Am J Cancer Res 2023;13(5):1826-1844
www.ajcr.us /ISSN:2156-6976/ajcr0149492

Original Article

Screening of four signature genes for clinical testing through bioinformatics and in vitro methods in head and neck squamous cell carcinoma

Muhammad Javed[1], Rimsha Sadia Bukhari[2], Rubab Rasool[3], Majid Alhomrani[4,5], Saleh A Alghamdi[6], Hamza Habeeballah[7], Faiqah Ramzan[8], Bandar K Baothman[9], Muhammad Kashif[10], Sarah Almaghrabi[11,12], Abdullah M Izmirly[13,14], Syed Yousaf Khalid[15]

[1]Primary and Secondary Health Care Department, Lahore, Pakistan; [2]Fatima Jinnah Medical University, Lahore, Pakistan; [3]Health Bridge Hospital, Lahore, Pakistan; [4]Department of Clinical Laboratories Sciences, The Faculty of Applied Medical Sciences, Taif University, Taif, Saudi Arabia; [5]Centre of Biomedical Sciences Research (CBSR), Deanship of Scientific Research, Taif University, Taif, Saudi Arabia; [6]Department of Clinical Laboratory Since, Medical Genetics, College of Applied Medical Sciences, Taif University, Taif, Saudi Arabia; [7]Faculty of Applied Medical Sciences in Rabigh, King Abdulaziz University, Jeddah 21589, Saudi Arabia; [8]Department of Animal and Poultry Production, Faculty of Veterinary and Animal Sciences, Gomal University, Dera Ismail Khan, Pakistan; [9]Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences in Rabigh, King Abdulaziz University, Jeddah 21589, Saudi Arabia; [10]Department of Clinical Sciences, Sub Campus Jhang, University of Veterinary and Animal Sciences, Lahore, Pakistan; [11]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah 21589, Saudi Arabia; [12]Center of Innovations in Personalized Medicine (CIPM), King Abdulaziz University, Jeddah 21589, Saudi Arabia; [13]Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, King Abdulaziz University, P.O. Box 80216, Jeddah 21589, Saudi Arabia; [14]Special Infectious Agents Unit-BSL3, King Fahd Medical Research Center, King Abdulaziz University, Jeddah 21589, Saudi Arabia; [15]Department of General Surgery, Letterkenny University Hospital, Ireland

Received February 3, 2023; Accepted March 23, 2023; Epub May 15, 2023; Published May 30, 2023

Abstract: Head and neck squamous cell carcinoma (HNSC) is the 6th most common cancer around the globe; its underlying molecular mechanisms and accurate molecular markers are still lacking. In this study, we explored hub genes and their potential signaling pathways through which these genes participate in the development of HNSC. The GSE23036 gene microarray dataset was attained from the GEO (Gene Expression Omnibus) database. Hub genes were identified via the Cytohubba plug-in application of the Cytoscape. The Cancer Genome Atlas (TCGA) datasets and cell lines (HOK and FuDu) were used to evaluate expression variations in the hub genes. Moreover, promoter methylation, genetic alteration, gene enrichment, miRNA network, and immunocyte infiltration analysis were also performed to confirm the oncogenic role and biomarker potential of the hub genes in HNSC patients. Based on the hub gene analysis results, four hub genes, including KNTC1 (Kinetochore Associated 1), CEP55 (Centrosomal protein of 55 kDa), AURKA (Aurora A Kinase), and ECT2 (Epithelial Cell Transforming 2), with the highest degree scores were denoted as hub genes. All these four genes were significantly up-regulated in HNSC clinical samples and cell lines relative to their counterparts. Overexpression of KNTC1, CEP55, AURKA, and ECT2 was also associated with poor survival and various clinical parameters of the HNSC patients. Methylation analysis through targeted bisulfite sequencing of HOK and FuDu cell lines revealed that the overexpression of KNTC1, CEP55, AURKA, and ECT2 hub genes was due to their promoter hypomethylation. Moreover, higher expressions of KNTC1, CEP55, AURKA, and ECT2 were positively correlated with the abundance of the CD4+ T cells and macrophage while with the reduction of CD8+ T cells in HNSC samples. Finally, gene enrichment analysis showed that all hub genes are involved in "nucleoplasm, centrosome, mitotic spindle, and cytosol" pathways. In conclusion, the KNTC1, CEP55, AURKA, and ECT2 genes could be potential biomarkers for HNSC patients and provide a novel insight into the diagnosis and treatment of the disease.

Keywords: HNSC, biomarker, TCGA, cell lines

Introduction

Head and neck squamous cell carcinoma (HNSC) ranked 6th as the most prevalent cancer around the globe [1]. Every year, around 300000 people die due to HNSC, and these numbers are expected to increase in the coming years [1]. Although the success ratio of HNSC treatment has obviously improved over the last few decades, the 5-year survival rate of HNSC patients is still below 40% worldwide [2]. The poor survival of HNSC patients is because of failure in the early detection of this disease. The improvement of survival rates in HNSC patients largely depends on the early diagnosis of this disease. Therefore, accurate diagnosis is very critical for successfully treating HNSC patients.

During the last few years, using gene microarray and next-generation sequencing (NGS) technologies, a huge variety of novel diagnostic, prognostic biomarkers or potential treatment targets have been reported in HNSC patients [3, 4]. However, in previous independent research, the utilization of small sample sizes, different screening platforms, and biomarker selection criteria have all contributed significantly to the biasness of the results. To solve this problem, integrative multi-omics research has been preferred to obtain more stable, sensitive, and accurate molecular biomarkers. Previously, HNSC biomarkers included P16INK4A, CK4, CDK6, HRAS, TP53, BRCA1, and PTEN [5-7]. However, the performance of these biomarkers is not up to mark.

In the current study, a single HNSC GSE23036 [8] gene microarray dataset was initially used to determine the differentially expressed genes (DEGs) and hub genes. Then, we used multiple HNSC TCGA datasets from various platforms (UALCAN, GEPIA, OncoDB, and GENT2) and HNSC cell lines (HOK and FuDu) to validate the expression profiles of the hub genes. In addition to this, the identified hub genes were also further investigate via a series of bioinformatics analyses to investigate their diagnostic and prognostic performances in the HNSC patients. Ultimately, the outcomes of the present study shed light on the novel diagnostic and prognostic roles of four novel genes in HNSC, including KNTC1 (Kinetochore Associated 1), CEP55 (Centrosomal protein of 55 kDa), AURKA (Aurora A Kinase), and ECT2 (Epithelial Cell Transforming 2).

Methodology

The HNSC gene microarray dataset collection

The HNSC gene microarray dataset (accession # GSE23036) was collected from the GEO database [9]. The GSE23036 [8] dataset contained mRNA expression profiles of total 68 samples, including 63 HNSC and 5 normal controls.

Data processing for DEGs analysis

Prior to the DEGs analysis, a matrix consisting of gene expression values in GSE23036 was converted with the help of R-language based log2 function [10], and the values were obtained as log2 transformed expression values. The quality of expression data for outlier values was checked through principal component analysis (PCa) [11]. To ensure the quality of the data only those genes whose expression was detected in at least 3 samples were considered for the further analyses. DEGs were determined with the help of R-language based "Limma" package [12]. Only genes having "|Log2FC| > 1.0, false discovery rate (FDR) < 0.05 and P < 0.05" were denoted as DEGs between HNSC and normal samples.

Construction of the protein-protein interaction (PPI) network and the selection of hub genes

The analysis of interactions and creation of the PPI between DEGs were carried out by STRING [13] using the DEGs acquired from the GSE23036. The created PPI network type was a full STRING network. After creating networks, the PPIs were visualized through the Cytoscape 3.7.1 software for module and hub gene identification [14]. The critical module was identified using the MCODE plug-in application of the Cytoscape, and the top four genes having the highest degree scores than other genes in the significant module were selected by Cytohubba plug-in application as potential hub genes [15].

UALCAN analysis

The expression testing of identified hub genes at the mRNA and protein levels across the TCGA HNSC dataset was performed using the UALCAN database [16]. The clinical parameter-

based expression analysis feature of this database also facilitated us to investigate clinical parameter-based expression profiles of the hub gene.

Validation and prognostic analyses

The GEPIA [17], OncoDb [18], and GENT2 [19] are online TCGA databases. In this study, these three databases were tested to validate the mRNA expressions of the hub gene across HNSC samples relative to controls. In addition, the "Survival Analysis" feature of the GEPIA database also facilitated us in checking the prognostic performance of the hub genes.

Methylation, genetic alterations, and co-express gene analysis

The MEXPRESS [20] and OncoDB [18] databases were tested in this study for exploring methylation statuses, while the cBioPortal [21] database was tested for gaining insight into the genetic alterations among hub genes and identifying their highly co-expressed genes in HNSC patients. The TCGA HNSC datasets were chosen for the mentioned analyses.

Gene enrichment and sub-cellular localization analyses

The gene ontology annotation of the hub genes for CC, BP, MF, and KEGG was done using the DAVID tool [22]. In addition to this, for studying the sub-cellular localization of the proteins encoded by the hub genes, the HPA database [23] was tested in this study.

Immune cells infiltration analysis

The TIMER database [24] was tested in this work in order to investigate correlations among infiltration levels of the immune cells (CD8+ T cells, CD4+ T cells, and macrophages) and expression levels of the identified hub genes in HNSC patients.

miRNA network and drug prediction analyses

For gaining insight into the miRNA network of the hub genes, we utilized ENCORI database to construct the miRNA network of the hub genes [25]. In order to use hub genes as the potential therapeutic targets, possible interactions of the possible drugs with hub genes were explored with the help of DrugBank database [26].

RNA-seq and targeted bisulfite-seq analysis based in vitro validation of the hub genes expression and methylation status

A total of one HNSC cell line, including FaDu, and one normal human oral keratinocyte (HOK) cell line were purchased from the ATCC (American Type Culture Collection). The purchased cell lines were cultured in DMEM (HyClone), supplemented with 10% fetal bovine serum (FBS; TBD), 1% glutamine, and 1% penicillin-streptomycin in 5% CO_2 at 37°C. Total RNA extraction from all these three cells lines was done using TRIzol® reagent method [27], while total DNA was extracted via organic method [28]. Finally, RNA and DNA samples were sent to Beijing Genomics Institute (BGI) company for RNA-seq bisulfite-seq analysis.

After RNA-seq analysis, the gene expression values of the hub genes were normalized using reads per kilo base million reads (RPKM) and fragments per kilo base million reads (FPKM). While, methylation values were normalized as beta values. The obtained FPKM, and beta values against hub genes in HNSC and normal oral keratinocyte (HOK) cell line were compared to identify differences in the expression and methylation levels.

Statistics details for in silico analyses

DEGs were identified using a t-test [29]. While for GO and KEGG enrichment analysis, we used Fisher's Exact test for computing statistical difference [30]. Correlational analyses were carried out using Pearson method. For comparisons, a student t-test was adopted in the current study. All the analyses were carried out in R version 3.6.3 software.

Results

The HNSC gene microarray dataset processing, DEGs determination, and hub gene analysis

Outlying microarray samples can cause huge biasness in the analysis results [31]. Therefore, the removal of such samples is critical prior to further analysis. In order to check the outlier sample, we firstly performed PCa analysis.

