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O TRANSTORNO DE DÉFICIT DE ATENÇÃO E HIPERATIVIDADE (TDAH): ESTUDO FUNCIONAL E DE ASSOCIAÇÃO COM O GENE *DRD4*

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Ao Fábio, pelo companheirismo, paciência e apoio
incondicional, mesmo à distância.

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RESUMO

O transtorno de déficit de atenção e hiperatividade (TDAH) é um dos transtornos psiquiátricos mais freqüentes da infância e adolescência, sendo caracterizado por sintomas de desatenção, hiperatividade e impulsividade. A contribuição genética na etiologia do TDAH é uma das mais altas já verificadas para transtornos psiquiátricos, com herdabilidade média estimada de 76%. Dentre os fatores genéticos que contribuiriam para o desenvolvimento da doença, genes que codificam componentes do sistema dopaminérgico estão entre os principais candidatos. Entre estes, o gene que codifica o receptor D4 de dopamina (*DRD4*) é o loco mais intensamente investigado nos estudos moleculares com o TDAH. O polimorfismo mais estudado no *DRD4* é um VNTR de 48 pb localizado no exon 3; porém outros polimorfismos, localizados na região promotora do gene – uma duplicação de 120pb e os SNPs -521C>T e -616C>G – também vêm sendo propostos como polimorfismos de suscetibilidade ao TDAH. Além desses, novas variantes em regiões regulatórias do gene, os SNPs rs11246227 e rs11246228, foram observados recentemente em associação com sintomas de desatenção do TDAH. O objetivo geral do presente trabalho foi aumentar a compreensão acerca da participação do gene *DRD4* na etiologia do TDAH na nossa população. Para tanto, foi testada inicialmente a possibilidade de associação do SNP rs11246227, sendo em seguida investigado o significado funcional dos SNPs rs11246227 e rs11246228, e sua possível relação com a doença, através de ferramentas de bioinformática. O estudo de associação foi realizado em uma amostra composta por 478 pacientes com TDAH, diagnosticados segundo os critérios do DSM-IV, e seus pais biológicos. O rs112466227 foi investigado por abordagens baseada em família (FBAT) e dimensional (PBAT, ANOVA). A possibilidade de desequilíbrio de ligação (DL) com polimorfismos previamente investigados na presente amostra foi estimada pelo programa MLocus. A análise *in silico* foi realizada utilizando diferentes bases de dados genômicos e programas de predição de sítios alvo para miRNAs e de funcionalidade. A análise pelo FBAT mostrou um desvio significativo da transmissão do alelo C nos pacientes do subtipo desatento. Foram observadas evidências de DL com a duplicação de 120bp e com o VNTR do exon 3. As análises de bioinformática mostraram que os SNPs rs11246227 e rs11246228 estão localizadas na região 3' do gene *DRD4*, e não na região 5', como previamente descrito. Diferenças entre os alelos, com perda ou ganho de sítios de ligação para diferentes miRNAs, foram detectados em ambos os SNPs pelos programas *MicroInspector*,

smiRNAdb e *miRecords*, e apenas no rs11246227 pelos programas *Human miRNA Target* e *Mirò*. A grande variabilidade e a complexidade genética marcante do gene *DRD4* aliada à heterogeneidade fenotípica do TDAH provavelmente contribuíram para nossos resultados de associação, divergentes dos descritos na literatura, os quais necessitam de replicação em estudos futuros. Nossos achados em bioinformática sugerem um possível envolvimento dos SNPs investigados com a ligação de miRNAs relacionados aos processos de neurogênese e neuroplasticidade. Genes envolvidos com estes processos vêm sendo identificados nos *genome-wide association studies* realizados com o TDAH, o que apóia nossos resultados *in silico*. Entretanto, mais estudos funcionais são necessários, tanto *in silico* como *in vitro*, para esclarecer o envolvimento dos polimorfismos analisados na regulação da expressão do gene *DRD4* via miRNAs e, consequentemente, do possível efeito desses elementos na etiologia da doença.

Palavras-chave: *DRD4,* TDAH, suscetibilidade, estudo de associação, estudo funcional, bioinformática, miRNAs.

ABSTRACT

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common psychiatric disorders of childhood and adolescence, characterized by inattentive, hyperactive and impulsive symptoms. Genetic contribution to ADHD etiology is one of the highest ever recorded for psychiatric disorders, with a mean heritability of 76%. Among genetic factors that could contribute to disorder development, genes encoding components from dopaminergic system are the main candidate. Of these, the dopamine D4 receptor gene (*DRD4*) is the most extensively investigated locus in molecular studies of ADHD. The most studied polymorphism in *DRD4* gene is a variable number of tandem repeats (VNTR) of 48bp, located at exon 3, although other polymorphisms, located in promoter region – a 120bp duplication and the SNPs -521C> T and-616C> G – have also been proposed as susceptibility polymorphisms for ADHD. In addition, new variants in regulatory regions, the SNPs rs11246227 and rs11246228, have recently been associated with inattentive symptoms of the disorder. The overall objective of this study was to increase the understanding on the involvement of *DRD4* gene in ADHD etiology in our population. For this purpose, the possibility of association with the SNP rs11246227 was initially tested, being afterwards investigated the functional effect of both rs11246227 and rs11246228 and their possible relation to ADHD through bioinformatics approach. The association study was performed in a sample composed by 478 ADHD patients, diagnosed according to DSM-IV criteria, and their biological parents. The rs112466227 was investigated by both family-based (FBAT) and dimensional (PBAT, ANOVA) approaches. The possibility of linkage disequilibrium (LD) with polymorphisms previously investigated in the present sample was estimated by MLocus software. *In silico* analysis was conducted using different genomic databases and programs to predict miRNA target sites and functionality. FBAT analysis showed a significant excess of C allele transmission in inattentive subtype patients. Evidences of LD with both 120bp tandem duplication and exon 3 VNTR were observed. Bioinformatics analyses showed that both SNPs rs11246227 and rs11246228 are located in the 3' region of *DRD4* gene, and not at 5' region, as previously described. Differences between alleles, with loss or gain of binding sites, were detected in both SNPs by *MicroInspector*, *smiRNAdb* and *miRecords*, and only in rs11246227 by *Human miRNA Targets* and *miRò*. *DRD4* huge variability and marked genetic complexity allied to ADHD phenotypic heterogeneity might have contributed to our

association results, distinct from the ones reported in literature, what needs to be replicated in future studies. Our bioinformatics findings suggest a possible involvement of investigated SNPs in binding properties of miRNAs related to processes of neurogenesis and neuronal plasticity. Genes involved in these processes have been identified in ADHD *genome-wide association studies*, reinforcing our *in silico* results. However, new functional studies, using both *in silico* and *in vitro* approaches, are needed to clarify the involvement of the investigated polymorphisms in *DRD4* expression control mediated by miRNAs and, consequently, the possible effect of these elements in ADHD etiology.

Keywords: *DRD4,* ADHD, susceptibility, association study, functional study, bioinformatics, miRNA.

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I. Introdução

1. Caracterização clínica e epidemiológica do TDAH

O transtorno de déficit de atenção e hiperatividade (TDAH) é um dos transtornos psiquiátricos mais comuns da infância e adolescência, afetando em torno de 5% das crianças em idade escolar (Polanczyk e cols., 2007) e sendo cerca de quatro vezes mais comum em meninos do que em meninas em amostras clínicas (Rohde e Halpern, 2004, Spencer e cols., 2007). Trata-se de uma patologia bastante heterogênea clinicamente, cujos sintomas, definidos pela quarta edição do Manual de Diagnóstico e Estatística de Transtornos Mentais, o DSM-IV (American Psychiatric Association, 1994), dividem-se nas áreas de desatenção e hiperatividade-impulsividade. Partindo dessa classificação, são reconhecidos três tipos clínicos do transtorno, conforme a presença de um mínimo de seis sintomas em uma ou ambas as áreas: predominantemente desatento, predominantemente hiperativo-impulsivo, e combinado. Esses tipos diferem quanto à freqüência – na maioria das amostras de pacientes referidos, o tipo combinado é o mais comum, o tipo desatento é intermediário, e o tipo hiperativo-impulsivo o menos freqüente – e quanto a características como prevalência entre os sexos, idade dos probandos, idade de início da doença, características clínicas e prognóstico (Rohde e Halpern, 2004; Spencer e cols., 2007).

Embora tenha sido considerado por muito tempo uma doença tipicamente infantil, o TDAH pode persistir até a adolescência ou idade adulta em cerca de 50% dos casos com início na infância (Biederman e cols., 2007; Lara e cols., 2009). Enquanto os sintomas de hiperatividade e impulsividade tendem a diminuir mais precocemente, os de desatenção, desorganização e distração são mais persistentes (Acosta e cols., 2004). Em geral, a continuidade dos sintomas se associa a uma série de disfunções significativas na vida do indivíduo, como dificuldades emocionais, de relacionamento e de ajustamento social, falhas acadêmicas e ocupacionais (Wilens e Dodson, 2004; De Graaf e cols., 2008).

Uma proporção significativa de crianças e adolescentes com TDAH apresenta comorbidades com outros transtornos psiquiátricos, como transtorno de oposição-desafio ou de conduta (em 50% dos casos de TDAH), transtornos de ansiedade (25% dos casos), de

humor (15-30% dos casos) e de aprendizado (20-30% dos casos) (Jensen e cols, 2001; Acosta e cols., 2004). Também é freqüente a ocorrência de comportamentos delinqüentes em adolescentes com TDAH (Acosta e cols., 2004; Cormier, 2008). Entre estes pacientes, o tabagismo atinge uma prevalência em torno de 30% em ambos os sexos (Sullivan e Rudnik-Levin, 2001; Galera e cols., 2005; McClernon e Kollins, 2008), estando geralmente associado ao uso de drogas ilícitas (Wilens e Dodson, 2004). Nos adultos com TDAH, além de problemas por abuso ou dependência de substâncias, é comum personalidade anti-social (Acosta e cols., 2004; Newcorn, 2008; Stein, 2008). A presença de comorbidades parece influenciar na continuidade dos sintomas, além de contribuir para o desenvolvimento de psicopatologias graves em idades posteriores (Wilens e Dodson, 2004; Newcorn, 2008).

2. Etiologia do TDAH

O TDAH é considerado um fenótipo complexo e de herança multifatorial, necessitando da participação tanto de genes como de agentes ambientais para a manifestação dos sintomas (Wallis e cols., 2008; Stergiakouli e Thapar, 2010). Conforme sugerido pela maioria dos estudos, a contribuição ambiental é devida principalmente a fatores não compartilhados (Waldman e Gizer, 2006; Wallis e cols., 2008; Stergiakouli e Thapar, 2010). Dentre os fatores que parecem ser importantes para o surgimento e manutenção da doença, podem ser ressaltados agentes psicossociais que atuam no funcionamento adaptativo e saúde emocional geral da criança, como desentendimentos familiares e baixo nível sócio-econômico (Counts e cols., 2005; Banerjee e cols., 2007). Além disso, complicações pré, peri e neonatais, tais como eclampsia, bem como a exposição intraútero à nicotina, parecem contribuir para o surgimento da doença (Banerjee e cols., 2007).

