Expression Profile and Prognostic Values of HOXA Family Members in Laryngeal Squamous Cell Cancer

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The homeobox A cluster (HOXA) gene family, comprising 11 members, is involved in a wide spectrum of biological functions in human cancers. However, there is little research on the expression profile and prognostic values of HOXA genes in laryngeal squamous cell cancer (LSCC). Based on updated public resources and integrative bioinformatics analysis, we assessed the expression profile and prognostic values of the HOXA family members. Expression and methylation data on HOXA family members were obtained from The Cancer Genome Atlas (TCGA). The prognostic values of HOXA members and clinical features were identified. A gene set enrichment analysis (GSEA) was conducted to explore the mechanism underlying the involvement of HOXA members in LSCC. The associations between tumor immune infiltrating cells (TIICs) and the HOXA family members were evaluated using the Tumor Immune Estimation Resource (TIMER) database. HOXA2 and HOXA4 were downregulated and HOXA7 and HOXA9–13 were upregulated in LSCC. Upregulation of HOXA10, HOXA11, and HOXA13, along with two clinical characteristics (M stage and gender), were associated with a poor LSCC prognosis based on the results of univariate and multivariate Cox proportional hazards regression analyses. Although there were no significant correlations between TIICs and HOXA members, the GSEA results indicated that HOXA members participate in multiple biological processes underlying tumorigenesis. This study comprehensively analyzed the HOXA members, providing insights for further investigation of the HOXA family members as potential targets in LSCC.

Keywords: HOXA family, TCGA, prognosis, GSEA, LSCC

INTRODUCTION

Laryngeal cancer is one of the most common malignancies in the head and neck region, and laryngeal squamous cell cancer (LSCC) accounts for more than 95% of cases (1). Despite progress regarding comprehensive therapeutic strategies to treat LSCC, the prognosis of LSCC remains unsatisfactory, as 30–40% of patients die within 5 years of diagnosis with advanced LSCC (2). Identification of reliable biomarkers for LSCC prognosis could facilitate individualized treatment.

The HOX gene family is one of the families of homeobox genes that function as developmental regulatory genes (3). In mammals, there are 39 HOX genes in four gene clusters named HOXA, HOXB, HOXC, and HOXD (4). The HOXA cluster comprises 11 genes (including HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXA6, HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13), which...
encode proteins that contain the DNA-binding homeobox motif (5). The molecular functions of the HOXA family cover a wide spectrum of biological processes, including differentiation, proliferation, migration and cell death. A substantial body of scientific evidence indicates that the expression of particular HOXA genes is dysregulated in certain types of carcinomas, which contributes to carcinogenesis (6–10). For instance, HOXA1 mRNA and protein expression is upregulated in breast cancer, and forced expression of HOXA1 in human breast cancer cells resulted in increased cell proliferation and doxorubicin resistance (11, 12). Aberrantly expressed HOXA6 and HOXA13 were also observed in breast cancer (13). In colorectal cancer, HOXA13 was expressed more in normal colons than in malignant colons, and it was more highly expressed on the left side of the normal colon compared to the right side, indicating that differential HOXA gene expression occurs in an organized manner (10). Additionally, several studies have reported that HOXA9 and HOXA10 can serve as predictive biomarkers of poor survival in glioblastoma multiforme (GBM) (14–16).

Collectively, the differential expression and prognostic values of the HOXA family members have been noticed in various types of cancers. Studying the differential expression of HOXA genes in LSCC provides an opportunity to advance our understanding of LSCC development and to develop new therapeutic agents. In this study, based on updated public resources and integrative bioinformatics analysis, the expression profile and prognostic values of the HOXA family members were comprehensively assessed.

MATERIALS AND METHODS

The Cancer Genome Atlas (TCGA) mRNA Expression Data of the HOXA Family

The TCGA program was conducted by the National Cancer Institute and National Human Genome Research Institute to molecularly characterize over 20,000 primary cancer samples and matched normal samples spanning 33 cancer types, including 528 cases of primary head and neck squamous carcinoma (HNSC), two cases of metastatic HNSC and 74 adjacent normal control samples. A total of 111 cases of laryngeal squamous cell cancer (LSCC) and 12 normal controls were included in the current study, after matching clinical parameters (including gender, age, smoking history, alcohol consumption, tumor (T) stage, node (N) stage, metastasis (M) stage, clinical stage and primary cancer sites). Subsequently, we used the Genomic Data Commons (GDC) Data Transfer Tool recommended by TCGA to download high-throughput sequencing (HTSeq) Fragments Per Kilobase of transcript per Million mapped reads (FPKM) data on the HOXA family.

