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IFN- γ Polymorphisms (IFN- γ +2109 and IFN- γ +3810) Are Associated with Severe Hepatic Fibrosis in Human Hepatic Schistosomiasis (*Schistosoma mansoni*)¹

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Schistosome infection is a major public health concern affecting millions of people living in tropical regions of Africa, Asia, and South America. Schistosomes cause mild clinical symptoms in most subjects, whereas a small proportion of individuals presents severe clinical disease (as periportal fibrosis (PPF)) that may lead to death. Severe PPF results from an abnormal deposition of extracellular matrix proteins in the periportal spaces due to a chronic inflammation triggered by eggs and schistosome Ags. Extracellular matrix protein production is regulated by a number of cytokines, including IFN- γ . We have now screened putative polymorphic sites within this gene in a population living in an endemic area for *Schistosoma mansoni*. Two polymorphisms located in the third intron of the IFN- γ gene are associated with PPF. The IFN- γ +2109 A/G polymorphism is associated with a higher risk for developing PPF, whereas the IFN- γ +3810 G/A polymorphism is associated with less PPF. The polymorphisms result in changes in nuclear protein interactions with the intronic regions of the gene, suggesting that they may modify IFN- γ mRNA expression. These results are consistent with the results of previous studies. Indeed, PPF is controlled by a major locus located on chromosome 6q22-q23, closely linked to the gene encoding the α -chain of the IFN- γ receptor, and low IFN- γ producers have been shown to have an increased risk of severe PPF. Together, these observations support the view that IFN- γ expression and subsequent signal transduction play a critical role in the control of PPF in human hepatic schistosome infection (*S. mansoni*). *The Journal of Immunology*, 2003, 171: 5596–5601.

Schistosomiasis is a worldwide public health problem. Millions of people in subtropical countries are affected, and it is estimated that 50,000–100,000 die annually (1). In *Schistosoma mansoni* infection, some eggs, trapped in hepatic sinusoids, induce an immune reaction that prevents toxic substances from diffusing from the eggs into the surrounding hepatic tissue

(2–5). Egg-derived products and mediators released at the site of the inflammation stimulate the differentiation of stellate cells into myofibroblasts. Fibroblasts secrete extracellular matrix proteins (ECMP)⁵ that deposit in the periportal space (6). In most infected subjects living in endemic areas, ECMP accumulation is well controlled, and these individuals develop minor pathological manifestations. However, in 5–10% of *S. mansoni*-infected subjects, ECMP accumulate in the portal space due to imbalance between fibrogenesis and fibrolysis, leading to extended periportal fibrosis (PPF), also called Symmers' pipestem fibrosis. PPF is a major cause of portal hypertension and its attendant sequelae, which include varices and abdominal ascites. Subjects with advanced PPF may die of hematemesis, coinfection, or heart failure (7, 8).

Various explanations have been put forward to explain the heterogeneous distribution of severe disease cases in endemic populations, including heavy infection, gender, and age (9–11). Our laboratory, in collaboration with colleagues of the University of Gezira, have analyzed the factors that could explain the development of PPF in a Sudanese population located in the highly *S. mansoni* endemic area of Gezira. This study demonstrated that the genetic makeup of the host plays an important role in susceptibility to PPF, and a major susceptibility gene has been mapped on chromosome 6 (6q22-q23) near the gene encoding the α -chain of the

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⁵ Abbreviations used in this paper: ECMP, extracellular matrix protein; CI, confidence interval; MMP, matrix metalloproteinase; OR, odd ratio; PPB, peripheral portal branch; PPF, periportal fibrosis; SNP, single nucleotide polymorphism; TIMP, tissue inhibitor of metalloprotease; TMP, tissue metalloprotease.

