may participate in the development of hepatocarcinoma in schistosomiasis patients.

Our report of an association between *CTGF* polymorphisms and severe fibrosis does not rule out the possibility that polymorphisms in other pathways also affect fibrosis progression. In this regard, there are several studies with various experimental models that show that IL-13 has a major profibrotic effect in mice infected with *S. mansoni*. Several human studies have also shown that polymorphisms in IL-13 or IL-13 receptor chains are associated with aggravated fibrosis, such as in sclerodermia (Granel et al., 2006a; Granel et al., 2006b). In human schistosomiasis, Joseph et al. (2004) have reported an increased production of IL-13 by white blood cells stimulated by schistosome egg antigens in cultures from subjects with advanced disease. IFN-γ production was associated with HF in *S. mansoni*–infected Sudanese subjects; also, polymorphisms in IFN-γ that reduce IFN-γ production were associated with increased HF (Henri et al., 2002;

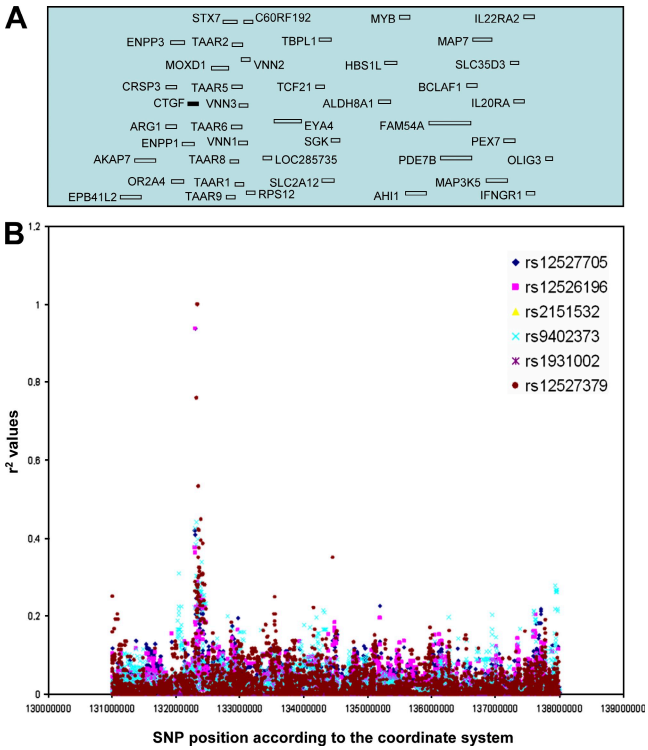


Figure 2. No other SNPs were correlated with SNPs rs12527705, rs12526196, rs9402373, and rs12527379 ($r^2 > 0.6$) for the whole 6q22–q23 region in Chinese subjects. (A) Physical map of the 6q22–q23 region, indicating all the genes. (B) Correlation (r^2) between SNPs rs12527705, rs12526196, rs9402373, and rs12527379 and the 6,896 recorded polymorphisms in the 6q22–q23 (7-Mb) region surrounding *CTGF*. The genotypes were downloaded from the HapMap project (Chinese reference population).

