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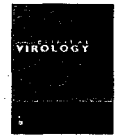

Prevalence of human gammaretrovirus XMRV in sporadic prostate cancer

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ABSTRACT

Background: We previously identified a novel exogenous gammaretrovirus (xenotropic murine leukemia virus-related gammaretrovirus (XMRV)) using a pan-viral microarray. XMRV is the first MLV-related virus found in human infection. Forty percent (8/20) of familial prostate cancer patients homozygous for a mutation in RNase L (R462Q) were positive for XMRV, while the virus was rarely (1/66) detected in familial prostate cancer patients heterozygous for R462Q or carrying the wild type allele.
Objectives: To determine the presence of XMRV in non-familial prostate cancer samples.
Study design: RNA from prostate tissue was analyzed for XMRV using nested RT-PCR. In all samples, RNase L (R462Q) genotyping was performed using an allele-specific PCR.
Results: XMRV-specific sequences were detected in one of 105 tissue samples from non-familial prostate cancer patients and from one of 70 tissue samples from men without prostate cancer. The two XMRV-positive patients were wild type or heterozygous for the R462Q mutation and thus carried at least one fully functional RNase L allele.
Conclusions: XMRV was rarely detected in non-familial prostate cancer samples from Northern European patients. The homozygous mutation R462Q (QQ) was significantly underrepresented (<6%) in this cohort when compared to other studies (11–17%).

1. Introduction

Prostate cancer is the most frequent cancer of men in North America and Europe. Well known factors contributing to the risk of prostate cancer are age, androgens, environmental and genetic factors.[1] Sporadic (non-familial) prostate cancer is the most common form of prostate cancer (80–90%) and its incidence increases with age. Familial prostate cancer, which accounts for 10–20% of all prostate cancer cases, occurs much earlier in life and is defined as prostate cancer occurring in individuals with three or more first degree relatives who had prostate cancer.

Recent work emphasizes that prostate cancer is frequently associated with chronic prostatic inflammation. A lesion called proliferative inflammatory atrophy is often found in the premalignant stages of the disease.[1] Viral infections may be triggers for the inflammatory process. However, epidemiological studies designed to detect links between specific viral infections and prostate cancer have been inconclusive.[2–9]

Recently, a new gammaretrovirus, xenotropic murine leukemia virus-related gammaretrovirus (XMRV), was discovered in prostatic tissue from patients with familial prostate cancer;[10] specifically in patients homozygous for a missense mutation in the RNase L gene, R462Q. Fluorescence in situ hybridization revealed that prostatic stroma cells were infected at low frequency (0.5–1.2%).

RNase L, an endoribonuclease of the antiviral defense pathway, was one of the first prostate cancer susceptibility genes recognized. The missense mutation R462Q has been linked to hereditary prostate cancer [11–13] and has been implicated in up to 13% of all prostate cancer cases in some studies.[11] Not all studies have confirmed this finding, perhaps because of differences in population genetics or environmental factors.[14–16]

In the present study, we analyzed 105 RNA samples from the prostate tissue of 87 sporadic prostate cancer patients and also biopsy samples from 70 healthy men without prostate cancer for the presence of XMRV.

Abbreviations: PCR, polymerase chain reaction; PCA, prostate cancer; SNP, single nucleotide polymorphism; XMRV, xenotropic murine leukemia virus-related virus; PSA, prostate specific antigen; PIA, proliferative inflammatory atrophy; HPC, hereditary prostate cancer.

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Table 1
Clinical and pathological data of all patients

