

A malignant prognostic indicator of Uterine Corpus Endometrial Carcinoma: CDKN2A

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Abstract. Mounting evidence have proved that the expression level of CDKN2A has a certain influence on the immune infiltration of tumor and the prognosis of cancer patients. Nevertheless, the association between CDKN2A and Uterine Corpus Endometrial Carcinoma (UCEC) still remains unclear. Therefore, the aim of this study is to investigate the effects of CDKN2A on the prognosis of UCEC patients and its relationship with immune infiltration. ONCOMINE, GEPIA 2, Kaplan-Meier Plotter, TIMER2.0, Metascape, STRING and cBioPortal were used in this study to explore the influence of CDKN2A expression on UCEC. Compared to adjacent normal tissue, we demonstrated that high CDKN2A expression was associated with poor survival of UCEC patients (OS:HR=2.9, Logrank $p=0.0055$; DFS:HR=2.3, Log rank $p=0.016$). Meanwhile, significant correlation between CDKN2A expression and immune infiltration was observed in UCEC tumor tissue, such as CD8⁺ T cells ($R=-0.431$, $P=2.72E-05$) and Monocyte ($R=0.295$, $P=5.20E-03$) etc. In addition, the enrichment analysis results showed that CDKN2A and its co-expressed genes were involved in the pathways of RNA metabolism, positive regulation of cell cycle process and TP53 activity. The main gene alteration of CDKN2A in UCEC patients was amplification, and the mutation rate was only 3%. In conclusion, CDKN2A is expected to become a novel prognostic indicator and a potential drug target for UCEC.

Keywords: CDKN2A; Bioinformatics analysis; UCEC.

1. Introduction

Uterine Corpus Endometrial Carcinoma (UCEC) is the most common malignant tumor of uterus, accounts for approximately 5% and 2% of cancer morbidity and mortality in women worldwide [1]. The incidence of UCEC has increased over the years, with approximately 319,600 cases and 76,200 deaths in 2012, which has become the sixth most common cancer in women worldwide and the 14th leading cause of cancer-related deaths [1]. Known risk factors for UCEC include diabetes, hypertension, family history of UCEC, obesity, and hormone-related factors etc. [2].

The CDKN2A gene (Located on chromosome 9P21) encodes proteins p14ARF and p16INK4a, which are involved in cell cycle regulation via p53 and Rb pathways [3]. P14ARF protein stabilizes and activates the p53 pathway [3]. P16INK4a induces allosteric conformational changes of CDK4 or 6 proteins and inhibits the binding of CDK4 or 6 to cyclin D to form complexes [4]. This results in hypo-phosphorylated status of Rb and thereby preventing G1/S cell cycle progression [5]. Interference of these pathways plays a key role in the progression of many cancers [6]. Abnormal gene silencing is closely related to changes in cell cycle regulation during carcinogenesis [5]. In particular, gene silencing of CDKN2A encoding p16INK4a protein is causally related to several different types of cancer [5]. CDKN2A mutations are the most common cause of inherited melanoma and are often associated with the development of familial atypical polynevis melanoma (FAMMM) syndrome [7]. Unlike familial melanoma cases lacking CDKN2A gene mutations, those with CDKN2A germline mutations developed at a younger age, which increased the number of melanoma cases in each family and were more likely to develop high frequency of multiple melanomas and other cancers, especially pancreatic cancer and breast cancer [8, 9]. Thus, CDKN2A was mostly regarded as a tumor suppressor gene in previous studies, and its occurrence of gene silence or mutation would accelerate disease progression. Interestingly, CDKN2A became an oncogenic gene in UCEC patients, and its high expression was associated with worse prognosis and survival.

