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ORIGINAL RESEARCH

Diverse Chromobox Family Members: Potential Prognostic Biomarkers and Therapeutic Targets in Head and Neck Squamous Cell Carcinoma

Kuan Hu[1], Lei Yao[1], Lei Zhou[2], Juanni Li[3,4]

[1]Department of Hepatobiliary Surgery, Xiangya Hospital, Central South University, Changsha, 410008, Hunan, People's Republic of China; [2]Department of Anesthesiology, Third Xiangya Hospital of Central South University, Changsha, 410008, Hunan, People's Republic of China; [3]Department of Pathology, Xiangya Hospital, Central South University, Changsha, 410008, Hunan, People's Republic of China; [4]National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, 410008, Hunan, People's Republic of China

Correspondence: Juanni Li, Department of Pathology, Xiangya Hospital, Central South University, Changsha, 410008, Hunan, People's Republic of China, Email lijuanni2014@csu.edu.cn

Background: The Chromobox (CBX) family members were involved in a variety of physiological and oncological processes through the regulation of the epigenetic modification of chromatin. However, the comprehensive analysis of the CBX family in head and neck squamous cell carcinoma (HNSC) is lacking.

Methods: In this work, we used multiple online databases and tools to investigate the roles of CBX family in aspects of gene expression, prognostic evaluation, genetic alteration, immune micro-environment of tumor, and status of methylation.

Results: The mRNA expression levels of CBX1, CBX3, and CBX5 were aberrantly increased in patients with HNSC, while CBX7 was aberrantly decreased. Higher expression of CBX7 was significantly associated with longer OS. Within the 5–11% of genetic alteration rate of CBXs, CBX3 ranked the highest and CBX5/7 ranked the lowest. SPRR1B, S100A7, CASP14, CDSN, LCE3D were the top 5 neighbor genes with the strongest association with CBXs in HNSC patients. Signaling pathways such as epidermal cell differentiation, cornification, and peptide cross-linking were demonstrated to have a strong association with CBX genes. The profiles of immune cell infiltration had high similarity for the group of HNSC patients stratified by expression of CBXs. The methylation levels of CBX1 and CBX5 significantly decreased, while that of CBX7 significantly increased in HNSC samples when compared with normal tissue.

Conclusion: In conclusion, the CBX family showed its valuation for further investigation in HNSC. Our research highlighted that CBX7 had the potential to be a novel diagnostic and prognostic biomarker for patients with HNSC.

Keywords: CBX, HNSC, expression, prognosis, immune infiltration

Introduction

Head and neck squamous cell carcinoma (HNSC) ranks 6th globally in the top morbidity list of all cancer types,[1–3] with more than 800,000 new cases diagnosed worldwide per year. HNSC are malignancies derived from the mucosal epithelium which is located in the oral cavity, paranasal sinuses, larynx, and pharynx.[4] With the well-known risk factors of smoking, alcohol drinking, and human papillomavirus (HPV) infections, HNSC presents high heterogeneity and complexity in pathogenesis which remains to be further investigated.[5] Hence, aiming to improve the diagnosis and prognosis of HNSC, it is valuable to identify pivotal and novel molecules that account for the tumorigenesis and progression.

The Chromobox (CBX) family consists of 8 members ranging from CBX1 to CBX8 in the human genome, with the common Polycomb Repressor Complex 1 (PCR1) in structure, an epigenetic regulatory complex that was intensively reported to regulate the epigenetic modification of chromatin and thereby take part in a variety of physiological (such as cell differentiation and DNA repair) and oncological processes (such as self-renewal of cancer stem cells).[6,7] The CBX family members can be further divided into two subgroups based on their protein structure: the heterochromatin protein 1

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group (including CBX1, CBX3, and CBX5) and the polycomb group (including CBX2, CBX4, CBX6, CBX7, and CBX8).[8] Currently, increasing evidence demonstrated that CBX family members were involved in the initiation and progression of various cancers including but no limited to liver cancer,[9] lung cancer,[10] ovarian cancer,[11] and breast cancer.[12] The differential expression and dysregulation of CBXs happened recently in multiple cancer types and play either oncogenic or tumor inhibitory roles based on their texture and downstream target genes.[13,14] For instance, CBX7 was proved to be a tumor suppressor gene in thyroid cancer,[15] however, it acted as an oncogene in gastric cancer.[16] To our knowledge, there have been rarely studies exploring the role of all eight CBX members together in HNSC until now, except some preliminary evidence supporting that high expression of CBX3 in HNSC is associated with poor prognosis.[17]

In this study, we aimed to investigate the roles of CBXs in HNSC in a fresh perspective with the usage of multiple online databases and tools. A comprehensive analysis was performed here to demonstrate the differential expression profiles, prognostic values, genetic alterations, and functional enrichment analysis of CBXs in HNSC, and to explore the correlation of CBXs to clinical parameters, immune cells infiltration, and methylation status as well.

Methods

Expression Profiles of CBX Family Members and Their Association with the Clinical Parameters in HNSC

The mRNA levels of the eight CBX family members were retrieved from UALCAN[18] and Oncomine,[19] two different online tools of a comprehensive analysis of cancer data from databases including TCGA, CPTAC, MET500, and GEO. The correlations of CBX family members with the clinical parameters were also analyzed through UALCAN database. The comparison of relative expression among the eight CBX genes was performed through GEPIA2 using Spearman correlation.[20,21] Student's *t*-test was performed to compare two independent samples. $P < 0.05$ was considered as statistically difference. The databases presented in this study were listed in Supplementary Table S1.

