Functional analysis of haplotypes and promoter activity at the 5' region of the DRD2 gene and associations with schizophrenia.

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Abstract

Background: In previous studies, we researched the association of the DRD2 gene promoter region SNP loci rs7116768, rs1047479195, rs1799732, rs1799978 and schizophrenia using Sanger sequencing. rs7116768 and rs1799978 were found to be slightly associated with schizophrenia. This study investigated the effects of haplotypes consisted of the four SNPs on protein expression level in vitro and identified the functional sequence in the 5’ regulatory region of DRD2 gene which has a potential link with schizophrenia.

Methods: Recombinant plasmids with haplotypes, SNPs and 13 recombinant vectors containing deletion fragments from the DRD2 gene 5' regulatory region were transfected into HEK293 and SK-N-SH cell lines. Relative luciferase activity of the haplotypes, SNPs and different sequences was compared using a dual luciferase reporter assay system.

Results: Haplotype H4(G-C-InsC-G) could significantly increase the gene expression in SK-N-SH cell lines. Allele C of rs7116768, allele A of rs1047479195 and allele del C of rs1799732 could up-regulate the gene expression. There were 5~7 functional regions in the promoter region of DRD2 gene that could affect the level of gene expression.

Conclusion: We cannot rule out the possibility that different haplotypes may influence DRD2 gene expression in vivo. We observed that allele C of rs7116768, allele A of rs1047479195 and allele del C of rs1799732 could up-regulate gene expression. The truncation results confirmed the existence of functional regions in the promoter region of DRD2 gene that could affect the level of gene expression.

Introduction

Schizophrenia is a serious mental illness. More than 21 million people worldwide suffer from schizophrenia, with a lifetime prevalence of more than 1 percent[1, 2]. Schizophrenia is a complex mental illness with unknown etiology. It is believed that environmental and genetic factors lead to this disease together. Many hypotheses have been proposed to explain the causes of schizophrenia. These hypotheses involve many neurotransmitters, such as dopamine, serotonin, and gamma-aminobutyric acid. Dopamine is an important neurotransmitter produced in the brain. The dopamine receptors acting together with dopamine belong to the G-protein-coupled receptor family and are involved in many nervous system activities, such as memory, learning, cognition, reward and neuroendocrine signal regulation [3]. Dopaminergic neurotransmission disorder is believed to be one of the causes of schizophrenia[4, 5]. Thus, genes associated with dopamine receptors are candidate genes of schizophrenia. The genome-wide association study (GWAS) demonstrated that DRD2 gene is one of the important candidate genes for schizophrenia[6]However, studies on the association between DRD2 gene polymorphisms and schizophrenia have not reached consistent conclusions. Taking the functional polymorphism site rs1799732 of the DRD2 gene promoter region as an example, Arinami et al. first discovered the polymorphism site rs1799732 in 1997, and proposed that the C del allele could reduce the expression of

DRD2 gene [7]But then consistent experimental conclusions can not be gotten in different countries and regions.

In previous studies, we researched the association of the DRD2 gene promoter region SNP loci rs7116768, rs1047479195, rs1799732, rs1799978 and schizophrenia using Sanger sequencing. Rs7116768 and rs1799978 were found to be slightly associated with schizophrenia[8]. The relative positions of the four SNPS are shown in Fig. 1, and the haplotype composed of the four SNPs is shown in Table 1.

In order to further determine the function of the DRD2 gene promoter region on gene expression, we used the dual-luciferase reporter assay to explore the effects of different haplotypes and different promoter region fragments on DRD2 gene expression.

Materials And Methods

Sample

Based on our previous analysis of haploid samples from different samples, we selected DNA samples with four haplotypes for follow-up study.

Construction of PGL-3 vector

The functional regions of the 5'-terminal regulatory region of DRD2 gene were predicted by Match^{TM}(http://www.gene-regulation.com/pub/programs.html). The predicted result is shown in Figure 2. Based on the predicted results, the influence of each SNP on gene expression was analyzed, and the truncation basis was provided for truncation experiments to avoid damaging the original functional areas. Primer 5.0 software and PubMed Blast were used to design sequence-specific primers. According to the existing polyclonal sites on the PGL-3 vector, we connected the recognition sequence of the selected restriction enzyme at the 5'-terminal of the primer(Table2). The PCR reaction used PrimeSTAR ® kit (Takara, Dalian, China). We used pGM-T Ligation®Kit (TIANGEN, Beijing,China) to link the target gene fragment with the pGM-T vector. The recombinant vector is transformed into JM109 competent cells by thermal excitation[9]. We used SanPrep®Col-umn Enodotoxin-Free Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai, China) to extract plasmids and then verified them by sequencing. We cut the aim gene fragment from pGM - T vector using the corresponding restriction enzymes (H1 used Kpn Iand Bgl II, P1 - P13 used Nhe Iand Bgl II) and connected to the pGL-3 luciferase reporter Basic Vector(Promega, Madison, Wisconsin, USA). We used SanPrep®Col-umn Enodotoxin-Free Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai, China) to extract plasmids and then verified them by sequencing.

Cell culture and transfection

HEK293 cell lines and SK-N-SH cells cell lines were used for double luciferase reporter gene assay. HEK293 cells were cultured in HyClone®DMEM/high glucose medium with 10% fetal bovine serum. SK-N-

SH cells were cultured in KeyGEN BioTECH®DMEM high glucose medium with 0.011 g/L sodium pyruvate containing 15% fetal bovine serum. When the cell density was above 90%, the cells were inoculated on the 24-well plates(1×10^5 cells per well). We transfected the recombinant PGL-3 plasmid(1ug) and PRL-TK (Promega) plasmid (100ng) into cells using Lipofectamine®3000 reagent (Invitrogen, California, USA). The cells were harvested after 29 hours. Finally, we standardized firefly luciferase activity (LUC value) using renin luciferase activity (TK value). Standardized activity intensity was used for comparison between different haplotypes or between different fragments. Each experiment was repeated independently in triplicate three times.

