MINI REVIEW

Linking human beta retrovirus infection with primary biliary cirrhosis

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Summary  Several environmental agents have been linked with primary biliary cirrhosis (PBC) that include bacteria, xenobiotics and viruses. A human beta retrovirus (HBRV) related to mouse mammary tumor virus has been cloned and characterized from patients with PBC. This agent can be detected in the majority of patients' perihepatic lymph nodes by immunochemistry and RT-PCR. The HBRV has recently been isolated in culture and integration sites have been identified in the genome of patients to provide convincing evidence of beta retrovirus infection in patients. Three lines of evidence support a role for the virus in PBC. First, the beta retrovirus is linked with aberrant expression of mitochondrial protein(s) on the biliary epithelium cell (BEC) surface, a disease specific phenotype. Second, the related agent, mouse mammary tumor virus has been linked with autoimmune biliary disease in the NOD.c3c4 mouse model for PBC. In this mouse model, the virus is localized to diseased biliary epithelium that also display aberrant expression of the mitochondrial autoantigens. In translational studies, both patients with PBC and NOD.c3c4 mice demonstrate significant improvement in biliary disease with combination antiviral therapy. An overview of the biological relevance of the beta retrovirus infection in PBC will be discussed in this review.

Primary biliary cirrhosis (PBC) is an enigmatic disease characterized by granulomatous destruction of interlobular bile ducts and the spontaneous generation of antimitochondrial antibodies (AMA). Patients make both humoral and cellular immune responses to the pyruvate dehydrogenase complex E2 (PDC-E2) and related oxo-acid proteins. These proteins are usually located on the mitochondrial inner membrane but patients with PBC display aberrant expression of PDC-E2 on the biliary epithelium cell (BEC) surface and in perihepatic lymph nodes [1]. However, the mechanism that triggers the mitochondrial phenotype and the immune mediated biliary destruction are subject to debate [2].

It is generally accepted that PBC is triggered by an environmental agent in a genetically susceptible individual. Multiple agents have been linked with PBC to date that include:

- xenobiotics that modify the PDC-E2 to trigger autoimmunity [3];
- bacteria, such as *Novosphingobium* species that metabolize xenobiotics and trigger autoimmunity by molecular mimicry [4,5];
- the human betaretrovirus (HBRV), related to mouse mammary tumor virus (MMTV), that triggers the PDC-E2 expression in normal BEC in culture [6,7].
Table 1 Potential disease association of each environmental factor that has been linked with the development of Primary biliary cirrhosis (PBC).

<table>
<thead>
<tr>
<th>Disease association</th>
<th>Bacteria: Novosphingobium</th>
<th>Xenobiotics</th>
<th>Betaretrovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated from PBC patients</td>
<td>No</td>
<td>N/A</td>
<td>Isolated in co-culture from PBC perihepatic lymph node in HS578T cells</td>
</tr>
<tr>
<td>Detected in PBC biliary epithelium</td>
<td>No</td>
<td>N/A</td>
<td>Visualized by electron microscopy Cloned from BEC cDNA library Integration sites in BEC DNA</td>
</tr>
<tr>
<td>Detected in PBC patients</td>
<td>25% of PBC patients and 25% of controls have evidence of Novosphingobium in faeces but not liver</td>
<td>N/A</td>
<td>Detected in the majority of PBC perihepatic lymph nodes by immunochemistry and RT-PCR</td>
</tr>
<tr>
<td>Serological reactivity</td>
<td>AMA recognize human and bacterial PDC-E2</td>
<td>AMA recognize xenobiotic modified PDC-E2 mimotopes</td>
<td>PBC sera recognize beta retrovirus envelope surface protein</td>
</tr>
<tr>
<td>Mitochondrial phenotype: aberrant PDC-E2 expression</td>
<td>It is thought that the protein in BEC is of human origin Bacterial PDC-E2 has not been linked with aberrant PDC-E2 expression</td>
<td>Xenobiotic metabolism has not been linked with aberrant PDC-E2 expression</td>
<td>Phenotype associated with viral infection in PBC perihepatic lymph nodes MMTV and conditioned media with HBRV triggers phenotype in normal BEC in vitro NOD.c3c4 biliary epithelium display MMTV ENV and Gag proteins and aberrant PDC-E2</td>
</tr>
<tr>
<td>Mouse models</td>
<td>Novosphingobium trigger AMA and hepatic inflammation in the NOD.1101 mouse model Lymphocyte transfer to syngeneic mouse causes similar disease</td>
<td>2-octynoic acid triggers AMA reactivity and hepatic inflammation in the C57BL/6 and NOD.1101 mouse models</td>
<td>Virus replicates in bile ducts and salivary epithelium in mice; sites effected in PBC Highly active antiviral therapy abrogates cholangitis in the NOD.c3c4 mouse model linked with MMTV infection Combivir alone results in viral resistance</td>
</tr>
<tr>
<td>Biological plausibility</td>
<td>PBC patients develop recurrent urinary tract infections Molecular mimicry is associated with AMA reactivity</td>
<td>The liver is responsible for metabolizing xenobiotics Xenobiotics may be incorporated into PDC-E2 complex</td>
<td>PBC patients have significant biochemical and histological response to Combivir but develop viral resistance Cyclosporine A is protective against recurrent PBC in liver transplant recipients and has demonstrable antiviral activity against beta retrovirus</td>
</tr>
<tr>
<td>Link to female preponderance</td>
<td>Women more prone to UTI</td>
<td>Women wear nail polish containing xenobiotics</td>
<td>Beta retroviral replication is stimulated by female hormones, especially during and following pregnancy</td>
</tr>
</tbody>
</table>

