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Original Article

SEC24D gene as a biomarker in human cancers and its association with CD8+ T cell immune cell infiltration

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Abstract: Objective: The SEC24D (SEC24 Homolog D, COPII Coat Complex Component) gene belongs to the SEC24 subfamily of genes. The protein encoded by this gene, along with its other binding partners, mediates the transport of newly-synthesized proteins from the endoplasmic reticulum to the Golgi apparatus. Methods: A pan-cancer analysis of this gene, as well as its diagnostic and prognostic implications, are lacking in the medical literature. First, we analyzed SEC24D gene expression, its prognostic effect, promoter methylation level, genetic alteration landscape, pathways, CD8+ T immune cell infiltration, and gene-drug network in various types of cancer through various online databases and bioinformatic tools. Then, we performed the expression and methylation validation analysis of the SEC24D gene on cell lines using RNA sequencing (RNA-seq) and targeted bisulfite sequencing (bisulfite-seq) techniques. Results: Bioinformatic analysis showed that the SEC24D gene was overexpressed in metastasis across Kidney Renal Clear Cell Carcinoma (KIRC), Lung Squamous Cell Carcinoma (LUSC), and Stomach Adenocarcinoma (STAD) patients and was a prognostic risk factor. Then, using RNA sequencing and targeted bisulfite sequencing analysis, it was validated in cell lines that SEC24D was overexpressed and hypomethylated in KIRC patients. Mutational analysis revealed that SEC24D was mutated less frequently in KIRC, LUSC, and STAD patients. It was further observed that CD8+ T cell infiltration levels were increased in SEC24D-overexpressed KIRC, LUSC, and STAD samples. Pathway enrichment analysis of SEC24D-associated genes revealed their participation in two important pathways. Moreover, we suggested a few valuable drugs for treating KIRC, LUSC, and STAD patients with respect to overexpressed SEC24D. Conclusion: This is the first pan-cancer study that details the oncogenic roles of SEC24D among different cancers.

Keywords: SEC24D, CNVs, OS, diagnostic

Introduction

Cancer is a group of 200 diseases that affect millions around the globe each year [1]. Older age, cancer-related family history, tobacco consumption, obesity, drinking alcohol, and different infectious agents such as viruses, bacteria, and parasites predispose to cancer [2-8]. Some viruses, such as Epstein-Barr virus (EBV) and Human papillomavirus (HPV) can disrupt normal cell signaling and force cells to grow abnormally [9, 10]. Moreover, some infections, such as those caused by Helicobacter pylori (H. pylori) and Chlamydia trachomatis, can impair the immune system, making the body unable to fight against cancer [11, 12].

Recently, the incidence of cancer has risen and it is a major health concern [13]. If signaling pathways associated with oncogensis are elucidated, they can be used as a diagnostic and prognostic biomarker for cancer patients. Therefore, it is important to continue studying the molecular mechanisms of cancer development in order to identify biomarkers for diagnosis and prognosis of different cancers.

The SEC24D (SEC24 Homolog D, COPII Coat Complex Component) is a SEC24 gene subfamily member [14]. The protein encoded by SEC24D is an essential component of the COPII (Coat Protein Complex II) which transports newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus [15]. It was observed in earlier investigations that when SEC24D was knocked out in zebrafish, the COPII was not formed and protein transport to the Golgi apparatus was halted, ultimately resulting in naive protein accumulation inside the endoplasmic reticulum (ER) [16]. Furthermore, a few recent studies have also associated SEC24D genetic mutations and expression variations with osteogenesis imperfecta and breast cancer [17, 18]. However, the implication of SEC24D in different cancer subtypes is not understood.

The emergence of next-generation sequencing (NGS) technology and the online availability of The Cancer Genome Atlas (TCGA) datasets has made multi-omics data public to researchers. Therefore, this is an ideal scenario for conducting a pan-cancer analysis to unveil novel biomarkers in cancer patients. In this study, we performed extensive *in silico* research to explore the role of SEC24D expression in a pan-cancer analysis among 24 different human cancers. Our study is the first pan-cancer study, that analyzed expression profiling and various other aspects (promoter methylation, genetic alteration, copy number variations (CNVs), CD8+ T immune cell infiltration, relevant pathways, and chemotherapeutic drug analysis) of the SEC24D gene. Ultimately, this will provide a novel insight into the roles of SEC24D.

Materials and methods

UALCAN and MEXPRESS-based analysis

The UALCAN and MEXPRESS web portals (http://ualcan.path.uab.edu/cgi-bin/ualcan-res.pl) are easy-to-use web resources for performing different kinds of analysis, including gene expression, survival, and promoter methylation analysis, based on the data taken from TCGA datasets [19]. In the current study, we used UALCAN platform for pan-cancer expression analysis of SEC24D in different cancers. In addition, we used the UALCAN platform for analyzing SEC24D expression in various selected cancer patients stratified by clinical variables. Moreover, using the MEXPRESS platform, promoter methylation level of SEC24D gene was checked among different cancers. Student's t-test was employed for UALCAN and MEXPRESS results, and a value of $P < 0.05$ was considered significant.

Survival and metastatic analysis

The KM plotter tool (https://kmplot.com/kmplot.com/analysis) was developed for survival analysis of 54 thousand genes related to different types of human cancers [20], while the TNMplot (https://www.tnmplot.com/) tool has the ability to explore the relative expression of any gene of interest across normal, cancerous, or metastatic tissue samples [21]. In our study, the significant values of SEC24D for determining the overall survival (OS) of different cancer patients were computed using the KM plotter, while the effect of higher SEC24D expression on metastasis of cancers was analyzed by the TNMplot. A *P*-value of < 0.05 was considered significant.

GENT2 analysis

The GENT2 (http://gent2.appex.kr/) platform was conceptually built to demonstrate the transcription expression level across different cancer tissues paired with controls [22]. Here in this study, using GENT2, we validated the SEC24D expression on new cohorts of different cancer patients. For this tool we used a Student t-test, and a *P*-value of < 0.05 was considered significant.

Human protein atlas (HPA) database

The SEC24D immunohistochemical (IHC) expression data from different cancer tissues along with normal controls were taken by HPA (https://www.proteinatlas.org/) [23] using a 63-year-old normal and male cancer specimens of KIRC, a 63-year-old normal female and 70-year-old female cancer specimens of LUAD, and a 48-year-old normal male and 62-year-old male cancer specimens of STAD. A polyclonal antibody (HPA041626) against SEC24D was used for IHC, and a *P*-value (< 0.05) was considered significant.

cBioportal analysis

The cBioPortal tool (https://www.cbioportal.org/) was developed to assist researchers in

exploring multidimensional cancer genomics-associated data from TCGA datasets [24]. We utilized this tool to explore and visualize SEC24D genetic alterations and copy number variations (CNVs) in the TCGA datasets of different cancers.

