Expert Opinion on Orphan Drugs

ISSN: (Print) (Online) Journal homepage: informahealthcare.com/journals/ieod20

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To cite this article: Ying Gao, Mian-Lin Zhong, Yang-Li Dai, Yong-Hui Jiang & Chao-Chun Zou (2023) Overview of genetic testing in Prader-Willi syndrome, Expert Opinion on Orphan Drugs, 11:1, 16-25, DOI: 10.1080/21678707.2023.2262104

To link to this article: https://doi.org/10.1080/21678707.2023.2262104

Published online: 28 Sep 2023.

EXPERT OPINION ON ORPHAN DRUGS
2023, VOL. 11, NO. 1, 16–25
https://doi.org/10.1080/21678707.2023.2262104

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REVIEW

Overview of genetic testing in Prader-Willi syndrome

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ABSTRACT

Introduction: Prader-Willi syndrome (PWS) is a complicated neurodevelopmental genetic disorder stemming from the loss of expression of imprinted genes within the 15q11-q13 region. It is characterized by impaired hypothalamic development and function. Infants with PWS typically present hypotonia and feeding difficulties, which in later stages of childhood progress to hyperphagia, obesity, and endocrine dysfunctions. However, early diagnosis and treatment have proven effective in mitigating obesity and related co-morbidities in patients with PWS. Moreover, the precise molecular classification of PWS is crucial to tailor the appropriate treatment strategies and provide valuable genetic counseling.

Areas covered: This review contains various conventional and novel PWS diagnostic methods, assessing each method's underlying mechanisms, advantages and disadvantages. Furthermore, our review presents a genetic testing workflow for PWS diagnosis and explores promising techniques for newborn and prenatal screening, which facilitate early diagnosis and intervention. This review synthesizes pertinent studies from 1990 to 2022, gathered from databases including PubMed, Web of Science, EBSCO, and Cochrane.

Expert opinion: Starting with MS-MLPA is the most efficient way to detect underlying genetic mechanisms. However, it is essential to note that certain rare instances, such as balanced chromosomal rearrangements, may require complementary diagnostic techniques to identify accurately.

ARTICLE HISTORY
Received 15 October 2022
Accepted 19 September 2023

KEYWORDS
Prader-Willi syndrome; diagnostic testing; newborn screening; prenatal screening; clinical diagnosis

1. Introduction

Prader-Willi syndrome (PWS) is a multisystem disorder caused by the absence of expression of the paternally active genes on chromosome 15q11-q13 region [1]. Conversely, Angelman syndrome (AS) is caused by the absence of the active maternal gene in chromosome 15 [2].

So far, Multiple genes have been identified and mapped within the PWS region, including *MKRN3*, *MAGEL2*, *NDN*, *PWRN1*, *NPAP1*, *SNURF-SNRPN* and small nucleolar RNA cluster (Figure 1). Due to genomic imprinting, *SNRPN*, *MAGEL2* and *NDN* genes are differentially methylated in CpG islands of their promoter regions [3]. This CpG island at the 5′ end is extensively methylated on the maternal chromosome and unmethylated on the paternal allele, leading to the transcriptional silencing of the maternal allele [4,5].

PWS affects males and females equally, with prevalence rates varying between 1 in 10,000 births to 1 in 25,000 births [7]. Hypothalamus dysfunction and abnormal development are primarily responsible for most PWS phenotypes [8]. Miller et al. identified seven nutritional phases in PWS. Phase 0 is associated with intrauterine growth restriction. Phase 1 is characterized by hypotonia. In phase 1a (0–9 months), the infant presents feeding difficulty with or without failure to thrive. While in phase 1b (9–25 months), the infant has a normal appetite and weight increase rate. Phase 2 is characterized by weight gain, with no significant change in appetite in subphase 2a (about 2–4.5 years) but an increased food interest in subphase 2b (about 4.5–8 years). In phase 3 (from 8 years to adult), the patients with PWS show marked hyperphagia and insatiable appetite. However, some adults may progress to phase 4, characterized by a return to normal appetite [9]. Additionally, characteristic craniofacial features, behavior problems, cognitive disabilities, spinal deformity, and hip dysplasia are also presented in PWS [2,10,11].

Due to hypothalamic dysfunction, most cases of PWS present endocrine dysfunctions, including growth hormone (GH) deficiency (GHD), central hypothyroidism, glucose metabolism disorders, corticotropin deficiency and hypogonadism. GHD is common in children with PWS (about 40–100%) and may be associated with short stature, excessive body fat, decreased muscle mass and energy expenditure [12,13]. Additionally, GHD and hypogonadism may cause a marked bone phenotype in PWS, including low bone mineral density, reduced bone mineral content, and a high frequency of osteoporosis and fractures [14].

