No evidence for the involvement of XMRV or MCV in the pathogenesis of breast cancer

G Khan*,[1], PS Philip[1], M Naase[2] and KMI Al Zarouni[1]

[1]Departments of Microbiology and Immunology, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates; [2]School of Health and Social Sciences, Middlesex University, London, UK

BACKGROUND: The aetiology of breast cancer remains elusive. A viral aetiology has been proposed, but to date no virus has been conclusively demonstrated to be involved. Recently, two new viruses, namely Merkel cell polyomavirus (MCV) and xenotropic murine leukaemia virus-related virus (XMRV) have been identified and implicated in the pathogenesis of Merkel cell carcinoma (MCC) and familial form of prostate cancer, respectively.

METHODS: We examined 204 samples from 58 different cases of breast cancer for presence of MCV or XMRV by PCR. Samples consisted of both malignant and non-malignant tissues. Additionally, we included 6 cases of MCC and 12 cases of prostate cancer as potential controls for MCV and XMRV, respectively.

RESULTS: All of the breast cancer samples examined were negative for both MCV and XMRV. However, 4/6 MCC and 2/12 prostate cancer samples were found to be positive for MCV and XMRV, respectively. Sequence analysis of the amplified products confirmed that these sequences belonged to MCV and XMRV.

CONCLUSION: We conclude that there is no evidence for the involvement of MCV or XMRV in the pathogenesis of breast cancer. What role these viruses have in the pathogenesis of MCC and prostate carcinomas remains to be demonstrated.

Breast cancer is one of the most common malignancies in women worldwide. In spite of extensive research, the aetiology of this malignancy remains unknown. However, a number of risk factors have been identified, including life style, environmental and genetic factors (Veronesi *et al*, 2005). In a proportion of cases, no identifiable risk factor can be identified, prompting the idea that an oncogenic virus may be involved (Amarante and Watanabe, 2009). Indeed, several viruses have been implicated over the years (Labrecque *et al*, 1995; Bonnet *et al*, 1999; Melana *et al*, 2007; Cox *et al*, 2010; Glenn *et al*, 2010; Ariad *et al*, 2011), but none have conclusively been demonstrated to be central to the disease process (Chu *et al*, 2001; Herrmann and Niedobitek, 2003; Murray, 2006; Larrey *et al*, 2010; Khan *et al*, 2011; Silva and da Silva, 2011).

Recently, two new viruses have been identified and shown to be involved in human malignancies. The first of these is a gammaretrovirus, termed xenotropic murine leukaemia virus-related virus (XMRV) discovered in human prostate carcinomas from patients who were homozygous for the anti-viral enzyme, ribonuclease L (Urisman *et al*, 2006). If confirmed, XMRV will become the fourth member of the retroviridae family to infect humans and the second to be associated with a human malignancy (Schlaberg *et al*, 2009; Knouf *et al*, 2009; Arnold *et al*, 2010). However, the role of XMRV in prostate cancer remains controversial with a number of studies reporting negative findings (Hohn *et al*, 2009; Furuta *et al*, 2011; Stieler *et al*, 2011). Similarly,

a role for XMRV in the pathogenesis of chronic fatigue syndrome was also reported (Lombardi *et al*, 2009), but this association has now been discredited and retracted (van der Meer *et al*, 2010; Paprotka *et al*, 2011; Steffen *et al*, 2011; Alberts, 2011). Furthermore, some studies have reported that XMRV is not an exogenous virus at all, but rather a mouse endogenous virus contaminant (Hue *et al*, 2010; Sato *et al*, 2010; Smith, 2010).

