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Polymorphic Lengths of Dinucleotide $(GT)^n$ Repeats in Upstream of Human nNOS Exon 1f Gene Play a Role in Modulating the nNOS Transcription: Clinical Implications

Mi-Kyung Shin[1], **Kyung-Nam Kim**[1], **Chul-Eung Kim**[2], **Sung-Keun Lee**[1,3], **Ju-Hee Kang**[1,3] **& Chang-Shin Park**[1,3]

[1]Department of Pharmacology, Center for Advanced Medical Education, Inha University College of Medicine by BK-21 Project, Korea
[2]Department of Psychiatry, Inha University Hospital, College of Medicine, Inha University, Korea
[3]MTRC, Inha Research Institute for Medical Science, College of Medicine, Inha University, Jungseok Building B-6F, Shinheungdong-3Ga, Jung-Gu, Incheon 400-712, Korea
Correspondence and requests for materials should be addressed to C. S. Park (parkshin@inha.ac.kr) or J. H. Kang (johykang@inha.ac.kr)

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Abstract

The expression of neuronal nitric oxide synthase (nNOS) is regulated by various spliced first exons (exon 1a-1i), sharing differentially common exon 2 in diverse human tissues. The highly complex structure and regulation of human nNOS gene gave limitations of information for the precise mechanism of nNOS regulation. In the present study, we report that the repeats of polymorphic dinucleotides $(GT)^nA(TG)^n$ repeats located in just upstream to the exon 1f in human nNOS gene play suppressive role in transcription, as shown in the characteristics of Z-DNA motif in other genes. In neuronal and trophoblast cells transfected transiently with luciferase construct without dinucleotide repeats at the 5′-flanking region of exon 1f in nNOS gene, the luciferase activity was increased markedly. However, the presence of the dinucleotide repeats dramatically suppressed the luciferase activity to the basal level, and which was dependent on the length of $(GT)^n$ and $(TG)^n$ repeats. More importantly, we found the polymorphisms in the length of dinucleotide repeats in human. Furthermore, we show for the first time here that there is a significant association of the lengths of polymorphic dinucleotide $(GT)^n$ and $(TG)^n$ repeats with the risk of schizophrenia.

Keywords: Neuronal NOS, Dinucleotide repeat, Schizophrenia

In central nervous systems, nitric oxide (NO) produced by three NO synthase (NOS) isoforms (nNOS; neuronal, iNOS; inducible, eNOS; endothelial NOS) is an important messenger that involves in a wide variety of physiological and pathological processes. As neuronal NO is produced constitutively to play appropriate roles of a diffusible signal messenger, NO has to be tightly regulated. Among three NOS isoforms, nNOS expression is precisely regulated and the structure of nNOS gene is extremely complicated[1,2]. Recently, many investigators have shown that nNOS expression is dynamically regulated by various physiological and pathological stimuli via transcription factors and/or protein modification[3].

Alternatively, the expression of nNOS is potentially regulated by various spliced first exons (exon 1a-1i), sharing differentially common exon 2 in diverse human tissues. In particular, as reported by Newton *et al*. exon 1c, 1f and 1g sequences were found in cDNA clones of human brain as the major splices[4]. Recent studies have shown that structural and allelic mRNA diversity is important to the regulation of nNOS[1,5,6]. However, owing to the high complexity of human nNOS gene structure and regulation of expression, little information for the precise mechanism of nNOS regulation was known yet. In the connection with the mechanisms controlling transcriptions of these mRNA diversities in human, the roles of 5′-flanking region of exon 1f expressed in human brain as a major splice are not fully understood. We found the suppressive effect of $(GT)^nA(TG)^n$ dinucleotide repeat sequences placed adjacently on the 5′-flanking region of exon 1f on the transcription of human nNOS gene. Moreover, we found that variable lengths of the $(GT)^n$ and $(TG)^n$ repeated sequences were present in human nNOS gene, and the length polymorphisms of dinucleotide