GH treatment could improve growth body composition, muscle strength, motor function and cognitive level [15]. Early GH therapy has more favorable outcomes in body composition without increasing adverse effects. In addition, early treatment has been proven to improve learning and speech problems [16–18]. Thus, early diagnosis and treatment are necessary for patients with PWS. The diagnosis of age has recently dropped significantly, with most cases diagnosed in the first year of life [19]. However, misdiagnosis and missed diagnoses are still noted in some countries [20]. This review

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Article highlights

- The importance of early diagnosis and treatment for Prader-Willi Syndrome.
- Advantages and disadvantages of each method.
- Genetic testing strategies for Prader-Willi syndrome.
- Prenatal and newborn screening is a promising prospect to be further studied.
- Measuring the expression of sno-lncRNAs in the blood is a novel tool to screen or diagnose PWS

aims to evaluate each method's underlying mechanisms, advantages and disadvantages, and explore an effective diagnostic strategy for PWS.

2. Literature search

This review comprehensively assessed relevant studies from 1990 to 2022, sourced from PubMed, Web of Science, EBSCO, and Cochrane databases, using the keywords: 'Prader-Willi syndrome' OR 'PWS' AND ['diagnosis' OR 'diagnose' OR 'genetic analysis' OR 'molecular analysis'].

3. Genetic typing

There are three main molecular classes in PWS. Approximately 70–75% of cases of PWS result from the deletion of paternal 15q11.2–13. The typical deletion is subdivided into two main subgroups, which involve a common distal breakpoint (BP3) and two proximal breakpoints (BP1, BP2) (Figure 1). Longer type I, approximately 6Mb in size, extends from BP1 to BP3. While shorter type II, about 5.3MB in size, spans from BP2 to BP3 [21,22]. Patients with deletion more frequently present feeding problems, hypopigmentation, sleep disturbance and speech articulation defects (Table 1) [23]. Furthermore, patients with type I deletion present more behavior and cognitive problems than type II due to the loss of four genes from BP1 to BP2 [24]. In addition, about 8% of the deletion subjects with PWS are caused by unique or atypical deletions (neither type I nor type II). A smaller or larger deletion could lead to a milder or more severe phenotype than the typical deletion [25].

About 25–30% of cases of PWS arise from maternal uniparental disomy (mUPD) of chromosome 15 (mUPD15) [26]. According to the pathogenetic mechanism, mUPD15 has three types: maternal heterodisomy, maternal isodisomy and segmental isodisomy [27]. Compared with typical deletions, PWS with mUPD have higher verbal IQ scores and better social skills but are more prone to autism [28]. However, hundreds of recessive genes are located on chromosome 15. If the mother carries some recessive disease genes on chromosome 15, individuals with mUPD15 have a higher risk of having other genetic diseases, including cardiac abnormalities, seizures, hearing loss, or metabolic defects [27]. Furthermore, the remaining cases of PWS are mainly due to imprinting defects (IDs), accounting for about 1–3% [29]. The most common cause of ID is epimutation, with a small recurrence risk [30].

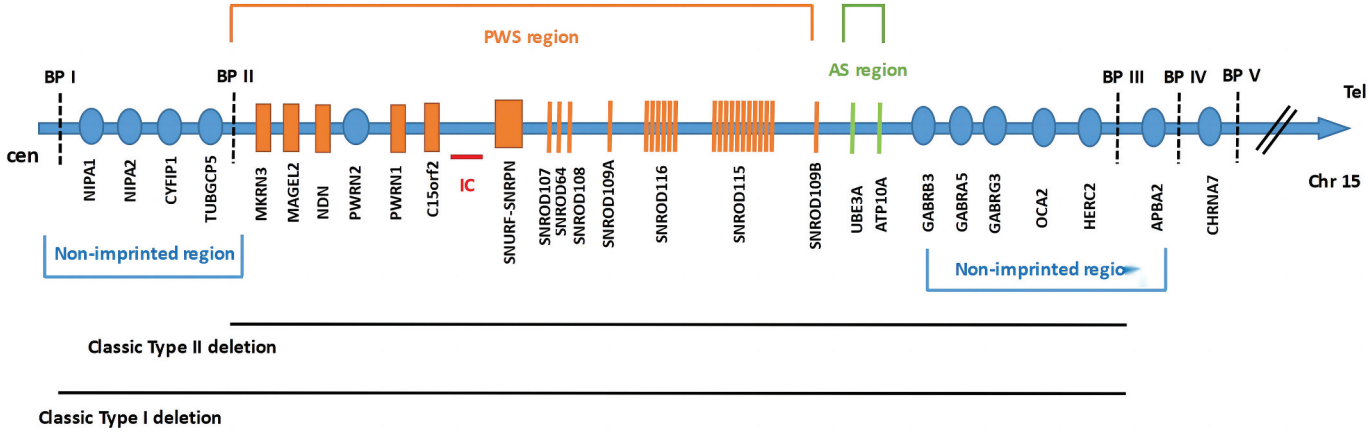

Figure 1. Overview of the critical region for PWS. Blue spots represent biallelically expressed genes, orange boxes/vertical lines represent paternally expressed genes, and green vertical lines represent maternally expressed genes. It is adapted from [6].

