Chromosomal microarray should be performed for cases of fetal short long bones detected prenatally

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May 5, 2020

Abstract

Objectives: To investigate the prevalence of pathogenic and likely-pathogenic variants detected by chromosomal microarray analysis (CMA), among pregnancies with fetal short long bones diagnosed by ultrasound. Design: A retrospective study. Setting: The study was based on national records from the Israeli Ministry of Health. Sample: Chromosomal microarray analyses performed nationwide, during January 2016 to March 2018, for the indication of prenatal diagnosis of short long bones (n=66). Methods: Clinical data was retrieved from genetic counselling summary letters and from patients' medical records. The CMA yield was compared to two cohorts that reported the background risk. Main outcome measure: Pathogenic/likely pathogenic CMA. Results: There were 4 cases with a pathogenic/likely pathogenic result (6%). The rate of chromosomal abnormalities was significantly higher compared to the background risk for copy number variations (CNVs) [P<0.001], [odds ratio (OR) 4.5, 95% CI 1.6-12.7], [OR 5.8, 95% CI 2-16.2], for both isolated [OR 6.1, 95% CI 1.4-26], [OR 7.8, 95% CI 1.8-33.5], and non-isolated cases[OR 10, 95% CI 2.2-44], [OR 12.8, 95% CI 2.9-57], , and for cases in which the lowest estimated bone length percentile was above the 3rd percentile (below 5th percentile) [OR 23, 95% CI 6.2-87], [OR 29.9, 95% CI 8-111], . Conclusion: The yield of CMA in cases with short long bones (both isolated and non-isolated) is significantly higher than the background risk for chromosomal anomalies in pregnancies with no sonographic anomalies. This suggests that CMA should be offered in pregnancies with a diagnosis of fetal short long bones.

Introduction

Routine fetal biometric evaluation includes femur length measurement. In cases where the femur length is 2 or more standard deviations below the normal range, the guidelines recommend measuring the length of the other long bones and to perform a thorough skeletal assessment.[1-3]

Short, fetal long bones may be constitutional and/or attributed to race/ethnicity. In these cases, no further testing is required. In some cases however, it may be the first sign of intrauterine growth retardation and placental insufficiency, resulting in an increased risk for pregnancy complications, including preterm delivery

and pregnancy-associated hypertension disorders.[4-6] Other causes for short long bones are chromosomal abnormalities and genetic syndromes including monogenic skeletal dysplasias.[7-10]A short femur and humerus have been linked to karyotype detectable aneuploidies; in particular trisomy 21.[8] Information regarding the rate of submicroscopic chromosomal aberrations detected by chromosomal microarray analysis (CMA) is limited to a few reports in the literature.[11, 12]

This study investigated the prevalence of pathogenic/likely pathogenic variants detected by CMA, among pregnancies complicated with short long bones in relation to other clinical characteristics.

Methods

This retrospective study was based on national records from the Israeli Ministry of Health (MOH). We searched the electronic database of the MOH for CMA tests performed from January 2016 to March 2018 for the indication of short fetal long bones, below the 5thpercentile for gestational age. All testing was financed by the MOH after approval by a clinical geneticist.

The study was approved by the Institutional Review Board for Human Subjects (September 6, 2016, registration number – MOH2016).

Clinical data were obtained from the Israeli national database and from patients' medical records. Detailed clinical information including maternal age, maternal chronic illness, familial background of genetic conditions, obstetrical history of recurrent spontaneous abortions, elevated nuchal translucency, biochemical screening results, gestational age at diagnosis, lowest bone length percentile recorded during the pregnancy, and the presence of additional sonographic findings were retrieved.

CMA findings were reviewed independently by two authors (R.S.H. and I.M.) and grouped independently into four categories:

1. Normal (including benign and variants of unknown significance - likely benign categories),
2. Pathogenic (P)/likely pathogenic (LP) variants,
3. Microdeletion/duplication with low penetrance, and
4. Variants of unknown clinical significance (VUS).

The categorization was based on laboratory reports, as well as on new information gained from the medical literature and from the authors' experience.

For VUS, only cases with deletions [?]1 Mb and duplications [?]2 Mb were included. These variants are reported by the lab, according to the guidelines determined by Israeli Society of Medical Geneticists.

Microarray results were also categorized into "karyotype-detectable" (i.e., copy number variants of at least 10 MB) or not "karyotype-detectable" in order to assess the incremental yield of CMA over karyotype.

