Title: Human cancers and mammalian retroviruses: should we worry about bovine leukaemia virus?

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Keywords

Virology

Breast cancer

XMRV

Zoonosis

HTLV

Main text (1348/1500 words)

There is considerable interest[1] in the possibility that a virus may be contributing to the aetiology of human breast cancer, with previous and ongoing work focusing on Epstein-Barr virus, human papillomavirus and a number of other candidates, including newly identified viruses present in dairy products (eg[2]). Recent work has suggested that bovine leukaemia virus (BLV) may be a risk factor for human breast cancer[3]. This deltaretrovirus of cattle causes B cell leukaemia in around 5% of infected hosts, is present in a large proportion of commercial dairy and beef herds, and is shed in milk (but inactivated by pasteurisation). The position of BLV in the pantheon of retroviruses linked to human cancers is, at present, controversial.

A number of retroviruses can cause or contribute to cancer in humans, with the clearest evidence found in simian retroviruses which have jumped the species barrier into humans. Human T lymphotropic viruses I and II (HTLV-I and HTLV-II) evolved from simian T lymphotropic viruses which infected humans as zoonoses and over time adapted to their human hosts. Transmitting horizontally and vertically between humans, HTLV-I causes cancer in a small number of infected individuals, and HTLV-II is associated with increased cancer risk[4]. HIV-1 indirectly promotes cancer development by reducing immune surveillance, particularly predisposing HIV-positive individuals to virus-driven cancers such as Kaposi's sarcoma. There is a long-standing interest in the potential role of other mammalian retroviruses in human oncogenesis[5]. Could BLV be the next retrovirus to join this list?

Recent reports have produced conflicting evidence for a link between BLV and human cancer. Buehring and colleagues compared healthy and diseased human mammary epithelial tissue, and found 29% of healthy, 38% of pre-malignant and 59% of malignant mammary epithelial samples to be BLV positive. This builds on earlier work showing the presence of BLV in the mammary epithelial cells of cattle[6]; and antibodies to BLV in human blood donors[7]. Consequently, the group propose BLV positivity as a risk factor for breast cancer. Buehring et al used in-situ PCR and immunohistochemistry to study formalin-fixed paraffin embedded (FFPE) mammary epithelial. Unfortunately, FFPE samples often give poor-quality, degraded DNA, which has made sequence analysis of the BLV present in these sample difficult; at present, the group have not published the sequences recovered, although they report that they are different to the control sequence used by the lab[8]. In contrast to this, RNA-seq analysis of 4433 tumours from 19 cancer types found no evidence of known or novel viruses infecting human breast cancers[9]. Similarly, retrovirus-specific enrichment of tumour samples[10] found no evidence of retroviral infections in human B cell lymphomas (where BLV might be found, given BLVs role in bovine B cell leukaemia); and exome sequencing and RNAseq of breast cancer FFPE samples from Belgium also found no evidence of BLV or other retroviruses[11]. Conversely, a multi-pathogen probe-based array found evidence of retroviruses in a proportion of screened FFPE breast cancer samples[12], as well as other viral and bacterial pathogens. If BLV is present in human breast cancer, these

conflicting findings could be explained by a number of different factors: BLV may only be present in breast cancer tissue at very low levels; it may be transcriptionally silent; or BLV may only be found in breast cancers from geographically-restricted areas.

Other non-human retroviruses have been linked to breast cancer. One of the longest-standing candidates for a viral cause of human breast cancer is mouse mammary tumour virus (MMTV), first identified as an extrachromosomal cause of murine mammary gland cancer in 1933[13], transmitted in milk. MMTV established the model of viral oncogenesis in breast cancer, and ever since scientists have searched for an analogous virus in humans. Decades of studies have generated a body of literature that is highly suggestive of a human analogue, but lacks definitive proof of its existence. Ultrastructural studies identified MMTV-like particles in human breast cancer samples[14] and in human breast milk[15]. More recently, genetic and molecular methods have been used but they have not resolved the controversy that surrounds MMTV and a putative human mammary tumour virus. A key difficulty with molecular studies is the presence in the human genome of human endogenous retroviruses (HERVs) with significant homology to MMTV. As a result, since the mid-1990s studies have used primers complementary to regions of the MMTV genome with low homology to known HERVs, commonly targeted to the *env* region of MMTV which has just 16% homology to HERVK-10, the most closely related HERV. A recent systematic review of these refined molecular studies concluded that, while many studies detected MMTV *env* sequences in malignant tissue, various methodological flaws meant that none of the studies with a positive result were convincing[16].

