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Avaliação citogenética molecular em portadores de cardiopatia congênita através da técnica de MLPA.

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RESUMO

Introdução: O desenvolvimento normal do coração compreende uma maquinaria de eventos genéticos altamente regulada, que envolve importantes fatores de transcrição. Alterações cardíacas estruturais, que caracterizam a cardiopatia congênita (CHD), são fortemente associadas a alterações cromossômicas e a CNVs, que são fatores de risco para o desenvolvimento da CHD. **Objetivo:** Investigar, através da técnica de MLPA, a presença de CNVs em genes de referência para o desenvolvimento cardíaco normal em portadores de CHD. **Material e Métodos:** Os genes *GATA4, NKX2-5, TBX5, BMP4* e *CRELD1* e a região cromossômica 22q11.2 foram analisadas em 207 pacientes portadores de CHD (idade 1 dia ± 13 anos), admitidos pela primeira vez na Unidade de Terapia Intensiva (UTI) de um hospital pediátrico, utilizando o kit SALSA P311-B1 CHD. **Resultados:** Foram detectadas CNVs em 7 pacientes (3,4%); 4 casos de deleção da região 22q11.2 (1,9%), 2 casos de deleção do *GATA4* (1%) e 1 caso de duplicação da região 22q11.2 (0,5%). Não foram identificadas alterações nos genes *NKX2-5, TBX5, BMP4* e *CRELD1*. **Conclusão:** Del*GATA4* parece estar presente em um número significativo de pacientes com CHD, especialmente os com defeitos septais, PLSVC, alterações da artéria pulmonar e achados extracardíacos. A triagem de *GATA4* parece ser mais efetiva quando direcionada a estes achados. A investigação de mutações em *GATA4* e de 22q11.2DS em portadores de CHD são importantes na antecipação do diagnóstico, contribuindo para o planejamento familiar.

Palavras-chave: defeito cardíaco congênito; MLPA; variação do número de cópias do DNA; fator de transcrição *GATA4*; síndrome de deleção 22q11.2.

ABSTRACT

Introduction: The normal development of the heart comprises a highly regulated machinery of genetic events, involving important transcriptional factors. Structural changes in the heart, which characterize congenital heart disease (CHD), have been strongly associated with chromosomal abnormalities and copy number variants (CNVs) which are risk factors for the development of CHD. **Aim:** To investigate, through the MLPA technique, the presence of CNVs in reference genes for normal cardiac development in patients with CHD. **Materials and methods:** *GATA4, NKX2-5, TBX5, BMP4* and *CRELD1* genes and 22q11.2 chromosome region were analyzed in 207 patients with CHD (age 1 day ± 13 years), admitted for the first time in a cardiac intensive care unit from a pediatric hospital, using MLPA SALSA P311-B1 CHD kit. **Results:** CNVs were detected in 7 patients (3.4%); 4 cases of 22q11.2 deletion syndrome (1.9%), 2 cases of *GATA4* deletion (1%) and 1 case of 22q11.2 duplication syndrome (0.5%). No patients with alterations in the *NKX2-5, TBX5, BMP4* and *CRELD1* genes were identified. **Conclusion:** Del*GATA4* appears to be present in a significant number of CHD patients, especially those with septal defects, PLSVC, pulmonary artery abnormalities and extracardiac findings. *GATA4* screening seems to be more effective when directed at these findings. The investigation of mutations in *GATA4* and 22q11.2DS in CHD patients is important in anticipating the diagnosis, contributing to family planning.

Keywords: congenital heart defects; MLPA; DNA copy number variation; GATA4 transcription factor; 22q11.2 deletion syndrome.

Lista de abreviaturas

AC: do inglês, *aortic coarctation*

AS: do inglês, *aortic stenosis*

ASD: do inglês, *atrial septal defect*

AVSD: do inglês, *atrioventricular septal defect.*

BAV: do inglês, *bicuspid aortic valve*

CGH: do inglês, *comparative genomic hybridization*

CHD: do inglês, *congenital heart disease*

CNV: do inglês, *copy number variation*

DNA: do inglês, *deoxyribonucleic acid*

DORV: do inglês, *double outlet right ventricle*

EDTA: do inglês, *ethylenediaminetetraacetic acid*

FISH: do inglês, *fluorescence in situ hybridization*

HCSA: Hospital da Criança Santo Antônio

HLHS: do inglês, *hypoplastic left heart syndrome*

HRHS: do inglês, *hypoplastic right heart syndrome*

IAA: do inglês, *interrupted aortic arch*

MA: do inglês, *mitral atresia*

MS: do inglês, *mitral stenosis*

MLPA: do inglês, *multiplex ligation-dependent probe amplification*

PA: do inglês, *pulmonary atresia*

PCR: do inglês, *polymerase chain reaction*

PDA: do inglês, *patent ductus arteriosus*

PS: do inglês, *pulmonary stenosis*

PTA: do inglês, *persistent truncus arteriosus*

PLSVC: do inglês, *persistent left superior vein cava*

QF-PCR: do inglês, *quantitative fluorescent polymerase chain reaction*

SNP: do inglês, *single nucleotide polymorphism*

TA: do inglês, *tricuspid atresia*

TAPVR: do inglês, *total anomalous pulmonary venous return*

TGA: do inglês, *transposition of the great arteries*

TOF: tetralogia de Fallot

UTI: Unidade de Terapia Intensiva

VSD: do inglês, *ventricular septal defect*

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SUMÁRIO

1. REFERENCIAL TEÓRICO

A cardiopatia congênita ou *congenital heart disease* (CHD) se refere a um conjunto de alterações estruturais/funcionais do coração e grandes vasos que surgem na embriogênese cardíaca[1]. A CHD é uma das principais causas de morbidade e mortalidade infantil, afetando cerca de 1% dos nativivos, com incidência de 19 a 75 casos a cada 1.000 recém-nascidos[2,3].

A etiologia da CHD é complexa, onde apenas 20% dos casos podem ser atribuídos a alterações cromossômicas, síndromes mendelianas, defeitos de gene único não sindrômicos ou teratógenos[4]. Alterações cromossômicas estão frequentemente associadas a ocorrência de lesões cardíacas complexas, gerando uma alta incidência de perdas fetais[5]. A combinação de fatores genéticos, epigenéticos e ambientais ocorre na maioria dos casos. Dentre os fatores externos destacam-se infecções maternas, deficiência nutricional e uso de álcool/drogas durante a gestação (Figura 1)[4].

O conhecimento relacionado a CHD avançou muito desde sua descrição e classificação, facilitando o diagnóstico preciso e possibilitando interferências durante o período pré-natal. Estratégias corretivas contribuíram para o aumento da expectativa de vida de crianças e adultos com CHD[6]. A sobrevivência dos pacientes varia de acordo com a gravidade da doença, sendo de 98% para CHD leve, 96% para CHD moderada e 56% para CHD grave. Os pacientes submetidos a procedimentos corretivos necessitam de monitoramento contínuo devido ao alto risco de complicações tardias que podem resultar em cirurgias adicionais[7].

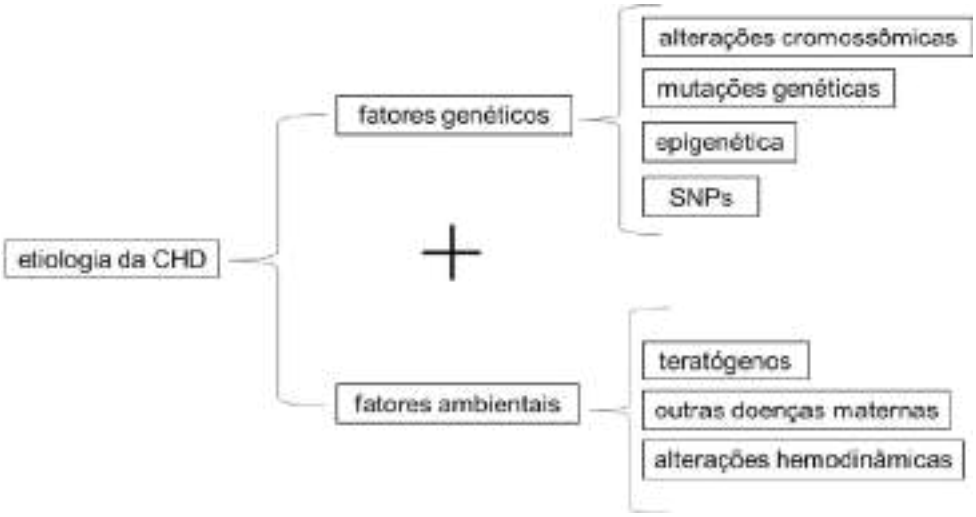

Figura 1 Etiologia da cardiopatia congênita. Adaptado de Blue et al., (2012)[4]

Em casos não sindrômicos, mutações em mais de 50 genes já foram descritas como diretamente implicadas com o aparecimento da CHD. Porém, estudos com camundongos hipotetizaram que alterações em mais de 500 genes podem causar defeitos cardíacos[8], fornecendo um indicativo para a investigação destes fatores e seu papel como causadores da doença em humanos.

Mutações em genes reguladores transcricionais essenciais, como *GATA4* e *NKX2-5,* são amplamente estudadas como fatores causais da CHD. Estas mutações impactam a expressão dos genes que coordenam o desenvolvimento cardíaco normal[9,10]. Além disso, mutações em genes distintos podem causar malformações idênticas, demonstrando a interdependência dos fatores responsáveis pelo desenvolvimento cardíaco[1].

Apesar da quantidade significativa de estudos que tentam estabelecer os fatores causais da CHD, delineando as vias moleculares principais da cardiogênese, ainda restam dúvidas do real papel destes genes e seus padrões de expressão na patologia das malformações cardíacas, bem como

sua correspondência com o fenótipo clínico do paciente. Assim, enfatiza-se a necessidade de testes genéticos em indivíduos afetados, objetivando o desenvolvimento de novas medidas profiláticas, estratégias de tratamento e até mesmo novos tratamentos.

1.1. Manifestações clínicas da CHD

As malformações cardíacas estão entre os defeitos mais frequentemente observados no período neonatal e são responsáveis por 3 a 5% das mortes nesse período. Cerca de 20 a 30% das crianças morrem no primeiro mês de vida, principalmente por insuficiência cardíaca[11].

A CHD pode ser classificada em cianótica ou não-cianótica devido à mistura, ou não, de sangue venoso e arterial. Crianças cianóticas apresentam tonalidade azulada nos lábios e pele e diminuição do fluxo pulmonar. As principais cardiopatias cianóticas incluem transposição das grandes artérias (TGA), tetralogia de Fallot (TOF), anomalia de Ebstein, atresia tricúspide (TA), atresia pulmonar (PA) e *truncus arteriosus* persistente (PTA). Na categoria de CHD não-cianótica, destacam-se as lesões obstrutivas do lado esquerdo como hipoplasia de ventrículo esquerdo (HLHS), estenose mitral (MS), aórtica (AS), coarctação da aorta (AC) e interrupção do arco aórtico (IAA); e ainda a CHD de defeitos de septação ventricular (VSD), atrial (ASD) ou atrioventricular (AVSD). Defeitos de válvula aórtica bicúspide (BAV) e de septação estão entre as cardiopatias mais comuns[3,12] (Figura 2).

