Identifying potential prognostic biomarkers in head and neck cancer based on the analysis of microRNA expression profiles in TCGA database

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Abstract. The present study aimed to identify sensitive, specific and independent prognostic biomarkers in head and neck cancer (HNC) based on microRNA expression profiles and other high-throughput sequencing data in The Cancer Genome Atlas (TCGA) database. Identification of such prognostic biomarkers could provide insight into HNC diagnosis and treatment. The differential expression profiles of microRNAs between HNC tissues and adjacent cancer tissues in the TCGA database were analyzed (log fold-change >2; P<0.01). Univariate and multivariate Cox regression analyses of the differentially expressed microRNAs were performed to determine those significantly related to the survival of patients with HNC. The identified microRNAs were verified by survival and receiver operating characteristic curve analyses. To better predict prognosis, a combined prognostic model (risk equation) was established based on the risk coefficient of each microRNA, calculated by a multivariate Cox regression analysis, and the risk score was calculated. To explore the signaling pathways related to prognosis, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses were performed on the differentially expressed genes between the high-risk and low-risk groups, grouped according to the median risk score. A total of 89 differentially expressed microRNAs between HNC and adjacent cancer tissues were screened, 11 of which were identified as risk factors related to HNC survival by the univariate Cox regression analysis (P<0.05). The multivariate Cox regression analysis showed that three of the 11 microRNAs, hsa-miR-99a, hsa-miR-499a and hsa-miR-1911 (all P<0.01), were identified as independent risk factors significantly related to patient survival. The risk equation used was as follows: Risk score=(-0.1597 x hsa-miR-99a) + (0.1871 x hsa-miR-499a) + (0.1033 x hsa-miR-1911). KEGG and GO analyses showed that the JAK-STAT signaling pathway and some metabolic pathways were associated with HNC prognosis. The present study suggested that hsa-miR-99a, hsa-miR-499a and hsa-miR-1911 may serve as potential prognostic biomarkers in HNC.

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Abbreviations: HNC, head and neck cancer; TCGA, The Cancer Genome Atlas; ROC, receiver operating characteristic; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus

Key words: HNC, TCGA database, microRNA, prognostic analysis

Introduction

Head and neck cancer (HNC) is the sixth most common malignant tumor in the world, with the main pathological type being head and neck squamous cell carcinoma (HNSCC) (1). HNSCC involves numerous organs, such as the oral cavity, nasopharynx, oropharynx, larynx and salivary glands. The pathogenesis of HNC has not been fully elucidated, however, a number of studies have shown that smoking, drinking and papillomavirus infection are important environmental factors for HNC initiation (2,3). Patients with HNC have poor quality of life, and the majority have difficulty in communication, breathing and swallowing (4). Although the effectiveness of treatment for patients with HNC has improved in recent years, the prognosis is still poor (5). HNC remains one of the most important diseases threatening life and health, and is a major health problem that needs to be solved.

With in-depth research on non-coding RNAs and increasing research on microRNAs, the relationship between microRNAs

and HNC prognosis has received unprecedented attention. Numerous studies have reported that the abnormal expression of microRNAs is closely related to the initiation, progression and prognosis of various tumors (6-10), including HNC (11,12). The screening and identification of microRNAs related to the prognosis of HNC will provide important clues for the prevention, intervention and treatment of the high-risk population. Moreover, it is helpful to find new targets for HNC-targeted therapy. The identification of potential prognostic biomarkers in HNC, based on the analysis of microRNA expression profiles and the exploration of appropriate interventions, may improve the prognosis of patients with HNC.

The Cancer Genome Atlas (TCGA) database covers abundant genetic information and detailed clinical information of 33 types of tumors. The database includes six genome-wide platforms that assayed tumor DNA (exome sequencing, DNA methylation, and copy number), RNA (mRNA and microRNA sequencing) and a cancer-relevant set of proteins and phosphoproteins (13). This data can be directly downloaded through the TCGA Data Portal (https://portal.gdc.cancer.gov/) and provide convenient multidimensional omics data resources for tumor researchers (14,15). The present study aimed to identify microRNAs that were closely related to HNSCC prognosis by integrating the genomic, transcriptomic and clinicopathological information in the TCGA database. It is expected that the results from the present study will provide clues for the diagnosis and treatment of patients with HNC.

Materials and methods

Collection and analysis of microRNA expression profiles of HNC tissues in the TCGA database to identify prognostic microRNAs. The collection and analysis of differential expression profiles of microRNAs between HNC tissues (525 cases) and adjacent cancer tissues (44 cases) in the TCGA database (http://cancergenome.nih.gov) were performed through the TCGA Data Portal and R software (https://www.r-project.org, v3.5.1, logFC >2; P<0.01). Univariate and multivariate Cox regression analyses were further performed on microRNAs with differential expression to determine the independent risk factors significantly associated with patient survival using SPSS 22.0 (IBM Corp.). The identified risk factors were then verified by survival and receiver operating characteristic (ROC) curve analyses. To better predict prognosis, a prognostic model (risk equation) was established: Risk score=(-0.1597 x hsa-miR-99a) + (0.1871 x hsa-miR-499a) + (0.1033 x hsa-miR-1911), according to the risk coefficient of each microRNA calculated by the multivariate Cox regression analysis.