A contribuição genética no desenvolvimento do TDAH, por outro lado, é uma das mais altas já verificadas para transtornos psiquiátricos (Mick e Faraone, 2008). Os diferentes estudos de gêmeos já realizados estimaram uma herdabilidade de 76% para esse transtorno (Biederman e Faraone, 2005; Mick e Faraone, 2008). Estudos com famílias e adotados mostraram uma prevalência da doença entre pais biológicos de crianças com TDAH cerca de três vezes maior do que entre pais adotivos, e um risco para a doença de duas a oito vezes maior nos pais das crianças afetadas do que na população em geral (Mick e Faraone, 2008;

Stergiakouli e Thapar, 2010). Embora já tenha sido sugerida a hipótese de um gene principal contribuindo para o TDAH em algumas análises de segregação complexa (Wallis e cols., 2008), acredita-se que a alta influência genética na variância deste fenótipo se deva à participação de muitos genes de pequeno efeito conferindo suscetibilidade ao TDAH, além de uma possível influência de genes modificadores. Assim, o desenvolvimento do transtorno dependeria da interação desses genes entre si e com diversos fatores ambientais (Waldman e Gizer, 2006; Wallis e cols., 2008; Smith e cols., 2009; Stergiakouli e Thapar, 2010). Embora a maioria dos estudos tenha sugerido apenas efeitos genéticos aditivos (Martin, 2005), o estudo de Hudziak e cols. (2005) separa a contribuição genética em um componente dominante e um componente aditivo, onde cada um deles explicaria 48% e 30% da variância, respectivamente, indicando a possibilidade de uma maior complexidade na contribuição genética para o TDAH.

A busca pelos genes de suscetibilidade ou modificadores no TDAH tem utilizado como estratégias principais a investigação de genes candidatos, com base em evidências de estudos neurobiológicos, e as varreduras genômicas, onde tanto a possibilidade de associação como de ligação são verificadas (Wallis e cols., 2008; Franke e cols., 2009; Smith e cols., 2009). A hipótese de que os sintomas do TDAH se originam em disfunções no funcionamento cerebral é amplamente aceita na literatura. Dados de estudos de neuroimagem sugerem que o TDAH seja uma doença fronto-estriato-cerebelar, uma vez que tais regiões parecem ter volume e atividade diminuídos em pacientes com essa patologia (Curatolo e cols., 2009, Makris e cols., 2009). Estes resultados são corroborados por estudos neuropsicológicos, que demonstraram que crianças com TDAH têm desempenho prejudicado em processos relacionados ao lobo frontal e áreas subcorticais, tais como atenção, percepção, planejamento e organização, além de falhas na inibição comportamental (Arnsten e Li, 2005; Nigg, 2005; Makris e cols., 2009). Considerando que essas regiões são primariamente inervadas por catecolaminas, genes que codificam componentes desses sistemas de neurotransmissores estão entre os principais candidatos para estudos moleculares com o TDAH (Arnsten e Li, 2005; Curatolo e cols., 2008).

3. O sistema dopaminérgico e o TDAH

A primeira teoria bioquímica proposta para explicar o desenvolvimento do TDAH foi a hipótese dopaminérgica. Esta hipótese foi formulada a partir de estudos farmacológicos e com animais, os quais sugeriram que uma deficiência de dopamina nas regiões corticais e no estriado seria responsável pela manifestação dos sintomas do transtorno (Levy, 1991; Makris e cols., 2009). Entretanto, investigações posteriores indicaram um envolvimento mais complexo desse sistema na etiologia do TDAH, possivelmente com diferentes alterações nos níveis de transmissão dopaminérgica em cada área implicada, as quais refletiriam em déficits cognitivos e nas funções executivas, e nos sintomas de hiperatividade motora e impulsividade (Levy e Swanson, 2001; Sagvolden e cols., 2005).

A ação dos fármacos comumente utilizados no tratamento do TDAH também evidencia a participação do sistema dopaminérgico. Vários estudos têm documentado a grande eficácia dos estimulantes em reduzir os sintomas e melhorar a função em uma série de domínios (The MTA Cooperative Group, 2004). Essa classe de fármacos é a primeira escolha para tratamento, sendo efetiva em cerca de 70% dos pacientes (The MTA Cooperative Group, 2004; Martin, 2005). O estimulante mais prescrito na prática clínica é o metilfenidato, que parece atuar como um agonista indireto das rotas catecolaminérgicas centrais, facilitando a ação da dopamina e da noradrenalina endógenas através do bloqueio de sua recaptação no neurônio pré-sináptico (Biederman e Spencer, 1999; Masellis e cols., 2002; Madras e cols., 2005). Especificamente, o metilfenidato tem uma alta afinidade pelo transportador de dopamina, inibindo essa proteína e, portanto, a recaptação da dopamina na fenda sináptica (Masellis e cols., 2002; Madras e cols., 2005). Considerando-se todas as evidências neurobiológicas e farmacológicas, genes que codificam componentes do sistema dopaminérgico têm sido os principais alvos dos estudos moleculares com o TDAH (Biederman e Faraone, 2005; Waldman e Gizer, 2006; Thapar e cols., 2007).

4. TDAH e o gene *DRD4*

Dentre os componentes do sistema dopaminérgico, o gene que codifica o receptor D4 de dopamina (*DRD4*) é o loco mais intensamente investigado nos estudos moleculares com o TDAH (Gizer e cols., 2009; Banaschewski *et al*., 2010). O grande interesse pelo gene *DRD4* surgiu a partir da observação de sua associação com a dimensão de personalidade denominada "busca de novidades", provavelmente relacionada com o TDAH (Waldman e Gizer, 2006). Além disto, o produto deste gene concentra-se em áreas do cérebro cujas funções são relacionadas com sintomas da doença (Wallis *et al*., 2008).

O gene *DRD4* possui aproximadamente 4 kb, está localizado no cromossomo 11p15.5 e é um dos genes humanos mais polimórficos que se conhece (Gelernter e cols. 1992; Petronis e cols., 1993; Li e cols., 2006), como se pode visualizar na figura 1. O loco *DRD4* é responsável pela expressão de uma proteína transmembrana de sete domínios, o receptor do tipo 4 de dopamina (D4). Esse é um dos mais importantes entre os cinco tipos que compõem a classe de receptores dopaminérgicos (D1, D2, D3, D4, D5), principalmente devido à sua alta afinidade pela dopamina e pela clozapina, um antipsicótico atípico. O D4 está localizado nos neurônios pós-sinápticos do sistema dopaminérgico, sendo especialmente concentrado no córtex frontal e sistema límbico. Quando ativado, o receptor acopla-se a proteínas G inibitórias, que inibem a enzima adenilil ciclase, responsável pela conversão de ATP à AMP cíclico, dando prosseguimento à resposta intracelular (Van Tol e cols., 1991).

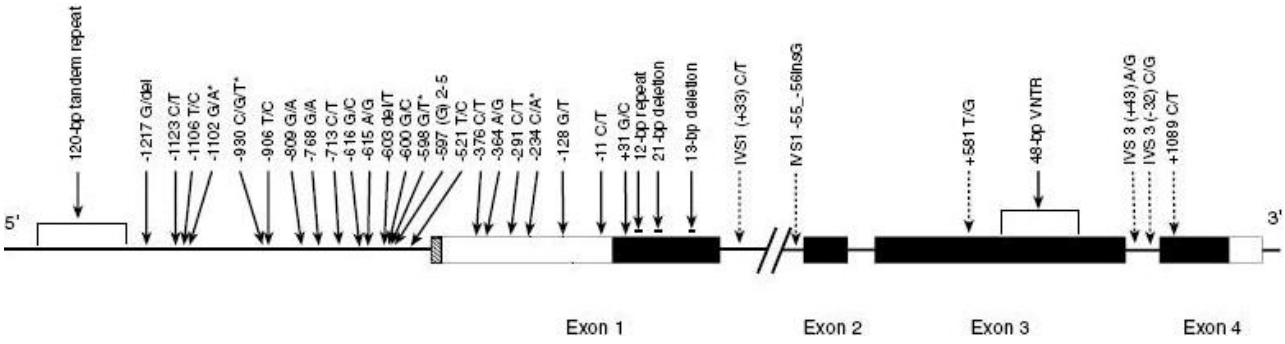

Figura 1- Representação esquemática de polimorfismos do gene *DRD4*. Em preto: regiões codificadoras; em branco: não codificadoras; em cinza, múltiplos sítios de iniciação. Adaptado de Mitsuyasu e cols. (2007).

O principal polimorfismo investigado no gene *DRD4* é um VNTR de 48 pares de bases (pb), localizado no terceiro exon do gene, região que supostamente codifica a terceira

alça intracelular do receptor. Acredita-se que seja através dessa alça que ocorra a ativação da resposta celular quando a dopamina se liga ao receptor extracelularmente (Waldman e Gizer, 2006). Onze alelos, contendo de duas a onze repetições, incluindo um alelo intermediário entre quatro e cinco repetições, já foram descritos (Wang e cols., 2004; Li e cols., 2006; Hattori e cols., 2009). LaHoste e colaboradores (1996), através de um estudo caso-controle, sugeriram que o alelo contendo sete repetições da sequência de 48 pb (alelo 7R), o mesmo associado com a dimensão busca de novidades, poderia ser um alelo de risco para o TDAH. Após este relato, diversos estudos foram realizados, utilizando diferentes estratégias e, embora várias investigações tenham replicado a associação com o gene *DRD4*, resultados negativos também são comuns (Mick e Faraone, 2008; Wallis *et al*., 2008). Por exemplo, Kirley e cols. (2004) obtiveram resultados negativos em suas amostras de TDAH como um todo, mas quando subgrupos particulares de pacientes foram considerados, tais como probandos com história familiar da doença, ou com transtorno de oposição-desafio comórbido, os resultados foram significantes. Apesar dessas inconsistências, uma metanálise confirmou um pequeno, mas significativo efeito desse polimorfismo no TDAH, com razão de chances de 1,45 (Intervalo de Confiança - IC - 95%: 1,27-1,65) para estudos de caso-controle e 1,16 (IC 95%: 1,03-1,31) para abordagens baseadas em famílias (Biederman and Faraone, 2005). Gizer e cols. (2009), em uma metanálise independente, corroboraram esses resultados, demonstrando uma razão de chances de 1,33 (IC 95%: 1,15-1,54) para o mesmo alelo, o alelo 7R. Com base nesses dados, o gene *DRD4* tem sido considerado um gene de suscetibilidade ao TDAH (Mick e Faraone, 2008; Smith e cols., 2009).

Estudos realizados *in vitro* com o objetivo de se determinar o significado funcional do VNTR também vêm apresentando resultados divergentes. Asghari e cols. (1995) demonstraram que a dopamina, ao se ligar ao receptor codificado pelo alelo 7R, apresentava capacidade de inibir a formação de cAMP reduzida à metade, em comparação aos receptores correspondentes aos alelos 2R e 4R. Porém, Jovanovic e cols. (1999) não observaram diferenças marcantes entre esses alelos, enquanto Wang e cols. (2004) sugeriram que ambos os alelos 2R e 7R têm eficiência reduzida em relação ao 4R.

