No Detection of the Retrovirus Xenotropic Murine Leukemia Virus-Related Virus in Individuals with Hemophilia

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Keywords

Hemophilia · Virus contamination · Plasma products ·
Virus safety · XMRV

Summary

Background: Xenotropic murine leukemia virus-related virus (XMRV) is a retrovirus that has recently been related to prostate cancers and chronic fatigue syndrome. Since other human-pathogenic retroviruses, such as HIV, human T-lymphotropic virus type I (HTLV-I) and –II, are known blood-transmitted pathogens, XMRV might present another hazard associated with products derived from in vitro cultures of human or animal origin, or blood component-based therapeutics. Here, we investigated whether XMRV was transmitted to individuals with hemophilia and frequent exposure to plasma-derived or recombinant clotting factors. **Methods:** We used highly sensitive real-time PCR to test plasma samples from 127 consecutive individuals with hemophilia who consulted our hemophilia center either for treatment or for a standard check-up. **Results:** From the 127 hemophiliacs, 80 had prior contact to persons with either hepatitis B (n = 30), hepatitis C (n = 74) and/or HIV (n = 21), and 30 were currently being treated with plasma-derived and 97 with recombinant factor concentrates. None of the individuals tested positive for XMRV. **Conclusions:** Independent of the ongoing discussion on whether the positive XMRV testing in initial reports was a result of reagent, sample, or tissue contamination, and whether XMRV is a real threat or a testing artifact, our data suggest that XMRV might not play an important role for hemophiliacs.

Introduction

Since its discovery in 2006 [1], the gamma-retrovirus xenotropic murine leukemia virus-related virus (XMRV) has provoked extensive discussion within the scientific community. XMRV was initially identified in prostate cancers and associated with their occurrence [1, 2]. Cancers with a higher grade of malignancy are more likely to contain detectable virus in their tissue than low-grade prostate cancers [2]. In 2009, the detection of XMRV sequences in the blood of patients with chronic fatigue syndrome alarmed the medical community; 67% of 101 patients with chronic fatigue syndrome were reported to be positive for XMRV compared to 4% of healthy controls [3]. Another study [4] confirmed this high prevalence in patients with chronic fatigue syndrome, and reported a prevalence of XMRV in 6.8% of healthy blood donors. Infectious particles have been reported in cellular and non-cellular components of the blood [3], but other methods of transmission, e.g. airborne [5] and the sexual [6] routes, have also been proposed. XMRV was also proposed to play a causative role in other diseases, including autism, fibromyalgia, multiple sclerosis, amyotrophic lateral sclerosis, and Parkinson's disease. However, the initial reports were soon followed by a large number of studies that could not confirm the associations of XMRV with the different diseases, including prostate cancer and chronic fatigue syndrome [7–19]. Other known human pathogenic retroviruses are HIV, human T-lymphotropic virus type I (HTLV I), and HTLV II. All 3 are associated with severe human pathology and known to be transmitted by blood transfusions. Consequently, in April 2010, Canada recommended as a preventive measure that in-

Table 1. Subjects' characteristics^a

	Number of subjects (%)
Hemophilia A	
Mild	59 (46)
Intermediate	12 (9)
Severe	40 (31)
All	111 (87)
Hemophilia B	
Mild	5 (4)
Intermediate	3 (3)
Severe	7 (6)
All	15 (12)
Acquired hemophilia	1 (1)
All currently treated with plasma-derived coagulation factors	30 (24)
All	127 (100)

^aMedian age (range): 48 (23–85) years.

dividuals with chronic fatigue syndrome should be discouraged from blood donations for fear of transmitting the disease via blood or blood products. Other blood services, including those in the USA, Australia, New Zealand, and the UK, followed soon after with comparable recommendations, which are still in effect today [20, 21].

XMRV also has the ability to infect different cell cultures of human and animal origin [10, 22] and, therefore, might be or might have been a contaminant of in vitro cell culture systems. So far, no viral transmission of XMRV by blood products or other biotechnological therapeutics has been confirmed. However, since prior to the introduction of virus inactivation, HIV, hepatitis C, and/or hepatitis B were transmitted to the majority of hemophilia patients under treatment, this population may be highly susceptible for exposure to XMRV, as for other retroviral pathogens. Even a low prevalence of XMRV within the blood donor population could have led to significant number of infections in hemophiliacs. We therefore established a highly sensitive real-time PCR method for detecting XMRV, and examined the blood of individuals with hemophilia.