Accordingly, none of the samples was detected as an outlier in the GSE23036 HNSC dataset (Supplementary Figure 1A). The boxplot-wise and heatmap-wise expression profiles of the samples included in the GSE23036 is given the Supplementary Figure 1A, 1B. Utilizing the "limma" package, in total, 2487 DEGs were determined between HNSC and normal tissue samples (Supplementary Figure 1C). The dispersion of DEGs was shown with the help of a volcano plot (Supplementary Figure 1D). Such gene expression profiles of the DEGs highlighted that HNSC and normal samples in the analyzed dataset significantly differ from each other in terms of dysregulated genes.

Employing the STRING database, a PPI of the top 250 DEGs with the lowest *p*-values was constructed and subjected to module and hub gene identification analysis (**Figure 1A**). The top identified module was comprised of 13 DEGs (**Figure 1B**, **1C**), and out of which KNTC1 (Kinetochore Associated 1), CEP55 (Centrosomal protein of 55 kDa), AURKA (Aurora A Kinase), and ECT2 (Epithelial Cell Transforming 2) genes with the highest degree scores were denoted as hub genes for further analysis (**Figure 1D**).

TCGA expression analysis of hub genes

In order to check the worthiness of hub genes as a novel diagnostic tool, we undertook the expression analysis of these genes in the TCGA dataset via the UALCAN. The expression analysis was carried at the both mRNA and protein levels across HNSC patients with different clinical variables and their corresponding control samples. As shown in **Figures 2**-**4**, the hub gene (KNTC1, CEP55, AURKA, and ECT2) expressions were notably ($P < 0.05$) higher in HNSC samples of different clinical parameters at the mRNA (**Figure 3**) as well as protein (**Figure 4**) levels relative to the controls.

Hub gene expressions verification and prognostic performance

A single TCGA dataset may not be sufficient to determine the worthiness of the KNTC1, CEP55, AURKA, and ECT2 as diagnostic tool. Thus, we utilized 3 more TCGA datasets from 3 different online sources, including GEPIA, OncoDB, and GENT2, for mRNA expression verification purposes. Analysis outcomes from these databases were consistent with the UALCAN results, i.e., the significant ($P < 0.05$) up-regulation of KNTC1, CEP55, AURKA, and ECT2 genes in HNSC samples compared to controls were also observed via the GEPIA, OncoDB, and GENT2 databases (**Figure 5A-C**). Moreover, to evaluate the prognostic significance of the KNTC1, CEP55, AURKA, and ECT2 genes, the "survival" module of the GEPIA was used, and results revealed that overexpression of the KNTC1, CEP55, AURKA, and ECT2 genes was associated with the poor prognosis in HNSC patients (**Figure 5D**). In other words, HNSC patients having the overexpression of the KNTC1, CEP55, AURKA, and ECT2 hub genes have shorter survival durations than those HNSC patients who has low expressions of the KNTC1, CEP55, AURKA, and ECT2 hub genes.

Methylation, genetic alterations, and co-express gene analysis

Promoter methylation patterns of the KNTC1, CEP55, AURKA, and ECT2 genes were obtained via MEXPRESS and OncoDB. Significant negative correlations were observed when promoter methylation patterns of the KNTC1, CEP55, AURKA, and ECT2 were compared between HNSC and controls (**Figure 6**). Methylation analysis findings revealed that higher expressions of KNTC1, CEP55, AURKA, and ECT2 across HNSC may be due to decreased methylation levels in their promoter regions.

The cBioPortal was utilized to assess KNTC1, CEP55, AURKA, and ECT2 genes' genetic alterations across the TCGA cohort of 530 HNSC samples. All these four hub genes were genetically altered in HNSC samples with varying frequencies ranging for the 0.4% (in case of AURKA) to 20% (in case of ECT2) (**Figure 7A**). The two most kinds of alterations in those genes were amplification and missense mutations (**Figure 7A**). Therefore, amplification may also be the reason behind the overexpression of KNTC1, CEP55, AURKA, and ECT2 in HNSC samples. Moreover, we further analyzed that the HNSC patient group harboring KNTC1, CEP55, AURKA, and ECT2 genetic alterations had poorer OS and DFs than unaltered HNSC patients (**Figure 7B**). Finally, the co-expressed gene analysis from the cBioPortal showed that along expressed genes in HNSC samples include TMPO, KIF11, UBE2C, and SMC4, respectively (**Figure 7C**).

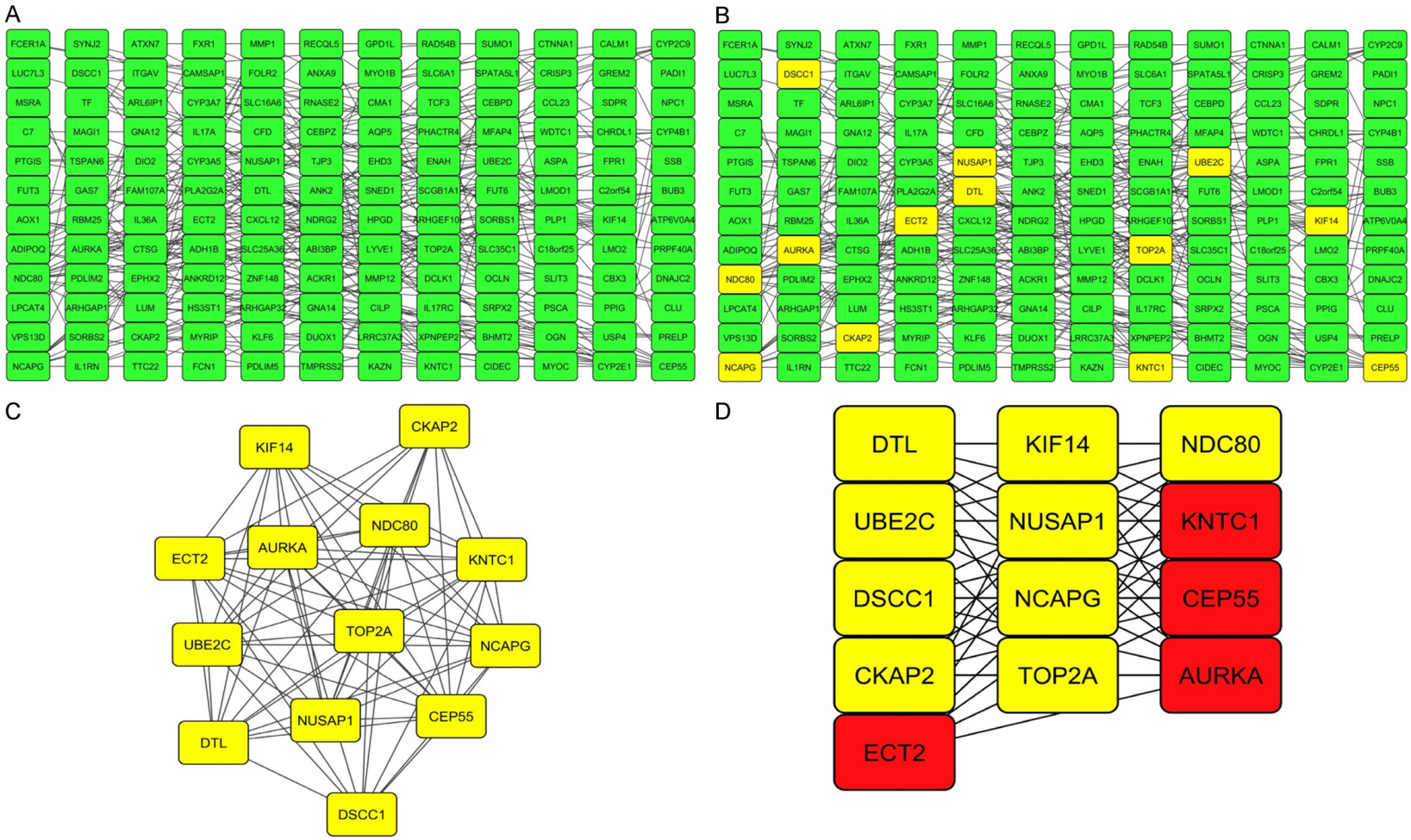


Figure 1. A PPI of the obtained 250 DEGs, a module, and a PPI of the denoted hub genes in GSE23036. (A) A PPI network of the top 250 DEGs in GSE23036 microarray dataset, (B, C) A PPI network of the most significant module, and (D) A PPI network of identified four hub genes. $P < 0.05$ = significant.

