XMRV, prostate cancer and chronic fatigue syndrome

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Background: A new retrovirus, xenotropic murine leukaemia virus-related virus (XMRV), was identified in 2006 and an association was claimed between it and a genetic polymorphism predisposing to cancer of the prostate. In 2009 the same virus was identified in a cohort of patients with chronic fatigue syndrome (CFS). In 2010 a second related virus was identified in a separate group of CFS patients. A series of studies from disparate geographical areas have failed to substantiate this work. Most recently several papers have suggested that the detection of these viruses was explained by laboratory contamination.

Sources of data: All papers including the wording XMRV were abstracted from the NIH library of medicine database and included in the analysis.

Areas of agreement: XMRV is a newly described retrovirus whose nucleic acid has been identified in samples from patients with both prostate cancer and CFS.

Areas of controversy: Opinions differ as to whether the detected nucleic acid indicates infection with this virus in this disease or whether laboratory contamination of samples accounts for its presence.

Growing points: An increasing number of papers now refute the association of XMRV with human disease in humans although there is some evidence of serological reactivity to the virus. While it is unlikely that XMRV is a major cause of either prostate cancer or CFS, it can infect human cells and might yet have a role in human disease.

Areas timely for developing research: Further studies to either prove or disprove the disease association of the virus are ongoing.

Keywords: XMRV/prostate cancer/chronic fatigue syndrome

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Is XMRV implicated in the acquisition and development of prostate cancer?

Cancer of the prostate is the most common non-skin cancer in men comprising 25% of all male cancers in the UK and 28% in the USA, where it is the second-leading cause of cancer-related death in this group. A major risk factor is age; 80% of males over the age of 80 have malignant cells in their prostate when examined post-mortem. In younger age groups, however, factors such as ethnic origin and family history are more important and it is estimated that 30–40% of early onset (<55 years) prostate cancer is due to inherited factors. Genes involved in the immune response, such as ELAC2 and MSR1, have been implicated raising the possibility that a dysfunctional response to an infectious agent may be involved. Several recent studies have shown a link between germline mutations in another immune response gene, the enzyme RNase L, and susceptibility to prostate cancer (references within). One such RNase L variant, which contains a glutamine instead of an arginine (R462Q), is commonly found within the population and has a 3-fold reduced enzymatic activity compared with the wild type. RNase L can degrade viral (and cellular) RNA and is part of the anti-viral type I interferon (IFN) response pathway. It is induced in response to IFN and activated in the presence of viral double-stranded RNA. Activation ultimately leads to apoptosis of virally infected cells. Cells homozygous for the R462Q allele have been shown to be impaired in their ability to apoptose; hence the increased incidence of prostate cancer in individuals homozygous for the R462Q allele was initially suggested to be simply due to a reduced susceptibility to apoptosis. Diminished RNase L activity could, however, also lead to impaired antiviral protection; viruses are estimated to cause ~20% of human cancers. Thus an otherwise harmless virus might become a plausible agent for tumour causation in this group.

The previously monumental task of seeking an unknown pathogen as a disease association has become tractable in recent years using array technology. Urisman et al. sought the presence of viral nucleic acid within prostate cancer tissue by extracting the RNA from some of their cohort of R462Q homozygous or wild-type RNase L prostate cancer samples and hybridized it to a DNA microarray containing conserved sequences from all known groups of animal, bacterial and plant viruses. Seven of eleven samples from patients homozygous for the R462Q mutation contained RNAs that hybridized to gammaretroviral DNA sequences, indicating the presence of a gammaretrovirus in these tissues. One sample of five from wild-type RNase L prostate cancer
tissue also contained gammaretroviral RNA. The agent was identified as a novel virus and termed xenotropic murine leukaemia virus-related virus (XMRV), and when the entire cohort was examined by nested RT–PCR, looking for the presence of XMRV, 40% (of 20) of tumours homozygous for R462Q were positive, whereas it was absent from all but 1 of 66 wild-type/heterozygous tumours. The authors used a combination of immune and fluorescent [fluorescence in situ hybridization (FISH)] staining techniques to visualize the infected cells, and found apparently infected cells in low numbers (≏1% of cells). Notably infection was exclusively localized to stromal cells, and a few haematopoietic cells. No prostate epithelial cells (tumorous or otherwise) appeared to contain the virus. The authors speculated that any oncogenic effect of the virus must be a paracrine one. This would be a novel method of tumour causation, as the tumour cells themselves are not infected with the virus. Concordant with this original study is another from Atlanta, USA, of 40 prostate cancer patients. Using a novel serological assay, alongside PCR and FISH, they found 27.5% of patients to be infected with XMRV and again an association between the RNase L genotype and the presence of the virus. A third report, that looked at both prostate cancer tissue and prostate tissue from healthy controls, also found XMRV DNA to be present in 6.3% of cancerous prostates and 2% of control prostates. When tissues were stained to seek specific viral protein, this rose to 23% of prostate cancer samples. However, in this study viral components were found only within malignant epithelium and not in stromal cells. This group also showed a correlation between the severity of the tumour and the presence of XMRV infection but paradoxically could detect no link between RNase L genotype and XMRV infection. A fourth study within the USA found 22% of cancerous prostate samples to contain XMRV; many of these patients also had detectable XMRV within healthy tissues, leading the authors to speculate that XMRV infection may precede and possibly promote tumourigenesis. However, they too failed to find an association between XMRV infection and RNase L genotype, and although not statistically significant, they observed a slight trend such that higher Gleason score tumours were more likely to be XMRV positive than lower grade tumours.

