

Original Article

Running Title: *RHEX and IKBKE as Emerging Endometrial Cancer Biomarkers*

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Bioinformatics Analysis and Experimental Assessment of RHEX and IKBKE in Endometrial Cancer

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Abstract

Background: The mortality rate from endometrial cancer remains high despite advances in treatment. The five-year relative survival rate of advanced and metastatic patients is less than 10%. The present study aimed to evaluate selected molecular markers and their possible associations with disease characteristics and clinical outcomes in endometrial cancer.

Methods: In this study, gene expression datasets of endometrial cancer and non-tumoral samples were obtained from The Cancer Genome Atlas (TCGA). To find effector genes associated with endometrial cancer, we used a feature selection technique based on the Random Forest–Recursive Feature Elimination algorithm. The reliability of the selected featured genes was determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The data were analyzed using GraphPad Prism version 8.0. A *P*-value less than 0.05 was considered statistically significant, and a *P*-value of less than 0.05 was considered statistically significant.

Results: Analytical focus on the TCGA dataset led to the identification of nine genes, *SMIM22*, *RHEX*, *UHFR1*, *IKBKE*, *H3C10*, *FXYD1*, *MYZAP*, *GPIHBP1*, and *MMP28*, of significance in endometrial cancer. These genes were overexpressed in the endometrial tissue of endometrial cancer compared with non-tumoral tissue. Two genes, *RHEX* and *IKBKE*, from the list of feature genes were prioritized for further analysis and qRT-PCR based on our criteria. Results from patient samples showed that both genes were upregulated compared with the non-tumoral group and exhibited increased expression with disease progression.

Conclusion: Our findings suggest that altered expression of *RHEX* and *IKBKE* may be linked to clinical features of endometrial cancer.

Keywords: Endometrial Neoplasms, Gene Expression Profiling, RHEX, NF-kappa B Kinase subunit Epsilon

Introduction

With over 66,000 new cases diagnosed each year over the last ten years, endometrial cancer (EC) is the sixth most common cancer worldwide, and accounts for 90% of uterine cancer cases¹. The mortality rate from this disease remains high despite advances in treatment. Patients with EC have a five-year relative survival rate of about 90% in the early stages, but in advanced and metastatic stages, it drops to less than 10%². Given the significant difference in survival rates, the need for optimal management of the disease is highlighted. The following definition of the EC substages was derived from available evidence: Stage I (SI): A noninvasive type of EC confined to the endometrium, involving less than 50% of the myometrium and without invasion into the lymphatic vascular space (LVSI), as well as noninvasive histological types involving 50% or more of

the myometrium without LVSI or focal LVSI. Stage II (SII): Noninvasive types that invade the cervical stroma. Stage III (SIII): Vaginal/parametrial invasion and pelvic peritoneal metastasis, micrometastases and macrometastases to pelvic and para-aortic lymph nodes. Stage IV (SIV): Locally advanced disease that includes distant metastases to the bladder and rectum. Resistance to therapy and survival rates decrease as the disease progresses³. EC is generally divided into two types. Type 1, which includes grades 1 (G1) and 2 (G2), accounts for 80 to 85 % of cases. Typically occurring in younger individuals, this subtype is associated with a favorable prognosis and generally considered to be indolent. Type 2 accounts for 10 to 15 % of cases and affects older individuals. It is aggressive and has a poor prognosis. Type 2 includes grade 3 (G3) and high-grade (HG)

tumors⁴. Therefore, optimal management of the disease highlights the need for precise molecular tools.

Despite good prognosis with early diagnosis, several key challenges remain in the clinical management of the disease. For instance, a significant minority of women relapse. There is currently no approved noninvasive test for diagnosis. In recent years, the discovery of blood biomarkers has been actively pursued to facilitate diagnosis and improve risk stratification of patients with EC, and many studies have identified candidate genes. However, to date, no standard biomarker has been validated for clinical use due to the molecular complexity and high heterogeneity of EC⁵. These scientific gaps have led to increased treatment failure and relapse rates.

Advances in bioinformatics technologies and gene expression data analysis have enabled the systematic identification of candidate genes with significant expression changes in EC⁶⁻⁸. These genes can be proposed as

emerging molecular markers and novel therapeutic targets. However, experimental confirmation and functional evaluation of these genes in clinical samples are of critical importance for validation. In our study, nine genes, Small integral membrane protein 22 (SMIM22), Regulator of hemoglobinization and erythroid cell expansion (RHEX), Ubiquitin like protein containing a plant homeodomain (PHD) and RING finger domains 1 (UHRF1), Inhibitor of nuclear factor kappa B kinase subunit epsilon (IKBKE), H3 clustered histone 10 (H3C10), FXYD domain containing ion transport regulator 1 (FXYD1), Myocardial zonula adherens protein (MYZAP), Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP1), and Matrix metallopeptidase 28 (MMP28), with altered expression in EC were identified using bioinformatics methods. Two of these genes, RHEX and IKBKE, were selected for further analysis

based on their significantly increased expression in EC, and their established validity and importance as biomarkers in breast cancer and non-small cell lung carcinoma⁹⁻¹¹. The precise role of these genes in EC remain notably unexplored, highlighting the importance of further investigation.

In this study, a sensitive qRT-PCR approach was employed to validate alterations in RHEX and IKBKE expression in clinical EC samples. This approach may support further investigation of molecular features and mechanisms associated with EC.

Materials and Methods

Gene expression data collection and preprocessing

Gene expression datasets for EC and non-tumoral samples were obtained from TCGA using the Bioconductor R package TCGA biolinks¹². The dataset consisted of 35 normal and 553 EC tumor samples. To ensure

comparability across samples, normalization was performed using the DESeq2 (1.50.2) package. The variance-stabilizing transformation was applied to make expression levels comparable across samples. Following normalization, genes with low variance across the dataset were filtered out to minimize background noise and enhance statistical power.