The Prognotic Analysis of the CBX Family in HNSC

Two individual databases Kaplan–Meier plotter[22] and UALCAN[18] were used for data extraction and analysis for the prognostic analysis of the CBX family in HNSC. Overall Survival was used as the primary endpoint here to evaluate the roles of CBXs on survival. Kaplan–Meier analysis was performed and the Log rank test was used. The *p*-value less than 0.05 meant data has statistical significance.

Status of Genetic Alteration and the Homologic Analysis of the CBXs Family

We used cBioPortal, a public website designed for deep analysis of genomics and clinical data from cancer-related databases, to illustrate the genetic alteration and the homologic analysis of the CBX family.[23,24] A dataset containing the clinical information of 496 HNSC patients was distracted (TCGA, Firehose Legacy), then the genetic alteration analysis was performed.

Identification of CBX-Associated Genes and the Functional Enrichment Analysis

Cytoscape application was used to filter out the 196 CBX-associated genes based on the dataset from cBioPortal (listed on Supplementary Table S2).[25] Then we use WebGestalt to perform the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for the purpose of identification of associated functions and pathways of CBX.[26]

Immune Cell Infiltration Analysis of HNSC with Altered Expression of CBXs

TIMER2.0 was enrolled to evaluate the correlation of immune cell infiltration with the expression of CBX members in HNSC.[27] Briefly, the "immune association" module of the TIMER2.0 was used to generate the scatterplots. Spearman correlations were applied here. $P < 0.05$ was considered as statistically significant. Infiltrated immune cells included CD4+ T cells, CD8+ T cells, dendritic cells, macrophages, B cells, and neutrophils.

DNA Methylation Analysis of CBXs in HNSC

The methylation levels of CBX family members were analyzed by the usage of DiseaseMeth2.0, a public online resource containing DNA methylation status of multiple genes in cancers.[28,29] Student's *t*-test was performed here, and the p-value less than 0.05 meant data has statistical significance.

Results

Aberrant Expression of CBXs in HNSC Patients

The mRNA expression profiles of 8 CBX genes in HNSC were retrieved with the usage of ONCOMINE and UALCAN databases. Data returned from ONCOMINE database indicated that CBX1, CBX3, and CBX5 were significantly over-expressed in HNSC compared with normal tissue, while lower expression of CBX7 was found in HNSC with statistical significance (Supplementary Figure S1). Data from UALCAN database showed more ambitious results, all of the 8 CBXs except CBX7 were found to be overexpressed in HNSC compared with normal tissue. Intriguingly CBX7 was the only molecule in the family that was down-regulated in HNSC (Figure 1, Supplementary Table S3). Taking comprehensive consideration of the results from these two different databases, the mRNA expression levels of CBX1, CBX3, and CBX5 were aberrantly increased in patients with HNSC, while the mRNA expression level of CBX7 was aberrantly decreased.

The Correlation of Each CBX Member with the Clinicopathological Parameters in HNSC Patients

Next, we wonder if there was any correlation between CBX family members and the clinicopathological parameters of HNSC patients. By achieving this, two clinicopathological parameters (cancer stages and tumor grades of HNSC individuals) that reflect the tumor progression were recruited through UALCAN. As a consequence, the mRNA expressions of most of the CBX members were associated with the cancer stage of HNSC individuals. In fact, the expressions of CBX1-5 and CBX8 trend to go higher when the cancer stage increased. While the highest expression of CBX7 was in cancer stage 1, then it decreased gradually when the cancer stage increased (Figure 2A). In the aspect of tumor grade, almost all the CBX members demonstrated increased trends of mRNA expressions when the tumor grade increased gradually. However, only CBX7