Analysis of the effect of clinicopathological characteristics on HNC prognosis. Univariate and multivariate Cox regression analyses were performed on clinicopathological characteristics to analyze their roles in HNC survival. These findings were then verified by Kaplan-Meier survival analysis and calculated using the log-rank test with GraphPad Prism7 (GraphPad Software Inc.). Clinicopathological characteristics analyzed included age, gender, tumor-node-metastasis (TNM) stage, Fuhrman grade, ethnicity, radiotherapy, human papillomavirus (HPV) status and mutation number.

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses to explore the signaling pathways related to HNC prognosis. HNC cases in TCGA database were divided into high-risk and low-risk groups according the median risk score calculated by the risk equation (cut-off value=1.03), and subsequently a differential gene expression analysis was performed. KEGG (https://www.genome.jp/kegg/) and GO (http://geneontology.org/) analyses were performed on the upregulated and downregulated genes to explore the signaling pathways related to the microRNAs aforementioned using R cluster Profiler package (16).

Results

Identification of three microRNAs as independent risk factors in HNC prognosis. The differential expression profiles of microRNAs in HNC tissues (525 cases) and adjacent tumor tissues (44 cases) in the TCGA database (logFC >2; P<0.01) were analyzed. A total of 89 differentially expressed microRNAs were found, of which 38 were upregulated and 51 were downregulated in HNC tissues compared with those in adjacent tumor tissues. After excluding two HNC cases that lacked clinicopathological data, 523 patients with HNC were randomly divided into the test group (n=300) and the validation group (n=223). The general clinicopathological data are shown in Table I.

In the test group, 11 of the 89 differentially expressed microRNAs were determined as risk factors through the univariate Cox regression analysis, as shown in Table II. To establish a more accurate prediction model, these 11 microRNAs were further analyzed by a multivariate Cox regression analysis. A total of three microRNAs, namely, hsa-miR-99a (P=0.0078), hsa-miR-499a (P=0.0023) and hsa-miR-1911 (P=0.0304), were identified as independent risk factors significantly associated with patient survival (Table III). These three microRNAs exhibited differential expression in HNC tissues and adjacent tumor tissues (Fig. 1A). Survival and ROC curve analyses were performed on the three microRNAs in the test group, and the results showed that they are closely related to prognosis (Fig. 1B and C).

Establishing a combined prognostic model/risk equation. To better predict prognosis, the three aforementioned microRNAs were combined to establish a prognostic model. The risk equation was as follows: Risk score=(-0.1597 x hsa-miR-99a) + (0.1871 x hsa-miR-499a) + (0.1033 x hsa-miR-1911), according to the risk coefficient of each microRNA calculated by the multivariate Cox regression analysis. A positive coefficient indicates that high expression is associated with a poor prognosis (for example, hsa-miR-499a and hsa-miR-1911), while a negative coefficient indicates that high expression is associated with a good prognosis (for example, hsa-miR-99a; Fig. 2A). Risk scores for each patient were calculated and ranked. The expression of hsa-miR-499a, hsa-miR-1911 and hsa-miR-99a and the distribution of the risk scores are shown in Fig. 2B. As a protective factor, hsa-miR-99a was highly expressed in the low-risk group, while as risk factors, hsa-miR-499a and hsa-miR-1911 were highly expressed in the high-risk group (Fig. 2B). Similar results were obtained in the validation

Table I. General clinicopathological data of head and neck cancer cases in The Cancer Genome Atlas database.

Characteristic	Test series n=300 (%)	Validation series n=223 (%)	Entire series n=523 (%)
Sex			
Male	209/297 (70.4)	170/222 (76.6)	379/519 (73.0)
Female	88/297 (29.6)	52/222 (23.4)	140/519 (27.0)
Age (years)			
≥60	171/297 (57.6)	115/222 (51.8)	286/519 (55.1)
<60	126/297 (42.4)	107/222 (48.2)	233/519 (44.9)
TNM stage			
I	20/252 (7.9)	7/198 (3.5)	27/450 (6.00)
II	50/252 (19.8)	26/198 (13.1)	76/450 (16.9)
III	35/252 (13.9)	43/198 (21.7)	78/450 (17.3)
IV	147/252 (58.3)	122/198 (61.6)	269/450 (59.8)
Fuhrman grade			
I	30/282 (10.6)	33/215 (15.3)	63/498 (12.7)
II	172/283 (60.8)	133/215 (61.9)	305/498 (61.2)
III	76/283 (26.9)	47/215 (21.9)	123/498 (24.7)
IV	5/283 (1.8)	2/215 (0.9)	7/498 (1.4)
Ethnicity			
Non-white	32/288 (11.1)	28/216 (13.0)	60/504 (11.9)
White	256/288 (88.9)	188/216 (87.0)	444/504 (88.1)
Radiation			
No	94/255 (36.9)	62/197 (31.5)	156/452 (34.5)
Yes	161/255 (63.1)	135/197 (68.5)	296/452 (65.5)

Some patients have incomplete clinical information, including age, gender and TNM stage, so the number of patients with each pathological feature is not consistent with the total number of patients.

Table II. Eleven microRNAs as risk factors of head and neck cancer prognosis.

