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# Effects of UVA1 Phototherapy on Expression of Human Endogenous Retroviral Sequence (HERV)-K10 *gag* in Morphea: A Preliminary Study

Authors' Contribution:  
Study Design A  
Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

ABCDEF 1 **Michał Jacek Kowalczyk**  
ABCDEF 2 **Ewa Teresiak-Mikołajczak**  
ADF 2 **Aleksandra Dańczak-Pazdrowska**  
ADFG 1 **Ryszard Żaba**  
ADFG 2 **Zygmunt Adamski**  
ABCDF 2 **Agnieszka Osmola-Mańkowska**

1 Department of Dermatology and Venereology, Psoriasis and Novel Therapies in Dermatology Unit, Poznań University of Medical Sciences, Poznań, Poland  
2 Department of Dermatology, Poznań University of Medical Sciences, Poznań, Poland

**Corresponding Author:** Ewa Teresiak-Mikołajczak, e-mail: [ewa.teresiak@wp.pl](mailto:ewa.teresiak@wp.pl)  
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**Background:** Morphea, also known as localized scleroderma, is a rare autoimmune connective tissue disease characterized by skin fibrosis. UVA1 phototherapy is an important asset in the reduction of clinical manifestations in morphea. There are studies claiming that UV light modulates the expression of some human endogenous retroviral sequences.

The aim of this study was to determine if the expression of HERV-K10 *gag* element is lowered by UVA1 phototherapy in morphea, a disease in which such irradiation has a soothing effect.

**Material/Methods:** The expression levels of the HERV-K10 *gag* were assessed by real-time PCR (polymerase chain reaction) in peripheral blood mononuclear cells (PBMC) and skin-punch biopsies of healthy volunteers and 9 morphea patients before and after phototherapy. Additionally, correlations between the HERV-K10 *gag* expression and age, disease duration, the Localized Scleroderma Skin Severity Index (LoSSI), and antinuclear antibody (ANA) titers were assessed.

**Results:** In PBMC, HERV-K10 *gag* mRNA was significantly elevated after UVA1 phototherapy compared to healthy controls. Most of the patients responded with an increased expression level of this sequence. However, we found no statistical evidence at this point that phototherapy indeed has an effect on the HERV-K10 *gag* expression (there were no statistical differences in PBMC of morphea patients before and after phototherapy). Similarly, there was no statistically relevant effect of the UVA1 on the expression of HERV-K10 *gag* in skin.

**Conclusions:** At this point, the effect of UVA1 phototherapy on the expression of HERV-K10 *gag* cannot be statistically confirmed.

**MeSH Keywords:** **Endogenous Retroviruses • Real-Time Polymerase Chain Reaction • Scleroderma, Localized**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/897985>

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## Background

Morphea, or localized scleroderma, is a relatively rare autoimmune connective tissue disease characterized mainly by skin fibrosis leading to dermal thickening. The pathogenesis of the disease remains unknown. Unlike systemic sclerosis (SS), which is characterized by the involvement of internal organs, morphea is believed to be limited to skin [1–7].

Unlike lupus erythematosus (LE), for example, in which UV radiation exerts a triggering, pathogenic effect, morphea is a disease in which it has a positive effect on the skin. UVA1 phototherapy is an important asset in the reduction of clinical manifestations in morphea. Most importantly, it down-regulates the inflammatory process and diminishes fibrosis, since the fibroblast activity results from the progression of inflammation in morphea [8,9].

Human endogenous retroviral sequences (HERV) are an integral part of the human genome formed through ancient retroviral infections of germ-line cells, and preserved in the genome by vertical gene transfer through successive host generations. Mutations acquired over time prevent them from forming infectious viral particles, although their original gene arrangement, including *gag*, *pol*, and *env* sequences, is mostly retained. On the other hand, due to their vast number in the genome, HERV seem to influence the cell by various mechanisms, such as through their localization near other genes or within promoter regions, splicing site alterations, direct production of truncated viral proteins, and many other [10–12].

There are studies reporting that UV light induces the expression of certain HERV in LE. Hohenadl et al. demonstrated that skin biopsies from UV-irradiated areas or skin lesions of LE patients showed elevated expression of some HERV sequences compared to controls. Many of these were HERV-K elements. The authors suggest that the combined direct effects of UV radiation on the induction of apoptosis and UV-driven transcriptional activation of HERV may lead to inappropriate presentation of autoantigens (including HERV proteins) leading to autoantibody production [9]. Wu et al. showed that UVB upregulates HERV-E expression in SLE by UVB-induced inhibition of its LTR methylation [13]. Schanab et al. demonstrated the upregulation of HERV-K expression in melanoma and non-melanoma cells *in vitro* after UV irradiation. The findings of the aforementioned studies indicate that HERV-K expression can be activated by UV light in certain skin conditions [14].