Table 1. Genotypes and phenotypes in PWS.

Genotypes	Incidence	Mechanism	Phenotypes
Deletions	70–75%	Type I: BP1-BP3 Type II: BP2-BP3 Atypical deletion	1. Type I deletion present more behavior and cognitive problems than type II 2. Atypical deletion leads to a milder or more severe phenotype than the typical deletion 3. More frequently present feeding problems, hypopigmentation, sleep disturbance and speech articulation defects than UPD
mUPD	25–30%	Maternal heterodisomy Maternal isodisomy Segmental isodisomy	1. Highly associated with advanced maternal age 2. Higher verbal IQ scores and better social skills but more prone to autism
ID	about 3%	Epimutation Microdeletion in the imprinting center	1. ID epimutation has a small recurrence risk 2. ID microdeletion has a 50% recurrence risk 3. The phenotype is similar to mUPD15
Rearrangements	<1%	Unbalanced Chr 15 rearrangements cause deletion Balanced Chr 15 rearrangements	1. Possibly have a 50% recurrence risk 2. Have a higher risk of having other genetic diseases

However, about 15% of IDs result from microdeletion in the imprinting center (IC), which is regulated by epigenetic modification and located at gene *SNRPN* and promoter. The recurrence risk in this situation is 50% [31,32]. The clinical features in ID are similar to mUPD15 [28].

Chromosome 15 rearrangements (translocation or inversions) are also reported in some sporadic cases [33]. In addition, about 1% of PWS deletion patients are caused by unbalanced chromosome 15 rearrangements, which could lead to a 50% recurrence risk when the rearrangement is paternally inherited [21].

4. Clinical diagnosis

Consensus clinical diagnostic criteria for PWS were established in 1993 before the availability of genetic diagnosis, as shown in Table 2 [34]. However, with the availability of genetic tests, the purpose of clinical diagnosis has gradually changed into raising diagnostic suspicion. Thus, Meral et al. proposed a revised criterion (Table 3) with a lower threshold to prompt DNA testing for PWS [11]. However, genetic methods are still necessary to diagnose PWS.

5. Cytogenetic diagnosis

5.1. High resolution cytogenetics

Before the availability of genetic tests, high resolution cytogenetics provided the first laboratory diagnostic test for PWS. However, it is less used nowadays. Deletions of paternal 15q11.2–13 were reported in about 60% of patients with PWS with high resolution cytogenetic techniques. In addition, about 3–5% of patients showed other chromosomal abnormalities involving chromosome 15. However, one-third of patients with PWS presented a normal karyotype in high resolution cytogenetics due to submicroscopic deletions and mUPD [35,36].

5.2. Fluorescence in-situ hybridization (FISH)

The fluorescence in-situ hybridization (FISH) technique is used to detect the absence of the PWS region on chromosome 15. It is based on the hybridization of single-stranded DNA to a complementary sequence of the PWS region. It can be performed on cells after routine cytogenetic analyses or fresh preparations. The probe labeled with fluorochrome hybridizes to the complementary site. Thus, a colored signal at the hybridization site can be visualized by fluorescence microscopy [37]. For unaffected individuals, signals show on both chromosomes 15, while in PWS patients with deletions, the signal shows only on one chromosome 15 [38]. In addition, FISH can distinguish type I from type II deletion with appropriate probes [39,40]. However, it could not be performed in a high throughput manner and fails to detect UPD or differentiate between PWS and AS deletions.

5.3. Chromosomal microarray analysis (CMA)

Chromosomal microarray analysis (CMA) detects microdeletions and microduplications effectively, which can distinguish

Table 2. Consensus clinical diagnostic criteria for PWS.

	Major criteria (1 point each)	Minor criteria (1/2 point each)	Supportive Findings (not score)
1	Hypotonia with poor suck	Decreased fetal movement/infantile lethargy/ weak cry	High pain threshold
2	Feeding problems and poor weight gain/failure to thrive	Characteristic behavior problems	Decreased vomiting
3	Excessive weight gain at 1–6 years and central obesity	Sleep disturbance	Temperature instability/altered temperature sensitivity
4	Characteristic facial features	Short stature	Scoliosis/kyphosis
5	Hypogonadism	Hypopigmentation	Early adrenarche
6	Global developmental delay	Small hands/feet	Osteoporosis
7	*Hyperphagia*	Narrow hands with straight ulnar border	Unusual skill
8	*Abnormality of the Prader-Willi chromosome region*	Eye abnormalities	Normal neuromuscular studies
9		Thick viscous saliva	
10		Speech articulation defects	
11		Skin picking	

For children ≤3 years of age: 5 points are necessary, and at least 4 points come from major criteria. For children >3 years of age: 8 points are required, and at least 5 points come from major criteria.