MicroRNA	Hazard ratio	z-score	P-value
hsa-miR-1911	1.134803307	3.034161031	0.002412056
hsa-miR-4510	0.693911755	-2.814364084	0.004887384
hsa-miR-99a	0.868724953	-2.712379627	0.006680204
hsa-miR-410	1.172183233	2.486637243	0.012895682
hsa-miR-381	1.182780455	2.405357775	0.016156640
hsa-miR-411	1.177476467	2.329398742	0.019837952
hsa-miR-499a	1.131263045	2.209846565	0.027115813
hsa-miR-548f-1	1.117557677	2.176162956	0.029543078
hsa-miR-1-1	1.056771872	2.151940931	0.031402007
hsa-miR-1-2	1.053267713	2.000451927	0.045451486
hsa-miR-4652	1.114090000	1.981273393	0.047560623

group and in the entire series, as shown in Fig. 2C and D. ROC curve (AUC=0.842; Fig. 2E) and survival analyses (Fig. 2F) were then carried out in the test group. Patients in the high-risk group had significantly shorter overall survival than those in the low-risk group (P<0.0001, Fig. 2F). Moreover, survival analysis was also carried out in the validation group and in the entire series, and the results were similar to those of the test group (Fig. 2G and H).

Table III. Three microRNAs as independent risk factors in head and neck cancer prognosis.

MicroRNA	Regression coefficient	Hazard ratio	P-value
hsa-miR-99a	-0.1597	0.8524	0.0078
hsa-miR-499a	0.1871	1.2057	0.0023
hsa-miR-1911	0.1033	1.1088	0.0304

Risk score used to predict prognosis is not affected by clinicopathological characteristics. Considering that the prognosis of patients with HNC is associated with clinicopathological characteristics, univariate and multivariate Cox regression analyses were conducted in the test group, the verification group and in the entire series based on the risk score and clinicopathological data. The results showed that age (entire series), gender (validation and entire series), TNM stage (test, validation and entire series), mutation number (entire series) and risk score (test, validation and entire series) were risk factors in the univariate Cox regression analysis. Contrastingly, radiotherapy

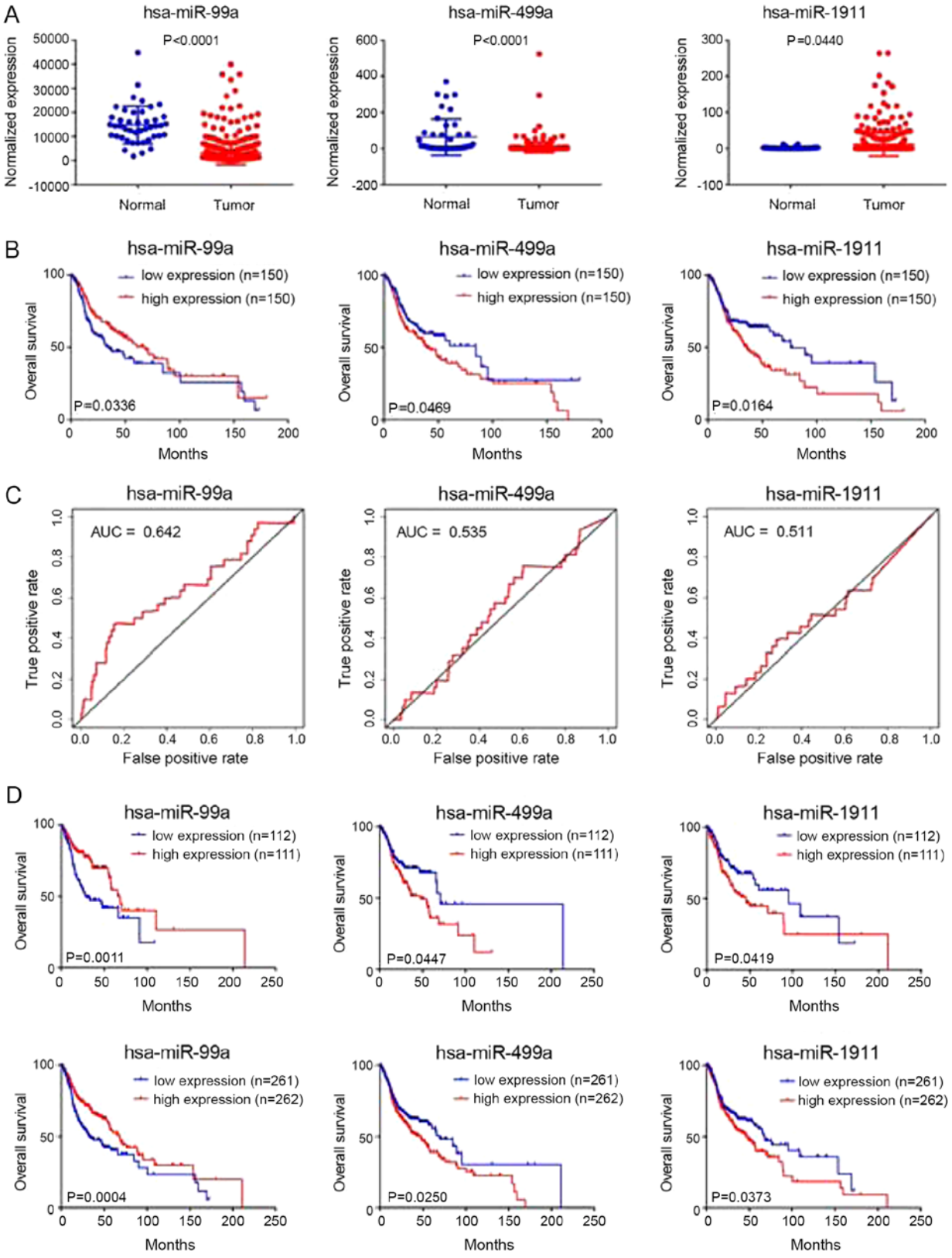

Figure 1. Novel microRNA biomarkers predict prognosis in HNC. (A) Differential expression patterns of the three microRNAs in HNC tissues and adjacent tumor tissues. (B) Survival curves of the three microRNAs in the test group (P<0.05). (C) ROC curve analysis of the three microRNAs. HNC, head and neck cancer; ROC, receiver operating characteristic. (D) Survival curves of the 3 microRNAs in the validation group (top) and entire group (bottom; P<0.05).