BEC: biliary epithelial cells; AMA: antimitochondrial antibodies; PDC-E2: pyruvate dehydrogenase complex E2; MMTV: mouse mammary tumor virus.
The relationship of xenobiotics and bacteria in PBC have been extensively discussed (Table 1 and [3,5] reviews) and in this review, we will concentrate on the evolving studies outlining the role of HBRV in patients with PBC.

Discovery of the human betaretrovirus in patients with primary biliary cirrhosis

Several complementary approaches were used to identify a retrovirus as a candidate agent associated with PBC. After finding no evidence of bacterial 16s ribosomal DNA in PBC liver samples, representational difference analysis studies were performed using liver and skin from a PBC patient, as the technique had recently been employed to discover the human herpes virus 8 associated with Kaposi's sarcoma [2,8,9]. Retroviral sequences were found and follow up Western blot studies using a retrovirus isolated from patients with Sjogrens syndrome showed antibody reactivity to retroviral proteins in PBC patients' serum [10,11]. In addition, electron microscopy of BEC showed virus-like particles in PBC patients [7]. A direct cloning approach capable of identifying any retroviral pol gene was used to amplify retroviral sequence from a PBC BEC cDNA library [7]. The full-length provirus was PCR-cloned from perihepatic lymph node DNA once this tissue compartment was found to be a major reservoir of viral infection in patients with PBC [7,12]. Basic local alignment search tool nucleotide (BLASTN) searches confirmed that clones of the 9,690 bp PBC viral genome shared 95 to 99% homology with MMTV and "human mammary tumor tissue" sequences derived from breast cancer tissue [12,13]. The "HBRV" was named in accordance with the International Committee on the taxonomy of viruses [14], as all the human and mouse sequences share marked betaretrovirus homology. Indeed, it is likely that the human infection resulted from a zoonosis as there are few functional and genetic differences between the mouse and human virus [7,12].

MMTV was first described as a transmissible extrachromosomal factor causing breast cancer in mice in 1936 by Bittner [15]. He found that the virus could be transmitted horizontally as an infectious agent or vertically in the mouse genome as an endogenous retrovirus [16]. Interest in this virus as a potential human pathogen came to a height in the 1970s, when Moore and colleagues visualized "B-type particles" resembling MMTV in 60% of milk samples derived from patients with breast cancer [17]. Since then, many groups have identified tissue and serologic evidence of infection and cloned "'human mammary tumor virus'" sequences from human breast cancer, lymphoma, peripheral blood mononuclear cells, liver, lymph node and serum [7,12,13,18–25], whereas others failed to confirm these findings [26–30]. Accordingly, the viral hypothesis of breast cancer has been subject to considerable controversy and referred to as a "'tumor virus'" [31].

