Diagnosis of the Genomic Imprinting Diseases by the Usage of Conventional and Molecular Analyses

Konvansiyonel Genetik ve Moleküler Genetik Analizler ile Genomik Damgalama Hastalıklarının Tanısı

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ABSTRACT

Objective: Prader-Willi (PWS) and Angelman syndromes (AS) are genomic imprinting diseases with intellectual disability. In approximately 70 % of the cases, there is a cytogenetic deletion involving the chromosome 15q11-q13 inherited from patient's father (in PWS) and from patient's mother (in AS). In approximately 20-25% and 3-7% of the cases, there is an uniparental disomy (UPD) of chromosome 15 in PWS and AS patients, respectively. The mutation ratio in AS patients is approximately 10 %, while the ratio is two percent in PWS patients.
Methods: In the first step cytogenetic analyses were performed by using high resolution banding (HRB) for 20 patients who were selected by diagnostic criteria (11 pre-diagnosed with AS and 9 pre-diagnosed with PWS). Then, via Fluorescent *in situ* Hybridization (FISH) technique, *SNRPN* gene (small nuclear ribonucleoprotein polypeptide N gene) locus for PWS, D15S10 locus for AS were searched. Finally the uniparental disomy of chromosome 15 along with the mutation/deletion of imprinting center were examined.
Results: HRB and FISH analyses revealed no deletion except in one AS patient whose D15S10 region was found deleted via FISH technique. Mutation/deletion of imprinting center analyses in all of them were evaluated as normal.
Conclusion: As a result, by this project patients with PWS and AS, who were selected by the diagnostic criteria for each syndrome, were evaluated via conventional genetic and molecular genetics methods and they were offered with the efficient genetic counseling.

Key Words: Angelman syndrome, FISH, genomic imprinting, intellectual disability, Prader-Willi syndrome, uniparental disomy

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ÖZET

Amaç: Prader-Willi (PWS) and Angelman sendromu (AS) entelektüel yetersizlik ile seyreden genomik damgalama hastalıklarındandır. Olguların yaklaşık %70'inde PWS'nda babadan kalıtılan, AS'nda anneden kalıtılan 15q11-q13 bölgesinde delesyon mevcuttur. PWS'nda %20-25, Angelman sendromunda ise %3-7 kromozom 15'in uniparental dizomisi (UPD) vardır. Mutasyon oranları ise AS'nda %10 iken Prader-willi sendromunda %2'dir.
Yöntem: İlk aşamada tanımlama kriterlerine göre seçilmiş 11 AS, 9 PWS ön tanılı 20 olgunun yüksek rezolüsyonlu bantlama (HRB) tekniği kullanılarak sitogenetik analizleri yapılmıştır. Sonra PWS için *SNRPN* gen (small nuclear ribonucleoprotein polypeptide N) lokusü, AS için D15S10 lokusü Floresan *in situ* Hibridizasyon (FISH) tekniği ile araştırılmıştır. En son aşamada ise 15. kromozomun UPD'si ve imprinting merkezinin mutasyonları araştırılmıştır.
Bulgular: FISH ile delesyonu tespit edilen bir AS hastası hariç, tüm hastaların HRB ve FISH analizlerinde herhangi bir delesyon saptanmamıştır. Tüm hastaların imprinting merkezinin mutasyon/delesyon çalışmaları da nomal bulunmuştur.
Sonuç: Sonuç olarak tanımlama kriterlerine göre PWS ve AS düşünülen hastalar konvansiyonel genetik ve moleküler genetik yöntemler ile araştırılmış ve elde edilen sonuçlar ışığında genetik danışmanlık almışlardır.

Anahtar Sözcükler: Angelman sendromu,entelektüel yetersizlik, FISH, genomik damgalama, Prader-Willi sendromu, uniparental dizomi

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INTRODUCTION

Angelman Syndrome (AS) is a rare neurogenetic disease which was named in 1965 by English doctor Harry Angelman, (1-4). Deletion of some part of DNA sequences is the most common reason of the disease. Larger deletions like 5 megabases can be detected via conventional cytogenetic techniques while for the smaller ones, called as microdeletions, Fluorescent *in situ* Hybridization (FISH) analysis or more advanced molecular analyses are required. AS is associated with the clinical features of intellectual disability, minimal or absent speech, epileptic seizures, inappropriate laughter and characteristic facial appearance. Due to inappropriate laughter of patients, disease was firstly called as 'happy puppet syndrome' (2). Except the deletion in maternal copy of chromosome 15q11-q13 in most cases, paternal uniparental disomy, imprinting center mutation and familial mutation in ubiquitin protein ligase E3A (*UBE3A*) gene can also cause this disease (2). In addition to these, telomeric loss of chromosome 22 can be rarely related with AS (3).

