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Distribution of Xenotropic Murine Leukemia Virus-Related Virus (XMRV) Infection in Chronic Fatigue Syndrome and Prostate Cancer

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Abstract

In 2006, sequences described as xenotropic murine leukemia virus-related virus (XMRV) were discovered in prostate cancer patients. In October 2009, we published the first direct isolation of infectious XMRV from humans and the detection of infectious XMRV in patients with chronic fatigue syndrome. In that study, a combination of classic retroviral methods were used including: DNA polymerase chain reaction and reverse transcriptase polymerase chain reaction for gag and env, full length genomic sequencing, immunoblotting for viral protein expression in activated peripheral blood mononuclear cells, passage of infectious virus in both plasma and peripheral blood mononuclear cells to indicator cell lines, and detection of antibodies to XMRV in plasma. A combination of these methods has since allowed us to confirm infection by XMRV in 85% of the 101 patients that were originally studied. Since 2009, seven studies, predominantly using DNA polymerase chain reaction of blood products or tumor tissue, have reported failures to detect XMRV infection in patients with either prostate cancer or chronic fatigue syndrome. A review of the current literature on XMRV supports the importance of applying multiple independent techniques in order to determine the presence of this virus. Detection methods based upon the biological and molecular amplification of XMRV, which is usually present at low levels in unstimulated blood cells and plasma, are more sensitive than assays for the virus by DNA polymerase chain reaction of unstimulated peripheral blood mononuclear cells. When we examined patient blood samples that had originally tested negative by DNA polymerase chain reaction by more sensitive methods, we observed that they were infected with XMRV; thus, the DNA polymerase chain reaction tests provided false negative results. Therefore, we conclude that molecular analyses using DNA from unstimulated peripheral blood mononuclear cells or from whole blood are not yet sufficient as stand-alone assays for the identification of XMRV-infected individuals. Complementary methods are reviewed, that if rigorously followed, will likely show a more accurate snapshot of the actual distribution of XMRV infection in humans. **(AIDS Rev. 2010;12:149-52)**

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Introduction

Recently, through ViroChip analysis, sequences corresponding to a previously unknown retrovirus were detected in samples of human prostate tissue[1]. Retroviral DNA was isolated from a minority of the samples in the nonmalignant (stromal) cell microenvironment. The DNA sequences were found to be distantly related to HIV-1 and human T-lymphotropic virus type 1 (HTLV-1), but very closely related to a type of retrovirus named xenotropic murine leukemia virus (X-MLV)[2,3]. As a result, the newly detected virus was named xenotropic murine leukemia virus-related virus (XMRV). Despite a high degree of sequence identity with X-MLV, XMRV is clearly distinct from all previously described X-MLV and is not present as an endogenous retrovirus in the mouse genome. Different isolates of XMRV from prostate cancer and chronic fatigue syndrome (CFS) patients published to date show very little sequence variation and form a distinct branch following phylogenetic analysis[4]. Additional evidence that XMRV is a human virus that can infect humans includes the mapping of viral integration sites within human chromosomes[5], the presence of viral antibodies in human plasma[4,6], and the presence of viral proteins and nucleic acids in fresh or frozen tissue[4,6,7].

XMRV in prostate cancer: positive and negative studies

The association between XMRV and prostate cancer was strengthened in two recent reports of the presence of XMRV proteins, as well as nucleic acid sequences, in prostate cancer tissue[7]. The study by Schlaberg, et al.[7], in contrast to the original report of XMRV in stromal cells of prostate tumors[1], reported that expression of XMRV was observed in the malignant cells in these samples, with evidence of XMRV infection found in almost 25% of samples analyzed. The principal methods for viral detection were either by polyclonal antisera to XMRV virions (23% positive) or by DNA PCR (6% positive) of the integrase region. Detection of XMRV proviral DNA via polymerase chain reaction (PCR) in this study supported the protein data, but was less sensitive. Subsequently, Arnold, et al.[6] described a novel serologic assay detecting neutralizing antibodies to XMRV, resulting in 27.5% seropositivity in prostate cancer patients. The authors confirmed the serologic results by PCR as well as fluorescence *in situ* hybridization (FISH), another unambiguous XMRV detection method, thus demonstrating the value of multiple independent methods.