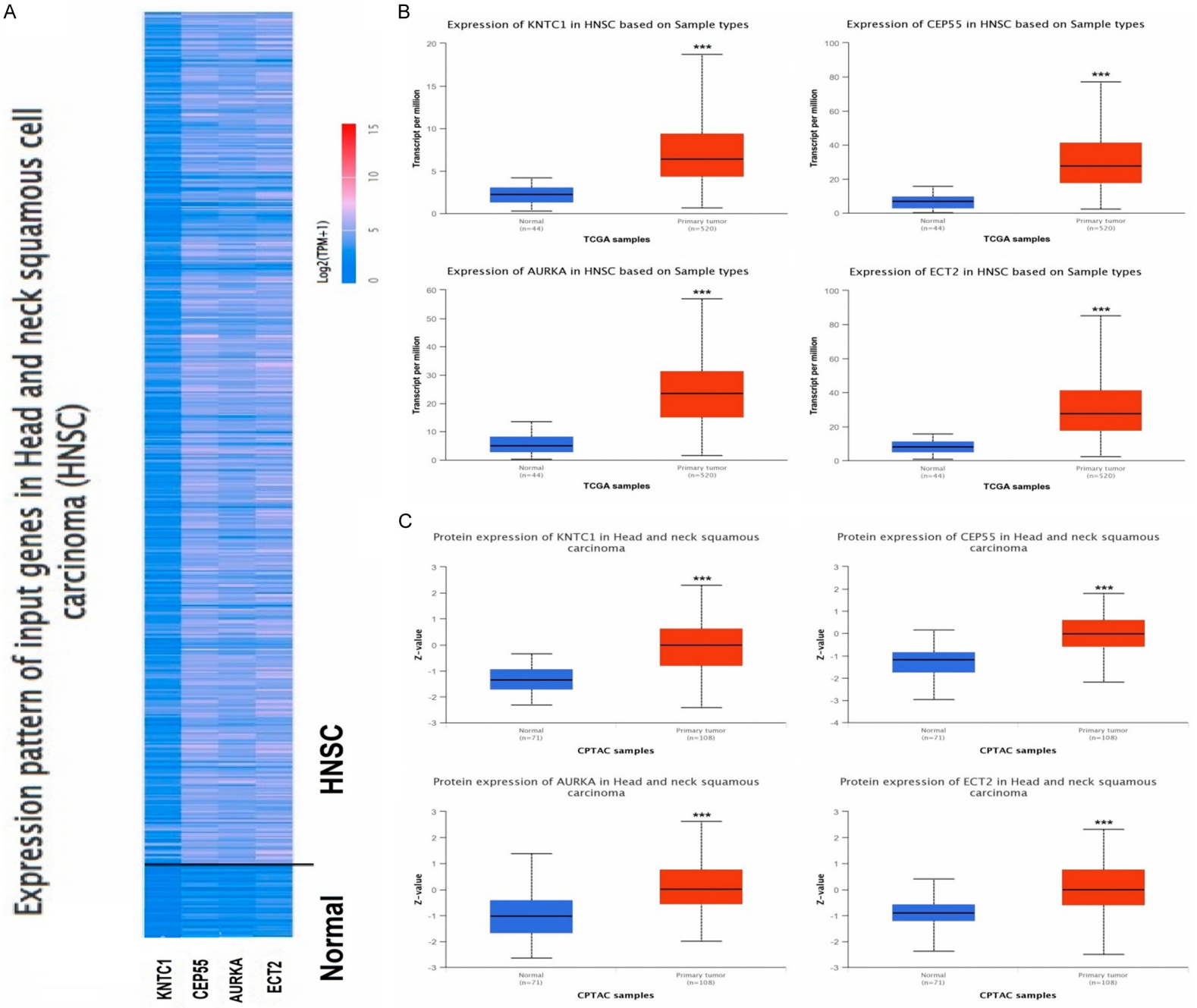

A
Expression pattern of input genes in Head and neck squamous cell carcinoma (HNSC)
Log2(TPM+1)
KNTC1
CEP55
AURKA
ECT2
HNSC
Normal
B
Expression of KNTC1 in HNSC based on Sample types
Expression of CEP55 in HNSC based on Sample types
Expression of AURKA in HNSC based on Sample types
Expression of ECT2 in HNSC based on Sample types
Transcript per million
Normal (n=44)
Primary tumor (n=520)
TCGA samples
C
Protein expression of KNTC1 in Head and neck squamous carcinoma
Protein expression of CEP55 in Head and neck squamous carcinoma
Protein expression of AURKA in Head and neck squamous carcinoma
Protein expression of ECT2 in Head and neck squamous carcinoma
Z-value
Normal (n=71)
Primary tumor (n=108)
CPTAC samples

Figure 2. mRNA and protein expression analysis of KNTC1, CEP55, AURKA, and ECT2 using UALCAN. (A) A heatmap of KNTC1, CEP55, AURKA, and ECT2 in HNSC normal samples group, (B) Box plot presentation of KNTC1, CEP55, AURKA, and ECT2 hub genes mRNA expression, and (C) Box plot presentation of KNTC1, CEP55, AURKA, and ECT2 hub genes protein expression. $P < 0.05$ = significant.

Gene enrichment and sub-cellular localization analyses

As reported by the DAVID tool, the KNTC1, CEP55, AURKA, and ECT2 along with their co-expressed genes were involved in the "germinal vesicle, centralspindlin complex, RZZ complex, female germ cell complex, chromosome passenger complex, and kinetochore microtubule" CC (Supplementary Figure 2A), "histone serine kinase activity, plus-end directed microtubule motor activity, lamin binding, and ubiquitin-like protein ligase binding" MF (Supplementary Figure 2B), "mitotic centrosome separation, centrosome separation, cytokinesis, mitotic sister chromatid segregation, mitotic spindle organization, and mitotic nuclear division" BPs (Supplementary Figure 2C), and "progesterone-mediated oocyte maturation, oocyte meiosis, and ubiquitin mediated proteolysis" KEGG terms (Supplementary Figure 2D).

As for the sub-cellular localization of the proteins encoded by the KNTC1, CEP55, AURKA, and ECT2 hub genes across HNSC cells, the KNTC1 was detected in "cytosol and plasma membrane" (Supplementary Figure 2E), CEP55 was seen in "plasma membrane, centriolar satellite, and midbody" (Supplementary Figure 2E), AURKA was found in "nucleoplasm, centrosome, mitotic spindle, and cytosol" (Supplementary Figure 2E), while ECT2 was detected in "nucleoplasm and cytosol" (Supplementary Figure 2E).

Immune cells infiltration analysis

Across the HNSC TCGA cohort, the TIMER database was used to evaluate correlations among CD8+ T cells, CD4+ T cells, and macrophage infiltration level and expression and expressions of the KNTC1, CEP55, AURKA, and ECT2 hub genes. As reported by the TIMER analysis, higher expressions of KNTC1, CEP55, AURKA, and ECT2 were positively correlated with the abundance of the CD4+ T cells and macrophages while with the reduction of CD8+ T cells across HNSC samples (Supplementary Figure 3).

miRNA network of the hub genes

Via ENCORI and Cytoscape, we constructed the lncRNA-miRNA-mRNA co-regulatory networks of the KNTC1, CEP55, AURKA, and ECT2. In the constructed networks, the total counts of miRNAs, and mRNAs were 182 and 4, respectively (Supplementary Figure 4). Based on the constructed networks, we have identified one miRNA (has-mir-16-5p), that targets all hub genes simultaneously. Therefore, we speculate that the identified miRNA (has-mir-16-5p), and hub genes (KNTC1, CEP55, AURKA, and ECT2) (Supplementary Figure 4) as an axis, might also be the potential inducers of the HNSC.

Drug prediction analysis of the hub genes

Through the DrugBank database, we selected a total of seven drugs (**Table 1**) which can potentially reduce hub gene expressions (KNTC1, CEP55, AURKA, and ECT2), including the two most common drugs, namely cyclosporine and dasatinib (**Table 1**). The identified drugs in this work may be utilize in the treatment of HNSC patients.

Experimental in vitro validation of the hub genes expression and methylation status

In the current study, by performing RNA-seq and targeted bisulfite-seq analyses of one HNSC (FaDu) and one normal human oral keratinocyte (HOK) cell lines, the expression and methylation levels of identified four hub genes were validated. The expression levels of these genes were validated using FPKM, while methylation level was validated using beta values. Both FPKM and beta are quantitative values with widespread use in the RNA-seq analysis. As shown in **Figure 8A**, it was noticed that KNTC1, CEP55, AURKA, and ECT2 hub genes were expressed in both cell lines and RPKM values of KNTC1, CEP55, AURKA, and ECT2 were notably higher in HNSC cell lines (FaDu) as compared to normal cell line (HOK) (**Figure 8A**). Similarly, the beta values of KNTC1, CEP55, AURKA, and ECT2 were higher in normal (HOK) cell line while lower in HNSC cell line (FuDu) (**Figure 8B**).

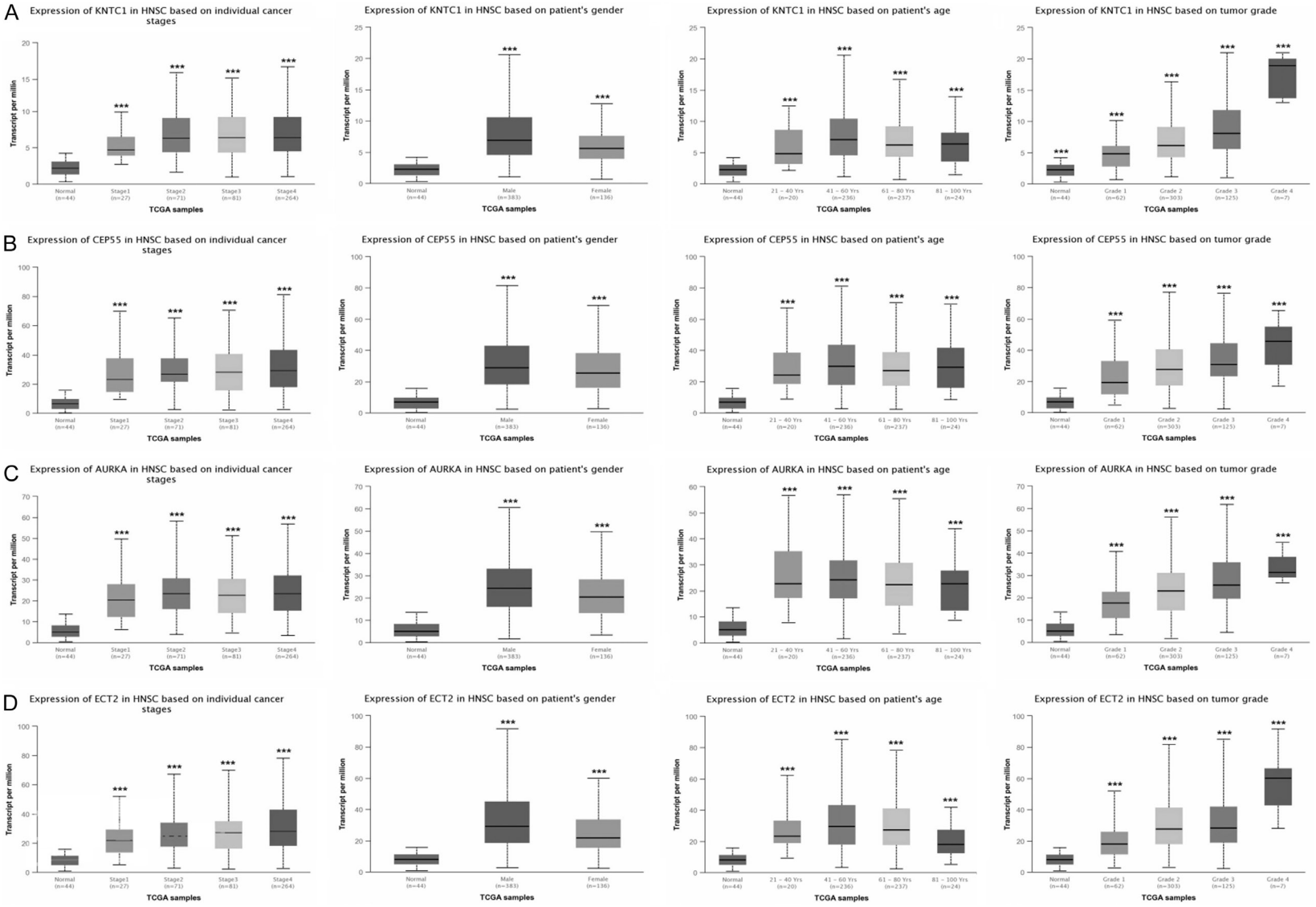


Figure 3. mRNA expression profiling of KNTC1, CEP55, AURKA, and ECT2 in HNSC samples of different clinical variables relative to controls using UALCAN. (A) mRNA expression of KNTC1, (B) mRNA expression of CEP55, (C) mRNA expression of AURKA, and (D) mRNA expression profiling of ECT2. $P < 0.05$ = significant.