Study of the biology of MMTV provides clues to potential pathogenicity in humans. Once weanling pups ingest MMTV in their mother's milk, the virus first infects lymphocytes in the Peyer's patches in the gut and is then transmitted to breast tissue and to a lesser extent in the salivary glands, liver and brain in mice [16,32]; sites associated with pathology in patients with PBC [33]. In the mother, viral replication is directly stimulated during pregnancy by activation of female hormone responsive elements in viral long terminal repeats (LTR) and upstream of the ENV gene [34,35]. The HBRV has the same female steroid responsive elements and it is tempting to speculate that the preponderance of PBC in women may be linked to the hormonal stimulation of betaretrovirus replication [16,32]. Also, a retroviral involvement in the pathogenesis of PBC helps to address the conundrum observed with liver transplantation. Recurrent PBC occurs earlier and is more severe with tacrolimus therapy as compared to the less potent immunosuppression with cyclosporin A in liver transplant recipients [36]. This appears counterintuitive for an autoimmune disease; however, from an infectious standpoint, it is known that cyclosporin A inhibits hepatitis C virus and HIV replication and we have recently demonstrated that betaretrovirus production is inhibited in vitro by cyclosporin A as well [37].

Prevalence of betaretrovirus infection in patients with primary biliary cirrhosis

Two studies have shown that the viremia can be detected in serum of approximately one in four patients with PBC as compared to 5% of control subjects [7,38]. RT-PCR and immunochemistry investigations found that the HBRV was predominantly distributed in lymphoid tissue rather than the liver in patients with PBC as 75% of lymph node samples from PBC patients were positive for viral protein and RNA but only a third of patients had viral RNA in the liver (Fig. 1). As virus has been visualized in biliary epithelium by electron microscopy, it is possible that RT-PCR studies of hepatic RNA lacked sensitivity to detect the HBRV in biliary epithelium. In fact, the gold standard for detecting retrovirus is the demonstration of viral integrations within the host genome as this provides incontrovertible evidence of infection. Indeed, using linker-mediated (LM)-PCR to detect beta retrovirus integration sites [39], preliminary studies of PBC patients have shown that up to 80% of PBC patients harbor HBRV in BEC [40].

![Figure 1](image)

Figure 1: Prevalence studies conducted in patients with primary biliary cirrhosis showing that the use of more sensitive techniques for identifying human beta retrovirus nucleic acid leads to a higher detection rate in separate tissue compartments [1–3,7,29,39].
Analogous to breast cancer, the viral hypothesis of PBC is controversial as the virus has a low level of replication and is difficult to detect. For example, Selmi et al. were unable to detect beta retrovirus DNA in the liver using a single round of PCR in PBC patients or control subjects [29], whereas Johal et al. found viral DNA in 5% of PBC liver samples using nested PCR in the liver [41]. Similarly, our laboratory could only find proviral DNA sequences in two of 12 hepatic DNA samples using a nested PCR technique [7]. Not surprisingly, the more sensitive techniques are able to identify a higher frequency of viral infection with 30% using RT-PCR and up to 80% using LM-PCR (Fig. 1).

Serological studies have been performed using MMTV Western blots. Selmi and colleagues found no serological evidence of infection to MMTV Western blots apart from what was described as nonspecific AMA reactivity [29]. In the studies conducted in our laboratory, PDC-E2 was used to block the AMA reactivity and antibody reactivity to MMTV envelope proteins was observed in the majority of PBC patients as well as AMA reactivity [42]. An ELISA assay has been established to resolve this issue, as more definitive serologic tests for HBRV will clearly be required to conduct epidemiological studies and diagnose infection.