Protein-protein interaction and enrichment analysis

STRING (https://string-db.org/cgi/network) is a dedicated database for drawing the PPI networks [17]. In our study, first, we drew a PPI network of the SEC24D to find its binding partners. Second, using Cytoscape software, we used visualization of the PPI network, and finally, an online tool, DAVID [25] was used for pathway enrichment analysis of the SEC24D enriched genes with the default settings.

Associations among SEC24D and CD8+ T immune cells

The TIMER database (http://timer.cistrome.org/) is intended to analyze immune infiltrates in different cancers [26]. Herein, using TIMER, a Spearman correlation analysis was performed to find the correlation between SEC24D expression and CD8+ T immune cells in different cancer patients. A *P*-value < 0.05 was considered significant.

Retrieval of SEC24D-associated chemotherapeutic drugs

The Comparative Toxicogenomics Database (CTD, http://ctdbase.org/) [27] was utilized to retrieve SEC24D-associated chemotherapeutic drugs.

RNA-seq and targeted bisulfite-seq analysis-based in vitro validation of SEC24D expression and methylation status

A total of 3 cell lines, including human RCC cell lines 786-0, A-498, 769-P, and normal renal tubular epithelial cell line HK-2, were purchased from the ATCC (American Type Culture Collection). The purchased cell lines were cultured in DMEM (HyClone), supplemented with 10% fetal bovine serum (FBS; TBD), 1% glutamine, and 1% penicillin-streptomycin in 5% CO_2 at 37°C. Total RNA extraction from all these three cells lines was done using TRIzol® reagent method [28], while total DNA was extracted by organic method [29]. Finally, RNA and DNA samples were sent to Beijing Genomics Institute (BGI) company for RNA-seq bisulfite-seq analysis.

After RNA-seq analysis, the gene expression values of the SEC24D were normalized using reads per kilobase million reads (RPKM) and fragments per kilobase million reads (FPKM). Methylation values were normalized as beta values. The obtained FPKM, and beta values against hub genes in RCC and a normal control cell line were compared to identify differences in the expression and methylation levels.

Results

Expression landscape of SEC24D in human cancers

The comparison of SEC24D mRNA expression between normal and cancerous tissues was evaluated using TCGA datasets of 24 cancer subtypes through pan-cancer analysis. Results showed that SEC24D was significantly ($P < 0.05$) up-regulated in all 24 analyzed human cancer samples compared to normal control samples (**Figure 1**).

Survival and metastatic analysis

Km plotter was applied to investigate the association of up-regulated SEC24D with OS durations in 24 cancer subtypes patients, while TNMplot was utilized to analyze the effect of up-regulated SEC24D on the metastasis. Results showed that overexpression of SEC24D was significantly ($P < 0.05$) associated with decreased OS duration, and with metastasis in KIRC, LUSC, and STAD patients (**Figure 2**) in 24 analyzed subtypes shown in **Figure 1**. Collectively, these results indicate that a higher expression of SEC24D is a key alteration associated with development and progression of KIRC, LUSC, and STAD.

SEC24D expression and clinical variables

Among distinct cancer subtypes (KIRC, LUSC, and STAD) the expression of SEC24D was re-analyzed to verify the significance of SEC24D expression in normal and cancerous samples with different clinical parameters including cancer stage, race, gender, and nodal metastasis status. Results of the analysis further highlighted that SEC24D was also significantly ($P < 0.05$) overexpressed in KIRC, LUSC, and STAD patients with these diverse clinical variables relative to control samples (**Figure 3**).

