FULL-LENGTH REPORT

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Targeted exome sequencing for the identification of a protective variant against Internet gaming disorder at rs2229910 of neurotrophic tyrosine kinase receptor, type 3 (NTRK3): A pilot study

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Background and aims: Internet gaming disorder (IGD) has gained recognition as a potential new diagnosis in the fifth revision of the Diagnostic and Statistical Manual of Mental Disorders, but genetic evidence supporting this disorder remains scarce. *Methods:* In this study, targeted exome sequencing was conducted in 30 IGD patients and 30 control subjects with a focus on genes linked to various neurotransmitters associated with substance and non-substance addictions, depression, and attention deficit hyperactivity disorder. *Results:* rs2229910 of neurotrophic tyrosine kinase receptor, type 3 (NTRK3) was the only single nucleotide polymorphism (SNP) that exhibited a significantly different minor allele frequency in IGD subjects compared to controls ($p = .01932$), suggesting that this SNP has a protective effect against IGD (odds ratio = 0.1541). The presence of this potentially protective allele was also associated with less time spent on Internet gaming and lower scores on the Young's Internet Addiction Test and Korean Internet Addiction Proneness Scale for Adults. *Conclusions:* The results of this first targeted exome sequencing study of IGD subjects indicate that rs2229910 of NTRK3 is a genetic variant that is significantly related to IGD. These findings may have significant implications for future research investigating the genetics of IGD and other behavioral addictions.

Keywords: Internet gaming disorder (IGD), targeted sequencing, exome sequencing, NTRK3

INTRODUCTION

Internet addiction, also known as excessive Internet use, began to gain public attention in Asian countries such as South Korea and Taiwan due to its high prevalence and rapid expansion in the early 2000s. Because Internet access is now easier to obtain and has become less expensive worldwide, its overuse has become a serious problem in Western societies and developing countries. Accordingly, scientific research in diverse fields such as psychology, sociology, psychiatry, and neuroscience is now being conducted. The perspective of Internet addiction as an epidemic and/or a social phenomenon is now shifting toward viewing this behavior as a potential mental health disorder, and as a result, Internet gaming disorder (IGD) is now included in Section III (Conditions for Further Study) of the fifth revision of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V; American Psychiatric Association, 2013). The DSM-V criteria for IGD were drawn from those for gambling and substance use disorders, which is suggestive of the similarities between the phenotypic aspects of IGD and other addictive disorders. However, additional information on the biological basis of IGD is necessary to fully understand this disorder.

To date, only a few articles investigating the genetics of IGD have been published. Twin studies from China (Li, Chen, Li, & Li, 2014) and the Netherlands (Vink, van Beijsterveldt, Huppertz, Bartels, & Boomsma, 2015) found that genetic factors can explain 48–66% of IGD subjects, indicating that there is a heritable nature to this disorder. Earlier association studies showed that the Taq1A1 allele of the dopamine (DA) D2 receptor gene (DRD2), methionine

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variant of the catecholamine-O-methyltransferase gene (COMT), which is the low activity allele (COMTL allele; Han et al., 2007), homozygous short allelic variant of the serotonin (5-HT) reuptake transporter gene (SS-5HTTLPR; Lee et al., 2008), and CC variant of rs1044396 on the cholinergic receptor, nicotinic, alpha 4 gene (CHRNA4; Montag, Kirsch, Sauer, Markett, & Reuter, 2012) are more prevalent in individuals with IGD compared to control subjects. The Taq1A1 allele of DRD2 and the COMTL allele are also related to other addictive disorders such as alcohol use disorder (Blum et al., 1990; Wang et al., 2001) and gambling disorders (Comings et al., 1996). SS-5HTTLPR is widely recognized for its association with depression, which is one of the disorders most commonly found to be comorbid with IGD (Blier & De Montigny, 1999), and rs1044396 of CHRNA4 is associated with nicotine dependence, attention, and working memory (Feng et al., 2004; Greenwood et al., 2009).

