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

G Khan*1, PS Philip1, M Naase2 and KMI Al Zarouni1

1Departments of Microbiology and Immunology, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates; 2School 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 MCC 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.

Keywords: MCV; XMRV; breast cancer; prostate cancer; Merkel cell carcinoma

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, 2009). 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; Kaee 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 malignancies, including...
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 μl 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 previously 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). Amplification reactions were carried out in 10 μl volumes containing 2 μl of DNA, 0.5 m M dNTPs, 1 × PCR reaction buffer, 2 μM MgCl₂, 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

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**Table 1** 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'-CATTCGTGATCAGAACCCTAC-3' | 411–432a | 195            | 55 °C                 |
|          | Reverse| 5'-AGTATCTGGAGAACCCTAAAG-3' | 609–588  |                |                       |
| MCV      | Forward| 5'-GACTTGGCAAAAACCTTTTTCTG-3' | 2022–045b | 141            | 61 °C                 |
|          | Reverse| 5'-CGCGCGTGTGGCAAAATGG-3'  | 2163–143 |                |                       |
| β-Globin | Forward| 5'-TGGTGGTTCTACCCCTTGACC-3' | 148–162c | 148            | 55 °C                 |
|          | Reverse| 5'-GAGTTGTTCGCCAGTGGACCA-3' | 296–277  |                |                       |

Abbreviation: β-G = human β-globin. Location in GeneBank accession number: *EF 185282.1, bEU375803.1, cNM000518.4.*
Absence of XMRV and MCV in breast carcinomas

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It is generally accepted that environmental factors have an increasing 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 MCC 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

![Figure 2](image-url)
Absence of XMRV and MCV in breast carcinomas
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![Sequence analysis of XMRV PCR product amplified from prostate sample 23c.](image)

**Figure 3**

Conflict of interest
The authors declare no conflict of interest.

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