The other oncogenic virus that has recently been identified is the Merkel cell polyomavirus (MCV) isolated from a relatively rare form of skin cancer called Merkel cell carcinoma (MCC) (Feng *et al*, 2008). Merkel cell polyomavirus sequences have been shown to be present in up to 80% of MCCs (Feng *et al*, 2008; Garneski *et al*, 2009; Kaae *et al*, 2010). Moreover, the virus has been shown to be clonally integrated in the tumour cells and probably has a role in the pathogenesis of this malignancy. More recent studies have shown that MCV is more prevalent than initially thought and that the virus can also be detected in non-tumour tissues (Gaynor *et al*, 2007; Pastrana *et al*, 2009; Babakir-Mina *et al*, 2010; Loyo *et al*, 2010). However, in contrast to non-tumour tissue, the MCV found in MCC is not only integrated into the host cell DNA but also crucially has mutations in the viral oncogene large T (LT) antigen (Shuda *et al*, 2008), prematurely truncating the MCV LT helicase and thereby preventing autoreactivation of integrated virus replication that would be detrimental to cell survival. Similar loss of full length LT in other animal polyomaviruses has been reported (Small *et al*, 1982; Manos and Gluzman, 1984), indicating that the loss of full length LT in tumour tissues is not an experimental artefact, but probably a mechanism of polyomavirus-mediated oncogenesis (Shuda *et al*, 2008). The potential role of MCV in the pathogenesis of other human malignances, including

Table I Details of the PCR primers used for the amplification of XMRV, MCV and β-globin

Target	Primer	Sequence	Location	Size of product	Annealing Temperature
XMRV	Forward	5′-CATTCTGTATCAGTTAACCTAC-3′	411–432[a]	195	55 °C
	Reverse	5′-ATGATCTCGAGAACACTTAAAG-3′	609–588		
MCV	Forward	5′-GACTTTGCAAAACCATTTCCTTGA-3′	2022–045[b]	141	61 °C
	Reverse	5′-CTGCGGCTTGTTGGCAAATGG-3′	2163–143		
hβ-G	Forward	5′-TGGTGGTCTACCCTTGGACC-3′	148–162[c]	148	55 °C
	Reverse	5′-GAGGTTGTCCAGGTGAGCCA-3′	296–277		

Abbreviation: Hβ-G = human β-globin Location in GeneBank Accession number. [a]EF 185282.1, [b]EU375803.1, [c]NM000518.4.

small cell carcinoma (Wetzels *et al*, 2009), prostate cancer (Bluemn *et al*, 2009) and mesotheliomas (Bhatia *et al*, 2010), is also currently being investigated. To date, no report has been published looking at MCV and XMRV in the pathogenesis of breast cancer in a larger series of cases.

METHODS

Clinical samples

Breast samples   A total of 204 formalin-fixed paraffin-embedded (FFPE) breast tissues from 58 female cases of breast carcinomas were retrieved from the Department of Pathology archives after receiving ethical approval from the Al Ain Medical District Human Research Ethics Committee (application number AAMD HREC 08/39). These cases have been previously studied and further details including ER, PR and HER2 status can be found in our previous publication (Khan *et al*, 2011).

Briefly, 55/58 cases had multiple tissues (between 2 and 9, benign and malignant) that could be studied. The mean age of our cases was 48 years (median 47, range 20–97 years). Tissues consisted of:

(a) breast tissues: 161 samples (116 with histological evidence of malignancy, 4 benign, 41 tumour-free);
(b) lymph nodes: 43 samples (34 with evidence of metastasis and 9 free of malignancy).

Prostate samples   A total of 12 FFPE cases of prostate carcinomas from the British African-Caribbean patients were available for inclusion into this study as potential positive controls for XMRV. The mean age of the patients was 71 years (median 70, range 64–84 years) with mean PSA value of 93.5 ng ml^{-1} (median 55).
Merkel cell carcinoma samples   Six FFPE cases of MCC from Germany were included as potential positive controls for MCV. Cases consisted of four females and two males, mean age 75 years (median 75 years, range 64–87 years).

Viral plasmid controls

A plasmid containing the entire XMRV sequence (XMRV VP62/pcDNA3) (Urisman *et al*, 2006; Dong *et al*, 2007) was obtained from Drs Robert H Silverman and Beihua Dong, through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID. Another plasmid containing MCV sequence (pcDNA.MCV350 (144–3696) (Feng *et al*, 2008) was obtained from Dr Patrick Moore, also through the NIH AIDS Research and Reference Reagent Program. These plasmids were used as positive controls and to establish our PCR protocol.

DNA extraction from clinical samples

DNA was extracted from FFPE clinical samples using standard phenol-chloroform extraction methodology previously described (Farrugia *et al*, 2010). For each sample, 4 × 5 μM sections were cut and placed in a screw-cap eppendorf and DNA extracted. The quantity and purity of the extracted DNA was determined by OD260/280 ratio using the Nanodrop-1000 instrument (PeqLab Biotechnologie GmbH, Erlangen, Germany).

