Universidade Federal do Rio Grande do Sul
Instituto de Biociências
Bacharelado em Ciências Biológicas

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Análise do polimorfismo de inserção/deleção de 14 pares de base na região 3'UTR do gene HLA-G no Transtorno do Espectro Autista

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2021

FICHA CATALOGRÁFICA

CIP - Catalogação na Publicação

Ziliotto, Marina
Análise do polimorfismo de inserção/deleção de 14 pares de base na região 3'UTR do gene HLA-G no Transtorno do Espectro Autista / Marina Ziliotto. -- 2021.
59 f.
Orientador: José Artur Bogo Chies.

Coorientadora: Bruna Kulmann-Leal.

Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Bacharelado em Ciências Biológicas, Porto Alegre, BR-RS, 2021.
1. Genética. 2. Imunogenética. 3. Transtorno do Espectro Autista. 4. HLA-G. 5. Sistema Imune. I. Bogo Chies, José Artur, orient. II. Kulmann-Leal, Bruna, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com osdados fornecidos pelo(a) autor(a).

FOLHA DE APROVAÇÃO

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Aprovada em: Porto Alegre, 12 de maio de 2021.

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AGRADECIMENTOS

Em tempos de pandemia, escrever o TCC se tornou o único ato simbólico de despedida da graduação, por isso, esses agradecimentos são também uma forma de finalização dessa etapa. Nos meus cinco anos de formação, a minha principal meta nunca foi me tornar uma bacharela em Ciências Biológicas, mas sim uma profissional que conseguisse contribuir socialmente e que pudesse trabalhar com o que acredita. Por isso, agradeço a todos que contribuíram das mais diversas formas nessa construção.

Aos meus pais, que não só apoiaram minha vinda à Porto Alegre, como também permitiram que eu vivesse meu sonho integralmente. Sou especialmente grata porque sei que vocês não tiveram a mesma chance que eu tive, e mesmo assim, desde a infância, investiram em mim e na minha educação. Mãe, te agradeço por ter me ensinado tanto sobre ser independente. Tua história e teu exemplo me incentivam a buscar meu próprio caminho e ir atrás do que eu acredito.

À minha família em Porto Alegre, Thales, Maria e Nelise. Grande parte da minha construção se deve as inúmeras conversas e trocas, aos eventos acadêmicos e também aos vinhos compartilhados. Obrigada por compartilharem um pouco de vocês comigo nas risadas, nos abraços, nos medos e nas frustrações. Tive sorte de viver um período tão importante ao lado de pessoas que admiro profundamente. Sempre que lembrar dos anos de faculdade, vou lembrar de vocês.

Ao meu orientador, José Artur Bogo Chies, que me acolheu desde o início dessa jornada e sempre me incentivou através do exemplo a buscar a ciência com o meu próprio olhar de curiosidade. Aos meus colegas de laboratório, agradeço por mostrar como fazer a ciência e como pensar criticamente. Vocês me inspiraram a sempre buscar olhar além e fazer o melhor. Todos os debates na hora do cafezinho agregaram muito na minha visão de cientista e moldaram meu caminho dentro da biologia e da pesquisa. Agradeço especialmente à Bruna Kulmann Leal, que me coorientou com tanta didática, atenção e paciência. Além de tudo, me inspirei muito em ti durante toda essa jornada final. Obrigada!

Às minhas amigas e amigos que fiz durante o período da graduação e aos que continuam comigo desde antes da biologia. Vocês me acolheram e me inspiraram das mais

diversas formas! Sem vocês tudo isso não teria graça e eu não teria histórias pra contar. Obrigada por me acompanharem em tantas fases e por serem partes de tantas memórias importantes.

Obrigada!

RESUMO

O Transtorno do Espectro Autista (TEA) é uma desordem do neurodesenvolvimento caracterizada por uma díade comportamental, tanto alterações na comunicação e interação social, quanto na presença de comportamentos repetitivos/esteriotipados ou interesses restritos, cujas causas não foram totalmente elucidadas. No entanto, sabe-se que fatores genéticos e ambientais estão envolvidos no desenvolvimento do transtorno. Considerando os fatores genéticos, polimorfismos em genes relacionados ao sistema imune já foram associados ao TEA. Nesse contexto, sabe-se que a desregulação do sistema imune materno durante o período pré-natal é um fator de risco para o desenvolvimento do TEA. O HLA-G é uma molécula que possui um papel de indução de tolerância imunológica e sua expressão é limitada a poucos tecidos, sendo bastante presente na placenta durante a gestação, onde, junto com outras moléculas, é responsável por proteger o feto de uma resposta imune materna. O polimorfismo de inserção/deleção de 14 pares de base na região 3'UTR do gene HLA-G (rs371194629) resulta em uma menor expressão da proteína HLA-G. Essa variante já foi associada a problemas gestacionais como pré-eclâmpsia e aborto espontâneo. Considerando essas conexões, analisamos a possível relação entre o polimorfismo de in/del de 14pb na região 3'UTR do HLA-G com o desenvolvimento de sintomatologia do TEA. Neste trabalho, avaliamos as frequências genotípicas deste polimorfismo em uma amostra de 188 pacientes com TEA diagnosticados pelo DSM-IV na ala neuropediátrica do Hospital de Clínicas de Porto Alegre. As amostras foram amplificadas por PCR convencional e genotipadas em gel de poliacrilamida 8%. O teste qui-quadrado foi utilizado para testar associações entre os genótipos e a sintomatologia. As amostras se encontram em equilíbrio de Hardy-Weinberg. Os genótipos de inserção foram inicialmente associados com o sintoma de hetero agressão, mas a associação foi perdida após a correção de Bonferroni. Embora a associação tenha sido perdida, ela aponta para uma possível contribuição desse polimorfismo para a heterogeneidade dos sintomas apresentados por indivíduos diagnosticados com TEA. Futuros estudos devem levar em conta essa associação inicial para que se construam análises mais abrangentes sobre o papel da interface materno-fetal, HLA-G e os possíveis desfechos desses cenários na sintomatologia do TEA.

Palavras-chave: HLA-G. Transtorno do Espectro Autista. Genética

ABSTRACT/RESUMEN/RÉSUMÉ

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by stereotyped and repetitive behaviors that affect communication and social interests, which causes have not been fully elucidated. However, it is known that genetic and environmental factors are involved in the development of the disorder. Considering the genetic factors, polymorphisms in genes related to the immune system have already been associated with ASD. In this context, it is known that the dysregulation of the maternal immune system during the prenatal period is a risk factor for the development of ASD. HLA-G is a molecule that has an immunological tolerance-inducing role and its expression is limited to a few tissues, being very present in the placenta during pregnancy, where, together with other molecules, it is responsible for protecting the fetus from a maternal immune response immune. The 14 base pair insertion/deletion polymorphism in the 3'UTR region of the HLA-G gene (rs371194629) results in less expression of the HLA-G protein. This variant has already been associated with gestational problems such as pre-eclampsia and spontaneous abortion. Considering these connections, we analyzed the possible relationship between the 14pb in/del polymorphism in the 3'UTR region of HLA-G with the development of TEA symptoms. In this study, we evaluated the genotypic frequencies of this polymorphism in a sample of 188 patients with ASD diagnosed by DSM-IV in the neuropediatric ward of Hospital de Clínicas de Porto Alegre. The samples were amplified by conventional PCR and genotyped on 8% polyacrylamide gel. The chi-square test was used to test associations between genotypes and symptoms. The samples are in Hardy-Weinberg equilibrium. The insertion genotypes were initially associated with the symptom of hetero aggression, but the association was lost after Bonferroni's correction. Although the association has been lost, it points to a possible contribution of this polymorphism to the heterogeneity of symptoms necessary for those diagnosed with ASD. Future studies should take this initial association into account in order to build more comprehensive analyzes on the role of the maternal-fetal interface, HLA-G and the possible outcomes of these scenarios in the symptoms of ASD.

Keywords: HLA-G. Autism Spectrum Disorder. Genetics.

