DOCTORAL THESIS

Analysis of single nucleotide polymorphisms in human μ opioid receptor gene and positive transcriptional regulation by poly(ADP-ribose) polymerase-1

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Chapter 3

Positive transcriptional regulation of the μ opioid receptor gene by poly(ADP-ribose) polymerase-1

List of Papers

1. Takeshi Ono, Akihiro Muto, Toshio Kaneda, Eri Arita, Tadashi Yoshida

Novel linkage disequilibrium of single nucleotide polymorphisms in the transcriptional regulatory region of μ opioid receptor gene in Japanese population, *Biol. Pharm. Bull.*, (in press)

This article was used in chapter 1.

2. Takeshi Ono, Toshio Kaneda, Akihiro Muto, Tadashi Yoshida

Positive Transcriptional Regulation of the Human μ Opioid Receptor Gene by Poly (ADP-ribose) Polymerase-1 and Increasing of its DNA Binding Affinity resulting from base substitution of -172G/T, *J. Biol. Chem.*, (received)

This article was used in chapter 2 and 3.

Abbreviations

In this thesis, the following abbreviations were used.

ADP:	Adenosine diphosphate
ANOVA:	Analysis of variance
ATP:	Adenosine triphosphate
B-Myb:	Myeloblastosis oncogene
BSA:	Bovine serum albumin
BZA:	Sodium benzoate
BZD:	Benzamide
CaMKII:	Ca^{2+}/calmodulin-dependent kinase II
C/EBP:	CCAAT/enhancer binding protein
cAMP:	Cyclic adenosine monophosphate
CDTA:	1,2-Cyclohexanediaminetetraacetic acid monohydrate
CREB:	cAMP responsive element binding protein
DAMGO:	[D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin
DMSO:	Dimethyl sulfoxide
DRD2:	Dopamine D_2 receptor
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
EGFP:	Enhanced green fluorescent protein
EGTA:	Ethylene glycol bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELK:	E-Twenty-six-specific sequence domain transcription factor

EMSA:	Electrophoretic mobility-shift assay.
ERK:	Extracellular signal-regulated kinase
GAPDH:	D-Glyceraledehyde-3-phosphate dehydrogenase
GATA:	Glutamyl-tRNA amidotransferase subunit A
HES1:	Hairy and enhancer of split 1
HRP:	Horseradish peroxidase
HTLV-Tax1:	Human T-cell leukemia virus type 1
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
IRES:	Internal ribosomal entry site
Ivs:	Intervening sequence
MALD-TOF:	Matrix-assisted laser desorption/ionization time-of-flight
MOR:	μ Opioid receptor
NAD^+:	Nicotinamide adenine dinucleotide
NFAT:	Nuclear factor of activated T cell
NF-κB:	Nuclear factor-kappa B
Oct-1:	Octamer-binding transcription factor 1
p-ABSF:	4-(2-Aminoethy)benzenesulfonyl fluoride
PARP-1:	Poly(ADP-ribose) polymerase-1
PBS:	Phosphate buffered saline
PVDF:	Polyvinylidinedifluoride
RT-PCR:	Reverse transcripion-polymerase chain reaction
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SNP:	Single nucleotide polymorphism
SREBP:	Sterol regulatory element binding protein 1
STAT:	Signal transducer and activator of transcription
TBE:	Tris borate EDTA
TK:	Thymidine kinase
TNF:	Tumor necrosis factor
TPA:	12-O-Tetradecanoylphorbol 13-acetate
WB:	Western blotting

General introduction

Opioids exert potent analgesic effects, which are mediated by the binding of agonists such as opioid alkaloids or opioid peptides to their endogenous receptors.[1-5] Pharmacological and clinical studies have shown that among all the known opioid receptors, the μ opioid receptor (MOR) exerts the most profound analgesic effect.[3-7] However, MOR also exerts side effects such as psychological and physical dependence. Therefore, some patients and health professionals are prejudiced against morphine.

Recent studies have identified the cDNA encoding μ, δ, and κ opioid receptors in rodents.[8-15] Identification of receptor subtypes has clarified that morphine induced analgesia is due to MOR. In addition, expression of MOR on cultured cells has clarified that enzymes such as adenylate cyclase or phospholipase C and K^+ or Ca^{2+} channels are related to the signal transduction mechanism via MOR.[16-22] The mechanism underlying the analgesic effect of morphine can be summarized as follows. Activation of MOR located on the presynaptic terminals of C fibers and A delta fibers indirectly inhibits the voltage-dependent calcium channels, thus decreasing the cAMP levels and blocking the release of pain neurotransmitters such as glutamate and substance P from nociceptive fibers. [23] Psychological dependence on morphine is caused by dopamine secretion by the activation of MOR. However, under pain, dopamine secretion is controlled by the κ opioid receptor activated by interaction with the endogenous opioid peptide; thus, psychological dependence is not caused under conditions of pain.[24-27] Tolerance to morphine is caused by the desensitization of G protein-coupled receptors in the phosphorylation of serine and threonine residues by

Ca^{2+}/calmodulin-dependent kinase II (CaMK II).[28-31]

These studies on opioid receptors not only clarified the antinociceptive potency of the receptors but also the mechanism underlying the dependence on and tolerance to these receptor ligands. Therefore, opioids were recognized as excellent pain-relieving drugs and have since then been widely used. However, it is difficult to determine an appropriate dose of morphine, because the efficacy of the drug dose depends on individual specificity. An opioid dose less than the appropriate dose causes mild side effects such as drowsy, vomitus, and obstipation, without exerting any analgesic effect. In contrast, an opioid dose more than the appropriate dose causes severe side effects such as respiratory depression. The appropriate dose of an analgesic may be different for each patient even if they are in the same stage of cancer progression, with same lifestyle and age, and of the same sex. Therefore, it is difficult to determine an appropriate dose of opioids.

Gene polymorphisms are one of the factors that explain the individual differences with regard to medicinal and side effects of a drug. Gene polymorphisms are individual differences in the genomic DNA with the frequency of $\geq$1%. Two types of gene polymorphisms single nucleotide polymorphisms (SNPs) and microsatellite polymorphisms are thought to provide useful information. The formers are substitutions of single nucleotides, and the latters are iterations in DNA sequences. Although the influence of SNPs is less than that of the microsatellite polymorphisms, there are more types of SNPs than microsatellite polymorphisms. It is considered that more than 2,000,000 types of SNPs and 100,000 types of microsatellite polymorphisms exist in the human genome. Therefore, SNPs are expected to be more useful in medical

practice than microsatellite polymorphisms. In general, an SNP is distinguished by the region where it exists: an rSNP exists in the transcriptional region, a cSNP in the translational region, and an iSNP in the intronic region. The rSNPs may alter the expression level of protein via gene transcription, and the cSNPs may alter the function of protein via amino acid substitution. The iSNPs are assumed to alter the expression level or the function of protein via splicing in gene transcription.[32, 33)]

Several studies on SNPs have clarified the relation between SNPs and individual differences with regard to drug treatment. For instance, because the productivity of leukotriene in a patient whose genome has an SNP in the transcriptional regulatory region of 5-lipoxygenase genes is low, it is clarified that the effect of lipoxygenase inhibitor on these patients is weak.[34-36)]

Similarly, in pain therapy, prediction of the effect of morphine based on SNP information is expected to enable treatment with morphine with minimum side effects. Recently, individual specificity was considered to be associated with the SNPs existing in the human MOR gene.[37-42)] Some of these SNPs are associated with the analgesic or side effects of morphine, while others influence the function or expression levels of the receptor.[43-48)]

This study demonstrated that the SNP on the transcript regulatory region of MOR gene increased the transcriptional control of MOR gene. The analysis of SNP detected 10 SNPs on transcriptional region and 4 exons of Japanese MOR gene. Moreover, linkage analysis revealed significant linkage, which was not observed in studies performed in other nations (chapter 1). The specific nuclear proteins were found to bind to neighborhood of -172G/T which is located on 172 bp upstream of the

translation initiation site, and its affinity to DNA was increased by base substitution from -172G to T. This protein was identified poly(ADP-ribose) polymerase-1 (PARP-1) by affinity purification and mass spectrometry (chapter 2).

PARP-1 was turned out as the positive regulator of MOR gene. Moreover, tumor necrosis factor (TNF)-α enhances MOR gene expression besides the increasing of PARP-1-binding to -172G/T region. These results suggest the MOR gene expression was enhanced by -172T substitution (chapter 3).

Chapter 1

Analysis of single nucleotide polymorphisms in Japanese MOR gene

Introduction

μ Opioid receptor (MOR) is molecular targets of analgesic opioids, such as morphine, fentanyl, and related drugs, which also mediate tolerance and dependence. MOR gene located on chromosome 6q24-25 is composed of 4 exons, 3 introns, and a transcriptional regulatory region, which is 3,000 bp upstream from the translation initiation site.[49] Greater than 40 kinds of single nucleotide polymorphisms (SNPs) have been reported on the MOR gene.[50] In clinical studies such as those involving case controls, certain SNPs have been reported to relate to the analgesic or side-effects of morphine.[51] An *in vitro* study demonstrated that certain SNPs influenced the receptor function of MOR by substitution of its amino acid, whereas other SNPs influenced the receptor expression levels by altering the MOR gene transcriptional regulation.[51]

One of the well-established polymorphisms involving the MOR gene is referred to as 118A/G; it causes amino acid exchange, referred to as 40Asn/Asp, in the extracellular region of the receptor.[52] This substitution leads to a change in the affinity of the receptor to its ligands, which increases β-endorphin binding; however, it causes a decrease in morphine-6-glucuronid activity.[43, 44] In other SNPs, 779G/A, 794G/A, or 802T/C in MOR exon 3 caused an amino acid change, i.e., 260Arg/His, 265Arg/His, or 268Ser/Pro, in the third intracellular loop of MOR, which decreases the receptor signaling activity.[45, 46] Furthermore, 802T/C (268Ser/Pro) lost CaMKII-induced receptor desensitization.[46] The frequency of occurrence of SNPs that cause amino acid substitution thereby influencing MOR function is extremely low, except for

118A/G.[50, 51] Therefore, it is expected that polymorphisms in the transcript regulatory region would contribute to the various responses of individuals to opioids. Although the SNPs of the MOR gene have been widely analyzed, the frequencies of SNPs on the MOR gene among the Japanese have not been established. In this study, the SNPs analysis of the Japanese MOR gene report newly identified linkage disequilibrium, namely, -1748G/A and -172G/T.

Materials and Methods

Subjects

The study subjects comprised of 52 healthy Japanese volunteers. This study was approved by the Institutional Review Committee of Hoshi University. Written informed consent to participate in this study was obtained from all subjects.

Genotyping

Genomic DNA samples were extracted from peripheral blood of each subject by using Wizard Genomic DNA Purification Kit (Promega, CA, USA). Samples were employed to analyze the MOR gene polymorphisms among members of the Japanese population. Polymerase chain reaction (PCR) was performed by using LA Taq (Takara Bio Inc, Shiga). The reaction mixtures contained 50 ng of genomic DNA in 50 μL (2.5 units of LA Taq, 1× LA PCR buffer, 0.4 mM deoxyribonucleotide triphosphate (dNTP) mix, 2.5 mM $MgCl_2$, 0.5 μM primers). Each PCR product was purified using SV Wizard gel and PCR purification kit (Promega).

DNA sequence of the PCR product was analyzed by direct sequence analysis using CEQ DTCS-Quick Start Kit (Beckman Coulter Inc, CA, USA). The detection of polymorphism in the MOR gene was based on the observations made from the genomic DNA sequence. Figure 1 or Table 1 presents the analyzed regions and the primer sequence used to amplify those regions.

Linkage disequilibrium

Linkage disequilibrium measures were calculated for SNP loci occurring with a frequency of 5% or greater in the MOR gene. The linkage disequilibrium observed between 2 points was analyzed for the detection of SNPs with at least 5% occurrence frequency. Linkage disequilibrium was calculated by using the QTL Haplo and calculation resource referred to in these reports.[53, 54)]

Results

Analysis of single nucleotide polymorphisms in Japanese MOR gene

This study analyzed the transcriptional regulatory region, the entire region of exons, and the intron 2 of MOR gene using genomic DNA samples. Some SNPs were observed in the transcriptional regulatory region (-1748G/A, -1565T/C, -1045A/G), in the untranslated region (-172G/T, -38C/A,) in the coding region (118A/G) and the intronic region (ivs2+31G/A, ivs2+691C/G, ivs4+274A/G, ivs4+435G/A). No tandem repeats, insertions, or deletions of nucleotides were found in this study. Table 2 summarizes the allele and genotype frequencies.

Linkage analysis of Japanese MOR gene

Subsequently, the linkage disequilibrium was analyzed between 2 points of 6 SNPs with an occurrence frequency of 5% or greater (-1748A, 7.7%; -1565C, 5.8%; -172T, 7.7%; -118G, 47.1%; ivs2+691G, 17.3%; ivs4+435A, 12.5%). Calculation of the linkage disequilibrium ($1 > D' > -1$, $1 > r^2$) revealed significant linkage in -1748G/A and -172G/T ($D' = 0.864$; $r^2 = 0.746$, Table 3). Genotype of -1748G/G did not have -172 T allele and similarly as for -172G/G genotype did not have -1748A allele (Table 4). Furthermore, forecast frequency of -172T/-1748A was higher than that of -172G/-1748A and -172T/-1748G (-172T/-1748A, 6.7%; -172G/-1748A, 1.0%; -172T/-1748G, 1.0%; Table 5).

Discussion

Since 10 years, various polymorphisms in MOR gene have been reported. SNP frequencies of Japanese MOR gene in regions other than in the transcript region were provided in a previous report.[55] In our study, 5 SNPs (-1748G/A, -1565T/C, -1045A/G, -172G/T, -38C/A) were detected in the transcriptional regulatory region. Frequently, DNA binding affinity to the transcription factor is influenced by base substitution in transcription factor binding region or its neighborhood. Subsequently, transcription factors that could possibly be bound to the DNA in the neighborhood of SNPs were assumed by using the computer program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCHJ.html). This program assessed that C/EBP or CREB binds to -1748G/A region, OCT-1 binds to -1565T/C region and -1045A/G region, and GATA, SREBP, and SP1 bind to -172G/T, respectively.

