


Data Mining Identifies Six Proteins that Can Act as Prognostic Markers for Head and Neck Squamous Cell Carcinoma

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a malignant tumor of the upper aerodigestive tract affecting the oral cavity, lips, paranasal sinuses, larynx, and nasopharynx. Proteogenomics combines proteomics and genomics and employs mass spectrometry and high-throughput sequencing technologies to identify novel peptides. The aim of this study was to identify potential protein biomarkers for clinical treatment of HNSCC. To achieve this, we utilized two sets of data, one on proteins from The Cancer Proteome Atlas (TCPA) and the other on gene expression from The Cancer Genome Atlas (TCGA) database, to evaluate dysfunctional proteogenomics microenvironment. Univariate Cox regression analysis was performed to examine the relationship between protein signatures and prognosis. A total of 19 proteins were significantly associated with overall survival (OS) of patients, of which E2F transcription factor 1 (E2F1; HR = 4.557, 95% CI = 1.810 to 11.469) and enhancer of zeste homolog 2 (EZH2; HR = 0.430, 95% CI = 0.187 to 0.984) were the most differentially expressed between patients with longer and shorter OS, respectively. Furthermore, multivariate Cox regression analysis on six proteins (ERALPHA, HER3, BRAF, P27, RAPTOR, and E2F1) was performed to build the prognostic model. The receiver operating characteristic curves were used to determine whether the expression pattern of survival-related proteins could provide an early prediction of the occurrence of HNSCC. Herein, we found an AUC of 0.720. Based on an online database, we identified novel protein markers for the prognosis of HNSCC. The findings of the present study may provide new insights into the development of new and reliable tools for precise cancer intervention.

Keywords

head and neck squamous cell carcinoma, proteomics, TCPA, TCGA, prognosis

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a malignant tumor of the upper aerodigestive tract affecting the oral cavity, lips, paranasal sinuses, larynx, and nasopharynx¹. Currently, it is the sixth most prevalent cancer globally and accounts for more than 650,000 new cases and 90,000 deaths annually². Known risk factors for the disease include smoking cigarettes, infection with human papillomavirus (HPV), and alcohol consumption³. One of the main challenges in HNSCC treatment is late diagnosis because symptoms tend to occur in the advanced stages of the disease. Consequently, the 5-yr survival rate of individuals with HNSCC is less than 50% and can drop to less than 35% in patients that develop local recurrence or metastasis⁴. Once in the advanced stages, treatment interventions can affect the structure and function of organs involved in speech, taste, smell, swallowing, as well as other vital organs of the body. These eventualities reduce the overall quality of life of the affected individuals^{5,6}.

Proteogenomics is a field of research that combines proteomics and genomics and utilizes mass spectrometry and high-throughput sequencing technologies to identify novel peptides. Mass spectrometry-based matching or

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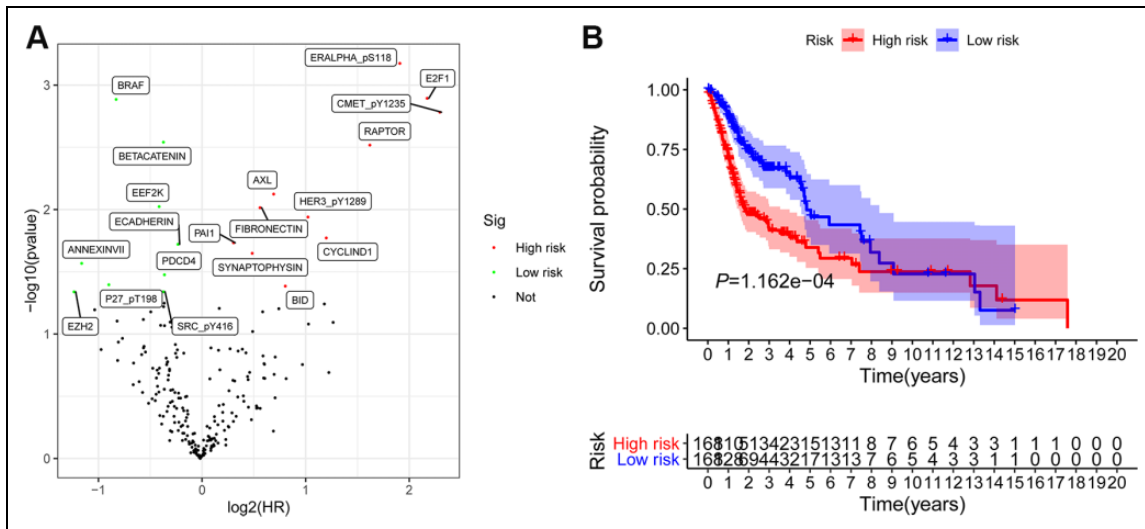