SUMÁRIO

1 INTRODUÇÃO

1.1 O TRANSTORNO DO ESPECTRO AUTISTA (TEA)

O termo Transtorno do Espectro Autista (TEA) é utilizado para descrever indivíduos que expressam, nos primeiros anos de vida, uma combinação de prejuízos na comunicação social e comportamentos sensoriais e motores restritos e repetitivos (Lord et al., 2018). Nos últimos 50 anos, esse transtorno passou de uma condição infantil específica e rara para um transtorno bastante heterogêneo e com uma prevalência elevada. O TEA engloba atualmente, de acordo com o Manual de Diagnóstico e Estatístico de Transtornos Mentais 5.ª edição (DSM-5), os Transtornos Invasivos do Desenvolvimento antes denominados separadamente de: Autismo Infantil Precoce; Autismo Infantil; Autismo de Kanner; Autismo de Alto Funcionamento; Autismo Atípico; Transtorno Global do Desenvolvimento sem Outra Especificação; Transtorno Desintegrativo da Infância; e Síndrome de Asperger.

O Transtorno do Espectro Autista é o mais conhecido entre os Transtornos Invasivos do Desenvolvimento (TID). Os TIDs são os transtornos de desenvolvimento mais comuns e referem-se ao desenvolvimento atípico de habilidades sociais, comunicativas e demais habilidades, tendo início precoce (Klin, 2006). O TEA é um transtorno do neurodesenvolvimento altamente herdado, embora não seja preponderante em alguns casos. A herdabilidade apresentada por essa desordem varia de 64% a 91%, de acordo com estudo recente (Tick et al, 2016) e é expresso de forma bastante heterogênea, sendo comum que o paciente manifeste outras comorbidades em conjunto, como hiperatividade, ansiedade, depressão, epilepsia e Transtorno do Déficit de Atenção com Hiperatividade (TDAH) (Lord et al., 2020).

Muitas causas neurobiológicas já foram apontadas e muitos estudos descreveram marcadores neurais e de comportamento que indicam um alto risco para o desenvolvimento do TEA (Gliga et al., 2014). Ainda sim, o diganóstico é essencialmente comportamental já que não existem marcadores biológico para risco ou diagnóstico de TEA. A neuroinflamação e disfunções neuro-imunes têm sido cada vez mais apontadas como fatores principais para o desenvolvimento do transtorno (Siniscalco et al., 2018). Sabe-se que pessoas autistas exibem padrões de conectividade alterados no sistema nervoso central, que são observados logo nos primeiros anos de vida (O' Reilly et al., 2017). Embora diversos fatores venham sendo

elucidados em relação ao seu desenvolvimento, ainda não é possível definir a etiologia desse transtorno.

1.2 HISTÓRICO DO TRANSTORNO DO ESPECTRO AUTISTA

Hora de celebrar as mulheres esquecidas pela ciência: Grunya Schukareva foi a primeira psiquiatra a descrever o autismo (conceituado por Bleuler) em crianças que apresentavam uma tendência autista para si. A descrição ocorreu cerca de 2 décadas antes da publicação dos estudos do Kanner e do Asperger. O psiquiatra suíço Paul Eugen Bleuler a utilizou a expressão “autismo” em seu livro chamado “Dementia Praecox” pela primeira vez em 1911 para descrever a perda de contato com a realidade que resultava em um prejuízo na comunicação. Em 1943 foram descritos 11 casos denominados como distúrbios autísticos do contato afetivo, em que se encontrava uma incapacidade dos casos de se relacionarem de forma usual, bem como respostas incomuns ao ambiente (Kanner, 1943). Em 1944, o pediatra suíço Hans Asperger, desconhecendo o trabalho de Kanner, também descreveu casos com características semelhantes ao autismo, porém seus pacientes apresentavam comprometimento nas habilidades cognitivas e verbais. (Gadia et al., 2004).

Entre os anos 1950 e 1960 a etiologia do autismo entrou em questão, sendo considerada uma resposta a pais emocionalmente não responsivos. Essa hipótese ficou muito conhecida como “hipótese da mãe geladeira” e as crianças foram expostas a tratamentos bastante aversivos. Essa hipótese foi abandonada e refutada à medida que novos estudos apontaram o autismo como um transtorno cerebral altamente herdado e presente desde a infância. O Transtorno do Espectro Autista aparece pela primeira vez no DSM-III em 1980, quando os TIDs são adicionados ao manual, abrangendo um espectro maior de prejuízos na comunicação (Klin, 2006). Desde então, essa classificação passou por várias redefinições. Atualmente, a denominação adequada de acordo com o DSM- 5 é Transtorno do Espectro Autista (TEA), por se tratar de um transtorno heterogêneo e de desenvolvimento complexo, com múltiplas etiologias e vários graus de severidade (Lord, 2020).

1.3 ETIOLOGIA

Embora a etiologia do TEA não seja completamente elucidada, sabe-se que diversos fatores influenciam o seu desenvolvimento. Estima-se que o desfecho do TEA seja causado

pela interação entre fatores genéticos e ambientais (Herbert, 2010) e foi classificado como um transtorno comum (Wang et al., 2017). Mais de 600 genes já foram relacionados com o autismo, mas para muitos deles, os tamanhos de efeito individuais são bastante pequenos. Muitas variantes comuns e raras podem contribuir aditivamente na herdabilidade do TEA. Mutações truncadas herdadas são encontradas em excesso em indivíduos autistas, incluindo genes implicados na codificação de proteínas para formação sináptica, regulação da transcrição, e vias de remodelação da cromatina (Waye et al., 2018).

Alguns dos fatores ambientais associados ao desenvolvimento de TEA incluem ativação imune materna, idade parental avançada, deficiência de vitamina D nos pacientes, uso de determinadas medicações durante períodos específicos da gestação (como o ácido valpróico) e complicações durante o parto associadas com trauma, isquemia e hipóxia. Além disso, associação com exposição a metais pesados também já foi verificada. Nenhuma relação com o uso de vacinas, tecnologias de reprodução assistida, tabagismo materno e uso de timerosal foram encontradas até o momento (Modabbernia et al., 2017). No entanto, Em 1988, uma publicação foi realizada sugerindo a relação entre a MMR com TEA, a qual foi completamente refutada, o artigo foi retratado e diversas publicações, até os dias atuais, demonstram que não há relação da vacinação com autismo. Inicialmente, o autor procurava estabelecer uma relação entre a vacina e distúrbios intestinais, o que o levou a acreditar que essa patologia poderia ocasionar uma toxicidade durante o desenvolvimento do sistema nervoso e, consequentemente, no desenvolvimento do autismo, a qual nunca foi estabelecida. Estudos posteriores, incluindo estudos dos mesmos autores, não conseguiram comprovar a hipótese e, ainda, vários estudos epidemiológicos posteriores indicam a segurança da vacina MMS (DeStefano & Shimabukuro, 2019).

É importante salientar que o estudo que propôs uma associação entre autismo e vacina contra MMR foi subsequentemente retratado pela revista por impropriedades no recrutamento de pacientes e conflitos financeiros de interesse do autor, mas, ainda assim, o estudo trouxe consequências sérias para a população, como o aumento de casos de sarampo devido ao fortalecimento de uma equivocada visão anti- vacinas. Além disso, contribuiu com a insegurança da população em termos de vacinação, e de modo mais geral, com o descrédito da ciência e do embasamento científico, que pode levar ao uso de tratamentos sem eficácia comprovada.

1.4 EPIDEMIOLOGIA

Nota-se que os números com TEA diagnosticados vem aumentando nas últimas décadas. Segundo dados recentes publicados pela Rede de Monitoramento de Deficiências Mentais e Autismo (ADDM) e pelo Centro de Controle e Prevenção de Doenças (CDC) nos Estados Unidos, uma em cada 54 crianças foi diagnosticada com TEA em 2016. Uma porcentagem elevada quando comparada àquela de 2014, em que foi diagnosticada uma em cada 59 crianças, e ainda maior quando comparada com os anos 2000, período em que a rede publicava seus primeiros dados, que indicavam o diagnóstico de TEA para uma em cada 149 crianças (Maenner et al., 2020). Estima-se que esse aumento esteja relacionado com a melhora do diagnóstico e também com a definição mais ampla do TEA. No entanto, esses fatores só explicam metade desse aumento, o que aponta para a importância dos fatores ambientais de risco, bem como nas interrelações desses fatores ambientais com fatores de predisposição genética. No Brasil, um estudo conduzido na cidade de Atibaia, São Paulo, avaliou a prevalência do Transtorno Global do Desenvolvimento, estimando diagnóstico de um a cada 267 habitantes (Paula et al., 2011). Em 2019, a lei 13.861/2019 foi sancionada, permitindo que os dados demográficos sobre TEA fossem incluídos no censo de 2020. Dessa forma, será possível estimar com mais propriedade a prevalência do transtorno na população brasileira. Porém, até o momento, a realização do censo demográfico nacional de 2020 é incerta devido a questões orçamentárias e políticas.