Retroviruses are ubiquitous agents. Some, such as HIV, the causative agent of AIDS, are highly pathogenic and easily transmissible. Many others are confined to the germline DNA and transmitted only vertically as endogenous retroviruses, the vast majority of which have no disease association in humans. There are several retrovirus classifications of which the most basic is the division between simple and complex retroviruses, based on the absence or presence respectively of accessory and regulatory genes. HIV, for example, is a complex
retrovirus with regulatory genes, such as tat, rev, nef, vpu etc. XMRV is a simple retrovirus. The term xenotropic refers to a murine virus’s ability to infect non-murine cell lines in laboratory culture. There are no known human pathogens similar to XMRV and it is very distantly related indeed to the two known human pathogenic retroviruses HIV and HTLV-1 (the approximate relatedness of the viruses is shown in Fig. 1).

Soon after these initial studies were published additional circumstantial evidence of the likely role of XMRV in malignancy was obtained. Some retroviruses are known to be able to transform cells in vitro, although the mechanism by which this occurs varies. Some retroviral genomes contain oncogenes, and are thus capable of directly transforming the cells they infect. XMRV does not contain an oncogene and lacks direct transforming activity, yet it has been seen to induce low rates of indirect transformation in vitro.\textsuperscript{12} Retroviruses can also induce transformation by insertional activation of cellular oncogenes, followed by outgrowth of cells in which there are multiple insertions of the retroviral genome. Like certain other retroviruses, XMRV displays a preference for insertion within transcriptional regulatory regions.\textsuperscript{13} The presence of a glucocorticoid response element within the U3 region might further enhance XMRV’s transcriptional activation properties while also making it responsive to the hormonal milieu of the

Fig. 1 Phylogenetic tree of major retrovirus families showing approximate relative relatedness. The length of individual lines indicates the relative similarity/difference in genetic sequence between viruses. The human pathogens HIV and HTLV-1 are seen within the complex viruses. The letters ERV refer to an endogenous retrovirus. The position of XMRV is shown, the dotted line indicating its position in the tree but not its sequence similarity to other viruses.
prostate. Multiple integration of the XMRV genome was found in 22Rv1 prostate carcinoma cells. However, it remains to be seen whether XMRV behaves in this manner *in vivo*; those studies claiming it can be found in prostate tissue observe very few copies of the XMRV genome in tens of thousands of patient cells, suggesting that it has not integrated multiple times nor readily induced transformation.

Despite the common ground that XMRV is present in prostate cancer tissue the four studies disagreed on several critical issues, such as the role of R462Q, and which cell types within the cancer are infected. Following the first of these papers several new studies on patients with prostate cancer began to appear, initially from within Europe, all of which showed a very low level, or even a complete absence, of XMRV infection within each cohort. Two were performed within Germany; in one, no XMRV infection was detected in 589 samples, and in the other ~1% of samples were XMRV positive, whether they were from prostate cancer patients or from healthy controls. In a Dutch cohort 4% of prostate cancer samples were XMRV positive, which was still low enough for the authors to conclude that XMRV infection is not associated with prostate cancer in the Netherlands.