Identification of the important genes

A Random Forest–Recursive Feature Elimination (RF-RFE) framework was applied to identify key genes, and all machine-learning procedures were conducted in the Python environment. To minimize the risk of overfitting and ensure methodological rigor, several essential steps were incorporated. First, the TCGA dataset (553 tumors, 35 normals) was randomly divided into training and test sets using a stratified 70/30 split to preserve class distribution. A Random Forest classifier with 500 decision trees, balanced class weighting to correct for

the substantial class imbalance, and a fixed random seed was used as the base estimator. To avoid information leakage during feature selection, nested cross-validation was implemented. Specifically, a 10-fold StratifiedKFold cross-validation was applied within the training set, and RF-RFE was executed independently within each fold to identify robust, non-overfit features. Features consistently selected across folds were retained as stable candidate genes to improve generalizability. The top 500 genes were selected as candidate features¹³. In addition, a systematic literature review was conducted to identify genes previously reported to be associated with EC. Correlation analysis was then performed between RF-RFE–identified genes and the literature-based gene set. Genes showing the highest number of significant correlations with the literature-derived genes, tumor protein p53 (TP53), phosphatase and tensin homolog (PTEN), DNA polymerase epsilon (POLE), DNA

mismatch repair protein (MSH 2,6), and human mutL homolog (MLH 1,2) were prioritized. The subcellular localization of their encoded proteins was then determined. Finally, nine genes that were simultaneously identified by RF-RFE, showed strong correlation with the literature-based set, and were classified as membrane or cytoplasmic proteins, were selected as candidates for further investigation. To evaluate the generalizability of the nine-gene signature identified from the TCGA cohort, we used an independent microarray dataset (GSE17025) for external validation. Expression values were log2-transformed, and probe sets were mapped to the selected nine genes. The Random Forest classifier trained on the TCGA data was then applied to this external cohort using only the nine-gene panel. For external validation of selected feature genes, the GeneCards (<https://www.genecards.org/>) and Human Protein Atlas (<https://www.proteinatlas.org/>) databases

were used to examine the subcellular localization of the identified genes. Furthermore, the Gene Expression Profiling Interactive Analysis (GEPIA) tool was employed to validate gene expression patterns in healthy and malignant tissues¹⁴.

Subcellular localization study

A comprehensive and integrative database, GeneCards (<https://www.genecards.org/>) offers brief genomic, transcriptomic, proteomic, genetic, and functional information on all known and anticipated human genes¹⁵. An initial assessment of each protein's subcellular localization was conducted using the GeneCards database.

Survival analysis

The association between mRNA expression levels of the selected genes and overall survival (OS) outcomes in EC patients was investigated using the GEPIA2 database (<http://gepia2.cancer-pku.cn/>)¹⁶. Statistical

significance was determined using a log-rank *P*-value of less than 0.05.

Study participants and sample collection

Tissue samples, from 50 EC patient (mean age \pm SD: 60.46 ± 7.89 years), were collected from newly diagnosed women based on clinical and pathological findings, along with 50 non-EC samples from age-matched, non-cancerous women (mean age \pm SD: 59.42 ± 8.147 years), who had undergone hysterectomy for tumor or non-tumor-related reasons. Due to limited availability of clinical samples, no formal sample size calculation was performed, and the number of samples in each group was determined based on feasibility. EC samples from patients with other malignancies, autoimmune diseases, a history of chemotherapy, hormone therapy, endometriosis, obesity, diabetes, radiotherapy or infectious diseases within one month before sampling were excluded from this study. Additionally, a family history of malignancy or autoimmune diseases in

first-degree relatives was regarded as the exclusion criteria for the non-cancerous group. The collected tissues were washed three times with cold phosphate-buffered saline (PBS), frozen in liquid nitrogen, and stored at -80°C until qRT-PCR analysis. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (ethical approval code IR.SUMS.REC.1403.114). In addition, written informed consent was obtained from the patients by the surgeon. The clinicopathological characteristics of the patients are summarized in Table 1.

RNA extraction and cDNA synthesis

Total RNA was extracted from endometrial tissue samples using the Total RNA Extraction Kit (Pars Tous, Iran; Cat No. A101231), according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDropTM Spectrophotometer (Thermo Fisher Scientific, USA), and RNA integrity was

assessed by electrophoresis on a 1% agarose gel. A total of 2 µg RNA was used for first-strand cDNA synthesis in a final volume of 10 µL using the Easy cDNA Synthesis Kit (Pars Tous; Iran Cat No. A101161), which includes a combination of random hexamer and oligo-dt primers, following the manufacturer's protocol.

qRT-PCR

qRT-PCR was performed using 2x Master Mix Green (Ampliqon, Denmark; ID No. 5000830), containing SYBR Green dye and TaqMan Hot Start DNA Polymerase, on an ABI StepOne qRT-PCR system (Applied Biosystems, USA). Each reaction was carried out in duplicate in a final volume of 20 µL. Primers were designed using AlleleID software (version 7), and their specificity was confirmed through basic local alignment search tool (BLAST) analysis against the human genome and transcriptome using the national center for biotechnology information (NCBI) database. To avoid

genomic DNA amplification, primers were designed to span exon-exon junctions where possible. All primers were synthesized by Metabion (Germany). β -actin was used as an internal reference gene. The sequences of the primers used for amplification are listed in Table 2.

Amplification was carried out in 48-well microtiter plates under the following conditions: initial denaturation at 95°C for 15 minutes, followed by 50 cycles of 95°C for 15 seconds and 61°C for 30 seconds. A melt curve analysis was performed at the end of each run to confirm the specificity of the PCR products. Threshold setting and baseline values were manually reviewed and adjusted where necessary, to ensure the accuracy of the cycle threshold (Ct) values. Ct values, obtained using StepOne Software version 2.3, were normalized with the CtNorm algorithm (<http://www.ctnorm.sums.ac>) ¹⁷. Subsequently, relative gene expression was quantified by applying the $2^{-\Delta \Delta CT}$ formula.

Statistical analysis

The results of the study were reported as the mean with SEM and median with 95% confidence interval (CI) or interquartile range (IQR). Data distribution was assessed using the D'Agostino-Pearson or Shapiro-Wilk test. The t-test, Mann-Whitney, and Kruskal-Wallis H Test were used to compare gene expression levels. Statistical analyses were performed using GraphPad Prism version 8.0. A *P*-value less than 0.05 was considered to be statistically significant.