Subsequently, SNP frequencies obtained in this study was compared with those in previous reports. The frequency of both -1748A and -172T was 7.7%, and significant linkage was observed between these 2 SNPs (Tables 2 and 3). The genotype of -1748G/G or -172G/G did not possess a -172T allele or -1748A allele (Table 4). The haplotype forecast frequency of -1748A/-172T was higher than that of -1748G/-172T or -1748A/-172 G (Table 5). Therefore, -1748A and -172T could possibly comprise the same haplotype. In a study performed on African-American populations, the observed frequency of -1748G/A or -172G/T was similar to this study (-1748G/A, 2.9%; -172G/T, 11.4%); however, the linkages -1748G/A and -172G/T were not observed.[50] In contrast, the frequency of 118A/G in our result (47.1%; Table 2) demonstrated a

greater similarity to the Asian populations than to the European or American ones (Malay, 42.0%; Indian, 47.4%; Chinese, 35.1%; Caucasian, 11.5%; African-American, 1.6%); further, the frequency of ivs2+691C/G reported in our result (17.3%; Table 2) demonstrated a greater similarity to the Asian population than to the European and American ones (Malay, 20.5%; Indian, 22.5%; Chinese, 18.3%; Caucasian, 40.0%; African-American, 50%).[56-58] Although SNP frequency of 118A/G or ivs2+691C/G demonstrated a similar frequency as that observed in Asians, the linkage of 118A/G and ivs2+691C/G observed in Asians was not observed in this study (Malay, $D' = 0.86$; Indian, $D' = 0.826$; Chinese, $D' = 0.91$; our result, $D' = -0.066$; Table 3).[59] Therefore, these results suggest that our results obtained from SNPs analysis of MOR in Japanese populations are different from those reported in other countries.

In addition to the importance of each SNP, linkage disequilibrium or haplotype analysis is assumed to be a useful means to analyze polymorphisms, because the phenotype is not only related to each SNP but also to the haplotype comprising of 2 or greater number of SNPs. In conclusion, significant linkage between -1748G/A and -172G/T was found. This linkage could possibly be related to individual specificity of opioid effects by altering MOR gene transcription. Therefore the possibility that SNPs on the transcript regulatory region including a novel linkage of -1748G/A and -172G/T contribute to the MOR gene expression was examined in the next chapter.

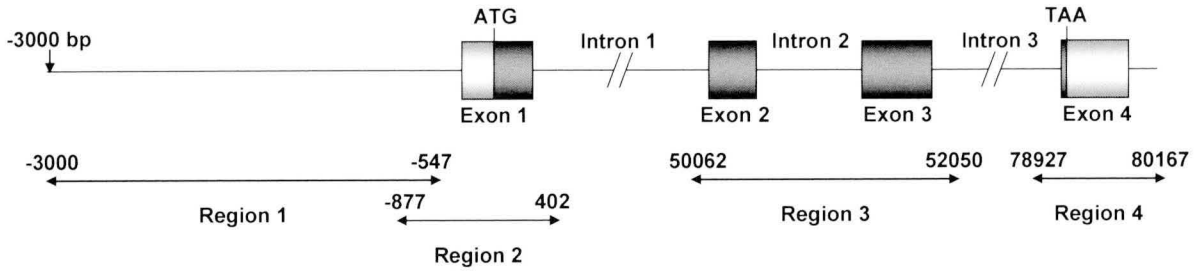

Fig. 1 Overview of the genomic MOR gene and analyzed regions

This outline shows the composition of and the areas subjected to SNPs analysis of MOR gene. Arrows show analyzed regions (Region 1, 2450 bp; Region 2, 1279 bp; Region 3, 1988 bp; Region 4, 1240 bp). These regions were amplified by using primers specific to each sequence (Table 1 provides the details).

Table 1. Analyzed regions and primer sequence

Region 1		
Locus number*	Direction	Nucleotide
-3000 ~ -2982	Forward	ATGGGGTTGCTCTTTGCCTG
-567 ~ -547	Reverse	CTAAAACCCCAATGCTCTTCC
Region 2		
Locus number*	Direction	Nucleotide
-877 ~ -857	Forward	TGCCACAACCTTCTGATTTCT
382 ~ 402	Reverse	TACCTCCCCTCTTTCATCCTC
Region 3		
Locus number*	Direction	Nucleotide
50062 ~ 50082	Forward	GAAACTCAACAAAGCAGCATC
52030 ~ 52050	Reverse	CTTCCCCTCTTCCCTCCATTC
Region 4		
Locus number*	Direction	Nucleotide
78927 ~ 78949	Forward	GTAGCATACACAAATGAAGAGC
80146 ~ 80167	Reverse	TCTGGCAATCAACAAAAACATC

(* Locus number shows the figure from the translation initiation site.)

Table 2. Genotype frequency of MOR gene polymorphisms

Locus	Genotype frequency			Allele frequency	
-1748	G/G 86.5%	G/A 11.5%	A/A 2.0%	G 92.3%	A 7.7%
-1565	T/T 88.5%	T/C 11.5%	C/C 0.0%	T 94.2%	C 5.8%
-1045	A/A 96.2%	A/G 3.8%	G/G 0.0%	A 98.1%	G 1.9%
-172	G/G 86.5%	G/T 11.5%	T/T 2.0%	G 92.3%	T 7.7%
-38	C/C 98.1%	C/A 1.9%	A/A 0.0%	C 99.0%	A 1.0%
118	A/A 40.4%	A/G 25.0%	G/G 34.6%	A 52.9%	G 47.1%
Ivs2+31	G/G 94.2%	G/A 5.8%	A/A 0.0%	G 97.1%	A 2.9%
Ivs2+691	C/C 67.3%	C/G 30.8%	G/G 1.9%	C 82.7%	G 17.3%
Ivs4+274	A/A 94.2%	A/G 5.8%	G/G 0.0%	A 97.1%	G 2.9%
Ivs4+435	G/G 75.0%	G/A 25.0%	A/A 0.0%	G 87.5%	A 12.5%

Table 3. Linkage disequilibrium of MOR gene polymorphism

Locus 1	Locus 2	D'	r^2
-1748	-1565	-1.000	0.005
-1748	-172	0.864	0.746
-1748	118	-0.281	0.006
-1748	Ivs2+691	0.656	0.171
-1748	Ivs4+435	0.228	0.030
-1565	-172	-1.000	0.005
-1565	118	-0.572	0.018
-1565	Ivs2+691	-0.098	0.001
-1565	Ivs4+435	-0.843	0.006
-172	118	0.074	0.001
-172	Ivs2+691	0.656	0.171
-172	Ivs4+435	0.380	0.084
118	Ivs2+691	-0.066	0.001
118	Ivs4+435	-0.139	0.003
Ivs2+691	Ivs4+435	0.585	0.234

Table 4. Combination genotype frequency of -1748G/A and -172G/T

Genotype		frequency
-1748	-172	
G/G	G/G	86.5%
G/A	G/T	11.5%
A/A	T/T	2.0%

Table 5. Forecast haplotype frequency of -1748G/A and -172G/T

-1748	-172	frequency
G	G	91.3%
G	T	1.0%
A	G	1.0%
A	T	6.7%

Chapter 2

Influence of base substitution in transcriptional region of MOR gene

Introduction

The expression level of the MOR gene is controlled by various transcriptional factors. The transcript of the MOR gene is regulated by the region from the translation beginning point to 2 kbp upstream.[60, 61] In human neuroblastoma cell line SH-SY5Y, (D-Ala^2, N-Me-Phe^4, Gly^5-ol) enkephalin (DAMGO) up-regulated MOR gene via SP1 and SP3, 12-O-tetradecanoylphorbol-13acetatate (TPA) up-regulated MOR gene via AP1, and interleukin (IL)-6 up-regulated via STAT1 and STAT3.[62-64] SNPs in promoter region could influence to MOR expression and following responsiveness to its agonists. In immuno-effector cells, IL-4 up-regulated MOR gene via STAT6 binding to -997. The -995C/A exists in the DNA binding site of STAT6. And the affinity of STAT6 to -995A was lower than that to -995C. TNF-α upregulated the MOR gene via NF-κB binding to -2174, -557, and -207. The -554G/A polymorphism is present on the DNA binding site of NF-κB. The affinity of NF-κB to -554A was lower than that to -554G. Therefore, either the -995C/A or the -554G/A polymorphism has the possibility of influencing the MOR gene expression that IL-4 or TNF-α causes through respective transcriptional factors.[47, 48] CXBK mice, a crossbred between C57BL/6By and BALB/cBy mice, [65] are known as MOR knockdown mice. It was reported that the base substitution at -202C/A detected in CXBK mice decreased the SP1 binding affinity to the MOR gene. [66]

In this chapter, the possibility that 5 SNPs (-1748G/A, -1565T/C, -1045A/G, -172G/T, -38C/A) on the transcript regulatory region which had been detected by the previous SNP analysis contributes to the MOR gene transcription were examined. In process of

these analyses, specific nuclear proteins bound to -172G/T region was found and its affinity was increased by base substitution of -172G/T. In this study, PARP-1 was shown to bind to -172T region in MOR gene, prefer to -172G, and positively regulated MOR gene expression.

Material and Methods

Cell culture

The human neuroblastoma cell line SH-SY5Y and the human embryonic kidney cell line HEK293T were cultivated in Dulbecco's modified Eagle's medium (WAKO Pure Chemical Industries, Ltd., Osaka) supplemented with 10% fetal bovine serum (GIBCO BRL, MD, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin: GIBCO BRL).

Nuclear extract preparation

Nuclear extracts were prepared from HEK293T or SH-SY5Y cells. All steps were performed at 4°C. The cells were resuspended in lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM p-ABSF, 2 μg/mL aprotinin, 2 μg/mL pepstatin, 2 μg/mL leupeptin). The lysate was centrifuged at 500 x g for 3 minutes to pellet the nuclei, which were washed with lysis buffer. The nuclei incubate in elution buffer (50 mM HEPES, 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 20% grycerol, 1 mM DTT, 1 mM p-ABSF, 2 μg/mL aprotinin, 2 μg/mL pepstatin, 2 μg/mL leupeptin) for 1 hour. Samples were centrifuged at 14,000 x g for 15 minutes. The supernatant liquids were used for nuclear extracts. Protein concentration of nuclear extracts was determined using the Coomassie Protein Assay Reagent Kit (PIERCE, Rockford, IL USA).

Electrophoretic mobility shift assays (EMSA)

All oligonucleotides were synthesized by Operon Biotechnologies (Tokyo). To obtain double-strand DNA, equimolar amounts of both strands were heated to 85°C for 15 minutes with 20 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$, and 50 mM NaCl. After heating, each sample was allowed to cool down to room temperature slowly.

Oligonucleotides used for EMSA were 5′ end labeled with T_4 kinase (TOYOBO, LTD., Osaka) and [γ-^{32}P] ATP (Perkin-Elmer Inc., MA, USA). After the labeling reaction, labeled oligonucleotides were purified with MicroSpin^{TM} G-25 Columns (GE Healthcare, BUCKS, UK) and diluted to 100 μL. Oligonucleotide sequences are shown as follows: -172G probe: 5′-AGAGGAGAATGTCAGATGCTCAGCTCGGTCCCCT CCGCCTGA-3′, -172T probe: 5′-AGAGGAGAATGTCAGATGCTCATCTCGGTCC CCTCCGCCTGA-3′. Nuclear extracts were preincubated for 15 minutes at room temperature in 10 μL of binding reaction mixture contained 1 μg of nuclear extracts and 2 μL of 5 x gel-shift binding buffer (Promega). Subsequently, 35 fmole of labeled probe was added, and incubation was continued for 30 minutes at room temperature. For competition assay, unlabeled probe was added to the binding reaction mixture before the addition of the labeled probe. For super-shift assay, anti-PARP-1 antibody (sc-8007; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dialyzed with 0.1 x PBS for two days. Subsequently, dialyzed antibody and buffer was concentrated on 8-fold by the vacuum. Two microliters of anti-PARP-1 antibody, dialyzed buffer or anti-SP1 antibody (sc-59; Santa Cruz) was added to the binding reaction and incubated for 1 hour at 4°C before the addition of the labeled probe. Samples were loaded on a 6% polyacrylamide gel in 0.5 x TBE buffer for 1 hour. After loading, gel was

dewatered, exposed to the imaging plate and analyzed with Typhoon9410 (GE Healthcare).

Affinity purification of DNA-binging protein

For blocking non-specific binding of protein, 10 μL of streptavidin agarose beads (Calbiochem, Darmstadt, Germany) were pre-incubated in 2% bovine serum albumin (BSA) and washed twice in the bufferd solution (10 mM Tris-HCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM DTT, and 50 mM NaCl). Biotin-labeled oligonucleotides (90 pmol) were added to nuclear extracts of 100 μg and incubated for 30 minutes at room temperature. Next, streptavidin agarose beads pre-treated by BSA were added to the DNA-protein complex and incubated for 1 hour at 4°C. Beads-DNA-protein complexes were washed twice in the buffered solution and then collected by centrifugation at 1,400 x g for 5 minutes. The sample for SDS-PAGE was prepared by heating precipitated complex with 100 μL of Laemmli buffer [62.5 mM Tris-HCL (pH 6.5), 2% SDS, 10% glycerol, and 0.00125% bromophenol blue] containing 5% β-mercaptoethanol.

Western blotting (WB)

Nuclear extracts containing 2 μg of protein were diluted to 100 μL with Laemmli buffer contained of 5% β-mercapt ethanol and heated for 5 minutes at 95°C. Samples were loaded on 8% polyacrylamide gel and transferred to PVDF membrane. The PARP-1 protein was detected by mouse monoclonal antibody that recognizes the C terminus of PARP-1, anti-mouse-HRP secondary antibodies and ECL reagent.

Reporter gene plasmids

All reporter plasmids are based on the pGL3 basic vector (Promega). Construction of pGL3 TK-172G or -172T was accomplished by inserting oligonucleotides used for EMSA into the pGL3 basic vector digested with *Sma* I (TaKaRa BIO INC). Next, thymidine kinase promoter sequence was obtained from the pRL TK vector (Promega) by digesting with *Bgl* II (TaKaRa BIO INC) and *Hind* III (TaKaRa BIO INC) and then inserting in all pGL3 vectors digested. Construction of pGL3-172G or -172T was accomplished by inserting the MOR promoter ranged from 3 kbp up-stream to translation initiation site into the pGL3 basic vector at *Xho* I and *Hind* III sites. The inserted -172G fragment was amplified from genomic DNA of the -172G/G genotype with PCR; PCR was performed using KOD plus (TOYOBO, LTD,) and the following set of primers: Forward 5′-tatctcgaggatggcggagtcttcgg-3′, Reverse 5′-tgcctcgagtacctctc ccaattaccacag-3′. The -172T fragment was amplified from the -172G fragment by mutagenesis that employed PCR. DNA fragments were purified using the Wizard PCR Preps DNA Purification System (Promega), and plasmid DNA was purified using the Genopure Plasmid Maxi Kit (Roche Diagnostics, Basel, Switzerland).