Sample	Age	Group	PSA (ng/ml)	Free PSA (%)	Tumor-stage	Gleason-score
1	36	B	0.33	51.52	NA	NA
2	45	B	0.25	84	NA	NA
3	48	B	0.6	53.33	NA	NA
4	51	B	0.45	37.87	NA	NA
5	34	B	0.2	95	NA	NA
6	33	B	0.22	50	NA	NA
7	46	B	0.57	54.39	NA	NA
8	45	B	0.81	38.27	NA	NA
9	43	B	0.6	53.33	NA	NA
10	40	B	0.44	52.27	NA	NA
11	57	B	0.2	30	NA	NA
12	56	B	0.35	25.71	NA	NA
13	57	B	0.49	30.61	NA	NA
14	63	B	0.75	24	NA	NA
15	56	B	1.01	50.5	NA	NA
16	57	B	1.12	31.25	NA	NA
17	55	B	0.81	24.69	NA	NA
18	56	B	0.42	35.71	NA	NA
19	52	B	0.63	49.21	NA	NA
20	57	B	0.82	21.95	NA	NA
21	55	B	0.83	24.1	NA	NA
22	65	B	0.5	NA	NA	NA
23	63	B	0.69	34.78	NA	NA
24	64	B	0.86	27.91	NA	NA
25	55	B	1.1	25.45	NA	NA
26	80	B	20.54	12.37	NA	NA
27	64	B	NA	NA	NA	NA
28	63	B	6.53	20.52	NA	NA
29	52	B	8.15	18.53	NA	NA
30	66	B	6.28	30.89	NA	NA
31	65	B	13.39	29.42	NA	NA
32	70	B	27.54	10.64	NA	NA
33	65	B	3.05	31.15	NA	NA
34	57	B	5.32	22.37	NA	NA
35	77	B	NA	NA	NA	NA
36	71	B	3.05	9.18	NA	NA
37	70	B	7.52	23.94	NA	NA
38	71	B	10.46	18.36	NA	NA
39	60	B	6.4	19.69	NA	NA
40	60	B	6.36	17.45	NA	NA
41	66	A	5.6	21.79	pT2c	3+3
42	65	A	2.85	21.05	pT2c	3+3
43	58	A	4.99	11.82	pT2c	3+3
44	67	A	4.07	23.83	pT2c	3+3
45	68	A	6.1	15.9	pT2a	3+3
46	62	A	3.14	14.01	pT2c	3+4
47	63	A	8.53	8.79	pT2c	3+4
48	65	A	NA	NA	NA	3+3
49	62	A	7.84	23.85	pT2c	3+3
50	50	A	6.42	12.31	pT2a	3+3
51	51	A	7.86	14.5	pT2c	3+3
52	50	A	3.69	25.75	pT2c	3+3
53	68	A	5.91	19.8	pT3b	4+3
54	69	A	3.67	16.08	pT2a	4+3
55	65	A	9.81	9.68	pT3a	4+3
56	68	A	11.35	8.11	pT3a	5+4
57	63	A	9.26	18.79	pT2c	4+3
58	54	A	5.8	8.1	pT2c	3+3
59	64	A	NA	NA	pT2c	3+4
60	52	A	3.61	13.57	pT2c	3+2
61	65	A	6.02	10.3	pT3a	3+4
62	64	A	2.68	23.88	pT2a	3+3
63	71	A	9.67	11.48	pT3a	4+3
64	62	A	6.4	6.72	pT3a	4+3
65	–	A	9.32	4.4	pT2c	3+2
66	60	A	NA	NA	pT2c	3+4
67	52	A	8.77	8.32	pT2c	3+4
68	–	A	3.34	15.27	pT3a	3+3
69	57	A	5.46	20.88	pT2c	3+3
70	57	A	5.33	14.45	pT3b	4+3
71	67	A	11.91	9.15	pT2c	4+3
72	53	A	3.04	15.46	pT2c	3+4

Table 1 (*Continued*)

Sample	Age	Group	PSA (ng/ml)	Free PSA (%)	Tumor-stage	Gleason-score
73	62	A	9.11	9.77	pT2c	3+3
74	63	A	9.38	6.18	pT2c	3+3
75	67	A	5.13	15.01	pT2c	3+3
76	66	A	12.27	14.83	pT3b	3+4
77	57	A	24.17	28.71	pT2c	3+3
78	66	A	3.7	12.16	pT2c	3+4
79	65	A	6.2	4.52	pT2c	3+4
80	54	A	9.53	5.14	pT2a	4+3
81	58	A	17.98	13.24	pT3a	4+3
82	53	A	5.52	13.22	pT2c	3+4
83	62	A	4.48	7.81	pT2a	3+4
84	70	A	3.56	18.82	pT2c	3+3
85	61	A	3.15	21.9	pT3a	3+4
86	58	A	6.48	6.48	pT2c	3+4
87	65	A	10.28	10.21	pT2c	3+4
88	54	A	22.62	4.02	pT3a	3+4
89	65	A	16.71	6.1	pT2a	4+4
90	71	A	6.19	16.16	pT2c	3+3
91	54	A	7.31	7.8	pT2c	3+4
92	62	A	7.85	6.62	pT2c	3+4
93	64	A	5.75	9.91	pT2c	3+3
94	63	A	5.04	8.5	NA	3+3
95	73	A	10.13	20.24	NA	3+4
96	69	A	6.22	9.8	NA	3+4
97	60	A	9.18	13.2	NA	4+4
98	69	A	17.98	1.7	NA	3+4
99	73	A	29.76	5.7	NA	3+4
100	57	A	7.09	14.1	NA	3+3
101	60	A	4.02	17.7	NA	3+3
102	75	A	6.75	21	NA	3+3
103	64	A	4.54	29.7	NA	3+3
104	72	A	6.94	1.2	NA	4+3
105	65	A	10.59	18.1	NA	3+3
106	59	A	8.67	18.2	NA	4+3
107	71	A	2.79	17.6	NA	3+3
108	49	A	1.12	24.1	NA	3+3
109	66	A	8.17	7.6	NA	5+3
110	65	A	NA	NA	pT3a	3+4
111	71	A	NA	NA	pT2c	4+3
112	48	A	NA	NA	pT2c	3+3
113	67	A	NA	NA	pT3b	4+3
114	62	A	NA	NA	pT3b	4+3
115	76	A	NA	NA	pT3b	3+4
116	58	A	NA	NA	pT3b	3+4
117	63	A	NA	NA	pT2c	3+4
118	59	A	NA	NA	pT3b	3+4
119	69	A	NA	NA	pT3b	NA
120	67	A	NA	NA	pT2b	3+3
121	70	A	NA	NA	pT3a	3+4
122	60	A	NA	NA	pT3b	3+4
123	70	A	NA	NA	pT3b	4+3
124	67	A	NA	NA	pT3b	3+4
125	67	A	NA	NA	pT2c	3+3
126	44	A	2.77	13	NA	NA
127	65	A	10.46	12.05	NA	NA
128	67	B	4.63	13.6	NA	NA
129	62	B	3.54	13.37	NA	NA
130	68	B	14.78	17.48	NA	NA
131	55	B	4.78	22.18	NA	NA
132	70	B	10.52	19.1	NA	NA
133	67	B	3.49	24.64	NA	NA
134	44	B	3.74	14.44	NA	NA
135	69	B	9.14	18.2	NA	NA
136	59	B	5.33	12.4	NA	NA
137	63	B	4.1	14.2	NA	NA
138	57	B	1	35	NA	NA
139	61	B	9.72	21.71	NA	NA
140	60	B	4.58	34.7	NA	NA
141	66	B	4.6	30.65	NA	NA
142	67	B	5.42	35.1	NA	NA
143	62	B	4.1	17.6	NA	NA
144	75	B	3.89	28.3	NA	NA