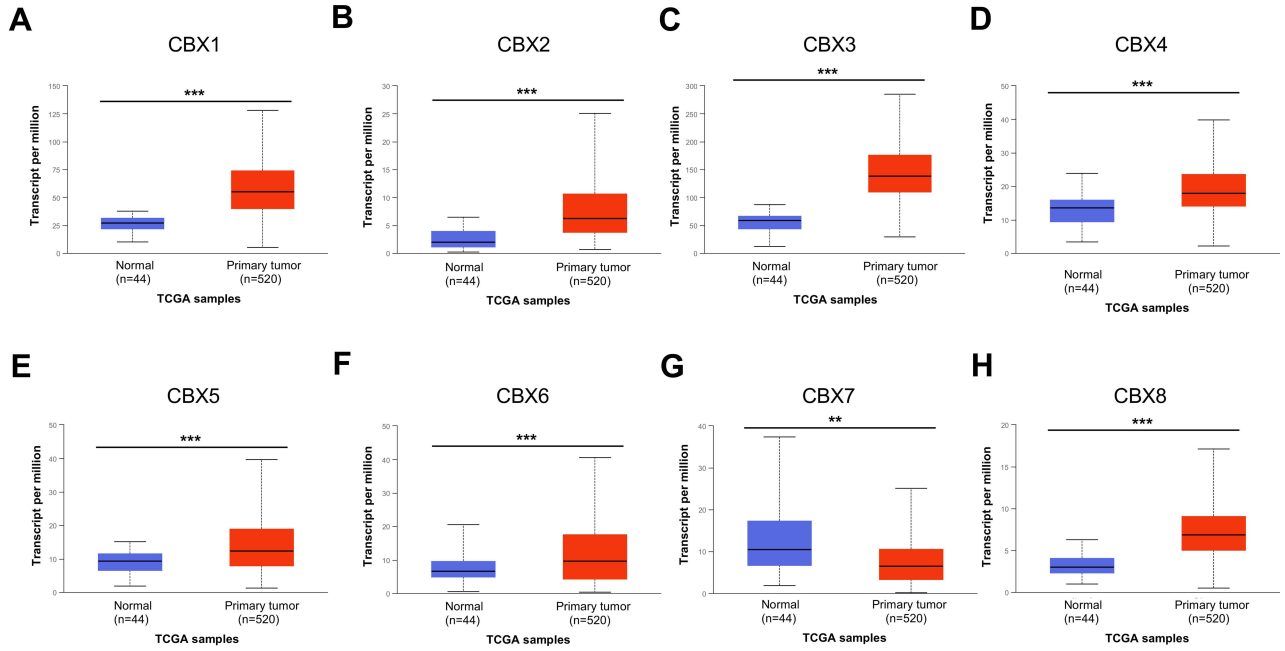

Figure 1 The mRNA expression profiles of the CBX family in HNSC. (**A**–**H**) data from the UALCAN database showing the mRNA expression levels of CBX1-8 between HNSC tumor tissues (n = 520) and normal tissues (n = 44). $^{**}p < 0.01$, $^{***}p < 0.001$.

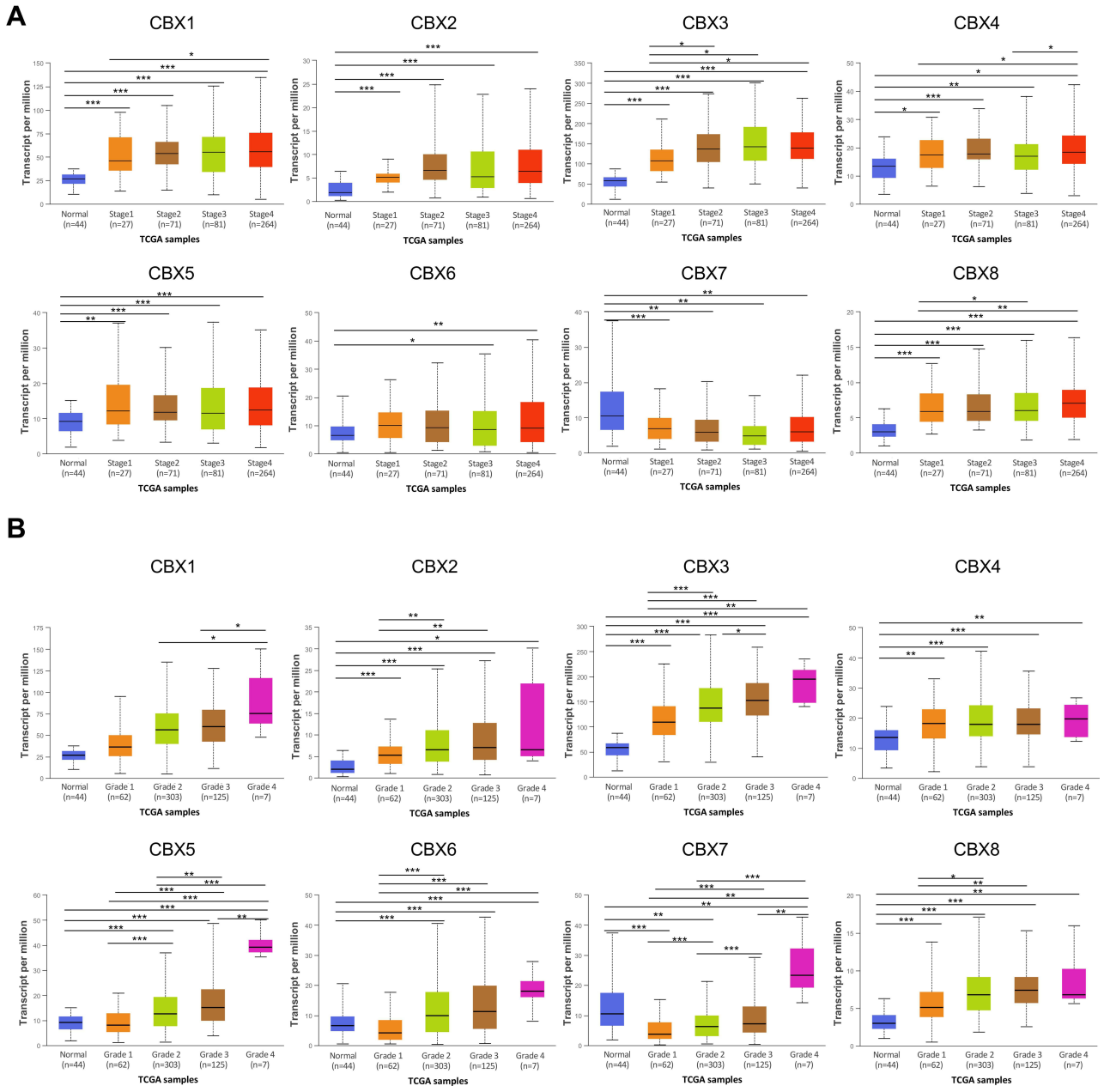

Figure 2 The correlation of CBXs with the clinical parameters of patients with HNSC. (**A**) results from UALCAN database showing the correlation of mRNA expression of CBXs with the cancer stages of HNSC (normal = 44, tumor = 443). (**B**) results from UALCAN database showing the correlation of mRNA expression of CBXs with the tumor grades of HNSC (normal = 44, tumor = 497). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

expression showed a decreased trend in tumor grade 1–3 that was opposite with the other CBX members (Figure 2B), giving the speculation that an extremely low level of tumor grade might reverse the expression of CBX7 by some unknown mechanism. The expression level of CBX7 in grade IV was higher compared with normal tissue (Figure 2B), which may because of the insufficient number of cases in tumor grade IV for a realistic comparison. Undoubtedly further investigations with more sample sizes are necessary to get rid of statistical bias.

