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ORIGINAL ARTICLE

Investigation of Mutations in H19, IGF2, CDKN1C, KCNQ1, and NSD1 Genes in Iranian Children Suspected of Silver-Russell Syndrome (SRS) and Beckwith-Wiedemann Syndrome (BWS) with MS-MLPA

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Abstract: *Background: Silver-Russell Syndrome (SRS) and Beckwith-Wiedemann Syndrome (BWS) are two syndromes that are poorly diagnosed in many affected people due to mild and subtle symptoms, genetic complexity, and lack of familiarity with the hallmarks. Objective: The present study was conducted with the aim of determining mutations in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes in Iranian children suspected of SRS and BWS by the MS-MLPA method. Methods: In this case series study, which was conducted in 2022 in Pars Genome Laboratory, Karaj, Iran, 10 children suspected of SRS or BWS syndrome were included. These 10 Iranian children were referred by pediatric endocrinologists. 5 ml of peripheral blood was taken per patient for DNA extraction. MS-MLPA method was undertaken for possible mutations (methylation and deletion) in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes. Results: The interpretation of MS-MLPA results showed that out of 10 children (4 boys and 6 girls) suspected of having SRS or BWS syndrome (based on the pediatric endocrinologist's diagnosis), only 3 children were definitively diagnosed with SRS or BWS syndrome. Based on this, methylation changes in the promoter of ICR1 and ICR2, which are related to the genes H19, IGF2, CDKN1C, KCNQ1, and NSD1, lead to the development of SRS or BWS syndrome. Conclusion: The present findings showed that methylation changes in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes are associated with the occurrence of SRS or BWS syndrome. In this study, we show that MS-MLPA can serve as a rapid, inexpensive, and reliable method for the molecular diagnosis of these syndromes.*

Keywords: *Silver-Russell Syndrome (SRS), Beckwith-Wiedemann Syndrome (BWS), Methylation, MS-MLPA*

INTRODUCTION

Silver-Russell Syndrome (SRS) is a rare disorder characterized by intrauterine growth restriction, poor postnatal growth, macrocephaly, a triangular facial appearance, prominent forehead, body asymmetry, and significant feeding difficulties [1]. Patients can have a wide range of clinical findings. The majority of people with SRS have normal intelligence but have motor or speech delays. SRS is genetically heterogeneous, which means that different genetic abnormalities cause this disorder [2]. In around 60% of people with SRS, the disease is related to mutations in chromosome 7 or 11. Genetic defects that may be responsible for this syndrome include uniparental disomy of maternal chromosome 11P15.5, deletion of chromosome 7, or duplication of chromosome 7 or mutation of the KCNQ1, H19 (located on 11p15) and NSD1 (located on 5p35). However, in about 40% of patients with SRS, the cause is unknown. In general, the diagnosis of SRS is based on clinical findings. Since many symptoms are subtle and nonspecific, SRS remains difficult to diagnose [3]. Consensus guidelines for the evaluation and diagnosis of SRS have recently been published based on the Netchine-Harbison clinical scoring system for SRS [4]. Testing for known genetic causes of SRS (chromosomes 7 and 11) can confirm the clinical diagnosis in up to 60% of individuals. Knowing the main genetic cause can also help to select the appropriate treatment approach [5]. Treatment of this syndrome focuses on managing the symptoms [6,7]. SRS is present at birth and many symptoms improve as the patient grows [8].

Beckwith-Wiedemann Syndrome (BWS) is an overgrowth syndrome that affects certain chromosomes of the fetus. People with this syndrome may experience mild to severe signs and symptoms. One out of every 11,000 infants is affected by BWS, which is equally common in both boys and girls. This syndrome causes excessive growth of some body parts. BWS affects many areas of the body and makes the child appear significantly larger than their peers. Patients show different signs and symptoms depending on the affected organs [9,10], including macrosomia, hemihyperplasia, macroglossia, hypoglycemia in infancy, and sometimes long-term hypoglycemia (due to high insulin). The management of this syndrome focuses on its common symptoms and complications.

Physicians monitor and check affected children for low blood sugar and increase the frequency of feeding to obtain optimal blood glucose levels. There are several genetic causes of BWS, usually resulting in changes in one or more genes in a region of chromosome 11 known as 11p15 [11,12]. In most cases, there is genetic mosaicism. Other genetic changes in this syndrome include methylation defects in the region of 11p15, uniparental disomy (UPD) in 11p15, or mutation of the CDKN1C and IGF2. The diagnosis of BWS is clinical and it is often confirmed by genetic testing. To assay these abnormalities, a blood sample or other tissue samples should be taken [13,14].

