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HTRA1 and *HTRA2* expression differentially modulate the clinical prognosis of cancer: a multi-omics analysis using bioinformatics approaches

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ABSTRACT

Globally the most common cause of human death is cancer. But still, all information is unavailable to detect cancer in the early stage, and the relation of groups of the gene with cancer has to explore. *HTRA* serine proteases facilitate cellular homeostasis. Oncogenesis and response to treatment have been connected to their dysfunction in several typical human tissues. The present study aimed to assess the expression pattern of *HTRA1* and *HTRA2* in different cancers and evaluate the prognostic outcome in cancer. Various bioinformatics databases and methodologies are used in this study, including Oncomine, Kaplan-Meier plotter, OncoLnc, R2 platform, PrognoScan databases, GEPIA, cBioPortal, STRING, KEGG, and Reactome pathways analyses. The expression of *HTRA1* and *HTRA2* was analyzed, and different web-based bioinformatics platforms were used to determine their functional protein partners and correlated genes. Moreover, the cross-cancer interaction between *HTRA1* and *HTRA2* with mutations and CNAs was explored. GO and pathway analysis was used to assess the impact of these associated characteristics on certain cancers. In prognosis analysis, a positive correlation was found between *HTRA1* overexpression and poor prognosis in pancreatic, kidney, colon, and rectum cancer. A significant positive relationship between *HTRA2* overexpression and poor survival was found in pancreatic, skin, and colon cancer patients. We found that *HTRA1* in individuals with pancreatic, kidney, and colon cancers might be targeted for cancer therapy, and *HTRA2* could be used as a prognostic biomarker for skin, pancreatic, and colon cancers. Meantime, both genes might be possible targets for colon and pancreatic cancer.

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INTRODUCTION

Cancer is a disorder characterized by irregular cell growth that leads to abnormal cells spreading to other body areas [1]. Chemical and physical carcinogens present in our environment and found in various occupations cause DNA damage, leading to unregulated cell division and finally developing into cancer. Cancer has become the most common cause of human death worldwide [2,3]. According to an estimate, cancer accounts for about one of every six deaths, making it the second most prevalent cause of human death worldwide.

However, cancer is still causing many deaths; the number of survivors increases because of advances in early detection and treatment [4]. But there is no specific treatment to cure all types of cancer. It could be an effective solution to lowering the health risk of cancer if we found a curative agent [5,6]. There are several characteristics of cancer cells. One of the main characteristics is that cancer cells' metabolism has changed compared to normal cells [7,8]. These metabolic changes are referred to as cancer metabolism, and they help

cancer cells acquire and retain their malignant properties [9,10]. Alteration in enzyme expression is a natural phenomenon among these different metabolic changes, and sometimes the genes related to metabolic pathways also change in the tumor cell. Several factors affect the alteration in enzyme expression, namely gene amplification, insertion, or deletion, and environmental factors such as epigenetic changes in the gene. Apart from the underlying mechanisms, changes in basal enzymatic activity led to potential susceptibilities targeted for cancer treatment [11–13].

The high-temperature requirement A (*HTRA*) protein family consists of serine proteases involved in various physiological activities, namely mitochondrial homeostasis, cell death, and cell signaling [14]. Several studies have shown the involvement of *HTRA* in the development of cancer and neurodegenerative disorders [15,16]. As a result, the *HTRA* family is becoming potential targets for cancer treatment. In humans, the *HTRA* serine protease family consists of four members: *HTRA1*, *HTRA2*, *HTRA3*, and *HTRA4* [17].

HTRA1 is located on the long (q) arm of human chromosome 10 in a region known as 10q26.2 [18]. In the amino-terminus end of *HTRA1* serine protease, a Kazal motif, a localization sequence, and an IGFBP domain are present. In contrast, at the carboxy-terminus, a PDZ (an acronym made out of the first letters of the first three proteins identified to have the same domain such as postsynaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein) domain, and a serine protease domain is present [19]. According to many findings, the *HTRA1* gene has a role in suppressing tumors, and it activates apoptosis. Under expression of *HTRA1* helps cancer cell survival, facilitating tumor proliferation, malignancy, and metastasis [20]. The *HTRA1* gene is significantly downregulated in metastatic melanoma, brain, breast, ovarian, and liver cancer [21,22].

On the contrary, *HTRA2* is located on the short (p) arm of chromosome 2 in a locus known as 2p13.1 [23]. In the amino-terminus end of *HTRA2*, a mitochondrial targeting signal, the alpha-helix domain of a transmembrane protein, an IBM (IAP/inhibitor of apoptosis protein-binding motif), and a serine protease domain is present, while at the carboxy-terminus, a PDZ domain present [15,19]. There are many examples of the role of *HTRA2* in oncogenesis. *HTRA2* is commonly expressed in several cell lines of cancer [22]. Previous studies have shown that *HTRA2* has an involvement with cancer development [19,24]. *HTRA2* is significantly downregulated in endometrial cancer tissues while upregulated in prostate cancer tissue compared to normal tissues [25- 26]. *HTRA2* was also upregulated in gastric cancer relative to normal gastric mucosal cells [27].

Previous studies have discussed the two genes separately, albeit to a lesser extent. In this study, we would like to analyze two genes involved in many possible cancers. This research aimed to determine the expression pattern of *HTRA1* and *HTRA2* to assess their prognostic value in different types of human cancer. Using many online bioinformatics tools and resources, we investigate the expression of *HTRA1* and *HTRA2* and their prognostic effects in various cancers. We systematically analyzed the expression of *HTRA1* and *HTRA2* and their prognostic importance, methylation of promoters, mutation status, and co-expressed genes. We also studied the gene ontology and signal transduction pathways of genes associated with *HTRA1* and *HTRA2*. Analysis of copy number alterations was also conducted systemically. Hence, these comprehensive studies finally determined whether the expression pattern of *HTRA1* and *HTRA2* can be employed as a prognostic biomarker of cancer.

METHODS AND MATERIALS

Analysis of mRNA expression through Oncomine

We obtained the expression of *HTRA1* and *HTRA2* for various forms of cancer utilizing Oncomine online databases (https://www.oncomine.org/) [28,29]. This platform has a broad range of separate data sets and thoughtfully organized data. Using the default settings for different filter classes, the query was performed with the threshold parameters of 10%, 2, and 0.01 for gene ranking, fold-change, and p-value, respectively.

Analysis of mRNA transcript through GEPIA

Gene Expression Profiling Interactive Analysis (GEPIA) is an online (http://gepia.cancer-pku.cn/) interactive and well-organized tool for analyzing mRNA expression through RNA sequencing data [30,31]. It promises access to a wide array of data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) project. This database offers boxplots for studying transcript expression analysis of *HTRA1* and *HTRA2* in various cancer types. These expression patterns were used primarily to cross-check the findings obtained from the Oncomine network

Promoter methylation analysis using UALCAN

UALCAN (http://ualcan.path.uab.edu/analysis.html) is an extensive, freely accessible, and responsive online-based platform exploring cancer data [32]. This platform generates graphs and plots showing the expression pattern, patient survival, and promoter methylation of target genes. We used the UALCAN data to investigate the promoter methylation of *HTRA1* and *HTRA2* genes.

Analysis of protein-protein interaction (PPI) network using STRING

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (https://string-db.org/) is an online platform that supplies information about predictable gene function and protein-protein interaction [33,34]. It analyzes gene entries and rates them based on functional assessments. It includes a broad collection of data on functional correlation, including exchanges of proteins and genes, pathways, and data on co-expression. This database comes up with 24,584,628 proteins of 5,090 species. The PPI network was constructed utilizing both proven and predicted correlations, with no network clustering through STRING.

Gene expression, mutation, and copy number alterations (CNAs) through cBioPortal

The cBioPortal (https://www.cbioportal.org/) is an interactive online database designed for visualizing and analyzing TCGA and Pan-Cancer datasets [35,36]. In this analysis, cBioPortal was implemented with proper parameter settings to evaluate the mutation status and copy number alterations of *HTRA1* and *HTRA2*. Besides, we also investigated the copy number alterations for both genes and genes that correlated with them, using the OncoPrint feature.

Analysis of survival through Kaplan-Meier plotter

An online platform, Kaplan-Meier plotter (http://kmplot.com/analysis/), allows us to analyze the patient survival of different cancer types [37]. It helps to investigate the survival effect of over 54,000 genes in 21 types of cancer, including breast, ovarian, lung, and gastric cancer. We used this tool to examine the relationships between gene expression and survival patterns with cancers of interest in this analysis.

Analysis of cancer prognosis using PrognoScan

PrognoScan (http://www.prognoscan.org/) is a newly developed meta-analyzing database to evaluate the patient prognosis of various genes [38]. This online platform investigates the link between transcript expression and prognostic value through many tumor microarrays. The survival and prognostic importance of the *HTRA1* and *HTRA2* genes are determined through PrognoScan. The Cox p-value <0.05 was considered significant for this analysis.

Analysis of survival using OncoLnc

The OncoLnc (http://www.oncolnc.org/), an online platform, allows examining the associations between survival and gene expression [39]. This tool uses TCGA data of up to 21 cancers as its data source. This platform was used to collect cox regression analysis results for *HTRA1* and *HTRA2* in different types of cancers. Then we used this result to produce Kaplan-Meier survival plots.