These strikingly different findings at first prompted speculation that geographic factors were at work and that North American and European populations may have been differentially exposed to XMRV. However, very recently, Aloia *et al.* have published a study of almost 800 prostate cancer patients from the USA. They found no evidence for XMRV infection, arguing strongly against a difference in geographical distribution of the virus and more in favour of methodological issues of virus detection being responsible for the differences in findings.

When XMRV is detected in prostate cancer tissue, it appears to be present at extremely low levels. This implies that one or more extremely sensitive techniques will need to be employed in order to reliably detect it. However, the more sensitive the techniques used, the greater the likelihood of generating false positive results and the current weight of evidence suggests that XMRV is not found commonly enough, at high enough levels, or in the correct cell types to be an important contributory factor in many (or possibly any) prostate cancers.

**XMRV and chronic fatigue**

The situation with XMRV in prostate cancer remains uncertain, with the balance of evidence swinging away from XMRV as a cause. However, after the reports implicating XMRV in prostate cancer the
whole field was rocked in 2009 by the reported association of the same virus with another common disease, chronic fatigue syndrome (CFS).\textsuperscript{25}

CFS is an illness for which there is no definitive clinical test, and which therefore relies for its diagnosis on a reasonably well-defined clinical picture including unexplained fatigue for \(>6\) months and one or more of a number of other symptoms including headache, myalgia, unrefreshing sleep, poor concentration and word finding difficulties (for an excellent review on the epidemiology of CFS see\textsuperscript{26}). The lack of any sort of serological or nucleic acid-based test for diagnosis frustrates both patients (perceived often as malingering or depressed) and doctors, who often need to investigate extensively to make what is effectively a diagnosis of exclusion. The first hints that a specific pathogen was associated with the illness and that a simple PCR-based test might emerge evoked powerful, and to some extent understandable, responses of vindication and demand from this patient community. CFS is common; estimates of prevalence of between 400 and 2500 per 100 000 of population are reported.\textsuperscript{26} Thus it is a significant financial burden on society and may be a major social handicap to patients and their families.

In October 2009, Lombardi \textit{et al}.\textsuperscript{25} published the results of their study of 101 CFS patients and 218 healthy controls. Remarkably, XMRV was detected in 67\% of patient samples, and 3.7\% of controls. They used a nested PCR to screen samples initially, and followed this by sequencing the full viral genome from two samples and comparing their sequences to those of other XMRV strains. The viruses were almost identical to strains of XMRV described in prostate cancer tissue, except for six mutations, suggesting that the prostate cancer-derived XMRV and the CFS-derived XMRV arose from independent infections. Furthermore, phylogenetic analysis showed that these XMRV strains cluster together, separately from endogenous MLV strains, which was taken as suggesting that the XMRV found in the CFS patients was not a laboratory contaminant. Two techniques and a range of antibodies were then used to show XMRV protein expression in the peripheral blood mononuclear cells (PBMCs) of CFS patients, specifically within activated B and T lymphocytes. Replication competent virus was isolated following co-culture of PBMC or patient plasma with a permissive cell line. In addition, serology demonstrated that half of the CFS patients tested had developed a specific immune response against XMRV. Such a high prevalence of XMRV infection within the CFS patients led to widespread hope of a simple explanation for the syndrome, and the corollary of potential treatment options in the form of the established licenced antiretroviral drugs.

Since Lombardi \textit{et al}.\textsuperscript{27} published these dramatic findings, other groups have failed to replicate them. In one of two British studies, no
XMRV was observed in 186 CFS patients, screened by nested PCR. The second study, by Groom et al.\textsuperscript{28} examined 170 CFS patient samples and 395 controls. The authors were not able to identify XMRV in any of the samples by PCR, but $\sim5\%$ of samples (almost all of which were from healthy controls) did contain antibodies that neutralized XMRV infection, suggestive of prior XMRV exposure. Upon further examination, most of these samples were found to neutralize other viruses also, although four samples did specifically neutralize XMRV over the other viruses tested. Thus while refuting a link between CFS and XMRV infection this study may suggest that XMRV infection does occur at low levels in the general population. A further European study, performed in the Netherlands\textsuperscript{29} using both real-time PCR and nested PCR, found no evidence of XMRV, and neither did studies performed by real-time PCR and RT–PCR on a Chinese cohort\textsuperscript{30} or on two US cohorts\textsuperscript{31,32} by a variety of methods. Many of these negative studies were deliberately blinded, and in fact, the study by Switzer \textit{et al.} was also repeated in three independent laboratories to control for differences in environment, equipment or operator.

