



Low expression of TMPRSS2—a SARS-CoV-2 internalization protease—associates with basal subtype of head and neck squamous cell carcinoma

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ABSTRACT

SARS-CoV-2 is a single-stranded RNA virus that has caused the ongoing COVID-19 pandemic. *ACE2* and other genes utilized by SARS-CoV-2 to enter human cells have been shown to express in Head and Neck Squamous Cell Carcinoma (HNSCC) patients. However, their expression pattern in different subtypes has not been investigated. Hence, in the current study, we have analyzed the expression of *ACE2*, *TMPRSS2* and *FURIN* in 649 HNSCC patients from two independent cohorts. Our analysis showed significantly lower expression of *TMPRSS2* while significantly increased expression of *ACE2* and *FURIN* in HPV-negative HNSCC. Comparison of expression of these genes in the three subtypes of HNSCC patients (basal, classical and inflamed/mesenchymal) showed no significant difference in the expression of *ACE2* among the three subtypes; however, the basal subtype showed significantly reduced expression of *TMPRSS2* but significantly increased expression of *FURIN*. Comparison of expression of these genes between the HPV-negative patients of basal subtype vs all others confirmed significantly lower expression of *TMPRSS2* in HPV-negative patients of basal subtype as compared to all others. Our study shows that the different subtypes of HNSCC patients have different expression patterns of genes utilized by the SARS-CoV-2 to enter human cells, and hence, their susceptibility to SARS-CoV-2 may also be different. As the expression of *TMPRSS2* is significantly lower in the HNSCC patients of the basal subtype, we predict that these patients would be less susceptible to SARS-CoV-2 infection than the patients of other subtypes. However, these findings need to be further validated.

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is an enveloped single-stranded positive-sense RNA virus that has caused the ongoing COVID-19 pandemic all over the world. It has affected around 219 countries, infecting approximately 513 million people and responsible for nearly six million deaths worldwide. It is the third highly pathogenic and contagious coronavirus that infects human beings with the upper respiratory tract being the primary transmission route [1,2]. The trimeric spike (S) protein on a viral surface is the key determinant for viral entry to the host cells. It consists of two domains, the N-terminal S1 and the C-terminal S2 domains, which are proteolytically activated at the S1/S2 boundary upon viral entry [3–5]. The S1 domain contains a

receptor-binding domain (RBD) that specifically recognizes host angiotensin-converting enzyme 2 (*ACE2*) as its cellular receptor, while the S2 subunit mediates viral cell membrane fusion. The binding of the virus to *ACE2* is a key step in establishing the infection [35]. Several host proteases, such as cell surface transmembrane serine protease –2 (*TMPRSS2*), *FURIN*, and Trypsin have been identified to be responsible for S protein cleavage during virus entry [35]. *TMPRSS2* targets the S2 cleavage site, while *FURIN* has been proposed to mediate cleavage of the S protein at the S1/S2 cleavage site. While several other proteins are predicted to play a role in SARS-CoV-2 infection, *ACE2*, *TMPRSS2* and *FURIN* are the most crucial human proteins for viral entry to host cells.

ACE2 and *TMPRSS2* receptors are expressed throughout the head and neck in multiple cell types [6]. Although SARS-CoV-2 infection

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causes respiratory and cardiovascular complications, loss of taste (dysgeusia) is one of the initial symptoms of infection. This is likely due to the binding of the virus to the ACE2 receptors which are broadly expressed in the tongue surface and oral cavity; hence, oral cavity acts as a gateway to infection of SARS-CoV-2 [7]. A recent study has shown the association of increased ACE2 expression in lung tissues with smoking [8]. A previous study has evaluated the expression of ACE2 and TMPRSS2 in HNSCC patients [9]; however, the expression pattern of these genes within the different subtypes of HNSCC patients has not been thoroughly investigated. Hence, in the current study, we have analyzed the expression pattern of ACE2, TMPRSS2 and FURIN in different expression subtypes of HNSCC samples of two independent cohorts.

