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META-ANALYSIS

Attention-deficit/hyperactivity disorder and dopamine receptor D4 (DRD4) exon 3 variable number of tandem repeats (VNTR) 2-repeat allele

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Abstract: To investigate the association of attention-deficit/hyperactivity disorder (ADHD) with the 48-base pair (bp) variable number of tandem repeats (VNTR) in exon 3 of the dopamine receptor D4 (DRD4) gene, we genotyped 240 ADHD patients and their parents from Hong Kong. The 4R allele was most common, followed by 2R. We examined association between the 2R allele (relative to 4R) and ADHD by Transmission Disequilibrium Test (TDT). The odds ratio (OR) (95% confidence interval) was 0.90 (0.64–1.3). The *p*-value was 0.6. Examining subgroups revealed nominally significant association of 2R with inattentive ADHD: OR = 0.33 (0.12–0.92) and $p = 0.03$. Because our study used TDT analysis, we meta-analyzed the association of 2R with ADHD in Asians (1329 patient alleles), revealing results similar to ours: OR = 0.97 (0.80–1.2) and $p = 0.8$. To examine the association of 2R with inattentive ADHD, we meta-analyzed all studies (regardless of analysis type or ethnicity, in order to increase statistical power): 702 patient alleles, 1420 control alleles, OR = 0.81 (0.57–1.1) and $p = 0.2$. Overall, there is no evidence of association between ADHD and the 2R allele, but the suggestive association with the inattentive type warrants further investigation.

KEYWORDS
ADHD, dopamine, dopamine receptors, genetics, meta-analysis, minisatellite repeats

1 | INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric condition of hyperactivity, inattention, and impulsive behavior. It is among the most common mental disorders, occurring in about 5–7% of children and 2.5–5% of adults (Akutagava-Martins et al., 2016; Faraone et al., 2021; Polanczyk et al., 2007). In childhood, about 75–90% of patients are male (Akutagava-Martins et al., 2016).

ADHD heritability estimated from twin studies is about 74% (Faraone & Larsson, 2019), but heritability estimated from common, single-nucleotide variants is only about 22%, and genome-wide association studies (GWAS) have only recently detected variants associated with ADHD, perhaps because the numbers of subjects in GWAS were too small before (Demontis et al., 2023; Grimm et al., 2020; Leung et al., 2017; Pujol-Gualdo et al., 2021; Yao et al., 2021). Another possible cause of the low heritability estimates for common, single-nucleotide variants is that other variants play a role in ADHD.

One such variant that has been examined in ADHD is the dopamine receptor type D4 (DRD4) gene exon 3 variable number of tandem repeats (VNTR), in which a 48-base pair (bp) segment occurs anywhere from 2 to 11 times (Bonvicini et al., 2020; LaHoste et al., 1996; Leung et al., 2017). The 7-repeat (7R) allele has been associated with increased risk of ADHD, as compared to the most common allele, 4R (Bonvicini et al., 2020; Leung et al., 2017). In Asian populations, studies found that 7R is rare, while 2R is common, and because both 2R and 7R were reported to respond less to dopamine than does 4R, the association of 2R with ADHD has been investigated in Asians (Leung et al., 2005).

To explore the association of the DRD4 exon 3 VNTR with ADHD, we genotyped this variant in boys with ADHD and in their parents in Hong Kong. We then performed meta-analyses combining our results with published results.

2 | MATERIALS AND METHODS

2.1 | Participants

Chinese ADHD probands and their biological parents were recruited from child psychiatry clinics in Hong Kong, as in a previous study (Leung et al., 2017). Because the vast majority of ADHD patients in these clinics are male, only males were included in the study in order to maximize statistical power and simplify analysis.

Child psychiatrists diagnosed ADHD according to the Diagnostic and Statistical Manual of Mental Disorders—4th Edition (DSM-IV). Diagnosis was confirmed by a structured diagnostic interview using the Parent-informant version of Diagnostic Interview Schedule of Children—4th Edition (P-DISC-4) for psychiatric disorders of childhood (Shaffer et al., 2000). Parents who agreed to join the study gave informed written consent for their own involvement and for their children. The research ethics of the study, which conforms to standards of the Declaration of Helsinki, were approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Ethics Committee (ethical approval reference number CRE-2011.108) and the Kowloon West Cluster Clinical Ethics Committee (ethical approval reference number KW/EX/11-043 (37-17)) in Hong Kong.

