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RESEARCH ARTICLE

Characterization of ERV-6 env as a Potential Biomarker and Driver of Migration in Multiple Cancer Lineages

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Abstract

Background: Metastasis is the primary cause of cancer mortality, and genomic elements such as endogenous retroviruses (ERVs) are increasingly recognized as potential drivers of this process. This study investigates the characterization of ERV-6 env as a biomarker and a regulator of migration across multiple cancer lineages. the ERV family (specifically ERV-6) remains in the human genome sequence and may still be biologically active, recent bioinformatics analyses in genome sequencing have investigated crucial roles of ERV-6 env in caner disease, for example breast, colon, pancreatic, and skin cancer. Thus, the map of ERV -6 sequencing is a key to explaining the role of disease and cancer related to ERV-6 activation. **Methods:** To detected the ERV-6 in different cancer cell lines (T24 –Bladder, MCF7-Breast, CDKN2A-pancreatic, H460-Lung, SKOV3- Ovarian, and B16F10-Melanoma). PCR and nested PCR were done and measure the metastatic rate by using migration assay to showed role ERV-6 env to induce migration cancer cell lines. **Results:** Our data investigate that ERV-6 env was showed in all breast, bladder, pancreatic, lung, ovarian and melanoma cancer cell lines. In addition, specific amplicons showed in cell line, potentially reflecting novel ERV-6 env insertions. Although, ERV-6 was increased level of migration in different points time, the B16F10 cell recorded a significantly aggressive migratory level. The gap is nearly completely filled by cells at the 48-hour mark, in contrast to other cancer cell lines. **Conclusion:** Therefore, an ERV-6 env significantly impacts the ability of cancer cells to migrate (metastatic) and ERV-6 env plays a critical role as a valuable diagnostic biomarker.

Keywords: ERV-6- migration- invasion- cancer cell lines

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Introduction

Metastasis remains the primary cause of cancer mortality, necessitating a deeper understanding of the genomic drivers that facilitate cellular dissemination. While cancer is broadly characterized by altered proliferation and signaling, recent evidence suggests that the genome harbors latent elements, such as endogenous retroviruses (ERVs), that may drive these processes. This is particularly relevant given the global burden of cancer, which reached approximately 19.2 million new cases annually according to the World Health Organization (WHO) [1]. The cancer cells are transferred to a metastatic phenotype and move to the blood and lymph nodes. Although this stage of cancer could be very difficult to treat, it could lead to the development of new treatment strategies and drugs to inhibit cancer cells. Many factors of cancer can be related to age, environment, obesity, lifestyle, and genetics. But the Endogenous retroviruses (ERVs) play an important role in different cancer diseases [2, 3].

ERVs are remnants of ancestral exogenous virus infections that integrated into the vertebrate germline over 50 million years ago, now comprising nearly 9% of the human genome. Although many ERVs are truncated, recent bioinformatics analyses have highlighted the biological activity of ERV-6 (specifically its RNA and proteins) in various malignancies, including breast, colon, and pancreatic cancers [4, 5]. Consequently, defining the expression profile and genomic location of ERV-6 is essential to elucidating its role in oncogenic activation

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and environmental response [6].

Specific loci, such as ERV-6 (3p21.31c) and REV-6 (19q13.43), have been found overexpressed in glioblastoma and prostate cancers, where they influence gene regulation and patient survival [7]. While CRISPR-Cas9 knockouts have demonstrated that reducing ERV env expression inhibits prostate cancer cell growth [2], the prevalence and migratory impact of ERV-6 across a broader range of cancer lineages remain insufficiently characterized. This study addresses this gap by detecting ERV-6 env in six distinct cancer cell lines and quantifying its contribution to cellular migration, thereby establishing a foundation for novel targeted therapies.

Materials and Methods

Cell culture

Cell culture work was carried out in a Class II laminar flow cabinet, with different cancer cell lines (T24 –Bladder, MCF7-Breast, CDKN2A-pancreatic, H460-Lung, SKOV3-Ovarian, and B16F10-Melanoma) were obtained from the American Type Culture Collection (ATCC) and in an incubator at 37 ºC, 5% CO_2, and 100% humidity and obtained from ATCC. All media (DMEM and RPMI-1640) had 10% v/v FBS and 1% penicillin-streptomycin (P-S) added to the media. Media was stored at 4 ºC to keep its and warmed to 37 ºC before to use. Cells were cultured in a T-75 Falcon Tissue Culture. Flasks and passaged at approximately 70% confluence. Cells were counted by using Countess Cell Counting Chamber Slide.