Prognosis and correlation study through R2 genomics

The R2 Genomics (https://r2.amc.nl/) is an online platform that can analyze a vast set of publicly available genomic data [40,41]. In this study, we used the Kaplan-Meier scanner feature of R2 to assess the survival of some cancer patients. We also retrieved a list of genes associated with *HTRA1* and *HTRA2* via R2 genomics. Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) was then utilized to draw the Venn diagram and determine the associated genes of certain cancers using the gene list. Then, to conduct analyses of gene ontologies (such as biological process 2018, molecular function 2018, and cellular component 2018) and pathways (such as Kyoto Encyclopedia of Genes and Genomes or KEGG human 2019, Reactome pathway 2016, and Panther pathway 2016) of widely associated genes using Enrichr (https://maayanlab.cloud/Enrichr/) [42].

Statistical analysis

We used the Oncomine, cBioPortal, UALCAN, and GEPIA databases to gather gene expression. The p-values < 0.01 were considered significant. The methylation box plots were obtained from the UALCAN database. All results are displayed with p-values obtained from a log-rank test. The levels of significance (p-values) of all servers were determined by the programs used for the analyses. Several online bioinformatics resources, including R2: Kaplan Meier Scanner, PrognoScan, OncoLnc, and Kaplan-Meier Plotter, were used to create the survival plots. The p-values < 0.05 were considered significant (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

RESULTS

Transcription levels of *HTRA1* and *HTRA2* in different types of cancer

We started the study by analyzing the transcription level of *HTRA1* and *HTRA2* in different cancers relative to the normal tissues. To evaluate the impact of *HTRA1* and *HTRA2* serine proteases in the progression of different types of human cancers, we used the Oncomine and The GEPIA database. We used 0.01, 2, and 10% for p-value, fold change, and gene ranking, respectively, as our criteria for analysis in the Oncomine database. We inquired about the Oncomine and GEPIA database with all these criteria and observed an overview of *HTRA1* and *HTRA2* expressions. *HTRA1* serine protease was extensively overexpressed in some cancers, such as breast cancer, leukemia, lymphoma, pancreatic cancer, etc., and moderately upregulated in others, compared to their expression patterns in normal tissues. Unlike *HTRA1*, there was a moderate upregulation such as in head and neck cancer and downregulation of *HTRA2* serine protease in specific cancer such as breast and colorectal cancer. Based on these findings, we observed that the *HTRA* serine proteases play a role as either oncogene or tumor suppressor gene (TSG), but *HTRA1* is more oncogenic than the *HTRA2* (Figure 1a).

Next, via the cBioPortal web, we accessed the TCGA data and queried the transcript expression for both serine proteases in 32 different cancer types. These findings indicate that *HTRA1* and *HTRA2* are expressed differently in different kinds of cancer (Figure 1b).

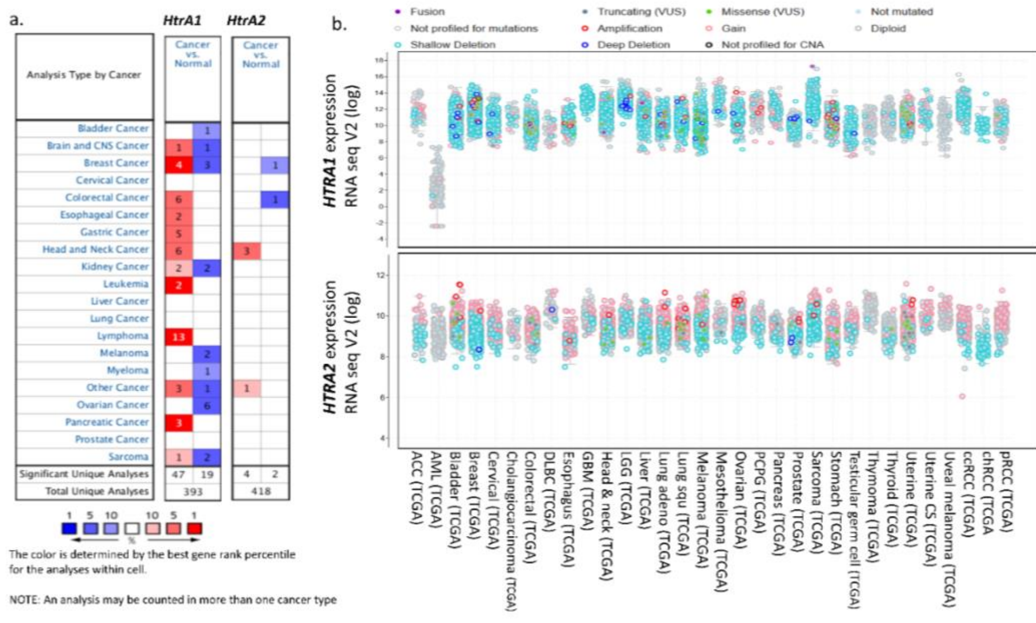

Figure 1. Transcription rates of *HTRA1* and *HTRA2* in various cancer types. (a) The above figure was obtained from the Oncomine database, showing statistically significant data of *HTRA1* and *HTRA2* mRNA with upregulation (indicated by red) and downregulation (indicated by blue) (cancer tissue vs. normal tissue). We used 0.01, 2, and 10% for p-value, fold change, and gene ranking, respectively, as our criteria for analysis. The number of studies that met these criteria is represented by the numbers in the boxes. (b) Study of mRNA levels for *HTRA1* and *HTRA2* in 32 forms of cancer utilizing cBioPortal online tool. Each point reflects an individual analysis.

Transcript expression analysis of *HTRA1* and *HTRA2*

To investigate the degree of differential expressions of *HTRA* serine protease, we performed a systemic analysis through the Oncomine platform in different types of human cancer. *HTRA1* and *HTRA2* transcript levels in various cancer cell lines compared to their normal tissues were examined in the Oncomine database. This study shows that

HTRA1 is overexpressed in the brain, B-cell lymphoma, pancreatic, and kidney cancers while underexpressed in ovarian, skin, colon, and rectum cancers in comparison with normal tissues [43,44] (Figure 2a [i-viii]).

To validate these results of *HTRA1* expression, we performed another study of this gene using a different web-based tool, Gene Expression Profiling Interactive Analysis (GEPIA). The results from GEPIA validated *HTRA1* overexpression in the brain, B-cell lymphoma, pancreatic, kidney cancers, and underexpression in ovarian, skin, colon, and rectum cancers (Figure 2b [i-viii]). *HTRA2* expression levels in various cancers vary considerably from *HTRA1* expression levels. This study showed that *HTRA2* is substantially upregulated in the brain, plasma cell, pancreatic, head, neck, kidney, skin, colon, and breast cancers. In contrast, it is under-expressed in ovarian cancer [45,46] (Figure 3a [i-viii]).

The above expression pattern of *HTRA2* in the brain, plasma cell, pancreatic, kidney, skin, colon, breast, and ovarian cancer has also been found from TCGA data on the GEPIA-based online platform, which we used for cross-checking the result retrieved from the Oncomine database (Figure 3b [i-viii]). Previous studies also support these results regarding *HTRA1* and *HTRA2* expression in different types of cancer. For instance, whereas the downregulation of *HTRA1* was noticed in ovarian, skin, and colorectal cancers [20,21,44,47], the upregulation was observed in brain cancer [43]. While the upregulated pattern of *HTRA2* was noticed in colorectal cancer [45], the downregulation was detected in ovarian cancer [46]. Our study showed that *HTRA1* and *HTRA2* exhibit distinct expression patterns in different human cancers, which is also supported by previous studies.

Analysis of promoter methylation in different cancer types

Methylation is a systematic alteration of biomolecules, and it is associated with gene expression and cancer development [48]. Next, we analyzed promoter methylation of *HTRA1* and *HTRA2* in different types of cancer based on sample types using the UALCAN database. The degree of DNA methylation is indicated by the beta value, ranging from 0 (unmethylated) to 1 (completely methylated). Diverse beta value cut-offs have been used to suggest hypermethylation (0.7-0.5) or hypomethylation (0.3-0.25) [49]. We observed that the promoter of the *HTRA1* is hypomethylated in several cancers, such as KIRC (kidney renal clear cell carcinoma) and BLCA (urothelial bladder carcinoma) (Figure 4a [iv, v, vii]). Still, it is hypermethylated in BRCA (invasive breast carcinoma), COAD (colon adenocarcinoma), ESCA (esophageal carcinoma), and LUAD (Lung adenocarcinoma), showed in figure 4a [i-iii, vi]. In contrast to *HTRA1*, the *HTRA2* gene is hypomethylated in many cancers, including BRCA (invasive breast carcinoma), KIRC (kidney renal clear cell carcinoma), PRAD (Prostate adenocarcinoma), BLCA (urothelial bladder carcinoma), and LIHC (Liver hepatocellular carcinoma), shown in figure 4b [i, iv-vii]. Still, it is hypermethylated in COAD (colon adenocarcinoma), ESCA (esophageal carcinoma) (Figure 4b [ii, iii]). Methylation in tumor suppressor genes' promoter regions silences them, and methylation inside the gene itself can cause mutational events. These pathways may play a key role in the development of a wide range of human cancers [50].