Um achado importante descrito por Gizer e cols. (2009) é a substancial heterogeneidade na magnitude de efeito desse polimorfismo no TDAH entre os diferentes estudos. Essa heterogeneidade marcante conduz à hipótese de que outros polimorfismos no locus *DRD4*, em desequilíbrio de ligação ou não com o VNTR de 48pb, poderiam contribuir com os efeitos desse gene no TDAH. Polimorfismos localizados em regiões regulatórias do

DRD4 tem sido particularmente investigados, uma vez que poderiam modificar a atividade transcricional do gene. Entre esses, o mais estudado é uma duplicação de 120pb localizada na região promotora do gene, sendo o alelo de 240pb sugerido como o alelo de risco para o TDAH na maioria dos estudos (McCracken e cols., 2000; Kustanovich e cols., 2004; Kereszturi e cols., 2007). Dois polimorfismos de nucleotideo único (SNPs) também localizados no promotor do gene, -616C>G (rs747302) e -521C>T (rs1800955), vêm sendo analisados em um pequeno número de estudos, relativamente recentes. Embora resultados controversos tenham sido observados para o -616C>G (Barr e cols., 2001; Lowe e cols., 2004), a maioria dos estudos com o -521C>T detectou resultados positivos (Bellgrove *et al*., 2005; Yang *et al*., 2008). O efeito potencial desta variante foi confirmado na metanálise de Gizer e cols. (2009), sendo a suscetibilidade ao TDAH conferida pelo alelo T, com razão de chances de 1,21 (IC 95%: 1,04-1,41).

A influência desses polimorfismos na funcionalidade do receptor também não é bem compreendida. Kamakura e cols. (1997) caracterizaram o promotor do gene *DRD4* em células de neuroblastoma e HeLa, identificando uma região entre os nucleotídeos -591 e -123 que provavelmente conferia expressão tecido-específica ao *DRD4*. Porém, tal efeito não foi confirmado por Kereszturi e cols. (2006), indicando a necessidade de estudos adicionais para explorar a possível influência dessa região na função do receptor. Okuyama e cols. (1999) avaliaram a influência do SNP -521C>T na expressão do *DRD4* em cultura de retinoblastoma humano, onde o gene é naturalmente expresso em abundância, e constataram que o alelo T, quando comparado ao C, seria responsável por uma atividade transcricional 40% menor. Contudo, Kereszturi e cols. (2006), ao tentarem replicar o possível efeito transcricional desse SNP usando um sistema repórter similar *in vitro*, não confirmaram essa diferença entre os alelos. O SNP -616C>G, por outro lado, teve a funcionalidade investigada somente através de ferramentas de bioinformática, tendo sido observadas diferenças potenciais entre os alelos em relação à ligação a fatores de transcrição, sugerindo um possível efeito do polimorfismo na atividade transcricional do gene (Barr e cols., 2001). Considerando a natureza altamente polimórfica do gene *DRD4*, que possui vários outros polimorfismos já identificados em regiões regulatórias, estudos abordando as diversas variantes e sua combinação em diferentes haplótipos podem ser necessários para o esclarecimento dos resultados contraditórios nos estudos de associação e de funcionalidade.

Além dos polimorfismos descritos, novos SNPs vêm sendo sugeridos em estudos mais recentes como passíveis de influência sobre a etiologia do TDAH. Um desses estudos é

o trabalho de Lasky-Su e cols (2008), cujo objetivo foi determinar se SNPs em genes candidatos ao TDAH estariam associados com fenótipos quantitativos gerados a partir de sintomas de desatenção e hiperatividade-impulsividade. Cento e quarenta e três SNPs foram selecionados em cinco genes: o gene do transportador de dopamina (*DAT1*), o gene do receptor D5 de dopamina (*DRD5*), o gene da Proteína 25 associada a sinaptossoma (*SNAP-25*), o gene do receptor 1B de serotonina (*HTR1B*), e o gene *DRD4*. O estudo utilizou como abordagem o Teste de Associação Baseado em Famílias - Componentes Principais (*Family-Based Association Test-Principal Components* - FBAT-PC), uma abordagem destinada a maximizar a informação genética quando múltiplos fenótipos são testados (Lange, van Steen *et al*. 2004). Duzentas e vinte e nove famílias com pacientes afetados por TDAH foram recrutadas através de diferentes estudos, sendo 90 obtidas a partir de um estudo longitudinal de famílias, 83 a partir de um estudo de ligação com pares de irmãos afetados com TDAH, 37 a partir de um estudo familiar de transtorno bipolar, 17 a partir de um estudo de famílias de adultos com TDAH, e 2 a partir de um estudo de TDAH e abuso de substâncias. Os SNPs adjacentes rs11246228 (hCV26775267, transição de T para C), e rs11246227 (hCV26775266, transição de C para T), localizados na região promotora do *DRD4*, demonstraram resultados significativos, sendo no rs11246228 o alelo C preferencialmente transmitido (P=0,012), e no rs11246227 o alelo T preferencialmente transmitido (P=0,012). Quando sintomas de desatenção foram usados para gerar um fenótipo univariado, utilizado na estatística FBAT-PC, a transmissão preferencial dos mesmos alelos se manteve significativa em ambos os SNPs (P=0,034 para o rs11246228 e P=0,017 para o rs11246227), enquanto que a significância foi perdida quando usados sintomas de hiperatividade-impulsividade. Da mesma forma, análises de correlação dos sintomas do TDAH com o fenótipo global evidenciaram as mais altas correlações com sintomas de desatenção, para ambos os polimorfismos. O estudo também verificou forte desequilíbrio de ligação (DL) entre os dois SNPs (D'=1,0, r2=1,0), e modesto DL dos mesmos com a variante de 7 repetições do VNTR do exon 3 (D'=0,93; r2=0,14).

Dessa forma, os achados de Lasky-Su e cols (2008) são consistentes com a hipótese do TDAH como uma doença geneticamente heterogênea, na qual diferentes polimorfismos contribuem para diferentes aspectos da doença. Estudos futuros são necessários para replicar os resultados de associação e examinar melhor a relação dos SNPs com o VNTR do exon 3, bem como investigar se esses SNPs possuem alguma relevância do ponto de vista funcional.

II. Justificativa

A elevada freqüência verificada para o TDAH na população aponta para a necessidade de estudos que visem a melhoria das condições de vida dos indivíduos com esta patologia. Nos Estados Unidos, o TDAH foi considerado prioridade em termos de saúde pública (National Institute of Health, 2000) devido aos prejuízos consideráveis que afetam as áreas acadêmica, social e emocional do indivíduo, ao alto nível de estresse gerado nas famílias e ao grande impacto que causa na sociedade (Barkley, 2002). Nesse sentido, um maior conhecimento acerca dos fatores genéticos relacionados à etiologia do TDAH é fundamental para melhor compreensão de todas as manifestações clínicas, o que poderá contribuir para a redução do impacto negativo não somente sobre os pacientes e suas famílias, mas também sobre a sociedade. Além disso, as informações genéticas poderão auxiliar o clínico a detectar mais precocemente a vulnerabilidade ao TDAH, podendo-se desenvolver, assim, estratégias de prevenção ao aparecimento e agravamento dos sintomas.

Os estudos com genes candidatos realizados até o momento não são suficientes para determinar quais genes e polimorfismos efetivamente conferem suscetibilidade à doença. Mesmo em relação ao gene *DRD4,* já aceito como um gene de suscetibilidade ao transtorno, ainda é necessário esclarecer quais as verdadeiras variantes que influenciam no aparecimento dos sintomas, qual o tamanho da sua contribuição, se seu efeito é específico para alguns aspectos da sintomatologia e quais seriam esses aspectos (Waldman e Gizer, 2006).

Na nossa população, diferentes genes já foram investigados no TDAH, incluindo o *DRD4*. Em um relato inicial, o alelo 7R do VNTR do exon 3 mostrou associação por uma abordagem caso-controle, mas não pelo método baseado em famílias (Roman e cols., 2001). Um efeito interessante deste polimorfismo foi ainda observado por Kieling e cols (2006): pacientes com TDAH submetidos a um teste neuropsicológico que avalia flexibilidade cognitiva (Teste de Desempenho Contínuo, CPT) tiveram um melhor desempenho, cometendo um número menor de erros, quando o alelo 4R estava em homozigose, enquanto que a presença de pelo menos um alelo 7R contribuiu para uma freqüência maior de erros. Em um estudo recente realizado com uma amostra consideravelmente maior (Akutagava-Martins e cols., 2011), não foi observado efeito do alelo 7R, seja pelo método baseado em famílias ou dimensional. Entretanto, foi possível detectar um déficit de transmissão de alelos 2R dos pais para a prole no grupo de pacientes do subtipo combinado, sugerindo um efeito protetor deste alelo. A investigação dos polimorfismos da região promotora descritos anteriormente

(duplicação de 120 pb, -616C>G e -521C>T), não evidenciou qualquer associação. Esses achados sugerem que o gene *DRD4* exerce influência sobre o TDAH na nossa população, embora de uma maneira complexa e ainda pouco compreendida.

Assim, é de fundamental importância uma investigação mais detalhada não somente dos polimorfismos já amplamente estudados, como também de variantes que tenham sido relacionadas mais recentemente ao transtorno. Nesse contexto, os SNPs associados aos sintomas de desatenção da doença, pelo estudo de Lasky-Su e cols. (2008), constituem um componente genético interessante para novos estudos, uma vez que são localizados em região regulatória e estão em DL com o VNTR.

Da mesma forma, a análise do significado funcional das variantes é fundamental para esclarecer a exata contribuição da genética na etiologia da doença. Como já mencionado, poucos estudos avaliando a funcionalidade dos supostos polimorfismos de risco do gene *DRD4* foram realizados. O conhecimento sobre a extensão em que polimorfismos de regiões regulatórias podem estar interferindo na atividade transcricional do gene, aumentando ou reduzindo a expressão, possibilitará um maior esclarecimento da influência de cada variante, da função do gene em si e, conseqüentemente, de sua participação na etiologia do TDAH.

III. Objetivos

1. Geral

Aumentar a compreensão acerca da participação do gene *DRD4* na etiologia do TDAH na nossa população, verificando a possibilidade de associação e analisando o efeito funcional de diferentes polimorfismos e sua relação com a doença.

2. Específicos

- Genotipar o SNP rs11246227 em uma amostra de pacientes com TDAH e seus pais biológicos.
- Testar a hipótese de associação do mesmo com o TDAH.
- Investigar a relação entre o SNP rs11246227 e outros polimorfismos do gene *DRD4* previamente estudados na presente amostra.
- Investigar, através de abordagens *in silico*, a possível funcionalidade dos SNPs rs11246227 e rs11246228 e sua possível relação com mecanismos patofisológicos do TDAH.

IV. Artigo

ASSOCIATION AND *IN SILICO* STUDY OF DOPAMINE D4 RECEPTOR GENE IN ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

Manuscrito em preparação.