Despite several further papers from the same group who initially described the XMRV CFS connection, suggesting that there were critical differences in the extent of and sensitivity of techniques used elsewhere, no other research team has been able to reproduce their findings. There has subsequently been criticism of the Lombardi \textit{et al.} study. The authors made no mention of whether CFS and control samples were processed suitably and identically in terms of their storage, handling and analysis. The samples were not blinded to the operator(s) and non-deliberate bias may have arisen as a result of this. Nor is it clear how the authors selected samples for follow-up analyses. Most concern, however, has focused on the relevance of the chosen cohort. Within the paper there is minimal discussion of the patients, and no mention of the demographics involved, duration of illness or assessment of medical and psychiatric conditions that should necessitate exclusion from the study. The control group is not described beyond ‘healthy’, so it is impossible to assess its suitability. It has since become apparent that the patient cohort was from an outbreak of CFS in Incline Village, Nevada, that has long been suggested to have a viral cause. The authors have suggested that patients were selected partly based on immunological abnormalities, such as abnormal levels of cytokines including IL-8 and MIP-1$\alpha$, and low IFN$\alpha$ levels. Such patients are atypical and do not represent the majority of CFS patients. A further concern at a scientific level is that the apparent level of viral infection in the patients’ peripheral blood was astonishingly high yet only half of them were able to mount a humoral immune response. There has never been a proven association of CFS with selective
antibody deficiency or panhypogammaglobulinaemia so a lack of antibody response to a symptomatic virus infection in humans on this scale would be unprecedented and difficult to explain.

A recent study at the US NIH raises further questions. Lo et al. examined a small cohort of patients for XMRV infection, and found 86.5% of CFS patient samples and 6.8% of controls to contain not XMRV but sequences from different, murine leukaemia viruses (MLVs) related to XMRV. The authors found significant variation between sequences they analysed, showing different types of MLV-related virus sequence that were more closely related to modified polytropic murine leukaemia viruses (MPMLVs) or polytropic murine leukaemia viruses (PMLVs) than they were to XMRV. The genetic variability between XMRV isolates examined in the various studies is illustrated in Figure 2. Complete genome sequences are not yet available. These findings could conceivably indicate that a genetically varied group of MLV-like retroviruses does infect humans and is associated with conditions such as CFS. Such variability in sequence could account for the lack of positive results observed to date; perhaps the techniques used were too specific and would only detect viruses with identical sequences to the XMRV strains already published. If XMRV is much more genetically diverse than originally observed, then perhaps the patient samples do contain an XMRV-like virus that was undetected.

This charitable view is difficult to sustain, and the presence of yet another retrovirus in these samples most likely illustrates how easily contamination of samples can occur. In prostate cancer research, the techniques used to detect XMRV needed to be incredibly sensitive in order to find such small amounts of viral material. However, the numerical data from Lombardi suggest that XMRV is abundant in the blood cells of CFS patients, yet no corroborating data has emerged from other laboratories. Detection of a virus using highly sensitive techniques and high-level detection of a new pathogen in a tissue where others cannot find a trace both point to the probability of contamination.

Supporting this are a recent series of studies from diverse laboratories which suggest that it is possible to amplify non-XMRV mouse-derived DNA using XMRV-specific primers and PCR conditions used in patient sample analysis. In one case, the contaminating mouse genetic material was found within a commercial RT–PCR kit, in others, patient samples were also screened for the presence of other retroviral elements, intracisternal A particle (IAP) long terminal repeat sequences, which are found in high copy numbers in mouse cells. All samples positive for XMRV also contained these IAP sequences, indicating the presence of mouse DNA contamination.
Fig. 2 Bayesian maximum clade credibility phylogeny of 22Rv1 cell line-and patient-derived XMRV gag gene sequences (1605 bp). XMRV sequences derived from prostate cancer patients (Urisman et al.) are indicated by white circles. XMRV sequences derived from chronic fatigue syndrome patients (Lombardi et al.) are indicated by grey (Lombardi et al.) and black (Lo et al.) circles. 22Rv1-derived XMRV clones are indicated by black squares (Hué et al.). Other endogenous murine leukaemia virus sequences were used as controls (see Hué et al.) The tree is rooted against the Moloney MLV sequence. Bayesian posterior probabilities >0.50 are indicated on the corresponding branches. The scale bar represents the number of nucleotide substitutions per site.
These results strongly argue that equally sensitive techniques must be used to examine patient samples, and to search for murine contamination. Further to this, sequence analysis of XMRV strains from patients, compared with cell-culture-derived XMRV shows one monophyletic cluster, with the cell-line-derived strains of XMRV interspersed throughout. This, again, suggests that the XMRV strains found in patient samples are likely to be derived from contaminating cell culture material. There is a strong possibility that PCR contamination has also affected the analysis of XMRV integration sites, as it has recently been shown that 2 of 14 integration sites from patient-derived samples are identical to sites cloned from experimentally infected DU145 cells, within the same laboratory.