Prader-Willi Syndrome (PWS) is also common microdeletion associated syndrome which is characterized by consecutive paternal gene deletion or loss of gene expression in the same chromosomal region with AS (15q11-q13) (5). PWS was defined firstly in 1956 with symptoms: infantile hypotonia, developmental delay, intellectual disability, behaviour disorder, characteristic facial appearance, obesity, hypogonadism and short stature (5). Underlying reasons behind PWS can also be maternal uniparental disomy and imprinting center mutations (5).

After comprehensive medical examination of pre-diagnosed PWS and AS patients, the first step of this study was the detailed analysis of patients' clinical findings due to National Center of Biotechnology Information (NCBI) - Online Mendelian Inheritance in Man (OMIM) database (6). Then, in all patients, high-quality and high resolution chromosomes had been obtained [550 and upper level, High Resolution Banding (HRB)] and then FISH technique with specific PWS and AS probes had been performed by using tissue culture preparates. For patients whose FISH results were negative, genotyping via silver staining method had been used in order to evaluate their genotype in terms of uniparental disomy. If silver staining method also did not give any significant result, as a last experimental procedure, methylation study using bisulfite protocol had been applied to those patient samples.

The aim of the study is easy diagnosis of patients with PWS and AS by using conventional genetics and molecular genetics techniques together with current clinical diagnosis criteria. Therefore it will be possible to give more precise diagnosis and genetic counseling to patients.

METHODS

The study was approved by Ethical Committee of Gazi University at 19th January 2009 (issue number 33). Diagnostic criteria for PWS and AS was used from National Center of Biotechnology Information (NCBI)- Online Mendelian Inheritance in Man (OMIM) database (6).

From each patient (11 pre-diagnosed with AS and 9 pre-diagnosed with PWS) who carry defined diagnostic criteria, 10 ml blood sample was taken: 5ml for DNA isolation (in tubes with EDTA) and 5ml for acquiring chromosomes (in tubes with heparin). High Resolution Banding tissue culture has been preferred for conventional chromosome analysis because of targeted high resolution banding pattern (550 bands or more). High-quality 20 metaphase plaques from each patient were analyzed.

The slides which were used in FISH technique had been obtained via tissue culture method. Interphase and metaphase plaques, whose details were explained below, were analyzed under microscope with fluorescence attachment.

The 10 polymorphic markers which are showed at Table 1, to 15q11-13 region were chosen as genotyping markers from 'Ensemble Database' considering heterogeneity incidences (www.ensemble.com) (7).

Table 1. The polymorphic markers addressing the 15q11-13 region

Marker	Primer	Primer Sequence
D15S1007	Forward	GGGGAACCTACACTTCCG
The length of amplification product: 165 bp*	Reverse	CCAGGAATCTCAAATGGCTT
D15S205	Forward	CTTAATGGTTTGGCAGGATA
The length of amplification product: 128 bp	Reverse	AGCTTAAAANCAAAATCTCCC
D15S1019	Forward	TTCTGGACCACGCATACTA
The length of amplification product: 202 bp	Reverse	ATCAGGCCATCTTTCATTGT
D15S130	Forward	TCTCAGGTTTGCTTTGTCAG
The length of amplification product: 218 bp	Reverse	GGCCTCCATGAACTAAACTG
D15S988	Forward	ATGAAAACATCTGGTTCAAA
The length of amplification product: 258 bp	Reverse	TCGCTTGCTGTAAGACCT
D15S979	Forward	TGCTGCCCAACATCCT
The length of amplification product: 135 bp	Reverse	CAGTGCTACATCCACGGAA
D15S128	Forward	GCTGTGTGTAAGTGTGTTTTATATC
The length of amplification product: 193 bp	Reverse	GCAAGCCAGTGGAGAG
D15S131	Forward	GAAAGGCACCTCATCTCG
The length of amplification product: 238 bp	Reverse	TTAAAAACTCTGGAGCAGCG
D15S986	Forward	GCAGGAATATGTCCAGGG
The length of amplification product: 182-196 bp	Reverse	CATGGCTGGTCTTTAGGTG
D15S822	Forward	TGAGTTTTTCCTATTGAGAGTCC
The length of amplification product: 258-306 bp	Reverse	GAAAGTCAACAGTCTCAGAGACC