Two cohorts were used to assess the background risk.

1. A large local cohort of 5,541 cases with normal prenatal ultrasounds in a large, hospital-based clinical laboratory.[13] The detection rate for this cohort was 1.4% (78 cases).
2. A second cohort of 10,614 cases was extrapolated from a meta-analysis by Srebniak et al.[14] We calculated the background risk by adding the risk for submicroscopic chromosomal abnormalities to the risk for karyotype detectable chromosomal abnormalities.[15] This risk was 1:384, based on the average maternal age of our cohort. The detection rate for this cohort was 1.1%.

Various platforms were used by 12 laboratories to perform CMA. Most medical center laboratories in Israel use the CytoScan 750K array, which is composed of 550,000 nonpolymorphic copy number variant probes and more than 200,000 single-nucleotide polymorphism probes, with an average resolution of 100 Kb.[16] One laboratory uses Infinium OmniExpress-24 v1.2 BeadChip, which includes 713,599 genome-wide markers at an average spacing of 4,080 bases and has a targeting minor allele frequency of 5%,[17] and one laboratory performs microarray analysis using a Cytochip ISCA 8360K format, BlueGnome.[18] Two additional centers (one previously working with the BlueGnome platform and one using BlueGnome and then Illumina) switched

to Affymetrix in 2017. One laboratory uses GenetiSure Unrestricted CGH + SNP (43180K) P/N G5976A Agilent. Genomic coordinates were evaluated in accordance with genome build GRCh37/hg19 in all laboratories. All analyses performed in the laboratories met the standards and guidelines of the American College of Genetics and Genomics for constitutional cytogenomic microarray analysis, including postnatal and prenatal applications,[13, 15] adopted by the recommendations of the Israeli Medical Genetics Association.[16]

Statistical analysis

Fisher's Exact Test or Chi-square were used to test the differences between CMA yield in relation to different parameters, and as compared to the background risk. $P<0.05$ was considered statistically significant. Python statistics library version 3.5.1 (scipy.stats) was used for statistical analysis.

Results

Characteristics of the study cohort

A total of 67 CMAs were performed as part of the short fetal long bones work-up, during the study period. We excluded 1 case of twin pregnancy.

The mean maternal age in the cohort was 30.9±7.8 years. Mean gestational age at diagnosis was 26.1±6.17 weeks, range 14.7–38 weeks. Mean gestational age at amniocentesis was 30.3±5.8 weeks. The mean lowest bone length percentile was 3.15±1.9. Our cohort included 16 cases with reported additional ultrasound findings.

Copy number variations (CNVs) detected by CMA (Table 1)

Among 66 cases included in the study cohort, 4 (6%) had a pathogenic/likely pathogenic CNV; one case of a pathogenic 15Mb terminal deletion at 18p11.32p11.21, [18p11.32p11.21(136227-15.170,636)x1]; one case of pathogenic 10Mb terminal deletion at 6q23.1q24.[16q23.1q24.1(74,897,270-85,159,980)x1]; one case of pathogenic 13 Mb interstitial deletion at 1p31.1 [1p31.1(66,634,291-80,128,969)X1] and a fourth case of 571kb duplication at 5q35.2, classified as likely pathogenic [5q35.2 (176,329,286-176,900,534) x 3]. This last case had a duplication of the *NSD1* gene. In 1 case, a variant of unknown clinical significance, inherited from the mother, was detected (Table 2). Three of the 4 pathogenic/likely-pathogenic CNVs were karyotype detectable. The incremental yield of CMA over karyotype was 1.5%. The rate of abnormal CMA results was 8% (2/25) in isolated cases and 12.5% (2/16) in non-isolated cases.

Testing for monogenic syndromes

Testing for the known *FGFR3* pathogenic variants was negative for all tested cases. Seven cases with normal CMA results were highly suspicious for skeletal dysplasia; therefore, additional testing was performed. Whole exome sequencing performed in 2 cases detected a VUS in the in the COLO1A1 gene (c.2519C>T; p.P840L), in one of the cases.

The yield of CMA according to different clinical characteristics (Table 2)

We did not find any correlation between the yield of CMA and bone length percentiles, gestational week, or detection of additional abnormal sonographic findings. Among several clinical characteristics tested, the only one that almost reached statistical significance was an abnormal fetal echocardiogram ($p = 0.05$).