ASSOCIATION AND *IN SILICO* STUDY OF DOPAMINE D4 RECEPTOR GENE IN ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

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Abstract

Dopamine D4 receptor gene (*DRD4*) is a widely investigated loci in attention deficit/hyperactivity disorder (ADHD), being accepted as a susceptibility gene. In the present study we investigated a possible association between ADHD and the Single Nucleotide Polymorphism (SNP) rs11246227 in *DRD4* gene. We also analyzed the functional role of the rs11246227 and rs11246228 through bioinformatics approach. Association study was performed in a sample composed of 478 ADHD patients, diagnosed according to DSM-IV criteria, and their biological parents. The rs112466227 was investigated by both family-based (FBAT) and dimensional (PBAT, ANOVA) approaches. Linkage disequilibrium (LD) considering different *DRD4* polymorphisms was estimated (MLocus). *In silico* analysis by bioinformatics was conducted using different databases and programs, in order to include different algorithms. FBAT analysis of inattentive subtype patients showed a significant excess of C allele transmission. MLocus demonstrated evidence of LD with both 120bp tandem duplication and exon 3 VNTR. Differences between alleles, with loss or gain of binding sites, were detected in both regions by *MicroInspector*, *smiRNAdb* and *miRecords*, and in the rs11246227 by *Human miRNA Targets* and *miRò*, agreeing with their location at 3' region. *DRD4* complexity allied to ADHD phenotypic heterogeneity probably contributes to divergent results in our association study compared to the literature. Our bioinformatics findings suggest a possible involvement of investigated SNPs in binding properties of miRNAs related to processes of neurogenesis and neuronal plasticity. However, new functional studies are needed to clarify their involvement in *DRD4* expression control mediated by miRNAs and, consequently, the possible effect in ADHD etiology.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a complex phenotype with multifactorial inheritance, affecting around 5% of school age children in different countries and cultures (Polanczyk *et al.*, 2007). According to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994), ADHD symptoms are divided into dimensions of inattention and hyperactivity/impulsivity, being recognized three clinical subtypes: predominantly inattentive, predominantly hyperactive/impulsive and combined (Biederman, 2007; Lara *et al.*, 2009). Etiologically, ADHD requires the involvement of both genes and environmental agents to manifest symptoms (Thapar *et al.*, 2007; Wallis *et al.*, 2008). Genetic contribution to the development of ADHD is one of the highest ever recorded for psychiatric disorders (Mick and Faraone, 2008), with heritability around 76% (Biederman and Faraone, 2005; Mick and Faraone, 2008). It is suggested that ADHD transmission occurs through several small effect genes that confer susceptibility to this disorder, also with a possible influence of modifying genes (Asherson and Image, 2004; Waldman and Gizer, 2006).

Data of neuroimaging studies suggests ADHD as a frontal-striatum-cerebellum disease (Curatolo *et al.*, 2009; Makris *et al.*, 2009). In this context, genes encoding components from dopaminergic system are the main candidates in molecular studies of ADHD (Biederman and Faraone, 2005; Waldman and Gizer, 2006; Thapar *et al.*, 2007). Among these, the dopamine D4 receptor gene (*DRD4*) is the most extensively investigated (Gizer *et al.*, 2009; Banaschewski *et al.*, 2010). The most studied polymorphism of *DRD4* gene is a variable number of tandem repeats (VNTR) of 48bp, located at exon 3 (Van Tol *et al.*, 1992; Waldman e Gizer, 2006). The first evidence of association between *DRD4* gene and ADHD comes from LaHoste *et al.* (1996), in a case-control study, suggesting the 7-repeat allele (7R) as the risk one. Several investigations replicated this association, as can be seen in metanalytic reviews by Biederman and Faraone (2005) and Gizer *et al* (2009). However, association detection and effect sizes seem to vary according to the approach used in each study as well as by other reasons, many times not identified (Waldman and Gizer, 2006). Recently, our group has reported a deficit of transmission of the 2R allele in patients of combined subtype, through a family-based approach (FBAT) (Akutagava-Martins *et al*, 2011). Therefore, it is possible that each VNTR allele has a different, unique effect on receptor function. This influence seems to depend not only on repeat number but also on variation within each repeat and around VNTR.

If so, different populations would have different alleles confering risk or protection to ADHD (Wang *et al.*, 2004; Waldman and Gizer, 2006; Tovo-Rodrigues *et al*, 2011).

The marked heterogeneity on the magnitude of VNTR effects on ADHD among different studies leads to the hypothesis that other polymorphisms at *DRD4* locus, in linkage disequilibrium (LD) or not, with the 48bp VNTR, might be responsible for the effects of this gene on ADHD. Polymorphisms located at the *DRD4* promoter region have been particularly investigated since they might modify transcription regulation of the gene. Among these, the most studied are a 120bp tandem duplication, with the 240bp allele being considered the risk allele for ADHD (McCracken *et al.*, 2000; Kustanovich *et al.*, 2004; Kereszturi *et al.*, 2007), and two single nucleotide polymorphisms (SNPs), -616C>G (rs747302) and -521C>T (rs1800955), respectively with contradictory (Barr *et al.*, 2001; Lowe *et al.*, 2004) and positive results in most studies (Bellgrove *et al.*, 2005; Yang *et al.*, 2008). Nevertheless, functional studies on these variants are still scarce, showing controversial results (Okuyama *et al.* 1999, Kereszturi *et al.*, 2006, Barr *et al.*, 2001).

Lasky-Su *et al.* (2008) applied a Family-Based Association Test-Principal Components (FBAT-PC) to the inattentive and hyperactive-impulsive symptom dimensions of ADHD, to determine whether SNPs in different candidate genes for ADHD were associated with quantitative phenotypes generated from such symptoms. Two hundred twenty-nine ADHD families were recruited through several ongoing research studies being conducted at Massachusetts General Hospital - Pediatric Psychopharmacology Clinic. Novel putative risk polymorphisms located at the promoter region of *DRD4*, the adjacent SNPs hCV26775267 (rs11246228, T>C) and hCV26775266 (rs11246227, C>T), were suggested as associated with ADHD, being the C allele at rs11246228 and the T allele at rs11246227 over-transmitted (P=0.012 and P=0.012, respectively). When inattentive symptoms were used to generate an univariate phenotype, the association with both SNPs was again detected (P=0.034 for rs11246228, and P=0.017 for rs11246227), with over-transmission of the same alleles. When hyperactive-impulsive symptoms were used, neither SNP remained significantly associated. Both SNPs were found to be in strong LD with each other, and were in modest LD with the exon 3 VNTR, 7-repeat variant. The results suggest that each SNP, or both together, may be important in influencing the inattentive symptoms of ADHD, although the role of an unknown functional variant in LD with these SNPs could not be excluded.

Considering DNA variations in ADHD related genes, some could influence physiological expression of the disorder, while others could alter the responses to

pharmacological and/or psychotherapeutic treatments. Because of the huge number of polymorphisms located in these genes, as in *DRD4*, and divergent results in association studies, functional studies seem to be essential. In this kind of approach, a selection of potentially functional variations, predicted by computational methods, is a necessary and crucial step. Therefore, in the present work we combined an association study and *in silico* analysis in the attempt to identify putative functional effects of the SNPs rs11246227 and rs11246228 in ADHD and specific symptoms of this disorder.

Material and Methods

Sample

The sample consisted of 478 ADHD children and/or adolescents and their biological parents, recruited through the Attention-Deficit/Hyperactivity Disorder Program (ProDAH) of Hospital de Clínicas de Porto Alegre (HCPA), and from public schools of Porto Alegre (capital of Rio Grande do Sul state, Brazil).

The patients enrolled through ProDAH consisted of 378 patients, being 16 single affected individuals (DNA from parents not available, therefore, included only in dimensional analyses), 93 duos (mother and patient) and 269 trios (mother, father and patient). This clinical sample were diagnosed according to DSM-IV criteria, following a three stage protocol, described in detail in Roman *et al.* (2001), Rohde (2002) and Polanczyk *et al.* (2007b). Briefly, the diagnostic procedure comprehended: a) evaluation with a semi-structured interview (Schedule for Affective Disorders and Schizophrenia for School-Age Children, Epidemiological Version – K-SADS-E) (Orvaschel, 1985), modified to evaluate DSM-IV criteria and filled in with the parents by trained research assistants; b) discussion of the derived diagnosis in a clinical committee, coordinated by a child and adolescent psychiatrist with large clinical experience; c) clinical evaluation of ADHD and comorbidities according to DSM-IV by a child and adolescent psychiatrist who had previously received K-SADS-E results and conducted the interviews with parents (generally the mother) and the patient. In case of a diagnostic discrepancy in the three stage procedure, preference was given to the diagnoses derived from clinical interviews. A cognitive evaluation based on cube and vocabulary subtests of Weschler Intelligence Scale – Third edition (WISC-III; WESCHLER, 1991) was performed by trained psychologists for estimating IQ. Also, parents filled in the Child Behavior Checklist (CBCL), Parent Report Form (Achenbach, 1991), a list of

symptoms that reports behavioral problems of the child and has a highly discriminatory power to clinical diagnosis of ADHD. Data concerning Swanson, Nolan and Pelham Scale - version IV (SNAP-IV), a scale that measures symptom scores in areas of inattention, hyperactivity, impulsivity and opposition (Swanson *et al.*, 2001), were also obtained in part of the sample. To the patients who were attending school, teachers completed the Attention Problem scale (CBCL – Teacher Report Form, TRF) (Achenbach, 1991), which include items related to ADHD in classroom. Social-demographic information was systematically collected from parents.

The sample obtained from public schools consisted of 100 patients of inattentive subtype only, coming from 27 duos and 73 trios. Initially, teachers were trained by a child and adolescent psychiatrist to detect symptoms of inattention in students. Individuals identified as possible ADHD cases from the predominantly inattentive subtype were invited to the diagnostic stage of the study, performed at ProDAH, following the same three stage procedure described above. More details about diagnosis and demographic data can be seen in Schmitz *et al.* (2006). Due to different origin and characteristics (age, ethnicity, ADHD subtype composition and SNAP scores) when compared to the clinical sample, these patients were included only in family-based analysis concerning the predominantly inattentive subtype, being excluded from other analyses.

This study was approved by the National Committee of Ethics in Research (CONEP) and by the Ethics Committee of HCPA. Parents gave a written informed agreement and the patients agreed verbally to participate of the study.

Genotyping procedures

A 5 mL blood sample was collected from each patient and, whenever possible, from biological parents. DNA was extracted from whole blood by a salting out method according to Lahiri and Nurnberger (1991). Only the SNP rs11246227 was analyzed, due to metodological issues. The studied polymorphism was genotyped by alellic discrimination system TaqMan SNP Genotyping Assay (Applied Biosystems) according to the manufacturer's recommended protocol.

Statistical analyses - Association study

Allele and genotype frequencies were obtained by counting. Hardy-Weinberg Equilibrium was tested with Genepop 4.0 software (Rousset, 2008). Association hypothesis

between *DRD4* markers and ADHD was verified by family-based approach using FBAT 2.0.2 software (Laird *et al.*, 2000). Through this methodology it is possible to determine if there is a preferential transmission of a particular allele or haplotype from the parents to the ADHD proband, thus detecting linkage and/or association. Family-based analysis using SNAP-IV scores as a quantitative phenotype was performed using FBAT-GEE statistics of PBAT 3.61 software (Lange *et al.*, 2004). Dimensional analyses were also performed by ANOVA one way, comparing SNAP-IV scores in patients with different genotypes, using SPSS 16.0 software. Linkage disequilibrium analysis was performed with MLocus software (Long, 1999). A significance level of 5% was accepted in all analyses.