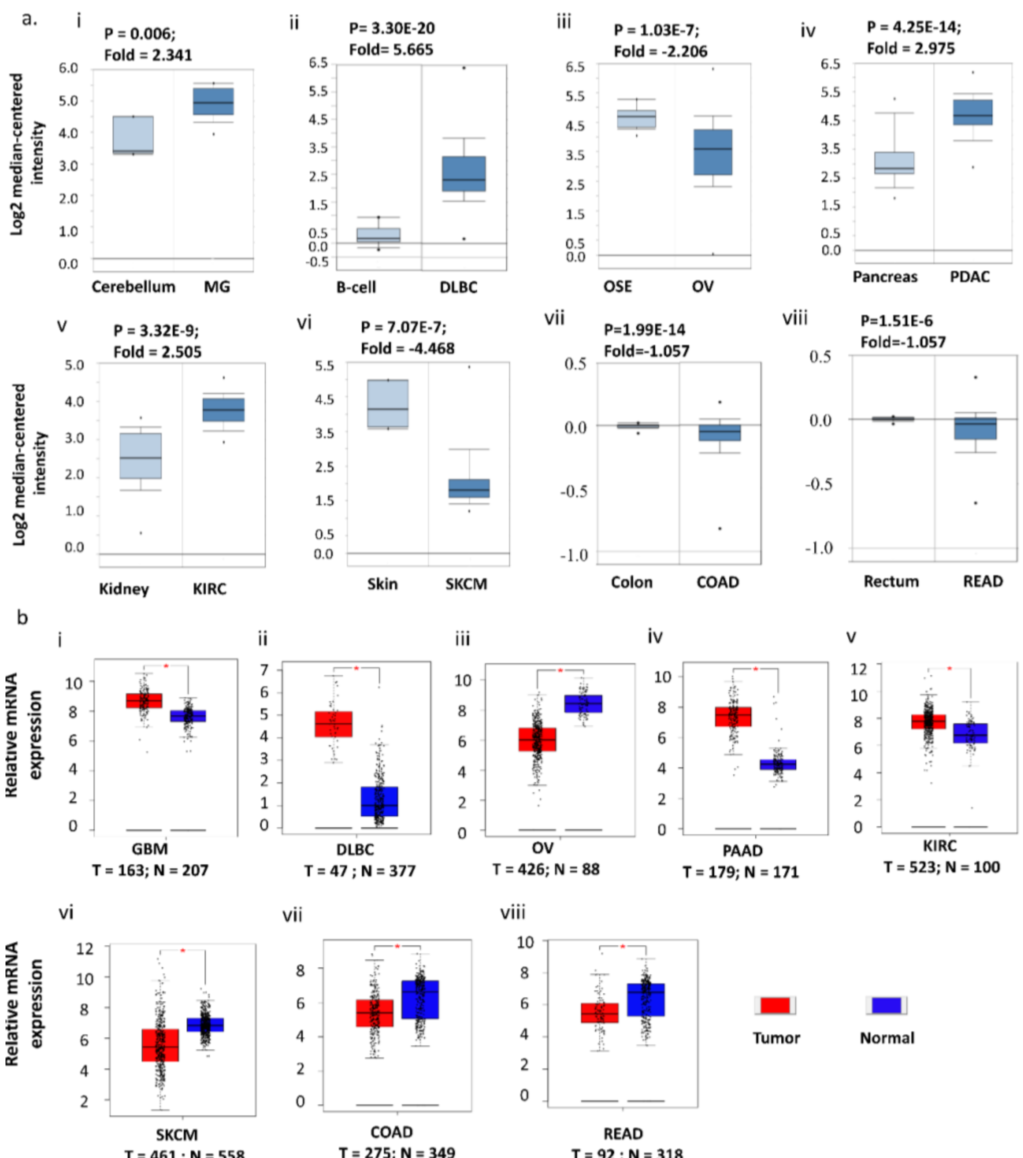

Figure 2. Analysis of *HTRA1* mRNA expression in different human cancers. (a) The *HTRA1* expression box plot was constructed from the Oncomine online tool. The left plot represents the normal tissue, while the cancer tissue is seen in the right plot. Analysis of MG (Malignant Glioma) compared with normal cerebellum (i), DLBC (Lymphoid Neoplasm Diffuse Large B-cell Lymphoma) compared with normal B-lymphocyte (ii), OV (Ovarian cancer) compared with normal ovarian surface epithelium (iii), PDAC (Pancreatic Ductal Adenocarcinoma) compared with the normal pancreas (iv), KIRC (Clear Cell Renal Cell Carcinoma compared with normal kidney cell (v), SKCM (Cutaneous Melanoma compared with normal skin (vi), COAD (Colon adenocarcinoma) compared with a normal colon (vii), READ (Rectum adenocarcinoma) compared with the normal rectum. (b) Analysis of *HTRA1* expression in The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTex) database. The box plot was generated using GEPIA to show the expression patterns of *HTRA1* in tumor (T) tissues compared to its normal (N) tissue. The left red plot represents tumor tissue, while the right blue blot reflects normal tissue (i-viii). The criteria of this analysis were 0.01 and 2 for p-value and fold change, respectively. GBM (Glioblastoma multiforme), DLBC (Lymphoid Neoplasm Diffuse Large B-cell Lymphoma), OV (Ovarian carcinoma), PAAD (Pancreatic adenocarcinoma), KIRC (Kidney renal clear cell carcinoma), SKCM (Skin Cutaneous Melanoma), COAD (Colon adenocarcinoma), READ (Rectum adenocarcinoma).

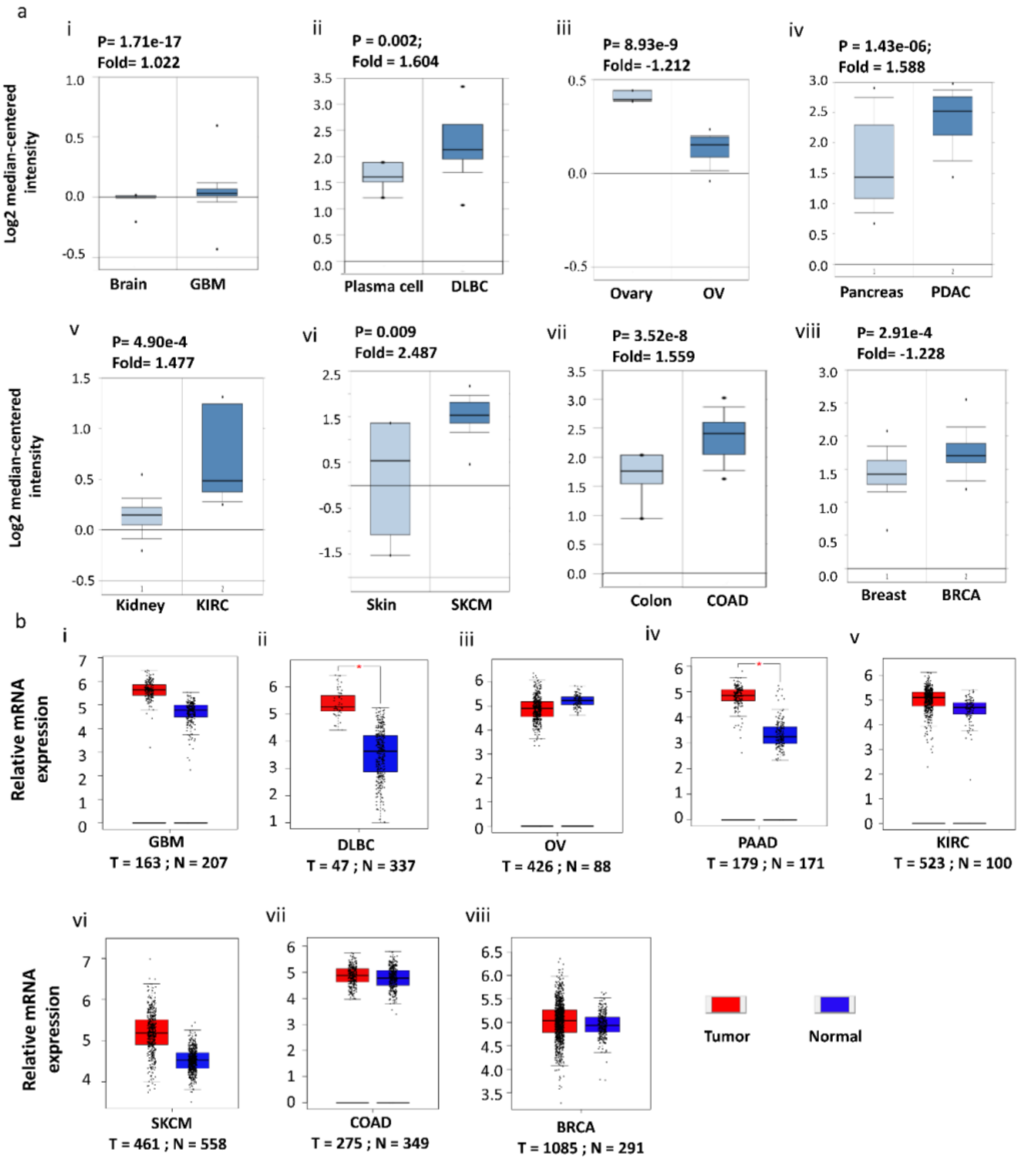

Figure 3. Analysis of *HTRA2* mRNA expression in different human cancers. (a) The *HTRA2* expression box plot was constructed from the Oncomine online tool. The left plot represents the normal tissue, while the cancer tissue is seen in the right plot. Analysis of GBM (Glioblastoma) compared with the cerebellum (i), DLBC (Lymphoid Neoplasm Diffuse Large B-cell Lymphoma) compared with B-lymphocyte (ii), OV (Ovarian cancer) compared with ovarian surface epithelium (iii), PDAC (Pancreatic Ductal Adenocarcinoma) compared with the pancreas (iv), ccRCC (Clear Cell Renal Cell Carcinoma) compared with kidney (v), SKCM (Skin Cutaneous Melanoma) compared with skin (vi), COAD (Colon adenocarcinoma) compared with a normal colon (vii), BRCA (Invasive breast carcinoma) compared with breast (viii). (b) Analysis of *HTRA2* expression in The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTex) database. The box plot was generated using GEPIA to show the expression patterns of *HTRA2* in tumor (T) tissues compared to its normal (N) tissue. The left red plot represents tumor tissue, while the right blue blot reflects normal tissue (i-viii). The criteria of this analysis were 0.01 and 2 for p-value and fold change, respectively. GBM (Glioblastoma multiforme), DLBC (Lymphoid Neoplasm Diffuse Large B-cell Lymphoma), OV (Ovarian carcinoma), PAAD (Pancreatic adenocarcinoma), KIRC (Kidney renal clear cell carcinoma), SKCM (Skin Cutaneous Melanoma), COAD (Colon adenocarcinoma), BRCA (Invasive breast carcinoma).