Other groups have made inroads into demonstrating that MMTV has the potential to be a human pathogen. Two recent studies have demonstrated that MMTV can infect human cells and spread in vitro [39,43,44]. Taking a virological approach to link beta retrovirus infection with PBC, our laboratory has recently isolated the agent from PBC patients lymph nodes and demonstrated viral integration sites in the cells infected in vitro as proof of infection [40]. Accordingly, steps have been taken that address the first two of Koch’s postulates to isolate the agent in vitro and demonstrate that the agent can be found in biliary epithelium, the site of disease in patients with PBC [40].

**Linking betaretrovirus infection with the mitochondrial phenotype of primary biliary cirrhosis**

Koch’s postulates are not applicable to chronic complex disorders with a strong genetic component. However, recapitulating a disease phenotype with viral infection in an animal model or in vitro can provide the necessary proof for a causal association. The aberrant expression of the mitochondrial antigens on the cell surface of biliary epithelium is both a disease specific phenotype and probably directly related to the development of the autoimmune response [1]. However, it is unknown why patients with PBC make autoantibodies and T cell responses to the PDC-E2 and whether these autoimmune responses result in tissue damage. However, it is likely that any environmental agent associated with PBC impacts on the development of the mitochondrial phenotype.

In order to directly address this question, we performed confocal microscopy studies with perihepatic lymph nodes using AMA and antiviral antibodies. In these studies, perinuclear betaretrovirus Capsid protein was observed in the same cells that also expressed aberrant PDC-E2 expression in PBC lymph nodes but not control tissue (Fig. 2a) [7]. Neither Xenobiotics nor bacteria have been linked to this mitochondrial phenotype in vivo (Table 1). To establish an in vitro model for PBC, we used perihepatic lymph nodes homogenates in co-culture with normal BEC, which subsequently developed cell surface PDC-E2 expression with samples from PBC patients but not from liver disease controls [6]. Subsequent serial passage of PBC-conditioned media resulted in the aberrant AMA reactivity in the normal biliary epithelium and this activity was abrogated by γ-irradiation, suggesting the transmissible factor contained nucleic acid [7]. The development of the PBC phenotype in BEC was accompanied with reactivity to anti-p27 MMTV Capsid antibodies and positive RT-PCR results with supernatants from passaged BEC cells [7]. To determine whether pure virus was triggering the phenotype, MMTV and other laboratory control viruses, such as herpes simplex, coxsackie and adenovirus, were incubated with the normal biliary epithelium and only those co-cultivated with MMTV developed the mitochondrial phenotype (Fig. 2b) [7].

Taken together, the in vivo and in vitro data provide robust and reproducible evidence that MMTV and the human betaretrovirus are associated with the mitochondrial phenotype of PBC. These observations suggest an alternative model to the molecular mimicry hypothesis that has been circulating for more than 40 years; to date, no one has ever convincingly linked bacterial antigens with the mitochondrial phenotype in vivo or BEC in vitro [5]. As the human betaretrovirus and MMTV share no amino acid homology with PDC-E2 or the related mitochondrial proteins [12], we conjecture that viral infection is a primary event in the process and the autoimmune response is induced by viral recruitment of an immune response to tissues expressing antigens that are usually sequestered in the mitochondrial inner membrane.

Our studies in mouse models of PBC have also linked betaretrovirus with aberrant PDC-E2 expression. Recently, an autoimmune biliary disease model has been created by partial introgression of chromosomes three and four into nonobese diabetic (NOD) mouse strain [45,46]. The NOD.c3c4 mouse develops spontaneous production of AMA as well as nonsuppurative granulomatous cholangitis. The spontaneous AMA production in germ free conditions suggested a hypothesis that the NOD.c3c4 mouse may be expressing endogenous MMTV to induce the disease [47]. Indeed, preliminary studies show that both MMTV Capsid and Envelope proteins are expressed in biliary epithelium of the NOD.c3c4 mouse accompanied by an apical distribution of aberrant PDC-E2 expression, similar to observations from patients with PBC [48]. Thus, the NOD.c3c4 mouse model of PBC provides the second example of betaretrovirus linked with the mitochondrial phenotype of PBC. In order to determine whether the cholangitis is related to viral infection in the NOD.c3c4 mouse, studies have been performed using highly active antiretroviral therapy (HAART) and anti-MMTV neutralizing antibodies. Both measures abrogated cholangitis in the NOD.c3c4 mouse, suggesting a central role of MMTV in triggering cholangitis [49]. It is hoped that mouse models will help us to identify the different immune and viral factors that combine to trigger idiopathic biliary disease and possibly new avenues of treating patients with PBC [47].
Figure 2  Human betaretrovirus is associated with the primary biliary cirrhosis (PBC) mitochondrial phenotype. (a) viral proteins are detected in cells with aberrant pyruvate dehydrogenase complex E2 expression in PBC, whereas the controls express the antimitochondrial antibodies reactivity in a mitochondrial distribution; (b) human beta retrovirus, mouse mammary tumor virus and PBC lymph node homogenates can trigger the mitochondrial phenotype in normal biliary epithelial cells in culture whereas control lymph nodes and viruses do not (adapted from [7] and reprinted with permission, Copyright 2003, National Academy of Sciences, USA).

