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Unmasking The Hidden Clues: Shedding Light On The Potential Of Novel Biomarkers In Endometrial Cancer, Comparing RT-Qpcr And Immunohistochemical Analysis In Clinical Specimens

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Abstract

Objective: The objective of this study was to compare the diagnostic accuracy of RT-qPCR in endometrial cancer using novel biomarkers in clinical specimens, keeping immunohistochemical analysis as the gold standard.

Methods: This cross-sectional validation study involved 210 endometrial biopsies, suspicious of endometrial carcinoma. RT-qPCR was performed, and relative gene expressions were calculated using the 2^-ΔΔCt method, with the endogenous control. Findings of the RT-qPCR were statistically analyzed keeping immunohistochemical analysis as the gold standard. Sensitivity, specificity, accuracy, negative predictive value (NPV) and positive predictive value (PPV) were calculated for the PCR technique. The clinical sensitivity of the test was determined by the Area under the curve (AUC)/Receiver Operating Curve (ROC).

Results: RT-qPCR diagnosed 61 patients while histochemical analysis diagnosed 60 patients with endometrial carcinoma. RT-qPCR has shown a sensitivity of 96.67%, specificity of 98% and diagnostic accuracy of 97.62%. PPV and NPV of Rt-qPCR were 95.08% and 98.66%.

Conclusions: RT-qPCR promises a highly sensitive and specific method for screening patients with endometrial carcinoma with expression of HE4, L1CAM, miR-200c, ARID1A, and PI3K.

Keywords: Endometrial cancer, Immunohistochemical, biomarkers, radiotherapy.

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1. Introduction

Endometrial carcinoma (EC) represents the leading malignancy among gynecologic cancers, with a predominant incidence in postmenopausal women.[1] EC is recorded as the sixth most prevalent cancer in women and has taken the fifteenth position in total incidences of cancers across the globe. A point of concern is that the preceding decades have witnessed a substantial upsurge in EC, with incidence rates escalating by 132%, establishing this malignancy as a global health concern. In 2020, endometrial cancer saw a worldwide incidence of 417,000 newly diagnosed cases, accompanied by a notable trend of doubled occurrence among women in their pre-menopausal years.[2] Consequently, the mortality attributable to EC has also exhibited an annual escalation. Projections for the United States in 2023 showed it at the fourth position in the incidences of cancer and the sixth primary cause of oncological mortality among the female population.[3] As far as the South Asian region is concerned, in Bangladesh and India, cervical cancer is the most common cancer among females in those countries. In Pakistan, Ovarian cancer is the most reported gynaecological malignancy, while cervical cancer comes just after it. Many tumors are reported very late at advanced stages and early reporting and diagnosis is now emphasized to reduce morbidity and mortality.[4] This indicates that challenges persist in the early diagnosis of EC and thereby stratification of patients for targeted therapies. The diagnostic methods for investigating endometrial cancer include transvaginal ultrasonography, endometrial biopsy, and, in rare circumstances, hysteroscopy. While these methods are generally sensitive in detecting endometrial cancer, they lack specificity, meaning they may sometimes yield false-positive results. Additionally, procedures like endometrial biopsy and hysteroscopy can be invasive and cause discomfort for the patient.[5]

The identification and validation of novel biomarkers hold a promising role in addressing these unmet clinical needs. Traditional methods like immunohistochemistry (IHC) have been instrumental in identifying protein expression in tissue samples.

However, recent advancements in molecular biology have identified numerous biomarkers that could revolutionize the diagnosis of this malignancy. In this scenario, the advent of quantitative reverse transcription polymerase chain reaction (RT-qPCR) offers a sensitive and quantitative approach to detecting gene expression.[6]

Among these biomarkers, Human Epididymis Protein 4 (HE4) is a novel biomarker that shows promise for the diagnosis and prognostic evaluation of various malignancies. HE4, a glycoprotein, has been extensively studied in cases of ovarian cancer as a marker for distinguishing ovarian cancers from benign gynaecological tumours. Some investigations have shown that HE4 has an important role in EC as HE4 has shown potential as a serum marker for EC, particularly in combination with some other important markers (including L1 Cell Adhesion Molecule (L1CAM), MicroRNAs (miR-200c), AT-Rich Interaction Domain 1A (ARID1A) and phosphatidylinositol 3-kinase (PI3K)). Overexpression of biomarker L1CAM has been linked to aggressive tumour behaviour and poor outcomes.[7]

While these biomarkers hold clinical potential, optimal methods for their assessment remain debated. RT-qPCR provides sensitive quantification of gene expression but lacks spatial context. Conversely, IHC enables visualization of protein localization and tumour heterogeneity but may be less quantitative.[8]

This study aims to determine the expression of endometrial cancer biomarkers using RT-qPCR, which quantifies the levels of the biomarkers and will compare the results with the findings of IHC, which visualizes protein expression in situ. The results of this study may help clinicians to opt for evidence-based methods for the diagnosis of EC.