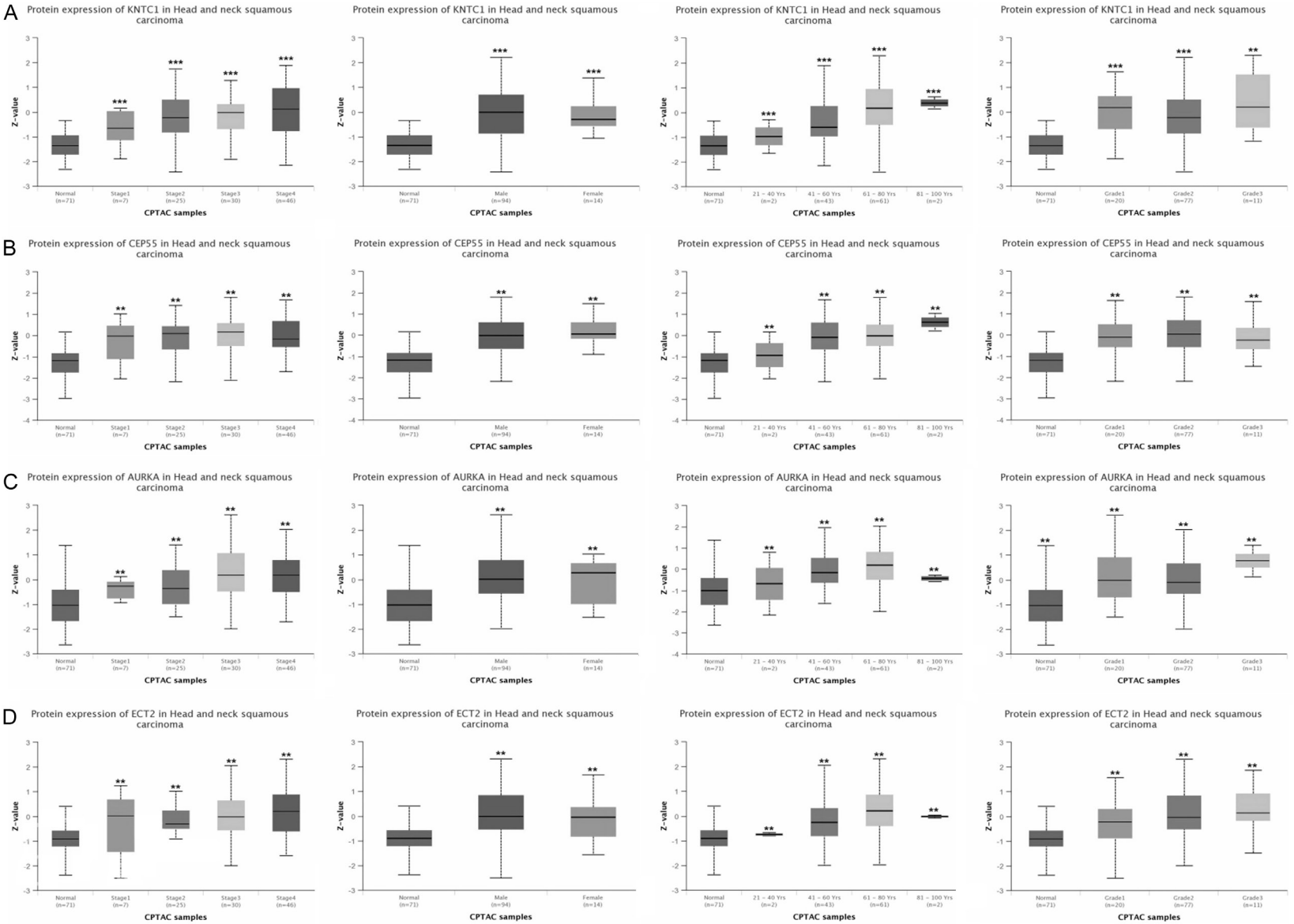


Figure 4. Protein expression profiling of KNTC1, CEP55, AURKA, and ECT2 in HNSC samples of different clinical variables relative to controls using UALCAN. (A) Protein expression of KNTC1, (B) Protein expression of CEP55, (C) Protein expression of AURKA in, and (D) Protein expression of ECT2. $P < 0.05$ = significant.

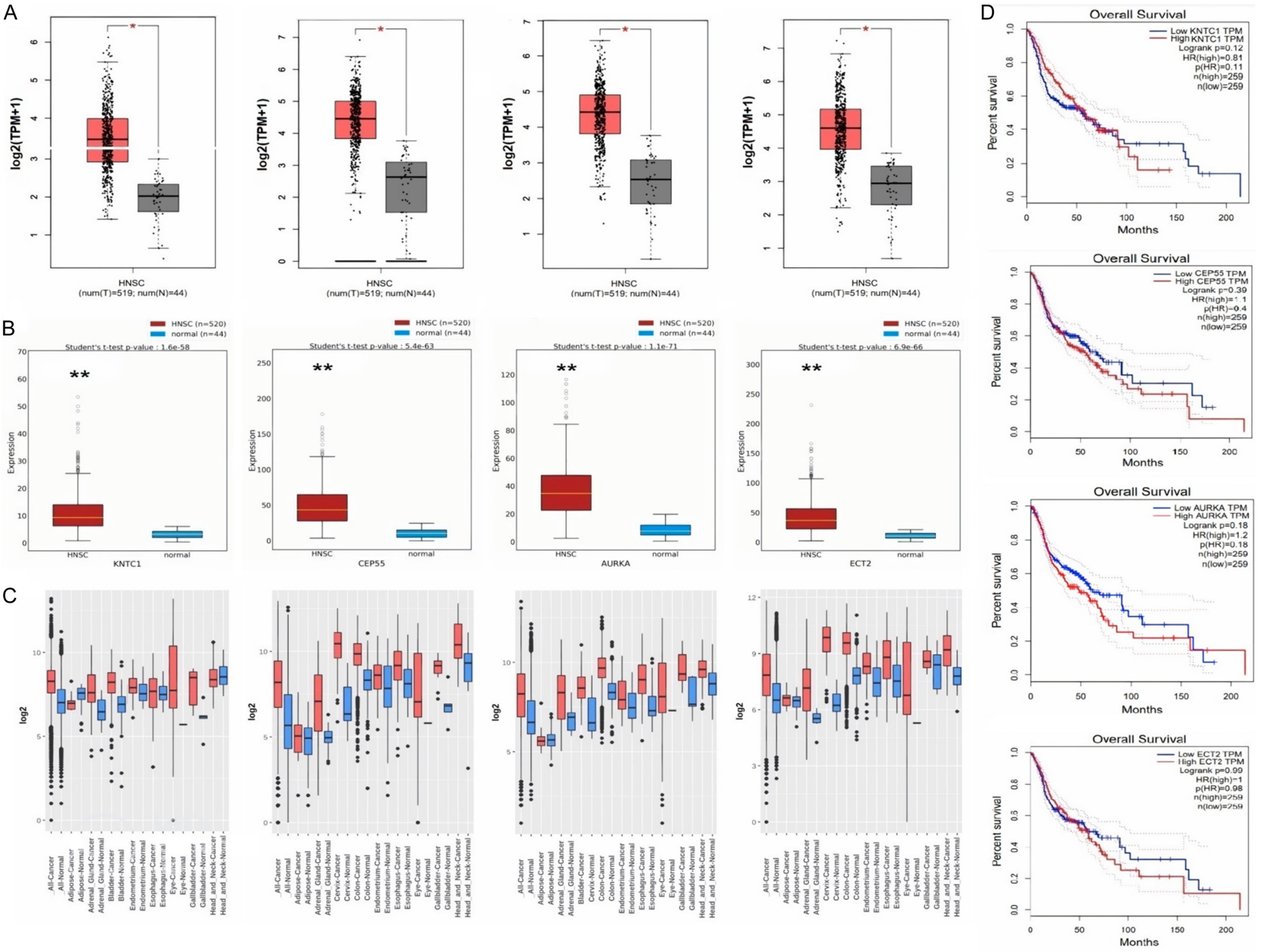

A
log2(TPM+1)
HNSC
(num(T)=519; num(N)=44)
B
HNSC (n=520)
normal (n=44)
Student's t-test p-value : 1.6e-58
Student's t-test p-value : 5.4e-63
Student's t-test p-value : 1.1e-71
Student's t-test p-value : 6.9e-66
Expression
KNTC1
CEP55
AURKA
ECT2
C
log2
D
Overall Survival
Low KNTC1 TPM
High KNTC1 TPM
Logrank p=0.12
HR(high)=0.81
p(HR)=0.11
n(high)=259
n(low)=259
Low CEP55 TPM
High CEP55 TPM
Logrank p=0.39
HR(high)=1.1
p(HR)=0.4
Low AURKA TPM
High AURKA TPM
Logrank p=0.18
HR(high)=1.2
p(HR)=0.18
Low ECT2 TPM
High ECT2 TPM
Logrank p=0.99
HR(high)=1
p(HR)=0.98
Percent survival
Months

Figure 5. Expression validation and survival analysis of KNTC1, CEP55, AURKA, and ECT2. (A) Expression validation of KNTC1, CEP55, AURKA, and ECT2 in HNSC and normal samples via GEPIA database, (B) Expression validation of KNTC1, CEP55, AURKA, and ECT2 in HNSC and normal samples via OncoDB database, (C) Expression validation of KNTC1, CEP55, AURKA, and ECT2 via GENT2 database, and (D) Survival analysis of KNTC1, CEP55, AURKA, and ECT2 in HNSC and normal samples via GEPIA database. P < 0.05 = significant.