PCR and sequencing

The PCR primers used for amplifying β-globin, XMRV and MCV have been previous described (Andres *et al*, 2010; Erlwein *et al*, 2010). Amplification was carried out using 1 U of *Taq* polymerase (Applied Biosystems Inc., Foster City, CA, USA), 0.5 mM dNTPs, 1 × PCR reaction buffer, 2 mM MgCl$_2$, 6 pmol of each forward and reverse primers and 200 ng of genomic DNA template in 30 μl reactions. The PCR was performed by an initial 5-min denaturation at 94 °C followed by 40 cycles of 94 °C for 60 s, 55 or 61 °C (depending on the primer set, Table 1) for 60 s and 72 °C for 60 s with a final elongation at 72 °C for 5 min. Each PCR run included a positive control and at least two negative controls. PCR reactions were carried out using an Applied Biosystems thermal cycler GeneAmp PCR System 2700. Amplified products were visualised on 2.5% agarose gel stained with ethidium bromide. All PCR amplified products clearly visible in the agarose gel were subsequently sequenced using the ABI Genetic Analyzer (3130 × 1) and the protocol of ABI Big Dye Terminator Reaction (Applied Biosystems Inc.). The sequence data were analysed using sequence analysis software v5.3 (Applied Biosystems Inc.) and compared with the reference sequences in the GenBank, accession number EF 185282.1 for XMRV and EU375803.1 for MCV.

RESULTS

PCR for β-globin

It is well known that the quality of DNA extracted from FFPE tissues is generally poor, irrespective of the extraction methodology used (Farrugia *et al*, 2010). Extracted DNA is usually fragmented and is only suitable for amplifying small fragments, typically below 300 bp (Coates *et al*, 1991). Taking this into consideration, we employed a PCR strategy that generated products below 200 bp. Additionally, we used a 'house-keeping gene' (β-globin) to assess the amplifiable quality of the extracted DNA. DNA from a total 204 samples (from 58 cases) was amplifiable for β-globin (Figure 1A) and subsequently tested for XMRV and MCV. A total of 15 samples that were negative for β-globin were excluded from further analysis.

PCR for XMRV and MCV using plasmid DNA

The PCR protocol for the detection of XMRV and MCV was initially optimised for sensitivity and specificity by using plasmids containing XMRV or MCV sequences serially diluted (10-fold) in 200 ng of DNA from BE(2)-M17 cell line (human neuroblastoma cell line, kind gift of Professor Omar El-Agnaf, United Arab Emirates University, UAE). We were reproducibly able to detect an estimated 700 copies of XMRV and 1000 copies of MCV DNA from 200 ng of genomic DNA (Figure 1B and C). The copy numbers were
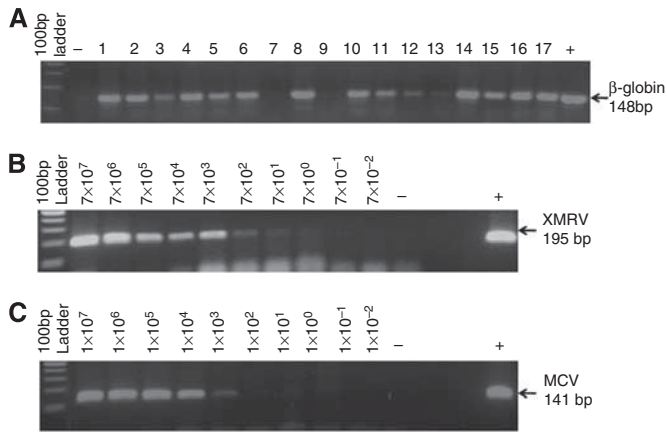
Molecular Diagnostics

Figure 1 PCR for (**A**) β-globin, (**B**) XMRV and (**C**) MCV. DNA extracted from FFPE tissues was assessed for its amplifiable quality by performing PCR for β-globin. (**A**) The 148 bp PCR product (arrow) was clearly visible in agarose gel in 204 of the 219 samples tested. Samples in which β-globin was not amplifiable, for example, samples in lane 7 and 9, were excluded for further analysis. (**B** and **C**) Show doubling dilutions of XMRV and MCV plasmid DNA in 200 ng of cellular DNA. The 100-bp DNA ladder is also indicated.