Although alternative methods, such as neutralization assays, enzyme-linked immunosorbent assays (ELISAs) immunohistochemistry and western blots, have been used to detect XMRV antibodies and proteins, these may also generate false positive results. These techniques rely on the detection of antibody binding to viral protein, and carry a strong possibility of antibody cross-reactivity with other viral or cellular proteins. This is exemplified by the fact that in one study, most of the samples shown to be XMRV positive by neutralization assay subsequently neutralized control viruses. Without possessing antibodies highly specific to XMRV, and proteins recognized only by anti-XMRV antibodies, it is difficult to draw XMRV-specific conclusions from these types of experiment.

XMRV appears to be a novel virus that is capable of replication in certain types of cultured cells, but there is little evidence to suggest that it infects human lymphocytes and more research is needed to investigate the replication kinetics of clinical isolates in these cells and infectious titres in human blood. Several reports suggest that XMRV can infect human lymphocytes in vitro. This is surprising as XMRV replication is restricted by the DNA-editing enzymes APOBEC 3G and 3F, which are expressed in PBMCs. It appears, however, that replication occurs at extremely low levels and that the XMRV genome soon becomes hypermutated by the APOBEC proteins, casting doubt on the relevance of these cells in the establishment of an XMRV infection.

Conclusions: XMRV—a novel virus but not a human pathogen?

The number of manuscripts published on XMRV (XMLV) has rocketed in the last year but what do they mean? The majority are concerned with the basic science of this novel virus. Does it actually infect humans at all? As mentioned, serological studies appear to show some
specific neutralizing ability in some individuals\textsuperscript{28} but without evidence of a temporal association with an infection the ever present phenomenon of cross reactivity with another virus remains. The low level of positives found by several studies, however, does make this a possibility. The numerical count of articles for and against XMRV being detected in association with disease, however, is shifting firmly into the negative (Fig. 3).

It seems increasingly unlikely that XMRV has a role in CFS, and probably not in prostate cancer; or if it does, this is restricted to a rare subset of patients. However, these are far from the only unexplained diseases for which a viral cause has been suspected and sought previously. In the wake of this work, studies have been published excluding XMRV as a cause of autism\textsuperscript{38} and amyotrophic lateral sclerosis\textsuperscript{39} (although there is support for a retrovirus). Nor is it more common in populations who might be assumed to be more susceptible to low-virulence organisms such as those infected with HIV\textsuperscript{38,40} or who have already acquired a blood-borne virus such as Hepatitis C,\textsuperscript{40} or those immunosuppressed through organ transplantation.\textsuperscript{31} XMRV was unsuccessfully sought in French children with a variety of unexplained medical conditions which were plausibly viral.\textsuperscript{41} New evidence suggesting that it can be found in respiratory secretions\textsuperscript{42} is haunted by the contamination evidence quoted above.

If XMRV is found to be causative of any human disease then the slight reassurance is that some, but not all, of our own innate defences seem quite good at inhibiting it\textsuperscript{43,44} and it does respond to certain drugs currently used to treat HIV.\textsuperscript{45,46}
The XMRV mini-saga has followed a well-trodden route associated with some ‘emerging’ infections, with initially positive findings in high-impact journals and then a large amount of effort in generating the more difficult to publish refuting studies. The door is not quite closed on XMRV as a human pathogen but without major new positive findings it seems likely to do so soon, leaving redoubled frustration for those patients with conditions like CFS where the pathogenesis remains obscure.

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