Transfection

Plasmid DNA transfection to HEK293T cells was performed in 24 well plate, using 2.4 μL of NP-OH transfection regent and 0.5 μg of plasmid DNA added to 12.5 μL of NaCl (40mM).[67] Mixture was incubated at room temperature for 20 minutes and used for each well. Transfection to SH-SY5Y cells was by using Fugene 6 (Roche Diagnostics).

Luciferase assay

HEK293T cells and SH-SY5Y cells were cultivated in 24-well plates at a density of 5 x 10^4 per well before transfection for 24 hours. Transient transfection construct was prepared using 0.25 μg of the pGL3 vector, 0.125 μg of the pEF-bos β-gal vector, and 0.125 μg of the pcDNA3-myc vector. The pEF-bos β-gal vector was used for β-galactosidase assay as an internal control of transfection efficacy. After incubation for 24 hours, cells were lysed in 200 μL of lysis solution [25 mM Tris-HCl (pH 8.0), 2 mM DTT, 2 mM CDTA (pH 7.0), 10% glycerol, 1% Triton X, 4 mM $MgCl_2$, and 4 mM EGTA)]. Each lysate (40 μL) was used for luciferase assay and for β-galactosidase assay. Luciferase activity was quantified in MiceoLumat Plus LB96Y (Berthold Technologies, Bad Wildbad, Germany) by measurements based on the Luciferase Reporter Assay System (Promega). These assays were performed 3 times in triplicate.

PARP-1 expression vector

The PARP-1 coding sequence was cloned from SH-SY5Y cells via PCR amplification; PCR was performed using KOD plus (TOYOBO, LTD) and the following set of primers: Forward 5′-tatctcgaggatggcggagtcttcgg-3′, Reverse 5′-tgcctcg agtacctctcccaattaccacag-3′. Following sequencing, the fragments were digested with *Xho* I (TaKaRa BIO INC) and inserted to expression vector. DNA fragments were purified using the Wizard PCR Preps DNA Purification System (Promega), and plasmid DNA was purified using the Genopure Plasmid Maxi Kit (Roche Diagnostics).

Statistical analysis

For statistical evaluation of the experiments, one-way ANOVA was performed. Asterisks indicate significantly different values (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$).

Results

Increasing of specific nuclear protein binding by the base substitution of -172G to -172T

To determine whether specific nuclear proteins bind to the -172G/T region, this study performed EMSA using nuclear proteins extracted from SH-SY5Y or HEK293T cells and ^{32}P-labeled oligonucleotide probes containing the -172G/T region. SH-SY5Y or HEK293T were respectively employed as MOR-expressing cells or as advantageous cells for gene transfer analysis. In each cell, specific nuclear protein binding to both the -172G and -172T probes was observed as doublet bands. More proteins displayed binding to the -172T probe than to the -172G probe (Fig. 2A). In order to compare binding affinity of specific nuclear proteins to the -172G or to the -172T probe, a cross competition assay was carried out using respective non-labeled probes as a competitor (Fig. 2B). Non-labeled -172T probe dose dependently interfered with specific nuclear protein binding to the -172G probe (Fig. 2B, lane 5-7). However, inhibitory effects by the non-labeled -172G probe to the -172T probe were comparatively weak (Fig. 2B, lane 9-11). Specific nuclear protein binding was similarly found in 4 SNPs region (-1748G/A, -1565T/C, -1045A/G, -38C/A) expect -172G/T, however the influence by the base substitution was not found (Fig. 3). These results were similar for both HEK293T and SH-SY5Y cells.

Transcriptional activity of -172G/T region and the influence of the base substitution in MOR promoter

The reporter vector of -172G/T TK or of -172G/T was used for the luciferase assay to monitor transcriptional activity of the MOR gene in SH-SY5Y or HEK293T cells. Oligonucleotide containing the -172G or -172T probe was cloned upstream of the thymidine kinase promoter sequence in the pGL3 TK vector. Luciferase activity of pGL3-172G TK or of pGL3-172T TK normalized by that of pGL3 TK was shown as the relative transcriptional activity of these sequences. As a result, both pGL3-172G TK and pGL3-172T TK showed the transcriptional activities, which in pGL3-172T TK was 1.7 times as high as that of pGL3-172G TK in SH-SY5Y or HEK293T cells (Fig. 4A). Similar to the results in pGL3-172G/T TK, the pGL3-172G/T containing the MOR promoter that ranged from the start site of transcription to -3 kb upstream indicated transcriptional activity; that of pGL3-172T was 2.0 to 2.5 times higher than that of pGL3-172G in SH-SY5Y or HEK293T cells (Fig. 4B). Results depicted in Figs. 2 and 4 indicate that the promoter containing -172T could possibly bind much more nuclear-protein and have higher transcriptional activity.

Purification of specific binding protein on -172G/T region using biotin-labeled -172T probe, and identification of that by MALDI-TOF mass spectrometry and by immunoblotting with specific antibody

For identification of binding proteins in the -172G/T region, the biotin-labeled -172T probe and streptavidin-agarose beads were applied to nuclear protein purification. Protein separated by SDS-PAGE was stained with Coomassie brilliant blue and observed as a single band at 120 kDa (Fig. 5A, lane 5). MALDI-TOF mass spectrometry revealed that the 120-kDa protein removed from acrylamide gel was poly(ADP-ribose) polymerase-1 (PARP-1), which was confirmed by WB with anti-PARP-1 antibody in both SH-SY5Y and HEK293T cells (Fig. 5B).

Enhancement of protein binding to -172T and of transcriptional activity by overexpressed PARP-1

PARP-1 overexpression was found in transfected HEK293T cells (Fig. 6A), and enhanced protein binding to the -172T probe and was detected as doublet bands in EMSA (Fig. 6B); it also increased transcriptional activity of pGL3-172T TK or of pGL3-172T (Fig. 6C). Similarly, increase in transcriptional activities was also observed in pGL3-172G TK or in pGL3-172G (data not shown).

Detection of PARP-1 binding sequence in -172T region

In super-shift analysis, anti-PARP-1 antibody specifically diminished PARP-1-derived doublet bands (Fig. 7; lane 3, 7). SP1 was initially predicted by computer program to bind -172G/T; however, anti-SP1 antibody did not influence protein-binding signals in -172T (Fig. 7; lanes 4, 8). These results clearly indicate that PARP-1 binds to the -172G/T region and activates transcription of MOR, which is influenced by base substitution.

For detection of PARP-1 binding sequence in the -172G/T region, a competition assay was performed using shortened or mutated -172T probes. In the analysis using shortened probes, mt 1 competed to -172T probe and diminished PARP-1 signals, but mt 2 did not have this effect. Among mutated probes of mt 1, mt 3 completely diminished PARP-1 signals, but inhibition by mt 4, mt 5, or mt 6 was partial (Fig. 8A). These results indicate that TGTCAGATG was the center of PARP-1 binding in the MOR gene, and the sequence CTCAT beside the -172T also related to its binding.

Discussion

SNP analysis of Japanese MOR gene found 5 SNPs (-1748G/A, -1565T/C, -1045A/G, -172G/T, -38C/A) on transcript regulatory region of MOR gene. To examine the influence that these SNPs exert to MOR gene expression, this study analyzed alteration of DNA binding affinity to nuclear protein by base substitution in the SNP region. In all SNPs regions, specific nuclear protein binding was shown. However, the influence by the base substitution was found in only -172G/T (Fig. 2, 3).

The -172G/T polymorphism in the MOR gene promoter is caused by base substitution from guanine to thymine, which is located on 172 bp upstream of the translation initiation site. In previous studies, no functional differences were found between the -172G and -172T probes on MOR promoter activity.[68] However, this study definitely detected such differences; i.e., the -172T probe displayed higher binding affinity to unknown nuclear protein and higher transcriptional activity than the -172G probe, thereby implying that this protein regulates MOR transcription at -172G/T (Fig. 2, 4). Therefore, we employed the computer program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCHJ.html) to forecast known transcription factors bindable to neighborhood of -172G/T region, which assessed that the SREBP, SP1 or GATA-1 possibly bound to -172G/T region. On the basis of the anticipated results, we attempted to compete with specific protein binding to the -172T probe by using oligoDNA-probes containing the consensus binding sequences for the anticipated transcription factors, but the binding was not influenced by these competitions (data not shown). Consequently, the specific nuclear protein bound to the -172T probe was purified and

identified PARP-1. WB using anti-PARP-1 antibody confirmed that this nuclear protein was definitely PARP-1 (Fig. 5B). The binding of PARP-1 to the -172G/T probe was also detected as doublet bands in EMSA (Fig. 2A, 4B), and anti-PARP-1 antibody diminished both bands in super-shift analysis (Fig. 7). Although it can only speculate about the details, PARP-1 might assume two kinds of binding forms to the -172G/T probe.

PARP-1 is a 116 kDa nuclear protein known to have DNA-binding activity and enzymatic activity of ADP ribosylation.[69] PARP-1 catalyzes the reaction that adds the ADP-ribose unit of NAD^+ to several nuclear proteins, including PARP-1 itself.[70] Initial study of PARP-1 implicated many biological functions, including DNA repair, recombination, apoptosis, and tumor genesis.[70] However, recent studies demonstrated that PARP-1 also contributed to gene transcription in several ways.

In the relationship between PARP-1-related transcriptional regulation and gene polymorphisms, it has been reported that -228G/T at the transcriptional region in the MARCB1 gene significantly increased PARP-1 binding affinity and reporter activity; furthermore, this SNP altered the level of SMARCB1 mRNA and protein expression in human acute lymphoblastic leukemia cell lines.[71] It was also reported that PARP-1 bound to NACP-Rep1 in microsatellite repeats located in the SNCA gene, which exhibited different transcriptional activities resulting from the varying individual length of NACP-Rep1 sequences.[72, 73]

Previously, many reports have shown the PARP-1 binding sequence, but some of these results are controversial e.g., those for the murine iNOS gene (5'-AATTATAATTT-3'), the rat Reg gene (5'-TGCCCCTCCCAT-3'), the mouse Tcirg

gene (5'-TTCCCACAGC-3'), the human SNCA gene [5'-$(TC)_{10}(T)_2(TC)_{10}(TA)_8$ $(CA)_{11}$-3'], and the human SMARCB1 gene (5'-CTTTTGTTTGAGCTGCGGCGC GCGCGTC-3').[71, 73-75] These reports enabled to speculate that PARP-1 tends to bind to the CCCC sequence like GC-box, but it is difficult to forecast the appropriate PARP-1 binding region from DNA sequences, as with other transcription factors. In many cases in previous studies, the PARP-1 binding region has been revealed only after the DNA protein binding to a specific DNA region was identified as PARP-1 by mass spectrometry.[76, 77] Such is also the case in our study.

In conclusion, this chapter demonstrated that PARP-1 bound to -172G/T region. PARP-1 binding affinity and transcriptional activity to -172T sequence was higher than that of -172G. Next examinations showed the contribution to the MOR gene transcript of PARP-1 in the following chapter.

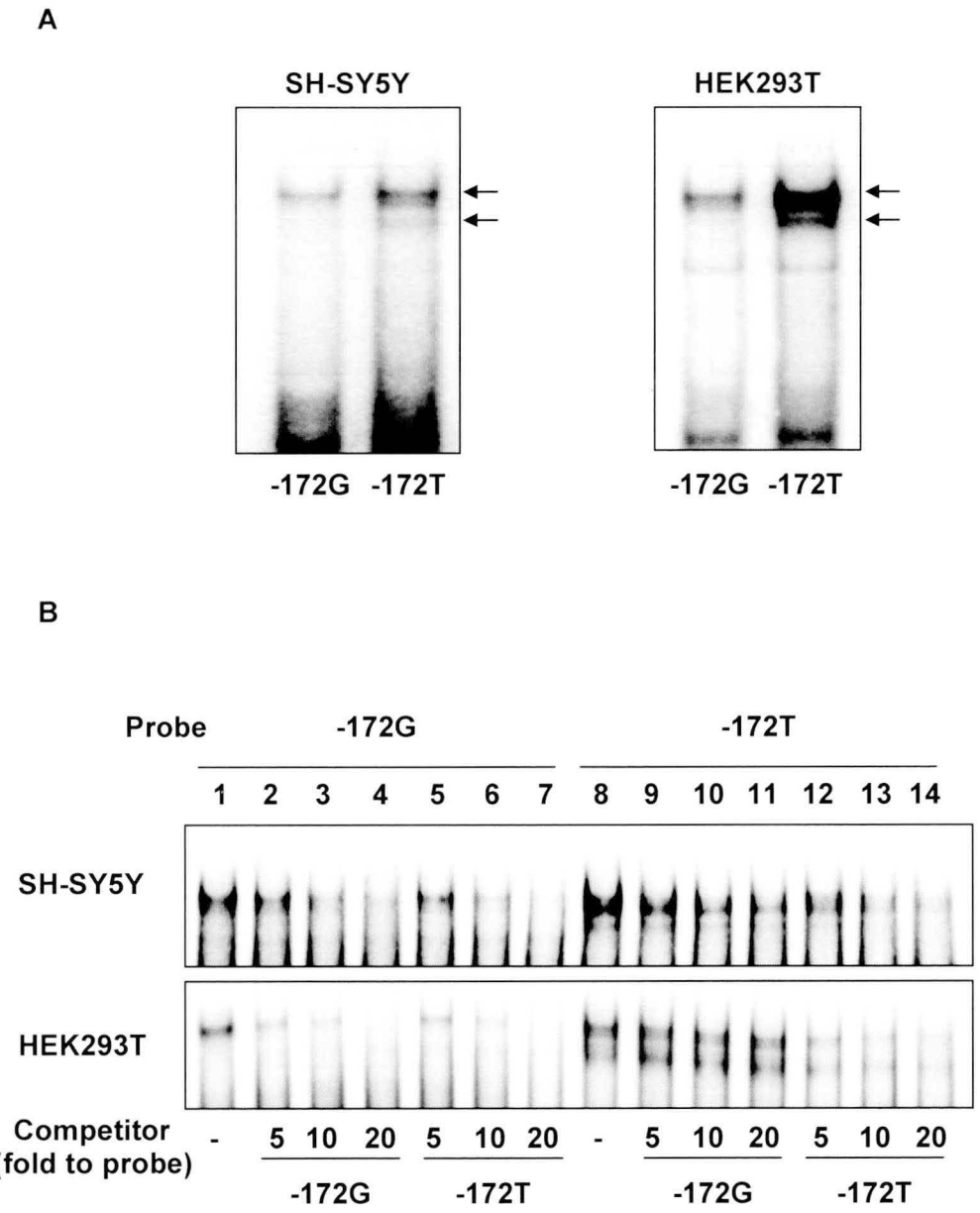

Figure 2. Specific nuclear protein binding to the -172G/T region in EMSA.