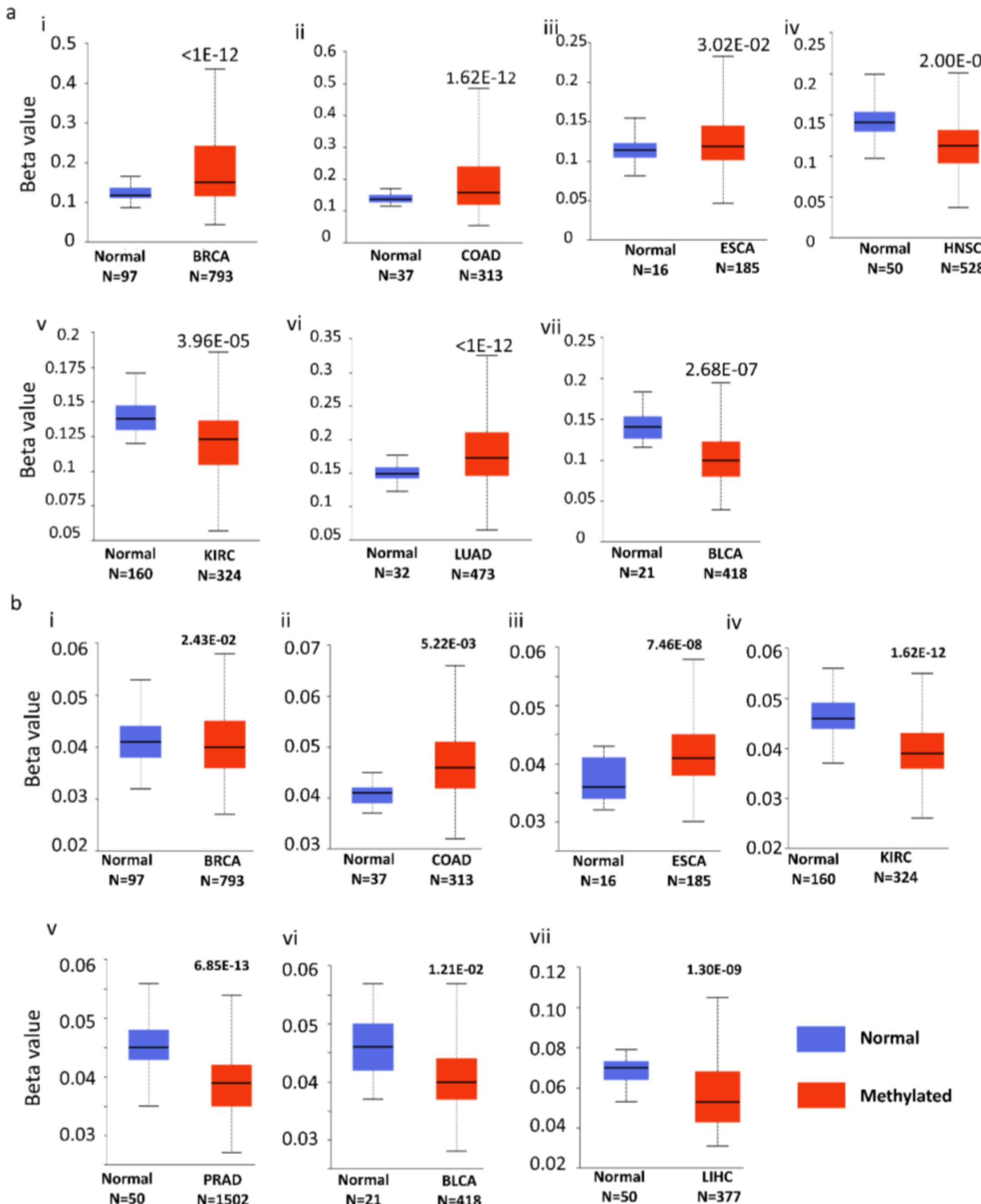

Figure 4. Analysis of promoter methylation in both *HTRA1* and *HTRA2* genes. The box plots were obtained from UALCAN online database. (a) The figure shows the methylation status of the *HTRA1* promoter in several human cancers (i-vii). (b) The figure shows the methylation status of *HTRA2* promoter in several human cancers (i-vii). BLCA, urothelial bladder carcinoma; BRCA, breast invasive carcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; HNSC, head, and neck squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; PRAD, prostate adenocarcinoma.

Predicting PPI networks of *HTRA1* and *HTRA2*

HTRA1 is a member of the *HTRA* family of serine proteases [21-51]. *HTRA1* was first extracted as a transformation-sensitive protein from fibroblasts because of its downregulation by SV40 [52]. *HTRA2* is also known as *L56/HTRA*, a structurally related protein to *HTRA1* expressed differently in human osteoarthritic cartilage 4. The serine protease domain and the PDZ domain in the carboxy terminus end are present in *HTRA1* and *HTRA2*. But what makes a difference is that *HTRA1* has an insulin-like growth factor binding proteins (IGFBP) domain and a signal sequence for secretion, which are missing in HTRA2 [53-54]. However, the molecular mechanism of *HTRA1* and *HTRA2* in cancer

progression is still unclear [55]. Our current study attempted to obtain comprehensive system-level information on *HTRA1* and *HTRA2* by constructing a PPI network with their closely correlated protein partners. We used the STRING database (https://string–db.org) to create the PPI network. STRING is an online database that provides the opportunity to analyze the functional and physical interaction of proteins [56]. We selected Homo sapiens as our target organism. The database was reviewed separately for both *HTRA1* and *HTRA2*, where nodes indicated the proteins, and the edges presented the interactions between two proteins.

Proteins operate their function in an assembled fashion, specifically through their interactions [57]. For further study, we identified significant functional protein partners for *HTRA1* and *HTRA2* (Figure 5a [i, ii]). The predicted protein partners of *HTRA1* are as follows: 60S ribosomal protein L34 (*RPL34*), aggrecan core protein (*ACAN*), ATP-dependent zinc metalloprotease YME1L1 (*YME1L1*), 60 kDa heat shock protein (*HSPD1*), ATP-dependent Clp protease proteolytic subunit (*CLPP*), puratrophin-1 (*PLEKHG4*), complement factor H (*CFH*), age-related maculopathy susceptibility 2 (*ARMS2*), ATP-dependent Clp protease ATP-binding subunit clpX-like (*CLPX*), chymotrypsin-C (*CTRC*) (Figure 5a [i]). The predicted protein partners of *HTRA2* are as follows: Baculoviral IAP repeat-containing protein 2 (*BIRC2*), baculoviral IAP repeat-containing protein 3 (*BIRC3*), endonuclease G (*ENDOG*), PTEN-induced kinase 1(*PINK1*), E3 ubiquitin-protein ligase parkin (*PARK2*), nucleolysin TIA-1 isoform p40 (*TIA1*), mitogen-activated protein kinase 14 (*MAPK14*), TNF receptor-associated factor 2 (*TRAF2*), diablo IAP-binding mitochondrial protein (DIABLO), X-linked inhibitor of apoptosis (*XIAP*) (Figure 5a [ii]). Combined with earlier research, the results reported above prove that *HTRA1* and *HTRA2* expression can play a role in cancer cell proliferation and survival through interactions with co-expressed genes.

Mutations and copy number alterations (CNAs) of *HTRA1* and *HTRA2*

We studied the mutations and copy number alterations (CNAs) of *HTRA1* and *HTRA2* in different types of cancer through the cBioPortal web tool. First, we examined the mutations of *HTRA1* in 83,154 samples of 80,247 individual patients from 211 studies that cover the data from all the accessible cancers. Among the samples, we found 219 mutations site in *HTRA1* with a 0.2% frequency of somatic mutation, and all are located between 0 to 480 amino acid residues. Among these mutations, there were 192 missense mutations, 19 truncating mutations, four in-frame mutations, and four fusion mutations (Figure 5a [i]). It is worth mentioning that there were also 17 repeat mutations in the 219 mutations. We have found that *HTRA1* mutations appeared predominantly in skin, breast, prostate, and colorectal cancer and spread across the PDZ domain (from 382 to 455 amino acid residues), with a hotspot in *V433I/A*, where six mutations were reported. The amino acid residue is changed due to frame-shift deletion (Figure 5a [i]).