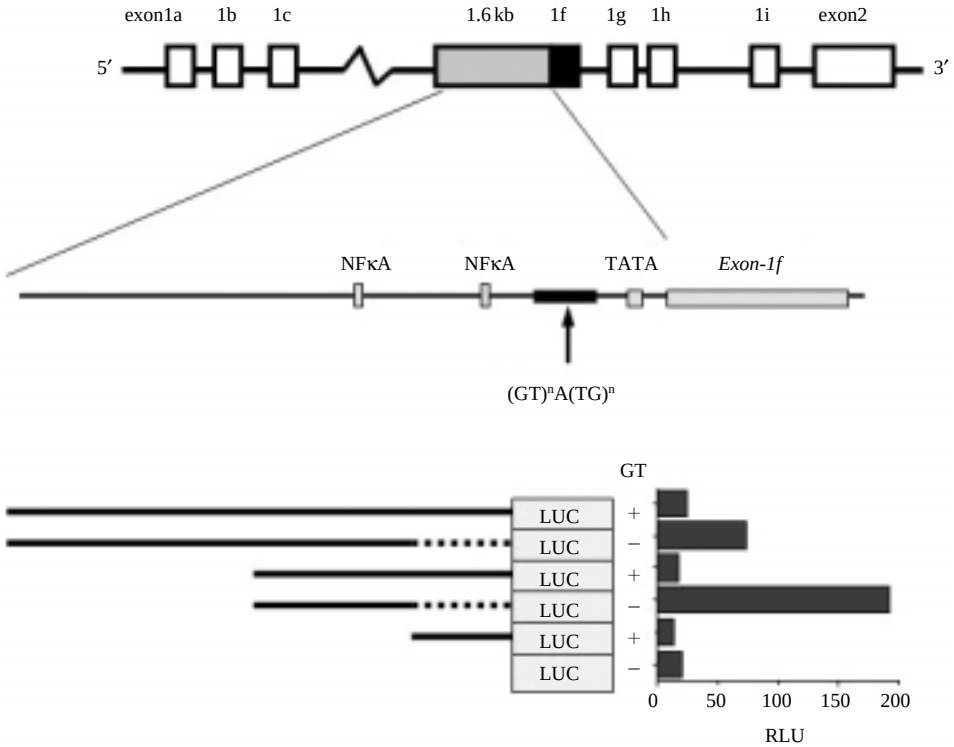

Figure 1. Location of $(GT)^n$ $A(TG)^n$ repeats within promoter sequences of Exon-*1f* of *nNOS*, and its reporter system with or without (deletion mutant) the repeat sequences and luciferase activity determined.

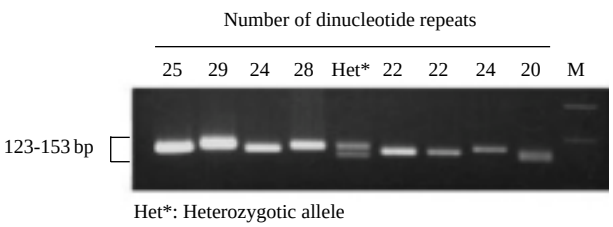

Figure 2. PCR products of differentially sized dinucleotides presented within the promoter sequences of Exon-*1f* of *nNOS*.

$(GT)^n$ and $(TG)^n$ repeats were significantly associated wih schizophrenia.