Materials and methods

Datasets- We included two datasets in the current study: (i) The RNASeq data of The Cancer Genome Atlas (TCGA) HNSCC patients was downloaded from GDAC Firehose (<http://gdac.broadinstitute.org>). It contained data from 522 patients, and out of these, we included 515 samples for further analysis. Based on the Human Papillomavirus (HPV) status samples were classified into HPV-negative, and HPV-positive groups, which included 438 and 77 samples, respectively [10]. Furthermore, HNSCC samples were categorized into three gene expression subtypes—namely, basal (BA), classical (CL) and inflamed/mesenchymal (IMS)—following the classification described previously [11]. 243 out of 515 samples were classified in the BA subtype, 142 in the CL, and the remaining 130 samples in the IMS subtype. (ii) Microarray data of 134 HNSCC patients was downloaded from Gene Expression Omnibus (GEO) with the expression ID (GSE40774, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40774>). This dataset contained 76 HPV-negative and 58 HPV-positive samples [11]. These samples were also classified into expression subtypes and the number of samples in each of these groups were: 44 in BA, 39 in CL, and 51 in IMS.

Analysis of ACE2, FURIN and TMPRSS2 gene expression- Gene

Expression scores for the SARS-CoV-2 related genes, namely, ACE2, FURIN and TMPRSS2 for all 515 TCGA and 134 UChicago samples, were obtained and analyzed. Scatter plots were prepared to analyze the correlation in the expression of these three genes. Heatmaps were prepared to show the distribution of samples across the three subtypes (BA, CL and IMS), HPV status and expression pattern of ACE2, FURIN and TMPRSS2 genes. In heatmaps, samples were clustered in two groups based on the expression of TMPRSS2 using k-means clustering. All the plots were plotted in RStudio (version 1.4.1717) using R (version 4.1.0). R packages ggplot2 and ggpubr were used to create the boxplots, heatmaps and scatter plots. Kolmogorov–Smirnov (KS) test in the stats package was used to compare different groups, and one-way analysis of variance (ANOVA) was used to compare the three different subtypes of HNSCC. Further, Tukey Honest Significant Differences (HSD) was used for multiple pairwise comparisons.

Results

In the present study, we have analyzed the expression of three major SARS-CoV-2 entry-linked genes in 649 HNSCC samples, including 515 from TCGA and 134 from UChicago datasets. The gene expression of ACE2 did not show any correlation with FURIN or TMPRSS2 in both datasets. However, the expression of TMPRSS2 showed a mild negative correlation with FURIN in both datasets (Fig 1). To test the hypothesis if the HPV-negative and HPV-positive HNSCC patients have a difference in the expression of SARS-CoV-2 related genes, we compared the expression of ACE2, FURIN and TMPRSS2 genes in both the groups of HNSCC patients. In both the datasets, we noticed significantly increased expression of ACE2 and FURIN genes in HPV-negative HNSCC patients as compared to the HPV-positives. However, the expression of the TMPRSS2 gene was significantly lower in HPV-negative HNSCC patients as compared to the HPV-positives (Fig 2A).

Next, we compared the expression of SARS-CoV-2 related genes among the three subtypes of HNSCC patients, i.e. basal (BA), classical (CL), and inflamed/mesenchymal (IMS). In both the datasets, there was no significant difference in the expression of ACE2 among the three