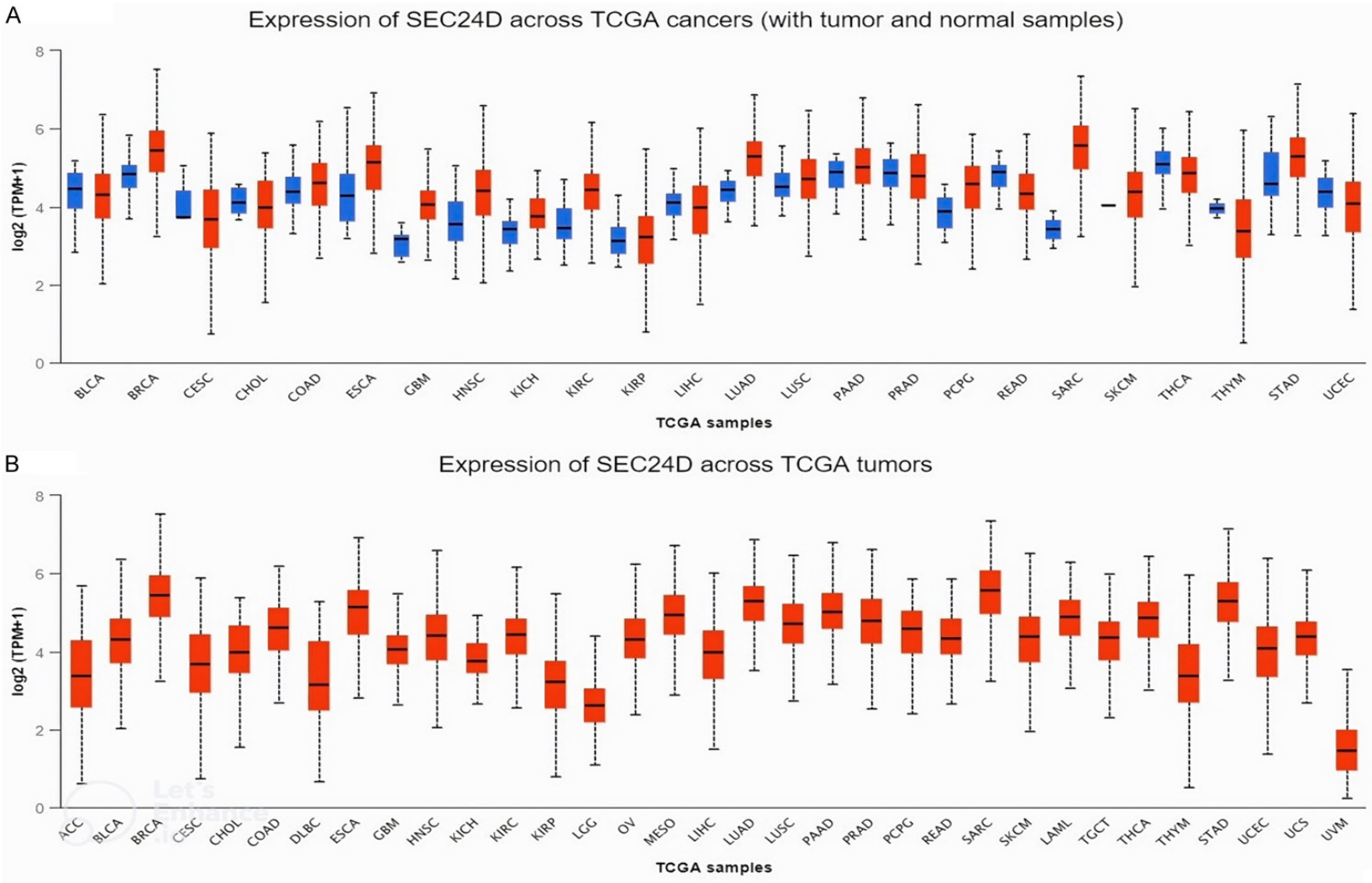

Figure 1. SEC24D expression profile in different types of human cancer tissues. (A) SEC24D expression profile in different cancer tissues, and (B) SEC24D expression profile in different cancer tissues paired with normal controls. A $^{*}P$-value of < 0.05 was selected as cutoff criterion.

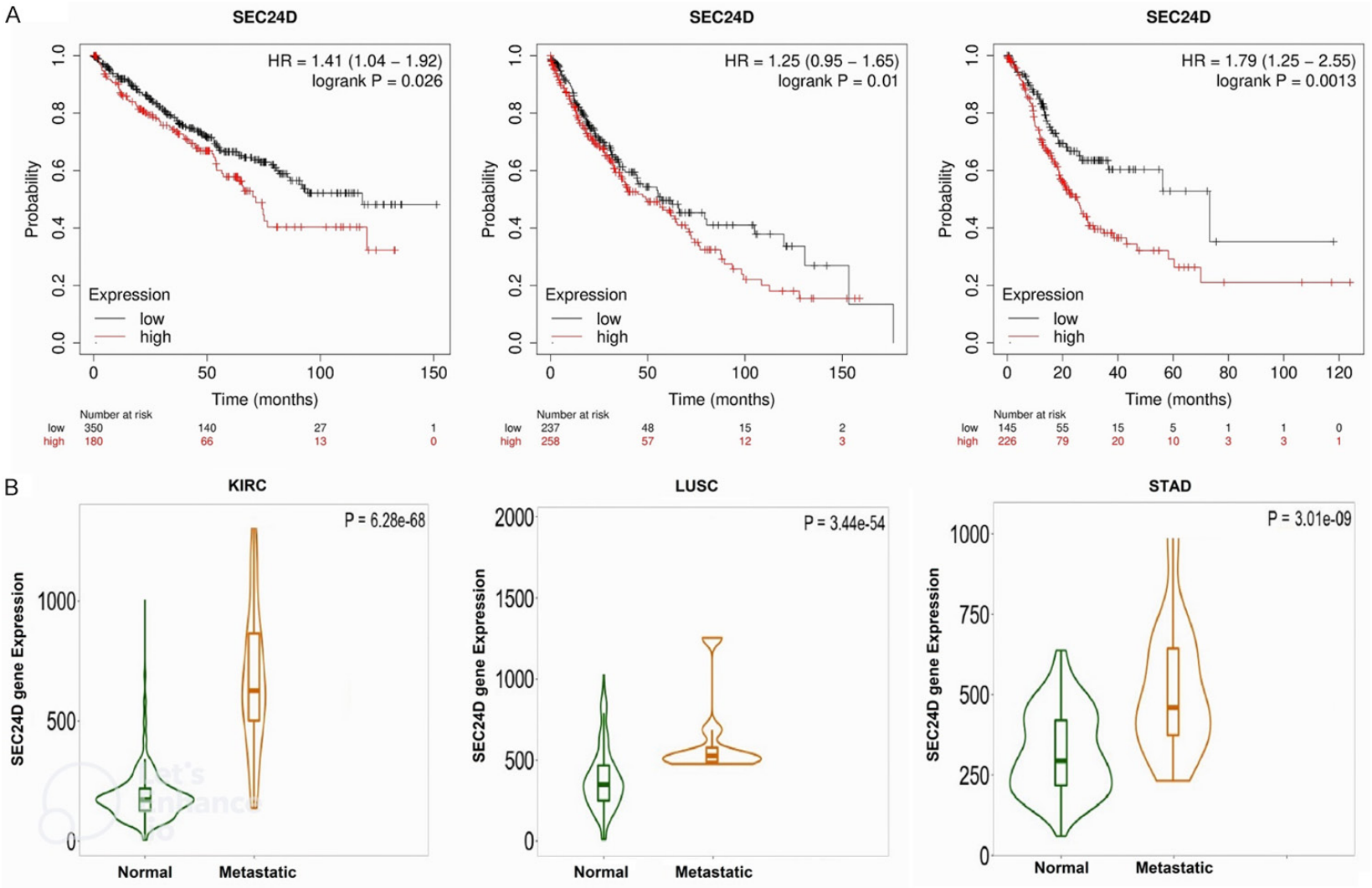

Figure 2. OS analysis and metastatic expression profiling of SEC24D in different human cancers. (A) OS analysis of SEC24D in KIRC, LUSC, and STAD and (B) metastatic expression profiling of SEC24D in KIRC, LUSC, and STAD. A $^{*}P$-value of < 0.05 was selected as cutoff criterion.

