The Exotic Biology of Xenotropic Murine Leukemia Related Viruses XMRVs

Pitfalls and New Concepts

Judy A Mikovits, PhD
November 20 ,2013

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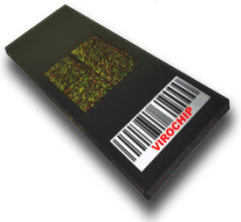
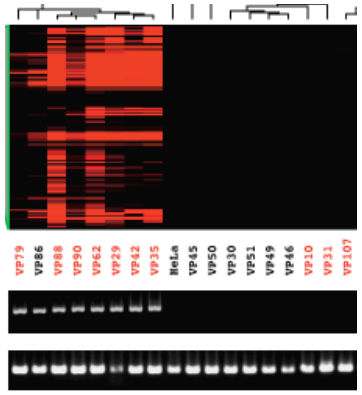
Identification of a Novel Gammaretrovirus in Prostate Tumors of Patients Homozygous for R462Q *RNASEL* Variant

Anatoly Urisman[1☯], Ross J. Molinaro[2,3☯], Nicole Fischer[4☯], Sarah J. Plummer[2], Graham Casey[2], Eric A. Klein[5], Krishnamurthy Malathi[2], Cristina Magi-Galluzzi[6], Raymond R. Tubbs[6], Don Ganem[4,7,8], Robert H. Silverman[2*], Joseph L. DeRisi[1,8*]

Prostate Cancer Tissue

DNA **Virochip**





Microarray

RT- PCR

- In 2006, scientists showed the presence of retroviral sequences in ~10% of prostate tumors
- Sequencing revealed that the sequences were most closely related to xenotropic murine leukemia virus (a gammaretrovirus)
- Named xenotropic murine leukemia-related virus (XMRV)

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PLOS | ONE

In-Depth Investigation of Archival and Prospectively Collected Samples Reveals No Evidence for XMRV Infection in Prostate Cancer

Deanna Lee[1,2], Jaydip Das Gupta[3], Christina Gaughan[3], Imke Steffen[4], Ning Tang[5], Ka-Cheung Luk[5], Xiaoxing Qiu[5], Anatoly Urisman[1], Nicole Fischer[6], Ross Molinaro[7], Miranda Broz[1], Gerald Schochetman[5], Eric A. Klein[3], Don Ganem[8], Joseph L. DeRisi[9,10], Graham Simmons[4], John Hackett Jr.[5], Robert H. Silverman[3], Charles Y. Chiu[1,2,11]*

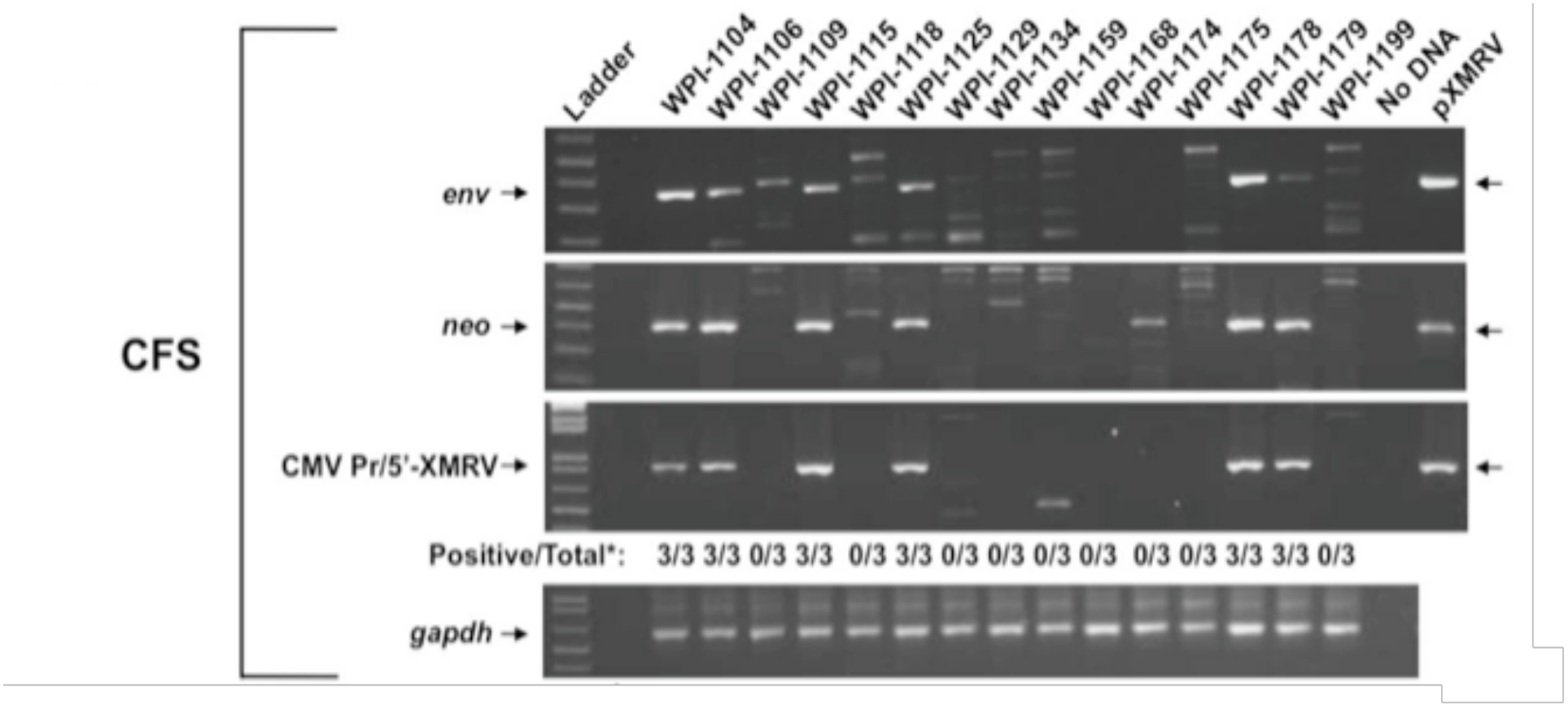
Received June 2, 2012; **Accepted** August 10, 2012; **Published** September 18, 2012

- Archival Tissue and RNA from original Prostate samples contained no XMRV
- Analysis of viral genomic and mitochondrial sequence revealed all previously identified strains are identical
- Archival RNA had been contaminated by an XMRV infected laboratory cell line

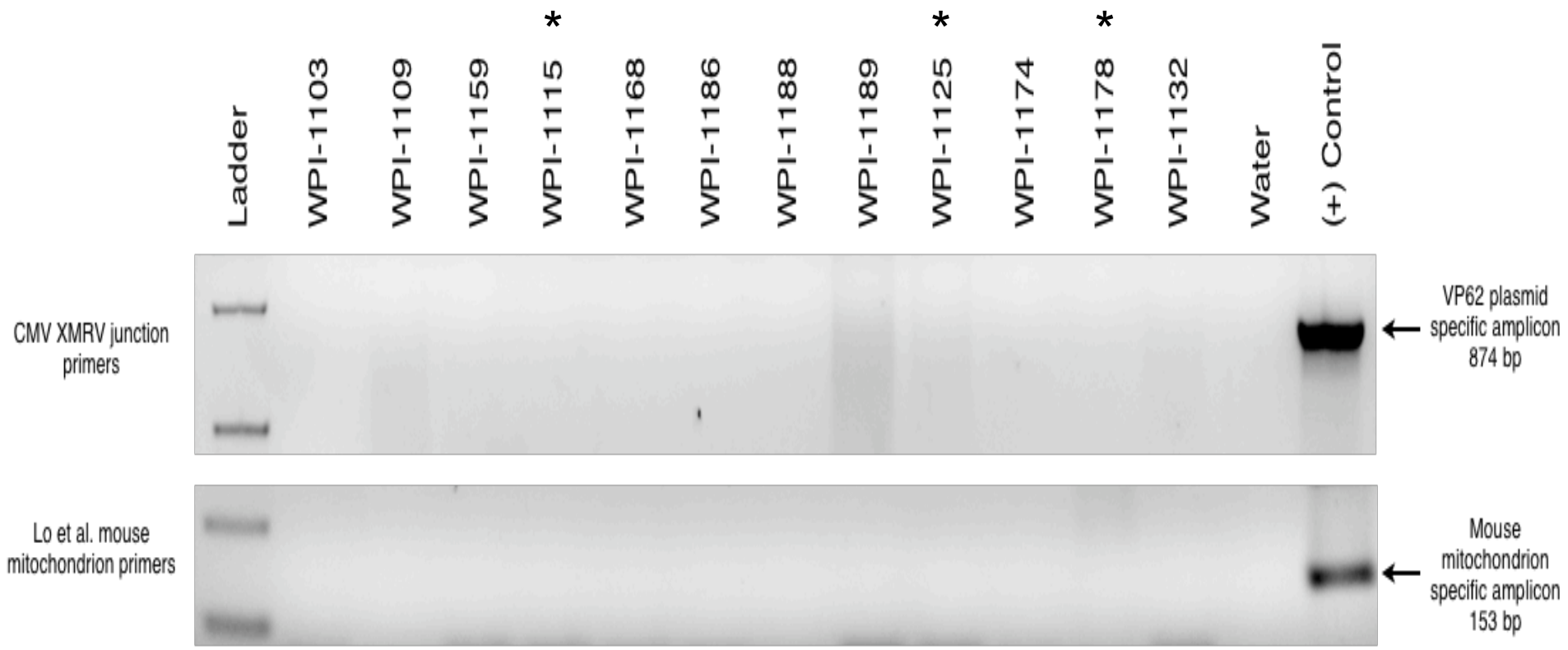
CONCLUSION:
XMRV is not a naturally occurring human or animal infections

- PITFALL : GARBAGE IN GARBAGE OUT!

