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RESEARCH ARTICLE

TRANSLATIONAL AND CLINICAL RESEARCH

CTHRC1 is associated with BRAF(V600E) mutation and correlates with prognosis, immune cell infiltration, and drug resistance in colon cancer, thyroid cancer, and melanoma

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ABSTRACT

Colon cancer, thyroid cancer, and melanoma are common malignant tumors that seriously threaten human health globally. The B-Raf proto-oncogene, serine/threonine kinase (BRAF)(V600E) mutation is an important driver gene mutation in these cancer types. In this study, we identified that collagen triple helix repeat containing 1 (CTHRC1) expression was associated with the BRAF(V600E) mutation in colon cancer, thyroid cancer, and melanoma. Based on database analysis and clinical tissue studies, CTHRC1 was verified to correlate with poor prognosis and worse clinicopathological features in colon cancer and thyroid cancer patients, but not in patients with melanoma. Several signaling pathways, immune cell infiltration, and immunotherapy markers were associated with CTHRC1 expression. Additionally, a high level of CTHRC1 was correlated with decreased sensitivity to antitumor drugs (vemurafenib, PLX-4720, dabrafenib, and SB-590885) targeting the BRAF(V600E) mutation. This study provides evidence of a significant correlation between CTHRC1 and the BRAF(V600E) mutation, suggesting its potential utility as a diagnostic and prognostic biomarker in human colon cancer, thyroid cancer, and melanoma.

Keywords: CTHRC1; BRAF(V600E); colon cancer; thyroid cancer; melanoma.

INTRODUCTION

Colon cancer is the most common malignant gastrointestinal tumor, leading cause of cancer-related death worldwide [1]. The five-year survival rate of patients with colon cancer is only 65% [2]. Thyroid cancer, especially papillary thyroid carcinoma [3], is one of most general malignant tumors in endocrine system, and its incidence rate has been steadily upward currently [4]. Melanoma is a cutaneous cancer caused by malignant melanocytes, ranks fifth in men and sixth in women among all cancers [5], with an increasing incidence rate in recent years [6]. Melanoma is highly malignant tumor with poor prognosis [7]. BRAF is a kind of serine/threonine protein kinase that activates the MAP kinase/ERK signaling pathway [2, 8], which acts as a driver gene in colon cancer, thyroid cancer and melanoma [9]. The main mutation type of BRAF is BRAF(V600E) [10]. BRAF(V600E) mutation has been found in 8-10% of metastatic colon cancer patients [11]; approximately half of papillary thyroid carcinomas and melanomas exhibit BRAF(V600E) mutation [12]. However, BRAF mutation is rarely observed in other type of human cancers. BRAF and MEK inhibitors (BRAFi and MEKi) represent a breakthrough in the treatment of BRAF(V600E)-mutant cancers, greatly improving outcomes for cancer patients [13, 14]. In melanoma, immunotherapy targeting BRAF(V600E) mutation represents a promising treatment option, especially for melanoma patients with traditional drug resistance [14, 15]. Besides traditional treatment including surgery, radioactive iodine and TSH suppression, BRAF(V600E) targeted therapy is another choice for thyroid cancer patients with poor prognosis [16, 17]. However, it arises another headache that primary and acquired drug resistance seriously impairs the effectiveness of

BRAF(V600E) targeted therapy [18]. Therefore, it is desirable to study the deep molecular mechanisms involved in BRAF(V600E) mutation of human colon cancer, thyroid cancer and melanoma.

Collagen triple helix repeat containing 1 (CTHRC1), locates in human chromosome 8q22.3, encodes an extracellular matrix glycoprotein with a molecular weight of 28 kDa [19, 20]. CTHRC1 was reported to play critical roles in various kinds of human cancers. Over-expression of CTHRC1 increased migration and invasion of ovarian cancer cells by activating the EGFR/ERK1/2/AKT signaling pathway [21]. Elevated CTHRC1 was demonstrated to be an independent factor of worse prognosis in gastric cancer [22, 23]. CTHRC1 promoted cell proliferation and invasion through PI3K/Akt signaling, and was a biomarker for prognosis prediction in bladder cancer [24, 25]. In colon cancer, CTHRC1 was proved to promote proliferation, migration and invasion via activation of Wnt/PCP pathway [26]. In papillary thyroid carcinoma, CTHRC1 was demonstrated to be correlated with tumor occurrence and malignant transformation, and it might play an crucial role in regulating EMT [27]. CTHRC1 has been reported to be overexpressed in melanoma cells, leading to melanoma metastasis [28, 29]. However, the systematical prognosis analysis of CTHRC1 and its relation with BRAF(V600E) mutation in human colon cancer, thyroid cancer and melanoma still need to be further carried out.

In this study, through database analysis and clinical tissue study, we demonstrated that expression of CTHRC1 was significantly associated with BRAF(V600E) mutation in colon cancer, thyroid cancer and melanoma. CTHRC1 expression was identified to be negatively

correlated with the overall survival (OS) rate in colon cancer and thyroid cancer patients and positively correlated with the OS rate in melanoma patients. Clinicopathological features including tumor infiltration depth/clinical stage, tumor size/lymph node metastasis/clinical stage was positively correlated with CTHRC1 expression levels in colon cancer and thyroid cancer respectively. Several signal pathways, immune cell infiltration and immunotherapy markers correlated to CTHRC1 expression in colon cancer and thyroid cancer, but not as significantly in melanoma. In clinical tissue study, colon cancer, thyroid cancer patients with BRAF(V600E) mutation showed elevated CTHRC1 expression and immune cell infiltrating. Moreover, high CTHRC1 expression level correlated with decreased sensitivity of BRAF(V600E) mutation targeted drugs. Therefore, we provided evidence that CTHRC1 was associated with BRAF(V600E) mutation, and could be used as a diagnostic and prognostic biomarker in human colon cancer, thyroid cancer and melanoma.

MATERIALS AND METHODS

Data collection based on TCGA database

Transcriptome RNA-seq data of colon cancer, thyroid cancer and melanoma samples with corresponding clinical characteristics, survival data and somatic mutation was collected from TCGA database. Cases with missing or flawed information were excluded. Therefore, 454 colon cancer samples, 476 thyroid cancer samples and 423 melanoma samples were involved in this study.

Correlation analysis of gene expression with BRAF(V600E) mutation

Correlation analysis of gene expression with BRAF(V600E) mutation was carried out essentially as described formerly by using limma (Version 3.52.3; http://www.bioconductor.org/ packages/release/bioc/html/limma.html) package of R software, ggplot2 R package (Version 3.3.6; https://cran.r-project.org/web/packages/ggplot2) and TIMER database-TIMER2.0 (http://timer. cistrome.org/) [30, 31]. For different gene expression analysis in BRAF(V600E) mutant samples compared with wild-type samples, log fold change (logFC) > 0.5 was considered statistically significant.

Clinical samples

In this study, 50 colon cancer, 50 thyroid cancer and 50 melanoma paraffin-embedded tissues were collected from the Department of Pathology, the First Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China). In the 50 colon cancer tissues, 15 of them were BRAF(V600E) mutant and 35 of them were wild-type (WT); in the 50 thyroid cancer tissues, 30 of them were BRAF(V600E) mutant and 20 of them were wild-type; in the 50 melanoma tissues, 20 of them were BRAF(V600E) mutant and 30 of them were wild-type. These tissue samples were from patients who underwent surgical resection between 2016 and 2021. The clinicopathological parameters (including age, gender, tumor size, tumor infiltration depth, lymph node metastasis, distant metastasis and clinical stage) of these patients were also collected. We performed this work in accordance with the World Medical Association's Code of Ethics (Declaration of Helsinki). This work has been approved by the Institutional Review Board of Anhui Medical University and informed consent has been obtained from all patients.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from paraffin-embedded tissues using a RNA-isolation kit (Thermo Fisher Scientific, USA). The mRNA levels of CTHRC1 were examined by qRT-PCR using SYBR green Master MIX (Applied Biosystem) essentially as described earlier [32, 33]. GAPDH was examined as an endogenous control. The primer sequences were as follows: CTHRC1: 5'- TCATCGCACTTCTTCTGTGGA -3' (forward) and 5'- GCCAACCCAGATAGCAACATC -3' (reverse) [32]; GAPDH: 5'- TGGCCATTATAGGACCGAGACTT -3' (forward) and 5'- CACCCTGTTGCTGTAGCCAAA -3' (reverse).

Overall survival (OS) rate and ROC curve analysis

Overall survival (OS) rates of colon cancer, thyroid cancer and melanoma patients were derived from the TCGA-Clinical Data Resource (CDR). Kaplan-Meier curves were analyzed by the "survival" packages in R. The optimal cut point for CTHRC1 was determined using the R package "survminer" based on the time of death of patients with colon cancer, thyroid cancer and melanoma.

The area under the curve (AUC) on the ROC curve was calculated and plotted to evaluate the diagnostic effect of CTHRC1 in colon cancer, thyroid cancer and melanoma respectively.

COX Regression Analysis

We used R version 4.1.2 software, the survival and survminer packages for COX regression analysis. Univariate COX regression analysis was performed to evaluate independent prognostic factors.

Protein-Protein Interaction (PPI) and molecular pathway enrichment analysis

STRING database was used for construction of protein-protein interaction (PPI) network of CTHRC1. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were used to reveal the molecular pathways related with CTHRC1 in colon cancer, thyroid cancer and melanoma respectively.

Correlation analysis of CTHRC1 expression with immune cells infiltrating

We collected data and analyze the correlation of CTHRC1 expression with immune cells infiltrating based on The TIMER database (TIMER2.0 (cistrome.org)). R version 4.1.2 software with ggplot2, tidyverse (Version: 1.3.2) and reshape2 (Version: 1.4.4) packages were used appropriately [35].

Immunohistochemistry

Immunohistochemistry (IHC) was performed to examine the protein levels of CTHRC1, Ki-67 and immune cell markers CD4, CD8, CD68 and CD69 essentially as described in previous studies by using the UltraSensitive-SP kit (Maixin-Bio, Fuzhou, China) [32]. CTHRC1 rabbit polyclonal antibody (Proteintech Group, Inc, Chicago, USA, 1:100), Ki-67, CD4, CD8, CD68 mouse monoclonal antibodies (Maixin-Bio, Fuzhou, China, 1:1), CD69 rabbit monoclonal antibody (Abcam, Cambridge, UK, 1:500) were used respectively. Stained sections were evaluated independently by two senior pathologists using an Olympus microscope (Olympus America, Inc., Melville, NY).