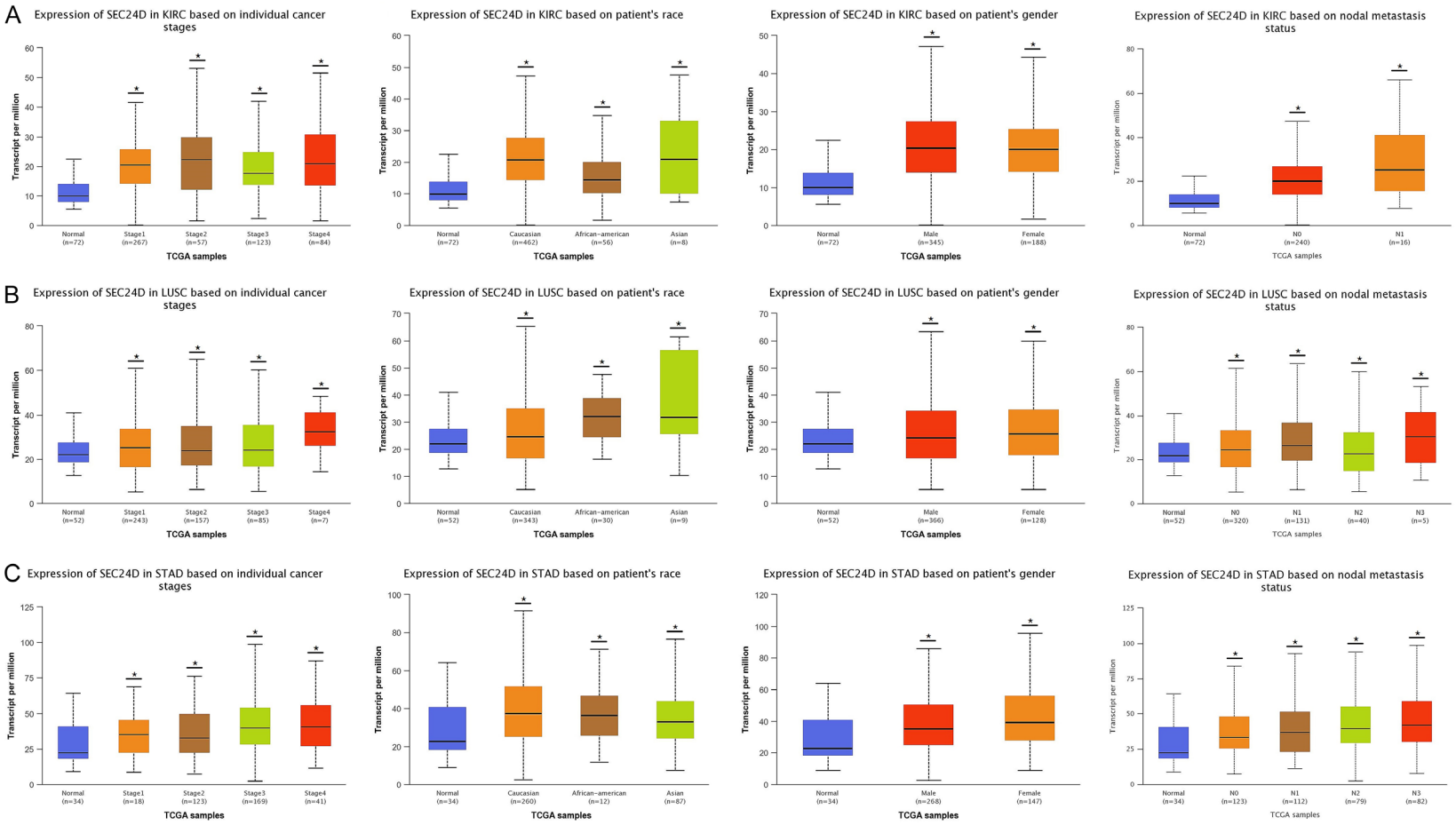

Figure 3. Expression profile of SEC24D in KIRC, LUSC, and STAD patients of different clinicopathologic values. (A) In KIRC patients classified based on different clinicopathologic values, (B) in LUSC patients classified based on different clinicopathologic values, and (C) in STAD patients classified based on different clinicopathologic values. A *P-value of < 0.05 was selected as cutoff criterion.

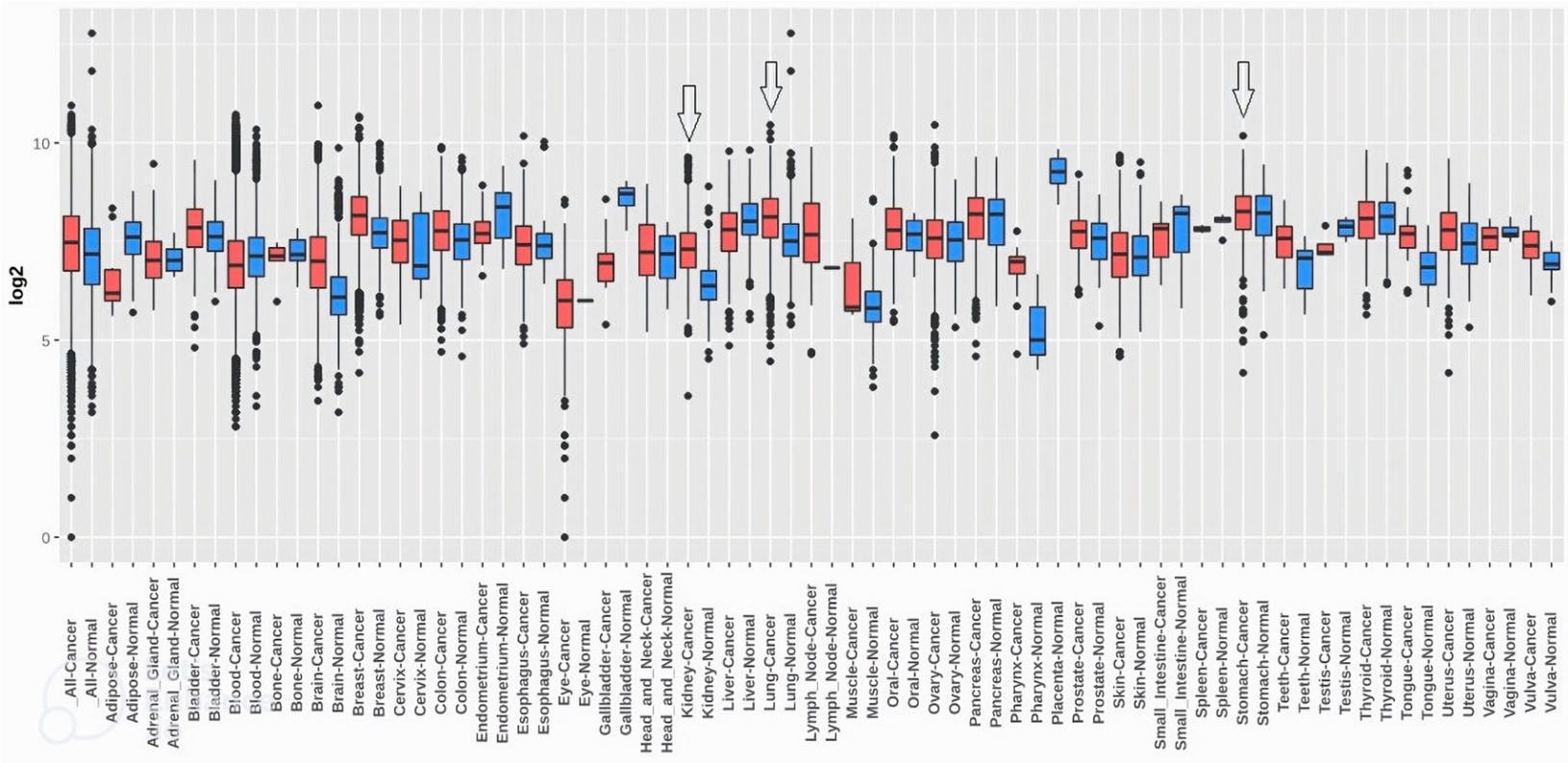

Figure 4. SEC24D transcription level validation in KIRC, LUSC, and STAD through GENT2. A *P*-value of < 0.05 was selected as cutoff criterion.

