Research Article

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The Retrovirus XMRV Is not Directly Involved in the Pathogenesis of Common Types of Lymphoid Malignancy

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Abstract

Background: A novel retrovirus, xenotropic murine leukemia virus-related virus (XMRV), has been detected in prostate cancer samples and in peripheral blood mononuclear cells (PBMC) from patients with chronic fatigue syndrome. In addition, the virus has been identified in PBMCs from healthy controls. These data suggest that XMRV is circulating in the human population. XMRV is closely related to murine leukemia viruses, which cause lymphoid malignancies in mice. The aim of this study was to determine whether XMRV is directly associated with common forms of human lymphoma or leukemia.

Methods: DNA samples from 368 patients with lymphoid malignancies and 139 patients with benign lymphadenopathy or other malignant disease were screened for XMRV, using three specific and sensitive quantitative PCR assays.

Results: XMRV was not detected in any sample using any of the three assays.

Conclusions: The data suggest that this virus is not directly involved in the pathogenesis of common types of lymphoid malignancy and that XMRV is not a prevalent blood borne infection, at least in the United Kingdom.

Impact: There is no evidence that XMRV is associated with lymphoid malignancies, and further studies should resolve inconsistencies in results of studies examining XMRV prevalence. *Cancer Epidemiol Biomarkers Prev. 20(10); 2232–6. ©2011 AACR.*

Introduction

In 2006, a novel retrovirus, xenotropic murine leukemia virus-related virus (XMRV), was detected in prostate cancer samples (1). Following the initial identification of XMRV sequences by Virochip analysis, complete genomic sequencing revealed that XMRV was closely related to, but distinct from, xenotropic murine leukemia viruses (1). Two subsequent studies from the United States corroborated these findings, detecting XMRV in 23% and 22% of samples by immunohistochemistry and PCR, respectively (2, 3). Other studies have not confirmed a strong association between XMRV and prostate cancer, as evidenced by negative findings in 2 large case series despite the use of similar methodology (4, 5).

Much recent attention has focused on a paper by Lombardi and colleagues (6) that reported the detection of XMRV in peripheral blood mononuclear cells (PBMC) from 68 of 101 (67.3%) patients with chronic fatigue syndrome (CFS). The virus was detected in both B- and T-lymphocytes and virus rescued from patient samples was able to infect lymphoid cell lines (6). In addition, XMRV was detected by PCR in 8 of 218 (3.7%) PBMC samples from healthy controls (6). In contrast, subsequent studies from the United Kingdom, the Netherlands, China, and the United States have not detected XMRV in blood or PBMC samples from a total of more than 600 CFS cases examined (7–11).

Current data on the detection of XMRV therefore lack consistency; however, the initial studies suggest that XMRV may be circulating in the human population. XMRV is a gammaretrovirus closely related to murine leukemia viruses (MLVs; ref. 1). Although xenotropic retroviruses have not, as yet, been shown to be pathogenic, ecotropic MLVs can cause leukemia in mice and gammaretroviruses are associated with leukemia/lymphoma in other species (12). The aim of this study was thus to determine whether XMRV is associated with common types of lymphoma and leukemia in humans.

Materials and Methods

DNA samples from 507 U.K. nonselected patients obtained between 1990 and 2009 were investigated (Table 1). Ethical approval for the study was obtained from a Research Ethics Committee; prior to analysis,

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Table 1. Samples assayed for presence of XMRV