IFN- γ receptor (12). Furthermore, analysis of the immune response of the subjects with severe PPF has demonstrated an association of advanced PPF with a reduction in IFN- γ production (13). Cytokines produced by egg-stimulated blood mononuclear cells from subjects with no or mild fibrosis and subjects with severe PPF were measured. Multivariate analysis of cytokine levels showed that high IFN- γ levels were associated with a marked reduction of the risk of fibrosis. These observations are supported by various studies that demonstrate that IFN- γ is a key antifibrogenic and profibrotic cytokine. Indeed, IFN- γ inhibits stellate cell differentiation and collagen production by myofibroblasts. Moreover, IFN- γ stimulates ECMP degradation by increasing tissue metalloproteases (TMP) and by inhibiting tissue inhibitors of metalloproteases (TIMP) (14–18). It was shown on mouse, in an in vivo model (liver injury induced by carbon tetrachloride), that IFN- γ inhibited lipocyte activation of liver injury, reduced collagen I mRNA. This results in an overall decrease in hepatic fibrosis (14). Jimenez et al. (15) had shown, in vitro, that IFN- α and IFN- γ inhibit collagen synthesis by human diploid fibroblasts mainly by decreasing collagen production rather than by impairment of secretion or increased extracellular degradation of the newly synthesized molecules. Moreover, in confluent human fibroblast microculture experiments, it was shown that IFN inhibit collagen production (16). Mallat et al. (17) had also shown that IFN- γ has a significant effect (inhibition) on newly synthesized collagen secretion by cultured Ito cells. Finally, Tamai et al. (18) had shown that IFN- γ coordinately up-regulates matrix metalloproteinase (MMP)-1 and MMP-3 gene expression in cultured keratinocytes. In contrast, it has no effect on TIMP-1 gene expression. These experiments suggest that IFN- γ enhances MMP gene expression at the posttranscriptional level. Thus, the altered MMP expression by IFN- γ without concomitant effect on TIMP gene expression potentially leads to imbalance between these proteases and their inhibitors, and enhanced proteolytic activity.

These results led us to test whether polymorphisms in the IFN- γ locus could alter the susceptibility to PPF. The results reported in this work show that two polymorphisms located in the IFN- γ gene are associated with PPF. These polymorphisms are shown to modify the binding of NFs to the IFN- γ genomic DNA, suggesting that they may modify the level of expression of this gene.

Materials and Methods

Study population

This study was performed on the population of two villages (Taweela/Umzukra) of the Gezira area, a region endemic for *S. mansoni* (Sudan). The entire population of the first village (Taweela: $n = 770$) and one-third of the population of the second village (Umzukra: $n = 700$) were studied. Pedigrees were drawn for both populations.

Ultrasound analysis

PPF was studied by ultrasound (portable Aloka SSD 500 Echo camera and 3.5-MHz convex probe). Liver size, peripheral portal branches (PPB), degree of PPF, thickness of PPB walls, spleen size, and splenic vein diameter were assessed, as previously described (19, 20). The disease was graded, as previously described (F0, F1, FII, and FIII). Briefly, grade 0 (F0) corresponds to normal liver with no thickening of the PPB wall and PPB diameters 2–3 mm. Grade I (F1) indicates a pattern of small stretches of fibrosis around secondary portal branches and PPB diameters 4 mm. FII still shows a patchy fibrosis, but a continuous fibrosis affects most second-order portal branches. PPB appears as long segments of fibrosis; PPB diameter is 5–6 mm. FIII shows a thickening of the walls of most PPBs; some branches are occluded, and long segment of fibrosis reaches the surface of the liver.

Ultrasound analysis is a valuable and noninvasive tool that allows assessment of liver size, PPB, the degree of PPF, thickness of PPB walls, spleen size, and splenic vein diameter. Portal hypertension was indicated by abnormal portal vein or splenic vein diameter (21, 22).

Subgroup design

Only unrelated subjects from these two villages were included in this study. When several unrelated individuals were present in a pedigree, the oldest subjects were selected because the risk of PPF increases markedly with age (12–20). These criteria allowed the selection of 105 unrelated individuals among those who volunteered to give blood. The selected subjects were classified under three subgroups. Subgroup A is including subjects with either no or mild or advanced fibrosis. Subgroup B is including subjects with either no or mild fibrosis. The final subgroup (C) is including patients with severe PPF.

Blood samples

A total of 5–15 ml of blood was collected on citrate as anticoagulant and kept at -20°C until DNA was extracted using the standard salting-out method (23).

Mutation detection

Polymorphism detection was conducted either by single-strand conformational polymorphism (24) or by digestion of the PCR product with restriction enzymes (25, 26). Briefly, for single-stranded conformational polymorphism analysis, 20 μl of PCR product was mixed with 20 μl of 0.2 N NaOH solution and denatured at 95°C for 5 min. A total of 20 μl of loading buffer was added to the denatured products before electrophoresis on mutation detection electrophoresis gel (0.5 \times) in Trizma base boric acid EDTA 1 \times at 7 mA (constant amperage) for 16 h at 4°C or room temperature, after which the gel was stained for 10 min in ethidium bromide solution (1 $\mu\text{g}/\text{ml}$). The analysis was performed at 4°C and at room temperature to increase the detection power of the analysis.

