Differential transcriptional expresión of the polymorphic myxovirus resistance protein A in response to interferon-alpha treatment

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Levels of myxovirus resistance protein A (MxA) mRNA were studied for a single nucleotide polymorphism in the promoter region at nucleotide position −88 of the gene to identify individual-specific responses to interferon (IFN)-α2 that might predict responsiveness to IFN-α therapy. We quantified MxA expression by reverse transcription and real-time polymerase chain reaction in peripheral blood mononuclear cells (PBMC) in vitro, induced by IFN-α2, from 22 healthy donors, in relation with G/T polymorphism located in the promoter of the MxA. MxA mRNA was significantly upregulated in all subjects (mean of 53-fold) in response to IFN-α2 in vitro (P < 0.01). Comparison of the inducibility of MxA mRNA expression in relation with G/T polymorphism showed a 4.26-fold higher induction of MxA mRNA levels in PBMC from carriers of the mutant allele (GT or TT) than homozygotes with the wild-type allele (GG) (P < 0.001). We propose that expression of the IFN-inducible MxA is affected by a single nucleotide polymorphism in the MxA promoter which can identify an individual response to IFN-α2. Pharmacogenetics 14:189–193 © 2004 Lippincott Williams & Wilkins

Keywords: quantitative MxA expression, −88G/T SNP, real time RT-PCR

Introduction

Since the discovery of interferons (IFNs) in 1957 as endogenous inhibitors of viral infection [1], considerable evidence has accumulated revealing that these molecules play an important role in the host’s defence response. IFNs induce the expression of a number of different proteins that mediate their antiviral, antiproliferative and immunomodulatory effects [2].

Many studies have described the beneficial effects of IFN type I (IFN-α and -β) in the treatment of patients with a wide variety of diseases, including multiple sclerosis (MS) [3,4], chronic hepatitis C (HCV) [5,6] and certain types of solid and haematological malignancies [7–9]. However, some individuals fail to respond to IFN-α therapy. Quantification of the myxovirus resistance protein A (MxA) transcript or protein is used as a marker of biological activity to monitor the clinical efficiency of treatment with IFN type I, because it is specifically induced in a dose-dependent manner by type I IFNs [4,10–12]. The promoter of the MxA contains two functional IFN-stimulated response elements (ISRE) near the transcription start site and both ISRE are essential for IFN responsiveness [13]. A single nucleotide polymorphism (SNP) (G/T at nt −88) in the first ISRE of the MxA promoter has been reported to correlate with the response of hepatitis C patients to IFN-α treatment [14].

We quantified MxA expression by real time reverse transcription-polymerase chain reaction (RT-PCR) in peripheral blood mononuclear cells (PBMC) induced by IFN-α2 from healthy volunteers and studied the G/T polymorphism located in the promoter (SNP −88) of the MxA gene. Our results showed a 4.26-fold higher upregulation of MxA mRNA levels in response to IFN-α in PBMC from carriers of a mutant allele (GT or TT) compared to homozygotes with the wild-type allele (GG).

Materials and methods

Subjects

To determine mRNA MxA expression in PBMC related with the different genotype in the MxA promoter, 22 healthy subjects (11 homozygotes GG, nine heterozygotes GT and two homozygotes TT) with a mean age of 22.2 ± 1.46 years were selected randomly. The two TT subjects were the only ones found among 150 genotyped from a healthy population.
Identification of MxA gene promoter genotypes

DNA was prepared using standard techniques. PCR and restriction fragment length polymorphism were performed according to the method of Hijikata et al. [14].

Cell culture

PBMC were isolated from freshly heparinized blood by Ficoll density-gradient centrifugation. Unfractionated PBMC (10^10) were resuspended in RPMI-1640 (BioWhittaker, Walkersville, Maryland, USA) containing 10% fetal bovine serum (BioWhittaker), 100 U/ml penicillin, 100 U/ml streptomycin and incubated at 37°C with 5% CO₂ in 24-well plates (Sarstedt Inc., Newton, North Carolina, USA) in the presence of: (i) 5 × 10^6 cells in supplemented medium only and (ii) 5 × 10^6 cells in supplemented medium plus 100 IU/ml IFN-α2b (Schering Plough, Brussels, Belgium) for 12 h to induce expression of MxA mRNA. The cells were then pelleted and subjected to RNA extraction and reverse transcription.

