TITLE: DNA Methylation Testing for Imprinting Disorders

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Hello, my name is Bradford Coffee. Welcome to this Pearl of Laboratory Medicine on "DNA Methylation Testing for Imprinting Disorders."

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Imprinting is defined as the exclusive or preferential expression of a gene from one of two parental alleles. There are approximately 75-100 known imprinted genes in the human genome. Imprinted genes are often found in clusters. Not all chromosomes have imprinted genes, only a subset do. Imprinted genes are located on chromosomes 6, 7, 11, 14, 15, 18, 19, and 20. Typically, both maternally expressed and paternally expressed genes are located in the same cluster. Imprinting is an epigenetic process in that there is a change in gene expression without a change in the DNA sequence. These changes are stable when transmitted from one generation to the next. Chromatin structure, controlled by epigenetics modifications, control imprinted gene expression.

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Epigenetic modifications include DNA methylation and histone modifications. DNA methylation occurs at the fifth position of the cytosine ring. Generally, DNA methylation is associated with repression of gene expression. It is found only in CG dinucleotides in somatic cells. Often, CG dinucleotides are referred to as CpGs. The reason for this is to indicate that the cytosine and guanine are located on the same strand of DNA (separated by a phosphate group) versus being located on opposite strands of DNA base paired together. There are about 50 million 5-methylcytosines in the human genome representing 4%-8% of the entire cytosine content in the genome. CpG dinucleotides are clustered in regions of the genome where there is a higher than average density of CpGs. These clusters are referred to as CpG islands. They are often found at the 5' end of genes and will contain the promoter as well as the first exon of a gene. About 50%-60% of genes in the human genome have a CpG island.

Histone modifications occur primarily in the N-terminal portion of the histone proteins. These N-terminal portions of the histones extend from the histone core octamer and are quite flexible. They interact very closely with the DNA that is wrapped around the histone octamer. Modifications to the histone tails that occur to various amino acids on the histone tails are: acetylation, methylation, phosphorylation, and ubiquitination. Different combinations of these histone modifications are associated with different chromatin states; either open, transcriptionally competent chromatin

(euchromatin) or closed, transcriptionally silent chromatin (heterochromatin). This is known as the histone code. The different combinations of histone modifications, along with DNA methylation, target different multimeric complexes which change the chromatin structure from euchromatin, where transcription can take place, to heterochromatin, where transcription is repressed, or vice versa.

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One of the best characterized imprinted gene clusters is located on chromosome 15 at 15q11-q13. This cluster has both paternally expressed genes and maternally expressed genes. The parent-of-origin specific expression of these genes is controlled by specific chromatin structure, which in turn is controlled by a combination of DNA methylation and histone modifications. The patterns of these epigenetic modifications on the maternally-inherited and paternally-inherited chromosome 15s is directed by the imprinting center (IC) located in the center of this cluster.

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Disruption of imprinted gene expression at 15q11-q13 results in two clinically distinct syndromes, Prader-Willi syndrome (PWS), and Angelman syndrome (AS). PWS is characterized by severe hypotonia in early infancy followed by development of an excessive eating behavior at around 2 years of age. These individuals have delayed motor milestones and language development. They are cognitively impaired, but less impaired than individuals with AS. Loss of paternal gene expression causes PWS. The loss of expression of a single gene does not cause the disorder, but the loss of several genes has been implicated, including *MAGEL2* and *HBII-85* snoRNAs. Therefore, the PWS phenotype is due to loss of expression of several contiguous imprinted genes. Pathogenic variant have recently been reported in *MAGEL2* to cause a PWS-like phenotype.

AS is characterized by severe developmental, cognitive, and language delays. Often, children with AS never acquire speech. They have gait ataxia and will often display an inappropriate happy demeanor that is characteristic of the disorder. AS is caused by loss of the maternally expressed *UBE3A* gene. *UBE3A* is expressed only from the maternally-inherited chromosome 15 in the brain and is expressed bi-allelically in other tissues.

For more information, Dr. Christina Lockwood's Pearl of Laboratory Medicine on "Prader-Willi and Angelman Syndromes" is available at www.traineecouncil.org.