DNA PCR has been used as the sole or primary assay method for XMRV in several studies that failed to detect XMRV in prostate cancer patients or tumors. In a study from Johns Hopkins, prostate tissue DNA from 338 prostate cancer patients was negative upon nested PCR for *gag* sequences[8]. Two independent studies of prostate cancer tissue samples from Germany found no XMRV using DNA PCR, reverse transcriptase (RT) PCR and an ELISA for XMRV Gag and Env antibodies[9,10]. Given the discordance between such findings, determining a role for XMRV in prostatic disease will require more extensive epidemiological examination of incidence in both specialized and general human populations as well as more sensitive assays for XMRV detection. Isolation of infectious XMRV from prostate cancer patients has not been published.

XMRV in chronic fatigue syndrome: positive and negative studies

Our recent study[4] found that sequences to XMRV *gag* and/or *env* were present in a high percentage (67%) of individuals with CFS, according to either single-round DNA PCR and/or nested RT PCR. Evidence of XMRV infection *in vivo* was detected in phytohemagglutinin and interleukin 2 (PHA/IL-2) activated leukocytes isolated from peripheral blood. Further studies revealed that patients' T-cells grown in IL-2 and B-cells grown in CD40L were infected *in vivo* with XMRV and that T-cell lines could be infected *in vitro* with XMRV. More importantly, this study also demonstrated that infectious virus was present in infected individuals; XMRV could be transmitted either cell-associated or cell-free from both activated lymphocytes and plasma from infected individuals by passage to lymph node carcinoma of the prostate (LNCaP), a prostate cancer cell line robust for XMRV replication[11,12]. Plasma from these individuals also had antibodies specific for the envelope protein of this type of retrovirus, showing that XMRV can elicit an immune response. In contrast to subsequent reports, our 2009 paper was not a survey of CFS using PCR or a search for antibodies to XMRV proteins in patients; instead, several different assays for the presence of XMRV nucleic acids, proteins, and infectious virus were performed. A list detailing the assays that were performed on each of the samples in the initial study has recently been published[13].

Three studies from Europe published in 2010 have not found XMRV in CFS patients. In the first study, scientists from the United Kingdom found no *gag* sequences using PCR on whole blood DNA from 186 CFS patients[14].

In the second study, PCR assays for *gag* and *env* using peripheral blood mononuclear cell (PBMC) DNA from UK CFS patients found all 170 samples negative by PCR[15]. Groom, et al.[15] also used a neutralizing viral assay and found that 26 of the sera had neutralizing activity. The 26 positive samples also neutralized Env proteins of other viruses; therefore this activity is not specific. In a Dutch study, PCR for *int* and *gag* sequences performed on PBMC DNA of 32 CFS patients was negative[16]. Examination of samples by DNA PCR *gag* and *pol* sequences for XMRV was also negative in a recent study reported by the Centers for Disease Control[17]. Plasma from the patients and controls was also examined for the presence of antisera that could react with MLV proteins, with largely negative results[17].

Differences between the studies of XMRV in human populations

As detailed above, we implemented four different ways of establishing XMRV infection in the blood products of CFS patients. We described detection of XMRV by single-round DNA PCR of unstimulated PBMC from our most viremic patients[4]. Although it is the least sensitive assay for XMRV, it is also the least likely to give false positive results resulting from contamination of samples with small amounts of mouse DNA, which contains endogenous retroviral sequences that are similar to XMRV. The fact that some patients exhibited readily detectable provirus with only single-round PCR provided strong evidence for XMRV infection.

Nested PCR is a more sensitive method than single-round PCR for detection of low amounts of viral sequence. Nevertheless, 96% of 210 normal samples tested in our 2009 study[4] were negative upon nested PCR. Of the 101 CFS samples, 11 were positive upon single-round DNA PCR for *gag* sequences. Of the 90 samples from which no viral sequences were obtained by single-round DNA PCR, 60 were positive for *gag* sequences by nested PCR from cDNA.

There could be multiple reasons for the disparity in results between different studies of the prevalence of XMRV in human populations. Geographical differences in the distribution of XMRV could account for at least some of the discordant results. For example, the prevalence of another retrovirus, HTLV-1, profoundly differs around the world[18,19].

