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ORIGINAL ARTICLE

Expression of the *pol* gene of human endogenous retroviruses HERV-K and -W in leukemia patients

Massimiliano Bergallo[1] · Paola Montanari[1] · Katia Mareschi[1,2] · Chiara Merlino[1] · Massimo Berger[2] · Ilaria Bini[2] · Valentina Daprà[1] · Ilaria Galliano[1] · Franca Fagioli[2]

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Abstract The human endogenous retroviruses (HERVs) are a family of endogenous retroviruses that integrated into the germ cell DNA of primates over 30 million years ago. HERV expression seems impaired in several diseases, ranging from autoimmune to neoplastic disorders. The purpose of this study was to evaluate the overall endogenous retroviral transcription profile in bone marrow (BM) samples. A total of 30 paediatric high-risk leukaemia patients (lymphoid and myeloid malignancies) were tested for HERVs virus gene expression. Our findings show that HERV-K expression was significantly higher in leukaemia patients when compared to healthy donors of a similar median age. We observed a significantly high expression of HERV-K in acute lymphoblastic leukemia (ALL) patients. In this study, we also found a relative overexpression of the endogenous retrovirus HERV-K in BM cells from the majority of leukemia samples analyzed, in particular in ALL. This overexpression might be related to lymphatic leukemogenesis and it warrants further investigations.

Introduction

The "HERVs" (human endogenous retroviruses) are a family of endogenous retroviruses that were integrated into the germ cell DNA of primates over 30 million years ago [1]. Although replication-competent ERVs are found in other mammals [2], the integration process led to post-insertional mutations, deletions, and recombinations that made the totality non-infectious in humans. Nowadays, HERVs are classified into 50-200 families depending on primer binding site (PBS) sequence complementary to the 3' end of a cellular tRNAs used for reverse transcription [3, 4]. Solitary long terminal repeats (LTRs) were generated from existing HERVs by the loss of internal sequences, after recombination between the 5' and 3' LTRs sequences within the HERV or between separate LTRs. However transcription of HERV-K and other HERV elements is usually suppressed by epigenetic factors such as DNA methylation and heterochromatin-silencing by histone modifications. Iinserted LTRs act as alternative promoters to stimulate expression of nearby genes causing the activation of oncogenes or inactivation of tumor suppressor genes [5, 6]. Moreover, HERVs expression seems impaired in several diseases, ranging from autoimmune to neoplastic disorders [2, 7]. However, various agents (such as chemical, radiation, other exogenous viral infections) may re-activate HERV transcription [8]. There are only few studies on the expression of endogenous retroviruses in pediatric leukemias [9–11], moreover data regarding the participation of HERV-K and W in the development of leukemia are contradictory [11–13]. Specifically, during haemato-oncological processes, several studies have reported the presence of antibodies against HERV-K, over-expression of HERV genes and also the presence of retroviral particles in primary leukemia cells [14].

These considerations led us to study the *pol* gene expression of HERV-K and HERV-W in bone marrow (BM) samples from a cohort of consecutive pediatric patients with leukemia. We expect that the results obtained will provide insight into the role of endogenous retroviruses in the pathogenesis of leukemia.

✉ Massimiliano Bergallo
Massimiliano.bergallo@unito.it

[1] Department of Public Health and Pediatric Sciences, University of Turin, Medical School, 10136 Turin, Italy

[2] Pediatric Onco-Hematology and Stem Cell Transplantation and Cell Therapy Division, City of Science and Health of Turin, 10136 Turin, Italy

Material and methods

Patients and samples

The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, http://www.wma.net/en/30publications/10policies/b3/index.html., and informed consent was obtained in each case from the parents or their legal guardians. BM cells were collected from 30 leukemia pediatric patients as reported in Table 1.

BM mononuclear cells were separated using Ficoll-Paque (Amersham, Bucks, UK) density gradient centrifugation and frozen in a solution containing dimethyl sulfoxide at 10% and stored in liquid nitrogen. As control, BM mononuclear cells were obtained from 20 different pediatric healthy individuals with a similar median age, using the same method.