Based on that phenomenon, we hypothesized that in contrast, the expression of some HERV-K elements might be lowered by UV light in diseases in which such irradiation has a soothing effect, such as morphea.

We chose HERV-K10 *gag* sequence for 2 reasons. First, this sequence had already been thoroughly studied in the past. Its elevated expression had been found in placenta, embryonic tissue, and *in vitro* cell lines. The sequence apparently plays a role in the pathogenesis of testicular and breast cancers [15,16]. Apart from that, this very sequence had not only been studied in SLE, systemic sclerosis, and psoriasis, but also in our previously published analysis on HERV expression in morphea [13,17,18]. Secondly, this sequence has a relatively high expression level in skin in general [18]. Morphea is a rare disease, so it was clear that collecting many skin samples would be a challenge. Skin is a quite demanding target for RNA isolation, and choosing a highly expressed sequence would ensure gaining results even in case of low RNA yields.

## Material and Methods

### Patients and control

Whole-blood samples and skin-punch biopsies of 9 patients were collected before and after medium-dose UVA1 treatment. The patients were diagnosed and treated at the Dermatology Clinic of Poznan University of Medical Sciences. Histopathologic examination was undertaken to confirm the diagnosis. The inflammatory margin of sclerotic lesions from non-sun-exposed skin was the punch biopsy location. Severe and disseminated morphea qualified a patient for phototherapy treatment [8]. The clinical subtypes of morphea included disseminated plaque morphea and linear morphea. Clinical severity was assessed prior to and after phototherapy with the use of the Localized Scleroderma Skin Severity Index (LoSSI) [19].

The control group consisted of 16 whole-blood samples obtained from healthy volunteers matched by age and sex to the patients group, and 13 skin biopsies from skin residues after flap reconstruction surgery. All patients and volunteers gave written consent. The study was approved by a local bioethics committee.

### Antinuclear antibodies

The fluorescent pattern and titer of antinuclear antibodies (ANA) were assessed with the use of indirect immunofluorescence method before and after UVA1 phototherapy on HEp-2 cells and ANA Profil3 (Euroimmun).

### UVA1 phototherapy

GP-24H (Cosmedico) irradiation system was used to perform UVA1 phototherapy. The system emits 350-400 nm radiation up to 115 mW/cm<sup>2</sup> intensity. The medium dose of UVA1 (MD UVA1) was 45.9 J/cm<sup>2</sup>. The average number of irradiations was

**Table 1.** Primers used for real-time PCR.

Primer name	Sequence (5'-3')	Amplicon length	Accession/location
<i>HERV-K10 gag F</i> <i>HERV-K10 gag R</i>	GTA ATG GCT CAG TCA ACG CA GCC CCA TTA ATT CTG GAC CT	103	GenBank: M14123 [16–17,20], 5q33.3
<i>ACTB F</i> <i>ACTB R</i>	GCACAGAGCCTCGCCTT GTTGTCGACGACGAGCG	93	ENST00000331789, NM_001101.3 [21], 7p22
<i>GAPD F</i> <i>GAPD R</i>	Proprietary sequence	119	Human GAPD Gene Assay – Universal Probe Library (Roche Applied Science), 12p13.31

31.9. The average number of total doses was 1457.7. The therapy was conducted in a 3-to-5 times a week scheme.

### RNA isolation

Mononuclear cells obtained by Ficoll gradient centrifugation of the EDTA-collected whole blood were aspirated and washed in PBS. Total cellular RNA was isolated with the use of Tri Reagent (MRC) [20]. The transparent upper-phase samples obtained by phenol-chloroform extraction were silica-column purified with High Pure RNA Isolation Kit (Roche Diagnostics). We used 1.5 µg of total RNA for a DNase I reaction with the DNA-free kit (Ambion).

The skin-punch biopsies were immediately frozen in liquid nitrogen upon collection and stored at –80°C for downstream processing. The frozen samples were rotor-stator homogenized in Tri Reagent as the medium, followed by immediate freezing at –20°C. Thawed samples were phenol-chloroform extracted and the transparent upper-phase was purified and cleared from genomic DNA contamination with the use of the NucleoSpin RNA XS kit (Macherey-Nagel).