Table 1 (*Continued*)

Sample	Age	Group	PSA (ng/ml)	Free PSA (%)	Tumor-stage	Gleason-score
145	56	B	9.23	21.9	NA	NA
146	65	B	4.94	13.3	NA	NA
147	64	B	15.58	10.14	NA	NA
148	71	B	7.52	17.6	NA	NA
149	71	B	6.17	23.82	NA	NA
150	72	B	2.79	34.4	NA	NA
151	47	B	2.76	17	NA	NA
152	67	B	2.87	35.5	NA	NA
153	53	B	5.5	18.9	NA	NA
154	65	B	5.84	36.5	NA	NA
155	62	B	4.94	21.1	NA	NA
156	65	B	9.02	21.1	NA	NA
157	54	B	5.97	13.1	NA	NA

NA: "not analyzed".

2. Methods

2.1. Tissue sampling and RNA isolation

We studied histological tumor-free prostate biopsies from 87 patients (Group A; samples 41–127) with confirmed cancer undergoing radical prostatectomy at the Urology Department of the University Hospital Hamburg-Eppendorf, and from 70 control donors (Group B). Group B samples 1–40 were from men defined as healthy according to the following parameters: serum PSA <1 ng/ml; no family history of prostate cancer; normal transrectal ultrasound or negative digital rectal examination. Group B samples 128–157 were from men with multiple negative biopsy series.

In some patients with a large prostate cancer, biopsies were taken from the cancerous region (T) as well as from the region without signs of cancer (N) as confirmed by histology.

Tissue specimens were collected strictly from the peripheral zone of the prostate by ultrasound-guided transrectal biopsy. Samples were fixed in RNAlater (Qiagen) and RNA was isolated using RNeasy-columns (Qiagen) followed by RNA quality control using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc.). Clinical and pathological data for all individuals are shown in Table 1. The study was approved by the local ethics committee (*no. OB-052-04*).

2.2. XMRV RT-PCR; sequencing and clustering of XMRV gag sequences

XMRV-specific RT-PCR was performed as described previously.[10] Briefly, total RNA extracted from tissue obtained by needle biopsy was analyzed in a nested RT-PCR reaction using XMRV specific primers. PCR fragments were gel purified using QIAEX II gel extraction kit (Qiagen) cloned into pCR2.1-TA vector (Invitrogen) and sequenced. For phylogenetic tree analysis, a published sequence set was used.[10] The gag sequences were aligned with ClustalX version 1.82 [17,18] using default settings.