It is surprising that only four relevant variants have been identified within 8 years of the initial genetic study of IGD (Han et al., 2007) considering the growing body of literature that involves brain imaging studies of IGD patients. This scarcity of genetic studies on IGD may be due to publication bias and limitations of candidate gene association studies, such as heterogeneity of illness and population stratification. Family-based studies could be helpful for eliminating artifacts due to population stratification, but the short history of domestic Internet generalization makes them difficult to conduct. In addition, the tremendous number of subjects required for the identification of a novel relevant variant in a genome-wide association study (GWAS) with a p value less than 5×10^{-8} and the high cost of whole genome sequencing (WGS) may have also hindered genetic studies investigating IGD.

As next-generation sequencing techniques are introduced, higher throughput and read length are obtainable in conjunction with lower costs and shorter experimental periods. However, the sequencing of entire genomes in a large human sample remains costly. The sequencing exome, which is the protein coding region that represents less than 1% of the whole genome, is not only inexpensive to investigate but its assessment can efficiently identify disease-causing variants. Furthermore, if the sequencing range is narrowed down to a specific region of interest, this targeted sequencing can achieve much higher coverage levels, which makes it an ideal tool for examining genes in specific pathways or for follow-up studies of GWAS or WGS studies. Thus, the present pilot study conducted targeted exome sequencing to discover novel genetic variants associated with IGD.

MATERIALS AND METHODS

Participants

This study included 60 male Korean participants; half ($n = 30$) of them were IGD patients who visited the psychiatric outpatient clinics of three university hospitals in Seoul, South Korea (Seoul St. Mary's Hospital at Catholic University Medical College, Seoul Metropolitan Government-Seoul National University Boramae Medical Center, and Gangnam Eulji Hospital at Eulji University) for the treatment of problematic Internet game use. The IGD patients were diagnosed according to DSM-V criteria by clinically experienced psychiatrists. The sample pool was restricted to male subjects because the prevalence of IGD is higher in males (Ko et al., 2005). Participants with past or current major medical, neurological, or psychiatric disorders were excluded from the study. Unrelated male individuals ($n = 30$) with no history of psychiatric disorders were recruited from the local community as control subjects.

Measures

Participants were asked to complete a questionnaire assessing age, years of education, marital state, occupation, and alcohol and cigarette use. In addition, each participant completed the Alcohol Use Disorder Identification Test (AUDIT) and Fagerstrom Test for Nicotine Dependence (FTND). The severity of IGD was determined based on the average daily hours of Internet gaming on weekdays and weekends, the standardized Korean version of Young's Internet Addiction Test (Y-IAT; Kim, Lee, & Oh, 2003), and the Korean Internet Addiction Proneness Scale for Adults (KS-A; Kim, Kim, & Hwang, 2012). The Y-IAT is a self-report scale consisting of 20 questions answered using a 5-point Likert scale ranging from 1 (not at all) to 5 (always); this scale is in accordance with the DSM-IV criteria for pathological gambling and is widely used by investigators worldwide (Kim et al., 2003). All of the IGD patients primarily used the Internet for online gaming. The KS-A is a self-report scale consisting of 15 items answered with a 4-point Likert scale ranging from 1 (not at all) to 4 (always). Each participant also completed the Beck Depression Inventory (BDI; Beck, Ward, Mendelson, Mock, & Erbaugh, 1961), Beck Anxiety Inventory (BAI; Beck, Epstein, Brown, & Steer, 1988), and Barratt's Impulsivity Scale version 11 (BIS-11; Patton et al., 1995) to assess depressive, anxiety, and impulsivity symptoms, respectively. All of the scales have been validated for use in Korean subjects.

Target gene selection

A 500 kb probe for targeted exome sequencing was custom-designed for this study. The targets included genes related to the production, action, and metabolism of neurotransmitters that are associated with other addictive disorders, including DA, 5-HT, norepinephrine, gamma-aminobutyric acid (GABA), acetylcholine, opioids, glycine, and glutamate. In addition to genes and single nucleotide polymorphisms (SNPs) that have previously been linked with IGD, gambling disorders, and alcohol and nicotine dependence, and genes linked with depression and attention deficit hyperactivity disorder (ADHD) were also included in the probe, because depressive and impulsive features are the most commonly observed traits and comorbid disorders in patients with IGD (Yen, Ko, Yen, Wu, & Yang, 2007). The probe in this study consisted of 159 genes and 83 SNPs (Supplementary Material).