All samples were assessed with a no-RT real-time PCR for genomic DNA contamination. Glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) was the target gene in these reactions.

### Reverse transcription and real-time PCR

Random hexamer primer reverse transcription of 1.0 µg purified RNA was done to generate cDNA using the Transcriptor First-Strand cDNA Synthesis kit (Roche Diagnostics).

All real-time PCR reactions were performed on a LightCycler 2.0 instrument in absolute analysis mode with 10-fold dilution standard curves.

The LightCycler TaqMan Master kit (Roche Diagnostics) was used for *GAPD* and the FastSTART DNA Master SYBR Green I kit (Roche Diagnostics) was used for all other sequences. All

real-time PCR batches were cross-calibrated. Amplicon quality was assessed by melting point analysis [18].

Beta-actin (*ACTB*), *GAPD*, and geometric mean (“GM”) of the two were used for HERV-K10 *gag* normalization. The choice of a particular reference gene was made based on the results of non-parametric Wilcoxon signed-rank tests of the absolute expression of reference genes or their geometric mean before and after UVA1 phototherapy separately for whole-blood and skin-punch biopsies. The expression level of the HERV-K10 *gag* was expressed as copies per million copies of *ACTB*, *GAPD*, or their geometric mean.

The primer sequences used in this study are presented in Table 1.

### Statistics

Due to low patient numbers and the general lack of normal distribution of data, non-parametric tests were used for the statistical analysis. The Wilcoxon signed-rank paired test was used to assess statistical significance of the influence of the UVA1 phototherapy in morphea patients, while the Kruskal-Wallis test was used to assess changes between controls and morphea patients before or after phototherapy. Spearman’s rank analysis was used to assess the correlation strength between HERV-K10 *gag* expression and age, disease duration, LoSSI, and ANA titers. The level of statistical significance was established at  $p < 0.05$ . All statistical tests were performed with the aid of PAST v. 2.03, while the graphs were generated with QtiPlot v. 0.9.7 software.

### Results

All patients completed the treatment cycle of UVA1 phototherapy. LoSSI score was assessed in 8 out of 9 patients. All assessed patients improved in their condition (median score before 15.5; median after 4.5,  $p = 0.01$ ).

**Table 2.** Analysis of expression of HERV-10 gag in PBMC.

	Healthy control	Before UVA1	After UVA1
N	16	9	9
Min	45254	38350	76027
Max	136576	163396	375758
Mean	78118	100420	139079
Median	69237	88912	126027
Median change	–	+28.5%*	+82.0%**
Geom._mean	75039	91747	122325

Values are expressed as copies of HERV-K10 gag per one million copies of GAPD. \* Significant only when ACTB and „GM” are reference; \*\* significant in any three references.

**Table 3.** Statistical analysis of the UVA1 influence on HERV-K10 gag expression in PBMC.

	Before UVA1	After UVA1
Healthy control	0.134*	0.005**
Before UVA1	–	0.659

Results of the Kruskal-Wallis test based on GAPD normalization. \* Significant only when ACTB and „GM” are reference; \*\* significant in any three references

**Reference gene analysis**

The p values of the Wilcoxon signed-rank tests of the absolute expression before and after phototherapy in whole-blood samples were 0.26, 0.11, and 0.11 for GAPD, ACTB and “GM”, respectively. All 3 were statistically unaffected by phototherapy (p>0.05), but since GAPD was the least affected, it was chosen to be the reference for HERV-K10 gag.

Analogically, the p values of the Wilcoxon signed-rank tests of the absolute expression before and after phototherapy in skin-punch biopsies were 0.68, 0.26, and 0.77 for GAPD, ACTB, and “GM”, respectively. Since the geometric mean value of the 2 reference genes was the least affected by phototherapy, it was chosen to be the reference for HERV-K10 gag. Since the p value of ACTB was quite different that the other 2, ACTB was not taken into consideration later in this study.

**Expression of HERV-10 gag in PBMC**

The results shown in Tables 2 and 3 relate to calculations in which GAPD was the gene of reference.