The Evaluation of Prognostic Role of CBX Expression in Patients with HNSC

We enrolled the overall survival (OS) as the primary endpoint in this section to evaluate the prognostic role of CBX expression in patients with HNSC. Two individual databases Kaplan–Meier plotter and UALCAN were used for data

extraction and analysis. As for the Consequence from Kaplan–Meier plotter, higher expression of CBX3 was significantly correlated with poorer OS in patients with HNSC; while higher expression of CBX5 and CBX7 was significantly associated with longer OS (Figure 3). Data returned from UALCAN showed that only higher expression of CBX7 was significantly associated with longer OS (Supplementary Figure S2 and Supplementary Table S3). Considering different resources of data collection of these two databases (UALCAN from TCGA, MET500, CPTAC, and CBTTC; Kaplan–Meier plotter database from GEO, EGA, and TCGA), the intersection was got to have a more reliable result. Thus, CBX7, the only CBX member that showed the association with longer OS from both databases, was considered to have the potential to become a novel biomarker that can predict a better prognosis for patients with HNSC.

Status of Genetic Alteration and the Homologic Analysis of the CBXs Family

Then we inquire about the status of genetic alteration of the 8 CBXs and their correlation with each other. Briefly, the genetic alteration rate of each CBX member was found in 496 sequenced HNSC samples. CBX3 was the gene with the highest alteration rate, while CBX5 and CBX7 ranked the top two genes with the lowest alteration rate (5%). In the aspect of the classification of genetic alteration, mRNA high became the major type in the CBX family genes, accompanied by a small amount of missense mutation and amplification (Figure 4A). Moreover, the correlation of different CBX members with each other was conducted using Spearman correlation by analyzing their mRNA expression. The results showed that CBX2 and CBX8, CBX4 and CBX8, CBX5 and CBX7, CBX6 and CBX7 were positively correlated with each other, respectively. Conversely, CBX3 was found to be negatively correlated with CBX7 (Figure 4B).

The Network of CBX-Associated Neighbor Genes and the Functional Enrichment Prediction

In this section, 119 genes that were intimately correlated with CBXs family members in HNSC patients were identified and shown as the network in Figure 5A through cBioportal and Cytoscape. Apparently, SPRR1B, S100A7, CASP14, CDSN, LCE3D were the top 5 neighbor genes with the strongest association with CBXs in HNSC patients. Then the

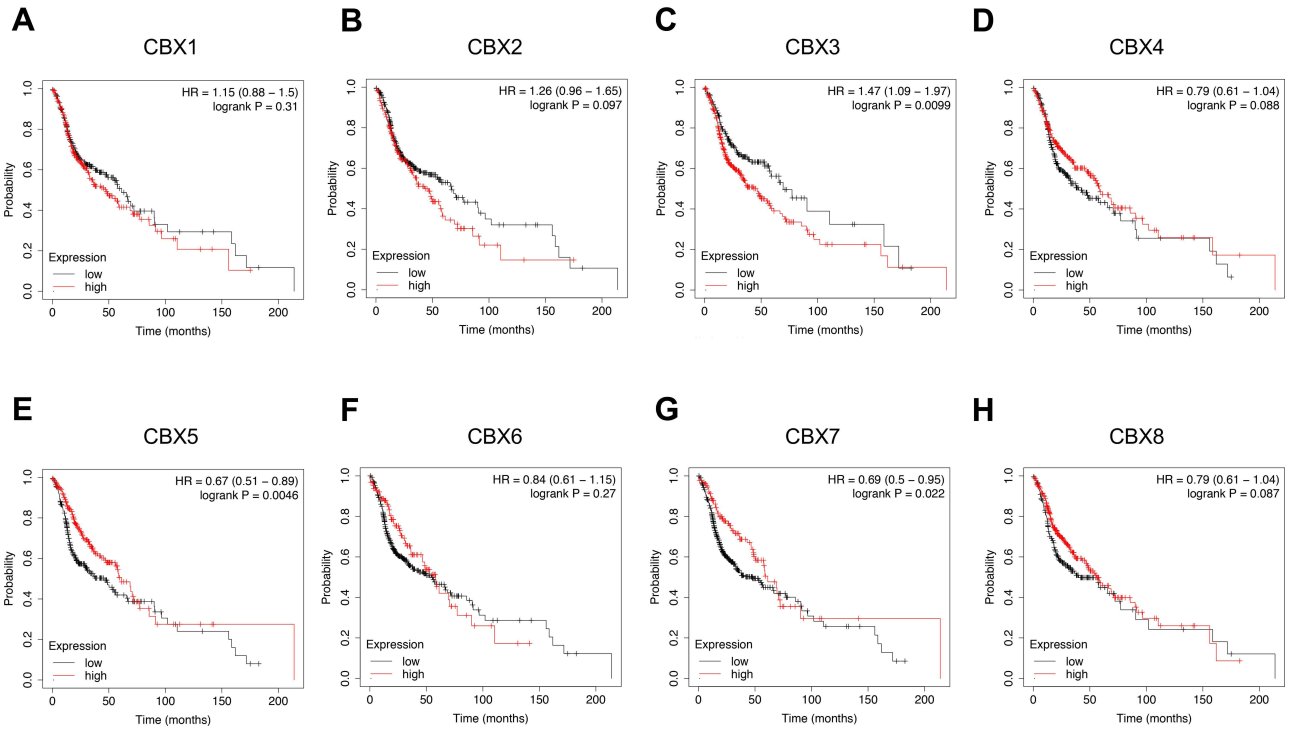

Figure 3 Prognostic assessment of the expression levels of CBXs in HNSC. The correlation of CBX mRNA expressions with overall survival (OS) of HNSC patients (n=499) was analyzed by using Kaplan-Meier plotter database.