Reverse transcription

Total RNA was extracted using the automated extractor Maxwell (Promega, Madison, WI) using the "simply RNA Blood Kit protocol" without modification. One microgram of total RNA was reverse-transcribed with 8 µl of buffer 10X, 4.8 µl of $MgCl_2$ 25 mM, 2 µl ImpromII (Promega), 1 µl of RNase inhibitor 20U/l, 0.4 µl random hexamers 250 µM (Promega), 2 µl mix dNTPs 100 mM (Promega) and dd-water in a final volume of 20 µl. The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems, Foster City, CA, USA) under

Table 1 Clinical data of patients entering in the study

Pt	Diagnosis	Gender	Age at diagnosis	Molecular characterization	BM blasts	Outcome
1	AML	F	15 y	FLT3-ITD	85	DOD +6 months from HSCT
2	AML	M	7 y	FLT3-ITD	69	A&W +5 yrs from HSCT
3	AML	F	3 y	None	47	A&W +1.4 yrs from HSCT
4	CML	F	12 y	BCR/ABL p210	5	A&W +5 yrs from HSCT
5	AML	M	7 m	MOZ/CBP	21	A&W +1.7 yrs from HSCT
6	ALL	F	11 y	BCR/ABL p210	93	A&W +4.5 yrs from HSCT
7	ALL	M	11 y	None	83	Relapse +1.7 yrs
8	AML	M	10 y	DEK/CAN	47	A&W +1.9 yrs from HSCT
9	AML	F	7 y	None	53	DOD +1 yrs from HSCT
10	CML	F	11 y	BCR/ABL p210	8	A&W +5.4 yrs from HSCT
11	AML	F	16 y	MLL/AF6	95	Relapse +4.5 yrs from HSCT
12	AML	M	2 y	FLT3-ITD e NPM1+	85	A&W +3.5 yrs from HSCT
13	ALL	F	1 y	BCR/ABL p190	88	A&W +1.8 yrs from HSCT
14	ALL	M	10 y	None	46	Relapse +1.5 yrs from HSCT
15	ALL	F	7 y	None	77	Relapse + 11 months
16	ALL	M	15 y	None	90	A&W +6 yrs from HSCT
17	CML	M	13 y	BCR/ABL p210	1	A&W +2.4 yrs from HSCT
18	AML	M	10 y	RUNX1-RUNX1T1 and del(9)	58	A&W +5.7 yrs from HSCT
19	ALL	F	16 y	BCR/ABL p190	86	DOD +6 months from HSCT
20	ALL	M	8 y	None	87	A&W +3.3 yrs from HSCT
21	AML	M	1 y	RUNX1-RUNX1T1	90	A&W +2.2 yrs from HSCT
22	AML	M	13 y	MOZ/CBP	92	A&W +1.1 yrs from HSCT
23	AML	M	5 y	HWAF1	60	A&W +2.8 yrs from HSCT
24	AML	M	4 y	None	62	A&W +3.8 yrs from HSCT
25	CML	F	4 y	BCR/ABL p210	1	A&W +2.4 yrs from HSCT
26	ALL	M	11 y	BCR/ABL p190	44	Relapse +1.4 yrs from HSCT
27	JMML	F	1 y	PTPN11	0.8	A&W +7.2 yrs from HSCT
28	AML	M	7 m	BCR/ABL p210 and *CBFB-MYH11*	85	DOD +6 months from HSCT
29	ALL	M	18 y	None	85	Relapse +22 months
30	AML	F	17	FLT-3 ITD	90	A&W +4.5 yrs from HSCT

F: female, M: male, y: years, m: months, BM: bone marrow, AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, CML: chronic myeloid leukemia, JMML: juvenile myelomonocytic leukemia, DOD: dead of disease, A&W: alive and well, HSCT: Hematopoietic Stem Cell Transplantation

the following conditions: 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C for the inactivation of enzyme; the subsequent cDNAs were stored at −80° until use.