Bidirectional communication between cells and their surrounding microenvironment is particularly important for the stability of normal tissues and the growth of tumors. Especially, the interaction between tumor cells and stroma cells has a profound impact on the occurrence, development and prognosis of tumors [10]. The complex and dynamic cellular environment in which tumors or cancer stem cells reside is called the tumor microenvironment (TME) [11]. In addition to endothelial cells, stromal cells and fibroblasts, the structural and functional components of the matrix in typical TME also include congenital (macrophages, neutrophils, NK cells, etc.) and adaptive immune cells (T cells and B cells), which could contribute to tumor progression [12]. These different cells communicate with each other through direct contact or release of cytokines and chemokines, controlling and regulating tumor growth in autocrine and paracrine ways [13]. Cytotoxic CD8+ memory T cells are a common type of T lymphocytes in TME that promote favorable cancer prognosis by recognizing specific antigens on tumor cells and stimulating an immune response to kill tumor cells [11, 14]. B cells are seen in TME and along the margin of tumor invasion, but are more often found in draining lymph nodes and adjacent lymphoid tissue [15]. It controls tumor progression by regulating the survival and proliferation of tumor cells and is attribute to the development of treatment resistance [11]. The main types of macrophages in TME include macrophages M1 and macrophages M2, among which macrophages M2 has immunosuppressive effect and promotes tumor development [11]. NK cells have the ability to recognize tumor cells by expressing a series of receptors that allow them to detect cellular targets without harming normal cells [16].

In this study, we investigated the effect of CDKN2A expression on the prognosis of UCEC patients and its relationship with immune infiltration. Enrichment analysis of CDKN2A and its co-expressed genes indicated their involvement on RNA metabolism, cell cycle and TP53 activity. The genetic alterations of CDKN2A of UCEC patients were also explored.

2. Results

2.1 Expression of CDKN2A in UCEC

Using data from the TCGA database, we first compared the difference of CDKN2A expression in UCEC tissues and the adjacent normal tissues. Compared with normal tissues (n=35), we found significant statistical increase in CDKN2A expression levels in primary tumors (n=546, $p<0.001$) (Figure 1A). Meanwhile, subgroup analysis based on individual cancer stages, patient's race, patient's weight, patient's age, Menopause stays, Histological subtypes, and TP53 mutation status were also performed. The expression of CDKN2A in different tumor stages was statistically significant (Figure 1B), which seemed to be positively correlated with the disease severity of UCEC. The expression of CDKN2A in UCEC also varied on patient's race ($p<0.001$), and the expression level was highest in African-American (n=107), followed by Caucasian (n=374), and Asian (n=20) (Figure 1C). Patient's weight was associated with the expression of CDKN2A ($p<0.001$), and the expression level of CDKN2A showed a decreasing trend in patients with normal weight (n=93), obese (n=189), extreme weight (n=113) and extreme obese (n=116). The expression of CDKN2A was statistically significant between the adjacent normal tissues and UCEC tissues of all ages ($p<0.001$), and the highest expression level was found in patients aged 61-80 (Figure 1E). Compared with normal, expression of CDKN2A in UCEC patients of different menopausal states was statistically significant ($P<0.001$), and the expression level of CDKN2A in post-Menopause patients was the highest (n=447) (Figure 1G). By comparing the CDKN2A expression levels of the three histological subtypes of UCEC patients (Endometrioid: n=409, Serous: n=115, Mixed serous and endometrioid: n=22), we found that all of them were statistically significant ($p<0.001$) (Figure 1H). Based on TP53 mutation status, the expression of CDKN2A had statistical significance among normal (n=35), TP53-NonMutant (n=345), and TP53-Mutant ($p<0.001$), and the expression level of CDKN2A was the highest in TP53-Mutant (n=196) (Figure 1H).