Some polymorphisms are creating or destroying restriction sites (25, 26). Some genotyping was done by restriction analysis. Briefly, 5 μl of PCR product was digested by specific restriction enzymes, as indicated previously (25, 26), under the conditions described by the manufacturer (New England Biolabs, Beverly, MA).

Statistical analysis

Statistical analysis (χ^2 or Fisher's tests) using SPSS software (Chicago, IL) was used to compare the study groups (subgroup A vs subgroup C, or subgroup B vs subgroup C). Linkage disequilibrium analysis was performed on the genepop website (<http://wbiomed.curtin.edu.au/genepop/index.html>).

Nuclear extract preparation

Nuclear extracts were prepared from PHA-stimulated fresh human peripheral blood T cells, as described previously, for 3 days, washed under acid conditions, and cultured in 10% RPMI 1640 for 2 days without PHA (27). Then cells were left unstimulated or T cells were stimulated with 10 ng/ml PMA + 1 $\mu\text{g}/\text{ml}$ ionomycin (I) for 30 min (see Fig. 1). Cell pellets were resuspended in lysis buffer (50 mM KCl, 25 mM HEPES, pH 7.8, 0.5% Nonidet P-40, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, 100 mM DTT) and subsequently incubated on ice for 5 min. Nuclei were collected by centrifugation at 2,000 rpm; the supernatant was harvested as the cytoplasmic protein fraction. Nuclei were washed in buffer A without Nonidet P-40 and harvested at 2,000 rpm. Nuclear pellets were resuspended in extraction buffer (500 mM KCl, 25 mM HEPES, pH 7.8, 10% glycerol, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, 100 mM DTT), frozen in dry ice, thawed slowly on ice, and finally centrifuged at 14,000 rpm for 10 min. The supernatant was harvested, and nuclear proteins were quantified with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

EMSA

Complementary single-stranded oligonucleotides were commercially synthesized to span ~ 10 bp on either side of the variant nucleotide, as follows: IFN- γ +2109F, 5'-TGA GGA AGA AGC (A/G)GG GAG TAC TG-3'; IFN- γ +2109R, 5'-CAG TAC TCC C(T/C)G CTT CTCCT-3'; IFN- γ +3810F, 5'-TGA TGC ATA CAG (G/A)AA AGA CTG AA-3'; IFN- γ +3810R, 5'-TTC AGT CTT T(C/T)C TGT ATG CAT-3'.

Complementary strands were annealed by combining 2 μg of each oligonucleotide and 6 μl of 10 \times annealing buffer (500 mM Tris, 100 mM

MgCl₂, and 50 mM DTT) in a 60 μ l reaction, placing in a boiling water bath for 5 min, and allowing to cool to room temperature. Then, 2 μ l of the double-stranded oligonucleotide probes was labeled with [³²P]dCTP. The DNA-protein-binding reaction was conducted in a 20 μ l reaction mixture consisting of 7 μ g of nuclear protein extract from each cell condition, 1 μ l of 1 mg poly(dI-dC) (Sigma-Aldrich, St. Louis, MO), 4 μ l of 5 \times binding buffer (60 mM HEPES, 7.5 mM MgCl₂, 300 mM KCl, 1 mM ethylenediamine tetra-acetic acid, 2.5 mM DTT, 50% glycerol, and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride), and 1.5 \times 10⁴ cpm of ³²P-labeled oligonucleotide probe (28). The DNA-protein-binding reaction was incubated at room temperature for 20 min, then loaded on a 6% nondenaturing polyacrylamide gel, and run for 2 h at 140 V.

Results

IFN- γ single nucleotide polymorphism analysis

A thorough search of the entire human IFN- γ gene for polymorphisms identified five single nucleotide polymorphisms (SNPs) (25, 26). Two SNPs were located within the promoter (IFN- γ -183 and IFN- γ -155) (25). Two additional single nucleotide substitutions were identified in intron 3 (IFN- γ +2109 and IFN- γ +3810), and a single substitution was found in the 3' untranslated region (IFN- γ +5134) (26). The frequency of these polymorphisms in each clinical fibrosis group (F0, FI, FII, or FIII) is shown in Table I.