The yield of CMA compared to the background risk

We assessed the yield of CMA in pregnancies complicated with short long bones compared to 2 cohorts that represented the background risk: a cohort of 5,541 uncomplicated pregnancies for which the yield of CMA was 1.4%[13] and a cohort of 10,614 cases extrapolated from a meta-analysis by Srebniak et al.,[14] for which the yield of CMA was 1.1%. The rate of chromosomal abnormalities was significantly higher for all short long bones cases as compared to the background population for both cohorts: 4.5 (95% CI 1.6-12.7; $P=0.0017$) and 5.7 (95% CI 2-16.2; $P<0.001$). Furthermore, the yield for both isolated and non-isolated cases was significantly higher than the background risk ($P<0.05$). The yield was higher than background risk for

cases diagnosed with short long bones after 22 weeks of gestation compared to both cohorts, but not for cases diagnosed after 24 weeks (Table 3). For cases in which the lowest estimated bone length percentile was above the 3rd percentile (but below the 5th percentile), the yield was also higher than background risk.

Discussion

Main findings

In our study of singleton pregnancies that underwent work-up for short long bones detected by ultrasonography, the yield of CMA for pathogenic/likely pathogenic CNV was 6%. This was significantly higher than the background population, suggesting this test should be offered as part of the work-up in these cases.

Strengths and Limitations

This study is one of the first to report the yield of CMA in a cohort of pregnancies with fetal short long bones. The study limitations relate to its retrospective nature, as well as the lack of data regarding neonatal outcomes. In addition due to the size of our cohort, we were underpowered to assess all relevant clinical characteristics that may have an impact on the yield of CMA testing in fetuses diagnosed with short long bones.

Interpretation

Data regarding the yield of CMA in pregnancies complicated with short long bones, are sparse. Liu et al,[11] recently reported a high yield of 15.6% for pathogenic/likely pathogenic CNV in cases with short femur length.[11] This might be explained by the cohort features, including a higher rate of cases with additional sonographic findings: 38/64 (59%) compared to 25/66 (38%) in our cohort. Shaffer et al, reported an incremental yield of 7.3% for CMA over the karyotype[19] compared to our incremental yield of only 1.5%.

The rate of abnormal testing in isolated cases, with no additional findings, in our cohort was 8%, which is comparable to the 9.5% reported by Liu et al.[11] Of the two isolated cases in which pathogenic CNVs were detected, one had a deletion at 18p11.32-p11.21, which does not include any gene related to reduced growth. However, Chen et al.[20] described a 13-year-old girl, who presented with Turner-like syndrome including short stature, with a 18p11.32-p11.21 deletion, identical to the deletion in our case. The other deletion in chromosome 16 encompasses the *MAF* gene (OMIM #601088). Heterozygous mutations in the *MAF* gene result in Ayme-Gripp syndrome (OMIM# 601088), in which reduced growth is part of the phenotype, along with other clinical features such as congenital cataracts, which were not reported in this case.[21]

The additional two cases of pathogenic/likely pathogenic CNV were found in non-isolated cases. The deletion in at 1p31.1 was detected in a fetus with suspected muscular ventricle septum defect. The deletion contains the *CDC42* gene (OMIM#616737), and the *RPL11* gene (OMIM #612562). Heterozygous mutations in the *CDC42* gene cause Takenouchi-Kosaki syndrome (OMIM #616737), an autosomal dominant multisystem disorder with cardiac and skeletal involvement.[22] Heterozygous mutations in the*RPL11* gene result in Diamond-Blackfan anemia (OMIM # 612562), a variable phenotype syndrome characterized by red blood cell aplasia, growth retardation, craniofacial, upper limb, heart, and urinary system congenital malformations.[23]

The second case of pathogenic/likely pathogenic CNV with non-isolated short long bones, was diagnosed with persistent right umbilical vein, which is considered a soft marker for increased risk for malformations.[24] Duplication of the *NSD1* gene was reported to be associated with clinical characteristics of short stature, specific facial features, and intellectual disability in a small number of patients.[25, 26] Interestingly, deletions and coding variants of the *NSD1* gene cause Sotos syndrome, which is characterized overgrowth, macrocephaly, typical facies and learning disability.[27, 28]

