INTRODUCTION

Wolf-Hirschhorn syndrome (WHS) is a contiguous gene syndrome, caused by partial or complete deletion of the short arm of chromosome 4, particularly of 4p16.3 band. This condition was described for the first time in 1965 by Wolf et al. and Hirschhorn et al. and is the first recognized subtelomeric deletion syndrome. The frequency is estimated at 1/20000-1/50000 births, with a female preponderence of 2:1.

Patients with WHS have a characteristic craniofacial phenotype resembling a Greek warrior helmet, with microcephaly, prominent glabella, hypertelorism, broad nasal bridge and high arched eyebrow. Typically they present postnatal growth retardation, developmental delay, mental retardation, muscular hypotonia, seizures, congenital heart defects and short stature. Identification of affected individuals strongly depends on the ability of the clinician to recognize the condition due to the high variability of the phenotype. Also, later in life the facial phenotype becomes less characteristic, making differentiation from other chromosomal anomalies more difficult.

In this paper we present four cases (three females and a male) with WHS with different clinical phenotypes, diagnosed using cytogenetic and molecular investigation performed in our genetics laboratory.

SUBJECTS AND METHODS

Patients

All children were referred to our Clinical Genetics Department, because of dysmorphic features with or without other anomalies, coming from non-consanguineous families. (Table 1) Every individual presented a classic WHS phenotype which required further investigation for confirmation of the clinical suspicion. Three cases were diagnosed by conventional cytogenetic technique - regular G-banding, while the...
remaining one required FISH (Fluorescence in situ hybridization) investigation for the WHS diagnosis confirmation. (Table 2).

Table 2. Cytogenetic results for cases 1-4.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Karyotype</th>
<th>FISH result</th>
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<tbody>
<tr>
<td>Case 1</td>
<td>46,XX,del(4)(p15.2→pter)</td>
<td>Not performed</td>
</tr>
<tr>
<td>Case 2</td>
<td>46,XX,ish del(4)(p16.3p16.3)(WHSCR-)</td>
<td></td>
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<tr>
<td>Case 3</td>
<td>46,XY,del(4)(p15.2→pter)</td>
<td>Not performed</td>
</tr>
<tr>
<td>Case 4</td>
<td>46,XX,del(4)(p15.2→pter)</td>
<td>Not performed</td>
</tr>
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Pictures of the patients’ phenotypes could not be presented in this work because the parents refused to give a written consent for the publishing of medical photographs.

CASE 1

The first patient, a female, was born by cesarean delivery after a gestation of 42 weeks with APGAR score 8. This was the first pregnancy of the family. The evaluation of the subject was taken at 5 months of age.

The clinical examination revealed the weight to be 5000g (<P5) and length 60cm (P10). The child presented striking abnormal cranio-facial features (microcephaly, hypertelorism, irian coloboma, downturned corners of the mouth and posterior rotated ears) associated with hypotonia, suggestive for WHS phenotype. (Table 1)

The cytogenetic investigation confirmed the initial clinical diagnosis. The karyotype result was 46,XX,del(4)(p15.2→pter). (Fig. 1) The exclusion of any potential chromosomal rearrangements for the parents was not possible because of the family’s lack of compliance.

CASE 2

A five months old female infant presenting dysmorphic features combined with important hypotonia and severe postnatal growth retardation with feeding difficulties, was referred for clinical assessment. She was born at term by cesarean delivery with APGAR score 9, birth weight 2400 g (<P5) and body length 48 cm (P25). A very particular aspect concerning the family history was the fact that the mother lost 10 pregnancies in the first trimester; also, the first child of the couple was born having a neural tube anomaly and died in early infancy.

The clinical examination of the patient revealed abnormal craniofacial features with hypertelorism and microcephaly (OFC = 37cm), left single palmar crease, hypotonia and failure to thrive. (Table 1) The weight at the moment of evaluation was 4000 g (<P5) and length 58 cm (<P5). Supplementary investigations showed the presence of atrial septal defect and renourinary
abnormalities. The clinical phenotype very suggestive for WHS and the complex family history compelled us to perform a complete cytogenetic evaluation. First step consisted of conventional chromosome analysis for the whole family (patient and her parents), with normal results. (Fig. 2) Because of the high score for clinical diagnostic criteria of WHS, the second step was fluorescence in-situ hybridization (FISH) testing for the patient. The analysis revealed a submicroscopical deletion in the WHS critical region. (Figs. 3,4) To rule out any possible criptical chromosomal rearrangement, we performed FISH testing for the parents as well, with normal results for both of them.

Figure 2. Kariogram of case 2, showing apparent normal result.

Figure 3. Partial ideogram of chromosome 4, with localization of the marked probe for WHSCR.

**CASE 3**

The third case, a prematurely born male child with young parents (mother of 32 years, father of 33 years), presented with multiple congenital anomalies. He was born by vaginal delivery with APGAR score 6 and manifested prenatal growth retardation. Birth weight was 2400 g (<P5) and length was 47 cm (P10). At the age of 5 weeks, the patient’s growth continued to be delayed, with little weight gain (2800 g, <P5) and length of 48 cm (<P5).