A diferença entre sexos é geralmente indicada com uma proporção de quatro meninos diagnosticados para cada menina com o diagnóstico. O motivo dessa diferença vem sendo muito investigado, e várias hipóteses acerca de questões biológicas durante o desenvolvimento foram levantadas, como a teoria do cérebro extremamente masculino, na qual o autismo seria uma exageração do perfil masculino, e o modelo de proteção feminina, em que mulheres com TEA teriam que apresentar um maior número de mutações genéticas para desenvolver o transtorno (Cohen, 2002; Jacquemont et al., 2014). Uma metanálise recente indicou que essa proporção é, na verdade, de 3:1 (Loomes et al., 2017). Ainda, é levantado um possível viés de diagnóstico, onde meninas que apresentam os critérios para serem diagnosticadas com TEA possuem mais risco de não serem, de fato, diagnosticadas. Essa diferença no diagnóstico pode estar ocorrendo porque as meninas apresentam um fenótipo de autismo distinto, que não corresponde inteiramente à conceitualização convencional do transtorno. Alguns estudos recentes observaram uma diferença nas características comportamentais e cognitivas entre meninas e meninos diagnosticados. Por

exemplo, as meninas demonstraram menos probabilidade de apresentar interesses restritos e uma menor habilidade cognitiva, o que dificultaria o fechamento do diagnóstico. Apesar das diferenças observadas, ainda são necessários mais estudos para estabelecer essa diferença com segurança, bem como a validade desses resultados. Além disso, o processo de "camuflagem", que consiste em mascarar os sintomas do transtorno para se encaixar em padrões neurotípicos, é mais comum em meninas. Esse processo pode ser um dos fatores que explicam a diferença observada no fenótipo autista entre sexos. O diagnóstico também pode ser influenciado pelos estereótipos de gênero, que mudam o olhar social perante ao paciente e, consequentemente, a interpretação dos sintomas. Isso prejudica não só o diagnóstico correto, como também a busca precoce de tratamento e identificação correta dos sintomas (Loomes et al., 2017; Frazier et al., 2012; Bargiela et al., 2016).

1.5 DIAGNÓSTICO

A base do diagnóstico para o TEA é a apresentação comportamental. O TEA é considerado um transtorno invasivo do desenvolvimento e, embora este seja bastante heterogêneo, existe um conjunto de características relacionadas à interação social, comunicação e comportamentos restritos ou repetitivos que é utilizado para o diagnóstico. A classificação utiliza critérios estabelecidos pelo DSM-5. Também são utilizadas como base as escalas de diagnóstico ADI-R (Autism Diagnostic Interview-Revised), uma escala de entrevista, e o ADOS-2 (Autism Diagnostic Observation Schedule, Second Edition) que é uma escala de observação. Estas escalas devem sempre ser aplicadas por profissionais capacitados (Risi et al., 2006; McPartland et al., 2012)

É importante que o diagnóstico seja feito precocemente, pois apresentará um impacto relevante no neurodesenvolvimento, podendo atrasar ou evitar que certas características surjam e trazer benefícios na qualidade de vida. Além disso, contribui para que a criança possa ter uma socialização adequada e não sofra reclusão por sua dificuldade de comunicação e interação, o que poderia restringir sua oportunidade de aprender. Nesse sentido, é necessária uma ação conjunta da comunidade para que esses indivíduos tenham tratamento e socialização de forma consciente (Lord et al., 2020).

1.6 TEA E GENÉTICA

A herdabilidade do TEA, estimada através de uma meta-análise envolvendo dados de gêmeos, é de 64 a 91% (Tick et al., 2016). Além disso, é muito comum o diagnóstico de TEA em conjunto com desordens cromossômicas e síndromes genéticas raras. As condições genéticas mais comumente associadas com o TEA são: a síndrome do X-frágil, esclerose tuberosa, neurofibromatose, Síndrome de Angelman e Síndrome de Rett. Até 25% dos casos diagnosticados possuem etiologia genética definida através de técnicas de avaliação de genética médica padrão. Alterações cromossômicas visíveis correspondem a aproximadamente 5% dos casos. As anormalidades mais frequentes são a duplicação 15q11-q13 e deleções 2q37, 22q11.2 e 22q.13.3 (Devlin e Scherer, 2012; Miles, 2011; Betancur, 2011).

A variabilidade genética é apontada como um dos fatores que contribuem com a heterogeneidade clínica do transtorno dentro do espectro do TEA em que o fator genético é preponderante. A arquitetura genética pode variar entre uma mutação penetrante que causa o transtorno por si só, até o acúmulo de muitos alelos de baixo risco (Masi et al., 2017). Variantes genéticas comuns têm sido apontadas como principais responsáveis pelo desenvolvimento do transtorno e são compartilhadas como fatores de risco com outros transtornos neuropsiquiátricos, como a esquizofrenia. Essas variantes possuem um baixo efeito individualmente, mas produzem, de forma conjunta, um background genético favorável ao desenvolvimento do transtorno. Mutações de novo, variações de número de cópias (CNVs) e mutações de ponto apresentam maiores efeitos individualmente, mas coletivamente são responsáveis por menos de 5% dos casos. As variantes comuns têm sido apontadas como principais responsáveis pela etiologia do transtorno, em consonância com outros transtornos psiquiátricos (Gaugler et al., 2014; Grove et al., 2019). Um estudo de GWAS realizado pelo Grupo de Trabalho com Transtorno do Espectro Autista do Consórcio de Genômica Psiquiátrica (2017) não identificou qualquer variante estatisticamente significativa, mas contribuiu com evidências sobre a participação das variantes comuns no desenvolvimento do TEA, além de demonstrar semelhanças genéticas entre TEA e Esquizofrenia.

1.7 TEA E O SISTEMA IMUNE

Em 1964, após uma epidemia de rubéola nos Estados Unidos, observou-se uma grande incidência de autismo nas crianças com síndrome congênita de rubéola (Chess, 1971). Após esse caso, outros patógenos foram investigados e associados com um maior risco de desenvolvimento do TEA. Sugeriu-se então, que um importante fator de risco seria a resposta imune contra o patógeno durante a gestação, que poderia alterar o desenvolvimento do sistema nervoso central do feto (Meltzer e Water, 2017). Desde então, a ativação imune materna tem sido apontada como um fator crítico para o desenvolvimento do TEA. A gestação apresenta diferentes perfis imunes e marcadores solúveis de inflamação ao longo do período, sendo essencial para o processo fisiológico natural. Entretanto, desvios nessas ativações imunes maternas podem gerar susceptibilidades para diferentes desordens neuropsiquiátricas, por causa da interação entre a resposta imune e fatores materno-fetais. Essas alterações são abservadas por alterações na expressão de citocinas e na ativação microglial e de astrócitos (Fontes-Dutra et al., 2020). Essa ativação pode ser gerada por infecções durante a gestação que geram ambiente imune inflamatório, induzindo a produção de citocinas materna, por uma resposta inflamatória generalizada ou pela perda da regulação imune (Meltzer e Water, 2017). Por isso, a regulação do sistema imune materno é essencial para o desenvolvimento do feto.

O sistema imune tem um papel muito importante no desenvolvimento do sistema nervoso central e periférico, pois além de participar em muitas atividades neurológicas, também regula a proliferação neuronal, formação de sinapse, plasticidade e remove neurônios apoptóticos. Já são reconhecidas muitas alterações nas respostas imunes de indivíduos com TEA, como maior ocorrência de infecções, asma e infecções intestinais persistentes. Além disso, há uma grande incidência de alergias e doenças autoimunes (Bjorklund et al., 2016; Mead e Ashwood, 2014).