2.2 | Genotyping

Blood was taken from each child and parent, and DNA was extracted. To amplify the *DRD4* exon 3 VNTR, 9 μL of PCR master mix—containing 5.5 μL water, 2 μL 5× Q solution (Qiagen), 1 μL of HotStarTaq DNA Polymerase 10× buffer (Qiagen), 0.05 μL each of 100 μM PCR primers GCGACTACGTGGTCTACTCG and AGGACCCTCATGGCCTTG, 0.2 μL of 10 mM dNTPs, and 0.2 μL of 5 units/μL HotStarTaq DNA Polymerase (Qiagen)—was added to each PCR tube, and then 1 μL of 50–300 ng/μL template DNA was added. Fresh aliquots (minimizing freeze-thaw cycles) of primers and dNTP, and good quality DNA (Swanson et al., 2007), aided successful PCR. Thermal cycling was performed with an initial denaturation step at 95°C for 15 min; then 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and finally 72°C for 10 min (Qian et al., 2018).

PCR products were observed by adding 1 μL of Midori Green Direct stain (Nippon Genetics), performing electrophoresis at 140 V for 30 min on 1% agarose gels in TBE, photographing gels under UV light, and comparing bands with a 100-bp ladder to determine the number of repeats, with 379 bp being 2R, 427 bp being 3R, 475 bp being 4R, and 523 bp being 5R. Slightly above the longer allele of heterozygotes was a third band, possibly a heteroduplex of the shorter and longer allele (Kaiser et al., 2002). A gel of several samples is shown in Figure 1. PCR was repeated on any

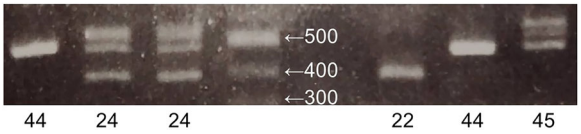

FIGURE 1 Gel illustrating genotyping of DRD4 exon 3 VNTR. Genotypes are shown below each lane. Bands in the ladder are labeled with their sizes in base pairs.

DNA sample producing an ambiguous genotype. Gels and TBE were re-used several times.

A total of 240 Chinese ADHD probands were successfully genotyped along with both of their parents. Information was available on 239 probands, aged between 6.2 and 13.0 years (mean = 9.3, SD = 1.6). By P-DISC-4 criteria, 66.1% were combined type, 21.3% were inattentive type, 6.3% were hyperactive-impulsive type, and 6.3% had no type classification.

For the haplotype relative risk (HRR) test, 240 pseudo-control genotypes were synthesized by selecting, for each parent, the allele that was not passed to the child. To determine alleles for transmission disequilibrium testing (TDT), for each parent a code was constructed, with the first digit being the allele transmitted to the child (2R, 3R, or other) and the second digit being the allele not transmitted to the child; these were counted in a table whose rows were transmitted alleles and whose columns were nontransmitted alleles, and McNemar's chi-square was calculated for 2R versus other alleles.

2.3 | Meta-analysis

We performed a literature search in PubMed for each of the two meta-analyses: one for ADHD and DRD4 VNTR with TDT data in Asians, and the other for ADHD and DRD4 VNTR with genotype data for the inattentive type. We repeatedly searched using different keywords until no additional studies appeared. For the latter meta-analysis, we recorded whether each study used TDT or case-control design. For each study, we noted the geographic origin of subjects and recorded the number of common alleles—2R, 4R, and 7R—and other alleles among transmitted alleles or in cases, and also among nontransmitted alleles or in controls, yielding six Asian ADHD TDT studies and six inattentive ADHD studies (Figure 2). Adding our own data produced seven studies to consider for each meta-analysis (Table 2).