(A) Each probe was radiolabeled (-172G, -172T), and 1.0 μg of nuclear protein was obtained from SH-SY5Y or HEK293T. Arrows indicate specific protein binding to the -172G/T region. (B) Competition assay using nonlabeled probe for investigation of relative protein binding affinity to -172G or -172T. Lane 1 to 7, radiolabeled -172G probe; lane 8 to 14, radiolabeled -172T probe; lane 1 and 8, noncompetition; lanes 2 to 4 and 9 to 11, 5- to 20-fold molar excess of -172G; lanes 5 to 7 and 12 to 14, 5- to 20-fold molar excess of -172T.

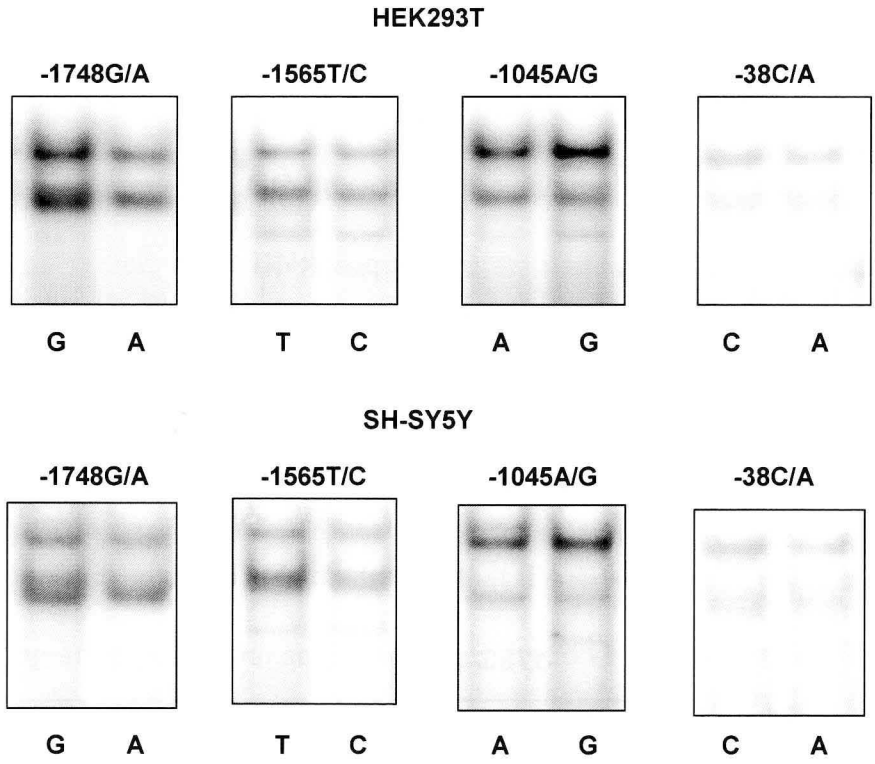

Fig. 3 Specific nuclear protein binding to each SNP region in EMSA

Radio-labeled each probe (-1748G or A, -1565T or C, -1045A or G, -38C or A) and 1.0 μg nuclear protein obtained from SH-SY5Y or HEK293T was used.

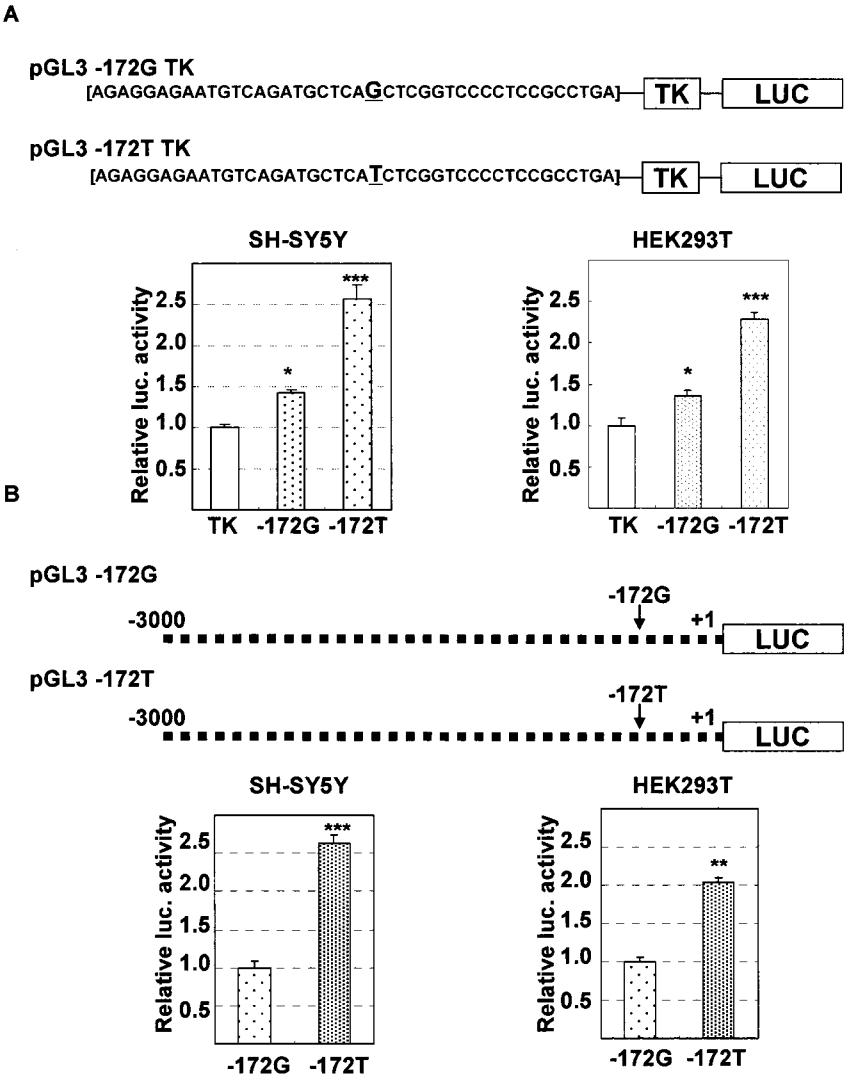

Figure 4. Transcriptional activity of -172G or -172T in MOR gene.

(A) pGL3-172G/T TK were constructed by insertion of 42 bp oligonucleotide containing -172G/T upstream of TK in the pGL3 TK vector. (B) pGL3-172T or pGL3-172G was constructed by insertion of respective MOR promoter gene, -3 kb upstream from transcriptional start site, to pGL3 basic vector.

Luciferase assay was performed 24 hours after each transfection of reporter vectors in HEK293T cells or SH-SY5Y cells. Relative luciferase activity of pGL3-172G or pGL3-172T was indicated against that of pGL3 TK (A), and that of pGL3-172T was expressed against that of pGL3-172G (B). Activities of coexpressed β-galactosidase were utilized for the correction of transfection activity in respective samples. Error bar indicates S.D. derived from three independent experiments. Asterisks indicate significantly different values (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$)

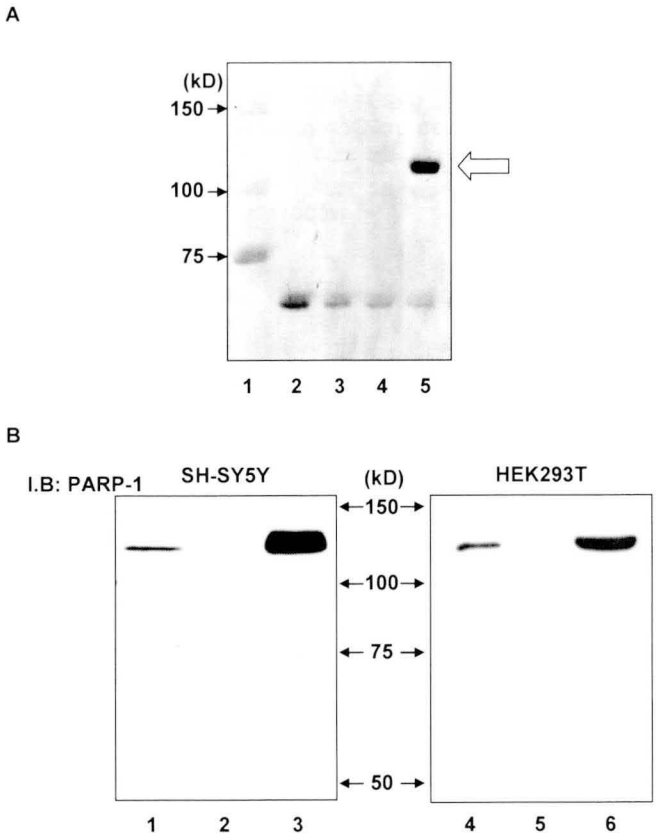

Figure 5. Purification of specific binding protein to the -172G/T region and confirmation of identified PARP-1 protein using specific antibody in Western blotting.

(A) Specific protein was purified using biotin-labeled -172T probe, avidin-agarose beads, and HEK293T-derived nuclear extract, and was applied to SDS-PAGE and stained with Coomassie brilliant blue. Avidin-agarose beads were pretreated by BSA in order to prevent nonspecific protein binding. Loaded sample was as follows: lane 1, protein molecular weight standards; lane 2, 1 μg of BSA; lane 3, purified protein with biotin-labeled -172T probe/without nuclear extracts; lane 4, purified protein with non-biotin-labeled -172T probe and HEK293T-derived nuclear extracts; lane 5, purified protein with biotin-labeled -172T probe and HEK293T-derived nuclear extracts. In lane 5 only, specific DNA binding protein was observed at 120 kDa, as indicated by the arrow. (B) Cell lysate or purified protein from SH-SY5Y or HEK293T was loaded to SDS-PAGE, and was investigated by immune-blotting using anti-PARP-1 antibody. Lane 1 to lane 3, nuclear protein was extracted from SH-SY5Y; lane 4 to 6; nuclear protein was extracted from HEK293T. Lane 1, 4: 1 μg of unpurified nuclear protein; lane 2, 5: purified protein with non-biotin labeled -172T probe; lane 3, 6: purified protein with biotin-labeled -172T probe.

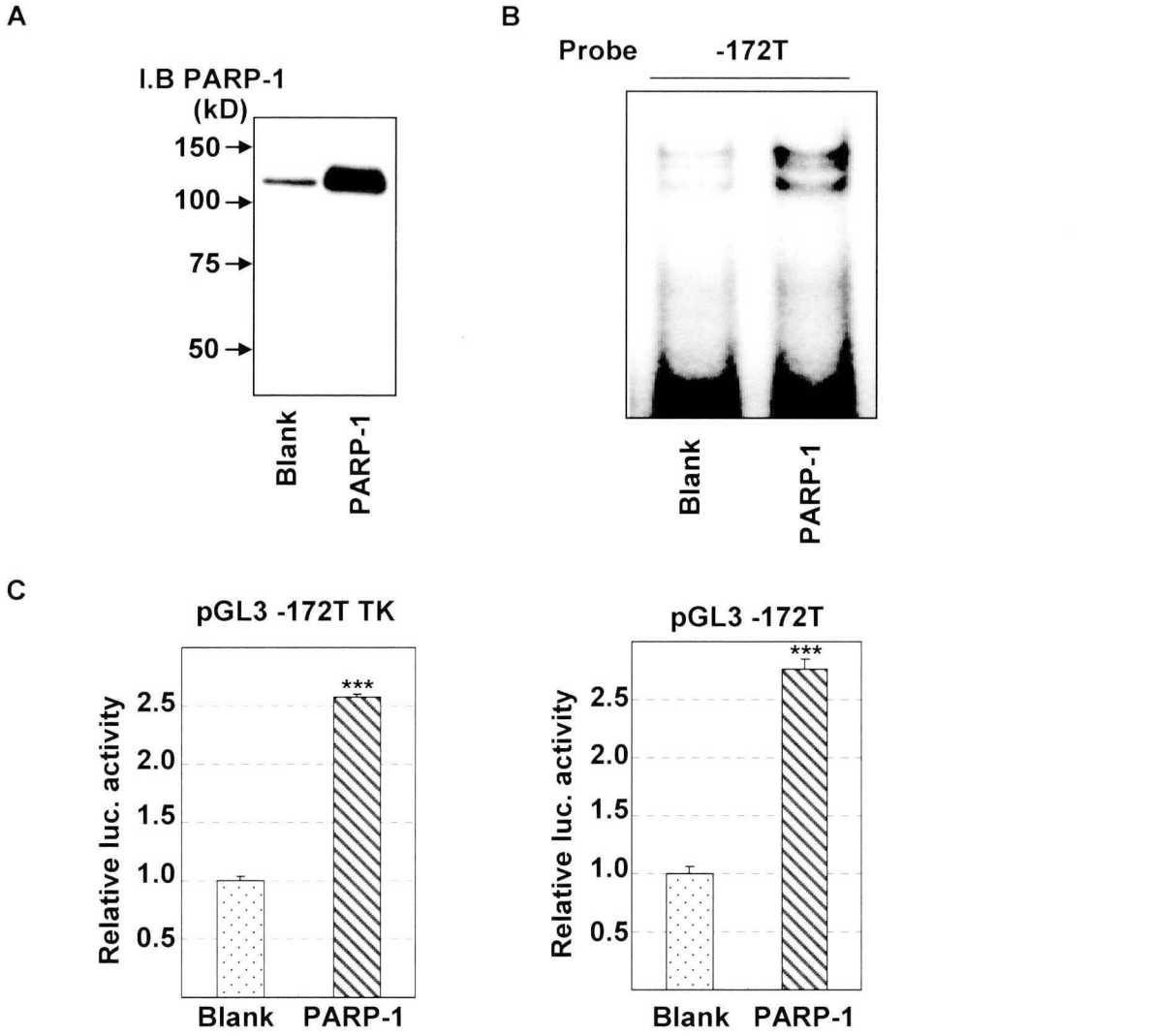

Figure 6. Enhancement of protein binding to the -172G/T region, and of transcription activity in MOR gene by PARP-1 overexpression.