Distribution of genotypes in study groups

One hundred and five unrelated subjects were studied in this study, as follows: 32 subjects had no fibrosis (F0), 24 had mild fibrosis (FI), 20 subjects had advanced fibrosis (FII), and 49 individuals displayed severe (FIII) fibrosis. Portal hypertension was observed in one-third of FII subjects and in all FIII subjects. For statistical analysis, three subgroups were designed: subgroup A, subjects with either F0, FI, or FII fibrosis; subgroup B, subjects with either F0 or FI fibrosis; and finally, subgroup C, subjects with severe (FIII) fibrosis. Statistical analysis of the frequencies of the various polymorphisms in subgroup A showed that these polymorphisms were in Hardy-Weinberg equilibrium ($\chi^2 < 5.99$). The frequency for IFN- γ polymorphisms in subgroups A and C is indicated in Table II.

IFN- γ +2109 A/G and +3810 G/A polymorphisms are associated with severe fibrosis

No statistically significant difference in genotype distribution, between the two clinical groups A and C, was observed for IFN- γ

-183, -155, and +5134 polymorphisms. Fisher's exact test, conducted with SPSS software, gave the following *p* values: 0.32 (IFN- γ -183), 0.27 (IFN- γ -155), and 1 (IFN- γ +5134) polymorphisms.

However, significant associations were detected among IFN- γ +2109 A (*p* = 0.035), IFN- γ +3810 G (*p* = 0.035), and FIII (Table II). These two polymorphisms are not in linkage disequilibrium (*p* = 0.246). The frequency of IFN- γ +2109 A/A genotype was higher (78.9 vs 58.6%) in subgroup A than in subjects with severe fibrosis (FIII; subgroup C). The IFN- γ +2109 G allele was associated with a higher risk of PPF (odds ratio (OR) = 2.6) (confidence interval (CI): 0.15–0.95). Thus, subjects carrying the IFN- γ +2109 A/G genotype have a risk of severe fibrosis that is on average 2.6 times higher than subjects with the IFN- γ +2109 G/G genotype. The frequency of the IFN- γ +3810 G/G genotype was lower (82.2 vs 100%) in subgroup A than in subjects with severe fibrosis (FIII; subgroup C). The IFN- γ +3810 A allele was associated with a reduced risk of PPF. The number of subjects exhibiting this allele was too small to estimate an OR.

As some FII subjects may progress to FIII, the analysis was repeated comparing FIII (subgroup C) with F0 + FI subjects (subgroup B). The result of this analysis supported the association of these IFN- γ polymorphisms with severe fibrosis (IFN- γ +2109, *p* = 0.05, OR = 0.39, CI = 0.15–1.03; IFN- γ +3810, *p* = 0.09).

Mutations IFN- γ +2109 and IFN- γ +3810 create new NF/DNA complexes

Polymorphisms are located in the third intron of the IFN- γ gene, a region that has been shown to be involved in transcriptional regulation (29). The transcription factor database, TRANSFAC (30), was used to analyze these polymorphic loci in the context of their adjacent nucleotides for potential differences in sequences similarity to known transcription factor-binding sites. Although computer analysis comparing wild-type and variant sequences in intron 3 (+2109 A/G) did not identify known/consensus protein-binding sites, the IFN- γ +3810 locus is similar to the human consensus CD28 RE/NF- κ B binding site. EMSA, using nuclear extracts from PHA-blasted or unblasted human peripheral T cells, was performed to determine whether DNA-protein interactions were altered by the polymorphisms at either site. The +2109 G

Table I. Frequency of IFN- γ polymorphisms in the population of Taweela Umzakra and in the various fibrosis grade groups^a

Polymorphisms	Genotypes	Whole Population	Clinical Groups			
			F0	FI	FII	FIII
IFN- γ -183	G/G	99/104 ^b (95.2) ^c	31/32 (96.9)	23/24 (95.8)	17/20 (85)	28/28 (100)
	G/T	5/104 (4.8)	1/32 (3.1)	1/24 (4.2)	3/20 (15)	0/28 (0)
	T/T	0/104 (0)	3/32 (0)	0/24 (0)	0/20 (0)	0/28 (0)
IFN- γ -155	A/A	103/104 (99)	32/32 (100)	24/24 (100)	20/20 (100)	27/28 (96.4)
	A/G	1/104 (1)	0/32 (0)	0/24 (0)	0/20 (0)	1/28 (3.6)
	G/G	0/104 (0)	0/32 (0)	0/24 (0)	0/20 (0)	0/28 (0)
IFN- γ -2109	A/A	77/105 (73.3)	24/32 (75)	20/24 (83.3)	16/20 (80)	17/29 (58.6)
	A/G	28/105 (26.7)	8/32 (25)	4/24 (16.7)	4/20 (20)	12/29 (41.4)
	G/G	0/105 (0)	0/32 (0)	0/24 (0)	0/20 (0)	0/29 (0)
IFN- γ +3810	G/G	85/98 (86.8)	25/30 (83.3)	22/24 (91.7)	13/19 (68.4)	25/25 (100)
	G/A	12/98 (12.2)	4/30 (23.3)	2/24 (8.3)	6/19 (31.6)	0/25 (0)
	A/A	1/98 (1)	1/30 (3.4)	0/24 (0)	0/19 (0)	0/25 (0)
IFN- γ -5134	G/G	94/98 (95.9)	29/32 (90.6)	24/24 (100)	19/19 (100)	22/23 (95.7)
	G/A	4/98 (4.1)	3/32 (9.4)	0/24 (0)	0/19 (0)	1/23 (4.3)
	A/A	0/100 (0)	0/32 (0)	0/24 (0)	0/19 (0)	0/23 (0)