Results

Bioinformatics-driven identification of key genes

To identify genes of potential importance in EC, gene expression data were extracted from the TCGA database. To achieve this, the RF-RFE algorithm was applied to assess the relative importance of genes. Based on the resulting importance scores, the top 500 genes were selected based on their

significance scores and subsequently subjected to further analysis. On the held-out test set, the model achieved an accuracy of 0.92 and an area under the receiver operating characteristic (ROC) curve (AUC) of 0.94. These results confirm that the top-500 gene signature was robust to discovery cohort. After identifying the 500 genes with the highest significance score, a rigorous refinement process was applied. The genes that showed the most significant correlation with key genes involved in EC were prioritized. In addition, special emphasis was placed on genes located in the cell membrane as well as the cytoplasm. These selected genes showed higher expression levels in cancer tissue compared with their expression in non-tumoral tissue. Based on these analyses, nine key genes were finally identified (Table 3, and supplementary Table 1). The nine-gene signature was validated on an independent cohort (GSE17025), achieving an accuracy of 0.81 and an AUC of

0.86. Among them, RHEX and IKBKE were prioritized for further analysis and validation using qRT-PCR.

Subcellular localization prediction

The GeneCards database was used to determine the subcellular localization of the identified proteins. Four proteins associated with genes SMIM22, RHEX, GPIHBP1, MYZAP and FXYD1 are located in the cell membrane. The IKBKE gene protein was predicted to be located in the cytoplasm. In contrast, H3C10 and UHRF1 were predicted to be located in the nucleus, while MMP28 was predicted to be located in the extracellular matrix.

Importance of the selected genes

Based on the findings obtained from the Kaplan-Meier plotter database analysis, it was observed that a higher expression of GPIHBP1 exhibited a significant association with unfavorable overall survival (OS) outcomes in EC (OS Hazard Ratio (HR) = 3.

2, log-rank p-value = 0.0031) as illustrated in Figure 1A. In contrast, a higher expression of UHRF1 was associated with favorable OS (OS HR=0.51, logrank *P*-value= 0.057, a trend toward significance) (Figure 1B). However, no statistically difference in OS was observed for the other genes.

qRT-PCR-based validation of top bioinformatically identified candidate gene

Based on bioinformatic analyses, two candidate genes, RHEX and IKBKE, were selected for experimental validation by qRT-PCR. To do so, two approaches were considered: molecular characterization, and clinical associations.

To explore the importance of RHEX and IKBKE gene expression, their expression levels were compared between non-tumoral tissue and G1 tumor, type 1 tumor (G1 and G2 tumor) and all tumor tissue data. The results showed that the RHEX gene was overexpressed in G1 (*P*-value: 0.06, Fold

change: 1.33), type 1 (*P*-value: 0.021, Fold change: 1.59), and all tumor patients (*P*-value: 0.000, Fold change: 2.77), compared with non-tumoral tissue (Table 4, Figure 2A).

The IKBKE gene was also overexpressed in G1 (*P*-value: 0.016, Fold change: 1.62), type 1 (*P*-value: 0.001, Fold change: 1.97), and all tumor patients (*P*-value< 0.0001, Fold change: 2.29), compared with non-tumor tissue (Table 5, Figure 2B).

To explore the association between RHEX and IKBKE expression and clinical characteristics, their expression levels were assessed according to the grade and stage of the disease. These studies were conducted in four comparative sections: type 1, SI vs. type 1, SIII/ G3, SI vs. G3, SIII/ HG, SI vs. HG, SIII, and type 1 vs. type 2. The results showed that the expression levels of the RHEX gene (Table 4, Figure 3A, B, C, and D) increased in patients with increasing grade and stage of the disease (*P*-value: 0.005, Fold change: 2.26/ exact *P*-value: 0.047, Fold change:

5.63/ *P*-value: 0.013, Fold change: 2.67 and *P*-value: 0.005, Fold change: 2.95, respectively). The IKBKE gene (Table 5, Figure 4A, B, C, and D) also showed increased expression in patients with increasing grade and stage of the disease (*P*-value: 0.004, Fold change: 1.91/ exact *P*-value: 0.044, Fold change: 7.73/ *P*-value: 0.700, Fold change: 1.25 and *P*-value: 0.013, Fold change: 2.1, respectively).

Also, their expression levels were examined. Comparisons between similar stages but different grades were considered important. Therefore, the present study included comparisons between G1, SI vs HG, SI, and G1, SIII vs HG, SIII. Both RHEX (*P*-value: 0.008, Fold change: 2.39 and exact *P*-value: 0.020, Fold change: 4.07, respectively) and IKBKE (*P*-value: 0.004, Fold change: 1.34 and exact *P*-value: 0.711, Fold change: 2.03, respectively) genes showed greater expression increases in higher grades of

disease (Table 5, Figure 5A and B for RHEX, C, and D for IKBKE).

Discussion

EC, the most prevalent gynecologic malignancy, is steadily increasing due to aging populations and obesity-related factors. An in-depth examination of the changes in gene expression that underlie tumor biology is made possible by the widespread usage of transcriptional data from public repositories such as TCGA in cancer research. These resources facilitate the identification of differentially expressed genes (DEGs). Following that, these genes may be thoroughly examined to identify key regulators and core genes with potential roles in EC clinical context. In this study, RNA expression data from TCGA were analyzed to identify DEGs between EC and non-tumoral samples. The up-regulated genes were then evaluated using a random forest algorithm to identify the most important genes. These key genes were further investigated based on

their higher expression in EC tissues compared with non-tumoral tissues. In total, 500 genes were screened for classification into tumor and non-tumor groups. After identifying the 500 genes with the highest significance score, a rigorous refinement process was applied. The genes that showed the most significant correlation with key genes involved in EC were prioritized. In addition, special emphasis was placed on genes located in the cell membrane as well as the cytoplasm. Based on these analyses, nine key genes, SMIM22, RHEX, UHRF1, IKBKE, H3C10, FXYD1, MYZAP, GPIHBP1, and MMP28, were ultimately identified (Table 3).

The microprotein SMIM22 has been identified as a contributing factor in breast cancer. This 10-kDa protein is predominantly expressed in hormone receptor-positive breast tumors. Reduced expression of SMIM22 leads to reduced cell proliferation in several breast cancer cell lines. SMIM22

interacts with the enzyme squalene epoxidase (SQLE), which plays a key role in cholesterol synthesis, and is known to act as oncogene in breast cancer. Increased expression of SMIM22 results in the accumulation of SQLE protein without affecting its mRNA level, leading to the accumulation of lipid droplets in the cell. These findings identify SMIM22 as a functional microprotein involved in carcinogenesis and cellular lipid homeostasis¹⁸. Although the role of the SMIM22 gene has been mainly investigated in breast cancer, our bioinformatics findings indicate that this gene is also overexpressed in EC, and is likely involved in cell proliferation, migration, and metabolic alterations in tumor cells.