2.3. RNase L genotyping and quantitative real-time PCR

Allele-specific PCR to detect single nucleotide polymorphism R462Q was performed as described.[11] Briefly, this allele-specific PCR utilized two primers, each with the 3′ terminal base complementary to one of the alleles to be identified. Two separate PCR amplification reactions using the same reverse primer were performed to detect each allele. For the quantitative real time PCR, 100 ng of total RNA was reverse transcribed in a total volume of 25 μl using random primers. 5 μl of cDNA were amplified using the Qiagen SyBr Green Master Mix on a Biorad iCycler according to manufacturer's instructions. Each experiment was performed in triplicate. The primers used were described recently.[19]

DU145 and LNCaP, two prostate cancer cell lines (American Type Culture Collection), were cultured in RPMI medium supplemented with 10% FCS.

3. Results

3.1. Low frequency of XMRV in sporadic prostate cancer

The gammaretrovirus XMRV was originally identified in RNase L-deficient prostate cancer tissue of patients with familial prostate cancer. In the absence of epidemiological data for XMRV, the present study was initiated to extend the search for XMRV-specific sequences to include patients with sporadic prostate cancer independent of the RNase L status. Only one sample of 105 obtained from the sporadic prostate cancer patients was positive for XMRV (0.95%) by RT-PCR. Additionally, XMRV sequences were detected in one of the 70 (1.42%) RNA samples from prostate tissue of healthy donors (Table 2).

To examine the relationship between the amplified sequences and those previously published for XMRV,[10] the gag region from both samples was amplified by nested RT-PCR and sequenced. The sequences were highly similar (Fig. 1), showing 98–99% sequence identity within a 390-bp region of gag, suggesting that the amplified sequences are indeed from the same virus, XMRV.

3.2. RNase L genotyping

Data from our earlier studies provide evidence that functional mutations in RNase L might be important for the acquisition of XMRV. In the present study, an allele-specific PCR [11] for the SNP R462Q within RNase L was performed (Fig. 2). Neither of the two XMRV-positive samples was homozygous for the R462Q mutation. The prostate cancer sample (sample 57) was heterozygous (QR) and the control sample (sample 6) displayed a wild type (RR) RNase L genotype. The results obtained by PCR were confirmed by sequencing (Fig. 2B). Table 3 summarizes the results of the RNase L SNP R462Q genotyping of all samples included in the study. Only a few of the samples (<6%) showed the homozygous QQ genotype previously reported to be present in 13–15% of control cases, sporadic prostate cancer samples and familial prostate cancer samples.[11,12,15,16,20–22] The distribution of wild type and heterozygous mutations was concordant with published results.

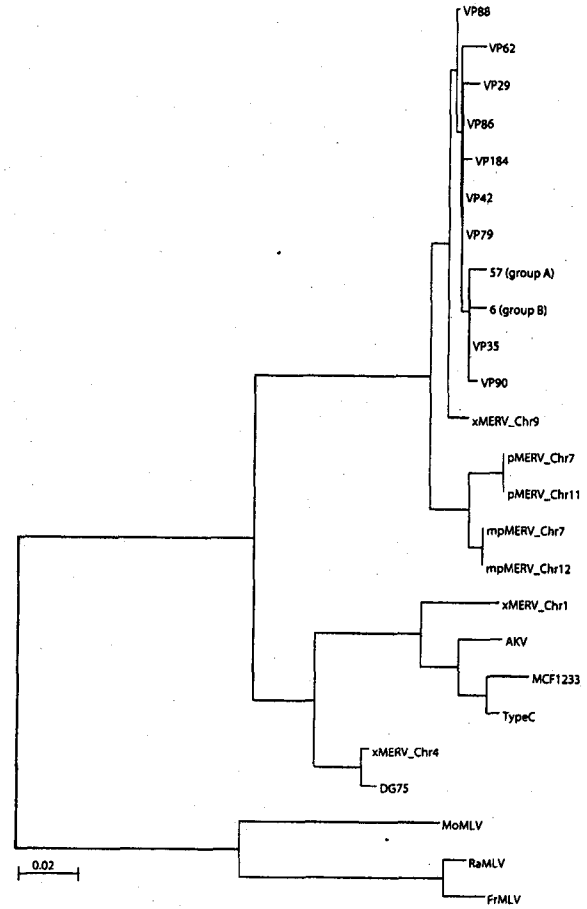

Fig. 1. XMRV gag sequences derived from sporadic and familial prostate cancer samples. Phylogenetic tree comparing a 390-nt RT-PCR gag fragment amplified from sporadic tumor samples (57 in Group A and 6 in Group B) with recently published XMRV sequences from familial prostate cancer patients.[10] The sequences were aligned using ClustalX and the tree was generated using the neighbor-joining method. Sequences are labeled as xenotropic (X), polytropic (P), modified polytropic (Pm), or ecotropic (E).