The mean, median, and geometric mean values suggest that the expression of HERV-K10 gag in PBMC is the lowest in healthy people, higher in morphea patients, and elevated even more by phototherapy (Table 2, Figure 1). However, the results of the Kruskal-Wallis test show a statistically significant difference only between healthy volunteers and patients after

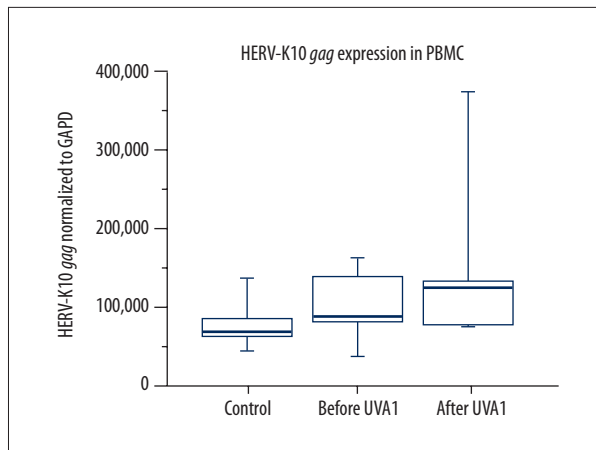
phototherapy. On the other hand, the p value in comparison of healthy volunteers and patients before phototherapy was 0.134, which is quite low (Table 3). Furthermore, normalization of the HERV-K10 gag expression to ACTB and “GM” results in lower, statistically significant p values (data not shown). One may therefore expect that HERV-K10 gag expression in patients either before or after phototherapy is statistically increased compared to healthy volunteers and the inconsistency in the data results from low number of patients.

The effect of the UVA1 treatment is a different situation. Although in 7 out of 9 patients, the expression of HERV-K10 gag was elevated rather than lowered (Figure 2), the Wilcoxon signed-rank test p value before and after treatment was 0.173 (and 0.659 for the Kruskal-Wallis test, Table 3) when normalized to GAPD. The normalization of the HERV-K10 gag expression to either ACTB or “GM” resulted in p=0.180. All these data suggest a statistically insignificant difference at this point.

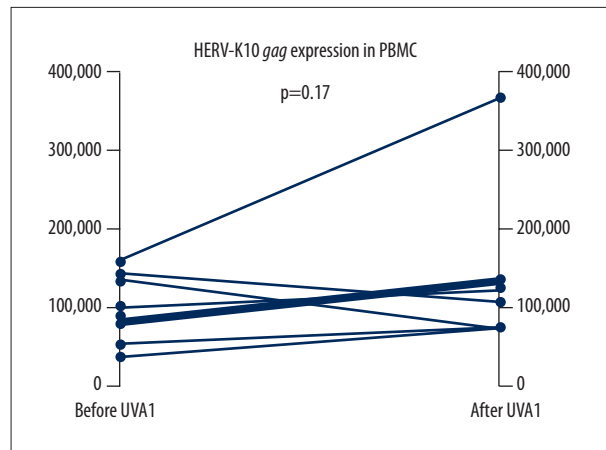
**Expression of HERV-10 gag in skin-punch biopsies**

The results provided in Tables 4 and 5 relate to calculations in which “GM” was the reference.

The mean, median, and geometric mean values suggest that the expression of HERV-K10 gag in skin-punch biopsies is the highest in healthy people, and decreases in morphea patients, with roughly similar values either before and after phototherapy (Table 4, Figure 3). The Kruskal-Wallis test does not confirm



**Figure 1.** Expression of HERV-10 *gag* in healthy volunteers and patients in PBMC. Middle lines represent the median, boxes represent 25–75 percentiles, and whiskers extend to minimum and maximum values.



**Figure 2.** The effect of UVA1 phototherapy in PBMC. Values normalized to *GAPD*.

**Table 4.** Analysis of expression of HERV-10 *gag* in skin.

	Healthy control	Before UVA1	After UVA1
N	13	9	9
Min	353329	306749	327752
Max	854136	692884	684707
Mean	574657	463190	467651
Median	554691	444778	406207
Median change	–	–19.8%	–26.8%
Geom._mean	551763	446825	455950
Stand._dev	171328	133856	115059

Values are expressed as copies of HERV-K10 *gag* per one million copies of “GM”.

