

Human epididymis protein 4 and Lewis y enhance chemotherapeutic resistance in epithelial ovarian cancer through the p38MAPK pathway

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Research

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Abstract

Background

Ovarian cancer has a high mortality rate due to difficulties in early detection and chemotherapy resistance. Human epididymal protein 4 (HE4) has been adopted as a novel serum biomarker for early ovarian cancer diagnosis, we have previously detected the presence of Lewis y antigen modifications on HE4 in ovarian cancer cell lines. In this study, we aimed to analyze the expression of HE4 and Lewis y antigen in human ovarian cancer in order to analyze their correlation with each other, as well as with the clinical pathological parameters of ovarian cancer patients.

Methods

We first used immunohistochemistry to detect the respective expressions of these compounds in two patient groups (chemotherapy-resistant and -sensitive) containing a total of 95 patients. Then, we adopted a bioinformatic approach and used online large-sample databases (TCGA, CCLE and GTEX; Metascape, Cytoscape) to explore the potential mechanisms of action of these compounds.

Results

Our results demonstrate that high HE4 and Lewis y expressions could be used as markers for chemotherapy-resistance and poor prognosis in ovarian cancer patients. These two expressions were widely correlated in various cancer tissues and are thought to act by activating the p38 MAPK pathway and inducing VEGFA, PTGS2, EGR1, and HIF1A, thereby promoting malignant biological behavior and resistance in ovarian cancer.

Conclusions

This finding not only reveals the possible mechanism by which HE4 and Lewis y antigen affect ovarian cancer, but also identifies a four-gene signature that could be very useful in ovarian cancer detection and/or the development of new targeted therapies.

Background

Ovarian cancer is a malignant disease in the female reproductive tract with a high mortality rate; more than 75% of ovarian cancer patients are diagnosed at advanced stages, which explains the low five-year survival rate of the disease (< 45%)¹. Difficulties in early detection and development of chemotherapy resistance are the prime culprits that affect the prognosis of this disease. Therefore, overcoming both obstacles will significantly improve patient survival rates.

Human epididymal protein 4 (HE4), also known as whey acidic protein (*WFDC2*), was first discovered in the distal epithelial cells of the epididymis and named accordingly. In recent years, HE4 has stirred some interest in the ovarian cancer research field and has been adopted as a novel serum biomarker for early ovarian cancer diagnosis². The ROMA index, an ovarian cancer risk prediction model that takes into account the levels of HE4 and CA125, can be used to assess the risk of ovarian cancer in premenopausal women^{3,4}. Lewis y antigen is a tumor-associated carbohydrate antigen that is part of multiple receptor structures on cellular surfaces. Our previous *in vivo* and *in vitro* studies demonstrated that Lewis y antigen is associated with the biological behaviors of tumors, including proliferation, adhesion, and chemotherapy resistance⁵⁻⁷. Our recent study showed the presence of Lewis y antigen modifications on the HE4 structure; such modifications were detected in ovarian cancer cell lines, cell culture media, and malignant ovarian tumor tissues⁸.

Although research on the effect of HE4 on the biological behavior of ovarian cancer has been on the rise as of late, the role of HE4 drug resistance and the underlying mechanisms still remain unclear. Besides, the effect of fucosylation on the biological function of HE4 and the relationship between HE4 and Lewis y have not been comprehensively explored yet. Therefore, the present study employed immunohistochemical techniques to determine the expression of HE4 and Lewis y antigen in chemotherapy-resistant and chemotherapy-sensitive epithelial ovarian cancer tissues. We further evaluated the correlation between the expression of these proteins and their association with chemotherapy resistance and poor prognosis in ovarian cancer. Moreover, we used different bioinformatic techniques to explore the possible mechanisms of action of HE4 and Lewis y.

Methods

Specimen collection, grouping, and characteristics

Specimens were collected from 95 patients with primary epithelial ovarian cancer, confirmed by surgery and pathology, between May 2005 and July 2009 at the Department of Obstetrics and Gynecology, Shengjing Hospital of China Medical University, at least one year after respective follow-ups. Patients underwent cytoreductive surgery for either advanced- or early-ovarian cancer, with dissection of two uterine appendages and pelvic and para-aortic lymph nodes, along with appendectomy and omentectomy. Moreover, a regimen consisting of 6 to 8 courses of paclitaxel + carboplatin (PC) chemotherapy was implemented, with patients required to be regularly followed up for more than one year. None of the patients had received radiotherapy, chemotherapy, or biological treatments before surgery. The ovarian tumors were classified according to the standards established by the WHO in 2009 and were distributed as follows: 61 cases of serous cystadenocarcinoma, 8 cases of mucinous cystadenocarcinoma, 4 cases of endometrioid carcinoma, 6 cases of clear cell carcinoma, and 16 cases of poorly differentiated adenocarcinoma. Pathological differentiation was as follows: 15 cases of Grade I, 39 cases of Grade II,

and 41 cases of Grade III. Moreover, clinical staging was performed according to the 2014 FIGO classification standards; 18 cases were Stage I, 13 cases were Stage II, 62 cases were Stage III, and 2 cases were Stage IV. Furthermore, resistance to chemotherapy was evaluated according to the guidelines of the National Comprehensive Cancer Network (NCCN)⁹; the ovarian cancer patients were divided into a chemotherapy-resistant group, a partially-sensitive group (partial sensitive group), and a sensitive group. Patients who received PC-based chemotherapy for the first time and obtained clinical remission along with patients suffering from treatment-induced recurrence during or within six months of the chemotherapy period were included in the drug resistance group; patients with recurrence 6–12 months after chemotherapy were included in the partially sensitive group; while patients with no recurrence or with recurrence 12 months or more after chemotherapy were included in the sensitive group. The main clinical features of ovarian cancer recurrence include: 1) a continuous increase in CA125; 2) a mass is noted during gynecological examination; 3) a mass is observed during imaging examination; 4) ascites is evident; 5) intestinal obstruction of unknown cause. The 95 patients in this study were grouped accordingly into the following: 35 patients were included in the chemotherapy-resistant group; whereas, the remaining 60 were included in the sensitive group, including six partially-sensitive patients. The overall mean age of patients was 54.36 ± 9.41 years (range: 24 to 78 years, median age: 53.0 years); the mean age of drug-resistant patients was 56.24 ± 9.50 years (range: 34 to 76 years, median age is 56 years); while that of the patients in the sensitive group was 53.31 ± 9.27 years (range: median age of 24 to 78 years is 53 years). There was no significant difference in age between the two groups ($F = 0.104$, $P = 0.147$).

