

Differential activity of interferon- α 8 promoter is regulated by Oct-1 and a SNP that dictates prognosis of glioma

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Abbreviations: SNP, single nucleotide polymorphism; IFN, interferon; EMSA, electrophoretic mobility shift assay; TLR, toll like receptor

We have previously reported that the single nucleotide polymorphism (SNP) rs12553612 in IFNA8 is associated with better overall survival of glioma patients with the AA-genotype compared with patients with the AC-genotype. As rs12553612 is located in the IFNA8 promoter, we hypothesized that the A-allele allows for an enhanced IFNA8 promoter activity compared with the C-allele. Reporter assays in the human monocyte derived THP-1 cell line demonstrated a superior promoter activity of the A-allele compared with the C-allele. Electrophoretic mobility shift assays (EMSA) further demonstrated that the A-genotype specifically binds to more nuclear proteins than the C-genotype, including the transcription factor Oct-1. Further, co-transfection of plasmids encoding Oct-1 and the reporter constructs revealed that Oct-1 enhanced the promoter activity with the A- but not the C-allele. Taken together, our data demonstrate that the A-allele in the rs12553612 SNP, which is associated with better glioma patient survival, allows for IFNA8 transcription by allowing for Oct-1 binding, which is absent in patients with C allele, and suggests a molecular mechanism of IFNA8 mediated immune-surveillance of glioma progression.

Introduction

Malignant gliomas are the most common primary brain tumors with dismal prognosis. As information about their etiology and the prognostic factors that influence patients' survival is still limited, it is critical to better understand the critical biological interactions that regulate glioma development and growth.

A growing line of evidence supports significant roles of immunosurveillance for prevention and regulation of cancer development. For example, tumor infiltrating T-cells are capable of killing tumor cells¹ and are a positive prognostic factor for cancer patients.¹ Among a variety of cytokines and their signaling pathways, the Type I interferons (IFNs), IFN α and IFN β , appear to play a key role in this regard. Although they have been long known to induce tumor cell apoptosis and angiogenesis inhibition,² hematopoietic cells in the host (rather than tumor cells) are the crucial targets of the antitumor activity of endogenous Type-1 IFNs.^{3,4} More recent studies with melanoma

have demonstrated that host type I IFNs are critical for the innate immune recognition of a growing melanoma through signaling on CD8 α ⁺ dendritic cells (DCs).^{5,6}

A SNP is a single nucleotide variation that occurs within a gene of members of the same species. Several SNPs in immune regulatory genes correlate with glioma risks and/or prognosis.⁷⁻⁹ Previous studies have shown a significant impact of SNPs in innate immune pathways, such as ones in Toll-like receptor (TLR) 3,^{10,11} TLR4¹² as well as interleukin-4 receptor (IL-4R α), which is associated with differential risk and prognosis of glioblastoma multiforme.^{8,13} Recently, we reported a previously undefined protective role of the Type-1 IFN pathway in the surveillance against de novo mouse gliomas and that SNPs in IFNAR1 and IFNA8 are associated with significantly altered overall survival of patients with WHO Grade II and III gliomas.⁷ Specifically, the SNP rs12553612 is located at 335 base pairs (bp) upstream of the IFNA8 initiation codon, which is in the putative IFNA8 promoter region. As a SNP in a promoter region may

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affect the promoter activity and therefore the gene expression levels,¹⁴ we hypothesized that the SNP in the IFNA8 promoter (rs12553612) affects the interaction of transcription factors with

the DNA region involving the SNP, thereby affecting the activity of the IFNA8 promoter.

Here we report that the A-genotype of rs12553612 in the IFNA8 promoter which is associated with favorable overall survival of glioma patients confers a better promoter activity than the C-genotype. While in silico analysis predicts multiple DNA binding proteins to bind to this site, we found that probable factors C-Krox and Elk-1 did not regulate promoter activity. However Oct-1 binds to the DNA promoter segment containing the A-genotype and overexpression of Oct-1 in THP-1 cells enhanced the promoter activity. These data suggest a possible biological mechanism underlying the enhanced IFNA8 expression in glioma patients with the A-SNP (rs12553612) in the IFNA8 promoter resulting in better prognosis.