Six WPI DNA Samples shown in Fig. 1 of the original study analyzed by the Silverman Lab in 2009 contained VP-62 plasmid

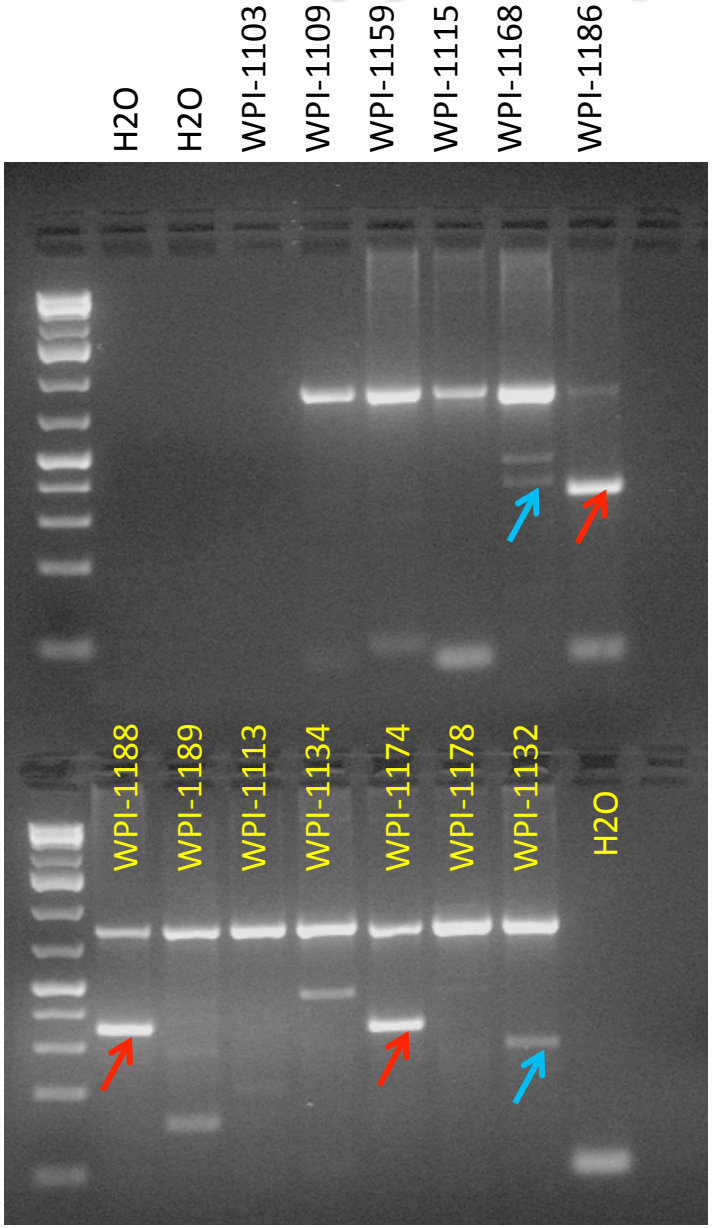


Original DNA Samples were negative for XMRV plasmid



- **Pitfall: Choose your collaborators wisely!!**

Independent Reanalysis of archival samples used in Original Study Detected XMRV gag without plasmid or mouse contamination



PCR performed with USB HotStart-IT FideliTaq Master Mix

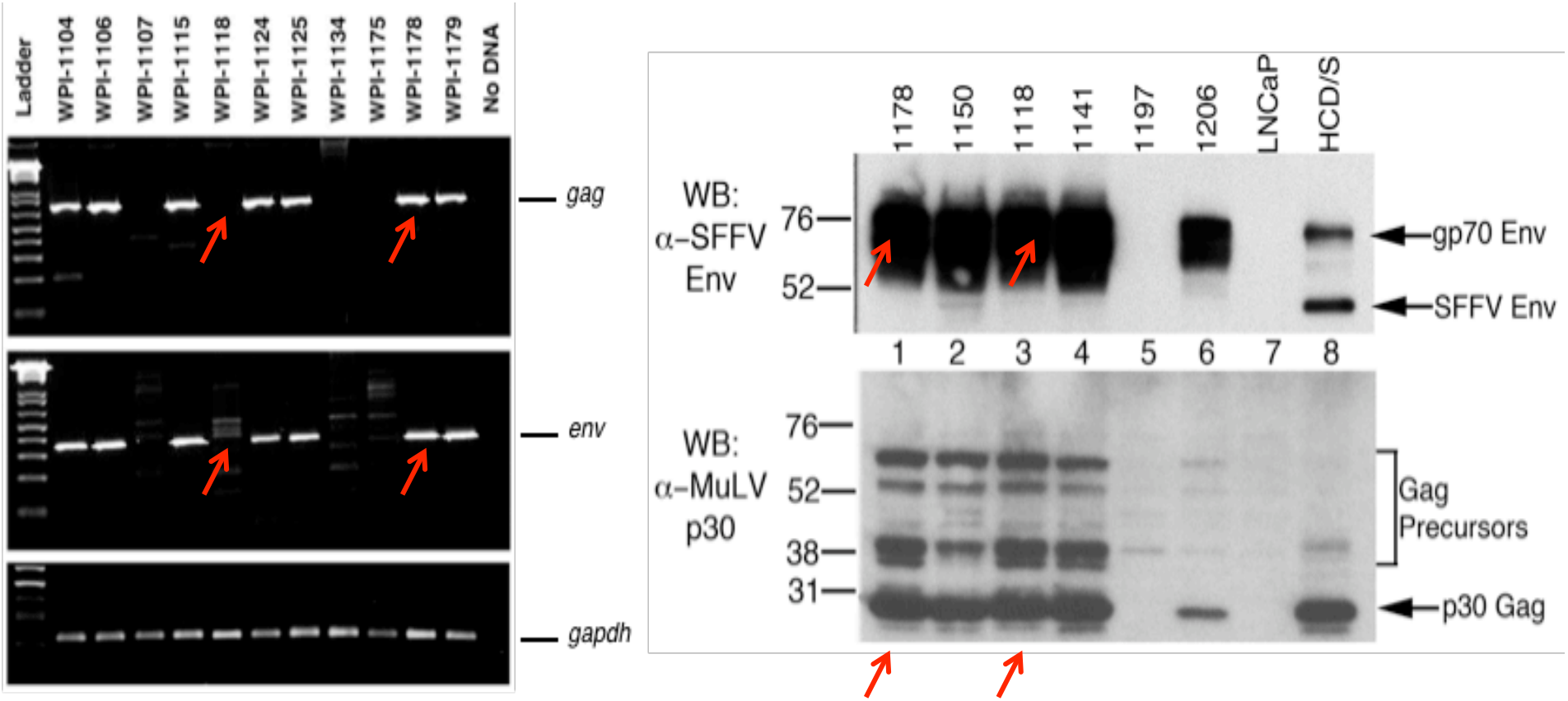
94°C 2 min
45 cycles:
94°C 30 sec, 54.8°C 30 sec, 72°C , 30 sec
72°C 3 min.

All three are negative for IAP and negative for CMV385F/XMRV528R primers for VP62 junction fragment

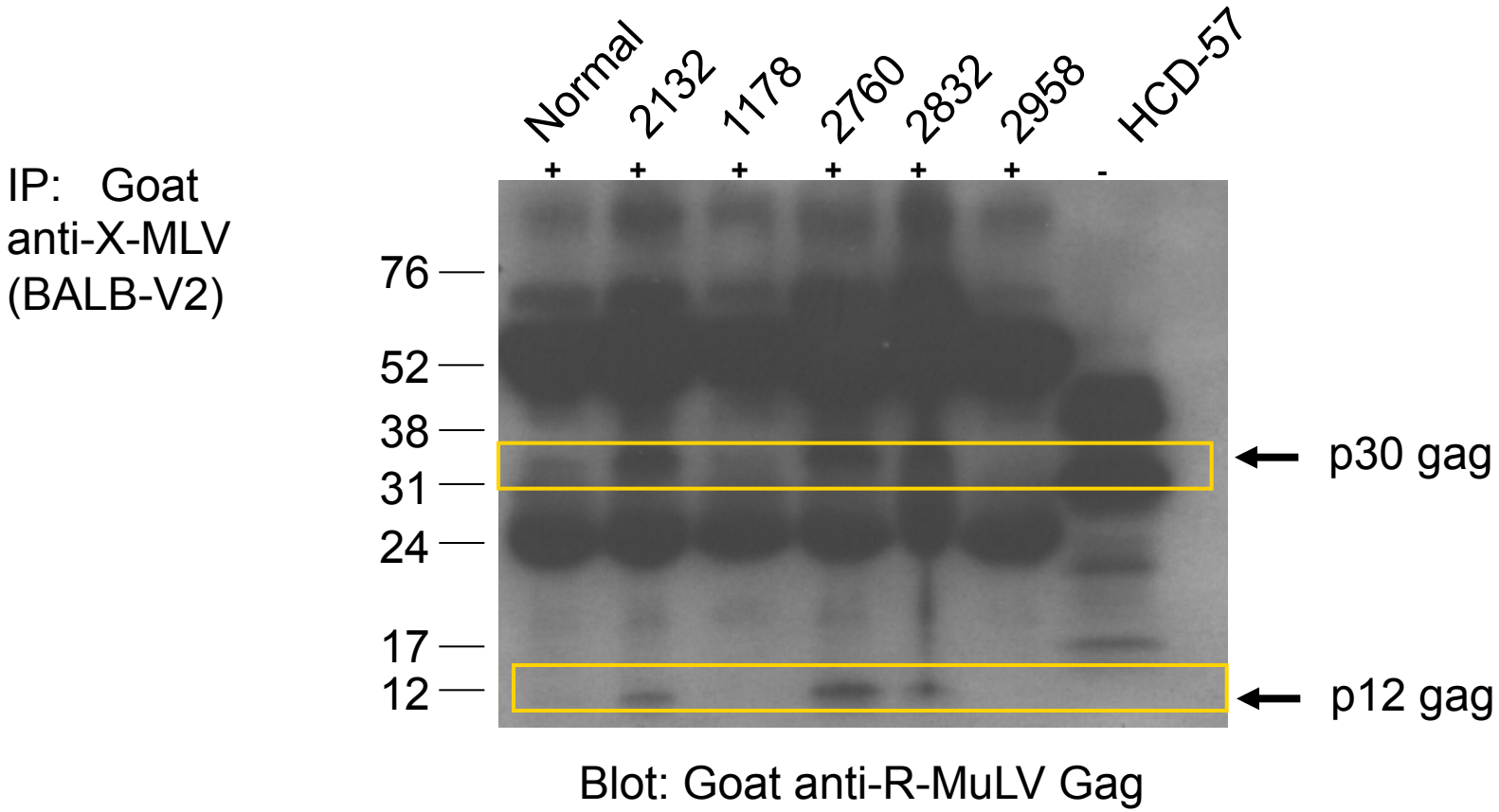
Sequencing of bands:

↗ Non-specific (Human DNA)
↗ XMRV Gag

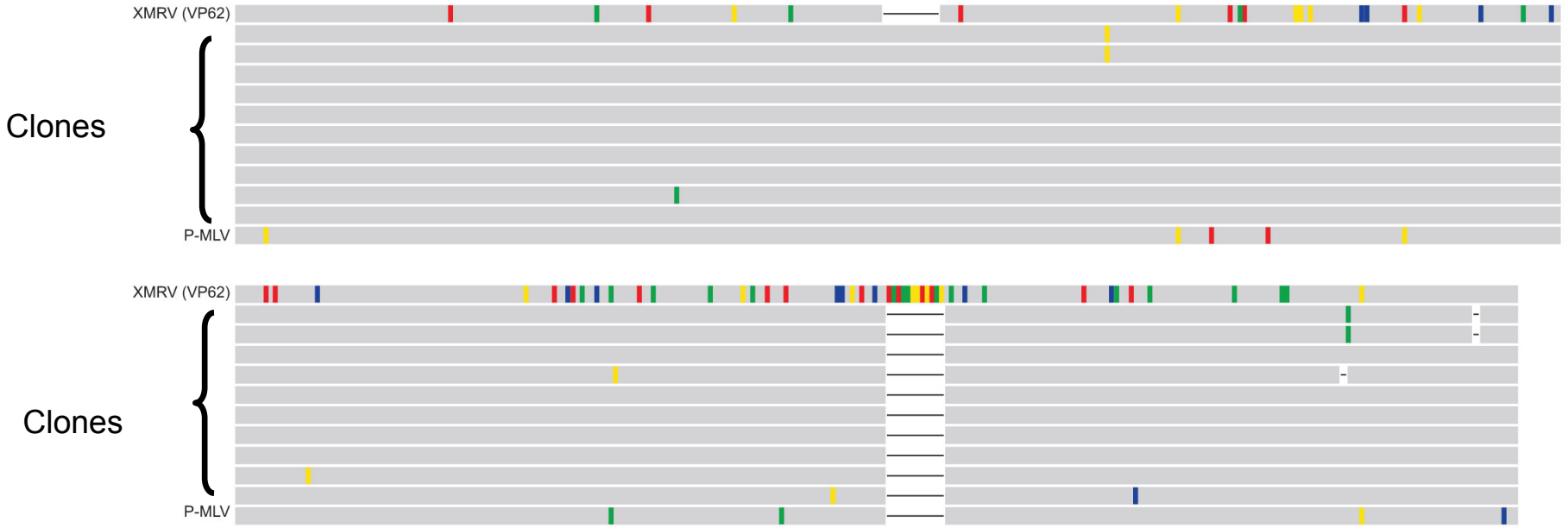
Cell-Free Transmission of XMRV from PCR-negative CFS Patients' Plasma to LNCaP cells



Direct Isolation of XMRV Protein From Plasma of CFS Patients By Immunoprecipitation with Anti-X-MLV Antibodies



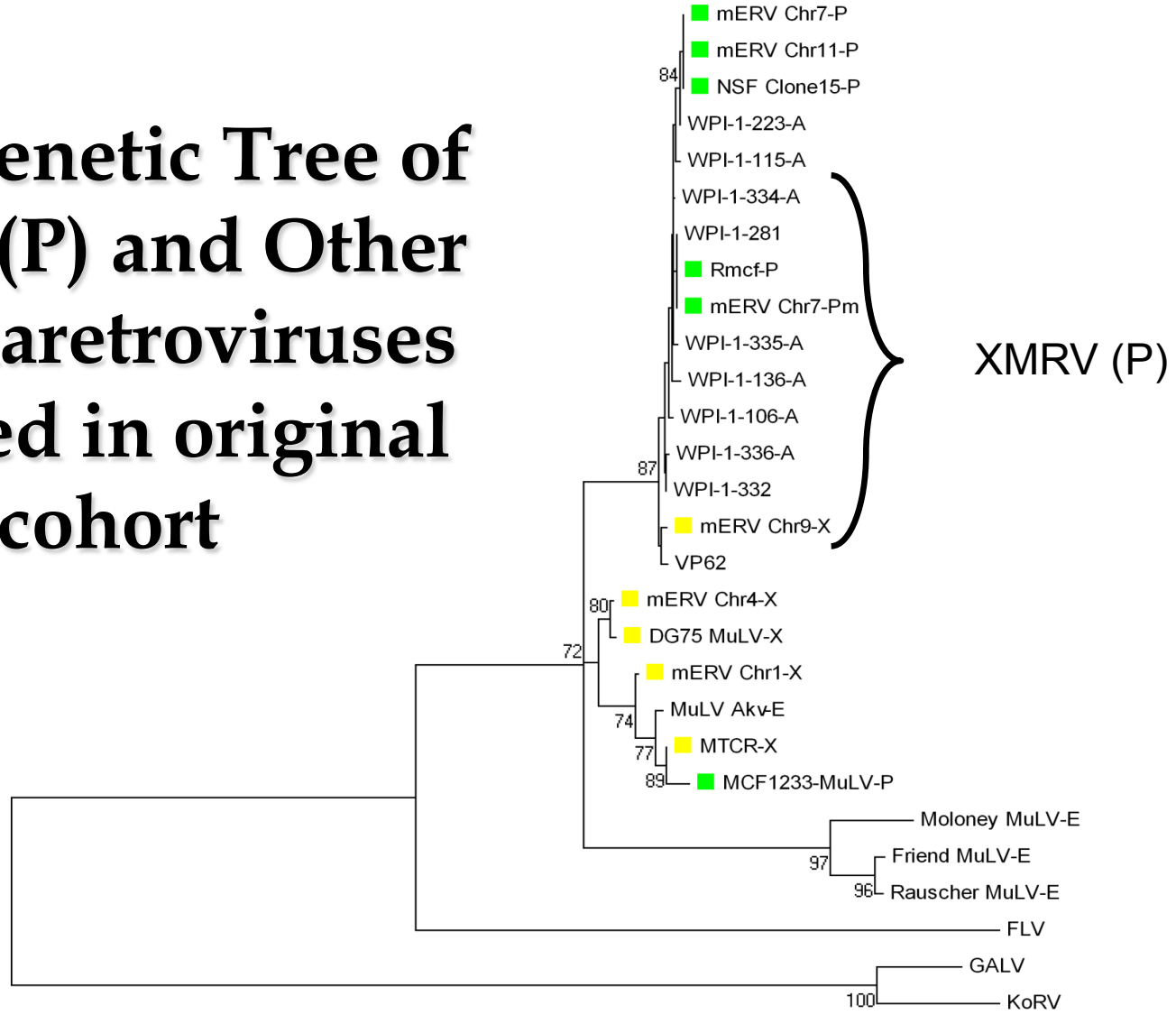
Clones of XMRV Env SU Similar to Polytropic XMRVs



- The main XMRV/ in this patient is unlikely to be VP-62

Pitfall: Extraordinary measures are required to rule out contamination!

Phylogenetic Tree of XMRV(P) and Other Gammaretroviruses detected in original cohort



Horizontal Spread of Gammaretroviruses in Tissue Culture

Table 4. Characterization of murine leukemia viruses (MLV) detected in human non-xenograft cultures in xenograft culture laboratories[1]

Cell line type	MLV positive cell lines[1]	MLV sequence homology[2]	RT Enzyme (nU/μl)	Mouse DNA[3]	Other sources or passages[4]	Source: Lab PI
NSCLC	NCI-H460	ND	Negative	-	Negative	C. Rudin
NSCLC	NCI-H1155	MLV N417	ND	-	ND	A. Gazdar (NCI)[6]
SCLC	NCI-H60	MLV N417	3.6×10^6	-	Negative	A. Gazdar (NCI)[6]
SCLC	NCI-H82	MLV NZB	1.3×10^6	-	Negative	C. Rudin
SCLC	NCI-H1092	MLV N417	8.0×10^3	-	Negative	A. Gazdar (NCI)[6]
SCLC	NCI-H182	MLV N417	ND	-	ND	A. Gazdar (NCI)[6]
SCLC	NCI-H289	MLV N417	ND	-	Negative	A. Gazdar (NCI)[6]
SCLC	NCI-H1514	MLV N417	ND	-	ND	A. Gazdar (NCI)[6]
Colon	RKO	XMRV	2.9×10^3	-	Negative	A. Maitra
Prostate	PrEC2	ND	ND	-	ND	J.T. Hsieh
Prostate	LNCaP	Multiple MLV strains[5]	ND	++++	Negative	J.T. Hsieh
Prostate	PC3	ND	ND	-/+	Negative	J.T. Hsieh
SCLC	NCI-H146	MLV NZB likely	7.2×10^5	-/+	Negative	C. Rudin

Zhang et al., Cancer, Biol. Ther. 2011, 12:617

PITFALL : ability of these viruses to spread to uninfected cells through aerosolization

Lack of reproducibility of sequence data closed the study of XMRV in human disease

TABLE 3 Equivalent levels of XMRV sequences and anti-XMRV antibodies in CFS (chronic fatigue syndrome) patients and matched controls

			CFS/ME cases ($n = 147$)		Controls ($n = 146$)	
Lab site	Analysis	Sample	Total studied	No. positive (%)	Total studied	No. positive (%)
CDC	RT-PCR	Plasma	147	0 (0.0)	146	0 (0.0)
FDA	RT-PCR	Plasma	121[a]	0 (0.0)	110[a]	0 (0.0)
	PCR	PBMC	121[a]	0 (0.0)	111[a]	0 (0.0)
Mikovits, Ruscetti, and Hanson	PCR of cultured PBMC	PBMC	117[b]	0 (0.0)	126[b]	0 (0.0)
Mikovits and Ruscetti	Serology	Plasma	147	9 (6.1)	146	9 (6.2)

[a] Numbers represent all samples available for analysis at that site.