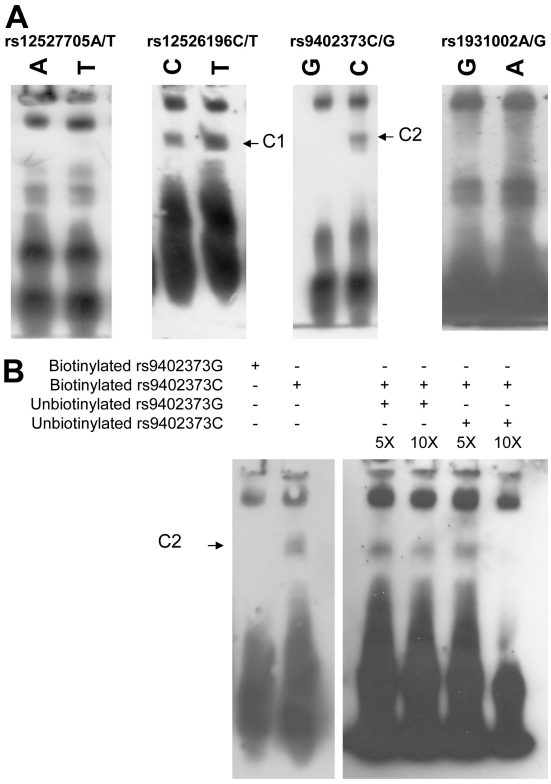


Figure 3. EMSA for SNPs rs12526196, rs9402373, rs12527705, and rs1931002. (A) EMSA was performed according to Materials and methods using nuclear extracts of a stimulated human hepatocyte cell line (HEPG2). The 12526196T allele bound nuclear factors (complex 1) with a higher affinity than the C allele. The SNP rs9402373C allele bound nuclear factors (complex 2) that were not bound by the G allele. No allele-specific binding was observed with SNPs rs12527705 and rs1931002. (B) Competitive reactions performed with 100 (5×) or 200 (10×) fmol of unbiotinylated rs9402373G and C probe. The unbiotinylated rs9402373G did not compete with the biotinylated rs9402373C probe for binding.

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Chevillard et al., 2003). Interestingly, the inhibitory effect of IFN-γ on stellate cell differentiation into myofibroblasts involves a Stat1-mediated down-regulation of *CTGF* expression (Fitzner et al., 2007).

In conclusion, this is the first report of the association between two independent polymorphisms and HF, identifying the most critical steps in disease development and indicating new therapeutic targets. It also provides valuable markers of HF progression.

MATERIALS AND METHODS

Study samples. Chinese fishermen were recruited (fisherman sample) on boats, whereas farmers (farmer sample) were recruited from hospital records (cases) or directly from their farms or villages (controls). Fishermen live on boats and/or on islands. They were highly exposed to infection, whereas most farmers were infected a long time ago, as parasite transmission in most fields was stopped 15 yr ago. Fishermen are mostly from Jiangshu province, and a few of them are from Hubei and Jiangxi. Farmers have been living in the Hunan province for several generations, and some of them originate from the mountain area. The Sudanese sample was recruited from farmers living in villages of the Gezira region (Dessein et al., 1999). The Brazilian sample was recruited in a village in northeast Brazil among subjects with high exposure to infected waters.

Evaluation of HF and case/control definition. HF was evaluated according to the World Health Organization (WHO) guidelines modified as indicated for *S. japonicum* infections (Arnaud et al., 2008).

In *S. japonicum* infections, HF may be central (thick central fibrosis refers to grades CLH, D, E, or F) or peripheral (thick peripheral fibrosis refers to grades GNH or GW). Advanced central and advanced peripheral fibrosis are noted as CLM and GNM, respectively.

In *S. mansoni* infections, HF is mostly central (thick central fibrosis refers to grades D, E, and F), and grade C indicates linear thickening of the portal vein. When schistosome transmission was lower, as in the Brazilian samples, the most advanced cases have grade C fibrosis and <2% of subjects exhibit grades D, E, or F. Enlarged portal vein diameter, varicose veins, and ascites can be used to identify the most severe cases unless study subjects have received many antischistosome treatments. An enlarged portal vein diameter can be taken as evidence of portal hypertension and, when combined with advanced or severe HF, indicates a severe disease. Likewise, the presence of varicose veins and/or ascite indicates portal hypertension and severe disease. The human protocols were approved by the Research Ethical Committees of the University of Gezira and the National Cancer Institute (Gezira, Sudan), of the Hunan Institute of Parasitic Diseases (Yueyang, China), and of the University do Triangulo Mineiro (Ubéraba, Brazil).