DNA extraction

DNA was extracted from the cell pellets at approximately 3 x 10^6 cells, and the blood and tissue DNA extraction kit on the Maxwell® 16 Instrument, following the manufacturer's protocol.

First PCR ERV-6

The reaction was done in 20µl final volume, with DNA cancer cell lines (5µl), 11.1x PCR buffer, 0.02 units / µl Taq DNA polymerase, and primers (0.5 µM). The primers were designed according ERV-6 map sequencing Figure 1, used to amplify 3' end ERV-6 ENV gene as shown in Table 1.

The amplification size product is 1130bp. This PCR experiment also contained a negative control where water (instead of DNA) was added in the PCR hood and positive control. The PCR conditions was 96°C 1 min, 96°C 30 sec, 60 °C 2 min, 72°C 2 min (34 cycle), 59°C 10 min, 15 °C forever.

Nested PCR

Nested PCR mix was prepared in 10 µl a final volume of 10 µl per reaction was included, 11.1 x PCR buffer, 0.4 units Taq DNA polymerase and primers CMK3LTR (Table 2) was used to specify the 3' end of the ERV-6 env gene with same revers primer that mentioned to amplify specific fragment of REV-6 (216bp) as illustrate in Figure 2.

This experiment also contained (hood negative control) where water was added in the PCR hood while a "bench negative control" where water was added instead of DNA on the bench. PCR conditions was the same as the first PCR.

To confirm that PCR reactions were successful in experiments and had produced a good yield of DNA from cancer cell lines. The PCR product was mixed with 5 x Loading dye and run out on 1% LE agarose gels. The molecular weight markers were used depending on the size of the product in the running gel. 100 bp, ϕX174HaeIII and 1 kb Ladders.

Migration assay

In vitro wound-healing migration assay was set up in three cancer cell lines (MCF7-Breast, SKOV3-Ovarian, and B16F10-Melanoma). The protocol was done according to the instructions of the company (culture insert, ibid, Germany) by using two well chambers. Briefly, cells (1×10^5 cells) were suspended in 100 µL of medium, then 70 µL of the cell suspension was added to each well. After 24 hours, the chamber was removed carefully, and the migration cells level at different points in time (0, 2, 6, 24, 48) hours, and the gap was calculated using ImageJ software and statistics analysis by Prism (Student's unpaired t test), * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$. All experiments were repeated twice, and each data point was measured in triplicate. Mean values and 95% confidence intervals (CIs) were calculated.

Results

For the detection of the ERV-6 env gene in six cancer cell lines. We extracted DNA from cancer cell lines under culture conditions, and checked the integrity and concentration of the DNA fragment. The PCR assay successfully amplified the target region, confirming the presence of ERV-6 in all tested cancer cell lines, including breast, bladder, pancreatic, lung, ovarian, and melanoma models (Figure 3). The experiment included appropriate controls: a negative control to rule out DNA

Table 1. Sequencing of Primers First PCR Assay

CMKENVF	CAGGTGTACCCAACAGCTC	Forward
CMKENVR	AAAGAGATCCCACTTCCATGCG	Revers

Table 2. Sequencing of Primers Nested PCR Assay

CMK3LTR	GATCCTCCATATGCTGAACG	Forward
CMKENVR	AAAGAGATCCCACTTCCATGCG	Revers

Figure 1. Mapping of ERV-6 Shows Design of First PCR Primers

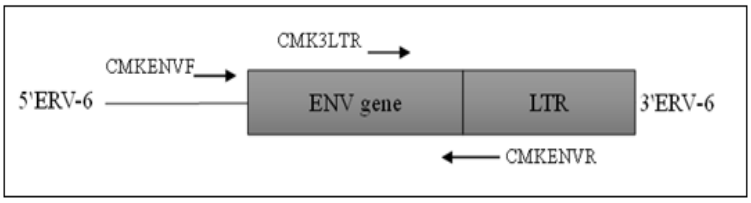

Figure 2. Amplification Specific Fragment of REV-6 (216bp) by Nested PCR

contamination, and a positive control to validate assay performance. The PCR reactions were fractionated by agarose gel electrophoresis as shown in Figure 3, ERV-6 was showed in all breast, bladder, pancreatic, lung, ovarian and melanoma cancer cell lines.