(A) PARP-1 overexpression was shown in Western blotting. (B) PARP-1 binding to the -172T probe was investigated in EMSA. HEK 293T cells were transfected with the PARP-1 expression vector, and a nuclear extract was prepared for EMSA 48 h after the transfection. (C) Transcript activity in luciferase assay with pGL3-172T TK or pGL3-172T. HEK 293T cells were transfected with the PARP-1 expression vector and respective reporter vector, and cell lysate was prepared for luciferase assay 48 h after the transfection. The error bar indicates S.D. derived from three independent experiments. β-galactosidase activities, a control for transfection efficiency, were used to normalize the data. Asterisks indicate significantly different values (***, $p < 0.001$).

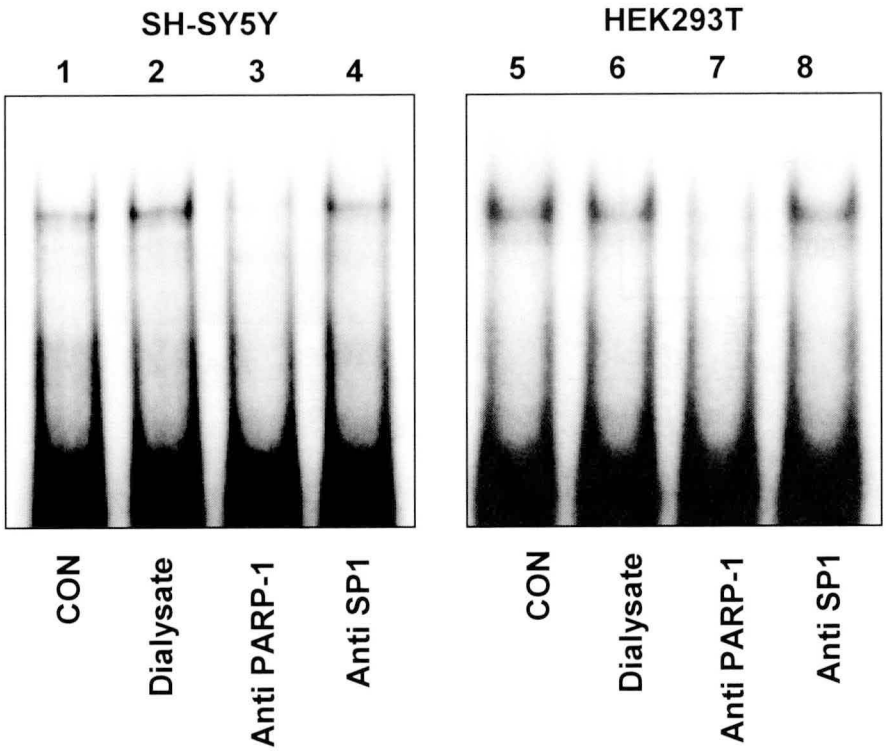

Figure 7. Super shift analysis with anti-PARP-1 antibody for reconfirmation of PARP-1 binding to the -172T probe.

Each sample, consisting of 1 μg of nuclear protein extracted from SH-SY5Y (lane 1 to 4) or HEK293T (lane 5 to 8), was loaded in EMSA. Anti-PARP-1 antibody was prepared after the concentration and the dialysis because the concentration of commercially available antibody was too low. Lane 1, 5: nuclear extract without any antibody or dialyzed buffer; lane 2, 6: nuclear extract with 2 μl of dialyzed buffer used for dialysis of antibody solution; lane 3, 7: nuclear extract with 2 μl of anti-PARP-1 antibody; lane 4, 8: nuclear extract with 2 μl of anti-SP1 antibody as a negative control.

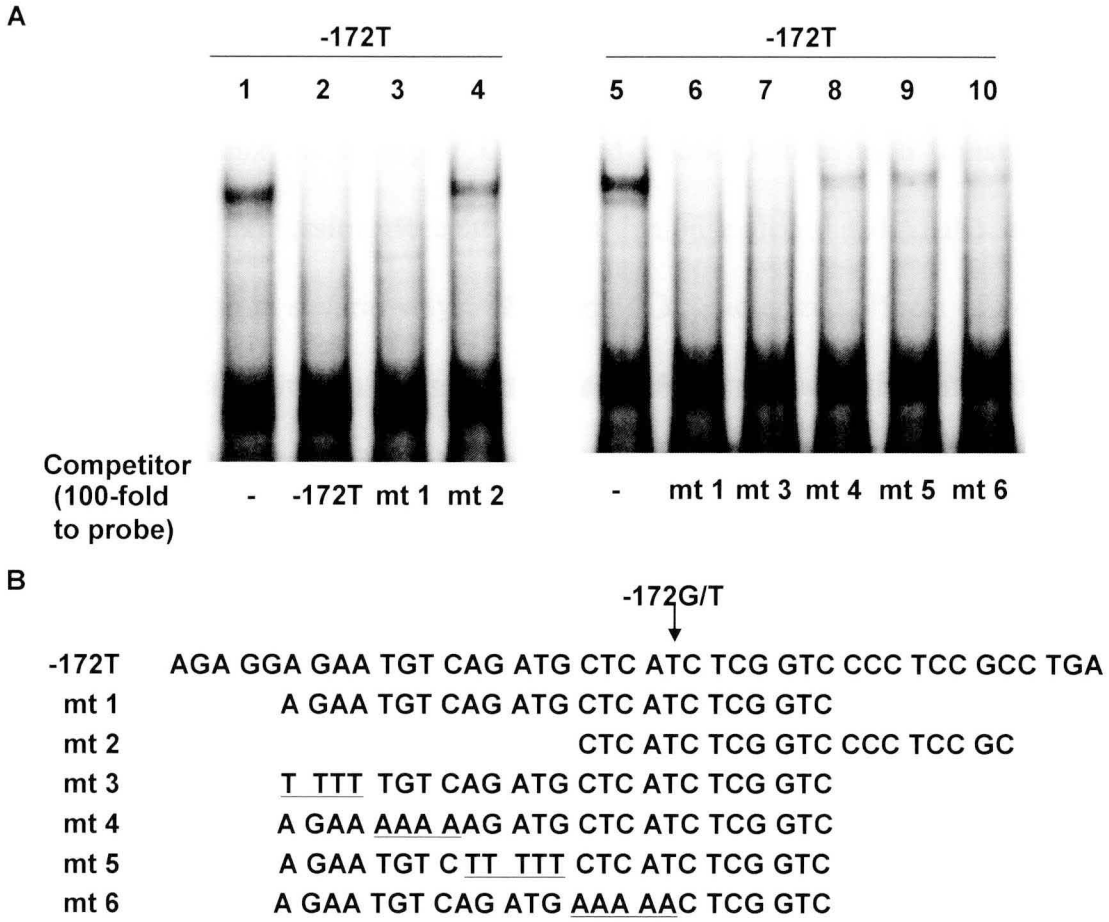

Figure 8. Analysis of PARP-1 binding sequence in the -172T probe by competition assay.

(A) Nuclear protein (2 μg/lane) extracted from HEK293T was applied to EMSA. Respective 100-fold nonlabeled oligo-DNAs were added for competition to labeled -172T probe. Lane 1, 5 was control sample without competitor, and in lane 2 to 4, 6 to 10 was with each competitor.
(B) Competitor sequences are described. Underlined bases indicate mutated sequence to clarify the PARP-1 binding region in -172T probe.

Chapter 3

Positive transcriptional regulation of the μ opioid receptor gene by poly (ADP-ribose) polymerase-1

Introduction

PARP-1 is an enzyme to repair DNA, and is also known as a transcription factor regulating gene expression. Recent studies demonstrated that PARP-1 contributed to gene transcription in several ways. The earliest characterized effects of PARP-1 on the genome were the modulation of chromatin structure and the ADP-ribosylation of histon.[70, 78-80] PARP-1 binding to nucleosomes in the absence of NAD^+ promotes the compaction of nucleosomal arrays and leads to repression of transcription. In the presence of saturating amounts of NAD^+, auto-modified PARP-1 releases from the chromatin, and leads to de-compaction of nucleosomal arrays and the restoration of transcription.[81, 82] Roles for PARP-1 as a coregulator (either a coactivator or a corepressor), such as NF-κB, HES1, B-Myb, Oct-1, HTLV Tax-1, SP1, NFAT, Elk1, and others have been reported.[78, 83-89] In some case, PARP-1 enzymatic activity is not required for its coregulatory activity (NF-κB, B-Myb, and HTLV Tax-1),[78, 83, 86] while in others it is required (HES1, SP1, NFAT, and ELK1).[84, 87-89] Furthermore, PARP-1 was shown to have other role, i.e., separate regions of PARP-1 interacted with AP-2α and independently regulated own transcriptional activation.[90] In direct effects of PARP-1 to the transcriptional regulation of target genes, PARP-1 binds to DNA sequence in the regulatory region of genes and functions as a classical enhancer or repressor-binding factor.[77, 91, 92] The mechanism of PARP-1 to regulate transcription as an enhancer or repressor-binding factor is unknown. Identification of genes regulated by direct effect of PARP-1 is expected to cause clarification to this mechanism.

In preceding chapter, PARP-1 was found to bind to -172G/T region in MOR gene, and its affinity was increased by base substitution of -172G/T. Continuously, this study examined that PARP-1 exerted on the MOR gene expression, and demonstrated that PARP-1 positively regulates to MOR gene expression.

Material and Methods

Cell culture

SH-SY5Y cells or HEK293T cells were cultivated following the method described in Chapter 2.

Reagent

Benzamide (BZD: WAKO Pure Chemical Industries, Ltd.), sodium benzoate (BZA: WAKO Pure Chemical Industries, Ltd.), and TNF-α (WAKO Pure Chemical Industries, Ltd.) were dissolved in DMSO (DMSO: WAKO Pure Chemical Industries, Ltd.). In all experiments, the same amount of DMSO was added to the control sample.

Transfection

Plasmid DNA was transfected to HEK293T or SH-SY5Y by using NP-OH or Fugene 6 (Roche Diagnostics) following described Chapter 2.

Luciferase assay

Reporter vector used pGL3-172G TK, pGL3-172T TK, pGL3-172G, or pGL3-172T. Vector construction and luciferase activity of reporter vectors were measured following the method described in Chapter 2.

EMSA

Purification of nuclear extract, oligonucleotides, or electrophoresis was following the method described in Chapter 2.

Western blotting

The method of the sample adjustment, electrophoresis, transfer, or the analysis following described in Chapter 2.

RT-PCR

Total RNA was prepared using Trizol (Invitrogen Co., Carlsbad, CA), and reverse transcription was performed in a final volume of 20 μL using 1 μg of RNA, 1 μL of oligo-dT_{15} primer (Promega), and 50 units of Rever Tra Ace (TOYOBO, LTD). The reaction conditions were as follows: 70°C for 10 minutes, 4°C for 5 minutes, 42°C for 60 minutes, and 99°C for 5 minutes.

PCR was carried out using an ABI PRISM 7000 system (Applied Biosystems, Carlsbad CA). Reactions were performed in a final volume of 25 μL using 5 μL of diluted cDNA, 12.5 pmol of each primer, and 0.625 units of Taq polymerase (Qiagen GmbH, Hilden, Germany). Amplification conditions were as follows: 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 minute. Primer sequences used are as follows:

MOR	Forward: 5'-TGCCACCAACATCTACATTTTCAACC-3'
	Reverse: 5'-GCCACCACCACCAGCACCATC-3'
GAPDH	Forward: 5'-GAACATCATCCCTGCCTCTACT-3'
	Reverse: 5'-CTTCCTCTTGTGCTCTTGCTG-3'

DRD2	Forward: 5'-ATCCCACCCAGCCACCACCAG-3'
	Reverse: 5'-CACGCCAAGCCCCACAAAGAG-3'
TNFR1A	Forward: 5'-TCAGTCCCGTGCCCAGTTCC-3'
	Reverse: 5'-TCTCGTGGTCGCTCAGCCCT-3'

Statistical Analysis

For statistical evaluation of the experiments, One-way or Two-way ANOVA was performed. Asterisks indicate significantly different values (*, $p < 0.05$; **, $p < 0.01$; ***, $p< 0.001$)

Results

Suppression of the binding to 172G/T region and of MOR gene expression by PARP-1 inhibitor

BZD, an inhibitor of PARP-1, was used for further confirmation of PARP-1 binding to the -172G/T region. Although neither the protein nor the mRNA expression of PARP-1 was affected by BZD (Fig. 9A, B), the quantity of PARP-1 binding in the -172T probe dose-dependently decreased 24 hours after the addition of BZD in SH-SY5Y cells (Fig. 9C). SH-SY5Y cells are known to express MOR constitutively. MOR mRNA expression detected by RT-PCR was suppressed by BZD in a dose-dependent manner. However, BZA, as a negative control for BZD, did not influence the tested mRNA expressions, including MOR (Fig. 9A). Increase in the dopamine D_2 receptor gene by BZD indicated that the inhibition of MOR mRNA expression by BZD was not merely caused by nonspecific toxic effects.

TNF-α increased PARP-1 binding to -172G/T region and MOR promoter activity

Next, this study searched for factors that increase PARP-1-binding to the -172G/T probe. As a result, it was found that TNF-α increased the binding of PARP-1 1 to 6 hour after the addition of TNF-α in SH-SY5Y (Fig. 10A). Moreover, the signals of PARP-1 binding to the -172T probe were higher than those of -172G probe with or without stimulation by TNF-α (Fig. 10A). Corresponding to the results of PARP-1 binding to the -172G/T probe, TNF-α preferentially increased transcriptional activity of pGL3-172T (3.0-fold) more than that of pGL3-172G (1.7-fold) (Fig. 10B).

Suppression of TNF-α-induced enhancement of the PARP-1 binding to 172G/T region and of MOR gene expression by PARP-1 inhibitor

Increase in MOR mRNA expression was observed at 1 hour or 6 hours after TNF-α stimulation in SH-SY5Y cells (Fig. 11A). During the increase in PARP-1 binding to -172G/T by TNF-α (Fig. 10A), the total amount of the PARP-1 protein in SH-SY5Y did not change (Fig. 11B). Moreover, both increase in PARP-1 binding to -172T and MOR expression induced by TNF-α stimulation were inhibited by pretreatment with BZD (Figs. 11C, D). These results indicate that PARP-1 also contributed to TNF-α-induced upregulation of the MOR gene.