Chen et al.[29] were the first to suggest a gene dose effect of the NSD1 gene, followed by a number of reported cases of which duplications of the 5q35 region encompassing the *NSD1* gene resulted in a phenotype of short stature and microcephaly, along with other phenotypic features, including learning disability, mild to

moderate intellectual disability, distinctive facies, delayed bone age, microcephaly, seizures, and failure to thrive.[25, 26, 30, 31]

In our cohort, seven cases presented with sonographic features suggestive of skeletal dysplasia. No clinically significant aberrations were identified by CMA among these cases. Subsequently, two of these cases underwent further genetic testing with whole exome sequencing with no pathogenic aberrations were found in these cases. Liu et al. reported the results of the genetic analysis performed for the 15 cases suggestive of skeletal dysplasia in their cohort.[11]Genetic aberrations were identified by CMA in two cases (2/15), 1 pathogenic/likely pathogenic CNV and one variant of unknown significance. Genetic sequencing, identified pathogenic aberrations in 53% (8/15) of these cases.[11]

These results emphasize the challenges of prenatal diagnosis in cases of short long bones. Major, significant disorders will not be diagnosed by CMA alone. Hence, in the presence of sonographic findings suggestive of skeletal dysplasia, with negative CMA, further investigation is recommended using whole exome sequencing or targeted gene panels. Figure 1 depicts a suggested protocol for pregnancies diagnosed with fetal short long bones.

A main issue addressed in our study was whether the risk for abnormal genetic analysis by CMA is significantly higher in cases of short long bones suspected by prenatal scanning, as compared to the background risk. We found that the rate of pathogenic/likely pathogenic CNVs in cases was significantly higher compared to the background population, in a local cohort[13] and in a cohort derived from a meta-analysis.[14] The fact that the specific CNVs detected are associated with skeletal anomalies emphasize the relevance of our findings.

In an attempt to better characterize groups that would benefit from genetic analysis, we grouped the cases by the presence of additional findings. The yield of CMA was significantly higher than the background risk, in both comparison groups. Additional analysis revealed that cases diagnosed after 22 weeks of gestation but not after 24 weeks had a significantly higher yield of CMA compared to the background risk, suggesting that cases with a diagnosis of short long bones detected after 24 weeks are more likely to be constitutional and genetic evaluation in these cases is less beneficial. However, larger cohorts are needed to determine the accuracy of this suggestion. The yield of CMA was also higher than the background risk in cases where the measured percentile was larger than the 3rd percentile, suggesting that testing cases in the lower portion of the normal range should also be considered.

Conclusion

The yield of CMA for clinically significant CNV in cases with short long bones is 6%. This is significantly higher than the background risk for chromosomal anomalies in normal pregnancies, suggesting that CMA should be performed in these cases.

Disclosure of Interests

No conflict of interest is apparent

Contribution to Authorship

K.T.G– Project development, Data collection and analysis, Manuscript writing.

A.S - Project development, Data collection, Manuscript editing.

I.M – Data analysis, Manuscript editing.

L.S.D - Data collection, Manuscript editing.

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M.F.Z - Data collection, Manuscript editing.

R.S.H- Project development, Data collection and analysis, Manuscript writing.

Details of Ethics Approval

The study was approved by the Institutional Review Board for Human Subjects (September 6, 2016, registration number – MOH2016).

Funding

This study received no funding.

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Figure legend

Figure 1. Suggested work-up for cases with prenatally diagnosed fetal short long bones.

CMA, chromosomal microarray analysis

#cases with femur length below the 5th percentile should also be included due to possible error in measurement and the potential decrease in percentile during the pregnancy.

**FGFR3* mutations - testing for achondroplasia and hypochondroplasia:

- c.1138G>A (p.Gly380Arg)
- c.1138G>C (p.Gly380Arg)
- c.1620C>A (p.Asn540Lys)
- c.1620C>G (Asn540Lys)

Table 1: Summary of the aberrant chromosomal microarray analysis findings.