**Table 5.** Statistical analysis of the UVA1 influence on HERV-K10 *gag* expression in skin.

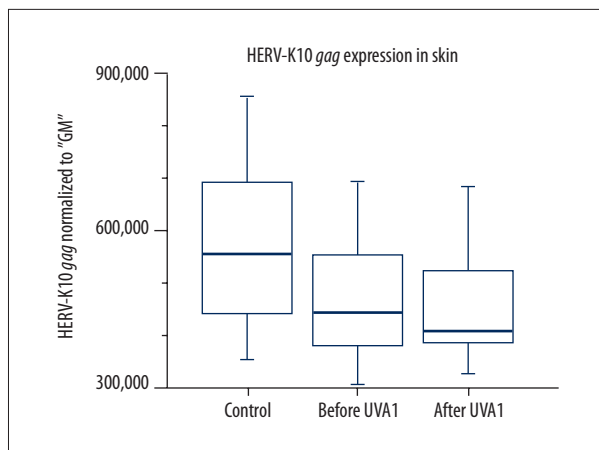
	Before UVA1	After UVA1
Healthy control	0.16	0.14
Before UVA1	–	0.72

Results of the Kruskal-Wallis test based on “GM” normalization.

such downregulation, although one has to bear in mind that values of 0.16 and 0.14 (before and after therapy, respectively), having just 9 pairs of data, are quite suggestive. When normalized to *GAPD*, the p values are 0.18 and 0.08. These difference might become statistically significant if the morphea group was larger.

Five out of 9 patients showed a slight decrease of HERV-K10 *gag* expression after UVA1 phototherapy (Figure 4). The Wilcoxon signed-rank test p value before and after treatment

was 0.68 (and 0.72 for the Kruskal-Wallis test, Table 5) when normalized to “GM”. Insignificant p values of both tests are also apparent for the *GAPD* normalization (data not shown). The results of the Wilcoxon signed-rank test when *ACTB* is the gene of reference suggest a restoration of the HERV-K10 *gag* expression after UVA1 phototherapy to the levels of healthy volunteers, yet this is apparently caused by the lowered *ACTB* expression after phototherapy, which was the cause of the initial rejection of *ACTB* as the gene of reference in skin-punch biopsies.



**Figure 3.** Expression of HERV-10 *gag* in healthy volunteers and patients in skin. Middle lines represent the median, boxes represent 25–75 percentiles, and whiskers extend to minimum and maximum values.

**Correlation assessment**

The Spearman’s rank correlation analysis revealed no statistically significant correlations between ANA titer and the expression of HERV-K10 *gag* sequence in morphea patients in PBMC and skin-punch biopsies, neither before nor after UVA1 phototherapy.

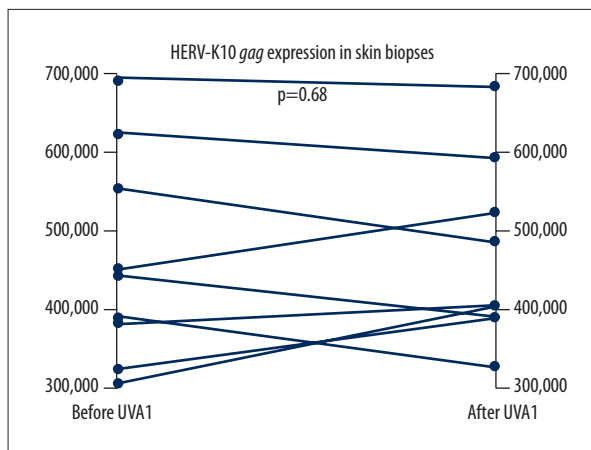
The HERV-K10 *gag* expression in PBMC shows a strong positive correlation with disease duration both before and after UVA1 therapy ( $R_s=0.85$ ,  $p=0.01$  and  $R_s=0.81$ ,  $p=0.02$ ; before and after UVA1, respectively).

The HERV-K10 *gag* expression in PBMC, both before and after treatment, shows a strong negative correlation only with LoSSI before treatment ( $R_s=0.84$ ,  $p=0.01$  and  $R_s=0.90$ ,  $p=0.005$ ; before and after UVA1, respectively). That correlation is lost when comparing HERV-K10 *gag* expression in PBMC and LoSSI assessed after treatment.

In skin-punch biopsies, the HERV-K10 *gag* expression correlates negatively with age before UVA1 treatment ( $R_s=-0.88$ ,  $p=0.01$ ). The correlation, although still apparent, is much weaker after the treatment ( $R_s=-0.57$ ,  $p=0.13$ ).