RNA extraction and cDNA synthesis

Total RNA was extracted by Aqua Pure RNA Isolation Kit (Bio-Rad, Hercules, California, USA) according to the manufacturer’s instructions. The amount of RNA was measured spectrophotometrically by absorbance at 260 nm. RNA was stored at −80°C until use. The reverse transcription reaction was carried out in a total volume of 20 μl containing 1 mmol deoxynucleoside triphosphates (dNTPs) (Promega, Madison, Wisconsin, USA), 60 U of Moloney murine leukemia virus reverse transcriptase (Promega), 10 U of RNasin ribonuclease inhibitor (Promega), 2.5 mmol of random hexamers (Promega), 10 mmol Tris-HCl, 50 mmol KCl, 1% Triton X-100, 1.5 mmol MgCl₂ (Promega). To this mixture, we added 2 μg of total RNA. The reaction was allowed to proceed for 60 min at 42°C followed by 5 min heating at 95°C and rapid cooling on ice. The cDNA was stored at −20°C until use.

Preparation of cDNA calibrators

cDNA calibrators were prepared by PCR amplification run to saturation with their specific primers (see below). We produced specific PCR amplicons (calibrators) to generate calibration curves suitable for quantification of amplified specific cDNAs. The PCR amplicons obtained were purified by Wizard MagneSil PCR clean-up system (Promega) and their quantity was determined by ultraviolet detection. After quantification, the calibrators were diluted several fold. Five calibrators were used for each calibration curve, from 10^5 to 10^1 copies.

Real-time PCR and quantification of mRNA MxA

MxA expression levels were analysed by reverse transcription and real-time PCR using SYBR Green I. The amplification conditions of the template were optimized for the iCycler iQ system (Bio-Rad) and the subsequent PCR runs showed a single PCR product during melting curve and electrophoresis analysis (data not shown). PCR reaction and data analysis were performed following the manufacturer’s guide. GenBank accession no. M30817 was used for designing MxA primers (Table 1). The primers selected for MxA (Table 1) were designed to distinguish cDNA and genomic DNA by using specific primer analysis software Primer Express (version 2.0; Applied Biosystems, Foster City, California, USA) and, for β-actin (Table 1), they were obtained as described by Øvstebø et al. [15]. We added 10 μl of cDNA sample or calibrator (calibration curve) to 200 nmol of each primer, 25 μl of 2 × iQ SYBR Green supermix (50 U/ml iTaq DNA polymerase, 20 nmol SYBR Green I, 0.4 mmol of each dNTPs, 6 mmol MgCl₂, 40 mmol TrisCl, 100 mmol CIK) (Bio-Rad), and PCR-grade water to a volume of 50 μl. MxA and β-actin mRNA were amplified in separate tubes using the following protocol: 95°C for 10 min, followed by 40 cycles at 95°C for 20 s and at 55°C (for MxA) or at 60°C (for β-actin) for 20 s. Each sample was subjected to triplicate PCR reactions and the mean threshold cycle (Cₜ) value of samples was adjusted by quantification of β-actin as an endogenous housekeeping gene. Cₜ is the cycle at which the increase in signal associated with an exponential growth of PCR products is first detected.

Table 1 Oligonucleotide polymerase chain reaction primers for human myxovirus resistance protein A (MxA) and β-actin

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Oligonucleotide primer sequence (5’ to 3’)</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP in MxA promoter*</td>
<td>AACA CAC CGT TTT CCA CCC TGG AGA GGC CAG</td>
<td>599</td>
</tr>
<tr>
<td>Forward primer</td>
<td>TGG GCA GTG CTG GAG TGC GGC CTC CGC TCT</td>
<td>189</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GGC ATC TCC ACC CTG AAG TA</td>
<td>203</td>
</tr>
<tr>
<td>MxA cDNA</td>
<td>GGCGATCGCTCACCCTGAAAGTTCTCCCTCTCAAA</td>
<td>599</td>
</tr>
<tr>
<td>Forward primer</td>
<td>ACA GCA AAT CAA GGCG ACG ACT GGA</td>
<td>189</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>CGG ATC AGC TCT CAA CCT CTC CCG</td>
<td>203</td>
</tr>
<tr>
<td>β-actin*</td>
<td>GGC ATC TCC ACC CTG AAG TA</td>
<td>599</td>
</tr>
<tr>
<td>Forward primer</td>
<td>GGCGATCGCTCACCCTGAAAGTTCTCCCTCTCAAA</td>
<td>189</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>ACA GCA AAT CAA GGCG ACG ACT GGA</td>
<td>203</td>
</tr>
</tbody>
</table>