Immunohistochemistry

The ovarian tissues of each group were sliced into 5 μm -thick serial sections. The expression of HE4 and Lewis y was detected by the streptavidin-peroxidase linked (SP) immunohistochemical method per the manufacturer's instructions [12]¹⁰. Each batch of sections was compared with positive and negative controls; normal human epididymis tissue was used as HE4 positive control; human gastric cancer tissue was used as Lewis y antigen positive control; while phosphate buffered saline (PBS) was added instead of primary antibodies in both negative controls (blanks). Blank buffered photographs were replaced with phosphate buffered saline (PBS) as the primary antibody. The working concentration of rabbit anti-human HE4 antibody (Abcam, UK) was 1:50 and that of mouse anti-human Lewis y antibody (Abcam, UK) was 1:200.

Analyzing the immunohistochemical results

After immunohistochemical staining, the appearance of brownish-yellow particles in the cell membranes and cytoplasm of the tissue samples was used to determine positivity. Moreover, samples were scored according to the intensity of the obtained color; no color, light yellow, brownish-yellow, and tan, were scored as 0, 1, 2, and 3, respectively. Additionally, for each slice, the average percentage (five consecutive observations) of colored cells in the visual field of a high-power field (HPF) microscope (400x) was calculated and the corresponding samples were assigned a score as follows: 0 points if the positive cell rate was < 5%; 1 point if the rate is between 5–25%, 2 points if the rate is between 26–50%, 3 points if the rate is between 51–75%, and 4 points if the rate is between > 75%. After that, the two obtained scores were multiplied to get the final score; 0 to 2 points represented negative expression (-), 3 to 4 points reflected weak positivity (+), 5 to 8 points represented moderate positivity (++), and 9 to 12 points reflected strong positivity (+++); (-) and (+) were defined as low expression, while (++) and (+++) were defined as high expression. Two different observers performed the visual inspections to avoid any errors.

Prognostic analysis

After referring to patient hospitalization data, we performed follow-up phone calls with all the patients; the last follow-up was performed on May 2019. Next, the overall survival (OS) time was analyzed, which refers to the time from the follow-up date to the death of the patient due to disease, or the follow-up deadline.

Correlation and large-sample-database analysis

The correlation between the expression of the two compounds in ovarian cancer tissues was analyzed. Simultaneously, in order to explore the universality of this correlation, we downloaded the pan-cancer mRNA expression data (<https://tcga-data.nci.nih.gov>) and the expression data of normal tissues from the Genotype-Tissue Expression project portal (GTEx, <https://www.gtexportal.org/home/>)¹¹; moreover, cancer cell line data was also acquired from the Cancer Cell Line Encyclopedia (CCLE)¹². We then analyzed the correlation between the mRNA expression of the associated genes (*WFDC2* and *FUT1*) in R software by Pearson correlation analysis.

Analyzing the microarray expression profile of HE4 and Lewis y gene transfection

In our previous studies, we analyzed changes in the genome-wide gene expression profile of epithelial ovarian cancer cell lines after transfection of HE4 and Lewis y genes (*WFDC2* and *FUT1*) and noted changes in many differentially expressed genes (DEGs)^{13–15}. In this study, we analyzed DEG profiles, up- and down-regulation, using the Venny-based online software (<http://jvenn.toulouse.inra.fr/app/example.html>). Moreover, we used the "VennDiagram" R package (RStudio, Version 1.2.5019) to illustrate our results.

Gene Ontology (GO) and cluster network plot analyses

The overlapped genes were uploaded to the online gene annotation and analysis resource Metascape¹⁶ to perform a GO enrichment analysis; this enrichment analysis tested all genes in the human genome in the following databases: KEGG pathway, GO Biological process, Reactome gene sets, Canonical pathways, and CORUM. The inclusion criteria for this analysis included a P value < 0.01, a minimum count of 3, and an enrichment factor > 1.5 and DEGs were grouped according to their similarity; the most statistically significant term in each cluster was used to represent said cluster. Further, in order to further study the relationship between terms, a subset of enriched terms (similarity > 0.3) was selected and presented as a network graph, by connecting the edges; Cytoscape (<https://cytoscape.org>) was used to visualize the enriched terms, where each point represents an enriched term, first colored by cluster ID, and then colored according to its P value.

Pathway analysis, integration of protein-protein interaction network, and module analysis

The list of overlapped DEGs was uploaded to the KOBAS website (<http://kobas.cbi.pku.edu.cn/kobas3>) to perform the KEGG pathway analysis online; the KEGG Mapper (<https://www.kegg.jp/kegg/mapper.html>) was used to visualize specific genes in the enriched pathways. Next, the list was uploaded to the STRING database (<http://string-db.org>) in order to form a protein interaction (PPI) network analysis; STRING is an online network that strictly evaluates the direct (physical) or indirect (functional) interactions between proteins. The results were then imported, via stringApp, to Cytoscape (<https://cytoscape.org>) to visualize molecular interaction networks and biological pathways; stringApp is a Cytoscape application that can easily import STRING networks into Cytoscape; it retains the many STRING features and integrates data from associated databases. In this study, two Cytoscape plugins (MCC plugin and MCODE plugin) were used to screen hub genes and cytoHubba, a Java plugin for Cytoscape, was utilized for identifying hub objects and sub-networks from complex interaction groups; it provides 11 topological analysis methods, of which MCC (Maximal Clique Centrality) has better accuracy in predicting key protein performance¹⁷. Whereas, MCODE clusters a given network based on topology to generate highly interconnected subgraphs of molecular complexes and partial paths.

Statistical analysis

Statistical analyses were performed using SPSS software (Version 26.0, SPSS, IBM, USA). Count data was tested by χ^2 test and measurement data was tested by t test. Spearman rank correlation analysis was used to analyze the correlation between the expression of these two proteins, while Pearson correlation analysis was performed to analyze the expression of the two proteins in the large sample databases and form a gene mRNA expression correlation. Moreover, Logistic Regression was used to analyze various clinical pathological parameters and the relationship between HE4 and Lewis y expression and chemotherapy resistance. The Kaplan-Meier estimator (Log-rank test) was used to perform a survival analysis. Meanwhile, the Cox model was used to analyze the relationship between various clinical pathological parameters and the expression of HE4 and Lewis y with prognosis (Methods: Enter); "Ggplot2," "forestplot" packages were used in R software (Version 3.6.1, RStudio Version 1.2.1335); $P < 0.05$ was considered statistically significant.