3.3. RNase L expression

Relative RNase L mRNA expression levels were assayed in XMRV-positive samples using quantitative real time RT-PCR. LNCaP cells, which have an inactivating deletion mutation in one allele of *RNASEL*,[20] These cells had a 20-fold reduction in RNase L expression levels compared to DU145 cells (Fig. 3). In contrast, the two XMRV-positive samples (6 and 57) did not have reduced RNase L expression when compared to DU145 cells. Randomly selected samples from our cohort representing Group A and Group B did not show major differences in RNase L expression. However, we observed a 50-fold difference in RNase L mRNA expression when comparing RNA from tumor cells (T) with normal tissue (N) from patient 117. Two other samples, 118 and 119, did not show major differences in RNase L expression between tumor cells and normal cells.

Table 2
XMRV detection using nested gag RT-PCR

	PCA (Group A)	Control (Group B)
XMRV RT (+)	1/105	1/70
Median age (yrs)	61.1	58.5

Total RNA isolated from prostate tissue from patients with prostate cancer (PCA) was analyzed for the presence of XMRV sequences using an RT-nested PCR. GAPDH was amplified in parallel as an internal control.

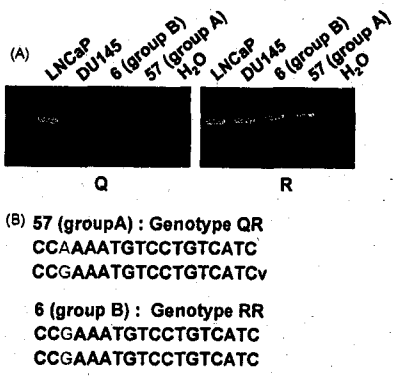

Fig. 2. Allele-specific PCR for the genotyping of the R462Q (A1385G) mutation in the RNase L gene. Ethidium bromide stained agarose gel showing PCR-positive fragments of RNase L using allele-specific forward primers Q (left) or R (right) in separate PCR reactions with a common reverse primer R. RNA from prostate cancer cell lines LNCaP (Genotype QR) and DU145 (Genotype RR) were used as controls. (B) Nucleotide sequence of the two XMRV positive PCR fragments from tumor samples shown in (A). Nucleotide exchange at position 1385 is shown in light grey.

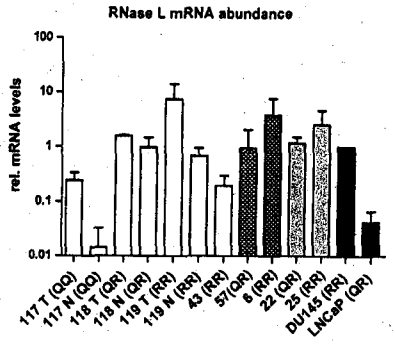

Fig. 3. RNase L mRNA expression in prostate tissue samples. RNase L mRNA expression was measured by quantitative real time PCR as described in Section 2. Standard deviations from three independent experiments (four replicates each) are shown. Prostate cancer cell lines DU145 and LNCaP (shown as black bars) were used as controls. RNase L mRNA levels from healthy control patients (Group B) are shown in light grey, samples from PCA patients (Group A) are shown as white bars. T indicates RNA from tumor region, N stands for RNA from normal tissue. The two XMRV-positive cases are indicated as speckled bars. The RNase L genotype of all samples is shown in brackets.

Table 3
RNase L genotyping of SNP R462Q

Study group	Number screened	RNASEL (SNP 462)			Sample type
		RR	RQ	QQ	
PCA (Group A)	87	51	29	7	Tissue DNA
Control (Group B)	70	42	24	4	Tissue DNA

DNA from prostate tissue from patients with prostate cancer (PCA) was analyzed for the presence of a single nucleotide polymorphism (SNP) at amino acid position 462 within the RNase L gene. RR, RQ, QQ: wild type, heterozygous and homozygous genotype, respectively.

4. Discussion

XMRV, a novel gammaretrovirus, was recently identified in familial prostate cancer samples using a pan-viral microarray.[10] Our earlier studies suggested that functional mutations in RNase L might be important for the acquisition of XMRV. Almost all XMRV-positive prostate cancer cases described so far carry a mutation within RNase L (R462Q), resulting in reduced RNase L activity.[10]

However, the current study found only a low prevalence of XMRV in non-familial prostate tissue of men in Northern Europe.