Results

The A-genotype leads to superior promoter activity compared with the C-genotype. Glioma patients with the AA-genotype in the rs12553612 SNP in the IFNA8 promoter exhibit prolonged overall survival compared with patients with the AC-genotype.⁷ Additionally, as type-I IFNs promote immune cell functions, we examined whether IFNA8 promoter activities in the A-genotype were superior to those in the C-genotype. To understand the underlying molecular basis, we created IFNA8 promoter luciferase constructs by cloning the promoter region of IFNA8 (-1528--27 upstream the IFNA8 precursor) with either A or C at position -335 into the pGL4.20 luciferase vector at the XhoI and HindIII sites in the multiple cloning site. Human monocyte derived THP-1 cells were co-transfected with these Firefly luciferase reporter plasmids with the A- or C- genotype (Fluc) and Renilla luciferase plasmid for internal control (Rluc). Relative luciferase activities (Fluc/Rluc) were obtained at 24 h after the co-transfection (Fig. 1). We found that the IFNA8 A-genotype reporter plasmid demonstrated significantly higher activity than the C- genotype. The immunoadjuvant polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose (poly-ICLC) has been shown to enhance the efficacy of glioma vaccines, as we

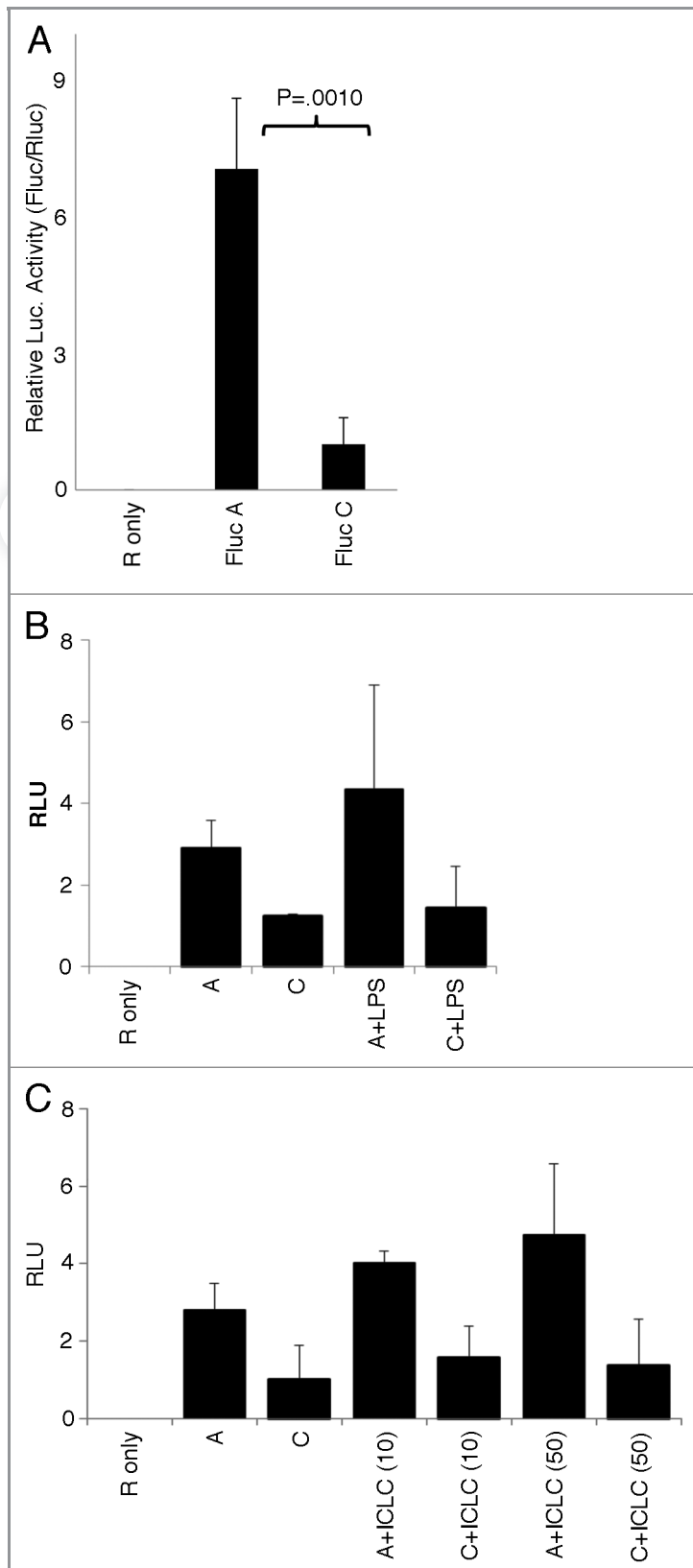


Figure 1. Interferon (IFN)-A8 promoter activity with the A-genotype at -335 is superior to that with the C-genotype. (A) 1×10^5 THP-1 cells were co-transfected with 0.02 μ g of pGL4 vector encoding Renilla luciferase (Rluc) as internal control and 0.18 μ g of pGL4 vector encoding Firefly luciferase downstream of IFNA8 promoter with A- or C-genotype (A-Fluc and C-Fluc). Twenty-four hours after the co-transfection, luc activity was measured from triplicate cell-lysates and relative luciferase was calculated (Fluc/Rluc). (B and C) Twenty-four hours after the co-transfection, the cells were stimulated with either (B) 10 μ g/ml of LPS or (C) 10 or 50 μ g/ml of poly-ICLC. Two hours after the stimulation, relative luciferase activity was measured. Results are from one of three experiments with similar results. The p value was calculated by an unpaired two-tailed Student's t-test. Error bars indicate standard deviation among triplicate samples.