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There are 4 known mechanisms that result in loss of imprinted gene expression at 15q11-q13 that cause PWS and AS. The same mutations can cause either PWS or AS depending upon which parental chromosome carries the mutation. For example, the most common mutation that causes both of these disorders is a 5-7 Mb deletion that is due to non-allelic homologous recombination (NAHR) mediated by low copy repeats that flank the 15q11-q13 imprinted region. For both PWS and AS, about 70% of the patients have disease due to these large deletions. If the deletion occurs on the paternal chromosome, causing loss of paternal-specific gene expression, the individual has PWS. If the deletion occurs on the maternal chromosome, causing loss of *UBE3A* gene expression in the brain, the individual has AS.

The second most common mechanism is uniparental disomy or UPD, i.e. inheritance of both copies of chromosome 15 from a single parent. If an individual has maternal UPD, there is loss of paternal-specific

gene expression and they will have PWS. If they have paternal UPD, there is loss of maternal *UBE3A* expression in the brain and they will have AS.

Single gene mutations generally do not cause PWS, though point mutations in specific paternally expressed genes result in PWS-like phenotypes. However, point mutations in the *UBE3A* gene cause about 10% of the cases of AS. For these mutations to cause AS, they must be located on the maternally inherited chromosome. If they are located on the paternally inherited chromosome, they will not cause disease.

A fourth mechanism for PWS and AS is imprinting errors. These errors are the failure of the establishment or the maintenance of the correct imprint (i.e. the correct parent-of-origin specific chromatin configuration) during gametogenesis. Approximately 85% -90% of these imprinting errors are epimutations, meaning there is not an identifiable underlying sequence change that is causing the imprinting error. It is thought that these errors are due to mistakes in establishing the correct epigenetic marks (DNA methylation and histone modifications) during gametogenesis. On the other hand, about 10%-15% of imprinting errors are due to small deletions that are restricted to either the PWS or AS IC, located immediately upstream of the *SNRPN* gene.

Three different mechanisms (the large 5-7 MB deletions, UPD 15, and imprinting center errors) can all be detected by DNA methylation analysis of the *SPRPN* gene. About 99% of case of PWS and 80% of cases of AS will be detected by *SNRPN* DNA methylation testing. For this reason, DNA methylation analysis of *SNRPN* is recommended as the first line test for patients suspected to have PWS or AS. If a methylation abnormality is identified, additional testing is required to determine the molecular mechanism so that accurate recurrence risk estimates can be provided to the family.

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The CpG island containing the promoter of the *SNRPN* gene is methylated on the maternal chromosome 15 and is unmethylated on the paternal chromosome 15. Therefore, *SNRPN* is expressed only from the paternal chromosome 15. The most commonly used test for *SNRPN* methyletion analysis is methylation-specific PCR that targets the CpG island located at the 5' end of the gene. An alternative method is Southern blot analysis with a methylation-sensitive restriction enzyme which is used by some labs. The first step in methylation-specific PCR is sodium bisulfite treatment of genomic DNA. Sodium bisulfite treatment results in the deamination of unmethylated cytosines to uracils. The presence of a methyl group on the cytosine inhibits this deamination. Thus, cytosines that are methylated will remain as cytosines after sodium bisulfite treatment, whereas unmethylated cytosines are converted to uracil.

PCR primers are designed to specifically hybridize to either methylated (unconverted) cytosine or to the uracil (converted cytosine). Primer pairs are multiplexed with one primer pair specific for the methylated *SNPRN* DNA that is derived from the paternal chromosome 15. This primer pair yields a 174 bp PCR product. The second primer pair is specific for the unmethylated *SNRPN* DNA, derived from the maternal chromosome 15. This primer pair yields a 100 bp product.

If both the methylated, maternal-specific PCR product and the unmethylated, paternal-specific PCR product is present, this is a normal result indicating that this individual does not have PWS or AS due to a 5-7 MB deletion, UPD 15, or an IC error. They still may have PWS or AS due to other types of mutations that are not detected by this analysis, such as a point mutation in *UBE3A*, that causes AS. If only the methylated, maternal-specific PCR product is present, this confirms a diagnosis of PWS in this individual.

If only the unmethlylated paternal-specific PCR product is present, this confirms a diagnosis of AS. For these cases, additional testing is required to determine the molecular mechanism of disease to determine the recurrence risk. If additional testing determines that the individual has either a 5-7 MB deletion or UPD 15, then the recurrence risk is low and the family can be counseled appropriately about this low recurrence risk. However, if the individual has an IC error, the recurrence risk for the family can be as high as 50%.