RNase L, an endoribonuclease of the antiviral defense pathway, was one of the first susceptibility genes discovered in prostate cancer. The HPC1 (hereditary prostate cancer) locus was linked to prostate cancer in several genetic linkage studies performed in North America and Finland.[11,12,20,23] This finding was not be confirmed in a large case control study recently conducted in Germany.[16] RNase L is implicated in the interferon-mediated antiviral defence pathway and has been shown to play a role in several models of viral infection including influenza A, West Nile virus and herpes simplex virus.[21,22,24–26]

In our previous study, XMRV was detected in familial prostate cancer patients homozygous for the R462Q variant (QQ) of RNase L.[10] Overall, 40% (8/20) of patients homozygous for the SNP R462Q (QQ) has XMRV infection, whereas only 1.5% (1/66) patients heterozygous (QR) or carrying the wild type allele (RR) were XMRV-positive. Subsequent in vitro experiments demonstrating that XMRV replication increases with reduced RNase L activity further corroborated our previous results.[27]

So far, there are no epidemiological data regarding the prevalence of XMRV in prostate tissue, independent of the RNase L status. The prevalence of XMRV in our cohort was low (1.14%). The two XMRV-positive patients were heterozygous (HR) or wild type (RR) genotype and showed no deficiency in RNase L expression. These results are in accord with our previous observation that XMRV sequences are predominantly found in prostate cancer patients with a deficiency in RNase L and only rarely found in prostate cancer patients with at least one fully functional RNase L allele.

Genotyping for the SNP R462Q in the C-terminal domain of RNase L revealed that homozygosity of R462Q (QQ) is a relatively rare event (<6%). These results are in contrast to previous studies that have reported homozygous R462Q (QQ) mutations in 11–17% of cases, independent of the genetic background of the population. At present, we do not have an explanation for this observation, since all of our patients are of Caucasian background and live in Northern Europe.

We were not able to look for SNP R462Q in the germline cells of our cohort, as such material was not available. However, a recent study comparing germline with somatic mutations of RNase L observed a similar distribution of homozygous, heterozygous or wild type allele frequency in both tissue types.[28]

In conclusion, our results suggest that XMRV is not associated with sporadic prostate cancer in Northern Europe. The availability of an XMRV antibody-screening test should greatly enhance epidemiological studies of the prevalence of XMRV in larger cohorts of prostate cancer patients as well as in the general population.

Conflict of interest

The authors declare they have no competing interest.

Acknowledgement

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Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients

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Abstract

Background: A novel gammaretrovirus named *xenotropic murine leukemia virus-related virus* (XMRV) has been recently identified and found to have a prevalence of 40% in prostate tumor samples from American patients carrying a homozygous R462Q mutation in the RNaseL gene. This mutation impairs the function of the innate antiviral type I interferon pathway and is a known susceptibility factor for prostate cancer. Here, we attempt to measure the prevalence of XMRV in prostate cancer cases in Germany and determine whether an analogous association with the R462Q polymorphism exists.

Results: 589 prostate tumor samples were genotyped by real-time PCR with regard to the RNaseL mutation. DNA and RNA samples from these patients were screened for the presence of XMRV-specific *gag* sequences using a highly sensitive nested PCR and RT-PCR approach. Furthermore, 146 sera samples from prostate tumor patients were tested for XMRV Gag and Env antibodies using a newly developed ELISA assay. In agreement with earlier data, 12.9% (76 samples) were shown to be of the QQ genotype. However, XMRV specific sequences were detected at neither the DNA nor the RNA level. Consistent with this result, none of the sera analyzed from prostate cancer patients contained XMRV-specific antibodies.

Conclusion: Our results indicate a much lower prevalence (or even complete absence) of XMRV in prostate tumor patients in Germany. One possible reason for this could be a geographically restricted incidence of XMRV infections.

Background

Prostate cancer (PCa) is currently the most commonly diagnosed cancer in European males and causes approximately 80,000 deaths per year [1]. Modern methods of diagnosis and extensive programs for early detection have increased the chances for successful treatment in recent

years, but there is still only limited knowledge concerning susceptibility and putative risk factors for PCa. In addition to age, the risk factors for developing PCa are thought to be diet, alcohol consumption, exposure to ultraviolet radiation [2], and genetic factors [3]. One of the first studies to investigate the hereditary factors associated with a predisposition for developing prostate cancer identified the HPC1 locus (hereditary prostate cancer locus-1) [4], which is now known to harbor the RNaseL gene. RNaseL codes for a endoribonuclease involved in the IFN-regulated antiviral defense pathway (reviewed by [5]). The significance of RNaseL gene polymorphisms for the development of prostate cancer is still under scrutiny. The R462Q (rs486907) polymorphism for example is implicated in up to 13% of all US prostate cancer cases [6] and three other variants contribute to familial prostate cancer risk in the Japanese population [7], whereas no significant association with disease risk could be found in the German population [8].

Recently, an analysis for viral sequences in prostate cancer stroma tissues using custom-made microarrays resulted in the discovery of a new gammaretrovirus named *xenotropic murine leukemia virus-related virus* (XMRV), [9,10]. XMRV was present in eight of twenty (40%) cases in patients with familial prostate cancer that were homozygous at the R462Q locus for the QQ allel. On the other hand, the virus could be detected in only 1.5% of carriers of the RQ or RR allels. In subsequent studies involving smaller cohorts of European prostate cancer patients, the prevalence and correlation of the QQ-phenotype with the presence of XMRV were either far less significant [11] or the virus could not be detected at all [12]. Very recently XMRV was recognized by immunohistochemistry in 23% of prostate cancers from US American donors, independent of the R462Q polymorphism [13].