previously demonstrated in glioma-bearing mice^{15,16} and humans.¹⁷ As poly-ICLC and lipopolysaccharide (LPS) are potent inducers of Type-I IFNs, we further examined whether they could enhance IFNA8 promoter activity of the A and C genotype. Following 2 h treatment with 10 µg/ml poly-ICLC or LPS, THP-1 cells still exhibited increased activity of the A-genotype IFNA8 promoter over the C-genotype in the presence of LPS (Fig. 1B) or poly-ICLC (Fig. 1C).

The A-genotype IFN-A8 promoter specifically binds more nuclear proteins than the C-genotype. We hypothesized that the observed differential activities of the promoter constructs reflecting the two SNPs were mediated by altered binding of transcription factors at the site of SNPs. We therefore extracted nuclear proteins from THP-1 cells and incubated them with biotin-labeled 40-mers derived from the IFNA8 promoter (nucleotide -354 to -314) with either A- or C- genotype. By using EMSA to detect protein bound DNA, we found that the probe with A-genotype SNP binds to either more protein and/or with a higher affinity than the C-genotype SNP (Fig. 2A) as seen in both the shifted blot and densitometry plot. To demonstrate that the observed binding is sequence-specific, a competition assay was conducted using 200-fold more non labeled (but otherwise identical) 40-mers added to the sample. Indeed, the protein-DNA interaction was specific as the non labeled competitive inhibitors blocked the binding of protein both with the A- and C- genotype (Fig. 2B).

Transcription factor Oct-1 binds and enhances the promoter activity of the IFNA8 A-genotype. On the basis of predicted binding sites to the promoter region, we next performed a supershift assay to determine which proteins bind in greater amounts to the A-genotype. We selected Oct-1 and Elk-1 as our *in silico* analysis with TFsearch¹⁸ predicted that Oct-1 or Elk-1 may bind to this region. Further, TFsearch predicted that Oct-1

would bind to the A- but not C- genotype. Surprisingly, although Elk-1 is expressed in THP-1 cells (not shown) we could not detect any supershift in either the A- or C-genotype when THP-1 nuclear protein extracts were pre-incubated with Elk-1 specific antibody (Fig. 3A). However, when the nuclear extracts were incubated with Oct-1 antibody we observed a supershift in the A- but not C- genotype, suggesting the binding of Oct-1 to the IFNA8 promoter, as predicted *in silico*. These results suggest that Oct-1 may be the transcription factor involved in the activation of IFNA8 promoter, and failure of Oct-1 to bind to the C-genotype results in lower IFNA8 activity.