**Discussion**

The results of our previous study on expression of HERV sequences in morphea are concordant with the data presented in this report [18]. Again, data variance is relatively large, which makes the statistical analysis less reliable; therefore, in both studies we are quite certain that the transcription level of HERV-K10 *gag* is elevated in PBMC of morphea patients,



**Figure 4.** The effect of UVA1 phototherapy in skin. Note: Values normalized to “GM”.

yet the p values are over 5%. As postulated by Wu et al., HERV expression seems cell line-specific, thus the picture of expression in the entire group of PBMC is obscured [13]. It is currently unknown which cells within all PBMCs generate the increased HERV-K10 *gag* expression in morphea patients.

Because the 25–75 percentiles (Figure 1) are quite similar, we cannot at this time prove that the expression of HERV-K10 *gag* in PBMC changes after UVA1 phototherapy, although patients that underwent such therapy do exhibit significantly up-regulated HERV-K10 expression compared to healthy controls. Because of the small sample size, it is difficult to show statistical significance and form strong conclusions. Perhaps the present study should be continued with larger sample sizes.

The analysis of correlation suggests that the positive outcome of UVA1 in terms of LoSSI does not reflect the change in HERV-K10 *gag* expression in PBMC, since even after the course of treatment this expression correlates with the LoSSI, but measured before the treatment. Perhaps the blood samples might have been collected too early after UVA1 irradiation to notice the change, if any.

In our previous study we found that the expression of HERV-K10 *gag* in skin biopsies of morphea patients was 34% lower than in controls [18]. The difference was barely below the  $p=0.05$  value and was based on much larger number of samples. In this study the downregulation was also noticeable, yet the statistical significance was unreliable, most likely due to small sample size.

Although there is strong evidence that HERV-K10 *gag* is down-regulated in the skin of morphea patients in general, we have no evidence that UVA1 affects it in any way. Again, if there is in fact a change, it gets blurred by the vast variety of cells from which RNA is isolated.



We could not match the location of skin biopsies between controls and subjects because the control skin samples were obtained from skin residues after flap reconstruction surgery. Perhaps studies including sun-exposed and non-sun-exposed areas in healthy volunteers and morphea patients could contribute to understanding the effects of UV light on cutaneous HERV expression in certain skin conditions. There are at present no studies comparing the influence of UVR on HERV expression in healthy individuals, probably due to ethical issues.

However, results of recent studies suggest that UVR induces the skin lesions through upregulation of some HERV sequences in UVR-exacerbated skin conditions [9,13]. Based on that phenomenon, we hypothesized that the expression of some HERV elements might be decreased in morphea patients in whom UVA irradiation has a beneficial effect.

HERV-K10 *gag* expression is more strongly correlated with age before the UVA1 treatment than it is after it, but along with the lack of direct evidence that UVA1 affects its expression, such a statement is neither informative nor definitive.

Recently, we have seen a noticeable growth in morphea studies based on modern molecular techniques such as real-time PCR or next-generation sequencing (NGS) [13,18,23–25]. It appears that though due to the close resemblance of many HERV, the specificity of the real-time PCR approach is not always sufficient, as many different targets get amplified from the same primer pairs. The recently used NGS strategy in the analysis

of HERV-K in teratocarcinoma the Tera-1 cell line can specifically analyze closely-related HERV in various experiments and clinical conditions [26]. We agree with the statement postulated by Wu et al. that the expression of different types of HERV probably depends on the cell type and particular disease [13]. It seems it is crucial not to purify RNA from patient tissues, but rather from specifically purified types of cells.

## Conclusions

Treatment of morphea is a major challenge. The mechanism of UVA1 is not fully understood, and the molecule that absorbs UVA1 remains unknown. There are only a few studies demonstrating elevated expression of some HERV-K sequences in skin biopsies from UV-irradiated areas or skin lesions of LE patients compared to controls. To the best of our knowledge, there has been no study of the potential effects of UVA1 phototherapy on human endogenous retroviruses in morphea, a disease in which such irradiation has a soothing effect. We had suspected that the expression of some HERV-K elements might be lowered by UV light in morphea patients. Demonstration of such effects would be consistent with clinical observations of excellent results of UVA1 phototherapy in morphea. However, we demonstrated no statistically significant differences in the HERV-K10 *gag* expression in PBMC or skin of morphea patients before or after phototherapy. Therefore, at present, the effect of UVA1 phototherapy on the expression of HERV-K10 *gag* in morphea cannot be statistically confirmed.

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