O sistema imune funciona como uma defesa para as mudanças do ambiente, por isso, sua composição genética e programação inicial in utero são determinantes para definir o quanto o indivíduo consegue suportar essas mudanças. Essa regulação não interfere apenas na saúde, mas também na comunicação entre os sistemas nervoso e imune e no processamento neural. Alterações do sistema imune podem levar a efeitos profundos no sistema nervoso, no desenvolvimento do cérebro e em questões referentes à cognição (Estes e McAllister, 2015).

1.8 TEA, SISTEMA IMUNE E HLA-G

O Complexo Principal de Histocompatibilidade (MHC), que em humanos é também denominado de Sistema Antígeno Leucocitário Humano (HLA), é um complexo de genes localizados no braço curto do cromossomo 6, que estão diretamente envolvidos na resposta imune através da apresentação de antígenos, da regulação da inflamação, do sistema complemento e das respostas imunológicas inatas e adaptativas. O complexo é expresso em todas as células somáticas nucleadas e se divide em dois grupos: MHC I e MHC II, que possuem estrutura tridimensional semelhante e têm a função de ativar e apresentar peptídeos na superfície celular para células T CD8 + e CD4 +, respectivamente (Matzaraki et al., 2017).

O HLA tem papel central na medicina de transplantes e na terapia de transfusões porque participa das interações celulares restritas e da histocompatibilidade do tecido por sua discriminação celular de ‘próprio’ e 'não-próprio'. O MHC é caracterizado por ser altamente polimórfico e possuir um padrão extensivo de desequilíbrio de ligação que difere entre populações. O número elevado de alelos implica em um conjunto de MHC quase exclusivo para cada indivíduo, conferindo a habilidade de se ligar a uma ampla gama de peptídeos diferentes. Nos últimos 50 anos, muitos estudos têm demonstrado a influência do polimorfismo desse complexo sobre traços biológicos e susceptibilidade a doenças infecciosas e autoimunes (Matzaraki et al., 2017; Sommer, 2005).

Além de regular a imunidade, os genes do MHC podem ter um papel na reprodução e no comportamento social, como manutenção da gravidez e seleção de parceiros. A região genômica do MHC também vem sendo associada com o desenvolvimento e plasticidade do Sistema Nervoso Central (SNC), com interações neurológicas de células, função e comportamento sináptico, especialização do hemisférico cerebral e também distúrbios neurológicos e psiquiátricos (Shiina et al., 2009).

Um dos primeiros estudos associando variação imunológica e o desenvolvimento de TEA observou que pais de crianças autistas compartilham haplótipos de HLA mais frequentemente quando comparados com os pais de crianças fora do espectro (Stubbs et al., 1985). Desde então, muitos haplótipos e alelos de HLA já foram associados ao desenvolvimento do TEA (Torres et al., 2016).

O HLA-G é uma molécula de classe I não clássica. Ao contrário das moléculas de HLA clássicas, o HLA-G apresenta poucos polimorfismos na região codificadora, uma expressão tecidual limitada e a estrutura do gene bastante homóloga. Além disso, o HLA-G apresenta o peptídeo situado mais profundamente na fenda entre os domínios alfa 1 e 2 e seus

polimorfismos estão distribuídos ao longo das três cadeias alfa, enquanto as moléculas de HLA clássicas apresentam quase todos os polimorfismos concentrados em torno do sítio de ligação aos peptídeos. Essas características indicam que essa molécula não desempenha um papel importante na apresentação de antígenos. O HLA-G possui função tolerogênica, interagindo com componentes do sistema imune inato e adaptativo, como as células natural killer e os linfócitos T CD8+ citotóxicos. O gene apresenta mecanismos específicos de splicing alternativo, gerando sete RNAs mensageiros que codificam isoformas da proteína, sendo quatro ligadas à membrana (HLA-G1, G2, G3, and G4) e três solúveis (HLA-G5, G6, and G7) (Brenol et al., 2012; Carosella et al., 2003).

A molécula de HLA-G foi identificada inicialmente na interface feto-placentária, sendo expressa na superfície dos trofoblastos. Durante a gestação, o HLA-G é expresso pela placenta principalmente durante a implantação e início do desenvolvimento do feto. A interface materno-fetal é composta por trofoblastos fetais, leucócitos maternos e células estromais e endoteliais que compõem a decídua, formando trofoblastos extravilosos. Problemas no desenvolvimento e funcionamento do trofoblasto podem levar a perda gestacional ou patologias associadas à gestação, como pré-eclâmpsia, por isso, a implantação é uma fase crítica para o desenvolvimento fetal. A expressão do HLA-G contribui para a correta implantação do trofoblasto extraviloso, diferenciação de células deciduais, remodelação vascular e manutenção do estado de imunossupressão. Sua ação é essencial para a indução de tolerância ao feto semialogênico. (Persson et al., 2019; Gregori et al., 2015)

O HLA-G possui polimorfismos em íntrons, região promotora e região 3’ não traduzida (3’UTR). O polimorfismo de inserção/deleção de 14 pares de base se encontra na região não traduzida 3’ (rs371194629) e possui uma frequência de 61% para deleção e 38% para a inserção em uma população brasileira (Vargas et al., 2011). O alelo contendo a inserção dos 14 pares de base sofre um splicing alternativo adicional que retira mais 92 bases do início do éxon 8 no transcrito, influenciando a estabilidade do RNA mensageiro (Hiby et al., 1999; Rousseau et al, 2003) e sua inserção está associada com uma redução nos níveis de HLA-G expressos (Hviid et al., 2003). Essa variante já foi associada a problemas gestacionais como pré-eclâmpsia e aborto espontâneo recorrente, que também são mais frequentes em mães de pacientes com TEA (Vargas et al., 2011; Meltzer and Water, 2017; Guerini et al., 2015). Uma resposta imune materna alterada durante a implantação do embrião pode interferir no desenvolvimento do sistema nervoso central, levando ao desenvolvimento do TEA (Knuesel et al., 2014).

Considerando o papel central da molécula de HLA-G para a implantação do embrião e seu desenvolvimento, e também a crescente produção científica apontando para a relação entre sistema imune e desenvolvimento de transtornos psiquiátricos, nesse trabalho procuramos avaliar o papel da variante de inserção/deleção de 14 pares de base na região 3'UTR do HLA-G no desfecho de sintomatologia dentro do Transtorno do Espectro Autista.

2 OBJETIVOS

2.1 OBETIVOS GERAIS

Avaliar a influência do polimorfismo de inserção/deleção de 14pb na região 3'UTR do HLA-G no desenvolvimento da sintomatologia do TEA em uma amostra de crianças diagnosticadas com este transtorno, provenientes de uma população do sul do Brasil.

2.2 OBJETIVOS ESPECÍFICOS

1) Genotipar o polimorfismo de inserção/deleção de 14pb na região 3'UTR do HLA-G em amostras de material genético de crianças com TEA, a fim de descrever suas frequências alélicas e genotípicas.

2) Investigar a influência dessa variante gênica na susceptibilidade ao desenvolvimento do TEA em uma população brasileira.

3) Avaliar a potencial associação desse polimorfismo com sintomas de comportamento repetitivo, ecolalia, convulsão, epilepsia, instabilidade de humor, auto e hetero agressão, pânico, hiperatividade e desordens do sono, comumente presentes no TEA.

3 TRABALHO EXPERIMENTAL EM FORMA DE ARTIGO CIENTÍFICO

O trabalho apresentado a seguir está no formato de Original Research Paper a ser submetido para a revista Brain, Behavior & Immunity [ISSN: 0889-1591]

Analysis of the 14 base pair insertion/deletion polymorphism in the 3'UTR region of the HLA-G gene in Autistic Spectrum Disorder in a Brazilian cohort

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Highlights

- 14bp indel polymorphism in the 3'UTR of HLA-G gene was investigated in a Brazilian ASD cohort.
- The insertion genotype (14bp+/14bp+) was initially associated with hetero aggression.
- Further analyses should expand the cohort and include other ASD features.