We also queried the database for *HTRA2* using the same settings used as for *HTRA1*. In this case, we found 170 mutations with a 0.2% frequency of somatic mutation, the same as *HTRA1*, and all are located between 0 to 458 amino acid residues. However, mutations in *HTRA2* are significantly different from *HTRA1*. Among the total 170 mutations, 131 are missense mutations, 37 are truncating mutations, and 2 are inframe mutations. It should be noted that there were 11 repeat mutations. The mutations have appeared mainly in the prostate, endometrium, uterine and colorectal cancer, with a hotspot in *A116Qfs*93* located between amino acids 100 and 150, where seven mutations were mentioned, and the amino acid residue is changed due to frame-shift deletion (Figure 5b [ii]). In this analysis, we retrieved a cancer types summary, which shows alterations in

different types of cancers, with a minimum of 110 samples with a mutation frequency of at least 1%. These indicate that the *HTRA1* and *HTRA2* genes have predominantly altered in skin cancer (melanoma for *HTRA1* and metastatic melanoma for *HTRA2*) (Figure 5b [i, ii]).

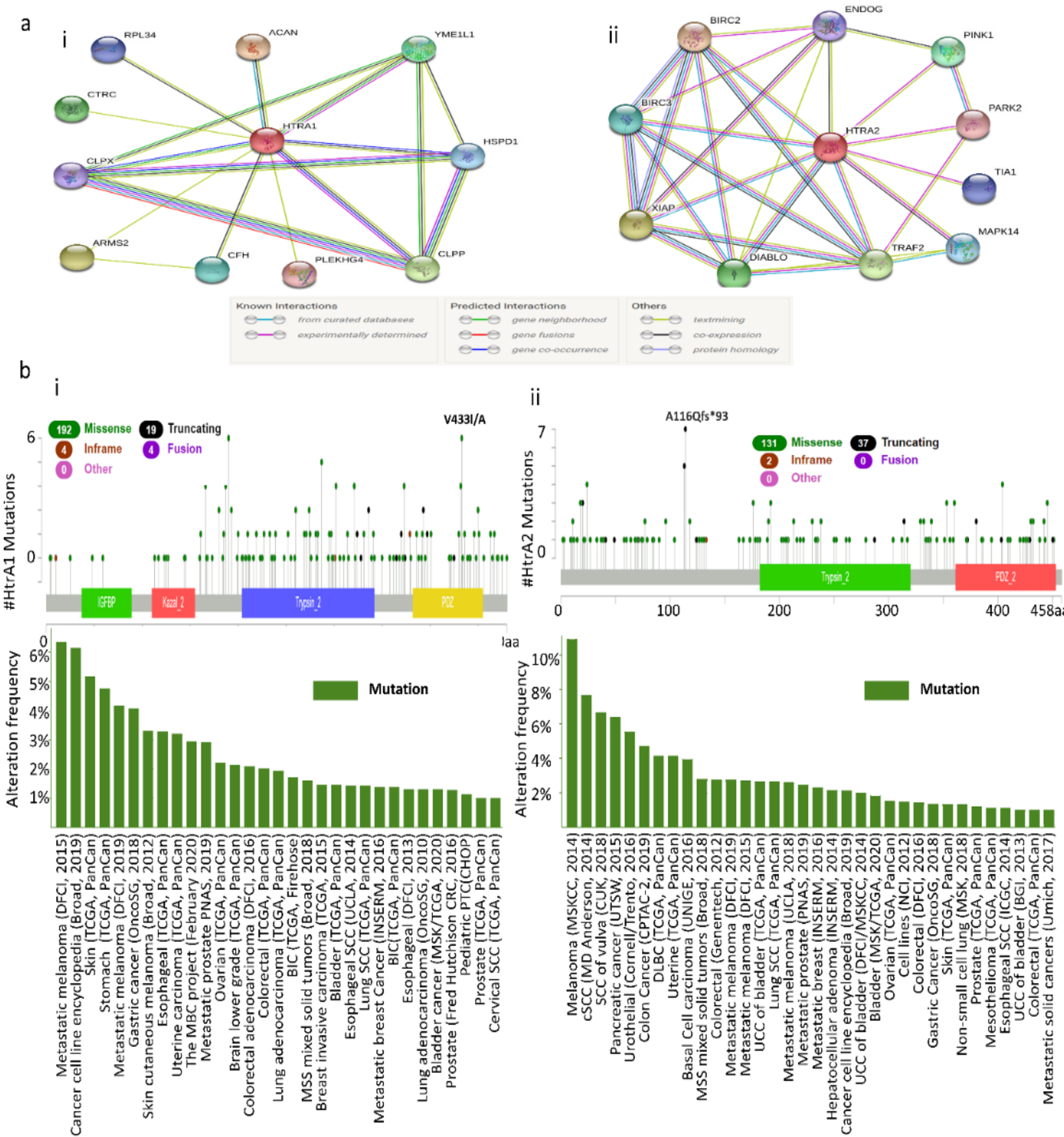

Figure 5. Recognition of protein-protein interactions necessary for *HTRA1* and *HTRA2* function. (a) The connecting nodes obtained using STRING are shown in circles. The predicted protein partners of *HTRA1* and *HTRA2* are shown in figures (i) and (ii), respectively. (b) This figure shows a total of 219 mutations, including 192 missense mutations, 19 truncating mutations, four in-frame mutations, and four fusion mutations. All the mutation sites are localized from 0 to 480 amino acids residue (i). A total of 170 mutations, including 131 missense mutations, 37 truncating mutations, and two in-frame mutations, are located between amino acids 0 and 458 (ii). The cancer types summary indicates that the *HTRA1* and *HTRA2* genes have predominantly altered in skin cancer (melanoma for *HTRA1* and metastatic melanoma for *HTRA2*).

Second, we examined the relationship of the mutation and copy number alterations with *HTRA1,* and its protein partners previously retrieved from the STRING database. We provided the gene list as *HTRA1*, *RPL34*, *ACAN*, *YME1L1*, *HSPD1*, *CLPP*, *PLEKHG4*, *CFH*, *ARMS2*, *CLPX*, *CTRC*, and investigated copy number alterations and mutations that cover all the cancer datasets available in the cBioPortal database (Figure 6a [i]). The findings revealed that the alteration frequency varied from 20% of 30 cases to 74.36% of 39 cases, including a minimum of 20% alterations. The *HTRA1*-centered-gene-signature alterations most commonly occur in cutaneous squamous cell carcinoma (SCC) and less in metastatic prostate cancer. Likewise, we studied the mutations and copy number alteration for *HTRA2* and its protein partners (*HTRA2*, *BIRC2*, *BIRC3*, *ENDOG*, *PINK1*, *PARK2*, *TIA1*, *MAPK14*, *TRAF2*, *DIABLO*, *XIAP*), which showed the alteration frequency varied from 15.19% of 236 cases to 73.47% of 147 cases, which includes a minimum of 15% alterations (Figure 6a [ii]). Unlike *HTRA1*, maximum alteration occurred in lung cancer for *HTRA2* and its protein partners, while less occurred in metastatic breast cancer. Whereas the alteration in SCC dominantly resulted from mutations (74.36% mutation) for *HTRA1* and its protein partners, and the alteration in gastric cancer from deep deletion (53.06% deep deletion, 0.68% mutation, 11.56% amplification, and 8.16% multiple alterations) for *HTRA2* and its protein partners.

Following that, we explored the OncoPrint feature to identify how genomic alterations are distributed through the *HTRA1* and its protein partners gene for the cutaneous squamous cell carcinoma (SCC) and through the *HTRA2* and its protein partners gene for lung cancer (Figure 6b [i, ii]). The fluctuation rate of genetic changes is substantially higher in the *HTRA1* and its protein partner genes than the *HTRA2* and its protein partner genes. The alterations mainly take place in the *CFH* (alteration frequency of 62%) and *ACAN* (alteration frequency of 44%) genes for the *HTRA1* and its protein partners genes (Figure 6b [i]). In comparison, the alteration dominantly occurred in the *XIAP* (alteration frequency of 47%) and *TAF2* (alteration frequency of 5%) for the *HTRA2* and its protein partners genes (Figure 6b [ii]).

Assessment of survival and prognosis of *HTRA1* and *HTRA2*

We have carried out the study with the use of various web-based tools, notably PrognoScan, R2, Kaplan-Meier Plotter, and OncoLnc, to examine the link between gene expression and prognostic value of *HTRA1* and *HTRA2*. We found a positive association between *HTRA1* and *HTRA2* upregulation with poor survival in pancreatic, kidney, colon, and rectum cancer (Figure 7a [iv, v, vii, viii]). On the contrary, the downregulation was positively linked to its high overall survival (OS) in the ovarian and kidney, as well as high relapse-free survival (RFS) in colon and rectum cancer (Figure 7a [iii, v, vii, viii]). In addition, there was a positive correlation between *HTRA1* downregulation with low overall survival in the brain and skin cancer, as well as relapse-free survival (RFS) in blood cancer (Figure 7a [i, vi, ii]). It should be emphasized that, in comparison with skin cancer, poor survival was worse in brain and blood cancer (Figure 7a [vi, i, ii]).

Depending on the samples and analyses, the Oncomine database revealed that the *HTRA1* is upregulated as well as downregulated in certain cancers, including the brain, breast, and kidney cancer (Figure 1a). We observed that most cancers have extremely distinct correlations with the expression of *HTRA2* and its effect on a patient's prognosis than with *HTRA1*. We found a substantial positive relationship between the *HTRA2* upregulation with poor survival in pancreatic, skin, and colon cancer (Figure 7b [iii, iv, vi, vii]). On the other hand, the upregulation of *HTRA2* was strongly linked to a high survival rate in the brain, blood, kidney, and breast cancer (Figure 7b [i, ii, v, viii]). In contrast to *HTRA1*, the Oncomine database could not supply particular patterns for

HTRA2 expression (Fig. 1a), even though the survival analysis showed both upregulation and downregulation in certain cancer types, namely the brain, blood, pancreatic, ovarian, and skin cancer.