Recently, the role of UHRF family genes, including UHRF1 and UHRF2, has been investigated in EC, and decreased UHRF2 expression and increased UHRF1 expression have been observed in atypical endometrial hyperplasia and EC. It has been reported that

decreased UHRF2 expression is significantly correlated with tumor histopathological grade. UHRF1 expression is also increased, especially in the peripheral tumor margins, suggesting that it may play a role in endometrial carcinogenesis by inhibiting PTEN expression through involvement in the processes of cell proliferation, migration, and metabolic changes of tumor cells¹⁹. Our bioinformatics analyses also indicate that the UHRF1 gene is overexpressed in EC.

MMPs play an important role in EC, and their abnormal expression is associated with tumor invasion and disease progression. MMP28, along with other MMPs, is overexpressed in patient tumor specimens and endometrial cell lines and may promote cancer progression by remodeling the extracellular matrix and facilitating Epithelial Mesenchymal Transition (EMT) ²⁰. Our bioinformatics analyses also indicate that the MMP28 gene is also overexpressed in EC. However, although reduced gene expression does not

necessarily correspond to reduced protein expression, our bioinformatics data suggest that this gene is downregulated in EC.

The expression of FXYD family genes was investigated in ovarian cancer tissues using TCGA data and independent clinical samples. FXYD1, FXYD5, and FXYD7 have been reported to be overexpressed in cancer tissues compared with non-tumoral tissues, and this increased expression is associated with poorer patient prognosis. Bioinformatics analyses also revealed that these genes are involved in signaling pathways related to tumor growth and metastasis. Based on these findings, FXYD1 has been proposed as a potential candidate for use as a diagnostic biomarker or therapeutic target in ovarian cancer²¹. Currently, there is limited information on the role of FXYD1 in EC, and further research is needed in this area. Our bioinformatics data show a decrease in the expression of this gene in EC.

Histone modifications play an important role in tumorigenesis and cancer progression. It has been shown that various histone modifications, including methylation, acetylation, and phosphorylation, can affect chromatin structure and gene expression. Histone modifications are also associated with the activation of oncogenic genes and the inactivation of tumor suppressor genes²². Given that the H3C10 gene encodes the histone H3 protein and plays a key role in chromatin regulation, it can be expected that this gene is involved in similar pathways, however, its role in EC has not yet been investigated. Our bioinformatics analyses indicate that the H3C10 gene is also overexpressed in EC. The role of two other genes, MYZAP and GPIHBP1, in EC has not been studied, and our bioinformatics results show downregulation of these genes in EC, suggesting their potential as biomarkers in this disease. In the survival analysis results of this study, increased GPIHBP1 was

associated with significantly lower OS, and had adverse effects on patient outcomes. This finding is consistent with previous studies showing that elevated GPIHBP1 expression in advanced stages of colon cancer is linked to immune evasion and tumor progression. In esophageal cancer, this gene also regulates triglyceride and fatty acid metabolism and significantly contributes to cancer progression²³. The bioinformatics results of this study indicate that GPIHBP1 expression is significantly reduced in EC tumor tissues. In line with this observation, one study reported that GPIHBP1 is downregulated in lung cancer tissues, although, for unknown reasons, its overexpression can promote lung cancer progression²⁴. There is very limited information about the MYZAP gene, suggesting that altered expression of this gene is directly related to tumor progression. Our study showed that the expression of this gene is reduced in tumor tissue.

The RHEX gene was initially identified as an important regulator of the EPO-EPOR pathway and erythroid cell proliferation²⁵. RHEX is highly expressed in EC tumor tissues and associated with clinical features of the disease, including International Federation of Obstetrics and Gynecology FIGO stage and tumor invasion. In endometrial cell models, functional investigations have demonstrated that decreased RHEX expression results in lower cell proliferation, inhibition of cell migration and invasion, increased apoptosis, and tumor development arrest. These results suggest that RHEX regulates cell migration, survival, and proliferation in EC and might be a good target for more biomarker studies²⁶.

IKBKE is a major activating kinase of the NF-κB pathway, which plays a key role in regulating inflammatory responses, cell survival, and treatment resistance²⁷. Chronic activation of NF-κB by IKBKE increases the expression of tumor-promoting genes and

facilitates cancer cell growth, migration, and survival²⁸. The IKBKE gene has been investigated as a potential biomarker in renal cell carcinoma (RCC). The results showed that IKBKE was highly expressed in tumor tissues and sunitinib-resistant cells and associated with clinical disease progression and reduced patient survival. Suppression of IKBKE resulted in cell cycle arrest in the G2/M phase, decreased cell proliferation and migration, and increased sensitivity to treatment. These findings suggest that further investigation of this gene in other cancers would be valuable. However, the role of IKBKE in EC has not yet been investigated²⁹.

In this study, two genes, RHEX and IKBKE were selected due to their biological significance, potential involvement in cancer-related pathways, and RNA-coding nature with detectable protein expression at the cell membrane or cytoplasmic level, and were subsequently analyzed by qRT-PCR. According to qRT-PCR analysis, the

expression levels of RHEX and IKBKE were significantly increased in G1 and type 1 endometrial tumor samples compared with non-tumoral tissues.

Our qRT-PCR results showed that RHEX and IKBKE expression was increased in G1 and type 1 tumor samples compared with the non-tumor group. Overall, 80–85% of newly diagnosed patients fall into the type 1 category, which is the sum of G1 and G2 patients. This pattern of increased expression was also observed in the analysis of all EC patients, including type 1 and type 2, compared with the non-tumor group (Table 4, Figure. 2). In addition, analyses in the EC patient group showed that samples from patients with disease progression or tumor metastasis showed increased RHEX and IKBKE expression. These analyses included comparisons between SI and SIII patients in the type 1 group, SI and SIII within the G3 patient group, and SI and SIII among HG patients, as well as an overall comparison

between type 1 and type 2 patients (Table 4, Figures. 3 and 4). Furthermore, analyses in the cohort of patients with EC showed that samples from patients with disease progression or tumor metastasis showed increased expression of RHEX and IKBKE.

In the next step of the analysis, and based on the clinical feature of differentiating patients with similar stage but different grades, two comparative analyses were performed (SI, G1 vs. SI, HG) and (SIII, G1 vs. SIII, HG). Within the same stage, patients with higher grades exhibited increased expression of RHEX and IKBKE (Table 4, Figure. 5). These findings further support the potential relevance of both genes in molecular pathways associated with EC, pending further validation.