Sample preparation and targeted gene sequencing

Each sequenced sample was prepared according to the Illumina protocols (Illumina, San Diego, CA, USA). Briefly, 1 μg of genomic DNA was extracted from peripheral blood samples and fragmented by nebulization. Following end-repair and adapter ligation, 350–400 base pair products were selected and amplified using a polymerase chain reaction (PCR) protocol, and the final product was validated using an Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Then, the target libraries were hybridized with the panel and enriched using the Illumina Exome Enrichment protocol, after which the purified and enriched libraries were sequenced using an Illumina HiSeq 2000 Sequencer.

Bioinformatics analysis

The paired-end sequence reads were first mapped to the human genome reference sequence (UCSC hg19) without unordered sequences or alternate haplotypes using a Burrow-Wheeler Aligner (BWA, version 0.5.9rc1; Li & Durbin, 2009). The raw alignment was sorted, and PCR 196 duplicates were removed by Picard (version 1.59; http://www.broadinstitute.org). Then the reads not across the targeted exonic regions were eliminated. The variants [SNPs and insertions and deletions (indels)] were called on each sample with the GATK HaplotypeCaller (version 3.4.46; http://www.broadinstitute.org) and the called variants were functionally annotated using ANNOVAR.

Statistical analyses

The quantitative and qualitative variables of the demographic and clinical data are expressed as means ± standard deviations (SDs) and percentages (%), respectively. The differences between groups were compared by Mann–Whitney *U* tests or Fisher's exact tests according to the characteristics of the variables with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The allelic association study was conducted using PLINK software (version 1.07, Purcell et al., 2007); some variants were filtered out according to the Hardy–Weinberg equilibrium test ($p < 1.0 \times 10^{-5}$) and minor allele frequency (MAF) thresholds (MAF < .001). The statistical significance of the association study was assessed using Chi-squared tests to compare MAFs between IGD patients and control subjects. The *p* values were adjusted using the genomic-control correction method to control for the inflation of test statistics (Devlin, Roeder, & Bacanu, 2001). For the most significant SNPs, models of inheritance, including additive, dominant, and recessive models, based on the minor allele were tested with a logistic regression analysis. A *p* value <.05 was considered statistically significant.

Ethics

All the study procedures were performed in accordance with the guidelines of the Declaration of Helsinki. The Institutional Review Boards of Seoul St. Mary's Hospital approved the study protocol, and all subjects provided written informed consent prior to participation.

RESULTS

Clinical characteristics

The demographic data and clinical features of the IGD patients and control subjects are shown in Table 1. Age, marital status, occupation, and alcohol and nicotine use did not differ between the two groups, but the IGD group played Internet games significantly longer than the control group on both weekdays and weekends. The Y-IAT, KS-A, BDI, BAI, and BIS-11 scores were all significantly higher in the IGD group than in the control group.

Genetic analyses

When the MAFs of the SNPs were compared between the IGD and control groups, 28 variants had an unadjusted *p* value <.05 (Table 2). However, only one SNP, rs2229910 of neurotrophic tyrosine kinase receptor, type 3 (NTRK3, also known as TRKC) remained significant [$p = .01932$, odds ratio (OR) = 0.1541] after adjusting the *p* value. Nine variants showed an adjusted *p* value <.1, which suggests a trend; the ORs for these SNPs were all <1, except for rs138128932 of myosin VB (MYO5B), which suggests a potential protective effect against IGD.

The distribution of genotypes for the most significant SNP, rs2229910 of NTRK3, is shown in Table 3, but a genotypic association test could not be performed because no subjects in the IGD group had a CC genotype. The dominant model was the most fit model of inheritance for the minor allele (C) of rs2229910 based on the logistic regression analysis ($p = .002032$, OR = 0.1346). The protective effects of the C allele became more prominent ($p = .023570$, OR = .005), when duration of education and the BDI, BAI, and BIS-11 scores were controlled for.

When the entire sample was divided into groups according to the GG and GC + CC genotypes, all of the variables related to IGD, such as the Y-IAT and KS-A scores and daily Internet gaming hours, were significantly lower in the GC + CC group that contained the potentially protective C allele. These differences in the Y-IAT and KS-A scores remained significant when the BDI, BAI, and BIS-11 scores were controlled for to exclude the influences of emotional factors (Table 4).

