Clinical Chemistry

Fragile X Mental Retardation 1 (FMR1) Intron 1 Methylation in Blood Predicts Verbal Cognitive Impairment in Female Carriers of Expanded FMR1 Alleles: Evidence from a Pilot Study

Bob Barrett: This is the podcast from *'Clinical Chemistry'.* I am Bob Barrett.

Fragile X Syndrome is the most common heritable form of intellectual disability. It's associated with a number of behavioral, cognitive, and physical problems, and it is a prominent cause of autism spectrum disorder.

Curiously, female children who carry the mutation of the gene linked to the syndrome, FMR1, have a 25-50% chance of cognitive impairment.

In the March 2012 issue of *'Clinical Chemistry'*, a research team led by Dr. David Godler, a senior postdoctoral research fellow from the Victorian Clinical Genetics Services, Murdoch Childrens Research Institute in Melbourne, Australia, uncovered a biomarker for cognitive status in females with mutations in the Fragile X mental retardation associated gene.

As a result the team has developed an innovative new test that could revolutionize the way Fragile X Syndrome is screened and diagnosed. Dr. Godler is our guest in this podcast.

Dr., could you please tell us what is Fragile X Syndrome and how significant is this problem?

Dr. David Godler: Fragile X Syndrome is the most common known single gene cause of inherited developmental disability and common with autism. The disorder usually results from abnormal methylation of the FMR1 gene, located on the X chromosome. Methylation of the FMR1 promoter is usually associated with trinucleotide CpG expansion greater than 200 repeats, and this is known as full mutation.

In the general population, roughly 1 in 3,600 males, and 1 in 5,000 females have this mutation. In most full mutation males, methylation of the FMR1 promotes the suppressive expression of the gene and production of its protein, FMRP. This protein is essential for normal development of neurons. Without this protein a range of developmental, physical, and behavioral problems occur.

There are also these more common small expansions within the FMR1 gene. These are known as premutation and gray zone. Premutation will use 55-200 repeats and are roughly found in 1 in 300 in the general population.

With gray zone, there are 45-54 repeats and are thought to be found roughly 1 in 13 general populations. So they are very common.

So these smaller expansions themselves do not cause the epigenetic silencing of FMR1 Fragile X, but they have been linked to late-onset disorders. However, not all carriers of these small expansions develop these late-onset conditions, and at this stage it's not known why some people do and others don't.

Furthermore, premutation alleles have the potential to expand to full mutation in children of premutation carriers. So there is a clear and causal and rational effect in Fragile X diagnostics.

Bob Barrett: How is Fragile X currently being diagnosed and are there shortcomings to that method?

Dr. David Godler: Laboratory testing for Fragile X generally occur in two stages. Stage one involves screening for small CGG repeats using PCR, with primers targeting sequences on either side of the lipid sequence.

General limit of amplification for these basic PCR is roughly a 130 repeats. Male samples with no PCR product, premutation positive males and females, and female samples with one amplification product go to the second stage testing.

The second stage testing involves Southern blot methylation analysis, and this is the current gold standard in Fragile X testing. It provides information on the size of the expansion beyond 200 repeats and methylation of the nearby methylation sensitive restriction site.

This restriction site is located within FMR1 CpG island. Fragile X is generally diagnosed if the patient has developmental delay or autism spectrum disorder of unknown cause, as well as hypermethylation of these methylation sensitive restriction site, and an expansion greater than 200 repeats.

There are however also cases where CGG repeat sizes slightly lower than 200 are hypermethylated. In these rare cases, methylation status is used to differentiate high premutation, unmethylated from local mutation, methylated alleles.

Furthermore, and importantly in females, because of the second X chromosome, X-inactivation, the phenotype is highly variable and molecular diagnosis using Southern blot methylation analysis is a lot more difficult in girls than in boys. This could be because X-inactivation varies between different tissues differently for different CpG sites.

But there is also some Southern blot targeting one methylation site in blood would not necessarily reflect what is happening with methylation for that same site and other sites of that FMR1 promoter in the brain.

Thus ultimately, Southern blot methylation analysis in one CpG site and blood does not necessarily reflect what is happening with FMRP production in the brain, and these can be highly problematic, particularly for early diagnosis of Fragile X in girls.

Bob Barrett: Tell us more about this test and if it can be used to diagnose Fragile X earlier in girls and is earlier diagnosis beneficial to girls and their families?

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Dr. David Godler: Our test uses PCR amplification of blood cells that convert the DNA and segmentation analysis, utilizing MALDI-TOF Mass Spectrometry. The target sequence is primarily intron DNA regions within the body of the FMR1 gene. Until now this region was thought to have no function or diagnostic significance to Fragile X.

The MALDI-TOF Mass approach allows for very high throughput and low cost analysis, as most of the processes are automated. The main advantages of a Southern blot of our approach are that samples that are of low quality and quantity that could not be used for Southern blot analysis can be still analyzed using our approach.

In fact, we have shown that we can successfully analyze DNA methylation in samples with as little as 70 picograms of DNA. For Southern blot, I think the minimum is around 5 micrograms per sample.

Also, there is no radiation and the method is not as laborious as Southern blot analysis.

Our methylation analysis protocol takes one and a half to two days from the original sample prep to the final result. And the system is very high throughput, as I said earlier, and it can perform up to five rounds per day, which equates to around 4,000 samples, making it ideal technology for Fragile X newborn screening, whereas Southern blot is not as suitable for this, because it's a much lower throughput test.