Results

HE4 and Lewis y antigen expression are correlated with ovarian cancer clinicopathological parameters, especially in chemotherapy-resistant tissues

HE4 was mainly expressed in the cell membranes and cytoplasm of tissue samples but was rarely expressed in the perinuclear region. The positive expression rates of HE4 and Lewis y in the ovarian cancer chemotherapy-resistant group were 97.1% and 88.6%, respectively, significantly higher than the expression rates of 76.7% and 68.3% in the sensitive group ($P = 0.008$ and 0.026 , respectively) (Figure 1, Table 1). We further explored the highest expression rates of the two groups and found that the HE4 and Lewis y antigen rates in the chemotherapy-resistant group (80.0%, 80.0%) were significantly higher than those in the chemotherapy-sensitive group (30.0%, 30.0%), as shown in Figure 1A and Table 1 ($P < 0.001$ and < 0.001 , respectively).

After exploring the relationship between HE4 and Lewis y expression and the clinicopathological parameters, we found that HE4 expression was related to FIGO stage, differentiation, and drug resistance ($P = 0.014$, 0.048 , and 0.008 , respectively); whereas, Lewis y expression was associated with lymph node metastasis and drug resistance ($P = 0.026$ and 0.026 , respectively). In terms of high expression rate, we found that high expression of both HE4 ($P < 0.001$ and 0.001 , respectively) and Lewis y ($P < 0.001$ and < 0.001 , respectively) were correlated with lymph node metastasis and drug resistance (Table 1).

HE4 and Lewis y antigen are independent risk factors of drug resistance in ovarian cancer

Age, FIGO stage, pathological grading and type, and HE4 and Lewis y antigen expression of ovarian cancer patients were included in the multivariate logistic regression analysis. As shown in Table 2, the FIGO stage, degree of differentiation, lymph node metastasis, and HE4 and Lewis y expression are independent risk factors for drug resistance (HR=2.882, 2.241, 6.118, 3.421, and 3.836; $P = 0.003$, 0.015 , < 0.001 , < 0.001 , and < 0.001 , respectively); as for the univariate analysis, factors with a P value less than 0.05 were included in the multivariate analysis. Our results suggest that poor differentiation and positive HE4 and Lewis y antigen expression are independent risk factors for chemotherapy resistance in ovarian cancer (HR=2.953, 2.972, and 3.254, respectively; $P = 0.022$, 0.004 , and 0.004 , respectively) (Table 2).

HE4 expression is an independent risk factor that affects prognosis in ovarian cancer

To further analyze the overall survival (OS) rate, we followed-up with the two ovarian cancer patient groups and saw that, among the 95 patients, 56 patients died, 35 survived, and 4 failed to follow-up; the overall median survival time of the patients was 48.00 ± 1.55 months (95% CI, 44.97-51.03 months).

Kaplan-Meier (KM) survival analysis showed that the median survival time of patients older than 60 years (33 ± 4.14 months) and those with FIGO III-IV (45 ± 3.53 months), chemotherapy-resistant (30 ± 3.86 months), and HE4-positive (46 ± 2.86 months) tumors was significantly lower than the median survival time of patients ≤ 60 years (50 ± 3.80 months) and those having FIGO stage I-II (56 ± 5.38 months), chemotherapy-sensitive (56 ± 4.09 months), and HE4-negative tumors (67 ± 0.00 months), respectively ($P = 0.010$, 0.042 , < 0.001 , and 0.007 , respectively) (Table 3 and Figure 1B).

Although the median survival time of patients with positive Lewis y expression was lower than that of patients with negative expression, the difference was not statistically significant (45.00 ± 2.76 months vs. 54.00 ± 5.18 months; $P = 0.016$) (Table 3); moreover, there was no correlation between pathological differentiation, pathological type, lymph node metastasis and patient OS ($P > 0.05$) (Table 3).

Further, only factors with a P value less than 0.05 after KM analysis were included in the Cox multivariate model analysis; results showed that HE4 positive expression and chemotherapy resistance were independent risk factors related to the prognosis of ovarian cancer patients (HR = 2.894 and 3.512, respectively; $P < 0.001$ and 0.045 , respectively) (Table 4).

Correlation between HE4 and Lewis y at protein and mRNA levels

Among the 95 ovarian cancer cases, 4 were negative for both HE4 and Lewis y antigen, while 29 cases were positive for both simultaneously as follows: 13 cases+, 11 cases++, 5 cases +++ (Figure 2A); the correlation between the expression of these two proteins was further confirmed by Spearman correlation ($R = 0.327$, $P = 0.001$).

To explore the general significance of the correlation between HE4 and Lewis y, we investigated the correlation between the mRNA expression of their respective genes (*WFDC2*, *FUT1*) using the large sample databases TCGA, CCLE, and GTEx. As shown Figure 2B, the correlation was confirmed via the TCGA pan-cancer ($R=0.351$, $P<0.001$) and the TCGA ovarian cancer tissue ($R=0.268$, $P<0.001$) databases; moreover, we noted similar results using the CCLE pan-cancer ($R=0.450$, $P<0.001$) and the CCLE ovarian cancer cell line ($R=0.478$, $P<0.001$) databases (Figure 2C), along with the GTEx database ($R= 0.410$, $P<0.001$) (Figure 2D). Therefore, the expression of HE4 and Lewis y were shown to have a positive correlation via all the aforementioned databases. This correlation has universal significance in various types of cancer tissues and cells, especially in ovarian cancer cells and tissues, and the potential interaction mechanism between them deserves further exploration.

Identification of hub genes by gene expression and enrichment analyses

During our previous analysis of gene expression sequences in ovarian cancer cells, we found several genes that were affected by HE4 and Lewis y expression. In the case of HE4, 1,113 genes were up-regulated with the increase in HE4 expression while 1,531 genes were down-regulated with its down-regulation¹⁴. As for Lewis y, 177 genes were up-regulated while 124 were down-regulated¹³. Here, we found through Venny analysis, 23 up-regulated and 9 down-regulated genes, that were commonly affected by the two proteins. The overlapped genes were then uploaded to Metascape for an enrichment analysis; we found extensive interactions between these overlapped genes (Figure 3B). Moreover, GO enrichment analysis revealed that the two proteins were involved in the positive regulation of different elements, including the p38MAPK cascade, cell cycle, and tumor necrosis factor, in addition to biological behaviors such as response and autophagy (Figure 3C, D).

Moreover, pathway analysis suggested that the overlapped DEGs are involved in pathways related to MAPK signaling, HIF-1 signaling, TNF signaling, and cell cycle regulation; the involved genes and associated P values are shown in Figure 4A. Interestingly, the MAPK pathway was the most significantly-enriched pathway and had the highest number of involved genes (Figure 4B), which suggests this pathway plays a big role in the malignant biological behavior of ovarian cancer.

