Detection of Murine Leukemia Virus (MLV)-related Virus Gene Sequences in Blood of Patients with Chronic Fatigue Syndrome and Blood Donors

-- The 1st International XMRV Workshop

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Chronicles of Finding Human Xenotropic Murine Leukemia Virus-related Virus (XMRV)

- **2006**— A mouse gammaretrovirus, XMRV, was found in tumor tissues of a subset of prostate cancer patients using multi-viruses microarray, ViroChip.

- **2007**— Both positive and negative finding of XMRVs in prostate cancer tissues were reported.

- **2009**— Finding of XMRVs in blood of 68 out of 101 (67%) patients with chronic fatigue syndrome (CFS) and 8 out of 218 (3.7%) normal blood donors. Studies revealed *near genetic identity of all XMRVs* from patients with CFS and tumor tissues of prostate cancers.

- **2010**— A host of studies revealed the absence of detection of XMRVs in patients with CFS and in blood donors.
Why are we studying XMRVs?

• The research studies of Lo’s lab at the Armed Forces Institute of Pathology (AFIP) in the early 1990’s led to the discovery and characterization of previously unknown human mycoplasmas in patients with AIDS.

• Subsequent studies by others reported that infections with these mycoplasmal agents were associated with CFS in the mid-1990s. Blood samples of CFS patients were then sent to us for investigation of possible mycoplasmal infections.

• We concluded that there was no association between with *Mycoplasma fermentans* or *M. penetrans* infections and CFS, nor could we culture any established or novel mycoplasma from the blood of these subjects.

• Portions of the CFS blood samples had been maintained in frozen storage at -80°C, provided a unique opportunity to study for the evidence, prompted by the conflicting reports, of XMRV infection in CFS patients.
Blood samples examined for evidence of infections with XMRV or MLV-related viruses

- Anthony Komaroff, M.D., Director of Chronic Fatigue Syndrome research center, Brigham & Women's hospital, the Simcox-Clifford-Higby professor of medicine, Harvard medical school sent 29 blood samples obtained from 25 CFS patients.

- Other CFS centers/clinicians provided 12 blood samples of CFS patients. A total of 41 blood samples obtained from 37 patients were kept in frozen storage at -80°C from mid 1990’s.

- Harvey Alter, M.D. and Richard Wang, Ph.D. provided previously frozen PBMCs from 44 volunteer blood donors collected between 2003-2006.

- **Summary of results:** 32 out of 37 (86.5%) patients tested positive by a nested PCR targeting MLV-like virus gag gene. In comparison, 3 out of 44 (6.8%) of volunteer healthy blood donors tested positive.
A. Primers: 419F/1154R
   1st round PCR
   (40 cycles)

B. Primers: NP116/NP117
   2nd round PCR
   (45 cycles)

C. Primers: NP116/NP117
   2nd round PCR
   (45 cycles)
Figure 2  The nested PCR amplification of MLV-like virus \textit{gag} gene from PBMC DNA of healthy blood donors.
Figure 2.
Presented at the 1st Intl. Workshop on XMRV
7-8 September 2010, Bethesda USA
Phylogenetic Analysis of MLV-related Virus *gag* Gene Sequences Amplified in The 1\textsuperscript{st} Round of Nested PCR -- 746 bp
Phylogenetic Analysis of MLV-related Virus gag Gene Sequences Amplified After The 2\textsuperscript{nd} Round of Nested PCR -- 380 bp
Figure 6.

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All PCR-based studies will and should have concerns of contaminations

In the study of XMRV and MLV-related viruses, there are three main concerns of contamination:

- Contamination by PCR amplicons
- Contamination by MLVs or viral vectors studied in the laboratories
- Contamination by mouse DNA—Mouse DNA genome contains endogenously many closely related proviruses of MLVs or mERVs.
Development of a semi-nested PCR targeting mouse-specific mitochondria DNA (mtDNA)

• It is necessary to verify that no contamination of mouse DNA in the assay system and in the clinical samples that tested positive for the MLV-related virus gene sequences.

• A highly sensitive nucleic acid PCR assay targeting mouse species-specific DNA sequences that are well-conserved and are present in multiple copies in the mouse cell will be the most ideal assay.