We further assessed whether overexpression of Elk-1 or Oct-1 could lead to enhanced activity of the IFNA8 promoter. We therefore performed a promoter luciferase assay using the IFNA8 promoter A- genotype. Consistently, overexpression of Oct-1 but not Elk-1 lead to a statistically significant increase in luciferase activity of the IFNA8 promoter (Fig. 3B), further supporting the role of Oct-1 in the differential IFNA8 promoter activities between the A- and C- genotype.

Discussion

We described herein that the A-genotype in the IFNA8 promoter SNP rs12553612 confers a better promoter activity compared with the C-SNP. As we previously reported that WHO Grade II-III glioma patients with the AA-genotype have better overall survival than patients with the AC-alleles, higher IFNα8 expression levels may indeed contribute to the better survival of patients. Through a series of experiments, we provide the following molecular mechanism to explain this observation. As depicted by Figure 4, the rs12553612 SNP results in a change of Oct-1 binding site of IFNA8 promoter at position -335, a change at this site from A to C allele causes substantial loss of

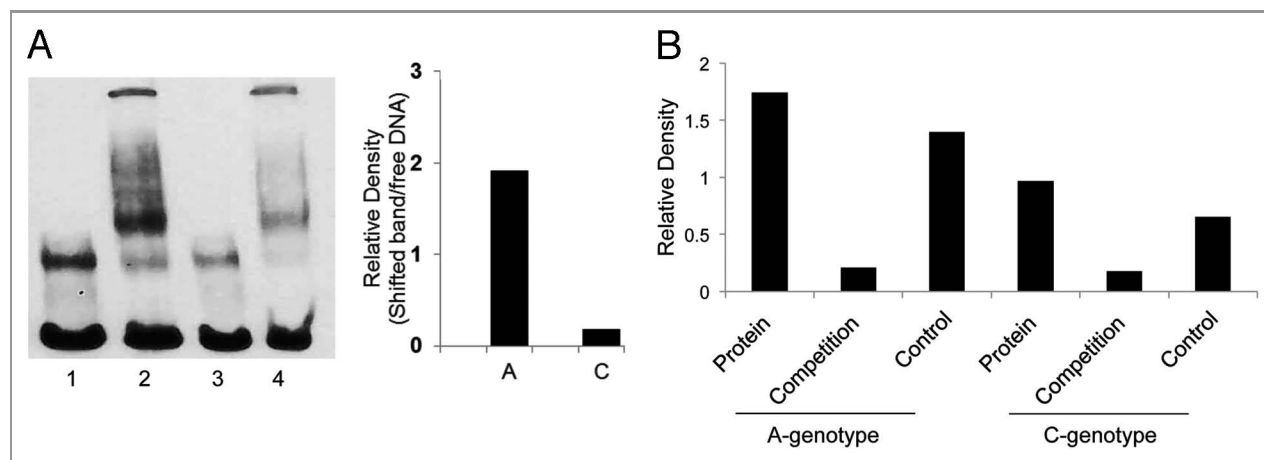


Figure 2. The DNA probe with the A-genotype in the IFNA8 promoter demonstrates higher binding to THP-1 nuclear lysate than one with the C-genotype by EMSA. (A) An EMSA was performed with biotin labeled DNA 40mers (20 fmol) with either the A-genotype or C-genotype SNP using THP-1-derived nuclear lysate (10 µg). Lane 1, A-genotype DNA alone; Lane 2, A-genotype DNA incubated with protein lysate; Lane 3, C-genotype DNA alone; Lane 4, C-genotype DNA incubated with protein lysate. Quantification of the bands in Lanes 2 and 4 was done using ImageJ (National Institutes of Health) software. (B) A competition assay with or without 200-fold excess non-labeled A- or C-genotype DNA over the biotin labeled SNP DNA to compete specifically with DNA binding site or EBNA DNA (control). Quantification of the bands was done using ImageJ (National Institutes of Health) software. Results are from one of three experiments with similar results.