Statistic analysis

We used single-factor LSD-t test for anova to compare the relative luciferase activity between different haplotypes and the relative luciferase activity between different lengths of target gene fragments.

Result

Confirmation of constructed haplotype vectors

The haplotype clone fragment region containing 4 SNPs was +447~-1623bp upstream of the 5'-terminal of DRD2 gene. It was linked to the PGL-3 vector by cloning and transfected into cells. The luciferase assay showed that the four haplotypes of PGL-3 recombinant vectors were all transcriptional active. The haplotype H1(G-C-InsC-A) with the highest frequency in previous studies was used as the reference haplotype.

Confirmation of the constructed truncated fragment vectors

We used cloning technology to connect segments with different lengths of the 5'-terminal regulatory region of DRD2 gene to the PGL-3 vector, respectively(Figure3). After transfection, luciferase reporter gene analysis showed that all truncated fragments had transcriptional activity.

Luciferase activity detection

There was no significant difference in the expression of 4 haplotypes in HEK293 cell lines(Figure 4a), while haplotype H4 was significantly higher than the other three in SK-N-SH cell lines, $p<0.01$(Figure 4b). In the HEK293 and SK-N-SH cell lines, the relative fluorescence intensity of the recombinant vector containing rs7116768 allele C was significantly higher than that of the recombinant vector containing allele G, p values were <0.001(Figure 5a)and 0.010(Figure 5b), respectively. In HEK293 and SK-N-SH cell lines, the relative luciferase activity of the recombinant vector containing allele A of rs1047479195 was significantly higher than that of the recombinant vector containing allele C, with $p <0.001$(Figure 5c and Figure 5d). The relative fluorescence intensity of the recombinant vector containing the allele del C of rs1799732 was significantly increased in HEK293 cell lines compared with that of the recombinant vector containing the allele ins C, $p<0.001$(Figure 5e). However, the changes in SK-N-SH cell lines were not

statistically significant(Figure 5f). In HEK293 and SK-N-SH cell lines, there was no significant difference between the recombinant vector of rs1799978 allele A and G (Figure 5g and Figure 5h). In HEK293 cell lines, compared with P13, the relative luciferase activity of P12 was enhanced, p<0.001; compared with P11, the relative luciferase activity of P10 decreased, p<0.001; compared with P10, the relative luciferase activity of P9 decreased, p<0.001(Figure 6a)，compared with P5, the relative luciferase activity of P4 decreased, p<0.001; compared with P4, the relative luciferase activity of P3 enhanced, p<0.001; compared with P3, the relative luciferase activity of P2 decreased, p<0.001; compared with P2, the relative luciferase activity of P1 enhanced, p<0.001(Figure 6c). In the SK-N-SH cell lines, Compared with P13, the relative luciferase activity of P12 decreased, p<0.001; the relative luciferase activity of P9 was lower than that of P10, p<0.001(Figure 7a); compared with P7, the relative luciferase intensity of P6 was enhanced, P=0.001(Figure 7b); compared with P5, the relative luciferase activity of P4 decreased, p<0.001; compared with P3, the relative luciferase activity of P2 enhanced, p<0.001(Figure 7c).

Discussion

According to the analysis of the results obtained in this experiment, haplotype H4 significantly enhanced relative luciferase activity in SK-N-SH cell lines, but this phenomenon was not observed in HEK293 cell lines. The difference in the expression of the same haplotype in different cell lines may be caused by the different transcription factors expressed in different cell lines. SK-N-SH cells are human neuroblastoma cells. The high expression of haplotype H4 in nerve cells can to some extent support the influence of dopamine D2 receptor as a neurotransmitter receptor in the nervous system. In order to explore whether this expression difference is due to the role of a single SNP or the combined role of multiple SNPs, we separately observed the effect of each SNP on gene expression. The results showed that allele C of rs7116768, allele A of rs1047479195 and allele del C of rs1799732 could up-regulate gene expression. This result is not completely consistent with the above allelic composition of G-A-Ins C-G of haplotype H4, indicating that the up-regulation effect of haplotype H4 on gene expression is a comprehensive effect between SNPs. In addition, the relative luciferase results of the three alleles were consistent with the results of the previous risk factor analysis(OR = 1.307 allele C of rs7116768; OR = 1.161 for allele A of rs1047479195; OR = 1.285 for the allele del C of rs1799732). As risk factors of schizophrenia, they may be involved in the occurrence of schizophrenia by up-regulating the expression of DRD2 gene.

The DRD2 5 '-end regulatory region − 121~-226 bp was shown to enhance relative luciferase activity in HEK293 cell lines and inhibit relative luciferase activity in SK-N-SH cell lines. The − 344~-468 bp region showed enhanced relative luciferase activity in HEK293 cell lines. The − 468~-539 bp region decreased relative luciferase activity in HEK293 and SK-N-SH cell lines. The − 720~-925 bp region showed enhanced relative luciferase activity in SK-N-SH cell lines. The − 1234~-1363 bp region decreased relative luciferase activity in HEK293 and SK-N-SH cell lines. The − 1363~-1457 bp region enhanced relative luciferase activity in HEK293 cell lines. The − 1457~-1549 bp region inhibited relative luciferase activity in HEK293 cell lines and enhanced relative luciferase activity in SK-N-SH cell lines. The − 1549~-1649 bp region showed enhanced relative luciferase activity in HEK293 cell lines. What's more, although we did not observe the effect of this region on gene expression in the two cell lines in the − 226~-344 bp region, there