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A second imprinted gene cluster associated with disease is at 11p15.5. Alterations in imprinted gene expression in this region of chromosome 11 cause the growth disorders Beckwith-Wiedemann syndrome (BWS), isolated hemihyperplasia, which is a milder form of BWS, and Russell-Silver syndrome (RSS).

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BWS is characterized by macrosomia or overgrowth, macroglossia or an enlarged tongue, abdominal wall defects (such as omphalocele or umbilical hernia), hemihyperplasia, and an increased risk of embryonal tumors (such as Wilm's tumor, hepatoblastoma, neuroblastoma, and rhabdomyosarcoma).

Conversely, RSS is characterized by growth retardation. Affected individuals have both prenatal and postnatal growth retardation. They typically are less than the 5th percentile in both height and weight. They have a triangular shaped face and will have a normal head circumference. This gives them the appearance of having a larger than normal head, referred to as pseudohydrocephalus, but the circumference of their head is normal for their age. RSS individuals often have 5th finger clinodactyly and hemihypotrophy or under growth of certain regions of their body. There is not an increased risk of cancer in these individuals.

BWS and RSS represent opposite ends of the growth phenotype spectrum and both are caused by alterations in imprinted gene expression at 11p15.5.

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As with PWS and AS, there are a variety of molecular mechanisms that alter imprinted gene expression at 11p15.5 that cause either BWS or RSS.

For BWS, the most common cause is loss of Differentially Methylated Region 2 (DMR2) methylation on the maternally inherited chromosome. This mechanism accounts for approximately 50% of the cases of BWS. DMR2, also known as IC2, regulates expression of the *KCNQ1OT1* (also known as Lit1) transcript. Loss of methylation at DMR2 results in expression of the *KCNQ1OT1* transcript from the maternal chromosome, where it is normally repressed. *KCNQ1OT1* silences in cis other adjacent genes, including the negative cell cycle regulator *CDKN1C*. It is thought that repression of the maternal copy of *CDKN1C*, through the expression of the normally silent *KCNQ1OT1* transcript, results in the BWS phenotype.

Approximately 20% of BWS are due to paternal UPD 11. Paternal UPD 11 is due to a post-zygotic somatic recombination that results in mosaicism for the UPD 11. The level of mosaicism can vary from tissue to tissue depending upon the timing and the specific origin of the recombination event.

Approximately 2%-7% of individuals with BWS have hypermethylation of DMR1 (also known IC1) on the maternally inherited chromosome. Hypermethylation of DMR1 inhibits binding of the chromatin insulator CTCF to this locus which allows the interaction of enhancer elements located telomeric of the H19 gene to interact with the promoter of IGF2, leading to increased expression of growth factor.

Approximately 10% of cases of BWS are due to point mutations in the *CDKN1C* gene. Similar to UBE3A mutations in Angelman syndrome, these mutations must be located in the maternally inherited copy of the gene to cause disease. Mutations in the paternal copy of *CDKN1C* do not cause BWS.

Large cytogenetically visible duplications and translocations cause approximately 1% of cases of BWS. These cytogenetically visible alterations lead to disruption of imprinted gene expression.

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Approximately 35%-65% of cases of RSS are due to hypomethylation of the paternal copy of DMR1. This hypomethylation leads to binding of the CTCF chromatin insulator which inhibits the interaction of the enhancers located telomeric to H19 with the IGF2 promoter, downregulating IGF2 expression. Approximately 10% of the cases of RSS are due to maternal UPD 7. The clinical phenotype with individuals with RSS due to hypomethylation of DMR1 upstream of the H19 gene on chromosome 11 is indistinguishable from the phenotype with RSS due to maternal UPD 7. Maternal UPD 7 is thought to result in the aberrant expression of imprinted gene(s) on chromosome 7; however, the specific genes responsible have not been identified.

Approximately 25% to 55% of cases of RSS are due to unknown etiology.