Fig. 1. (A) Volcano plot showing the survival-related proteins (red dots indicate high-risk proteins and green dots indicate low-risk proteins). (B) Overall survival analysis indicating that the prognosis of HNSCC in the high-risk group was better than that in the low-risk group. AXL: AXL receptor tyrosine kinase; BID: BH3 interacting domain death agonist; BRAF: B-Raf proto-oncogene serine/threonine kinase; CMET_pY1235: The MET transmembrane tyrosine kinase; CYCLIND1: Cyclin D1; E2F1: E2F transcription factor 1; ECADHERIN: E-cadherin; EEF2K: eukaryotic elongation factor 2 kinase; ERALPHA_pS118: estrogen receptor alpha; EZH2: enhancer of zeste homolog 2; HER3_pY1289: Erb-b2 receptor tyrosine kinase 3; HNSCC: head and neck squamous cell carcinoma; HR: hazard ratio; P27_pT198: cyclin-dependent kinase inhibitor p27; PAI1: plasminogen activator inhibitor type; PDCD4: programmed cell death 4; RAPTOR: mTOR regulation-related protein; SRC_pY416: SRC proto-oncogene.

identification of homologous peptides provides evidence of gene expression at protein level⁷. As such, it allows in-depth analysis of interactions between host and respective pathogens. Combining advanced genomics with proteomics has the potential of revealing useful biomarkers that can drive the development of new diagnostic techniques and therapies. Many studies have explored the role of proteogenomics in assessing the prognosis of cancers, but even so, the mechanism with which tumors progress remains unclear. Ma et al.⁸ found that C-C motif chemokine ligand protein mediates progression and angiogenesis of Epstein Barr virus (EBV)-mediated nasopharyngeal carcinoma. Moreover, patients with oral squamous cell carcinoma exhibit overexpressed CD44 molecule *S100A7*, and *S100P* proteins. Consistently, these proteins are considered as potential biomarkers for early detection of malignant tumors⁹. A few integrative tools for complete proteogenomics analyses have been developed; however, there are currently no high-throughput technologies for evaluating protein biomarkers that can predict the overall survival (OS) or response to HNSCC immunotherapy. Because high-throughput expression data are available, it is feasible to use global information on gene expression to analyze the association between genomics and prognoses of tumors. In this study, we utilized two sets of data, one on proteins, from The Cancer Proteome Atlas (TCPA), and the other on gene expression, from The Cancer Genome Atlas (TCGA) database, to evaluate dysfunctional proteogenomics microenvironment with an

aim of identifying useful protein biomarkers for clinical treatment of HNSCC.

Materials and Methods

Data Collection

Data was collected from TCPA, which contained a protein profile of 8,167 tumor samples. These proteins were primarily reflective of the gene expression of genetic maps in TCGA database. They provide a unique opportunity to validate the reliability of data in TCGA and identify model cell lines for functional research¹⁰. TCGA has generated many platforms for data on cancer genomics as well as proteomics using the Reverse Phase Protein Array, which measures the level of about 150 proteins and 50 phosphoproteins in tumors¹¹. Data on proteomics was therefore downloaded from TCPA (level 4), together with gene expression profiles from TCGA.

Establishing Prognostic Related Gene Signatures

Univariate Cox regression analysis was used to identify the prognostic genes and establish genetic characteristics. The gene signature was calculated as risk score = (Coefficient_{mRNA1} × expression of mRNA1) + (Coefficient_{mRNA2} × expression of mRNA2) + ... + (Coefficient_{mRNA_n} × expression mRNA_n). Based on the median, each parameter was categorized into a low-risk and a high-risk group. Kaplan-Meier