Furthermore, Cytoscape analysis was performed to identify the hub genes among the overlapped genes. We used the MCC algorithm in the cytoHubba plug-in to analyze the PPI and ranked the genes according to their obtained scores; the top 10 genes with the highest scores were considered hub genes (Figure 4C). The MCODE plug-in was used to further analyze the PPI; from the module analysis, we obtained a module containing four nodes and six edges. The obtained module was composed of four up-regulated genes, which were among the 10 hub genes obtained by cytoHubba analysis (Figure 4D). Therefore, we confirmed that *VEGFA*, *EGR1*, *HIF1A*, and *PTGS2* were hub genes that play a core role in the network diagram.

Discussion

Ovarian cancer is a common malignant tumor with the third-highest morbidity rate and the highest mortality rate among female reproductive system cancers; what is even more unsettling is the low five-year survival rate, which does not exceed 45%. Further, more than 75% of ovarian cancer patients are discovered at advanced stages of the disease¹, which is one of the main reasons behind the poor prognosis of ovarian cancer, along with chemotherapy resistance. Therefore, developing efficient methods for early screening and drug resistance prediction is crucial; of late, many studies have been aimed at exploring the mechanism of drug resistance in ovarian cancer and developing treatment strategies to overcome this menacing phenomenon.

Human epididymal protein 4 (HE4), also known as *WFDC2*, is a member of the Whey acidic protein family that was first discovered in human distal epididymal epithelial cells. In 2008, the US Food and Drug Administration (FDA) identified HE4 as a serum marker for monitoring recurrence and progression in epithelial ovarian cancer (EOC) patients. Thereafter, HE4 started gradually stirring more and more attention; several studies have demonstrated the usefulness of HE4 in the early diagnosis of EOC and the identification of pelvic masses, especially when used in combination with the risk of ovarian malignancy algorithm (ROMA); moreover, HE4 exhibited higher sensitivity and specificity than its predecessors, including CA125^{3,18}. In terms of function and mechanism, HE4 can promote the proliferation, invasion, metastasis, and drug resistance of ovarian cancer cells^{10,19,20}. This protein also forms the HE4-ANXA2 complex by combining with membrane-associated protein A2 (ANXA2) and promotes malignant behaviors such as cell proliferation, adhesion, invasion, and metastasis through MAPK and focal adhesion signaling pathways^{20,21}. Notably, HE4 knockdown can inhibit the invasive, cell growth, and tumor progression of ovarian cancer by inhibiting the JAK / STAT3 pathway²².

Recent studies have found that HE4 is associated with chemotherapy resistance in tumor cells²³⁻²⁵; HE4 could induce drug resistance in ovarian cancer cells by activating the AKT and ERK pathways and was associated with poor prognosis in ovarian cancer patients²⁴; HE4 could also interact with EGFR, IGFR, and, the transcription factor, HIF1 α , thereby promoting ovarian cancer cell invasiveness and drug resistance and, consequently, worsening the prognosis of ovarian patients²⁶; the over expression of HE4 was also noted to promote cisplatin- and paclitaxel-resistance in ovarian cancer, where the down-regulation of HE4 reversed resistance to many drugs.

In this study, we used paraffin sections of 95 ovarian cancer patient tissues and evaluated the predictive value of HE4 in chemotherapy-resistance by immunohistochemistry. We found that HE4 positive expression was significantly higher in tissues from drug-resistant patients than in tissues from the sensitive group. Notably, HE4 was an independent risk factor for chemotherapy resistance whose high expression significantly lowered patient survival; interestingly, HE4 expression and chemotherapy resistance were both independent risk factors for patient prognosis. These results indicate that HE4 may be

a predictor of chemotherapy resistance and poor prognosis in ovarian cancer and may provide a reference for future studies aiming to further explore the role of HE4 in chemotherapy-resistant ovarian cancer.

Sugar complexes are an important part of the cell membrane; exposed sugar chains are involved in important processes such as cell growth and differentiation²⁷. Lewis y antigen, a tumor-associated carbohydrate antigen (TACA), is a double fucosylated oligosaccharide that is over-expressed in epithelial malignancies including ovarian, pancreatic, prostate, rectal, and non-small cell lung cancers. Because the Lewis y antigen is located on the cell surface, it can modify the structure of cell surface membrane proteins, thereby affecting cellular functions. Previous studies by our group have proven that Lewis y is an important component of TGF- β , EGFR, ANXA2, CD44, CD147, MUC1 and other cell membrane proteins²⁸⁻³⁰; moreover, Lewis y glycosylation further promoted malignant biological behaviors in ovarian cancer such as cell proliferation, invasion, metastasis, drug resistance, autophagy, and apoptosis^{6,10,31}. Furthermore, we have also identified Lewis y antigen modifications on the HE4 structure⁸. In the current study, we found that Lewis y antigen is an independent risk factor of chemotherapy resistance whose expression in the resistant group was significantly higher than that in the sensitive group, similar to what we saw with HE4; the two proteins, HE4 and Lewis y, were positively correlated. Next, after analyzing the correlation between the mRNA expression of HE4 and Lewis y using large-sample databases (TCGA, CCLE, GTEx), we confirmed that these compounds were positively correlated at the mRNA level. These findings provide some evidence regarding the relationship between HE4 and Lewis y; however, further pre-clinical studies are needed to prove the potential correlation between HE4 and Lewis y. Nonetheless, HE4 and Lewis y play an important role in chemotherapy-resistant ovarian cancer and may have potential applications in clinical practice.

In order to further explore the potential underlying mechanisms behind the effect of HE4 and Lewis y on the biological behavior of ovarian cancer, we conducted bioinformatic analyses of these two indicators. Although we have previously explored gene expression alterations in ovarian cancer cell lines after transfection with *WFDC2* and *FUT1*, we did not explore how the transfected genes altered cellular genetic profiles; however, commonly-altered genes can provide essential information regarding the mechanism by which HE4 and Lewis y affect the malignant biological behavior of ovarian cancer. Therefore, we performed Venny analysis and found 32 overlapped genes, 23 of which were up-regulated while nine were down-regulated. We went further and analyzed these genes using Metascape in order to identify the most commonly-enriched GO terms; among the different pathways highlighted, several were related to oncogenetic processes, such as: "positive regulation of p38MAPK cascade," "positive regulation of cell cycle," "response to tumor necrosis factor," and "autophagy." These results confirmed that HE4 and Lewis y participate in tumorigenesis and drug resistance in epithelial ovarian carcinomas. In addition, through online pathway analysis, we found that these overlapped DEGs are involved in important signaling pathways such as MAPK, VEGF, HIF-1, and TNF, among which MAPK was the most significantly-activated pathway.