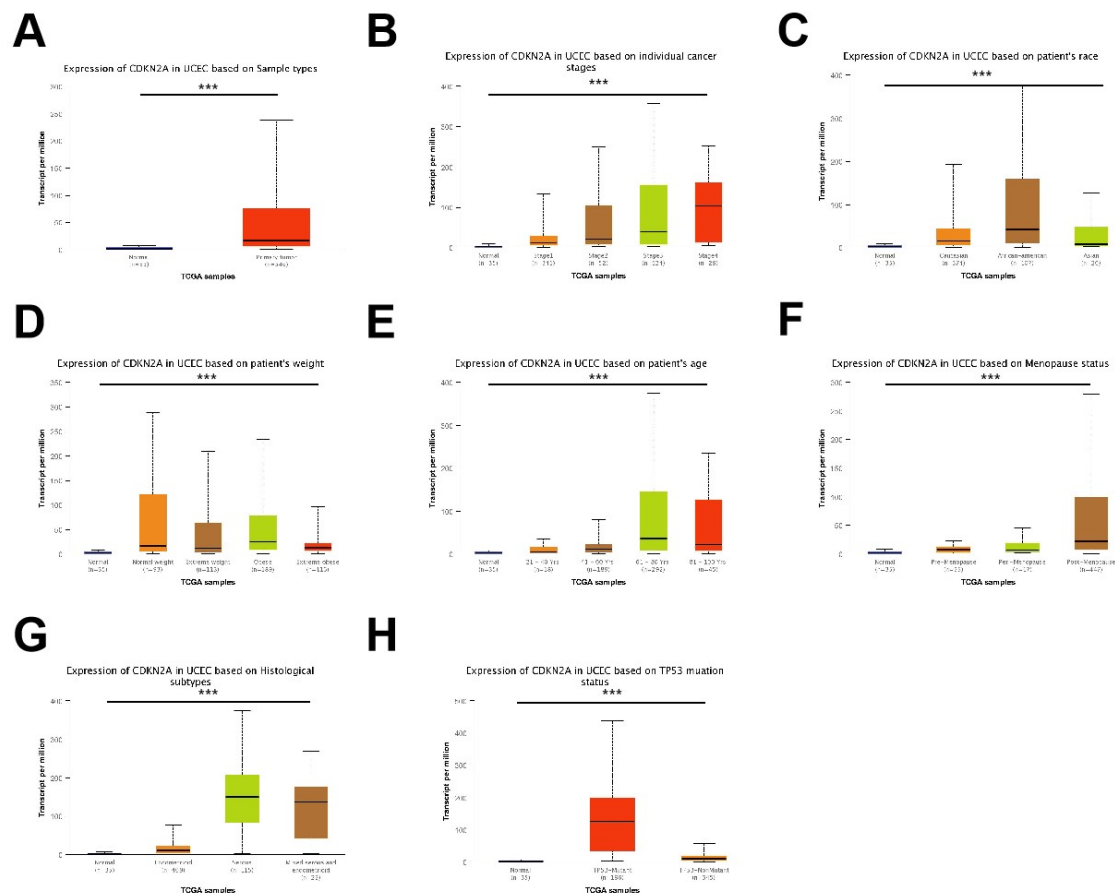


Figure 1. Expression of CDKN2A in UCEC based on (A) sample types, (B) individual cancer stages, (C) patient's race, (D) patient's weight, (E) patient's age, (F) Menopause stays, (G) Histological subtypes, (H) TP53 mutation status. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

2.2 Effects of CDKN2A expression on UCEC prognosis

By using GEPIA 2 [17] which is based on TCGA database, we further explored CDKN2A-related survival rates (overall survival: OS and disease free survival: DFS). We found that CDKN2A is a detrimental prognostic factor for UCEC, and its high expression is associated with poor survival in patients. (OS: HR = 2.9, logrank $P = 0.0055$; RFS, HR = 2.3, logrank $P = 0.016$) (Figures 2A-2B). In addition, as shown in Table 2, Kaplan-Meier Plotter [18] was used to study the correlation between CDKN2A mRNA expression and OS of UCEC patients with different clinicopathological features: female ($P < 0.001$, HR = 2.32, 95% CI from 1.50 to 3.58), White ($P = 0.0093$, HR = 1.92, 95% CI from 1.17 to 3.18), Black/African American ($P = 0.013$, HR = 4.23, 95% CI from 1.23 to 14.55), Grade 2 ($P = 0.24$, HR = 1.93, 95% CI from 0.63 to 5.92), Grade 3 ($P < 0.001$, HR = 2.64, 95% CI from 1.59 to 4.38), Mutation burden low ($P = 0.00039$, HR = 2.73, 95% CI from 1.53 to 4.87), Mutation burden high ($P = 0.28$, HR = 1.46, 95% CI from 0.73 to 2.91), CD4+ T-cells ($P = 0.047$, HR = 2.83, 95% CI from 0.97 to 8.25), CD8+ T-cells ($P = 0.035$, HR = 5.11, 95% CI from 0.98 to 26.63), B cells decreased ($P = 0.02$, HR = 3.48, 95% CI from 1.13 to 10.69), Basophils enriched ($P = 0.0029$, HR = 3.45, 95% CI from 1.45 to 8.20), Macrophages enriched ($P = 0.033$, HR = 4.01, 95% CI from 1.01 to 15.86), Natural killer T-cells enriched ($P = 0.0029$, HR = 5.61, 95% CI from 1.57 to 20.06), Type 1 T-helper cells enriched ($P = 0.013$, HR = 2.61, 95% CI from 1.19 to 5.75), Type 2 T-helper cells enriched ($P = 0.0057$, HR = 3.42, 95% CI from 1.35 to 8.66). In summary, we found that UCEC patients with up-regulated CDKN2A expression had poor prognosis. Subgroup analysis of UCEC patients from the perspective of different clinicopathological features showed that the expression of CDKN2A mRNA in many subgroups was associated with OS in UCEC patients.