Diagnosis	*N*	Age range (y)	Sex		Amount of DNA assayed
			Male	Female	
Lymphoid tissue samples					
Diffuse large B-cell lymphoma	58	10–95	27	31	1 μg
Follicular lymphoma	59	35–90	28	31	1 μg
Mantle cell lymphoma	9	50–90	8	1	1 μg
Small lymphocytic lymphoma/chronic lymphocytic leukemia	12	40–90	7	5	1 μg
T-cell lymphoma NOS	11	15–85	5	6	1 μg
Non-Hodgkin lymphoma NOS[a]	21	5–85	9	12	1 μg
Classical Hodgkin lymphoma	20	20–85	10	10	1 μg
Nodular lymphocyte predominant Hodgkin lymphoma	22	10–70	12	10	1 μg
Total lymphoma	212	5–95	106	106	
Benign lymphadenopathy	58	5–85	29	29	1 μg
Other malignancy	16	35–80	6	10	1 μg
Total lymphoid tissue	286	5–95	141	145	
Blood or bone marrow samples					
Chronic lymphocytic leukemia	6	60–80	4	2	1 μg
Childhood B-cell precursor acute lymphoblastic leukemia[b]	52	1–13	29	23	500 ng
Acute lymphoblastic leukemia NOS[c]	6	5–10	3	3	1 μg
Total leukemic samples	64	1–80	36	28	
Classical Hodgkin lymphoma	82	20–80	43	39	1 μg
Nodular lymphocyte predominant Hodgkin lymphoma	9	25-80	6	3	1 μg
Lymphoma NOS	1	80	0	1	1 μg
Other childhood malignancy[d]	65	0.7–14	36	29	500 ng
Total blood or bone marrow samples	221	0.7–80	121	100	
Total	507	0.7–95	262	245	

Abbreviation: NOS, not otherwise specified.

[a]Lymphoma NOS includes marginal zone cell lymphoma, Burkitt lymphoma, primary mediastinal B-cell lymphoma, and posttransplant lymphoproliferative disease.

[b]All samples of common acute lymphoblastic leukemia were predominantly leukemic blasts and 32 of these samples were derived from bone marrow.

[c]Two samples were derived from bone marrow.

[d]Includes samples from children with neuroblastoma ($n = 14$), osteosarcoma ($n = 9$), rhabdomyosarcoma ($n = 12$), and Wilms' tumor ($n = 30$).

samples were anonymized or pseudonymized (coded) where prior consent for viral investigative studies had been obtained. Information relating to diagnosis, sample site, age (in 5-year ranges), sex, and year of sampling were retained in the anonymization process but all other information was deleted. The 507 samples included 286 samples derived from secondary lymphoid tissue and, of these, 212 had lymphoma involvement. The remaining 221 samples were derived from bone marrow ($n = 34$) or blood (whole blood, $n = 15$; buffy coat, $n = 71$; PBMCs, $n = 101$); 64 of these samples, including all the bone marrow samples, were from patients with leukemia and contained leukemic cells. There were 368 samples from adults and 139 from children; 262 were from males and 245 from females (Table 1).

Real-time quantitative PCR (qPCR) was used to screen the samples for XMRV. Available XMRV sequences were aligned with those of other MLVs and regions of the *GAG*, *POL*, and *ENV* genes that were conserved among the XMRV isolates were identified. Primers and probes, derived from each of these conserved regions, were selected by using the Primer Express software program v2.0 (Applied Biosystems) and are detailed in Table 2.

To optimize the assay and determine sensitivity, replicates of 10-fold dilutions of commercially available 22Rv1 XMRV-infected prostate cancer cell line DNA (LGC

Table 2. Primers and probes used in quantitative PCR

Primer/probe	Nucleotide position	Sequence
β-Globin 5′ primer	–	GGCAACCCTAAGGTGAAGGC
β-Globin 3′ primer	–	GGTGAGCCAGGCCATCACTA
β-Globin probe	–	CATGGCAAGAAAGTGCTCGGTGCCT
XMRV *GAG* 5′ primer	716	AAGAGGCGCTGGGTTACCTT
XMRV *GAG* 3′ primer	770	TCCTGAGGCCATCCTACATTG
XMRV *GAG* probe	726	TGTTCCGCCGAATGGCCAACTT
XMRV *POL* 5′ primer	4489	CCAGGACATCAAAAAGGAAACAG
XMRV *POL* 3′ primer	4556	TCTCGGGCTGCTTGATCTG
XMRV *POL* probe	4514	CTGAGGCCAGAGGCAACCGTATG
XMRV *ENV* 5′ primer	5950	TGACAGACACTTTCCCTAAACTATATTTTG
XMRV *ENV* 3′ primer	6019	TCCGGGTCATCCCAGTTG
XMRV *ENV* probe	5981	CTTGTGTGATTTAGTTGGAG

NOTE: Nucleotide positions relate to the VP62 XMRV sequence (GenBank: DQ399707).