Regarding the RHEX gene, our findings are not only consistent with previous studies but also extend them. To date, only one study has reported that increased RHEX expression is associated with lymph node metastasis in

early-stage EC²⁶. Our results expand this observation, showing that elevated RHEX expression is not only limited to lymphatic metastasis but also correlated with overall disease progression and higher tumor stages. RHEX has been shown to facilitate cell proliferation and promote angiogenesis through activation of the JAK2–STAT signaling pathway³⁰. Consistent with this, our data revealed significantly higher RHEX expression in advanced grades and stages of EC, suggesting its role in tumor invasion and progression. Regarding IKBKE, previous studies have demonstrated its role in NF-κB activation and its contribution to drug resistance in breast, lung, and esophageal cancers^{9,11}. Our findings show, for the first time, that IKBKE is overexpressed in EC and that its expression correlates directly with tumor grade and disease stage. The observed upregulation of IKBKE in patients with higher grades and stages supports the hypothesis that this gene may play a critical

role in tumor invasion and therapeutic resistance in EC.

The simultaneous increase in RHEX and IKBKE expression in early and later stages of the disease suggests that these two genes may have a synergistic effect in establishing the tumor microenvironment (TME). This process can promote tumor growth and metastasis and enhance tumor survival.

Conclusion

The observed upregulation of RHEX and IKBKE in EC, and their correlation with disease grade and stage, indicate potential relevance in molecular and clinical features of EC, although further studies are needed for validation. Furthermore, given the localization of proteins in the cell membrane and cytoplasm, our data highlight these gene as potential molecular targets. Targeting the corresponding proteins, particularly those involved in the Jak2 and NF-κB pathways, may warrant further investigation, although

the therapeutic relevance remains to be validated.

One limitation of this study concerns sample selection. To reduce the impact of important metabolic comorbidities, participants with severe obesity, diabetes, or Inch syndrome were excluded from both the tumor and non-tumor groups. This made the two groups comparable in terms of key metabolic risks and reduced the possibility of confounding effects. However, we are aware that this selection may limit the generalizability of the results to the entire EC patient population. The number of samples examined at higher stages was also limited due to lower prevalence. Additionally, this study focused solely on gene expression levels, and no functional assays were conducted to establish causal relationships. Therefore, future research with larger sample sizes and complementary functional analyses is warranted.

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Authors' Contributions

Abbas Ghaderi: Conceived and supervised the study, guided experimental and analytical procedure, interpreted the results, and critically revised and finalized the manuscript; **Mohammad Reza Haghshenas:** Conceived and supervised the study, guided experimental and analytical procedure, interpreted the results, and critically revised and finalized the manuscript; **Ahmad Tahmasebi:** Contributed to the design of bioinformatics analysis, performed and interpreted bioinformatics data, and provided critical revision of the manuscript; **Amin Ramezani: corresponding author**, supervised the real-time PCR experiments and critically revised the manuscript; **Zahra Shirvani, Fatemeh Sadat Najib and Maryam Aghdaki:** Provided clinical samples and contributed patient clinical data; **Mahintaj Dara:** Assisted in raw data acquisition and **Zohreh Koohini:** Sample collection, performed

experimental procedure, data analysis and interpretation, manuscript writing and critical review. **Nafiseh Maghsoodi:** Final review and improvement of the manuscript before submission.

All authors have read and approved the final version, and agree to be accountable for all aspects of the work and to ensure that questions regarding the accuracy or integrity of any part of the work are appropriately reviewed and resolved.

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Conflict of Interest

None declared.

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Table 1. Clinicopathological characteristics of EC patients (n= 50), grouped by tumor stage (I-IV, A, B, C) and tumor grade (well, moderate, poorly)

CPC	Factors	Number (%)
Histologic stage	I (A, B and C)	34 (64 %)
	II (A and B)	3 (6 %)
	III (A, B and C)	13(26 %)
	IV (A and B)	-
Histologic grade	Well-differentiated (Grade 1)	25 (50 %)
	Moderately-differentiated (Grade 2)	12 (24 %)
	Poorly-differentiated (Grade 3 and High grade)	13 (26 %)

CPC: Clinicopathological characteristics, EC: Endometrial cancer

Table 2. Characteristics of primers used for target genes, including sequence, annealing temperature, and product length

Target	Sequence	Annealing temperature	PCR product lengths
β-actin	F: GCCTTGCCGATCCGC R: GCCGTAGCCGTTGTCG	61 °C	90
RHEX	F: TCACCGCCATCAACTACCT R: CCTGGGAACCTGGAGACTG	61 °C	86
IKBKE	F: ACATCCCTCCTTACCTCAGC R: GCAGCCGCCTTCAGTTCT	61 °C	92

RHEX: Regulator of hemoglobinization and erythroid cell expansion, IKBKE: Inhibitor of nuclear factor kappa-B kinase subunit epsilon, PCR: Polymerase chain reaction

Table 3. Characteristics of selected genes identified by bioinformatics analysis in EC, including ensemble ID, protein name, function, and subcellular localization

ENS ID	Aliases	Protein's name	Protein function and cellular location
ENSG00000267795	SMIM22	Small integral membrane protein 22	➤ Modulate lipid droplet formation, positive regulation of cell migration ➤ cell membrane
ENSG00000263961*	RHEX *	Regulator of hemoglobinization and erythroid cell expansion protein	➤ Signaling transduction factor ➤ cell membrane
ENSG00000276043	UHRF1	Hemi-methylated DNA-binding	➤ DNA methylation ➤ nucleus
ENSG00000263528*	IKBKE *	Inhibitor of nuclear factor kappa-B kinase subunit epsilon	➤ Showing features for immune binding site ➤ cytoplasm
ENSG00000278828	H3C10	Histone H3.1	➤ Core component of nucleosome ➤ nucleus
ENSG00000266964	FXYD1	Phospholemman	➤ Regulatory subunit of the sodium/potassium-transporting ATPase ➤ cell membrane
ENSG00000263155	MYZAP	Myocardial zonula adherens protein	➤ Cellular signaling ➤ cytoplasm and membrane
ENSG00000277494	GPIHBP1	Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1	➤ Mediates the transport of LPL ➤ cell membrane
ENSG00000271447	MMP28	Matrix metalloproteinase-28 Protein	➤ Cofactor ➤ ECM

ENSG: Ensemble Gene ID, EC: Endometrial cancer, ECM: Extra cellular matrix, SMIM22: Small integral membrane protein 22, RHEX: Regulator of hemoglobinization and erythroid Cell expansion, UHRF1: Ubiquitin like protein containing a plant homeodomain (PHD) and RING finger domains 1, IKBKE: Inhibitor of nuclear factor kappa B kinase Subunit epsilon, H3C10: H3 Clustered Histone 10, FXYD1: FXYD domain containing ion transport regulator 1, MYZAP: Myocardial zonula adherens protein, GPIHBP1: Glycosylphosphatidylinositol anchored high density lipoprotein binding Protein 1, and MMP28: Matrix metallopeptidase 28