is an Ins C allele of rs1799732 that can reduce gene expression in this region, It can be inferred that there may be a functional element that up-regulates gene expression in the − 226 ~ 344 bp region, and when rs1799732 is inserted into C, it can be combined with transcription factors to up-regulate gene expression. Heat shock factor HSF is the main regulatory factor of human cells under stress conditions. In addition, HSF is associated with many genes that do not up-regulate during heat shock. There are at least three binding sites of HSF in the upstream region of heat shock protein gene promoter, and there are also three consecutive binding sites of HSF in the upstream region of DRD2 gene, it may bind to HSF during gene expression to change the level of gene expression. In this study, the up-regulation of P6 ~ P7, i.e. -720~-925 bp, in the SK-N-SH cell line also proved this. Transcription factor Evi-1 can affect gene expression in tumor cells of the blood system and play a promoting role in the proliferation and metastasis of tumor cells. Combined with the predicted results of functional elements in promoter region, the up-regulation effect of -720~-925 bp region on the expression in SK-N-SH cell lines indicated that there was a functional region in -720~-925 bp region that could up-regulate gene expression. Transcription factor Pax-4 is highly expressed in islet cells and has the function of transcriptional inhibition. In this study, two binding sites of Pax-4 were predicted in the − 600~-619 bp region through the prediction of functional elements in the promoter region, The results of dual-luciferase reporter assay also showed that the truncated region P8 ~ P9, i.e. -539~-637 bp, could down-regulate the gene expression level in HEK293 cell line, this indicates that there is a functional region in the − 539~-637 bp region that can down-regulate gene expression. We found that the same haplotype or the same fragment in the regulation region of DRD2 gene 5 ' end did not have exactly the same effect on gene expression in different cell lines. Atwod BK et al. showed that HEK-293 cell lines expressed DRD2 gene to a moderate degree [10].

SK-N-SH cell lines belong to nerve cells and were used to study the functional regions of the DRD2 gene 3 ' terminal [11]. This indicates that the cell lines selected in this study are reasonable. Differences in results between different cell lines are likely due to heterogeneity between cell lines. Different transcription factors expressed in different cell lines may lead to inconsistencies in gene expression levels. Although we did not find a significant association between SNPs in the DRD2 gene promoter region and haplotypes and schizophrenia in case-control studies, haplotype H4 was observed to enhance relative luciferase activity in SK-N-SH cell lines. This suggests that we cannot rule out the possibility that different haplotypes may influence DRD2 gene expression in vivo. We observed that allele C of rs7116768, allele A of rs1047479195 and allele del C of rs1799732 could up-regulate gene expression. On the other hand, the truncation results confirmed the existence of functional regions in the promoter region of DRD2 gene that could affect the level of gene expression. This study provides a reference for the association between DRD2 gene and schizophrenia and other psychiatric disorders related to dopamine system disorders. In order to further clarify the causal relationship between them, more experiments are needed to flesh out our conclusions.

Conclusion

This suggests that we cannot rule out the possibility that different haplotypes may influence DRD2 gene expression in vivo. We observed that allele C of rs7116768, allele A of rs1047479195 and allele del C of rs1799732 could up-regulate gene expression. The truncation results, on the other hand, confirmed the existence of functional regions in the promoter region of DRD2 gene that could affect the level of gene expression. This study provides a reference for the association between DRD2 gene and schizophrenia and other psychiatric disorders related to dopamine system disorders. In order to further clarify the causal relationship between them, more experiments are needed to flesh out our conclusions.

Declarations

Author contributions

X.C.Z. wrote the initial manuscript, X.C.Z., Y.L., Y.P.L. and J.Y conducted the experiment, X.C.Z., J.Y., J.F.X., J.X.X., X.X.,M.D., B.W. analyzed the results and modified the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards

All participants gave their informed consent in writing after the study aims and procedures were carefully explained to them in their own language. The study was approved by the ethical review board of the China Medical University, Shenyang Liaoning Province, People's Republic of China and in accordance with the standards of the Declaration of Helsinki.

Conflict of interest

The authors report no conflicts of interest in this work.

Consent for publication

The authors are consent for the publication of this manuscript.

Declaration

This manuscript has no more support data than the data mentioned in the manuscript.

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Tables

Table.1 The polymorphism distribution of four constructed haplotypes containing four SNPs.

	rs7116768	rs1047479195	rs1799732	rs1799978
H1	G	C	insC	A
H2	G	C	insC	G
H3	C	C	delC	A
H4	G	A	insC	G

Table2. Primers used for clonging

Prime	Sequence
H-F	5' GGGGTACCACTGACTACATAGATTC3'
H-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P1-F	5' CTAGCTAGCTAGTAATGGCTGATCC3'
P1-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P2-F	5' CTAGCTAGCTAGTCAGCAAGATCCA3'
P2-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P3-F	5' CTAGCTAGCTAGCTGCATTTGAGAG3'
P3-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P4-F	5' CTAGCTAGCTAGGGACTCAGTTCTC3'
P4-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P5-F	5' CTAGCTAGCTAGAGAGGCTGGATTC3'
P5-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P6-F	5' CTAGCTAGCTAGATTGCTGCAGAAG3'
P6-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P7-F	5' CTAGCTAGCTAGCCAAATGACAGTG3'
P7-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P8-F	5' CTAGCTAGCTAGCACCTCAGTTTGG3'
P8-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P9-F	5' CTAGCTAGCTAGCCAGCAATAGAAC3'
P9-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P10-F	5' CTAGCTAGCTAGCCTCCTCTCCTTG3'
P10-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P11-F	5' CTAGCTAGCTAGTCTGGGTGTGGGT3'
P11-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P12-F	5' CTAGCTAGCTAGACAGCTCCTTTGG3'
P12-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P13-F	5' CTAGCTAGCTAGTTCAGTGCCGAAC3'
P13-R	5' GAAGATCTTCAAACTTCTGGTCCTG3'
P14-F	5' CTAGCTAGCTAGTACCCGTTCAGGC 3'
P14-R	5' GAAGATCTTCAAACTTCTGGTCCTG 3'
P15-F	5' CTAGCTAGCTAGTACCCGTTCCAGGC 3'
P15-R	5' GAAGATCTTCAAACTTCTGGTCCTG 3'

F represents the upstream primer, and R represents the downstream primer. The amplification primer pair of haplotype H1~4 was primer pair H. The amplification primer pair of rs1799978 was primer pair H. The amplification primer pair of allele delC of rs1799732 was primer pair P14.The amplification primer pair of allele insC of rs1799732 was primer pair P15.The amplification primer pair of rs1047479195 was primer pair P12.The amplification primer pair of rs7116768 was primer pair P12.