Functional studies

SNPs data and location were extracted from the following databases: *NCBI dbSNP* (http://www.ncbi.nlm.nih.gov/snp), *Ensembl dbSNP* (http://www.ensembl.org), *UCSC Genome Browser* (http://genome.ucsc.edu/cgi-bin/hgGateway), *SNPper* (http://snpper.chip.org/bio), and *HapMap* (http://hapmap.ncbi.nlm.nih.gov/). These initial analyses showed the SNPs are actually located at 3' portion of *DRD4* gene, thus we focused our functional analysis in the search for binding sites for microRNAs (miRNAs).

At first, we explored two well-known "seed-based" algorithms that identify targets based on complementarity between the miRNA seed sequence and the mRNA predicted by the gene: *miRanda* (http://www.microrna.org/microrna/home.do) and *PicTar* (http://pictar.mdc-berlin.de/) (John *et al.*, 2004; Krek *et al.*, 2005).

We searched targets also by "genome scanning" algorithms. Among these, we utilized *miRAlign* (http://bioinfo.uni-plovdiv.bg/microinspector/) to predict miRNAs based on sequence similarity between miRNAs of closely related species (Wang *et al.*, 2005); and *Human miRNA Target* (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl), based in targets that are conserved between human and mouse or rat (John *et al*, 2004); and *smiRNAdb* (http://www.mirz.unibas.ch/cloningprofiles/), an algorithm for exclude deep sequencing data for non-coding RNAs (Landgraf *et al.*, 2007).

Among the programs that differ slightly from the main prediction algorithms, we utilized *MicroInspector* (http://bioinfo.uni-plovdiv.bg/microinspector/) to identify weaker miRNA/mRNA interactions that are normally not picked up by seed-based models (Rusinov *et al.*, 2005); and *DIANA-microT* (http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi) to provide

confidence scores for each prediction which correlate with protein expression levels (Maragkakis *et al.*, 2009).

Identification of miRNA targets independently of cross species homology can be performed using programs classified as "machine learning" algorithms (Majer *et al.*, 2010); for this purpose, we used *miTarget2* (http://mirdb.org), based in microarray data from two different studies (Wang and El Naqa, 2008).

We also used additional resources that combine more than one target prediction program to identify potential targets of miRNAs: the Predicted Targets component of *miRecords* (http://mirecords.biolead.org/prediction_query.php) to compare targets from 11 miRNA target prediction programs (Xiao *et al.*, 2009); and *miRò* (http://ferrolab.dmi.unict.it/miro/), which provides miRNA functional annotations inferred through their validated and predicted targets (Laganà *et al.*, 2009).

Finally, we explored methods that contain experimentally validated miRNA target pairs reported in the literature: *miRNAMap 2.0* (http://miRNAMap.mbc.nctu.edu.tw), and *miRecords Validated Targets* component (http://mirecords.biolead.org/index.php) (Hsu *et al.*, 2008; Xiao *et al.*, 2009).

miRAlign, *MicroInspector* and *smiRNAdb* programs allow direct comparison between the sequences of different alleles of the SNPs in study. For other programs, the steps of identification of target sites, the alignment with the sequence and the search for differences between alleles had to be performed independently. The alignments were performed through the computer software *Mega5* (Tamura *et al.*, 2008); the genic sequences were extracted from the *Ensembl dbSNP*, and miRNA sequences were extracted from the *miRBase* (www.mirbase.org) (Griffiths-Jones *et al.*, 2006).

To conclude the prediction of miRNA target sites, we also investigated which miRNAs are expressed in the CNS tissues; for this, we explored the tissue-based search available in *microRNA.org* (www.microrna.org), that currently contain a large amount of miRNA experimental data for various tissues and cell types (Betel *et al.*, 2008).

We also used the *SNPexpress* database (available in http://people.chgv.lsrc.duke.edu/~dg48//SNPExpress/index.php), which enables us to identify what SNPs can influence the expression of human genes, that is, which one could be an expression quantitative trait locus (eQTL), separately in brain and Peripheral Blood Mononuclear Cell (PBMC) (Sanders *et al.*, 2008). Then, we tested the possibility of linkage disequilibrium between the potential eQTL and the SNPs in question (rs11246227 and

rs11246228), through the International *HapMap* Project (http://hapmap.ncbi.nlm.nih.gov/) (The International HapMap Consortium. 2003).

Results

Three hundred seventy eight individuals were included in the clinical sample. Patients were predominantly male (79.1%) from European ascent (86.3%), with a mean age of 10.1(±3.0) years and mean IQ of 92.5 (±13.8). Combined subtype of the disorder was the most prevalent (66.8%), followed by predominantly inattentive subtype (26.1%) and predominantly hyperactive/impulsive subtype (7.1%). Oppositional defiant disorder was the most common comorbidity (47.5%), but anxiety disorders (27.8%), conduct disorder (14.4%) and mood disorders (13.3%) were also frequent. Some of these demographic and clinical caractheristics, as age and ethnicity, were different in school sample and can be seen in Schmitz *et al.* (2006).

Allele frequencies were calculated considering only unrelated patients from European ascent (n=299). The C allele from rs11246227 was slightly more frequent (0.582) than T allele (0.417). Both allelic and genotypic frequencies are in agreement with literature for this ethnic group (Lasky-su *et al*, 2008). Genotypic frequencies did not significantly deviate from expected according to Hardy-Weinberg Equilibrium (data not shown). Allele and genotype distributions for the school sample did not differ significantly from clinical sample (data not shown, but avaliable upon request).

Association study

Association analyses through FBAT were performed for the following groups: clinical sample (patients obtained from the hospital and of all clinical subtypes), patients of combined subtype (obtained from the hospital only), patients of inattentive subtype (obtained from hospital and schools) and probands presenting oppositional defiant disorder (ODD) and/or conduct disorder (CD) comorbidities (obtained from the hospital only). Results are presented in Table I. FBAT analysis of inattentive subtype patients showed a significant excess of C allele transmission (P=0.040). There was no evidence of association with clinical sample, patients of combined subtype or patients presenting ODD and/or CD.

Linkage disequilibrium (LD) analysis with polymorphisms previoulsy studied by Akutagava-Martins *et al.* (2011) confirmed a very complex genetic structure for *DRD4* gene. Between rs11246227 and SNPs -616C>G and -521C>T, there was no LD (P=0.305/

D'=0.041 and *P*=0.145/ *D'*=0.052, respectively). The comparison of the rs11246227 and 120bp tandem duplication showed an evidence of moderate LD between the non-duplicated allele and the T allele and between the duplicated allele and the C allele (*P*≤0.001/ *D'*=0.467). For rs11246227 and VNTR there was evidence of moderate LD between C allele and 2R allele (*P*=0.002/ D'=0.419) and T allele and 4R allele (*P*≤0.001/ D'=0.683), and strong LD for C allele and 7R allele (*P*≤0.001/ D'=0.903).

Estimation of possible haplotypes between VNTR and rs11246227 was obtained from FBAT. A total of 19 combinations were observed, being the haplotype composed by T allele from rs11246227 and 4R allele from 3' VNTR the most common (36.7%). Haplotype analyses considered only those combinations with a frequency higher than 5%, which resulted in eight valid haplotypes. Analyses were performed for the same groups of patients described above and only through FBAT software. There was no evidence of association between any of the analyzed groups and possible haplotypes, with *P* values ranging from 0.137 to 0.994 (data not shown, but available upon request).

Dimensional analyses were performed through FBAT-GEE statics of PBAT, by comparing inattention, hyperactivity, opposition and total SNAP-IV scores between patients of three different genotype groups: the two possible homozygous and the heterozygous. Next, dimensional analyses were performed by ANOVA one way, comparing SNAP-IV scores in patients with the same genotype groups. Age and ADHD subtype were defined as covariates in all analyses. SNAP-IV scores of inattention and opposition were statistically transformed to reach normal distribution. There was no association between SNAP-IV scores and genotypes in neither approach, with *P* values ranging from 0.149 to 0.388 (data not shown, but available upon request).

Functional study

NCBI dbSNP, *Ensembl dbSNP*, *UCSC Genome Browser* and *SNPper* databases demonstrated that the SNPs rs11246227 and rs11246228 are located in the 3' region of the *DRD4* gene, more specifically to 4243 bp and 4258 bp from *DRD4* transcription initiation site, respectively. Thus, we suggest that the SNPs happen to be called, according to their locations and base exchanges, as +4243C> T (rs11246227) and +4258T>C (rs11246228), based in *Ensembl* position in relation to the *DRD4* gene.

As we had situated these SNPs in the 3' portion of the *DRD4*, we decided to look for potential miRNA binding sites around the polymorphisms in question, using different

bioinformatics tools. *PicTar, DIANA-microT, miTarget2* and *miRNAMap* programs were unable to predict miRNA target sites in the *DRD4* gene or region of concern. No differences between the alleles of both polymorphisms were detected by *miRalign* and *miRanda* programs, that is, the same miRNAs would bind in this *DRD4* region independently of the SNPs. *Human miRNA Targets* and *miRò* identified differences between the alleles of +4243C>T (rs11246227), regarding the presence or absence of binding to specific miRNAs. According to this programs, identical miRNAs linked to both alleles at +4258T>C (rs11246228). Differences between alleles in both SNPs were detected by *MicroInspector*, *smiRNAdb* and *miRecords*, with loss or gain of binding sites for different miRNAs, according to allele. Finally, no miRNA validated target sites in *DRD4* gene were detected by *miRNAMap 2.0* or *miRecords Validated Targets*.

The investigation of which miRNAs are expressed in the CNS tissues, through the *Tissue-based Search* for miRNA expression in *microRNA.org*, indicated significant expression of miRNAs hsa-let-7a, hsa-let-7b, hsa-miR-16, hsa-miR-124, hsa-miR-125b and hsa-miR-126 in adult cerebellum, adult frontal cortex, and four different lineages of neuroblastoma. Of these miRNAs, alignments revealed that miRNAs hsa-miR-125b and hsa-miR-126 would bind equally on both alleles of both SNPs, and hsa-miR-16 would bind only to the T allele of +4243C>T (rs11246227). The positive results are presented in Table II.