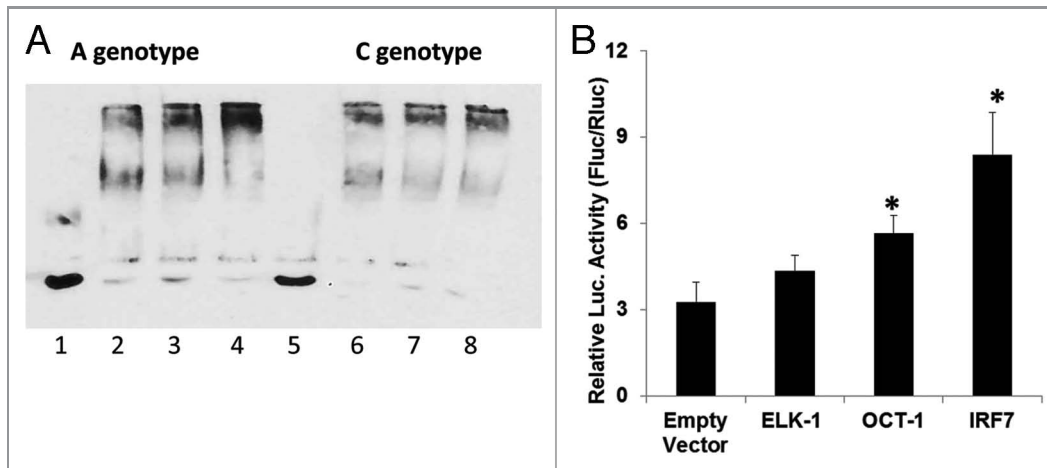


Figure 3. The A-genotype demonstrates superior binding to OCT-1 compared with the C-genotype. (A) Supershift assay was performed by pre-incubation of THP-1 cell nuclear lysate with either anti-ELK-1 or OCT-1mAb prior to DNA binding assay. Lanes 1–4, A-genotype DNA probe; Lanes 5–8, C-genotype DNA probe, Lanes 1 and 5, DNA probes alone; Lanes 2 and 6, DNA probes incubated with THP-1 lysate alone; Lanes 3 and 7, DNA probes with THP-1 lysate preincubated with anti-ELK-1 mAb; Lanes 4 and 8, DNA probes with THP-1 lysate preincubated with anti-OCT-1 mAb. The supershifted bands are marked with arrows. (B) THP-1 cells were transfected with the A-genotype Fluc-reporter plasmid and the internal control Rluc plasmid as well as an expression plasmid encoding either ELK-1, OCT- or IRF-7 as a positive control. Relative Luciferase was calculated as Fluc/Rluc. Results are from one of two experiments with similar results. * Indicates that the values were statistically different ($p < 0.05$) from the control samples with the empty vector by unpaired two-tailed Student's t-test. Error bars indicate standard deviation among triplicate sample

transcription factor Oct-1 binding affinity to the promoter resulting in downregulation of IFNA8 transcription. As we have shown before, Type I IFN signaling plays a major role in promoting anti-glioma immune surveillance.⁷ Thus a decreased IFNA8 transcription activity may potentially affect the immune surveillance resulting in lower survival. Using overexpression experiments, we demonstrated that Oct-1 but not Elk-1 regulates the IFNA8 promoter activity in the A- but not C- genotype.

Little is known about the roles and regulation of the individual IFNA genes of which there are 14 in humans. To date the primary activators of IFNA promoters that have been described are interferon regulatory factor (IRF) family members.¹⁹ For example, upon Newcastle disease virus (NDV) infection, infected cells overexpress IRF-5 that induced IFNA8.²⁰ Therefore, IRF-5

may be a key transcription factor for IFNA8. However, IRF-5 can act as both an activator and a repressor of IFN gene induction dependent on the IRF-interacting partner including IRF-3 and IRF-7.²⁰ Further, IFNA8 has been suggested to have the most potent anti-tumor activity in chronic myelogenous leukemia (CML)-derived cell lines²¹ suggesting the importance of IFNA8.