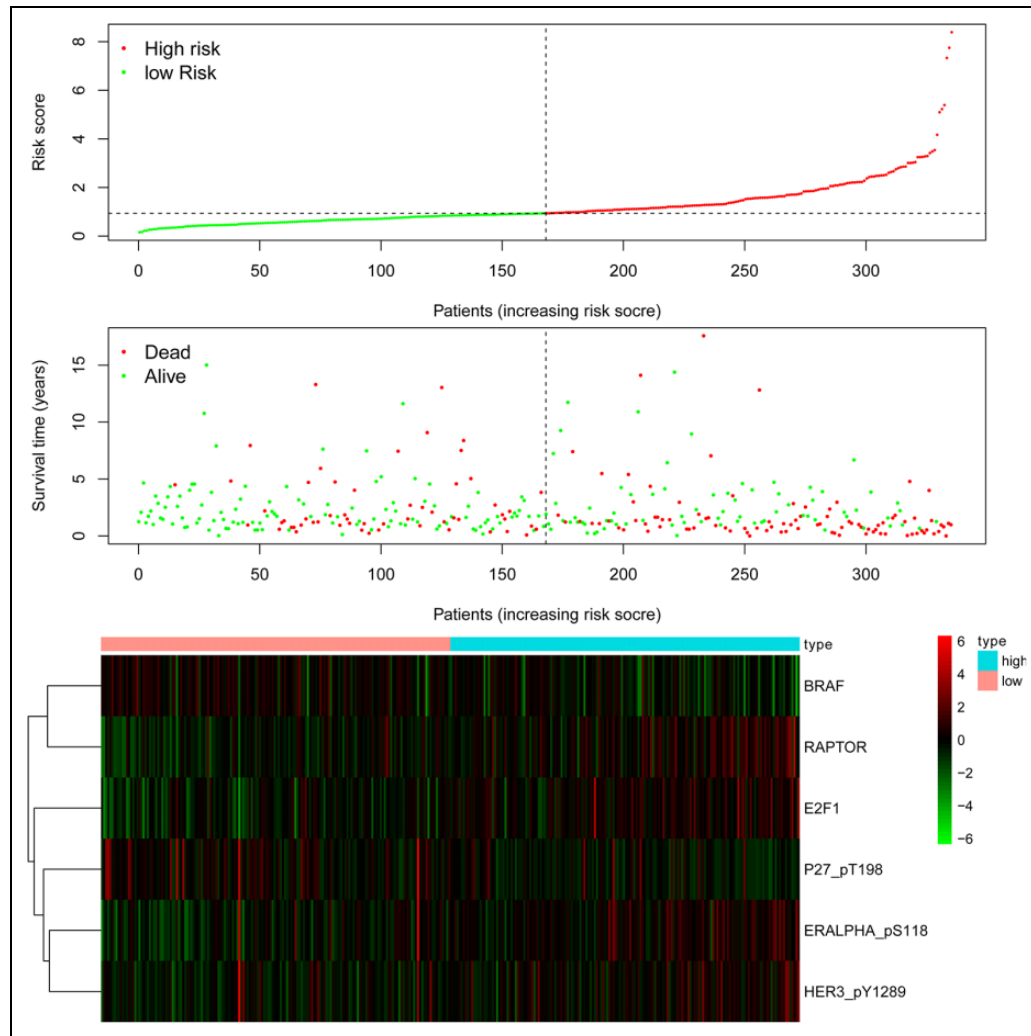


Fig. 2. Detailed prognostic signature information of HNSCC groups. BRAF: B-Raf proto-oncogene serine/threoninekinase; E2F1: E2F transcription factor 1; ERALPHA_pS118: estrogen receptor alpha; HER3_pY1289: Erb-b2 receptor tyrosine kinase 3; HNSCC: head and neck squamous cell carcinoma; P27_pT198: cyclin-dependent kinase inhibitor p27; RAPTOR: mTOR regulation-related protein.

survival analysis was performed to compare the survival rate between the study group and the control group.

Establishing a Predictive Nomogram

Nomograms are often used to determine the prognosis of cancer because they can simplify statistical prediction models and provide individualized numerical assessment of the likelihood of an event (such as relapse or death)¹². In the present study, a receiver operating characteristic (ROC) curve was plotted to evaluate the accuracy of the proteins as prognostic markers for HNSCC. Univariate and multifactorial Cox regression analyses were performed to compare the association between the expression of specific proteins and clinicopathological variables. Data with missing values were deleted from the survey. These data were analyzed using R packages in R (version 3.5.3 <http://www.r-project.org/>). Statistical

significance regarding Perl language for data matrix and all data processing was set at $P < 0.05$.