The *SNPexpress* database showed for *DRD4* gene, 15 SNPs in Peripheral Blood Mononuclear Cell (PBMC) and 16 SNPs in brain that would be classified as eQTLs, with a p-value threshold of 0.05 (data not shown, but avaliable upon request). Among these functional variants, the SNP rs3758653, located in promoter region of gene, has been shown to be in LD with +4258T>C (rs11246228), according to *HapMap project.*

Discussion

The present report is the first attempt at replication of Lasky-Su *et al* (2008) findings, being able to detect an effect of rs11246227 in ADHD inattentive subtype patients. However, our family-based analysis showed a significant excess of C allele transmission (P=0.040). These results are opposed to the findings of Lasky-Su *et al.* (2008), where inattentive symptoms of ADHD were associated with T allele. These differences could be related to the distinct statistical approaches used in each study: while those researchers applied a Family-Based Association Test-Principal Components (FBAT-PC), we used the general family based association test (FBAT). In other words, they verified an association with a quantitative

phenotype, while here the observed effect was on a categorical phenotype. The discrepancy in association detection and effect sizes according to analysis approach is well recognized in the literature (Waldman and Gizer, 2006; Gizer *et al.*, 2009). However, the possibility of alpha and beta errors due to the sample size when detecting association with C allele and no association with T allele, respectively, cannot be ruled out. It is important to note that in Lasky-Su' study not only the rs11246227, but also the adjacent SNP rs11246228, was observed as associated with inattentive symptoms. These SNPs were found to be in strong LD, suggesting that either SNP, or both, might be influencing the inattentive symptoms of ADHD. Since only rs11246227 was evaluated here, it is possible that the true susceptibility effect has been missed.

Another explanation is that, in distinct populations, different variants of the same gene could confer susceptibility to a disease, what can be understood as allele heterogeneity. This possibility was already verified in our population for the dopamine transporter gene (*DAT1*) (Genro *et al*, 2008), and could be applied to *DRD4* locus in the light of its unusual complexity and structure. In the work from Akutagava-Martins *et al*. (2011), no association with the 7-repeat allele generated by the exon 3 VNTR was detected in the present sample, contrasting with most of literature reports. According to Tovo-Rodrigues *et al.* (2011) this is not totally unexpected, since an excess of rare variants were observed in the 7-repeat alleles in our ADHD patients when compared to controls, showing a particular variation pattern for *DRD4* gene in our population. The LD analysis performed in the present study also confirmed a very complex genetic structure for *DRD4* gene. We found evidences that rs11246227 is in moderate LD with 2R and 4R allele of 48bp VNTR, and in strong LD with 7R allele of the same polymorphism, while Lasky-Su *et al.* (2008) observed modest LD only with the 7-repeat variant. This marked heterogeneity could also explain our results in detecting association with a different allele of a previously associated SNP.

The dimensional analyses performed in this study failed in revealing any association or trend. This may be due to the reduced power of these analyses, since SNAP-IV scores used as quantitative phenotypes were available for only 283 patients. Quantitative family-based analysis, performed using PBAT, considered an even smaller number of patients (142 families), due to the inclusion of informative families only. Considering the robustness of the method, the lack of association could be a false-negative result. However, the dimensional analyses performed using ANOVA, a less restrictive test, confirmed this negative association of *DRD4* with a quantitative ADHD phenotype.

There is a rapidly growing interest for polymorphisms in microRNA (miRNA) target sites (PolymiRTS) in genetic analyses as several studies have suggested association between polymiRTS and human diseases (Sethupathy and Collins, 2008). The specific binding between a miRNA and its target mRNA is established by the complementarity of the miRNA seed site (positions 2–7 from the 5' end of the mature miRNA) and the target sequence in the mRNA 3'UTR (Rahman *et al.*, 2010, Saunders *et al.*, 2007). Considering that a single nucleotide change in the mRNA target site can considerably affect the translational regulation of the coded protein, a SNP being a PolymiRTSs may naturally be responsible for a functional alteration (Sethupathy and Collins, 2008). Recently, there have been a number of findings showing that SNPs in 3'UTRs can affect gene expression due to reduction or even elimination of miRNAs binding according to the allele (Abelson *et al.*, 2005; Clop *et al.*, 2006; Sethupathy and Collins, 2008; Wang *et al.*, 2008). Some mutations in 3′ portion of the candidate disease genes that disrupt miRNA binding sites can also impact diseases through reduced or total loss of miRNA-mediated regulation. For example, some patients who suffer from Tourettes' syndrome have mutations in the miR-189 target site within the 3' UTR of the *SLITRK1* gene (SLIT and NTRK-like family, member 1), which encodes a member of the SLITRK protein family, involved in neurite outgrowth (Abelson *et al.*, 2005; O'Roak *et al.*, 2010). Several polymorphisms have been identified within the 3' region of ADHD-associated genes, such as the dopamine transporter gene (*DAT1*). The 40bp variable number repeat (VNTR) polymorphism located in the 3'UTR of this gene has been extensively studied, being implicated in the regulation of gene expression (Mavroconstanti, 2010). Thus, 3' variations in other ADHD genes might present functional effects as well.

In attempting to clarify the role of each SNP in association results, we investigated both rs11246227 and rs11246228 using bioinformatics tools. Since our preliminary findings situated these SNPs in the 3' portion of *DRD4*, we decided to look for potential miRNA binding sites around the polymorphisms in question, using different bioinformatics tools. A substantial number of target sites were detected by *smiRNAdb*, *MicroInspector*, *miRecords* and *MiRò*, which also identified several differences between alleles of both SNPs. These programs present some particular characteristics that can be advantageous in comparison with other softwares. For example, *miRecords* and *MiRò* combine more than one target prediction algoritm to identify potential targets of miRNAs, possibly increasing the predictive power of miRNA binding sites (Xiao *et al.*, 2009; Laganà *et al.*, 2009; Majer *et al*, 2010); *MicroInspector* is able to identify miRNA/mRNA interactions picked up by seed-based

models, so it would extend the prediction (Rusinov *et al.*, 2005, Baev *et al.*, 2008). Most target genes have multiple miRNA target sites, indicating that cooperative binding may be essential for formation of inhibitory complexes (Doench and Sharp 2004, John *et al.*, 2004). So, we believe that the relatively high number of binding sites detected by these programs represent the reality, being not due to false positives.

On the other hand, while *miRanda* found a single target site that could bind to both SNPs, *PicTar* was unable to detect miRNAs for *DRD4* gene. However, as both *miRanda* and *PicTar* rely highly on cross-species conservation, their accuracy in predicting species-specific miRNA target sites is limited (Majer *et al.*, 2010). Although the rule of seed pairing has been successfully used to predict target sites with statistical support, the seed matches are not always sufficient for repression, implying that additional features would be required for reliable target selection (Grimson *et al.*, 2007). In this sense, *miRAlign* genome scanning algorithm detected only one miRNA target site, identical for two alleles in both SNPs, but this "low performance" might be due to the frequent inability of this kind of program to identify non-conserved or tissue-specific miRNAs (Majer *et al.*, 2010). *Human miRNA Target* detected binding sites only to +4243C>T (rs11246227), and was unable to find any target site to +4258T>C (rs11246228), possibly by the same limitations. The programs unable to predict miRNA target sites for the *DRD4* gene or region of concern besides *PicTar,* i.e., *DIANA-microT, miTarget2* and *miRNAMap*, fit into different approaches. Thus, we present here results of programs with similar approaches, predicting or not binding sites for miRNAs in gene or region studied.

For a project requiring information about miRNA targets, the possibilities are limited to either running one of the available algorithms, or looking for sets of precomputed targets (Mazière *et al.*, 2007). Most softwares used for prediction of target genes regulated by miRNAs includes different features, as base pairing of the miRNA seed sequence to the complementary 3′ UTR site, conservation of the miRNA binding site, favourable minimum free energy (MFE) of local miRNA–mRNA interaction, and structural accessibility of the neighbouring mRNA sequence, but the "weight" given to each feature may be apportioned differently (Majer and Booth, 2010). Algorithms used in these predictions are based on similar assumptions, and all prediction softwares suffer from limitations (Alexiou *et al.*, 2009). According to studies that compared some microRNA target prediction tools, insufficient experimental information does not allow to assess an accurate benchmark performance of the tools (Doran and Strauss, 2007). For that reason, many researchers utilize

at least two programs and use the intersecting list of targets between the programs to increase specificity, therefore decreasing sensitivity (Saba *et al.*, 2008; Alexiou *et al.*, 2009; Hua *et al.*, 2009; Wang and Li, 2009).

A SNP in the 3' UTR may create a sequence match to the seed of a miRNA that previously was not associated with the given mRNA. However, the presence of such target at the primary sequence level is not biologically relevant, unless the appropriate cognate miRNA is coexpressed (temporally and spatially) with the given mRNA (Saunders *et al.*, 2006). For this reason, attempting to refine our findings, we seek possible validated target sites by different ways. Analyzing *DRD4* mRNA, no miRNA validated target sites were detected by *miRNAMap 2.0* or *miRecords Validated Targets*, as expected by the absence of evidence in literature. Through the tissue-based search for miRNA expression in *microRNA.org*, we found significant expression of some human miRNAs in Central Nervous System (CNS) tissues: adult cerebellum, adult frontal cortex, and four different lineages of neuroblastoma. In the present work, three of these miRNAs were found to be able to bind in the studied SNPs region: hsa-miR-125b, hsa-miR-126 and hsa-miR-16. Findings related to hsa-miR-125b, which has binding site on both alleles of both SNPs, are identical to those found for this miRNA by *MiRò*, what could be an additional evidence of this miRNA target site in region of interest. The other two have no coincidences with the programs previously used, therefore the hsa-miR-16 can represent a potential miRNA to help understand our association results, since this miRNA binds only to the T allele of +4243C>T (rs11246227). In other words, the C allele might prevent the binding of this miRNA to the *DRD4* mRNA. Hypothetically, the presence of an active mRNA and/or a mRNA of longer lifetime could then be related to the presence of inattentive symptoms. It is important to note that hsa-miR-16 is expressed in cerebellum and frontal cortex. Both areas seem to be involved in ADHD; the association between frontal cortex and inattentive symptoms has increasingly been supported (Curatolo *et al.*, 2009; Makris *et al.*, 2009). For the other SNP, +4258T>C (rs11246228), we found evidences of LD with rs3758653, a functional SNP in promoter region of *DRD4* gene, pointed by *SNPexpress* as an probable eQTL with potential effect on exon expression level, in peripheral blood mononuclear cells. Thus, once again we cannot discard the possibility of some of the *DRD4* SNPs be just a marker.

Some recent evidences showing involvement of miRNAs in central nervous system have been pointed in literature (Christensen and Schratt, 2009; Kocerha *et al*., 2009), as the hsa-miR-17, that we showed by *miRecords* that would bind only to T allele in +4243C>T

(rs11246227) and only to C allele in +4258T>C (rs11246228). The hsa-miR-17 members expression declines between late embryonic and postnatal life (Hebert *et al.*, 2009), which correlates with the decline in neurogenesis that occurs during this period (Caviness *et al.*, 2009, Zhao *et al.*, 2008), suggesting that hsa-miR-17 members may be involved in promoting both proliferation and neurogenesis. Other interesting findings are hsa-miR-125a-3p, that would bind only to the T allele of +4258T>C (rs11246228), according *smiRNAdb*, and hsa-miR-125a-5p, that would bind to both alleles of the same SNP, according *miRecords* and *MiRò*. Cortez *et al.*, (2010) showed that hsa-miR-125a is downregulated in glioblastoma, and it would regulate PDNP (Phosphodiesterase I/ Nucleotide Pyrophosphatase 3), a putative marker of neural stem cells. The hsa-miR-106b was detected in our investigation by *smiRNAdb*, binding only in T allele from +4258T>C (rs11246228). The hsa-miR-106b~25 members, in which hsa-miR-106b is included, are known to modulate embryonic stem cell differentiation, and promote reprogramming of mouse embryonic fibroblasts into induced pluripotent stem cells, modulating neural stem/progenital cells proliferation and differentiation (Foshay *et al.*, 2009; Brett *et al.*, 2011; Li *et al.*, 2011). Other miRNAs recently found to regulate adult neural stem cells *in vivo* and *in vitro* includes miR-124 and let-7b, highly expressed in nervous tissues according *Tissue-based Search* for miRNA expression in *microRNA.org*. The hsa-miR-125b detected by this last dataset and by *MiRò*, which equally binds in both alleles of both SNPs, is up-regulated in astrogliosis, possibly contributing to this disease and to defects in the cell cycle that are characteristic of degenerating brain tissues (Pogue *et al.*, 2010).