A nested PCR was set up with primers specific for ERV-6 3'LTRs (as mentioned in methods). After thermal cycling, PCR products were fractionated by agarose gel electrophoresis (Figure 4). Successful amplification of ERV-6 3'LTRs was indicated by diagnostic size bands in each cell line. These experiments confirmed that ERV-6 was amplified from the genomes of cancer cells. As a result, specific amplicons were observed in each cell line, potentially reflecting novel ERV-6 env insertions.

ERV6env gene correlation with migration cancer cell lines

Our data demonstrate that the ERV-6 env gene is present in all cancer cell lines (breast, bladder, pancreatic, lung, ovarian, and melanoma). So, it therefore wanted to examine the ERV-6 env gene that can reflect cell migration, and so employed a wound-healing migration assay in three cancer cell lines (MCF7-Breast, SKOV3-Ovarian, and B16F10-Melanoma). In this assay, the time it takes for the gap to close is monitored, as can be seen in Figure 5: A. MCF7 cell shows the slowest migration level among three time points (0,6 and 24) hours. But, at 48 hours, a significant ($p < 0.001$), clear gap remains visible in the center of the wound compared with 2 hours. Moreover, SKOV3 cells recorded moderate migratory progress. At 2 hours, the non-significant migration level, the gap became considerably closer and became highly significant ($p < 0.001$) by the 48-hour compared to the 0h start point time, but at the same time, it is still not fully closed. Our data showed that the B16F10 cell recorded a significantly aggressive migratory level. The gap is nearly completely filled by cells at the 48-hour mark, in contrast to 0 hours, indicating a highly metastatic cell line. Therefore, an ERV-6 env significantly impacts the ability of cancer cells to migrate (metastatic). It is important to note that the migration of cancer cells is generally associated with ERV-6env gene (Figure 5: B). These results suggest that ERV-6 env plays a critical role in metastatic cancer cell lines characteristics. As a result, specific amplicons showed in cell line, potentially reflecting novel ERV-6 insertions, and the indicated loci of ERV-6 may play an important role in the causes or development of cancer and can be used as a biomarker.

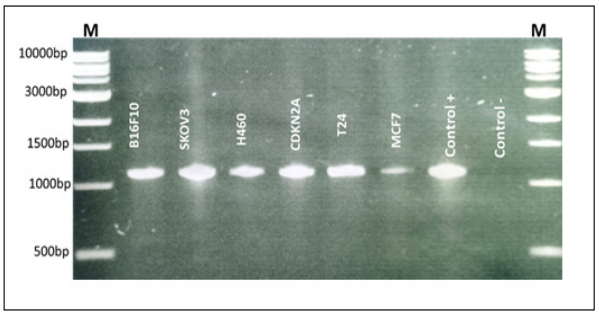

Figure 3. The PCR Assay was Performed to Detect ERV-6 env Gene in T24 –Bladder, MCF7-Breast, CDKN2A-pancreatic, H460-Lung, SKOV3- Ovarian and B16F10-Melanoma). Amplification 1130bp in length. The experiment included two controls, negative to exclude DNA contamination and positive control (+). 1 kb marker(M) was used.

Discussion

Human endogenous retroviruses (ERVs) are integral components of the human genome with significant oncogenic potential [8]. Historically regarded as "junk DNA" these elements are now recognized as active participants in cancer development. The sequencing in RNA encodes very important requirements proteins for survival in the cell, such as envelope proteins. The mature form of retrovirus, integrated into the DNA of the cell as provirus, then replicates by the reverse transcriptase process. In addition, ERV genetic sequencing is essential to study the clear history of discovery or the evolution of