In addition to organ growth defects, BWS patients are susceptible to other diseases, including cancer (such as Wilms' kidney tumor, hepatoblastoma, and neuroblastoma), heart disease (pericarditis, cardiomyopathy, etc.), hypoglycemia, hemihyperplasia, and digestive disorders [15]. Conducting investigations of H19, IGF2, CDKN1C, KCNQ1 (located 11p15), and NSD1 (located 5p35) genes in these syndromes and increasing information in this field can lead to a more accurate and precise diagnosis; preventing secondary diseases including cancer; and also be effective in preventing the transfer of these genes to the fetus before and during pregnancy [3,13,14]. Considering that no study in this field has been done in Iran, the present research aims to investigate mutations in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes in children suspected of SRS and BWS with Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) method.

MATERIALS AND METHODS

Study procedures

In this case series study that was conducted in 2022 at Pars Genome Laboratory in Karaj, Iran, 10 children suspected of SRS or BWS were included. These 10 Iranian children were referred to us by a pediatric endocrinologist for final confirmation of the diagnosis. 5 ml of peripheral blood was taken for DNA extraction from these patients. MS-MLPA method was undertaken to identify mutations (methylation and deletion) in H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes [16].

Inclusion criteria

The pediatric endocrinologists referred children under 12 years of age and clinically suspicious for the diagnosis of SRS or BWS. Written informed consent from parents and guardians of the children was documented.

Exclusion criteria

Children with multiple comorbidities

DNA extraction and purification

5 ml of peripheral blood was taken from each patient. DNA extraction from peripheral blood leukocytes was performed for all children using a DNA extraction kit (DENA Zist Asia, Cot.No:S-1031-1, made in Iran). Then, the quantity of extracted DNA was determined and confirmed with the nanodrop device, and the quality of the DNA samples was confirmed by using 1% agarose gel.

MS-MLPA

MS-MLPA probe mixture (ME030BWS/SRS) was purchased from the manufacturer (MRC-Holland, Amsterdam/NL).

This mix contains 42 probes, 26 of which are specific for the BWS/SRS region in 11p15. Ten of the 11p15 probes are methylation-specific since they contain a HhaI recognition site. Two probes for NSD1 and 13 reference probes (without HhaI recognition site) and one digestion control probe.

We used a total of 200 ng of genomic DNA extracted from peripheral blood by the salting-out procedure. 10 Mmol/l Tris-EDTA (pH 8.0) was added to DNA if dilution was necessary. After 16 h of hybridization at 60C, samples were equally split into two aliquots. The first aliquot underwent ligation only, whereas the second one underwent ligation followed by enzymatic digestion with HhaI [a restriction enzyme recognizing only unmethylated DNA] and PCR amplification using universal primers.

In the latter case, amplification products were obtained and detected by capillary electrophoresis only if the CpG included in the HhaI site was methylated. Ligation, enzymatic digestion, and PCR amplification were performed according to the manufacturer's instructions.

All samples were run in duplicate and at least two normal samples were analyzed in each MS-MLPA experiment, as controls. PCR products (1 ml) were mixed with 0.4 ml of internal size standard (500LIZ Genescan; Applied Biosystems)

and 9 ml of deionized formamide and injected into an ABI-3130 genetic analyzer (Applied Biosystems) equipped with a 36 cm, four capillaries array using POP7 as polymer. The samples were injected for 20 s at 1.5 kV and run for a total time of 1200 s at 15 kV. Genemarker v1.95 software is used for analysis, which performs all steps of data normalization and sloping data correction.

Ethical considerations

This study was approved by the ethics committee of Azad University of Tehran Medical Sciences (code: IR.IAU.PS.REC.1401.062). Throughout the study, researchers adhered to the Declaration of Helsinki. Written informed consent was obtained from the patients in this research. All information related to children was kept confidential. This study did not interfere with the process of providing medical services and care by physicians in the clinic. This research did not impose any additional costs on the patient.

RESULTS

The interpretation of MS-MLPA findings demonstrated that out of 10 children (4 boys and 6 girls) suspected of SRS or BWS (based on the diagnosis of a pediatric endocrinologist), only 3 were definitively diagnosed with these syndromes. None of the children were the result of a consanguine marriage. No deletion or addition changes were observed in any of the children, especially in the NSD1 gene.

The first patient

A newborn baby girl was diagnosed by an endocrinologist with suspicion of BWS. DNA was extracted from the blood sample and molecular analysis was done with MS MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments and therefore deemed normal (Figure 1A). Methylation analysis showed that hypomethylation of IC2 occurred in the 11p15 section [Figure 1B]. Therefore, this infant indeed had BWS.