DISCUSSION

In this study, the only genetic variant with a significant protective effect against IGD was rs2229910 of NTRK3. The NTRK3 gene encodes a membrane-bound receptor with a high affinity for neurotrophic factor 3 (NTF3, also known as NT-3), and both are involved in the myelination and apoptosis of neuronal and glial cells (Beltaifa et al., 2005; Cosgaya, Chan, & Shooter, 2002; Nikoletopoulou et al., 2010). In this study, the MAF of NTRK3 at rs2229910 (the C allele) in the control group was 0.3167, which is similar to the MAF (0.3610) provided by the Exome Aggregation Consortium (ExAC) study that included 60,706 human subjects of various ethnicities (Lek et al., 2016). The MAF of rs2229910 has yet to be reported for the Korean population or for other homogenous ethnic groups.

Table 1. Demographics and clinical characteristics of the study subjects

Variables	IGD ($n = 30$, male)	NC ($n = 30$, male)	p value
Age (years), mean (*SD*)	23.1 (6.4)	24.3 (3.8)	.094
Duration of education (years), mean (*SD*)	13.0 (2.3)	14.3 (2.2)	.034*
Marital status, *n* (%)			
Unmarried	25 (92.6)	29 (96.7)	.599
Married	2 (7.4)	1 (3.3)	
Occupation, *n* (%)			
Yes	5 (25)	4 (13.8)	.542
No	20 (75)	25 (86.2)	
Smoking, *n* (%)			
Non-smoker	19 (70.4)	22 (75.9)	.765
Current smoker	8 (29.6)	7 (24.1)	
Alcohol, *n* (%)			
Non-drinker	11 (40.7)	7 (24.1)	.254
Drinker	16 (59.3)	22 (75.9)	
AUDIT, mean (*SD*)	8.94 (7.05)	5.93 (3.22)	.142
FTND, mean (*SD*)	3.25 (1.98)	1.00 (1.00)	.100
Weekday – daily Internet gaming hours (hr), mean (*SD*)	6.62 (3.15)	1.39 (0.70)	3.154×10^{-8}*
Weekend – daily Internet gaming hours (hr), mean (*SD*)	8.50 (3.71)	1.93 (1.10)	1.887×10^{-8}*
Y-IAT, mean (*SD*)	73.57 (9.39)	31.37 (9.69)	9.119×10^{-11}*
KS-A, mean (*SD*)	50.04 (7.41)	26.07 (8.05)	3.301×10^{-10}*
BDI, mean (*SD*)	20.17 (12.09)	4.76 (4.25)	1.523×10^{-6}*
BAI, mean (*SD*)	18.78 (14.40)	5.83 (5.17)	2.389×10^{-4}*
BIS-11, mean (*SD*)	68.68 (11.31)	54.38 (9.23)	1.696×10^{-5}*

Note. IGD, Internet gaming disorder patients; NC, normal control; SD, standard deviation; AUDIT, Alcohol Use Disorder Identification Test; FTND, Fagerstrom Test for Nicotine Dependence; Y-IAT, Young's Internet Addiction Test; KS-A, Korean Internet Addiction Proneness Scale for Adults; BDI, Beck Depression Inventory; BAI, Beck Anxiety Inventory; BIS-11, Barratt's Impulsivity Scale version 11. *$p < .05$ (Mann–Whitney U test).

This study evaluated target genes such as NTF1, NTF2, NTF3, NTF4, NTRK1, NTRK2, and NTRK3, which are genes that encode neurotrophic factors and related receptors, because each had been previously associated with neurodevelopmental disorders, including ADHD (Conner et al., 2008; Ribases et al., 2008). Previous studies of additional variants of NTRK3 have shown that this gene is associated with several psychiatric disorders including panic/anxiety disorder (Dierssen et al., 2006; Muinos-Gimeno et al., 2009; Santos et al., 2013), depressive and bipolar disorders (Athanasiu et al., 2011; Feng et al., 2008; Verma et al., 2008), schizophrenia (Otnaess et al., 2009), obsessive–compulsive disorder (Alonso et al., 2008; Muinos-Gimeno et al., 2009), and eating disorders (Mercader et al., 2008). However, the present study was the first to investigate the role that variants of NTRK3 play in influencing one's susceptibility to substance and non-substance addictive disorders.