Figure 2 PCR for MCV in (**A**) breast and (**B**) Merkel cell carcinoma. No MCV-specific amplification product was observed in any of the 204 samples from breast cancer patients. However, 4/6 MCC were found to be positive for MCV.

calculated using the online calculator (Staroscik, 2004). Bands from dilutions with 70 copies of XMRV and 100 copies of MCV were also visible, but were very weak. Thus, our single-round PCR method had a detection sensitivity of 70–700 copies for XMRV and 100–1000 copies for MCV.

PCR analysis for XMRV and MCV in clinical samples

The optimised PCR protocol was used for screening XMRV and MCV in breast cancer. None of the breast tissues (malignant or non-malignant) were found to be positive for XMRV or MCV (Figure 2A). Plasmid controls were consistently positive. Additionally, we examined 12 cases of prostate cancer and 6 cases of MCC as potential positive controls for XMRV and MCV, respectively. Amplification products of the expected size were visible on agarose gels for 2/12 prostate samples and 4/6 MCC samples (Figure 2B).

Sequencing PCR amplified products

To confirm the identity of the PCR bands observed in the prostate and MCC samples, the PCR products were sequenced. For sequencing, sufficient DNA was available from 1/2 XMRV-positive prostate cases and 4/4 MCV-positive MCC cases. Sequence analysis confirmed the products to be of XMRV or MCV origin. The XMRV sequence amplified from the prostate case was 98% homologous to the sequence in the GenBank (accession number EF 185282.1). The prostate XMRV sequence had a single nucleotide deletion at position 469 and two single nucleotide substitutions at positions 553 and 563 (Figure 3). The MCV sequences amplified from the four MCC cases (across regions 2083–2163) were 100% homologous to the MCV strain, MKL-1 (accession number EU375803.1).

DISCUSSION

Breast cancer is a leading cause of death in woman worldwide and recent studies indicate that the incidence of this malignancy is increasing by approximately 3% per year (Forouzanfar *et al*, 2011). It is genera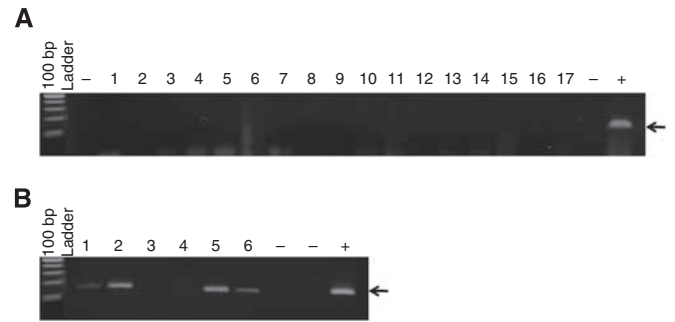lly accepted that environmental factors have an important role in the aetiology of breast cancer. Of the environmental factors, viruses have received considerable attention. Indeed, a number of viruses have been implicated in the pathogenesis of breast cancer, including mouse mammary tumour virus (Fernandez-Cobo *et al*, 2006; Indik *et al*, 2007), human papillomavirus (Damin *et al*, 2004; Akil *et al*, 2008) and Epstein-Barr virus (Preciado *et al*, 2005; Mazouni *et al*, 2011). However, no known virus has yet been conclusively demonstrated to be central in the pathogenesis of this malignancy. Xenotropic murine leukaemia virus-related virus and MCV are two relatively new viruses that have been associated with human malignancies. We have examined the possibility that one of these viruses may be linked to the pathogenesis of breast cancer. We found no evidence for the involvement of these viruses. We did, however, find evidence for the presence of XMRV and MCV in a proportion of prostate and MCC cases, respectively, confirming previous findings (Urisman *et al*, 2006; Feng *et al*, 2008).