Interestingly, our findings for *DRD4* suggest a possible involvement of miRNAs related to processes of neurogenesis, considered a prominent part of a wider process called neuroplasticity. Recently, it has been postulated that a dysfunction of neuroplasticity mechanisms can be involved in the pathophysiology of ADHD and other common neuropsychiatric disorders (Jensen *et al.*, 2009; Forero *et al.*, 2006; Ramocki *et al.*, 2008). In this respect, a review suggested that variations in some candidate genes such as *SNAP25*, *SLC6A4*, *HTR1B*, *BDNF* and *SLC6A2*, genes well known to be involved in neurotransmission and neural plasticity (Abayrak *et al.*, 2008), could be important risk factors for ADHD in different populations (Faraone *et al.*, 2005; Banaschewski *et al.*, 2010). Recent genome-wide association studies of ADHD and related phenotypes support the role of neurodevelopment and neuroplasticity genes in the etiology of this disorder (Roman *et al.*, 2009; Neale *et al.*, 2010; Lantieri e cols., 2010). In this context, Pluess, Belsky, & Neuman (2009) suggested that

DRD4 could actually be a "plasticity gene" rather than an ADHD vulnerability gene, that is, a gene that would modify the plasticity individuals have to environmental conditions. This would be consistent with a hypothesis previously proposed by Belsky and colleagues, who suggested some genetic alleles (including *DRD4* 7-repeat allele) could cause individuals to be more responsive to both positive and negative environmental influences (Belsky *et al.*, 2009). Considering *DRD4* as a gene involved in neuroplasticity, the miRNAs indicated in our study might represent some of the ways by which the *DRD4* gene could be influencing in neuroplasticity and neurogenesis, and consequently in ADHD. This idea, nevertheless, must be further investigated by both association and functional studies.

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Conflict of interest

Dr Rohde was on the speakers' bureau and/or acted as consultant for Eli-Lilly, Janssen-Cilag, and Novartis in the last three years. Currently, his only industry related activity is taking part in the advisory board/speakers bureau for Eli Lilly, Novartis and Shire. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by him received unrestricted educational and research support from the following pharmaceutical companies in the last three years: Abbott, Bristol-Myers Squibb, Eli-Lilly, Janssen-Cilag, and Novartis.

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Table I. FBAT analyses for rs11246227 in the clinical sample, patients of combined subtype, patients of inattentive subtype and ADHD probands with oppositional defiant disorder (ODD) and/or conduct disorder (CD).

Sample	Allele	N[1]	Observed[2]	Expected[3]	*Z*	*P*
Clinical sample	C	187	217	214	0.372	0.709
	T	187	167	170	-0.372	0.709
Combined subtype	C	116	127	130.5	-0.559	0.576
	T	116	109	105.5	0.559	0.576
Inattentive subtype	C	108	137	124.5	2.048	0.040
	T	108	87	99.5	-2.048	0.040
ADHD + ODD and/or CD	C	94	101	104	-0.535	0.592
	T	94	89	86	0.535	0.592

[1] Number of informative families included in the test.
[2] Observed number of transmissions.
[3] Expected number of transmissions under the null hypothesis of association.

Table II. Main binding sites for miRNA, found by softwares containing information about the 3' region of *DRD4* gene.

Software	+4243C>T (rs11246227)	+4258T>C (rs11246228)
miRanda	hsa-miR-342-5p in both alleles.	hsa-miR-342-5p in both alleles, but binding in different positions.
miRAlign	hsa-mir-345 in both alleles.	hsa-mir-345 in both alleles.
Human miRNA targets	hsa-miR-133a in both alleles, hsa-miR-133b only in T.	No binding site in this SNP.
smiRNAdb	hsa-miR-31, hsa-miR-296-3p and hsa-miR-1201 only in C allele.	hsa-miR-20a, hsa-miR-20b, hsa-miR-93, hsa-miR-106a, hsa-miR-106b, hsa-miR-125a-3p, hsa-miR-513a-3p, hsa-miR-519d, hsa-miR-548k, hsa-miR-552, hsa-miR-561 and hsa-miR-1252 only in T allele; hsa-miR-665 and hsa-miR-1294 only in C allele.
MicroInspector	hsa-miR-373, hsa-miR-575, and hsa-miR-601 only in C allele; hsa-miR-149-star only in T allele; hsa-miR-1180 in both alleles.	hsa-miR-188-3p and hsa-miR-662 only in C allele; hsa-miR-628-3p only in T allele; hsa-miR-145, hsa-miR-551b, hsa-miR-564 and hsa-miR-671-3p in both alleles.
miRecords	hsa-miR-9, hsa-miR-133a, hsa-miR-518d-3p, hsa-miR-518e, hsa-miR-524-5p and hsa-miR-593 in both alleles; hsa-miR-17, hsa-miR-106a, hsa-miR-133b, hsa-miR-518c, and hsa-miR-518d-5p only in T allele.	hsa-miR-106, hsa-miR-125a-5p, hsa-miR-518b, hsa-miR-518c, hsa-miR-518e, hsa-miR-518d-5p, and hsa-miR-520d-5p in both alleles; hsa-miR-637 only in T allele; hsa-miR-17 only in C allele.
miRò	hsa-miR-125b, hsa-miR-133a and hsa-miR-518d-3p in both alleles; hsa-miR-133b only in T allele.	hsa-miR-125a-5p, hsa-miR-125b and hsa-miR-518b in both alleles.
Tissue-based Search in microRNA.org	hsa-miR-125b and hsa-miR-126 in both alleles; hsa-miR-16 only in the T allele	hsa-miR-125b and hsa-miR-126 in both alleles

V. Discussão

Os resultados do estudo de associação obtidos neste trabalho, conforme discutido, enfatizam a suposta heterogeneidade genética e/ou alélica que existe no TDAH. Particularmente em relação ao gene *DRD4*, eles concordam com achados prévios para a mesma amostra (Akutagava-Martins e cols., 2011; Tovo-Rodrigues e cols., 2011). O mesmo tem sido observado para outros genes analisados pelo nosso grupo, tais como o gene do receptor D2 de dopamina (*DRD2*) (resultados preliminares, ainda não publicados) e do transportador de dopamina (*DAT1*) (Genro e cols., 2008). Esta possível heterogeneidade genética e/ou alélica poderia ser explicada pelo background genético da nossa população (Zembrzuski e cols., 2006). Sugere-se que amostras de TDAH de diferentes países podem diferir quanto à composição genética, devido a particularidades étnico-populacionais, o que pode contribuir para erros tipo I e tipo II nos estudos moleculares (Wang e cols., 2004; Waldman e cols., 2006). Em relação ao *DRD4*, os resultados contraditórios podem ser produtos destas diferenças, e é possível que a grande variabilidade populacional não esteja restrita ao VNTR, mas sim a todo o gene (Chang e cols., 1996; Hutz e cols., 2000). Por outro lado, é necessário enfatizar que embora com polimorfismos e/ou alelos diferentes, associações com os mesmos genes sugeridos na literatura tem sido detectadas na nossa amostra, o que nos faz acreditar que os resultados obtidos não são apenas falso-positivos. Por exemplo, nossas análises mostraram associação com a área de desatenção, mesmo subgrupo de sintomas onde o efeito observado por Lasky-Su e cols. (2008) foi verificado. Ou seja, o nosso resultado corrobora de certa forma o envolvimento do gene *DRD4* especificamente com sintomas de desatenção, o que seria consistente com a localização deste receptor no cérebro (Matsuomoto e cols, 1995; Barkley, 1997; Oak e cols., 2000).

Conforme já salientado, estudos funcionais parecem ser essenciais para compreender as diferenças nos achados de associação, uma vez que poderão determinar quais as variantes que de fato influenciam na função gênica e, consequentemente, nos processos neurobiológicos que embasam a doença. Neste sentido, muito se discute sobre qual seria a melhor abordagem, já que estudos *in vivo* se limitam praticamente a estudos de neuroimagem, cujas logísticas são em geral bastante complexas e com uma série de limitações éticas (Szobot e cols., 2005; 2010). Estudos *in vitro* com diferentes linhagens de células humanas aparecem como alternativas viáveis para a investigação da função de genes cerebrais. Porém, estes estudos envolvem metodologias longas, com muito trabalho de bancada e sujeitas a muitas falhas.

Isso também acaba dificultando a sua realização, o que pode ser evidenciado pelo reduzido número de publicações na área (Okuyama e cols., 1999; Wang e cols., 2004; Kereszturi e cols., 2006).

A concentração de esforços, tempo e recursos neste tipo de estudo pode ser otimizada pelos estudos *in silico*, ou através de ferramentas da bioinformática, com a determinação prévia dos polimorfismos mais promissores a serem testados *in vitro* (Mooney e cols., 2010). Além disso, a bioinformática permite examinar as variantes em diferentes cenários funcionais, e não apenas um, como ocorre nos experimentos *in vitro*. Esta vantagem ficou evidente neste trabalho. Inicialmente, observamos que os SNPs descritos como localizados na região promotora do gene *DRD4* estavam, na verdade, na região 3' UTR. Então, concentramos nossa pesquisa em elementos e mecanismos regulatórios relacionados à região 3'dos genes. A busca por resultados de bioinformártica a partir da localização anterior teria levado a resultados totalmente diferentes, provavelmente equivocados, e poderia representar o insucesso de um experimento *in vitro* já a partir de seu delineamento.

A localização dos SNPs em porções gênicas diferentes do originalmente descrito levanta outras questões, acerca das limitações da bioinformática. Nós interpretamos que o achado de Lasky-Su e cols. (2008), embora incorreto, foi o que poderia ter sido obtido à época. Todo o conhecimento acerca do papel regulatório de regiões 3' é ainda escasso, especialmente no que concerne a genes humanos. O mesmo pode ser dito sobre os miRNAs e outros elementos regulatórios supostamente atuantes nessas regiões. Assim, os achados obtidos num determinado projeto são totalmente dependentes dos bancos de dados e programas disponíveis naquele momento. Essa limitação impediu que o presente trabalho avançasse mais, chegando a observações e conclusões mais definitivas. Os dados que existem são ainda bastante inconsistentes, o que provavelmente colaborou para as diferenças nos resultados obtidos de acordo com os diferentes programas e análises utilizados.