Examination of data for inconsistencies revealed two studies in the inattentive ADHD meta-analysis. Rather than contacting the authors for clarification, we resolved the discrepancies as follows. The first study (Qian et al., 2004) displayed a table of allele and genotype frequencies, with the allele frequencies that could be calculated from the displayed genotype frequencies differing from the displayed allele frequencies. We supposed that the genotype frequencies were closer to the raw data and were thus more likely to be correct; therefore, we used allele frequencies that we calculated from displayed genotype frequencies. The second study with inconsistencies (ElBaz Mohamed et al., 2017) reported that 2R allele frequencies were 44% in patients and 0% in controls; such an extreme difference suggests technical error or ethnic stratification. Hardy–Weinberg equilibrium analysis (lumping non-2R alleles together) gave $p = 6e\text{-}6$ for patients and $p = 1.0$ for controls. In controls, the paper reported that 3R and 5R comprised 60% of alleles, far higher than any other ethnic group in a worldwide survey of this variant (the closest were Finns, at 17%) (Chang et al., 1996). Thus we omitted this study from meta-analysis. For other studies, we did not test for deviation from Hardy–Weinberg equilibrium because only allele data were available for the TDT study used in the meta-analysis of Asians, and genotype data were not published for three of the studies in the meta-analysis of inattentive ADHD. We therefore used five published studies and our own data for this meta-analysis.

Meta-analysis was performed with the metafor software package (Viechtbauer, 2010). The I^2 and Q tests were performed to detect between-study heterogeneity. Both the Mantel–Haenszel fixed-effects model and random-effects model were used. Neither adjustment for environmental effects, sensitivity analysis, nor assessment for the effects of population stratification were conducted since environmental effects or population stratification were not apparent. We completed the Annals of Human Genetics checklist required for meta-analysis reports.

3 | RESULTS

3.1 | This study

Genotypes of the DRD4 exon 3 VNTR are shown in Table 1 for ADHD children and their biological parents. The 4R allele was most common, followed by 2R, while other alleles, including the 7R allele (which is the most common allele in Europeans), were rare.

Examining whether the predominant minor allele, 2R, was associated with risk of ADHD, HRR and TDT analysis did not demonstrate a significant effect. Performing association analysis with subgroups (not shown) revealed a tendency toward association with the inattentive type (Table 1), particularly when using stringent criteria to classify patients as the inattentive type: classification by parents and the Chinese version of the Strengths and

TABLE 1 DRD4 exon 3 VNTR genotype and allele frequencies for ADHD parent-child trios.

Genotypes	All			Inattentive			Inattentive +		
or alleles	Parents	ADHD subjects	Pseudocontrols	ADHD subjects	Pseudocontrols	Noninattentive	ADHD subjects	Pseudocontrols	Nonin.+
22	23 (5%)	11 (5%)	10 (4%)	2 (4%)	1 (2%)	9 (5%)		1 (3%)	11 (5%)
23	3 (1%)								
24	126 (26%)	62 (26%)	72 (30%)	10 (19%)	17 (33%)	52 (28%)	6 (16%)	14 (38%)	56 (28%)
25	4 (1%)	2 (1%)	1 (0%)			2 (1%)			2 (1%)
34	7 (1%)	6 (3%)	4 (2%)			6 (3%)			6 (3%)
44	303 (63%)	151 (63%)	146 (61%)	38 (73%)	34 (65%)	113 (60%)	29 (78%)	22 (59%)	122(60%)
45	14 (3%)	8 (3%)	7 (3%)	2 (4%)		6 (3%)	2 (5%)		6 (3%)
Total	480	240	240	52	52	188	37	37	203
2	179 (19%)	86 (18%)	93 (19%)	14 (13%)	19 (18%)	72 (19%)	6 (8%)	16 (22%)	80 (20%)
3	10 (1%)	6 (1%)	4 (1%)			6 (2%)			6 (1%)
4	753 (78%)	378 (79%)	375 (78%)	88 (85%)	85 (82%)	290 (77%)	66 (89%)	58 (78%)	312(77%)
5	18 (2%)	10 (2%)	8 (2%)	2 (2%)		8 (2%)	2 (3%)		8 (2%)
Total	960	480	480	104	104	376	74	74	406
HRR: OR (95% CI), P		0.91 (0.66–1.3) 0.6		0.70 (0.33–1.5) 0.3			0.32 (0.12–0.87) 0.03		
TDT: OR (95% CI), P		0.90 (0.64–1.3) 0.6		0.60 (0.26–1.4) 0.3			0.33 (0.12–0.92) 0.04		