Figures

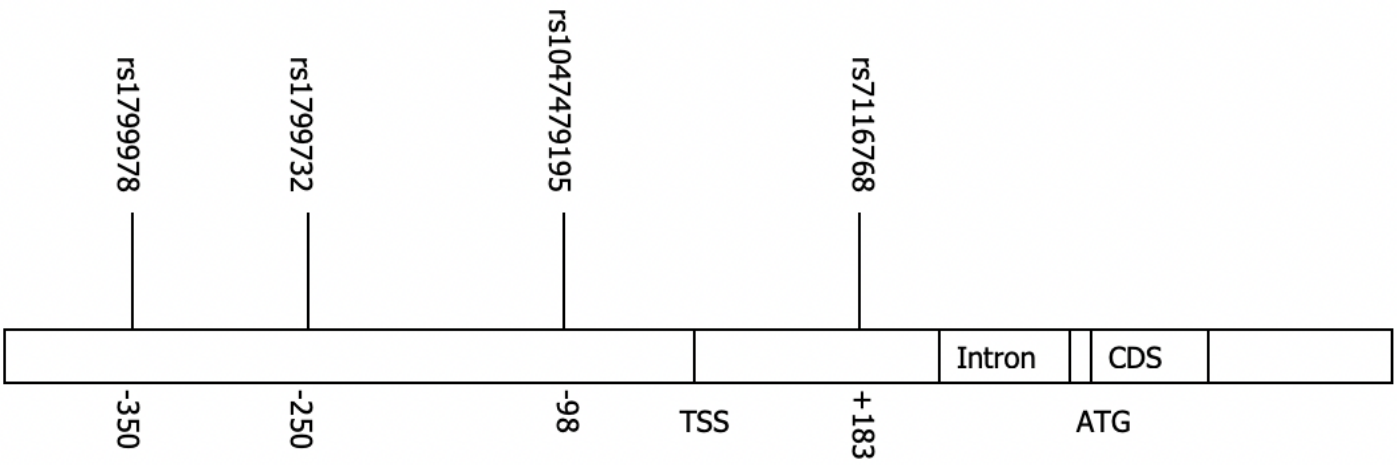

Figure 1

The relative positions of rs7116768, rs1047479195, rs1799732 and rs1799978 in DRD2 gene.