The MAPK signaling pathway regulates almost all cellular processes, including gene expression, cell cycle, cell survival, cell death, and cell movement³². There are multiple mechanisms underlying HE4-mediated chemoresistance, including dysregulation of MAPK signaling (inhibition of *EGR1* and p38) and changes in the level or stability of tubulin; notably, recombinant HE4 up-regulates the expression levels of α -tubulin, β -tubulin, and microtubule-associated protein tau (MAPT)³³. Moreover, TGF- β 1 regulates the expression of Lewis y by activating the MAPK/c-Fos pathway, thereby further affecting the proliferation, invasion, and prognosis of malignant epithelial tumors³⁴. In malignant melanoma, siRNA inhibition of Lewis y expression can prevent the activation of the EGFR/MAPK pathway and inhibit cell proliferation³⁵. The above-mentioned studies support our findings regarding the relationship of HE4 and Lewis y and the MAPK signaling pathway. Therefore, we speculate that Lewis y modified HE4 promotes the chemotherapy-resistant biological behaviors of ovarian cancer through the p38MAPK signaling pathway. We then constructed a PPI network, using the MCC and MCODE algorithms, and identified four hub genes among the overlapped genes (*VEGFA*, *EGR1*, *HIF1A*, and *PTGS2*); analyzing the known functions of these genes can provide ideas and breakthroughs for prospective studies on HE4 and Lewis y.

VEGF-mediated MAPK/ERK and PI3K/AKT signaling pathways play an important role in regulating the growth, proliferation, migration, and angiogenesis of tumor cells and vascular endothelial cells³⁶; moreover, *VEGFA* has a known role in regulating tumor cell chemotherapeutic drug sensitivity. In addition to its important function in reproductive health^{37,38}, *EGR1* is bidirectional regulator of pathological process in malignant tumors; in breast cancer and lung cancer tissues, the *EGR1* down-regulation plays a tumor-suppressive role and inhibits the proliferation and migration of tumor cells^{39,40}; whereas, in colorectal and prostate cancers, *EGR1* plays an oncogenic role where its significant up-regulation promotes the proliferation and migration of tumors^{41,42}. Recent studies have found that *EGR1* is associated with drug resistance⁴³ and metastasis⁴⁴ in breast cancer, as well as autophagy⁴⁵. Numerous studies have suggested a correlation between *HIF1a* and drug resistance in tumors, such as ovarian cancer^{46,47}. Besides, HIF1 α has been also shown to affect drug resistance in tumors by regulating apoptosis and autophagy^{47,48}. *PTGS2*, also known as *COX-2*, plays an important role in inflammation and cancer development; its expression plays a vital role during various stages of a large number of cancer types⁴⁹⁻⁵¹. Recent studies have found that *COX-2* is associated with drug resistance in breast cancer⁵⁰ and bladder cancer⁵¹. Studies on a variety of malignant tumors have shown that autophagy is related to tumor drug resistance⁵²⁻⁵⁴; however, this effect is bidirectional. Moreover, autophagy can also promote the death of drug-resistant cells^{55,56}. Liang F et al. showed that in ovarian cancer, MAPK and PI3K/AKT-mediated pathways regulate autophagy, which in turn affects the metastasis of ovarian cancer cells and cisplatin resistance⁵³.

Conclusions

In conclusion, we speculate that the modification of Lewis y residues on HE4 can promote chemotherapy resistance in ovarian cancer by activating p38MAPK signaling. Furthermore, we believe that the four hub genes *VEGFA*, *EGR1*, *PTGS2*, and *HIF1A* play an important role in the pathogenesis of ovarian cancer and may open the door for better screening methods and/or therapeutic targets after further investigations.

Abbreviations

HE4
Human epididymal protein 4;
TGCA
The Cancer Genome Atlas;
CCLE
the Cancer Cell Line Encyclopedia;
GETx
the Genotype-Tissue Expression project;
MAPK
mitogen-activated protein kinases;
OS
overall survival;
HR
Hazardratio;
DEGs
differentially expressed genes;
GO
Gene Ontology;
KEGG
Kyoto Encyclopedia of Genes and Genomes;
PPI
Protein-protein interaction.

Declarations

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Not applicable

Authors' contributions:

Jian Gao and Liancheng Zhu contributed equally to this work.

JG and LZ conceived, designed the study, wrote and drafted the manuscript. JG and HZ performed the experiment in immunohistochemical staining and data analysis. LZ performed the bioinformatics analysis. SJ, DL and SG interpreted the results and reviewed the manuscript. BL reviewed the manuscript and support the funding. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The present study was approval from the Ethical Committee of Shengjing Hospital affiliated to China Medical University (number of approval: 2010PS84K).

Consent for publication

Not applicable.

Competing interests

All authors declare no conflict of interest in this study.

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Tables

Table 1 Comparison of clinicopathological parameters of HE4 and Lewis y expression in patients with chemotherapy-resistant and -sensitive ovarian cancer.

	Cases					HE4		Lewis y antigen									
	95	-	+	++	+++	Positive (%)	P	High (%)	P	-	+	++	+++	Positive (%)	P	High (%)	P
Age							0.115		0.518						0.414		0.625
≤60	68	13	20	21	14	80.9		51.8		18	16	28	6	73.5		50.0	
>60	27	2	14	5	6	92.6		40.7		5	10	10	2	81.5		44.4	
FIGO							0.014		0.187						0.800		0.379
I-II	31	9	10	7	5	71.0		38.7		8	10	12	1	74.2		41.9	
III-IV	64	6	24	19	15	90.6		53.1		15	16	26	7	76.6		51.6	
Differentiation							0.048		0.634						0.654		0.634
Well-moderate	54	12	17	14	11	77.8		46.3		14	15	22	3	74.1		46.3	
poor	41	3	17	12	9	92.7		51.2		9	11	16	5	78.0		51.2	
Pathological subtypes							0.711		0.291						0.701		0.138
Serous	61	9	20	17	15	85.2		52.5		14	14	26	7	77.0		54.1	
Other types	34	6	14	9	5	82.4		41.2		9	12	12	1	73.5		38.2	
Lymphatic metastasis							0.141		<0.001						0.026		<0.001
Yes	60	12	30	13	5	80.0		30.0		19	22	16	3	68.3		31.7	
No	35	3	4	13	15	91.4		80.0		4	4	22	5	88.6		77.1	
Drug reaction							0.008		<0.001						0.026		<0.001
Resistance	35	1	6	14	14	97.1		80.0		4	3	20	8	88.6		80.0	
Sensitive	60	14	28	12	6	76.7		30.0		19	23	18	0	68.3		30.0	

Table 2. Multiple logistic regression analysis of the relationship between clinicopathological parameters, immunohistochemical expression, and chemotherapy resistance.