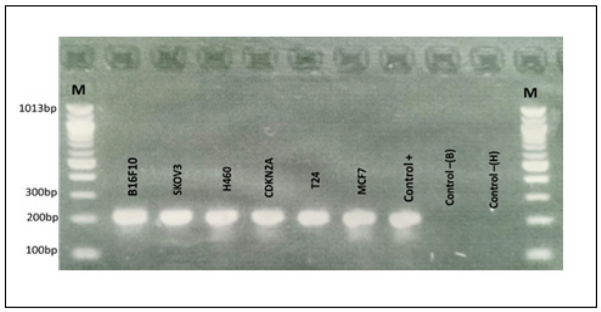

Figure 4. Specific an Amplification env Gene in Cancer Cell Lines. The PCR product was input to the Nested PCR assay using CMK3LTR (primer specific for ERV-6 3'LTRs) and CMKENVR (primer specific for env gene). The Nested PCR products were size fractionated through 2% agarose gels. The experiment included two negative controls. ϕX174HaeIII marker (M) was used.

ERV [9]. In 1960, the first diagnosis of ERV in chickens and mice was made. Laboratory techniques are used to identify ERVs, such as co-cultivation with Living cells, hybridization with retroviral probes, and PCR [1]. The PCR process efforts that investigate ERV across a living cell, from insects, snakes, turtles, and mammals, especially humans [10].

It is noted that the human genome is a complex genetic sequence, with retroviral infections of germline cells millions of years ago. Although most ERVs have been inactivated by mutations and epigenetic factors, the ERV family (specifically ERV-6) remains in the human genome sequence and may still be biologically active, as a result of their reactivation, which causes cancer disease [8]. The World Health Organization (WHO) has recorded a significant increase in cancer, nearly 19.2 million. So, it is important for understanding these ERV activation and drivers of cancer [5, 8].

Our finding that ERV-6 env is present in all six tested cancer cell lines (Bladder, Breast, Pancreatic, Lung, Ovarian, and Melanoma), confirmed by both standard and nested PCR, aligns with previous reports identifying ERV-6 integration or expression across diverse tumor types [2, 11] investigated that expression of the A172 cell line (glioblastoma) showed a high expression of ERV-6 at chromosome 19q13.43b. In addition, the researchers demonstrated HML-6 env overexpression in glioblastoma patients compared to the control group by using RNA in situ hybridization.

Stricker, et al. [11] confirmed the positive correlation of ERV-6 levels and urothelial cancer, which is highly expressed in it. The potential reactivation of ERV-6\ env through hypomethylation of the Long Terminal Repeat (LTR) region, leading to increased transcription [5], provides a plausible mechanism for the observed expression. [5], provides a plausible mechanism for the observed expression in our study. This reactivation may alter cellular signaling pathways, such as the Notch pathway, thereby facilitating tumor progression [4]. Our results are similar to those reported by [2, 3, 12]. All of them mentioned that the ERV env gene is found in most cancer diseases and may be associated with increased metastatic cancer cells.

Breast, pancreatic, and ovarian cancers have also demonstrated a role of ERV-6, which holds the view that shRNA interference was used to knock down ERV-6 in pancreatic and breast cancer cell lines, resulting in decreased expression of the env gene and inhibited pancreatic and breast cancer cell lines proliferation [13]. Ovarian cancer patients showed significantly increased mRNA levels of ERV-6 compared to healthy individuals. Additionally, env protein expression in breast, pancreatic cancer, and melanoma was at an increased level by using flow cytometry and immunohistochemistry compared to the control group [7, 14].

Possible explanation for this might be that env with a panel of different cancer cell lines increased expression because it may be related to a change in some factors, such as the development of cancer or drugs [5]. Also, the biological attributes of ERV- 6, the insertion or deletion sequence in a specific locus, may influence the increased transcription factors and lead to a highly significant transcription process.

Some papers investigated that env protein recorded significantly increased in pancreatic patients as well as pancreatic cell lines, in contrast to the control group. The knockdown RNAi of cancer cells in a mouse model reduced env expression and decreased proliferation of the cancer cell lines and metastasis [2].