Note: "Inattentive +" is ADHD classed as inattentive by parents and the Chinese version of the Strengths and Weaknesses of ADHD Symptoms and Normal Behaviors (TSWAN). For analysis of 2R allele versus others, HRR is haplotype relative risk, TDT is transmission disequilibrium test with Yates continuity correction, OR is odds ratio, and CI is confidence interval.[Correction added on 02/05/2024, after first online publication: column headings have been realigned for accuracy in this version]

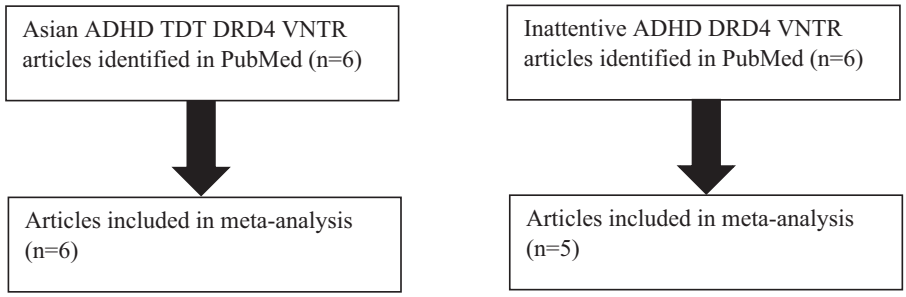

FIGURE 2 Flow diagram of search strategy of the meta-analyses.

Weaknesses of ADHD Symptoms and Normal Behaviors (TSWAN). This tendency toward association of the 2R allele was consistent whether inattentive ADHD children were compared with their own parents or with children with ADHD of other types: OR = 0.66 (95% confidence interval [95% CI]: 0.35–1.2), $p = 0.2$ for inattentive ADHD; OR = 0.36 (95% CI: 0.15–0.86), $p = 0.02$ when using stringent criteria.

3.2 | Meta-analysis

3.2.1 | 2R and ADHD in Asians

Because allele frequencies in our data were similar to those of other studies in Asians, with the 4R allele being the most common and the 2R allele the most common minor allele, we conducted a meta-analysis examining the association of the 2R allele with ADHD in Asians. Because we performed TDT analysis, we limited our meta-analysis to studies reporting TDT analysis. Including our data, we found seven studies, totaling 1329 transmitted and 1329 nontransmitted alleles (Tables 2 and 3).

The odd number of alleles was due to one study (Kim et al., 2005) which could not genotype one of the alleles of one patient as an integral-numbered allele (it was genotyped as "4.5R").

We examined heterogeneity among the studies using I^2 and Q tests (Table 3). Meta-analysis using neither the fixed-effects nor random-effects models revealed significant association of the 2R allele with ADHD (Table 3 and Figure 3).

Among those subjects with ADHD, the proportion of males was over 80% in all studies.

3.2.2 | 2R and inattentive ADHD

Since our data suggested an association of the 2R allele with reduced risk of inattentive ADHD, we conducted a meta-analysis examining the association of the 2R allele with inattentive ADHD. However, many studies did not report data on the types of ADHD, or on the 2R allele (instead, only reporting the presence or absence of the 7R allele). Therefore, few studies reported the data required for our meta-analysis. We thus sought to maximize statistical power by including all studies, regardless of analysis type or ethnicity. Including our data, we found seven studies, totaling 702 case alleles and 1420 control alleles (Tables 2 and 3).

One paper reported numbers of alleles that did not match alleles counted from genotypes; therefore, we used alleles counted from genotypes (Qian et al., 2004). Another paper reported that 2R allele frequencies were 44% in patients and 0% in controls; such an extreme difference suggests error, such as in genotyping or in collecting controls of the same ethnicity as the patients, thus we omitted this study from meta-analysis (ElBaz Mohamed et al., 2017).