Discussion

The preceding chapter demonstrated that PARP-1 bound to the neighborhood of -172G/T. In this chapter, the contribution of PARP-1 to the MOR gene expression and the possibility of the base substitution of -172G/T to increase MOR gene expression were shown.

In a report concerning the mouse MOR gene, PARP-1 bound to the poly (C) sequences (5'-CTTCTGCTCCCCCCCCCCCTACCC-3'; -430 to -407 bp) and repressed MOR gene expression in mouse neuroblastoma NS20Y cells.[93] In contrast to that report, my results show that PARP-1 increases human MOR gene transcriptional activity (Fig. 6C), and BZD, an inhibitor of PARP-1, decreases MOR gene expression in human neuroblastoma SH-SY5Y cells (Fig. 9A). When an unknown protein binding to the -172T probe was revealed as PARP-1, it was expected that PARP-1 bound to the CCCC sequence of these probes located 162-165 bp upstream from the translation start site of the human MOR gene. However, results in Fig. 8 clearly show that PARP-1 binds to TGTCAGATG within the -172T probe [5'-AGAGGAGAATGTCAGATGCT -CA (G/T) CTCGGCCCCTCCGCCTGA-3'; -194 to -153 bp] but not to CCCC. Thus, human MOR gene transcription regulated by PARP-1 might be in different mechanism from that of the mouse because the human MOR regulatory region including the -172G/T probe did not have a region similar to the poly (C) sequences in the mouse MOR gene.

This study explored the inducing-factors for MOR expression, as well as those for PARP-1-binding to the -172G/T region, and it was found that TNF-α increased MOR

transcription and PARP-1 binding to that region. TNF-α was reported as the inducer of MOR transcription in human immune effector cells through NF-κB.[48] In this study, increasing of MOR gene expression by TNF-α (Fig. 11A) was suppressed by BZD in SH-SY5Y cells (Fig. 11D). These results suggest that TNF-α induces MOR transcription in SH-SY5Y cells not only through NF-κB but also through PARP-1. Both increasing of PARP-1 binding by TNF-α and decreasing of PARP-1 binding by BZD did not involve changing the amount of PARP-1 protein level. Furthermore, anti-PARP-1 antibody that recognized the PARP-1 C terminus containing the catalytic domain completely diminished bands of PARP-1 binding to the -172G/T region in EMSA, but did not shift it upward. Considering that BZD inhibits PARP-1 catalytic activity, and that anti-PARP-1 antibody recognizes the PARP-1 C terminus containing the catalytic domain, catalytic activity of PARP-1 might be crucial for its own binding to the -172G/T region, as well as for MOR gene transcription.

In this study, TNF-α remarkably increased PARP-1 binding to -172T probe, but presently the details of mechanism for this binding was not known, which might result from the post-translational modification of PARP-1 through auto-PARylation, acetylation, or phosphorylation.[94-97] PARP-1 dependent transcription was reported to be regulated by cellular signaling pathways. Actually, direct phosphorylation of PARP-1 by ERK1/2 enhanced PARP-1 catalytic activity, but the effect of that on transcriptional activity was obscure.[96] Although TNF-α-induced signaling to PARP-1 was indistinct, the effects on cytotoxicity by TNF-α were reported to be mediated by ADP-ribosylaton in murine fibroblast cell line L929 cells.[98] Moreover, previous studies reported that DNA binding activity of PARP-1 was altered due to its catalytic

activity by NAD^+ in *in vitro* experiments.[93] Therefore, the regulation of nuclear NAD^+ synthesis might be critical for PARP-1 dependent gene regulation including MOR expression.

In summary, this study demonstrated that PARP-1 binds to the -172G/T region and up-regulates MOR gene transcription. PARP-1 binding affinity to -172T sequence was higher than that of -172G, and the transcriptional activity with or without TNF-α was also enhanced by -172T substitution, which was speculated to relate to own catalytic activity. In conclusion, PARP-1 positively regulates MOR gene transcription via -172G/T, which possibly influences individual specificity in therapeutic opioid effect, furthermore, which might provide useful information for inflammatory pain therapy.

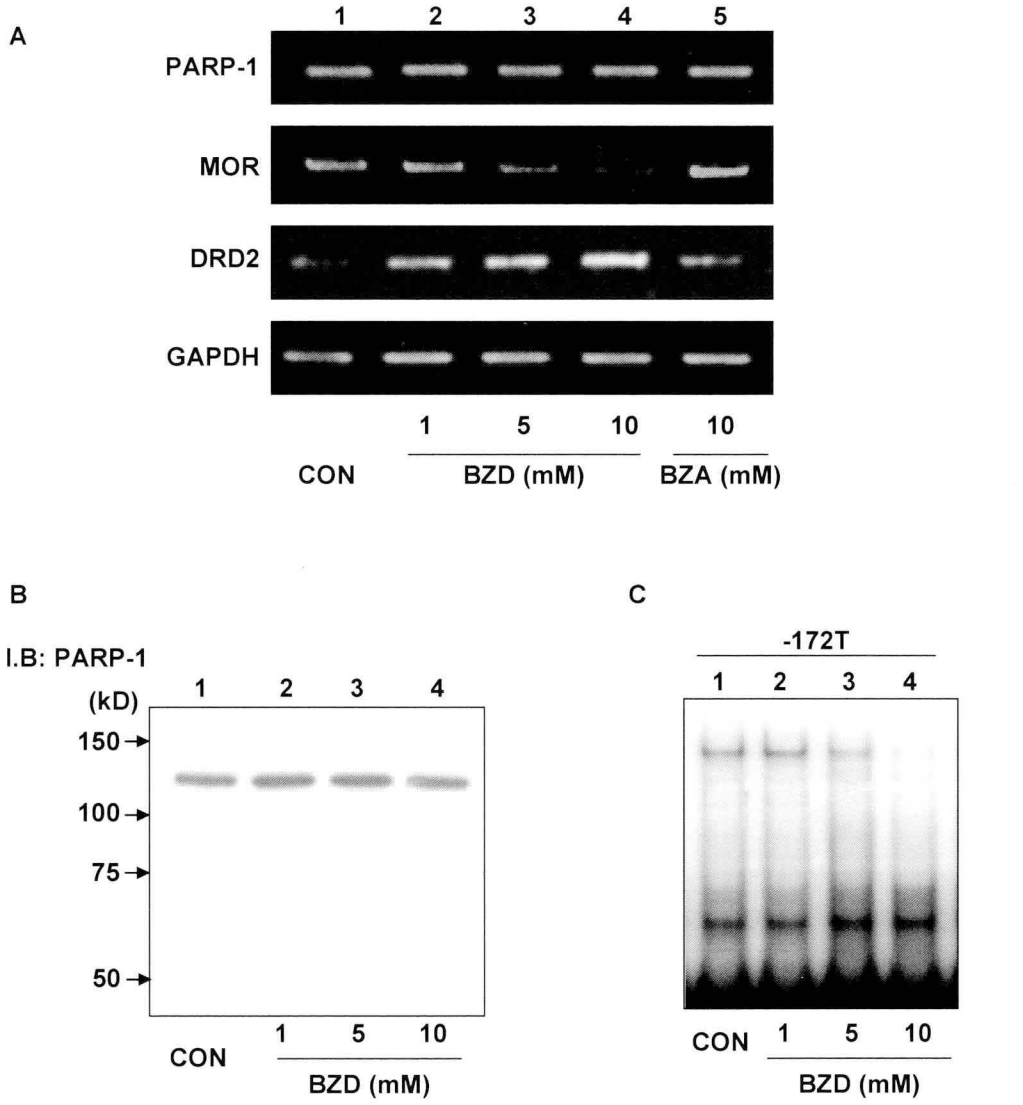

Figure 9. The inhibition of MOR mRNA and PARP-1 binding expression by the treatment of PARP-1 inhibitor in SH-SY5Ycell.

(A) Total RNA in SH-SY5Y was extracted 24 hours after the addition of DMSO, BZD, or BZA. Then, MOR mRNA expression was investigated by RT-PCR. Lane 1: DMSO as a vehicle control; lane 2 to 4: BZD (1, 5, and 10 mM); lane 6: BZA as a negative control of BZD. PCR cycles of each gene were as follows: MOR: 26, PARP-1: 25, DRD2: 28, GAPDH: 20.
(B) PARP-1 protein expression detected by anti-PARP-1 antibody.
(C) BZD inhibited PARP-1 binding to the -172T probe. Nuclear protein in BZD-treated SH-SY5T cells was extracted 24 hours after addition of DMSO (vehicle control) or various concentrations of BZD. Lane 1: nuclear protein treated with DMSO as a vehicle control; lane 2 to 4: nuclear protein treated with BZD (1, 5, and 10 mM).

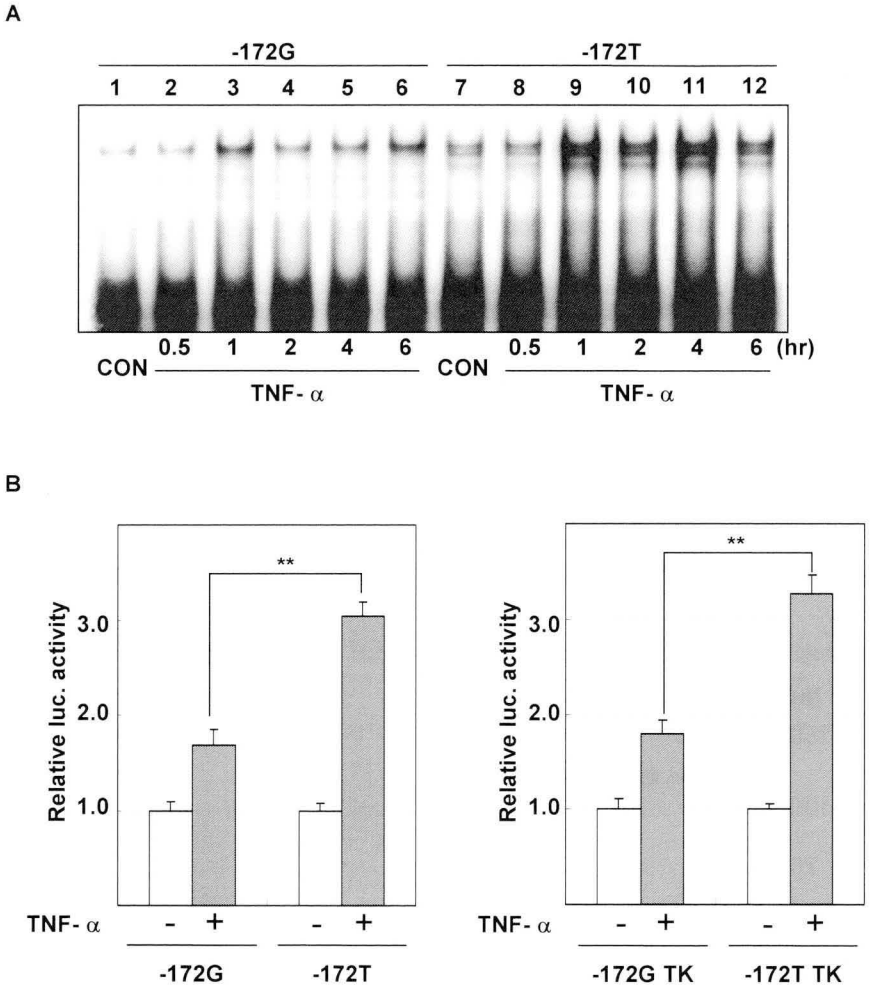

Figure 10. TNF-α-induced enhancement of PARP-1 binding to the -172G/T region and transcription activity of MOR gene in SH-SY5Y cells

Nuclear protein or cell lysate in SH-SY5Y was prepared at respective times after stimulation with TNF-α (10 ng/ml). (A) PARP-1 binding to the -172G or the -172T probe after the stimulation of TNF-α was investigated by EMSA. (B) SH-SY5Y cells transfected with pGL3-172G/T or with pGL3-172G/T TK were stimulated by TNF-α and were prepared 12 hours after TNF-α stimulation for luciferase assay. Luciferase activity in -172T was represented as relative value against that in -172G. Error bar indicates S.D. derived from three independent experiments. β-galactosidase activities, as controls for transfection efficiency, were used to normalize the data. Asterisks indicate significantly different values (**, $p < 0.01$).