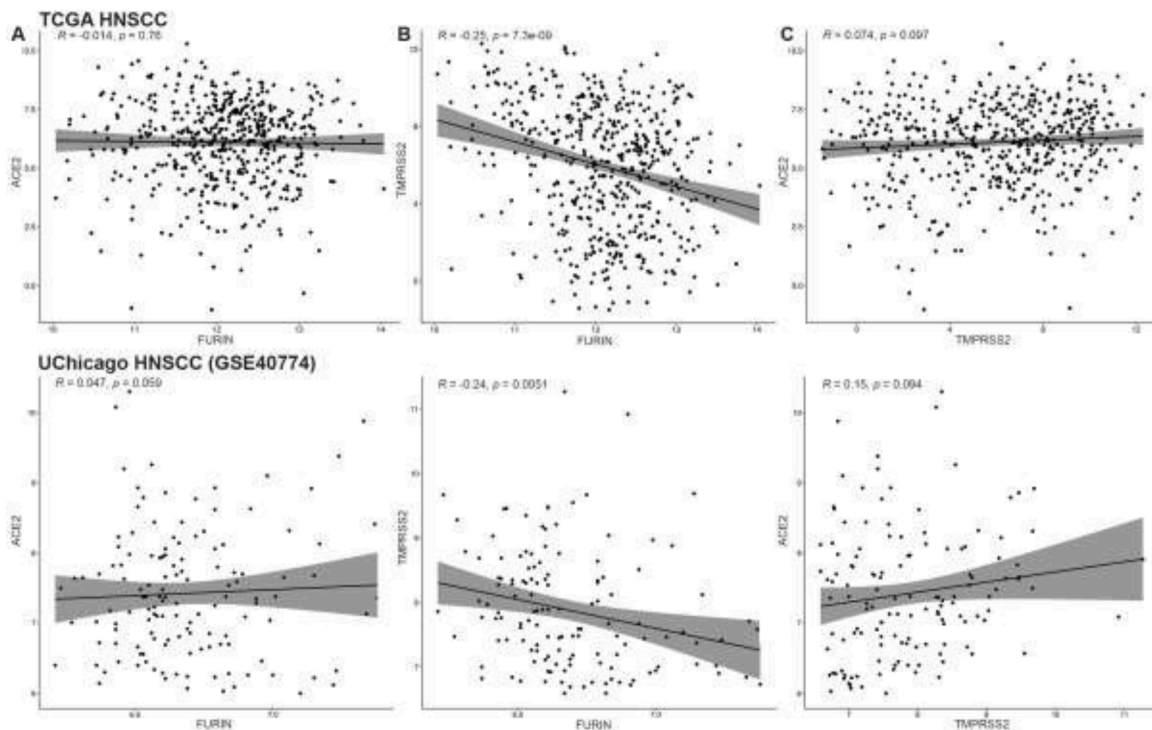


Fig. 1. Correlation between the expression of: (A) ACE2 and FURIN, (B) TMPRSS2 and FURIN, and (C) ACE2 and TMPRSS2 in TCGA HNSCC and UChicago HNSCC (GSE40774) datasets.