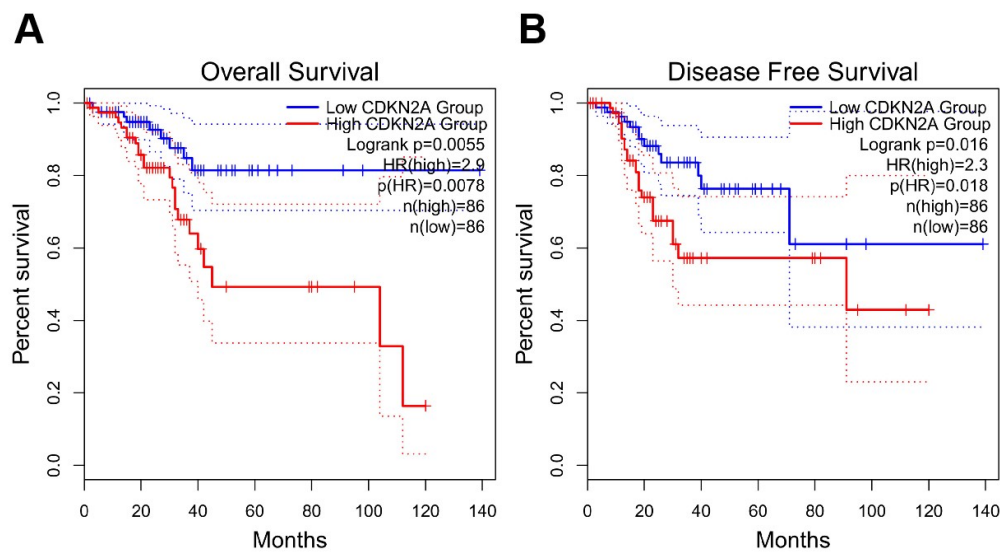


Figure 2. Kaplan-Meier curve comparing the effect of high and low expression of CDKN2A in UCEC on (A) OS and (B) DFS.