To understand more about clinical outcomes associated with both genes, we used the PrognoScan database to look at the prognostic characteristics of *HTRA1* and *HTRA2* expression patterns for various cancer tissues. This study discovered that *HTRA1* was linked to a poor prognosis in brain and blood cancer and that its underexpression was substantially correlated with poor survival. We cross-checked the *HTRA1* expression level in brain and blood cells; the upregulation of these genes according to the Oncomine platform was confirmed through the GEPIA database.

We found the overexpression of *HTRA2* to be related to a very poor prognosis in skin cancer. The *HTRA2* underexpression was linked to high OS against this cancer. On the other hand, upregulation of *HTRA2* in breast cancer was linked to the high OS, while the downregulation of *HTRA2* was linked to the poor OS in this cancer. Regarding the expression and survival pattern of *HTRA1*, *HTRA2*, and their interacting protein partners, this result could assist us in understanding the fundamental biological targets for the prognosis of different types of cancer.

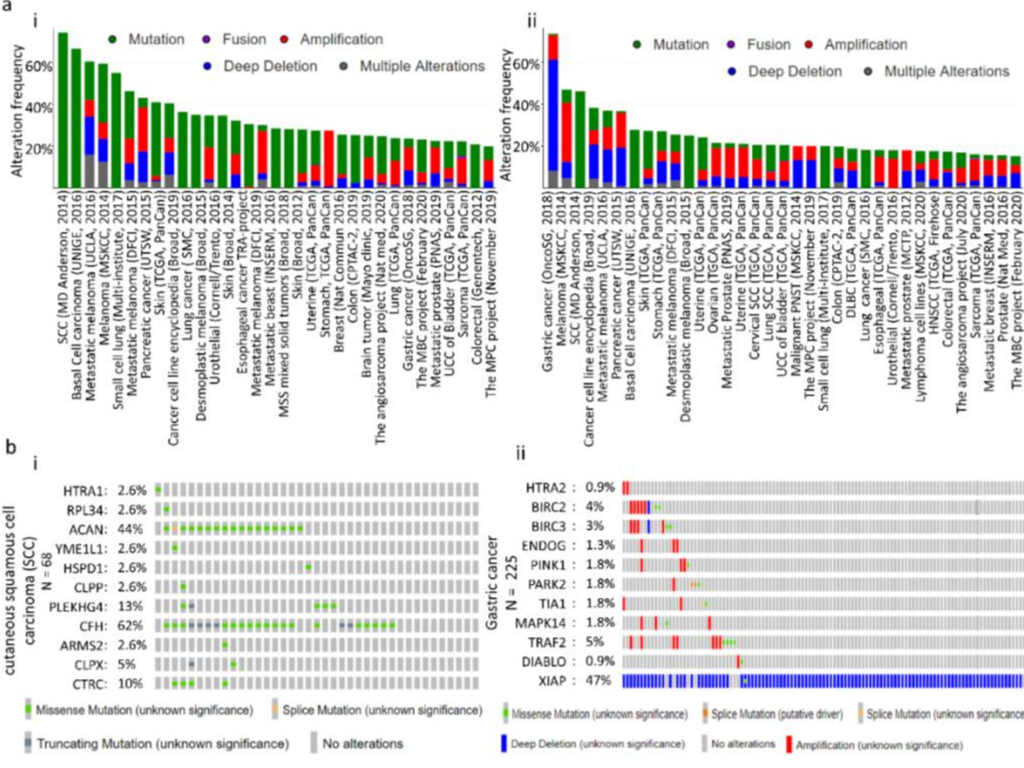

Figure 6. (a) Frequency of genetic changes in *HTRA1*-centered-signature-gene (*HTRA1, RPL34, ACAN, YME1L1, HSPD1, CLPP, PLEKHG4, CFH, ARMS2, CLPX, CTRC*) was retrieved from cBioPortal database, which includes a minimum of 20% alterations. The color of the bar indicates the alteration types. Green color indicates mutation, purple indicates fusion, red indicates amplification, blue indicates deep deletion, and grey indicates multiple alterations in the alteration frequency (i). The alteration frequency of *HTRA2*-centered-signature-gene (*HTRA2, BIRC2, BIRC3, ENDOG, PINK1, PARK2, TIA1, MAPK14, TRAF2, DIABLO, XIAP*) was retrieved from cBioPortal database which includes a minimum of 15% alterations. The color of the bar indicates the alteration types. Green color indicates mutation, purple indicates fusion, red indicates amplification, blue indicates deep deletion, and grey indicates multiple alterations in the alteration frequency (ii). (b) The *HTRA1*-centered-gene-signature alterations most commonly occur in cutaneous squamous cell carcinoma (SCC). We explored the OncoPrint feature to identify how genomic alterations are distributed through the *HTRA1* and its protein partners gene within cutaneous squamous cell carcinoma. A missense mutation (green), splice fusion (yellow), truncating mutation (purple), and no alterations (grey) were included in the alteration frequency (i). The

HTRA2-centered-gene-signature alterations most commonly occur in gastric cancer. We explored the OncoPrint feature in order to identify how genomic alterations are distributed through the *HTRA1* and its protein partners gene within gastric cancer. A missense mutation (green), splice mutation (yellow), deep deletion (blue), amplification (red), and no alterations (grey) were included in the alteration frequency (ii).

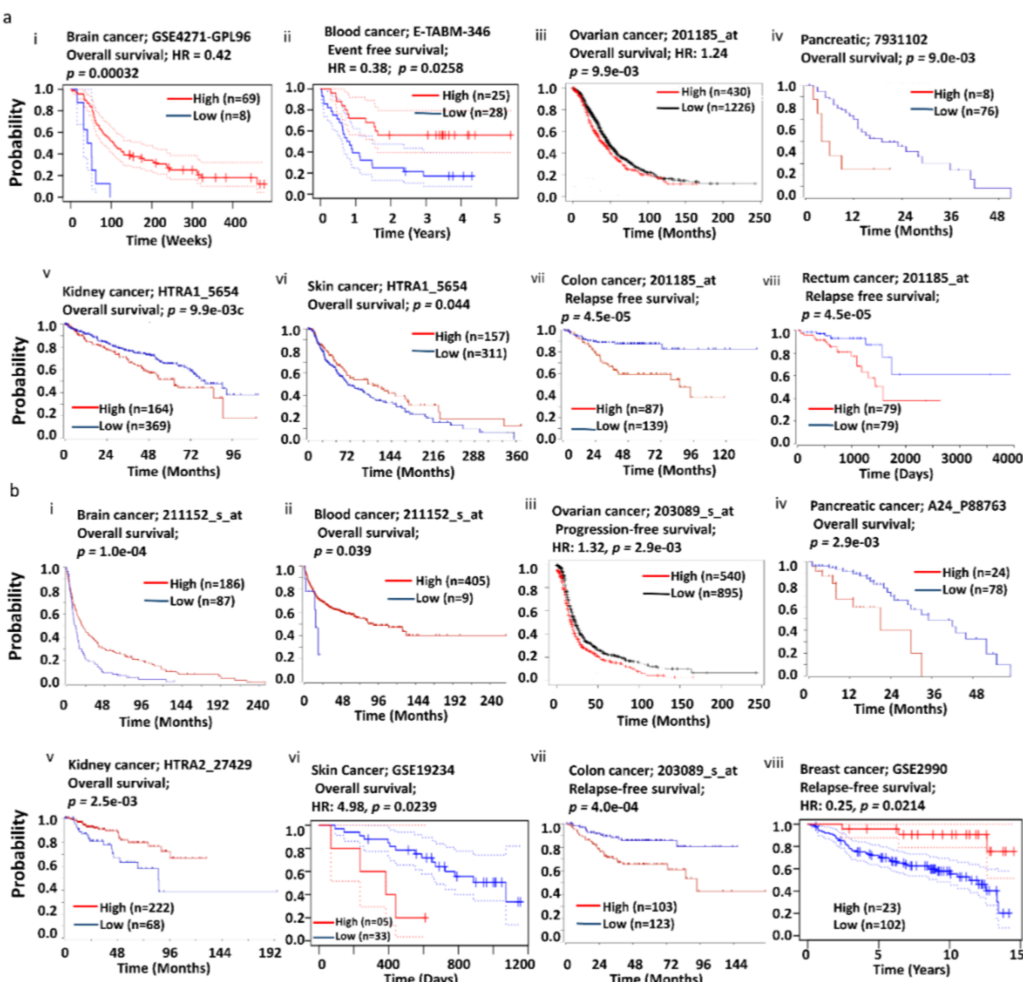

Figure 7. *HTRA1* and *HTRA2* gene expression with their clinical significance. (a) Survival plot generated from PrognoScan with *HTRA1* expression pattern (red, high expression; blue, low expression) for (i) brain cancer, (ii) blood cancer. Survival plot generated from Kaplan-Meier Plotter with *HTRA1* expression pattern (red, high expression; black, low expression) for (iii) ovarian cancer. Survival plot generated from R2: Kaplan Meier scanner with *HTRA1* expression pattern (red, high expression; blue, low expression) for (vi) Pancreatic cancer, (v) kidney cancer, (vii) skin cancer, (vii) colon cancer. Survival plot generated from OncoLnc with *HTRA1* expression pattern (red, high expression; blue, low expression) for (ix) rectum cancer. The analysis threshold was Cox p <0.05 for all databases. (b) Survival plot generated from R2: Kaplan Meier scanner with *HTRA2* expression pattern (red, high expression; blue, low expression) for (i) brain cancer, (ii) blood cancer, (iii) pancreatic cancer, (v) kidney cancer, (vii) colon cancer. Survival plot generated from Kaplan-Meier plotter with *HTRA2* expression pattern (red, high expression; black, low expression) for (iii) ovarian cancer. Survival plot generated from PrognoScan with *HTRA2* expression pattern (red, high expression; blue, low expression) for (vi) skin cancer, (viii) breast cancer. The analysis threshold was Cox p <0.05 for all databases.