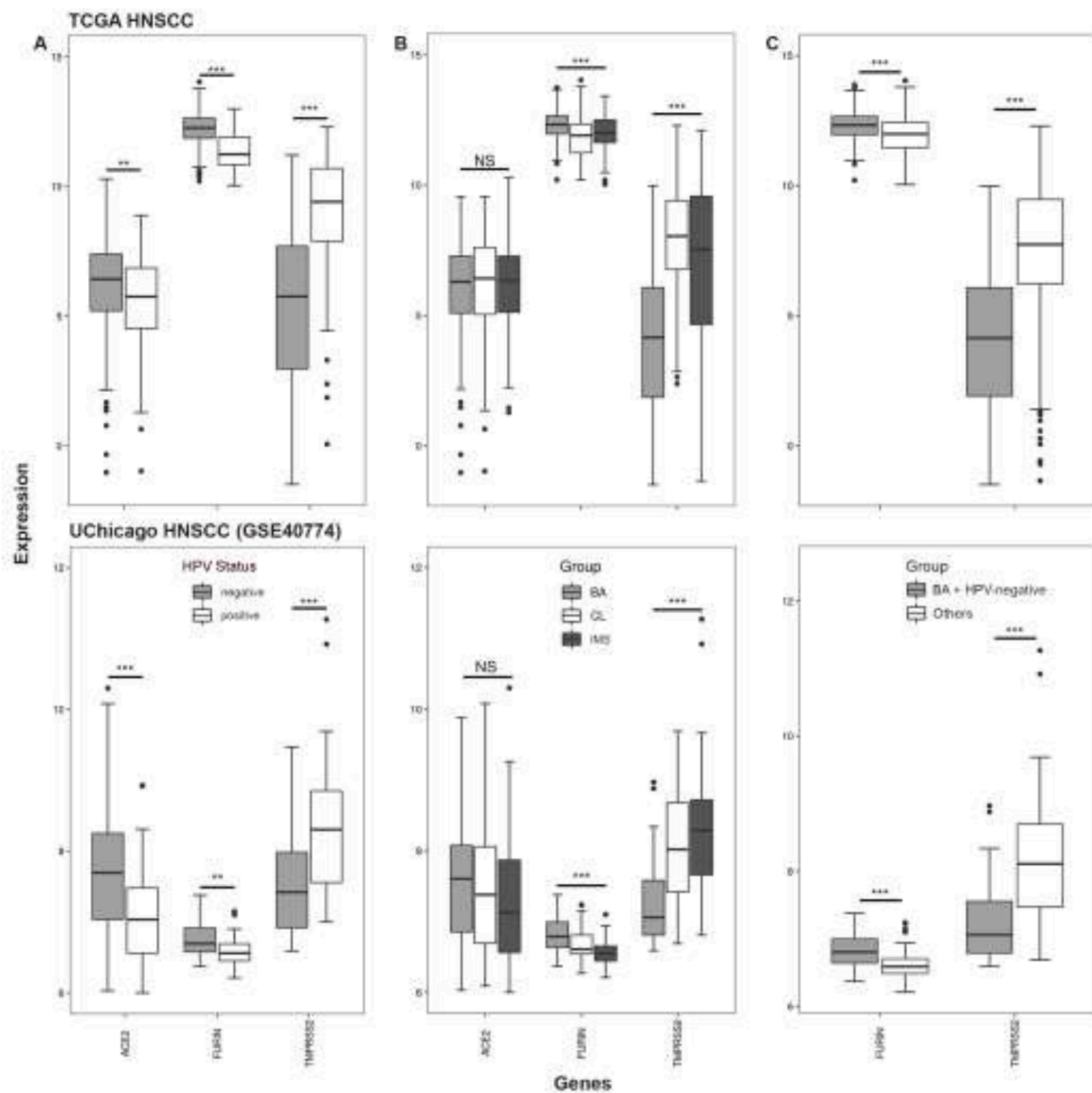


Fig. 2. Expression of *ACE2*, *FURIN* and *TMPRSS2* in different subtypes of HNSCC patients: (A) Expression in HPV-negative vs HPV-positive HNSCC patients, (B) Expression BA, CL and IMS subtypes of HNSCC patients, (C) Expression in BA+HPV-negative HNSCC patients vs all others. Asterisks (*) indicate significance based on p-values. *** represents p-value < 0.001, ** represents p-value between 0.001 to <0.01, * represents p-value between 0.01 <0.05, and finally NS represents non-significant p-value.

subtypes of HNSCC patients. The expression of the *FURIN* gene showed the least variance among the three SARS-CoV-2 genes (Table 1, Fig 2B, 3). Further, the expression of *FURIN* was significantly different among the three subtypes. The comparison of CL vs BA and IMS vs BA groups showed significantly higher expression of *FURIN* in the BA subtype; however, the comparison of IMS vs CL did not show a significant difference in the TCGA dataset but a marginally significant difference in the UChicago dataset (Table 2, Fig 2B).

In both the datasets, the comparison of the *TMPRSS2* gene expression

Table 1

Expression landscape of *ACE2*, *FURIN* and *TMPRSS2* genes. *FURIN* shows the least Interquartile range (IQR) and variance among the three genes.

Gene Name	TCGA HNSCC		Uchicago HNSCC (GSE40774)	
	IQR	Variance	IQR	Variance
<i>FURIN</i>	0.8771773	0.4875826	0.29	0.06133624
<i>TMPRSS2</i>	4.870246	10.12317	1.2675	0.8429796
<i>ACE2</i>	2.244773	3.103106	1.255	0.8248581

among the three subtypes showed significantly lower expression of *TMPRSS2* in the BA subtype as compared to the other two subtypes (Fig 3, Table 2). When the expression of the *TMPRSS2* gene was independently compared between all the three subtypes, the pattern of significantly lower expression in the BA subtype was evident: the comparison of CL vs BA and IMS vs BA subtypes showed significantly lower expression of the *TMPRSS2* gene in the BA subtype in both the datasets. Furthermore, the comparison of IMS vs CL showed significantly lower expression in the IMS subtype in the TCGA dataset, but in the UChicago dataset, this difference was not significant. (Table 2, Fig 2B). HNSCC patients of the BA subtype were predominantly HPV-negative (Fig 3). To inspect the combined effect of the BA subtype and HPV status, we compared the expression of these genes between the HPV-negative patients of the BA subtype vs all others. This analysis confirmed significantly lower expression of *TMPRSS2* in HPV-negative patients of BA subtype as compared to all others. A similar analysis of *FURIN* showed higher expression in HPV-negative patients of the BA subtype as compared to all others (Fig 2C). Although both of these comparisons showed a highly significant difference, the magnitude of the difference