Results

Prognostic Protein Signatures

Data on proteomics were first retrieved from TCGA. The corresponding genomic data on clinical survival was downloaded from TCGA database. Univariate Cox regression analysis identified 19 proteins that were significantly associated with OS of patients, of which *E2F transcription factor 1* (*E2F1*; hazard ratio (HR) = 4.557, 95% confidence interval (CI) = 1.810 to 11.469) and *enhancer of zeste homolog 2* (*EZH2*; HR = 0.430, 95% CI = 0.187 to 0.984) were the most differentially expressed between patients with longer and shorter OS, respectively (Table S1). The volcano plot was drawn to visualize the expression profile of survival-related proteins. The red and green dots indicate high-risk

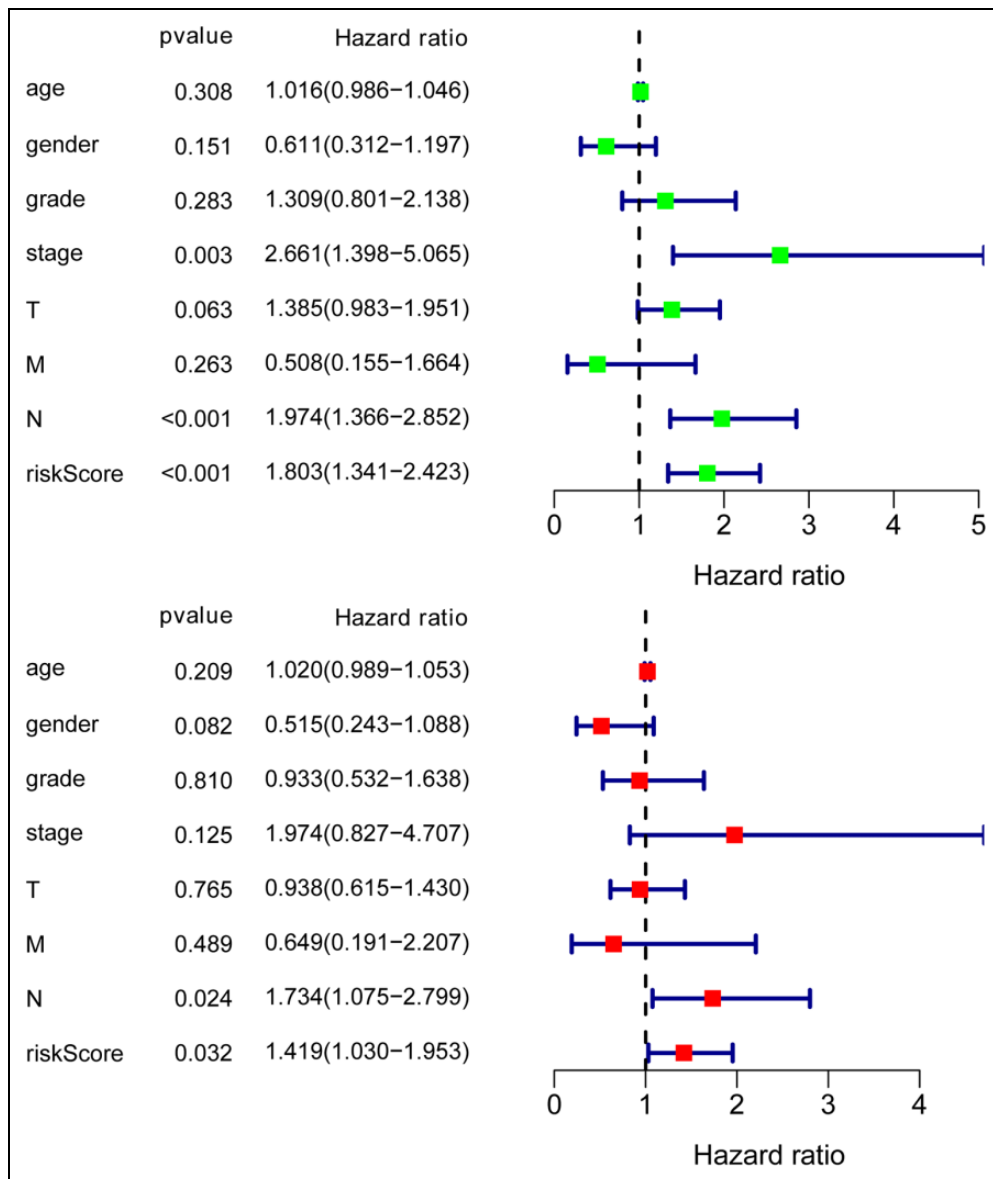


Fig. 3. The results of Cox regression analysis indicate that our prognostic model is an independent prognostic factor for overall survival.