Standards) were assayed. Dilutions contained 100 ng to 1 pg DNA; because this cell line is estimated to contain at least 10 integrated copies of XMRV per cell, dilutions contained 1.5×10^5 or more to more than 1.5 copies of the XMRV genome per reaction (13). To further determine assay sensitivity, the coding sequence of the XMRV (VP62 sequence) gag matrix protein was synthesized and cloned (DNA 2.0). Multiple replicates of 2-fold dilutions of the resulting plasmid, containing from 64 to 8 copies in a background of 1 μg human placental DNA, were tested by using the *GAG* assay.

qPCR was carried out by TaqMan methodology (Applied Biosystems). All samples were screened for amplifiability by using a human β-globin TaqMan assay (ref. 14; Table 2) before testing with the 3 XMRV assays. Reactions were carried out in a total volume of either 50 or 25 μL and included either 500 ng or 1 μg of DNA (Table 1), each primer at 300 nmol/L, probe at 200 nmol/L, and 1× TaqMan Universal PCR Mastermix without UNG (Applied Biosystems). Replicate dilutions of the positive control, 22Rv1 DNA, were included in each assay to generate a standard curve and a "no template control" was included after every two test samples. Amplification and analysis were carried out on a 7500 Real-Time PCR System by Sequence Detection Software v1.4 (Applied Biosystems), using the default parameters for 40 cycles.

Extensive measures were taken to avoid PCR contamination. Sample processing, DNA extraction, and PCR setup were carried out in a laboratory that had never handled MLVs or known XMRV-positive cell lines or samples. A single-round, closed-tube PCR assay was used and positive control DNA was added to reaction mixes by a second operator in a second location.

Results

We first assessed the sensitivity of our qPCR assays to ensure that they could detect low copy number XMRV genomes. All 3 assays detected XMRV genomes in DNA extracted from the 22Rv1 cell line with similar profiles indicating that the 3 assays have similar sensitivity. Positive results were consistently obtained by using 1 pg template DNA, suggesting that the 22Rv1 cell line contains more than 10 copies of XMRV per cell, as reported by others (4). The *GAG* assay was consistently able to detect 16 copies of XMRV *GAG* plasmid in a background of 1 μg human placental DNA in 6 replicates (Fig. 1). Eight copies were detected in 5 of 6 replicates. The complete genome of the virus present in the DNA from the 22Rv1 cell line has not been sequenced, thus these data also confirmed the ability of the assays to detect XMRV from different sources.

All samples included in the final analysis were satisfactorily amplified by the human β-globin assay; of the 507 samples, 446 (88%) contained at least 500 ng of amplifiable DNA. Each sample was tested for XMRV with the 3 assays: *GAG*, *POL*, and *ENV*. None of the samples was positive in any of the assays. All positive controls were positive and all "no template controls" were negative.

Discussion

This study found no evidence that XMRV is directly involved in the pathogenesis of common types of human lymphoid malignancy. Gammaretroviruses generally cause tumors by insertional activation and outgrowth of a clonal population of infected cells; the virus is therefore present in every tumor cell and should be readily detectable by PCR. We screened 170 samples from a range of non-Hodgkin lymphomas, including 59 follicular lymphomas and 58 diffuse large B-cell lymphomas, with negative results. The striking rise in the incidence of non-Hodgkin lymphoma observed in the latter part of the 20th century cannot, therefore, be explained by the introduction of XMRV into the general population (15, 16). We also investigated XMRV involvement in classical Hodgkin lymphoma and childhood B-cell precursor acute