It is adapted from Yang-Li D et al [20].

Table 3. Revised criteria to prompt DNA testing for PWS.

Age at Assessment	Clinical Features of Prompt DNA Testing
<2 years	1. Hypotonia with poor suck
2–6 years	1. Hypotonia with a history of poor suck 2. Developmental delay
6–12 years	1. History of hypotonia with poor suck 2. Developmental delay 3. Excessive eating with central obesity
≥13 years	1. Cognitive impairment 2. Excessive eating with central obesity 3. Hypothalamic hypogonadism/typical behavior problems

It is adapted from Gunay-Aygun et al [11].

changes as small as 100 to 200 kb [41]. It has two main techniques: comparative genomic hybridization (CGH) and single nucleotide polymorphisms (SNP). The array CGH (aCGH) compares the patient's DNA sample to the reference sample to identify copy number changes. The fragment of the patient's and control DNA samples, labeled with distinct fluorescent colors such as green and red, are mixed in equal proportions and hybridized to the probe on the array. By digital imaging software, the fluorescence intensity of every probe would be measured. Thus, deletion or duplication will be differentiated by the ratio of the fluorescence intensities. In addition, the resolution of aCGH depends on the number, type, and distribution of probes [42].

In the SNP array, the patient's sample is labeled and hybridized into the probe, which is selected from the known DNA locations distributed across the human genome. The copy number changes and single nucleotide polymorphism will be detected by comparing the absolute fluorescence probe intensities to the normal control. In addition, the SNP array can detect long contiguous stretches of homozygosity (LCSH) to distinguish UPD [43].

CMA is proficient in detecting deletion size and additional chromosomal anomalies. However, balanced chromosomal rearrangements (such as balanced translocations or inversions) cannot be identified [44].

6. Molecular genetic diagnosis

6.1. Southern blotting

In Southern blot methylation analysis, it is necessary to choose probes to assess the differential methylation status of the gene *SNRPN* rather than any other locus. The DNA sample was digested with restriction endonucleases, followed by gel electrophoresis for separation. Subsequently, the mixture would be transferred to the porous membrane. Specific sequences would be detected by hybridizing with labeled probes [45]. Thus, Southern blot analysis can identify large deletions and UPD for PWS and AS. However, the site of restriction endonucleases could be affected by a rare restriction fragment length polymorphism, which leads to false positive results [4]. In addition, it is time-consuming and gradually replaced by polymerase chain reaction (PCR).

6.2. Polymerase chain reaction

6.2.1. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a sensitive method to detect low-abundance RNA and analyze gene expression. Rachel and Francke used RT-PCR to test the expression of the *SNRPN* gene to diagnose PWS. The *SNRPN* expression could be detected in unaffected people while negative in whatever forms of patients with PWS [46]. However, Muralidhar et al. reported weak *SNRPN* expression in two PWS subjects, and no noticeable clinical features were identified between the weak and negative gene expression [47]. Except for the *SNRPN* gene, many RNA transcripts exist in the PWS region. Nevertheless, unreliable RNA extraction and unstable RNA limit its application.

6.2.2. PCR after restriction endonuclease digestion

Based on differential digestion of expressed *SNRPN* sequences, Chotai and Payne developed a novel PCR test to diagnose PWS and AS. The methylation requiring nuclease *Mcr*BC digests methylated sequences. While the methylation-sensitive endonuclease *Not*I digests unmethylated DNA. Following treatment with either *Not*I or *Mcr*BC, the DNA samples are amplified by PCR. After *Mcr*BC digestion, the *SNRPN* sequence is absent in patients with PWS. Conversely, the SNRPN sequence is absent in AS patients after *NotI* digestion. The potential advantages of this method are rapid, simple and cost-effective. However, a false negative is the most critical problem in this method because of partial digests. In addition, this method misses PWS or AS patients with chromosomal abnormalities and fails to distinguish between deletions, UPD or ID [48].