Table 4. Summary of experimental analyses of RHEX gene expression evaluated by qRT-PCR

Comparison	RHEX					Sample size
	P-value	Adjusted p	Effect size (r)	Effect size Cohens d (95% CI)	Fold Change	
Non-tumoral vs G1	0.060	0.18	≈ 0.23	--	1.33	n: 49 vs n: 16
Non-tumoral vs type 1	0.02 *	0.06	≈ 0.27	--	1.59	n: 49 vs n: 24
Non-tumoral vs all tumors	0.00 ***	0.00 ***	≈ 0.38	--	2.77	n: 49 vs n: 45
Type 1, SI vs type 1, SIII	0.00 **	--	--	0.38 (95% CI 0.35 to 1.76)	2.29	n: 21 vs n: 5
G3, SI vs G3, SIII	0.04 *	--	≈ 0.82	--	5.63	n: 8 vs n: 2
HG, SI vs HG, SIII	0.01 *	--	--	0.60 (95% CI 0.55 to 5.262)	2.67	n: 5 vs n: 4
Type 1 vs type 2	0.00 **	--	≈ 0.41	--	2.95	n: 28 vs n: 21
G1, SI vs HG, SI	0.00 **	--	--	0.28 (95%CI 0.18 to 2.315)	2.39	n: 17 vs n: 6
G1, SIII vs HG, SIII	0.02 *	--	≈ 0.80	--	4.07	n: 4 vs n: 3

*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, and ****: $P < 0.0001$, G: Grade, S: Stage, CI: Confidence interval, RHEX: Regulator of hemoglobinization and erythroid cell expansion, IKBKE: Inhibitor of nuclear factor kappa-B kinase subunit epsilon

Table 5. Summary of experimental analyses of IKBKE gene expression evaluated by qRT-PCR

Comparison	IKBKE					Sample size
	P-value	Adjusted p	Effect size (r)	Effect size (Cohens d, 95% CI)	Fold Change	
Non-tumoral vs G1	0.01 *	0.04 *	≈ 0.30	--	1.62	n: 44 vs n: 18
Non-tumoral vs type 1	P< 0.01 **	P< 0.01 **	≈ 0.39	--	1.97	n: 44 vs n: 25
Non-tumoral vs all tumors	< 0.0001 ****	< 0.0001 ****	≈ 0.45	--	2.29	n: 44 vs n: 48
Type 1, SI vs type 1, SIII	P< 0.01 **	--	--	0.33 (95% CI 0.2973 to 1.385)	1.91	n: 18 vs n: 5
G3, SI vs G3, SIII	0.04 *	--	≈ 0.82	--	7.73	n: 8 vs n: 2
HG, SI vs HG, SIII	0.70	--	--	0.02 (95% CI -2.529 to 3.560)	1.25	n: 5 vs n: 4
Type 1 vs type 2	0.01 *	--	≈ 0.35	--	2.1	n: 25 vs n: 23
G1, SI vs HG, SI	P< 0.01 **	--	--	0.33 (95% CI 0.33 to 1.63)	1.34	n: 16 vs n: 6
G1, SIII vs HG, SIII	0.71	--	0	--	2.03	n: 4 vs n: 3

*: P<0.05, **: P<0.01, ***: P<0.001, and ****: P<0.0001, G: Grade, S: Stage, CI: Confidence interval, RHEX: Regulator of hemoglobinization and erythroid cell expansion, IKBKE: Inhibitor of nuclear factor kappa-B kinase subunit epsilon

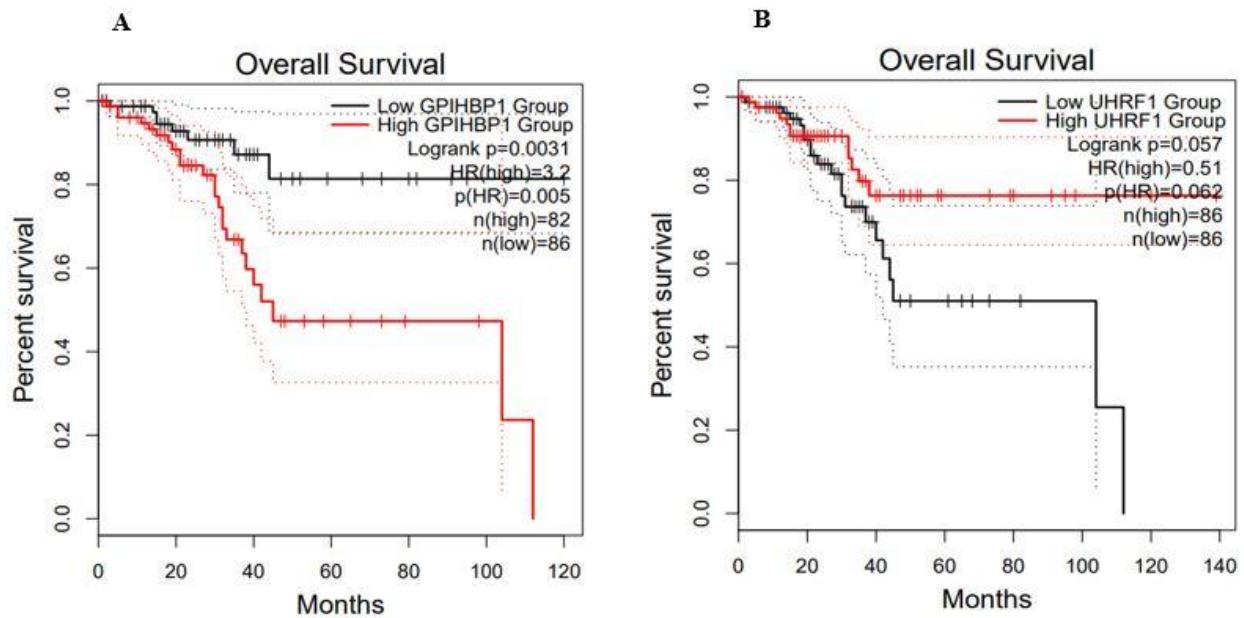


Figure 1: Survival analysis of query genes in EC patients from the Kaplan-Meier plotter database. Overall survival curves of (A) GPIHBP1, and (B) UHRF1. A log-rank *P*-value below the 0.05 threshold indicates a statistically significant association. HR, Hazard ratio, GPIHBP1: High density lipoprotein binding protein 1, UHRF1: Ubiquitin like protein containing a plant homeodomain (PHD) and RING finger domains 1