*bp:base pair

After obtaining genomic DNA of parents and patients, interested regions were amplified using PCR method (GeneAmp PCR System 9700). 10 primer pairs were used for each case. Amplification products were run in %6-7 denaturated polyacrylamide gel electophoresis with 1600-2000 Volt and so alleles were distinguished according to their polymorphic character (Life technologies S2001).Then gels were stained via silver staining technique and the genotypes were recorded by using direct photographing technique.

For methylation study, bisulfite method and then PCR were used to analyze methylation status of patients' genome, which occurs with addition of methyl groups to CpG islands of DNA.

RESULTS

High resolution cytogenetic analysis of 20 patients revealed normal constitutional karyotypes (Table 2). Examination of two different probes which are specific to 15q11.2 locus, D15S10 probe for AS and small nuclear ribonucleoprotein polypeptide N (*SNRPN* gene probe) probe for PWS, via FISH technique revealed only one deletion in a patient who is the fifteen-month pre-diagnosed with AS (case 5) having significant developmental retardation, microcephalia, behavioral problems and especially lighter hair color according to other family members (Table 2, Figure 1).

The uniparental disomy and methylation procedures were determined as normal in all patients (Table 2).

Table 2: The results of cytogenetic and molecular analysis of the PWS and AS patients

Cases	Chromosome Analysis	FISH	Metilation Specific PCR	Uniparental Disomy
Case 1*	46,XX	ish 15q11.2(D15S10x2), ish subtel (41x2)	Normal	None
Case 2*	46,XX	ish 15q11.2(D15S10x2), ish subtel (41x2)	Normal	None
Case 3	46,XX	ish 15q11.2(D15S10x2)	Normal	None
Case 4	46,XY	ish 15q11.2(D15S10Ax2)	Normal	None
Case 5**	46,XY	ish del(15)(q11.2q11.2)(D15S10-)	-	-
Case 6	46,XY	ish 15q11.2(D15S10x2)	Normal	None
Case 7	46,XY	ish 15q11.2(D15S10x2)	Normal	None
Case 8	46,XY	ish 15q11.2(D15S10x2)	Normal	None
Case 9	46,XY	ish 15q11.2(D15S10x2)	Normal	None
Case 10	46,XX	ish 15q11.2(D15S10x2)	Normal	None
Case 11*	46,XX	ish 15q11.2(D15S10x2), ish subtel (41x2)	Normal	None
Case 12	46,XX	ish 15q11.2(SNRPNx2)	Normal	None
Case 13	46,XX	ish 15q11.2(SNRPNx2)	Normal	None
Case 14	46,XX	ish 15q11.2(SNRPNx2)	Normal	None
Case 15	46,XY	ish 15q11.2(SNRPNx2)	Normal	None
Case 16	46,XX	ish 15q11.2(SNRPNx2)	Normal	None
Case 17	46,XY	ish 15q11.2(SNRPNx2)	Normal	None
Case 18	46,XY	ish 15q11.2(SNRPNx2)	Normal	None
Case 19*	46,XY	ish 15q11.2(SNRPNx2), ish subtel (41x2)	Normal	None
Case 20	46,XY	ish 15q11.2(SNRPNx2)	Normal	None

*Because of the dysmorphic signs addition to FISH study of AS and PWS critical regions, all the short and long arm of telemoric regions were also examined via FISH technique and all of them found normal.
**Deletion of D15S10 region via FISH technique confirmed the diagnosis of AS in case 5.