Some reports have also shown that XMRV (Lo *et al*, 2010; Fischer *et al*, 2010) and MCV (Kean *et al*, 2009; Tolstov *et al*, 2009; Pancaldi *et al*, 2011) are not restricted to tumours only and can also be found in healthy individuals and normal tissues in tumour-affected patients. Our data does not support this. We tested both malignant and non-malignant tissues, breast and lymph nodes from breast cancer patients, but failed to find viral sequences in any of the 204 samples tested. It is possible that these viruses are present in cells other than those of the breast and lymph nodes that we examined (Pancaldi *et al*, 2011). It is also possible that viral sequences are present, but at very low copy numbers (Pancaldi *et al*, 2011) and beyond the detection limit of the PCR method used in this study. We used a standard single round PCR approach rather than nested PCR, on the premises that if XMRV or MCV is involved in the pathogenesis of breast cancer then the virus would be expected to be present in all of the malignant cells and therefore easily detected by a standard single round PCR methodology. This is indeed what we found with MCV in MCC, where 4/6 cases were clearly positive for the virus. This single round PCR approach also reduces the chances of contamination and false positives.

Although, numerous studies have confirmed the association between MCV and MCC, the relation between XMRV and prostate cancer is far from clear. In fact, the very existence of XMRV as an exogenous human gammaretrovirus has been questioned (Paprotka *et al*, 2011; Knox *et al*, 2011; Cingöz *et al*, 2011). In this study, we found 2 of the 12 prostate samples to be positive for XMRV. One of the two XMRV amplified products was subsequently sequenced and clearly identified as belonging to XMRV VP62 genome. However, the sequence amplified in our case had several mutations compared to XMRV VP62 genome, suggesting that the source of XMRV in this sample was not due to contamination from plasmid XMRV VP62 used as a positive control. We had limited

```
XMRV VP62    461 5'-TTGGGGGACG AGAGACAGAG ACACTTCCCG CCCCCGTCTG AATTTTTGCT-3'
Sample 23c       .........-. .......... .......... .......... ..........

             511 5'-TTCGGTTTTA CGCCGAAACC GCGCCGCGCG TCTGATTTGT TTTGTTGTTC-3'
                 .......... .......... .......... .......... ..g.......

             561 5'-TTCTGTTCTT CGTTAGTTTT CTTCTGTCTT TAAGTGTTCT-3'
                 ..t....... .......... .......... ..........
```

Figure 3 Sequence analysis of XMRV PCR product amplified from prostate sample 23c. Sequence of the region 461 to 600 nucleotides is represented and compared with the XMRV VP62 isolate (GenBank accession number: EF185282). The one deletion and two single nucleotide mutations are shown.

material from these two XMRV-positive prostate samples, and as such we were not able to confirm our findings using alternative primers targeting separate regions of XMRV. Thus, the possibility that the single nucleotide differences found in our case is due to sequencing errors cannot be excluded.

In chronic fatigue syndrome, it is now accepted that the detection of XMRV was most likely due to laboratory contamination and the original paper has now been retracted (Alberts, 2011; Cingöz et al, 2011; Knox et al, 2011; Paprotka et al, 2011; Steffen et al, 2011). Some studies have reported viral particles by electron microscopy as well as XMRV protein expression by immunohistochemistry (Schlaberg et al, 2009; Rodriguez and Goff, 2010; Stieler et al, 2010), indicating that XMRV is transcriptionally active and replication competent. From our data, we cannot, however, draw any conclusions as to whether XMRV represents endogenous or exogenous sequences. Further investigations are required to clarify this controversy and what role this virus has in the pathogenesis of prostate cancer.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Akil N, Yasmeen A, Kassab A, Ghabreau L, Darnel AD, Al Moustafa A-E (2008) High-risk human papillomavirus infections in breast cancer in Syrian women and their association with Id-1 expression: a tissue microarray study. *Br J Cancer* 99: 404–407

Alberts B (2011) Retraction. *Science* 334: 1636

Amarante MK, Watanabe MAE (2009) The possible involvement of virus in breast cancer. *J Cancer Res Clin Oncol* 135: 329–337

Andres C, Belloni B, Puchta U, Sander CA, Flaig MJ (2010) Prevalence of MCPyV in Merkel cell carcinoma and non-MCC tumors. *J Cutan Pathol* 37: 28–34

Ariad S, Milk N, Bolotin A, Gopas J, Sion-Vardy N, Benharoch D (2011) Measles virus antigens in breast cancer. *Anticancer Res* 31: 913–920

Arnold RS, Makarova NV, Osunkoya AO, Suppiah S, Scott TA, Johnson NA, Bhosle SM, Liotta D, Hunter E, Marshall FF, Ly H, Molinaro RJ, Blackwell JL, Petros JA (2010) XMRV infection in patients with prostate cancer: novel serologic assay and correlation with PCR and FISH. *Urology* 75: 755–761