[b] Fifty samples (30 cases; 20 controls) were unable to be assayed because at least one of two aliquots from each set of subject PBMC did not grow in tissue culture.

recent data says NOT SO FAST

Partial molecular cloning of the JHK retrovirus using gammaretrovirus consensus PCR primers

Brian D Halligan[1], Hai-Yuan Sun[2], Vladimir M Kushnaryov[2] & Sidney E Grossberg*[2]

[1]Biotechnology & Bioengineering Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA

[2]Department of Microbiology & Molecular Genetics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA

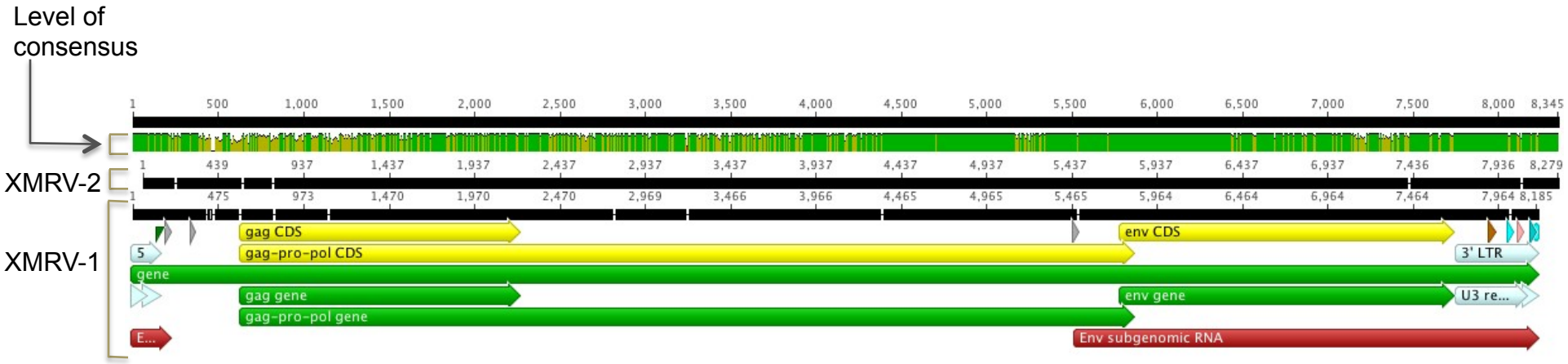
*Author for correspondence: Tel.: +1 414 276 8194 ▪ segrossb@gmail.com

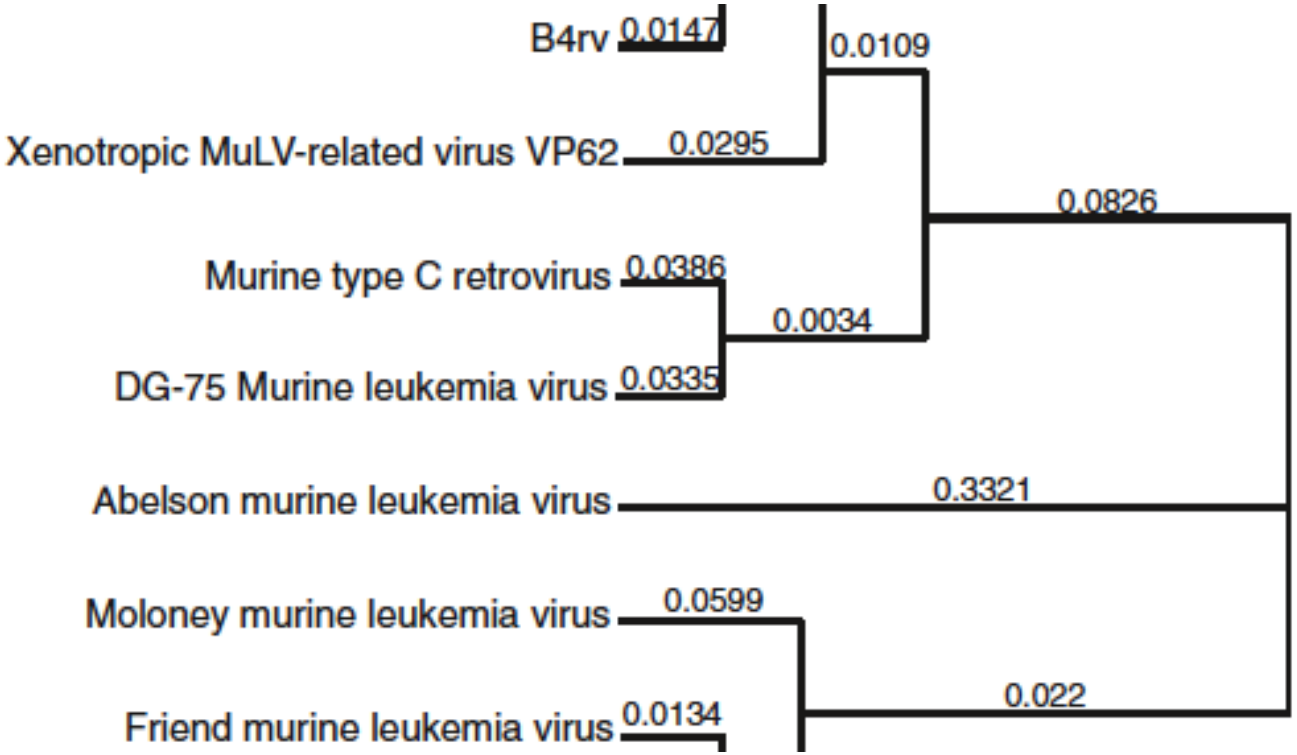
The JHK virus (JHKV) was previously described as a type C retrovirus that has some distinctive ultrastructural features and replicates constitutively in a human B-lymphoblastoid cell line, JHK-3. In order to facilitate the cloning of sequences

Future Virology

August 2013

Sequence and phylogenetic analysis of a novel xenotropic XMRV-like MLV B4rv,

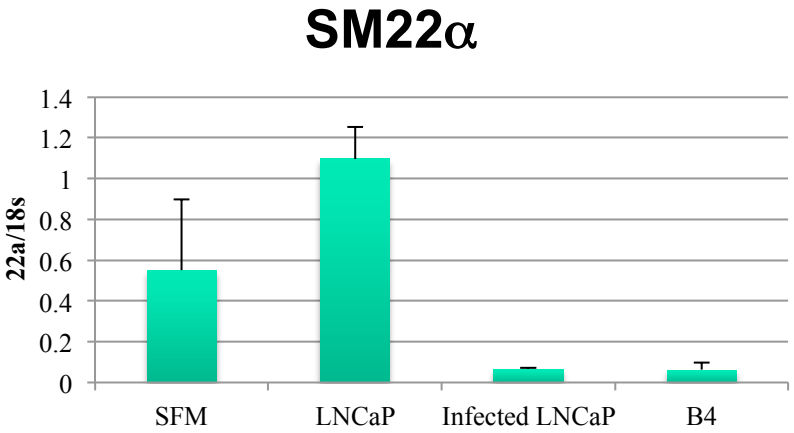


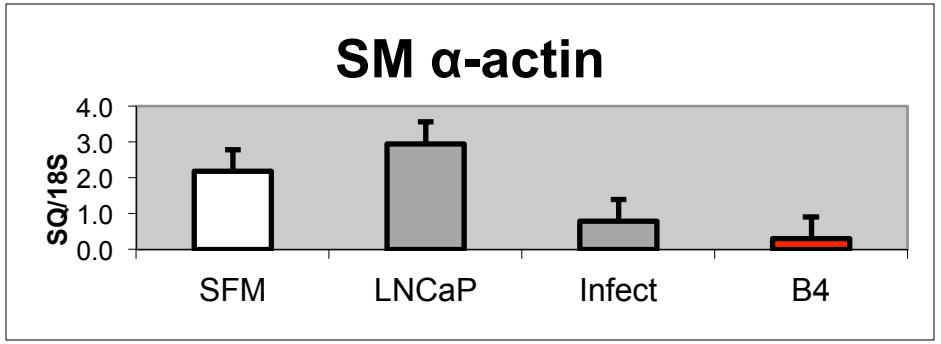


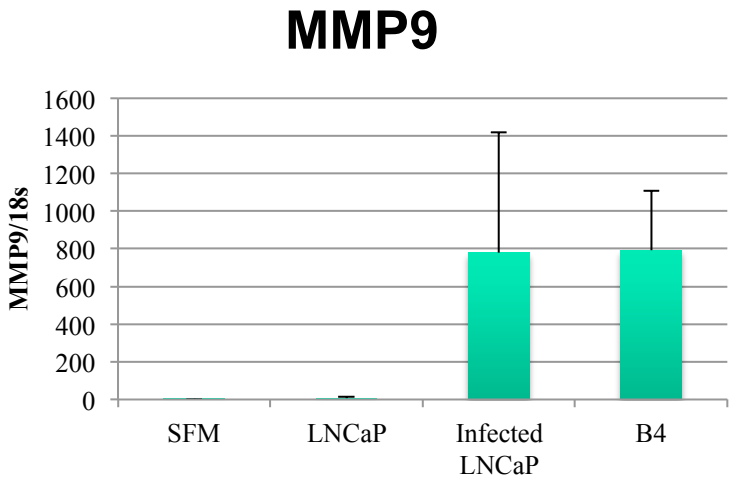
93% overall homology

Gary Owens lab
November 10, 2009
Published 2013

XMRV-2 (B4RV) Infected LNCaP Cells Secreted Factors that Repressed Expression of SMC Marker Genes but Activated MMP9

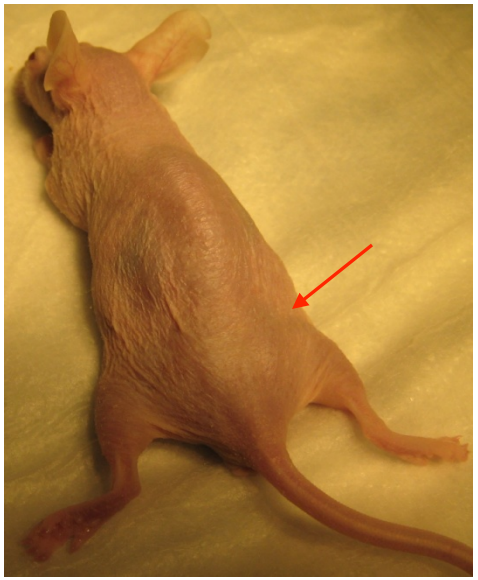


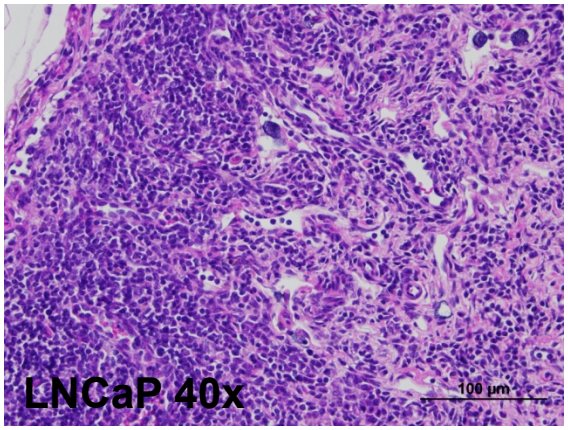




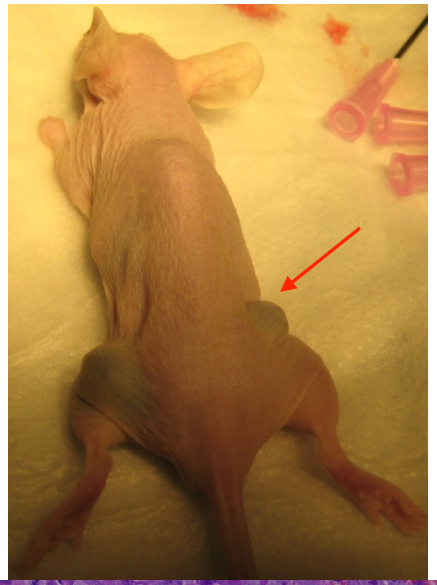
XMRV2 (B4RV) Infected Tumors were Hemorrhagic