Because NTRK3 is an essential modulator of myelination throughout the lifespan (Beltaifa et al., 2005; Cosgaya et al., 2002), rs2229910 may modify the vulnerability of an individual to IGD via its influence on white matter integrity. Recent diffusion tensor imaging studies of NTRK3 observed increased myelination in right-sided frontal fiber tracts (Jeong, Han, Kim, Lee, & Renshaw, 2015) as well as the thalamus and left posterior cingulate cortex (Dong, DeVito, Huang, & Du, 2012). rs2229910 of NTRK3 is a putatively synonymous SNP. It is generally thought that synonymous SNPs do not affect phenotypes or disease manifestation because they do not change the amino acid composition of encoding proteins. However, recent evidence suggest that synonymous mutations can influence the expression levels of genes by modulating mRNA splicing patterns (Pagani, Raponi, & Baralle, 2005), mRNA stability (Sauna & Kimchi-Sarfaty, 2011), protein translation efficiency, protein folding (Kimchi-Sarfaty et al., 2007), and microRNA regulation (Wang, Qiu, & Cui, 2015).

In this study, several SNPs, including gamma aminobutyric acid-B receptor 2 (GABBR2), nerve growth factor receptor (NGFR), zinc finger protein 91-ciliary neurotrophic factor (ZFP91-CNTF), and dopa decarboxylase (DDC), had p values <.09. DDC and GABBR2 have been implicated in the production and action of dopamine and GABA, respectively, which are neurotransmitters widely recognized for their important roles in the pathophysiology of substance and non-substance addictions. The present findings regarding NGFR and ZFP91-CNTF suggest that, along with NTRK3, various neurotrophic factors and their receptors may play key roles in the susceptibility to IGD. Thus, future studies should investigate variants in these genes as candidates for the genetic and pathophysiological basis of IGD.

This study had several limitations that should be noted. First, the small sample size was a major limitation, and the surprisingly low OR (0.15) for rs2229910 was likely due to the very low number of GC ($n = 4$) and CC ($n = 0$)

Table 2. SNPs with statistically different ($p < .05$) MAFs between Internet gaming disorder patients and normal control subjects

CHR	SNP	Gene	Region	Major allele[a]	Minor allele[a]	MAF (IGD)	MAF (NC)	χ2	*p*	*p′*	OR
15	rs2229910[b]	NTRK3	Exon	G	C	0.06667	0.3167	12.1	.000504	.01932	0.1541
9	rs1000441	GABBR2	Intron	T	T	0.06667	0.25	7.566	.005947	.06435	0.2143
17	rs11466133	NGFR	Intron	A	AC	0.3	0.5333	6.72	.009534	.0813	0.375
11	rs1800169	ZFP91-CNTF	ncRNA-intron[d]	G	A	0.1	0.2833	6.508	.01074	.08625	0.281
7	rs3735273	DDC	Intron	C	T	0.2167	0.4333	6.42	.01129	.08841	0.3617
7	rs6950777	DDC	Intron	G	A	0.2167	0.4333	6.42	.01129	.08841	0.3617
18	rs138128932[c]	MYO5B	Exon	A	G	0.1	0	6.316	.01197	.09103	NA
7	rs5884156	DDC	Intron	A	AG	0.2333	0.45	6.261	.01234	.09244	0.372
1	rs1874044	CSMD2	Intron	T	C	0.25	0.4667	6.125	.01333	.09606	0.381
1	rs1874045[c]	CSMD2	Exon	T	C	0.25	0.4667	6.125	.01333	.09606	0.381
12	rs3741475[b]	NOS1	Exon	G	A	0.3833	0.1833	5.91	.01506	.1021	2.769
6	rs6297	HTR1B	Downstream	T	C	0.1333	0.01667	5.886	.01526	.1028	9.077
12	rs11832738	CACNA1C	Intron	A	G	0.2	0.4	5.714	.01683	.1079	0.375
9	rs2289656	NTRK2	Intron	G	A	0	0.08333	5.217	.02236	.1245	0
1	rs35761029[c]	CSMD2	Exon	T	A	0.05	0.1833	5.175	.02291	.1261	0.2344
5	rs5868607	CARTPT	UTR3	CA	C	0.15	0.03333	4.904	.02679	.1364	5.118
11	rs1042577	GAL	UTR3	C	T	0.2	0.3833	4.881	.02716	.1374	0.4022
4	rs72681567	TDO2	Intron	A	G	0.01667	0.1167	4.821	.02811	.1398	0.1283
7	rs5573[b]	NPY	Exon	A	G	0.2167	0.4	4.728	.02967	.1437	0.4149
12	rs11062272	CACNA1C	Intron	G	T	0.15	0.3167	4.658	.0309	.1467	0.3808
12	rs2293051	NOS1	Intron	G	C	0.2667	0.45	4.385	.03625	.1591	0.4444
14	rs17832998[c]	DACT1	Exon	C	T	0.1167	0.2667	4.357	.03686	.1604	0.3632
1	rs10798976[b]	CSMD2	Exon	G	A	0.05	0.1667	4.227	.03978	.1668	0.2632
11	rs58224139[b]	ANKK1	Exon	C	T	0	0.06667	4.138	.04193	.1713	0
5	rs112846276[c]	HTR1A	Exon	G	A	0	0.06667	4.138	.04193	.1713	0
20	rs3827020	CHRNA4	Intron	T	C	0.3833	0.5667	4.043	.04434	.1763	0.4754
5	rs11959820[c]	PPARGC1B	Exon	C	A	0.1333	0.03333	3.927	.04751	.1827	4.462
8	rs2270637[c]	SLC18A1	Exon	C	G	0.3	0.15	3.871	.04913	.1858	2.429