This present study describes the development and use of sensitive PCR and RT-PCR assays to test DNA and RNA from 589 PCa tumor samples obtained from the Charité hospital in Berlin (Germany) for the presence of proviral XMRV DNA and corresponding viral transcripts. In addition, we used an ELISA based on recombinant XMRV proteins to screen 146 PCa patient sera for viral Env- and Gag-specific antibodies. Neither in the 76 specimens homozygous for the QQ allele, nor in any of the other samples could XMRV or a related gammaretrovirus be detected. Furthermore, none of the sera contained antibodies specific for the XMRV Env or Gag proteins.

Methods

Patients

Tissue samples were collected from 589 patients undergoing radical prostatectomy for histologically proven primary prostate cancer at the Department of Urology, Charité - Universitätsmedizin Berlin, between 2000 and 2006. Institutional review board approval for this study was obtained and all patients gave their informed consent prior to surgery. Tissue samples were obtained immediately after surgery, snap-frozen in liquid nitrogen and stored at -80°C. Histopathologic classification of the samples was based on the World Health Organization and 1997 TNM classification guidelines (International Union Against Cancer, 1997). The patient's median age was 63 years (range 43 - 80). The serum PSA levels were measured prior to surgery and ranged from 0.1 to 100 ng/ml (median 7.5 ng/ml). 405 of 589 patients (69%) had organ-confined disease (pT2) while the remaining 31% had non organ-confined disease (pT3 and pT4). Using the Gleason-score (GS) system, the sample population was divided into low-grade tumors (GS 2-6, n = 282), intermediate cases (GS 7, n = 175), and high-grade prostate carcinomas (GS 8-10, n = 68).

Nucleic acid isolation

Frozen tissues were mechanically sliced and immediately lysed in DNA- or RNA-lysis buffer, column-purified, and eluted (50-200 µl) according to the manufacturers instructions (QIAamp DNA Mini Kit, RNeasy Mini Kit, QIAGEN GmbH, Hilden, Germany). The $OD_{260/280}$ ratio and nucleic acid concentrations were determined using the Nanodrop-1000 instrument (PeqLab Biotechnologie GmbH, Erlangen, Germany). In addition, RNA samples were checked for integrity using a Bioanalzyer-2100 (Agilent Technologies, Inc., Santa Clara CA, United States). No additional macro-/micro-dissections were performed on the prostate tissues because viral nucleic acids were expected to be present preferentially in the stromal compartments.

Diagnostic PCR

A nested PCR was developed for the detection of XMRV sequences that amplifies regions upstream of the *gag* start codon, harboring the unique 24 nt deletion [10]. First, we constructed by fusion-PCR a synthetic gene representing the region from nucleotide 1 to 800 of the MLV DG-75 (Genbank acc. number AF221065). This fragment was cloned into the pCR4-TOPO vector (Invitrogen, Karlsruhe, Germany), and the identity of the fragment was confirmed by sequencing. The same procedure was used to clone a corresponding 800 nt fragment for use as a positive control of the XMRV genome (Genbank acc. num. EF185282). Conditions of first round PCR for the detection of proviral sequences were: 100 ng patient DNA, primer Out-For 5'-CCGTGTTCCCAATAAAGCCT-3', Out-Rev 5'-TGACATCCACAGACTGGTTG-3', (30 sec @ 94°C, 30 sec @ 60°C, 30 sec @ 72°C) × 20 cycles. Using $1/10^{th}$ of the first reaction and primer In-For 5'-GCAGCCCTGGGAGACGTC-3' and In-Rev 5'-CGGCGCGGTTTCGGCG-3' the second round PCR is able to detect any XMRV-like sequences, e.g. MLV DG-75. In addition, using a primer spanning the XMRV-specific deletion 5'-CCCCAACAAAGCCACTCCAAAA-3' we were able to distinguish between XMRV and DG75 sequences. Second round PCR reaction was performed at an elevated annealing temperature of 64°C for 35 cycles.

A nested-PCR strategy was used to detect XMRV-specific viral RNAs in the total RNA of prostate tissue samples in which the first round RT-PCR was performed as described above using *In-For* and *In-Rev* followed by a quantitative real-time PCR published by Dong *et al.*, 2007 [9,14]. As an internal control, a human GAPDH specific primer and probe set were included in which the primers for the outer RT-PCR were the same as for the inner PCR: forward 5'-GGCGATGCTGGCGCTGAGTAC-3' reverse 5'-TGGTCCACACCCATGACGA-3' and the probe 5'-YAK-TTCACCACCATGGAGAAGGCTGGG-Eclipse Dark quencher-3' [15].