(test and entire series) and HPV infection (entire series) were protective factors. The multivariate Cox regression analysis of these factors showed that the risk score (test, validation and entire series) and TNM stage (test, validation and entire series) were independent risk factors, while radiotherapy (test, validation and entire series) was an independent protective factor (Table IV). Kaplan-Meier survival analysis was then performed on age, gender, TNM stage, grade, ethnicity,

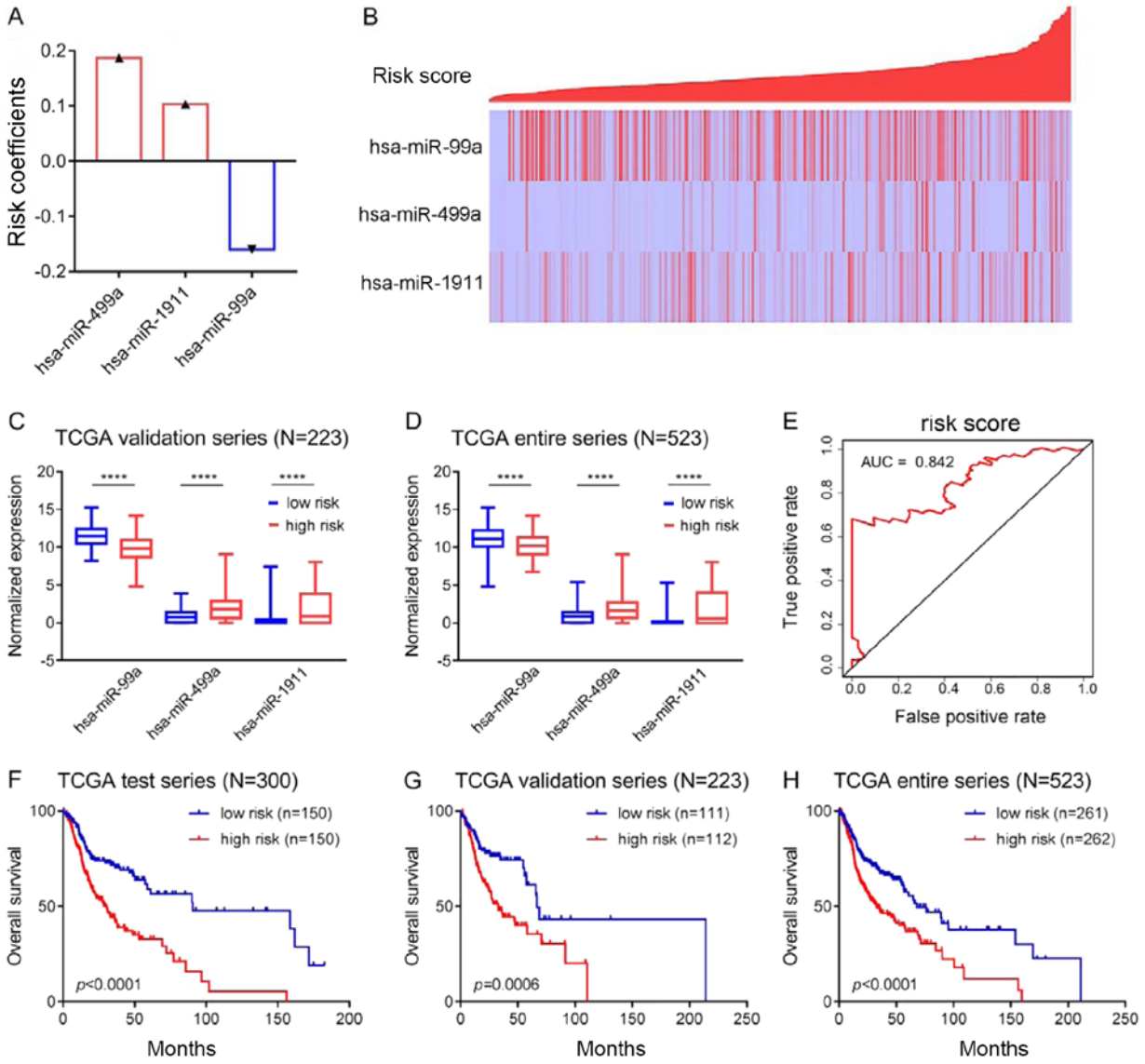

Figure 2. Three-microRNA prognostic model. (A) Risk coefficients of the three microRNAs screened as independent risk factors for HNC. A positive value indicates a risk factor, while a negative value indicates a protective factor. (B) Expression of hsa-miR-499a, hsa-miR-1911, hsa-miR-99a and the distribution of risk scores in the test group. Expression of the three microRNAs in (C) the validation group and the (D) entire series (****P<0.0001). (E) ROC curve analysis based on the three microRNA risk scores in the test group. Survival curves of patients in (F) the test and (G) validation groups as well as in (H) the entire series. HNC, head and neck cancer; ROC, receiver operating characteristic; TCGA, The Cancer Genome Atlas.

radiotherapy, HPV status and mutation number in the entire series to verify the above conclusions. The results are shown in Fig. 3A-H.