and low-risk proteins, respectively (Fig. 1A). Multivariate Cox regression analysis on the six proteins Estrogen receptor alpha (*ERALPHA*), Erb-b2 receptor tyrosine kinase 3 (*HER3*), B-Raf proto-oncogene serine/threoninekinase (*BRAF*), Cyclin-dependent kinase inhibitor p27 (*P27*), mTOR regulation-related protein (*RAPTOR*), and E2F transcription factor 1 (*E2F1*) was performed to build the prognostic model. The formula for our model was: Risk Score = $0.84 \times ERALPHA + 0.89 \times HER3 + (-0.64) \times BRAF + (-0.86) \times P27 + 1.79 \times RAPTOR + 1.33 \times E2F1$. Significant differences in expression levels of *ERALPHA* ($P = 2.934e-12$), *HER3* ($P = 3.779e-07$), *BRAF* ($P = 7.187e-08$), *P27* ($P = 1.059e-06$), *RAPTOR* ($P = 2.694e-11$), and *E2F1* ($P = 6.311e-12$) were found between the high-risk and the low-risk groups.

Survival Results and Multivariate Examination. OS analysis showed that the expression of high-risk proteins was associated with better prognosis of HNSCC compared with low-risk proteins ($P < 0.01$; Fig. 1B). Also, the comprehensive prognostic signature model of the HNSCC revealed that HNSCC patients expressing high-risk proteins exhibited lower mortality rates and high OS (Fig. 2). Cox regression analysis showed that our prognostic model was an independent factor for OS (Fig. 3). The ROC curves were used to determine whether the expression pattern of survival-related proteins could provide an early prediction for the occurrence of HNSCC. We found an area under roc curve (AUC) of 0.720, which indicated that the sensitivity and specificity of this prognostic model were moderate. Clinicopathological factors associated with HNSCC were also explored. The

AUC of age, gender, grade, stage, tumor (T), metastasis (M), and node (N) were 0.546, 0.476, 0.526, 0.639, 0.578, 0.444, and 0.698, respectively (Fig. 4).

Creating Predictive Nomogram. A nomogram was created by combining clinical factors and the prognostic model. The final model comprised seven factors captured in TCGA, which included age, gender, grade, stage, T, M, and N (Fig. 5). Combining the prognostic model with clinical factors improved the accuracy of predicting OS for 1, 2, and 3 yr. Analysis of co-expression of the six proteins identified 24 negatively correlated and 68 positive correlated proteins (Table S2).

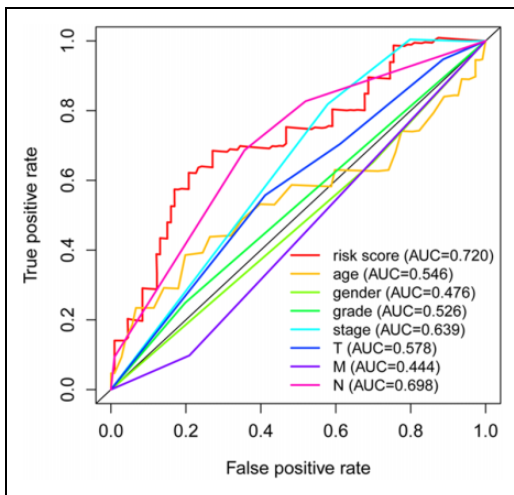


Fig. 4. The results of our model and the clinicopathological predictors of HNSCC by ROC. AUC: area under roc curve; HNSCC: head and neck squamous cell carcinoma; ROC: receiver operating characteristic.

Discussion

Analysis of cancer proteomics provides new insights into changes that occur in the early stages of tumorigenesis, and therefore assist in the identification of novel biomarkers for early disease diagnosis and prognosis. Tumor cells present unique proteomic characteristics regarding their growth and survival compared to normal cells. These unique proteomic signaling pathways can be used as prognostic markers for the occurrence and development of cancer. Also, they can act as therapeutic targets in cancer treatment. Herein, we identified new and reliable prognostic proteomics signatures based on online datasets. These proteomic profiles are likely to manifest in patients with HNSCC and thus can be utilized as biomarkers and targets for therapeutic interventions.