RNaseL genotyping

A real-time PCR setup designed by Olfert Landt/TIB MOLBIOL, Berlin was used for RNaseL genotyping of tumor samples which detects the single nucleotide polymorphism G1385A (rs486907) responsible for the R462Q mutation. PCR was carried out with R462Q_F CCTATTAAGATGTTTTGTGGTTGCAG, R462Q_A GGAAGATGTGGAAAATGAGGAAG and the probes R462Q_(A) YAK-ATTTGCCCAAAATGTCCTGTCATC-BBQ and R462Q_(G) FAM-ATTTGCCCGAAATGTCCTGTCATC-BBQ following a two-step protocol with 95°C for 20 sec and 60°C for 1 min. Positive controls were constructed by fusion PCR, starting with 40 mer oligonucleotides, of the two 297 bp fragments corresponding to the "R"- and "Q" versions of the RNAseL genomic region and cloning these into the pCR4-TOPO vector (Invitrogen). For each PCR, positive control plasmids containing the R- or Q-sequence were included.

Recombinant Proteins, Immunization

Recombinant proteins of XMRV pr65 (Gag) and gp70 (Env/SU) were generated for immunization and for the ELISA assays. For XMRV Env/SU, a fragment containing the amino acids 1-245 of the surface unit (gp70) was amplified, cloned in pET16b vector (Novagen, Gibbstown, USA) and expressed in BL21 *E. coli*. For XMRV Gag (pr65), two fragments (amino acids 1-272 and 259-535) that overlap by 14 amino acids were constructed. The expressed proteins were affinity purified using a Ni-NTA column and eluted in 8 M urea, and proteins for immunization were subsequently dialyzed against phosphate buffered saline. BALB/c mice were immunized with the recombinant fragments of the Envelope or Gag proteins, and sera were collected throughout the period of four immunizations. All animal experiments were performed in accordance with institutional and state guidelines.

ELISA

Two weeks after the last immunization the mice were bled, and serum antibodies were measured by solid phase enzyme-linked immunosorbent assay (ELISA). Briefly, bacterially expressed and purified (via His-tag) protein fragments were coated overnight on Probind-96-well plates (Becton Dickinson Labware Europe, Le Pont de Claix, France) at room temperature in equimolar amounts. The plates were blocked with 2% Marvel milk powder in phosphate buffered saline (PBS) for 2 h at 37°C, washed three times with PBS, 0.05% Tween 20 and serial diluted mouse sera or patient sera at a 1:200 dilution in PBS with 2% milk powder and 0.05% Tween20 added into each well. After incubation for 1 hour at 37°C, each well was again washed three times and a 1:1000 dilution of a goat anti-mouse IgG-HRP conjugate (Sigma Aldrich, Munich, Germany) in PBS, 2% milk powder, 0.05% Tween 20 (Serva, Heidelberg, Germany) was added. After further incubation for 1 hour at 37°C, each well was again washed three times. The chromogen orthophenylendiamin (OPD) in 0.05 M phosphate-citrate buffer, pH 5.0 containing 4 µl of a 30% solution of the hydrogen peroxide substrate per 10 ml was then added. After 5-10 minutes the color development was stopped by addition of sulphuric acid and the absorbance at 492 nm/620 nm was measured in a microplate reader.

Patient sera were tested for XMRV-Gag or -Env binding antibodies in the same way, using a goat anti-human IgG-HRP conjugate as secondary antibody (Sigma Aldrich, Munich, Germany). Out of the 146 sera samples only from 30 patients the corresponding nucleic acids were included in the 589 DNA/RNA samples.

Immunofluorescence microscopy

Cells were grown on gelatine (0.3% coldwater fish gelatine in distilled water) coated glass slides in 12-well plates and 24 h after seeding were transfected using Polyfect Reagent (Qiagen) with the full length molecular clone pCDNA3.1-VP62 or with the pTH-XMRV-coEnv or pTH-XMRV-coGAG plasmids containing codon optimized synthetic full-length genes of the XMRV *env* or *gag* under control of the CMV promoter. 48 h after transfection the cells were fixed with 2% formaldehyde (Sigma) in PBS. Cells were rinsed briefly in PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min and washed 3 times with PBS. After 30 min incubation with blocking buffer (2% Marvel milk powder in PBS) cells were incubated for 60 min at 37°C with the mouse or human antisera diluted 1:200 in blocking buffer. The slides were washed extensively with PBS. The secondary antibodies conjugated to fluorophores were added for 30 min. After thorough