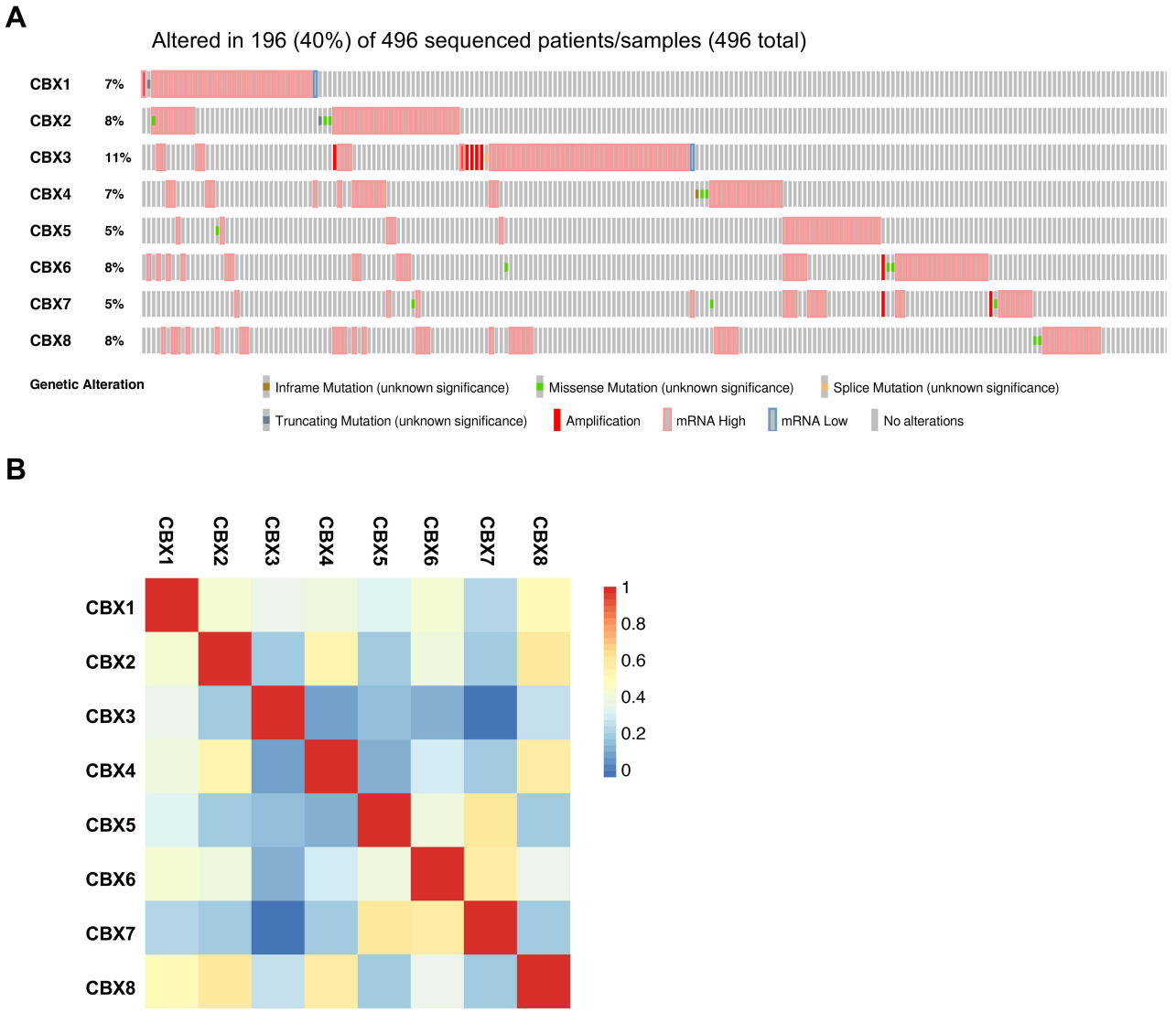

Figure 4 The genetic alterations and correlation analysis of CBX family in HNSC. (**A**) The frequency and types of genetic alteration of CBX family in HNSC performed by cBioPortal (496 sequenced samples). (**B**) The correlation analysis within the CBX family in HNSC by using GEPIA2.

functional enrichment prediction of the 119 CBX-associated neighbor genes was further analyzed through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The GO annotation returned from the WebGestalt database revealed their function in biological process, cellular component, and molecular function. The most relevant biological processes involved were the multicellular organismal process, biological regulation, metabolic process, response to stimulus, and developmental process (Figure 5B, left panel). Furthermore, cellular components associated with CBXs were the membrane, extracellular space, cytosol, vesicle, endomembrane system, and nucleus (Figure 5B, middle panel). In the molecular function part affected by CBXs, protein binding, ion binding, hydrolase activity, structural molecule activity, and transferase activity were on the list (Figure 5B, right panel). In addition, KEGG analysis figured out 10 pathways that may be involved by CBXs. Among them, cornification, peptide cross-linking, keratinization, keratinocyte differentiation, and epidermal cell differentiation ranked ahead (Figure 5C).