	Probe Name	Bin Size	1734-030-2_B1
1	CDKN1C-exon1	347.1	1.536
2	CDKN1C_exon1	196.6	1.137
3	CDKN1C_exon3	445.1	1.121
4	Ctrl-2p25	460.6	0.881
5	Ctrl_02q24	309.5	0.995
6	Ctrl_07q31	154.5	0.823
7	Ctrl_10q21	382.6	1.023
8	Ctrl_10q25	127.3	0.904
9	Ctrl_12q13	208.4	1.144
10	Ctrl_14q24	148.2	1.057
11	Ctrl_16q22	337.3	0.941
12	Ctrl_17p12	258.8	1.107
13	Ctrl_18q21	425.5	0.969
14	Ctrl_22q11	202.4	0.978
15	Ctrl_3q29	292.4	1.009
16	Ctrl_9q21	177.2	0.839
17	H19-upstream	214.3	0.874
18	H19-upstream	451.7	0.940
19	H19DMR/IC1	184.1	0.934
20	H19DMR/IC1	300.1	1.008
21	H19DMR/IC1	133.7	0.957
22	H19DMR/IC1	239.9	0.968
23	H19_exon5	228.5	1.108
24	H19_upstream	190.5	1.032
25	H19_upstream	160.0	0.977
26	IGF2_EXON4	171.3	1.025
27	IGF2_exon7	283.8	1.024
28	KCNQ1_ex13	267.4	0.979
29	KCNQ1_ex15	373.4	1.144
30	KCNQ1_ex17	409.9	1.156
31	KCNQ1_exon2	434.7	1.029
32	KCNQ1_exon3	221.9	0.975
33	KCNQ1_exon7	328.1	1.292
34	KCNQ1_exon8	363.0	1.115
35	KCNQ1_exon9	399.4	0.921
36	KVDMR/IC2	139.4	1.078
37	KvDMR/IC2	274.4	1.278
38	KvDMR/IC2	165.2	1.003
39	KvDMR/IC2	391.7	1.191
40	NSD1-EXON23	317.8	1.020
41	NSD1-exon21	417.4	0.900
42	Y-fragment	106.0	0.000
43	digestion cont	354.8	1.135
44	x-fragment	101.5	0.990

A. Copy number analysis

	Probe Name	Bin Size	1734D-030-5_E
1	CDKN1C-exon1	347.1	0.878
2	CDKN1C_exon1	196.6	1.204
3	CDKN1C_exon3	445.1	0.975
4	Ctrl-2p25	460.6	1.029
5	Ctrl_02q24	309.5	1.078
6	Ctrl_07q31	154.5	0.824
7	Ctrl_10q21	382.6	0.929
8	Ctrl_10q25	127.3	0.923
9	Ctrl_12q13	208.4	1.244
10	Ctrl_14q24	148.2	1.027
11	Ctrl_16q22	337.3	1.033
12	Ctrl_17p12	258.8	1.293
13	Ctrl_18q21	425.5	0.968
14	Ctrl_22q11	202.4	1.039
15	Ctrl_3q29	292.4	1.072
16	Ctrl_9q21	177.2	0.902
17	H19-upstream	214.3	0.893
18	H19-upstream	451.7	0.932
19	H19DMR/IC1	184.1	0.956
20	H19DMR/IC1	300.1	1.100
21	H19DMR/IC1	133.7	0.951
22	H19DMR/IC1	239.9	1.011
23	H19_exon5	228.5	1.099
24	H19_upstream	190.5	1.067
25	H19_upstream	160.0	0.855
26	IGF2_EXON4	171.3	0.000
27	IGF2_exon7	283.8	1.084
28	KCNQ1_ex13	267.4	1.027
29	KCNQ1_ex15	373.4	1.243
30	KCNQ1_ex17	409.9	1.116
31	KCNQ1_exon2	434.7	1.014
32	KCNQ1_exon3	221.9	1.074
33	KCNQ1_exon7	328.1	1.446
34	KCNQ1_exon8	363.0	1.050
35	KCNQ1_exon9	399.4	0.948
36	KVDMR/IC2	139.4	0.315
37	KvDMR/IC2	274.4	0.404
38	KvDMR/IC2	165.2	0.290
39	KvDMR/IC2	391.7	0.301
40	NSD1-EXON23	317.8	1.028
41	NSD1-exon21	417.4	0.960
42	Y-fragment	106.0	0.000
43	digestion cont	354.8	-1
44	x-fragment	101.5	1.072

B. Methylation analysis

Figure 1: MLPA genetic test findings related to the first patient

The second patient

A 21-month-old girl, with the examination of an endocrinologist, was diagnosed with suspected SRS. DNA was extracted from the blood sample and molecular analysis was done with MS-MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments, hence a normal finding (Figure 2A). Methylation analysis showed that hypomethylation of IC1

occurred in the 11p15 segment. This hypomethylation occurred in probes 19 to 22 (Figure 2B). Therefore, this baby definitely had SRS.