Relative quantification by real-time PCR

Relative quantification of mRNA expression of selected genes was achieved by means of Taqman qPCR amplification with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, which was chosen as a reference gene, using the ABI PRISM 7500 real time system (Life technologies, Texas, USA). The expression of HERV-K and W *pol* gene and GAPDH was quantified by real-time PCR. Approximately 100 ng of cDNA was amplified in a 20 µl total volume reaction containing 2.5 U ampli-Taq Gold DNA polymerase (Applied Biosystem, USA), 1.25 mmol/l MgCl2 and 900 nmol of specific HERV-K primers (KPOLF-5’-CCACTGTAGAGCCTCCTAAACCC-3’) (KPOLR-5’-TTGGTAGCGGCCACTGATTT-3’) and probe (KPOLP-6FAM-CCCACACCGGTTTTTCTGTTTTCCAAGTTA A-TAMRA) or HERV-W primers (WPOLF-5’-ACMTG-GAYKRTYTTRCCCCAA-3’) (WPOLR-5’- GTAAATCAT CCACMTAYYGAAGGAYMA-3’) and probe (WPOLP-6FAM-TYAGGGATAGCCCYCATCTRTTTGGYCAGGCA -TAMRA) or GAPDH specific primers (GAPDHF-5’-CCA AGGTCATCCATGACAAC-3’) (GAPDHR-5’- GTGGCA GTGATGGCATGGAC-3’) and probe (GAPDH-6FAM-TGGTATCGTGGAAGGA-3’ MGB). The amplifications were in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. Each experiment was repeated in triplicate. Relative quantification of the target gene expression in patients was compared with normal samples using the ΔΔCt method and the relative results were expressed in corresponding arbitrary units (AU).

Statistical analysis

Differences in mRNA expression levels between leukemia samples and controls were evaluated by unpaired two-tailed Student’s t test with Welch’s correction using the Prism software (GraphPad Software, La Jolla, CA). In all analyses, $P < 0.05$ was taken to indicate a statistical significance between the analyzed groups.

Results

For quantitative evaluation of *pol* gene expression of HERV-K and HERV-W, real-time PCR (using a TaqMan system) was used. The threshold value Ct, computed for each of the genes as the average of three determinations, was used to measure the amount of resulting PCR product.

The study group enrolled 30 pediatric leukemia patients (15 acute myeloid leukemia (AML) 10 acute lymphoblastic leukemia (ALL), four chronic myeloid leukemia (CML) and one juvenile myelomonocytic leukemia (JMML) diagnosed and treated in our center. Table 1 summarizes the patient’s characteristics. The median age of the patient cohort and healthy cohort were 10.3 years (range 0.8-18.6) and 10.3 years (range 3.9-16.4), respectively.

Because retro-transcription efficiency was equivalent in all samples, as suggested by GAPDH expression, we made the hypothesis that the variations in signal intensity between mononuclear cells from leukemia patients and normal BM cells might be related to differences in HERV-K and –W *pol* transcriptional levels.

Relative expression of the HERV-K and -W *pol* gene in leukemia samples vs healthy donors

As shown in Figure 1 HERV-K expression was significantly higher in the leukemia patients than in the healthy subjects ($p = 0.0109$). In particular, 18 samples out of 30 (60%) showed higher HERV-K levels than the average levels seen in healthy subjects. Conversely 22 of 30 samples (73.3%) showed a higher expression of HERV-W in comparison to the healthy donors; however, the difference was not statistically significance ($p = 0.9841$).

