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Research Article

Association between Human Endogenous Retrovirus K gene expression and breast cancer

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ABSTRACT

Breast cancer is one of the most common cancers known, and it is also a significant cause of death in women. If breast cancer is diagnosed in the early stages of the disease and treated appropriately, we can see an increase in life expectancy for more than 90% of patients. Research on molecular biomarkers with enough sensitivity and specificity can be a good solution for rapid diagnosis in the clinical stage. Meanwhile, endogenous retroviral biomarkers can have good functional benefits. Human Endogenous Retroviruses as heterochromatin fragments of the genome usually lack expression, but in several types of human cancers, including breast cancer, HERV-Kenv mRNA is significantly increased. This study used RT-PCR to detect the expression of HERV-K mRNA and tried to introduce screening tools for the early detection of breast cancer. In this case-control study, blood samples of 50 patients with hospitalized breast cancer and 50 healthy individuals were designed to evaluate the expression of HERV-Kenv mRNA using specific primers and were analyzed by RT-PCR. PCR test was optimized as a positive control using *Hela* cancer cell line (cervical adenocarcinoma), which expresses the HERV-Kenv gene. Studies on both patient and control groups showed that the increase in mRNA expression was positive in 64% of patients with breast cancer and negative in all healthy individuals. The results indicate an increase in the expression of endogenous human retroviruses (HERVs) in breast cancer. Because the amount of HERV-Kenv mRNA in the blood of breast cancer patients increases dramatically, it is predicted that these mobile genetic elements could be used as a diagnostic biomarker.

1. Introduction

Breast cancer is the most common and deadly type of cancer among women. This cancer is considered one of the most critical factors in women's health in the world [1]. Although this type of cancer is curable and its prevalence is increasing globally, the main problem is the lack of accurate, rapid, and early diagnosis [2]. However, there are several diagnostic methods for detecting breast cancer, such as mammography and clinical trials of tissue differentiation antigens (ER, PR, and Her2) [3]. Still, there are shortcomings with these methods, such as the incidence of false positives or negatives in young women, complications caused by

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radiation during mammography, and the low sensitivity of these biomarkers in the early stages of the disease can be a reason to find new diagnostic methods [4]. Research on molecular biomarkers with enough sensitivity and specificity can be a good solution for rapid diagnosis in the clinical stage [5]. Meanwhile, endogenous retroviral biomarkers can have good functional benefits [6].

A group of viruses that have recently emerged due to their association with cancer is the Human Endogenous Retrovirus (HERV) family, which is referred to as a subset of Mobile Genetic Elements [7]. These viral sequences were inserted into the human genome millions of years ago, and studies have shown that these viral sequences have infected approximately 8% of the human genome [8]. These retroviruses have long-end replicas that contain the coding region for reverse transcriptase [9]. They have also lost their ability to infect and cannot move along the genome due to multiple mutations [10]. Most HERV loci are silenced by DNA hypermethylation, but in many cancers, the genomes of these loci are activated and expressed [11].

Endogenous retroviruses are divided into three different categories based on their similarities with external retroviruses [12]. The HERV-K group belongs to the second category of the HERV family, which is very similar to β-retroviruses. This family is divided into 11 different subgroups (HML-1 to HML-11)[13]. The expression of HERV-Kenv in most breast cancer tissues is significantly higher than that in normal tissues. There is also a significant relationship between estrogen and progesterone stimulation and the transcription rate of HERV-Kenv in breast cancer tissues [14].

Gene expression analysis showed that increased HERV gene expression was affected by external stimuli, such as chemicals, radiation, ultraviolet light, smoking, other viruses, and internal factors such as estrogen and cytokines [15-17]. Also, in breast cancer tissues, the HERV K RT (reverse transcriptase) enzyme expression has shown a significant increase [17, 18].

Examination of HERV-Kenv mRNA expression in patients' serum indicates that the protein was moderately or strongly expressed in cancer tissues [19]. However, no expression of HERV-Kenv protein was found in normal tissues. HERV-Kenv mRNA assay in patients' serum can be used as a screening tool to diagnose early non-invasive breast cancer. This assay promotes early treatment and prevents cancer from spreading to other tissues [14]. Therefore, in this study, the expression of the human endogenous retrovirus K gene in breast cancer patients and healthy individuals was studied to introduce new screening tools for the early detection of breast cancer.

2. Materials and methods

2.1 Patients

To evaluate the expression of HERV-Kenv mRNA, blood samples of 50 breast cancer patients admitted to the hospital within six months from April to September 2020 were examined. All patients had tumors less than 5cm. In addition, 50 individuals who were referred to the mammography department and were diagnosed with no breast cancer were used as control groups. After obtaining the consent of all participants, 3ml of blood was prepared from them and was transferred to the laboratory in a test tube containing EDTA for RNA extraction.