	Probe Name	Bin Size	D07_4-769-2-03
1	CDKN1C-exon1	347.1	0.729
2	CDKN1C_exon1	196.1	0.944
3	CDKN1C_exon3	442.4	0.907
4	Ctrl-2p25	459.7	1.155
5	Ctrl_02q24	311.0	0.907
6	Ctrl_07q31	154.0	1.047
7	Ctrl_10q21	381.6	1.010
8	Ctrl_10q26	127.2	0.893
9	Ctrl_12q13	207.5	1.003
10	Ctrl_14q24	147.6	0.958
11	Ctrl_16q22	338.9	1.041
12	Ctrl_17p12	259.8	0.912
13	Ctrl_18q21	423.6	1.013
14	Ctrl_22q11	202.1	0.988
15	Ctrl_3q29	293.7	1.033
16	Ctrl_9q21	177.3	1.023
17	H19-upstream	215.0	1.002
18	H19-upstream	450.4	1.046
19	H19DMR/IC1	183.7	1.010
20	H19DMR/IC1	301.5	1.068
21	H19DMR/IC1	133.0	0.987
22	H19DMR/IC1	241.1	1.026
23	H19_exon5	229.5	1.045
24	H19_upstream	190.4	1.004
25	H19_upstream	160.2	0.985
26	IGF2_EXON4	170.2	0.975
27	IGF2_exon7	285.4	0.975
28	KCNQ1_ex13	269.4	1.130
29	KCNQ1_ex15	372.4	0.847
30	KCNQ1_ex17	407.8	0.982
31	KCNQ1_exon2	433.3	1.019
32	KCNQ1_exon3	223.0	1.007
33	KCNQ1_exon7	329.3	0.909
34	KCNQ1_exon8	361.0	1.017
35	KCNQ1_exon9	398.8	1.133
36	KVDMR/IC2	138.6	0.961
37	KvDMR/IC2	276.0	1.002
38	KvDMR/IC2	165.1	1.024
39	KvDMR/IC2	390.9	0.950
40	NSD1-EXON23	319.9	1.060
41	NSD1-exon21	417.1	0.993
42	Y-fragment	105.9	0.000
43	digestion cont	353.5	0.975
44	x-fragment	101.4	0.976

A. Copy number analysis

	Probe Name	Bin Size	F07_6-769B-03
1	CDKN1C-exon1	347.1	1.188
2	CDKN1C_exon1	196.1	0.945
3	CDKN1C_exon3	442.4	0.960
4	Ctrl-2p25	459.7	1.089
5	Ctrl_02q24	311.0	0.893
6	Ctrl_07q31	154.0	1.106
7	Ctrl_10q21	381.6	0.975
8	Ctrl_10q26	127.2	1.014
9	Ctrl_12q13	207.5	1.033
10	Ctrl_14q24	147.6	0.986
11	Ctrl_16q22	338.9	1.058
12	Ctrl_17p12	259.8	0.911
13	Ctrl_18q21	423.6	1.039
14	Ctrl_22q11	202.1	1.012
15	Ctrl_3q29	293.7	0.993
16	Ctrl_9q21	177.3	1.018
17	H19-upstream	215.0	1.106
18	H19-upstream	450.4	1.030
19	H19DMR/IC1	183.7	0.433
20	H19DMR/IC1	301.5	0.383
21	H19DMR/IC1	133.0	0.594
22	H19DMR/IC1	241.1	0.500
23	H19_exon5	229.5	1.024
24	H19_upstream	190.4	0.996
25	H19_upstream	160.2	1.077
26	IGF2_EXON4	170.2	0.000
27	IGF2_exon7	285.4	0.973
28	KCNQ1_ex13	269.4	1.097
29	KCNQ1_ex15	372.4	0.854
30	KCNQ1_ex17	407.8	1.087
31	KCNQ1_exon2	433.3	0.912
32	KCNQ1_exon3	223.0	0.957
33	KCNQ1_exon7	329.3	0.928
34	KCNQ1_exon8	361.0	0.957
35	KCNQ1_exon9	398.8	0.946
36	KVDMR/IC2	138.6	1.089
37	KvDMR/IC2	276.0	1.000
38	KvDMR/IC2	165.1	1.078
39	KvDMR/IC2	390.9	0.975
40	NSD1-EXON23	319.9	0.966
41	NSD1-exon21	417.1	0.964
42	Y-fragment	105.9	0.000
43	digestion cont	353.5	x1
44	x-fragment	101.4	1.095