Babakir-Mina M, Ciccozzi M, Presti AL, Greco F, Perno CF, Ciotti M (2010) Identification of Merkel cell polyomavirus in the lower respiratory tract of Italian patients. *J Med Virol* 82: 505–509

Bhatia K, Modali R, Goedert JJ (2010) Merkel cell polyomavirus is not detected in mesotheliomas. *J Clin Virol* 47: 196–198

Bluemn EG, Paulson KG, Higgins EE, Sun Y, Nghiem P, Nelson PS (2009) Merkel cell polyomavirus is not detected in prostate cancers, surrounding stroma, or benign prostate controls. *J Clin Virol* 44: 164–166

Bonnet M, Guinebretiere JM, Kremmer E, Grunewald V, Benhamou E, Contesso G, Joab I (1999) Detection of Epstein-Barr virus in invasive breast cancers. *J Natl Cancer Inst* 91: 1376–1381

Chu PG, Chang KL, Chen YY, Chen WG, Weiss LM (2001) No significant association of Epstein-Barr virus infection with invasive breast carcinoma. *Am J Pathol* 159: 571–578

Cingöz O, Paprotka T, Delviks-Frankenberry KA, Wildt S, Hu W-S, Pathak VK, Coffin JM (2011) Characterization, mapping and distribution of the two XMRV parental proviruses. *J Virol* 86: 328–338

Coates PJ, d'Ardenne AJ, Khan G, Kangro HO, Slavin G (1991) Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. *J Clin Pathol* 44: 115–118

Cox B, Richardson A, Graham P, Gislefoss RE, Jellum E, Rollag H (2010) Breast cancer, cytomegalovirus and Epstein-Barr virus: a nested case-control study. *Br J Cancer* 102: 1665–1669

Damin APS, Karam R, Zettler CG, Caleffi M, Alexandre COP (2004) Evidence for an association of human papillomavirus and breast carcinomas. *Breast Cancer Res Treat* 84: 131–137

Dong B, Kim S, Hong S, Das Gupta J, Malathi K, Klein EA, Ganem D, Derisi JL, Chow SA, Silverman RH (2007) An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors. *Proc Natl Acad Sci USA* 104: 1655–1660

Erlwein O, Kaye S, McClure MO, Weber J, Wills G, Collier D, Wessely S, Cleare A (2010) Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome. *PLoS ONE* 5: e8519

Farrugia A, Keyser C, Ludes B (2010) Efficiency evaluation of a DNA extraction and purification protocol on archival formalin-fixed and paraffin-embedded tissue. *Forensic Sci Int* 194: e25–e28

Feng H, Shuda M, Chang Y, Moore PS (2008) Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* 319: 1096–1100

Fernandez-Cobo M, Melana SM, Holland JF, Pogo BGT (2006) Transcription profile of a human breast cancer cell line expressing MMTV-like sequences. *Infect Agents Cancer* 1: 7

Fischer N, Schulz C, Stieler K, Hohn O, Lange C, Drosten C, Aepfelbacher M (2010) Xenotropic murine leukemia virus-related gammaretrovirus in respiratory tract. *Emerg Infect Dis* 16: 1000–1002

Forouzanfar MH, Foreman KJ, Delossantos AM, Lozano R, Lopez AD, Murray CJL, Naghavi M (2011) Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis. *Lancet* 378: 1461–1484

Furuta RA, Miyazawa T, Sugiyama T, Kuratsune H, Ikeda Y, Sato E, Misawa N, Nakatomi Y, Sakuma R, Yasui K, Yamaguti K, Hirayama F (2011) No association of xenotropic murine leukemia virus-related virus with prostate cancer or chronic fatigue syndrome in Japan. *Retrovirology* 8: 20

Garneski KM, Warcola AH, Feng Q, Kiviat NB, Leonard JH, Nghiem P (2009) Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors. *J Invest Dermatol* 129: 246–248

Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, Brennan DC, Storch GA, Sloots TP, Wang D (2007) Identification of a novel polyomavirus from patients with acute respiratory tract infections. *PLoS Pathog* 3: e64