In vitro Effects of $(GT)^nA(TG)^n$ Repeated Sequences on nNOS Transcription

In the experiment of evaluating the effect of $(GT)^n$ $A(TG)^n$ sequences on the nNOS transcription *in vitro*, the construct containing variable sizes of (GT)A(TG) repeats [pGL2(GT+)] showed remarkable suppression of transcription activity compared to the activity of $(GT)^nA(TG)^n$ deleted constructs [pGL2(GT-)] (Figure 1). These results indicated that the $(GT)^nA(TG)^n$ repeated sequences may structurally repress the transcription of nNOS gene as suggested by previous studies[2,7,8]. The $(GT)^n$ purine-pyrimidine alternating repeats upstream to nNOS exon 1f were found with variable sizes from <20 repeats to up to 30 repeats in human (Figure 2). Therefore, we evaluated the precise effects of the size of the dinucleotide repeats on the nNOS transcriptional activity. As shown in Figure 3, the luciferase activities of constructs containing a long $(GT)^n$ and $(TG)^n$ repeated sequences were lower than those of constructs containing relatively short sequences. This result indicated that the basal transcription activity of nNOS may be significantly under tight control of $(GT)^n$ and $(TG)^n$ repeated sequence length. More importantly, our data suggested that the polymorphic lengths of the repeated sequences found in human lead to inter-individual variation of nNOS transcriptional levels or activity.

Association of Length Polymorphisms of $(GT)^n$ Repeats with Schizophrenia

In a very interesting recent study[9], a microsatellite repeat length polymorphism in promoter of the human N-methyl-D-aspartate (NMDA) receptor 2A subunit (GRIN2A) was reported to associate with the development and severity of psychiatric disorders. The repeats length of $(GT)^n$ and $(TG)^n$ in this study was also polymorphic and the difference of the length between patients and controls was quite small but significant. Therefore, the microsatellite repeat length polymorphism in promotor of the human nNOS exon 1f is possible to have great significance in brain physiolo-

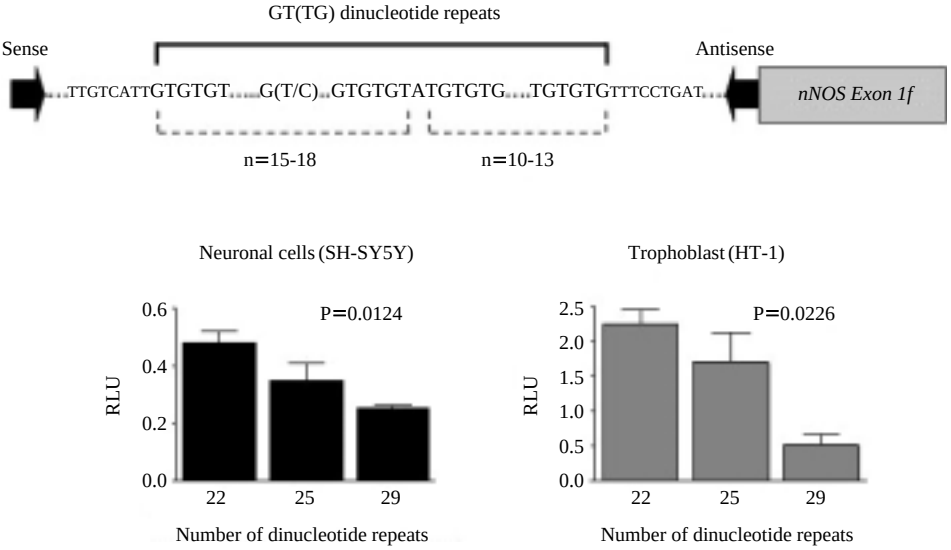

Figure 3. Sequence pattern of PCR products of $(GT)^nA(TG)^n$ repeats located at front of Exon-*1f* of *nNOS*, and difference in dinucleotide-dependent luciferase activity analysed in two cell lines. products of differentially sized dinucleotides presented within the promoter sequences of Exon-*1f* of *nNOS*.