Comparison of the mRNA Expression of the HOXA Family in LSCC and Normal Tissues

Using Perl 5.26 software, the mRNA expression levels of the HOXA family were obtained from the HTSeq level 3 data on genome mRNA expression. The differential expression of the HOXA family in LSCC tissues compared to normal tissues was analyzed utilizing the limma package in R 3.6.0 software. The results were visualized using the pheatmap package.

Correlation Between mRNA Expression and Methylation of the HOXA Family in LSCC

We used the GDC Data Transfer Tool recommended by TCGA to download data from Illumina HumanMethylation 450K on the methylation levels of cg sites in the gene promoter regions of differentially expressed HOXA members in LSCC tissues. Thereafter, we utilized the corrplot package to further explore the correlation between methylation and HOXA expression in LSCC. The information on cg sites from Illumina HumanMethylation 450K were annotated using the annotation file from the official Illumina website (https://support.illumina.com/downloads/~infinium_humannmethylation450_product_files.html).

Survival Analysis of HOXA Members in LSCC

The prognostic values of the HOXA members were investigated using the following two steps: (1) the associations between HOXA members, as well as each clinical parameter, and overall survival among LSCC patients were assessed using univariate Cox proportional hazards regression analyses and (2) using multivariate Cox proportional hazards regression analysis, the independent prognostic values of the HOXA members were then obtained by controlling for the significant clinical parameters from step 1. All the analyses were performed using the survival package in R 3.6.0 software.

Associations Between Tumor Immune Infiltrating Cells (TIICs) and the HOXA Family Using the Tumor Immune Estimation Resource (TIMER) Database

Tumor cells and TIICs interact through multiple genes and pathways during cancer progression. To explore the correlations between TIICs and HOXA members, we utilized the TIMER platform (https://cistrome.shinyapps.io/timer/), which is an online tool for assessing the specific gene(s) associated with TIICs (17). In TIMER, the TIICs include B-cells, CD4+ T-cells, CD8+ T-cells, dendritic cells, macrophages and neutrophils.

Gene Set Enrichment Analysis (GSEA)

To evaluate the potential mechanism underlying the involvement of HOXA members in the carcinogenesis of LSCC, we performed GSEA (version 4.0.1; http://software.broadinstitute.org/gsea/index.jsp) to identify the to identify the pathways related to the differential HOXA expression in the TCGA LSCC tissues (18). The annotated gene set file c2.cp.kegg.v7.0.symbols.gmt (from the Msig database) was used as the reference. GSEA was performed using a
random combination number of 1,000 permutations and a false discovery rate (FDR) <0.05 to identify the significantly enriched pathways.

**Statistical Analysis**
The HTSeq FPKM mRNA data from the TCGA database was handled using Perl 5.26 software. The *limma* package was applied to analyze the expression of HOXA members in LSCC tissues, the *corrplot* package was used for the correlation between methylation and expression of HOXA members, the *survival* package was used for the analysis of prognostic values, the *ggplot2* package was used to plot forest plots related to the multivariate Cox proportional hazards regression analysis.

**RESULTS**

**Expression Status of HOXA Members in LSCC Tissues**
First of all, the mRNA expression data on HOXA members (HOXA1–13) from 111 LSCC samples and 12 normal control samples, which originated from TCGA, were obtained using Perl software. Pearson’s correlation of HOXA family genes were calculated and used to assess whether these genes were correlated with each other using the *corrplot* package. As shown in Figure 1, the HOXA family genes were correlated to a significant degree.

Thereafter, the differentially expressed HOXA members were analyzed using the *limma* package and visualized

![Figure 1](https://www.frontiersin.org/article/10.3389/fonc.2020.00368)

**FIGURE 1** Associations between HOXA family members.
FIGURE 2 | Expression profile of HOXA members in LSCC represented by a heatmap (A), and histograms (B).
Li et al. Differentially Expressed HOXA Genes in LSCC using the **pheatmap** package, as shown in **Figure 2A**. As shown in **Figure 2B**, HOXA2 and HOXA4 were significantly downregulated in LSCC tissues compared to control tissues, while HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13 were significantly upregulated in LSCC tissues. There were no significant differences in HOXA1, HOXA3, HOXA5, and HOXA6 expression between LSCC and control tissues.