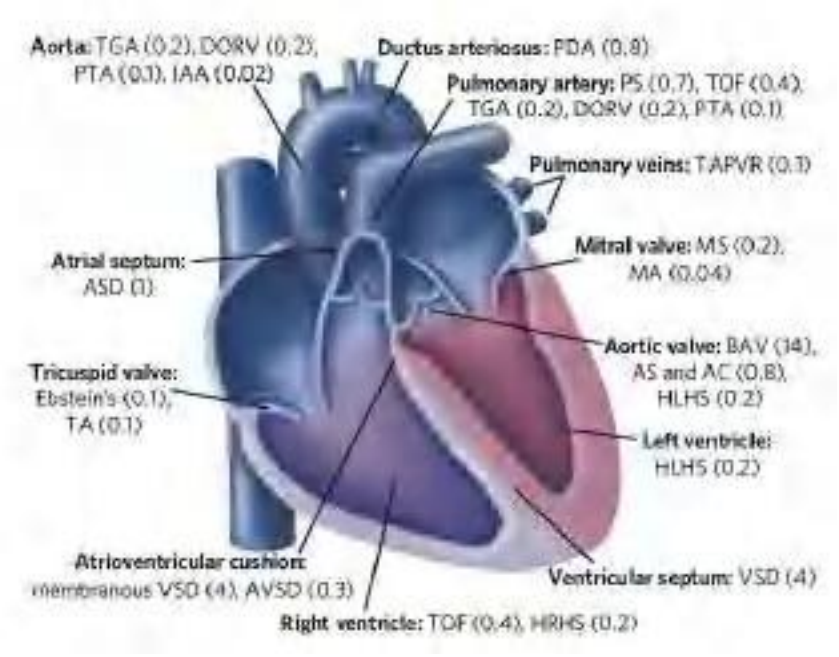

Figura 2 Malformações cardíacas usualmente identificadas na infância.

Adaptado de Bruneau, 2008.

*Incidência por 1.000 nascidos vivos indicado entre parênteses. (AC) coarctação da aorta; (AS) estenose aórtica; (ASD) defeito do septo atrial; (AVSD) defeito do septo atrioventricular; (BAV) defeitos de válvula aórtica bicúspide; (DORV) dupla via de saída do ventrículo direito; (Ebstein's) anomalia de Ebstein; (HLHS) hipoplasia do ventrículo esquerdo; (HRHS) hipoplasia do ventrículo direito; (IAA) interrupção do arco aórtico; (MA) atresia mitral; (MS) estenose mitral; (PDA) persistência do canal arterial; (PS) estenose pulmonar; (PTA) *truncus arteriosus* persistente; (TA) atresia tricúspide; (TAPVR) anomalia total do retorno venoso pulmonar; (TGA) transposição das grandes artérias; (TOF) tetralogia de Fallot; (VSD) defeito do septo ventricular.

Os diferentes tipos de malformação também são classificados de acordo com sua complexidade, sendo eles, grave, moderada e leve. Pacientes com CHD grave possuem pior prognóstico e mais frequentemente desenvolvem complicações. Apesar da baixa frequência populacional (4%), as alterações menores também devem ser investigadas pois atuam como marcadores externos de lesões complexas, facilitando o diagnóstico correto do paciente[13,14]. Na Europa e na América Latina, a incidência de CHD grave é de ~1,5 casos por 1.000 nascidos vivos[15,16]. Estes dados podem ter sido estimados erroneamente, pois os casos registrados correspondem a

malformações diagnosticadas apenas ao nascimento, excluindo qualquer outro defeito congênito que possa ser identificado durante a primeira infância[15].

Segundo Miller et al., (2007)[17], recém-nascidos com CHD também podem apresentar distúrbios neurológicos generalizados, semelhantes a neonatos prematuros, refletindo o desenvolvimento cerebral anormal em virtude do comprometimento do suprimento de oxigênio intrauterino.

1.2. Aspectos genético e molecular da CHD

A CHD tem sua etiologia determinada por alterações cromossômicas, desordens não sindrômicas envolvendo genes únicos (20%) ou por associação de genes e fatores ambientais na grande maioria dos casos (80%). A biologia molecular e as novas técnicas disponíveis têm possibilitado a descoberta de genes que podem interagir entre si ou com fatores externos gerando uma pré-disposição ao desenvolvimento da doença[2-4,18].

Mudanças no número de cópia de segmentos específicos do DNA são frequentemente associados à etiologia ou pré-disposição para doenças. Tais alterações podem incluir desde a presença de uma cópia extra de um cromossomo inteiro, a deleções e duplicações de inúmeros pares de bases ou pequenos fragmentos cromossômicos envolvendo apenas um único éxon[19]. Esta perda ou ganho de material genético desencadeia um efeito direto sobre a dosagem gênica, aumentando ou diminuindo o padrão de expressão dos genes afetados[4].

A ocorrência de CHD juntamente a outras malformações ou como parte de uma síndrome ocorre em 25 a 40% dos casos. Dentre os indivíduos que possuem alterações cromossômicas estruturais ou numéricas, 30% também

apresentam CHD[18,20,21]. São encontradas proporções significativas de CHD associada a aneuploidias, sendo que, 50% dos indivíduos com trissomia do 21 (síndrome de Down), 80% com trissomia do 13 (síndrome de Patau) e 90-100% com trissomia do 18 (síndrome de Edwards) apresentam algum defeito cardíaco. As síndromes de Turner e Klinefelter são acompanhadas de alguma alteração em 35% e 50% dos casos, respectivamente[21,22]. Síndromes de microdeleção e microduplicação, como a síndrome Velocardiofacial ou DiGeorge (22q11.2DS), síndrome de Williams (del7p11.23) e a síndrome de microduplicação 22q11.2 (22q11.2DupS), que incluem CHD como fenótipo também são frequentemente estudadas[8,23].

O desenvolvimento cardíaco normal é regulado estritamente pela interação de fatores de transcrição e seus reguladores em vias de sinalização específicas. Diante disso, muitos estudos descrevem conjuntos de genes candidatos e vias moleculares que, quando afetadas, provocam erros na maquinaria molecular e se manifestam através de alterações cardíacas. As CNVs (*copy number variation*), mutações somáticas, alterações nas taxas transcricionais e microRNA estão entre os mecanismos que podem explicar a base molecular da CHD[24].

As CNVs, presentes em 12% do genoma humano, incluem duplicações e deleções cromossômicas com tamanho variado de 1 Kb a vários Mb e são utilizadas para identificar genes candidatos. As CNVs detectadas em pacientes com CHD são investigadas, buscando a sua associação com a doença. Outras abordagens genéticas, como SNPs (*single nucleotide polymorphism*), também são utilizadas focando na detecção de polimorfismos em genes candidatos para a CHD[8,21].

A maioria dos portadores de CHD não apresentam defeitos de gene único, direcionando a investigação para possíveis combinações entre mutações em genes múltiplos ou para a combinação de mutação e fatores externos que resultam no amplo espectro fenotípico[21]. Estudos sugerem que um fator familiar, como casamento consanguíneo pode participar da etiologia multifatorial da doença[24,25].

Apesar dos grandes avanços no diagnóstico e tratamento o conhecimento sobre a etiologia e herdabilidade da CHD ainda é limitado. Estudos sobre o mecanismo molecular associado ao desenvolvimento cardíaco normal têm ajudado na identificação de fatores causais da CHD, fornecendo evidências da participação crucial de genes na morfogênese cardíaca. Os principais genes envolvidos são reguladores transcricionais, moléculas sinalizadoras e genes estruturais[20,21].

1.3 Genes associados a CHD sindrômica e não-sindrômica

A CHD está associada a causas genéticas e ambientais e muitas das comorbidades apresentadas por portadores desta doença, como insuficiência cardíaca, arritmia e alterações neurocognitivas, podem ser atribuídas a mutações genéticas. Em 35% dos casos é identificado alguma causa genética significativa. Os genes responsáveis pelo desenvolvimento cardíaco são os mais afetados por estas mutações, atualmente, mais de 50 genes são potenciais candidatos a serem estudados[8,26]. As mutações podem ser herdadas ou ocorrer de forma esporádica ou *de novo*[26].

A CHD ocorre associada a uma síndrome ou de forma isolada. Distúrbios na dosagem gênica, defeitos de gene único, grandes

deleções/duplicações ou microdeleções/microduplicações estão relacionados a CHD sindrômica. A forma isolada é a mais prevalente e mutações em fatores de transcrição são os mais comuns, outros genes afetados participam das vias de sinalização ou são componentes estruturais do coração. As mutações em genes causadores da doença são classificadas como de alta penetrância, de baixa penetrância para genes de susceptibilidade e variações comuns em genes de risco para a CHD[24].

Inúmeras CNVs já foram caracterizadas e associadas a síndromes clínicas que incluem CHD, uma das primeiras a ser descrita foi a deleção de 3 Mb do braço longo do cromossomo 22 (del22q11). A síndrome 22q11.2DS é a microdeleção humana mais comum e apresenta um fenótipo variável que inclui a cardiopatia congênita, hipocalcemia, imunodeficiência, características faciais clássicas e desordens de neurodesenvolvimento[27]. A região deletada do cromossomo 22 abrange o fator de transcrição *TBX1,* afetando seus níveis de expressão. A haploinsuficiência deste gene está relacionado ao desenvolvimento anormal da faringe, corroborando com a clínica apresentada por portadores da síndrome. Outros estudos ainda mostram que *TBX1* está fortemente associado à remodelação da cromatina, sugerindo a utilização de drogas epigenéticas no resgate de fenótipos que incluem este tipo de alteração genética[28,29].

A deleção do braço curto do cromossomo 8 (del8p23), também associada a CHD juntamente com atraso de desenvolvimento, abrange o fator de transcrição *GATA4*, que é um dos principais genes reguladores da função e do desenvolvimento cardíaco. Zhou e colaboradores (2017)[30] destacaram o papel essencial de *GATA4* no que eles chamaram de *‘second heart field’*,

envolvendo um subgrupo de células progenitoras responsáveis pela septação cardíaca. Foram identificadas também duas vias de sinalização, a *Pten* e a de *Hedgehog*, que parecem agir de forma independente de *GATA4*, sugerindo uma possível forma de restauração do defeito cardíaco. A haploinsuficiência de *GATA4* resulta em tipos comuns de CHD mostrando seu papel essencial na separação funcional das quatro câmaras cardíacas[9].

Além do *GATA4* e *TBX1*, destacam-se por seu papel funcional na cardiogênese, os genes fatores de transcrição *NKX2-5 e TBX5*. O *NKX2-5* (5q35) é um marcador conhecido de células progenitoras do miocárdio, sendo crítico para o desenvolvimento cardíaco em mamíferos. Mutações envolvendo este gene foram uma das primeiras a ser descrita por ter uma associação direta com a CHD[26]. A mutação em *NKX2-5* altera a regulação da formação do septo durante a morfogênese cardíaca, podendo apresentar diferentes quadros de malformações[10,31].

Defeitos de gene único que levam a síndromes associadas com a CHD são frequentemente investigados. Mutações no gene *TBX5* (12q24) causam duas condições marcadas por defeitos septais, a rara síndrome de Holt-Oram e Tetralogia de Fallot. Este fator de transcrição tem papel bem definido no desenvolvimento do coração e dos membros superiores[32,33]. *TBX5* é um regulador do desenvolvimento cardíaco, que contribui para a septação do coração através da interação com o complexo de desacetilase e remodelação do nucleossomo (NuRD). Quando mutado, este gene altera esta interação *TBX5*-NuRD, reprimindo a expressão de alguns genes importantes, provocando a CHD[34].

Garg e colaboradores (2003)[9], indicam que a formação de um complexo entre *GATA4* e *TBX5* são causadores das malformações do septo. Estes genes interagem durante a formação da mesoderme cardíaca e essa relação é interrompida em portadores de CHD. Acredita-se que *GATA4, TBX5* e *NKX2-5* atuem em conjunto regulando outros genes necessários para a formação do septo. Qualquer rompimento dessa interação, causada por mutações nestes genes, pode acarretar em defeitos congênitos específicos.

CRELD1 (3p25) é um fator de crescimento epidérmico que participa da adesão celular, juntamente com o *BMP4* (14q22) atuam na regulação do desenvolvimento do coxim endocárdico, estrutura que serve de sustentação para o septo cardíaco. Alterações nestes genes estão fortemente associadas a AVSD. Variações deletérias foram encontradas em *CRELD1* em portadores e não portadores de Síndrome de Down onde os autores conseguiram demonstrar a associação específica de *CRELD1* e AVSD[35-38].