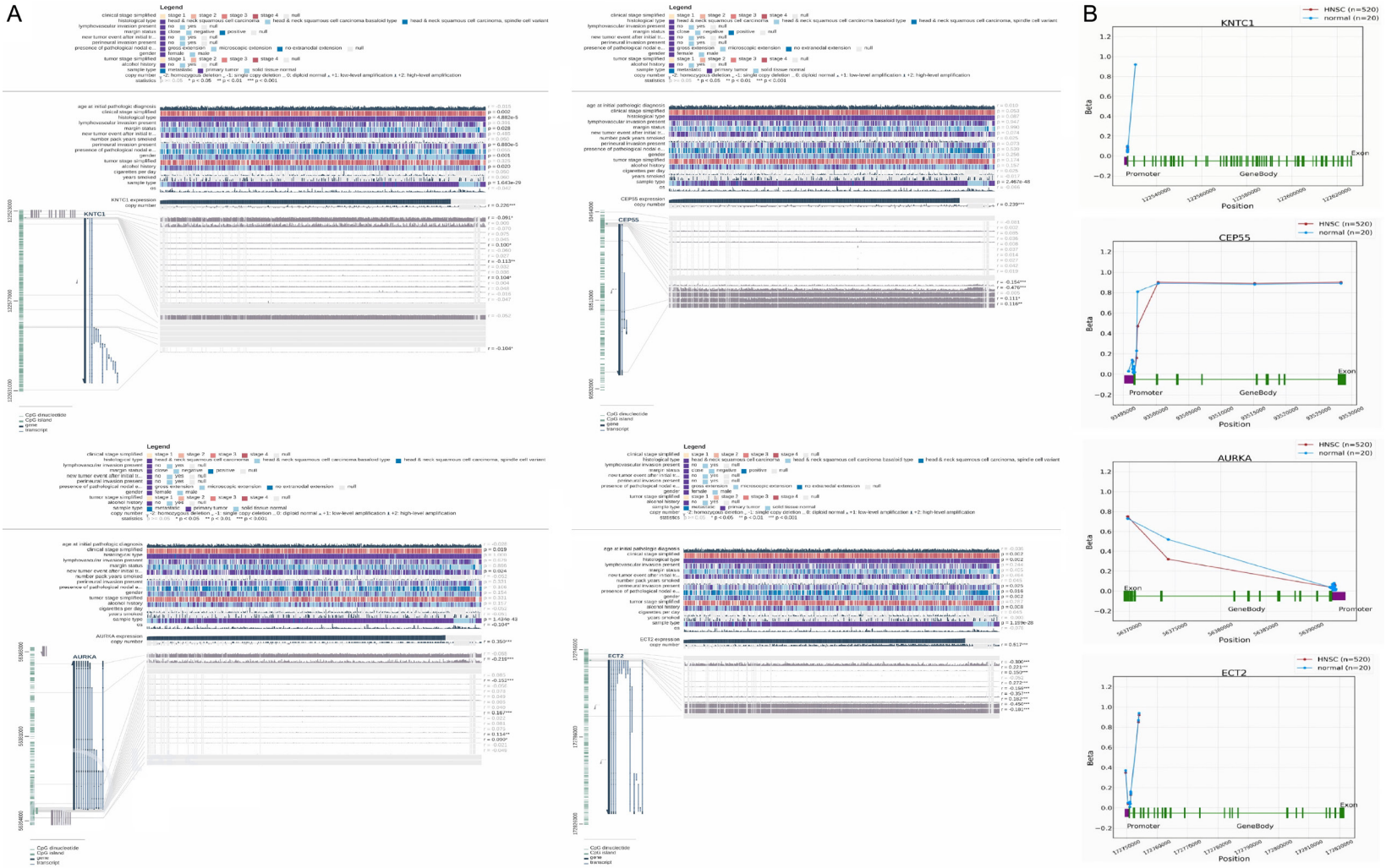
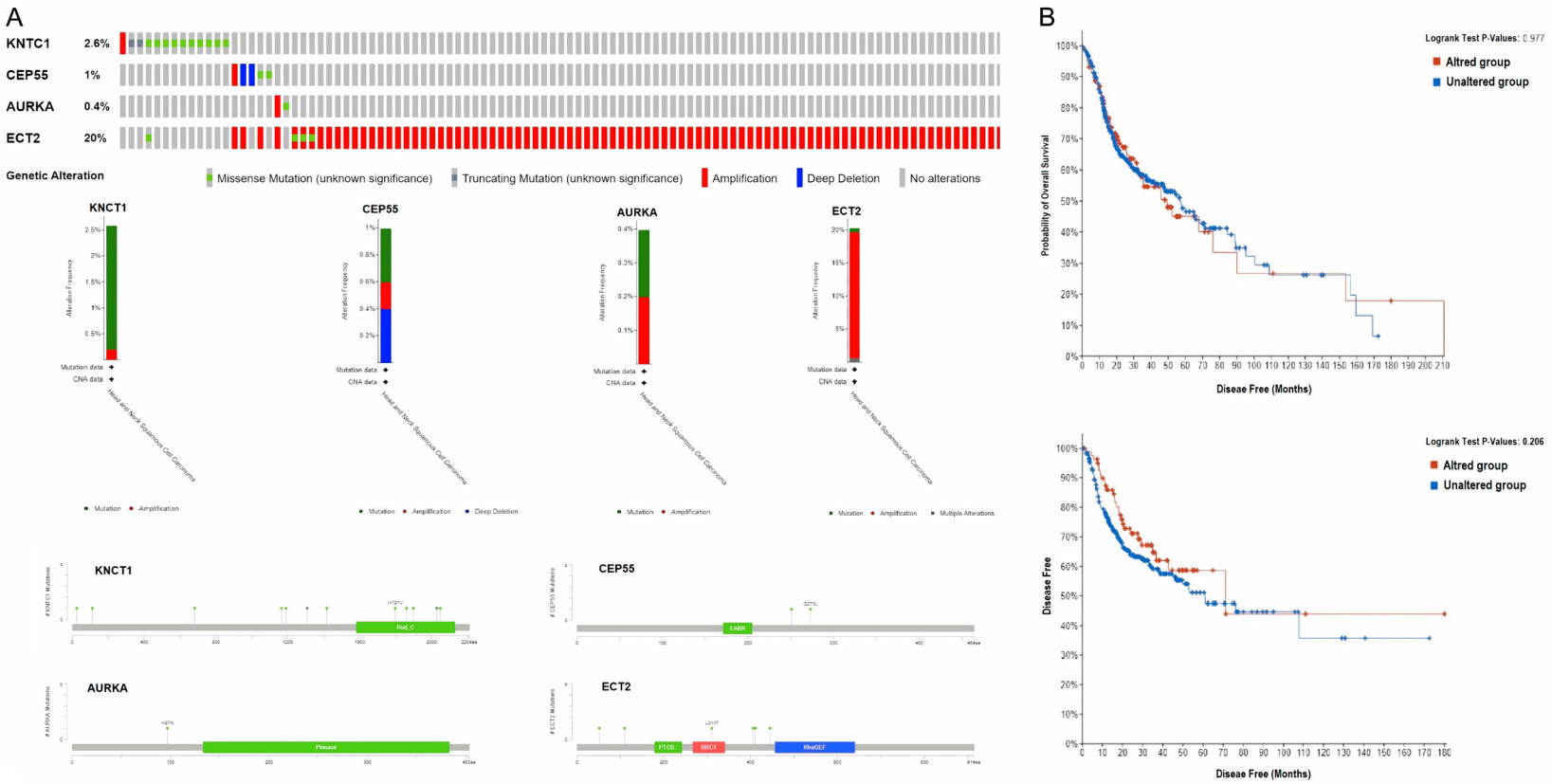


Figure 6. Methylation status exploration of KNTC1, CEP55, AURKA, and ECT2 via MEXPRESS and OncoDB in HNSC and normal samples. (A) Methylation status exploration via MEXPRESS, and (B) Methylation status exploration via OncoDB. P < 0.05 = significant.


A
KNTC1 2.6%
CEP55 1%
AURKA 0.4%
ECT2 20%
Genetic Alteration
Missense Mutation (unknown significance)
Truncating Mutation (unknown significance)
Amplification
Deep Deletion
No alterations
KNCT1
CEP55
AURKA
ECT2
Alteration Frequency
Mutation data
CNA data
Head and Neck Squamous Cell Carcinoma
Mutation
Amplification
Deep Deletion
Multiple Alterations
KNCT1
CEP55
AURKA
ECT2
RodC
EABR
Pkinase
PTCB
BRCT
RhoGEF
B
Logrank Test P-Values: 0.977
Altred group
Unaltered group
Probability of Overall Survival
Disease Free (Months)
Logrank Test P-Values: 0.206
Altred group
Unaltered group
Disease Free
Disease Free (Months)

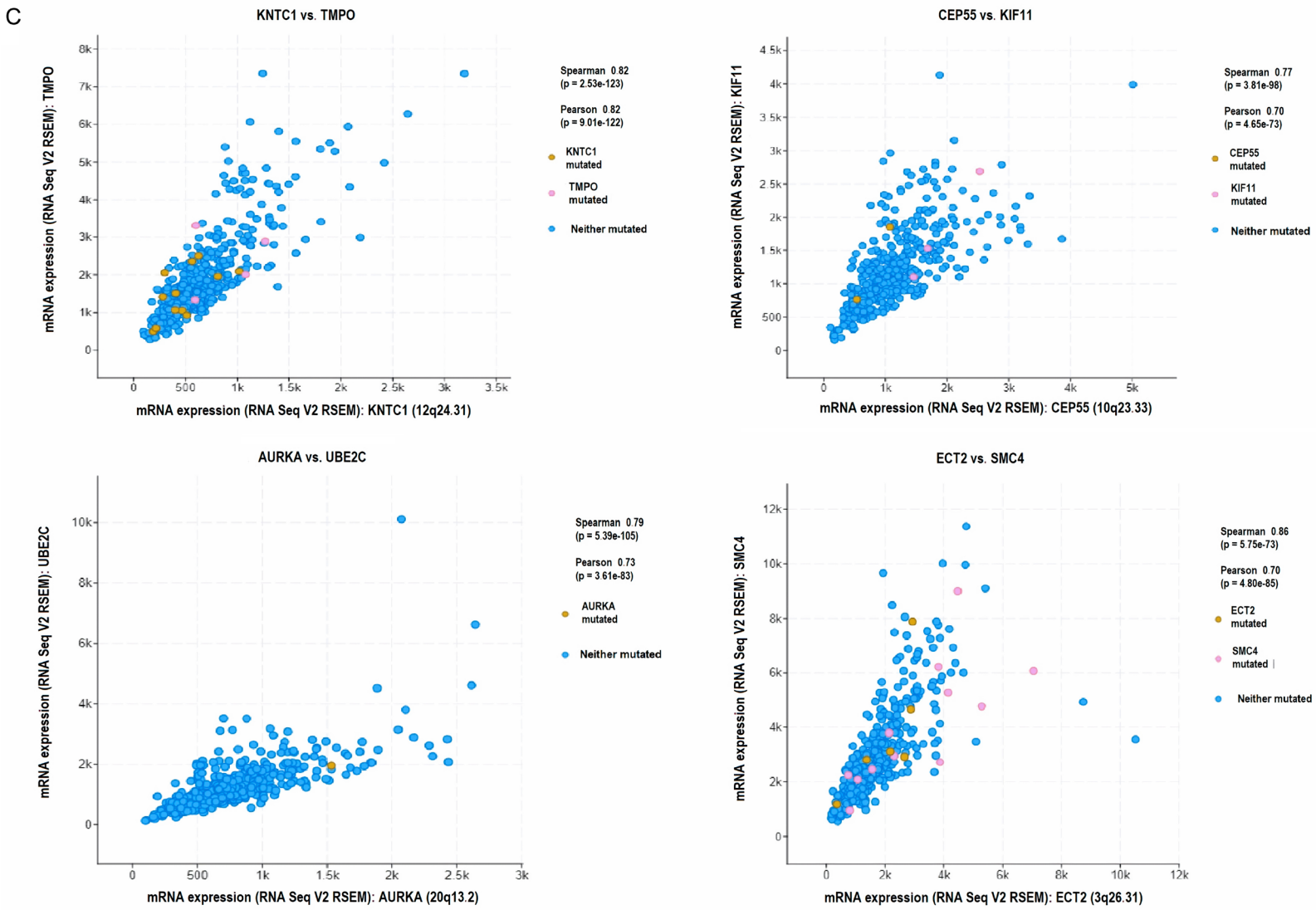


Figure 7. Exploration of genetic alteration frequencies, effect of the genetic mutations on the survival, and co-expressed gene analysis via cBioPortal. (A) Types, frequencies, and location of the genetic alterations in KNTC1, CEP55, AURKA, and ECT2, (B) Effect of the genetic mutations on the OS and DFs of the HNSC patients, and (C) Identification of co-expressed genes with KNTC1, CEP55, AURKA, and ECT2. $P < 0.05$ = significant.