• A semi-nested PCR assay targeting mouse mtDNA (~200 - 1800 DNA copies/cell) was developed using the unique sequences absent in human nuclear and mtDNA as the PCR primers.
Mouse-specific mtDNA semi-nested PCR

1st round of PCR: mt15982F/mt16267R (40 cycles)

Mouse DNA concentration in human DNA (35 ng):
- H₂O
- 0 fg
- 2.5 fg
- 5 fg
- 10 fg
- 50 fg
- 0.1 pg
- 0.2 pg
- 0.5 pg
- M

MLV-like virus gag gene specific PCR

1st round of PCR: 419F/1154R (40 cycles)

B.

2nd round of semi-nested PCR:
- mt16115F/mt16267R (45 cycles)

2nd round of nested PCR:
- GAG-I-F/GAG-I-R (45 cycles)

Mouse mitochondrion specific amplicon:
- 286 bp

MLV gag gene specific amplicon:
- 730 bp

Mouse mitochondrion specific amplicon:
- 153 bp

MLV gag gene specific amplicon:
- 413 bp

Figure S1

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A. Mouse DNA concentration in 35 ng of human DNA:

0fg 1fg 2.5fg 5fg 10fg 50fg M

CFS patients: 8 17 20 25 H2O

Healthy blood donors: BD-22 BD-26 BD-28 BD-21 BD-23 H2O

Mouse mitochondrion specific amplicon: 286 bp

B. 2nd round of nested PCR: mt16115F/mt16267R (45 cycles)

8 17 20 25 BD-22 * BD-26 * BD-28 * BD-21 BD-23 H2O

Mouse mitochondrion specific amplicon: 153 bp

Figure S2

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Why did many other studies have different findings--

• There could be a difference in the prevalence of the viral agents among patient groups in different geographic areas.

• Heterogeneity of CFS patient groups could be significant.

• Variations of clinical sample preparations could affect PCR amplification effectiveness and assay sensitivity.

• Variations of PCR protocols, primers, reagents or assay designs may have different sensitivity in detecting the diverse group of MLV-related virus gene sequences in the clinical samples.

• The nature of low grade infections with low titers of the virus or low copy numbers of the viral target genes in patients’ blood may likely account for the inconsistency and the disparity of PCR assays performed.
### CDC/FDA Reciprocal Sample Testing

<table>
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<th>CDC Sample Description (N)</th>
<th>CDC Result #pos/#reported</th>
<th>FDA Result #pos/#reported</th>
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</thead>
<tbody>
<tr>
<td>CFS Cases (34)</td>
<td>0/34</td>
<td>0/31*</td>
</tr>
<tr>
<td>Matched Healthy Controls (28)</td>
<td>0/28</td>
<td>0/26*</td>
</tr>
<tr>
<td>Spiked Positive Control – XMRV (8) [143 – 143,000 copies/μg]</td>
<td>(8/8)</td>
<td>8/8</td>
</tr>
<tr>
<td>Spiked Positive Control – MLV (2) [14,300 copies/μg]</td>
<td>(2/2)</td>
<td>2/2</td>
</tr>
<tr>
<td>Normal Human DNA (10 replicates)</td>
<td>Negative</td>
<td>5/5*</td>
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<table>
<thead>
<tr>
<th>FDA Sample Description (N)</th>
<th>CDC Result #pos/#reported</th>
<th>FDA Result #pos/#reported</th>
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</thead>
<tbody>
<tr>
<td>Blood Donors (4)</td>
<td>0/4</td>
<td>2/4</td>
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</tbody>
</table>

*Indeterminate results were obtained for 3 CFS cases, 2 healthy controls, and 5 replicates of normal human DNA.*
Conclusions:

• The study supports the earlier finding of MLV-related virus gene sequences in blood of many patients with CFS.

• The viral sequences can also be detected in a small fraction of volunteer healthy blood donors.

• Differing from the reported finding of near genetic identity of all XMRVs in patients with CFS, prostate cancers and in blood donors, our analysis of the viral gene sequences revealed a more genetically diverse group of MLV-like viruses. The viral gene sequences were more closely related to those of polytropic MLVs.

• Although it is not clear at all about the viral pathogenesis, our study suggests that MLV-related viruses are infecting some people. Disease association and possibility of blood transmission of these otherwise well-known MLV-like retroviruses in human warrant further studies.
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