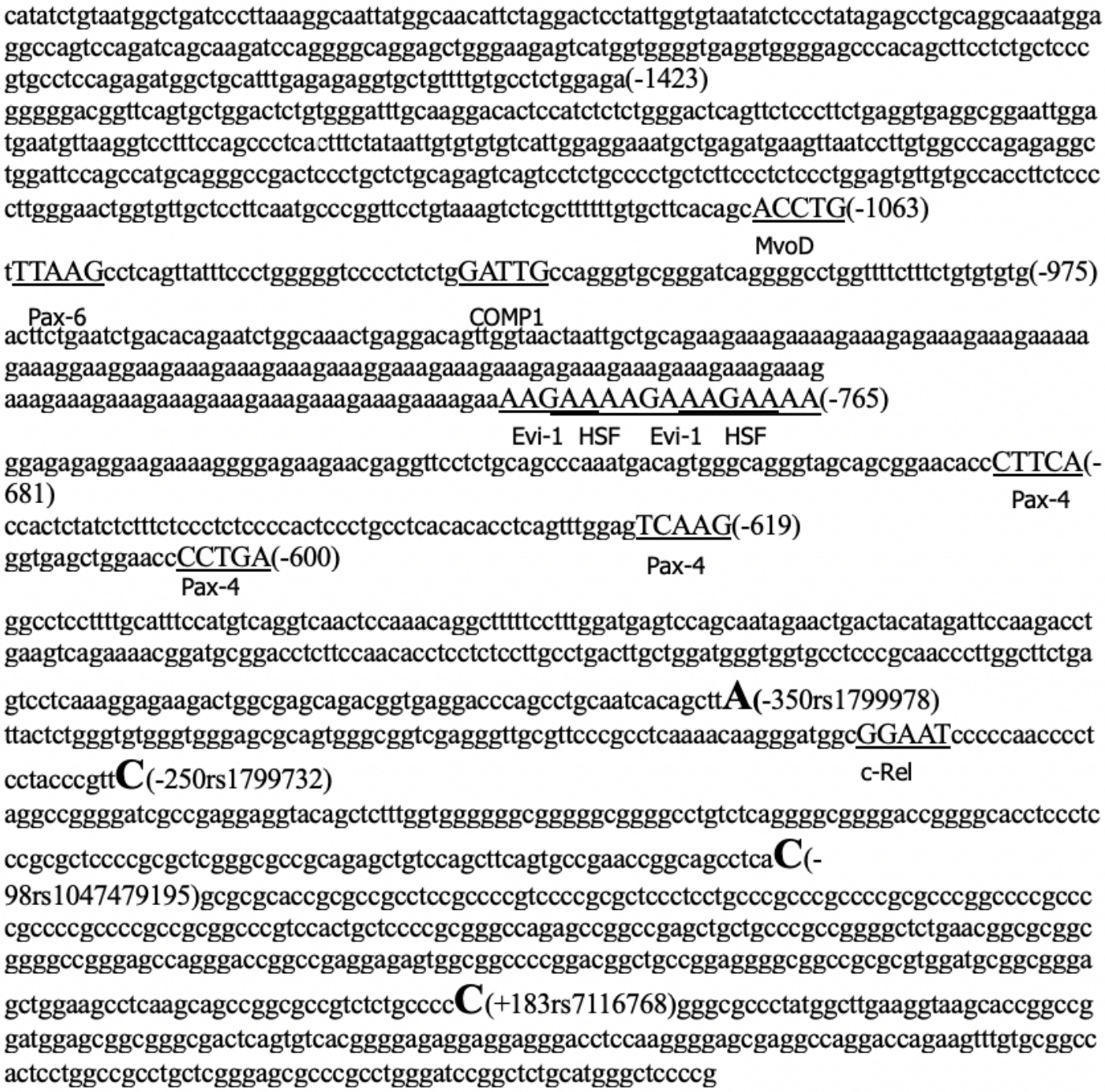

Figure 2

Prediction of the functional area of DRD2 regulatory area by MatchTM

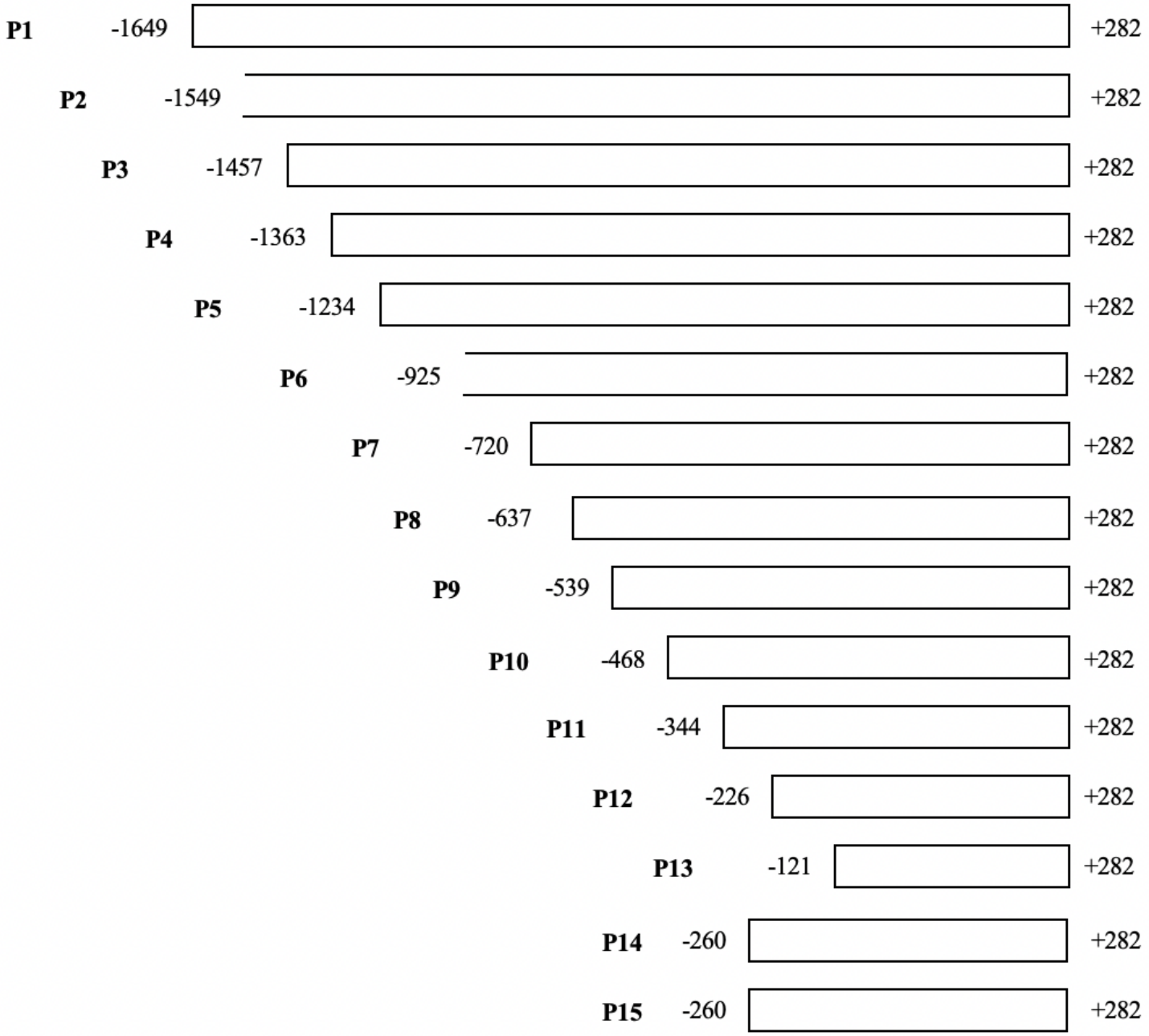

Figure 3

The length and relative position of all amplified fragments in the 5'-flanking sequence of the DRD2 gene.