6.2.3. Methylation-sensitive polymerase chain reaction (MS-PCR)

MS-PCR is considered a rapid and analytically sensitive technique. By treating DNA with sodium bisulfite, unmethylated cytosine converts to uracil, while methylated cytosine remains non-reactive. Thus, the differential methylation status of gene *SNRPN* is converted into sequence differences, followed by amplification with primers specific for methylated and unmethylated DNA in PCR reactions [49]. Zeschnigk et al. developed a method with one common primer binding to parental alleles and two different primers specifically binding to paternal or maternal alleles [50]. Compared with Southern blotting, this approach is economical and avoids restriction enzymes. It detects deletions, UPD and ID and correctly detects more than 99% of suspected cases, although it does not provide more information on molecular mechanisms, and further molecular genetic tests are necessary to distinguish the underlying molecular cause. However, Morandi et al. reported that a mosaic mUPD 15 girl with incomplete PWS presented a negative result of MS-PCR [51]. Thus, Baumer et al. used MS-PCR followed by denaturing high performance liquid chromatography (DHPLC) to detect low cell mosaicisms [52]. Due to single base variants or small deletions, DNA sequence analysis is needed for negative MS-PCR results. In addition, allelic dropout due to the rare presence of polymorphisms could cause a false-positive result, while the competition for reagents contributes to false-negative results [53,54].

6.3. Pyrosequencing

Pyrosequencing technology is a robust, high-throughput sequencing method that analyzes short- to medium-length DNA sequences. The sequence of methylated and unmethylated alleles is different after treating with bisulfite. Pyrosequencing distinguishes sequence differences by detecting the signal of pyrophosphate release, which was produced by incorporating nucleotides into the template strand [55,56]. Thus, White et al. used pyrosequencing assays to quantify CpG islands within the *SNRPN* gene to identify PWS and AS. This method could be used individually and in combination to diagnose PWS and AS. In

addition, it could also detect cases of mosaicism, and the cost and time are similar to the MS-PCR [57]. However, absolute quantification is affected by DNA concentration, PCR amplification bias, and bisulfite treatment.

6.4. Melting curve analysis

The melting curve analysis is a powerful method based on PCR. The difference in nucleic acid melting temperature (Tm) highly depends on the sequence variants. Thus, Worm et al. first described the methylation-specific melting curve analysis (MS-MA) that integrates bisulfite-treated DNA amplification and melting analysis to detect PWS and AS [58]. Bisulfite-treated unmethylated cytosine is converted to uracil, decreasing the stability of the heteroduplex structure and Tm. Methylated alleles had high Tm than the unmethylated alleles. Therefore, characteristic melting curves of PWS and AS are acquired. Compared with normal individuals with two marked peaks, PWS individuals only show a single peak at the higher melting temperature (corresponding to the maternal allele) [59,60].

To detect the deletion and non-deletion genotype, Wang et al. used the *LIS1* gene as a reference gene for PWS melting curve analysis. The *LIS1* gene is located on chromosome 17p13.3. The copy number changes of *LIS1* are rare, and the clinical phenotypes are distinct from PWS. Thus, deletion or non-deletion PWS could be identified by comparing relative peak height ratios of maternal *SNRPN*: *LIS1*. The non-deletion patients with PWS had a higher ratio than the deletion patients with PWS. However, peak height ratio variants were observed within each genotype in the replicate assay run, and positive control samples are recommended to be set for each assay run [61]. In addition, Hung et al. used real-time PCR with melting curve analysis to distinguish between deletion and non-deletion PWS individuals [59].

In MS-MA, DNA binding dye SYBR Green is unsaturated and inhibits PCR amplification. While EvaGreen is a saturated concentration dye and can be used at much higher dye concentrations without inhibiting PCR amplification. Thus, White et al. established a methylation-sensitive high-resolution melting-curve analysis (MS-HRM) with EvaGreen to distinguish PWS, which detected a lower abundance of methylated DNA. In MS-HRM, mutation or mosaicism may cause unusual melting curve shapes for PWS and AS samples. However, one PWS sample with a variant melting curve was not detected as mosaicism or mutation [62,63]. Thus, samples that cannot be unambiguously assigned to three diagnostic categories should be further investigated.

HRM is a nondestructive test that other techniques can subsequently analyze. In addition, as a post-PCR technique, HRM could decrease the risk of PCR contamination and dispense electrophoresis gel analysis [64].