Our data demonstrate that Oct-1 can bind and promote IFNA8 promoter activity. Oct-1, also known as POU domain Class 2 transcription factor 1 (POU2F1) is known to be post transcriptionally regulated at least in part by p34cdc2-related protein kinase which is active during mitosis as well as multiple other kinases and phosphatases.²² Thus activated proliferating immune cells may have enhanced Oct-1 activity which can lead directly to Type-I IFN production. Other studies have demonstrated additional mechanisms by which Oct-1 function is regulated, such as Hydrogen peroxide which can stimulate the nuclear import of Oct-1²³ and the glucocorticoid receptor (GR) which can synergize with Oct-1 and promote recruitment of the complex (Oct-1-GR) to glucocorticoid response elements on DNA.²⁴ Oct-1 can also be activated in response to DNA damage.²⁵ These may still be partial mechanisms with which Oct-1 is regulated in glioma tissues and IFNA8 is induced in patients with the A-genotype. Interestingly, Oct-1 can inhibit IRF-7- and IRF-3-mediated IFNA11 expression in a virus infection model.²⁶ Further investigations are warranted to evaluate the role of Oct-1 in the entire Type-I IFN families and anti-tumor immunity.

Further investigations using samples obtained from human donors with AA-, AC- or CC-genotype would have strengthened our study. However, unfortunately, analysis of patient samples was not feasible as we have previously reported that of about 300 patients analyzed few patients

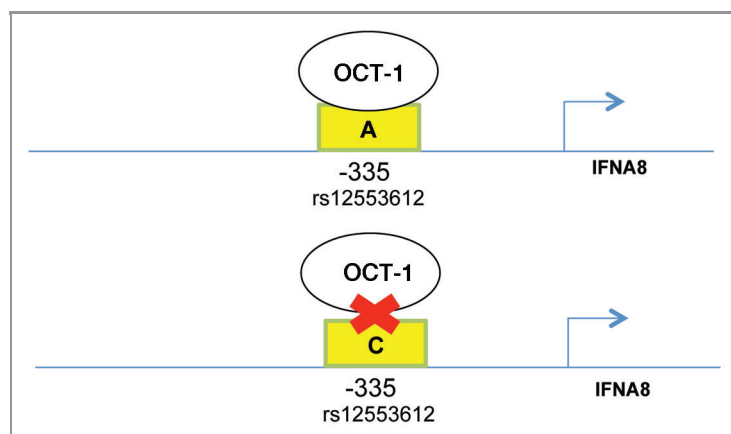


Figure 4. Schematic demonstrating the OCT-1 binding ability to the IFNA8 promoter region containing the rs12553612 SNP.

(n = 9) have the AC-genotype and we identified no individuals with the CC-genotype.⁷ Accordingly, the National Center for Biotechnology Information (NCBI) database for the current SNP (rs12553612) indicates that among a total 947 individuals analyzed AA-, AC- and CC-genotypes were found in 719, 122 and 14 individuals, respectively, with a dominant prevalence of AC and CC genotypes in Asian populations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=12553612#Diversity).

Taken together, our data suggest that Oct-1 can regulate IFNA8 promoter in the A-genotype but not the C-genotype allele. We predict based on our data that patients with the AA-genotype should have higher expression of IFNA8 than patients with the AC- or CC-genotype.

Materials and Methods

Reagents. RPMI 1640, fetal bovine serum (FBS), L-glutamine, sodium pyruvate, 2-mercaptoethanol, nonessential amino acids, and penicillin/streptomycin and all reagents for DNA transfection were purchased from Invitrogen, including Lipofectamine 2000 (11668-02), with the exception of Luciferase reporter genes and dual-Luciferase reporter assay system, which were from Promega (E1910). Plasmids containing human cDNA clones were purchased from Origene. Oligonucleotides for the IFNA8 electrophoretic mobility shift assays (EMSA) were obtained from Integrated DNA technologies; EMSA were done using the Thermo LightShift Chemiluminescent EMSA Kit (20148). Oct-1 (clone C-21, sc-232) and Elk-1 (clone I-20, sc-355) antibodies were purchased from Santa Cruz Biotechnology.

Cell culture. The THP-1 human monocyte cell line was maintained in complete medium (RPMI 1640) supplemented with 10% heat-inactivated FBS, 50 units/mL penicillin, 50 µg/mL streptomycin, 1 mM sodium pyruvate, 55 µM 2-mercaptoethanol, and MEM non-essential amino acids (Life Technologies Invitrogen) in a humidified incubator in 5% CO₂ at 37°C.