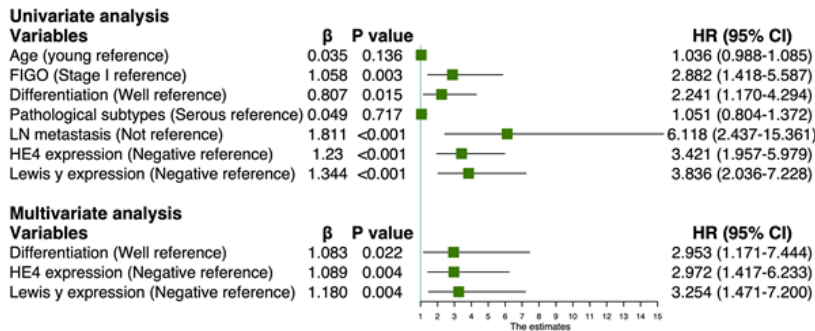
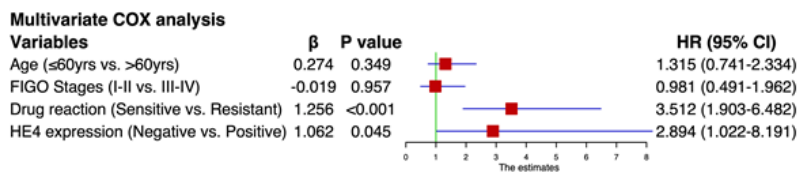


Table 3. Kaplan-Meier analysis of the relationship between various clinicopathological parameters, HE4 and Lewis y expression, and prognosis.

	N	median±SD	χ^2	P
Age			6.649	0.010
≤60	68	50.00±3.80		
>60	27	33.00±4.14		
FIGO			4.122	0.042
I-II	31	56.00±5.38		
III-IV	64	45.00±3.53		
Differentiation			0.067	0.795
Well-moderate	54	48.00±4.00		
poor	41	47.00±1.81		
Pathological subtypes			0.130	0.719
Serous	61	48.00±4.17		
Other types	34	47.00±2.13		
Lymphatic metastasis			1.837	0.175
Yes	60	48.00±1.97		
No	35	40.00±9.73		
Drug reaction			27.997	<0.001
Resistance	35	30.00±3.86		
Sensitive	60	56.00±4.09		
HE4 expression			7.174	0.007
Negative	15	67.00±0.00		
Positive	80	46.00±2.86		
Lewis y expression			2.476	0.116
Negative	23	54.00±5.18		
Positive	72	45.00±2.76		

Table4. COX regression analysis of prognosis.



Figures

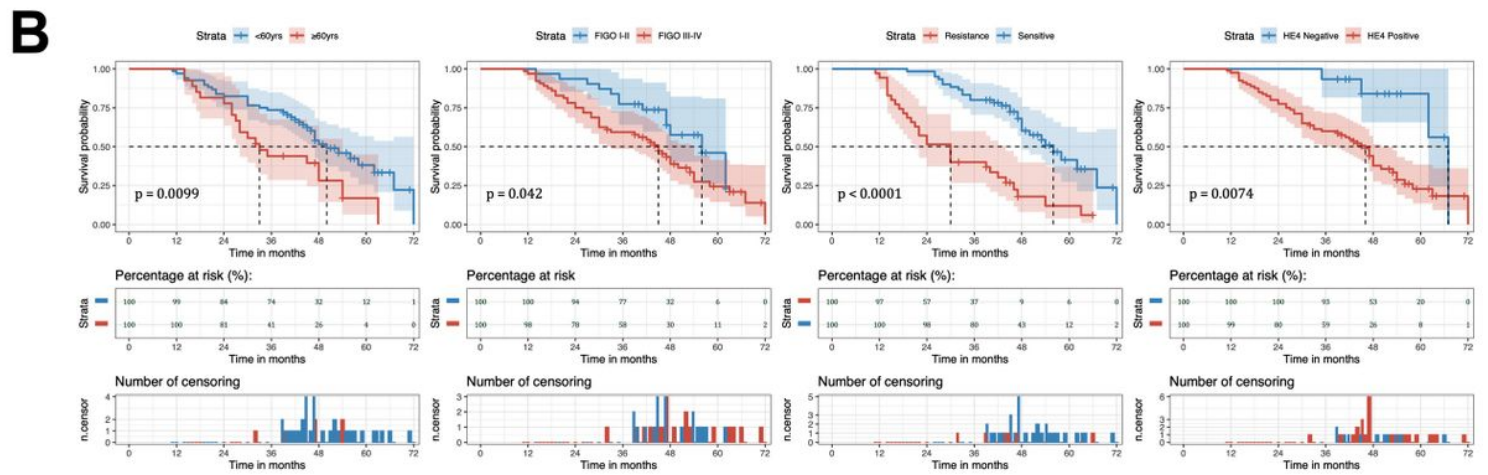
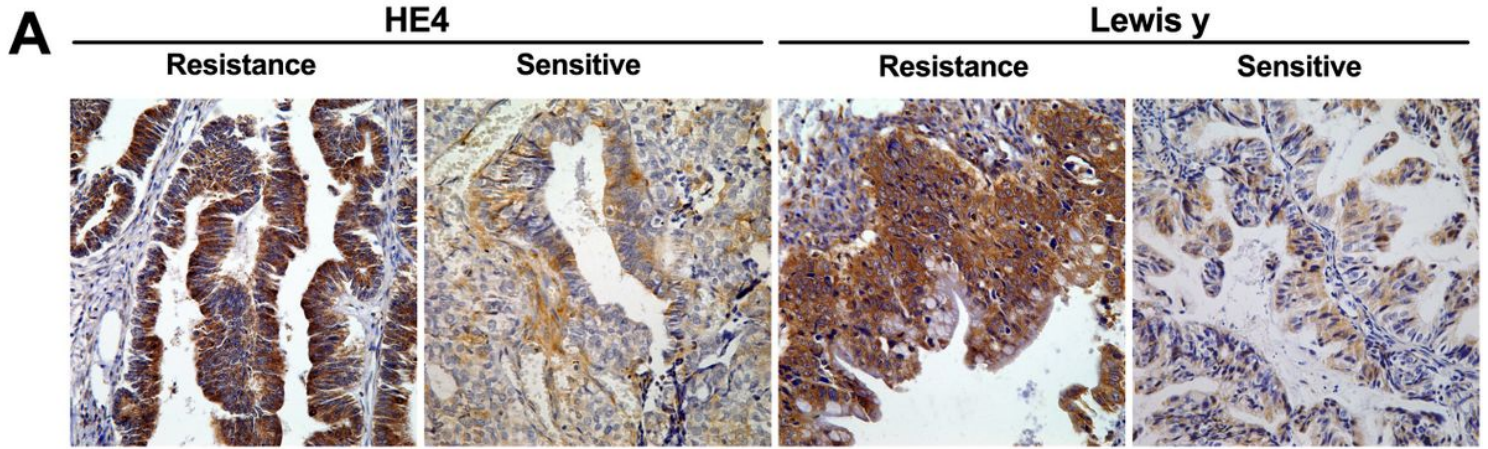