Abstract

Autism Spectrum Disorder (ASD) is a highly inheritable neurodevelopmental disorder characterized by deficits in social communication as well as by repetitive patterns of behaviors and interests. The etiology of this disorder its not yet fully understood, but it has already been associated with altered immune response during pregnancy. HLA-G is a non-classical HLA class I molecule, expressed by the trophoblast at the maternal/fetal interface, and induces allogenic tolerance against the fetus. A 14-bp insertion in the HLA-G 3'UTR (rs371194629) associates with reduced levels of HLA-G mRNA and soluble HLA-G, hampering the efficacy of immune tolerance during pregnancy. To understand how this in/del polymorphism could participate in the ASD symptomatology, we evaluated the genotype frequencies of this polymorphism in a sample of 188 ASD patients. The individuals were diagnosed by DSM-IV in the neuropediatric ward of Hospital de Clínicas de Porto Alegre. Chi-square tests were performed to verify associations between genotypes and symptomatology. The genotype frequencies observed on ASD individuals were in accordance to the Hardy-Weinberg equilibrium. The insertion genotype (14bp+/14bp+) was initially associated with hetero aggression, but the association was lost after Bonferroni's correction. However, this study points to a possible contribution of this polymorphism to the clinical heterogeneity of ASD and new studies should be conducted considering the contribution of maternal-fetal interface and HLA-G to different scenarios of ASD outcome.

Key Words: Autism Spectrum Disorder, ASD, immunogenetics, immune system, HLA-G, HLA-G 3'UTR, polymorphism, maternal/fetal interface, pregnancy.

Introduction

Autism Spectrum Disorder (ASD) is a childhood-onset neurodevelopmental disorder involving persistent deficits in social communication and social interaction across multiple contexts. According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), ASD characteristically encompasses restricted, repetitive patterns of behavior, interests, or activities. The most recent data published in the Network for Monitoring Mental Disabilities and Autism (ADDM) and by the Center for Disease Control and Prevention (CDC) in the United States, points that one in each fifty-four children was diagnosed with autism in 2016. Even though ASD etiology remains unknown, this is a highly heritable disorder and, over the last four decades, several studies have shown associations between altered immune responses and impairment of the central nervous system (CNS). In this sense, both these conditions may be implied in the development or regulation of ASD pathogenesis and symptomatology (Bjorklund et al., 2016).

It is well established that the immune system influences neurodevelopmental processes at different levels, including the regulation of neuronal proliferation, synapse formation, remotion of apoptotic neurons and neural plasticity (Mead & Ashwood, 2015). Several levels of immune dysregulation were already observed in ASD patients, including increased proinflammatory cytokine Th1/Th2 ratios, altered immune cell populations including skewed T-cell and NK-cell responses, and the presence of anti-brain autoantibodies, which were associated with impaired development (Meltzer & Water, 2017). These responses are often associated with impaired behavior and with the degree of severity affecting ASD core symptoms (Sarasella et al., 2009). The maternal genetic background and the early immune system programming in utero seems to be key factors to determine the responses to environmental changes during the lifetime of each individual (Estes & McAllister, 2015). Indeed, some of the genetic variations associated with ASD are in genes involved in immune processes, and some of those participate in both immune and nervous systems. One of the first associations between ASD and the immune system was that parents of children with ASD shared HLA antigens more often than parents of non-affected children (Stubbs et al., 1985). Since this first observation, a considerable number of studies associated HLA alleles, genes and haplotypes with ASD etiology (Torres et al, 2016).

The Human Leukocyte Antigen system (HLA), that correspond to the Major Histocompatibility Complex (MHC) in humans, is a complex of genes located in the short arm of chromosome 6 that are directly involved in immune responses through antigen presentation, inflammation regulation, activation of the complement system, as well as other innate and adaptive processes. The classical MHC molecules participate in cellular communication processes and in tissue histocompatibility, being characterized by a high level of polymorphism (Matzaraki et al., 2017). HLA seems also to participate in social behavior construction, in processes related to reproduction, and in pregnancy maintenance. Putting together, these characteristics highlight the importance of HLA genes and molecules, in a range of infectious, autoimmune and immune-mediated diseases (Sommer, 2005).

There are two main MHC classes, MHC I and MHC II, which share similar three-dimensional structures and function, activating and presenting peptides on cell surface to CD8+ and CD4+ T cells, respectively. HLA-G is a non-classical class I molecule. Unlike

classic HLA molecules, HLA-G has few polymorphic sites in the gene coding region and presents limited tissue expression. Besides, HLA-G has specific alternative splicing mechanisms, generating seven different mRNAs that encode distinct protein isoforms, four of which are membrane-bound (HLA-G1, G2, G3, and G4) and three soluble (HLA-G5, G6, and G7) (Carosella et al., 2003). In addition, HLA-G presents peptides that are located more deeply in the cleft between the alpha domains 1 and 2, and its polymorphisms are distributed along the three alpha chains, while classic HLA molecules have almost all polymorphisms concentrated around the site of binding to peptides (Brenol et al., 2012).

The HLA-G molecule was first identified at the feto-placental interface, being expressed on the surface of the trophoblasts. The maternal-fetal interface is composed of fetal trophoblasts, maternal leukocytes, stromal and endothelial cells that make up the decidua. At this compartment, HLA-G has a tolerogenic function, interacting with molecules of the innate and adaptive immune system, such as natural killer cells and cytotoxic T lymphocytes. HLA-G gene is located at 6p21.3 chromosome region, within the class I major histocompatibility complex (MHC) gene cluster (Geraghty et al., 1987), and presents polymorphisms distributed along introns, the promoter region and also in the 3' untranslated region (3'UTR), which is the case for the 14 base pair insertion polymorphism (rs371194629). The allele containing the 14 base pair insertion undergoes an additional alternative splicing, removing 92 extra bases from the beginning of exon 8 in the transcript. The additional splicing influences mRNA stability (Hiby et al., 1999; Rousseau et al, 2003), and is associated with a significant reduction in both membrane bound and soluble HLA-G levels (Hviid et al., 2003; Hylenius et al., 2004). Interestingly, some studies suggested an association of the 14 base pair insertion polymorphism and the development of ASD in the Italian population (Guerini et al., 2015; Guerini et al., 2017).

Considering the central role of the HLA-G molecule in embryo implantation and its development, and also the growing scientific production pointing to the relationship between the immune system and the development of psychiatric disorders, this study aims to evaluate the potential role of the insertion/deletion variant of 14 base pairs in the 3'UTR region of HLA-G on Autistic Spectrum Disorder children, both concerning susceptibility and clinical features.

Methods

Samples

A total of 488 individuals were recruited to this study, being 188 children diagnosed with ASD, 172 mothers and 128 fathers of the diagnosed children. Of these, 149 families were previously collected and described by Longo et al. (2009) and the remaining patients were assessed and described by Schuch et al. (2014) at the Hospital de Clínicas de Porto Alegre (HCPA, Porto Alegre, Rio Grande do Sul, Brazil), in association with the Psychology Department of the Universidade Federal Rio Grande do Sul (UFRGS, Porto Alegre, Rio Grande do Sul, Brazil). In this study, DNA samples from those 188 children diagnosed with ASD were evaluated.

The presence of fragile X syndrome or other genetic conditions, chromosomal abnormalities and SNC lesional abnormalities were used as exclusion criteria, following the research protocol of Slongo et al. (2009). The probands were submitted to an evaluation by neuro pediatricians at the Neuropediatric Outpatient Unit from HCPA, where the diagnosis was attributed. The probands included were diagnosed as idiopathic ASD cases, according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) and fit the criteria for autistic disorder, Asperger disorder or PDD-NOS. Data on the presence or absence of commonly seen clinical ASD symptoms, such as repetitive behaviors, echolalia, seizures and epilepsy (at least 2 unprovoked seizures), mood instability, aggression (including unprovoked and recurrent aggressive behavior toward self and/or others), psychomotor agitation and sleep disorders, were also collected during regular appointments, inquiring the parents or caregivers for the presence of symptoms. The answer was considered when the patient showed any of the described symptoms before treatment with prescribed drugs. ASQ (Autism Screening Questionnaire) and CARS (Childhood Autism Rating Scale) scores were evaluated in some patients. These scores were used to assess severity of autistic behavioral symptoms. Social-demographic data of families were collected from clinical files. Schuch et al. (2014) and Longo et al. (2009) present more detailed information about exclusion criteria, tools for diagnostic and other clinical and socio-demographic data.