A investigação de genes candidatos em pacientes com CHD pode ajudar no diagnóstico precoce e no manejo dos sintomas extracardíacos. A compreensão do desenvolvimento da CHD poderá auxiliar na incorporação de intervenções terapêuticas e preventivas que utilizem abordagens moleculares como método de triagem, ampliando o papel da genética no atendimento clínico, além de promover aconselhamento genético individualizado para os pacientes e seus familiares.

1.4 Estratégias de análise genética e suas aplicações no estudo da cardiopatia

As metodologias de citogenética molecular melhoraram a capacidade de detecção de CNVs das sequências cromossômicas, possibilitando a obtenção

rápida de resultados para pacientes com CHD. Os testes citogenéticos atuais, que incluem hibridização *in situ* fluorescente (FISH), *arrays* genômicos como SNP/CGH-*array* (hibridização genômica comparativa e polimorfismos de genes únicos baseado em microarranjos) e MLPA (*Multiplex Ligation-dependent Probe Amplification)*, podem auxiliar no diagnóstico clínico destes pacientes[20,39].

O exame de cariótipo com bandeamento G é o padrão-ouro para o diagnóstico de alterações cromossômicas, especialmente aneuploidias e grandes rearranjos estruturais (>5-10 Mb). Possui baixo custo e alto rendimento diagnóstico, apesar de não detectar alterações muito pequenas, é a técnica de primeira linha na investigação de pacientes sindrômicos[20,40].

Por conta disso, na década de 1980, foi criada a FISH, que através da hibridização do DNA alvo com sondas fluorescentes[41] aumentou o poder de resolução dos testes citogenéticos, melhorando a análise de cromossomos inteiros e sendo capaz de detectar alterações genéticas pequenas (microdeleções/microduplicações) e rearranjos cromossômicos complexos (>40 kb)[42]. Sua principal limitação se dá pela detecção restrita de alterações, em virtude do conjunto de sondas disponíveis no mercado. Por ser uma investigação pontual, o teste diagnóstico deve ser direcionado de acordo com a suspeita clínica do paciente[43].

Já o *array*-CGH, possibilitou a análise do genoma inteiro e em alta resolução, detectando variações até então desconhecidas [39]. Esta técnica permite detectar rearranjos cromossômicos desbalanceados muito pequenos em todo o genoma, porém o alto custo ainda limita a utilização deste método pelas instituições de saúde[44].

Entre as diferentes abordagens utilizadas para a análise de alterações cromossômicas, uma em particular vem se destacando. A técnica de MLPA é indicada para caracterizar desordens congênitas e hereditárias, onde é possível detectar CNVs em genes específicos e pequenos rearranjos intragênicos[19,39,45]. Este método tornou-se muito utilizado na investigação molecular de doenças genéticas, tendo ampla aplicação e inúmeras vantagens em relação a outras técnicas, como alto rendimento, baixo custo, não necessita de cultivo celular, possibilidade de análise de até 96 amostras simultaneamente e resultados em 24h. Permite o estudo de várias regiões do genoma em uma única reação (mais de 40 sequências) e com sequências alvo curtas (60-80nt) pode identificar deleções e duplicações de genes únicos extremamente pequenas (<40 kb) que não são detectadas por FISH, por exemplo. Mais de 300 conjuntos de sondas (*probes*) estão sendo comercializadas, direcionadas à investigação e diagnóstico de várias doenças genéticas[19,44]. A limitação desta técnica se dá na incapacidade de detectar rearranjos balanceados e mosaicismos baixos, além de ser muito sensível em relação à qualidade do DNA utilizado[46].

A identificação de alterações cromossômicas é importante no acompanhamento pré-natal e na antecipação de diagnósticos, contribuindo para o planejamento familiar por meio de aconselhamento genético adequado. A MLPA é uma boa candidata na detecção de desordens comuns, devido a ampla gama de doenças que sua análise é capaz de detectar. Esta técnica, utilizada como método de triagem genética, atua como uma ferramenta de confirmação diagnóstica. A MLPA na investigação de CHD poderá auxiliar na

melhor compreensão acerca da gênese das cardiopatias, podendo auxiliar também no melhor manejo terapêutico.

2. REFERÊNCIAS BIBLIOGRÁFICAS

1. Fahed AC, Gelb BD, Seidman JG, Seidman CE. Genetics of congenital heart disease: the glass half empty. Circ Res. 2013;112(4):707–20.

2. Hoffman JIE, Kaplan S. The incidence of congenital heart disease. J Amer Col Card. 2002;39(12):1890–00.

3. Bruneau BG. The developmental genetics of congenital heart disease. Nature. 2008;451(7181):943–8.

4. Blue GM, Kirk EP, Sholler GF, Harvey RP, Winlaw DS. Congenital heart disease: Current knowledge about causes and inheritance. Med J Aust. 2012;197(3):155–9.

5. Hoffman JIE. Incidence of congenital heart disease: II. Prenatal incidence. Ped Card. 1995;16(4):155–65.

6. Gilboa SM, Salemi JL, Nembhard WN, Fixler DE, Correa A. Mortality resulting from congenital heart disease among children and adults in the United States, 1999 to 2006. Circulation. 2010;122:2254–63.

7. Bhatt AB, Foster E, Kuehl K, Alpert J, Brabeck S, Crumb S, et al. Congenital heart disease in the older adult: A scientific statement from the American heart association. Circulation. 2015;131:1884–31.

8. Andersen TA, Troelsen KLL, Larsen LA. Of mice and men: molecular genetics of congenital heart disease. Cell Mol Life Sci. 2014;71(8):1327–52.

9. Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA, et al. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature. 2003;424(6947):443–7.

10. Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science. 1998;281(5373):108–11.

11. Santana MVT. Cardiopatias congênitas no recém-nascido: Diagnóstico e tratamento. 3 ed. São Paulo:Atheneu;2014.

12. Mendis S, Puska P, Norrving B. Global atlas on cardiovascular disease prevention and control. WHO. 2011;2–14.

13. Hoyme HE. Minor anomalies: Diagnostic clues to aberrant human morphogenesis. Genetica. 1993;89(1-3):307–15.

14. Trevisan P, Rosa RFM, Koshiyama DB, Zen TD, Paskulin GA, Zen PRG. Congenital heart disease and chromossomopathies detected by the karyotype. Rev Paul Pediatr. 2014;32(2):262–71.

15. Bernier PL, Stefanescu A, Samoukovic G, Tchervenkov CI. The challenge of congenital heart disease worldwide: epidemiologic and demographic facts. Semin Thorac Cardiovasc Surg Pediatr Card Surg Annu. 2010;13(1):26-4.

16. van der Bom T, Zomer AC, Zwinderman AH, Meijboom FJ, Bouma BJ, Mulder BJM. The changing epidemiology of congenital heart disease. Nat Rev Cardiol. 2011;8(1):50–60.

17. Miller SP, McQuillen PS, Hamrick S, Xu D, Glidden DV, Charlton N, et al. Abnormal brain development in newborns with congenital heart disease. The N Engl J Med. 2007;357(9):1928-38.

18. Payne RM, Johnson MC, Grant JW, Strauss AW. Toward a molecular understanding of congenital heart disease. Circulation. 1995;91(2):494–04.

19. Schouten JP, McElgunn CJ, Waaijer R. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002;30(12):e57.

20. Pierpont ME, Basson CT, Benson DW Jr, Gelb BD, Giglia TM, Goldmuntz E, et al. Genetic basis for congenital heart defects: current knowledge a scientific statement from the American Heart Association congenital cardiac defects committee, council on cardiovascular disease in the young: endorsed by the American Academy of Pediatrics. Circulation. 2007;115(23):3015–38.

21. Richards AA, Garg V. Genetics of congenital heart disease. Curr Cardiol Rev. 2010;6(2):91–7.

22. Carvalho JJM. Aspectos preventivos em cardiologia. Arq Bras Cardiol. 1988;59–67.

23. Ou Z, Berg JS, Yonath H, Enciso VB, Miller DT, Picker J. Microduplications of 22q11.2 are frequently inherited and are associated with variable phenotypes. Genet Med. 2008;10(4):267–77.

24. Fahed CA, Nemer MG. Genetic causes of syndromic and non-syndromic congenital heart disease. In: Mutations in Human Genetic Disease. IntechOpen. 2012;119–48.

25. Yunis K, Mumtaz G, Bitar F, Chamseddine F, Kassar M, Rashkidi J, et al. Consanguineous marriage and congenital heart defects: A case-control study in the neonatal period. Am J Med Genet A. 2006; 140(14):1524–30.

26. Zaidi S, Brueckner M. Genetics and Genomics of Congenital Heart Disease. Circ Res. 2017;120(6):923-40.

27. Simmons MA, Brueckner M. The genetics of congenital heart disease…understanding and improving long-term outcomes in congenital heart disease: a review for the general cardiologist and primary care physician. Curr Opin Pediatr. 2017;29(5):520-8.

28. Jeromi LA, Papaioannou VE. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. Nat Genet. 2001;27(3):286-91.

29. Fulcoli FG, Franzese M, Liu X, Zhang Z, Angelini C, Baldini A. Rebalancing gene haploinsufficiency in vivo by targeting chromatin. Nat Commun. 2016;7:11688.

30. Zhou L, Liu J, Xiang M, Olson P, Guzzetta A, Zhang K, et al. Gata4 potentiates second heart field proliferation and Hedgehog signaling for cardiac septation. Proc Natl Acad Sci U S A. 2017;114(8):e1422-31.

31. Stallmeyer B, Fenge H, Nowak-Gottl U, Schulze-Bahr E. Mutational spectrum in the cardiac transcription factor gene NKX2.5 (CSX) associated with congenital heart disease. Clin Genet. 2010;78(6):533–40.

32. Mori AD, Bruneau BG. TBX5 mutations and congenital heart disease: Holt-Oram syndrome revealed. Curr Opin Cardiol. 2004;19(3):211–15.

33. Baban A, Postma AV, Marini M, Trocchio G, Santilli A, Pelegrini M, et al. Identification of TBX5 mutations in a series of 94 patients with Tetralogy of Fallot. Am J Med Genet A. 2014;164(12):3100–07.

34. Waldron L, Steimle JD, Greco TM, Gomez NC, Dorr KM, Kweon J, et al. The cardiac TBX5 interactome reveals a chromatin remodeling network essential for cardiac septation. Dev Cell. 2016;36(3):262-75.

35. Li H, Cherry S, Klinedinst D, DeLeon V, Redig J, Reshey B, et al. Genetic modifiers predisposing to congenital heart disease in the sensitized Down syndrome population. Circ Cardiovasc Genet. 2012;5(3):301–8.

36. Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, et al. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes Dis. 2014;1(1):87–105.

37. Jiao K, Kulessa H, Tompkins K, Zhou Y, Batts L, Baldwin HS, et al. An essential role of Bmp4 in the atrioventricular septation of the mouse heart. Genes Dev. 2003;17(19):2362– 7.

38. Robinson SW, Morris CD, Goldmuntz E, Reller MD, Jones MA, Steiner RD, et al. Missense mutations in CRELD1 are associated with cardiac atrioventricular septal defects. Am J Hum Genet. 2003;72(4):1047–52.

39. Lee C, Iafrate AJ, Brothman AR. Copy number variations and clinical cytogenetic diagnosis of constitutional disorders. Nat Gen. 2007;39(7 Suppl):S48–S54.

40. Bui TH. Prenatal cytogenetic diagnosis: Gone FISHing, BAC soon! Ultrasound Obstet Gynecol. 2007;30(3):247–51.

41. Rudkin GT, Stollar BD. High resolution detection of DNA-RNA hybrids in situ by indirect immunofluorescence. Nature. 1977;265(5593):472–3.

42. Jensen E. Technical Review: In Situ Hybridization. Anat Rec. 2014;297(8):1349-53.

43. Gozetti A, Le Beau MM. Fluorescence in situ hybridization: uses and limitations. Semin Hematol. 2000;37(4):320-33.

44. Stuppia L, Antonucci I, Palka G, Gatta V. Use of the MLPA assay in the molecular diagnosis of gene copy number alterations in human genetic diseases. Int J Mol Sci. 2012;13(3): 3245–76.