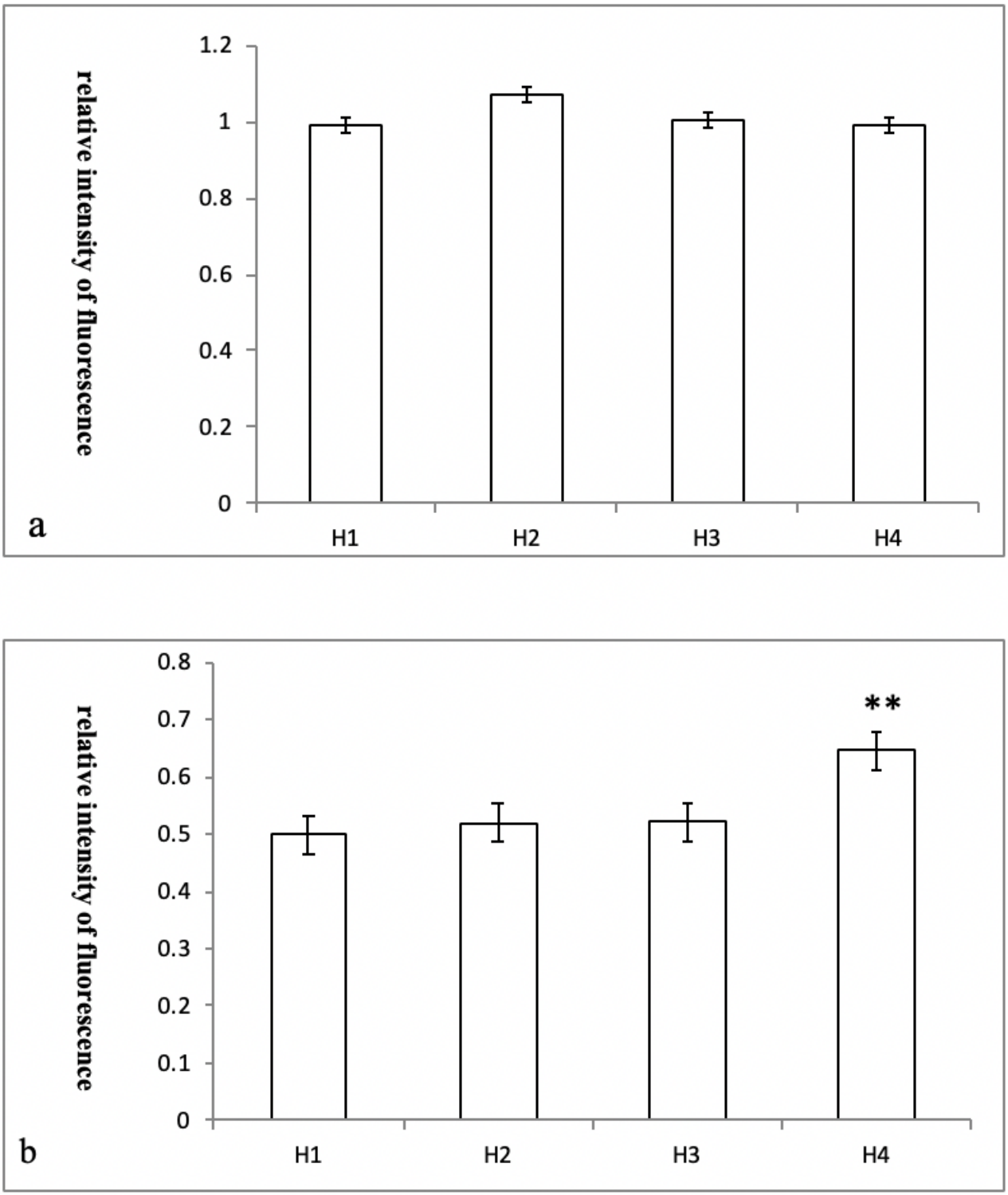

Figure 4

Relative luciferase activity of four constructed haplotypes in HEK293 and SK-N-SH cell lines. Normalized activities of all the test clones (H2–H4) were compared with the reference haplotype (H1). **represents $p < 0.01$. The error bars are standard deviationof the mean. a: Relative luciferase activity of four constructed haplotypes in HEK293 cell lines. b: Relative luciferase activity of four constructed haplotypes in SK-N-SH cell lines.