Pathways and functional gene ontology

Gene expressed with other genes in a signal transduction pathway plays an important role in developing human cancer [58,59]. At this point, we found some genes that are correlated positively and negatively with *HTRA1* and *HTRA2*. To find correlated genes in selected cancers, we performed a systematic study utilizing the R2 platform. Based on the overexpression nature retrieved from the Oncomine database, we selected two sets

of cancer, each set containing the top four cancer for both *HTRA1* and *HTRA2*. In the R2 database, we first selected the gene expression data for different types of cancer. Based on upregulation, the following four cancer forms for *HTRA1* were individually considered: leukemia, lymphoma, breast, and pancreatic cancer. In this study, the threshold was designed to a p-value < 0.01, and the correction was a false discovery rate (FDR) correction. We found that 819, 3573, 3239, and 965 genes were positively correlated with *HTRA1* in leukemia, lymphoma, breast, and pancreatic cancer, respectively. A gene cluster of our positively correlated genes (henceforth mentioned as "*HTRA1*-correlated gene cluster") with *HTRA1* was common in four types of cancer (Figure 8a [i]). No common genes have correlated negatively with *HTRA1* in these four Cancer types (Figure 8b [i]).

Next, we conducted a similar study for *HTRA2*, considering lymphoma, kidney, skin, and pancreatic cancer on overexpression. We observed that 165, 2029, 3578, and 2882 genes were positively correlated with *HTRA2* in lymphoma, kidney, skin, and pancreatic cancer, respectively. Compared to *HTRA1*, there were 23 positively correlated genes (henceforth mentioned as "*HTRA2*-correlated gene cluster") with *HTRA2* were common in four types of cancer (Figure 8b [i]). Furthermore, in all cancers studied, a shortlist of three genes correlated negatively with *HTRA2* (Figure 8b [ii]). The above correlation study showed that *HTRA1* and *HTRA2* exhibit distinct patterns of correlation with many other genes in selected cancers. The findings indicate that *HTRA1* and *HTRA2* are involved in different similar regulatory pathways in the genes.

After, we conducted gene ontology (GO) and pathway analysis for both *HTRA1*-correlated gene clusters and *HTRA2*-correlated genes that contribute to the progression of different cancers in humans using a web-based tool, Enrichr. We included results from three databases for pathway analysis (Figure 9a, 9b).

We observed various significant pathways for the *HTRA1*-correlated gene cluster, including N-glycan biosynthesis, breast cancer, protein processing in the endoplasmic reticulum, human papillomavirus infection, and cancer pathway, etc. in KEGG human 2019 database (Figure 9a [i]). Likewise, Reactome pathways 2016 showed the pathways related to N-glycan trimming and elongation in the cis-Golgi, intra-Golgi traffic, signaling pathways occurred by NOTCH1, degradation of extracellular matrix, etc. (Figure 9a [ii]). Analysis of Panther pathway 2016 also showed the notch signaling like Reactome 2016 (Figure 9a [iii]). For the *HTRA2*-correlated gene cluster, the study of KEGG human 2019 showed several critical pathways, including spliceosome, necroptosis, RNA polymerase, proteasome, drug metabolism, etc. (Figure 9b [i]). The Reactome pathways 2016 showed the pathways related to mRNA splicing, major and minor mRNA splicing, processing of capped intron-containing pre-mRNA, pathways associated with HIV infection, etc. (Figure 9b [ii]). Analysis of Panther pathway 2016 showed the pathways including FAS signaling, ubiquitin-proteasome, and Wnt signaling pathway (Figure 9b [iii]).

Next, we analyzed the GO (gene ontology) for both *HTRA1* and *HTRA2* gene clusters. For the *HTRA1*-correlated gene cluster, the GO biological process 2018 mainly includes epithelial to mesenchymal transition involved in endocardial cushion formation, atrioventricular valve development, pulmonary valve development, negative regulation of androgen receptor signaling pathway, etc. (Figure 9a [iv]). Satellite DNA binding, mannosyl-oligosaccharide mannosidase activity, transcription factor activity, serine-type peptidase activity, etc., significant functions are suggested by GO molecular function 2018 (Figure 9a [v]). The GO cellular component 2018 was most significantly associated with an integral part of the Golgi membrane, Golgi sub-compartment, etc. (Figure 9a [vi]). Last, we analyzed the GO process of human cancer by using the list of co-expressed genes

of *HTRA2*. In this instance, we observed that the *HTRA2*-correlated gene was most significantly associated with regulation of cell death, regulation of mitochondrial autophagy, ubiquitin-dependent ERAD pathway, RNA and mRNA splicing, mitotic nuclear division, etc. in the case of GO biological process 2018 (Figure 9b [iv]). GO molecular function 2018 suggested ubiquitin-binding, RNA binding, coupled ATPase activity, interleukin-1 binding, annealing helicase activity, etc. (Figure 9b [v]). The GO cellular component 2018 was most significantly associated with mitochondrial intermembrane space, mitochondrial envelop, DNA-directed RNA polymerase II, nuclear proteasome complex, death-inducing signaling complex, cytosolic proteasome complex, and CD40 receptor complex, etc. (Figure 9b [vi]).

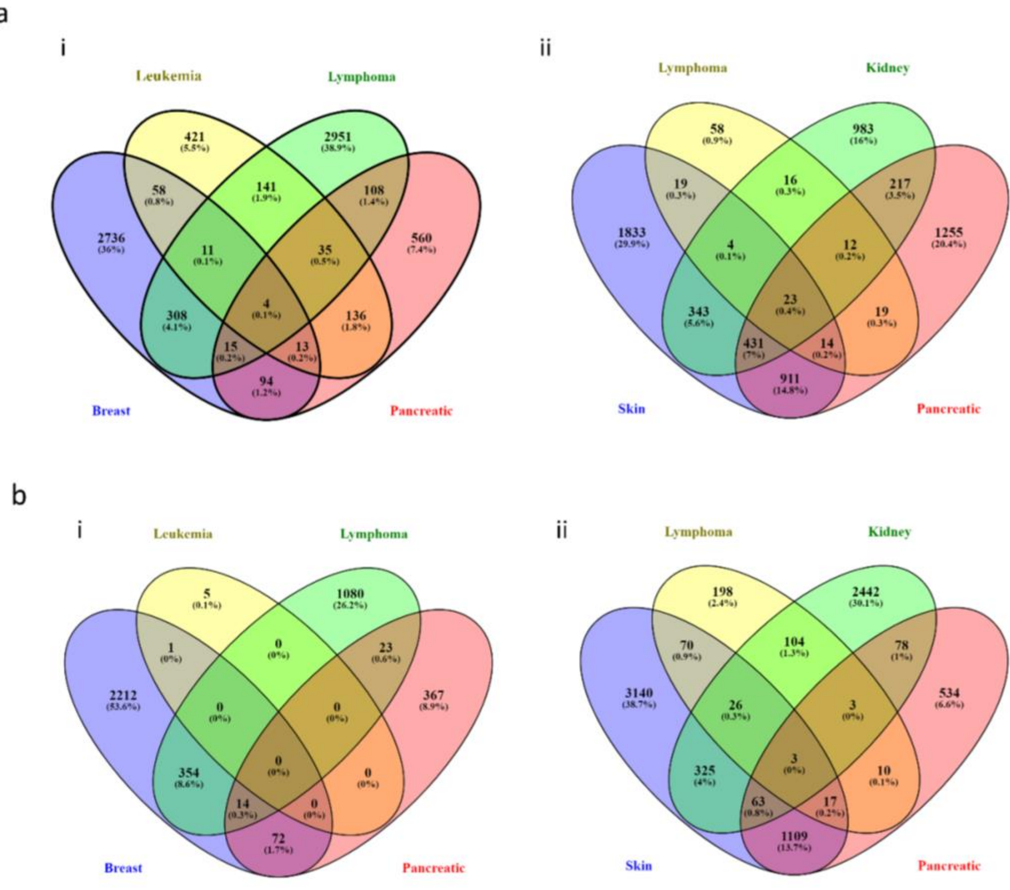

Figure 8. Study of the correlated gene in selected four types of cancer. These correlated genes were obtained from the R2 database. Venny 2.1 was used to create the Venn diagram for identifying the common genes. (a) The Venn diagram shows the genes positively correlated with *HTRA1* in leukemia, lymphoma, breast, and pancreatic cancer (i). The Venn diagram shows the genes positively associated with *HTRA2* in lymphoma, skin, kidney, and pancreatic cancer (ii). (b) Venn diagram of the negatively correlated gene with *HTRA1* (i) and *HTRA2* (ii) in selected four cancers.