Relative expression of the HERV-K and -W *pol* gene in different types of leukemia

HERV-K expression was significantly higher in the ALL patients in comparison to the healthy donors (figure 2A) and the ML patients (Figure 2C) ($p = 0.0110$ and 0.0461 respectively). This significant difference in HERV-K expression was not observed when we compared expression in myeloid malignancies patients with healthy donors ($p = 0.2170$, Figure 2B). The comparison of HERV-W expression between the ALL patients and the healthy donors (Figure 2D), between patients with myeloid malignancies and healthy donors (Figure 2E) and between ALL and myeloid malignancy patients (Figure 2F) did not show any significant differences ($p = 0.5916$, 0.7359 and $p = 0.1684$ respectively).

Relative expression of the HERV-K and -W *pol* gene in different karyotypes

Subsequently, we re-analyzed data obtained from the amplification of the *pol* gene of HERV-K and -W in specific subgroups of patients with reference to altered karyotypes. We compared HERV expression in BCR/ABL, FLT3 mutated patients and patients showing other translocations (t(6,11),(10,11), (8,16)) with expression in leukemia patients without mutations. We observed a trend of higher expression

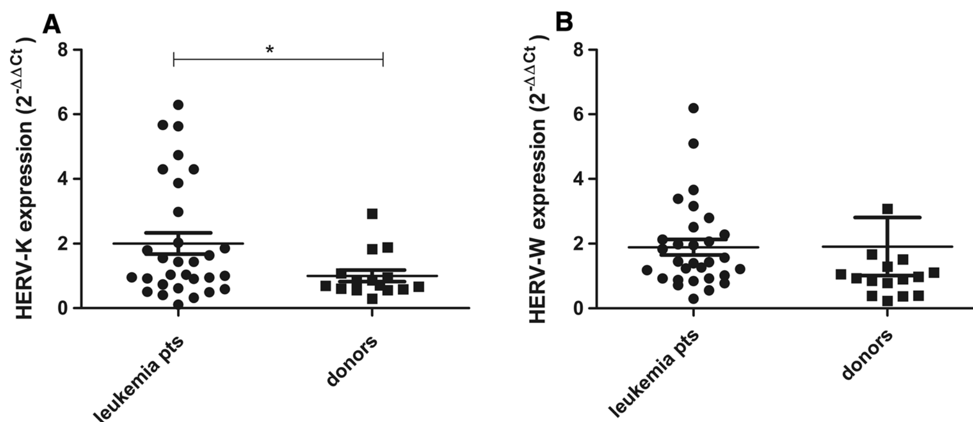

Fig. 1 Expression of the *pol* gene of HERV-K (A) and -W (B) was evaluated by quantitative PCR real time in the leukemia patients and the healthy donors, expressed as $2^{-\Delta\Delta Ct}$. HERV-K and HERV-W expression was higher in the leukemia patients than in healthy subjects. The symbol * indicates a statistically significant difference ($p < 0.05$) calculated by an unpaired t test with Welch's correction for the expression of HERV-K in the leukemia patients, in comparison to healthy donors