DNA transfection. Mixture of plasmid-DNA (pDNA) was prepared as 0.08–0.2 µg (total pDNA value)/ 25 µl/well. Usually, 0.02 µg/well pGL4.73 (hRluc/SV40) vector (Rluc) as internal control and 0.06–0.18 µg/well pGL4.20 (luc2/Puro) vector (Fluc) containing the A- or C-genotype of IFNA8 promoter gene were combined with diluted Lipofectamine 2000 (0.5 µl/well), and incubated for 20 min at room temperature. After the incubation, the complexes were added to each well containing 1×10^5 THP-1 cells and incubated at 37°C in a CO₂ incubator for 24 h. During the incubation, 100–150 µl/well complete medium was added 18–20 h after the transfection. When using stimulant such as poly-ICLC (Oncovir, Inc.) and LPS, the stimulant was added 2 h before stopping the incubation.

Dual-luciferase assay. Cultured cells with complexes for DNA transfection were centrifuged at three times at 1,500 rpm for 2–3 min. 80 µl/well passive lysis buffer (PLB) was added into the well containing the pellet and incubated for 15 min at room temperature. Aliquots of PLB lysate (20 µl) were transferred into luminometer tube containing 70 µl of LARII and inserted into luminometer machine to measure Fluc activity.

After addition of 100 µl of Stop&Glo Reagent, Rluc activity was measured immediately. Relative Luciferase activity [A- (or C-) Fluc/A- (or C-) Rluc]/(untreated C-Fluc/untreated C-Rluc) was measured using OD data from triplicate cell-lysates.

Electrophoretic mobility shift assay (EMSA). The non-radioactive LightShift Chemiluminescent EMSA Kit (Thermo, 20148) was used to detect DNA-protein interactions. The 5' biotin end-labeled DNA oligonucleotides each containing one SNP in the IFNA8 promoter was used as a probe for the EMSA. The probe DNAs were 40-bp double stranded DNA made by annealing (95°C for 5 min) single stranded oligonucleotides for the A SNP (5'-Biotin-TAGGAATGTAGTACATTCAAA TATGT GCATAATATATCTG and 5'-Biotin CAGATATA TTATGCACATATTTG AATGTACTACATTCCTA) and the C SNP (5'-Biotin-TAGGAATGTAGTACATT CACATATGT GCATAATATATCTG and 5'-Biotin-CAGATATATTATGCA CAT ATGTGAATGTACTACATTCCTA). Specificity was determined by a competition assay with the addition of 200 molar excess of unlabeled double stranded IFNA8 promoter oligonucleotide.

Nuclear extracts (10 µg) were isolated from the THP-1 human monocyte cell line using the Thermo Subcellular Protein Fractionation Kit (Thermo, 7884) and protein concentration was determined by the Bradford assay. Aliquots of nuclear extracts (10 µg) were incubated with 20 femtomole (fM) AT or GC IFNA8 probe in 1× binding buffer, 500 mM KCl, 0.1% NP40, 2.5% glycerol, 50 ng/µl poly dI-dC and 5 mM MgCl₂ for 20 min and were then electrophoresed through a 6% DNA retardation gel at 70V for ~45 min. For the supershift assay, nuclear extracts were incubated with antibodies for Elk-1 or Oct-1 for 2 h on ice prior to incubation with probes. The gels were electrophoretically transferred at 380 mA for 1 h on ice to a positively charged nylon membrane and immediately cross-linked for 15 min with a UV transilluminator equipped with a 312 nm bulb. Streptavidin-horseradish peroxidase conjugate and the LightShift Chemiluminescent Substrate (Thermo, 89880) were used to detect the biotin end-labeled DNA. The nylon membranes were exposed to X-ray film for 0.5–2 min for detection of the signal.

Statistical analyses. The statistical significance (p value) of differences between groups was calculated by unpaired two-tailed Student's t-test. Differences were considered as significant when $p < 0.05$. All statistical analyses were performed on Graphpad Prism software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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