Glenn WK, Salmons B, Lawson JS, Whitaker NJ (2010) Mouse mammary tumor-like virus and human breast cancer. *Breast Cancer Res Treat* 123: 907–909

Molecular Diagnostics

Herrmann K, Niedobitek G (2003) Epstein-Barr virus-associated carcinomas: facts and fiction. *J Pathol* 199: 140–145

Hohn O, Krause H, Barbarotto P, Niederstadt L, Beimforde N, Denner J, Miller K, Kurth R, Bannert N (2009) Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients. *Retrovirology* 6: 92

Hue S, Gray ER, Gall A, Katzourakis A, Tan CP, Houldcroft CJ, McLaren S, Pillay D, Futreal A, Garson JA, Pybus OG, Kellam P, Towers GJ (2010) Disease-associated XMRV sequences are consistent with laboratory contamination. *Retrovirology* 7: 111

Indik S, Günzburg WH, Kulich P, Salmons B, Rouault F (2007) Rapid spread of mouse mammary tumor virus in cultured human breast cells. *Retrovirology* 4: 73

Kaae J, Hansen AV, Biggar RJ, Boyd HA, Moore PS, Wohlfahrt J, Melbye M (2010) Merkel cell carcinoma: incidence, mortality, and risk of other cancers. *J Natl Cancer Inst* 102: 793–801

Kean JM, Rao S, Wang M, Garcea RL (2009) Seroepidemiology of human polyomaviruses. *PLoS Pathog* 5: e1000363

Khan G, Philip PS, Al Ashari M, Houcinat Y, Daoud S (2011) Localization of Epstein-Barr virus to infiltrating lymphocytes in breast carcinomas and not malignant cells. *Exp Mol Pathol* 91: 466–470

Knouf EC, Metzger MJ, Mitchell PS, Arroyo JD, Chevillet JR, Tewari M, Miller AD (2009) Multiple integrated copies and high-level production of the human retrovirus XMRV (xenotropic murine leukemia virus-related virus) from 22Rv1 prostate carcinoma cells. *J Virol* 83: 7353–7356

Knox K, Carrigan D, Simmons G, Teque F, Zhou Y, Hackett Jr J, Qiu X, Luk K-C, Schochetman G, Knox A, Kogelnik AM, Levy JA (2011) No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected. *Science* 333: 94–97

Labrecque LG, Barnes DM, Fentiman IS, Griffin BE (1995) Epstein-Barr virus in epithelial cell tumors: a breast cancer study. *Cancer Res* 55: 39–45

Larrey D, Bozonnat M-C, Kain I, Pageaux G-P, Assenat E (2010) Is chronic hepatitis C virus infection a risk factor for breast cancer? *World J Gastroenterol* 16: 3687–3691

Lo S-C, Pripuzova N, Li B, Komaroff AL, Hung G-C, Wang R, Alter HJ (2010) Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors. *Proc Natl Acad Sci USA* 107: 15874–15879

Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL, Ruscetti SK, Bagni RK, Petrow-Sadowski C, Gold B, Dean M, Silverman RH, Mikovits JA (2009) Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. *Science* 326: 585–589

Loyo M, Guerrero-Preston R, Brait M, Hoque MO, Chuang A, Kim MS, Sharma R, Liégeois NJ, Koch WM, Califano JA, Westra WH, Sidransky D (2010) Quantitative detection of Merkel cell virus in human tissues and possible mode of transmission. *Int J Cancer* 126: 2991–2996

Manos MM, Gluzman Y (1984) Simian virus 40 large T-antigen point mutants that are defective in viral DNA replication but competent in oncogenic transformation. *Mol Cell Biol* 4: 1125–1133

Mazouni C, Fina F, Romain S, Ouafik L, Bonnier P, Brandone J-M, Martin P-M (2011) Epstein-Barr virus as a marker of biological aggressiveness in breast cancer. *Br J Cancer* 104: 332–337

Melana SM, Nepomnaschy I, Sakalian M, Abbott A, Hasa J, Holland JF, Pogo BGT (2007) Characterization of viral particles isolated from primary cultures of human breast cancer cells. *Cancer Res* 67: 8960–8965

Murray PG (2006) Epstein-Barr virus in breast cancer: artefact or aetiological agent? *J Pathol* 209: 427–429