The Immune Cell Infiltration in HNSC Varies According to the Expression of CBXs

The profiles of immune cell infiltration inside or around the tumor tissue reflected the immune efficacy and immune response of the human body against the tumor, and affect the tumor progression and metastasis as well. Therefore,

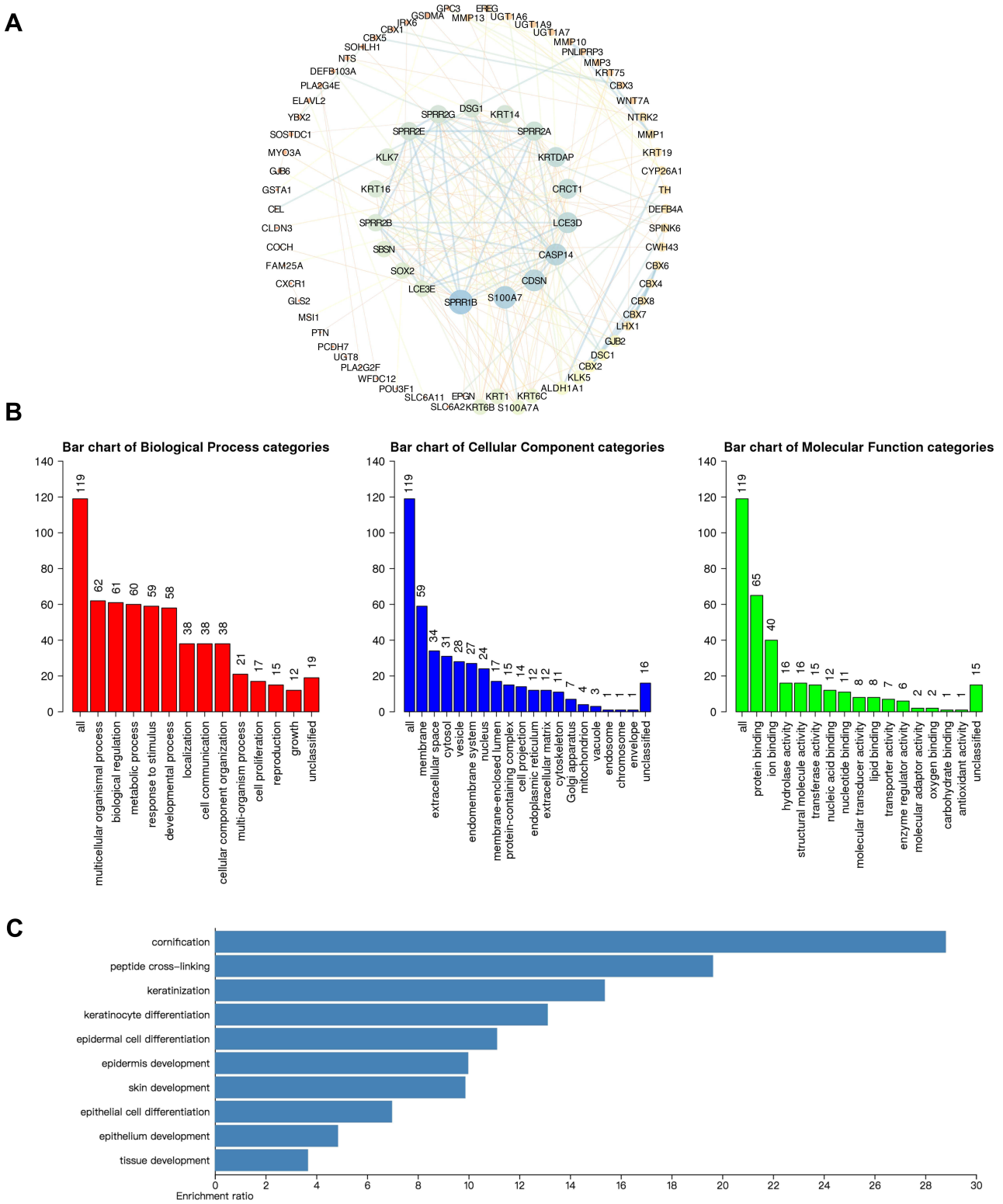

Figure 5 Identification of CBX-associated genes in HNSC and their functional prediction. (**A**) 196 CBX-associated genes in HNSC were filtered by using cBioPortal and Cytoscape. (**B**) The Gene Ontology (GO) analysis was performed to predict the biological functions and processes of CBXs. (**C**) Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis was performed to predict the possible signaling pathways related to CBXs.

immune cell infiltration was a pivotal factor in the tumor microenvironment. Here, we sought to explore the potential relationship between CBXs expression and immune cell infiltration in HNSC. Data obtained from TIMER2.0 database indicated that almost all the CBX family members showed surprisingly accordance in their relationship with each type of

infiltrated immune cell (Figure 6, Supplementary Figure S3). In detail, all the 8 CBX members were significantly correlated with CD8+ T cell and CD4+ T cell infiltration. 8 CBXs except CBX4 were found to be negatively associated with B cells. 8 CBXs except CBX4 were negatively correlated with dendritic cells. 8 CBXs apart from CBX2 and CBX8