Figure 9. Pathways and gene ontologies related to *HTRA1*, *HTRA2*, and correlated genes. Pathways are obtained from Enrichr online database. The color gradient and the bar length reflect the significance level of the study (brighter color represents more significance). (a) GO and pathways of *HTRA1*. (i) KEGG human 2019, (ii) Reactome 2016, (iii) Panther 2016, (iv) GO biological process 2018, (v) GO molecular function 2018, and (vi) GO cellular component 2018. (b) GO and pathways of *HTRA2*. (i) KEGG human 2019, (ii) Reactome 2016, (iii) Panther 2016, (iv) GO biological process 2018, (v) GO molecular function 2018, and (vi) GO cellular component 2018.

DISCUSSION

Numerous investigations demonstrated that *HTRA* serine proteases contribute significantly to the development of different types of human cancer [43,44,60,61]. *HTRA1* can be used as a promising tissue marker in various cancer [62]. Mitochondrial homeostasis is regulated by *HTRA2* and plays a critical role in apoptosis induction [55]. Also, multiple studies have contended that targeted therapies controlled by *HTRA1* and *HTRA2* are promising molecular medicines that prevent the growth of human cancer [17,19,63]. However, *HTRA1* and *HTRA2* are yet unrecognized in their function in the progression and prognosis of human cancer. To understand the role of *HTRA1* and *HTRA2* in the progression and prognosis of human cancer, we conducted a systemic data mining study on several gene expression datasets of publicly available databases. In cancer and their respective normal tissues, *HTRA1* and *HTRA2* have been found to be differentially expressed. According to Oncomine and GEPIA-based mRNA transcript analysis, we found that the degree of their expression of these genes was also different depending on the tissue. Based on Oncomine data, we discovered that *HTRA1* was overexpressed in various cancer, including the brain, B-cell, breast, pancreatic, and kidney cancers, but was underexpressed in ovarian, skin, colon, and rectum cancers compared to that in normal tissue.

On the contrary, *HTRA2* was overexpressed in the brain, plasma cells, pancreatic, head and neck, kidney, skin, colon, and breast cancers. Still, it was underexpressed in ovarian cancer relative to respective normal tissues. In addition, we found the same result of expression for both genes in these cancers when we validated the Oncomine data through the GEPIA database.

Next, we examined the mutation and copy number alterations (CNAs) of both the *HTRA1* and *HTRA2* genes to evaluate the impact of CNAs on the development of cancer. Many human cancers are related to CNAs, and it plays an essential role in cancer development [64,65]. Thus, this structural aberration can be employed to develop molecular cancer therapeutics. Earlier studies have demonstrated that clinical outcomes in several cancers, including breast, pancreatic, colorectal cancer, etc., were linked to the structural changes in genes like *PIK3CA*, *PTEN*, *KRAS*, *NRAS*, *TP53*, etc. [66–69]. Instead of these studies, the essential roles of copy number alterations and mutation for a gene in the prognosis of human cancer remain unclear. Therefore, to assess CNAs and mutations related to human cancer for both *HTRA1* and *HTRA2* genes, we used the cBioPortal web. There were 219 mutations found in *HTRA1*, including missense, truncation, fusion, and inframe mutations. Most of the mutations spanned over the PDZ domain and appeared mainly in skin, breast, prostate, and colorectal cancer. We also found a mutation hotspot in V433I/A. Unlike *HTRA1*, the mutation in *HTRA2* includes missense, truncating, and in-frame mutation. These mutations primarily appeared in the prostate, endometrium, uterine and colorectal cancer.

The mutation hotspot *A116Qfs*93* is localized from 100 to 150 amino acid residues. The degree of mutation in HTRA2 was significantly different than in *HTRA1* [70,71]. Such mutations may contribute to the regulation of cancer growth and prognosis, although this remains to be confirmed. Most of the activities in the biological system, cell-cell signal transduction, and disease progression are controlled by protein-protein interactions (PPIs) [72]. Firstly, we identified the eleven gene signature significantly correlated with *HTRA1* and *HTRA2* using STRING. In subsequent analysis through cBioPortal, we found that the genetic alterations in the HTRA1-centered gene mainly occurred in cutaneous squamous cell carcinoma (SCC) with an alteration frequency of 20%-74.36%. The alterations in SCC primarily occurred due to mutation [73,74]. On the contrary, the genetic alterations in the *HTRA2*-centered gene predominantly occurred in gastric cancer

with an alteration frequency of 15.19%-73.47%. Unlike *HTRA1*, the alterations in gastric cancer mainly occurred due to mutation, amplification, multiple alterations, and deep deletion [75].

Signaling pathways of the human body play an essential role in the biological process, including cell proliferation, cell development, cancer development, etc. [58]. Previous studies demonstrated that signaling pathways like the Wnt pathway, Notch pathway, TGF-beta pathway, etc., were associated with cancer [76–78]. Therefore, we used the R2 platform to identify the significantly correlated gene with *HTRA1* and *HTRA2* for pathway and GO analysis in certain selected cancers. These *HTRA* serine proteases are overexpressed highly. For *HTRA1*, we found a significant number of genes with a positive correlation in four types of cancer, namely leukemia, lymphoma, breast, and pancreatic cancer. Among these genes, four were common in all kinds of cancer. For *HTRA2*, positively correlated genes were found in leukemia, kidney, skin, and pancreatic cancers; among these, 23 genes are common in all cancers. The common genes were identified using Venny 2.1. We used an online platform, Enrichr, for these common genes for functional enrichment, pathway, and GO analysis. We found that the pathways related to *HTRA1* and *HTRA2* were significantly different. For *HTRA1*, we observed several pathways, including N-glycan biosynthesis, breast cancer, signaling pathways that occurred by NOTCH1, etc., and the common genes were most notable linked with epithelial to mesenchymal transition in biological process, satellite DNA binding in molecular function, and integral component of Golgi membrane in cellular components [79]. For *HTRA2*, we found many important pathways, including spliceosome, mRNA splicing, FAS signaling, ubiquitin-proteasome, etc., which were significantly dissimilar from *HTRA2* related pathways [80].

Then, we examined the prognostic value of both genes based on expression levels of *HTRA1* and *HTRA2* in various selected cancers using publicly available online tools such as Kaplan-Meier plotter, R2, PrognoScan, and OncoLnc. We observed that a high expression level of *HTRA1* was linked to poor survival in pancreatic, kidney, and colon cancers. The overexpression of *HTRA1* resulted in high survival in brain, blood, and skin cancer. On the contrary, the high expression of *HTRA2* was linked to a poor prognosis in pancreatic, skin, and colon cancer. But high *HTRA1* expression was linked to high survival in brain, blood, kidney, and breast cancer. Some of the findings of our investigation are also supported by previous studies [81–83]. Thus. We concluded that *HTRA1* shows the oncogenic feature in pancreatic, kidney, and colon cancers. Likewise, *HTRA2* plays an oncogenic role in different types of cancer, including pancreatic, skin, and colon cancers.

On the other hand, *HTRA1* plays an anti-oncogenic role in the brain, blood, and skin cancer. *HTRA2* exhibits an anti-oncogenic feature in the brain, blood, kidney, and breast cancer. It is worth mentioning that both *HTRA1* and *HTRA2* show oncogenic effects in pancreatic and colon cancer and exhibit anti-oncogenic features in brain and blood cancer. Previous studies also supported these results [84].

In summary, we investigated and assessed the expression, mutation status, and copy number alteration patterns of *HTRA1* and *HTRA2* genes using a comprehensive statistical analysis, including freely available expression and medical evidence. For different types of cancer, this study was only capable of predicting the expression level of both genes. The findings of this study suggest that these serine proteases can be transformed into medical practices and may impact the clinical outcomes of certain cancers.

CONCLUSION

According to the study, we utilized various web-based bioinformatics tools and software to investigate the expression pattern, promoter methylation of genes, protein-protein interaction (PPI) partners, linked genes, and patient prognosis related to the expression of other *HTRA1* and *HTRA2* genes in different types of human cancers. This study showed that the expression of *HTRA1* and *HTRA2* affect the patient's survival and clinical outcomes in different ways. Our multi-omics analysis uncovered that *HTRA1* and *HTRA2* play a role in cancer improvement and differentially modulate cancer's clinical outcomes. Whereas *HTRA1* in individuals with pancreatic, kidney, and colon cancers might be targeted for cancer therapy, *HTRA2* could be used as a prognostic biomarker for skin, pancreatic, and colon cancers. These two genes were previously explored separately in only a few human cancers. We have tried to look at the effect of these genes in the highest number of cancers from publicly available data using different bioinformatics tools. We also tried to determine if both genes would act as biomarkers in the same type of cancer. We found that these two genes can serve as prognostic biomarkers for various cancers separately. Meantime, both *HTRA1* and *HTRA2* are possible targets for colon and pancreatic cancer.

In conclusion, our multi-omics findings could help us better understand the link between *HTRA1* and *HTRA2* expression and clinical prognosis. They also give fresh insights into cancer's molecular pathways, assisting in translating genetic information into treatment. The findings of this study offer some insight into the molecular and clinical features of various cancers and hence might be utilized to aid in the translation of genetic information into cancer therapy. More practical, exploratory, and clinical tests may be required in the future to approve these results since overfit and underfit outcomes may appear in bioinformatics research.

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AUTHOR CONTRIBUTIONS

AR and MAHMJ designed the research project. AR analyzed the data and drafted the paper. RAH wrote the PPI result of the article. The manuscript was evaluated and modified by MAHMJ, SA, MSR, and MTET. MAHMJ supervised the research study. The text was edited and approved for final submission by all authors.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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