All participants signed an informed consent prior to their inclusion in the study. The study was in accordance with the ethical standards established in the 1964 Declaration of Helsinki and its later amendments and was approved by the Ethics Committee of HCPA and the Psychology Department (protocol numbers 05-451, 06-237 and 06632012.4.0000.5334, respectively).

Genotyping

DNA extraction was performed in whole blood samples utilizing a salting out procedure (Lahiri & Nurnberger, 1991). Genotyping for the 14bp+/14bp polymorphism in the 3' UTR of the HLA-G gene was performed by polymerase chain reaction (PCR) using sense HLA-G8F (5' TGTGAAACAGCTGCCCTGTGT 3') (Castelli et al., 2010) and antisense GmiRNA primers (5' CTGGTGGGACAAGGTTCTACTG 3') (Cordero et al., 2009). PCR

products for the HLA-G 14bp polymorphisms were genotyped in 8% polyacrylamide gel stained with ethidium bromide. This resulted in products of either 537 bp, when the insertion was present, or 523 bp when the 14 bp was absent.

Statistical Analysis

To verify whether the genotype frequencies met the Hardy–Weinberg Equilibrium, chi-square analysis was performed. To evaluate associations of the different symptomatology (repetitive behaviors, echolalia, seizures and epilepsy, mood instability, self and hetero aggression, psychomotor agitation and sleep disorders) of the ASD children and the HLA-G*14bp insertion/deletion polymorphism, chi-square analysis was also chosen, with p-values under 0.05 considered as statistically significant. Statistical analyses were carried out using SPSS 18 for Windows. The results found have gone through Bonferroni correction (n = 10)

due to the multiple hypothesis tests. A p-value <0.05 after Bonferroni correction for degree of freedom (pc) was considered statistically significant. We used a recessive model for our analyses, where the heterozygotes were combined with the less frequent homozygotes (homozygotes for the 14bp insertion).

Results

*Allelic and genotypic frequencies of the HLA-G*14bp insertion/deletion polymorphism*

The demographic data of children diagnosed with ASD is shown in table 1. Our sample showed a majority of male (80.9%) and Caucasoid (74.5%) patients, with a mean age of 9.74 ± 5.104 years. Table 2 shows both genotypic and allele distribution of the HLA-G*14bp insertion/deletion polymorphism in our sample. The genotypic frequency observed was 38.3% of probands homozygous for the deletion, 48.9% heterozygous individuals and 12.8% homozygous for insertion patients. The genotypic distribution was in agreement with the Hardy–Weinberg equilibrium (p>0.05).

*Association of the HLA-G*14bp insertion/deletion polymorphism with clinical symptoms*

The chi-square analyses showed an initial association between the HLA-G*14bp insertion allele and hetero aggression (p= 0.035), but after Bonferroni correction, the association was lost (pc= 0.35). No significant association was observed between the polymorphism and other symptoms, as shown on Table 3.

Discussion

During pregnancy, HLA-G is expressed in the placenta. Because of its tolerogenic function, the expression of HLA-G is essential for inducing tolerance to the semi-allogeneic fetus, mainly during implantation and early development, preventing the destruction of the fetal tissues by the maternal immune system and contributing to the correct implantation of extravillous trophoblast, differentiation of deciduous cells, vascular remodeling and maintenance of the state of immunosuppression (Carosella et al., 2003). In fact, implantation is a critical phase for fetal development, and HLA-G expression during this period is essential.

Failures in the normal development of the trophoblast can lead to loss of pregnancy or pathologies associated with pregnancy, such as pre-eclampsia (Gregori et al., 2015). It is interesting to notice that, in our sample, 38% of mothers presented some pregnancy complication (data not shown). Moreover, lower plasma HLA-G protein concentrations in first and second semester of gestation were observed in patients who developed preeclampsia (Yie et al., 2005) and the HLA-G*14bp insertion/deletion polymorphism was associated to recurrent spontaneous abortions (Hviid et al., 2002). Preeclampsia and repetitive abortions are gestational problems related to exacerbated immune activation and are seen more frequently

in mothers of children with ASD (Dachew et al., 2018; Funderbuck et al., 1983; Gardener et al., 2009).

The HLA-G*14bp insertion/deletion polymorphism in the 3'UTR region of the HLA-G gene is known to decrease the expression of HLA-G mRNAs, being also associated to lower levels of the soluble HLA-G molecule. Therefore, less HLA-G molecules expression on the trophoblast can lead to a more activated immune system during pregnancy. Maternal immune activation (mIA) is known to be a risk factor to neurochemical and behavioral abnormalities, and increasing evidence shows that it could be a shared environmental risk factor for CNS disorders, including autism and schizophrenia (Knuesel et al., 2014).

Recent studies in the Italian population found an association between ASD development and the HLA-G*14bp, where both 14bp+/14bp+ genotype and 14bp+ allele were statistically more frequent in children diagnosed with ASD and in their mothers, when compared to the general population (Guerini et al., 2015). The HLA-G*01:05N allele was also significantly more frequent in ASD patients (Guerini et al., 2017). The HLA-G*14bp insertion and activating KIR-HLA-C complex, which is also associated with ASD and suggested to correlate with inflammation during fetal development, were evaluated for a possible association with cognitive and behavioral scores and EEG profile in 119 ASD children. Analyses showed that children with KIR2DS1-HLAC2+/HLA14bp+ pattern had higher scores in the Childhood Autism Rating Scale (CARS) and Autistic Core Behaviour (Guerini et al; 2017).

In the present study, we observed an association between the 14*bp insertion in the 3'UTR of HLA-G gene and hetero agression ($p=0.035$), but this association was lost after Bonferroni correction. Aggressive behavior is a common symptom in ASD children and seems to be more present in severe cases (Giacomo et al., 2016), and both auto and hetero aggressive behaviors are present in other neuropsychiatric disorders, such as schizophrenia and attention deficit hyperactivity disorder (ADHD) (Connor et al., 2002; Raine et al., 2002) The exact relation between aggressive behavior and autism is not yet well established, but the treatment consists in pharmacological approaches (Parickh et al., 2008). Increasing evidence suggest that the immune system is involved with responses to social stress, wich can lead to aggressive behaviour. Patological levels of aggression showed an elevated proinflammatory cytokines in individuals diagnosed with intermittent explosive disorder (IED) or psychosis (Coccaro et al., 2014; Das et al., 2016). Also, individuals with high aggression traits showed higher circulating cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP) when compared with non-aggressive individuals (Takahashi et al., 2018). These results point to a possible collaboration of the immune system in the development of behavioral traits of symptoms related to the diagnosis of autism spectrum disorder.

Of our knowledge, no other study has associated the HLA-G*14bp insertion/deletion polymorphism with ASD symptomatology. Also, no study has yet evaluated the frequency of this polymorphism in an ASD cohort in a Brazilian population. Herein, we present data that could contribute to an even deeper discussion about how the genetics of the immune system contributes to ASD development and its clinical heterogeneity and new information about the distribution of this polymorphism in a Brazilian ASD cohort.

Conclusion

Although our analyses did not find a statistically significant association between HLA-G*14bp insertion/deletion polymorphism and symptoms related to the pathophysiology of ASD after Bonferroni correction, this study contributes to a better understanding of the multifactorial and multigenic genetic architecture of ASD pathogenesis, and points to new approaches in the crescent field of the immune system and ASD. Further analyses of the influence of the HLA-G*14bp insertion/deletion polymorphism and ASD development should include bigger cohorts and also analyze genotypes of the mothers. Future studies should also consider this polymorphism influence on clinical heterogeneity, especially concerning aggressive behaviors. Also, more studies must be conducted to investigate the genetic aspects that influence ASD development.