2.2 RNA extraction and primer design

In order to extract RNA from patients' blood samples, all steps were performed according to the protocol in the Hybrid-RTM Blood RNA kit (GeneAll, South Korea). The kit is designed for rapid extraction of high-purity RNA from blood samples. After extraction, the DNase I enzyme (Cat#Nr.18068-015, Thermo Fisher Scientific, USA) was used to remove any contamination with genomic DNA. To ensure that the obtained RNA was not contaminated with genomic DNA, the extracted product was examined using conventional PCR. The final product was elucidated in RNase-free tubes and stored at -80°C. A nanodrop device was used to evaluate the quality of the extracted RNA.

In this study, two primers (forward and reverse) were designed for the HERV-Kenv

gene by referring to sequences in the NCBI database. The β-actin gene primers were also designed as primers of a reference gene (housekeeping gene). The length of HERV-Kenv and β-actin primers were 20 and 21 nucleotides, respectively. After the design, the primer sequences were also blasted by NCBI and Gene Runner to examine their specificity thoroughly. Primers were manufactured by Remington and Winchester, USA. The sequence of primers is shown in Table 1.

Table1. Primer sequences and cycling conditions

Genes	F/R	Primer Sequence (5'→3')
HERV-Kenv	Forward	CACAACTAAAGAAGCTGACG
	Reverse	CATAGGCCCAGTTGGTATAG
β-actin	Forward	ATCAGCAAGCAGGAGTACGAT
	Reverse	AAAGGGTGTAAAACGCAGCTC

2.3 RT-PCR

One-step RT-PCR method was used for RT-PCR. In this method, cDNA production and PCR reaction are performed simultaneously in one step. For this purpose, a ready-made HyperScript kit (Gene All, South Korea) was used. In order to perform the reaction, the 96-well thermocycler (BIO-RAD, USA) was used. 1µl (10Pmol) of primer, 2µl of extracted RNA sample, and 7µl of RNase-free water were added to 10µl of Mastermix. The reaction temperature program was performed at 55°C for 60 minutes, the primary denaturation stage was at 94°C for 5 minutes, 35 cycles with a temperature program of 94°C for 30 seconds (secondary denaturation), 59°C for 30 seconds (for primers annealing), 72°C for 45 seconds (extension) and finally 8 minutes at 72°C for a final extension.

PCR test was optimized as a positive control using Hela cancer cell line (cervical adenocarcinoma), which expresses the HERV-Kenv gene. After electrophoresis of PCR product, the specific band obtained from HERV-Kenv primers (168 bp) and β-actin primers (94 bp) were examined. To ensure the PCR test's optimization and get a specific sample band and positive internal control, the optimized PCR test was used for 50 patient samples and 50 control samples. Finally, the accuracy of the RT-PCR reaction was evaluated by 2% agarose gel.

3. Results

A total of 100 people were enrolled in this study, of which 50 breast cancer patients belonged to the case group, and 50 healthy people without breast cancer belonged to the control group. To measure the concentration of extracted RNA with a nano-drop device, the ratio of 260 to 280 should be between 1.8 and 2.2. In addition, the ratio of 260 to 230 for RNA should be higher than 2, which was consistent with the results of this study. The PCR product of the extracted RNA was loaded on a 2% agarose gel, and no band was obtained, indicating that the extracted RNA was not contaminated by genomic DNA. In addition, to optimize the PCR process and the accuracy of primers, the Hela cell genome (cervical cancer cell line) was used as a positive control sample.

The expression of HERV-Kenv mRNA showed that in 32 samples (64%) out of 50 blood samples of breast cancer patients RT-PCR test was positive and in the remaining 18 samples (36%) RT-PCR test was negative (Table 2). However, the evaluation of 50 control blood samples by RT-PCR was completely negative. The expression of 60% of the HERV-Kenv gene in the sample indicates that the gene is more likely to be expressed during cancer or disease progression. In Figure 1, the RT-PCR product of the HERV-Kenv gene and β-actin reference gene in some patients and controls is shown on gel electrophoresis.

Table2. HERV-Kenv gene expression in case group and study group

HERV-Kenv gene expression	Case group (n=50)	Control group (n=50)	P-value
Expressed (positive)	32 (64%)	0 (0%)	0.0015
Non- expressed (negative)	16 (36%)	50 (100%)	0.022

