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**Title:** Molecular karyotyping in anorectal malformations: Could *DGCR6* gene haploinsufficiency cause anal atresia in 22q11 deletion syndrome?

**Running Title:** Anorectal malformations in 22q11 deletion syndrome

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Abstract

**Background/aim:** Anorectal malformations (ARM) are classified as multifactorial diseases. The etiology of ARM is still not clear because of the complexity of the pathologic changes.

**Materials and methods:** In the present study, the array-CGH results of 10 patients with ARM not associated with a specific syndrome despite having some additional findings were evaluated. Array-CGH analysis was performed using the 8x60K ISCA, Agilent® microarray platform. Pathogenic CNVs were further confirmed using fluorescence in situ hybridization (FISH) or quantitative real-time polymerase chain reaction (qPCR).

**Results:** Chromosome 22q11.2 deletion was detected in two patients. One of these patients had anal stenosis, minor cardiac abnormalities, and a small 0.89-Mb deletion. The second patient had anal atresia, immune deficiency, inguinal hernia, and a 2.7-Mb cryptic deletion. The overlapping genes in the deletion regions of the two patients were **DGCR5**, **DGCR6** and **PRODH**.

**Conclusion:** **DGCR6**, alters the expression of important genes such as **TBX1** and affects neural crest migration. Considering that ARMs are caused by abnormalities in neural crest cell migration, it is thought that these genes may play a role in the etiology of ARM. To our knowledge, this region is one of the smallest interstitial deletions in the chromosome 22q11.2 region to be published to date. Further research on the **DGCR6** gene, which may be a candidate gene responsible for anal atresia, will clarify this point.

**Keywords:** anorectal malformation; anal atresia; 22q11 deletion syndrome; microarray; **DGCR6**; **PRODH**; **DGCR5**
1. Introduction

Anorectal malformations (ARM) are among the most common congenital defects that can be treated with surgical methods (1). Its prevalence is 3.6 per 10,000 births (2). It is classified as a multifactorial disease due to the coexistence of genetic causes and environmental factors. It can be seen in a wide range, ranging from an anteriorly displaced anus, which can be easily corrected surgically, to a cloaca anomaly, which requires much more complex and repetitive surgeries (3). The prevalence in boys is higher than in girls, the male/female ratio is reported to be about 1.2-1.6 (4). ARMs can be seen in the same family members and twins, it is thought that environmental and genetic factors play a prominent role in the development of ARM (5).

It has been previously determined that environmental factors such as drug use in pregnancy, smoking, maternal diabetes mellitus, and retinoic acid embryopathy can cause ARM (6,7). An additional congenital anomaly is seen in 40% to 70% of children who are born with ARM (8). VACTERL association (MIM #192350) and CHARGE syndrome (MIM #214800) should be considered in the presence of additional major components in the genetic approach in ARM (9). Approximately 50% of all ARMs are associated with a syndrome, complex multiple anomalies, or a chromosomal disorder (10). Although the genetic etiology of ARM has not been fully elucidated, chromosomal disease such as Down syndrome (MIM #190685), 22q11 deletion syndrome (MIM #611867), 22q11 duplication syndrome (MIM #608363), cat eye syndrome (MIM #115470), and single-gene disorders, such as Currarino syndrome (MIM #176450), and FG syndrome (MIM #145410) are well-known causes of ARM (9).

Submicroscopic deletions and duplications have been frequently reported in patients with ARM (11). Molecular karyotyping is important in determining the underlying cause in patients diagnosed with ARM because of this common association, especially if they are accompanied by additional findings and/or dysmorphic features of other systems. Array comparative genomic hybridization (array-CGH) allows the examination of the entire genome at the same time and enables the determination of the submicroscopic microdeletion and microduplications in patients with ARM (12).
In this study, we aimed to evaluate the molecular cytogenetic analysis results of 10 patients with ARM using related databases and literature data.

2. Materials and methods
Ethics Committee approval for this retrospective study was obtained from the Ethics Committee of Keçiören Training and Research Hospital, dated 13.01.2016, with the decision number 15/1056.