According to the aforementioned results, age, gender, TNM stage, radiotherapy, HPV status and mutation number can affect the prognosis of patients with HNC. Therefore, a further stratified analysis was conducted to analyze whether these clinicopathological characteristics can interfere with the prediction effect of the risk score. The results are shown in Fig. 4. With the exception of the HPV-positive infection group, patients with a high-risk score in each group had a poor prognosis, while patients with a low-risk score had an improved prognosis. As shown in Fig. 4A-D, patients with high-risk scores had a poor prognosis regardless of age (≥60 or <60) or gender (female or male). As shown in Fig. 4E-H, patients with high-risk scores had short overall survival regardless of whether the patients were in the early or late TNM stage or whether they had received radiotherapy. As shown in Fig. 4I and J, the two groups with more or less mutations (divided by median mutation number) were divided into two groups according to prognosis (good prognosis and poor prognosis) by risk score. Similarly, patients with a high-risk score in the HPV-negative group had a short survival time (Fig. 4L). Although the P-value in the HPV-positive group was not significant, it can be seen from Fig. 4K that patients with a high-risk score had a shorter survival period, while those with a low-risk score had a longer survival time.

Signaling pathways associated with HNC prognosis. To explore the signaling pathways related to the aforementioned microRNAs identified, the HNC cases in the TCGA database were divided into high-risk and low-risk groups according the risk score, calculated by the risk equation.

Table IV. Risk score and clinicopathological characteristics affect head and neck cancer prognosis.

A, Test series (n=300)

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Risk score (High vs. low)	2.435	1.691-3.508	<0.0001	2.020	1.262-3.233	0.0030
Age (≥60 vs. <60)	1.432	0.997-2.057	0.0520	-	-	-
Gender (Male vs. female)	0.762	0.531-1.094	0.1410	-	-	-
TNM (I, II, III, IV)	1.384	1.121-1.709	0.0020	1.797	1.329-2.431	<0.0001
Grade	0.735	0.507-1.065	0.1040	-	-	-
Ethnicity (White vs. non-white)	0.759	0.459-1.253	0.2810	-	-	-
Radiation (Yes vs. no)	0.602	0.403-0.901	0.0140	0.395	0.242-0.644	<0.0001

B, Validation series (n=223)

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Risk score (High vs. low)	2.006	1.298-3.101	0.0020	2.045	1.204-3.476	0.0080
Grade	0.960	0.597-1.544	0.8680	-	-	-
Age (≥60 vs. <60)	1.188	0.776-1.816	0.4280	-	-	-
Gender (Male vs. female)	0.616	0.389-0.977	0.0400	-	-	-
TNM (I, II, III, IV)	1.530	1.113-2.104	0.0090	2.654	1.713-4.112	<0.0001
Ethnicity (White vs. non-white)	1.009	0.659-1.545	0.9670	-	-	-
Radiation (Yes vs. no)	0.664	0.409-1.078	0.0980	0.333	0.188-0.591	<0.0001

Entire series (n=523)

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Risk score (High vs. low)	1.869	1.422-2.457	<0.0001	1.799	1.252-2.586	0.002
Age (≥60 vs. <60)	1.327	1.009-1.745	0.043	-	-	-
Gender (Male vs. female)	0.704	0.530-0.934	0.015	0.652	0.450-0.944	0.024
Radiation (Yes vs. no)	0.628	0.462-0.855	0.003	0.368	0.246-0.549	<0.0001
HPV status (Positive vs. negative)	0.484	0.294-0.795	0.004	-	-	-
Mutation count (≥48 vs. <48)	1.797	1.144-2.821	0.011	-	-	-
Ethnicity (White vs. non-white)	0.759	0.523-1.100	0.145	-	-	-
Grade	0.817	0.610-1.093	0.174	-	-	-
TNM (I, II, III, IV)	1.421	1.192-1.694	<0.0001	2.108	1.615-2.752	<0.0001

P<0.05 was considered to indicate a statistically significant difference. '-' represents not applicable. HR, hazard ratio; CI, confidence interval; HPV, human papillomavirus; TNM, tumor-node-metastasis.

A total of 556 differentially expressed genes, including 356 upregulated genes and 200 downregulated genes, were found between the high-risk and low-risk groups through differential gene expression analysis (logFC >1; P<0.05). The differentially expressed genes between HNC tissues and adjacent cancer tissues in the TCGA database (logFC >1; P<0.05) were also analyzed. The upregulated genes in HNC tissues were intersected with the upregulated genes of the high-risk group, and 106 upregulated genes were obtained. Similarly, the downregulated genes in HNC tissues were intersected with the downregulated genes of the high-risk group, and 46 downregulated genes were obtained, as shown in Fig. 5A and B. Then, KEGG and GO analyses of the intersecting upregulated and downregulated genes were performed, as shown in Fig. 5C-F. The results showed that genes in the high-risk group were mainly enriched in