Pancaldi C, Corazzari V, Maniero S, Mazzoni E, Comar M, Martini F, Tognon M (2011) Merkel cell polyomavirus DNA sequences in the buffy coats of healthy blood donors. *Blood* 117: 7099–7101

Paprotka T, Delviks-Frankenberry KA, Cingöz O, Martinez A, Kung H-J, Tepper CG, Hu W-S, Fivash Jr MJ, Coffin JM, Pathak VK (2011) Recombinant origin of the retrovirus XMRV. *Science* 333: 97–101

Pastrana DV, Tolstov YL, Becker JC, Moore PS, Chang Y, Buck CB (2009) Quantitation of human seroresponsiveness to Merkel cell polyomavirus. *PLoS Pathog* 5: e1000578

Preciado MV, Chabay PA, De Matteo EN, Gonzalez P, Grinstein S, Actis A, Gass HD (2005) Epstein-Barr virus in breast carcinoma in Argentina. *Arch Pathol Lab Med* 129: 377–381

Rodriguez JJ, Goff SP (2010) Xenotropic murine leukemia virus-related virus establishes an efficient spreading infection and exhibits enhanced transcriptional activity in prostate carcinoma cells. *J Virol* 84: 2556–2562

Sato E, Furuta RA, Miyazawa T (2010) An endogenous murine leukemia viral genome contaminant in a commercial RT-PCR Kit is amplified using standard primers for XMRV. *Retrovirology* 7: 110

Schlaberg R, Choe DJ, Brown KR, Thaker HM, Singh IR (2009) XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors. *Proc Natl Acad Sci USA* 106: 16351–16356

Shuda M, Feng H, Kwun HJ, Rosen ST, Gjoerup O, Moore PS, Chang Y (2008) T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. *Proc Natl Acad Sci USA* 105: 16272–16277

Silva Jr RG, da Silva BB (2011) No evidence for an association of human papillomavirus and breast carcinoma. *Breast Cancer Res Treat* 125: 261–264

Small MB, Gluzman Y, Ozer HL (1982) Enhanced transformation of human fibroblasts by origin-defective simian virus 40. *Nature* 296: 671–672

Smith RA (2010) Contamination of clinical specimens with MLV-encoding nucleic acids: implications for XMRV and other candidate human retroviruses. *Retrovirology* 7: 112

Staroscik A (2004) Calculator for determining the number of copies of a template. http://www.uri.edu/research/gsc/resources/cndna.html. (accessed 9 January 2012)

Steffen I, Tyrrell DL, Stein E, Montalvo L, Lee T-H, Zhou Y, Lu K, Switzer WM, Tang S, Jia H, Hockman D, Santer DM, Logan M, Landi A, Law J, Houghton M, Simmons G (2011) No evidence for XMRV nucleic acids, infectious virus or anti-XMRV antibodies in Canadian patients with chronic fatigue syndrome. *PLoS ONE* 6: e27870

Stieler K, Schindler S, Schlomm T, Hohn O, Bannert N, Simon R, Minner S, Schindler M, Fischer N (2011) No detection of XMRV in blood samples and tissue sections from prostate cancer patients in Northern Europe. *PLoS One* 6: e25592

Stieler K, Schulz C, Lavanya M, Aepfelbacher M, Stocking C, Fischer N (2010) Host range and cellular tropism of the human exogenous gammaretrovirus XMRV. *Virology* 399: 23–30

Tolstov YL, Pastrana DV, Feng H, Becker JC, Jenkins FJ, Moschos S, Chang Y, Buck CB, Moore PS (2009) Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays. *Int J Cancer* 125: 1250–1256

Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, Klein EA, Malathi K, Magi-Galluzzi C, Tubbs RR, Ganem D, Silverman RH, DeRisi JL (2006) Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. *PLoS Pathog* 2: e25

van der Meer JWM, Netea MG, Galama JMD, van Kuppeveld FJM (2010) Comment on 'Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome'. *Science* 328: 825

Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G (2005) Breast cancer. *Lancet* 365: 1727–1741

Wetzels CTAH, Hoefnagel JGM, Bakkers JMJE, Dijkman HBPM, Blokx WAM, Melchers WJG (2009) Ultrastructural proof of polyomavirus in Merkel cell carcinoma tumour cells and its absence in small cell carcinoma of the lung. *PLoS ONE* 4: e4958

Molecular Diagnostics