2.1. Patients
In this study, we analyzed children who were diagnosed as having ARM with minor additional anomalies and normal karyotype at Gazi University Hospital from January 2012 to December 2014. Eight boys and two girls with ARM who were aged between 12 days and 9 years were assessed. The clinical diagnosis of ARM was made based on phenotypic and radiographic evaluation by a pediatric surgeon. Anal stenosis was present in two of the patients, anal atresia was present in the remaining eight patients, and anal fistula was present in only one patient. All patients were referred for evaluation of genetic etiology. None of the patients had a history of ARM in other family members. Only one patient's parents were consanguineous. Dysmorphic and additional findings of all patients are evaluated and summarized in Table 1. Although there were some additional findings, nine of the 10 patients were neither associated with a specific single-gene disease nor with an association after a detailed dysmorphologic examination. The coexistence of anal atresia and distal sacral agenesis in patient #4 met two findings of theCurrarino syndrome triad. Therefore, HLB9 gene sequence analysis was planned for this patient if the molecular karyotyping result was normal. Array-CGH analysis was performed. Pathogenic CNVs were further confirmed using fluorescence in situ hybridization (FISH) or quantitative real-time polymerase chain reaction (qPCR).

2.2. Molecular karyotyping
After the patients were evaluated clinically, blood samples were taken for routine genetic studies. Genomic DNA was isolated from peripheral blood using the salting-out method. Array-

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CGH analysis was performed using the 8x60K ISCA, Agilent® microarray platform (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s protocol. Data analysis was performed using the CytoGenomics software (v. 2.0.6.0) (Agilent Technologies, USA).

2.3. Variant Interpretation

For interpretation of copy number variants (CNVs), we used the standard probe cut-off levels used in routine diagnostics at the Department of Medical Genetics, Gazi University Hospital: (i) CNVs of 100 kb and over, (ii) including the gene, (iii) absolute log2-ratios above 0.25, (iv) CNVs with a minimum of three consecutive probes. CNVs which are smaller than 100 kb, if they contain genes associated with ARM have been confirmed by a second method, and then included in the results.

After analyzing the extracted data according to these criteria, CNVs were compared with variants reported in the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation), Database of Chromosomal Imbalance and Phenotype in Humans Ensembl Resources (DECIPHER, https://decipher.sanger.ac.uk), ClinVar and the in-house database at the Department of Medical Genetics, Gazi University Hospital. CNVs were classified as benign if they overlapped with CNVs reported in the DGV (more than three studies or more than 1% reported in the normal population) or if they were frequently observed in the in-house database. CNVs were interpreted as pathogenic if they overlapped with the critical region of a microdeletion or microduplication syndrome or contained a dose-sensitive gene according to the ClinGen Dosing Sensitivity Map (https://dosage.clinicalgenome.org/). CNVs were interpreted as variant of unknown significance (VOUS) when it was not known whether the genes contained were dosage-sensitive. Various databases have been used to identify CNVs that may be associated with ARM etiology (UCSC Genome Bioinformatics, PubMed (U.S. National Library of Medicine), OMIM (Online Mendelian Inheritance in Man), Uniprot, Genecards).
2.4. Fluorescence in situ hybridization (FISH)

FISH analysis was performed from peripheral blood samples using a Vysis DiGeorge region probe (LSI TUPLE1 SpectrumOrange/LSI ARSA SpectrumGreen probe, Abbot, USA) to confirm the 22q11 deletion, according to the manufacturer’s instructions.

2.5. Quantitative PCR

Quantitative real-time PCR (qPCR) was performed using EvaGreen® (Biotium, USA) on a BIO-RAD CFX96 Touch real-time PCR system (Bio-Rad Laboratories, Inc., USA) with the recommended PCR conditions. All experiments were repeated three times. The primers were 5’ GATGGCTTTTTCTGCTGTA 3’ and 3’ ACCTTATGCTGACAGAGATGTGA 5’. The cycle of threshold (CT) values of patient #1 are available on request.

3. Results

Demographic characteristics and examination findings of the patients with ARM are summarized in Table 1. Five of 10 patient’s results did not have a CNV that met the evaluation criteria. ARM-related CNV was found in two patients (patient #1 and patient #8). VOUS was found in two patients (patient #1 and patient #2) and likely benign changes were determined in two patients (patient #7 and patient #9). The molecular karyotyping results of the patients with ARM are shown in Table 2.