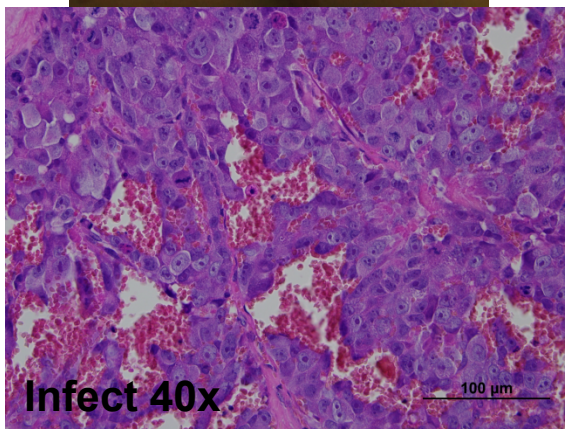
LNCaP





LNCaP-XMRV2 Infect





Owens Conclusions Nov. 2009:

1. B4 tumor cells harbor a retrovirus sharing ~93% homology to XMRV-1 - we have designated it XMRV-2.

2. XMRV-2 is capable of infecting and stably integrating into the genome of a human prostate tumor cell line LNCaP which contains a loss of function deletion mutation within the RNAaseL familial prostate cancer susceptibility gene-1.

3. XMRV-2 infected LNCaP cells show multiple functional changes associated with increased tumorgenicity and/or metastasis including increased growth, altered migration and adhesion, and secretion of factors that decrease vascular SMC differentiation.

Replication competent Retroviruses in 10 Days!

Generation of Multiple Replication-Competent Retroviruses through Recombination between PreXMRV-1 and PreXMRV-2

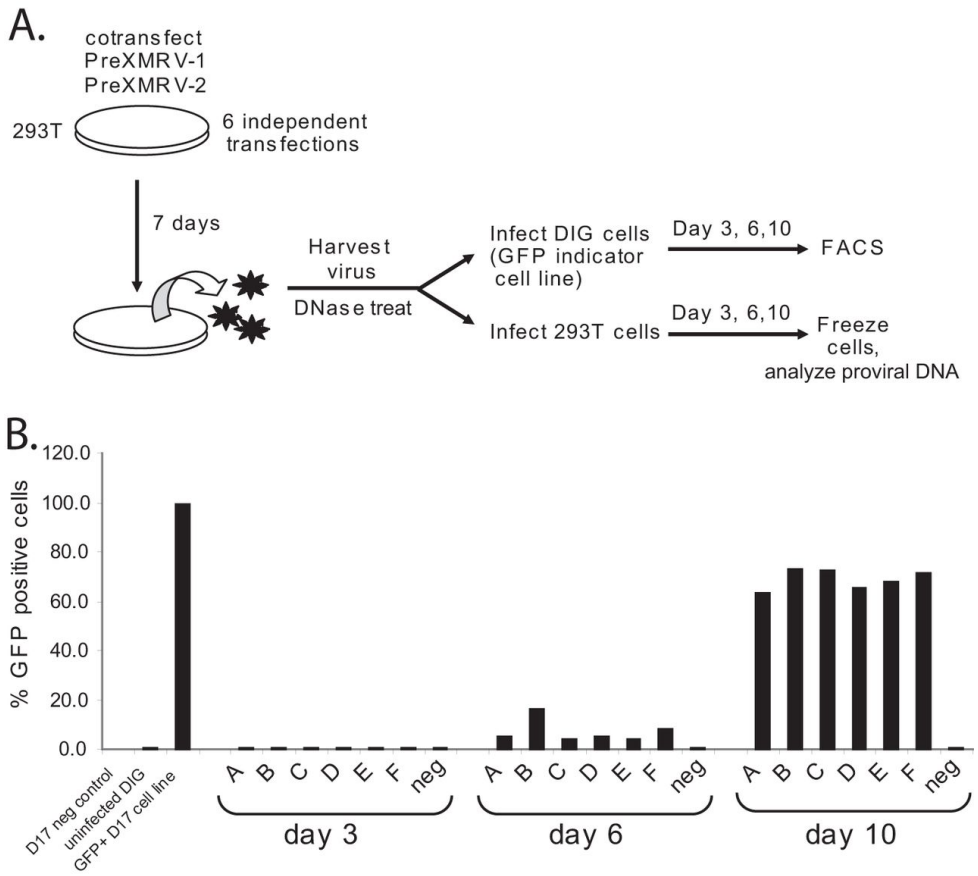
Krista Delviks-Frankenberry,[a] Tobias Paprotka,[a]* Oya Cingöz,[c]* Sheryl Wildt,[d] Wei-Shau Hu,[b] John M. Coffin,[c] Vinay K. Pathak[a]

Viral Mutation Section[a] and Viral Recombination Section,[b] HIV Drug Resistance Program, National Cancer Institute—Frederick, Frederick, Maryland, USA; Program in Genetics, Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, Massachusetts, USA[c]; Harlan Laboratories, Indianapolis, Indiana, USA[d]

- Are two RCRs made by passing human prostate tissue through mouse; XMRV, BRV4 (second recombinant infectious virus occurring in human cells)
- Additional XMRV-like viruses may exist

- They do not have to be the exact sequence of XMRV (VP62)

Cotransfection of PreXMRV-1 and PreXMRV-2 produces replication-competent virus.



Delviks-Frankenberry K et al. J. Virol. 2013;87:11525-11537

Pitfall: RCR XMRVs are not unique or infrequent!

Murgai *et al. Retrovirology* 2013, **10**:34
http://www.retrovirology.com/content/10/1/34

RETROVIROLOGY

RESEARCH Open Access

Xenotropic MLV envelope proteins induce tumor cells to secrete factors that promote the formation of immature blood vessels

Meera Murgai[1], James Thomas[2], Olga Cherepanova[1], Krista Delviks-Frankenberry[4], Paul Deeble[3], Vinay K Pathak[4], David Rekosh[5] and Gary Owens[1*]

Although it is highly unlikely that either XMRV VP62 or B4Rv themselves infect humans and are pathogenic, the results suggest that xenograft approaches commonly used in these studies of human cancer promote the evolution of novel retroviruses with pathogenic properties.

Similar retroviruses may have evolved to infect humans!

Conclusions Owens et al.

- ENV proteins from both viruses impact tumor pathogenesis (change microvasculature)
- Similarities to Vascular Pathologies seen in ME/CFS
- These Microvasculature aberrations caused solely by XMRV ENV protein

Antibodies to SFFV ENV Reproducibly Detected in Human Population

TABLE 3 Equivalent levels of XMRV sequences and anti-XMRV antibodies in CFS (chronic fatigue syndrome) patients and matched controls

			CFS/ME cases ($n = 147$)		Controls ($n = 146$)	
Lab site	Analysis	Sample	Total studied	No. positive (%)	Total studied	No. positive (%)
CDC	RT-PCR	Plasma	147	0 (0.0)	146	0 (0.0)
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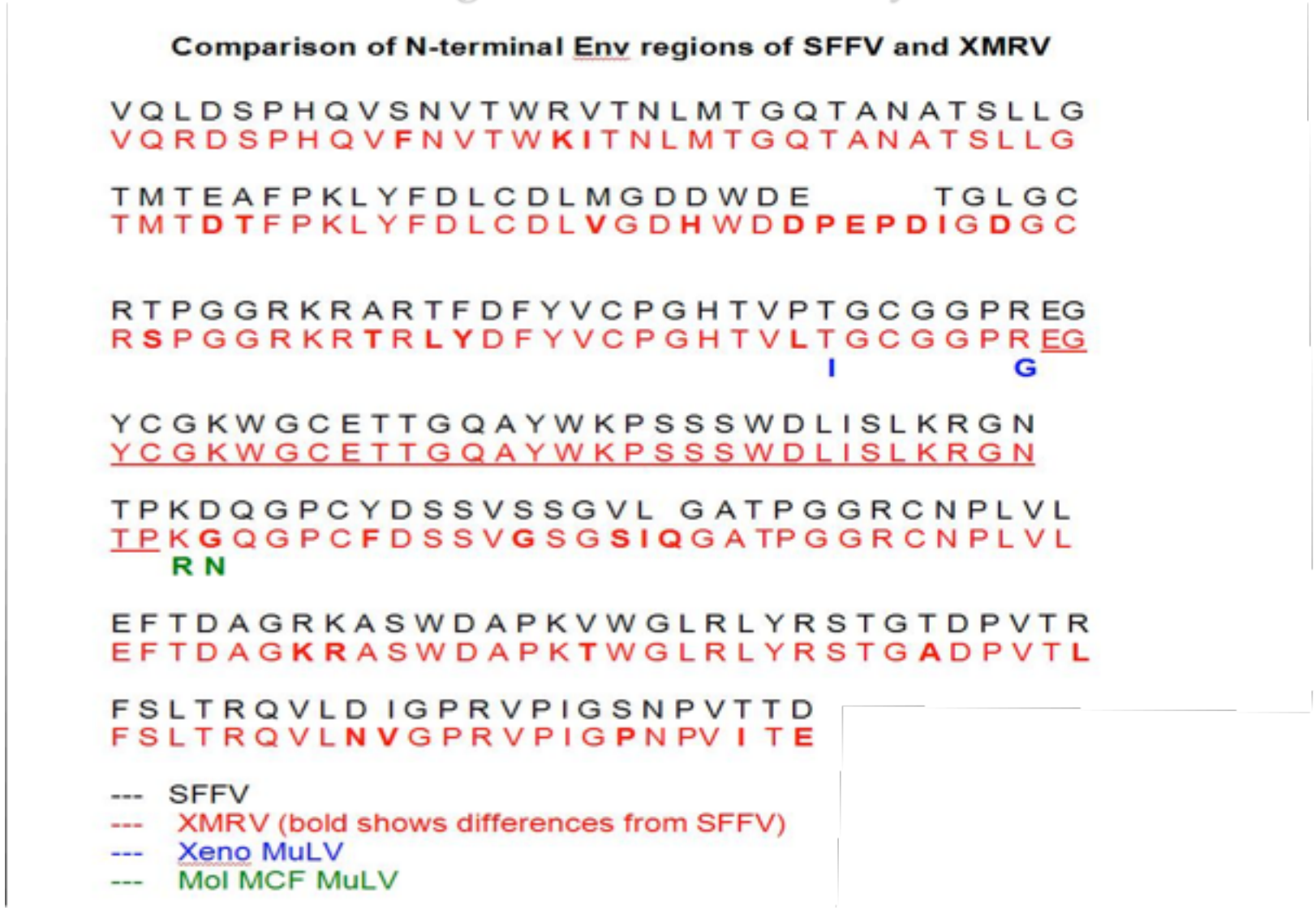
[a] Numbers represent all samples available for analysis at that site.