B. Methylation analysis

Figure 2: MLPA genetic test findings related to the second patient

The third patient

A 2-year-old boy was suspected of having BWS according to the examination of an endocrinologist. DNA was extracted from the blood sample and molecular analysis was done with MS-MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments and therefore, it was deemed normal [Figure 3A]. Methylation analysis showed that hypermethylation of IC1 occurred in the 11p15 segment. This hypomethylation occurred in probes 36, 38, and 39. IC2 hypomethylation was detected in the 11p15 segment. This hypomethylation occurred in probes 19 to 22 (Figure 3B). Therefore, this boy definitely had BWS. Uniparental disomy occurred in this patient and he inherited both chromosomes from his father.

The fourth patient

A 9-year-old boy was suspected of SRS according to the examination of an endocrinologist. DNA was extracted from the blood sample and molecular analysis was done with MS-MLPA genetic test. Copy number analysis showed that no deletions or duplications occurred in the chromosomal segments and therefore, it was deemed normal (Figure 4A). Methylation analysis showed a normal pattern in the IC1 and IC2 (Figure 4B). Therefore, this child did not suffer from either SRS or BWS.

Fifth to tenth patients

The fifth to seventh patients were a 5-year-old girl, an 8-year-old girl, and a 12-year-old girl, respectively, suspected of SRS. The eighth patient was a 4-year-old boy, the ninth patient was a 6-year-old girl, and the tenth patient was a 7-year-old boy who was suspected of having BWS; according to MS-MLPA, it was found that none had either SRS or BWS.

DISCUSSION

The main reason for investigating mutations and methylation changes in H19, IGF2, CDKN1C, and KCNQ1 (located on 11p15) and NSD1 (located on 5q35.3) genes lies in their function. Alterations in these genes affect function and can give rise to the phenotypes associated with SRS or BWS. The gene CDKN1C (Cyclin Dependent Kinase Inhibitor 1C) is a tumor suppressor and a strong inhibitor and a negative regulator of cell

proliferation.

	Probe Name	Bin Size	756-030-3_C08
1	CDKN1C-exon1	347.2	0.933
2	CDKN1C_exon1	196.6	1.037
3	CDKN1C_exon3	444.4	1.000
4	Ctrl-3p26	460.2	1.021
5	Ctrl_82q24	309.9	0.887
6	Ctrl_87q31	154.6	1.043
7	Ctrl_18q21	382.3	0.968
8	Ctrl_18q26	127.3	1.031
9	Ctrl_12q13	208.5	0.900
10	Ctrl_14q24	148.2	1.024
11	Ctrl_16q22	337.9	0.927
12	Ctrl_17p12	259.4	0.999
13	Ctrl_18q21	424.9	1.032
14	Ctrl_22q11	202.7	0.995
15	Ctrl_3q29	292.7	0.940
16	Ctrl_9q21	177.5	0.957
17	H19-upstream	214.8	1.027
18	H19-upstream	451.4	1.061
19	H19DMR/IC1	184.2	1.004
20	H19DMR/IC1	350.4	1.128
21	H19DMR/IC1	133.6	1.064
22	H19DMR/IC1	240.6	1.097
23	H19_exon5	229.0	0.992
24	H19_upstream	190.8	0.926
25	H19_upstream	160.3	0.977
26	IGF2_EXON4	171.3	1.037
27	IGF2_exon7	284.3	1.007
28	KCNQ1_ex13	268.1	1.013
29	KCNQ1_ex15	373.2	1.027
30	KCNQ1_ex17	409.3	0.973
31	KCNQ1_exon2	434.2	1.029
32	KCNQ1_exon3	222.6	0.990
33	KCNQ1_exon7	328.5	1.001
34	KCNQ1_exon8	382.4	1.006
35	KCNQ1_exon9	399.1	0.991
36	KVDMR/IC2	139.3	0.962
37	KVDMR/IC2	274.9	1.021
38	KvDMR/IC2	165.4	1.049
39	KvDMR/IC2	391.4	0.955
40	NSD1-EXON23	318.4	0.958
41	NSD1-exon21	417.3	0.969
42	Y-fragment	106.0	1.019
43	digestion cont	354.4	1.015
44	x-fragment	101.6	0.959