We examined heterogeneity among studies using I^2 and Q tests (Table 3). For the odds ratio of the association of the 2R allele with ADHD, the 95% CI was 0.59–1.03 for the fixed-effects model and 0.57–1.14 for the random-effects model (Table 3 and Figure 4).

Among those subjects with ADHD, most studies did not specify the proportion who were males.

3.2.3 | 7R and inattentive ADHD

Because functional research suggested that both the 7R and 2R forms of DRD4 are less active than the 4R form (Asghari et al., 2002; Oak et al., 2000; Wang et al., 2004), we investigated whether the 7R allele displayed an association with reduced risk of inattentive ADHD, similar to the association we observed with the 2R allele. Table 2 displays all the identified studies which reported DRD4 VNTR alleles in inattentive ADHD. Only two studies reported more than zero alleles in both cases and controls, therefore we did not attempt meta-analysis.

TABLE 2 Basic characteristics of eligible studies.

Author year	Origin	Study	2	4	7	Total	2	4	7	Total
Asian ADHD TDT studies			Transmitted alleles				Nontransmitted alleles			
(Qian et al., 2004)	Beijing	TDT	47	250	0	320	45	256	0	320
(Brookes et al., 2005)	Taipei	TDT	46	149	0	198	48	146	0	198
(Kim et al., 2005)	Korea	TDT	31	45	0	89	34	37	1	89
(Cheuk et al., 2006)	Hong Kong	TDT	13	102	1	122	16	94	1	122
(Das et al., 2011)	Kolkata	TDT	13	22	4	54	15	27	2	54
(Leung et al., 2017)	Hong Kong	TDT	22	41	0	66	13	51	0	66
This study	Hong Kong	TDT	86	378	0	480	93	375	0	480
Inattentive ADHD studies			Case alleles				Control alleles			
(Rowe et al., 1998)	US	CC	3	48	20	74	13	80	15	116
(Todd et al., 2001)	Missouri	TDT	19	44	37	108	13	51	32	108
(Qian et al., 2004)	Beijing	CC[a]	51	269	0	330	64	263	0	330
(Cheuk et al., 2006)	Hong Kong	CC	1	29	1	32	23	100	0	128
(ElBaz Mohamed et al., 2017)	Cairo	CC[a]	27	13	0	62	0	31	9	100
(Hong et al., 2018)	Korea	CC	12	49	0	64	112	498	2	644
This study	Hong Kong	TDT	12	81	0	94	18	76	0	94

[a]Inconsistent allele counts in paper.

CC, case-control; TDT, transmission disequilibrium test.

TABLE 3 Results of meta-analysis of association between DRD4 exon 3 VNTR and ADHD.

Analysis (number of studies)	Case/control	Model	OR (*p*)	95% CI	Heterogeneity
2R vs. others in Asians; TDT (7)	1329/1329	Fixed-effects	0.97 (0.77)	0.80–1.18	$Q = 4.2$, $p = 0.65$; $I^2 = 0\%$
		Random-effects	0.97 (0.76)	0.80–1.18	$\tau^2 = 0.00$ (95% CI: 0.00, 0.36)
2R vs. others in inattentive (6)	702/1420	Fixed-effects	0.78 (0.08)	0.59–1.03	$Q = 8.7$, $p = 0.12$; $I^2 = 43\%$
		Random-effects	0.81 (0.23)	0.57–1.14	$\tau^2 = 0.037$ (95% CI: 0.0, 3.8)

Note: Q is Cochran's heterogeneity test statistic; I^2 is a measure of heterogeneity defined as total heterogeneity divided by total variability; τ^2 is estimated variance in OR across studies.

OR, odds ratio; CI, confidence interval; TDT, transmission disequilibrium test.