Patient #1: A male, aged 3 years, who had anal stenosis, minor cardiac anomaly (secundum ASD and PFO) and mild spinal enlargement at the 10th thoracic vertebral level in the spinal cord, had 89 kb monoallelic deletion in the 22q11.21 region (arr(hg19) 22q11.21(18,894,835-18,984,519) ×1). It was represented by three probes and included three genes (DGCR6, DGCR5 and PRODH). Although the deletion was smaller than 100 kb, because it contained 22q11 deletion syndrome critical region genes and could be associated with the ARM phenotype, it was confirmed using qPCR. That CNV was reported in healthy individuals in the DGV database, but the 115 kb deletion (patient #332402) containing PRODH and DGCR6 genes was classified as pathogenic in the DECIPHER database. In the ClinVar database, three CNVs
containing the \textit{PRODH} and \textit{DGCR6} genes, the smallest 30 kb and the largest 109 kb, were interpreted as pathogenic (patient #59070, #4005, and #583475). This CNV was classified as VOUS according to the recommendation of the American College of Medical Genetics and Genomics (ACMG) and Clinical Genome Resource (ClinGen) (13).

Patient #8: A 12-day-old male with no symptoms other than frequent infection, anal atresia, and a history of an undescended testicle. As a result of the molecular karyotyping of patient #8, we observed a 2.7-Mb monoallelic deletion in the 22q11 region, arr(hg19) 22q11.21(18,628,019-21,505,417), which was classified as pathogenic, and the patient was diagnosed as having 22q11 deletion syndrome after it was confirmed using FISH. FISH analysis for 22q11 of his parents was also performed and found to be normal.

In patient #1, there was also a 102 kb deletion in the 2q13 region containing the \textit{NPHP1} and \textit{MALL} genes. This deletion, which can be found in healthy individuals according to the DGV database, is represented by 35 probes. ARM has not been reported among patients with deletion of a similar region in the DECIPHER database. The \textit{NPHP1} gene encodes a protein involved in cell division, which is mostly expressed in the kidneys, eyes, testes, and ovaries. Homozygous or compound heterozygous missense mutations in the \textit{NPHP1} gene have been associated with Joubert syndrome type 4 (JBTS4, MIM #604583), Senior-Loken syndrome (SLSL1, MIM #266900), and autosomal recessive nephronophthisis 1, juvenile (NPH1, MIM# 256100). The patient has been evaluated as a carrier for the related diseases because of the heterozygous deletion of the region. Clinical findings are not expected in heterozygous carriers. Finally, the current duplication was not associated with ARM and was classified as VOUS.

Patient #2 had a 710 kb duplication in the 1q31.1 region, including the \textit{PLA2G4A} gene (phospholipase A2, group 4A, MIM# 600522). The duplication is not a polymorphic CNV according to the DGV database. According to the UCSC database, the duplication has been classified as VOUS. In DECIPHER, there are no patients with anorectal malformation and

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duplication of the same region. Based on this information, we classified the duplication in patient #2 as a VOUS and concluded that it was not associated with ARM.

Patient #7 had a 160 kb duplication in the chromosome 10q26.3 region including LOC619207, SCART1, CYP2E1, SYCE1, SPRNP1 genes, which was reported as polymorphic CNV according to the DGV database. In the DECIPHER database, similar-sized CNVs have been reported as benign in patients #285904, #286222, and #294706. This CNV, which was detected in patient #7, could not be associated with ARM and was classified as likely benign.

As a result of the molecular karyotyping of patient #9, a 111-kb duplication represented by three probes was detected on the short arm of chromosome 5. This duplication, including the CEP72 and TPPP genes, has been reported in healthy individuals in the DGV database. Similar duplications are reported as benign in the DECIPHER database. Therefore, this CNV, which could not be associated with the clinic of patient #9, was evaluated as likely benign.

4. Discussion
We evaluated the array CGH results of 10 patients with ARM in terms of their relation with ARM by searching databases and the literature. CNVs that could not affect the phenotype were detected in eight of the 10 patients, and it was thought that CNVs detected in two patients might be related to ARM.

In patient #8, a 2.8-Mb deletion in chromosome 22q11.21 was detected and confirmed using FISH. This region includes the critical region of 22q11 deletion syndrome, which is a well-known microdeletion syndrome. Although 90% of patients with deletion 22q11 syndrome are de novo, a small portion is inherited from a parent. For (14) this reason, deletion analysis was performed on the parents of patient #8 using FISH and it was determined that they did not carry the deletion. Patient #8 did not show the characteristic findings of 22q11 deletion syndrome such as heart anomaly. Of the findings seen in 22q11 deletion syndrome in our patient, there...
were only anal atresia, undescended testis, and frequent infection. Undescended testis occurs as a finding of the syndrome in approximately 6% of patients (15).