Material and Methods

Study Subjects

Following approval by the institutional review board (ethical committee) of the medical faculty of the Johann Wolfgang Goethe University, Frankfurt, 127 consecutive individuals with hemophilia who consulted the hemophilia center for adults at the university clinics, either for treatment or a standard check-up, were included in this study. A description of the study population is shown in table 1. Plasma samples remaining from coagulation testing were aliquoted and stored at –80 °C. Patient samples and data were pseudonymized. The identifier and patient information remained within the hemophilia center, while sample testing and data analysis was performed independently.

Viral RNA Preparation and in vitro Transcription

Plasma (100 µl) from each individual included in the study was subjected to nucleic acid extraction using a viral RNA mini Kit (Qiagen, Hilden, Germany). An in vitro-transcribed RNA fragment was added as an internal control, and was co-extracted to monitor the extraction process and subsequent real-time amplification. From the total eluted volume of 75 µl, 10-µl aliquots were subjected to PCR amplification for XMRV-RNA. Extracts were stored at –20 °C for repeat testing.

XMRV Sequence Amplification and Detection

Real-time amplification of XMRV-RNA was performed using 2 previously published primer and probe sets targeting the env and the gag regions of XMRV-RNA, respectively [6]. For the assay targeting the env gene of XMRV, the following sequences were used: forward primer [Q7472F], 5'-TCAGGACAAGGGTGGTTTGAG-3'; reverse primer [Q7527R], 5'-GGCCCATATGGTGGATATCA-3'; and fluorescent probe [ENV-PRO], (6-FAM) 5'-TTAACAGGTCCCCATGGT-TCACGACC-3' (BHQ1). For the gag assay, the sequences used were: forward primer [Q445T], 5'-GGACTTTTTGGAGTGGCTTTGTT-3'; reverse primer [Q528T], 5'-GCGTAAAACCGAAAGCAAAAAT-3'; and fluorescent probe [F480PRO-BHQ], (6-FAM) 5'-ACAGAGACACTTCCCGCCCCCG-3' (BHQ1). For both assays, primers and a VIC-TAMRA-labeled fluorescent probe were added for non-competitive amplification and detection of the internal control RNA. Both PCR assays were carried out in a total volume of 25 µl consisting of QuantiTect Multiplex RT-PCR master mix, 6 U reverse transcriptase, 400 nmol/l each of the forward and the reverse primers, 100 nmol/l of both probes, and 10 µl template RNA. The PCR assays were performed on a 7300 sequence detection system (Applied Biosystems, Foster City, CA). Amplification was carried out under the following conditions: 55 °C for 20 min, 95 °C for 15 min, and 50 cycles of 95 °C for 10 s and 56 °C for 40 s. In each extraction and amplification run at least 2 positive and negative controls were processed. As positive controls XMRV cell-culture supernatant was used (kindly provided by Indira K. Hewlett, FDA, DC).

Results

Hemophilia Subjects

Individuals with hemophilia who consulted the participating hemophilia center for treatment or for standard check-up were consecutively included in the study. Of the 127 participants, 111 individuals were affected by hemophilia A and 15 by hemophilia B. 1 subject had an acquired hemophilia caused by inhibitory antibodies against factor VIII. 2 additional subjects with congenital hemophilia A also suffered from inhibitory antibodies against factor VIII. 47 patients had the severe form (factor activity < 1% of normal in plasma), 15 the intermediate form (1–5% activity), and 64 the mild form of either hemophilia A or B. An overview is shown in table 1. The median age was 48 years (range 23–85 years). Most of the subjects had had repeated exposure to plasma-derived clotting factors in the past, and there was a high prevalence of blood-transmitted infections in the study group. 30 of the study participants (24%) were still undergoing regular treatment with plasma-derived clotting factors from various suppliers. Of the 127 participants, 30 had contact with hepatitis B and 74 with hepatitis C. A chronic infection was present in 5 individuals

Table 2. Subjects infected with or after contact with blood transmitted pathogens

	Number of subjects (%)
No infection	47 (37)
Hepatitis B and/or C and/or HIV	80 (63)
Hepatitis B	4 (3)
Hepatitis C	32 (25)
HIV	1 (1)
Hepatitis B + C	23 (18)
Hepatitis B + HIV	1 (1)
Hepatitis C + HIV	17 (13)
Hepatitis B +C +HIV	2 (2)
All	127 (100)

with hepatitis B, and 50 subjects were suffering from hepatitis C. 21 subjects were infected by HIV. The infection rates of the group are summarized in table 2.

XMRV-Testing

A highly sensitive real-time PCR method was employed to test plasma samples for XMRV sequences. XMRV particles from cell culture were spiked into control samples and were used as positive controls. An in vitro-transcribed RNA fragment, as internal control, was added as a quality control for the extraction process and to exclude false-negative testing by PCR inhibition. None of the 127 subjects was tested positive for XMRV.