Table 1. DrugBank-based hub genes-associated drugs

Sr. No	Hub gene	Drug name	Effect	Reference	Group
1	KNTC1	Dasatinib	Decrease expression of KNTC1 mRNA	A21899	Approved
		Cyclosporine		A21092	
		Dronabinol		A22083	
		Troglitazone		A24633	
2	CEP55	Dasatinib	Decrease expression of CEP55 mRNA	A21899	Approved
		Tretinoin		A24453	
		Palbociclib		A23569	
		Cyclosporine		A21092	
3	AURKA	Cyclosporine	Decrease expression of AURKA mRNA	A21092	Approved
		Calcitriol		A22301	
		Afimoxifene		A20479	
		Dasatinib		A21899	
4	ECT2	Cyclosporine	Decrease expression of ECT2 mRNA	A21092	Approved
		Dasatinib		A21899	

Discussion

The identification of novel potential molecular biomarkers to achieve accurate HNSC diagnosis at the early stages and discovering appropriate chemotherapeutic drugs and drug targets for treating HNSC patients is highly demanded at present. This current study initially concentrated on the GSE23036 HNSC gene expression microarray dataset for exploring possible biomarkers and therapeutic targets in HNSC patients. After identifying potential signature genes (hub genes), we attempted to integrate various multi-omics TCGA datasets and utilized HNSC cell lines to achieve the possible best quality in terms of validating hub gene expressions and investigating their tumor-causing roles. After a detailed analysis of the GSE23036 HNSC gene expression microarray dataset, we obtained the KNTC1, CEP55, AURKA, and ECT2 genes as potential biomarkers in HNSC. After expression analysis using TCGA HNSC cohorts and cell lines (HOK and FuDu), it was apparent that the KNTC1, CEP55, AURKA, and ECT2 hub genes were up-regulated in HNSC relative to the control samples. Moreover, mutational and methylation analysis revealed that amplification and promoter hypomethylation in these genes notably contribute to the overexpression of these genes in HNSC patients relative to their normal counterparts.

The KNTC1, an evolutionary conserved gene, is one of the major mitotic checkpoint components, that promote proper chromosomal segregation during the cell division process [32, 33]. It is earlier shown by various studies that most of the genes involving the mitosis process are overexpressed in different human malignancies, including cancer, and some of these genes are oncogenes [34, 35]. In this view, the higher expression of KNTC1 was noticed by prior studies in a wide variety of human cancers, including colorectal, breast cancer, esophageal cancer, hepatocellular carcinoma, gastric cancer, and neuroblastoma [36-40]. However, to the best of our knowledge, the KNTC1 role in the pathogenesis of HNSC has not been reported earlier in the medical literature.

The CEP55 protein plays a vital role in the mitosis process, specifically by binding with CDK1, ERK2, and PLK1 proteins [41]. The overexpression of CEP55 is earlier documented in different human cancers, including the cancers of the breast [42], thyroid cancer [43], prostate cancer [44], kidney and so on.

Prior clinical studies revealed that CEP55 overexpression is a potential molecular biomarker for different cancers. Such as, Nina Hauptman et al. [45] highlighted through bioinformatics analysis that CEP55 up-regulation is a marker of diagnosis for colorectal cancer patients. In esophageal squamous cell carcinoma (ESCC) patients, Yang Jia et al. [46] found that overexpression of CEP55 mRNA is correlated with poor survival. Moreover, the 5-year survival of cancer patients with normal CEP55 expression

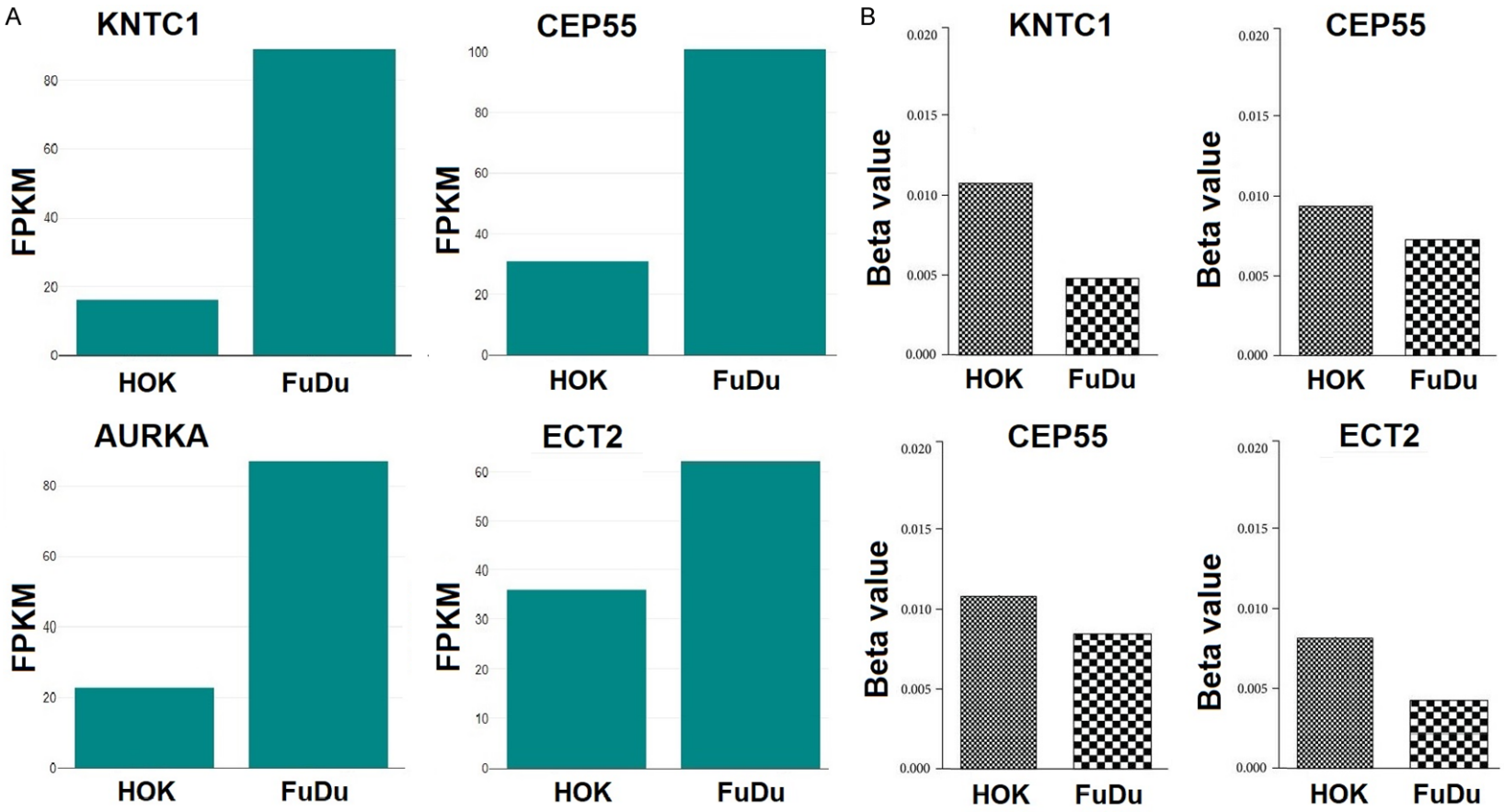


Figure 8. Validating KNTC1, CEP55, AURKA, and ECT2 expressions and methylation status using HOK and FaDu cell lines via RNA-seq and targeted bisulfite-seq analyses. (A) FPKM values based expression plots of the KNTC1, CEP55, AURKA, and ECT2, and (B) Beta values based methylation plots of the KNTC1, CEP55, AURKA, and ECT2.

was notably higher than that of those having overexpression of CEP55 [47]. Regarding the use of CEP55 overexpression as a biomarker in LUAD and LUSC, the results of the previous studies are conflicting [47].

AURKA is an important molecule for cell cycle progression [48, 49]. It has been frequently found overexpressed or mutated in various types of human tumors [39, 40]. For example, Tanaka et al. [41] investigated 33 cases of BRIC and found overexpression of AURKA in 94% of the cases. According to Du et al. AURKA overexpression was correlated with the poor survivals of colorectal, esophageal, and lung cancer patients [50]. Miyoshi et al. observed AURKA overexpression in 64% of the BRIC cases using reverse transcription-polymerase chain reaction (RT-PCR) in a group of 47 patients [42]. However, comparatively, a larger study including 112 BRIC patients did not find any association between AURKA expression and BRIC patients' survival [43]. However, Nadler et al. have observed the elevated expression of AURKA in breast tumors and related it with OS [44].

The ECT2 gene codes for a guanine nucleotide exchange factor whose overexpression is associated with the development of cancer [51]. In certain cancer subtypes, the overexpression of the ECT2 gene was observed in previous studies, and therefore this gene is denoted as the oncogene [52, 53]. The ECT2 mRNA and protein are reported to be significantly overexpressed in ovarian cancer cells relative to normal cells. The overexpressed ECT2 protein was mainly detected in the nucleus of the ovarian cancer cells leading to the higher cell proliferation [54]. Moreover, ECT2 overexpression serves as a major contributor to the occurrence of breast cancer [55]. However, the molecular subtype based cancer driving role of ECT2 in breast cancer was unclear. In addition to this, the dysregulation of ECT2 was also revealed as an oncogenic factor in colorectal [56], esophageal [57], and lung cancers [58].

A huge number of recently conducted researches have claimed that the infiltration of immune cells could significantly accelerate the development of cancer [59]. For example, Hu et al. showed that overexpressed OGN gene can enhance CD8+ T imunne cells infiltration, and thus inhibit blood vessels formation in colon cancer [60]. Keeping this vital information in view, we investigated the relationships among hub genes and a few important immune cell infiltrations. Interestingly, higher expressions of the KNTC1, CEP55, AURKA, and ECT2 were positively correlated with the abundance of the CD4+ T cells and macrophages while with the reduction of CD8+ T cells across HNSC samples, indicating that these genes may be involved in regulating HNSC at immunological level. KEGG analysis results further support this hypothesis; KNTC1, CEP55, AURKA, and ECT2 hub genes were enriched in the "progesterone-mediated oocyte maturation, oocyte meiosis, and ubiquitin mediated proteolysis" immune-related pathways. However, still there is a need to conduct more experiments for validating hub gene associations with immune infiltration in HNSC. We further noticed that KNTC1, CEP55, AURKA, and ECT2 hub genes' expression were regulated simultaneously by hsa-mir-16-5p miRNA in HNSC patients. Previously, the dysregulation of hsa-mir-16-5p in multiple human cancers has been reported in published studies, for example in breast cancer, bladder cancer, glioblastoma, and lung cancer [61, 62]. However, any tumor suppressor or tumor-causing role of hsa-mir-16-5p in HNSC is not reported anywhere. Therefore, the exploration of the hsa-mir-16-5p oncogenic role in HNSC development with further biological experiments will be highly valuable.

Conclusion

In conclusion, by utilizing detailed bioinformatics RNA-seq, and targeted bisulfite-seq methodologies, four hub genes (KNTC1, CEP55, AURKA, and ECT2) were identified as potential markers of the HNSC. All these four hub genes were overexpressed in HNSC samples. The overexpression of KNTC1, CEP55, AURKA, and ECT2 was the outcome of gene amplification and promoter hypomethylation. The KNTC1, CEP55, AURKA, and ECT2 hub genes were involved in immunological regulation across HNSC tissue samples, which further needs to be verified through biological experiments.

Acknowledgements

Majid alhomrani would like to acknowledge Taif University Researchers Supporting project number (TURSP 2020/257), Taif University, Saudi Arabia.