A. Copy number analysis

	Probe Name	Bin Size	756D-030-7_G08
1	CDKN1C-exon1	347.3	0.959
2	CDKN1C_exon1	196.6	1.041
3	CDKN1C_exon3	444.4	1.025
4	Ctrl-3p26	460.3	1.068
5	Ctrl_82q24	309.9	0.976
6	Ctrl_87q31	154.6	1.017
7	Ctrl_18q21	382.3	1.116
8	Ctrl_18q26	127.3	1.032
9	Ctrl_12q13	208.4	0.946
10	Ctrl_14q24	148.3	0.977
11	Ctrl_16q22	337.9	1.007
12	Ctrl_17p12	259.4	0.991
13	Ctrl_18q21	424.9	1.020
14	Ctrl_22q11	202.6	1.012
15	Ctrl_3q29	292.7	0.931
16	Ctrl_9q21	177.4	1.021
17	H19-upstream	214.7	1.084
18	H19-upstream	451.4	1.013
19	H19DMR/IC1	184.1	1.614
20	H19DMR/IC1	350.4	1.824
21	H19DMR/IC1	133.5	1.715
22	H19DMR/IC1	240.6	1.792
23	H19_exon5	229.0	1.029
24	H19_upstream	190.7	0.963
25	H19_upstream	160.2	1.068
26	IGF2_EXON4	171.3	0.888
27	IGF2_exon7	284.3	1.028
28	KCNQ1_ex13	268.1	1.026
29	KCNQ1_ex15	373.2	0.975
30	KCNQ1_ex17	409.3	1.095
31	KCNQ1_exon2	434.2	1.081
32	KCNQ1_exon3	222.6	1.017
33	KCNQ1_exon7	328.5	0.931
34	KCNQ1_exon8	382.4	1.057
35	KCNQ1_exon9	399.1	0.999
36	KVDMR/IC2	139.2	0.487
37	KvDMR/IC2	274.9	0.725
38	KvDMR/IC2	165.4	0.393
39	KvDMR/IC2	391.4	0.478
40	NSD1-EXON23	318.4	1.044
41	NSD1-exon21	417.3	1.042
42	Y-fragment	106.0	1.096
43	digestion cont	354.4	-1
44	x-fragment	101.6	0.564

B. Methylation analysis

Figure 3: MLPA genetic test findings related to the third patient

Mutation in this gene can cause abnormal height and abnormal bladder structure and function. It also provides instructions for making proteins that help growth regulation [17]. NSD1 (Nuclear Receptor Binding SET Domain Protein 1) acts as a basic transcription factor and a regulator in transcription. Mutation in this gene can cause childhood acute myeloid leukemia and abnormal body height [18]. KCNQ1 [potassium voltage-gated channel subfamily Q member 1] gene belongs to a large family of genes responsible for making potassium channels [19]. The IGF2 gene (insulin-like growth factor 2) plays an essential role in the growth and development of the fetus before birth, and studies also showed that IGF2 causes the growth and division (multiplication) of cells in many different tissues [20]. The H19 gene is located near the IGF2 gene and provides instructions for making a non-coding RNA. Despite many genes, the H19 gene does not contain instructions for making a protein. The function of this non-coding RNA produced from this gene is unknown, but researchers believe it may act as a tumor suppressor. The H19 gene is highly active before birth in various tissues and appears to play an important role in early development [21].

The molecular defects of BWS and SRS are very heterogeneous. Many of these abnormalities involve epigenetic or copy number changes within the 11p15.5 gene cluster. Identification of these defects usually requires the use of multiple assays. Molecular diagnosis of SRS and BWS is currently possible only in one-third of patients [22] The aim of this study was to evaluate the methylation status and copy number of genes H19, IGF2, CDKN1C, KCNQ1, and NSD1 in blood samples of 10 Iranian children suspected of SRS or BWS.

Analysis of methylation, deletion, and addition in the mentioned genes in 10 children suspected of SRS or BWS, led to the diagnosis of SRS in one child and BWS in 2 children. In line with the present study, Gaston et al. showed that analysis of KvDMR1 methylation in leukocyte DNA alone identified BWS in 60% of all patients (64% of patients with severe form and 53% of patients with incomplete form of BWS). The analysis of the H19 gene methylation identified 13 other BWS patients with H19 hypermethylation, which confirms the effect of methylation in the occurrence of BWS in the two mentioned genes in line with our study [23].

Therefore, KCNQ1OT and H19 gene analysis is useful in routine

evaluation and leads to the diagnosis of BWS or SRS in children suspected of these syndromes [24].