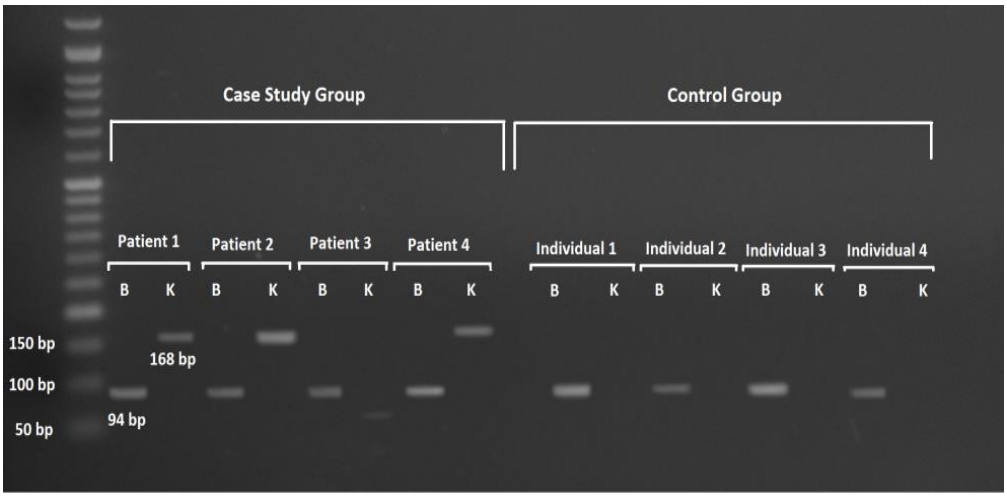

Fig. 1. RT-PCR product of HERV-Kenv gene and β-actin reference gene in case study group and control group; B means β-actin and K means HERV-Kenv

4. Discussion

The results of this study showed that a 64% increase in HERV-K mRNA expression indicates an increase in the expression of this gene in breast cancer patients. Breast cancer is one of the chronic non-communicable diseases that endanger the health of family members and affect the physical, mental, economic, and social status of individuals. The high prevalence, severity, complications, and possibility of breast cancer intervention require effective measures against breast cancer [20]. Breast cancer is the most common cancer among women, and its heterogeneous manifestations and treatability emphasize the necessity of early detection [21].

Although there are a variety of diagnostic methods, such as mammography, ultrasound, MRI, and tissue differentiation antigen clinical tests to diagnose breast cancer, so far, a simple, accurate and reliable test has been designed to analyze the early stage of the disease [21]. In some cancers, screening techniques for early detection are based on widely accepted biomarkers [22].

A group of viruses that have recently emerged due to their association with cancer is the human endogenous retrovirus (HERV) family, which remnants of old germ cell infections and are now part of the human genome. The retroviral elements of HERV-K are silenced due to their presence in heterochromatin fragments and antiviral defense mechanisms. In the case of cancer, these retrotransposons will exit from a dormant state and resume their activity [23]. Studies have shown that HERV transcription increases in various human cancers, including ovarian cancer, lymphoma, sarcoma, bladder cancer, skin cancer, and breast cancer cell lines [24].

Wang-Johanning *et al.* [25] examined the expression of HERV-Kenv in ovarian cancer cells and confirmed the increased expression of the env gene in ovarian cancer. This study also confirmed the increased expression of other HERV classes in ovarian cancer. Golan *et al*. [26] identified the human endogenous retrovirus reverse transcriptase enzyme (HERV) as a prognostic biomarker for breast cancer. Also, Zhao *et al*. [27] evaluated HERV-Kenv expression by immunohistochemistry. They concluded that HERV-Kenv expression occurs in breast cancer tissues as opposed to healthy tissue. In addition, env expression is associated with cancer progression and metastasis. Therefore, HERV-Kenv protein can be used as a prognostic marker for breast cancer. Rhyu *et al*. [28] also examined the level of mRNA and HERV-KHML protein in blood plasma. In this study, they confirmed the effect of chemotherapy on reducing HERV-Kenv gene expression. Wang-Johanning *et al*. [29] identified antibodies against HERV K and mRNAs of its various genes, especially env, as a serum biomarker of breast cancer.

5. Conclusion

Breast cancer is the most common and deadly type of cancer among women. This

cancer is considered one of the most critical factors in women's health in the world . Although this type of cancer is curable and its prevalence is increasing globally, the main problem is the lack of accurate, rapid, and early diagnosis. Research on molecular biomarkers with enough sensitivity and specificity can be a good solution for rapid diagnosis in the clinical stage. Meanwhile, endogenous retroviral biomarkers can have good functional benefits. Therefore, in this study, the expression of the human endogenous retrovirus K gene in breast cancer patients and healthy individuals was studied to introduce new screening tools for the early detection of breast cancer. The results of this study show that serum HERV-Kenv mRNA can indicate the presence of breast cancer in at least some women because, in this study, it was found that the expression of HERV-Kenv mRNA in the serum of a group of women with breast cancer increases. Results can conclude that more detailed studies on this biomarker can become the most promising biomarkers in breast cancer diagnosis. If this biomarker enters the clinical space, it can be used as a supplement for mammography. Due to the low level of HERV-Kenv mRNA in the blood, the design of more sensitive and accurate tests such as Real-Time PCR is suggested in future studies.

Conflict of interest

None of the authors have any conflict of interest to declare.

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None of the authors have any conflict of interest to declare

Consent for publications

All authors approved the final manuscript for publication.

Availability of data and material

The authors have embedded all data in the manuscript.

Authors' contributions

M.T. designed the idea and helped for doing, L.F. helped for doing and helped in manuscript draft writing, Y.Z. helped in data collection, helped in data analysis, and R.S.S. helped in study design, sampling and data collection, doing and article drafting.

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Ethics approval and consent to participate

This study design was approved by ethical code IR.KMU.AH.REC.1397.152 in the research unit. Also, consent forms were completed for all participants.

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