Translational studies using antiretroviral therapy in patients with primary biliary cirrhosis

Pilot studies and randomized-controlled trials with antiretroviral therapy have been conducted to address the hypothesis that the human betaretrovirus causes cholangitis in patients with PBC. At the time the pilot studies were conducted, lamivudine therapy had proven to be an effective and safe treatment for patients with hepatitis B virus infection. Yet, PBC patients treated with lamivudine monotherapy derived little demonstrable biochemical or histological benefit [50]. The pilot study using Combivir (lamivudine and zidovudine) showed more promise. Significant reductions in hepatic biochemistry were observed with clinically relevant reduction in total necro-inflammatory scores on liver biopsy. Still, the most striking observation was the reversal of ductopenia, which has not been achieved by any other treatments for PBC to date [50].

As 40% of the patients in pilot study had normalized their hepatic biochemistry levels after 1-year’s therapy in the pilot study, normalization and half normalization of alkaline phosphatase levels to baseline were chosen as endpoints in a randomized-controlled trial. PBC patients stabilized on standard ursodeoxycholic acid therapy received Combivir or placebo for 6 months and the biochemical endpoints were adopted in order to avoid the need for liver biopsies [51]. However, the endpoints turned out to be far too stringent for this randomized-controlled trial as none of the patients normalized alkaline phosphatase. While almost double the
population in the treatment arm halved their alkaline phosphatase levels towards normal (31% versus 17%), there were insufficient patients for this to reach significance [52]. However, a significant if not substantial impact was observed in patients on Combivir, who showed considerable improvement in alkaline phosphatase levels compared to those on placebo at all time points (Fig. 3) [52]. These differences merit attention, as a 10% or greater reduction of mean alkaline phosphatase levels compared to placebo was considered a clinically meaningful outcome in a recent international multicenter trial using obeticholic acid to treat PBC [53]. Also, it is noteworthy that patients on Combivir experienced significant improvements in symptoms such as fatigue, as well [52]. Despite the demonstrable improvements in biochemistry and histology, the use of Combivir is not recommended for treating PBC. For example, the zidovudine prodrug is not efficiently activated in the liver and the long-term use is associated with untoward side effects as well as virological resistance to therapy [54,55]. For example, some patients with developed biochemical rebound associated with increased viral load in serum, suggestive of the development of virological resistance to Combivir [51]. While samples were lacking to detect viral mutations in PBC patients, a proportion of NOD.c3c4 mice on Combivir therapy developed increased levels in hepatic MMTV as well as mutations in the YMDD (YMDD is peptide sequence in the polymerase protein tyrosine, methionine, aspartic acid, aspartic acid) region of the reverse transcriptase protein [49]. The amino acid substitution is comparable to mutations described with hepatitis B virus and human immunodeficiency with lamivudine or Combivir therapy, respectively [56].

In order to avoid viral resistance in patients, more robust and well-tolerated antiviral regimens should be tested in vivo. A further consideration is that HIV infection was only adequately controlled once HAART became available and well-tolerated antiviral regimens should be tested in PBC patients in order to prevent progression to liver failure in patients unresponsive to ursodeoxycholic acid.

Conflict of interest statement

The authors declare no other conflicts of interest.

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