**Correlation of HOXA Expression and Methylation in LSCC**

Methylation of gene promoter regions is one of the most common mechanisms that influences gene expression during the progression of human cancer. We identified seven differentially expressed HOXA members in LSCC (downregulated HOXA2 and HOXA4 and upregulated HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13). The Pearson’s correlation results showed that six of seven differentially expressed HOXA members (including HOXA4, HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13) was negative associated with methylation level (**Figure S1**), and only five of the 32 assessed CG sites in the promoter region of HOXA2 exhibited negative correlation with HOXA2 expression in LSCC (**Figure 3**). These results indicated the inverse correlation between expression and methylation level of HOXA members in LSCC.

**FIGURE 3 |** Pearson’s correlation between methylation levels and expression of HOXA2.
Prognostic Values of HOXA Members in LSCC

Subsequently, the prognostic values of HOXA members were analyzed. First, the predictive capabilities of differentially expressed HOXA members (HOXA2, HOXA4, HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13) and clinical features were assessed by univariate Cox proportional hazards regression analyses. The results showed that the expression of three HOXA members (HOXA10, HOXA11, and HOXA13) and two clinical features (M stage and male) were associated with poor outcome of LSCC patients (hazard ratio [HR] for HOXA10: 1.379 (1.081–1.759); HR for HOXA11: 1.179 (1.000–1.391); HR for HOXA13: 1.129 (0.999–1.277); HR for M stage: 8.225 (1.901–35.594); and HR for male: 3.367 [1.708–6.639]) (Table 1). Second, the independent prognostic values of HOXA10, HOXA11, and HOXA13 were assessed using multivariate Cox proportional hazards regression analysis to control for the prognostic effects of the clinical features. The results showed that the expression of HOXA10, HOXA11, and HOXA13 and two clinical parameters (M stage and gender) were independent prognostic biomarkers of LSCC outcome. The results of the multivariate Cox proportional hazards regression analysis are exhibited in forest plots in Figure 4.

Correlations Between TIICs and HOXA Members

Considering the increasing evidence on the associations between immunological features and prognosis in cancer, we further

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**TABLE 1** | Univariate Cox proportional hazards regression analyses of HOXA members and clinical features in LSCC.

| Parameter          | Univariate analysis |              |              |              |              |              |
|--------------------|---------------------|--------------|--------------|--------------|--------------|--------------|
|                    | Hazard ratio        | 95% CI       | P            |              |              |              |
| Age                | 1.004               | 0.969–1.041  | 0.811        |              |              |              |
| Smoking history    | 0.659               | 0.366–1.185  | 0.164        |              |              |              |
| Alcohol consumption| 0.688               | 0.377–1.1827 | 0.166        |              |              |              |
| M stage            | 8.225               | 1.901–35.594 | 0.005        |              |              |              |
| N stage            | 1.305               | 0.744–2.289  | 0.354        |              |              |              |
| T stage            | 0.702               | 0.348–1.4145 | 0.322        |              |              |              |
| Stage              | 0.894               | 0.379–2.108  | 0.797        |              |              |              |
| Gender             | 3.367               | 1.708–6.639  | 4.564E–04    |              |              |              |
| Grade              | 0.886               | 0.581–1.351  | 0.572        |              |              |              |
| HOXA1 expression   | 1.384               | 1.042–1.837  | 0.025        |              |              |              |
| HOXA2 expression   | 1.059               | 0.828–1.356  | 0.646        |              |              |              |
| HOXA3 expression   | 1.238               | 0.932–1.647  | 0.140        |              |              |              |
| HOXA4 expression   | 1.174               | 0.857–1.608  | 0.317        |              |              |              |
| HOXA5 expression   | 1.143               | 0.885–1.477  | 0.304        |              |              |              |
| HOXA6 expression   | 1.105               | 0.915–1.334  | 0.299        |              |              |              |
| HOXA7 expression   | 1.149               | 0.93–1.419   | 0.198        |              |              |              |
| HOXA9 expression   | 1.115               | 0.993–1.252  | 0.065        |              |              |              |
| HOXA10 expression  | 1.379               | 1.081–1.759  | 0.0097       |              |              |              |
| HOXA11 expression  | 1.179               | 1.000–1.391  | 0.0498       |              |              |              |
| HOXA13 expression  | 1.129               | 0.999–1.277  | 0.051        |              |              |              |

Bold means P < 0.05.

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**FIGURE 4** | Forest plots of the results of multivariate Cox regression analyses of significant prognostic factors: HOXA10 (A), HOXA11 (B), and HOXA13 (C). *stands for P < 0.05; **stands for P < 0.01.
explored the correlations between TlICs and HOXA members. The TIMER database is a public resource used to explore the associations between certain gene products and immune cells around tumor cells. The first column in Figure 5 shows scatterplots of the expression of HOXA members against tumor purity. HOXA members with high expression in the microenvironment cells are expected to have a negative association with tumor purity, while HOXA members with high expression in tumor cells are expected to have a positive association with tumor purity (17). In accordance with our aforementioned findings, HOXA7, HOXA10, and HOXA13 were highly expressed in LSCC tissues, with positive associations with tumor purity (Figure 5). However, there were no significant correlations between TlICs and HOXA members (Figure S2).