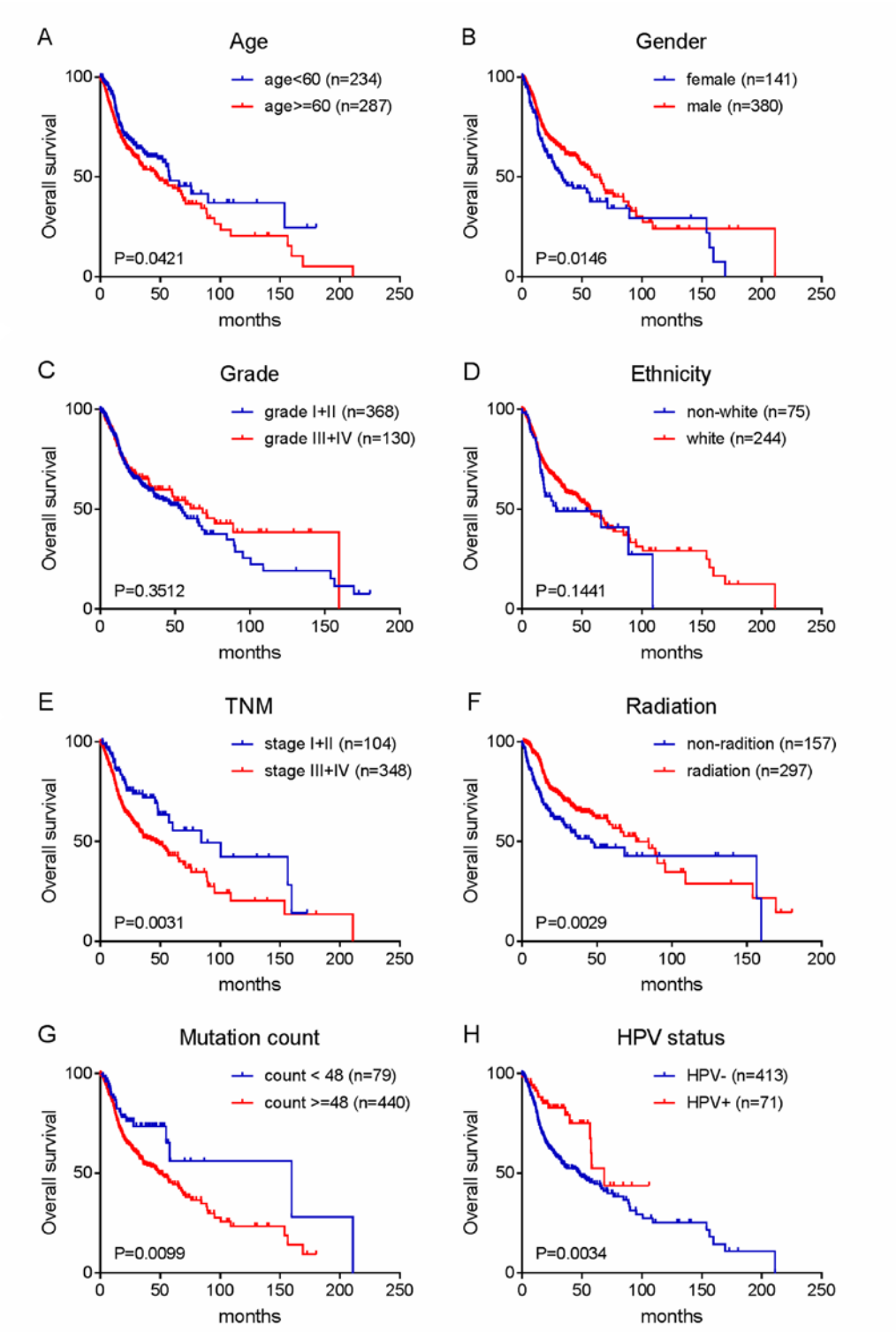

Figure 3. Survival analysis based on clinicopathological characteristics. (A) Age, (B) sex, (E) TNM stage, and (G) mutation number are risk factors in HNC prognosis, while (F) radiotherapy and (H) HPV infection are protective factors. (C) Grade and (D) Ethnicity have no significantly associated with HNC prognosis. TNM, tumor-node-metastasis; HNC, head and neck cancer; HPV, human papillomavirus.

'Regulation of action cytoskeleton', 'Parkinson's disease', 'JAK-STAT signaling pathway' and others. The genes in the low-risk group were mainly enriched in 'Tyrosine metabolism', 'hormone biosynthesis' and others, which suggested that The JAK-STAT signaling pathway and some metabolic pathways, such as tryptophan metabolism may associated with HNC prognosis.

Discussion

MicroRNAs are non-coding RNA 18-25 nucleotides in length that can pair with a complementary base of a specific mRNA, causing degradation or dysfunction of the mRNA (17). MicroRNAs play an important role in the regulation of gene expression and can affect the