	Probe Name	Bin Size	731-030-2_B08_
1	CDKN1C-exon1	347.2	1.373
2	CDKN1C_exon1	196.6	0.997
3	CDKN1C_exon3	444.4	1.085
4	Ctrl-2p25	460.2	0.934
5	Ctrl_02q24	309.9	0.900
6	Ctrl_07q31	154.6	0.778
7	Ctrl_10q21	382.3	0.992
8	Ctrl_10q25	127.3	0.847
9	Ctrl_12q13	208.5	1.031
10	Ctrl_14q24	148.2	0.961
11	Ctrl_16q22	337.9	0.950
12	Ctrl_17p12	259.4	1.028
13	Ctrl_18q21	424.9	1.100
14	Ctrl_22q11	202.7	0.998
15	Ctrl_3q29	292.7	1.054
16	Ctrl_9q21	177.5	0.820
17	H19-upstream	214.8	0.884
18	H19-upstream	451.4	1.123
19	H19DMR/IC1	184.2	0.846
20	H19DMR/IC1	300.4	1.011
21	H19DMR/IC1	133.6	0.850
22	H19DMR/IC1	240.6	0.902
23	H19_exon5	229.0	1.099
24	H19_upstream	190.8	0.989
25	H19_upstream	160.3	0.769
26	IGF2_EXON4	171.3	0.928
27	IGF2_exon7	284.3	1.009
28	KCNQ1_ex13	268.1	0.908
29	KCNQ1_ex15	373.2	1.150
30	KCNQ1_ex17	489.3	1.081
31	KCNQ1_exon2	434.2	1.023
32	KCNQ1_exon3	222.5	0.919
33	KCNQ1_exon7	328.5	1.190
34	KCNQ1_exon8	382.4	1.131
35	KCNQ1_exon9	399.1	0.978
36	KvDMR/IC2	139.3	0.851
37	KvDMR/IC2	274.9	1.109
38	KvDMR/IC2	165.4	0.917
39	KvDMR/IC2	391.4	1.116
40	NSD1-EXON23	318.4	1.033
41	NSD1-exon21	417.3	1.014
42	Y-fragment	186.0	0.943
43	digestion contr	354.4	0.972
44	x-fragment	101.6	0.525

	Probe Name	Bin Size	731D-030-6_F08
1	CDKN1C-exon1	347.3	1.050
2	CDKN1C_exon1	196.6	1.067
3	CDKN1C_exon3	444.4	0.928
4	Ctrl-2p25	460.3	0.851
5	Ctrl_02q24	309.9	1.033
6	Ctrl_07q31	154.6	0.805
7	Ctrl_10q21	382.3	0.961
8	Ctrl_10q25	127.3	0.934
9	Ctrl_12q13	208.4	1.157
10	Ctrl_14q24	148.3	1.104
11	Ctrl_16q22	337.9	0.976
12	Ctrl_17p12	259.4	1.069
13	Ctrl_18q21	424.9	0.984
14	Ctrl_22q11	202.6	1.036
15	Ctrl_3q29	292.7	0.939
16	Ctrl_9q21	177.4	0.943
17	H19-upstream	214.7	0.937
18	H19-upstream	451.4	0.871
19	H19DMR/IC1	184.1	0.984
20	H19DMR/IC1	300.4	0.960
21	H19DMR/IC1	133.5	0.958
22	H19DMR/IC1	240.6	0.935
23	H19_exon5	229.0	1.101
24	H19_upstream	190.7	1.049
25	H19_upstream	160.2	0.846
26	IGF2_EXON4	171.3	0.863
27	IGF2_exon7	284.3	0.992
28	KCNQ1_ex13	268.1	0.940
29	KCNQ1_ex15	373.2	1.065
30	KCNQ1_ex17	489.3	1.019
31	KCNQ1_exon2	434.2	0.931
32	KCNQ1_exon3	222.6	0.957
33	KCNQ1_exon7	328.5	1.134
34	KCNQ1_exon8	382.4	1.029
35	KCNQ1_exon9	399.1	0.837
36	KvDMR/IC2	139.2	0.981
37	KvDMR/IC2	274.9	0.976
38	KvDMR/IC2	165.4	0.888
39	KvDMR/IC2	391.4	0.867
40	NSD1-EXON23	318.4	0.996
41	NSD1-exon21	417.3	0.914
42	Y-fragment	186.8	1.099
43	digestion contr	354.4	-1
44	x-fragment	101.6	0.564

A. Copy number analysis B. Methylation analysis

Figure 4: MLPA genetic test findings related to the fourth patient

Gaston et al. showed complete or nearly complete demethylation (KvDMR1 MI >90%) in 70% of BWS patients, but about one-third showed partial demethylation, suggesting that this abnormality can occur with mosaicism. These findings were supported by the analysis of other pathological tissues that show different demethylation patterns from blood cells. For example, KvDMR1 is incompletely demethylated in the tongue tissue samples of patients while it is fully demethylated in blood cells, indicating that the analysis of blood samples was sufficient to diagnose BWS [23]. Gaston et al., observed that demethylation of the KCNQ1OT gene was never associated with abnormal methylation of the H19 gene [23] except in patients with UPD 11p15, indicating at least two imprinting centers at 11p15.5. These findings were consistent with a recent study by Engel et al [12].