6.5. Methylation sensitive multiplex ligation-dependent probe amplification (MS-MLPA)

MS-MLPA could simultaneously detect methylation status and copy number changes of the 15q11-q13 region. The reactions were divided into two parts. One is treated with probe ligation and *Hha*I endonuclease for methylation detection, and the other is only treated with probe ligation for copy number changes [65]. The *Hha*I enzyme only digests paternal unmethylated genomic DNA, and no PCR product will be generated. In contrast, the methylated sequences will prevent the digestion and amplify in the subsequent PCR. In addition, additional methylation-sensitive control probes outside chromosome 15 are needed to ensure complete digestion [60]. Therefore, the methylation status and copy number changes could be detected by comparing probe-height ratios. In unaffected individuals, the probe-height ratio in *Hhal* digested samples is half that in undigested samples (0.5/1). However, PWS samples with typical deletions show a half reduction for probes within the 15q11-q13 region, whether digested or not (0.5/0.5). For type I deletions, the number of probes decreases in the region from BP1 to BP3. While for type II deletions, the copy number is normal from BP1 to BP2 [66]. Patients with PWS with UPD have two methylated allies. Thus, the number of probes in undigested samples is similar to that in digested samples (1/1). Furthermore, IC microdeletion can also be detected with appropriate probes [67]. If probes could not detect the microdeletion in IC, however, MS-MLPA will not distinguish UPD from ID, and further microsatellite analysis is necessary to perform. In addition, single-base variations in the probe-binding regions or the restriction enzyme recognition site would lead to false positive or negative results [60].

6.6. Microsatellite analysis (MSA)

Microsatellites, also named short tandem repeats (STR), are short, highly polymorphous and tandemly repeated simple sequences ranging in size from 1 to 6 base pairs. With the occurrence of PCR, the high sensitive microsatellite analysis has been widely used in discovering genes and diagnosing diseases, which is fast and requires less DNA [68]. STR markers are located in the typical PWS and AS deletion region and the distal region near the telomere. Microsatellite loci are identified by primers labeled with fluorescence and amplified with PCR. However, the proband and parental DNA samples are all required, which is impractical in some cases [69,70]. In addition, an interpretation of the result should build on more than one informative marker. Microsatellite analysis is a time-costuming method that could distinguish between UPD heterodisomy and deletion. Nevertheless, it failed to identify deletion and UPD isodisomy.

6.7. RNA

Due to the unreliable and unstable extracted RNA, it is uncommonly used for PWS diagnosis. However, Yin et al. discovered long non-coding RNAs (lncRNAs) with small nuclear RNAs (snoRNAs) ends and named snoRNA-related lncRNAs (sno-lncRNAs), which were processed from intron. Moreover, the PWS critical region encodes five classes of sno-lncRNAs, which are highly expressed in unaffected individuals while not expressed in patients with PWS [71]. Depletion of PWS region sno-lncRNAs not changes gene expression but leads to altered alternative splicing of several hundred mRNAs [72]. In

addition, sno-lncRNAs are reliable and have slow degradation compared to other RNAs. These findings suggest that measuring the expression of sno-lncRNAs in the blood is a promising tool for diagnosing PWS.

7. Prenatal screening

Prenatal screening for PWS is a promising prospect, which may lead to early treatment with improved quality of life. In addition, it is essential for those PWS families with high recurrence risks.

Prenatal characteristics associated with PWS include abnormal fetal growth, polyhydramnios, and notably decreased fetal movements [73,74]. While these features are nonspecific, the simultaneous presence of abnormal fetal growth alongside decreased fetal movements or polyhydramnios should raise high suspicions for PWS.

Chorionic villus sampling and amniocentesis are the most common sources of genetic testing in prenatal diagnosis. However, these samples are more hypomethylated than other tissue, which makes methylation analysis difficult in PWS prenatal screening. Glenn et al. found that the *SNRPN* gene maintains the imprint throughout a wide range of tissue (including chorionic villus and amniocentesis) and is suitable for methylation analysis [75].

Noninvasive prenatal testing (NIPT) could detect chromosome aneuploidies with high sensitivity and specificity, which utilizes cell-fetal DNA from maternal plasma or serum and is safe for the fetus. Wapner et al. utilized the SNP-based NIPT to detect five fetal microdeletion syndromes (including PWS and AS) [76]. However, a positive predictive value is expected to be low because of these rare diseases, and the application of NIPT is required to be further studied before it is widely adopted [77]. In addition, preimplantation genetic testing (PGT) could be used in some families with IC microdeletions.

If trisomy 15 or trisomy 15 mosaicism is detected, PWS with maternal UPD or AS with paternal UPD should be alerted due to the trisomic rescue event in early pregnancy. In this instance, DNA methylation or CMA should be highly considered [78]. If 15q12 missing was detected, FISH, CMA, or MS-MLPA should be considered due to PWS/AS critical deletion [79].