45. Slater HR, Bruno DL, Ren H, Pertile M, Schouten JP, Choo KH. Rapid, high throughput prenatal detection of aneuploidy using a novel quantitative method (MLPA). J Med Genet. 2003;40(12):907–12.

46. Kozlowski P, Jasinska AJ, Kwiatkowski DJ. New applications and developments in the use of multiplex ligation-dependent probe amplification. Electrophoresis. 2008;29(23):4627–36.

3. OBJETIVOS

3.1 Objetivo geral

Investigar, através da técnica de MLPA, a presença de *CNVs* em genes de referência para o desenvolvimento cardíaco normal em portadores de CHD.

3.2 Objetivos específicos

I. Detectar CNVs em genes de referência para CHD com kit específico de MLPA (P311-B1).

II. Associar as alterações encontradas ao fenótipo clínico dos pacientes;

III. Avaliar a contribuição da técnica de MLPA na triagem molecular de alterações genéticas dentro de uma amostra de pacientes com CHD.

4. ARTIGO CIENTÍFICO

Investigation and prevalence of copy number variation in GATA4 gene and 22q11.2 region in patients with congenital heart disease in a reference hospital in Brazil

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Investigation and prevalence of copy number variation in GATA4 gene and 22q11.2 region in patients with congenital heart disease in a reference hospital in Brazil

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Abstract

Background: The normal development of the heart comprises a highly regulated machinery of genetic events, involving important transcriptional factors. Structural changes in the heart, which characterize congenital heart disease (CHD), have been strongly associated with chromosomal abnormalities and copy number variants (CNVs) which are risk factors for the development of CHD. Our goal was to investigate through the Multiplex ligation-dependent probe amplification (MLPA) technique, the presence of CNVs in reference genes for normal cardiac development in patients with CHD. **Methods:** *GATA4, NKX2-5, TBX5, BMP4* and *CRELDl* genes and 22q11.2 chromosome region were analyzed in 207 patients with CHD (age 1 day±13 years) admitted for the first time in a cardiac intensive care unit from a pediatric hospital, using MLPA SALSA P311-B1 CHD kit. **Results:** CNVs were detected in 7 patients (3.4%); 4 cases of 22q11.2 deletion syndrome (1.9%), 2 cases of *GATA4* deletion (1%) and 1 case of 22q11.2 duplication syndrome (0.5%). No patients with alterations in the *NKX2-5*, *TBX5, BMP4* and *CRELDl* genes were identified. **Conclusion:** Del*GATA4* appears to be present in a significant number of CHD patients, especially those with septal defects, PLSVC, pulmonary artery abnormalities and extracardiac findings. *GATA4* screening seems to be more effective when directed at these findings. The investigation of mutations in *GATA4* and 22q11.2DS in CHD patients is important in anticipating the diagnosis, contributing to family planning.

Keywords: congenital heart defects; MLPA; copy number variation; GATA4 transcription factor; 22q11.2 deletion syndrome.

Background

Congenital heart disease (CHD) consists of structural changes in the heart and large vessels and is recognized as the leading cause of neonatal mortality, affecting about 1% of newborns [1,2]. Regarding of CHDs etiology, 20% are attributed to chromosomal and/or to single-gene alterations; the remaining is a combination of genetic, epigenetic and environmental factors. Chromosomal abnormalities and copy number variants (CNVs) contribute to the risk of CHD [3].

The CNVs identification in *GATA4*, *NKX2-5* and *TBX5* are among mechanisms that may explain the CHD molecular basis since these transcription factors (TFs) are strongly involved in cardiogenesis. The altered expression of *GATA4* (OMIM: 600576) results in common CHDs, such as atrial (ASD) and ventricular septal defects (VSD) and pulmonary stenosis (PS). Facial dysmorphisms and mental retardation may also be present [4-6]. It is believed that all TFs acts together by regulating the cardiac septum formation, whereas its haploinsufficiency results in developmental heart disorders [7]. Several clinical syndromes associated with CHDs have already been related to several CNVs. The first to be described was the 22q11 deletion syndrome (22q11DS) (OMIM:188400) that is among the most common human microdeletion syndromes [8].

Multiplex ligation-dependent probe amplification (MLPA) is a quantitative genomic scanning method used to identify changes in the DNA number copies from deletions and duplications [9]. The investigation of CNVs in patients with CHD through this technique can help in the early diagnosis and identification of the specific cause of the disease.

Thus, we used the MLPA technique to investigate the presence of 22q11DS and CNVs in reference genes for normal cardiac development, in CHD patients at a referral hospital in Southern Brazil.

Methods

All participants were recruited from cardiac intensive care unit (ICU) of the Hospital da Criança Santo Antônio (HCSA), Porto Alegre, RS, Brazil, during the period of 1 year. This hospital belongs to the Santa Casa of Misericórdia of Porto Alegre (SCMPA) and is a reference center for the evaluation and treatment of patients with CHD.

Peripheral blood was collected from all participants for genomic DNA extraction from lymphocytes by standard protocol. The MLPA assay was performed by SALSA P311-B1 CHD kit for CNVs screening in *GATA4, NKX2-5, TBX5, BMP4* and *CRELDl* genes and 22q11.2 chromosome region following the manufacturer's recommendations (MRC-Holland, Amsterdam, The Netherlands). The fragments obtained by ABI3130 sequencer were analyzed with the use of the Coffalyser software (MRC-Holland), which normalizes the signals from all probes and compare them with reference samples. For each comparative analysis, three normal controls were used.

These patients belong to the study developed by Rosa et al. [10], in which high resolution GTG-Banding karyotype and fluorescence *in situ* hybridization (FISH) technique for 22q11 microdeletion were performed, using the DiGeorge/VCFS Region Probe (TUPLE1) (Vysis, Abbott Laboratories, AbbottPark, IL), following a standard codenaturation protocol.

All patients also underwent a physical examination performed by only one clinical geneticist (RFMR) that classified the patients based on their dysmorphisms in syndromic or non-syndromic. The CHDs were described based on the results of the echocardiography, cardiac catheterization and surgical description, following the classification suggested by Botto et al. [11]. Family history of CHD was noted when present. The study was approved by the institutional Ethics Committee.

Results

A total of 210 patients were hospitalized in the cardiac ICU of the HCSA during the period of the study. From them, 3 individuals were excluded due to lack of DNA sample for the MLPA analysis. Thus, our final sample consisted of 207 patients, 110 males (52.4%), with ages ranging from 1 day to 13 years (median of 220 days, ranging from 1 day to 13.5 years). Most of them were of Caucasian origin (79%) and were hospitalized for performance of cardiac surgery (74.8%), cardiologic evaluation (14.1%) and cardiac catheterization (9.2%). The main CHDs observed consisted of ventricular septal defects (VSDs) (17.4%), atrial septal defects (ASDs) (15.9%), tetralogy of Fallot (11.1%), coarctation of the aorta (10.6%) and atrioventricular septal defects (9.7%). Sixty-four patients (30.9%) were classified as syndromic. Familial recurrence of CHD was observed in 15.7% of the sample individuals.

Chromosomal anomalies detected through karyotype were detected in 29 patients (14%) and consisted of trisomy 21 (n=24), trisomy 18 (n=2), triple X (n=1), 17p duplication (n=1) and additional chromosome material next to 18p (n=1). The 22qq11 microdeletion was identified through FISH in 4 patients (1.9%).

MLPA assay detected alterations in 7 patients (3.4%) (Table 1). We identified a heterozygous deletion of the seven exons of *GATA4* (del*GATA4*) in 2 patients (0.96%) (Figure 1A). The clinical findings presented by the patients can be seen in Table 1. Both were male patients. One child had a VSD and the other a pulmonary stenosis (PS). It is noteworthy that both presented an associated persistence of left superior vena cava (PLSVC) and an ostium secundum-type ASD. Moreover, they also had a membranous-type VSD and an abnormality of the pulmonary artery (a bicuspid pulmonary valve and a PS, respectively). Only one was considered syndromic through the evaluation by physical exam. It is noteworthy that this patient had a previous suspicion of Williams-

Beuren syndrome (WBS) (patient 2) (Figure 1A). However, the additional analysis through MLPA for abnormalities in 7q11.23 (the region of WBS) did not confirm this associated diagnosis. Complementary evaluations disclosed the presence of an asymptomatic ectopic kidney in the patient 1. Thus, both presented associated extracardiac findings, however, their phenotypes seem to be different. Perhaps, this could be explained by different sizes of the deletions involving the *GATA4* presented by the patients, since the MLPA only evaluated the involvement of this gene. None of them had a family history of CHD (Table 1). The frequency of del*GATA4* among patients with VSDs (n=36) was 2.8% and among those with pulmonary stenosis/atresia (n=9) was 11.1%. From 14 patients with PLSVC of the total sample (6.8%), 2 (14.3%) had del*GATA4*. It is noteworthy that from 4 patients with PLSVC associated to an abnormality of the pulmonary artery, 2 (50%) presented del*GATA4*.

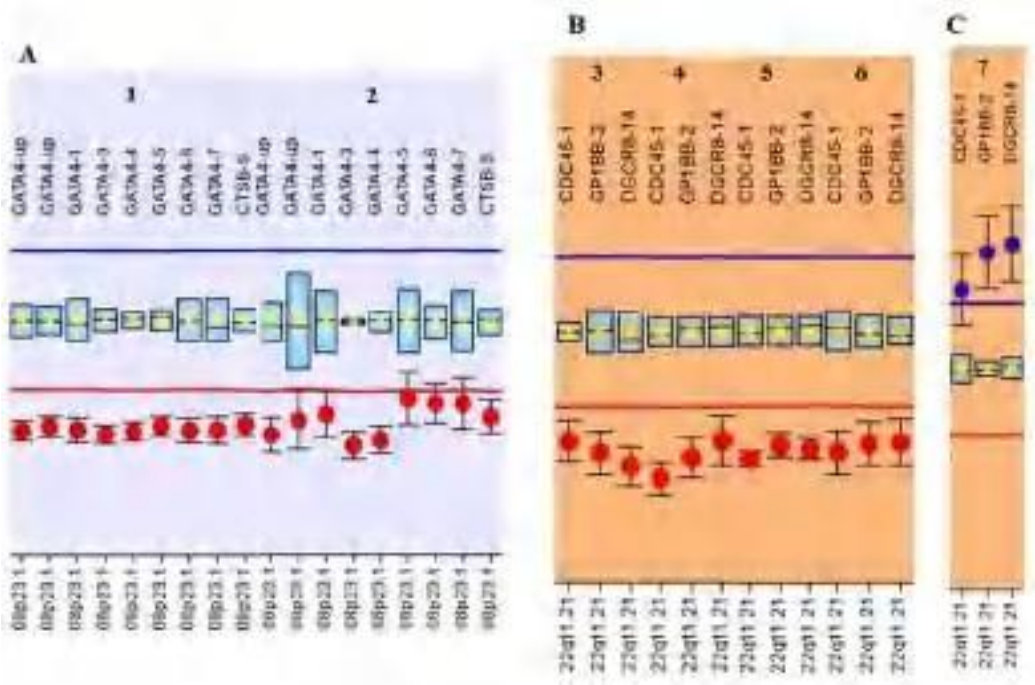
In addition, the most prevalent CNV was the heterozygous deletion in the 22q11.2 (22q11DS) identified in 4 patients (1.9%) (Figure 1B). These results confirm those previously found by Rosa et al. [10] through fluorescence *in situ* hybridization (FISH) technique. However, 1 patient had a heterozygous duplication in the 22q11.2 region (22q11DupS) (0.5%) (Figure 1C), which was not detected by FISH in the previous study. Duplication occurred in a region close to FISH probes hybridization site. No alterations were identified in the *NKX2-5*, *TBX5*, *BMP4* and *CRELDl* genes.