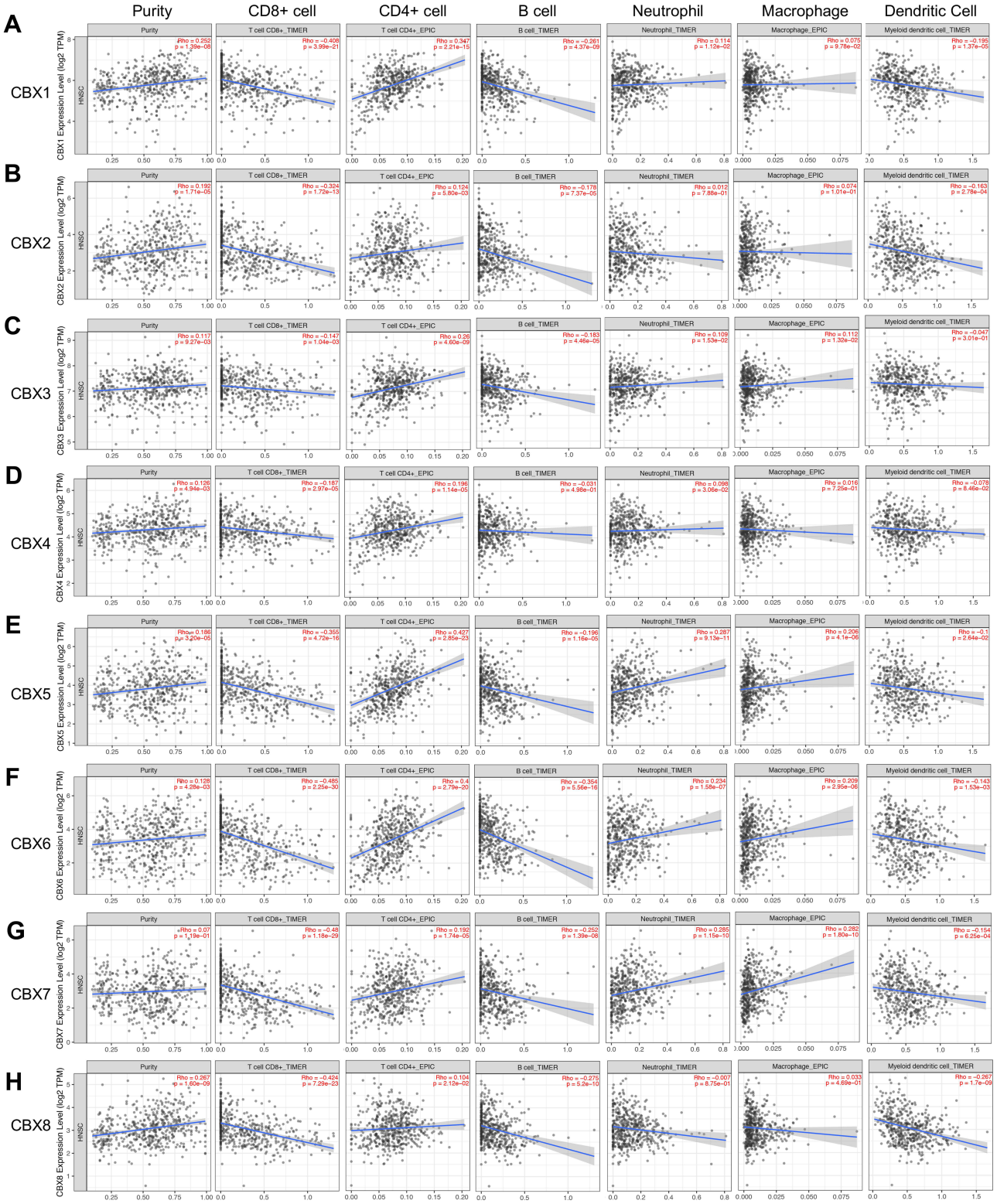

Figure 6 The profiles of immune cell infiltration in HNSC patients with altered expression of CBXs. The association of (**A**) CBX1, (**B**) CBX2, (**C**) CBX3, (**D**) CBX4, (**E**) CBX5, (**F**)CBX6, (**G**) CBX7, and (**H**) CBX8 with different kinds of infiltrated immune cell in HNSC patients (n = 522) was shown.

were found to be positively associated with B cells. CBX3 and CBX5-7 were shown to have strong positive correlation with macrophage. These results taken together suggested that the profiles of immune cell infiltration had high similarity for the group of HNSC patients stratified by expression of CBXs.

DNA Methylation Evaluation of the CBXs in Patients with HNSC

As an important mechanism of post-transcriptional modification in epigenetics, DNA methylation was well recognized to regulate the initiation and development of multiple types of cancer including HNSC by resulting in the silence of the target genes. DiseaseMeth database was enrolled here to detect if there was any difference in the DNA methylation level of CBXs between HNSC samples and normal tissues. As a consequence presented in Supplementary Figure S4, the methylation levels of CBX1 and CBX5 significantly decreased, while that of CBX7 significantly increased in HNSC samples when compared with normal tissue. Remind of the high expression of CBX1 and CBX5, low expression of CBX7, these altered methylations might be the underline mechanism that account for the differentiated mRNA expression of CBX genes.

Discussion

Epigenetic regulation has been well known as an unequivocally molecular mechanism underneath a variety of biological processes these years, with the characteristics of high occurrence especially in the process of multiple cancers.[30,31] Increasing evidence illustrated that the dysregulation of epigenetic regulation including histone modifications, DNA methylation, and the non-coding RNAs contributed to the initiation and development of cancer.[32,33] CBX family members, as the indispensable component of PRC1, exerted their roles of targeting PRC1 to chromatin, thereby onset the following biological functions ranging from cancer stem cell recognition to tumorigenesis.[6,34] Recently the continually raising research aiming at CBXs in different kinds of cancer offered us new insight into the development of therapeutic targets and novel biomarkers.[35,36] However, as far as our concerned, none of the CBX genes has been investigated in HNSC. Hence, this study was the first to discuss the roles of CBX family in HNSC in the pattern of comprehensive analysis.