Figure 1

Expression of HE4 and Lewis y in resistant and sensitive ovarian cancer groups and their relationship with prognosis. A. Photographs were acquired by optical microscopy (40x). B. The association between age >60 years, advanced FIGO stage, chemotherapy resistance, and positive expression of HE4 and poor OS in patients by Kaplan-Meier analysis ($P < 0.05$)

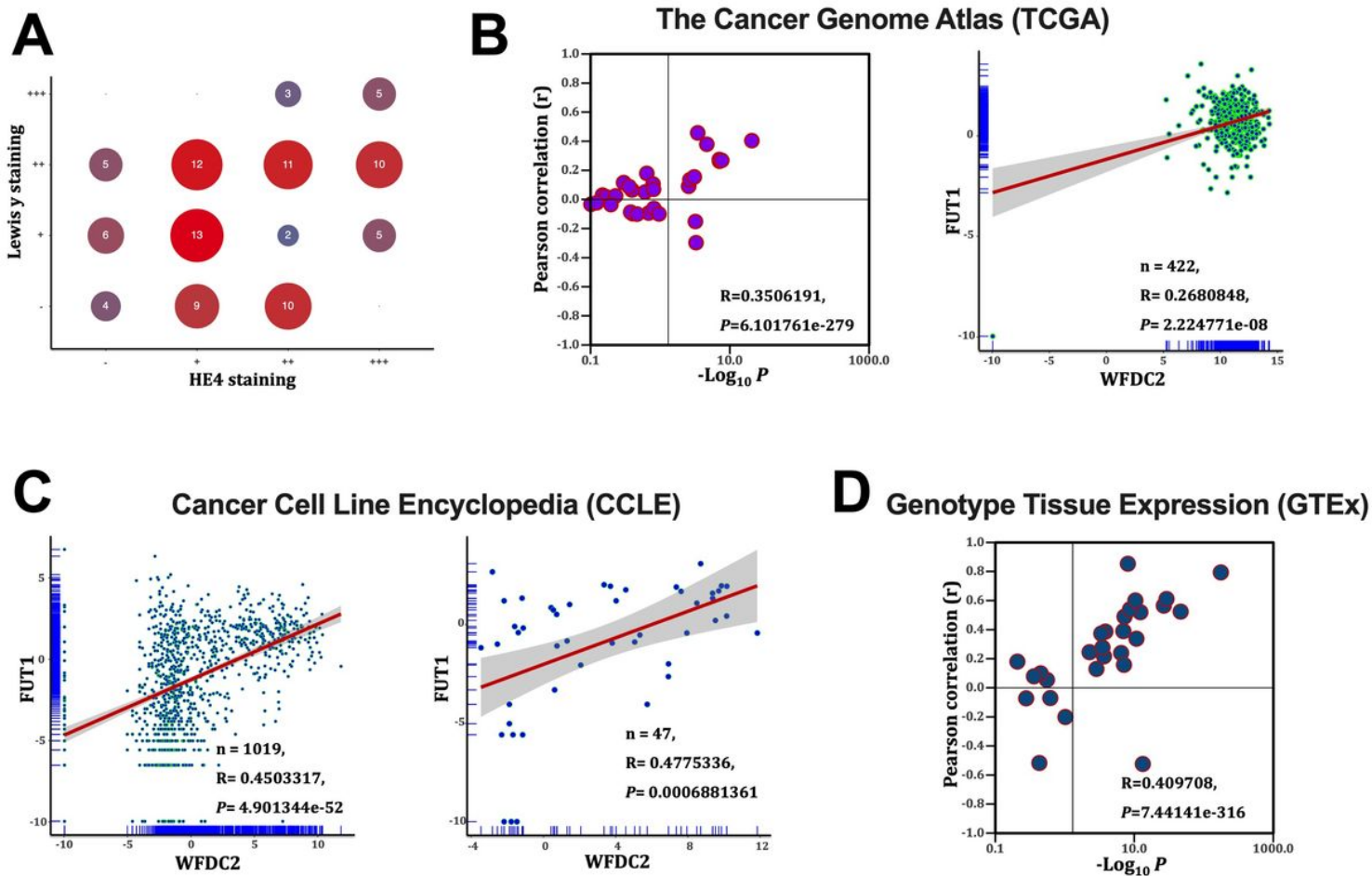


Figure 2

Correlation analysis of HE4 and Lewis y in ovarian cancer tissues at both protein and mRNA levels by TCGA, CCLE, and GTEx large-sample databases.