Table 1. Copy number variations (CNVs) detected by multiplex ligation dependent probe amplification (MLPA) and clinical phenotype of congenital heart disease (CHD) patients.

	PATIENT		
CLINICAL AND GENETIC FEATURES	1	2	7
CNV	delGATA4	delGATA4	dup22q11
Syndromic	N	Y	N
Age	1y 1m 17d	1y 10m 17d	3y 3m 13d
Growth retardation			+
Microcephaly	+		
Craniofacial features			
High forehead		+	
Broad foreahead			+
Telecanthus			+
Upslanting palpebral fissures			
Epicantic folds		+	+
Hypoplastic nares	+		
Long philtrum	+	+	
Thin upper lip	+		
High arched palate		+	
Micrognathia	+	+	
Overfold helix		+	
Preauricular pits			+
Congenital Heart Defects			
ASD	+	+	
VSD	+	+	
PLSVC	+	+	
PVS		+	
Subvalvular aortic ring			+
Renal anomalies			
Ectopic Kidney	+		
Mental retardation			+

CNV: copy number variation; N: No; Y: Yes; y: years; m: month; d: days; ASD: atrial septal defect; VSD: ventricular septal defect; PLSVC: persistent left superior vena cava; PVS: pulmonary valve stenosis.

Figure 1. Multiplex ligation dependent probe amplification P311-B1 CHD analysis. MLPA analysis (A) positive for deletion of the *GATA4* (patients 1 and 2); (B) positive for 22q11.2DS (patients 3,4,5 and 6) and (C) positive for 22q11DupS (patient 7).



Legends: Reference values: DQ (dosage quotient); Normal (between red and blue lines) 0.80 < DQ < 1.2; heterozygous deletion (red dots) 0.40 < DQ < 0.65; heterozygous duplication (blue dots) 1.30 < DQ < 1.65.

Discussion

The emergence of molecular cytogenetic techniques, as MLPA, improved the detection of CNVs. Panels of nucleotide variants and CNVs when combined have demonstrated efficiency as first line diagnostic tests in constitutional imbalances with a detection potential of 15-30% of CHD-causing variants [12-13]. Here, genomic scanning detected CNVs in 3.4% of the patients tested, a similar result found by Sorensen et al. [14] (3.2%) and below the frequencies described by Monteiro et al. [15] (23.4%) and Campos et al. [16] (17.9%). The authors believe that these variations probably were caused by the criteria for patient selection.

It is known that CNVs involving *GATA4*, located at 8p23.1, are associated with CHDs, where the mechanism of pathogenicity leads to gene haploinsufficiency. The most frequent defects are VSDs and ASDs. Our study identified del*GATA4* (~151kb) in 2 patients (0.96%) (Figure 1A). Pulmonary stenosis, a CHD also associated to del*GATA4*, was observed in one of our patients. Other complex defects, as Tetralogy of Fallot and double-outlet right ventricle, have been also described in individuals with del*GATA4*. Facial dysmorphisms and mental retardation may be present, especially in patients with larger 8p deletions involving *GATA4* [5,6] (our 2 patients had facial dysmorphisms, in special the patient 2). Our data agree with the report made by El Malti et al. [13] who detected 1 case among 154 patients (0.7%). Moreover, recent studies have not detected patients with *GATA4* alterations, despite the similarity of the clinical phenotype of the patients [17-19].

Del*GATA4* are described in CHD familial cases with overlapping of phenotypes and cardiac septum defects. In these cases, the interaction of *GATA4*, *TBX5* and *NKX2-5* is interrupted [4,5], which lead to proliferation defects of cardiomyocytes during embryogenesis [20]. In our sample, however, none of our patients with del*GATA4* had a family history of CHD.

Among the 207 patients evaluated, 4 cases presented 22q11DS (~630kb) [22q11:17,847,478-18,477,850 (hg18)], covering *CDC45*, *GPlBB* and *DGCR8* genes (Figure 1B). In addition, 1 case presented 22q11DupS (Figure 1C and Table 1). The 22q11DS has a very heterogeneous phenotype that includes CHD, hypocalcemia, immunodeficiency, facial dysmorphic and neurodevelopment disorders [8], corroborating with the clinical findings of this study. Rosa et al. [10] previously described the clinical features of our 22q11DS patients. The 22q11DS is among the main causes of CHD [10], and it was the most frequent alteration found in our study

(1.9%). This finding agrees with the literature, where the detection rate described has ranged from 0.4% to 8.5% [11,13-15,18]. The detection of 22q11DupS is reported as less common, ranging from 0.7% to 2.5% [13,14,16], a similar frequency to us (0.5%). The identification of 22q11DupS has increased due to the screening techniques currently used. However, its prevalence and definitive phenotype remain unknown [21].

Conclusions

Thus, del*GATA4* seems to be present in a significant number of patients with CHD, especially those with septal defects, PLSVC, an abnormality of the pulmonary artery and extracardiac findings (perhaps, features that recalling patients with WBS, and renal abnormalities). Maybe, the testing for del*GATA4* should be directed to patients with these findings. The frequency of abnormalities involving *GATA4* can be even higher and depends on the technique used for its detection. Based on all this, we believe that the inclusion of mutations research involving *GATA4* gene should be considered in the screening of patients with CHD.

Investigation of *GATA4* mutations and of 22q11.2 deletion syndrome in CHD patients are important in prenatal care and in anticipation of diagnoses, contributing to family planning through appropriate genetic counseling. The identification of them is important, because it may assist in the early diagnosis and management of extracardiac symptoms, reducing the consequences of the disease. Understanding the etiology of CHD will aid in the incorporation of therapeutic and preventive interventions that use molecular approaches as a screening method, increasing the role of genetics in clinical care.

List of abbreviations

22q11DS	22q11 deletion syndrome
22q11DupS	22q11 duplication syndrome
ASD	Atrial septal defect
CHD	Congenital heart disease
CNV	Copy number variation
FISH	Fluorescence *in situ* hybridization
MLPA	Multiplex ligation-dependent probe amplification
PCR	Polymerase chain reaction
PLSVC	Persistent left superior vena cava
PS	Pulmonary stenosis
VSD	Ventricular septal defect

Ethics approval and consent to participate: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication: Not applicable

Availability of data and material: The datasets generated and analyzed during the current study are not publicly available due they contain personal information from participants and family members, being a hospital database, but are available from the corresponding author on reasonable request.

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

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Author contributions: PRGZ conceptualized the research, acquired funds and performed the critical analysis of the data; MAF performed the experiments, data critical analyzes and wrote the manuscript; ABG assisted in the application of the methodology; GA was responsible for the formal analysis of the data and manipulation of the equipment; LD and RFR supervised and contributed to the writing and review of the article.

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REFERENCES

1. Hoffman JIE, Kaplan S. The incidence of congenital heart disease. J Am Coll Cardiol. 2002;39(12):1890–00; doi:10.1016/s0735-1097(02)01886-7.

2. Bruneau BG. The developmental genetics of congenital heart disease. Nature. 2008;451(7181):943–8; doi:10.1038/nature06801.

3. Blue GM, Kirk EP, Sholler GF, Harvey RP, Winlaw DS. Congenital heart disease: Current knowledge about causes and inheritance. Med J Aust. 2012;197(3):155–9; doi:10.5694/mja12.10811.

4. Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA et al. *GATA4* mutations cause human congenital heart defects and reveal an interaction with *TBX5*. Nature. 2003;424(6947): 443–7; doi:10.1038/nature01827.

5. Kinnunen S, Välimäki M, Tölli M, Wohlfahrt G, Darwich R, Komati H et al. Nuclear receptor-like structure and interaction of congenital heart disease-associated factors GATA4 and NKX2-5. PLoS One. 2015;10(12):e0144145; doi:10.1371/journal.pone.0144145.

6. Matsuoka R. *GATA4* mutation and congenital cardiovascular diseases: Importance of phenotype and genetic background clarification. J Mol Cell Card. 2007;43(6):667-9; doi:10.1016/j.yjmcc.2007.08.017.

7. Srivastava D. Genetic regulation of cardiogenesis and congenital heart disease. Annu Rev Pathol. 2006;1:199-213; doi:10.1146/annurev.pathol.1.110304.100039.

8. Simmons MA, Brueckner M. The genetics of congenital heart disease… understanding and improving long-term outcomes in congenital heart disease: a review for the general cardiologist and primary care physician. Curr Opin Pediat. 2017;29(5):520-8; doi:10.1097/MOP.0000000000000538.

9. Schouten JP, McElgunn CJ, Waaijer R. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002;30(12):e57; doi:10.1093/nar/gnf056.

10. Rosa RF, Pilla CB, Pereira VL, Flores JA, Golendziner E, Koshiyama DB et al. 22q11.2 deletion syndrome in patients admitted to a cardiac pediatric intensive care unit in Brazil. Am J Med Genet A. 2008;146A(13):1655-61; doi:10.1002/ajmg.a.32378.

11. Botto, L.D.; Correa, A.; Erickson, J.D. Racial and temporal variations in the prevalence of heart defects, Pediatrics. 107 (2001) E32; doi: 10.1542/peds.107.3.e32.

12. Pohovski LM, Dumic KK, Odak L, Barisic I. Multiplex ligation-dependent probe amplification workflow for the detection of submicroscopic chromosomal abnormalities in patients with developmental delay/intellectual disability. Mol Cytogenet. 2013;6(1):7; doi:10.1186/1755-8166-6-7.

13. El Malti R, Liu H, Doray B, Thauvin C, Maltret A, Dauphin C et al. A systematic variant screening in familial cases of congenital heart defects demonstrates the usefulness of molecular genetics in this field. Eur J Hum Genet. 2016;24(2):228-36; doi:10.1038/ejhg.2015.105.

14. Sørensen KM, El-Segaier M, Fernlund E, Errami A, Bouvagnet P, Nehme N et al. Screening of congenital heart disease patients using multiplex ligation-dependent probe amplification: early diagnosis of syndromic patients. Am J Med Genet A. 2012;158A(4):720-5; doi:10.1002/ajmg.a.35214.

15. Monteiro RAC, de Freitas ML, Vianna GS, de Oliveira VT, Pietra RX, Ferreira LCA et al. Major contribution of genomic copy number variation in syndromic congenital heart disease: the use of MLPA as the first genetic test. Mol Syndromol. 2017;8(5):227-35; doi:10.1159/000477226.

16. Campos CM, Zanardo EA, Dutra RL, Kulikowski LD, Kim CA. Investigation of copy number variation in children with conotruncal heart defects. Arq Bras Cardiol. 2015;104(1);24-31; doi:10.5935/abc.20140169.

17. Liu Z, Wang J, Liu S, Deng Y, Liu H, Li N et al. Copy number variation of GATA4 and NKX2-5 in Chinese fetuses with congenital heart disease. Pediatr Int. 2015;57(2):234-8; doi:10.1111/ped.12489.

18. Aguayo-Gómez A, Arteaga-Vázquez J, Svyryd Y, Calderón-Colmenero J, Zamora-González C, Vargas-Alarcón G et al. Identification of Copy Number Variations in Isolated Tetralogy of Fallot. Pediatr Cardiol. 2015;36(8):1642-6; doi:10.1007/s00246-015-1210-9.

19. Mutlu ET, Aykan HH, Karagöz T. Analysis of gene copy number variations in patients with congenital heart disease using multiplex ligation-dependent probe amplification. Anatol J Cardiol. 2018;20(1):9-15; doi:10.14744/AnatolJCardiol.2018.70481.

20. Ang YS, Rivas RN, Ribeiro AJS, Srivas R, Rivera J, Stone NR, et al. Disease model of GATA4 mutation reveals transcription factor cooperativity in human cardiogenesis. Cell. 2016;167(7):1734-49; doi:10.1016/j.cell.2016.11.033.

21. Wenger TL, Miller JS, DePolo LM, Marchena AB, Clements CC, Emanuel BS et al. 22q11.2 duplication syndrome: elevated rate of autism spectrum disorder and need for medical screening. Mol Autism. 2016;7:27; doi:10.1186/s13229-016-0090-z.