Another attractive feature of our approach is that it allows for an option to simultaneously analyze methylation of more sites within a region as large as 600 base pairs. This flexibility allows for accurate determination of which site or combination of sites might be used as an optimal biomarker.

Bob Barrett: How early is Fragile X currently being diagnosed using the current protocols, and would there be benefits or even treatments available for earlier diagnosis?

Dr. David Godler: Typically, if Fragile X phenotype is not obvious until later in the childhood, and despite of the robust protocols using CGG testing, in most cases the disorder is still only being diagnosed at the age of three or even later.

The main benefits of earlier diagnosis for the families is that parents may be able to get a head start from birth or early infancy. This could be years before clinical features become apparent and maybe particularly important to girls, where the clinical features are highly variable.

For instance, we have seen also families that have as many as three children with Fragile X before they even know that their first child has the disorder, and this is particularly important if the first child is a girl.

Thus, earlier diagnosis is not only important for the affected child from the early intervention perspective, but also for the parents.

In terms of early intervention for the identified children, current treatments available are behavioral interventions and psychotropic medications.

More specific therapies for Fragile X that target downstream biological processes associated with FMRP deficiency in the brain are now also being developed by big pharma and are in clinical trials.

An important paper recently published in *'Science Translational Medicine'* in 2011 from one of these trials raises the possibility, quite an exciting possibility, for personalized medicine in Fragile X, which is based on methylation rather than CGG size.

Bob Barrett: Well, do you see a day when this test could be included in newborn screening, which would of course give us the earliest possible diagnosis at birth?

Dr. David Godler: Excellent question! Yes, the assay has the potential to be included as part of newborn screening, because it overcomes a number of ethical and technical barriers preventing other high-throughput Fragile X test from being applied in these settings.

Primarily our approach is specific for Fragile X phenotype, and that's what makes it special in males and females, and the test will not identify premutation and gray zone carriers

and will not identify full mutation carrier girls that are asymptomatic.

Secondly, our methylation test works just as well in newborn archival blood spots as in DNA from fresh blood, and this is quite exciting for us in being now prepared for another publication.

Thirdly, our test is inexpensive enough to be used in newborn screening techniques.

Bob Barrett: Well, compared to the other Fragile X tests available now, what are the advantages and the limitations to your test? And do you think this will replace the existing Fragile X tests?

Dr. David Godler: Well, in terms of other tests, no other high throughput Fragile X tests have being related to Fragile X phenotype in females with sufficiently high sensitivity and specificity. Our test appears to be especially advantageous for diagnosis and screening in females, and we have also shown a specificity and sensitivity approaching a 100 in females, which would identify full mutation in females with IQ less than 70.

We think that the most unique feature of our assay in compared to others is the primer design, which targets a unique intronic DNA sequence, previously thought to have no function or significance or utility in Fragile X testing.

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And in this *'Clinical Chemistry'* manuscript we have shown that methylation of these specific intronic sites in blood were significantly correlated with the type and severity of cognitive involvement in full mutation females.

While in exactly the same samples we saw no significant relationship with these clinical features when we examined methylation using the gold standard, Southern blot, in the CpG island.

In terms of limitations, we think that the main limitation that it might have a limited impact on prevention of the disorder, as it will not pick up directly premutation alleles, which are themselves unmethylated.

But at the same time, this limitation can be viewed as an advantage, as ethical issues will be avoided, which are associated with detection of small expansion allele in the context of newborn screening.

Furthermore, the male or female problems with Fragile X will be identified using our method, will point to other related risk of full mutation or premutation in their families. We think this will open the possibility of counseling and subsequent cascade testing depending on the wishes of the families.

Another limitation of our approach is that, and some say this is the main limitation, is that the MALDI-TOF MS system is expensive and not everyone can afford to buy the specialized equipment.

However, we all acknowledge that incorporation of the technique into newborn screening would require acquisition and new technology and upskilling of laboratory staff. Mass spectrometry has already been using newborn screening citing, so the sources required, we think, are not out of keeping compared to those currently present for disorders that's already included in newborn screening.

Finally, I do not think that our methylation test will replace existing Fragile X test, but we think that our test will complement them.

The proposed technique can be also combined with any of the latest CGG test that can amplify repeats up to full mutation. And this combination can be used, if desired, to detect all categories of FMR1 alleles in relation to both repeat expansion size and methylation status in both males and females.

Bob Barrett: Finally, Dr. Godler, let's look to the future, where do you see your research going in this area, and do you anticipate your test to be available soon for general Fragile X testing?

Dr. David Godler: Well, larger studies are now underway internationally to sort of validate our findings published in *'Clinical Chemistry'*. These studies will also examine association of biomarker region with clinical parameters other than cognitive involvement.

These may be just as relevant to early diagnosis of Fragile X Syndrome and early intervention. We anticipate that these studies will be completed by the end of 2012.

Based on the results, of course, from these studies, the test may become available for Fragile X testing within a year or two.

Bob Barrett: Dr. David Godler is a senior postdoctoral research fellow from the Victorian Clinical Genetic Services, Murdoch Childrens Research Institute in Melbourne, Australia. He has been our guest in this podcast from *'Clinical Chemistry'*.

I am Bob Barrett. Thanks for listening!

Total Duration: 13 Minutes