Disclosure of conflict of interest

None.

Address correspondence to: Syed Yousaf Khalid, Department of General Surgery, Letterkenny University Hospital, Ireland. E-mail: syed_yousaf@yahoo.com; Faiqah Ramzan, Department of Animal and Poultry Production, Faculty of Veterinary and Animal Sciences, Gomal University, Dera Ismail Khan, Pakistan. E-mail: faiqah.ramzan@gmail.com

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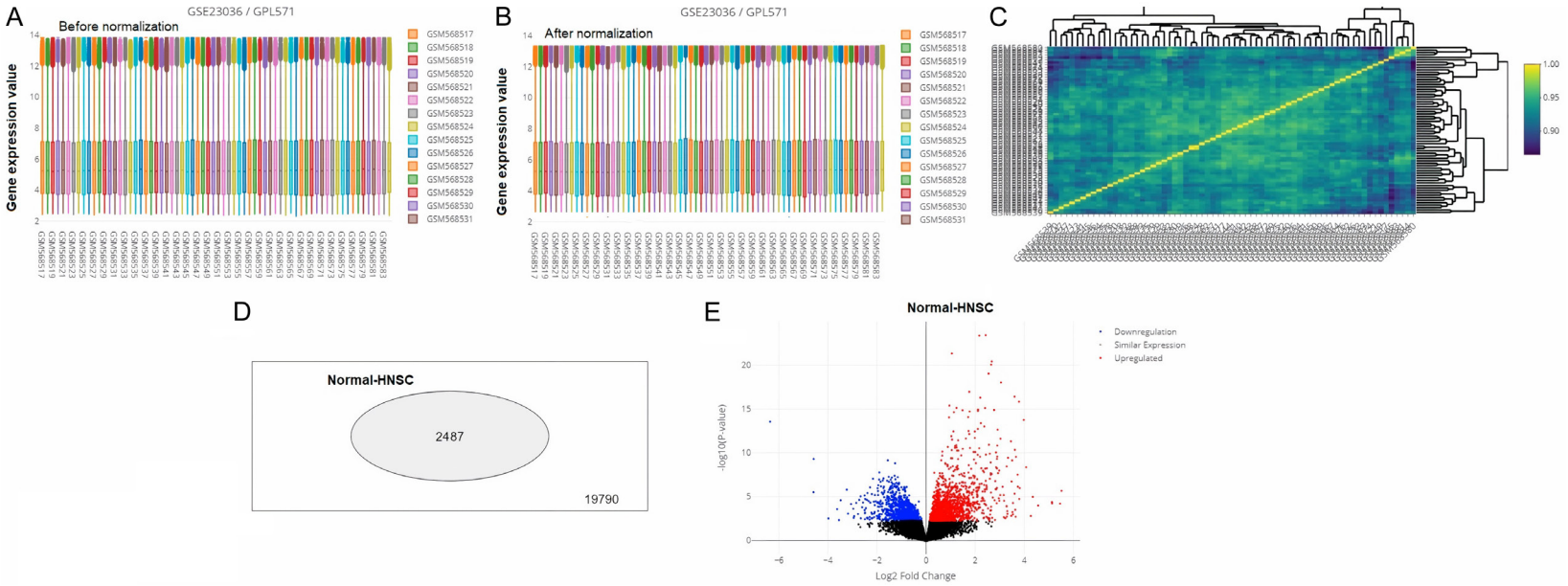
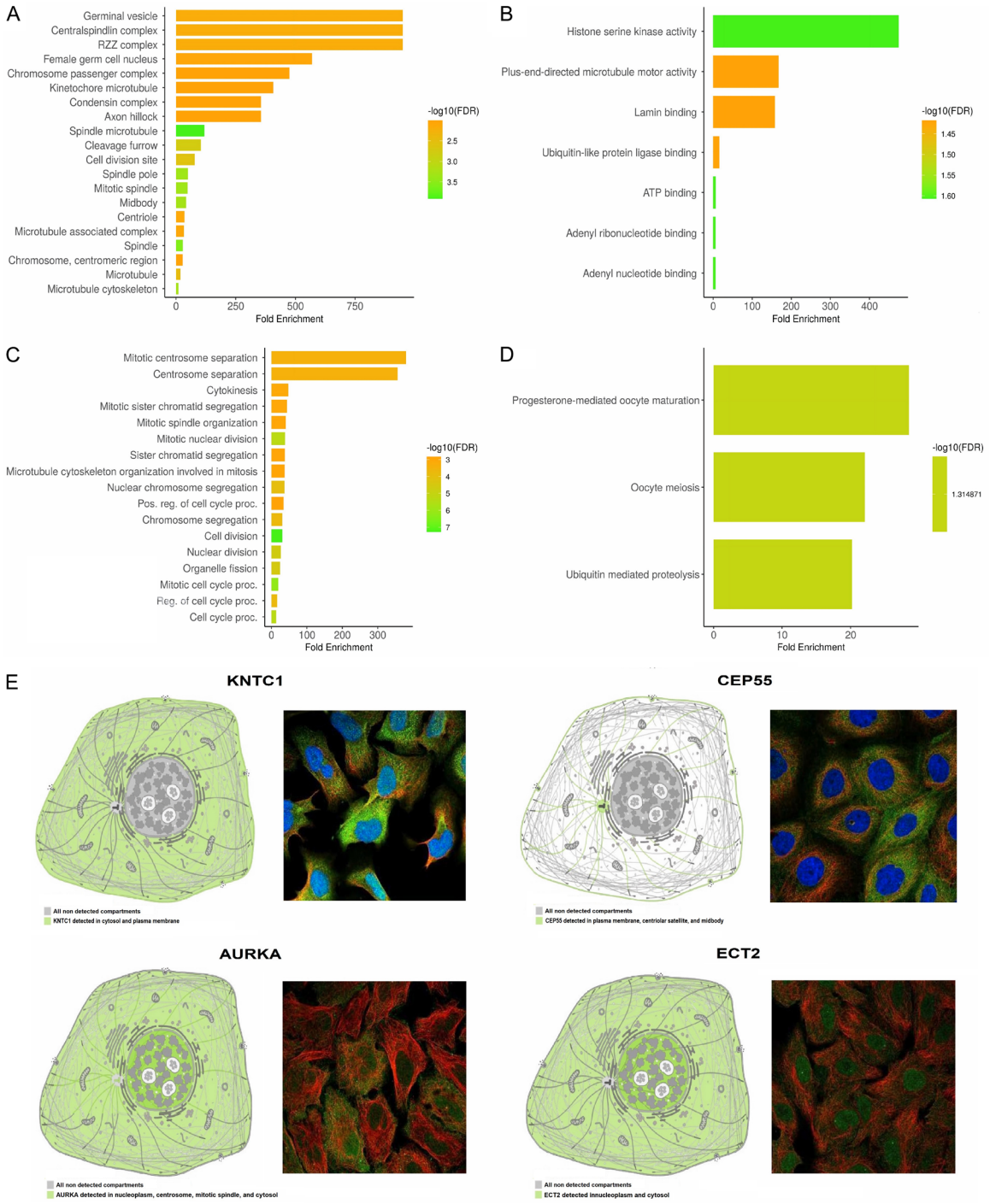
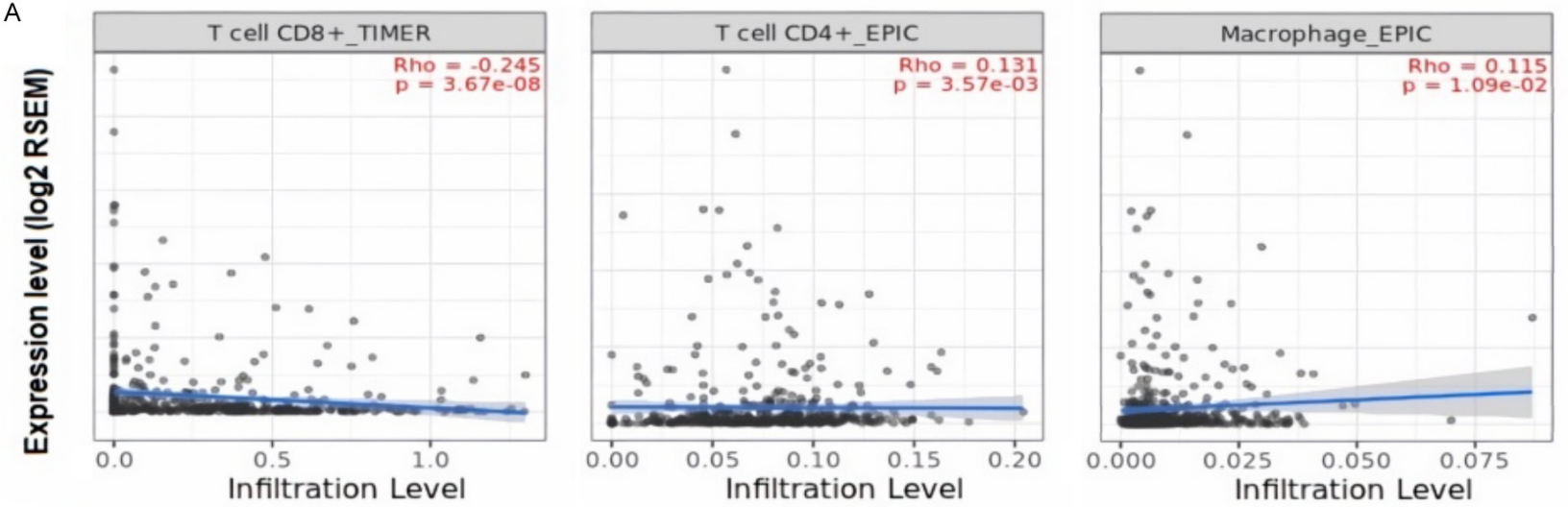


Supplementary Figure 1. A comparison between expression profiles of samples, volcano graphs of DEGs, and a total count of DEGs in GSE23036 microarray dataset. (A, B) A comparison between expression profiles of samples in GSE23036 microarray dataset, (C) A total count of DEGs and non-DEGs in GSE23036 microarray dataset, (D) A volcano graph of the DEGs observed in GSE23036 microarray dataset, and (E) Volcano graph of the DEGs, identified in the GSE23036 microarray dataset.