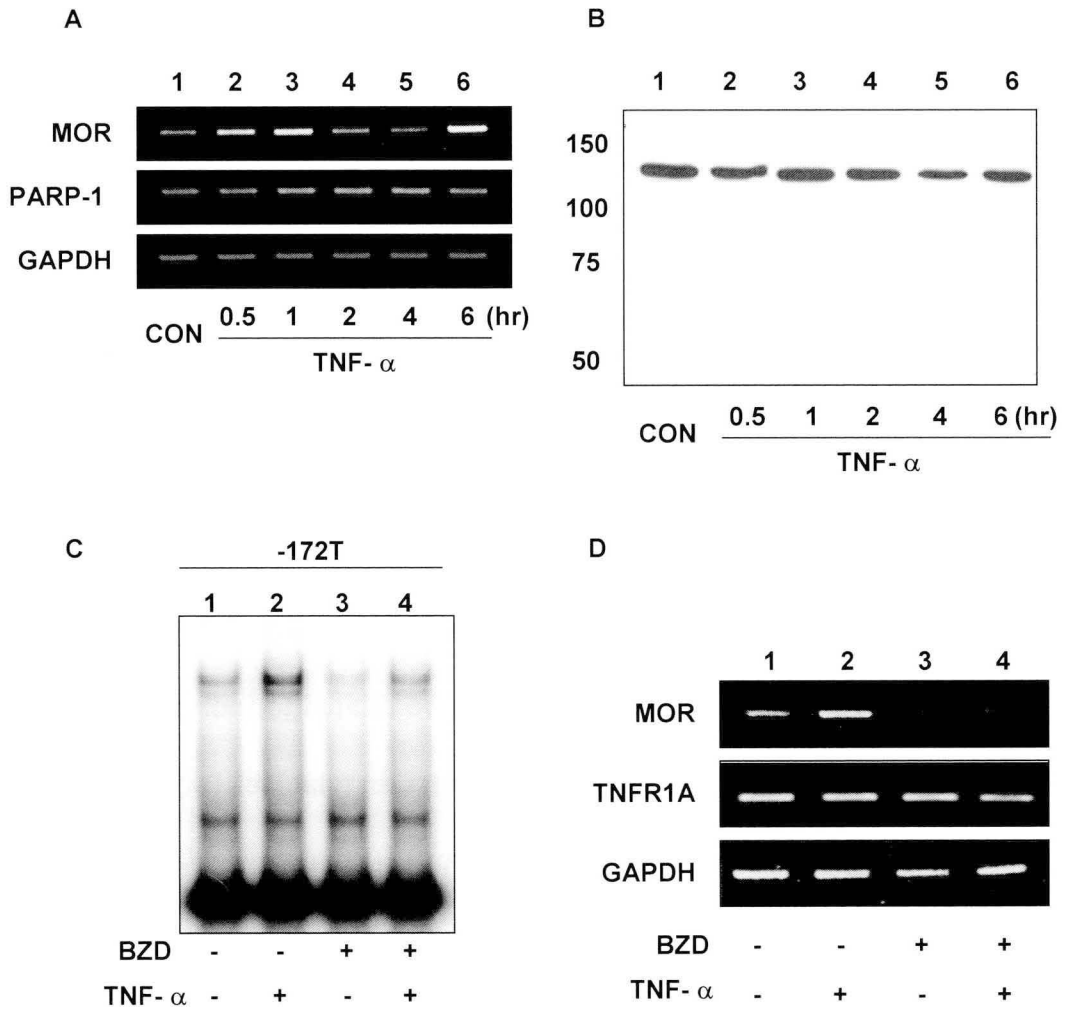

Figure 11. Inhibition of TNF-α-induced PARP-1 binding to the -172T probe, and of MOR mRNA expression, by PARP-1 inhibitor, BZD, in SH-SY5Y cells.

Total RNA, nuclear protein, or cell lysate in SH-SY5Y was prepared at respective times after the stimulation with TNF-α. (A) MOR or PARP-1 gene expression was investigated by RT-PCR. (B) PARP-1 protein expression detected by anti-PARP-1 antibody. BZD was pretreated in SH-SY5Y 24 hours before TNF-α stimulation. (C) Influence of BZD to the TNF-α enhanced PARP-1 binding to the -172T probe was investigated by EMSA. (D) Influence of BZD to TNF-α enhanced MOR mRNA expression, or to TNFR1A gene expression, was monitored by RT-PCR. Lane 1: DMSO as a vehicle control; lane 2: 10 ng/ml of TNF-α; lane 3: 10 mM of BZD; and lane 4: 10 mM of BZD and 10 ng/ml of TNF-α. PCR cycles of each gene were as follows: MOR: 26; PARP-1: 25; TNFR1A: 31; GAPDH: 20.

General conclusion

In conclusion, this study found novel linkage between -1748G/A and -172G/T in analysis of Japanese MOR gene. Furthermore, PARP-1 bound to the neighborhood of -172G/T increased the expression level of the MOR gene and was suggested to have a possibility to relate to MOR gene transcript regulation by the inflammatory cytokine such as TNF-α. It is expected that MOR gene expression is increased by -172G/T, because affinity to PARP-1 binding and transcription activity of -172T were higher than those of -172G regardless of the stimulation of TNF-α.

This study showed that -172G/T could possibly be related to individual specificity of opioid effects by increasing MOR gene transcription via PARP-1, further, it could provide useful information for medical practice.

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References

1) Lord J.A., Waterfield A.A., Hughes J., Kosterlitz H.W., Endogenous opioid peptides: multiple agonists and receptors, *Nature,* **267,** 495-9 (1977).

2) Martin W.R., Eades C.G., Thompson J.A., Huppler R.E., Gilbert P.E., The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog, *J Pharmacol Exp Ther,* **197**, 517-32 (1976).

3) Wolozin B.L., Pasternak G.W., Classification of multiple morphine and enkephalin binding sites in the central nervous system, *Proc Natl Acad Sc. USA,* **78,** 6181-5 (1981).

4) Su T.P., Further demonstration of kappa opioid binding sites in the brain: evidence for heterogeneity, *J Pharmacol Exp Ther,* **232,** 144-8 (1985).

5) Stefano G.B., Melchiorri P., Negri L., Hughes T.K. Jr., Scharrer B., [D-Ala2]deltorphin I binding and pharmacological evidence for a special subtype of delta opioid receptor on human and invertebrate immune cells, *Proc Natl Acad Sci USA,* **89,** 9316-20 (1992).

6) Clark J.A., Liu L., Price M., Hersh B., Edelson M., Pasternak G.W., Kappa opiate receptor multiplicity: evidence for two U50,488-sensitive kappa 1 subtypes and a novel kappa 3 subtype, *J Pharmacol Exp Ther,* **251,** 461-8 (1989)..

7) Rothman R.B., Bykov V., de Costa B.R., Jacobson A.E., Rice K.C., Brady L.S., Interaction of endogenous opioid peptides and other drugs with four kappa opioid binding sites in guinea pig brain, *Peptides,* **11,** 311-31 (1990).

8) Wang J.B., Imai Y., Eppler C.M., Gregor P., Spivak C.E., Uhl G.R., mu opiate receptor: cDNA cloning and expression, *Proc Natl Acad Sci USA,* **90,** 10230-4 (1993).

9) Chen Y., Mestek A., Liu J., Hurley J.A., Yu L., Molecular cloning and functional expression of a mu-opioid receptor from rat brain, *Mol Pharmacol,* **44,** 8-12 (1993).

10) Fukuda K., Kato S., Mori K., Nishi M., Takeshima H., Primary structures and expression from cDNAs of rat opioid receptor delta- and mu-subtypes, *FEBS Lett,* **327,** 311-4 (1993).

11) Evans C.J., Keith D.E., Jr., Morrison H., Magendzo K., Edwards R.H., Cloning of a delta opioid receptor by functional expression, *Science,* **258,** 1952-5 (1992).

12) Kieffer B.L., Befort K., Gaveriaux-Ruff C., Hirth C.G., The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization, *Proc Natl Acad Sci USA,* **89,** 12048-52 (1992).

13) Yasuda K., Raynor K., Kong H., Breder C.D., Takeda J., Reisine T., Bell G.I., Cloning and functional comparison of kappa and delta opioid receptors from mouse brain, *Proc Natl Acad Sci USA,* **90,** 6736-40 (1993).

14) Minami M., Toya T., Katao Y., Maekawa K., Nakamura S., Onogi T., Kaneko S.,Satoh M., Cloning and expression of a cDNA for the rat kappa-opioid receptor, *FEBS Lett,* **329,** 291-5 (1993).

15) Nishi M., Takeshima H., Fukuda K., Kato S., Mori K., cDNA cloning and pharmacological characterization of an opioid receptor with high affinities for kappa-subtype-selective ligands, *FEBS Lett,* **330,** 77-80 (1993).

16) Koski G., Klee W.A., Opiates inhibit adenylate cyclase by stimulating GTP hydrolysis, *Proc Natl Acad Sci USA,* **78,** 4185-9 (1981).

17) Koski G., Streaty R.A., Klee W.A., Modulation of sodium-sensitive GTPase by partial opiate agonists. An explanation for the dual requirement for Na+ and GTP in inhibitory regulation of adenylate cyclase, *J Biol Chem,* **257,** 14035-40 (1982).

18) Blume A.J., Interaction of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides, *Proc Natl Acad Sci USA,* **75,** 1713-7 (1978).

19) Frances B., Moisand C., Meunier J.C., Na+ ions and Gpp(NH)p selectively inhibit agonist interactions at mu- and kappa-opioid receptor sites in rabbit and guinea-pig cerebellum membranes, *Eur J Pharmacol,* **117,** 223-32 (1985).

20) Demoliou-Mason C.D., Barnard E.A., Distinct subtypes of the opioid receptor with allosteric interactions in brain membranes, *J Neurochem,* **46,** 1118-28 (1986).

21) Makman M.H., Dvorkin B., Crain S.M., Modulation of adenylate cyclase activity of mouse spinal cord-ganglion explants by opioids, serotonin and pertussis toxin, *Brain Res,* **445,** 303-13 (1988).

22) Miyake M., Christie M.J., North R.A., Single potassium channels opened by opioids in rat locus ceruleus neurons, *Proc Natl Acad Sci USA,* **86,** 3419-22 (1989).

23) Johnson P.S., Wang J.B., Wang W.F., Uhl G.R., Expressed mu opiate receptor couples to adenylate cyclase and phosphatidyl inositol turnover, *Neuroreport,* **5,** 507-9 (1994).

24) Suzuki T., Kishimoto Y., Misawa M., Formalin- and carrageenan-induced inflammation attenuates place preferences produced by morphine, methamphetamine and cocaine, *Life Sci,* **59,** 1667-74 (1996)

25) Funada M., Suzuki T., Narita M., Misawa M., Nagase H., Blockade of morphine reward through the activation of kappa-opioid receptors in mice, *Neuropharmacology,* **32,** 1315-23 (1993).

26) Suzuki T., Narita M., Takahashi Y., Misawa M., Nagase H., Effects of nor-binaltorphimine on the development of analgesic tolerance to and physical dependence on morphine, *Eur J Pharmacol,* **213,** 91-7 (1992).

27) Green P.G., Lee N.M., Dynorphin A- (1-13) attenuates withdrawal in morphine-dependent rats: effect of route of administration, *Eur J Pharmacol,* **145,** 267-72 (1988).

28) Koch T., Kroslak T., Mayer P., Raulf E., Hollt V., Site mutation in the rat mu-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein kinase II in agonist-mediated desensitization, *J Neurochem,* **69,** 1767-70 (1997).

29) Koch T., Schulz S., Schroder H., Wolf R., Raulf E., Hollt V., Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization, *J Biol Chem,* **273,** 13652-7 (1998).

30) McDonald B.J., Moss S.J., Differential phosphorylation of intracellular domains of gamma-aminobutyric acid type A receptor subunits by calcium/calmodulin type 2-dependent protein kinase and cGMP-dependent protein kinase, *J Biol Chem,* **269,** 18111-7 (1994).

31) Mestek A., Hurley J.H., Bye L.S., Campbell A.D., Chen Y., Tian M., Liu J., Schulman H., Yu L., The human mu opioid receptor: modulation of functional desensitization by calcium/calmodulin-dependent protein kinase and protein kinase C, *J Neurosci,* **15,** 2396-406 (1995).

32) Poonkuzhali B., Lamba J., Strom S., Sparreboom A., Thummel K., Watkins P., Schuetz E., Association of breast cancer resistance protein/ABCG2 phenotypes and novel promoter and intron 1 single nucleotide polymorphisms, *Drug Metab Dispos,* **36,** 780-95 (2008)

33) Zhang Y., Bertolino A., Fazio L., Blasi G., Rampino A., Romano R., Lee M.L., Xiao T., Papp A., Wang D., Sadée W., Polymorphisms in human dopamine D2 receptor gene affect gene expression, splicing, and neuronal activity during working memory, *Proc Natl Acad Sci USA,* **104**, 20552-7 (2007).

34) Assimes T.L., Knowles J.W., Priest J.R., Basu A., Volcik K.A., Southwick A., Tabor H.K., Hartiala J., Allayee H., Grove M.L., Tabibiazar R., Sidney S., Fortmann S.P., Go A., Hlatky M., Iribarren C., Boerwinkle E., Myers R., Risch N., Quertermous T., Common polymorphisms of ALOX5 and ALOX5AP and risk of coronary artery disease, *Hum Genet,* **123,** 399-408 (2008)

35) Al-Shemari H., Bossé Y., Hudson T.J., Cabaluna M., Duval M., Lemire M., Vallee-Smedja S., Frenkiel S., Desrosiers M. Influence of leukotriene gene polymorphisms on chronic rhinosinusitis, *BMC Med Genet,* **26,** 9:21 (2008).

36) Arthur A.T., Armati P.J., Bye C., Southern M.S., Genetics Consortium, Heard R.N., Stewart G.J., Pollard J.D., Booth D.R, Genes implicated in multiple sclerosis pathogenesis from consilience of genotyping and expression profiles in relapse and remission, *BMC Med Genet,* **19**, 9-17 (2008).

37) Hirota T., Ieiri I., Takane H., Sano H., Kawamoto K., Aono H., Yamasaki A., Takeuchi H., Masada M., Shimizu E., Higuchi S., Otsubo K., Sequence variability and candidate gene analysis in two cancer patients with complex clinical outcomes during morphine therapy, *Drug Metab Dispos,* **31,** 677-80 (2003).

38) Klepstad P., Rakvag T.T., Kaasa S., Holthe M., Dale O., Borchgrevink P.C., Baar C., Vikan T., Krokan H.E., Skorpen F., The 118 A > G polymorphism in the human mu-opioid receptor gene may increase morphine requirements in patients with pain caused by malignant disease, *Acta Anaesthesiol Scand,* **48,** 1232-9 (2004).

39) Lotsch J., Zimmermann M., Darimont J., Marx C., Dudziak R., Skarke C., Geisslinger G., Does the A118G polymorphism at the mu-opioid receptor gene protect against morphine-6-glucuronide toxicity?, *Anesthesiology,* **97,** 814-9 (2002).

40) Lotsch J., Skarke C., Grosch S., Darimont J., Schmidt H., Geisslinger G., The polymorphism A118G of the human mu-opioid receptor gene decreases the pupil constrictory effect of morphine-6-glucuronide but not that of morphine, *Pharmacogenetics,* **12,** 3-9 (2002).

41) Romberg R., Olofsen E., Sarton E., den Hartigh J., Taschner P.E., Dahan A., Pharmacokinetic-pharmacodynamic modeling of morphine-6-glucuronide-induced analgesia in healthy volunteers: absence of sex differences, *Anesthesiology,* **100,** 120-33 (2004).

42) Skarke C., Darimont J., Schmidt H., Geisslinger G., Lotsch J., Analgesic effects of morphine and morphine-6-glucuronide in a transcutaneous electrical pain model in healthy volunteers, *Clin Pharmacol Ther,* **73,** 107-21 (2003).

43) Bond C., LaForge K.S., Tian M., Melia D., Zhang S., Borg L., Gong J., Schluger J., Strong J.A., Leal S.M., Tischfield J.A., Kreek M.J., Yu L., Single-nucleotide polymorphism in the human mu opioid receptor gene alters beta-endorphin binding and activity: possible implications for opiate addiction, *Proc Natl Acad Sci USA,* **95,** 9608-13 (1998).