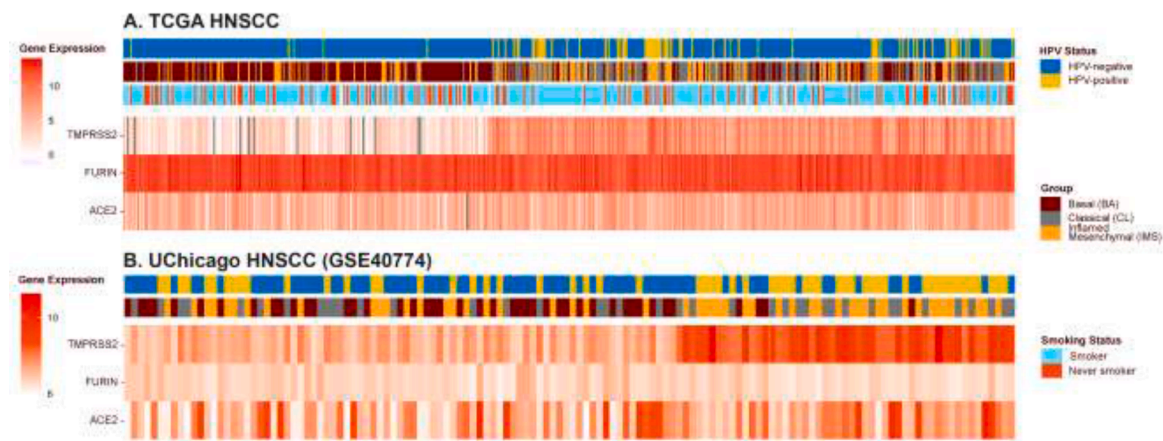


Fig. 3. Heatmap showing expression of *ACE2*, *FURIN* and *TMPRSS2* genes in (A) TCGA HNSCC and (B) UChicago HNSCC (GSE40774) datasets. Samples are clustered by the expression of the *TMPRSS2* gene. Subtype information and HPV status of each sample is also shown.

Table 2

Tukey-HSD test results for the comparison of expression of *ACE2*, *FURIN* and *TMPRSS2* genes between CL vs BA, IMS vs BA and IMS vs CL subtypes in TCGA HNSCC and UChicago HNSCC (GSE40774) datasets. Values marked in boldface denote statistical significance at $p < 0.05$ level.

S. No	Gene	Differene in Mean (95% Confidence Interval) Adjusted p-value			
		CL vs BA	IMS vs BA	IMS vs CL	
1	TCGA	<i>ACE2</i>	0.206	0.024	-0.182
			(-0.235	(-0.433	(-0.688
			0.647) 0.515	0.481) 0.991	0.324) 0.675
2	TCGA	<i>FURIN</i>	-0.461	-0.345	0.115
			(-0.628	(-0.519	(-0.076
			-0.294)	-0.172)	0.307) 0.334
3	TCGA	<i>TMPRSS2</i>	3.811 (3.138	2.849 (2.152	-0.962
			4.483) 0.000	3.546) 0.000	(-1.733
					-0.190)
1	UChicago	<i>ACE2</i>	-0.024	-0.236	-0.212
			(-0.498	-0.679 0.207	(-0.670
			0.449) 0.992	0.419	0.246) 0.519
2	UChicago	<i>FURIN</i>	-0.142	-0.256	-0.114
			(-0.259	-0.366	(-0.228
			-0.025)	-0.146 0.000	(-0.001)
3	UChicago	<i>TMPRSS2</i>	0.837 (0.415	1.002 0.607	0.164
			1.259) 0.000	1.397 0.000	(-0.244
					0.573) 0.607

was much higher in the case of *TMPRSS2*: in the TCGA dataset, the difference of medians between HPV-negative BA subtype patients vs all others was 3.59 and 0.3 for *TMPRSS2* and *FURIN*, respectively. In the UChicago dataset, these differences were 1.05 and 0.21, respectively.

As the *TMPRSS2* expression showed maximum variance among the three SARS-CoV-2 entry linked genes and its expression showed a significant difference in gene expression subtypes, we performed K-means clustering ($K = 2$) of HNSCC patients based on the expression of *TMPRSS2* gene. Interestingly, in the TCGA dataset, almost all the samples with *TMPRSS2*-low expression were HPV-negative and belonged to either BA or CL subtypes (Fig 3A). However, in the UChicago dataset, when samples were clustered based on the *TMPRSS2* expression, most of the *TMPRSS2*-high expressing samples were found to be HPV-positive and belonged to either CL or IMS subtype (Fig 3B).