In the present study, the analysis of H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes was normal in 7 out of 10 children. In line with these findings, Gaston et al reported that the analysis of KCNQ1OT and H19 genes was normal for 26 patients (26.8%). CDKN1C gene sequencing was performed in 10 of these patients [5 sporadic forms and 5 patients from families]. No abnormalities were found in sporadic cases. CDKN1C mutations were found in the families of 2 patients, and both patients had exomphalos [23]. Other unknown 11p15 abnormalities may account for other cases. Various mechanisms may cause over-growth syndrome by inducing the activity of growth factors such as IGF2. Several transgenic mouse models have provided evidence for this by showing increased levels of bioactive IGF2 induced by inhibition of the IGF type 2 receptor [IGF2R] or glypican 3, two agents known to bind and downregulate IGF2 expression, making it less accessible to the IGF1R [25]. Further studies on 11p15 genes and IGF2-related regulators in overgrowth syndromes should be conducted to determine the association between genotype and phenotype and to elucidate functional mechanisms.

In a study by Jurkiewicz et al., the MS-MLPA screening test identified the triplication of the IGF2/H19 domain for all probes of these genes in the ME030 kit, although this result was contrary to the results of our study because we did not find any duplication or deletion in individuals. They also

showed that the H19 (ICR1) region had increased methylation and also showed normal methylation patterns for the IGF2R KCNQ1OT1 genes, which are in line with the results of our study [26].

In the present study, MS-MLPA was used for the molecular diagnosis of BWS and SRS. The results showed that MS-MLPA is a sensitive and reliable tool for further identification of molecular defects of BWS and SRS. These findings are in line with the study of Priolo et al., which was conducted in Italy [27]. Most epigenetic defects of BWS and SRS occur sporadically and exist in the mosaic form in individuals [28,29]. Therefore, it is important to use a sensitive diagnostic method to identify all mosaic cases. MS-MLPA method for the detection of 11p15.5 defects is very accurate and sensitive [27].

The great advantage of MS-MLPA is that this method simultaneously determines the copy number and methylation level of the loci. This is clearly shown in the third patient who had both hypomethylation and hypermethylation. While the ligation-only reaction revealed an increase in copy number, the ligation-digestion reaction provided information about the parental origin of the amplified region. Another study also confirmed that MS-MLPA can be considered a robust assay for the analysis of chromosome 11p5.5 defects [30]. In conclusion, our experience with these 10 suspected children showed that MS-MLPA is an easy, low-cost, and reliable method for the molecular diagnosis of BWS/SRS cases with im-printed and repetitive defects on chromosome 11p15. However, due to the small sample size as one limitation in this study, these results need to be confirmed in a larger series of patients. Also, for 7 children who were not confirmed to be suffering from BWS and RSS syndromes in this study, we suggest performing a WES test for them because the doctor may have made a mistake in diagnosing their symptoms and they may be suffering from another disease.

CONCLUSIONS

This was the first study to document these two syndromes in Iran. The present findings showed that methylation changes in the control regions of ICR1 and ICR2, which are related to H19, IGF2, CDKN1C, KCNQ1, and NSD1 genes, lead to the development of SRS or BWS. A diagnosis of the two syndromes is not accurate solely based solely on clinical symptoms and without the use of cellular and molecular genetic testing. MS-MLPA is suggested as a low-cost, reliable, and rapid method for the molecular diagnosis of BWS/SRS with imprinting and duplication defects on chromosome 11P15.

Conflicts of interest and sources of funding

The authors declare no conflict of interest.

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Authors' contribution

MAE, ME, OM, NS, and SS were responsible for the study concept and design. MAE, ME, OM, NS, and SS led data collection. MAE, ME, OM, NS, and SS were responsible for the analysis and interpretation of data. MAE, ME, OM, NS, and SS wrote the draft.

Ethics approval and consent to participate

This study was approved by the ethics committee of Azaz University of Medical Sciences [code: IR.IAU.PS.REC.1401.062]. Throughout the study, researchers adhered to the Declaration of Helsinki. Written informed consent was obtained from the children's parents in this research.

Patient consent for publication

Informed consent was obtained from all subjects involved in the study.

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