8. Newborn screening

Whole blood is the most typical DNA source in many methods for detecting PWS and AS. However, it is difficult for whole blood to massively screen newborns. Dried blood spot (DBS), a part of newborn screening programs in many countries, is simple to collect and transport [80]. Thus, Mahmoud et al. used MS-PCR and MS-MLPA to diagnose PWS on DBS. This pilot study showed that PWS could be correctly identified once high-quality DNA was extracted successfully. However, some samples extracted low DNA concentration and did not meet the requirements for methylation analysis. Although, these samples have a long storage time of 8–10 years, which may be affected by inappropriate storage conditions and bacteria containment [81]. In addition, Ferreira et al. used MS-HRM to screen PWS on DBS and assessed three different DNA isolation methods from DBS (Qiagen-DBS, Mem-DBS, and Chellex-DBS). In MS-HIRM, none of the isolation methods significantly changed the melting temperature curve. Mem-DBS and Chellex-DBS methods provided high DNA concentration, while the Qiagen-DBS method showed high DNA purity and quality, which benefits high amplification in MS-HRM [82]. In addition, Godlier et al. used methylation-specific quantitative melt analysis (MS-QMA) to screen chromosome 15 imprinting disorders in DBS [83]. The MS-QMA combines HRM and real-time PCR (RT-PCR) to provide quantification of DNA methylation [84]. Thus, it is an emerging field for PWS newborn screening.

9. Conclusion

Early diagnosis and treatment of PWS could effectively mitigate obesity and related co-morbidities, significantly improving life quality for patients with PWS and their families. In this review, we concluded the advantages and disadvantages of each method, as shown in Table 4. Furthermore, prenatal and newborn screening could potentially improve diagnostic age [85,86]. In addition, the discovery of sno-lncRNAs offers a novel approach for screening or diagnosing PWS due to their stability and slow degradation. Combining sno-lncRNA with neonatal screening is a promising prospect to be further studied.

10. Expert opinion

Recently, the age of diagnosis for PWS has significantly decreased, with most cases diagnosed within the first year of life, leading to early human growth hormone treatment and management for PWS. However, there are four keys to be considered in PWS clinical and laboratory diagnosis. Firstly, it is essential to distinguish the relatively few patients with PWS from the vast majority of laboratory referrals that present with similar symptoms, both at birth and later in life. Secondly, after diagnosing PWS, it is necessary to identify those few patients with a high recurrence risk (IC deletion) from most patients with a low recurrence risk (deletions and UPD). Thirdly, the testing process should be as 'user-friendly' as possible. It is notoriously difficult to obtain blood or tissues from probands and their families, and family members are not always available for additional genetic testing. Fourthly, the testing cost and time-consuming should be recognized. The fewer tests performed, the more cost-effective and time-efficient the diagnosis becomes [40].

Therefore, we present a genetic testing workflow for PWS diagnosis (Figure 2). The initial requirement is to identify or recognize suspected patients. Classic clinical features of abnormal growth, polyhydramnios, decreased fetal movements, hypotonia with poor suck, excessive eating with obesity, and global developmental delay could help to identify suspected patients. When patients are suspected to be PWS, starting with MS-MLPA is the most efficient way. This method could detect more than 99% of patients with PWS and exclude AS immediately. MS-MLPA could detect deletion (including large deletion and IC microdeletion), providing a definitive diagnosis for about 70%-75% of patients with PWS. If IC microdeletion is confirmed, deletion analysis

Table 4. Advantages and limitations of PWS diagnosis.

	Uses	Limitations
Cytogenetic diagnosis		
FISH	Can detect deletion and chromosomal rearrangement, could detect about 65–75% of PWS	Cannot distinguish normal and UPD, ID Cannot distinguish PWS and AS deletions Cannot give information about the whole PWS critical region and the other chromosomes
CMA (CGH +SNP)	Can detect deletion (including deletion size), UPD isodisomy and additional chromosomal anomalies and could detect about 80–90% of PWS	Cannot identify chromosomal rearrangement Cannot distinguish UPD heterodisomy and ID epimutation Cannot distinguish PWS and AS deletions
Molecular genetic diagnosis		
MS-PCR	Can detect more than 99% of PWS (including deletion, UPD and ID)	Cannot distinguish the underlying genetic mechanism Cannot identify chromosomal rearrangement
MS-MLPA	Can detect more than 99% of PWS (including deletion, UPD and ID) Can distinguish PWS and AS deletion deletion Can distinguish deletion and nondeletion Can distinguish Type I and Type II deletion	Cannot distinguish UPD and ID Cannot identify chromosomal rearrangement
MS-MA/MS-HRM	Can detect deletion, UPD and ID Can distinguish deletion and nondeletion Can decrease PCR contamination risk and dispense electrophoresis gel analysis	Cannot distinguish the underlying genetic mechanism The melting curve that cannot be unambiguously assigned should be further investigated
Pyrosequencing	Can detect including deletion, UPD and ID Can quantify SRNPN gene Methylation Can detect PWS with mosaic mUPD	Cannot distinguish the underlying genetic mechanism Affected by DNA centration, PCR amplification bias, and bisulfite treatment
MSA	Can detect deletion (especially for microdeletion) and UPD heterodisomy	Not a first-line test, performed after methylation analysis diagnoses PWS Cannot distinguish UPD isodisomy and ID Should require both proband and parents' DNA samples