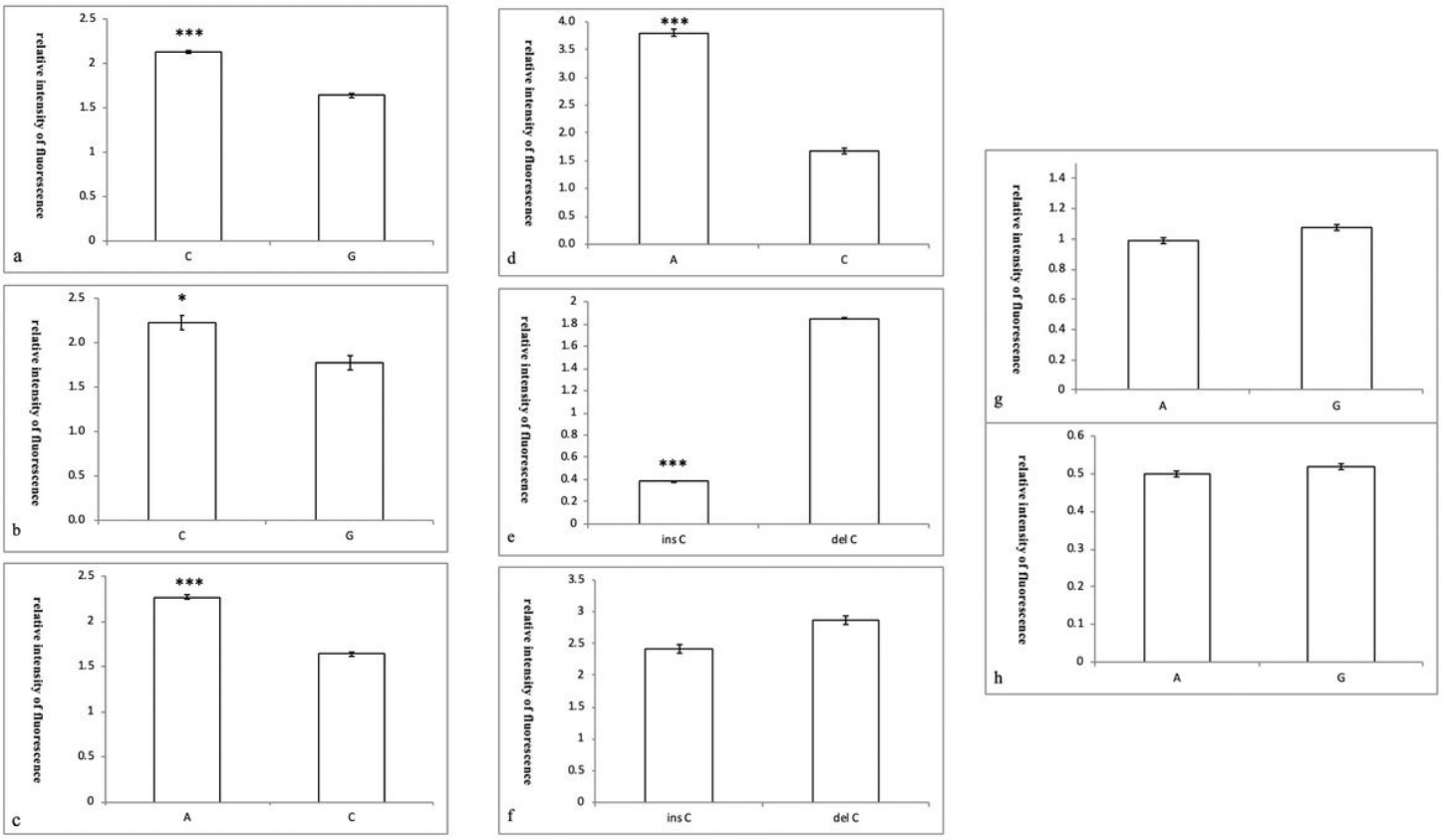

Figure 5

The relative luciferase activity of different alleles of four SNPs in HEK293 and SK-N-SH cell lines. ***represents $p < 0.001$ and *represents $p < 0.05$ .The error bars are standard deviation of the mean. a: The relative luciferase activity of alleles C and G of rs7116768 in the HEK293 cell lines. b: The relative luciferase activity of alleles C and G of rs7116768 in the SK-N-SH cell lines. c: The relative luciferase activity of alleles A and C of rs1047479195 in the HEK293 cell lines. d: The relative luciferase activity of alleles A and C of rs1047479195 in the SK-N-SH cell lines. e: The relative luciferase activity of alleles ins C and del C of rs1799732 in the HEK293 cell lines. f: The relative luciferase activity of alleles ins C and del C of rs1799732 in the SK-N-SH cell lines. g: The relative luciferase activity of alleles A and G of rs1799978 in the HEK293 cell lines. h: The relative luciferase activity of alleles A and G of rs1799978 in the SK-N-SH cell lines.