5. CONCLUSÕES

Este estudo demonstrou que a investigação de mutações em *GATA4* e de 22q11.2DS em pacientes com CHD são importantes no cuidado pré-natal e na antecipação do diagnóstico, contribuindo para o planejamento familiar através de um aconselhamento genético apropriado.

Assim, a del*GATA4* parece estar presente em um número significativo de pacientes com CHD, especialmente aqueles com defeitos septais, veia cava superior esquerda persistente (PLSCV), alterações da artéria pulmonar e achados extracardíacos (talvez, características que lembrem pacientes com SWB e alterações renais). Ainda, o teste para del*GATA4* parece ser mais efetivo quando direcionado para pacientes com estes achados. A frequência de alterações envolvendo *GATA4* pode ser ainda maior e depende da técnica utilizada para sua detecção. Com base nisso, acredita-se que a inclusão de mutações envolvendo o gene *GATA4* deve ser considerada na triagem de pacientes com CHD. Essa identificação é importante, pois pode auxiliar no diagnóstico precoce e no manejo dos sintomas extracardíacos, reduzindo as consequências da doença.

A CHD ainda representa um problema de saúde pública, pois muitas malformações complexas necessitam de serviços especializados. Por isso, compreender a etiologia da CHD fornecerá novos conhecimentos para o tratamento individualizado de cada paciente e ajudará na incorporação de intervenções terapêuticas e preventivas que usam abordagens moleculares como método de triagem, aumentando o papel da genética no atendimento clínico.

6. ANEXOS

6.1 Parecer do Comitê de Ética da UFCSPA

UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estudo molecular e citogenético molecular de pacientes portadores de cardiopatia congênita: em busca de novas etiologias

Pesquisador: Rafael Fabiano Machado Rosa

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP.);

Versão: 2

CAAE: 61389516.0.0000.5345

Instituição Proponente: Universidade Federal de Ciências da Saúde de Porto Alegre

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.871.676

Apresentação do Projeto:

A cardiopatia congênita é um conjunto de anormalidades estruturais e funcionais do coração e grandes vasos, que podem surgir durante a embriogênese cardíaca. É a alteração congênita mais comum e uma das principais causas de morbidade infantil relacionadas a malformações congênitas. Com incidência de 19 a 75 casos a cada mil nascidos vivo, está presente em maiores proporções em casos de aborto com tendência para uma maior ocorrência de lesões complexas, estando algumas destas malformações relacionadas com anormalidades cromossômica. A etiologia da CC é complexa e multifatorial, com cerca de 80% das cardiopatias congênitas surgindo através da combinação de fatores genéticos e ambientais. Cerca de 20% dos casos podem ser atribuídos a anomalias cromossômicas, síndromes mendelianas, desordens genéticas não
sindrômicas ou teratogenos. As malformações cardíacas estão entre as anomalias mais frequentemente observadas no período neonatal, sendo responsáveis por 3 a 5% das mortes nesse período. Cerca de 20 a 30% das crianças morrem no primeiro mês de vida, por insuficiência cardíaca ou crises de hipóxia. A apresentação e evolução dos sintomas dependem da natureza e gravidade do defeito anatômico e das alterações fisiológicas cardiovasculares. Muitas

6.2 Parecer do Comitê de Ética da ISCMPA

Irmandade da Santa Casa de Misericórdia de Porto Alegre

Rua Prof. Annes Dias, 285 - Telefone: (51) 3214.8000 - Fax: (51) 3214.8585
CEP: 90020-090 - Porto Alegre - Rio Grande do Sul - CNPJ: 92.883.780/0001-05
Site: www.santacasa.org.br - E-mail: medicina@santacasa.tche.br

Dr. Giorgio A. Paskulin
Pesquisador Responsável
Projeto 1014/05

Carta nº 004/06 CEP/ISCMPA Porto Alegre, 10 de março 2006.

O Comitê de Ética em Pesquisa da Irmandade da Santa Casa de Misericórdia de Porto Alegre, em resposta à solicitação do pesquisador responsável, referente ao projeto de pesquisa intitulado: *"Prevalência e caracterização clínica dos pacientes portadores de microdeleção 22q11 detectados através de técnica de hibridização in situ fluorescente (FISH) que internam por suspeita de cardiopatia congênita numa Unidade de Tratamento Intensivo de um hospital pediátrico"*, que está sendo conduzido em nossa Instituição sob a responsabilidade do Dr. Giorgio A. Paskulin, emiti a seguinte informação:

"A Conep após o envio do presente projeto para avaliação, informou que o referido estudo não necessita da aprovação da mesma, sendo de responsabilidade do CEP a aprovação final. O presente Comitê após esta análise, não encontra óbices ao desenvolvimento do estudo em nossa Instituição".

Sendo o que tínhamos para o momento, reafirmamos nossos protestos de apreço e consideração.

Atenciosamente,

Dr. Cláudio Telöken
Coordenador do CEP/ISCMPA

6.3 Produção bibliográfica

1. Floriani MA., Bau AEK, Silva RP, Graziadio C, Dorfman LE, Zen TD et al. Applications of electron microscopy in health: the example of epidermolysis bullosa. J Bras Patol Med Lab. 2017; 53(1):65-67. http://dx.doi.org/10.5935/1676-2444.20170011

2. Ferreira MAT, Gonzales JFO, Diniz BL, Floriani MA, Bau AEK, Rosa RCM et al. Description of clinical aspects and microscopy of the hair shaft of a carrier of familial monilethrix. J Bras Patol Med Lab. 2018; 54(5):333-335. http://dx.doi.org/10.5935/1676-2444.20180055.

3. Santos AS, Bassani NC, Floriani MA, Fell PRK, Dietrich C, Rosa EB et al. Evaluation of the fetal urine sample through vesicocentesis: an approach to diagnostic and therapeutic application. J Bras Patol Med Lab. 2017; 53(4):258-260. http://dx.doi.org/10.5935/1676-2444.20170040.

4. Diniz BL, Floriani MA, Ferreira MAT, Gonzales JFO, Lisboa NH, Jakimiu AR et al. Increased levels of chitotriosidase in a patient with Alagille syndrome: association or coincidence? J Bras Patol Med Lab. 2018; 54(1):37-39. http://dx.doi.org/10.5935/1676-2444.20180008.

5. Floriani MA, Vilas Boas MR, Rosa RFM, Trevisan P, Dorfman LE, Rosa RCM et al. Report of a patient with fragile X syndrome unexpectedly identified by karyotype analysis. J Bras Patol Med Lab. 2017; 53(2):108-109. http://dx.doi.org/10.5935/1676-2444.20170017.

6. Dorfman LE, Floriani MA, Oliveira TMRDR, Cunegatto B, Rosa RFM, Zen PRG. The role of cytogenetics and molecular biology in the diagnosis, treatment and monitoring of patients with chronic myeloid leukemia. J Bras Patol Med Lab. 2018; 54(2):83-91.

7. Floriani MA, Dorfman LE, Koshiyama DB, Zen TD, Telles JAB, Cunha AC et al. Use of the fluid obtained by puncture of cystic hygroma: an alternative method for fetal karyotyping. J Bras Patol Med Lab. 2016; 52(6):391-392. http://dx.doi.org/10.5935/1676-2444.20160062.

Citação em editoriais:

1. Chauffaille ML. The tip of the iceberg: what is hidden under the fragile X. J Bras Patol Med Lab. 2017; 53(2):80-80. http://dx.doi.org/10.5935/1676-2444.20170015.

2. Campregher P. Standards for the diagnosis and treatment of chronic myeloid leukemia. J Bras Patol Med Lab. 2018; 54(2):68-68. http://dx.doi.org/10.5935/1676-2444.20180023.

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10.5935/1676-2444.20170011

Applications of electron microscopy in health: the example of epidermolysis bullosa

Aplicações da microscopia eletrônica em saúde: o exemplo da epidermólise bolhosa

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ABSTRACT

We report the case of a patient with dystrophic epidermolysis bullosa (DEB) diagnosed by transmission electron microscopy (TEM), emphasizing the applications and importance of this technique in the health area. The patient was a male, the only child of young and non-consanguineous parents without similar cases in the family. The patient underwent a cutaneous biopsy in which TEM revealed sub-basal membrane involvement, confirming the diagnosis of DEB. Despite technological advances, TEM continues to play an important role in diagnosis and clinical research and is considered the best option for confirmation of diagnosis and subtypes of diseases such as epidermolysis bullosa (EB).

Key words: epidermolysis bullosa; epidermolysis bullosa dystrophica; epidermis; basement membrane; electron microscopy.

INTRODUCTION

Our aim was to report the case of a patient with dystrophic epidermolysis bullosa (DEB), whose diagnosis was reached through transmission electron microscopy (TEM), highlighting the applications and importance of this technique in health. The patient was an 18-year-old Caucasian man, the only child of young and non-consanguineous parents without similar cases in the family. According to his mother, the bullous lesions appeared after 15 days of life, affecting the skin of hands, shoulders, elbows, back, knees, ankles and feet, besides oral and nasal mucosa. Some of the lesions had blood inside and the developed scars were atrophic (erythematous in the region of the knees and feet). The lesions frequently get infected, causing itching when dry (**Figure 1**). When the patient was 8 years old he underwent a skin biopsy, whose histopathological analysis showed findings indicative of a subepithelial blister, with only one intrapapillary projection in the central area. The adjacent dermis showed mild edema and minimal lymphocytic infiltrate. TEM performed from the biopsy of a bullous lesion caused by skin friction showed sub-basement membrane involvement, confirming the diagnosis of epidermolysis bullosa (EB) of the dystrophic type (**Figure 2**). An increased number of lesions appeared with the patient growth, especially after puberty. At age 13, epidermal cysts (milia) and dystrophic nails were also present. Dysphagia for solid foods and bleeding of the oral mucosa were also reported. Endoscopy and colonoscopy were normal. He presented abdominal pain at age 17 years, being diagnosed with secondary duodenal stenosis due to an aorto-mesenteric impingement. The patient underwent a duodenal-jejunal anastomosis surgery that progressed well, without the occurrence of stenosis or constipation.

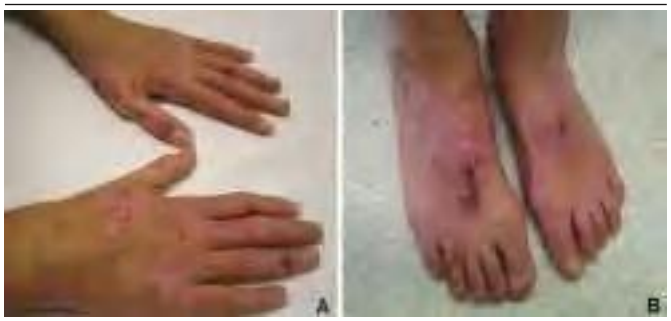

FIGURE 1 – *Images of the patient's hands and feet showing blistering and scarring skin lesions, in addition to dystrophic and missing nails (especially in the feet), secondary to EB EB: epidermolysis bullosa.*

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Description of clinical aspects and microscopy of the hair shaft of a carrier of familial monilethrix

Descrição dos aspectos clínicos e da microscopia da haste capilar de um portador de monilétrix familiar

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ABSTRACT

Monilethrix is a genetic condition that affects the hair shaft. We describe a family with this disease, focusing on its clinical aspects and microscopic hair characteristics. The patient was a 10-year-old female with history of hypotrichosis. In addition to diffuse alopecia, there was brittle hair, with ruptures in the hair shaft at different levels. The hair had a nodular appearance at naked eye. Other family members had the same symptoms, what indicates an autosomal dominant pattern of inheritance. Microscopic analysis revealed capillary fibers with areas of elliptical nodular appearance interspersed with regions of dystrophic constriction.

Key words: monilethrix; hair; hypotrichosis; inheritance patterns; microscopy.