2. Materials & Methods

This cross-sectional validation study was performed at the Department of Medicine, in collaboration with the oncology and gynaecology department of CMH Multan. The duration of this study was 1 year, from the 1st of June 2023 to the 31st of May 2024. This study was approved by the institutional review board. The estimated sample size was 209, while.

Five-year prevalence of EC in suspected cancer cases = 34.7%[9] Sensitivity of RT-qPCR in the diagnosis of EC genes = 95%

Specificity of RT-qPCR in the diagnosis of EC genes = 91%[16].

With a 5% margin of error for both sensitivity and specificity, the estimated sample size was 209.

After written informed consent, a total of 210 endometrial tissue biopsy samples obtained from patients suspected of endometrial cancer through clinical evaluation were included in this study through consecutive sampling. Patients with concurrent inflammatory diseases, endometrial hyperplasia, autoimmune disorders, hormonal therapy, non-endometrial primary tumours, hereditary cancer syndromes, inadequate samples or treatments that could affect biomarker expression were excluded from the study.

Total RNA was extracted from the endometrial tissue samples using the TRIzol® reagent. The quantity and purity of the extracted RNA were assessed using a NanoDrop™ spectrophotometer. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit. Reverse Transcription quantitative PCR (RT-qPCR) was conducted by the standard protocol. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The relative gene expression levels were calculated using the 2^-ΔΔCt method, with the endogenous control. To validate the RT-qPCR results, samples were analyzed using multiplex technology. In this approach, the wells of the microfluidic card contained fluorogenic 5' nuclease assays developed by Applied Biosystems, which enabled the detection of real-time amplification for the selected target genes. The relative expression levels of the genes were determined from the fluorescence data generated during the PCR process, utilizing the Relative Quantification Detection System (7900HT SDS). The expression levels of genes analyzed using RT-qPCR included HE4, L1CAM, miR-200c, ARID1A and PI3K. Genes exhibiting differential expression were confirmed through rigorous statistical analysis using a modified t-test.

The endometrial cancer was also confirmed in these samples by histopathological examination according to the IFGO staging system.

SPSS version 25.0 was used to analyze the data. The normality of data was determined with the Shapiro-Wilk test. Quantitative variables were expressed as Mean±SD, while qualitative variables were displayed as frequency

and percentages. Findings of the RT-qPCR were statistically analyzed in terms of Sensitivity, specificity, accuracy, and negative and positive predictive values while immunohistochemistry was kept as the gold standard technique. Clinical sensitivity of the RT-qPCR for different biomarkers was determined by the Area under the Curve (AUC)/Receiver Operating Test (ROC).

3. Results

The average age of study participants was 53.66±8.26 years with an age range of 41 to 72 years. The majority of females were married for more than 10 years and were multiparous. Most of the females were postmenopausal and had abnormal uterine bleeding while they didn't have a family history of endometrial carcinoma. The majority of females were overweight-obese. Table 1

Table 1: Demographic Characteristics of Study Population (n=209)

Variables		n(%age)
Marital status	Married	204 (97)
	Unmarried	2 (1)
	Widow	4 (2)
Years of marriage	≤5 year	3 (1.4)
	6-10 year	13 (6.2)
	>10 year	192 (91.4)
	N/A	2 (1)
Parity	Nulliparous	41 (19.5)
	Primiparous	37 (17.5)
	Multiparous	132 (63)
BMI	≤25	69 (33)
	26-29	88 (42)
	≥30	53 (25.2)
Menopausal status	Pre-menopause	32 (15.24)
	Post-Menopause	178 (84.8)
Family history of EC	Yes	26 (12.4)
	No	184 (87.62)
Co-morbidities	History of PCOS	42 (20)
	Diabetes	45 (21.4)
	Hypertension	29 (13.8)
Presenting symptoms	Abnormal uterine bleeding	104 (49.52)
	Abnormal vaginal discharge	63 (30)
	Pelvic pain	43 (20.5)

Tissue samples were treated with RT-qPCR and histochemical analysis, RT-qPCR diagnosed 61 patients with EC while histochemical analysis diagnosed 60 patients with EC. The RT-qPCR showed a high level of sensitivity, specificity and diagnostic accuracy in the diagnosis of EC. Table 2, Figure 1

Table 2: Comparison of Endometrial Carcinoma with RT-qPCR and Immunohistochemistry (n=200)