^a The study group is described in *Materials and Methods*. The genotype was determined as described (see Refs. 15, 16).

^b Number of subjects with the indicated genotype/number of subjects genotyped.

^c Percentage of subjects with the indicated genotype.

Table II. *IFN-γ genotype distribution and genotype frequencies in clinical groups^a*

Polymorphisms	Genotypes	Clinical Groups		p Value
		F0, F1, and FII (subgroup A)	FIII (subgroup C)	
IFN-γ -183 Hardy-Weinberg test (χ^2)	G/G	71/76 ^b (93.4) ^c	28/28 (100)	0.320
	G/T or T/T	5/76 (6.6) 0.15	0/28 (0)	
IFN-γ -155 Hardy-Weinberg test (χ^2)	A/A	76/76 (100)	27/28 (96.4)	0.269
	A/G or G/G	0/76 (0) 0	1/28 (3.6)	
IFN-γ +2109 Hardy-Weinberg test (χ^2)	A/A	60/76 (78.9)	17/29 (58.6)	0.035
	A/G or G/G	16/76 (21.1) 1.19	12/29 (41.4)	
IFN-γ +3810 Hardy-Weinberg test (χ^2)	G/G	60/73 (82.2)	25/25 (100)	0.035
	G/A or A/A	13/73 (17.8) 0.20	0/25 (0)	
IFN-γ +5134 Hardy-Weinberg test (χ^2)	G/G	72/75 (96)	22/23 (95.7)	1
	G/A or A/A	3/75 (4) 0.031	1/23 (4.3)	

^a The genotype distributions in the F0-I-II clinical group (subgroup A) of the five polymorphisms did not deviate from the Hardy-Weinberg equilibrium. Fisher's exact tests were carried out with SPSS software, to compare the two study groups (subgroup A vs subgroup C).

^b Number of subjects with the indicated genotype/number of subjects genotyped.

^c Percentage of subjects with the indicated genotype.

allele formed two DNA-protein complexes (complexes 1 and 2) (Fig. 1A). Complex 1 is common to both +2109 A and +2109 G alleles (Fig. 1A), whereas complex 2 was only formed by the +2109 G allele (Fig. 1A). Analysis of the +3810 G/A polymorphism revealed that the A-bearing allele formed a specific complex (complex 3; Fig. 1B) that is not shared with the +3810 G allele.