Correlation analysis of CTHRC1 expression with drug sensitivity

Gene expression and drug susceptibility data for the same cancer samples were collected based on the CellMiner database [36]. Data out of Clinical laboratory validation and FDA standard certification were excluded. Pearson correlation test was performed to analyze the correlation between CTHRC1 expression and respective drug sensitivity.

Ethical statement

The studies involving human participants were reviewed and approved by the ethics committee of Anhui Medical University. The patients/participants provided their informed consent to participate in this study.

Statistical analysis

We performed statistical analysis using SPSS 20.0 and R version 4.1.2 software. Proportional risk hypothesis testing and fitted survival regressions were performed in the survival analysis using the survival package, and the results were visualized using the survminer package as well as the ggplot2 package. AUC analysis for evaluating the prognostic diagnostic performance of CTHRC1 for COAD, THCA and SKCM. The Kolmogorov-Smirnov test was used to test the normality of the distribution. T-tests were performed for data that fit a normal distribution, and Mann-Whitney U-tests were performed for data that did not fit a normal distribution or had a small amount of data (sample size less than 30). We used univariate Cox regression analysis, in which we classified a variety of continuous variables based on pathological examination of gross specimens after surgery to determine the TNM stage of the patient's tumor, as well as the clinical approach to tumor staging. For example, colon cancer and melanoma are prevalent

around 50-55 years of age, and thyroid cancer is prevalent above 45 years of age. In pathology, the depth of infiltration of colon cancer T1-T2 indicates that the tumor is confined to the mucosal layer and basal layer, and T3-T4 indicates that the tumor invades to the plasma membrane and subplasma membrane; the T-staging of thyroid cancer focuses on the volume of the tumor, with T1-T2 indicating that the tumor volume is less than 4 cm and confined to the thyroid gland, and the T3-T4 staging indicating that the tumor volume is greater than 4 cm and accompanied by the invasion of external tissues; and the T1-T2 of melanoma indicates that the diameter of the tumor is less than 5 cm and without metastasis, and T3-T4 indicates tumor diameter greater than 2 cm with metastasis. Clinical staging was based on TNM staging, with grades I-II indicating that the lesion was confined to the primary site without metastasis, and grades III-IV indicating the presence of lymph nodes or distant metastasis along the border of the trunk. we used the minimum cutoff value method of the R package "survminer" to classify high and low CTHRC1 expression. The Pearson chi-square test was used to analyze the differences in clinicopathological parameters between the high and low CTHRC1 expression groups. $P<0.05$ was considered to be statistically significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

RESULTS

Elevated CTHRC1 was associated with BRAF(V600E) mutation in colon cancer, thyroid cancer, and melanoma patients

BRAF(V600E) is a common gene mutation and important in prognosis and treatment options of human colon cancer, thyroid cancer and melanoma. To explore the correlation between BRAF(V600E) and gene differential expression in colon cancer, thyroid cancer and melanoma,

we analyzed gene expression in BRAF(V600E) mutant group and wild-type BRAF group of colon cancer, thyroid cancer and melanoma patients base on TCGA database. As shown in Figure 1A, a lot of dysregulated genes were observed in BRAF(V600E) mutant group compared with wild-type BRAF group of colon cancer, thyroid cancer and melanoma respectively. Over-lapping the elevated gene lists in BRAF(V600E) mutant colon cancer, thyroid cancer and melanoma, 20 genes were screened out to be over-expressed in BRAF(V600E) mutant group of all three tumor types (Figure 1B). Among these 20 genes, TIMP1, IL27RA, LIF, ETV1, CTHRC1 were the top 5 most significantly correlated with BRAF(V600E) mutation in human colon cancer, thyroid cancer and melanoma; and only CTHRC1 was identified to be statistically significantly associated with the prognosis of all three tumor types (data will be shown later) (Figure 1B). Moreover, the correlation between CTHRC1 expression and BRAF(V600E) mutation in respective colon cancer, thyroid cancer and melanoma were further analyzed. As shown in Figure 2A, CTHRC1 expression and BRAF(V600E) mutation were positively correlated in these three kinds of tumors and the correlation coefficients were 0.139 in colon cancer (COAD, $P<0.05$), 0.794 in thyroid cancer (THCA, $P<0.05$) and 0.179 in melanoma (SKCM, $P<0.05$) respectively. Figure 2B showed the expression levels of CTHRC1 were significantly higher in BRAF(V600E) mutant group compared with wild-type BRAF group of colon cancer, thyroid cancer and melanoma based on public database. Moreover, we collected respective clinical tissue samples and examined expression levels of CTHRC1 in colon cancer, thyroid cancer and melanoma with/without BRAF(V600E) mutation by qRT-PCR. As shown in Figure 2C, the RNA levels of CTHRC1

were concordantly higher in tumor tissues with BRAF(V600E) mutation compared with those without BRAF(V600E) mutation in colon cancer, thyroid cancer and melanoma. Therefore, our data suggested elevated CTHRC1 expression was positively associated with BRAF(V600E) mutation in colon cancer, thyroid cancer and melanoma patients.

Association of CTHRC1 expression with prognosis in colon cancer, thyroid cancer, and melanoma patients

For further study, the correlation between CTHRC1 expression and survival rates in colon cancer, thyroid cancer and melanoma patients were analyzed by means of Kaplan-Meier curves based on TCGA database. As shown in Figure 3A, the overall survival (OS) rates of colon cancer patients with high CTHRC1 expression were significantly lower than that of colon cancer patients with low CTHRC1 expression (P=0.035); similarly, it exhibited a consistent trend in thyroid cancer patients (P=0.020) (Figure 3B). However, the OS rates of melanoma patients with high CTHRC1 expression were significantly higher than that of melanoma patients with low CTHRC1 expression (P=0.016) (Figure 3C).

Moreover, we performed univariate COX regression analysis between prognostic risk factors and OS rates in colon cancer, thyroid cancer and melanoma patients respectively. As shown in Table 1, in colon cancer patients, significant correlations were observed between the OS rates and risk factors including depth of tumor infiltration (HR=3.23) (P=0.006), lymph node metastasis (HR=2.73) (P<0.001), tumor distant metastasis (HR=3.4) (P<0.001), clinical stage (HR=3.04) (P<0.001) and CTHRC1 expression level (Low and High) (HR=1.56) (P=0.036); in thyroid cancer patients, there were significant correlations between the OS rates and risk

factors including tumor size (HR=3.07) (P=0.038), clinical stage (HR=7.1) (P<0.001) and CTHRC1 expression level (Low and High) (HR=3.03) (P=0.027) (Table 2); in melanoma patients, there were significant correlations between the OS rates and risk factors including age (HR=1.74) (P<0.001), tumor size (HR=1.96) (P<0.001), lymph node metastasis (HR=1.82) (P<0.001), distant metastasis (HR=1.88) (P=0.043), clinical stage (HR=1.73) (P<0.001) and CTHRC1 expression level (Low and High) (HR=0.71) (P=0.017) (Table 3).

To analyze the diagnostic value of CTHRC1 in colon cancer, thyroid cancer and melanoma patients, we performed ROC curve analysis. As shown in Figure 3D, 3E and 3F, the AUC was 0.977 for diagnosis of colon cancer, 0.654 for diagnosis of thyroid cancer and 0.596 for diagnosis of melanoma.

Therefore, the results of survival analysis showed that CTHRC1 expression was significantly associated with survival in patients with colon cancer, thyroid cancer, and melanoma; the ROC curves indicated that CTHRC1 is an important risk factor for adjuvant diagnosis of these three tumors, with the highest value in colon cancer.

Association of CTHRC1 expression with clinicopathological features in colon cancer, thyroid cancer, and melanoma patients

Next, the association of CTHRC1 expression with clinicopathological parameters in colon cancer, thyroid cancer and melanoma patients (including age, gender, tumor size, tumor infiltration depth, lymph node metastasis, distant metastasis and clinical stage) was analyzed according to the TCGA data. CTHRC1 expression levels were designated as high group and low group by using the "sur.cut" value calculated by "survminer" package as a cutoff

classification. In colon cancer patients, expression of CTHRC1 was positively correlated with tumor infiltration depth (P=0.019) and clinical stage (P=0.034), and there was no significant correlation between CTHRC1 expression and patients' age, gender, lymph node metastasis or distant metastasis (P>0.05) (Table 4). Expression of CTHRC1 was positively correlated with patient tumor size (P<0.001), lymph node metastasis (P<0.001) and clinical stage (P<0.001), and there was no significant correlation between CTHRC1 expression and patients' age, gender or distant metastasis (P>0.05) in patients with thyroid cancer (Table 5). In melanoma patients, CTHRC1 expression was correlated with patient age (P=0.026), and there was no significant correlation between CTHRC1 expression and patients' gender, tumor size, lymph node metastasis, distant metastasis or clinical stage (P>0.05) (Supplementary Table 1).

Furthermore, we collected 50 colon cancer tissues, 50 thyroid cancer tissues and 50 melanoma tissues for clinical analysis from the Department of Pathology, the First Affiliated Hospital of Anhui Medical University, and the RNA levels of CTHRC1 were examined by qRT-PCR. The association of CTHRC1 expression with clinicopathological parameters were also studied. In colon cancer patients, expression of CTHRC1 was positively correlated with patient tumor infiltration depth (P=0.034) and clinical stage (P=0.034), but was not significantly correlated with patients' age, gender, lymph node metastasis or distant metastasis (P>0.05) (Table 6). In thyroid cancer patients, CTHRC1 expression was positively correlated with tumor size (P=0.018) and lymph node metastasis (P=0.045), but there was no significant correlation between CTHRC1 expression and patients' age, gender, distant metastasis or clinical stage (P>0.05) (Table 7). In melanoma patients, CTHRC1 expression was not obviously associated

with patients' age, gender, tumor size, lymph node metastasis, distant metastasis or clinical stage (P>0.05) (Supplementary Table 2).

Collectively, these data suggested that CTHRC1 was associated with poor prognosis in human colon cancer and thyroid cancer, but the association between CTHRC1 and patient prognosis was not significant in human melanoma.

Molecular interactions and signal pathway analysis of CTHRC1

Based on the data from TCGA, molecular interactions and signal pathways related with CTHRC1 were analyzed. As shown in Figure 4A, the potential co-expression genes of CTHRC1 were identified by STRING tool and CTHRC1, DVL1, DVL2, DVL3, FZD3, ROR2, FZD5, FZD6, WNT3A, NTN4, VANGL2 formed a network in human cancers.