Endometrial Carcinoma Diagnosis	RT-qPCR	Histochemical analysis
Positive n (%)	61 (29)	60 (28.6)
Negative n (%)	149 (71)	150 (71.43)
Sensitivity	96.67%	-
Specificity	98%	-
Diagnostic accuracy	97.62%	-
Positive predictive value	95.08%	-
Negative predictive value	98.66%	-

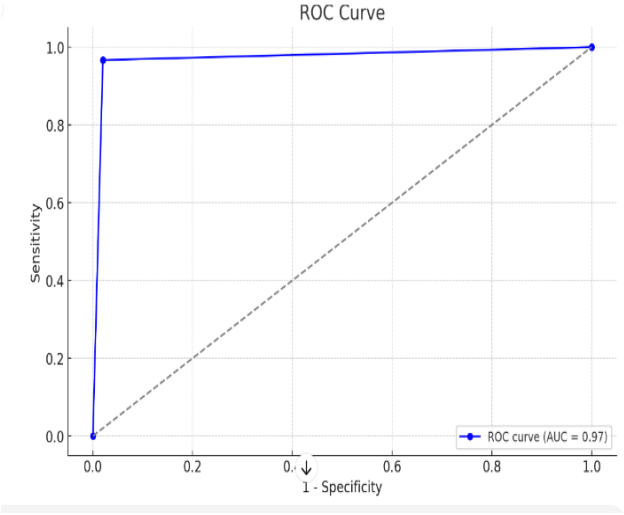

Figure 1: Area under the curve/ROC curve for RT-qPCR, depicting sensitivity of 97%.

Table 3 presents the results of immunochemistry, showing the presence of carcinoma in these blood samples. This indicates their clinical significance in the diagnosis and management of endometrial carcinoma.

Table 3: Determination of True positive and True negative cases in comparison with RT-qPCR and Immunohistochemistry

RT-qPCR	Slide examination		Total
	Positive	Negative	
Positive	58 (TP)	3 (FP)	**61**
Negative	0 (FN)	149 (TN)	**149**
Total	**58**	**152**	**210**

Various tumour markers were analyzed in patients' blood samples, their mean and standard deviations were determined in the overall study population and the cut-off value was determined with ROC curve. Figure 2, Table 4.

Table 2: Biochemical analysis of blood samples for tumour biomarkers (n=209).

Biomarkers	Levels (Mean±SD)
HE4 pmol/L	173.77±5.78
L1CAM copies/µg RNA	33.02±3.2
miR-200c cpm	30.93±4.16
ARID1A tpm	27.8±4.3
PI3K tpm	26.76±2.7

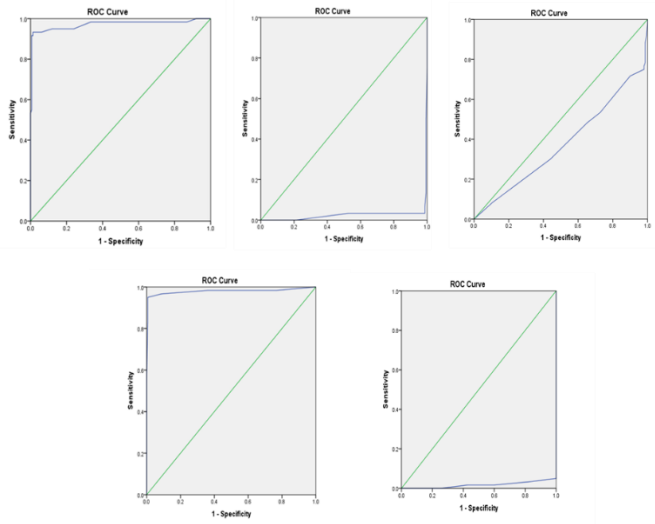

Figure 2: ROC curves for cutoff values of biomarkers for endometrial carcinoma: (a) cutoff value for HE4=56.5 pmol/L. (b) cutoff value for miR-200c = 24.50, (c) cutoff value for L1CAM copies/µg RNA (Ct value) = 22.50, (d) cutoff value for ARID1A=22.5, (e) cutoff value for PI3K was 26.5

4. Discussion

With the new biomarkers and gene expression levels for diagnosing cancer, the use of RT-qPCR for detecting novel genes has been discussed in several studies during the last few years.