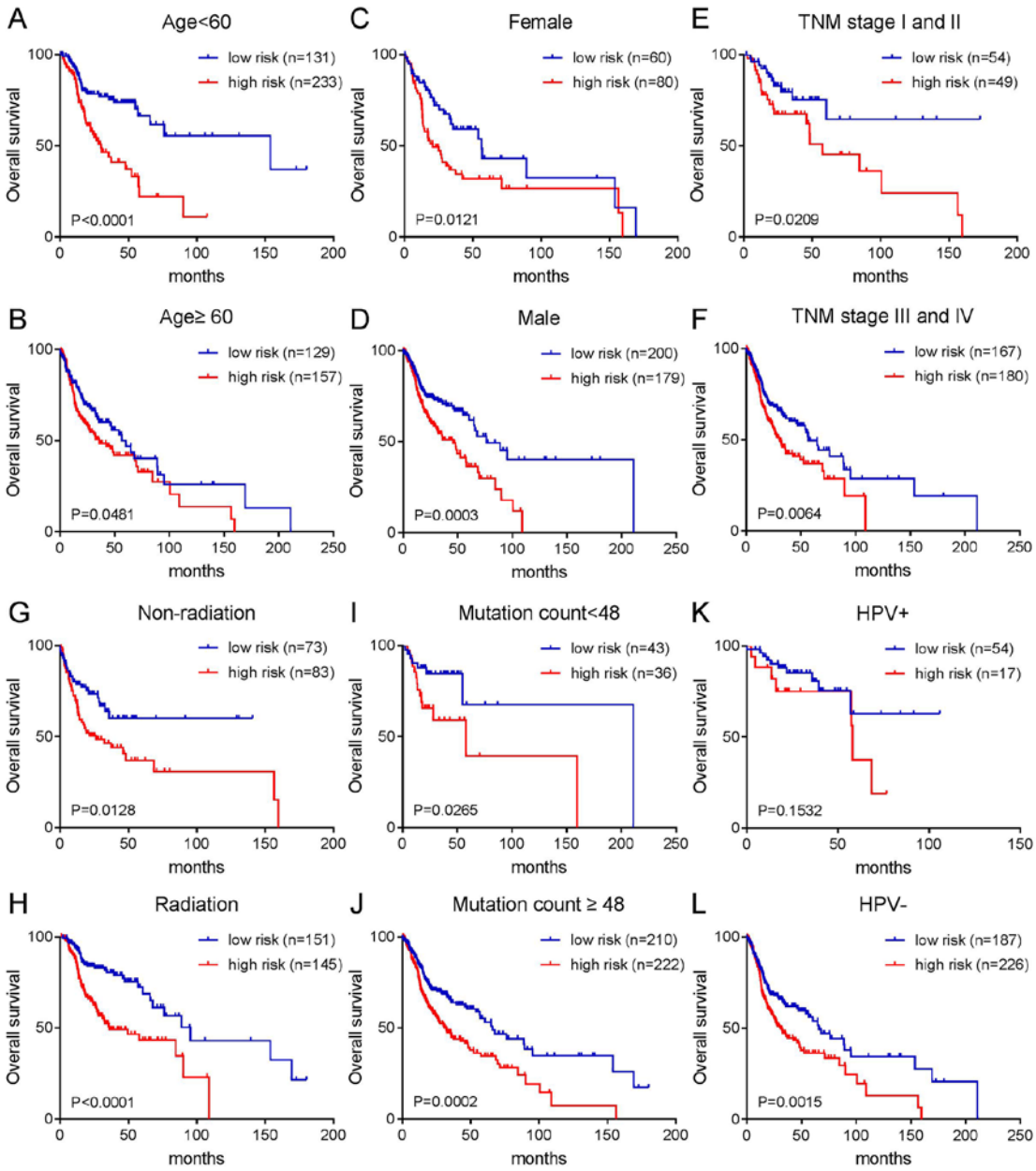

Figure 4. Survival analysis of clinicopathological characteristics grouped by risk score, Including age (A) <60 or (B) ≥60, (C) female or (D) male, TNM (E) stage I and II and (F) stage III and IV, (G) non-radiation and (H) radiation, mutation count (I) <48 and (J) ≥48, (K) HPV+ and (L) HPV-. Except for the HPV-positive group, patients with a high-risk score in each group had a poor prognosis, while patients with a low-risk score had a good prognosis. HPV, human papillomavirus.

differentiation, proliferation, apoptosis, migration and invasion of tumor cells (18-21). Numerous studies have shown that the initiation, progression and metastasis of HNC are often accompanied by the abnormal expression of specific microRNAs. Tran *et al* found that 33 microRNAs were upregulated and 22 microRNAs were downregulated in HNC cell lines compared with normal cell lines (22). The present study further explored microRNA expression profiles in HNC tissue specimens based on the TCGA database, providing more sensitive, specific and independent prognostic biomarkers for HNC. Therefore, the present study provided new clues for individual treatment, which may improve the prognosis of patients with HNC.

The TCGA project began in 2006 and aimed to create a comprehensive 'atlas' of the cancer genome through large-scale genome sequencing. To date, the TCGA has included multidimensional genomics, epigenomics and proteomics data of ≥20 types of cancer (15). The TCGA database is freely accessible to the public, providing big data support for researchers to explore new microRNAs and to study their functions. The present study identified three microRNAs, namely, hsa-miR-99a, hsa-miR-499a and hsa-miR-1911, which are significantly related to the prognosis of patients with HNSCC. These findings were based on microRNA expression profiles in the TCGA database, Cox regression analyses and other bioinformatics analyses, and have also been reported in other tumors (23).