In the present study, proteomic data was downloaded from TCGA (level 4), together with clinical survival data from TCGA. In total, 19 survival-related proteins were identified. The expression of *E2F1* protein correlated with high OS. *E2F1* (also known as *E2F-1*, *RBAP1*, *RBBP3*, *RBP3*) is a member of the E2F transcription factor family. The E2F family plays a vital role in controlling the cell cycle and inhibiting tumor-enhancing proteins. A recent study found that *E2F1* activates lncRNA small nucleolar RNA host gene 3 (*SNHG3*), thereby promoting the migration and proliferation of cells in individuals with non-small-cell lung cancer by transforming the growth factor- β pathway¹³. Similarly, therapeutic targets that suppress the myeloid zinc finger 1 (*MZF1*) and *MZF1* antisense RNA 1 (*MZF1-AS1*)/poly(-ADP-ribose) polymerase 1 (*PARP1*)/*E2F1* axis can inhibit the synthesis of proline and progression of neuroblastoma¹⁴. A study identified miRNA-205 as a putative HNSCC oncogene and regulator of *E2F1* protein expression¹⁵. Notably, HNSCC caused by HPV infection expresses E6 and E7 viral

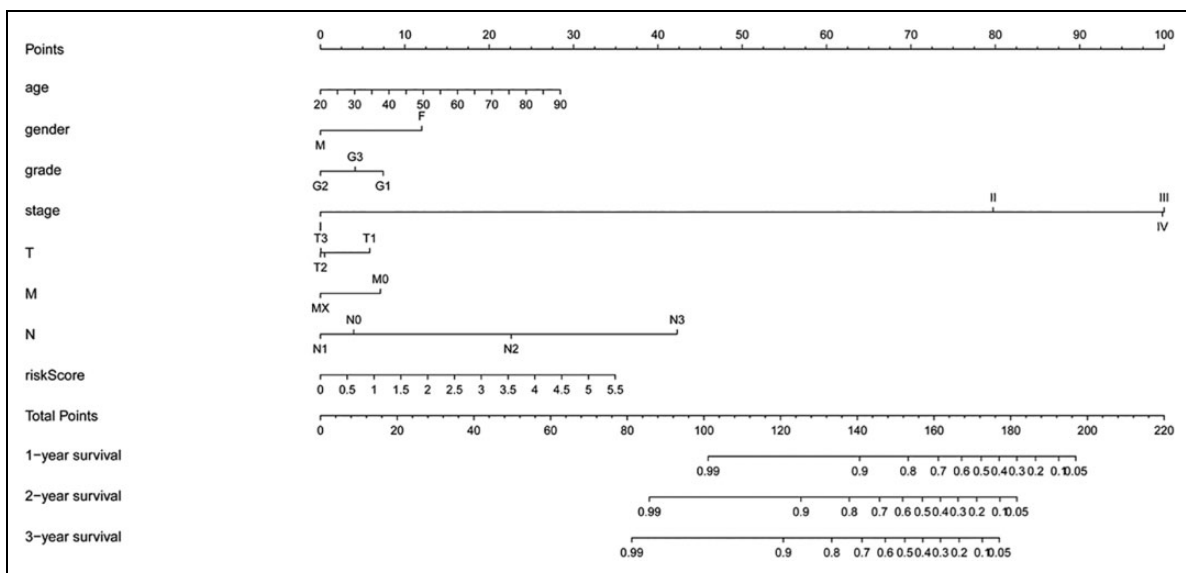


Fig. 5. Nomogram constructed using the clinical pathology and prognosis models.

oncogenes. These proteins suppress tumor protein p53 (*TP53*) and retinoblastoma 1 (*RBI*) and activate *E2F1*, a cell cycle regulator¹⁶. At present, the role of *E2F1* in HNSCC has not been fully elucidated. Given this, we aimed to explore the relationship between the expression of *E2F1* and the occurrence and progression of HNSCC. *EZH2* and the catalytic component of Polycomb inhibition complex 2 are associated with the expression of homologous genes (*Hox*) and early stages of inactivating X chromosome¹⁷. Overexpression of *EZH2* is thus associated with high incidence and poor prognosis of tumors. Maintaining the stability of mitochondrial membrane potential in HNSCC is dependent on *EZH2*¹⁸. Studies have shown that signal transducer and activator of transcription 3 (*STAT3*) signaling through overexpression/activation of phosphatidylinositol-3-kinase (*PI3K*) regulates HOX transcript antisense RNA (*HOTAIR*) and *EZH2* in HNSCC, thus promoting the progression of the disease¹⁹.