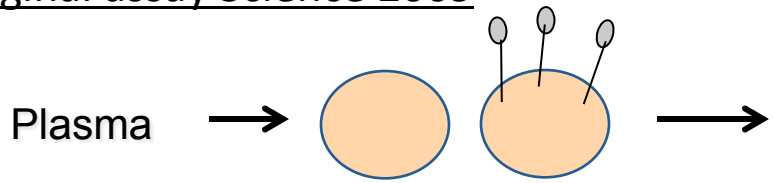
[b] Fifty samples (30 cases; 20 controls) were unable to be assayed because at least one of two aliquots from each set of subject PBMC did not grow in tissue culture.

N-Terminus of SFFV ENV allows recognition of most potential XMRVs using monoclonal antibody 7C10

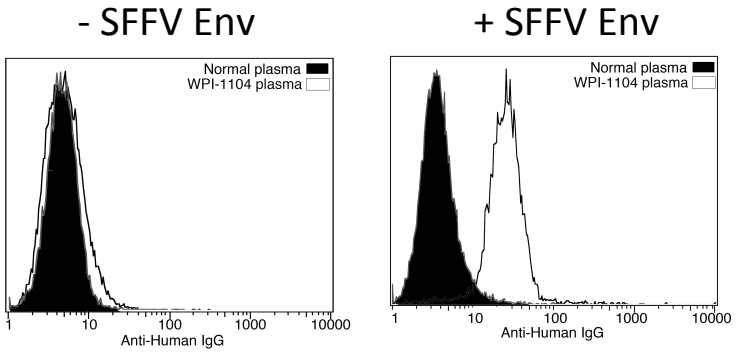


Patient Selection: the biomarker for patient population in our studies is the antibody to gamma retrovirus–ENV

Original assay Science 2009



FACS analysis of Murine Cell Line
+/- SFFV Env



Plasma from CFS patients block binding of SFFV Env rat mAb to the B cell line expressing SFFV Env, demonstrating specificity

An ANTIBODY POSITVE RESULT DOES NOT NECESSARILY SHOW THE PRESENCE OF A REPLCIATION COMEPTENT RETROVIRUS

What Could be the basis of SFFV ENV Reactivity in Man?

Plasmacytoid Dendritic Cells in the Duodenum of Individuals Diagnosed with Myalgic Encephalomyelitis Are Uniquely Immunoreactive to Antibodies to Human Endogenous Retroviral Proteins

KENNY L. DE MEIRLEIR[3] et al., In Vivo, 27:177 (2013)

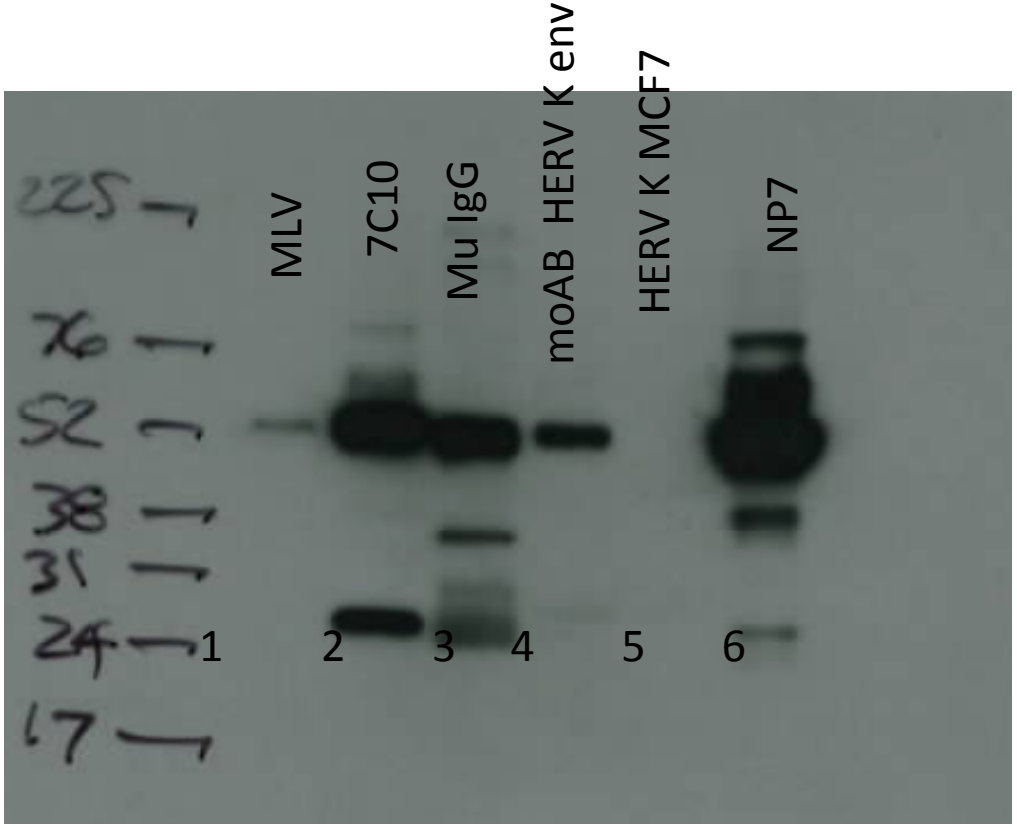
This manuscript claims that SFFV antibodies cross react with human endogenous retroviral proteins like HERV-K env

The only data in the paper using anti HERV protein antibodies is Fig 1, which shows that HERV-K env, HERV-K gag, HERV-FRD env and HERV-R env reactivity. These are the only and last data showing any reactivity with HERV antibodies

In Fig 2, they show reactivity using a rat monoclonal SFFV ENV and goat anti MLV gag) used in the science paper. There is only one cell in which they show these reactivity merge!!!. The rest of the paper is done only with anti SFFV Env

In the results the authors state that this suggests that the anti gammaretroviral antibodies were cross-reactive with the HERV antigens. No a compelling evidence is given to support this statement is true. They then proceed to use only anti SFFV as a marker for HERV antigen reactivity in the rest of the paper.

Lack of cross reactivity between Herv K and SFFV Env



Lane 1-4= NP7 IP with 7C10
Blot with Indicated antibodies

Lane 5 and 6 = cell lysates

NP-7 - mouse line expressing lots of SFFV gp55
MCF-7 human breast line expressing HervK env
7C10 rat monoclonal Antibody against SFFV gp55

The replicative activity of human endogenous retrovirus K102 (HERV-K102) with HIV viremia

Marian P. Laderoute[a,b], Antonio Giulivi[a,b], Louise Larocque[a], Deana Bellfoy[a], Yangxun Hou[a], Hong-Xing Wu[a], Keith Fowke[c], Jun Wu[a] and Francisco Diaz-Mitoma[d]

Results: Both the peptide serology and ddCt qPCR excess ratio methods suggested the activation of HERV-K102 in about 70–80% of HIV viremic cases whereas only 2–3% of normal healthy adults had marginally activated HERV-K102 ($P < 0.0001$). Moreover, by

Conclusions: Our work uniquely suggests the common activation of HERV-K102 with HIV viremia and may be first to directly demonstrate HERV-K102 cDNA production *in vivo*. The potential implications of the induction of HERV-K102 activation and replication for the prevention and control of HIV are discussed.

AIDS 2007, **21**:2417–2424

ARVs provide therapeutic benefit in some patients with autoimmune, Neuroimmune Disease and Cancer

Beneficial Effects could be against:

- An exogenous Replication Competent Retroviruses
- An expressed endogenous virus in an immune compromised individual
- A defective virus expressing only viral proteins
- Aberrantly expressed cellular RNA including microRNA (regulatory)

We found retroviruses in 85 percent of the sample pools. Again, it is very difficult to know whether or not this is clinically significant or not. And given the previous experience with retroviruses in chronic fatigue, I am going to be very clear in telling you, although I am reporting them in Professor Montoya's samples, neither he, nor we, have concluded that there is a relationship to disease.[582]

Dr. Ian Lipkin in a public conference call with the Centers for Disease Control on September 10, 2013

Pitfall : Unsupported conclusions hurt the field

Pitfall : findings were reproducible using different technologies?