Acknowledgements

MZ receives a scholarship from Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brazil). BKL receives a fellow scholarship from CNPq (Brazil). GTN receives a scholarship from CNPq (Brazil). JABC receives a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

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Table 1. Demographic and clinical characteristics of ASD samples (n=188).

Age (years)[1]		9.74 ± 5.104
Gender (male)[2]		80.9% (152)
Ethnicity[2]	Caucasoid	74.5% (140)
	Afro-American	7.4% (14)
	Others	17.6% (33)
Symptoms[2,3]	Epilepsy	11.2% (21)
	Sleep disorders	56.4% (106)
	Mood instability	48.9% (92)
	Hetero-aggressive behavior	37.8% (71)
	Self-aggressive behavior	45.7% (86)
	Repetitive Behavior	75.5% (142)
	Convulsion	25.5% (48)
	Hyperactivity	60,6% (114)
	Echolalia	62.2% (117)
	Panic	30.3% (57)
ASQ[1]		22.019 ± 5.4342
CARS[1]		35.667 ± 5.3384

Table 2. HLA-G 14bp insertion/deletion (14bp+/14bp) genotype and allele distribution in 188 ASD children.

	ASD children n(%)
Genotype	
14bp-/14bp-	72(38.3)
14bp+/14bp-	92(48.9)
14bp+/14bp+	24(12.8)
Total	188(100)
Allele	
14bp-	236(62.8)
14bp+	140(37.2)
Total	376(100)

Table 3. Associations between HLA-G/14 bp insertion/deletion polymorphism and ASD related behaviors (n=188).

Symptom	Genotype	Absence, n (%)	Presence, n (%)	OR (CI 95%)	*p*	p_c
Panic	14bp-/14bp-	54 (41.2)	18 (31.6)			
	14bp+/14bp- + 14bp+/14bp+	77 (58.8)	39 (68.4)	OR 0.65 (0.34-1.27)	0.211	n.s.
Convulsion	14bp-/14bp-	57 (40.7)	15 (31.2)			
	14bp+/14bp+ + 14bp+/14bp-	83 (59.3)	33 (68.8)	OR 0.66 (0.33-1.32)	0.244	n.s.
Self-aggressive behavior	14bp-/14bp-	41 (40.2)	31 (36)			
	14bp+/14bp- + 14bp+/14bp+	61 (59.2)	55 (64)	OR 0.83 (0.46-1.51)	0.560	n.s.
Hyperactivity	14bp-/14bp-	32 (43.2)	40 (35.1)			
	14bp+/14bp+ + 14bp+/14bp-	42 (56.8)	74 (64.9)	OR 0.70 (0.39-1.29)	0.261	n.s.
Echolalia	14bp-/14bp-	29 (40.8)	43 (36.8)			
	14bp+/14bp- + 14bp+/14bp+	42 (59.2)	74 (63.2)	OR 0.84 (0.46-1.54)	0.576	n.s.
Mood instability	14bp-/14bp-	35 (36.5)	37 (40.2)			
	14bp+/14bp+ + 14bp+/14bp-	61 (63.5)	55 (59.8)	OR 1.17 (0.65-2.11)	0.596	n.s.
Hetero-aggressive behavior	14bp-/14bp-	38 (32.5)	34 (47.9)			
	14bp+/14bp- + 14bp+/14bp+	79 (67.5)	37 (52.1)	OR 1.91 (1.04-3.50)	**0.035**	0.35

Sleep disorders	14bp-/14bp-	31 (37.8)	41 (38.7)			
	14bp+/14bp+ + 14bp+/14bp-	51 (62.2)	65 (61.3)	OR 1.03 (0.57-1.87)	0.903	n.s.
Repetitive Behavior	14bp-/14bp-	16 (34.8)	56 (39.4)			
	14bp+/14bp- + 14bp+/14bp+	30 (65.2)	86 (60.6)	OR 1.22 (0.61-2.44)	0.573	n.s.
Epilepsy	14bp-/14bp-	65 (38.9)	7 (33.3)			
	14bp+/14bp+ + 14bp+/14bp-	102 (61.1)	14 (66,7)	OR 0.78 (0.30-2.04)	0.619	n.s.

4 CONCLUSÃO

Neste trabalho, investigamos a relação do polimorfismo inserção/deleção de 14pb na região 3'UTR do HLA-G com desfechos clínicos de sintomatologia em pacientes diagnosticados com TEA. Considerando nossas informações, este foi o primeiro trabalho a investigar essa relação em uma população brasileira. A construção do conhecimento de aspectos genéticos no desenvolvimento de transtornos em diferentes populações é essencial para que possamos de fato entender como elas são constituídas e quais são suas especificidades. Esse trabalho faz parte de um recente projeto que vem sendo realizado no Laboratório de Imunobiologia e Imunogenética, onde analisamos a possível relação do TEA com diversas variantes do sistema imune. Dessa forma, este estudo contribui para a construção desse conhecimento e também para novos questionamentos que originarão futuros projetos.

Estudos que incluem a associação de polimorfismos com o desenvolvimento dos sintomas podem contribuir para o desenvolvimento de um tratamento específico para cada paciente. Além disso, densifica a discussão sobre a contribuição de variantes para o desenvolvimento do TEA, que é conhecido por ser altamente herdável e clinicamente heterogêneo.

Mesmo que nossa análise não tenha apresentado uma relação estatisticamente significativa após a correção de Bonferroni entre o polimorfismo estudado e a sintomatologia do TEA, apontou para uma possível associação com um dos sintomas analisados, a hetero agressão. Portanto, seria interessante aumentar o tamanho amostral e analisar como essa associação se comporta. Para um maior entendimento da relação do gene HLA-G com o desenvolvimento do TEA, o sequenciamento da região 3'UTR poderia ser realizado, possibilitando a análise de outras variantes da região. Investigar as frequências desse polimorfismo nas mães também apresentaria dados relevantes, pois a região da placenta apresenta células fetais e maternais, e isso poderia influenciar no eventual desfecho. Além disso, temos perspectivas de construir e incluir um grupo controle, de forma que possamos comparar as frequências alélicas e genotípicas em análises futuras.

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We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article.
To find out more, please visit the Preparation section below.

INTRODUCTION

Brain, Behavior, and Immunity, founded in 1987, is the official journal of the Psychoneuroimmunology Research Society (PNIRS). This innovative journal publishes peer-reviewed basic, experimental, and clinical studies dealing with behavioral, neural, endocrine, and immune system interactions in humans and animals. It is an international, interdisciplinary journal devoted to original research in neuroscience, immunology, integrative physiology, behavioral biology, psychiatry, psychology, and clinical medicine and is inclusive of research at the molecular, cellular, social, and whole organism level. The journal features online submission and review. Manuscripts are typically peer-reviewed and returned to authors within 30 days of submission, leading to timely publication of experimental results. There are no submission fees or page charges for *Brain, Behavior, and Immunity*, which is published eight times a year. Detailed instructions for authors can be found at https://www.editorialmanager.com/bbi/default.aspx.

Research areas include: Physiological mechanisms that convey messages between the immune and nervous systems and regulate their functions Stress and immunity, including the role of stress-related hormones and neurotransmitters on the immune system. Actions of cytokines, growth factors and PAMP activation on neuronal and glial cells that regulate behavior, learning, memory and neurogenesis Role of hormones, growth factors and cytokines in the immune and central or peripheral nervous systems Interactions between the immune system and brain that are involved in development of neurological, psychiatric and mental health disordersRole of immunological processes in neurodegenerative disordersThe effects of psychotropic medications on immunological mechanisms and their potential relevance to therapeutic interventionsNeuroimaging studies examining how immunological mechanisms affect brain structure and functionClinical trials and experimental studies testing the effects on both immune stimulation and immune suppression on brain and behaviorThe role of microglia in pain, psychological processes and in psychiatric disordersImmunological mechanisms involved in traumatic brain injury and its resolution Immunologic disorders, infection and behavior Role of the immune system in development and maintenance of inflammatory and chronic pain Immune mechanisms that regulate the blood-brain-interface (BBI) Immune factors that affect health psychology Sleep, exercise, immunity and health Immune system interactions that affect behavior following use of psychotropic drugs, alcohol and other drugs of abuse Healthy aging of the immune system and brain Role of inflammation and stress during perinatal development Cancer and its treatment, stem cells and their effects on brain behavior and immunity Reciprocal communication between the microbiome, immune and nervous systems Regulation of nerve injury and repair by the immune system Psychosocial, behavioral, and neuroendocrine influences on immunity and on the development and progression of immunologically-mediated diseases Nutrition, inflammation, obesity and behavior Genomics of behavior and immunity

Types of Article
Original full-length research reports, full-length review articles,short communications, brief commentaries, and letters to the editor will be considered for publication.