The potential of utilizing the pattern of survival-related proteins in early prediction of future occurrence of HNSCC was examined using the ROC curve. The AUC was 0.720. We examined six proteins (*ERALPHA*, *HER3*, *BRAF*, *P27*, *RAPTOR*, and *E2F1*) in the multivariate Cox regression analysis to build the prognostic model. Estrogen receptor genes (*ERalpha* and *ERbeta*) play an important role in the progression of tumors, particularly breast cancer. Notably, the *ERbeta* gene has been shown to play a more significant role than *ERalpha* in the etiology of colorectal cancer²⁰. In breast cancer, the expression of *ERbeta* alone is an independent risk factor for poor prognosis of patients undergoing tamoxifen adjuvant therapy²¹. The expression of *ERalpha* and *ERbeta* in HNSCC upregulates the estrogen receptor ligands, thus activating transcription and transduction of cytoplasmic signals²². Also, *ERalpha* was shown to be associated with epidermal growth factor receptor (*EGFR*) in HNSCC cells treated with dasatinib²³. *HER3* (*Erb-b2 receptor tyrosine kinase 3*) is a member of the human *EGFR* family and consists of four type-1 transmembrane tyrosine kinase receptors: *HER1* to *HER4*. The upregulation of *HER3* is associated with poor survival rates in various malignancies, including HNSCC and breast, colorectal, gastric, and prostate cancer^{24–26}. Studies have also found that upregulating *HER3* using cetuximab hinders *EGFR*-mediated inhibition of HNSCC²⁷. The small nuclear ribonucleoprotein polypeptide E (*B-Raf*) proto-oncogene serine/threonine kinase (*BRAF*) encodes a protein belonging to the serine/threonine-protein kinase family. Mutation in *BRAF* is a marker for response to respective targeted therapy in patients with HNSCC²⁸. Therefore, factors that promote *BRAF* mutation drive oncogenesis. Accordingly, *BRAF* mutations are frequent in thyroid cancer²⁹. *P27* is a cyclin-dependent kinase inhibitor and plays an essential role in the negative regulation of the cell cycle. Overexpression of *p27* inhibits the proliferation of cancer cells, increases apoptosis, and arrests cell cycle in HNSCC³⁰. The expression of *p27* can be used to predict how HNSCC responds to chemotherapy³¹.

RAPTOR (mechanistic target of rapamycin (mTOR) regulation-related protein) is a 150 kDa mTOR-binding protein that is essential for TOR signaling *in vivo*³². Activation of the serine-threonine protein kinase (Akt)-mTOR pathway is a common phenomenon in HPV-associated HNSCC. As such, mTOR inhibitors present attractive candidates for the treatment of HPV-positive squamous neoplastic lesions³³. Similarly, activation of mTOR and auxiliary inhibition of mTOR in HPV-HNSCC disease can enhance primary site resistance, reduce the number of metastatic cells, and limit distant metastases³⁴.

The signatures established in this study could reflect proteomic microenvironmental diseases and provide potential biomarkers for proteomic treatment and treatment response prediction. However, these signatures should be verified in more independent cohorts and functional experiments that predict proteins.

This study had two main limitations. First, the results were not validated using clinical samples, and second, the results may not be reliable because of the small sample size.

Conclusions

Based on the online database, we identified novel protein markers for the prognosis of HNSCC. The findings of the present study may provide new insights into the development of new and reliable tools for precise cancer treatment.

Authors' Contributions

W.Z.H. designed and analyzed the research study; W.Z.H. and T.Y. wrote and revised the manuscript, W.Z.H. and C.Q. collected the data; and all authors contributed to and approved the final version of the manuscript.

Availability of Data and Materials

A part of this study is based upon data generated by TCGA Research Network: <https://www.cancer.gov/tcga>, and the data are also available in TCGA.

Ethical Approval

As the work is a bioinformatics analysis article, ethical approval was not necessary and all the data were retrieved from the free online databases.

Statement of Human and Animals Rights

This article does not contain studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.


Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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