Different technologies yield different results

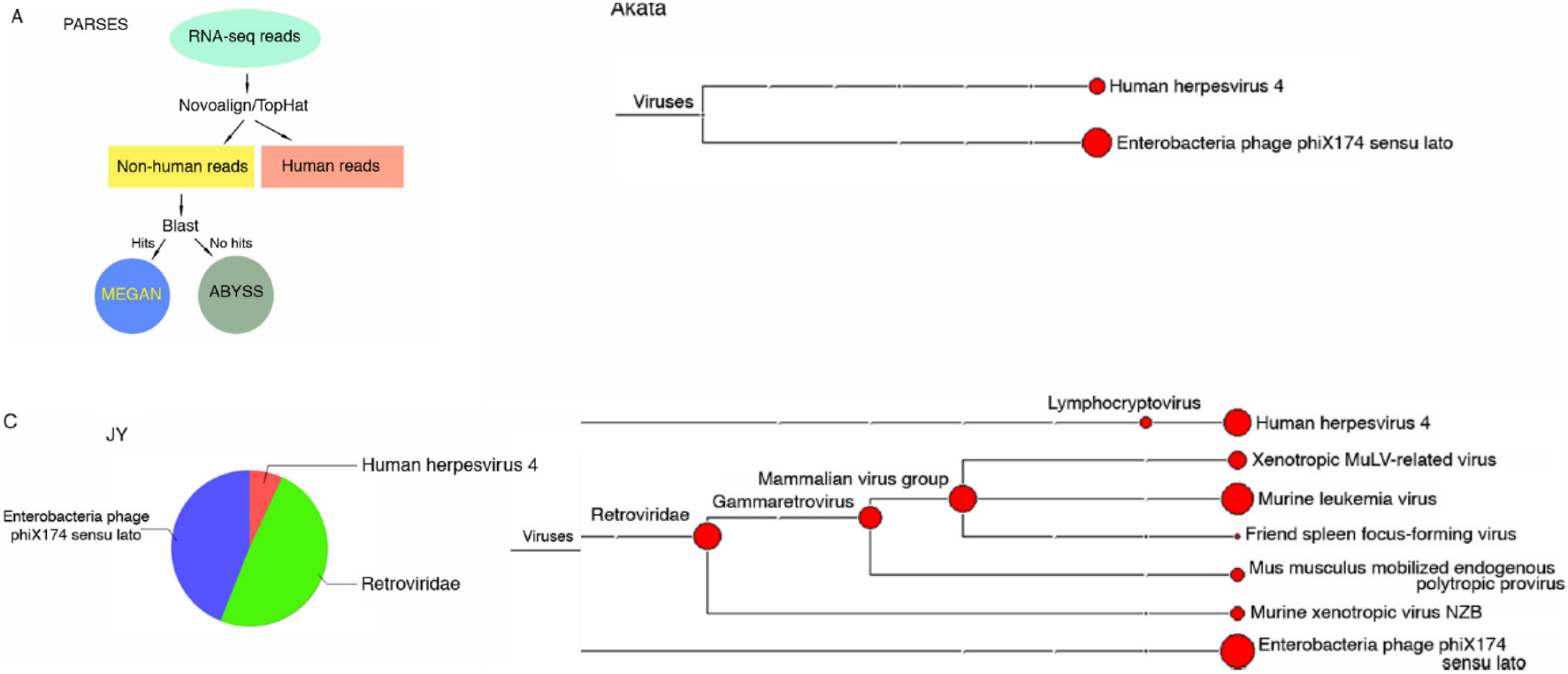
Would more sensitive technologies find retroviruses in other well –characterized CFS cohorts ?

JVI
Journals.ASM.org

Detection of Murine Leukemia Virus in the Epstein-Barr Virus-Positive Human B-Cell Line JY, Using a Computational RNA-Seq-Based Exogenous Agent Detection Pipeline, PARSES

Zhen Lin,[a] Adriane Puetter,[a] Joseph Coco,[b] Guorong Xu,[b] Michael J. Strong,[a] Xia Wang,[a] Claire Fewell,[a] Melody Baddoo,[a] Christopher Taylor,[b] and Erik K. Flemington[a]

Tulane University Health Sciences Center and Tulane Cancer Center, New Orleans, Louisiana, USA,[a] and University of New Orleans, New Orleans, Louisiana, USA[b]



3 B-Cell Lines Derived Directly From CFS Patients' PBMCs

- **CFS patient PBMCs were cultured; 3 samples developed into immortalized cell lines**
- **All three showed high CD20+ expression and two showed high CD23+ expression.**
- **All three showed strong similarity to B cells seen in patients.**

Marker	MCL	WPI 1125	WPI 1186	WPI 1143
CD5	+	+	+	+
CD23	-	-	+	+
CD19	+	+	+	+
CD20	+	+	+	+
FMC7	+	+	-	-
CD3	-	-	-	-
CD4	-	-	-	-
CD7	+	-	-	-
CD8	-	-	-	-
CD10	-	-	-	-
CD38	+	+	+	+
CD45	+	+	+	+
CD56	-	-	-	-
CD122	-	-	-	-
HLA-DR	+	+	+	+
Lambda	+	+	-	-
Kappa	+	+	+	+

These Cell lines were developed from CFS patients. One, (1125) developed MCL; one (1186) was developed from a bone marrow biopsy, 3rd a CLL

In Chronic Disease, Viruses Rarely Travel Alone

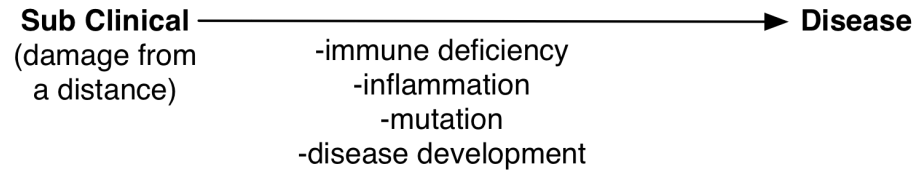
Table 1. Relations between coinfecting viruses and HIV-1

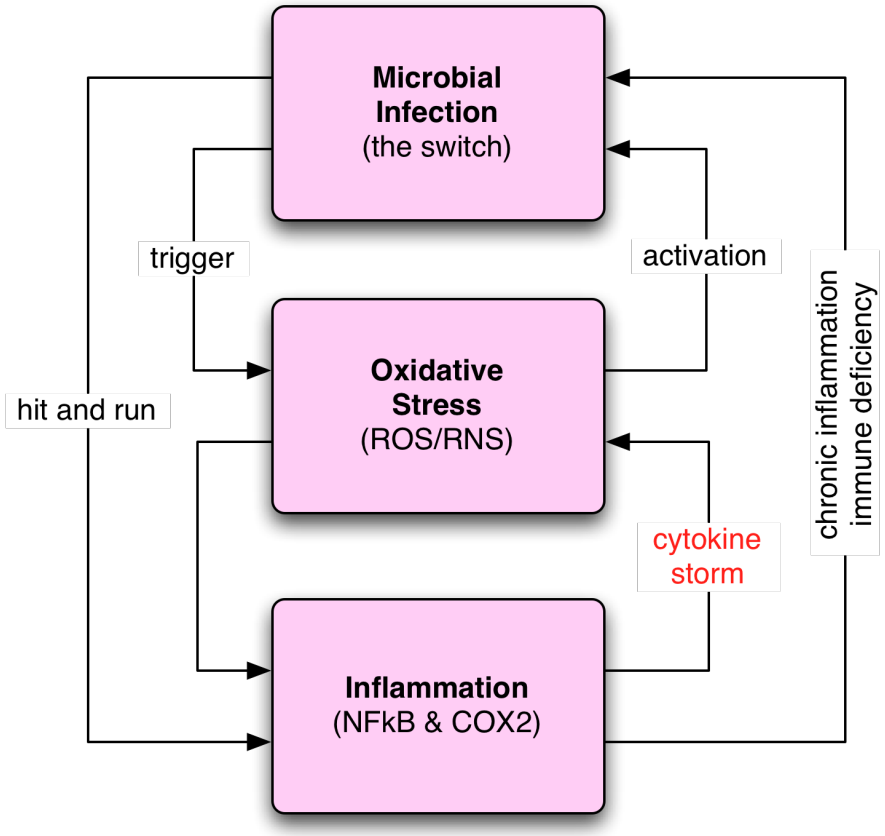
Virus	Mechanisms	Potential effects on HIV-1*
HBV	Several anti-HBV drugs are also HIV-1 RT inhibitors	HBV treatment also decreases HIV replication; emergence of HIV-1 RT-resistant mutants
HCV	Systemic immune activation	Facilitation of HIV-1 replication
GBV-C	CCR5 and CXCR4 downregulation; induction of RANTES and SDF-1	Suppression of HIV-1 replication
HTLV-1	LTR transactivation; induction of CC chemokines	Facilitation of HIV-1 replication; suppression of HIV-1 R5 replication
HTLV-2	Induction of CC chemokines; decreased systemic immune activation	Suppression of HIV-1 replication
HIV-2	Cross-reactive immune response; induction of CC chemokines	Decreased HIV-1 acquisition; suppression of HIV-1 R5 replication
JCV	Suppression of Tat functions	Suppression of HIV-1 replication in vitro
Measles virus	Induction of RANTES; blockage of CD4 T-cell cycle	Suppression of HIV-1 replication
HSV-2	Genital ulceration; increased LTR transactivation	Increased HIV-1 transmission; facilitation of HIV-1 replication
CMV	Increased HIV-1 load in semen; induction of chemokines, virokines, and viroceptors	Increased HIV-1 transmission; variable results were reported for the net effect
HHV-6	Induction of RANTES, virokines, LTR transactivation, CD3 and CD46 downregulation	Decreased replication of HIV-1 R5 ex vivo; net effect on HIV-1 replication in vivo to be studied
HHV-7	Downregulation of CD4	Decreased replication of HIV-1 R5 ex vivo; net effect on HIV-1 replication in vivo to be studied
HHV-8	Chemokines and virokines	Net effect of HIV-1 replication in vivo to be studied