Immunodeficiency has been defined in 70% of patients with 22q11 deletion syndrome. Pneumonia, especially in the neonatal period, is an important reason for hospitalization. The frequency of infections that continue until late childhood decreases with age (16). Although chronic constipation is a common feature seen in 35%, ARM is a rare finding of 22q11 deletion syndrome (17). Anal atresia was reported for the first time in DiGeorge syndrome in 1979 (18). Subsequently, anteriorly displaced anus has been defined in one of 44 patients with 22q11 deletion (19), then anteriorly displaced anus was reported in two more patients in another series of 55 patients (20). Worthington et al. reported three patients with ARM without cardiac anomalies in 1997. All three patients had hypernasal speech, uvula anomaly, speech delay, and developmental delay. The authors suggested that there was a common mechanism in the pathogenesis of Velocardo-facial syndrome (VCFS) and ARM because both occurred due to abnormal migration of mesenchymal cells (21). In support of this hypothesis, the most common microdeletion syndrome observed in patients with ARM is 22q11 deletion syndrome. In addition, ARM is an important finding in cat eye syndrome caused by overexpression of the 22q region. All these associations led to the intensification of genetic studies on ARM in the long arm of the 22nd chromosome. In genome-wide association studies related to ARM, the most featured region is still the 22q11 region (22).

Deletion of 22q11.21 containing the DGCR6, DGCR, and PRODH genes was detected in patient #1 and confirmed using qPCR. To the best of our knowledge, it is the smallest deletion detected in patients with anal atresia in the literature to date. Anal stenosis and minor cardiac anomalies, which were among the phenotypic findings of patient #1, have also been described in 22q11 deletion syndrome. It is not known which of the genes located in the critical region of 22q11 deletion syndrome causes ARM. It is possible that one or more of these genes whose deletions were determined in our two patients (patients #1 and #8) may cause the ARMs seen in 22q11 deletion syndrome.
In patient #1 and patient #8, different sizes deletions were detected in the 22q11.22 region and the DGCR6, DGCR5, and PRODH genes overlapped in both patients. When we searched the DGCR6, DGCR5, and PRODH gene deletions in the DECIPHER database, we found that in four patients, much larger deletions including these genes were reported in patients with anal atresia (patients #396778, #396780, #396830, and #398087). Anal atresia and chromosome 22q11.21 deletion were detected in two more patients reported by Dworschak et al. (23). In both patients, the deleted region was approximately 2 Mb in size and included the DGCR6, DGCR5, and PRODH genes (shown in Figure 1).

The DGCR6 gene, at the proximal part of the DiGeorge critical region, is one of the candidate genes thought to cause the DiGeorge syndrome phenotype (24). The dosage of the DGCR6 gene has been analyzed in 50 control samples using qPCR and found to be normal (25). Heart, liver, and skeletal muscle are the tissues with the highest DGCR6 protein expression (26). Chicken embryos with reduced DGCR6 expression have been found to have cardiovascular anomalies similar to DiGeorge syndrome (27).

The probability of the DGCR6 gene causing ARM is higher than the other two genes whose deletion was detected in patient #1 because the DGCR6 gene decreases TBX1 gene expression and regulates neural crest migration. TBX1 affects neural crest migration in the pharyngeal region (28) and alterations in the levels of TBX1 impact the severity of the congenital problems in 22q11 deletion syndrome (29).

Other genes in the common region were DGCR5 and PRODH. Neither of these two genes has been previously associated with ARM. The DGCR5 does not encode proteins but encodes various RNAs that have important regulatory functions. Biallelic deletions and mutations of the PRODH gene cause autosomal recessive hyperprolinemia type 1 disease. Deletions in a single allele have been associated with schizophrenia seen in 22q11 deletion syndrome (30). PRODH and DGCR6 gene deletions have previously been associated with congenital heart disease (29).
Although there have been studies that previously performed a genotype-phenotype correlation for 22q11 deletion syndrome, this is difficult because it is a contiguous deletion syndrome. It is not known which of the genes lost in 22q11 deletion syndrome causes ARM.

Although it is known that there is a common mechanism in the pathogenesis of VCFS and ARM and that both result from abnormal migration of mesenchymal cells, we showed for the first time that DGCR6 gene dosage alterations might be related to ARM. Our findings may contribute to the understanding of the etiology of ARM and future studies in patients with ARM and 22q11 deletion syndrome may clarify this candidate gene connection. The limitations of our study are the lack of whole-exome sequencing for the patients, the inclusion of a small number of patients, and the use of a low-resolution array platform. New studies with more patients using higher-resolution array platforms are needed to say with certainty that the change in the DGCR6 gene is associated with anal atresia.