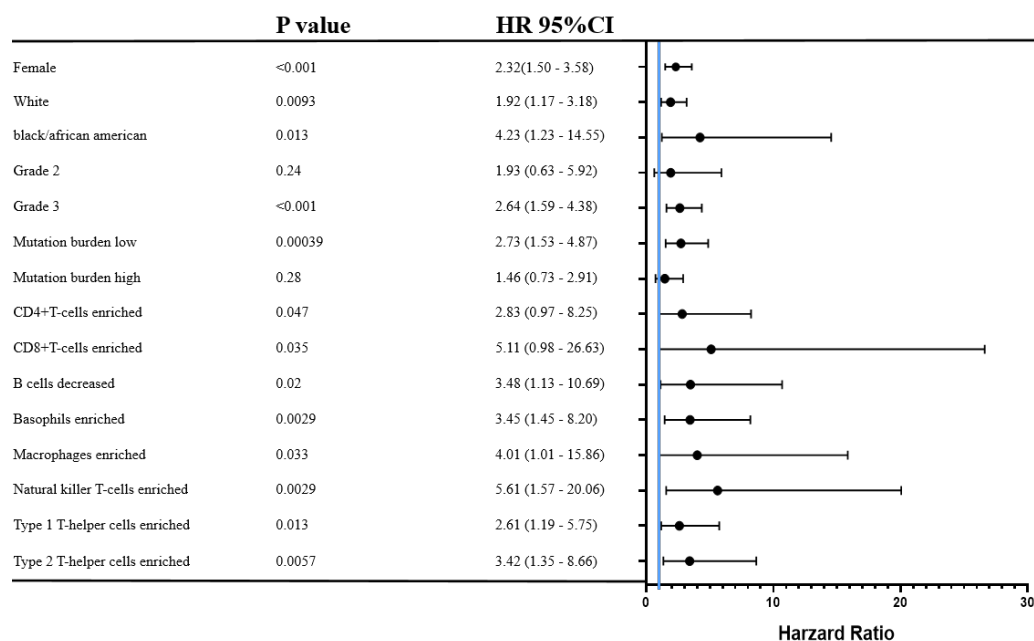


Figure 3. Forest plot of CDKN2A expression and overall survival of UCEC patients with different clinicopathological features. Blue line indicates the value of 1. Black dots represent hazard ratio.

2.3 Correlations between CDKN2A expression and immune infiltration

Immune cells in TME were reported to have an important impact on the survival of patients [11], so it is of great significance to explore the relationship between CDKN2A expression and immune infiltration in UCEC TME. Therefore, we calculated the coefficient of CDKN2A expression and immune infiltration level in UCEC patients (n=545) through TIMER2.0 [19], and determined whether CDKN2A expression in UCEC was related to immune infiltration level. The results showed that CDKN2A expression was significantly negative correlated with CD8+ T cells, NK cell, Macrophage M2. In addition, the expression of CDKN2A was weakly positively correlated B cells and Monocyte in UCEC (Figure 4).

For UCEC, the expression of CDKN2A was weakly positively correlated with B cell (R=0.222, P=3.80E-02), Monocyte (R=0.295, P=5.20E-03) (Figure 3A). On the contrary, the CDKN2A expression had significant negative correlations with the infiltration levels of CD8+ T-cells

($R=-0.431$, $P=2.72E-05$), Macrophage M2 ($R=-0.155$, $P=7.90E-03$), NK cell ($R=-0.234$, $P=2.81E-02$) (Figure 3B). Furthermore, there was no correlation between CDKN2A expression and Tregs infiltration level ($R=0.195$, $P=6.87E-02$) (Figure 3A). Different types of immune cell invasion have different effects on tumor progression [20]. In many human malignancies, the presence of T cells in tumor lesions is associated with better patient outcomes, and CD8+ T cells are considered to be the primary driver of anti-tumor immunity [21]. Extensive clinical and experimental evidence suggests that in most cases, the activity of macrophages is oncogenic and contributes to the progression of malignancy [22]. Macrophages in tumor microenvironment promote tumor-related angiogenesis and decomposition and remodeling of extracellular matrix, improve tumor cell motility and promote tumor progression and metastasis [23]. B cells play an important role in the body's anti-tumor immunity [11]. It has been proved that B cells in tumor tissues of patients with melanoma and renal cell carcinoma are closely related to patients' response to immune checkpoint blockade (ICB) treatment [24]. NK and NKT cells recognize cellular targets through inhibition, adhesion, activation and cytokine receptors, rapidly respond to the presence of tumor cells and participate in anti-tumor immune responses [16].

These results strongly demonstrated the relationship between CDKN2A expression and immune infiltration in UCEC. They may be related to anti-tumor immune response, tumor cell survival and metastasis, tumor treatment and patient prognosis, but the specific effects need to be further studied