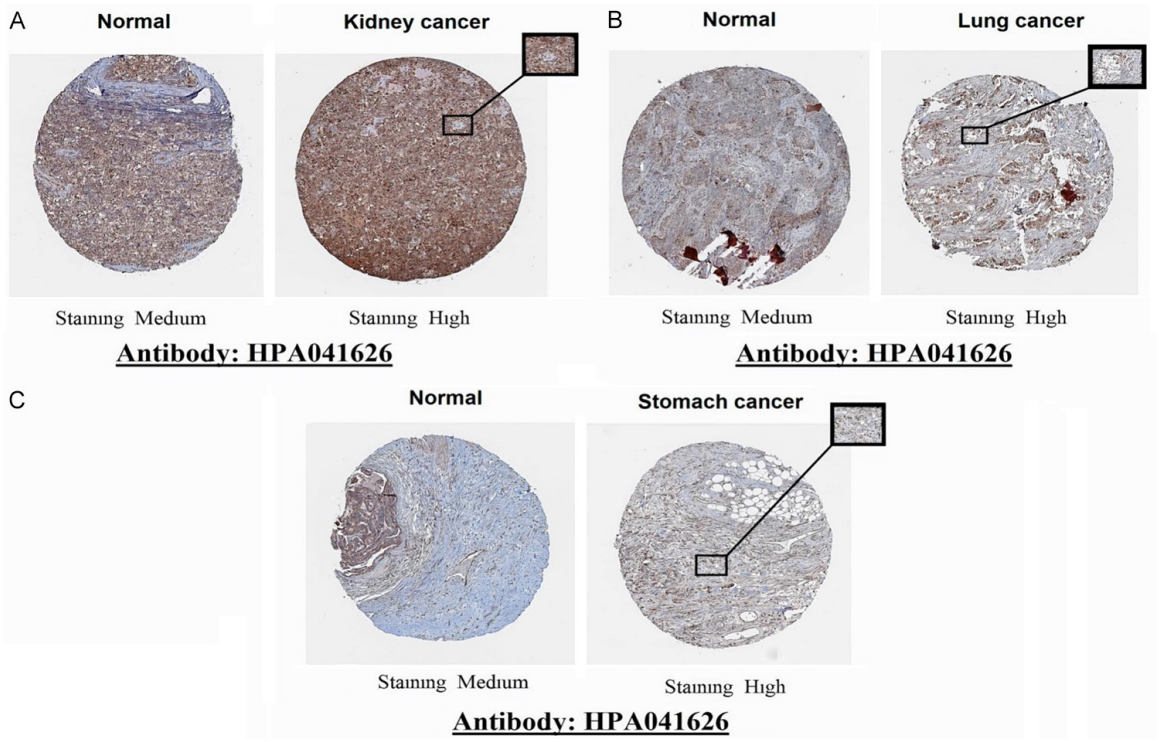

Figure 5. Immunohistochemistry images of SEC24D protein expression in different cancer and normal samples taken from HPA (×200). (A) SEC24D protein expression images in kidney cancer, (B) SEC24D protein expression images in lung cancer, and (C) SEC24D protein expression images in stomach cancer.

SEC24D transcription expression validation

SEC24D transcriptional expression level was further validated using KIRC, LUSC, and STAD samples by utilizing the new independent cohorts from the GENT2 database. As a result, we further validated a significantly ($P < 0.05$) higher expression of SEC24D in the tissue samples of these cancer patients relative to control samples (**Figure 4**).

Proteomics analysis of SEC24D

The proteomic expression of SEC24D was obtained from HPA database. Results revealed that SEC24D was expressed at a medium level (medium staining) in normal kidney, lung, and stomach tissues. However, when compared to normal controls, the SEC24D protein level was up-regulated (high staining) across kidney, lung, and stomach cancer tissues (**Figure 5**).

DNA promoter methylation of SEC24D

Using MEXPRESS, we explored the correlations across KIRC, ESCA, and STAD samples among SEC24D expression and its DNA promoter methylation. The results highlighted the high ($P < 0.05$) DNA promoter methylation level of the SEC24D gene in normal control samples relative to KIRC, ESCA, and STAD samples (**Figure 6**). This analysis showed that SEC24D gene was significantly hypomethylated in KIRC, ESCA, and STAD samples.

Genetic alterations in SEC24D

Results of cBioPortal analysis showed that SEC24D encompasses genetic alterations (deep deletion and splice mutations) in only 1.8% cases of the KIRC patients, and 1.7% cases of the LUSC patients, with maximum cases of missense mutations, and 2% cases of the STAD patients, with maximum missense mutations and deep deletion CNVs (**Figure 7**).

PPI and enrichment analysis

To further explore associations between the SEC24D gene and its other binding partners, we utilized the STRING database and Cytoscape for retrieving and displaying a PPI of SEC24D and its associated genes. The obtained PPI network highlighted that the SEC24D gene inter-

Figure 6. Promoter methylation analysis of SEC24D in KIRC, LUSC, and STAD. (A) In KIRC, (B) in LUSC, and (C) in STAD. A **P*-value of < 0.05 was selected as cutoff criterion.

Figure 7. Copy number variations (CNVs) and genetic alterations analysis of the SEC24D in TCGA KIRC, LUSC, and STAD datasets, (A) in TCGA KIRC dataset, (B) in TCGA LUSC dataset, and (C) in TCGA STAD dataset.

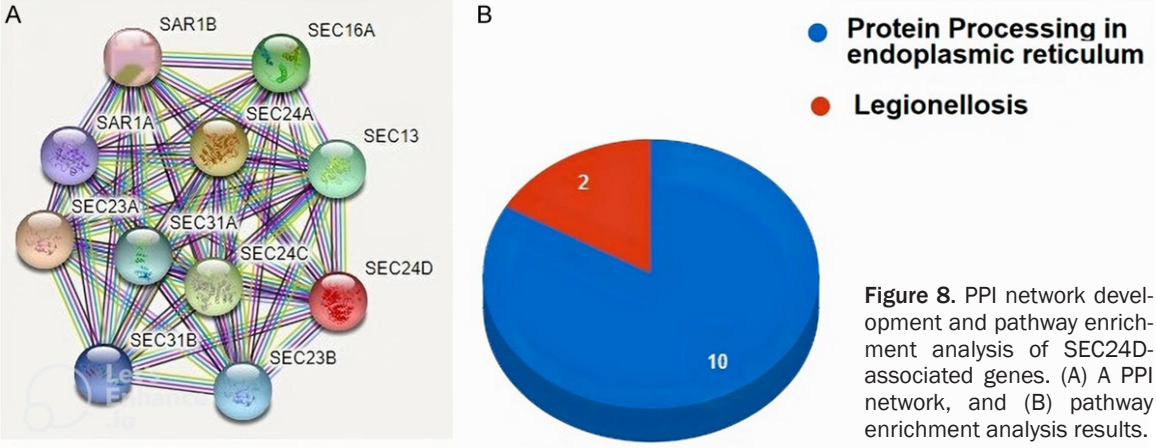

Figure 8. PPI network development and pathway enrichment analysis of SEC24D-associated genes. (A) A PPI network, and (B) pathway enrichment analysis results.