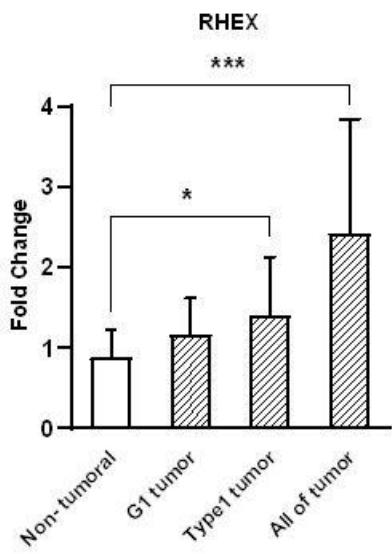
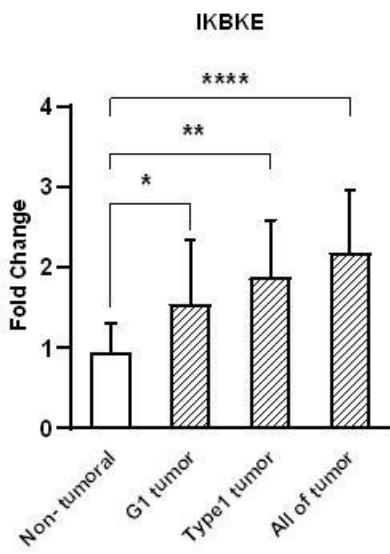
A**B**

Figure 2. Comparative analysis of RHEX (A) and IKBKE gene expression in EC (B). Sample size for each group in 2A are; n= 49 for non-tumoral, n= 16 for G1 tumor, n= 24 for type1 tumor, and n= 45 for all of tumor group. Sample size for each group in 2B are; n= 44 for non-tumoral, n= 18 for G1 tumor, n= 25 for type1 tumor, and n= 43 for all of tumor group Data are presented as median with interquartile range. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, and ****: $P < 0.0001$. G: Grade, S: Stage, EC: Endometrial cancer, RHEX: Regulator of hemoglobinization and erythroid cell expansion, IKBKE: Inhibitor of nuclear factor kappa-B kinase subunit epsilon

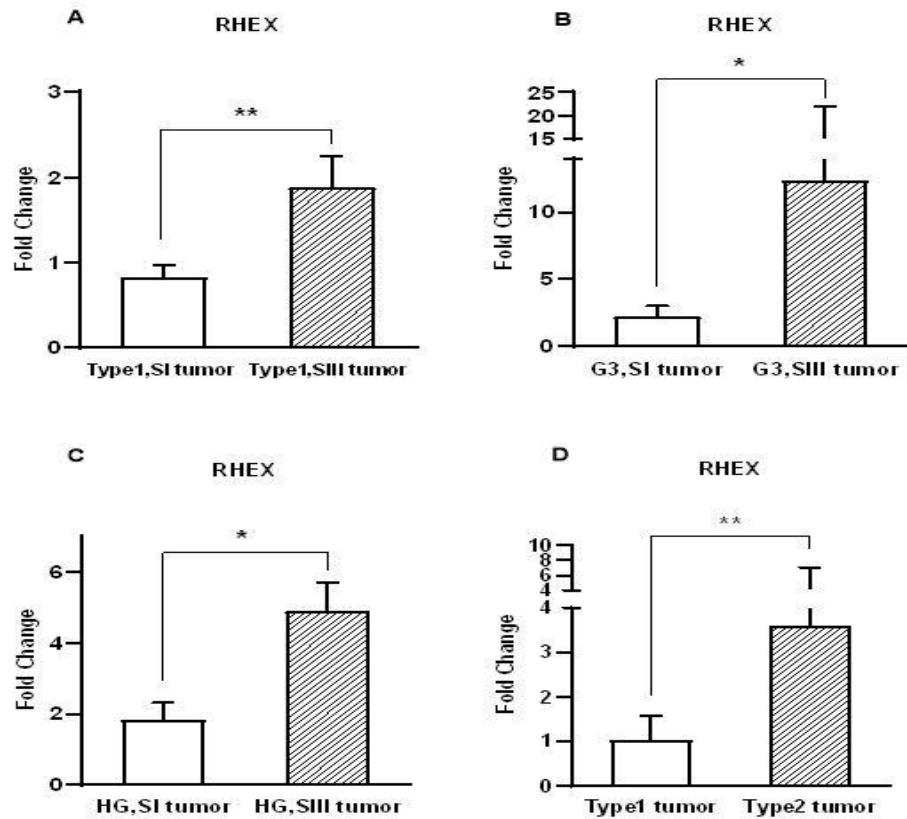


Figure 3. Comparative evaluation of RHEX expression with respect to EC clinical features. Sample size for each group in 3A are; n= 21 for type1, SI tumor, n= 5 for type1, SIII tumor. Sample size for each group in 3B are; n= 8 for G3, SI tumor, n= 2 for G3, SIII tumor. Sample size for each group in 3C are; n= 5 for HG, SI tumor, n= 4 for HG, SIII tumor. Sample size for each group in 3D are; n= 28 for type1 tumor, n= 21 for type2 tumor. Data are presented as mean with SEM or median with 95% CI. *: $P < 0.05$, and **: $P < 0.01$, G: Grade, S: Stage, EC: Endometrial cancer, CI: Confidence interval, RHEX: Regulator of hemoglobinization and erythroid cell expansion, IKBKE: Inhibitor of nuclear factor kappa-B kinase subunit epsilon