Discussion

In our study, no XMRV was detected in the plasma from a group of individuals with hemophilia and with a high risk of blood-transmitted diseases due to prior and current exposure to plasma-derived coagulation factors. This is apparently good news since, in the past, the majority of individuals with hemophilia had been shown to be affected by hepatitis and HIV-1, and a similar novel pathogen could have widespread implications.

Considering our results and the ongoing discussions about XMRV, there are some important open questions that we want to discuss in some more detail: Is XMRV a sincere threat, or is it a laboratory artifact? Is our methodology adequate to detect XMRV? Could XMRV be transmitted by coagulation factor concentrates derived from plasma fractionation or by recombinant production? And, ultimately, is XMRV a danger for hemophiliacs?

Although initial publications seemed to provide strong evidence for a role of XMRV in prostate cancer and chronic fatigue syndrome, other research teams failed to detect XMRV in samples of patients or healthy control persons [7–19, 23]. Persistent questioning of the initial studies even led to a partial retraction of the original paper on XMRV and

chronic fatigue syndrome [24]. Contaminations of reagents [25], tissues [26], and blood samples [27] have been described. XMRV is closely related to the murine leukemia virus (MLV) and cross-contamination with murine material seemed to have occurred in research laboratories as well as in the reagent production process [28, 29]. Suspicion of sample contamination was further supported by an independently conducted investigation in which none of 9 laboratories leading the field of XMRV was able to correctly detect the virus in coded replicate samples of blood from 15 subjects previously reported to be XMRV positive and 15 healthy control donors [30]. Although most researchers follow the contamination theory, the discussion is still ongoing, and some voices still persist in arguing in favor of a role for XMRV or other MLV-like gamma-retroviruses in human disease [21, 31].

Difficulties in detecting XMRV have been attributed to the transient nature of anti-XMRV antibodies [32] and to tissue- or cell type-specific persistence [3] of XMRV. Here, we tested plasma samples using an in-house nucleic acid amplification testing (NAT) system. We would not have been able to detect viral genomes in prostatic tissue or in cellular parts of the blood by this method. However, we believe that our methodology has several advantages for the purpose of this study. The individual donation NAT system employed is very sensitive and we would be able to detect XMRV even at minimal viral loads. Further, we directly detect the pathogen by amplifying part of its genome. If XMRV was transmitted through plasma products in the past, viral particles would have to be found in the plasma, and this was not the case.

Unlike the case for cellular blood products or fresh frozen plasma, in which novel retroviruses might present an actual risk, XMRV is unlikely to be a contaminant of modern coagulation factors. Viral safety of factor concentrates does not rely exclusively on preventive measures like donor selection and laboratory testing of donor samples or of the producer cell lines for already-known viruses. It also includes reliable pathogen inactivation, which has basically led to the absence of virus infections since its introduction [33]. The different methods in use have been extensively tested for their ability to inactivate retroviruses. The risk of XMRV transmission by modern coagulation factors therefore seems minimal.

Thus, negative testing in younger individuals was expected. On the other hand, the absence of XMRV in the plasma of patients who had received multiple plasma-derived products prior to the introduction of these safety measures argues against an increased infectious risk in individuals with hemophilia.

The growing amount of evidence in the literature that associations of XMRV with human diseases are most likely artifacts caused by sample contaminations or unspecific amplification using nested PCR methods, and our findings not detecting XMRV in the plasma of individuals with hemophilia, all point to the conclusion that XMRV is very unlikely to be a risk in hemophilia therapy.

Acknowledgments

The authors wish to thank Indira K. Hewlett, FDA, DC, for providing XMRV-positive controls. Research was supported by the 'Stiftung Hämotherapie-Forschung' [Hemotherapy Research Foundation] to J.S. and by the German Red Cross Blood Donor Service Baden-Württemberg-Hessen. E.S. received funding through Excellence Cluster Cardio-Pulmonary System (ECCPS, through the German Research Society). E.S. and J.S. received support from the LOEWE Center for Cell and Gene Therapy, Frankfurt, funded by the Hessisches Ministerium für Wissenschaft und Kunst, funding reference no.: III L 4- 518/17,004 (2010). Au-

thor contributions: J.S. and M.K.H. planned experiments, designed research, analyzed data and wrote the manuscript, S.A. and W.M. provided and collected patient samples and information, E.S. and M.S. planned experiments and revised the manuscript.

Disclosure Statement

J.S. is employee of BIOTEST AG. There are no conflicts of interest to disclose.

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