Table 1. Details of pathway enrichment analysis of SEC24D-associated genes

Pathway ID	Pathway Name	Gene count	*P*-value	Gene name
hsa04141	Protein processing in endoplasmic reticulum	10	< 0.05	SEC23A, SEC23B, SEC13, SEC24A, SAR1A, SAR1B, SEC24D, SEC24C, SEC23B, SEC31B, SEC31A
hsa05134	Legionellosis	2	< 0.05	SAR1A, SAR1B

acts with 10 other different genes (**Figure 8A**). Moreover, to further explore the possible underpinning pathways related to these genes, we performed pathway enrichment analysis and concluded that SEC24D-associated genes were mainly enriched in two major pathways, including "Protein processing in endoplasmic reticulum", and "Legionellosis" (**Figure 8B**; **Table 1**).

SEC24D and infiltrating level of CD8+ T immune cells

A systematic evaluation of the correlation between SEC24D gene expression and CD8+ T immune cell infiltration via the TIMER database revealed a notable ($P < 0.05$) positive correlation between SEC24D expression and CD8+ T immune cell infiltration in KIRC and LUSC samples, while a negative ($P < 0.05$) correlation was found between these two parameters in STAD (**Figure 9**).

Retrieval of SEC24D-associated drugs

Different drugs capable of regulating the expression of any gene(s) of interest can be explored by the CTD database. Using this resource, we retrieved a few SEC24D-associated drugs through a gene-drug interaction network that may affect the expression of SEC24D. For example, cyclosporine and valproic acid can enhance the expression of SEC24D, while oxaliplatin and topotecan can reduce SEC24D expression (**Figure 10**).

Experimental in vitro validation of SEC24D expression and methylation status

By performing RNA-seq and targeted bisulfite-seq analyses of 3 RCC cell lines, including 786-0, A-498, 769-P, and normal renal tubular epithelial cell line HK-2, the expression and methylation levels of SEC24D gene were validated. The expression levels of SEC24D were validated using FPKM, while methylation level was validated using beta values. As shown in **Figure 11A**, it SEC24D gene was expressed in both types of cell lines (normal and RCC), and FPKM values of SEC24D were higher in RCC cell lines (786-0, A-498, 769-P) as compared to normal cell line (HK-2) (**Figure 11A**). Moreover, the beta values of PROM2 were higher in the normal (HK-2) cell line but lower in the RCC cell line (786-0, A-498, 769-P) (**Figure 11B**).

Discussion

For processing and modification, newly-synthesized proteins in the ER get transported to the Golgi apparatus through small vesicles made up of COPII. After delivery, these proteins are modified and secreted to the specific cell organelles where they perform their assigned functions. Therefore, COPII is a key player in the transport of intracellular proteins [30, 31].

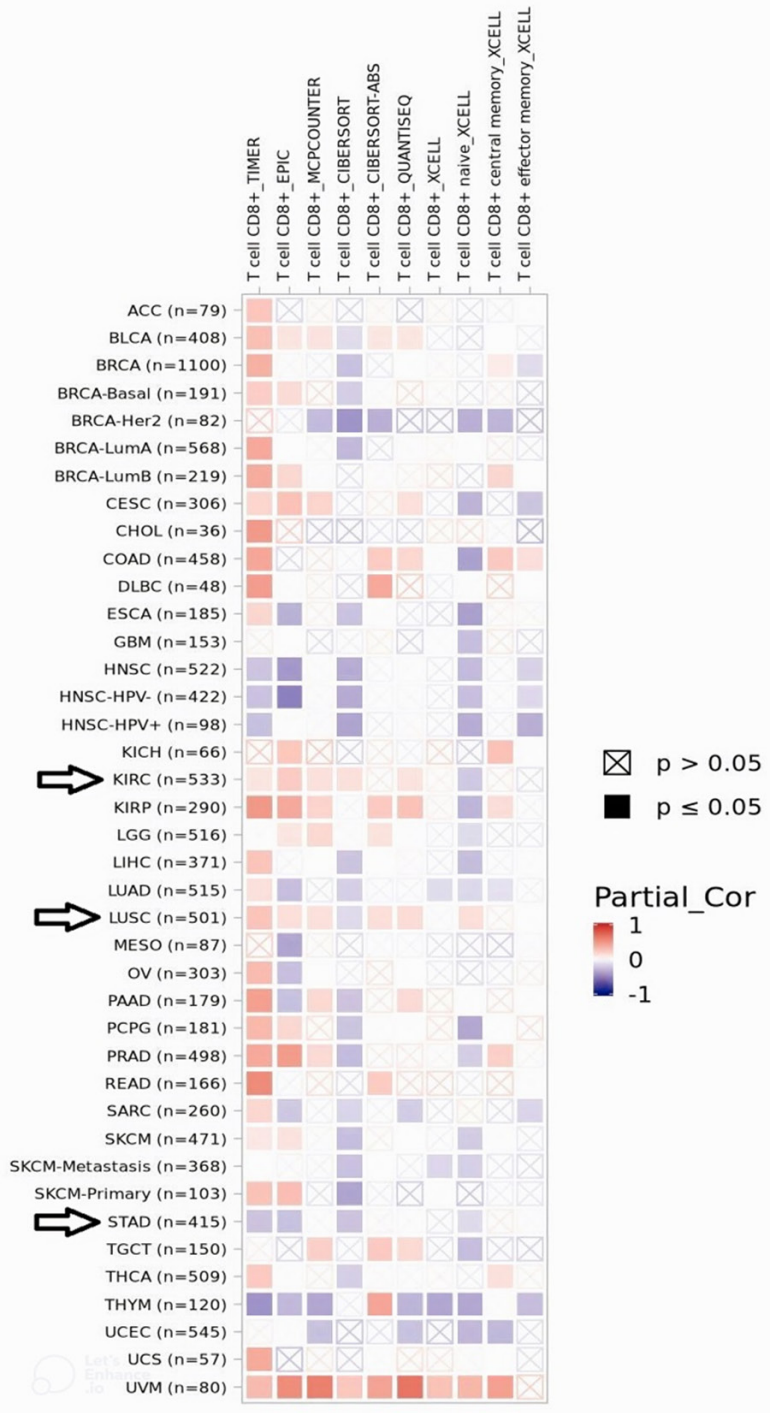

Figure 9. Results of the correlational analysis among SEC24D expression and CD8+ T immune cells infiltration in KIRC, LUSC, and STAD using TIMER. *P*-value < 0.05 was considered a significant result.