OMIM gene related to growth/ related disorder	**CMA Classification**	**CMA result (ISCN*) array GRCh37/hg19**	**Additional US findings**	**Percentile**	**Diagnosis week**	**Case**
Reported in a girl with Turner like syndrome^{++} Chen et al.	Pathogenic	18p11.32-p11.21 terminal deletion (136227-15.170,636) x1	None	4	–	1
MAF gene (*601088) - Ayme-Gripp syndrome.	Pathogenic	16q23.1q24.1 terminal deletion (74,897,270-85,159,980x1	None	3	28	2
CDC42 gene (*616737) - Takenouchi-Kosaki syndrome RPL11 gene (*612562) - Diamond-Blackfan anemia	Pathogenic	1p31.1interstitial deletion (66634291-80128969)x1	Muscular VSD	5	16.4	3
NSD1 Duplication syndrome	Likely pathogenic	5q35.2 duplication (176329286-176900534)	PRUV	4.5	24	4

OMIM gene related to growth/ related disorder	CMA Classification	CMA result (ISCN*) array GRCh37/hg19	Additional US findings	Percentile	Diagnosis week	Case
	Variant of unknown clinical significance	5p15.33 duplication (113,576-1,697,973) x3 Maternally inherited	Bell shape thorax		16	5

US, ultrasound. CMA, chromosomal microarray analysis; VSD, ventricular septal defect; PRUV, persistent right umbilical vein^{++}suggested by one report. [20]

Table 2: The yield of CMA according to clinical parameters

Parameter	CMA	CMA	CMA	P-value
N (%)	Normal Abnormal N (%)	Total		
Isolated SLB	23 (92)	2 (8)	25	0.63
Non-isolated SLB	14 (87.5)	2 (12.5)	16	
SLB with non-skeletal structural findings SLB skeletal structural findings	55 (93.2) 7 (100)	4 (6.8) 0	59 7	0.47
Normal fetal echo	34 (97.1)	1 (2.9)	35	0.055
Abnormal fetal echo	0	1	1	
Diagnosed >24 GW	23	1	24	0.57
Diagnosed [?]24 GW	16	2	18	
Diagnosed >26 GW	22	1	23	0.58
Diagnosed [?]26 GW	17	2	19	
Diagnosed >28 GW	18	0	18	0.24
Diagnosed [?]28 GW	21	3	24	
Lowest percentile [?]3% Lowest percentile >3%	20 (95.2) 9 (75)	1 (4.8) 3 (25)	21 12	0.125
Lowest percentile [?]1%	6 (100)	0	6	1
Lowest percentile >1%	23 (85.2)	4	27	

Parameter	CMA	CMA	CMA	P-value
Maternal age [?]40 years	2 (66.7)	1 (33.3)	3	0.7
Maternal age <40 years	50 (94.3)	3 (5.7)	53	
Normal screening test$^{\pm}$	25 (92.6)	2 (7.4)	27	0.55
Abnormal screening test$^{\pm}$	7 (87.5)	1 (12.5	8	

$^{\pm}$First or second trimester screening test for Down syndrome

SLB, short long bones; GW, gestational week; IUGR, intrauterine growth retardation; Hx, history

Table 3: The yield of CMA for short long bones cases compared to background risk

Compared to a background risk of 1.1% in a population of 10,614 cases[14] with normal ultrasound^{++++} OR (95% CI) P-value	Compared to a background risk of 1.4% in a population of 5541 cases with normal ultrasound[13] OR (95% CI) P-value	Clinically significant CMA Results - No (%)	No. of cases	Parameter
5.8 (95% CI 2-16.2) P=0.00015	**4.5 (95% CI 1.6-12.7) P=0.0017**	4 (6)	66	All SLB cases
7.8 (95% CI 1.8-33.5) P=0.001	**6.1 (95% CI 1.4-26) P=0.0057**	2 (8)	25	Isolated SLB
12.8 (95% CI 2.9-57) P<0.0001	**10 (95% CI 2.2-44) P=0.0002**	2 (12.5)	16	Non-Isolated SLB
3.9 (95% CI 0.5-29) P=0.15	3 (95% CI 1.6-12.7) P=0.25	1 (4.17)	24	Diagnosis >24 weeks gestation
5.98 (95% CI 1.4-25.3) P=0.006	**4.6 (95% CI 1.09-19.8) P=0.02**	2 (6.25)	32	Diagnosis >22 weeks gestation
15.6 (95% CI 5.3-45.8) P<0.0001	**12 (95% CI 4.1-36) P<0.0001**	4 (14.8)	27	Lowest percentile >1%
29.9 (95% CI 8-111) P<0.0001	**23 (95% CI 6.2-87) P<0.0001**	3 (25)	12	Lowest percentile >3%

$^{++++}$ The background risk was calculated as the risk for submicroscopic chromosomal abnormalities plus the risk for chromosomal abnormalities (1:384)[15] based on the average maternal age of the cohort.