Moreover, Stricker, et al. [11] mentioned that overexpression of ERV-6 in melanoma cell lines and tissues has been associated with high levels of

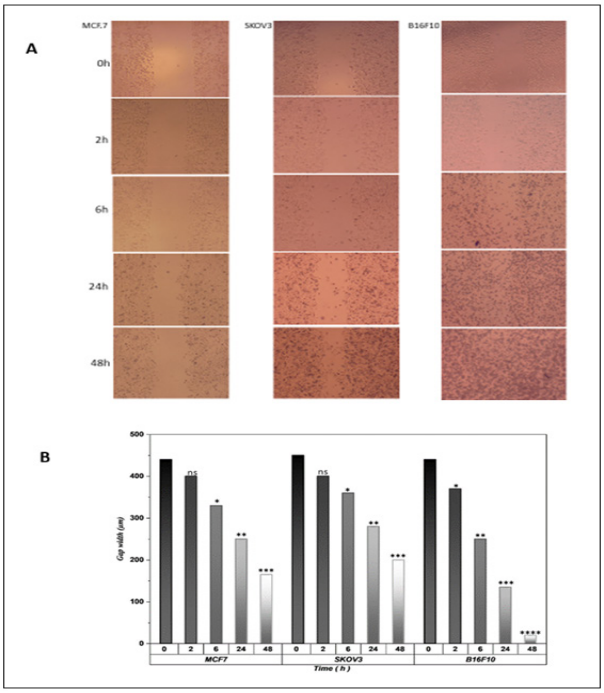

Figure 5. Cancer Cell Migration Assay. A: Three cancer cell lines were used for the migration assay. The pictures were taken at time 0,2,6,24 and at 48 hours. All experiment was repeated twice, and each data point was measured in triplicate. Scales bars, 450 µm. (B). The Gap of wounds was measured in each image and calculated the gap by imageJ software and statistics analysis by prism (Student's unpaired t test), * p< 0.05, ** p< 0.01, *** p< 0.001, **** p< 0.0001.

antibodies of ERV-6, which target the antigen in env. Although it can be used to administer the BCG vaccine-associated ERV-6 to reduce melanoma proliferation and use as a targeting to treat melanomas, it still remains an unclear strategy of treatment.

Taken together, all the published papers concerning bladder, breast, pancreatic, lung, ovarian, and melanoma cancer noted that ERV-6 detection can be used as a biomarker to diagnose different types of cancer and led to the discovery of a new drug to inhibit cancer cells.

Although, our data demonstrate the ERV-6 env gene in all cancer cell lines (breast, bladder, pancreatic, lung, ovarian, and melanoma), we wanted to show that the ERV-6 env gene can reflect cell migration, and so employed a wound-healing migration assay. In this assay, the closing gap is monitored for the level of migration (metastasis) of cancer cells.

These results are similar to those reported by Shah, et al. [2]. They investigated ERV-Np9 expression in teratocarcinoma cells, which reduced the gap closure at 24 hours by knocking it down with CRISPR/Cas [9]. Therefore, a low Np9 expression in teratocarcinoma cells may affect the metastasis of cells. The explanation for this might be that ERV-envelope proteins can stimulate the Epithelial-Mesenchymal Transition (EMT), by losing their polarity and adhesion to cells, resulting in the migratory rate. Many papers noted that ERV-6 env is significantly correlated with increasing metastasis rate in different cancer cells [1, 6].

The another evidence of result can be clearly seen in the silencing env RNA in breast cancer cell lines showed reducing migration and aggressive morphology. It may be that the ERV-6 env gene impacts signaling pathways such as those of Ras, p-RSK, and p-ERK, which play an important role in the migration and invasion phenotype [8].

The limitation of this paper, protein detection is necessary to analysis of ERVK-6 activation and use more types cancer cell line.

As a result, the specific amplicons observed in our tested cell lines potentially reflect novel ERV-6 insertions, suggesting that the indicated loci of ERV-6 may play an important role in the causes or development of cancer and can be used as a biomarker As a result, the specific amplicons observed in our tested cell lines potentially reflect novel ERV-6 insertions, suggesting that the indicated loci of ERV-6 may play an important role in the causes or development of cancer and can be used as a biomarker.

In conclusion, the study of ERV-6 represents a new process that may open the door to diagnosing cancer diseases and treatment. Our results confirm that ERV-6 env is detected in most cancer cell lines by two methods (PCR and Nested PCR). Also, it plays an important role as a regulator of migration, particularly in our cancer cell lines. Therefore, it is a key and potentially interesting target for future therapeutics and radiotherapy.

Acknowledgments

Statement of Transparency and Principles

- Author declares no conflict of interest
- Study was approved by Research Ethic Committee of author affiliated Institute.
- Study's data is available upon a reasonable request.
- All authors have contributed to implementation of this research.

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