Secondly, the existence of divergent XMRV or related viruses is possible. Variant viruses could easily be missed by many of the assays; PCR data is particularly susceptible to sequence variation. Although a PCR assay may work well when a known viral sequence is used as positive control, the sensitivity of the assay may be far less in clinical samples, where sequence variation may confound results. Unfortunately, the primers and PCR assays used in the reports that failed to find XMRV in clinical samples did not utilize certified positive clinical samples as positive controls, even though authors in three of four negative studies were offered samples from Lombardi, et al.[4]. Notably, the genetic variation between full-length XMRV sequences currently available is 0.03%, despite the fact that they are obtained from samples from patients exhibiting two vastly different diseases from geographically distinct areas. This variation is smaller than the variation observed between HTLV-1 isolates[20]. Analysis of many more samples will be needed to determine the actual extent of sequence variation in XMRV that infects human populations.

Thirdly, whether individuals infected with XMRV form antibodies to the viral proteins, and if so, to which viral proteins, remains an open question. Lombardi, et al. detected antibodies specific to XMRV Env[4,13] in 20-80% of CFS patient plasma tested vs. healthy controls in whom the presence of antibodies to Env are between 2-7%. Further experiments need to be performed to determine at what frequency individuals infected with XMRV exhibit detectable antibodies to XMRV proteins; thus, the presence or absence of antibodies to MLV proteins cannot presently be used to determine whether or not an individual is infected with XMRV.

Fourth, the clinical criteria for patient selection vary widely in the CFS field. Lack of uniformity between patient populations likely plays a major role in discordant results. In one study, Van Kuppeweld, et al.[16] employed the older Oxford criteria[21]. The Oxford definition does not define myalgic encephalomyelitis/chronic fatigue immune deficiency syndrome, but instead defines idiopathic chronic fatigue, i.e. tiredness. The patients selected in two negative studies were diagnosed according to the 1994 International "Fukuda" criteria[22]. The criteria for the 1994 case definition are based primarily on symptoms and not on physical signs or chemical or immunological tests. Suggestions have been made to subgroup CFS based on clinical and immunological data[23] such as those discussed in Lombardi ,et al., which also used the most rigorous case definition established, the Canadian Consensus Criteria (CCC)[22-24]. Patients who satisfy the CCC also meet the less rigorous Fukuda criteria, but given the heterogeneity and complexity of the disease, are unlikely to define homogenous subgroups and thus there are likely to be significant differences within patient populations in the negative studies, which clearly confound the interpretation of these

data. To further confound the ability to draw meaningful conclusions regarding XMRV in CFS patients, the subjects defined as patients in Switzer, et al.[17] were identified through Georgia and Wichita registries of population-based studies that were described as meeting criteria clearly not that of Fukuda, et al.[22]. Moreover, the authors selected study subjects that were not diagnosed by a physician and did not satisfy the more rigorous CCC.

Additional patient data at the time of sample collection, such as immune status, may be useful. For example, XMRV sequences were found in the respiratory tract secretions of 2-3% of healthy donors and 10% of immuno-compromised donors[25], suggesting that the immune status of individuals could play a role in XMRV detection.

Conclusion

Despite the association of XMRV with both prostate cancer and CFS, many questions remain regarding the prevalence of XMRV in the human population and the incidence of XMRV in disease. We have found that the most sensitive blood-based assays for XMRV detection in decreasing order are: (i) plasma or activated PBMC co-cultured with LNCaP followed by virus isolation and sequencing or nested PCR for *gag;* (ii) detection of viral proteins in activated PBMC by flow cytometry (FACS) or in tissue by immunohistochemistry[4]; (iii) or plasma antibodies for Env; (iv) *in situ* hybridization by FISH[6], RT PCR in plasma or cDNA from PBMC, with direct DNA from inactivated PBMC being the least sensitive method. We remain confident that applying multiple methods and rigorously following established protocols that have successfully detected XMRV will reveal a wider distribution of XMRV infection in humans than has currently been reported. The study of XMRV is in its infancy and much more information is needed concerning replication and pathogenesis of this virus in humans. Priorities for research are development of sensitive nucleic acid and serologic tests for high-throughput screening and the development of therapeutics for clinical testing. The development of standardized, highly sensitive assays for XMRV detection and the existence of a panel of positive clinical samples for use as controls are clearly essential to make progress in determining the prevalence of XMRV in various human populations and its possible role in disease.

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