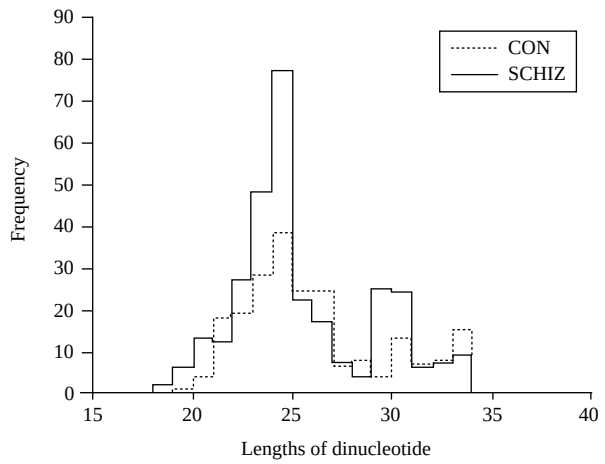

Figure 4. Significant difference in distribution of the lengths of $(GT)^nA(TG)^n$ repeats between normal and schizophrenia patients (chi-square=11.71, df=3, p=0.0084).

gy and/or pathophysiology. The NMDA receptor and neuronal NO is closely correlated to function as neuronal homeostasis or signal transmission.

To further support our *in vitro* results, we conducted a clinical gene-disease association study in normal volunteers and patients with schizophrenia. As shown in Figure 4, overall distribution of lengths of $(GT)^n$ and $(TG)^n$ repeats appears to be similar between control and patients. However, we found that there is a significant differences in distribution of sizes of $(GT)^n$ and $(TG)^n$ repeats polymorphisms and schizophrenia (chi-square=11.71, df=3, p=0.0084) when subjects were grouped according to their genotypes of $(GT)^n$ and $(TG)^n$ sizes ($\leq$20, 21-25, 26-30 and $>$30). In consistent with the previous study, our results might have significant influence on the future studies for the association of polymorphisms in nNOS gene regulation with pathophysiology in neuropsychiatric disorders.

Discussion

Until now, some investigators reported the significant or negligible association between the genetic polymorphisms of nNOS and neuron-psychiatric and cardiac diseases[10-14]. Recently, Lo *et al*. reported the positive association between the allele size variation from CAATT boxes to TATA element containing the $(GT)^n$ and $(TG)^n$ dinucleotide repeats and the prevalence of Parkinson's disease[10]. They suggested that the allele sizes of parkinsonism patients were smaller than those of sex- and age-matched controls, and then smaller allele sizes was prone to attend the higher oxidative/nitrogenous stress. However, they could not explain the meanings of the inter-individual allele size variation in respect to the effect of allele size on the regulation of nNOS expression. In human nNOS gene, 3 microsatellites dinucleotide repeats have been found at the 5′-flanking region of exon 1f, intron 2 and exon 29[10,12]. Taking consideration into the complexity in transcriptional control of nNOS and the extremely tight control of NO production in CNS, inter-individual variation of nNOS expression and/or activity resulted from microsatellite polymorphism may have pathophysiological significance on the development of neurodegenerative or psychological disorders.

In the present study, we found that the $(GT)^n$ and

Table 1. Oligonucleotide sequences used for the development of constructs or PCR amplifications of target site in *nNOS* gene.

Clone	Oligonucleotide sequences
pGL-1629/-1	P-1629: 5′-*GGCTCGA**GCTTGTGCTC CCAGAGAGGAAG-3′ P/AS-1: 5′-*GGGAAGCTT*GGGCTCCAA AGCATACATC-3′
PGL-1629/-154	P-1629: 5′-*GGCTCGA*GCTTGTGCTCC CAGAGAGGAAG-3′ P/AS-154: 5′-*GGGAAGCTT*CTGGGAG CCTGGATAGGGCT-3′
PGL-1629/-654	P-1629: 5′-*GGCTCGA*GCTTGTGCTCC CAGAGAGGAAG-3′ P/AS-654: 5′-*GGGAAGCTT*CTCCCCGT TGCTGTTGCTGC-3′
PGL-1629/-954	P-1629: 5′- *GGCTCGA*GCTTGTGCTCC CAGAGAGGAAG-3′ P/AS-954: 5′-*GGGAAGCTT*GGGTGTG TGACTTTGGGGGC-3′
pGL-154/-1	P-154: 5′-*GGCTCGA*GCCCTATCCAGG CTCCCAG-3′ P/AS-1: 5′-5′-*GGGAAGCTT*GGGCTCC AAAGCATACATC-3′

*The underlined sequences were added into the original nNOS sequences for making up the restriction enzymes recognition sites (*Xho*I and *Hind*III) for subcloning of respective constructs.