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We thank to patients and her family for their permission. We would like to thank Gazi University Pediatric Surgery Faculty Member Prof. Dr. Billur Demiroğulları (B.D.), who helped us reach the patients and passed away as a result of a bad illness.

Statement of Ethics:
The study was approved by the Ethics Committee of Keçiören Training and Research Hospital, Ankara (15/1056). This study was performed in accordance with the Declaration of Helsinki Principles. Written informed consent was obtained from the patient for the publication of this article and images.

Conflict of Interest Statement:
The authors have no conflicts of interest to declare.

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There is no funding sources to report.

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Table 1: Demographic characteristics, examination findings and test results of the patients

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Patient 7</th>
<th>Patient 8</th>
<th>Patient 9</th>
<th>Patient 10</th>
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</thead>
<tbody>
<tr>
<td>Age/Gender</td>
<td>3.5 y/M</td>
<td>4 y/M</td>
<td>4.5 y/M</td>
<td>7 y/M</td>
<td>5 m/M</td>
<td>9.5 y/M</td>
<td>5.5 y/M</td>
<td>12 day/M</td>
<td>7 m/F</td>
<td>7 y/F</td>
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<td>Prenatally</td>
<td>Maternal cigarette (3 of a day)</td>
<td>uneventful</td>
<td>High risk of NTD</td>
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<td>uneventful</td>
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<td>Natal</td>
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<td>NSV, term</td>
<td>NSVD, term</td>
<td>C/S, 35 week</td>
<td>NSV, term</td>
<td>NSVD, term</td>
<td>NSVD, term</td>
<td>C/S, term</td>
<td>NSV, term</td>
<td>NSV, term</td>
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<td>Consanguinity</td>
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<td>No</td>
<td>No</td>
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<td>Same village</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>3rd degree</td>
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<td>Dysmorphic features</td>
<td>Prominent forehead, metopic ridge, sparse eyebrows, dysplastic ears</td>
<td>Obesity, fusiform fingers</td>
<td>Broad arched eyebrows, thin upper lip, smooth philtrum hypertrophy</td>
<td>Synophis, long eyelashes, uplift earlobe</td>
<td>Uplifting palpebral fissures</td>
<td>Wide earlobe</td>
<td>Sparse eyebrows, narrow palpebral fissures, small mouth</td>
<td>Uplifting palpebral fissures, small mouth</td>
<td>Hirsutism, high nasal bridge, posterior rotated ears</td>
<td></td>
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<tr>
<td>Cardiac anomaly</td>
<td>ASD, PFO</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Secundum ASD</td>
<td>Secundum ASD</td>
<td>Secundum ASD, PFO</td>
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<tr>
<td>Abdomen USG</td>
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<td>Normal</td>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Skeletal anomaly</td>
<td>Spinal cord enlargement at T10-11</td>
<td>Normal</td>
<td>Normal</td>
<td>Distal sacral agenesis, coccygeal agenesis and syringohydromelia</td>
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### Table 2: Molecular karyotyping results of the ARM patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Chromosome</th>
<th>Deletion/Duplication</th>
<th>Genomic coordinates (Grch37/hg19)</th>
<th>Size</th>
<th>Prob</th>
<th>Classification</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22q11.21</td>
<td>Deletion</td>
<td>18894835-18984519</td>
<td>89 kb</td>
<td>3</td>
<td>VOUS</td>
<td>DGCR6, DGCR5, PRODH</td>
</tr>
<tr>
<td>1</td>
<td>2q13</td>
<td>Deletion</td>
<td>110862477-110964737</td>
<td>102 kb</td>
<td>35</td>
<td>VOUS</td>
<td>MALL, NPHP1</td>
</tr>
<tr>
<td>2</td>
<td>1q31.1</td>
<td>Duplication</td>
<td>186762066-187472702</td>
<td>710 kb</td>
<td>14</td>
<td>VOUS</td>
<td>PLA2G4A</td>
</tr>
<tr>
<td>7</td>
<td>10q26.3</td>
<td>Duplication</td>
<td>135243049-135404523</td>
<td>161 kb</td>
<td>30</td>
<td>Likely benign</td>
<td>LOC619207, SCART1, CYP2E1, SYCE1, SPRNP1</td>
</tr>
</tbody>
</table>

M; male, F; female, NTD; neural tube defect, C/S; cesarian section, NSVD; normal spontaneous vaginal delivery, ASD; atrial septal defect, PFO; patent foramen ovale, USG; ultrasonography
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