Lin B discussed that while blood samples are suitable for early cancer diagnosis, no effective serum biomarker existed for early endometrial cancer (EC) detection, except HE4 with some diagnostic value. Researchers have explored circulating tumor components like cfDNA, ctDNA, and miRNAs. The use of RT-qPCR detected mutations in 33% of 48 EC patients. Hence the RT-qPCR analysis of biomarkers like HE4 aided in EC diagnosis from blood samples.[10]

Behrouzi R in his review mentioned HE4 as the most promising for endometrial cancer, potentially aiding diagnosis, prognosis, hormone therapy response prediction, and recurrence monitoring. Combining HE4 with CA125 or other biomarkers has shown some level of improvement in diagnosing EC. HE4 may enable personalized management, avoiding unnecessary procedures. As molecular classification becomes routine, HE4's role in treatment and follow-up strategies is considered important, however, there's no consensus on optimal serum cut-off values.[11]

A study by Colas discovered molecular biomarkers for EC and proved their usefulness in uterine aspirate analysis, exhibiting high diagnostic performance with AUC values between 0.74-0.95, including early-stage EC detection. The findings paved the way for developing a highly sensitive and minimally invasive method of screening using uterine aspirates and the identified biomarkers, enabling accurate and early EC detection through a minimally invasive approach.[12]

A study explored gene expression profiles in endometrial cancer development using next-generation RNA sequencing RT-qPCR. The study identified 10 important genes from the RNA-seq data and subsequently confirmed their diagnostic utility for endometrial cancer using RT-qPCR.[6]

A study by Enroth S utilized PEA proteomics (PCR-based) and Olink Multiplex assays to identify potential diagnostic biomarkers EC and other gynaecological malignancies. The researchers compared cases of malignancy and healthy controls and women with benign tumours. In the discovery phase, they evaluated 441 plasma proteins, where 16 potential biomarkers emerged to be relevant. Subsequently, 9 out of these 16 potential protein biomarkers showed validated diagnostic value in a replication cohort. The results showed sensitivities and specificities above 64% and 67%, respectively, in distinguishing EC from healthy individuals or those having benign tumours.[13]

In their review, Donkers H found RT-qPCR biomarker profiling promising but noted the main limitations of using biomarkers like miRNAs to be inconsistent in results and challenges were present in comparing data across studies.[14]

In a study, RT PCR evaluated the expression levels of specific genes. The results revealed that certain genes were commonly downregulated, while others were upregulated in the endometrial cancer (EC) tissue samples compared to controls. Based on these gene expressions in EC, the researchers selected three upregulated genes, namely TRA2B, CYR61, and HIF-1α, for decisive diagnostic analysis.[15]

A recently published study by Van den Heerik used RT-qPCR assay to detect 11 pathogenic variants in the POLE gene associated with EC. The performance of this

assay was evaluated in 282 EC cases, and its feasibility and accuracy were externally validated. After DNA sequencing, the qPCR assay demonstrated a sensitivity of 96.0% and a specificity of 100% in detecting the POLE gene variants in EC samples.[16]

The mean age of women in our study was 53.66±8.26 years with an age range of 41 to 72 years. RT-qPCR diagnosed 61(29%) patients with EC while histochemical analysis diagnosed 60 (28.6%) patients with EC. RT-qPCR has shown a sensitivity of 96.67%, specificity of 98% and diagnostic accuracy of 97.62%. PPV was 95.08% and NPV was 98.66% in diagnosing EC. The best cutoff values for the biomarkers HE4, L1CAM, miR-200c, ARID1A and PI3K in the context of EC biomarkers were 56.5 pmol/L, 22.5 copies/µg, 24.50 cpm, 22.5 tpm and 26.5 tpm respectively. These value offers a high sensitivity while maintaining a relatively high specificity, making it an optimal point for differentiating between positive and negative cases in clinical specimens.

These results are consistent with the studies discussed above and confirm the utility of RT-qPCR as a sensitive and specific technique for diagnosing EC by detecting novel biomarkers associated with this EC. The limitations included the involvement of a small data set.

5. Conclusion

RT-qPCR has proven to be a sensitive technique for quantifying gene expression levels of novel biomarkers for diagnosing and treating EC. Its utilization will provide clinicians with a simple, less time-consuming and reliable method to facilitate diagnosis.

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Contributions:

S.F, A.N, A.S, I.A, S.N, M.A - Conception of study
S.F, A.N, A.S, I.A, S.N, M.A - Experimentation/Study Conduction
S.F, A.N, A.S, I.A, S.N, M.A - Analysis/Interpretation/Discussion
S.F, A.N, A.S, I.A, S.N, M.A - Manuscript Writing
S.F, A.N, A.S, I.A, S.N, M.A - Critical Review
S.F, A.N, A.S, I.A, S.N, M.A - Facilitation and Material analysis

All authors approved the final version to be published & agreed to be accountable for all aspects of the work.

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