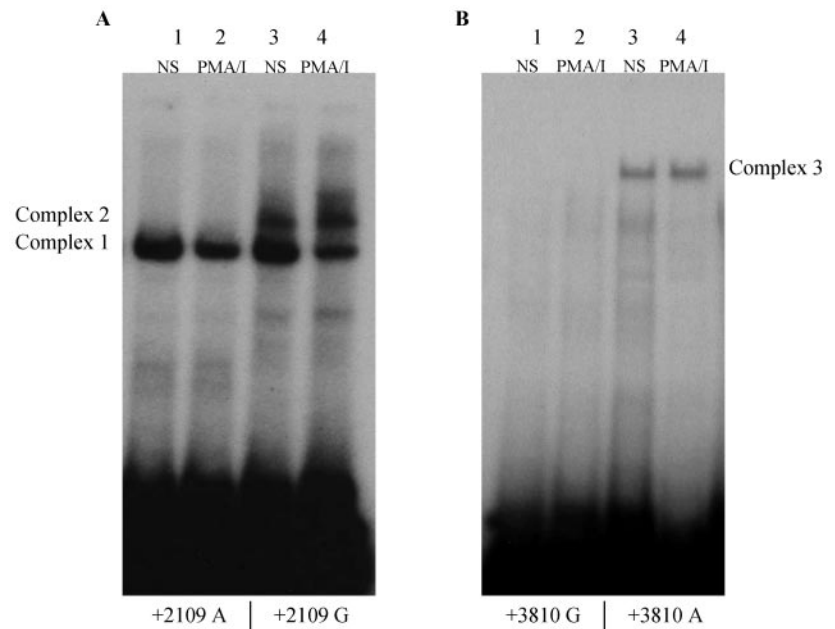
Discussion

In regions endemic for *S. mansoni*, 5–10% of infected subjects develop a severe hepatic disease characterized by PPF and portal hypertension. Previous studies in humans have suggested that difference in IFN-γ production could account, at least in part, for the different rate of fibrosis progression in populations in endemic areas (13). This led us to investigate whether any SNPs in the IFN-γ gene could be associated with severe fibrosis. We have shown in this work that two polymorphisms (IFN-γ +2109 A/G and IFN-γ +3810 A/G) are associated with severe PPF: the +2109 G allele

was associated with a higher risk of PPF, whereas the +3810 A allele was associated with protection against PPF.

Putative sequence changes in noncoding regions of a gene may influence gene expression due to the creation or alteration of DNA-binding sites for transcription factors. Several reports have suggested that specific regulatory elements located in the first, second, and third intron of the IFN-γ gene bind nuclear proteins that may have a role in the control of IFN-γ transcription (29, 31, 32). EMSA comparing wild-type (+2109 A and +3810 G) and variant sequences (+2109 G and +3810 A) in the third intron has detected differences in DNA-protein interaction at both sites using nuclear extracts from PHA-blasted human peripheral T cells (Fig. 1). Two protein complexes (complexes 1 and 2) were detected that bind to DNA containing the +2109 G allele, whereas DNA containing the +2109 A formed only one complex (complex 1). This finding suggests that this mutation might affect the gene transcription. Additional experiments, presently in progress, are being performed to

FIGURE 1. EMSA analysis of the +2109 A/G and +3810 G/A polymorphisms. This analysis compared DNA-nuclear protein interactions with oligonucleotides from either the common IFN-γ intron 3 (+2109 A and +3810 G) or the variants (+2109 G and +3810 A). Both variants formed specific complexes with nuclear extracts from stimulated (PMA/I) or unstimulated (NS) human peripheral T cells.



check this question. Nevertheless, the possibility that another polymorphism, undetected in this study, is in linkage disequilibrium with the present polymorphism cannot be totally excluded.

The +3810 A allele (AGAAAAGA) has sequence homology with the CD28-RE (AAAGAAATTC) that binds NF-AT (binding site GGAAA) and NF- κ B proteins (binding site GGGANTYYCC) (29, 33); the bold underlined A is the mutated base (polymorphism). The cooperation between NF-AT and NF- κ B proteins induces maximal transcription of the IFN- γ gene, resulting from a synergistic activity between the gene promoter and intronic enhancers (26). Therefore, the allelic variant (+3810 A) may result in more robust transcription of the IFN- γ gene than seen with the +3810 G allele.

This report of an association between genetic polymorphisms and severe PPF is consistent with the results of previous studies. These studies showed that PPF development is associated with a region located on chromosome 6q22-q23, closely linked to the gene encoding the α -chain of the IFN- γ receptor (12, 20). Moreover, low IFN- γ producers have been shown to have an increased risk of severe PPF (13). Studies in mice have also shown that inflammation and collagen deposition in the experimental hepatic granuloma are down-regulated by IFN- γ (34, 35). IFN- γ also has been reported to inhibit fibrogenesis and to increase fibrolysis (35, 36) by regulating TIMP and TIMP (14–18).

Altogether, these observations support the view that IFN- γ plays a critical role in the control of PPF in human schistosome infection. A few studies have associated alterations of the IFN- γ pathway with diabetes type 1 (37, 38), arthritis (39), lupus (40, 41), multiple sclerosis (40, 41), hepatitis B infection (42), malaria (43), and bacterial infection (44). Otherwise, susceptibility to nontuberculous mycobacteria or to bacillus Calmette Guérin vaccine has been associated with mutations in the IFN- γ receptor ligand-binding chain (IFNGR1), IFN- γ receptor signaling chain (IFNGR2), STAT1, IL-12 p40 subunit (NKSF2), and IL-12R β 1 chain (IL12RB1) genes (see Ref. 45 for review). Thus, genetic alterations in the IFN- γ gene or the IFN- γ signal transduction pathway may result in an altered clinical course of disease progression.

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