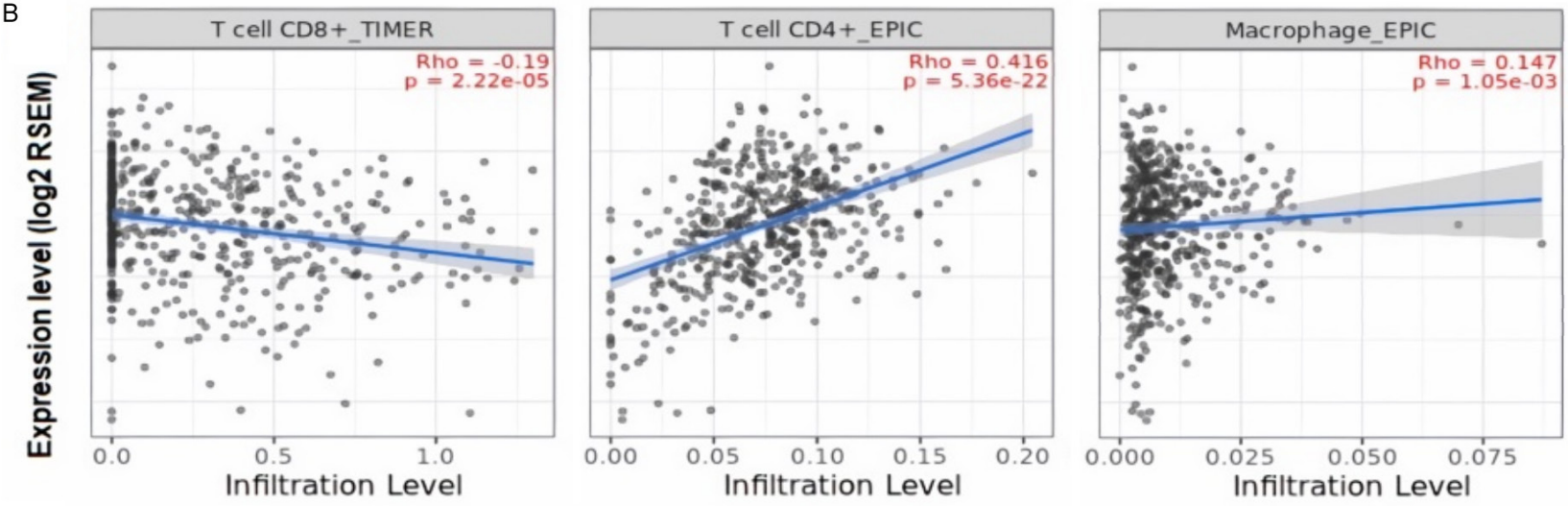


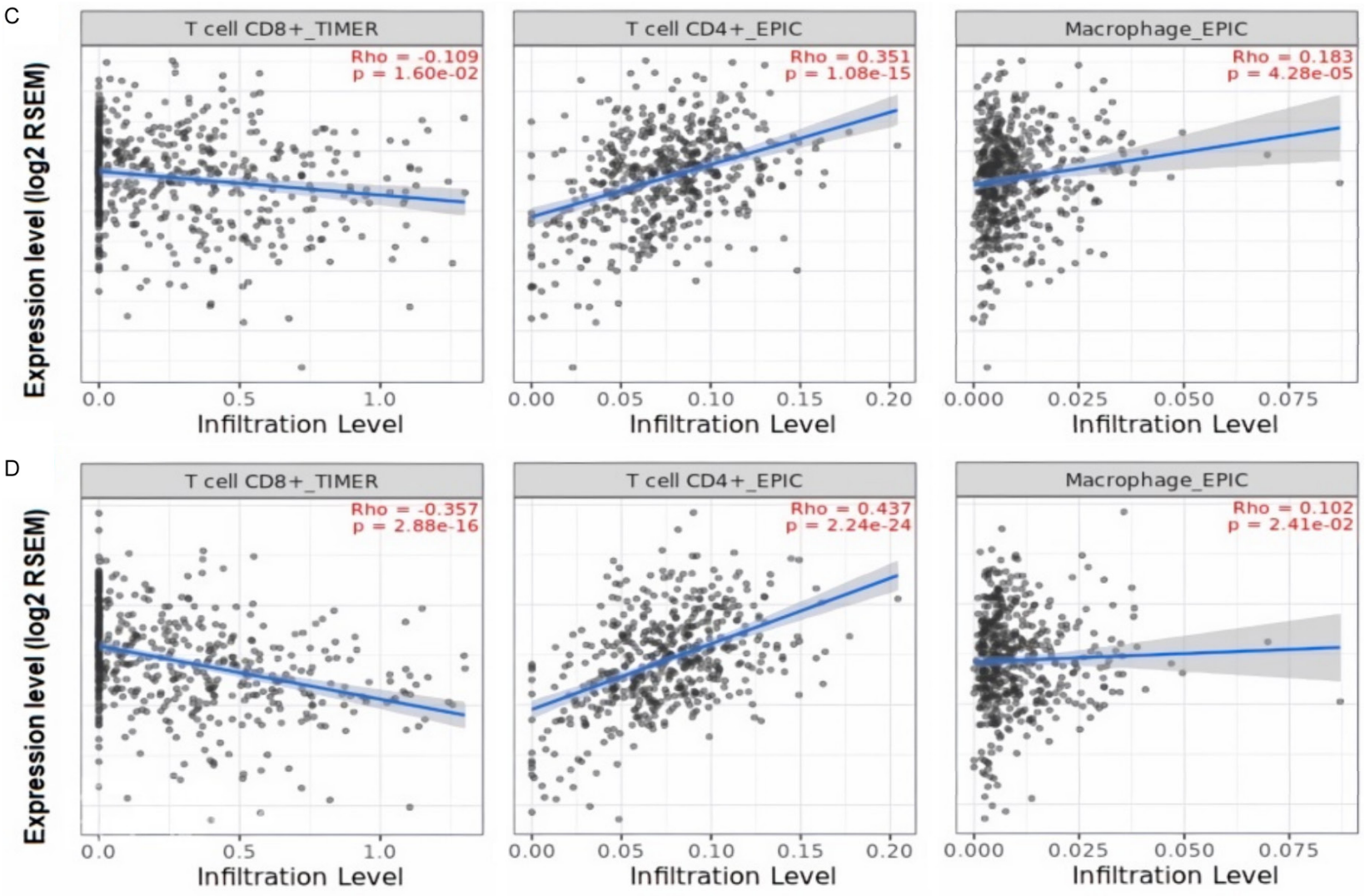
Supplementary Figure 2. Gene enrichment and subcellular localization analysis of KNTC1, CEP55, AURKA, and ECT2. (A) KNTC1, CEP55, AURKA, and ECT2 associated CC terms, (B) KNTC1, CEP55, AURKA, and ECT2 associated MF terms, (C) KNTC1, CEP55, AURKA, and ECT2 BP terms, (D) KNTC1, CEP55, AURKA, and ECT2 KEGG terms, and (E) Subcellular localization of KNTC1, CEP55, AURKA, and ECT2 in HNSC tissues.

A

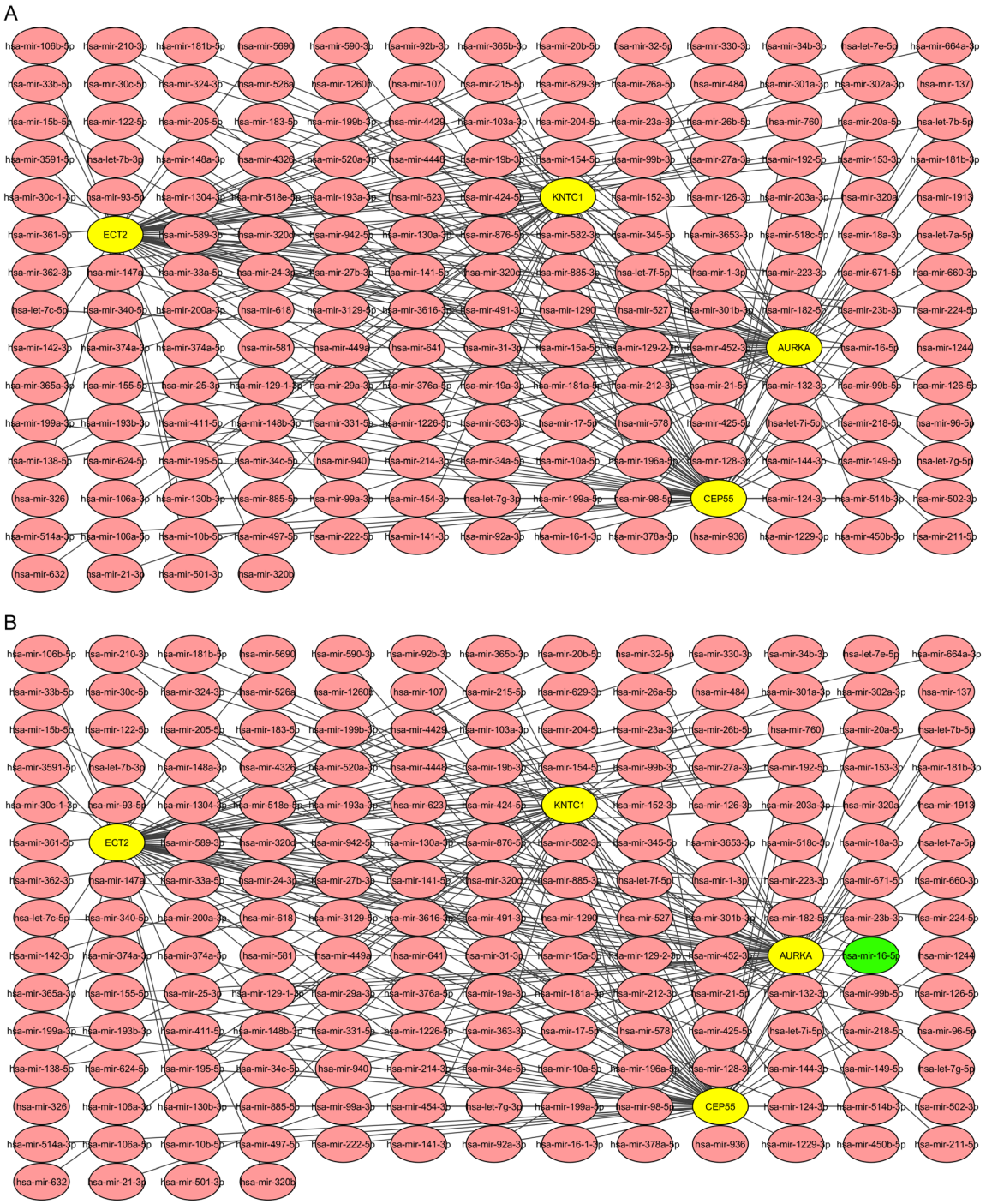

T cell CD8+_TIMER
Rho = -0.245
p = 3.67e-08
T cell CD4+_EPIC
Rho = 0.131
p = 3.57e-03
Macrophage_EPIC
Rho = 0.115
p = 1.09e-02
Expression level (log2 RSEM)
Infiltration Level

B


T cell CD8+_TIMER
Rho = -0.19
p = 2.22e-05
T cell CD4+_EPIC
Rho = 0.416
p = 5.36e-22
Macrophage_EPIC
Rho = 0.147
p = 1.05e-03
Expression level (log2 RSEM)
Infiltration Level



Supplementary Figure 3. Correlation analysis of KNTC1, CEP55, AURKA, and ECT2 hub genes expression with different immune cells (CD8+ T, CD4+ T, and Macrophages) infiltration level. (A) KNTC1, (B) CEP55, (C) AURKA, and (D) ECT2.



Supplementary Figure 4. miRNA-mRNA co-regulatory network of KNTC1, CEP55, AURKA, and ECT2 hub genes. (A) A PPI of miRNAs targeting hub genes, and (B) A PPI highlighting most important miRNA (hsa-mir-16-5p) targeting all hub genes.