The clinical examination revealed distinctive facies with microcephaly (OFC = 32.5 cm, <P5), asymmetric facial features, frontal bossing, hypertelorism, broad nasal bridge, asymmetric ears, microstomia and micrognatia. Other particular features consisted of severe hypotonia, pectus excavatum, talus valgus, micropenis and a large abdominal scar, secondary to a surgical intervention for bladder extrophy. (Table 1) Echocardiographic investigation showed an atrial septal defect. The karyotype result indicated a visible deletion of the short arm of chromosome 4 (46,XY,del(4)(p15.2→pter)). (Fig. 5) Cytogenetic analysis for the parents could not be performed.

Figure 4. FISH result on metaphase spreads for case 2 shows the presence of only one red signal (WHSCR), demonstrating the deletion of WHSCR on chromosome 4 and confirming the initial clinical diagnosis.

Figure 5. Partial karyogram of patient 3, showing a large deletion on chromosome 4.

**CASE 4**

This individual, a female of 2 weeks of age, the first child of the family, was referred for examination because of the association of dysmorphic features and other various internal anomalies. She was born prematurely (35 weeks of gestational age) by vaginal
delivery, with APGAR score 8, birth weight 2420 g and length 46 cm. The facial features were very suggestive for WHS, with the typical ‘Greek warrior helmet appearance’. The complete echographical examination revealed hypoplasia of corpus callosum, atrial septal defect and renal hypoplasia. (Table 1). The karyotype result was 46,XX,del(4)(p15.2→pter). (Fig. 5) Parental investigation was refused.

**Figure 6.** Partial karyogram of patient 4, showing a large deletion on chromosome 4.

**CYTOGENETIC INVESTIGATIONS**

Peripheral blood specimens were collected and cytogenetic analysis was performed on GTG-banded metaphase spreads prepared from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes, grown in commercially available lymphocyte medium (PB-MAX™, Invitrogen™, UK). The harvesting of cultures was done after 72 h of incubation and for each case 30 GTG-banded metaphases were karyotyped using IKAROS (MetaSystems) and LUCIA Cytogenetics System; chromosomes were analyzed according to guidelines provided by the International System for Human Cytogenetic Nomenclature (ISCN 2009). Cytogenetic analyses were performed for all cases, but only three karyotype results were abnormal.

**FLUORESCENCE IN SITU HYBRIDIZATION (FISH)**

FISH analysis was performed on metaphases obtained from peripheral blood cultures, using commercially available WHSCR-specific probes (Aquarius® Wolf-Hirschhorn Syndrome Critical Region Probe with Subtelomere Specific Probe (4qter), Cytocell®, UK), following the producer’s protocol. The images obtained were processed using ISIS (MetaSystems) software. FISH investigation was performed for the only case with the normal karyotype result and for the child’s parents.

**RESULTS**

All patients presented the minimal phenotype for WHS. Three cases (cases 2, 3 and 4) also had other major malformations such as congenital heart defect and genitourinary tract abnormalities. (Table 1) The karyotype revealed that cases 1, 3 and 4 had a similar visible deletion on the short arm of chromosome 4, confirming the initial clinical diagnosis. (Table 2) For case 2, considering the high clinical score for WHS and the normal karyotype result, FISH investigation was necessary. The result showed the presence of a submicroscopical deletion on chromosome 4, encompassing the WHS critical region (WHSCR). (Table 2) Parental investigation was possible only for the parents of patient 2, with normal results.

Seizures characterize the full WHS phenotype and are a major cause of death. None of the cases described here presented clinical seizures. This might be due to the young age of the patients at the time of the clinical evaluation. Studies show that seizures in WHS start mostly in the first year of life, with the age of onset varying between 3-23 months and a peak between 6-12 months.

We observed no significant genotype-phenotype correlations. For patient 1, although the deletion was visible on conventional karyotype and resembled the ones found for cases 3 and 4, the girl manifested the minimal WHS phenotype, with no other major malformations. (Table 1) Case 2 presented similar phenotype to that observed for cases 3 and 4 that had larger, visible deletions.

**DISCUSSION**

According to the size of the deletion and the phenotypical variability, Zollino et al.(2008) proposed a new classification of the WHS phenotypes. The authors suggested three phenotypical categories: a mild form associated with a deletion of 3.5 Mb or smaller, presenting mild mental retardation and some of the major malformations, a more common form caused by 5-18 Mb deletions characterized by severe mental retardation and major malformations, and a severe phenotype resulting from a deletion exceeding 22-25 Mb, with less resemblance to the typical WHS phenotype and characterized by severe mental retardation, seizures, prenatal growth delay, midline defects, hypotonia and psychotic behavior.