$(TG)^n$ dinucleotide repeats in the 5′-flanking region of the human nNOS exon 1f plays suppressive role in the transcription, as shown in the characteristics of Z-DNA motif in other genes[7,8]. In consistent with previous studies, the length of $(GT)^n$ and $(TG)^n$ repeats is longer, the transcription is lower. More importantly, we found that the $(GT)^n$ and $(TG)^n$ dinucleotide repeats in the 5′-flanking region of the human nNOS exon 1f distributed individually with polymorphic fashion in human and are significantly associated with development of schizophrenia. Therefore, the clinical significance of nNOS activity regulated by $(GT)^n$ and $(TG)^n$ dinucleotide repeats length polymorphism should be further evaluated in the future.

Methods

Reporter Gene Assay

For the experiment of reporter gene assay for the transcriptional modulation of nNOS gene expression, human catecholaminergic SH-SY5Y cells or trophoblast cell line (HT-1) were transfected with clone of human nNOS exon 1f promoter sequences (nt-1, -154, -654, or -954 to -1629) obtained from PCR amplification of human genomic DNA. The sequences of primers for PCR amplification of genomic DNA were designed from 5′-flanking region of exon 1f in human nNOS gene as shown in Table 1. The underlined sequences were added into the original nNOS sequences for making up the restriction enzymes recognition sites (*Xho*I and *Hind*III) for subcloning of respective constructs. The amplified fragments were digested with *Xho*I and *Hind*III, subcloned into TOTO TA cloning vector (Invitrogen) and subjected to DNA sequence analysis. The luciferase reporter plasmid (0.95 μg) with or without nNOS promoter sequence [pGL2(~nt-1629), or pGL2-basic respectively; Promega] was transiently cotransfected with 0.05 μg of pRL-null vector (Promega) by lipid mediated transfer LipofectAMINE and Plus reagent (Invitrogen) as described in manufacturer's instruction when cells were 60-70% confluent in 6-well plates. The transfected cells were harvested by treatment with lysis buffer (Promega) and luciferase activities were measured in a TD-20/20 Luminometer (TURNER DESIGNS, Promega) by using the Dual Luciferase Reporter Assay. To evaluate the effects of variable sizes of (GT)A(TG) repeat sequences on nNOS expression, respective constructs containing $(GT)^{22}$, $(GT)^{25}$, or $(GT)^{29}$ repeats found in human gene (Figure 2) were transfected in SH-SY5Y and luciferase activities were measured as described above. The sizes of (GT)A(TG) repeats were evaluated by PCR amplification of human genomic DNA from schizophrenia patients using the primers designed from 5′-flanking region of nNOS exon 1f (-1~-154) as shown in Table 1.

Clinical Study

A total of 524 unrelated Koreans participated in the nNOS genotype-disease association study. They consisted of 306 patients who had been diagnosed with schizophrenia by DSM-IV diagnostic criteria at the Inha University Hospital in Incheon (Republic of Korea) and 218 control (specific disease-free) volunteers. The study protocol was approved by the Institutional Review Board of Inha University Hospital, and all volunteers provided their written informed consent. To genotype the length of $(GT)^n$ repeats in nNOS gene, we used a simple PCR amplification using primers described in Table 1. The amplified fragments were visualized under transillumniation after 2% agarose-electrophoresis, as shown in Figure 2.

Acknowledgements

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