Full-length research reports: The chief criteria for the acceptance of submitted papers are the quality, originality, and clarity of the work reported, addressing one or more of the research areas reported above. There is no word limit on full length research reports, but papers should be consisely written and most should be able to articulate their findings within approximately 6,000 words.

Reviews: The journal publishes invited or unsolicited reviews on a contemporary topic, discussed authoritatively with the aim of providing a solid, and often novel, interpretation of research evidence, and of integrating a mechanistic model when applicable. Reviews consist of approximately 6,000 words of text and no more than 100 scientific references. Reviews must contain at least one figure highlighting the key aspects of the article, complete with explanatory figure legends. If appropriate,

a color version of the figure can be published in the online publication, with a black-and-white figure in the print version. If the author chooses this option, the figure legend must be self-explanatory in the absence of color-coding.

Short communications: Manuscripts published as short communications are, primarily, reports of novel, solid, important findings on contemporary, fast-moving topics. Small replication studies or incomplete data that do not move the field forward, and descriptions of methods and techniques, are not appropriate for this format. Papers will be considered short communications if the text, references, and a maximum of two tables or figures (or one of each) are limited to 3,500 words. Authors may elect to include additional illustrations, but the limitation to 3,500 words will remain.

Commentaries: These are short pieces written to accompany the publication of impactful full-length research reports. Invited by the Editor, they are limited to 900-1000 words and 5-10 references (including a refence to the relevant published report).

Viewpoints: These are opinion pieces that provide a personal view on broad, contemporary topics relevant to the interaction between health, brain, behaviour and immunity. Invited by the Editor, they are limited to 900-1000 words and 5-10 references, and will generally be immediately 'open-access' at no costs to the auhors.

Letters to the editor: These should be of high scientific quality, contain less than 500 words, and cite no more than 5 scientific references. If the letter is directed to a paper published in Brain, Behavior, and Immunity, the author of that paper will be provided an opportunity to respond. Both the letter to the editor and the author's response will be published simultaneously.

Announcements: *Brain, Behavior, and Immunity* will consider for publication announcements of interest to the readership such as notices of scientific meetings.

Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Poor standard of grammar or spelling will lead to the paper being sent back to Authors without peer-review. Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Study design and statistical reporting

BBI aspires to publish papers with the highest standards of reporting and presentation of methodological details, including the study design and the statistics used.

Study Design: State whether: 1) samples/animals were assigned randomly to various experimental groups (and the specific method of randomization); 2) the data collected was processed randomly and appropriately blocked; 3) experimenters were blind to group assignment and outcome assignment; and 4) an appropriate sample size was computed when the study was being designed.

Data Handling: Clearly state the numbers of participants, animals, or samples included in the study. Provide detailed explanations of the reasons for any attrition in the study. Explain how outliers are defined and handled and any data removed before analysis must be reported. Report how often each experiment was performed and whether the results were substantiated by repetition under a range of conditions. Sufficient information about sample collection must be provided to distinguish between independent biological data points and technical replicates.

Statistical reporting: Authors should identify the precise statistical tests used. In addition, planned comparisons, details of controls and power analyses to determine sample sizes, if applicable, should be reported. Complete results of the statistical analyses, including p values (rather than ranges), degrees of freedom and any estimates of effects size, should be reported in full in the Results section, including all within- and between-subject factors. For multiple comparisons and multiple correlations, define measures taken to reduce Type 1 errors. For neuroimaging studies, methods for controlling for multiple comparisons and the cluster-forming statistical threshold used must be reported. For ANOVAs, and other multivariate analyses, define measures taken to control for violation of the sphericity assumption and how you report results of corrected degrees of freedom statistics. Finally, state the name and version of the statistical software that was used.

Addressing Sex as a Biological Variable: We ask all authors to ensure proper consideration of sex as a biological variable. For example, any papers utilizing subjects (cells, animals, humans) of only one sex must state the sex of the samples in the title and abstract of the paper, with the obvious exception of sex-specific issues (e.g., prostate or ovarian function). Authors must also state the rationale for using samples from one sex rather than from both. For cellular work, the sex of origin of cells used should be reported, or if cells or tissue from both sexes were used without regard to sex, this fact should be indicated. Finally, the inability for any reason to study sex differences where they may exist should be discussed as a study limitation.

Format

Manuscripts should be prepared using a 12-point font, double-spaced throughout (including tables, footnotes, references, and figure captions) with 1-in. margins on all sides. Unusual typeface is acceptable only if it is clear and legible. For initial submission, all manuscripts must be prepared and submitted in one of the following formats: Microsoft Word (.doc), WordPerfect (.wps), or Rich Text Format (.rtf). All figures and tables should be clearly labeled at the top.

Revised manuscripts should not be marked using underlined or bolded words to indicate changes from the original submission. Instead, changes in the revised manuscript must be explained in a rebuttal letter. Submission of all revised manuscripts requires both figures and tables to be submitted separately from the manuscript text: do not insert figures and tables at the end of the text for revised manuscripts. Instead, the electronic submission system requires identification and submission of figures and tables separate from the text of revised manuscripts (see information below for graphs, scans, and illustrations). For more information, please also see the Author Gateway Web page for *Brain, Behavior, and Immunity* available through the journal home page at https://www.elsevier.com/locate/ybrbi.

Contact details for submission

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Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

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• All tables (including titles, description, footnotes)
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• Journal policies detailed in this guide have been reviewed
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BEFORE YOU BEGIN

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Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Studies in humans and animals

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double anonymized) or the manuscript file (if single anonymized). If there are no interests to declare then please state this:

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Authorship

While the journal does not request details of authors contribution, in accordance with the Consensus Statement on Surgery Journals Authorship (2005) we expect that all authors meet all three of the following conditions: 1) Authors make substantial contributions to conception and design, and/or acquisition of data, and/or analysis and interpretation of data; 2) Authors participate in drafting the article or revising it critically for important intellectual content; and 3) Authors give final approval of the version to be submitted and any revised version.

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Randomized controlled trials should be presented according to the CONSORT guidelines. At manuscript submission, authors must provide the CONSORT checklist accompanied by a flow diagram that illustrates the progress of patients through the trial, including recruitment, enrollment, randomization, withdrawal and completion, and a detailed description of the randomization procedure. The CONSORT checklist and template flow diagram are available online.

Registration of clinical trials
Registration in a public trials registry is a condition for publication of clinical trials in this journal in accordance with International Committee of Medical Journal Editors recommendations. Trials must register at or before the onset of patient enrolment. The clinical trial registration number should be included at the end of the abstract of the article. A clinical trial is defined as any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects of health outcomes. Health-related interventions include any intervention used to modify a biomedical or health-related outcome (for example drugs, surgical procedures, devices, behavioural treatments, dietary interventions, and process-of-care changes). Health outcomes include any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events. Purely observational studies (those in which the assignment of the medical intervention is not at the discretion of the investigator) will not require registration.

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References
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements
There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.
If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.
Divide the article into clearly defined sections.

Figures and tables embedded in text
Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure or table.

REVISED SUBMISSIONS

Use of word processing software
Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results
Results should be clear and concise.

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This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract
A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords
A list of up to 10 **keywords** or phrases suitable for indexing should be provided.

Abbreviations
Do not use periods after abbreviations of measure (cm, s, kg, mA, etc.) in text or tables, except for "in." (inch). The American Chemical Society *Style Guide* should be used as a reference for proper abbreviations.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

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If no funding has been provided for the research, please include the following sentence:

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Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

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• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
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• Please note that individual figure files larger than 10 MB must be provided in separate source files.

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You are urged to visit this site; some excerpts from the detailed information are given here.
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TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
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References
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Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

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As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

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