4 | DISCUSSION

The 7R allele increases ADHD risk compared to 4R, the most common allele, according to published studies (Bonvicini et al., 2020). Several studies reported that the D4 receptor produced by the 7R allele, compared to that produced by the 4R allele, is less responsive to dopamine (Asghari et al., 2002; Oak et al., 2000; Wang et al., 2004). The 2R form of the receptor also may be less responsive to dopamine (Asghari et al., 2002; Wang et al., 2004), and LD of flanking sequence variants of the DRD4 exon 3 VNTR suggests 2R is derived from the 7R allele, therefore one might expect that the 2R allele also increases ADHD risk. But another study reported that the 7R form may be more responsive to dopamine than 4R, while not reporting on 2R (Ferré et al., 2022).

However, no major association of ADHD with 2R, compared to 4R, was seen in our study (OR 95% CI: 0.64–1.3 for TDT) or in meta-analysis (OR 95% CI: 0.80–1.18) (Table 3 and Figure 3). A possible explanation is that the 2R receptor is much closer to the dopamine responsiveness of 4R protein than is the 7R, making any effect of 2R on ADHD risk much smaller and harder to detect (Asghari et al., 2002; Wang et al., 2004).

But examining only the inattentive type of ADHD showed a trend toward a protective effect of 2R (OR 95% CI: 0.26–1.4 for TDT), which was more prominent when using a more restricted definition of inattentive ADHD (OR 95% CI: 0.12–0.92 for TDT). Meta-analysis of only the inattentive type of ADHD found a trend consistent with this protective effect of 2R (OR 95% CI: 0.59–1.03 or 0.57–1.14; Table 3 and Figure 4). Perhaps DRD4 plays a larger role in this type of ADHD than in other types.

The 7R allele is positively associated with impulsivity, promiscuity, gambling, and even distance that ethnic groups had migrated; thus 7R may be related to short time orientation (Chen et al., 1999; Chen & Moyzis, 2018; Minkov & Bond, 2015). We therefore looked at the role of