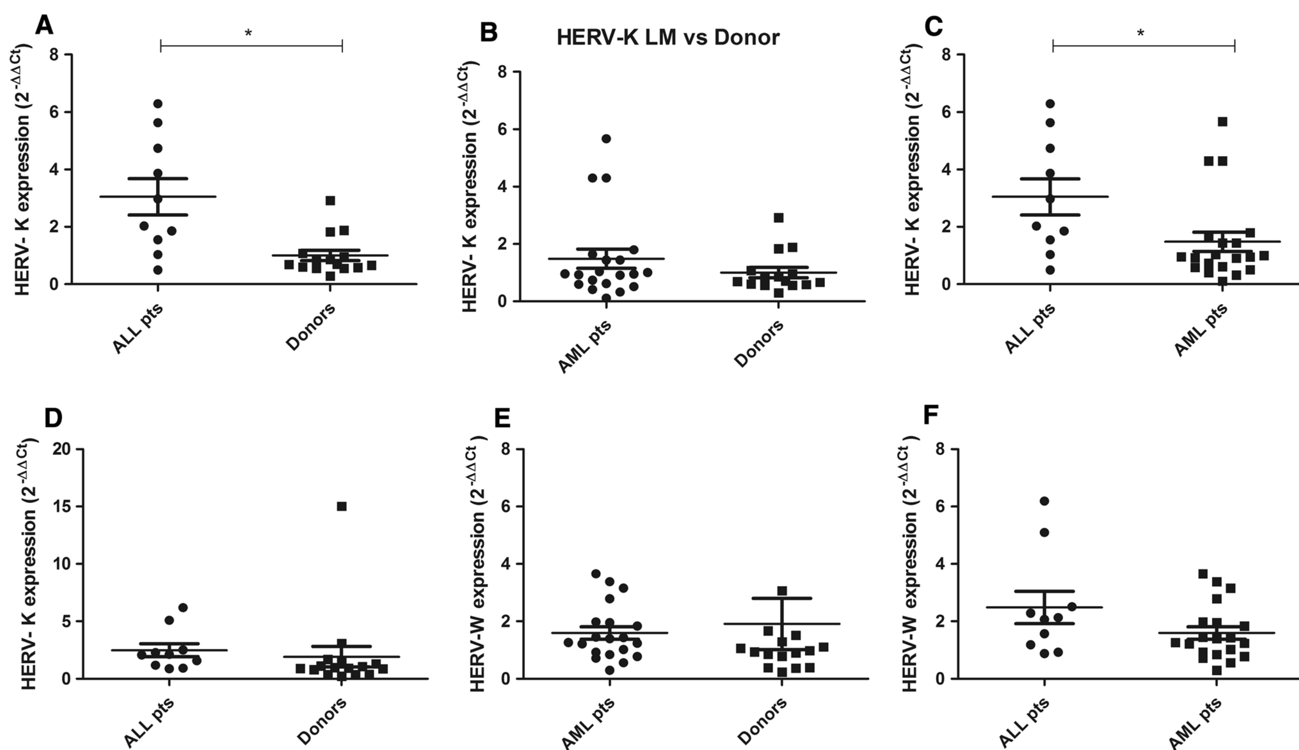

Fig. 2 Expression of the *pol* gene of HERV-K (A, B, C), and -W (D, E, F) expression evaluated by quantitative real time PCR. A) and D) Comparison of *pol* gene expression between ALL patients and healthy donor subjects; B) and E) Comparison between myeloid malignancies patients and healthy subjects; C) and F) Comparison between ALL and myeloid malignancies patients. The symbol * indicates a statistically significant difference ($p < 0.05$) calculated by an unpaired t test with Welch's correction for the HERV-K expression in ALL patients

of HERVs in subgroups with an altered karyotype, as shown in Figure 3, but this was not statistically significant ($p = 0.4$, $p = 0.5$ and 0.72 for HERV-K and $p = 0.25$, 0.50 and 0.77 for HERV-W, respectively).

Discussion

The re-activation of HERV transcription as a consequence of various factors (such as chemicals, radiation or other