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Various methods that quantify DNA methylation and/or copy number are used to test for BWS and RSS. Methylation-specific MLPA (MS-MLPA) is currently the preferred method utilized to test for these disorders. MS-MLPA simultaneously tests for methylation abnormalities (epimutations) and copy number variation at 11p15.5. This is important because in a small, but a significant, number of cases of both BWS and RSS are caused by deletions or duplications at 11p15.5. The recurrence risk for BWS and RSS varies dramatically depending upon the mechanism of disease. If BWS or RSS is caused by an epimutation, a DNA methylation defect due to the failure in the establishment or maintenance of DNA methylation (and other associated epigenetic marks), the recurrence risk is relatively low and is equal to the general population risk of the disease. However, if BWS or RSS is caused by a copy number variant, the recurrence risk can be as high as 50% for that family. MS-MLPA is a quantitative assay that can measure partial loss or gain of methylation. Since it is a quantitative assay, the appropriate normal range of methylation must be determined in a cohort of unaffected individuals. Significant deviation from this normal range, typically greater than 2 standard deviations from the mean, is considered abnormal.

The first step of MS-MLPA is to denature the genomic DNA and hybridize MLPA probes that target specific CpGs located in the 11p15.5 region. After hybridization, the reaction is split into two parts, one part for copy number analysis by traditional MLPA and the second part for methylation analysis. For the copy number analysis, only ligase is added to the reaction followed by amplification with the universal primers that flank the probes. Comparison of peak areas with a normal sample run in parallel allows for the detection of copy number changes.

For the methylation analysis, both ligase and the methylation sensitive enzyme Hha1 are added simultaneously. The methylation sensitive enzyme Hha1 will digest the unmethylated genomic DNA/probe DNA complex but will not digest the hemi-methylated genomic DNA/probe complex. After ligation and digestion, the reaction is amplified with the universal primers.

The products from both reactions are separated by capillary electrophoresis and the peak areas are measured. Each peak represents a single targeted site. For targets that have a Hha1 site, if the DNA is not methylated, no amplification will take place because the template DNA is cleaved, separating the universal primer binding sites onto different molecules. If the DNA is methylated, the Hha1 enzyme will not cleave the DNA, leaving the target DNA intact and allowing amplification with the universal primers. The peak areas are then compared between the Hha1 digested and undigested reactions. If there is imprinting at a specific target (i.e. one of the two alleles or 50% of the DNA is methylated), you will see reduction in the peak area for the digested reaction of about 50%. If there is loss of DNA methylation at that site, the reduction in the peak area for the digested reaction is significantly less than 50%. If there is complete loss of methylation, then there is no PCR product generated for the digested reaction.

Conversely, if there is a gain of methylation, then the peak area for the digested reaction will be significantly greater than 50% of the peak area than the undigested reaction. If there is complete gain of methylation (i.e. the normally unmethylated allele is completely methylated), then the peak area of the digested reaction will equal the peak area of the undigested reaction.

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Methylation-specific PCR can also be used to test for uniparental disomy if a differentially methylated region (DMR) is identified on that chromosome. We have developed a methylation-specific PCR test for chromosome 7 to test for maternal UPD 7 in RSS. The test targets two different DMRs on chromosome 7, one located in the *GRB10* gene, located on the p arm of chromosome 7, and the other DMR is located in the *PEG1/MEST* gene located on the q arm of chromosome 7. The advantage of methylation specific PCR analysis for UPDs is that parental samples are not required for the analysis, greatly simplifying the logistics in testing in a clinical laboratory. Also, because testing does not require parental samples, as does traditional UPD testing that analyzes transmission of polymorphic markers from both parents to the child, methylation-specific PCR analysis allows testing of individuals where both biological parents are not available, such as testing children who are adopted.

The methylation-specific PCR for chromosome 7 (or any other chromosome with an imprinting cluster containing a DMR) is as it is for chromosome 15 for PWS and AS testing. Again, the first step of the assay is sodium bisulfite treatment of the DNA followed by amplification with primers that are specific for amplifying DNA where the CpGs are methylated (not converted to uracil during sodium bisulfite treatment) or unmethylated (converted to uracil during bisulfite treatment). The methylated DNA-specific primer pair and unmethhylated DNA specific primer pair are designed to yield discrete size products after PCR amplification that are separated by agarose gel electrophoresis.

Allele drop-out due to presence of a rare SNP in the primer binding site has been documented to cause false-positive results in methylation specific PCR assays. By designing two different methylation-specific PCR assays targeting two different DMRs, the risk of a false positive due to allele drop-out is minimized.

Slide 14: References

Slide 15: Disclosures

Slide 16: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "DNA Methylation Testing for Imprinting Disorders." I am Bradford Coffee.