Moreover, enrichment analysis showed CTHRC1 was associated with signal pathways including osteoclast differentiation, staphylococcus aureus infection, phagosome, heparin binding, glycosaminoglycan binding, extracellular matrix structural constituent, extracellular matrix component, collagen trimer, collagen-containing extracellular matrix, collagen fibril organization, extracellular structure organization and extracellular matrix organization in colon cancer. In thyroid cancer, CTHRC1 was associated with signal pathways including ECM-receptor interaction, staphylococcus aureus infection, hematopoietic cell lineage, cytokine binding, extracellular matrix structural constituent conferring tensile strength, extracellular matrix structural constituent, external side of plasma membrane, collagen trimer, collagen-containing extracellular matrix, T cell activation, extracellular structure organization and extracellular matrix organization; CTHRC1 was associated with signal pathways including

focal adhesion, PI3K-Akt signaling pathway, ECM-receptor interaction, growth factor binding, glycosaminoglycan binding, extracellular matrix structural constituent, endoplasmic reticulum lumen, collagen trimer, collagen-containing extracellular matrix, aminoglycan metabolic process, extracellular structure organization and extracellular matrix organization in melanoma (Figure 4B).

With GSEA analysis, NABA matrisome, NABA matrisome associated, reactome adaptive immune system, reactome cytokine signaling in immune system and reactome hemostasis were found to be associated with CTHRC1 in colon cancer; reactome signaling by GPCR, reactome hemostasis, NABA matrisome associated, reactome neutrophil degranulation and reactome signaling by Rho-GTPases were associated with CTHRC1 in thyroid cancer; a association was observed between NABA matrisome, reactome signaling by receptor tyrosine kinases, reactome hemostasis, NABA matrisome associated and reactome signaling by GPCR and CTHRC1 in melanoma (Figure 4C).

Association of CTHRC1 expression with immune cell infiltrating and immunotherapy markers in colon cancer, thyroid cancer, and melanoma

Furthermore, we analyzed the correlation between CTHRC1 expression and immune cell infiltrating in colon cancer, thyroid cancer and melanoma. In colon cancer, many types of immune cells infiltrating (especially macrophages, neutrophils, dendritic cells, CD4+ T cells and CD8+ T cells) was positively correlated with CTHRC1 expression; a few types of immune cells including Th17 cells and NK CD56 bright cells infiltrating was negatively correlated with CTHRC1 expression (Figures 5A and 5B). In thyroid cancer, immune cells (especially

neutrophils, dendritic cells, B cells, CD4+ T cells and macrophages) infiltrating was also found to be positively correlated with CTHRC1 expression; Th17 cells infiltrating was negatively correlated with CTHRC1 expression (Figures 5A and 5B). In melanoma, immune cells infiltrating such as neutrophils and macrophages was found to be positively correlated with CTHRC1 expression, but the correlation of CTHRC1 expression with immune cells infiltrating was not as significant as that in colon cancer and thyroid cancer (respective correlation coefficients were smaller) (Figures 5A and 5B).

Moreover, we examined the correlation between CTHRC1 expression and immunotherapy markers including CD274, PDCD1, CTLA4 and LAG3 in colon cancer, thyroid cancer and melanoma. As shown in Figure 5C, CD274, PDCD1, CTLA4 and LAG3 were significantly positively correlated with CTHRC1 expression in colon cancer and thyroid cancer; only CTLA4 was significantly positively correlated with CTHRC1 expression in melanoma.

Therefore, our data indicated CTHRC1 expression was significantly correlated with multiple types of immune cells infiltrating and immunotherapy marker expression in colon cancer and thyroid cancer, but not as significant in melanoma; CTHRC1 might be important in immunotherapy for colon cancer and thyroid cancer.

Patients with BRAF(V600E) mutation showed elevated CTHRC1 expression and immune cell infiltrating in clinical tissues of colon cancer and thyroid cancer

For further study, we collected 15 colon cancer tissues with BRAF(V600E) mutation, 15 wild-type BRAF colon cancer tissues(WT), 15 thyroid cancer tissues with BRAF(V600E) mutation, 15 wild-type BRAF thyroid cancer tissues (WT), 15 melanoma tissues with BRAF(V600E)

mutation, 15 wild-type BRAF melanoma tissues (WT) and examined the protein levels of CTHRC1, immune cell markers such as CD4, CD8, CD68 and CD69, and proliferation marker Ki-67 by immunohistochemistry. As shown in Figure 6 and Table 8, protein levels of CTHRC1 were concordant higher in BRAF(V600E) mutant tissues compared with wild-type tissues (P=0.021), and CD4 positive cells (P=0.027), CD8 positive cells (P=0.003), CD69 positive cells (neutrophils) (P=0.025) were enriched more in BRAF(V600E) mutant tissues compared with wild-type tissues. However, there were no significant differences of CD68 positive cells (macrophages) and Ki-67 positive cells between BRAF(V600E) mutant colon cancer tissues and wild-type colon cancer tissues (P>0.05) in colon cancer. In thyroid cancer, protein levels of CTHRC1 were also concordant higher in BRAF(V600E) mutant tissues compared with wild-type tissues (P=0.009), and CD4 positive cells (P=0.021), CD8 positive cells (P=0.025), CD69 positive cells (neutrophils) (P=0.025) were enriched more in BRAF(V600E) mutant tissues compared with wild-type tissues. However, there were no significant differences of CD68 positive cells (macrophages) and Ki-67 positive cells between BRAF(V600E) mutant thyroid cancer tissues and wild-type thyroid cancer tissues (P>0.05), as shown in Figure 6 and Table 9. However, as demonstrated in Supplementary Figure 1 and Supplementary Table 3, the protein levels of CTHRC1, immune cell markers such as CD4, CD8, CD68 and CD69, and proliferation marker Ki-67 were not significantly different between BRAF (V600E) mutant melanoma tissues and wild-type melanoma tissues (P>0.05).

Therefore, BRAF(V600E) mutation was associated with CTHRC1 expression and they both correlated with immune cell infiltrating including CD4+ T cells, CD8+ T cells and neutrophils in colon cancer and thyroid cancer patients.

High level of CTHRC1 correlated with decreased sensitivity of BRAF(V600E) mutation targeted drugs

Based on database, we examined the correlation of CTHRC1 expression with response of anti-tumor drugs. Vemurafenib, PLX-4720, Dabrafenib and SB-590885 were 4 commonly used targeted drugs for BRAF(V600E) mutant tumors. As shown in Figure 7, the IC50 values of Vemurafenib, PLX-4720, Dabrafenib and SB-590885 were all significantly positively correlated with CTHRC1 expression (Vemurafenib, cor=0.436, PLX-4720, cor=0.422, Dabrafenib, cor=0.352, SB-590885, cor=0.365, all $P<0.01$). Therefore, high CTHRC1 expression level might be correlated with decreased sensitivity of BRAF(V600E) mutation targeted drugs.

Moreover, with the analysis of other drugs, the IC50 values of PI-103, Hypothemycin and OSI-027 also showed positive correlation with CTHRC1 expression in human cancers; the IC50 values of AFP464 and Aminoflavone showed negative correlation with CTHRC1 expression in human cancers. Therefore, these data suggested that CTHRC1 was an important biomarker for anti-tumor drug sensitivity evaluation.

DISCUSSION

In this study, we demonstrated that CTHRC1 was positively correlated with BRAF(V600E) mutation in human colon cancer, thyroid cancer and melanoma. In colon cancer and thyroid

cancer patients, CTHRC1 expression level was negatively associated with the overall survival (OS) rate, univariate COX regression analysis showed CTHRC1 was an important risk factor correlated with patient OS rates. Expression level of CTHRC1 was significantly correlated with tumor infiltration depth, clinical stage in colon cancer patients and with tumor size, lymph node metastasis, clinical stage in thyroid cancer patients. These results suggested that CTHRC1 was an oncogenic biomarker in colon cancer and thyroid cancer. As reported in previous study, CTHRC1 promoted human colorectal cancer cell proliferation and invasiveness by activating Wnt/PCP signaling [26]; CTHRC1 promoted liver metastasis of colorectal cancer through TGF-β pathway [37]. In addition, Pang et al. demonstrated that CTHRC1 is a potential diagnostic and prognostic indicator of colon adenocarcinoma [38], and overexpression of CTHRC1 was associated with poor prognosis in colorectal cancer patients [39]. Recent studies have reported that CTHRC1 is associated with the onset and malignant transformation of papillary thyroid carcinoma [27]. Furthermore, CTHRC1 has been shown to promote cell proliferation and inhibit apoptosis by activating the ERK1/2 signaling pathway in papillary thyroid carcinoma [40]. These findings are consistent with our current results. We provide compelling evidence that CTHRC1 is linked to the BRAF(V600E) mutation in both colon and thyroid cancers, further highlighting the significant role of CTHRC1 in influencing patient prognosis. On the other hand, though CTHRC1 was positively correlated with BRAF(V600E) mutation in melanoma, CTHRC1 expression level was positively associated with the OS rate in melanoma patients, which was opposite with colon cancer and thyroid cancer. Additionally, there was no significant correlation between CTHRC1 expression and patients' gender, tumor

size, lymph node metastasis, distant metastasis and clinical stage in melanoma. Despite BRAF(V600E) targeted drugs were well used in human melanoma [41], BRAF(V600E) mutation was actually more frequent in melanocytic nevi compared with melanoma [42]. W.E. Damsky et al. reported that BRAF(V600E) mutation induced mole formation in mice, but rarely melanoma formation [43]. Therefore, though CTHRC1 and BRAF(V600E) mutation were diagnostic and prognostic biomarkers in melanoma, they could not just simply considered to be oncogenic biomarkers, which exhibited tissue speciality.