INTRODUCTION

Hair abnormalities happen in childhood with some frequency due to acquired and congenital conditions. When the problem is in the hair shaft, these dysfunctions can be differentiated by the presence or not of increased fragility and breakage. Monilethrix is an abnormality characterized by increased fragility of the hair shaft[1]. Its name originates from the combination of two words: the Latin word "monile" (necklace), and the Greek "thrix" (hair), referring to the beaded aspect of the hair, which can be observed under light microscopy[2].

Monilethrix is a rare and non-syndromic genetic condition which can present two different patterns of inheritance: an autosomal dominant (OMIM 158000) and an autosomal recessive pattern (OMIM 252200)[3]. It is clinically characterized by short and fragile hair that breaks spontaneously or by friction[1, 4, 5]. Hair is normal at birth. However, the stem gains beaded or moniliform appearance during the first months of life[1, 4, 5], leading to periodic changes in its diameter[3]. The hairline presents areas of normal thickness with elliptical nodes alternated with regions of dystrophic constrictions. These internodal regions are prone to breakdown, which weakens the hair and may lead to alopecia[6].

Our aim was to report the rare case of a family with monilethrix, highlighting their clinical findings and, mainly, the microscopic characteristics of the hair. A 10-year-old patient was referred for evaluation due to sparse hair. She was daughter of a 35-year-old father and a 33-year-old mother. The father had a similar hair disorder. In addition, the paternal grandfather, an aunt, and two paternal uncles were also affected (**Figure 1**). The child was born by vaginal delivery, at term, weighing 3,830 grams and measuring 50 cm. She was born cyanotic, requiring mechanical ventilation. Her neuropsychomotor and speech development was normal for her age. On physical examination, at the age of 10 years, she presented adequate anthropometric measurements (weight, height and head circumference), as well as diffuse and irregular alopecia with hair rupture at different levels, which gave an appearance of hypotrichosis. Her hair had a nodular appearance and was brittle (**Figure 2**). Her eyebrows and eyelashes were normal, as well as her nails and skin. Microscopic analysis of the hair showed stenosis areas, many of them close to the root.

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EDITORIAL

The tip of the iceberg: what is hidden under the fragile X

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The case report published in this issue by Floriani *et al*. (2017)[1], describing a fragile X syndrome (FXS) unexpectedly detected by G-banding karyotype (G-bands after trypsin and Giemsa), seems like the view of the tip of the iceberg.

The karyotype was the only available diagnostic test until 1990, when the *FMR1* gene located at Xq27.3 was described. The deoxyribonucleic acid (DNA) test replaced the karyotype and became the gold standard as it detects more the 99% of cases.

Fragile X is a constriction, gap or break detected on metaphase chromosome obtained from cultures with acid folic depleted medium. Probably the methotrexate, an anti-folate drug, used to obtain high-resolution chromosomes explains the fragile site fortuitous detection in this case. The aberration was then confirmed with appropriate medium culture, allowing the diagnosis.

Diagnosis with a preliminary test like karyotype means a shortcut and the reduction of costs with additional tests besides the early reference of the family to genetic counselling. However, the molecular test must still be performed in the mother.

In fact, FXS is the most common inherited genetic syndrome causing mental retardation and autism spectrum in boys. Its prevalence in males is around 1:4000 and in females up to 1:6000. The higher frequency in males is because all men with the *FMR1* gene full mutation will present the disease, while some females will not have the physical, behavioral and cognitive features.

The genetic cause is a mutation of the *FMR1* gene: a trinucleotide repeat expansion [cytosine-guanine-guanine (CGG), or triplets] to more than 200 repeats [detected by polymerase chain reaction (PCR)] and abnormal methylation. The hypermethylation (detected by Southern blot test) silences the fragile X mental retardation protein (FMRP) synthesis.

In normal individuals, the triplets are between 6 and 45. However, when an allele size from 55 to 200 is detected, it is named premutation. Females with premutation are at risk of having children (male and female) with FXS according to the size of the triplets: the larger the number, the higher the risk of expansion to full mutation.

Premutation are unmethylated, so male with permutation do not present FXS, but suffer a neurodegenerative disorder: the fragile X tremor ataxia syndrome (FXTAS), manifested later in life. Around 25% of female with premutation present primary ovarian failure (POF) (menopause before 40 years) and present infertility (FXPOI).

Besides that, around 2% of the individuals present an intermediate allele size or grey area with 45-54 repeats that do not cause disease. Intermediate allele may rarely expand to premutation in future generation.

Therefore, the genetic counselling of the family is of fundamental importance in order to avoid new cases, to orient the female relatives about infertility or early menopause, to detect adult neurological symptoms of FXTAS and to detect male and female relatives with developmental, cognitive and learning disabilities.

Moreover, a FXS child is eligible for special education including speech and language, behavior and cognition, among others.

Fortunately, the karyotype provided the view of tip of the iceberg, now its size and move require to be monitored by additional medical attention.

REFERENCES

1. Floriani MA, Vilas Boas MR, Rosa RFM, et al. Report of a patient with fragile X syndrome unexpected identified by karyotype analysis. J Bras Patol Med Lab. 2017; 53(2): 108-9.

10.5935/1676-2444.20170015

EDITORIAL

Standards for the diagnosis and treatment of chronic myeloid leukemia

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Myeloproliferative disorders are a group of clonal myeloid neoplasms characterized by increased proliferation of myeloid cells with preserved cell differentiation. The molecular features of myeloproliferative neoplasms have been efficiently mapped in the last decades[1]. Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm defined by the presence of t(9;22)(q34;q11), a *BCR-ABL1* gene fusion, and characterized by three distinct clinical-laboratorial phases: a chronic phase (CP) with leukocytosis, left shift, basophilia, eosinophilia, thrombocytosis, and no increase in blast counts; an accelerated phase (AP) characterized by increasing in blast count and additional cytogenetic changes; and a blastic phase (BP) characterized by more than 20% blasts in the bone marrow. The management of CML represents one of the major advances in the history of medicine with the transformation of a highly lethal condition into a chronic disease managed, most of the times, with one pill a day. Although rare, with an incidence of 1.6 per 100,000[2], the estimated 8-year survival of CML in CP used to be 6% before 1975. After the discovery of tyrosine kinase inhibitors for CML treatment the 8-year survival became 87% since 2001[3]. The correct diagnosis and monitoring of CML involve distinct laboratorial techniques such as complete blood counts, bone marrow morphological analysis, conventional cytogenetics, fluorescence *in situ* hybridization and real time polymerase chain reaction (PCR). In order to achieve this highly successful treatment strategy, the correct diagnosis and laboratorial monitoring are crucial.

In this edition of the *Jornal Brasileiro de Patologia e Medicina Laboratorial* (JBPML), the paper of Dorfman *et al*. (2018)[4] presents a review of several aspects of CML. The paper covers diagnostic approaches, the importance of cytogenetic and molecular analysis, the three clinical phases of the disease, a summary of the three generations of tyrosine kinase inhibitors approved for CML treatment and monitoring strategies, including molecular criteria for determining treatment success and failure. The authors also include the cytogenetic and molecular anatomy of t(9;22)(q34;q11) and *BCR-ABL1* gene fusion with some of their variants. This information is essential for professionals working with diagnosis and monitoring of these patients, since discrepancies between cytogenetic and molecular analysis often pose a challenge to clinical pathologists in the diagnosis and also for treatment definition by hematologists[5], and a deep understanding of the possible molecular variations can be helpful in this context.

In summary, the review presented by Dorfman *et al*. (2018)[4] is a helpful resource for hematologists and pathologists dealing with diagnosis, monitoring and treatment of CML patients. It also serves as a useful source for students and professionals in need of a first contact with CML, since it comprehensively describe the main clinical and laboratorial aspects of this disease.

REFERENCES

1. Campregher PV, Santos FPS, Perini GP, Hamerschlak N. Molecular biology of Philadelphia-negative myeloproliferative neoplasms. Rev Bras Hematol Hemoter. 2012; 34(2): 150-5.

2. NIH. National Institutes of Health. Cancer stat facts: chronic myeloid leukemia (CML) [Internet]. 2017 [cited 2017 Jun 26]. Available at: http://seer.cancer.gov/statfacts/html/cmyl.html.

3. Kantarjian H, O'Brien S, Jabbour E, et al. Improved survival in chronic myeloid leukemia since the introduction of imatinib therapy: a single-institution historical experience. Blood. 2012 Mar 1; 119(9): 1981-7.

4. Dorfman LE, Floriani MA, Oliveira TMRDR, Cunegatto B, Rosa RFM, Zen PRG. The role of cytogenetics and molecular biology in the diagnosis, treatment and monitoring of patients with chronic myeloid leukemia. J Bras Patol Med Lab. 2018; 54(2): 83-91.

5. Luatti S, Baldazzi C, Marzocchi G, et al. Cryptic BCR-ABL fusion gene as variant rearrangement in chronic myeloid leukemia: molecular cytogenetic characterization and influence on TKIs therapy. Oncotarget. 2017 May 2; 8(18): 29906-13.

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Evaluation of the fetal urine sample through vesicocentesis: an approach to diagnostic and therapeutic application

Avaliação da urina fetal por meio da vesicocentese: uma abordagem com aplicação diagnóstica e terapêutica

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ABSTRACT

We report the case of a fetus with mega-bladder and suspected lower urinary tract obstruction (LUTO). The 20-week pregnancy ultrasound scan showed absence of amniotic fluid (anhydramnios), enlarged bladder, and narrowing of the urethra in the proximal region. At 21 weeks of gestational age, vesicocentesis was performed for relief of obstruction and analysis of biochemical of the fetal urine and karyotyping was carried out, which presented normal result (46,XY). This technique is indicated in cases of severe oligohydramnios or difficulty of placental access and has diagnostic and therapeutic function.

Key words: karyotype; urethral diseases; nephropathy; urological diagnostic techniques.

CASE REPORT

V. G. was a 21-year-old pregnant woman for the first time referred for the high-risk prenatal care due to an alteration in obstetrical ultrasound. There was an image suggestive of mega-bladder. The 20-week ultrasonography (USG) examination showed absence of the amniotic fluid (anhydramnios) and an enlarged bladder with narrowing of the urethra in the proximal region, suggestive of lower urinary tract obstruction (LUTO). At 21 weeks of gestation the fetal bladder was punctured for relief of obstruction, and biochemical and karyotype analysis of the material was performed. The volume of fetal urine removed was 282 ml. There were no complications during or after the procedure. The karyotype was normal (46,XY). The biochemical analysis showed the following results: potassium: 3 mEq/l (reference value: < 3 mEq/l); sodium: 109 mEq/l (reference value: < 100 mEq/l), and chlorides: 93 mEq/l (reference value: < 90 mEq/l), indicative of renal damage. Second-trimester ultrasound showed absence of amniotic fluid; increased echogenicity of the renal parenchyma, and distended bladder with "keyhole signal" (**Figure 1**). Fetal echocardiography was normal. The 25-week ultrasound also showed severe ascites, pericardial effusion, fetal bladder walls thickening, and the right kidney with diminished dimensions (**Figure 2**). The infant was born through vaginal delivery, at 27 weeks of pregnancy, weighing 1,960 grams and Apgar scores of 1 at first and fifth minutes. The infant had a significant abdominal distention and upper and lower limb deformities (**Figure 3**). He presented respiratory distress and evolved to death about 2 hours after birth.

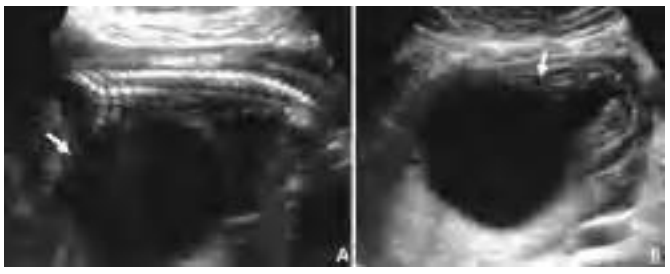

FIGURE 1 – *22-week ultrasound scan showing distended bladder (A) and the keyhole sign (B), suggestive of the LUTO (see white arrows)*

LUTO: lower urinary tract obstruction.