Several methods are used to confirm the clinical diagnosis. Conventional cytogenetic analysis (GTG-banding) can detect large deletions on the short arm of chromosome 4 but its relatively low resolution does not permit the identification of submicroscopic deletions. Fluorescence in situ hybridization is a molecular technique able to detect the smaller deletions not seen on conventional karyotype and can be applied
for the confirmation of about 95% of deletions. Most individuals (50-60%) carry a de novo deletion with no other discernable cytogenetic anomaly. About 25-30% of patients have a de novo FISH-detected microdeletion and only 15% of cases are found to have an unbalanced chromosomal rearrangement, either occurring de novo or inherited from a familial translocation.9

Studies revealed the presence of two critical regions for this condition: the WHS critical region 1 (WHSCR1), containing the entire WHS candidate gene 2 (WHSC2) and part of WHS candidate gene 1 (WHSC1), and WHS critical region 2 (WHSCR2), containing the LETM1 gene and part of WHSC1 gene.10,11

It has been proposed that deletion of WHSC1, a gene coding for a chromatin remodeling enzyme, is responsible for the characteristic facial features observed in WHS patients.12,13 Engbers et al (2009) reported a patient with a terminal 4p16.3 deletion that did not present the typical Greek helmet facial appearance but had other minor facial features indicating WHS, suggesting that other genes might contribute to the observed facial phenotype of this syndrome (such as short philtrum and downturned corners of the mouth).14

Hemizygosity of LETM1, coding for a ubiquitous Ca\textsuperscript{2+} binding protein involved in Ca\textsuperscript{2+} homeostasis, has been suggested to be the cause of the seizures characteristic of the full phenotype.11 Epileptic seizures occur in 50-100% of children with WHS, with a variable age of onset.15 The LETM1-encoded protein's function is not yet well understood; it is primarily active in mitochondria and studies suggest that loss of the LETM1 gene alters the structure of the mitochondria, although it is not yet clear how these alterations relate to the seizures observed in WHS patients.

Individuals with WHS often present mild to severe clinical manifestations. Several mechanisms have been proposed to explain the clinical phenotype variability observed between cases. Firstly, it has been proposed that the size of the deletion might be responsible for the differences observed. Early studies revealed no relationship between the size of the deleted material and phenotype. This could be due to the usage of low-resolution techniques and possibly missing some patients with smaller deletions. Recently, newly developed techniques allowed more precise detection and characterization of the size and regions deleted for patients with submicroscopic deletions and offered new evidence supporting the existence of a partial genotype-phenotype correlation in this syndrome. Wieczorek et al. (2000) found a link between standard deviations of birth weight, birth length, postnatal head circumference and mental retardation with the size of the deletion.16 In 2001, Shannon et al. observed a statistically significant relationship between the amount of the missing material on 4p and the overall risk of death in de novo deletions.17

Considering genotype-phenotype correlations, Chen (2006) stressed the importance of the characteristic facial appearance for the individuals with a large 4p16.3 deletion, in addition to severe mental and growth retardation, major malformations and seizures. For the patients with a 4p16.3 microdeletion the same author described a milder phenotype with lack of congenital malformations.18

From a clinical point of view, two of our cases (case 1 and 2) presented particular phenotype. This aspect was not consistent with the description made by Chen (2006); the patient with the larger deletion (case 1) manifested a slightly milder phenotype than the individual with a microdeletion (case 2). (Table 1) This dissimilarity should be investigated using molecular techniques of higher resolution that FISH to determine the precise extent of the deletion.

Although the amount of missing material on 4p could be responsible for the majority of the variability observed, many patients do not present a strict correlation between the size of the deletion on 4p and the severity of the clinical manifestations; this could be due to polymorphisms in the remaining alleles of the deleted genes, mutations in modifier genes located outside the deleted region or unbalanced translocations resulting in 4p deletion and a partial trisomy.19,20 Also, position effects, telomere silencing and environmental influences or post-zygotic mutational events could be responsible for the variable aspects of this condition.20 A minimal WHS phenotype has been described, consisting of typical facial appearance, congenital hypotonia, mental retardation and growth delay.9,13 Other variable features include clinical seizures, feeding difficulties, antibody deficiency and major congenital anomalies (heart lesions, skeletal anomalies, deafness, genitourinary tract defects, cleft palate).3

**CONCLUSIONS**

There are a number of cases on record where the initial chromosome investigation has been normal and a reexamination of the patient and a complete lab investigation has enabled the diagnosis to be confirmed. Most likely to be missed are patients carrying smaller sized deletions that present milder, less striking clinical features and that have an
apparently normal karyotype. For these individuals, molecular testing, such as FISH or MLPA (Multiplex Ligation-dependent Probe Amplification), should be performed for the confirmation or exclusion of the diagnosis. Therefore, we continued the investigations using molecular procedures for one of our cases with normal karyotype result but with suggestive WHS phenotype. In addition, it should be considered genetic testing for the parents of all the diagnosed patients when possible because of the high risk of recurrence due to a potential balanced translocation.

REFERENCES