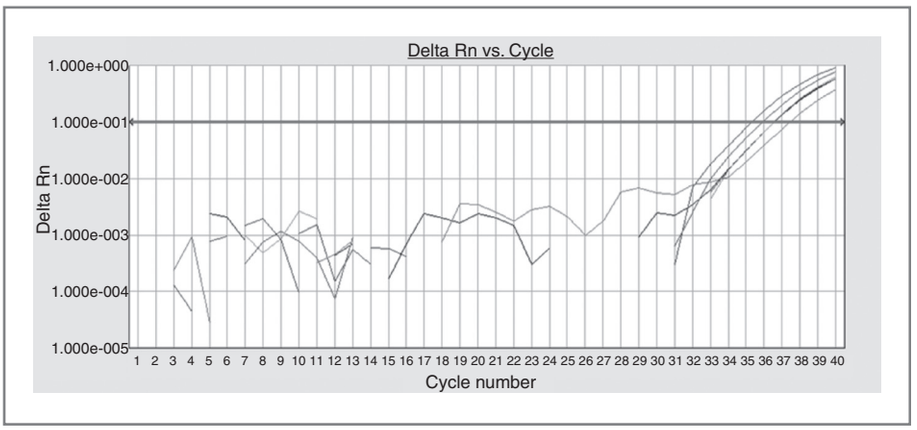

Figure 1. Detection of XMRV by quantitative PCR. Detection of XMRV in 6 replicate PCRs, each containing 16 copies of XMRV *GAG* plasmid in a background of 1 μg high molecular weight DNA, using the *GAG* quantitative PCR assay. Delta Rn: The magnitude of the signal generated by the given set of PCR conditions.

lymphoblastic leukemia, 2 diseases with suspected involvement of infectious agents (17, 18), but found no evidence of XMRV. We cannot exclude the possibility that XMRV is associated with a rare type(s) of lymphoma, analogous to the situation with human herpesvirus 8 and primary effusion lymphoma (19).

A previous study reported that XMRV was detectable in 3.7% of PBMC samples from healthy individuals (6). The present investigation was not designed to determine the prevalence of XMRV in PBMCs; however, a large number of samples of lymphoid tissue and blood or PBMCs were tested in this study. The lymphoid tissue samples included 58 benign lymphadenopathies and 92 of the adult blood samples were from lymphoma patients. In addition, the majority of the lymphoma samples would be expected to contain significant numbers of normal lymphocytes. Because we tested a larger amount of sample DNA (1 μg) than previous studies and our assays have similar sensitivity to those used by others, we anticipated some positive results. Our findings, therefore, do not appear consistent with the DNA PCR results reported by Lombardi and colleagues (6). Taken together with other U.K. studies investigating XMRV in blood samples, these data suggest that XMRV is not a prevalent, blood borne infection in the United Kingdom (7, 8, 20).

There are a number of possible reasons for inconsistencies in data relating to XMRV detection in blood and tissue samples. There may be geographic variation in the prevalence of XMRV. Positive results have largely been reported by groups from the United States (although not all U.S. groups report positive results) whereas Northern European studies have generally been negative (1–9, 11, 21). Although the available data suggest that XMRV is well conserved between isolates, failure to detect the virus could result from nucleotide sequence variation. To reduce the possibility of false-positive results due to primer/probe mismatches, we used 3 assays targeting different regions of the XMRV genome. Technical issues including type of assay, contamination, sensitivity, and sampling error could also explain some discrepancies. In this study we deliberately chose to use a single-round, closed-tube DNA assay rather than a nested analysis and extensive additional measures were taken to avoid contamination. To reduce sampling error, most of our reactions contained 1 μg of DNA and 3 PCRs were performed on each case; although we cannot exclude low-level infection, this sample amount was greater than that assayed in most other studies. cDNA PCR and cell culture may have advantages of sensitivity and allow a larger sample to be investigated (22), but these techniques require more sample manipulation and are therefore potentially more prone to contamination. Differences in DNA extraction methods and PCR methodologies are unlikely to explain all discrepancies in results because XMRV has also been detected by Virochip analysis and virus isolation (1, 6) and distinct viral integration sites have been shown in prostate cancers (23).

More recently, PCR contamination was proposed as the source of patient-derived XMRV integration sites (24). This is consistent with previous data suggesting that the presence of detectable XMRV is likely to be a PCR contaminant (25). Whether XMRV is genuinely circulating in the human population remains to be resolved and further studies, including the exchange of samples and assay comparisons using standardized templates, are ongoing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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