In molecular network study, we found that DVL1, DVL2, DVL3, FZD3, ROR2, FZD5, FZD6, WNT3A, NTN4, VANGL2 were involved in CTHRC1 pathway. Remarkably, three members of FZDs proteins (belonging to the frizzled transmembrane receptor family) FZD3, FZD5 and FZD6 were associated with CTHRC1. As reported previously, FZD3 was oncogenic in melanoma; high expression of FZD3 was correlated with poor OS in melanoma patients [44]. FZD5 was reported to be regulated through Wnt signaling pathway affecting Paneth cell differentiation, playing a promoting role in tumor development [45-47]. In Luo et al's study, FZD6 was identified to be a potential biomarker for anaplastic thyroid cancer [48]; FZD6 was correlated with VEGFA expression, promoting vascularization and primary tumor spread in uveal melanoma [49]. Therefore, the CTHRC1/FZDs involved pathway might play important roles in colon cancer, thyroid cancer and melanoma. Moreover, the network member WNT3A was involved in the induction of transcriptional co-activator YAP/TAZ, promoted the Wnt-FZD signaling axis, and acted as a key role in gene expression, osteogenic differentiation, and cell migration [51]. NTN4 was reported to enhance non-medullary thyroid cancer susceptibility

[51], promote melanoma cell invasion [52], but inhibit primary and metastatic colorectal tumor progression [53]. ROR2, VANGL2 and DVLs were demonstrated to be involved in WNT pathway [54, 55], serving as a pivotal regulator in proliferation, metastasis and development of colon cancer, thyroid cancer and melanoma [56-59]. Therefore, the CTHRC1 network might play crucial roles in human colon cancer, thyroid cancer and melanoma. However, the deep mechanisms and functions of CTHRC1 network in these cancers should be studied further.

Moreover, we examined that many kinds of immune cell infiltrating and immunotherapy markers were positively correlated with CTHRC1 expression in colon cancer, thyroid cancer and melanoma (macrophages, neutrophils, dendritic cells, CD4+ T cells, CD8+ T cells / CD274, PDCD1, CTLA4, LAG3 in colon cancer; neutrophils, dendritic cells, B cells, CD4+ T cells, macrophages / CD274, PDCD1, CTLA4, LAG3 in thyroid cancer; neutrophils, macrophages / CTLA4 in melanoma). As reported previously, CD274 and PDCD1 were widely-recognized biomarkers for efficacy prediction of PD-1/PD-L1 immune checkpoint based immunotherapy in human tumors [60]. Additionally, the phagocytic ability of tumor-associated macrophage to tumor cells was negatively correlated with PDCD1 expression in colon cancer [61]. Metastatic melanoma secreted CD274-loading extracellular vesicles to inhibit the function of CD8+ T cells and promoted tumor growth [62]. In previous study, expression of CTLA4 was demonstrated to suppress immune cell activation in colon cancer [63], positively correlated with multiple immune cells infiltration in papillary thyroid carcinoma [64], and potentially regulated lymph node T cell proliferation in the early immune response of melanoma [65]. LAG3 was another biomarker for immunotherapy, and antagonists

of LAG-3 enhance the antitumor efficacy of PD-L1 blockade therapy in colon cancer and melanoma [66, 67]. As the evidence we have provided, CTHRC1 expression was significantly associated with immune cell infiltrating and immunotherapy markers, we could induce that CTHRC1 crucially participated in the immune reaction of colon cancer, thyroid cancer and melanoma, and CTHRC1 could be used as a potential target for immunotherapy.

BRAF(V600E) targeted drugs (including Vemurafenib, PLX-4720, Dabrafenib and SB-590885) were efficiently used for therapy of colon cancer, thyroid cancer and melanoma with BRAF(V600E) mutation [68-71]. However, drug resistance made it difficult for patients to achieve the desired therapeutic effect [72-75]. The mechanisms of BRAF(V600E) targeted drug resistance involved were complicated. It has been reported NRG-1β activated ErbB-3 to promote Vemurafenib resistance in BRAF(V600E) colon cancer stem cells (CSCs) [76]. Overexpression of HMGB1 attenuated the sensitivity of BRAF(V600E) mutant thyroid cancer cells to Vemurafenib by increasing cell viability and decreasing apoptosis and caspase-3 activity [73]. RAC1 amplification was reported to be related with Dabrafenib resistance in papillary thyroid carcinoma [77]. In this study, we provided evidence that the IC50 values of Vemurafenib, PLX-4720, Dabrafenib and SB-590885 were all significantly positively correlated with CTHRC1 expression level. Therefore, high expression of CTHRC1 was correlated with the resistance of BRAF(V600E) targeted drugs; CTHRC1 inhibitors could be potentially used as adjuvant treatment drugs for BRAF(V600E) targeted drugs in BRAF(V600E) mutant colon cancer, thyroid cancer and melanoma.

CONCLUSION

In summary, this study demonstrated that CTHRC1 was correlated with BRAF(V600E), prognosis and clinicopathological features in colon cancer, thyroid cancer and melanoma. CTHRC1 expression was also associated with immune cell infiltrating, immunotherapy markers and BRAF(V600E) targeted drug resistance. Therefore, CTHRC1 could be used as a diagnostic, prognostic and adjuvant therapeutic biomarker in human colon cancer, thyroid cancer and melanoma.

Data availability

All data generated or analyzed during this study are included in this article. The original experimental data related to the research results in the article will be provided without reservation if necessary.

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TABLES AND FIGURES WITH LEGENDS

TABLE 1. COX regression analysis between prognostic risk factors and overall survival of colon cancer patients.

Characteristics	Total (*n*)	Univariate analysis	
		Hazard ratio (95% CI)	*P*
Age			0.275
≤ 55	94	Reference	
> 55	358	1.34 (0.79-2.27)	
Gender			0.675
Male	239	Reference	
Female	213	1.09 (0.73-1.64)	
Tumor infiltration depth			**0.006**
≤ T2	89	Reference	
T3-T4	363	3.23 (1.41-7.4)	
Lymph node metastasis			**<0.001**
No	267	Reference	
Yes	185	2.73 (1.8-4.15)	
Distant metastasis			**<0.001**
No	331	Reference	
Yes	114	3.4 (2.23-5.19)	
Stage			**<0.001**
I - II	252	Reference	
III - IV	189	3.04 (1.96-4.73)	
CTHRC1			**0.036**
Low	305	Reference	
High	149	1.56 (1.03-2.36)	

CI: Confidence interval; CTHRC1: Collagen triple helix repeat containing 1.

TABLE 2. COX regression analysis between prognostic risk factors and overall survival of thyroid cancer patients.

Characteristics	Total (*n*)	Univariate analysis	
		Hazard ratio (95% CI)	*P*
Age			0.997
≤ 45	236	Reference	
> 45	266	737240933.9 (0-Inf)	
Gender			0.22
Male	138	Reference	
Female	367	1.89 (0.68-5.22)	
Tumor size (cm)			**0.038**
< 4	307	Reference	
≥ 4	196	3.07 (1.06-8.84)	
Lymph node metastasis			0.516
No	227	Reference	
Yes	229	1.45 (0.47-4.44)	
Distant metastasis			0.898
No	286	Reference	
Yes	218	0.94 (0.35-2.53)	
Stage			**<0.001**
I - II	83	Reference	
III - IV	167	7.1 (2.28-22.07)	
CTHRC1			**0.027**
Low	391	Reference	
High	114	3.03 (1.14-8.1)	

CI: Confidence interval; CTHRC1: Collagen triple helix repeat containing 1.

TABLE 3. COX regression analysis between prognostic risk factors and overall survival of melanoma patients.

Characteristics	Total (*n*)	Univariate analysis	
		Hazard ratio (95% CI)	*P*
Age			**<0.001**
≤ 55	116	Reference	
> 55	266	1.74 (1.32-2.3)	
Gender			0.354
Male	292	Reference	
Female	179	1.14 (0.86-1.51)	
Tumor size (cm)			**<0.001**
< 2	151	Reference	
≥ 2	244	1.96 (1.45-2.65)	
Lymph node metastasis			**<0.001**
No	235	Reference	
Yes	179	1.82 (1.36-2.43)	
Distant metastasis			**0.043**
No	418	Reference	
Yes	25	1.88 (1.02-3.46)	
Stage			**<0.001**
I - II	238	Reference	
III - IV	195	1.73 (1.3-2.31)	
CTHRC1			**0.017**
Low	286	Reference	
High	185	0.71 (0.54-0.94)	

CI: Confidence interval; CTHRC1: Collagen triple helix repeat containing 1.

TABLE 4. Association of CTHRC1 expression with clinicopathological parameters in colon cancer patients based on TCGA database.

Characteristics	Low expression of CTHRC1 (%)	High expression of CTHRC1 (%)	χ^2	*P*
n	305	149		
Age			0.246	0.620
≤ 55	61 (20.1)	33 (22.1)		
> 55	242 (79.9)	116 (77.9)		
Gender			0.025	0.875
Male	161 (53.1)	78 (52.3)		
Female	142 (46.9)	71 (47.7)		
Tumor infiltration depth			5.521	**0.019**
≤ T2	69 (22.8)	20 (13.4)		
T3-T4	234 (77.2)	129 (86.6)		
Lymph node metastasis			2.660	0.103
No	187 (61.7)	80 (53.7)		
Yes	116 (38.3)	69 (46.3)		
Distant metastasis			0.069	0.757
No	223 (74.8)	108 (73.5)		
Yes	75 (25.2)	39 (26.5)		
Stage			4.221	**0.040**
I	59 (19.7)	16 (11.7)		
II-IV	241 (80.3)	121 (88.3)		

CTHRC1: Collagen triple helix repeat containing 1.

TABLE 5. Association of CTHRC1 expression with clinicopathological parameters in thyroid cancer patients based on TCGA database.

Characteristics	Low expression of CTHRC1 (%)	High expression of CTHRC1 (%)	χ^2	*P*
n	391	114		
Age			3.642	0.056
≤ 45	194 (49.6)	45 (39.5)		
> 45	197 (50.4)	69 (60.5)		
Gender			3.513	0.061
Male	99 (25.3)	39 (34.2)		
Female	292 (74.7)	75 (65.8)		
Tumor size (mm)			23.161	**<0.001**
≤ 4	260 (66.7)	47 (41.6)		
> 4	130 (33.3)	66 (58.4)		
Lymph node metastasis			17.891	**<0.001**
No	192 (55.3)	35 (32.1)		
Yes	155 (44.7)	74 (67.9)		
Distant metastasis			3.189	0.074
No	213 (54.6)	73 (64.0)		
Yes	177 (45.4)	41 (36.0)		
Stage			22.880	**<0.001**
I - II	281 (72.2)	55 (48.2)		
III - IV	108 (27.8)	59 (51.8)		

CTHRC1: Collagen triple helix repeat containing 1.

TABLE 6. Association of CTHRC1 expression with clinicopathological parameters in colon cancer patients based on clinical tissues.