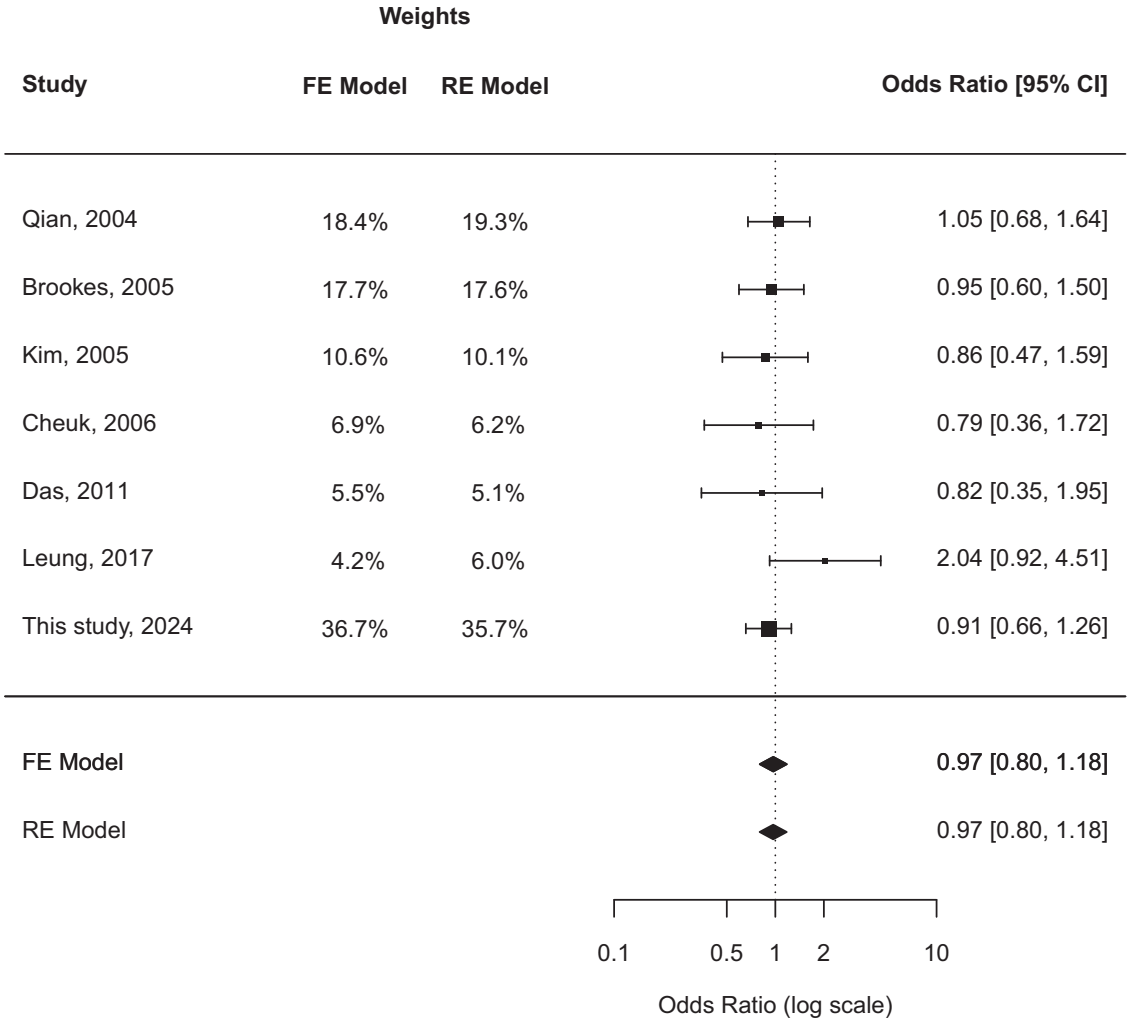

FIGURE 3 Forest plot of the association between the 2R allele and ADHD in Asians. FE, fixed-effects; RE, random-effects; CI, confidence interval.

7R in inattentive ADHD. However, although many studies examined 7R in ADHD, too few studies have so far reported their data in the inattentive type to draw a conclusion (Table 2).

Potential limitations of the meta-analyses were the small number of studies, the mixing of TDT and case-control studies, the mixing of ethnic populations varying widely in allele frequencies, the mixing of the sexes and predominance of males, and classification procedures for ADHD and inattentive type that may vary among studies. However, heterogeneity analysis did not show significant heterogeneity among studies, and both random-effects and fixed-effects models produced similar results, suggesting that the papers we included may be successfully meta-analyzed despite their differing characteristics.

It would be useful for studies to stratify results by sex so that meta-analysis could be performed separately for males and females. However, in clinic-derived samples, females comprise a small proportion of ADHD patients, less than 20% in the studies comprising our first meta-analysis, limiting the power of analysis in females. In contrast, in community epidemiological surveys, females comprise around ¼ to 1/3 of people with ADHD, and perhaps somewhat more for inattentive ADHD (Ayano et al., 2023; Willcutt, 2012), which may be less likely to cause disruption and to be referred to the clinic. Studies based on population cohorts may be required to achieve adequate statistical power to analyze males and females independently, which might reveal informative differences between sexes in genetic associations.

While our meta-analysis of the inattentive type might have been able to detect a large effect size, additional samples are needed in order to observe a modest effect size. Therefore, it would be interesting for authors of DRD4 VNTR genotyping studies who did not report