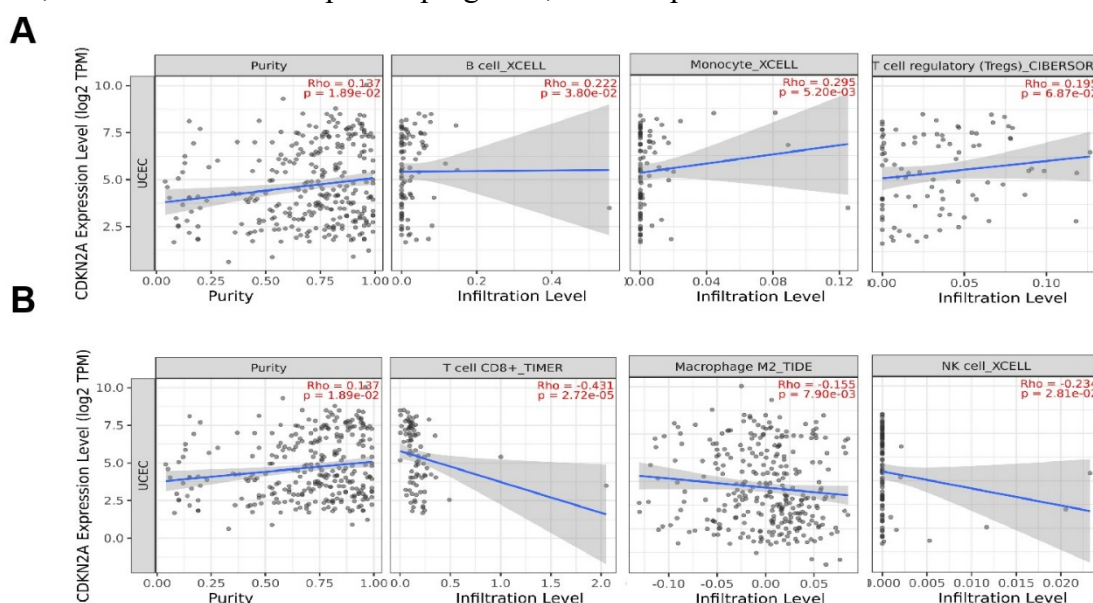


Figure 4. Correlation between CDKN2A expression and immune infiltration level in UCEC. (A) CDKN2A expression showed weak positive correlation with B cell and monocyte infiltration level, and T cell regulatory (Tregs) infiltration level. (B) CDKN2A expression was significantly negatively correlated with infiltration level of CD8+ T cells, NK cells and Macrophage M2. $P<0.05$ is considered as statistical significant.

2.4 Enrichment analysis of CDKN2A co-expressed genes

GEPIA 2 was used to search for top 300 genes with similar expression patterns or characteristics to CDKN2A in different cancer types and tissues. The top 20 pathways obtained after enrichment analysis of 300 CDKN2A co-expressed genes collected from GEPIA 2 by Metscape are shown in Figure 4A. To further capture the relationships between the terms, a subset of enriched terms has been selected and rendered as a network plot, where terms with a similarity > 0.3 are connected by edges (Figure 4B). These findings suggest that CDKN2A co-expressed genes were involved in many physiological activities related to cancer, including cell cycle regulation, RNA transport and metabolism. For each given gene list, protein-protein interaction enrichment analysis has been carried out with the STRING databases (Figure 5C).