*Primers for a genotyped single nucleotide polymorphism (SBP) in MxA promoter were obtained as described by Hijikata et al. [14]. *From Øvstebø et al. [15].
The calibration curve was generated using five calibrators (serial 10-fold dilution of purified PCR amplicons, 10^5–10^1 copies) and was created automatically by iCycler iQ software (version 3.0) by plotting the C_t value of samples to the calibration curve and normalizing them with respect to β-actin mRNA. They were then expressed as a number of copies (relative expression).

Statistical analysis
Comparison of target gene expression between GG, GT and TT genotype groups was performed using Student’s t-test. In all statistical analyses, a two-tailed P < 0.05 was considered statistically significant.

Results
Level of MxA mRNA in PBMC
MxA expression was examined in PBMC from 22 healthy donors by real-time RT-PCR. In PBMC cultured in basal conditions, all subjects expressed a detectable level of MxA mRNA, with a mean (range) value of 34.01 ± 21.63 (14.2–97.6) copies. MxA transcript was rapidly upregulated by IFN-α in human PBMC. After 12h of treatment with IFN-α2b, in vitro, MxA transcript expression in PBMC was increased in each of the 22 subjects. When the amount of MxA mRNA was compared between untreated and treated cells, a significant induction [mean (range) value of 1957.26 ± 1847.58 (160–5810) copies] was observed in response to IFN-α2b (P < 0.001). Indeed, the level of MxA mRNA was approximately 57-fold higher in PBMC induced by IFN-α than in basal PBMC.

Level of MxA mRNA in PBMC according to SNP at nt –88
The basal level of MxA mRNA expression in the GG group appeared to be lower than that of the GT group (31.55 ± 19.66 versus 40.06 ± 25.42) but the difference was not statistically significant (Table 2).

When the PBMC were stimulated in vitro with IFN-α2b, the levels of MxA mRNA rose in all three genotype groups, GG, GT and TT (Table 2). In the GG group, a moderate significant enhancement, 23.54 times (1.37 log_{10}), was observed with a mean (range) value of 742.99 ± 926.49 (160–2410) copies (P < 0.05). The increase in the GT group was higher (79.62 times), with a mean (range) value of 3189.77 ± 1939.85 (922–5810) copies (P < 0.001). In both individuals with the TT genotype, MxA expression also rose in PBMC induced by IFN-α2b (152.94-fold) but no statistical analysis was possible because of the low number of cases.

Table 2 MxA mRNA expression in peripheral blood mononuclear cells (PBMC) from healthy donors cultures in basal conditions and after interferon (IFN)-induction

<table>
<thead>
<tr>
<th>Genotype groups</th>
<th>GG (n = 11)</th>
<th>GT (n = 9)</th>
<th>TT (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA MxA basal (mean ± SD)</td>
<td>31.55 ± 19.66</td>
<td>40.04 ± 25.42 (NS)*</td>
<td>17.7–22.7</td>
</tr>
<tr>
<td>mRNA MxA induced by IFN (mean ± SD)</td>
<td>742.99 ± 926.49</td>
<td>3189.77 ± 1939.85</td>
<td>2569–3610</td>
</tr>
<tr>
<td>P-values for basal versus induced</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.01*</td>
<td>IN</td>
</tr>
<tr>
<td>Relative increase of MxA expression (’fold’)</td>
<td>23.54</td>
<td>79.62 (P &lt; 0.01)*</td>
<td>152.29</td>
</tr>
</tbody>
</table>

*P-values for GG group versus GT group. NS, Not significant; IN, insufficient number.

Discussion
Following the first report by Hoofnagle et al. [16] of the beneficial effects of IFN-α in chronic hepatitis C, thousands of patients have been treated with IFN-α. However, the efficacy of IFN-α therapy varies greatly within groups of patients treated for chronic viral hepatitis C. Many researchers have analysed the expression of IFN-inducible genes in an attempt to understand the molecular mechanism of IFN-α resistance. MxA expression is currently assumed to be the most specific surrogate parameter for measuring biological activity of both endogenous and exogenous IFN-α [10–12,17,18].