Characteristics	Low expression of CTHRC1 (%)	High expression of CTHRC1 (%)	χ^2	*P*
n	25	25		
Age			0.368	0.544
≤ 55	7 (28.0)	9 (36.0)		
> 55	18 (72.0)	16 (64.0)		
Gender			0.058	0.771
Male	16 (64.0)	15 (60.0)		
Female	9 (36.0)	10 (40.0)		
Tumor infiltration depth			4.504	**0.034**
≤ T2	12 (48.0)	4 (16.0)		
T3-T4	13 (52.0)	21 (84.0)		
Lymph node metastasis			0.347	0.556
No	17 (68.0)	15 (60.0)		
Yes	8 (32.0)	10 (40.0)		
Distant metastasis			0.149	0.700
No	22 (88.0)	20 (80.0)		
Yes	3 (12.0)	5 (20.0)		
Stage			4.504	**0.034**
I	12 (48.0)	4 (16.0)		
II - IV	13 (52.0)	21 (84.0)		

CTHRC1: Collagen triple helix repeat containing 1.

TABLE 7. Association of CTHRC1 expression with clinicopathological parameters in thyroid cancer patients based on clinical tissues.

Characteristics	Low expression of CTHRC1 (%)	High expression of CTHRC1 (%)	χ^2	*P*
n	25	25		
Age			0.081	0.777
≤ 45	11 (44.0)	12 (48.0)		
> 45	14 (56.0)	13 (52.0)		
Gender			1.389	0.239
Male	7 (28.0)	11 (44.0)		
Female	18 (72.0)	14 (56.0)		
Tumor size (cm)			5.556	**0.018**
< 4	20 (80.0)	12 (48.0)		
≥ 4	5 (20.0)	13 (52.0)		
Lymph node metastasis			4.023	**0.045**
No	14 (56.0)	7 (28.0)		
Yes	11 (44.0)	18 (72.0)		
Distant metastasis			0.125	0.724
No	21 (84.0)	19 (76.0)		
Yes	4 (16.0)	6 (24.0)		
Stage			1.282	0.258
I - II	15 (60.0)	11 (44.0)		
III - IV	10 (40.0)	14 (56.0)		

CTHRC1: Collagen triple helix repeat containing 1.

TABLE 8. Protein levels of respective markers in BRAF(V6000E) mutant (Mut) and wild type (WT) colon cancer tissues examined by immunohistochemistry.

Group	*n*	CTHRC1 expression		CD4 expression		CD8 expression		CD68 expression		CD69 expression		Ki-67 expression	
		low	high	low	high	low	high	low	high	low	high	low	high
WT	15	9	6	11	4	11	4	7	8	12	3	6	9
Mut	15	2	13	4	11	2	13	6	9	5	10	7	8
P		**0.021**†		**0.027**†		**0.003**†		1.000†		**0.025**†		1.000†	

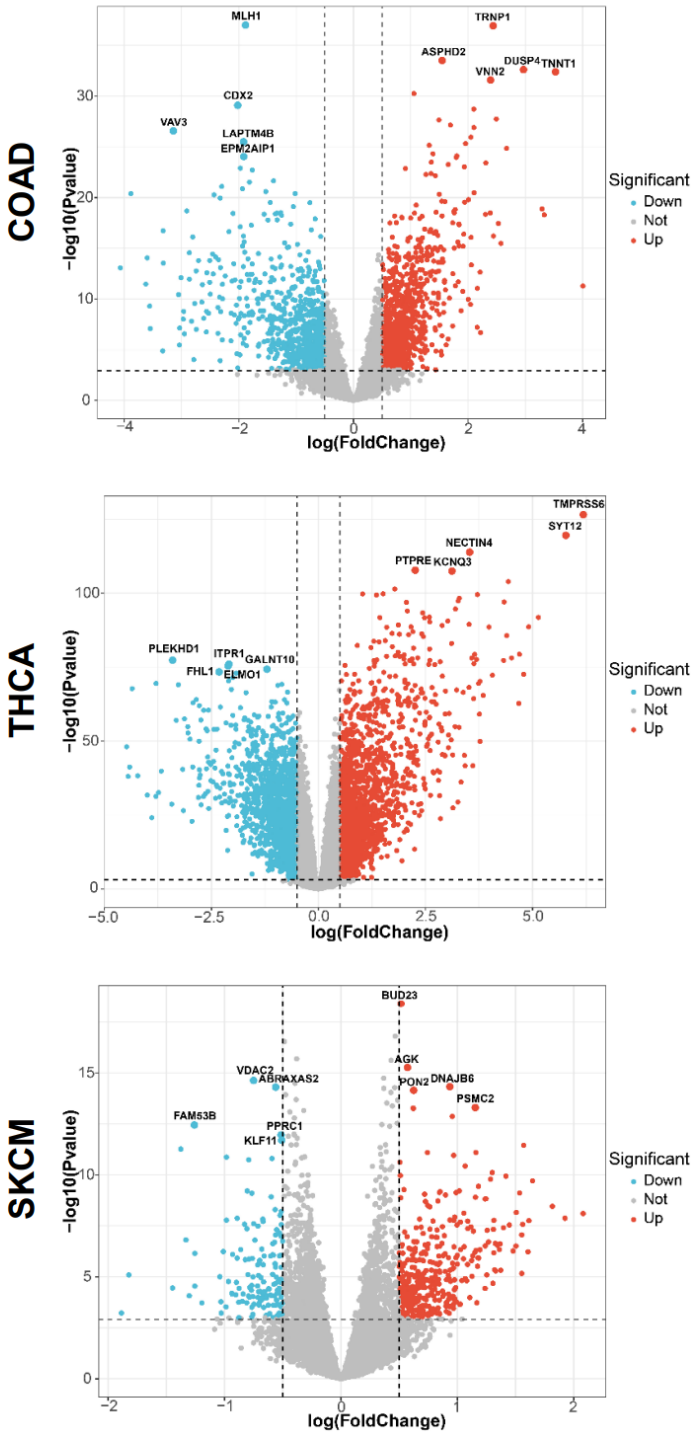
†for MWU test.

TABLE 9. Protein levels of respective markers in BRAF(V6000E) mutant (Mut) and wild type (WT) thyroid cancer tissues examined by immunohistochemistry.

Group	*n*	CTHRC1 expression		CD4 expression		CD8 expression		CD68 expression		CD69 expression		Ki-67 expression	
		low	high	low	high	low	high	low	high	low	high	low	high
WT	15	11	4	13	2	12	3	11	4	12	3	11	4
Mut	15	3	12	4	11	5	10	8	7	5	10	6	9
P		**0.009**†		**0.021**†		**0.025**†		0.450†		**0.025**†		0.139†	

†for MWU test.

A


COAD
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log(FoldChange)
MLH1
CDX2
VAV3
LAPTM4B
EPM2AIP1
TRNP1
ASPHD2
VNN2
DUSP4
TNNT1
Significant
Down
Not
Up
THCA
−log10(Pvalue)
log(FoldChange)
PLEKHD1
ITPR1
GALNT10
FHL1
ELMO1
TMPRSS6
SYT12
NECTIN4
PTPRE
KCNQ3
Significant
Down
Not
Up
SKCM
−log10(Pvalue)
log(FoldChange)
FAM53B
VDAC2
ABRAXAS2
PPRC1
KLF11
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AGK
PON2
DNAJB6
PSMC2
Significant
Down
Not
Up

B

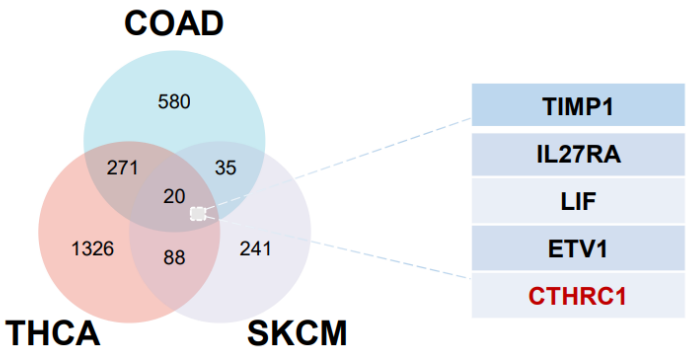
COAD
580
271
35
20
1326
88
241
THCA
SKCM
TIMP1
IL27RA
LIF
ETV1
CTHRC1

FIGURE 1. Correlation analysis of BRAF(V600E) mutation with gene expression in colon cancer, thyroid cancer and melanoma based on TCGA database. (A) Volcano plots showed genes down or up expressed in BRAF(V600E) mutant tissues compared with wild-type tissues of colon cancer, thyroid cancer and melanoma respectively (COAD, colon cancer; THCA, thyroid cancer; SKCM, melanoma); (B) Intersection analysis of the genes up expressed in BRAF(V600E) mutant colon cancer, thyroid cancer and melanoma tissues, and 5 potential oncogenes were screened out for further study.