In our study, we first checked the expression profiles of CBXs, and many evidence existed that supported our finding. CBX1 was proved to be over-expressed in prostate cancer and breast cancer and might be involved in the regulation of tri-methylation levels of histone H3K9.[37,38] Besides, overexpression of CBX1 could promote the proliferation and migration of hepatocellular carcinoma cells by activating the Wnt/β-Catenin signaling pathway.[39] Aberrantly high expression of CBX3 was observed frequently in various cancers such as non-small cell lung cancer[40] and tongue squamous cell carcinoma.[17] In the case of CBX5, its high expression was found to have a strong association with poor survival and increased risk of metastasis of breast cancer.[41] The role of CBX7 in different types of cancer was complicated and contradictory based on the published studies.[42] Therefore, specific mechanistic researches on CBX7 and HNSC were imperative on the basis of the remarkably decreased expression of CBX7 we found in HNSC.

Our study is the first to demonstrate the change of CBXs expression in HNSC patients with different cancer stages and tumor grades. Apparently increased trends of CBX1-6 and CBX8 expressions and decreased trend of CBX7 expression when cancer stages and tumor grades elevated, which is concurrent with the specific expression profiles of CBXs in HNSC when compared with normal tissue. These findings collectively suggested that CBXs might reflect the continuous process of HNSC development.

Dating back to several published papers, CBXs was proved to have prognostic value in many kinds of cancers. For instance, high expression of CBX3 was correlated with poor overall survival in tongue squamous cell carcinoma, genitourinary cancer, lung cancer, and digestive cancer.[43] Furthermore, an eight-gene signature containing CBX3 demonstrated prognostic value in head and neck squamous carcinoma.[44] The promotion role of the CBX3-p21 axis in regulating G1/S phase in tumor proliferation was reported to account for the potential mechanism in tongue squamous cell carcinoma.[45] In this study, we found that only a higher expression of CBX7 was significantly associated with longer OS from both databases. Taken together with the remarkably decreased expression in HNSC, CBX7 might become the potential tumor suppressor gene for better predicting the clinical outcome in CBXs family. Considering there was no published study exploring the role of CBX7 in HNSC, some kind of research about CBX7 in other cancers might serve as references. As we mentioned above, the role CBX7 was intricate in different types of cancers. It acted as the tumor suppressor gene in cancers originally located in colon,[46] bladder,[47] thyroid,[15] and

lung.[48] The possible mechanism may be partly due to the antagonism of CBX7 against CCNE and SPP1, two genes that were well known for tumor proliferation and migration.[42] Besides, it also served as the oncogene in prostate cancer and ovarian cancer.[36,49] It also has been reported that miR-18a could promote the proliferation and migration of hepatocellular carcinoma cells through silencing of CBX7, thereby partially resulting in the shorter OS of HCC patients.[50,51] In terms of the role of CBX7 in HNSC, certainly, deep mechanistic investigation was necessary to provide enough evidence before CBX7 could finally become a novel independent risk factor and therapeutic target against HNSC.

The filtration of co-expression genes and the functional enrichment analysis were important for the identification of the research direction and preliminary mechanistic research. Among the molecules and potential pathways we figured out which were probably associated with CBX7, S110A7 and epidermal cell differentiation draw our great attention. S110A7 was located at the region on human chromosome 1q21 named "epidermal differentiation complex". This protein was proved to play essential roles in the pathogenesis of HNSC and psoriatic. It was reported that accumulation of S100A7 in the nucleus of cells from HNSC indicated poorer clinical outcomes and thereby served as a biomarker for prognostic prediction.[52] Another published paper demonstrated that S100A7 played an oncogenic role in HNSC through the activation of RAB2A and p38/MAPK pathway.[53] Very recently, it was suggested that S100A7 could act as a good hallmark for the early detection of oral squamous cell carcinoma.[54] Taken consideration of the information collected above, we were optimistic about the further exploration of the association between CBXs, S100A7, and epidermal differentiation.

Immune cell infiltration was regarded as an independent signature for the evaluation of the immune status of the body against tumor these years, and also reflect the progression and relapse of cancer.[55,56] There were no studies until now focused on the association between immune cell infiltration and CBX in HNSC. In the present study, we found that the highly consistent signature of the infiltrated immune cells in HNSC subgroups which were classified by the expression of each CBX member. These findings gave us the speculation that CBX-mediated epidemic regulation might influence the immunological micro-environment of HNSC through various infiltrated immune cells. In addition, the DNA methylation status of CBXs raised a hypothesis that altered methylation levels of CBX1, CBX5, and CBX7 might induce the expression alteration of these three genes, respectively.

Some limitations still existed in this research. Most results were obtained from public online databases, which made the conclusions less credible unless further experimental verification shows consistent results. In our next research step, more solid experiments will be performed. In addition, advanced mechanistic investigations are also indispensable for a better explanation of the diagnostic and prognostic value of CBXs in HNSC.

Conclusion

In conclusion, the CBX family showed its valuation for further investigation in HNSC. Some particular combination of CBX members may serve as a novel integrated signature for clinical usage in the judgment of the condition of the HNSC, under the premise of further evidence supporting in the future. Our research highlighted that CBX7 had the potential to be a novel diagnostic and prognostic biomarker for patients with HNSC.

Data Sharing Statement

All data generated or analyzed during this study are included in the manuscript and Supplementary Materials.

Ethics Approval

All our data belong to public databases and online website, which are based on open source. The patients involved in the database have obtained ethical approval. Users can download relevant data for free for research and publish relevant articles, so there are no ethical issues. The waived ethics approval has been approved by the Ethic Committee of the Xiangya Hospital of Central South University.

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Disclosure

The authors declare no conflicts of interest for this work.

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