Fimereli et al. harnessed multiple *in silico* techniques to analyse breast cancer transcriptomes for viral gene expression, and concluded that no viruses were expressed highly enough to be considered causative, furthermore MMTV-like sequences were not detected[11]. As discussed above, RNAseq and deep sequencing studies of breast cancer samples have produced mixed support for the presence of a retrovirus in human breast cancer. However, as has been suggested for BLV, viral gene expression may not be necessary for MMTV oncogenesis. The story of MMTV or a human mammary tumour virus as oncogenic agents in human breast cancer remains unfinished: despite

over 40 years of research, nobody has delivered definitive proof. Indeed it is a lesson in the challenges of generating irrefutable evidence of causation, many of which will likely apply to BLV.

Trying to identify new pathogens that contribute to human oncogenesis is a fraught process, and a cautionary tale involves another mammalian retrovirus. In 2006, a murine leukaemia virus (MLV), xenotropic murine leukaemia virus-like retrovirus (XMRV), was detected in prostate cancer biopsies from men with mutations in gene *RNASEL*[17]. Later, XMRV and related MLVs were detected in the blood of people with chronic fatigue syndrome, although those studies were later retracted. A careful and co-ordinated international effort identified that XMRV and MLVs were detected in cancer biopsies[18], cell lines[19] and chronic fatigue syndrome blood samples[20] due to laboratory contamination from a range of sources[21, 22]. Murine-derived laboratory reagents are not the only source of contaminating viral and bacterial material: multiple studies have found laboratory kits, reagents and even water supplies to be contaminated with a wide range of nucleic acids[23, 24]. As molecular methods become more sensitive, the risk of very low level contamination generating false-positive results grows. PCR may be particularly sensitive to this problem.

The story of XMRV's rise and fall as a possible human pathogen has a number of important lessons for the study of BLV in human breast cancer. Molecular genetic screening, careful use of control samples, and stringent checks for contamination will be required to identify whether BLV is circulating in the human population. Detection of BLV in blinded samples analysed at multiple laboratories, ideally from different tissue collections sourced from different geographical areas would be key to gaining support for BLVs involvement in breast cancer.

Although not essential, identification of BLV in breast cancer through unbiased methods such as RNAseq or cancer exome sequencing would provide additional evidence. Analysis of microRNAs (miRs) may provide a further method for detecting putatively BLV-infected breast cancers. BLV is unusual among viruses in expressing miRs, which are more commonly associated with DNA viruses. One of the better-characterised BLV miRs (BLV-miR-B4) has properties associated with B cell tumorigenesis in a mouse

model. Low BLV mRNA and protein expression in infected bovine cells may also be a property of zoonotic BLV infections[25], reducing the chance of detection by methods such as RNAseq. In light of this, examination of the miR profiles of human breast cancers for retroviral miRs is a further productive avenue of research.

Future perspectives on BLV and breast cancer studies

The possibility of a novel mammalian retrovirus driving a proportion of human breast cancers continues to be contentious, whether the candidate is of bovine, murine or human origin. Distinguishing novel or rare human pathogens from laboratory artefacts will require extensive replication efforts. If the presence of BLV in human breast cancer can be unambiguously identified, further, larger case-control studies such as the one conducted by Buehring and colleagues[3] would clarify whether BLV is a risk factor or biomarker for breast cancer. The lessons of the XMRV story show how quickly an international, collaborative effort can characterise the molecular origins of a novel virus if there is sufficient interest among the scientific community. Until that time, BLV's identity as a human tumour virus or human 'rumour virus' remains unclear.

Funding

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