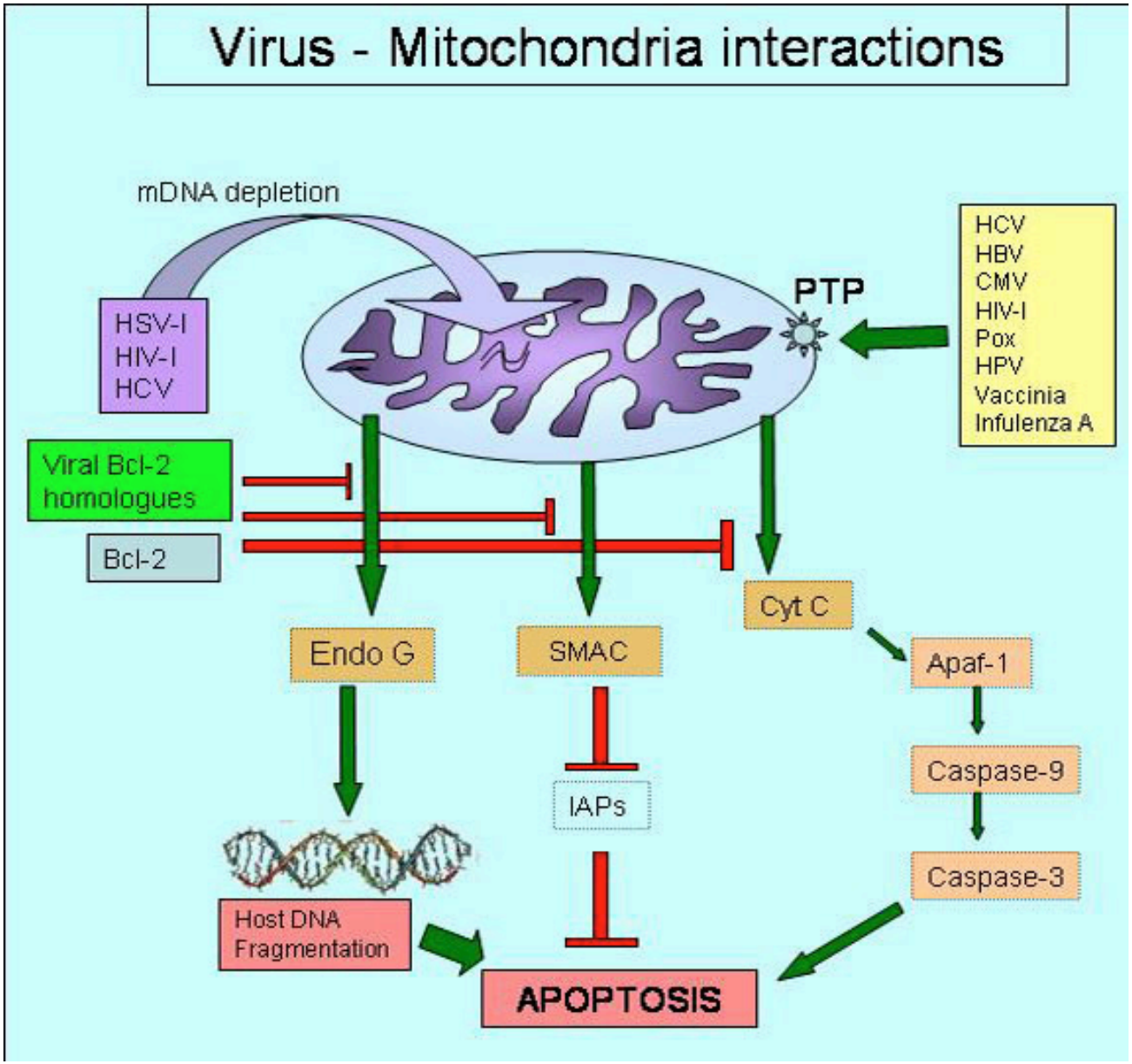
Hypothesis:

A family of human gamma retroviruses that selectively infect individuals with loss of function polymorphisms/mutations of the cancer (disease) susceptibility gene RNASEL, and that infection increases abnormal properties such as growth and/or the metastatic properties of the tumor, stromal cells and immune cells.

Key Contributors to Chronic Diseases







Recent advance in genomic technologies have identified ~1000 nuclear genes that regulate mitochondrial function …

New Technologies: Comprehensive Sequence Analysis of Nuclear mitochondrial genes

- NGS for variants in the nuclear mitochondrial exome that contribute to neurological disorders whose symptoms resemble mitochondrial disease.

Case Report Results:

- Abnormal autosomal dominant Variant was found in SCN4A gene that is likely a pathological mutation
- Pathological mutations found in two other patients also with multiple functional conditions (ME/CFS)

Incidental finding:

This patient has three variants in *RNASEL*. Mutations in this gene have been associated with predisposition to prostate cancer and this gene is a candidate for the hereditary prostate cancer 1 (HPC1) allele. One of these variants, p.E265*, has been reported in the literature in 4 brothers with prostate cancer.

New Concepts: drugs targeting channelopathies (Diamox) and key mitochondrial targets mTOR

Chronic innate immune activation leads to inflammation and immune dysregulation

- Presence of CD20+ CD23+ B cells, not normally seen in healthy subjects, and activated APCs in some ME/CFS, CLD patients are similar to the myeloid and B cell defects described in other retroviral associated Diseases.
- The significant changes in the myeloid compartment including phenotypes are suggestive of activation of Antigen Presenting Cells (APCs) .
- Increased , γδT Cells clonality in ME/CFS, CLD, CLL, MCL
- Increased NKT compartment together with increased NKT to NK ratio.
- Major changes in inflammasome

Conclusion

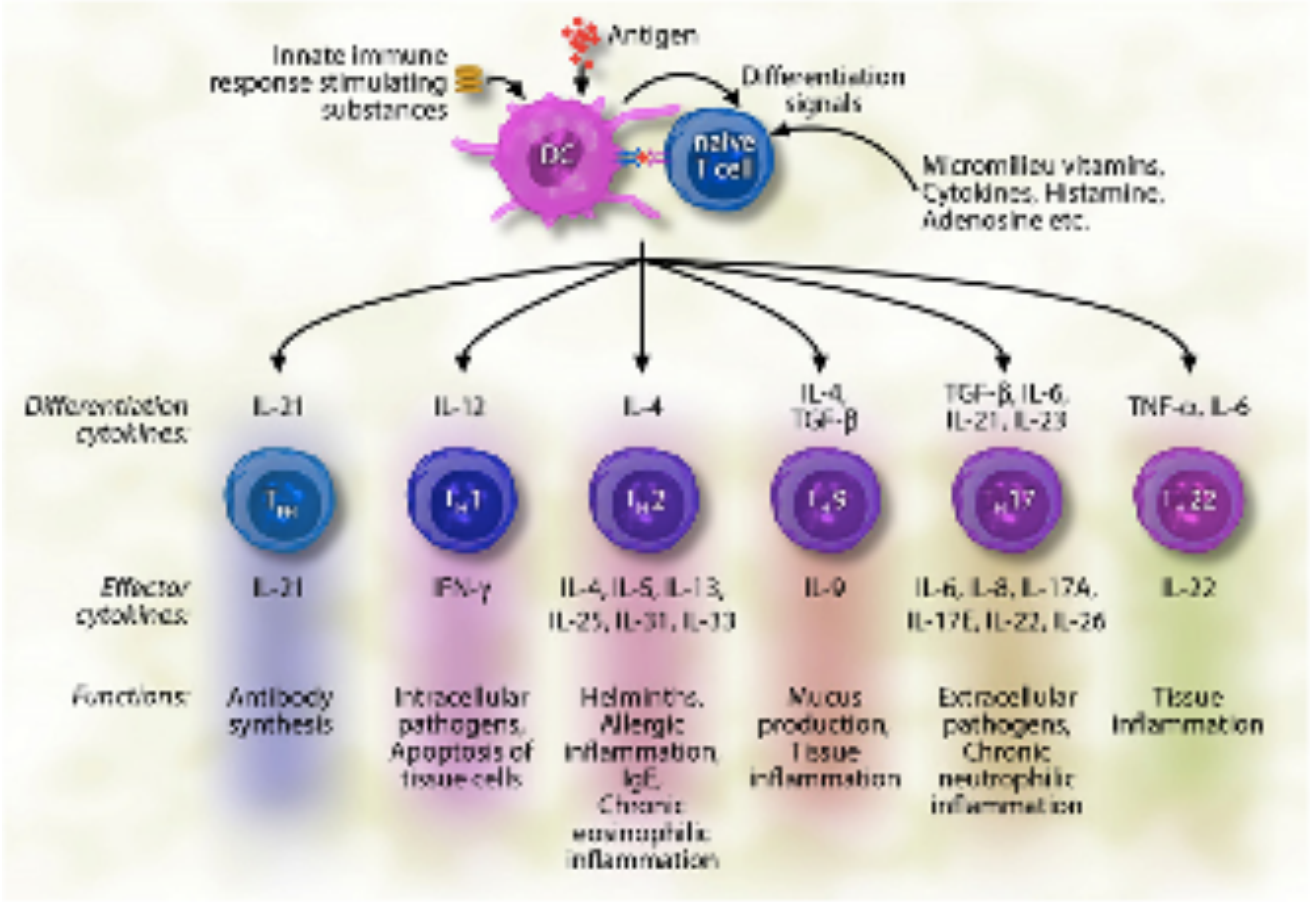
Results suggests a similar Disease cycle of chronic innate immune activation leading to an immune dysregulation and chronic immunosuppression and may guide future research towards the development of biomarkers and treatment targets

Emerging Concepts

- Recombination events in animal and human cells can generate families of infectious related gamma retroviruses
- Greatest concern is that they may acquire the ability to infect humans
- Are XMRV sequences and proteins important in human disease pathogenesis?
- Therapies to counteract environmentally induced aberrant gene RCR expression, inflammation immune dysregulation urgently need to be addressed

Thank you

Cytokine signatures can serve as a diagnostic fingerprint of pathogens and
Biomarkers for therapy



Additional Distinct Signatures in subgroups of ME/CFS

IL9	T and Mast Cell growth factor Inhibits Th1 cytokines Proliferation of CD8 T cells Chemokine production Mucus production in Bronchial epithelial cells	Helminth infections Hodgkins lymphoma Asthma Food Allergy
IL16	Chemotaxis Modulation of T cell response	Increased in inflammatory disease including: RA, IBD, Chron's Disease, hepatitis C infection, tuberculosis Inhibits HIV
IL17 (five isoforms)	Induction proinflammatory cytokines, chemokines, metalloproteinases Recruitment of neutrophils	RA, IBD, MS, allergic asthma Inflammatory cardiomyopathy
IL18	Induction of IFNg in presence of IL12 Enhances NK cell cytotoxicity	Autoimmune diseases/Inflammatory disorders: MS, RA, psoriasis, type I diabetes
*IL-21	Role in adaptive B/ T cell function Including antibody production	Cancer SLE, RA
*IL-22	Pathogen defense Wound Healing Tissue Regeneration	Psoriasis, IBD, Cancer

Pathways of Retrovirus Elicited Pathogenesis

- Inflammation / hormone regulation
- ROS / RNS
- Immune deficiency
- Epigenetics — change in gene expression without a DNA change
- Insertional mutagenesis
- RVs can be vertically transmitted
- RVs can recombine with aberrantly expressed endogenous RVs creating RCRs

November 2012

LETTER

doi:10.1038/nature11599

Resurrection of endogenous retroviruses in antibody-deficient mice

George R. Young[1], Urszula Eksmond[1], Rosalba Salcedo[2], Lena Alexopoulou[3], Jonathan P. Stoye[4] & George Kassiotis[1]

Our results shed light onto a previously unappreciated role for immunity in the control of ERVs and provide a potential mechanistic link between immune activation by microbial triggers and a range of pathologies associated with ERVs, including cancer.

Case report 3058 : plasma Gag RNA + and seropositive CFS/CLL