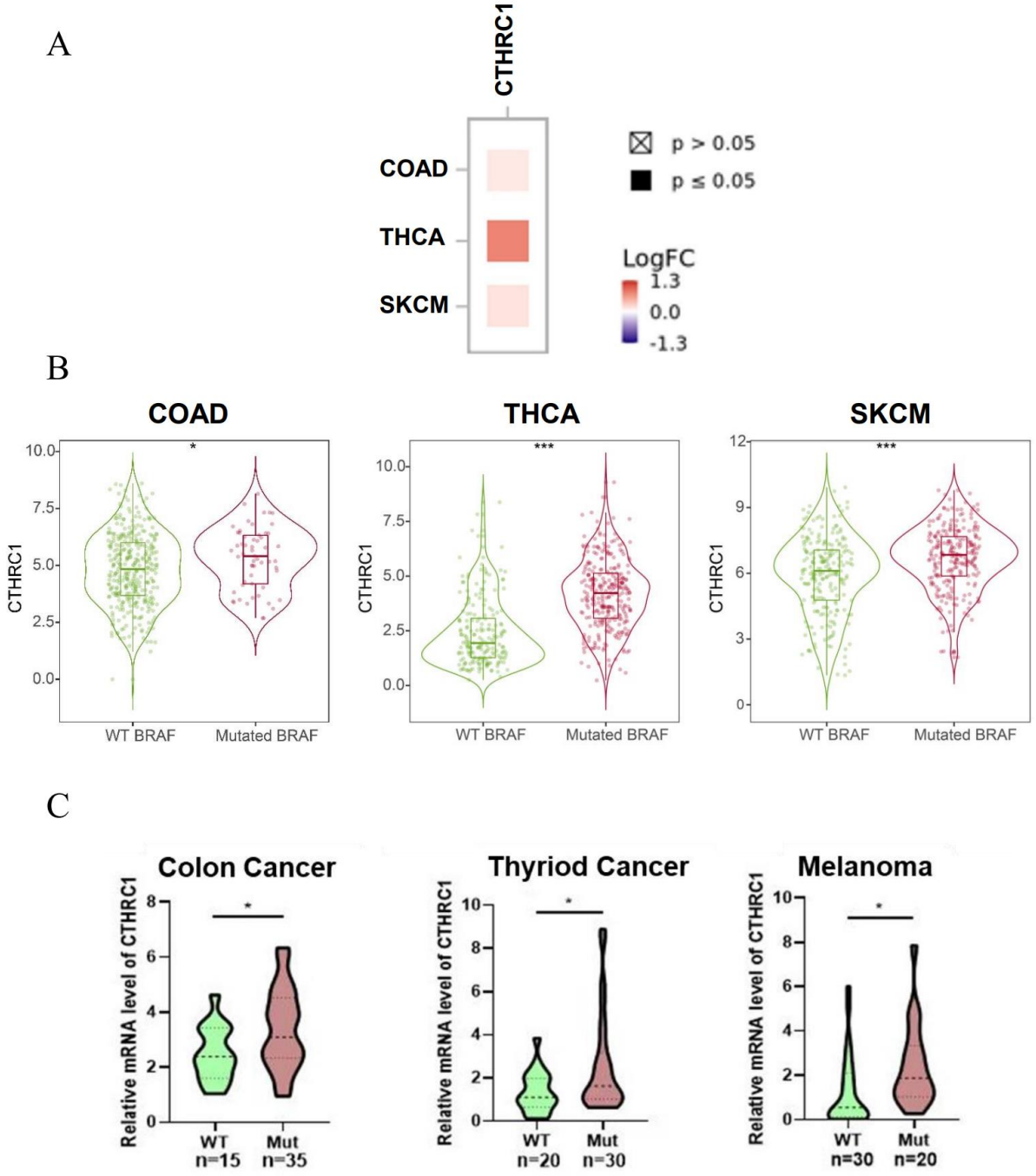

FIGURE 2. CTHRC1 was highly expressed in BRAF(V600E) mutant colon cancer, thyroid cancer and melanoma tissues. (A) Correlation of CTHRC1 expression and BRAF(V600E) mutation in colon cancer, thyroid cancer and melanoma tissues based on TCGA database; (B) CTHRC1 mRNA expression in BRAF(V600E) mutant and wild-type colon cancer, thyroid cancer and melanoma tissues based on TCGA database; (C) CTHRC1 mRNA expression in BRAF(V600E) mutant and wild-type colon cancer, thyroid cancer and melanoma clinical tissues we collected in the Department of

Pathology, the First Affiliated Hospital of Anhui Medical University , which was examined by qRT-PCR. *$P < 0.05$; ***$P < 0.001$.

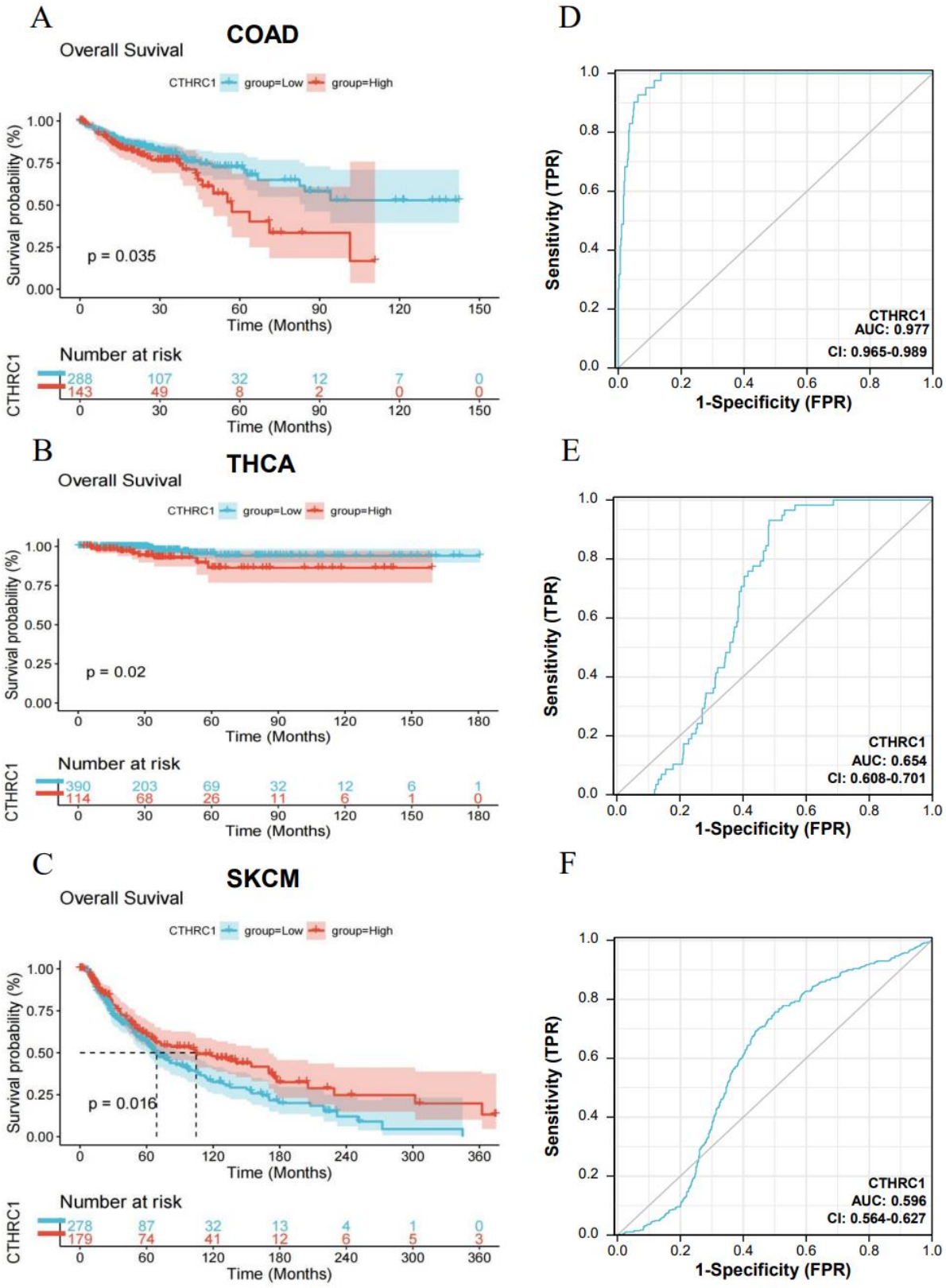

FIGURE 3. Overall survival (OS) rate and ROC curve analyses of CTHRC1 in colon cancer, thyroid cancer and melanoma based on TCGA database. (A-C) Patient OS rates of CTHRC1 high and low groups were analyzed by Kaplan-Meier analysis in colon cancer, thyroid cancer, and melanoma

respectively; (D-F) The (receiver operating characteristic) ROC curves of CTHRC1 for diagnosis of colon cancer, thyroid cancer, and melanoma respectively.

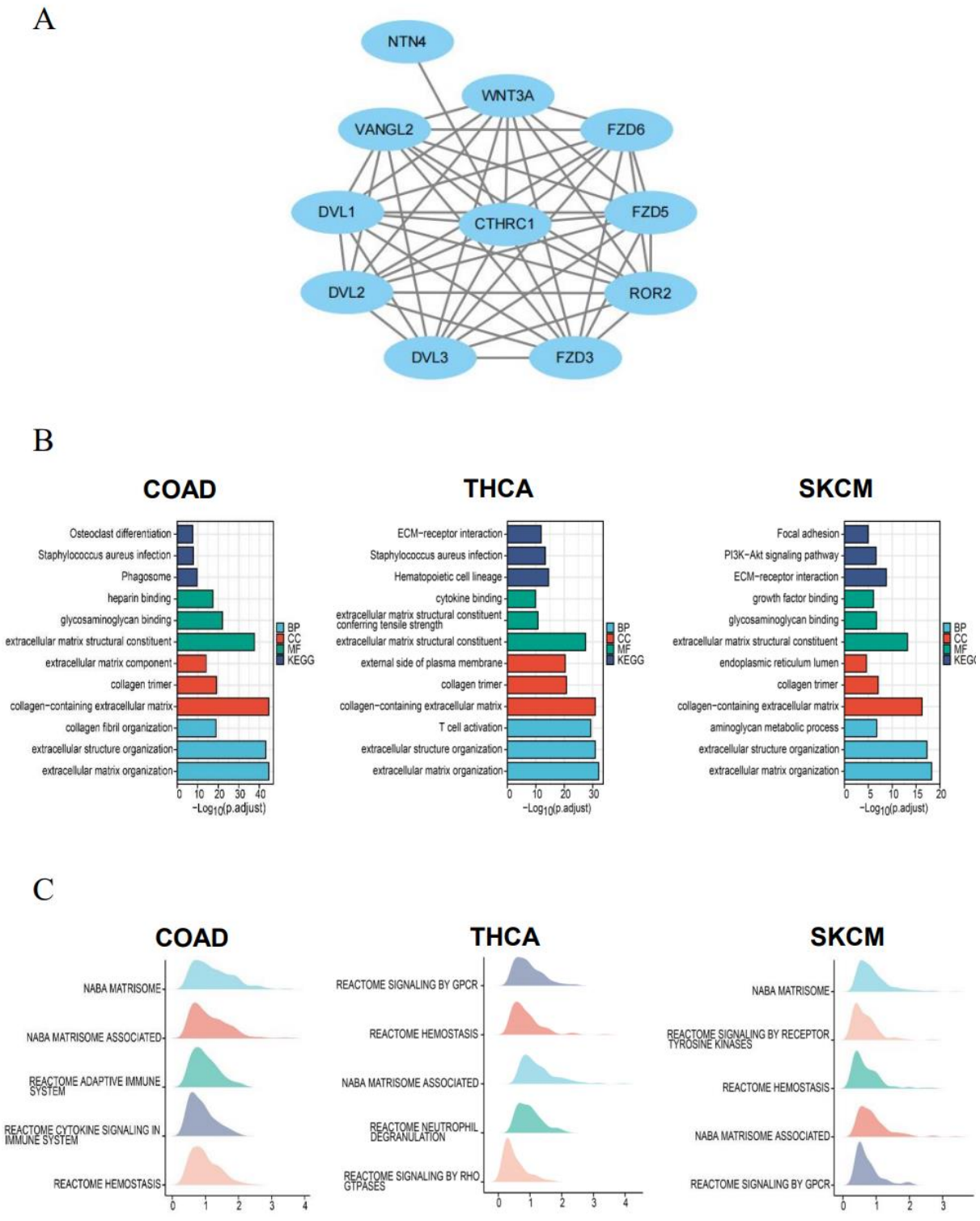

FIGURE 4. Protein-protein interaction and molecular signaling pathway enrichment analyses of CTHRC1 in colon cancer, thyroid cancer, and melanoma. (A) Protein-protein interaction network of CTHRC1 was analyzed by using STRING tool; (B) Histogram showing the GO and KEGG pathway analyses of CTHRC1; (C) Top5 GSEA (gene set enrichment analysis) results of CTHRC1.