Note. CHR, chromosome; SNP, single nucleotide polymorphism; MAF, minor allele frequency; OR, odds ratio; *p*, unadjusted *p*-value from χ^2 test; *p′*, genomic-control corrected *p*-values; NA, not available.
[a]Major and minor alleles in the whole sample; [b]synonymous SNP; [c]nonsynonymous SNP; [d]intronic region of non-coding RNA.

Table 3. Distribution of genotypes for the rs2229910 of NTRK3 in Internet gaming disorder patients and normal control subjects

	Genotype			
Group	GG	GC	CC	Total
IGD	26	4	0	30
NC	14	13	3	30
Total	40	17	3	60

Note. IGD, Internet gaming disorder patients; NC, normal control.

Table 4. Comparison of Internet gaming-associated clinical variables based on the genotypes for rs2229910 of NTRK3

Genotypes of NTRK3	GG ($n = 40$)	GC + CC ($n = 20$)	*p*	*p′*
Y-IAT	58.82 (22.36)	37.21 (18.16)	.004*	.008**
K-scale	41.95 (13.86)	28.79 (10.99)	.001*	.010**
Weekday – daily Internet gaming hours (hr)	4.76 (3.70)	2.39 (2.30)	.017*	.121
Weekend – daily Internet gaming hours (hr)	6.11 (4.49)	3.28 (3.14)	.020*	.057

*$p < .05$ (Mann–Whitney *U* test); **$p < .05$ when BDI, BAI, and BIS-11 scores are controlled for.

genotypes in the IGD group (Table 3). There were also some SNPs with a MAF of 0 in the IGD and control groups, including rs2289656 of NTRK2 and rs138128932 of MYO5B (Table 2). These findings may be different when a larger sample is used. Additionally, the statistical controlling method used for multiple tests may be considered another limitation of this study. The adjusted *p* value for rs2229910 of NTRK3 was significant ($p = .01932$) when genomic-control correction method was used but the significance was not maintained when other correction methods such as the Bonferroni correction ($p = .7212$) or the false discovery rate ($p = .5262$) were applied. Therefore, a replication of this study using a larger and independent sample is essential to validate the present results. In addition, future studies should explore whether the presence of the C allele of rs2229910 increases or decreases the expression level of NTRK3 to further elucidate the precise role of NTRK3 in IGD patients.

CONCLUSIONS

This pilot study is the first to use targeted exome sequencing in an attempt to discover novel candidate genes related to IGD. Although it was a pilot study conducted with a relatively small sample, the targeted exome sequencing was feasible and valuable and revealed that rs2229910 of NTRK3 may be a protective SNP against IGD. Furthermore, these findings suggest various implications for future research investigating the genetics of IGD and other behavioral addictions.

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Conflict of interest: The authors declare that they have no conflict of interest.

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