44) Lötsch J., Skarke C., Grösch S., Darimont J., Schmidt H., Geisslinger G., The polymorphism A118G of the human mu-opioid receptor gene decreases the pupil constrictory effect of morphine-6-glucuronide but not that of morphine, *Pharmacogenetics,* **12,** 3-9 (2002).

45) Wang D., Quillan JM., Winans K., Lucas JL., Sadee W., Single nucleotide polymorphisms in the human mu opioid receptor gene alter basal G protein coupling and calmodulin binding, *J Biol Chem,* **276,** 34624-30 (2001).

46) Koch T., Kroslak T., Averbeck M., Mayer P., Schroder H., Raulf E., Hollt V., Allelic variation S268P of the human mu-opioid receptor affects both desensitization and G protein coupling, *Mol Pharmaco,.* **58,** 328-34 (2000).

47) Kraus J., Borner C., Giannini E., Hickfang K., Braun H., Mayer P., Hoehe M.R., Ambrosch A., Konig W., Hollt V., Regulation of mu-opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site, *J Biol Chem,* **276,** 43901-8 (2001).

48) Kraus J., Borner C., Giannini E., Hollt V., The role of nuclear factor kappa B in tumor necrosis factor-regulated transcription of the human mu-opioid receptor gene, *Mol Pharmacol,* **64,** 876-84 (2003).

49) Wang J.B., Johnson P.S., Persico A.M., Hawkins A.L., Griffin C.A., Uhl G.R., Human mu opiate receptor; cDNA and genomic clones, pharmacologic characterization and chromosomal assignment, *FEBS Lett,* **338,** 217-22 (1994).

50) Hoehe M.R., Köpke K., Wendel B., Rohde K., Flachmeier C., Kidd K.K., Berrettini W.H., Church G.M., Sequence variability and candidate gene analysis in complex disease: association of mu opioid receptor gene variation with substance dependence, *Hum Mol Genet,* **9,** 2895-908 (2000).

51) Lotsch J., Geisslinger G., Are mu-opioid receptor polymorphisms important for clinical opioid therapy? *Trends Mol Med,* **11,** 82-9 (2005).

52) Bergen A.W., Kokoszka J., Peterson R., Long J.C., Virkkunen M., Linnoila M., Goldman D., Mu opioid receptor gene variants: lack of association with alcohol dependence, *Mol Psychiatry,* **2,** 490-4 (1997).

53) Shibata K., Ito T., Kitamura Y., Iwasaki N., Tanaka H., Kamatani N., Simultaneous estimation of haplotype frequencies and quantitative trait parameters: applications to the test of association between phenotype and diplotype configuration, *Genetics,* **168,** 525-39 (2004).

54) Ito T., Inoue E., Kamatani N., Association test algorithm between a qualitative phenotype and a haplotype or haplotype set using simultaneous estimation of haplotype frequencies, diplotype configurations and diplotype-based penetrances, *Genetics,* **168,** 2339-48 (2004).

55) Ide S., Kobayashi H., Ujike H., Ozaki N., Sekine Y., Inada T., Harano M., Komiyama T., Yamada M., Iyo M., Iwata N., Tanaka K., Shen H., Iwahashi K., Itokawa M., Minami M., Satoh M., Ikeda K., Sora I., Linkage disequilibrium and association with methamphetamine dependence/psychosis of μ-opioid receptor gene polymorphisms, *Pharmacogenomics J,* **6,** 179-88 (2006).

56) Shi J., Hui L., Xu Y., Wang F., Huang W., Hu G., Sequence variations in the mu-opioid receptor gene (OPRM1) associated with human addiction to heroin, *Hum Mutat,* **19,** 459-60 (2002).

57) Crowley J.J., Oslin D.W., Patkar A.A., Gottheil E., DeMaria P.A. Jr, O'Brien C.P., Berrettini W.H., Grice D.E., A genetic association study of the mu opioid receptor and severe opioid dependence, *Psychiatr Genet,* **13,** 169-73 (2003).

58) Tan E.C., Tan C.H., Karupathivan U., Yap E.P., Mu opioid receptor gene polymorphisms and heroin dependence in Asian populations, *Neuroreport,* **14,** 569-72 (2003).

59) Szeto C.Y., Tang N.L., Lee D.T., Stadlin A., Association between mu opioid receptor gene polymorphisms and Chinese heroin addicts, *Neuroreport,* **12,** 1103-6 (2001).

60) Xu Y., Carr L.G., Functional characterization of the promoter region of the human mu opioid receptor (hMOR) gene: identification of activating and inhibitory regions, *Cell Mol Biol (Noisy-le-grand),* **47,** 29-38 (2001).

61) Xu Y., Carr L.G., Transcriptional regulation of the human mu opioid receptor (hMOR) gene: evidence of positive and negative cis-acting elements in the proximal promoterand presence of a distal promoter, *DNA Cell Biol,* **20,** 391-402 (2001).

62) Xu Y., Carr L.G., Binding of Sp1/Sp3 to the proximal promoter of the hMOR gene is enhanced by DAMGO, *Gene,* **274,** 119-28 (2001).

63) Borner C., Hollt V., Kraus J., Involvement of activator protein-1 in transcriptional regulation of the humanmu-opioid receptor gene, *Mol Pharmacol,* **61,** 800-5 (2002).

64) Borner C., Kraus J., Schroder H., Ammer H., Hollt V., Transcriptional regulation of the human mu-opioid receptor gene byinterleukin-6, *Mol Pharmacol,* **66,** 1719-26 (2004).

65) Bailey, D.W., Cumulative effect or independent effect?, *Transplantation,* **11,** 419-22 (1971).

66) Lee P.W., Wu S., Lee Y.M., Differential expression of mu-opioid receptor gene in CXBK and B6 mice by Sp1, *Mol Pharmacol,* **66,** 1580-4 (2004).

67) Hattori Y, Ding W.X., Maitani Y, Highly efficient cationic hydroxyethylatedcholesterol-based nanoparticle-mediated gene transfer in vivo and in vitro in prostate carcinoma PC-3 cells, *J Control Release,* **120,** 122-30 (2007).

68) Bayerer B., Stamer U., Hoeft A., Stüber F., Genomic variations and transcriptional regulation of the human mu-opioid receptor gene, *Eur J Pain,* **11,** 421-7 (2007).

69) Soldatenkov V.A., Chasovskikh S., Potaman V.N., Trofimova I., Smulson M.E., Dritschilo A., Transcriptional repression by binding of poly (ADP-ribose) polymerase to promoter sequences, *J Biol Chem,* **277,** 665-670 (2002).

70) D'Amours D., Desnoyers S., D'Silva I., Poirier G.G., Poly (ADP-ribosyl) ation reactions in the regulation of nuclear functions, *Biochem J,* **342,** 249-268 (1999).

71) Pottier N., Cheok M.H., Yang W., Assem M., Tracey L., Obenauer J.C., Panetta J.C., Relling M.V., Evans W.E., Expression of SMARCB1 modulates steroid sensitivity in human lymphoblastoid cells: identification of a promoter SNP that alters PARP1 binding and SMARCB1 expression, *Hum Mol Genet,* **19,** 2261-71. (2007).

72) Chiba-Falek O., Nussbaum R.L., Effect of allelic variation at the NACP-Rep1 repeat upstream of the alpha-synuclein gene (SNCA) on transcription in a cell culture luciferase reporter system, *Hum Mol Genet,* **26**, 3101-9 (2007).

73) Chiba-Falek O., Kowalak J.A., Smulson M.E., Nussbaum R.L., Regulation of alpha-synuclein expression by poly (ADP ribose) polymerase-1 (PARP-1) binding to the NACP-Rep1 polymorphic site upstream of the SNCA gene, *Am J Hum Genet,* **76** 478-92 (2005).

74) Shiokawa M., Masutani M., Fujihara H., Ueki K., Nishikawa R., Sugimura T., Kubo H., Nakagama H., Genetic alteration of poly(ADP-ribose) polymerase-1 in human germ cell tumors, *Jpn J Clin Oncol,* **35,** 97-102 (2005).

75) Beranger G.E., Momier D., Rochet N., Quincey D., Guigonis J.M., Samson M., Carle G.F., Scimeca J.C., RANKL treatment releases the negative regulation of the poly(ADP-ribose) polymerase-1 on Tcirg1 gene expression during osteoclastogenesis, *J Bone Miner Res,* **21,** 1757-69 (2006).

76) Huang K., Tidyman W.E., Le K.U., Kirsten E., Kun E., Ordahl C.P., Analysis of nucleotide sequence-dependent DNA binding of poly(ADP-ribose) polymerase in a purified system, *Biochemistry,* **43**, 217-228 (2004).

77) Zhang Z., Hildebrandt E.F., Simbulan-Rosenthal C.M., Anderson M.G., Sequence-specific binding of poly(ADP-ribose) polymerase-1 to the human T cell leukemia virus type-I tax responsive element, *Virology,* **295**, 107-116 (2002).

78) Kraus W.L., Lis J.T., PARP goes transcription, *Cell,* **113,** 677-683 (2003).

79) Poirier G.G., de Murcia G., Jongstra-Bilen J., Niedergang C., Mandel P., Poly (ADP-ribosyl) ation of polynucleosomes causes relaxation of chromatin structure, *Proc Natl Acad Sci USA,* **79,** 3423-7 (1982).

80) Huletsky A., de Murcia G., Muller S., Hengartner M., Menard L., Lamarre D., Poirier G.G., The effect of poly (ADP-ribosyl) ation on native and H1-depleted chromatin. A role of poly (ADP-ribosyl) ation on core nucleosome structure, *J Biol Chem,* **264,** 8878-86 (1989).

81) Kim M.Y., Mauro S., Gevry N., Lis J.T., Kraus W.L., NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1, *Cell,* **119,** 803-14 (2004).

82) Wacker D.A., Ruhl D.D., Balagamwala E.H., Hope K.M., Zhang T., Kraus W.L., The DNA binding and catalytic domains of poly(ADP-ribose) polymerase 1 cooperate in the regulation of chromatin structure and transcription, *Mol Cell Biol,* **27,** 7475-85 (2007).

83) Kim M.Y., Zhang T., Kraus W.L., Poly (ADP-ribosyl) ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal, *Genes Dev,* **19,** 1951-67 (2005).

84) Ju B.G., Solum D., Song E.J., Lee K.J., Rose D.W., Glass C.K., Rosenfeld M.G., Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway, *Cell,* **119,** 815-29 (2004).

85) Pavri R., Lewis B., Kim T.K., Dilworth F.J., Erdjument-Bromage H., Tempst P., de Murcia G., Evans R., Chambon P.,Reinberg D., PARP-1 determines specificity in a retinoid signaling pathway via direct modulation of mediator, *Mol Cell,* **18,** 83-96 (2005).

86) Hassa P.O., Hottiger M.O., The functional role of poly (ADP-ribose) polymerase 1 as novel coactivator of NF-kappaB in inflammatory disorders, *Cell Mol Life Sci,* **59,** 1534-53 (2002).

87) Olabisi O.A., Soto-Nieves N., Nieves E., Yang T.T., Yang X., Yu R.Y., Suk H.Y., Macian F., Chow C.W., Regulation of transcription factor NFAT by ADP-ribosylation, *Mol Cell Biol,* **28,** 2860-71 (2008).

88) Zaniolo K., Desnoyers S., Leclerc S., Guerin S.L., Regulation of poly(ADP-ribose) polymerase-1 (PARP-1) gene expression through the post-translational modification of Sp1: a nuclear target protein of PARP-1, *BMC Mol Biol,* **8,** 96 (2007).

89) Cohen-Armon M., Visochek L., Rozensal D., Kalal A., Geistrikh I., Klein R., Bendetz-Nezer S., Yao Z., Seger R., DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation, *Mol Cell,* **25,** 297-308 (2007).

90) Li M., Naidu P., Yu Y., Berger N.A., Kannan P., Dual regulation of AP-2α transcriptional activation by poly(ADP-ribose) polymerase-1, *Biochem J,* **382,** 323-329 (2004).

91) Butler A.J., Ordahl C.P., Poly (ADP-ribose) polymerase binds with transcription enhancer factor 1 to MCAT1 elements to regulate muscle-specific transcription, *Mol Cell Biol,* **19,** 296-306 (1999).

92) Akiyama T., Takasawa S., Nata K., Kobayashi S., Abe M., Shervani N.J., Ikeda T., Nakagawa K., Unno M., Matsuno S., Okamoto H., Activation of Reg gene, a gene for insulin-producing beta -cell regeneration: poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly(ADP-ribosyl)ation, *Proc Natl Acad Sci USA,* **98,** 48-53 (2001).

93) Choi H.S., Hwang C.K., Kim C.S., Song K.Y., Law P.Y., Loh H.H., Wei L.N., Transcriptional regulation of mouse mu opioid receptor gene in neuronal cells by Poly(ADP-ribose) polymerase-1, *J Cell Mol Med,* (in press).

94) Cohen-Armon M., Visochek L., Rozensal D., Kalal A., Geistrikh I., Klein R., Bendetz-Nezer S., Yao Z., Seger R., PARP-1 activation by phosphorylated ERK2 increases Elk 1 activity: a link to histone acetylation, *Mol Cell,* **2,** 297-308 (2007).

95) Hassa P.O., Haenni S.S., Buerki C., Meier N.I., Lane W.S., Owen H., Gersbach M., Imhof R., Hottiger M.O., Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription, *J Biol Chem,* **49,** 40450-64 (2005).

96) Kauppinen T.M., Chan W.Y., Suh S.W., Wiggins A.K., Huang E.J., Swanson R.A., Direct phosphorylation and regulation of poly(ADP-ribose) polymerase-1 by extracellular signal-regulated kinases 1/2, *Proc Natl Acad Sci USA,* **18**, 7136-41 (2006).

97) Zhang S., Lin Y., Kim Y.S., Hande M.P., Liu Z.G., Shen H.M., c-Jun N-terminal kinase mediates hydrogen peroxide-induced cell death via sustained poly(ADP-ribose) polymerase-1 activation, *Cell Death Differ,* **5**, 1001-10 (2007).

98) Agarwal S., Drysdale B.E., Shin H.S., Tumor necrosis factor-mediated cytotoxicity involves ADP-ribosylation, *J Immunol,* **12**, 4187-92 (1988).