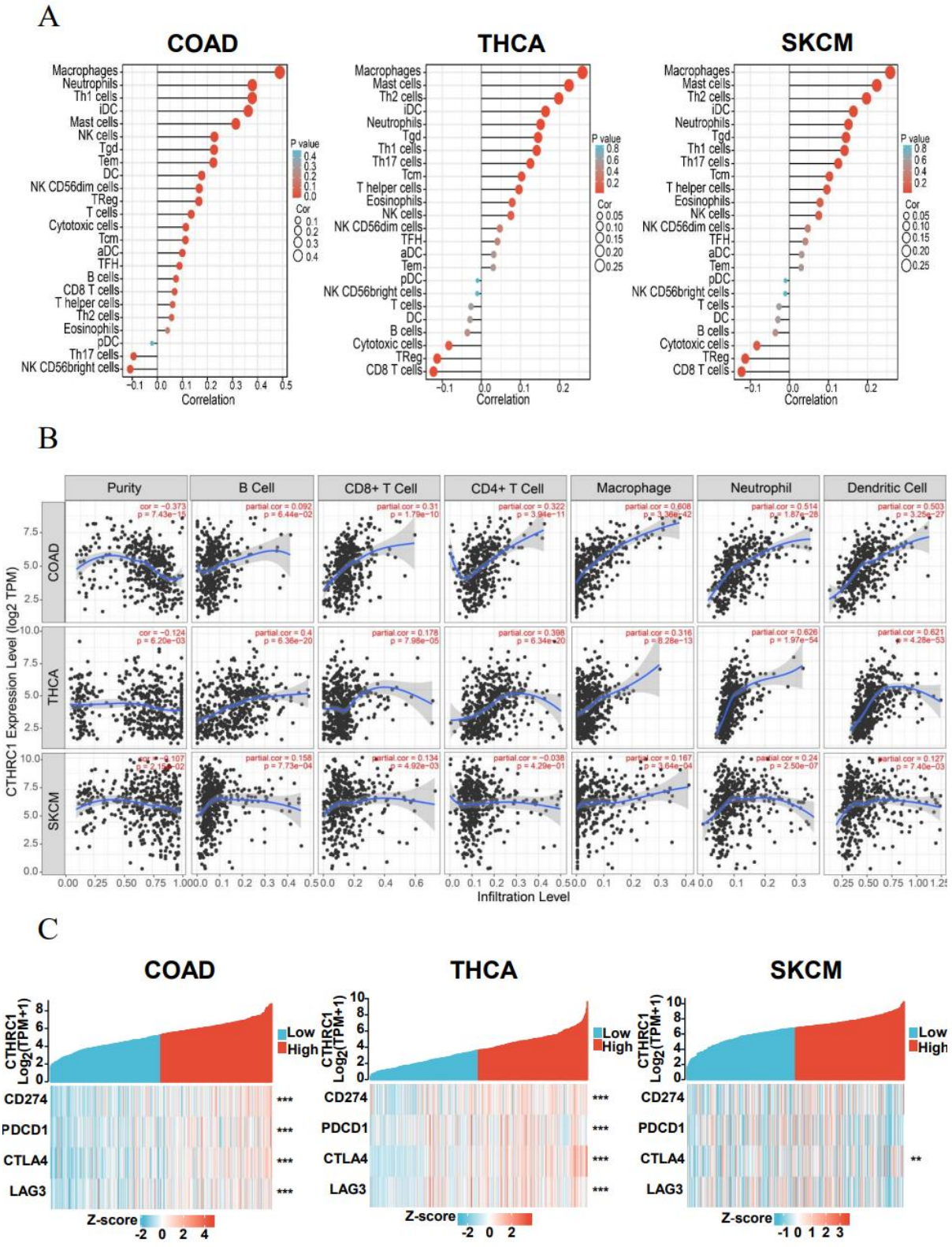

FIGURE 5. Correlation analysis of CTHRC1 expression with immune cell infiltrating and immunotherapy markers in colon cancer, thyroid cancer, and melanoma. (A) Correlation of CTHRC1 expression with 24 kinds of immune cells was calculated by Cibersort; (B) Correlation of CTHRC1 expression with respective immune cells based on TIMER database; (C) Heatmap of

CTHRC1 associated immunotherapy markers CD274, PDCD1, CTLA4 and LAG3. $^{**}P < 0.01$; $^{***}P < 0.001$.

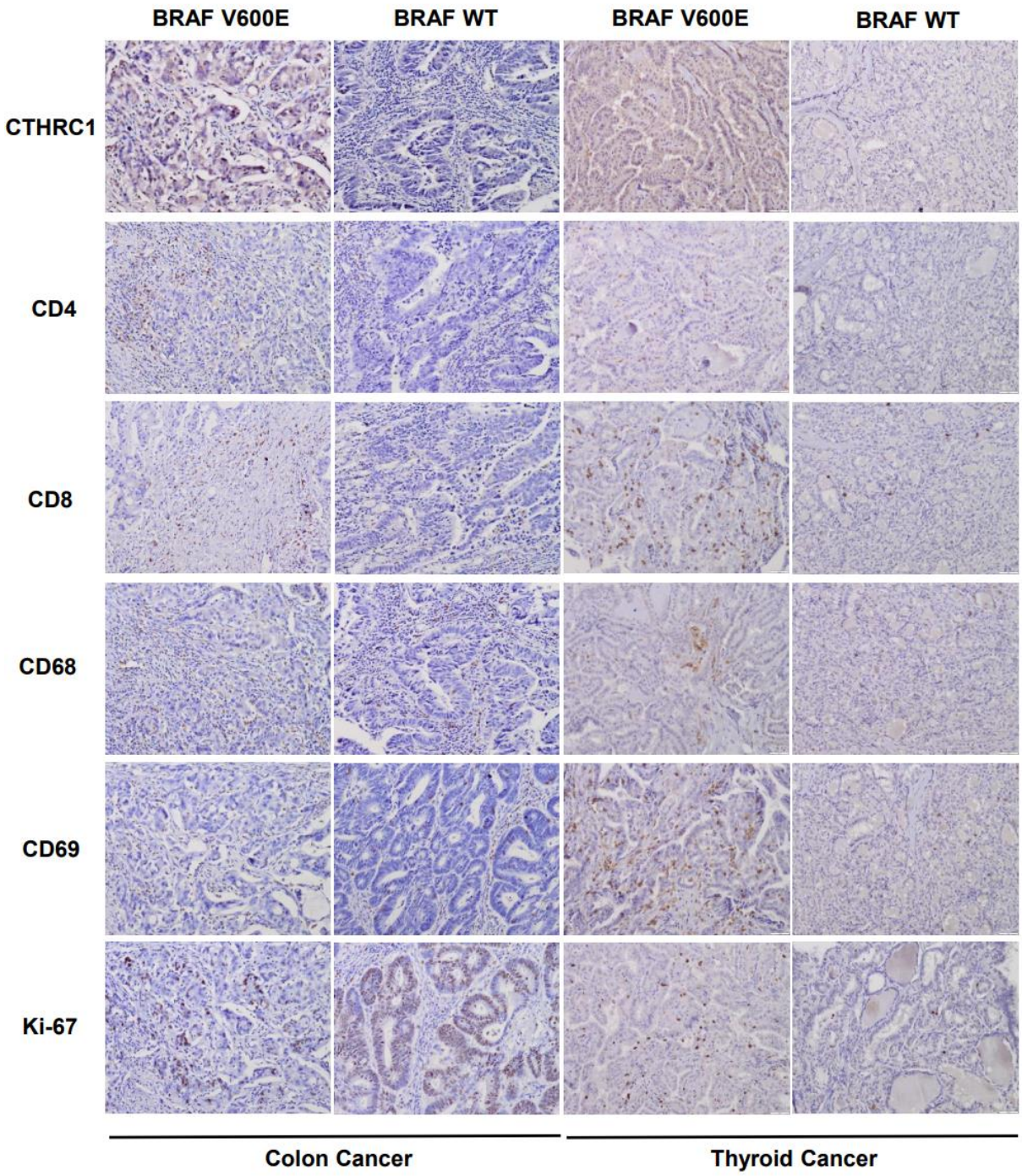

FIGURE 6. CTHRC1 and immune cell marker levels in colon cancer and thyroid cancer clinical tissues with/without BRAF(V600E) mutation. Immunohistochemistry to show CTHRC1, CD4, CD8, CD68, CD69, and Ki-67 protein levels in colon cancer and thyroid cancer clinical tissues with/without BRAF(V600E) mutation. Representative photographs were showed. BRAF(V600E), BRAF(V600E) mutation; BRAF WT, BRAF wild-type.