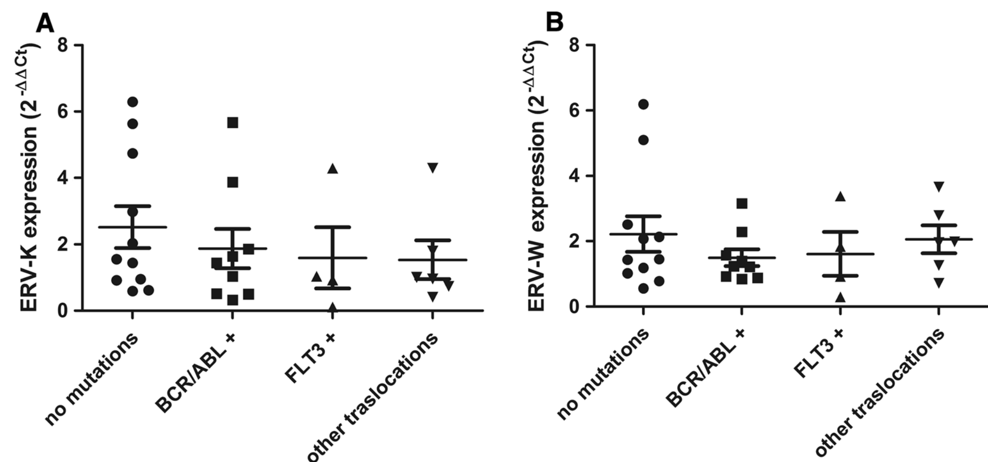

Fig. 3 Expression of the *pol* gene of HERV-K (A), and -W (B), evaluated by quantitative PCR real time, in leukemia patients in the different subgroups : no mutations and altered karyotypes (BCR/ABL +, FLT3 +, other mutations). Leukemia patients with altered karyotypes showed a higher HERV-K and W expression than leukemia patients with no mutations, but without statistical significance

exogenous viral infections) could act as a promoter for the development of several malignancies through the modification of cell gene expression. HERVs are integrated into the human genome and transmitted by the Mendelian mechanism. HERVs are generally defective with regards to specific gene function and replication capacity; however, they can be activated by environmental factors such as radiation, drugs and inflammatory cytokines [15]. The physiological role of HERV is still unknown, although some HERVs have been found to show organ-specific expression, suggesting an association between HERV and the development and differentiation of human tissues [16]. In malignancy, a relationship between HERV family type and histological type has been suggested [10].

The purpose of this study was to evaluate the overall endogenous retroviral transcription profile in BM samples from a representative cohort of high risk pediatric leukemia patients. Our findings show that HERV-K expression was higher in leukemia patients versus healthy subjects of a similar median age. We observed a significantly increased expression of HERV-K in ALL patients. These findings suggest that the HERV *pol* gene could play a role in lymphoblastic leukemogenesis and cell proliferation activity. This specific expression in leukemia patients also suggests that the HERV *pol* gene could be considered as a target for gene therapy.

The few papers in the literature exploring the role of HERV-K and -W in leukemia patients show contradictory data. Januszkiewicz-Lewandowska and colleagues speculate that high relative expression of the *env* gene in myeloid maligancies may suggest participation of env sequences in the pathogenesis of leukemia [11]. To the contrary Fischer and colleagues recently showed that only 2 out of 25 chronic lymphocytic leukemia patients exhibited elevated HERV-K *gag* expression (5-fold greater than healthy donors) [14]. In contrast to the observations by Januszkiewicz-Lewandowska and colleagues we showed an overexpression of HERV-K in ALL rather than the myeloid subsets. To the best of our knowledge, this is the first study of HERV-K *pol* mRNA expression in pediatric ALL showing that this molecule is over-expressed in the BM cells of patients when compared to healthy donors. In this study, we found a relative overexpression of the endogenous retrovirus HERV-K in the BM cells from the majority of leukemia samples analyzed. After considering the different mechanisms potentially involving HERVs in carcinogenesis, we suggest that overexpression of HERVs might participate in leukemogenesis, especially in ALL. Moreover, despite not achieving statistical significance, we observed that specific leukemia karyotypes, such as Philadelphia positive or FLT-3 mutated cells, were associated with a different expression of HERVs, showing how HERVs might have a role in this type of leukemogenesis. Furthermore, precise and larger quantitative studies are needed for the possible application of HERVs *pol* gene expression as a clinical marker for leukemia,

HERV expression studies could be supplemented by tests such as Western blot or other related immune assays using anti-HERV-K or W antibodies to support the aforementioned results. The relative overexpression of HERV-K and –W sequences reported herein is concordant with the description by Denner et al [17] of an antibody response against HERV-K peptides in leukemia patients. More studies are warranted to evaluate the potential impact of HERV *pol* expression on leukemia patients and their role in the regulation of signaling pathways involved in lymphoblastic leukemogenesis.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of University of Turin research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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