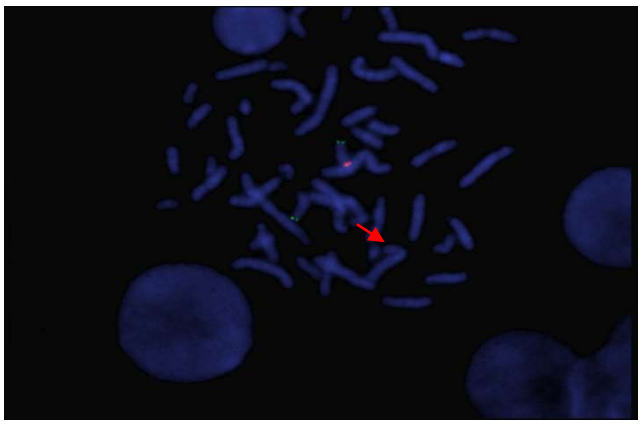

Figure 1. FISH painting of case 5. Green signals indicating the long arm telemoric region of chromosome 15, red signal is indicating the critical region (D15S10) of Angelman syndrome. Mark that one of the chromosome 15 had no red signal (D15S10 -).

DISCUSSION

AS and PWS are rare neurogenetic diseases related with several genetic mechanism in etiology because of that the detailed clinical examinations with genetic techniques are the key points for the diagnosis (1-3, 5). Microdeletions are the vast majority of the etiological factors with the ratio of approximately ¾. Concordant with this, one of the patient pre-diagnosed with AS in this study (Case 5) had syndrome specific deletion confirmed by FISH (Table 2, Figure 1). The case was a thirteen month old boy who had been followed due to repeating fever and developmental retardation. As we met in the literature, there are four AS cases, who have similar fever attacks without any reason (8). Microcephaly, developmental retardation, abnormal gestures are classical features of AS but thermoregulation problems are generally related with PWS probably due to hypothalamic dysfunctioning (9). Obesity, increased appetite, eating obsession were added to diagnosing criteria of AS in 2005 and this fact implies that there can be hypothalamic dysfunctioning in AS similar to PWS (2). Increased number of AS patients with thermoregulation dysfunctioning will eventually help revealing new genes regulating hypothalamus functioning and better understanding of thermoregulation physiopathology. The presence of hypothalamic dysfunction in both disease groups (AS and PWS) brings to mind that; most probably the mutation of genes that express biallelically on shared region (which is exposed to deletion) can have more influence on this dysfunction rather than the deletion of imprinted genes.

Microsatellite analysis was used to detect the uniparental disomy with the determination of heterodisomy and isodisomy. In this study, the results of patients, whose microsatellite analysis had been performed, were all found normal. The other reason in etiology of disease is the imprinting center mutation. *UBE3A* and *SNRPN* are mostly known imprinted genes, related with AS and PWS, respectively. No imprinting center mutation was found but the *UBE3A* gene deletion was confirmed in one of the patient (case 5) via FISH technique by the usage of D15S10 probe (Table 2, Figure 1).

As a genetically heterogeneous disease group, in AS and PWS, it is suggested that following to chromosome analysis firstly methylation analysis should be performed (10). By the usage of advanced molecular techniques regarding the methylation study, deletion, UPD, and imprinting errors which take role in the etiopathogenesis of the disease can be determined. In this study, high experience on FISH analyses and the low cost of the tests, following the chromosome study, FISH technique was performed secondly. The patients with no deletion were evaluated by using microsatellite analysis and methylation specific PCR. It was a chance to observe that all methods that were specific for the disease could be applied in case of the requirement and feasibilty of the laboratory, and also it was observed that the results were supported by each other. By considering the cost of the tests in further planned studies, observation of steps that are suggested in literature is going to be tried to prioritize.

CONCLUSION

As a result before being oriented to the other diseases whose clinical findings are concordant with AS and PWS, these well-known two microdeletion syndromes should be studied via with efficient, reliable, low-cost techniques. If in the result of tests that are performed; genetic modification that is concordant with AS and PWS is identified, genetic counseling in an appropriate way for each patient should be offered. If AS and PWS are eliminated with applied cytogenetic and molecular tests; patients should be evaluated from the aspect of other diseases/syndromes which are mimic these syndromes.

As a consequence it is indicated that only clinical diagnosis criteria are not sufficient to determine the diagnosis of AS and PWS; and before offering a genetic counseling, certainly cytogenetic and molecular analyses should be performed.

Conflict of interest
No conflict of interest was declared by the authors.

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