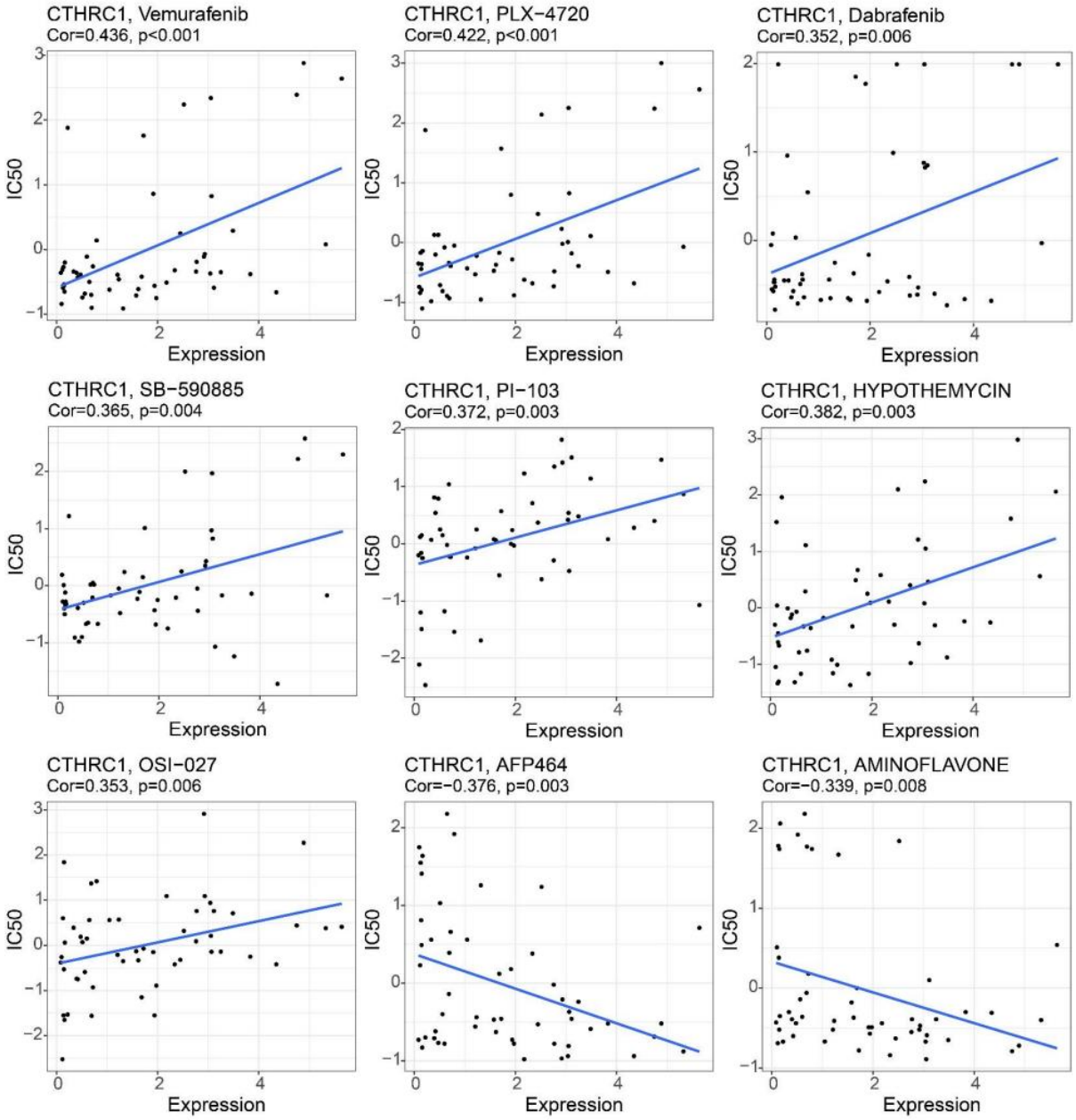

FIGURE 7. Correlation analysis of CTHRC1 expression with anticancer drug sensitivity. The correlations of CTHRC1 expression with anticancer drug (Vemurafenib, PLX-4720, Dabrafenib, SB-590885, PI-103, Hypothemycin, OSI-027, AFP464, Aminoflavone) sensitivity were analyzed based on CellMiner database.

SUPPLEMENTAL DATA

TABLE S1. Association of CTHRC1 expression with clinicopathological features in melanoma patients based on TCGA database.

Characteristics	Low expression of CTHRC1 (%)	High expression of CTHRC1 (%)	χ^2	*P*
n	286	185		
Age			4.951	**0.026**
≤ 55	108 (38.4)	89 (48.9)		
> 55	173 (61.6)	93 (51.1)		
Gender			1.692	0.193
Male	184 (64.3)	108 (58.4)		
Female	102 (35.6)	77 (41.6)		
Tumor size (mm)			2.549	0.110
< 2	85 (35.1)	66 (43.1)		
≥ 2	157 (64.9)	87 (56.9)		
Lymph node metastasis			0.150	0.699
No	140 (56.0)	95 (57.9)		
Yes	110 (44.0)	69 (42.1)		
Distant metastasis			0.800	0.371
No	255 (95.1)	163 (93.1)		
Yes	13 (4.9)	12 (6.9)		
Stage			0.071	0.790
I - II	147 (55.5)	91 (54.2)		
III - IV	118 (44.5)	77 (45.8)		

CTHRC1: Collagen triple helix repeat containing 1.

TABLE S2: Association of CTHRC1 expression with clinicopathological parameters in melanoma patients based on clinical tissues.

Characteristics	Low expression of CTHRC1(%)	High expression of CTHRC1(%)	χ^2	*P*
n	25	25		
Age			0.739	0.390
≤ 55	9 (36.0)	12 (48.0)		
> 55	16 (64.0)	13 (52.0)		
Gender			0.085	0.771
Male	15 (60.0)	16 (64.0)		
Female	10 (40.0)	9 (36.0)		
Tumor size (cm)			0.764	0.382
< 2	8 (32.0)	11 (44.0)		
≥ 2	17 (68.0)	14 (56.0)		
Lymph node metastasis			0.439	0.508
No	18 (72.0)	20 (80.0)		
Yes	7 (28.0)	5 (20.0)		
Distant metastasis			0.466	0.495
No	21 (84.0)	18 (72.0)		
Yes	4 (16.0)	7 (18.0)		
Stage			0.325	0.569
I - II	13 (52.0)	15 (60.0)		
III - IV	12 (48.0)	10 (40.0)		

CTHRC1: Collagen triple helix repeat containing 1

TABLE S3. Protein levels of respective markers in BRAF(V6000E) mutant (Mut) and wild type (WT) melanoma tissues examined by immunohistochemistry.

Group	*n*	CTHRC1 expression		CD4 expression		CD8 expression		CD68 expression		CD69 expression		Ki-67 expression	
		low	high	low	high	low	high	low	high	low	high	low	high
WT	15	10	5	9	6	10	5	10	5	8	7	5	10
Mut	15	5	10	8	7	8	7	9	6	6	9	4	11
P		0.143[†]		1.000[†]		0.710[†]		1.000[†]		0.715[†]		1.000[†]	

[†]For MWU test.

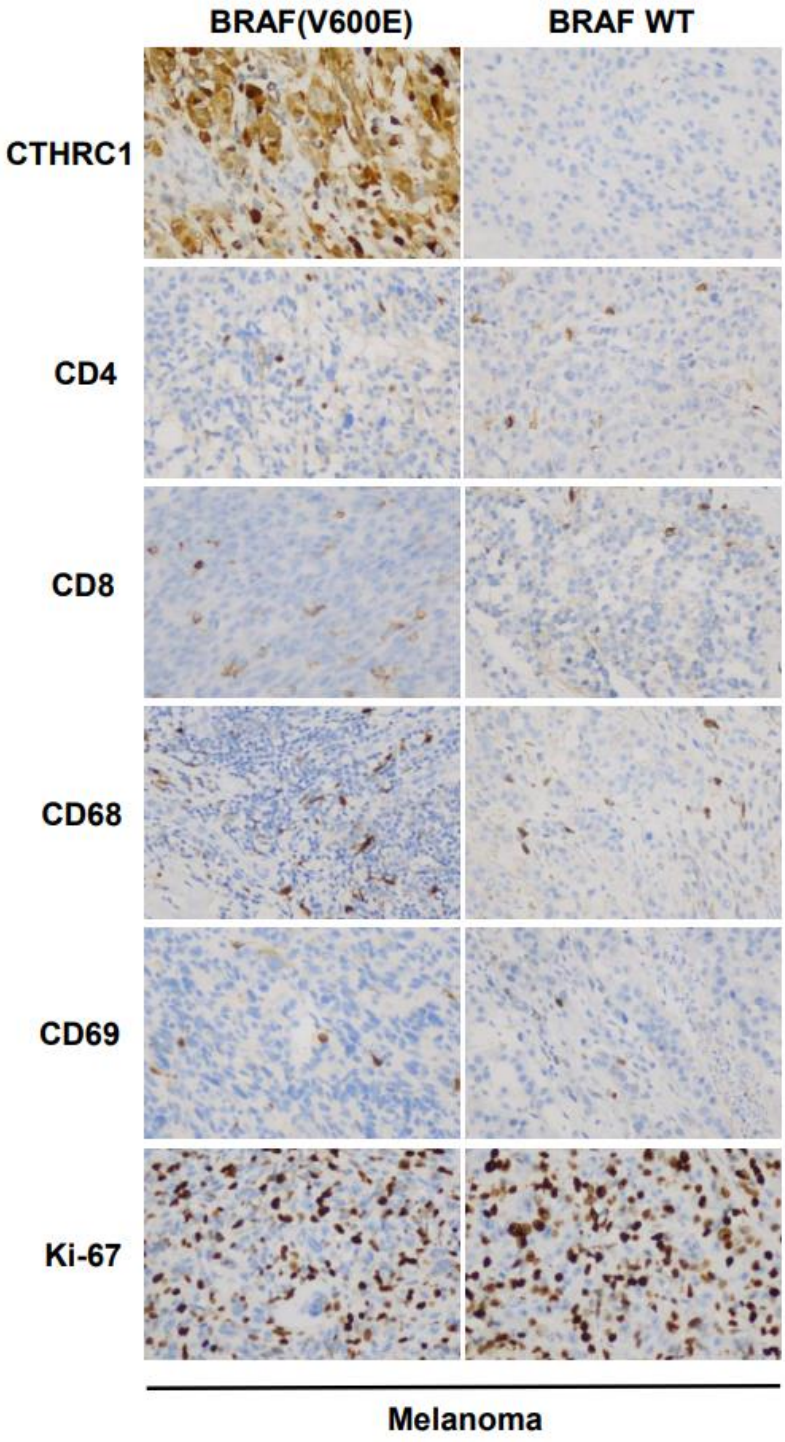

FIGURE S1. CTHRC1 and immune cell marker levels in melanoma tissues with/without BRAF(V600E) mutation. Immunohistochemistry to show CTHRC1, CD4, CD8, CD68, CD69, and Ki-67 protein levels in melanoma tissues with/without BRAF(V600E) mutation. Representative photographs were showed. BRAF(V600E), BRAF(V600E) mutation; BRAF WT, BRAF wild-type.