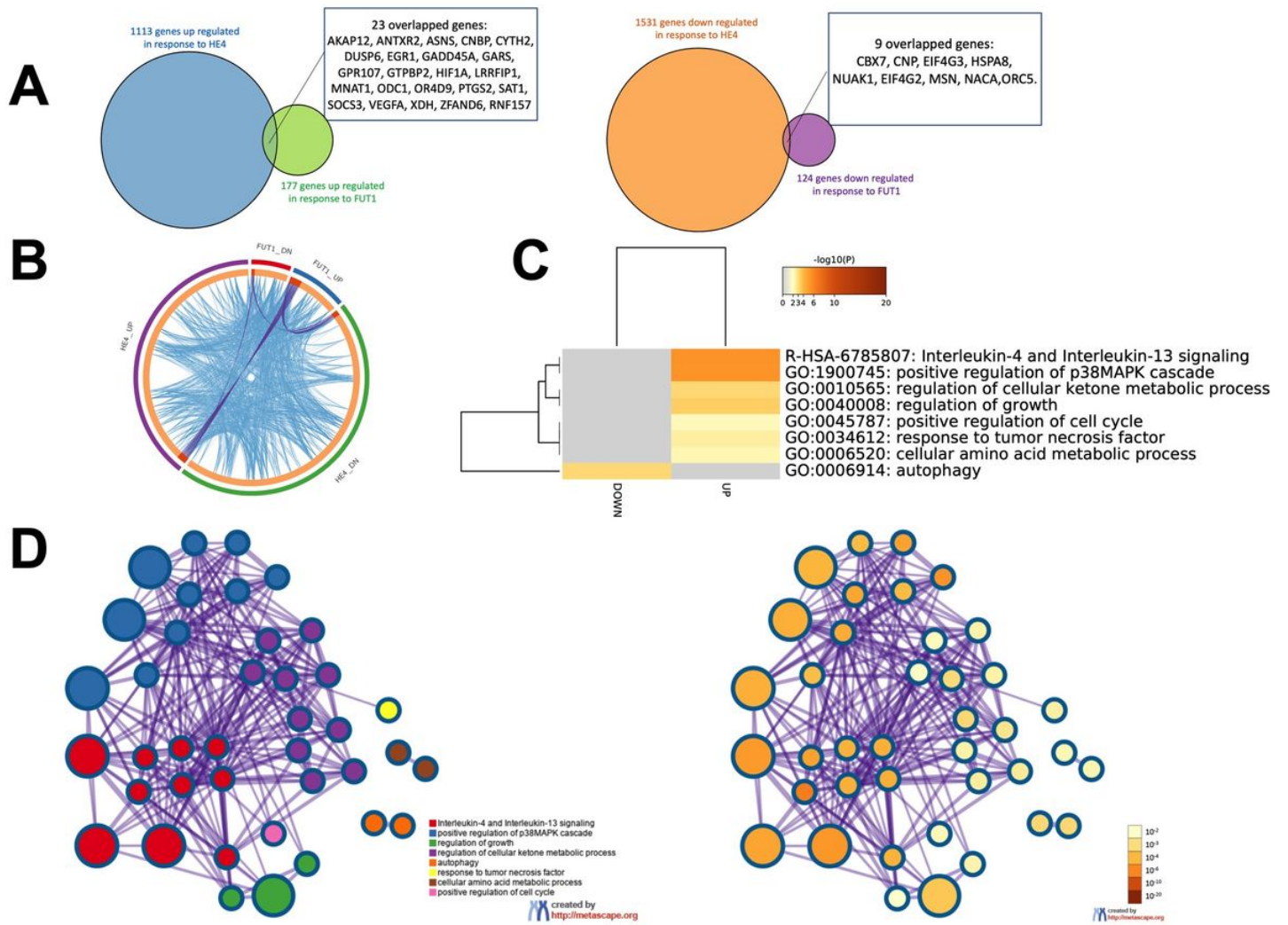


Figure 3

Venny analysis and GO enrichment analysis. Through Venny analysis, we found 23 overlapped genes that increased together with HE4 and Lewis y, and 124 overlapped genes that decreased together with HE4 and Lewis y (panel A). We performed an online Metascape analysis of these differentially expressed gene lists and noted the presence of an interaction between them (panel B); GO analysis suggested that these differentially expressed genes are involved in important pathways including MAPK, cell cycle, etc. (panel C). The obtained terms and their respective P values were used to construct a network graph (panel D), where the left side is sorted by enriched terms and the right side is sorted by P value.

A

#Term	P-Value	Corrected P-Value	Genes enriched in
MAPK signaling pathway	0.0001339	0.0127847	GADD45A, DUSP6, VEGFA, HSPA8
VEGF signaling pathway	0.0013219	0.0229533	PTGS2, VEGFA
HIF-1 signaling pathway	0.0042908	0.0435359	HIF1A, VEGFA
TNF signaling pathway	0.0045195	0.0437078	PTGS2, SOCS3
Cell cycle	0.0054896	0.0442746	ORC5, GADD45A

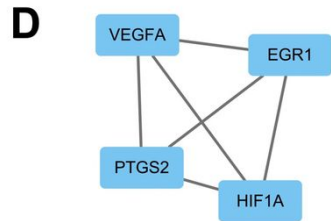
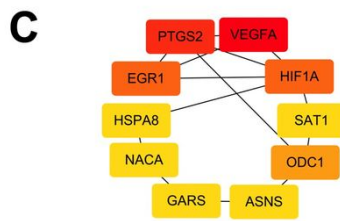
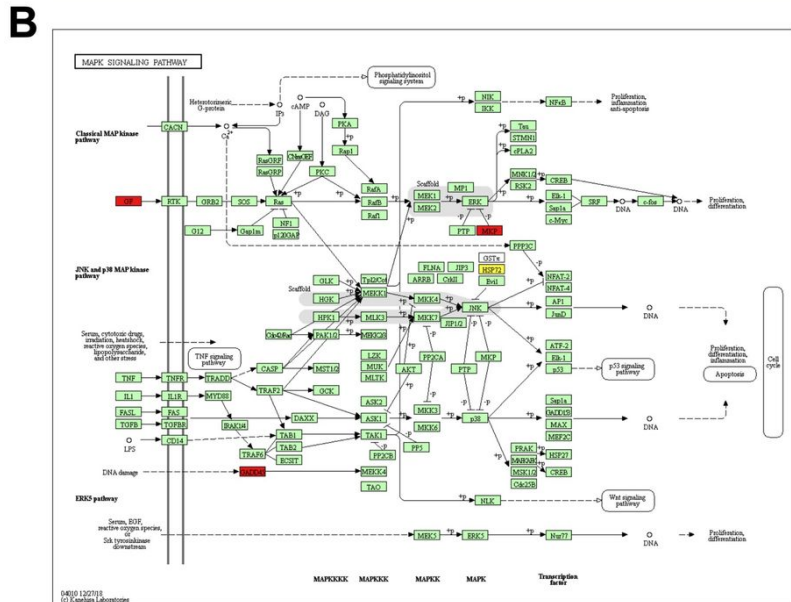


Figure 4

KEGG pathway analysis of the overlapped differentially expressed genes related to HE4 and Lewis y and confirmation of hub genes. A. The KEGG pathways associated with the overlapped differentially expressed genes were examined using an online database; the top 5 enriched pathways related to cancer were listed. B. Example map of the MAPK pathway, where genes marked in red are up-regulated and those marked in yellow are down-regulated. C. The top 10 genes with the highest scores found through the MCC algorithm in the cytoHubba plug-in. The higher the intensity of the reddish color in the figure the higher the score of the gene; that is, the closer the gene is to the other genes in the protein interaction network (has a core gene role). D. The MCODE plug-in was used to analyze the PPI module and identify four hub genes, including VEGFA, PTGS2, EGR1, HIF1A.