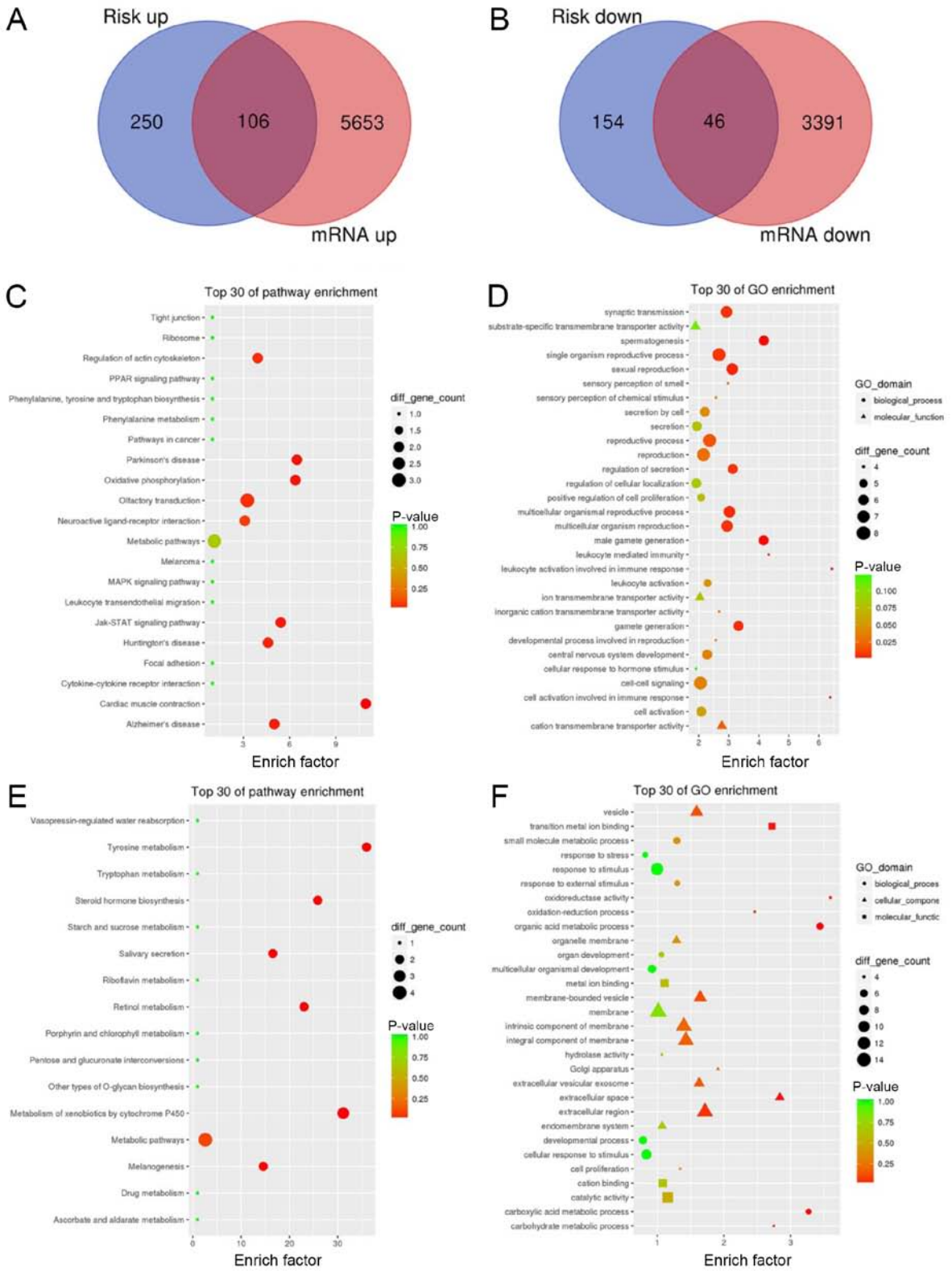

Figure 5. Signaling pathways associated with HNC prognosis. (A) Venn diagram of the intersections of genes associated with a high-risk score and upregulated in HNSCC. (B) Venn diagram of the intersections of genes associated with a low-risk score and downregulated in HNSCC. (C) KEGG and (D) GO signaling pathways analyses of the intersections of genes associated with a high-risk score and upregulated in HNSCC. (E) KEGG and (F) GO signaling pathways analyses of the intersections of genes associated with a low-risk score and downregulated in HNSCC. KEGG and GO signaling pathways analyses were performed using online website (http://enrich.shbio.com) which performed using R clusterprofiler package from GO and KEGG databases. HNSCC, head and neck squamous cell carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

hsa-miR-99a is a member of the miR-99 family, and studies have shown that it can significantly inhibit the invasion and migration of lung cancer and oral cancer cells (24,25). However, the role of miR-99a in the invasion and migration of HNC cells is rarely reported in the literature (26) In addition, it has been reported that miR-99b in the miR-99 family may play

an important role in the initiation and progression of cervical cancer. The upregulated expression of miR-99b can inhibit proliferation and induce apoptosis of cervical cancer cells, and may serve as a therapeutic target in cervical cancer. Studies have also reported that hsa-miR-499a and hsa-miR-1911 are associated with cancer susceptibility and prognosis, as in glioma (27,28). Yang and Wang (29) found that hsa-miR-1911 plays a key role in the occurrence and development of glioma. Therefore, hsa-miR-99a, hsa-miR-499a and hsa-miR-1911 may all play important roles in the genesis and development of HNSCC, and an accurate clarification of their functions will be beneficial for HNC treatment.

In conclusion, the present study screened three microRNAs related to HNSCC prognosis by integrating genomic and transcriptomic information in TCGA, and provided insight for subsequent analysis and research. Moreover, it was found that age, gender, TNM stage, radiotherapy, HPV status and mutation number can affect the prognosis of patients with HNC by effectively analyzing and summarizing clinicopathological data through stratified and survival analyses. This study also had some limitations. In the screening process of the data model, there was a certain extent of false positive and false negative results, which need to be further verified by similar experimental data to provide a reliable basis for tumor research.

Overall, the present study revealed that hsa-miR-99a, hsa-miR-499a and hsa-miR-1911 were closely associated with HNC prognosis, and their combined risk equation may be more effective in predicting HNC prognosis. The specific roles and biological functions of the aforementioned miRNAs in the initiation and progression of HNC require further investigation.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in TCGA database (http://cancergenome.nih.gov).

Authors' contributions

MZ and CD designed the research. XW, ZY and YZ carried out the research and drafted the manuscript. MH analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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