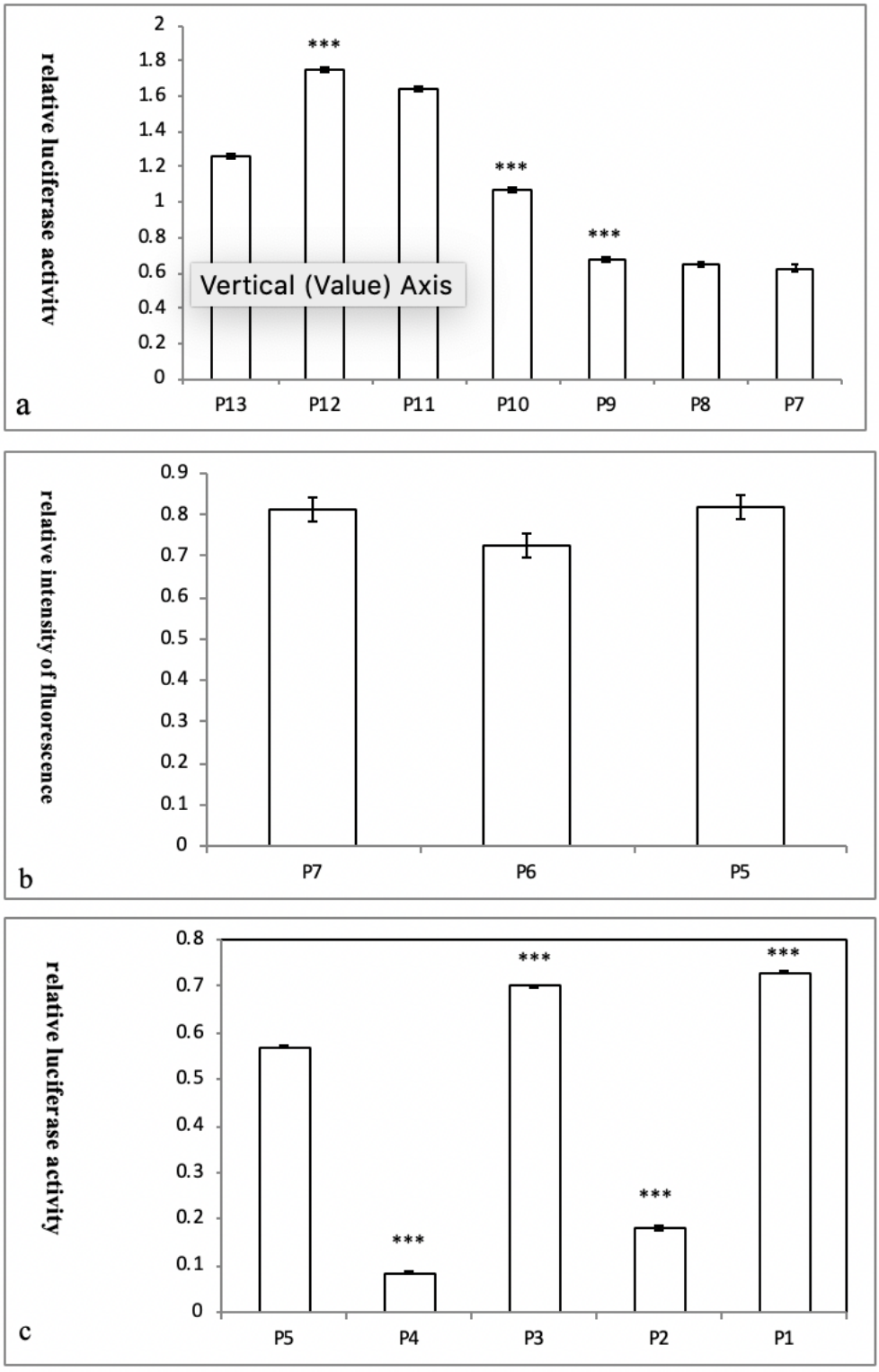

Figure 6

The relative luciferase activity of the recombinant vectors with different truncates in the 5 '-terminal of DRD2 gene in HEK293 cell lines. ***Represents $p < 0.001$. The error bars are standard deviation of the mean. a: The relative luciferase activity of P13,P12, P11,P10,P9,P8 and P7 in the HEK293 cell lines. b: The relative luciferase activity of P7,P6 and P5 in the HEK293 cell lines. c: The relative luciferase activity of P5,P4, P3 ,P2and P1 in the HEK293 cell lines.

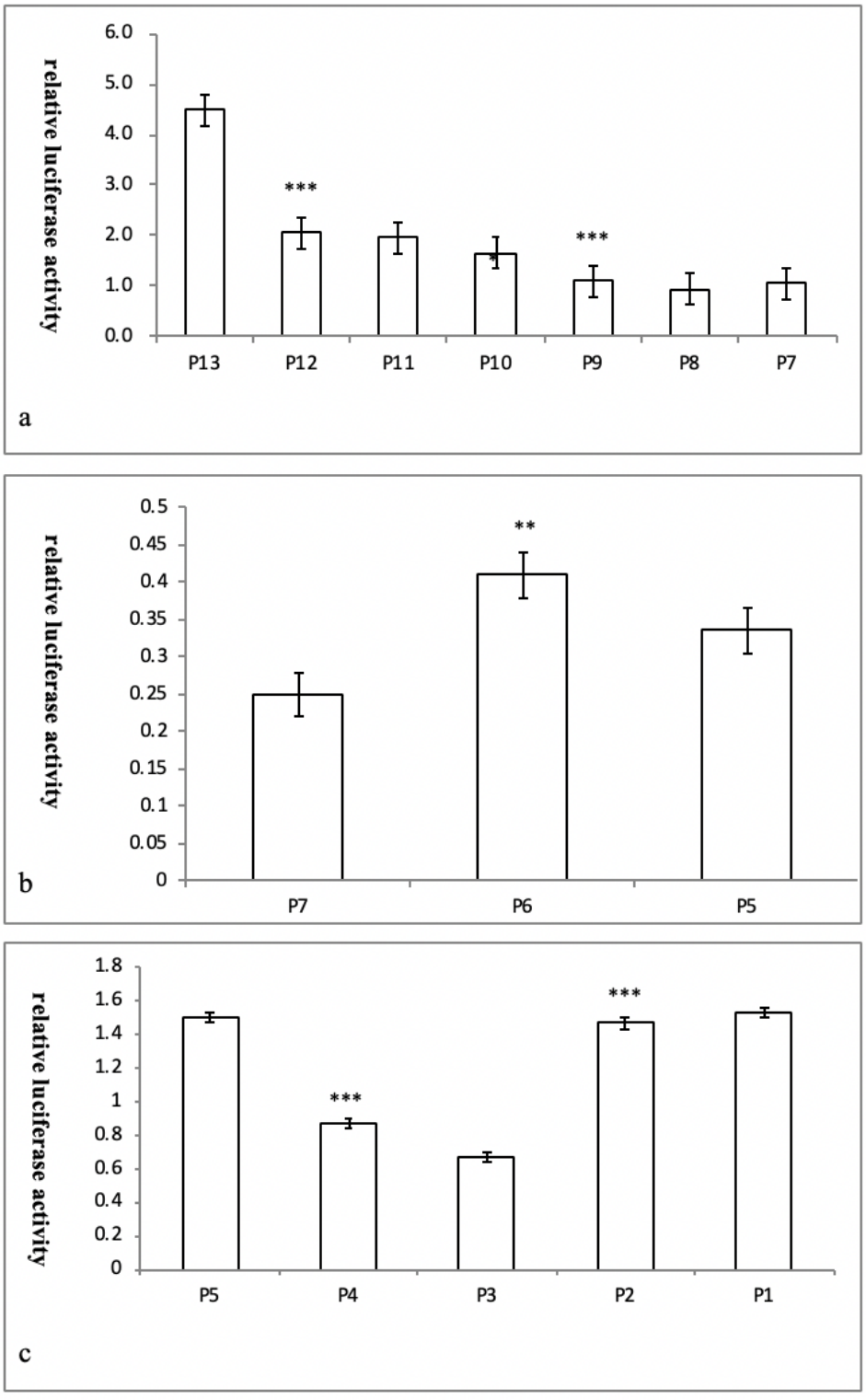

Figure 7

The relative luciferase activity of the recombinant vectors with different truncates in the 5 '-terminal of DRD2 gene in SK-N-SH cell lines. ***Represents $p < 0.001$ and **Represents $p < 0.01$. The error bars are standard deviation of the mean. a: The relative luciferase activity of P13,P12, P11,P10,P9,P8 and P7 in the SK-N-SH cell lines. b: The relative luciferase activity of P7,P6 and P5 in the SK-N-SH cell lines. c: The relative luciferase activity of P5,P4, P3 ,P2and P1 in the SK-N-SH cell lines.