Discussion

In the last two decades, coronaviruses have caused two outbreaks before causing the global COVID-19 pandemic in 2019 that is still ongoing. The first outbreak, SARS (Severe Acquired Respiratory Syndrome), occurred in 2003 in China, followed by MERS (Middle Eastern Respiratory Syndrome) outbreak in 2012 in Saudi Arabia, with fatality rates of approximately 9.2% and 37%, respectively. The COVID-19 disease has spread at an unprecedented rate and overburdened the already weak healthcare systems of several low- and middle-income countries. Individuals of low- and middle-income countries are deprived of adequate medical services and cannot access medical facilities promptly, even for ailments unrelated to SARS-CoV-2 [12]. A large proportion of individuals infected with COVID19 do not develop severe symptoms; however, patients with comorbidities like diabetes, cardiac condition, cancer and old age are more vulnerable. Cancer patients with prior treatment with chemotherapy and radiation are immunocompromised, and chances of developing severe symptoms after SARS-CoV-2 exposure are much higher.

The oral cavity acts as a gateway to infection of SARS-CoV-2 primarily because ACE2 receptors—which are utilized by the virus to gain entry to human cells—are broadly expressed in the tongue surface and oral cavity. [7]. Loss of taste (dysgeusia) is one of the initial symptoms of infection, likely due to the binding of the virus to the ACE2 receptors. In the current study, we have analyzed the expression of SARS-CoV-2 related genes in HNSCC patients. The expression of *FURIN* showed the least variance compared to the other genes, whereas *TMPRSS2* showed maximum variance (Table 1, Fig 2, and 3). As compared to the other two genes, in the TCGA dataset, the expression of *FURIN* was relatively high; however, in the UChicago dataset, its expression was relatively low. This difference is most likely due to the different platforms utilized for measuring expression. Interestingly, even though the relative expression of *FURIN* was different, it followed a similar trend in both the datasets. We noticed significantly increased expression of *ACE2* and *FURIN* genes in HPV-negative HNSCC patients as compared to the HPV-positives. However, the expression of the *TMPRSS2* gene was significantly lower in HPV-negative HNSCC patients as compared to the HPV-positives (Fig 2A). The comparison of expression of these genes among different subtypes showed significantly lower expression of *TMPRSS2* in the HNSCC patients of the BA subtype compared to the other two subtypes; however, the expression of *ACE2* was not different among different subtypes in both the datasets. The BA subtype is predominantly HPV-negative, enriched in EGFR signaling and has a hypoxic tumor microenvironment [11].

ACE2 receptors on the host cells are utilized by SARS-CoV-2 for entry into host cells and *TMPRSS2*, a transmembrane serine protease, is

required for priming of the Spike protein for entry. Moreover, inhibitors of TMPRSS2 block the viral entry into the cells [13]. As the expression of *TMPRSS2* is significantly lower in the HNSCC patients of the BA subtype, we can predict that these patients would be less susceptible to SARS-CoV-2 infection than the patients of other subtypes. However, the expression of *TMPRSS2* and other genes involved in SARS-CoV-2 entry to human cells needs to be validated at the protein level to validate these findings. Furthermore, *FURIN*, which is also a protease, was upregulated in the BA subtype patients. SARS-CoV-2 has a *FURIN* cleavage site, which is generally not present in other group members, and its loss can make the virus less pathogenic [14]. Hence, it needs to be seen if BA subtype HNSCC patients are less susceptible to COVID-19 or not. As the expression of *FURIN* had the least variance among the three genes, we predict that its upregulation may not contribute as significantly as the downregulation of *TMPRSS2* in the patients of the BA subtype. Our study shows that the different subtypes of HNSCC patients have different expression pattern of genes utilized by the SARS-CoV-2 to enter human cells, and hence, their susceptibility to COVID-19 may also be different. These results should be further explored and validated in a laboratory setting.

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Author contribution statement

AK conceptualized the study, prepared figures and tables and wrote the manuscript. JKT collected the data, carried out the analysis, prepared the figures and tables, and edited the manuscript. NT, SS and SKJ participated in drafting and editing the manuscript. RSS and SK participated in revising and editing the manuscript.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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