Através de análises *in silico*, nós também investigamos um possível papel de outros SNPs localizados na região promotora do gene *DRD4*, -616C>G (rs747302) e -521C>T (rs1800955), previamente analisados em abordagem de associação (Akutagava-Martins e cols., 2011). Estes resultados estão descritos no capítulo VII - Anexo. Para isso, avaliamos a possível presença de sítios de ligação a fatores de transcrição nesses SNPs, e conseguimos detectar algumas diferenças interessantes entre os alelos, com alguns fatores ligando-se somente num ou noutro. É interessante observar que alguns desses fatores de transcrição são relevantes no sistema nervoso central, e estão relacionados a processos de

neurodesenvolvimento e neuroplasticidade, concordando com nossos achados em relação aos miRNAs e indicando um caminho interessante a ser explorado em estudos subseqüentes.

A utilização das ferramentas de bioinformática é um meio bastante promissor para a busca das reais variantes funcionais e de seu impacto na funcionalidade do gene em questão, embora o avanço dessas ferramentas ainda seja necessário para conclusões mais definitivas. Uma vez entendido o mecanismo molecular, o caminho entre este e os sintomas de uma doença complexa como o TDAH, entretanto, ainda deverá ser percorrido.

VI. Referências bibliográficas

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VII. Anexo

Análise *in silico* de SNPs na região promotora do gene *DRD4*: resultados e discussão preliminares

Como já comentado na introdução desta Dissertação, dentre os polimorfismos localizados em regiões regulatórias do *DRD4*, dois polimorfismos de nucleotídeo único (SNPs), -616C>G (rs747302) e -521C>T (rs1800955) vêm sendo analisados quanto à possível influência na atividade transcricional do gene em um pequeno número de estudos (Kamakura e cols., 1997; Okuyama e cols., 1999; Barr e cols., 2001; Kereszturi e cols., 2006). Assim, nós realizamos um estudo de funcionalidade através de ferramentas de bioinformática, com o objetivo de investigar um possível papel desses SNPs no controle da expressão do gene *DRD4* e, consequentemente, na etiologia do TDAH.

Inicialmente, realizamos uma análise do promotor do *DRD4* através do navegador genômico da *UCSC Genome Browser* (http://genome.ucsc.edu/cgi-bin/hgGateway). Verificamos que a região onde estão situados os SNPs -521C>T (rs1800955) e -616C>G (rs747302) é uma região potencialmente regulatória para pelo menos sete espécies. A ferramenta também detectou um sinal da base de dados *ORegAnno*: *Open Regulatory Annotation* (Montgomery , Griffith e cols. 2006), na posição do SNP -521C>T (rs1800955). Este sinal corresponde ao estudo *in vitro* de Okuyama e cols. (1999), que detectou diferença na funcionalidade entre os alelos T e C, cujos resultados foram replicados pelo mesmo autor posteriormente (Okuyama e cols., 2000).

Através da base de dados *CisRED* (*cis-regulatory element database*: http://www.cisred.org/; Robertson e cols., 2006) identificamos os *motifs* presentes no gene *DRD4*, e em seguida investigamos a complementaridade dos *motifs* e respectivos fatores de transcrição à região do SNP pelo programa *Mega5* (Tamura *et al*., 2008). Verificamos que um dos *motifs* listados pelo *CisRED*, específico para ligação ao fator de transcrição NRF-1 (fator respiratório nuclear 1), alinhou perfeitamente na região do SNP -521C>T. Entretanto, o fator é capaz de reconhecer qualquer pirimidina, portanto a troca de Citosina por Timina não alteraria o sítio de ligação no promotor do gene. Para o -616C>G, além da presença de *motifs*

para ligação a fatores não nomeados (13201, 13234, 13227), verificamos o possível alinhamento do fator de transcrição AP-2alphaA (ativador ligado a proteína 2-alfa), porém este fator reconheceria tanto o alelo C como o G do SNP.

Nós também utilizamos a ferramenta *rVista* do programa *Vista* (*Vista-Tools for Comparative Genomics:* http://genome.lbl.gov/vista/index.shtml, Frazer e cols., 2004) para comparar os alelos de ambos os SNPs. Para tal, delimitamos uma seqüência de 40pb em torno dos SNPs e submetemos separadamente cada um dos alelos de cada SNP a todos os fatores de transcrição disponíveis no programa. Em seguida comparamos os alelos, quanto à presença de ligação na posição dos respectivos SNPs, com o objetivo de verificar a perda de anelamento naquele ponto, que poderia resultar em redução da afinidade do fator de transcrição pelo sítio alvo. No caso do -521C>T, pudemos observar que, para o alelo C, os fatores E2F1 (proteína 1 associada ao retinoblastoma) e P53 (antígeno tumoral celular p53) apresentavam alinhamento na posição do SNP, tanto considerando as sequências alinhadas, como conservadas. No alelo T do -521C>T, o alinhamento na posição do SNP para os fatores descritos acima desaparece. Por outro lado, este alelo mostrou na posição do SNP alinhamentos para os fatores AR (Receptor Androgênico), STAT3 (sinal transdutor e ativador da transcrição 3) e VMAF (oncogene de fobrosarcoma músculo-aponeurótico homólogo v-maf) que estavam ausentes no alelo C. Para o -616G>C, os fatores E2F1DP1, E2F1DP2 (E2F1 complexado aos cofatores DP1 ou DP2, respectivamente), PAX3 e PAX4 (proteína box pareada 3 e 4, respectivamente) apresentaram alinhamento na posição do SNP apenas no alelo C. Já os fatores AR (receptor androgênico), COUPTF (fator transcricional COUP), DR4 (antígeno de histocompatibilidade classe II HLA-DRB1), PPAR (receptor ativado por proliferadores de peroxissoma), SP1 (fator transcricional Sp1) e AP2 (ativador ligado a proteína 2) apresentaram o ponto de alinhamento no SNP apenas quando o alelo T do SNP -616C>G foi testado.

Através do programa TESS (*Transcription Element Search System*, http://www.cbil.upenn.edu/cgi-bin/tess/tess, Schug e Overton, 1997), nós restringimos a análise às sequências de 22pb em torno dos SNPs. O programa permite comparar os alelos entre si e apresenta os miRNAs que se ligam a cada uma das sequências submetidas. Para o SNP -521C>T, foi demonstrada a possível ligação dos fatores de transcrição ZF5 (proteína 5 homóloga dedo de zinco), CF1 (fator comum 1), Sp1 e E2F-DRTF (E2F complexado ao DP1, também conhecido como DRTF) somente ao alelo C. Os fatores TFII-I (fator de transcrição geral II-I), CAC-binding (carnitina/acilcarnitina translocase) e MEP-1 (proteína 1 do

metilossoma) ligariam somente ao alelo T. Para o SNP -616G>C, o fator de transcrição Zeste (proteína regulatória zeste) se ligaria somente ao alelo C e o fator CTCF (repressor transcricional CTCF), somente ao alelo T. Os resultados dessa análise estão resumidos na tabela anexa.

Dentre os fatores apontados pelos programas, é interessante observar que E2F1 é um fator associado ao retinoblastoma, linhagem na qual o gene *DRD4* é expresso abundantemente. O E2F1 atua como um ativador de transcrição que se liga ao DNA, ligando-se preferencialmente à proteína RB1 (proteína codificada pelo gene do retinoblastoma), de modo dependente do ciclo celular, e pode mediar a proliferação celular e apoptose p53-dependente. Em tempo, a família gênica do retinoblastoma é diferencialmente expressa durante a embriogênese, tendo sido detectada transcrição abundante de *RB1* durante todo o processo de neurogênese (Jiang e cols., 1997). No cérebro em desenvolvimento, tanto a proteína RB1 como o fator E2F1 são expressos em células precursoras em divisão e nos neurônios pós-mitóticos (Callaghan e cols, 1999; Ferguson e cols., 2002). Quando complexado aos cofatores DP1 ou DP2, E2F1 parece apresentar papel potencial em aspectos do desenvolvimento e diferenciação neural, estando entre as isoformas particulares de E2F e DP que desempenham uma função tecido-específica na diferenciação e maturação do tecido nervoso (Kusak e cols., 2001). Outros fatores também apresentam funções relacionadas ao sistema nervoso central e neurodesenvolvimento. Por exemplo, AR é um fator de transcrição ativado por ligante, sendo expresso em cultura de células-tronco neurais embrionárias e adultas, e está presente também no epitélio do ventrículo esquerdo durante o desenvolvimento, e no giro denteado no cérebro adulto (Galea, 2008). STAT3 (sinal transdutor e ativador de transcrição 3) desempenha papel crucial na diferenciação de células-tronco neurais (NSC), e a eliminação de STAT3 em cultura de NSC promove a neurogênese e inibe a astrogliogênese (Cao e cols., 2010). PAX3 regula a migração das células da crista neural (Nakazaki e cols., 2009), COUP-TF está envolvido na migração celular tangencial no cérebro em desenvolvimento e no desenvolvimento cerebelar (Tripodi e cols., 2004; Qin e cols., 2007) e SP1 participa da regulação da transcrição em vários tipos celulares do tubo neural (Lee e cols., 2004). Outro fator apontado que seria bastante interessante de ser considerado é o AP-2, visto que os fatores de transcrição da família AP-2 constituem fatores críticos na regulação da expressão gênica neural e desenvolvimento neuronal. Vários genes nos sistemas monoaminérgicos exibem os sítios de ligação a AP-2 em regiões regulatórias. A

família AP-2 está envolvida na regulação dos sistemas monoaminérgicos nos períodos pré e pós-natal, e, portanto, pode estar envolvida na fisiopatologia de doenças neuropsiquiátricas (Damberg, 2005). Ainda podemos ressaltar o fator CTCF, que desempenha um papel na regulação da expressão de toda uma rede de genes que sofrem *imprinting*, que poderiam contribuir para as deficiências no desenvolvimento e função cerebral (Cunningham e cols., 2010).

Considerando as evidências que sugerem um possível papel dos fatores de transcrição que se ligariam à região promotora do *DRD4* no desenvolvimento e funcionamento do sistema nervoso central, estudos investigando mais especificamente cada um desses fatores e comprovando as diferenças de ligação entre os alelos dos polimorfismos do promotor poderiam ajudar a compreender a complexidade dessa região e do gene em si.

Tabela Anexa. Principais diferenças entre os alelos dos SNPs -521C>T (rs1800955) e -616C>G (rs747302) quanto à presença de sítios de ligação para fatores de transcrição.

Programa	**-521C>T (rs1800955)**	**-616C>G (rs747302)**
rVista	*E2F1, P53* somente no alelo C*; AR, STAT3, VNAF* somente no alelo T.	*E2F1DP1, E2F1DP2, PAX3, PAX4* somente no alelo C; *AR, COUPTF, DR4, PPAR, SP1, AP2* somente no alelo G.
TESS	*ZF5, CF1, Sp1, E2* somente no alelo C; *TFII-I, CAC-binding, MEP-1* somente no alelo T.	*Zeste* somente no alelo C; *CTCF* somente no alelo G.

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