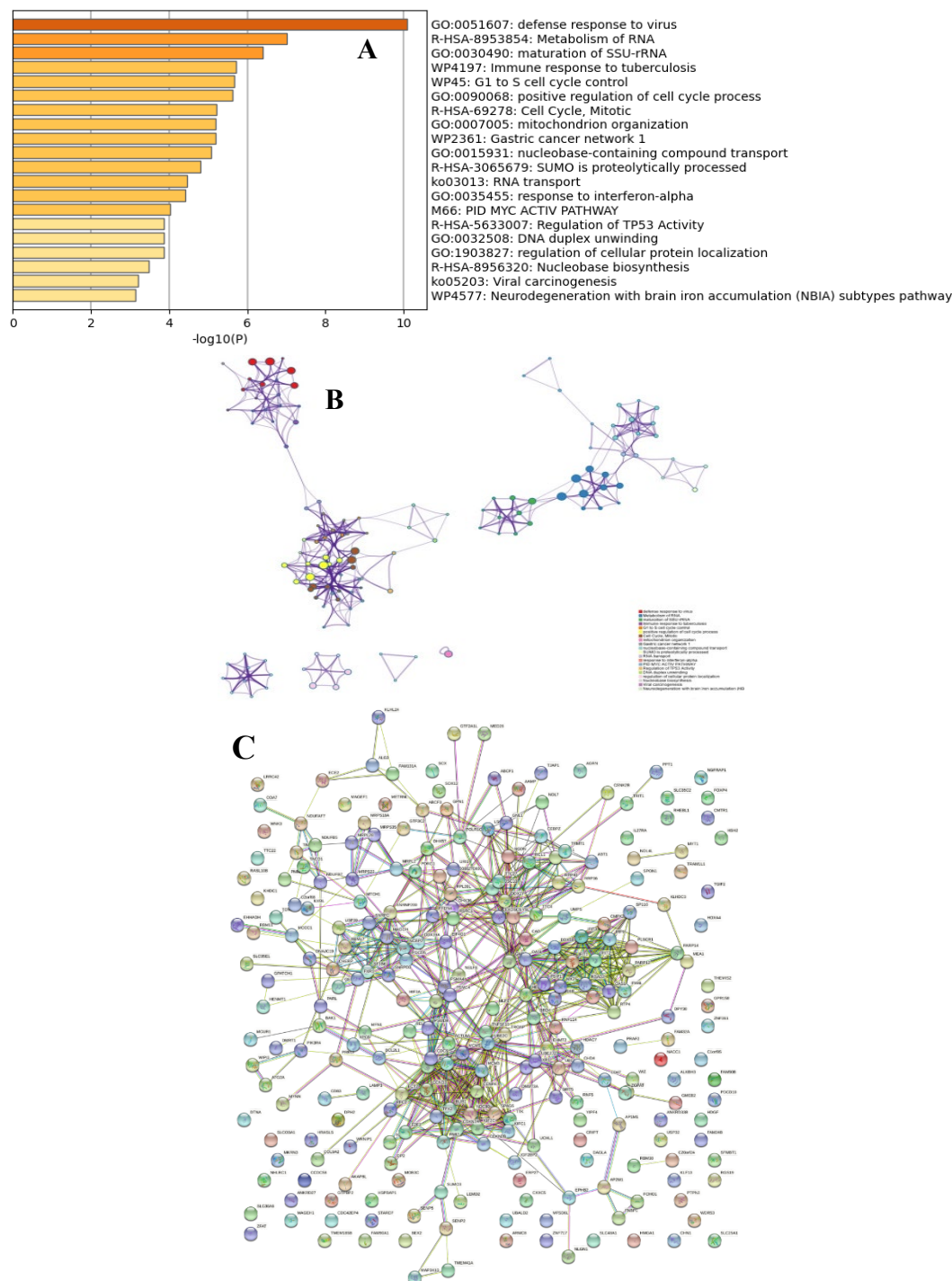
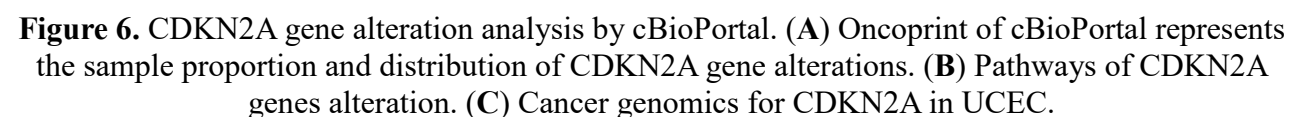


Figure 5. (A) Bar Graph Summary of top 20 significant pathways of enrichment analysis by Metascape. (B) Network of enriched terms, colored by cluster ID, where nodes that share the same cluster ID are typically close to each other. (C) Protein-protein interaction network conducted by STRING. Network nodes represent proteins and edges represent protein-protein associations.

2.5 The analysis of gene alterations of CDKN2A in UCEC

Alteration frequency of CDKN2A in UCEC was analyzed by using cBioPortal (Figure 6A, 6C). CDKN2A was observed to be altered in 1.89% of 529 cases, alteration types include mutation (1.13%, 6 cases) and amplification (0.76%, 4 cases) (Figure 6A). CDKN2A genes alteration are not common in UCEC patients. Significant pathways of CDKN2A genes alteration including TP53 and cell cycle (Figure 6B). A total of eight mutations were found in UCEC patients/samples, the mutation type of one was splice, one was Frame_Shift_Ins and the rest were missense.



In this study, we explored CDKN2A as an oncogenic gene, the high expression of it is associated with worse prognosis in UCEC patients. Moreover, we found that the increased expression level of CDKN2A would affect the infiltration of various immune cells in TME. Meanwhile, enrichment analysis of CDKN2A and its co-expressed genes showed that they were involved in regulating cell cycle, RNA metabolism and TP53 activity in UCEC, which was also consistent with the function of CDKN2A in regulating cell cycle in previous studies. Therefore, CDKN2A has the potential to be a prognostic biomarker for UCEC or as a new therapeutic target for UCEC.

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