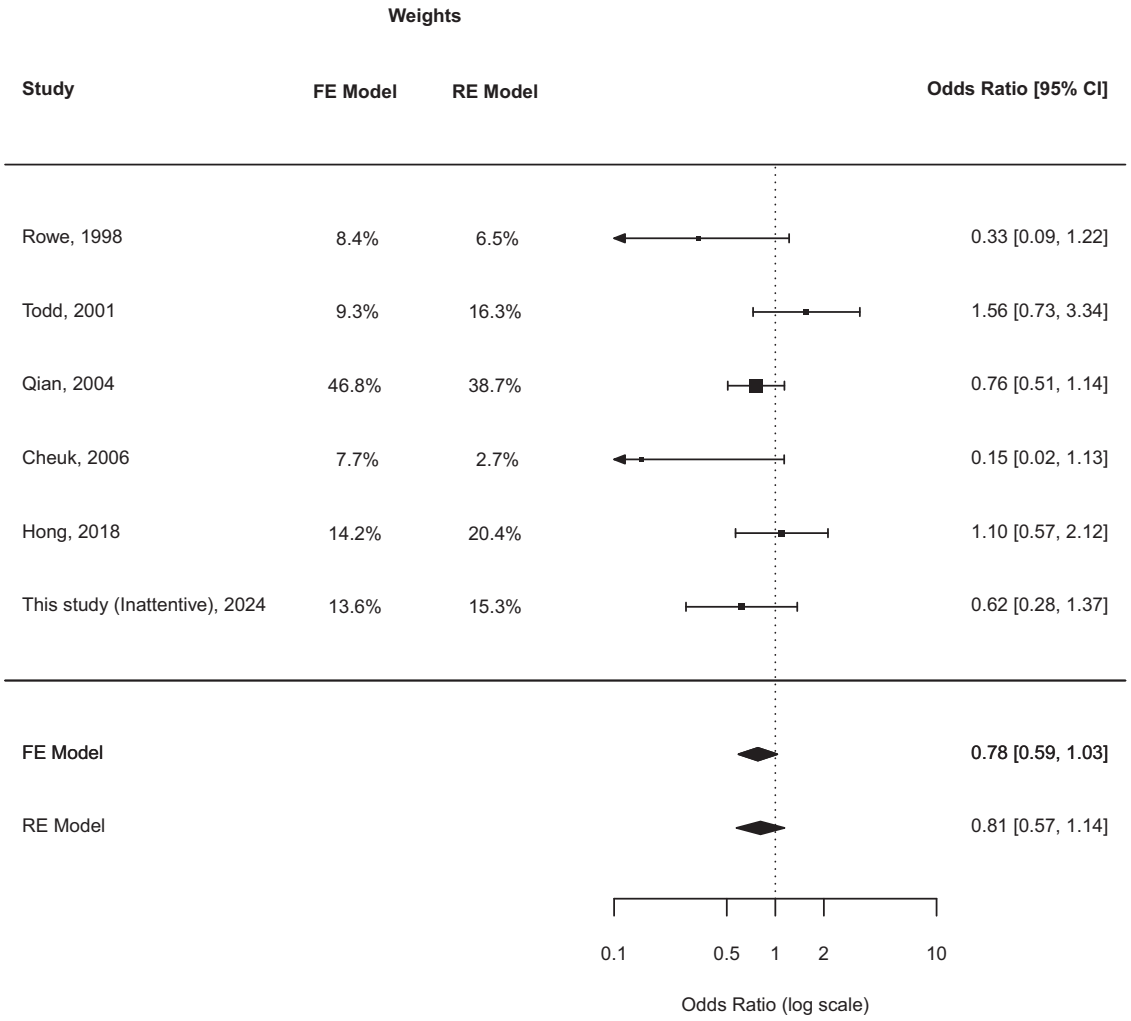

FIGURE 4 Forest plot of the association between the 2R allele and inattentive ADHD. FE, fixed-effects; RE, random-effects; CI, confidence interval.

alleles stratified by type of ADHD, or who did not report 2R alleles, but who do have data on ADHD type and complete data on genotype frequency, to now analyze and publish the allele association in inattentive ADHD. These additional data would increase the power of future meta-analysis to settle the question of whether 2R protects against inattentive ADHD.

AUTHOR CONTRIBUTIONS

Larry Baum designed the study, genotyped samples, analyzed data, searched the literature, performed statistical analysis, and wrote the article. Chi Chiu Lee recruited participants, genotyped samples, and revised the article. Rui Ye, James M Swanson, and Robert K Moyzis revised the article. Se Fong Hung, Chun Pan Tang, and Ting Pong Ho recruited participants and revised the article. Pak-Chung Sham designed the study and revised the article. Patrick Wing-Leung Leung recruited participants, designed the study, and revised the article.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PATIENT CONSENT STATEMENT

Parents who agreed to join the study gave informed written consent for their own involvement and for their children.

DATA AVAILABILITY STATEMENT

Our genotype data are available in Table 1. Data for meta-analyses are from published papers available from https://pubmed.ncbi.nlm.nih.gov/.

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