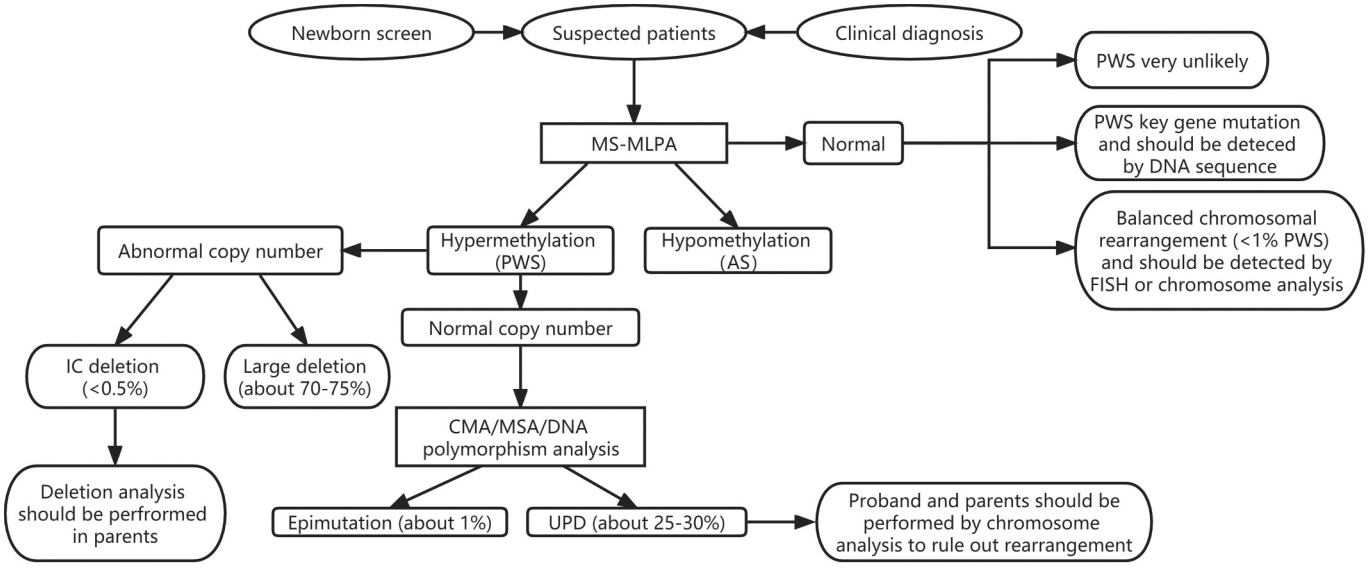

Figure 2. Genetic testing strategies for the Prader-Willi syndrome.

should be performed in families (especially for the father) to rule out familiar IC deletion.

If the result shows a normal copy number with abnormal methylation status, it may indicate UPD or ID. Then CMA, MSA, or DNA polymorphisms should be performed to distinguish UPD and ID. If PWS with mUPD is confirmed, the chromosome analysis should be performed both in probands and parents to rule out cytogenetic rearrangement. However, this instance is rare. If the ID is performed, the recurrence risk is small due to ID epimutation.

If the result of methylation analysis is normal, PWS is very unlikely. However, methylation analysis could not detect balanced chromosomal rearrangement and key gene mutation in PWS critical region (<1% PWS), which should be detected by chromosome analysis and DNA sequence [20].

In addition, PWS screening is increasingly being recognized as a promising prospect for further study. In prenatal screening, prenatal features are critical for clinicians to conduct appropriate diagnostic analysis as soon as possible. In addition, there is a growing interest in expanding NIPT due to its safety in prenatal screening. However, NIPT is more demanding and requires further research before it is widely adopted. In newborn screening, the combination of methylation analysis and DBS gradually becomes an emerging field due to its convenience for sample collection and transportation. Pilot studies on neonatal screening

could be carried out in countries with large populations first.

Due to the reliance and slow degradation of sno-lncRNAs, measuring the expression of sno-lncRNA in blood presents a novel tool for screening or diagnosis of PWS. However, the method of extracting sno-lncRNAs is technically demanding, requiring further research before it can be widely implemented.

Funding

This work was supported by the National Natural Science Foundation (81371215 and 81670786) and Key R & D Projects of Zhejiang Provincial Science and Technology Agency (2021C03094).

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or material discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or mending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Authors' contributions

CCZ conceptualized and designed the study and reviewed and revised the manuscript. YG drafted the initial manuscript. MLZ, YLD, and YHJ collected and reviewed the information about PWS diagnosis. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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