Statistical analysis. Multivariate logistic regression (SPSS statistical software version 10.0) was used to analyze the relationship between the probability of an individual developing hepatic disease and genetic variants. Covariates that are known to affect HF in infected individuals were also added in the analysis subjects, as follows. (a) Age for all samples but Brazilians. (b) Gender for Chinese (Sudanese and Brazilian cases and controls were matched for gender). (c) The number of Praziquantel (Bayer Pharma) treatments (only in Chinese fishermen, because all Chinese farmers had received a large number of treatments that could not be accurately evaluated; only a few Brazilians and Sudanese had received a few treatments, and this had no detectable impact on HF). (d) Exposure was measurable with Chinese fishermen but not with subjects from other samples who have been exposed very frequently to infection because of their daily farming activity. Nevertheless, the place of farming (whether it was still endemic or not) was a significant covariate for HF in Chinese farmers. (e) HBV and HCV infections could only be evaluated in Chinese farmers (HBV and HCV infections were tested if treatment could be provided by the local health authority); three Chinese farmers were infected with HCV and were not included, whereas half of the farmers had an active or cured HBV infection. Covariates affecting HF in fishermen were exposure, gender, and the number of antischistosome treatments. Exposure included two covariates: the number of years fishing and being born on a boat. Covariates affecting HF in Chinese farmers were age, gender, HBV infections, and whether the farming place was endemic. The covariate affecting HF in Sudanese was age only. No covariates were identified in Brazilian cases and controls who were matched for age, gender, and exposure.

The meta-analysis was performed with Comprehensive Meta Analysis Version 2 (available at www.Meta-Analysis.com) on data obtained without including covariates; the strength of the recorded genetic associations was slightly lower in the absence of the nongenetic covariates.

DNA. DNA was extracted from 3-ml blood samples using the standard salting-out method (Sambrook et al., 1989). In a few cases, buccal cell samples were collected and DNA was extracted and preamplified as previously described (He et al., 2007, 2008).

Sequencing. Purified PCR products were sequenced using a cycle sequencing system (BigDye Terminator; Applied Biosystems) on an automatic sequencer (Prism; Applied Biosystems). Sequencing reactions were performed on both strands (Table S2). Sequencing was performed by GATC Biotech.

Polymorphism genotyping. The SNPs rs9321314, rs9321315, rs6910279, rs6940184, rs12523697, rs9493149, rs12529636, rs12527705, rs12526196, rs6917644, rs9399005, rs6918698, rs9493150, rs2151532, rs3037970, rs928501, rs11966728, rs7747601, rs12198610, rs9402373, 132319925 D/I, rs1931002, rs12527379, rs9483364, and rs2095252 were genotyped using probe assays (TaqMan; Applied Biosystems). Polymorphisms rs6926879, rs341188377, rs1931003, rs12191459, rs12206863, rs7768619, rs10872386, and rs2327184 were genotyped by restriction enzyme analysis (Table S3).

Nuclear extract preparation. Nuclear extracts were prepared using nuclear and cytoplasmic component extraction reagents (NE-PER; Thermo Fisher Scientific) from a human hepatocyte cell line (HEPG2), stimulated for 1 h with 1 mM dexamethasone (Kobayashi et al., 2005; Gressner et al., 2007).

EMSA Complementary single-stranded oligonucleotides were commercially synthesized to span ~10 bp on either side of the variant nucleotide, as follows: rs9402373C, 5′-GCTCTCAAAACTAAGCCCAACTC-3′; rs9402373G, 5′-GCTCTCAAAAGTAAGCCCAACTC-3′; rs12527705A, 5′-GTAATATAGAAGATGGGTCTA-3′; rs12527705T, 5′-GTAATATAGATGATGGGTCTA-3′; rs12526196C, 5′-GAATATACAACGAATATGGGC-3′; rs12526196T, 5′-GAATATACAATGAATATGGGC-3′; rs1931002A, 5′-TGGATGATTCAAACAACTTGG-3′; and rs1931002G, 5′-TGGATGATTCGAACAACTTGG-3′. Experiments follow a previously described protocol (He et al., 2008).

Online supplemental material. HF was evaluated according to the WHO guidelines. The criteria for cases and control definitions are described in Table S1. We sequenced *CTGF* (3.2 kb) and 15.3 kb of sequence upstream from the start codon and 14.1 kb of sequence in the 3′ untranslated region in eight cases and two controls: sequencing revealed 61 SNPs, 50 of which had been described previously. The sequencing primers are described in Table S2. Several polymorphisms were genotyped by restriction enzyme analysis under standard conditions. Primers are described in Table S3. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090383/DC1.

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