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Increased levels of chitotriosidase in a patient with Alagille syndrome: association or coincidence?

Aumento dos níveis de quitotriosidase em um paciente com síndrome de Alagille: associação ou coincidência?

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ABSTRACT

We describe a case of a patient with Alagille syndrome (AS) presenting an increased level of the enzyme chitotriosidase (ChT), evaluating factors that could justify the relationship between AS and ChT. He was a male patient with cholestatic jaundice, facial dysmorphia and congenital heart disease who presented a brief septicemia. He underwent liver biopsy and analyses for inborn errors of metabolism that respectively showed ductopenia and increased levels of ChT. This increase could be potentially explained by inflammatory and infectious processes, or even by AS itself.

Key words: chitinase; cholestasis; inflammation; Alagille syndrome; sepsis.

INTRODUTION

Alagille syndrome (AS; OMIM 118450), also known as Alagille-Watson syndrome or arteriohepatic dysplasia, is an autosomal dominant genetic disease with variable clinical manifestations that may involve different organs and systems[1, 2]. Its first description occurred in 1969, by Alagille *et al*.[3], and it was also subsequently reported by Watson and Miller, in 1973[4]. However, the diagnostic criteria were only established by Alagille *et al*. in 1975[2]. Originally, the prevalence of the syndrome was estimated at 1:70,000 live births, but considering individuals without hepatic involvement, this frequency increases to 1:30,000[1, 2]. This disease is mainly caused by mutations in JAG1 (AS type 1) (about 90% of cases) and NOTCH2 genes (AS type 2)[2]. The first prominent clinical feature in most patients is the presence of neonatal liver disease with conjugated hyperbilirubinemia (cholestasis)[1,2]. Our aim was to describe a patient with AS presenting an increased level of chitotriosidase (ChT), evaluating factors that could explain the relationship between AS and ChT.

CASE REPORT

The patient was a boy aged 1 month and 3 days, with a history of cholestatic jaundice. He was the first child of a young and non-consanguineous couple, with no family history of genetic diseases. The mother has a previous history of a stillbirth and two gestational losses. The gestation of the patient was uneventful. His prenatal serologies were negative. The mother reported smoking throughout pregnancy (mean of 10 cigarettes per day) and occasional alcohol intake. The patient was born prematurely, at 33 weeks and 3 days, by cesarean section due to fetal distress, weighing 1,460 grams, measuring 42 cm, with head circumference of 24.5 cm and Apgar scores of 5 and 7 at first and fifth minutes, respectively. At birth, septicemia and respiratory failure were verified. Cholestasis was also diagnosed soon. Septicemia was treated with antibiotic therapy during 10 days. The patient presented alterations in liver function tests, with an increase in the levels of aspartate aminotransferase (AST or GOT) (150 U/l – reference values: 14 to 42 U/l), glutamic pyruvic

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Report of a patient with fragile X syndrome unexpectedly identified by karyotype analysis

Relato de um paciente com a síndrome do X frágil identificada de forma inesperada por meio do cariótipo

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ABSTRACT

Fragile X syndrome is considered the main known cause of inherited learning disabilities and it is characterized by mutations in the *FMR1* gene. Our aim was to report an unexpected detection of a patient with fragile X syndrome by GTG-Banding karyotype analysis (G-bands after trypsin and Giemsa). The karyotype analysis identified Xq27.3 fragility in 17% of the metaphases analyzed and in 54% when using TC 199, consistent with the cytogenetic diagnosis of the syndrome. This case was the sole one to present the fra(X) tests in the high-resolution karyotype analysis in our care service, contributing to future diagnoses of patients with history of developmental delay.

Key words: karyotype; fragile X syndrome; intellectual disability; chromosomal fragile sites.

INTRODUCTION

Fragile X syndrome is considered the main known cause of inherited learning disability and it is characterized by mutations in the *FMR1* gene[1]. Our aim was to report an unexpected detection of a patient with fragile X syndrome by GTG-Banding karyotype analysis (G-bands after trypsin and Giemsa). The patient was a 2-year-old boy with history of neuropsychomotor developmental delay. He was the second child of young parents, with no similar family history. His pregnancy was uneventful. The child was born by normal delivery at term, weighing 3,200 g and measuring 49 cm. He evolved with developmental and speech delay. Physical examination showed broad forehead, triangular face, epicanthal folds, and prominent ears. High resolution GTG-Banding karyotype analysis (≥ 550 bands) made from peripheral blood by the modified method of Yunis (1981)[2], using culture medium Roswell Park Memorial Institute medium (RPMI) 1640 (Invitrogen), identified spontaneous fragility of the region q27.3 of X chromosome in 17% of the 53 metaphases analyzed, which was consistent with the diagnosis of fragile X syndrome[3]. Further study using low folic acid culture medium (TC 199) showed the same fragile site in 54% of the metaphases analyzed, which confirmed the diagnosis (**Figure**)[3].

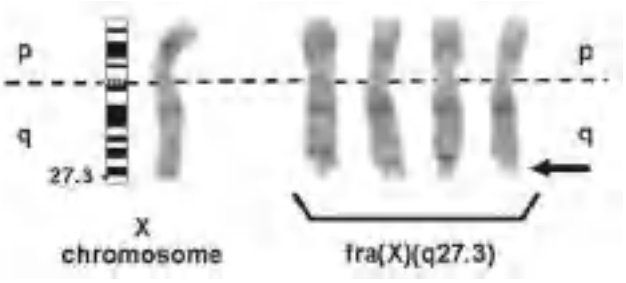

FIGURE – *Partial GTG-Banding karyotypes (≥ 550 bands) showing a normal X and fragile X [fra(X)(q27.3)] chromosomes*

GTG: G-bands after trypsin and Giemsa; p: short arm; q: long arm.

Lubs (1969) was the first to identify this chromosomal abnormality in individuals with fragile X syndrome, which he called "X chromosome marker"[4]. This alteration was more frequently verified when using the TC 199 medium[5]. Currently, the diagnosis of fragile X syndrome is more frequently performed by polymerase chain reaction (PCR) and southern blot; techniques

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The role of cytogenetics and molecular biology in the diagnosis, treatment and monitoring of patients with chronic myeloid leukemia

O papel da citogenética e da biologia molecular no diagnóstico, no tratamento e no monitoramento de pacientes com leucemia mieloide crônica

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ABSTRACT

Chronic myeloid leukemia (CML) is the most common myeloproliferative disorder among chronic neoplasms. The history of this disease joins with the development of cytogenetic analysis techniques in human. CML was the first cancer to be associated with a recurrent chromosomal alteration, a reciprocal translocation between the long arms of chromosomes 9 and 22 – Philadelphia chromosome. This work is an updated review on CML, which highlights the importance of cytogenetics analysis in the continuous monitoring and therapeutic orientation of this disease. The search for scientific articles was carried out in the PubMed electronic database, using the descriptors "leukemia", "chronic myeloid leukemia", "treatment", "diagnosis", "karyotype" and "cytogenetics". Specialized books and websites were also included. Detailed cytogenetic and molecular monitoring can assist in choosing the most effective drug for each patient, optimizing the treatment. Cytogenetics plays a key role in the detection of chromosomal abnormalities associated with malignancies, as well as the characterization of new alterations that allow more research and increase knowledge about the genetic aspects of these diseases. The development of new drugs, through the understanding of the molecular mechanisms involved, will allow a possible improvement in the survival of these patients.

Key words: BCR-ABL positive chronic myeloid leukemia; leukemia; cytogenetics; karyotype; Philadelphia chromosome; therapeutics.

INTRODUCTION

Chronic myeloid leukemia (CML) is a rare type of neoplasia, with incidence of 1-2 cases per 100,000 individuals annually. CML is the most common chronic myeloproliferative neoplasms, representing 0.5% of all new cancer cases in the United States[1]. This represents 15% of all leukemia in adults and is more common in men than women (1.3-1.7 compared to 1.0)[2]. The National Institutes of Health (NIH) estimates that, in 2017, there will be 8,950 new cases of CML and approximately 1,080 people will die from this disease[1, 3]. CML is most frequently diagnosed among individuals with 40-60 years of age, with mean age of 55 years and only 10% of patients are diagnosed with less than 20 years of age[2, 4].

CML is characterized by clonal expansion of hematopoietic progenitor cells, resulting in increased circulating cells of granulocytic lineage. The characteristic symptoms of this disease are chronic fatigue, weight loss, bleeding and fever; whereas signals are anemia, granulocytosis, and immature granulocytes, presence of basophils, thrombocytosis and splenomegaly[3].

The first treatments for CML provided a significant improvement in the quality of life at the chronic phase disease. For several years, the only treatment available for patients was hematopoietic stem cells transplantation[5]. The development of cytogenetic research involving the Philadelphia chromosome and molecular alterations enabled the emergence of a new perspective for the CML treatment, making it more accurate and effective. The aim of this article is an updated review about CML, highlighting

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Use of the fluid obtained by puncture of cystic hygroma: an alternative method for fetal karyotyping

Uso do fluido obtido por punção do higroma cístico: método alternativo para cariotipagem fetal

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ABSTRACT

The aim of our study aim was to report the case of a fetus with Turner syndrome (TS) diagnosed by karyotype from cystic hygroma (CH) fluid, highlighting the applications and importance of this procedure. First-trimester screening revealed an increased nuchal translucency measurement, cervical cystic hygroma and head and trunk subcutaneous edema. The presence of oligohydramnios prevented the performance of amniocentesis. We performed puncture of the CH for fetal karyotyping, which revealed X-chromosome monosomy (45,X), compatible with TS. Therefore, the use of CH fluid as an alternative sample for fetal karyotyping may be considered when conventional invasive procedures can not be performed.

Key words: karyotype; cystic lymphangioma; Turner syndrome; chromosome abnormalities; genetic counseling.

CASE REPORT

The aim of our study was to report a fetus with Turner syndrome (TS) diagnosed by karyotype from cystic hygroma (CH) fluid, emphasizing the applications and importance of this procedure. A 38-year-old healthy pregnant woman was in her third pregnancy and had a history of two previous miscarriages (all occurred during the first trimester). There was no family history of birth defects or genetic disorders. First-trimester screening revealed an increased nuchal translucency measurement (7 mm), cervical cystic hygroma and head and trunk subcutaneous edema. Ascites was also noted later. There was oligohydramnios, which prevented the execution of amniocentesis. Therefore, at 18 weeks of pregnancy, a puncture of the CH was held for fetal karyotyping, following a long-term culture. Chromosomal analysis showed a X-chromosome monosomy - 45,X[21], consistent with TS. At 21 weeks, there was no fetal heartbeat and spontaneous elimination of the fetus occurred two days later.

TS is a multisystemic disorder characterized by partial or total X-chromosome monosomy, seen in 1:2,500-3,000 born alive females. It can be diagnosed at different life stages, including the intrauterine period. Ultrasound findings, such as CH and hydrops, may assist in its detection[1]. Prenatal diagnosis is usually performed by karyotype analysis from amniotic fluid samples. However, as found in the present report, this type of sample or even fetal blood by cordocentesis becomes unable to obtain due to obstructions by large cysts or oligohydramnios[2].

The use of CH fluid, obtained through direct hygroma puncture, can be used as an alternative procedure[2]. Fetal CH is characterized by single or multiple lymphatic congenital cysts and is often found associated with TS[3]. The procedure is similar to amniocentesis and has low complication rate. If the CH is multiloculated, it is possible to puncture and analyze different regions (especially due to the possibility of mosaicism)[4]. The karyotype is performed through lymphocyte culture from cystic cells that has a mitotic index and metaphases quality compatible with a blood lymphocyte culture. The karyotype test results using CH puncture are obtained in four days, while by amniocentesis they range from 14-24 days[5] (**Figures 1** and **2**).

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