Several authors have monitored the expression of MxA in PBMC from HCV patients treated with IFN-α, and high levels of MxA protein or mRNA were found in most of the IFN-treated hepatitis C patients, in contrast to minimal levels before treatment [10,12,18–20]. However, this increase in MxA mRNA was only significant in virological responders compared to non-respon-
The extent of MxA expression clearly varies considerably between subjects. At present, there is no explanation for this different response to IFN. Both viral and host-specific factors may contribute. It is known that HCV core and envelope E2 proteins interfere with the host immune response [24,25]. Our study, which excluded viral factors by using only healthy controls, demonstrated a different response in MxA expression to stimulus with IFN-α2: mRNA MxA induction in subjects with the T allele (87.01-fold) was significantly higher (P < 0.001) than in homozygous GG subjects (23.54-fold). This suggests that host genetic factors influence the individual response to IFN-α2.

The results of the study reported herein reflect a quantitative variation in response to interferon depending on the genotype of the G/T polymorphism in each individual. Because the effect of interferon therapy is dose-dependent and, according to our results, the difference in the response can vary four or five-fold depending on the presence or absence of the T allele, we may infer that, apart from any possible immunological or virological influence, the genotype of the patient may be the determinant in this dose–response effect. Although it is undoubtedly not the only genetic factor influencing an individual response to interferon due to the many other IFN-inducible genes, polymorphism in the promoter region at nucleotide position –88 of the MxA promoter which is activated by IFN [13]. Hijikata et al. [14] described a SNP at nt –88 (G or T) in the promoter region of the gene, which correlated with different responses of HCV-infected patients to IFN therapy. Although they found that the frequency of GG homozygotes with respect to the SNP at nt –88 was significantly higher in non-responders than in sustained responders, they did not quantify MxA expression. In an experiment performed in vitro, they demonstrated that two SNP in the MxA promoter, with A at –123 and T at –88, showed an approximately four-fold higher activity of upregulating the down-stream reporter gene than that with C at –123 and G at –88 in a luciferase reporter assay [23]. In our study, we quantified MxA expression according to the different SNP genotypes at –88 in healthy control PBMC treated with IFN-α2. In GG subjects, we found an upregulation, with a mean value that was approximately 23-fold higher than that for untreated PBMC and, in carriers of a T allele, a mean value that was approximately 87-fold higher, with a statistically significant difference for both groups (P < 0.001) (Fig. 1). These results suggest individual differences in the response to interferon.

Gilli et al. [12] reported that two different groups of HCV patients could be recognized with respect to MxA induction 12 h after initiation of IFN-α therapy; one expressing a sharp increase and the other only a moderate increase in the amount of MxA mRNA [12]. These two groups bear a strong relation to those of our study: the sharp increase corresponds to carriers of the T allele and the moderate increase to homozygous GG subjects. Our results are also in line with those of Schlaak et al. [22], who observed considerable quantitative variation in the ‘fold’ inductions of MxA in the response to IFN-α2 between different cell types (e.g. PBMC: 48.3-fold versus dendritic cells: 26.6-fold) and in the same cell type from different donors (e.g. in T cells, with values ranging from 0.7-fold to 52-fold).

Fig. 1

Relative MxA mRNA expression (copies)

<table>
<thead>
<tr>
<th>Relative MxA mRNA expression (copies)</th>
<th>Homozygote wild-type allele (G) (n = 11)</th>
<th>Mutant allele (T) (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000</td>
<td></td>
<td></td>
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<tr>
<td>1000</td>
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<td>100</td>
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</table>

Relative myxovirus resistance protein A (MxA) mRNA expression in peripheral blood mononuclear cells (PBMC) from healthy individuals treated with interferon (IFN-α2). MxA mRNA was measured by real-time reverse transcription-polymerase chain reaction in PBMC from 11 subjects with homozygous wild-type (wt) allele G and from 11 subjects with the mutant allele T. Comparison of the inductibility of MxA mRNA expression between both groups showed a statistically significant difference (P < 0.001). Scale bar = mean value.
gene may be a factor to bear in mind as a marker, and for its relevance with respect to treatment efficacy.

Acknowledgement
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References