COPII is made up of two major proteins, the SEC23A and SEC24D. The dysregulation of the SEC24D gene affects the formation of COPII, thus impeding intracellular protein transport [32]. The rate of protein synthesis and transportation in cancer cells is particularly high. Therefore, we proposed that the involvement of SEC24D dysregulation may be a major factor in cancer development through diverse pathways.

Recent cancer research has mainly focused on cell proliferation and metastasis inhibition, apoptosis induction, and signal transduction [33-35]. Previously, there was not enough reported in the medical literature regarding SEC24D dysregulation in human cancers. However, genetic mutations in SEC24D and its up-regulation were rarely observed in a few conditions, such as osteogenesis imperfecta and breast cancer [17, 18]. Since SEC24D dysregulation can be a major factor in abnormal protein transport, this gene may play an active role in tumor cells. Therefore, this study aimed to comprehensively investigate SEC24D genes in 24 human cancers and to identify a few SEC24D-associated specific cancer subtypes.

We revealed that SEC24D was up-regulated in 24 human cancers and that its overexpression was significantly correlated with the decreased OS durations and metastasis in KIRC, LUSC, and STAD patients. We also explored that SEC24D was significantly overexpressed in KIRC, LUSC, and STAD patients with different clinicopathologic features. Moreover, SEC24D was found to be enriched in the deep deletion, deep amplification genetic abnormalities, and splice and missense mutations in the insignificant proportions (1.8%, 1.7%, and 2% cases) of the analyzed KIRC, LUSC, and STAD patients, respectively. Hence, we speculated that copy number variations and genetic mutations might participate

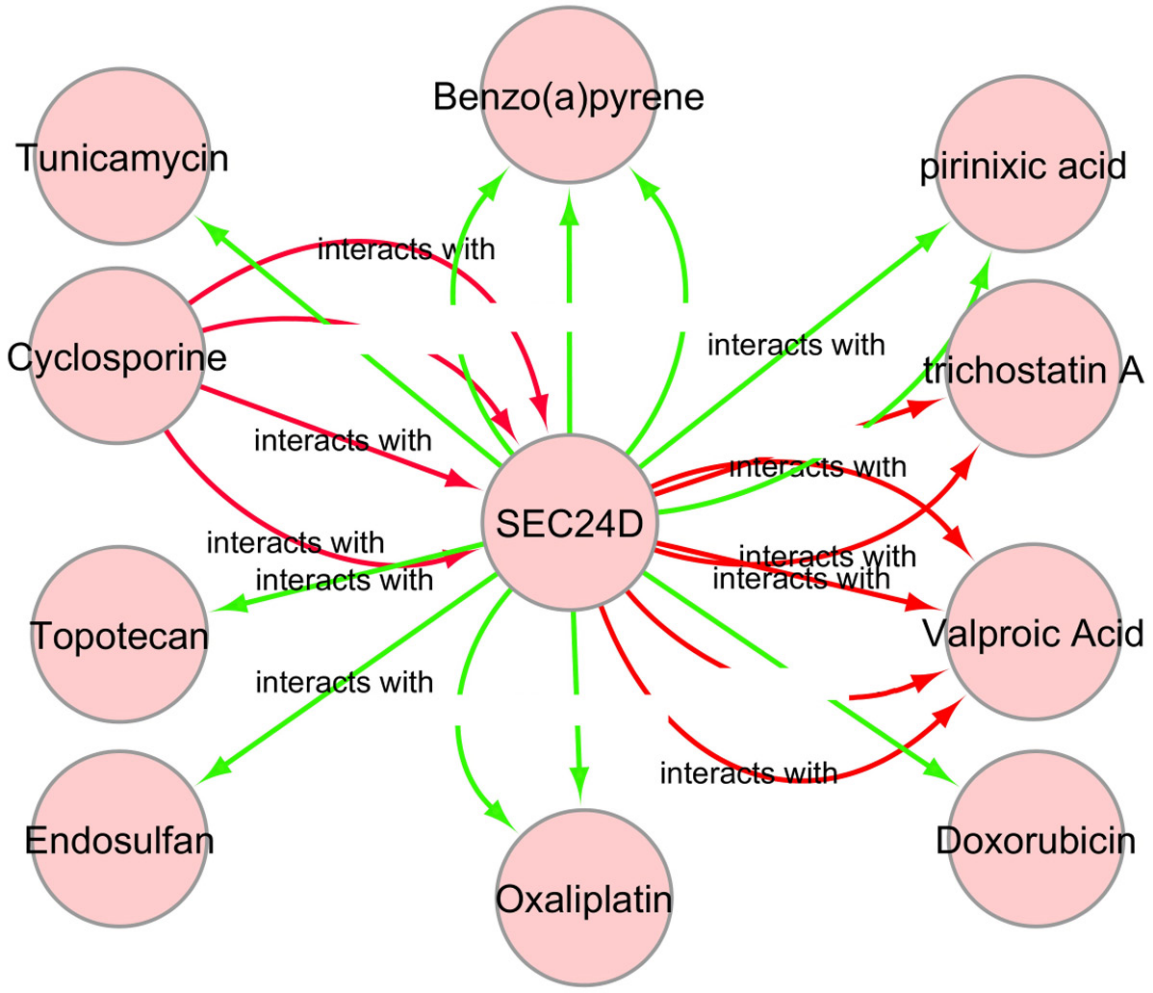

Figure 10. A SEC24D-associated gene-drug interaction network showing chemotherapeutic drugs capable to regulate its expression. Red arrows: drugs that can increase SEC24D expression, and green arrows: drugs that can decrease SEC24D expression. A count of arrows between drugs and SEC24D gene in the network represent the supported numbers of studies by the literature.

at a lower level in the expression regulation of the SEC24D. Furthermore, SEC24D DNA promoter methylation level was lower in KIRC, LUSC, and STAD samples relative to controls and this scenario of SEC24D promoter methylation showed that SEC24D gene was hypomethylated in cancer samples relative to normal controls.