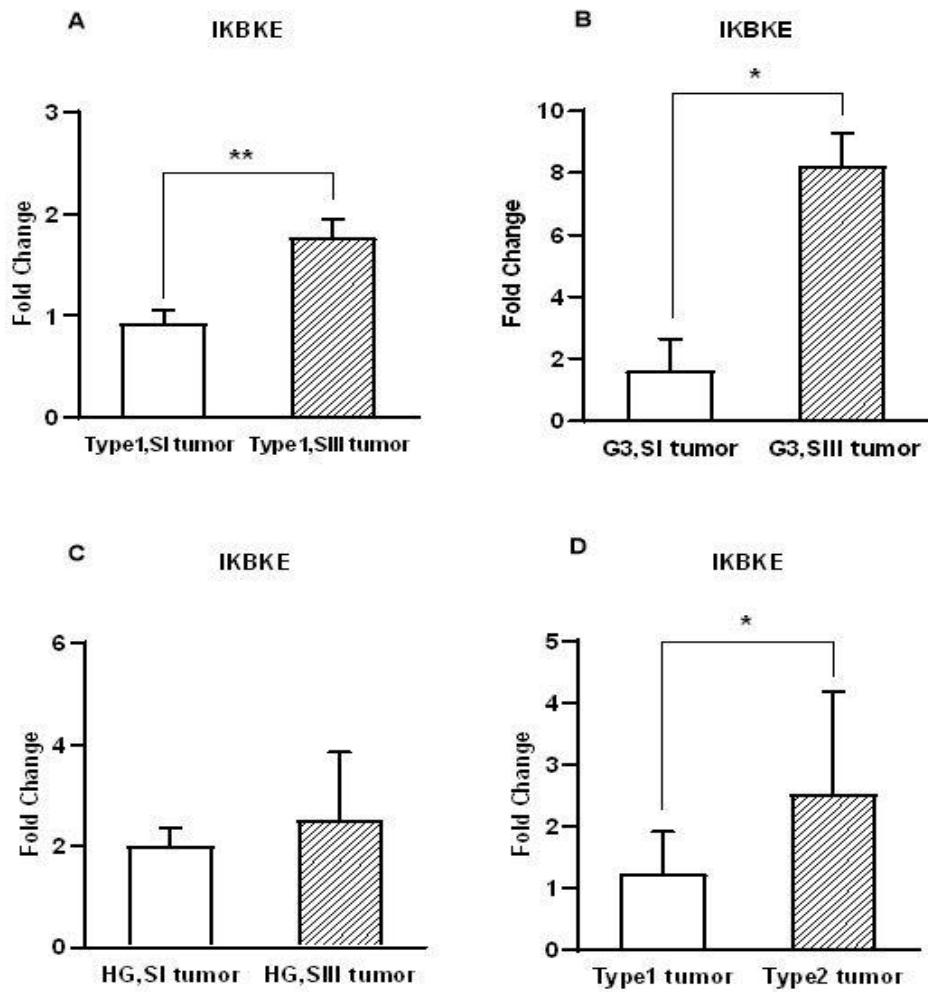


Figure 4. Comparative evaluation of IKBKE expression with respect to EC clinical features. Sample size for each group in 4A are; n= 18 for type1, SI tumor, n= 5 for type1, SIII tumor. Sample size for each group in 4B are; n= 8 for G3, SI tumor, n= 2 for G3, SIII tumor. Sample size for each group in 4C are; n= 5 for HG, SI tumor, n= 4 for HG, SIII tumor. Sample size for each group in 4D are; n= 25 for type1 tumor, n= 23 for type2 tumor. Data are presented as mean with SEM or median with 95% CI. *: $P < 0.05$, and **: $P < 0.01$, G: Grade, S: Stage, EC: Endometrial cancer, CI: Confidence interval, RHEX: Regulator of hemoglobinization and erythroid cell expansion, IKBKE: Inhibitor of nuclear factor kappa-B kinase subunit epsilon

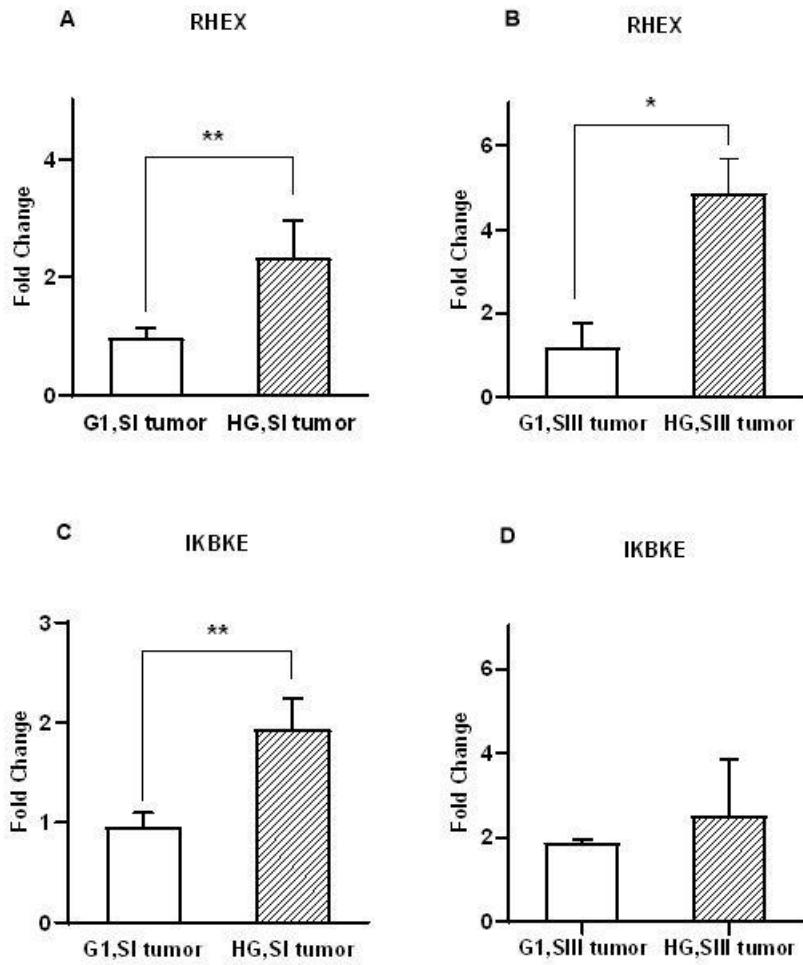


Figure 5. Comparative analysis of the clinical association of RHEX (A and B) and IKBKE (B and D). Sample size for each group in 5A are; n= 17 for G1, SI tumor, n= 6 for HG, SI tumor. Sample size for each group in 5B are; n= 4 for G1, SIII tumor, n= 3 for HG, SIII tumor. Sample size for each group in 5C are; n= 16 for G1, SI tumor, n= 6 for HG, SI tumor. Sample size for each group in 5D are; n= 4 for G1, SIII tumor, n= 3 for HG, SIII tumor. Data are presented as mean with SEM or median with 95% CI. *: $P < 0.05$, **: $P < 0.01$, G: Grade, S: Stage, CI: Confidence interval, RHEX: Regulator of hemoglobinization and erythroid cell expansion, IKBKE: Inhibitor of nuclear factor kappa-B kinase subunit epsilon