The expression variation in various genes, such as ACAA1, ACADSB, C3, C3AR1, HLA-DRA, and HLA-E has recently been proposed as a biomarker in KIRC patients [36, 37]. So far, many attempts have also been made to explore possible LUSC-associated molecular biomarkers [38, 39]. For example, YMS, CCNB2, RFC MCM6, EZH2, CDK4, TPX2, and PRC1 gene expression have recently been recognized as possible biomarkers in LUSC patients. Similarly, several studies have been carried out to reveal STAD biomarkers. For example, the dysregulation of JAK3 and TYK2 genes were suggested as diagnostic and prognostic STAD biomarkers by *Man et al.* [39]. Similarly, COL10A1, WNT2, ESM1, genes and RP11-598F7.5, FOXD2-AS1, and LINC01235 long non-coding RNAs (lncRNAs) have also been highlighted as possible diagnostic biomarkers for STAD [40]. Despite all these efforts, however, none of these or any other biomarker has been generalized so far in KIRC, LUSC, or STAD patients with different clinicopathologic features. In this study, we revealed the notable up-regulation of SEC24D expression in KIRC, LUSC, and STAD patients

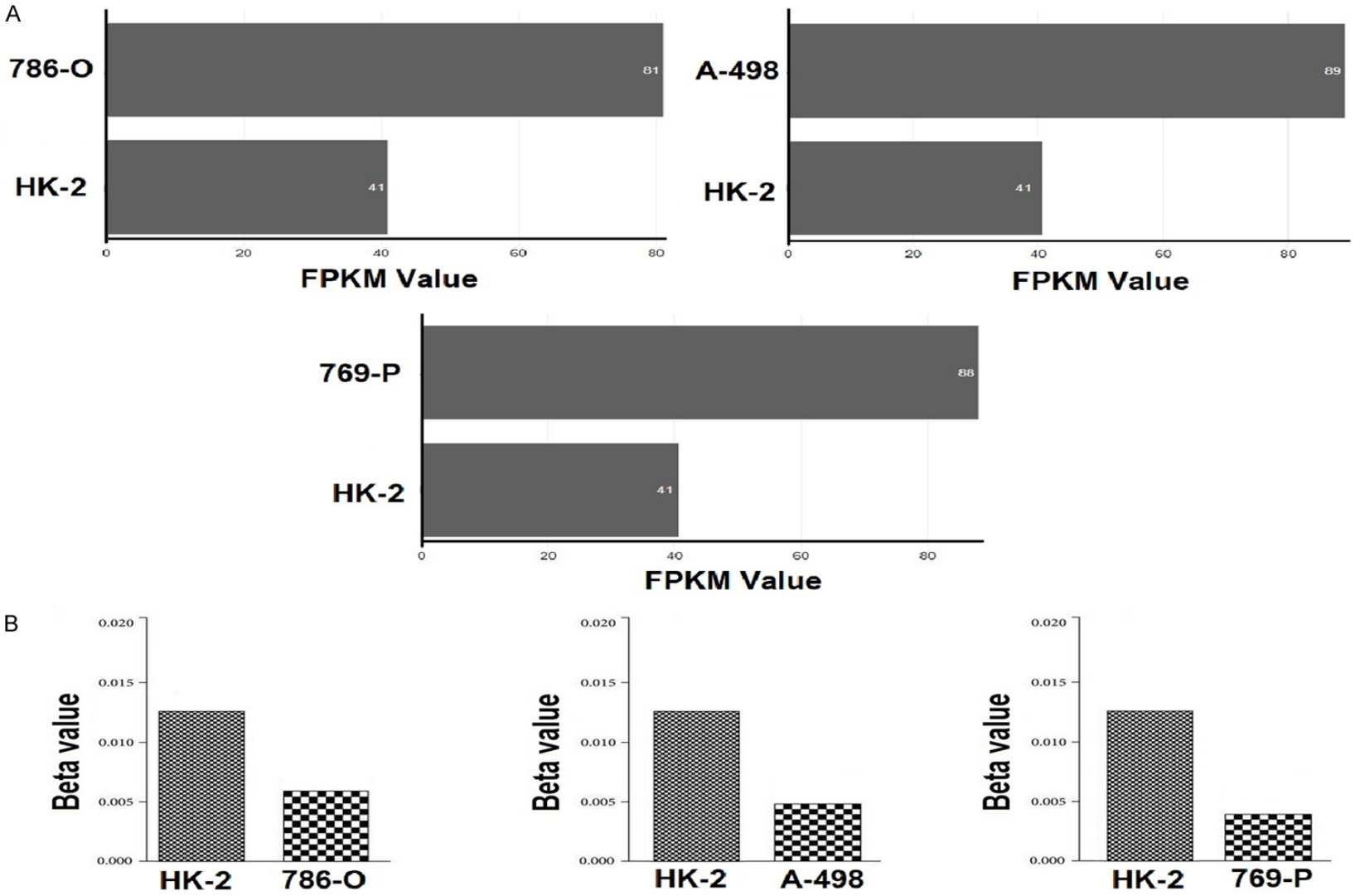

Figure 11. Validating SEC24D expression and methylation status using HK-2, 786-0, and A-498, 769-P cell lines by RNA-seq and targeted bisulfite-seq analyses. (A) FPKM values-based expression plots of SEC24D, and (B) beta values-based methylation plots of SEC24D.

with different clinicopathologic features (cancer stages, race, gender, and nodal metastasis) relative to controls. We also showed that SEC24D overexpression was linked to decreased OS of KIRC, LUSC, and STAD patients. Therefore, SEC24D up-regulation may be used as a new biomarker for KIRC, LUSC, and STAD patients.

The CD8+ T immune cells provide anti-cancer immunity [41]. Interestingly, it was observed in this study that SEC24D gene expression had a significant positive correlation with CD8+ T immune cells level in KIRC and LUSC but a significant negative correlation was observed in STAD. Such associations between SEC24D expression and CD8+ T immune cells in the tumorigenesis of KIRC, LUSC, and STAD have not been reported in prior studies. Moreover, based on pathway enrichment analysis of the SEC24D genes, we explored these genes' participation in "Protein processing in endoplasmic reticulum", and "Legionellosis" pathways. Lastly, our study also provided some useful drugs for treating KIRC, LUSC, and STAD patients with higher expression of the SEC24D gene.

Conclusion

We have systematically explored the shared diagnostic role as well as prognostic effects of SEC24D in KIRC, LUSC, and STAD through detailed bioinformatics and in vitro analyses. Given its clinical significance, the SEC24D gene can serve as a novel molecular biomarker and therapeutic target, as well as a predictive factor of immunotherapy for treating KIRC, LUSC, and STAD patients with different clinicopathologic variables.

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Disclosure of conflict of interest

None.

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