Potential Mechanism Underlying the Effects of Prognostic HOXA Members on LSCC Carcinogenesis

A GSEA of differentially expressed HOXA members with statistical prognostic value was conducted to evaluate the potential biological mechanism by which differential expression of HOXA10, HOXA11, and HOXA13 affects the carcinogenesis of LSCC. The GSEA indicated that high expression of HOXA10 was related to “WNT signaling pathway,” “pathway in cancer,” “basal cell carcinoma,” “cell cycle,” “mismatch repair,” and “DNA replication” (Figure 6A), high expression of HOXA11 was related to “DNA replication,” “mismatch repair,” and “nucleotide excision repair” (Figure 6B) and high expression of HOXA13 was related to “colorectal cancer” and “WNT signaling pathway” (Figure 6C).

DISCUSSION

Homeobox genes were first identified in the fruit fly Drosophila (19). A total of 39 Hox genes are located on various chromosomes, which are clustered into four clusters, namely HOXA, HOXB, HOXC and HOXD (4). The genes in these four cluster each encode a 61-amino acid homeodomain, and these genes are key components of master regulatory pathways during normal embryonic development (3). A typical characteristic of the homeodomain is its DNA-binding nature; the proteins function as transcription factors by binding to the promoters of various target genes (20). Increasing evidence has shown that the protein products of HOXA genes not only act as transcriptional factors promoting carcinogenesis but also serve as tumor-suppressor factors, based on their aberrant expression patterns in certain organs. Increasing published or public genomic data and multiple online platforms provide us the opportunity for exploring the expression profiles of families of genes in human cancers and their clinical practice value. This study demonstrated the distinct expression profile and methylation profile, prognostic values and biological processes related to HOXA members in LSCC.

Previous research has shown that, according to expression data, HOXA genes contribute to the development of human cancers. Reverse transcriptase-polymerase chain reaction (RT-PCR) showed that HOXA7 and HOXA9 mRNAs were significantly overexpressed in esophageal squamous cell carcinoma tissues compared to non-cancerous surrounding tissues (21), while HOXA9 was epigenetically downregulated in lung cancer (22). HOXA13 expression increased in breast cancer (13), whereas it was downregulated in colorectal cancer (10). However, the expression of the entire HOXA family in LSCC was not previously comprehensively investigated. This in silico study demonstrated the expression profile of HOXA members in LSCC and showed that HOXA2 and HOXA4 were downregulated in LSCC tissues compared to normal control tissues. In contrast, HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13 were upregulated in LSCC tissues compared to normal control tissues. Unfortunately, no significant differences in the mRNA expression of HOXA1, HOXA3, HOXA5, and HOXA6 were identified in LSCC tissues compared to normal control tissues.

According to the Pearson’s correlation between HOXA mRNA expression and the methylation level of cg sites in the promoter regions in LSCC, among the seven differentially expressed HOXA members (HOXA2, HOXA4, HOXA7, HOXA9, HOXA10, HOXA11, and HOXA13), most expression levels, particularly regarding HOXA4 and HOXA9, are affected by the methylation level. These results are in accordance with previous findings showing a negative correlation between HOXA4 methylation and expression in patients with acute myeloid leukemia (23).

Several reports have identified HOXA gene signatures in GBM, and high expression of HOXA9 and HOXA10 were reported to be predictors of poor outcome in patients with GBM (14, 15). Moreover, it was reported that novel methylation markers in HOXA9 also served as an independent indicator of prognosis in invasive bladder cancer (24). Additionally, multiple highly expressed HOXA members were reported to be significantly correlated with poor overall survival in patients with acute myeloid leukemia (25). In this study, univariate Cox proportional hazards regression analyses were performed to analyze the prognostic values of HOXA members in LSCC. In fact, four HOXA members were significantly associated with poor clinical outcomes in LSCC (HOXA1, HOXA10, HOXA11, and HOXA13). Thus, although no significant differential expression of HOXA1 was found in LSCC, the univariate Cox proportional hazards regression showed that HOXA1 expression was significantly associated with prognosis. The predictive potential of HOXA has also been reported in breast cancer (12). In breast cancer, HOXA1 knockdown inhibited cell proliferation and increased apoptosis and cell cycle arrest by influencing the aberrant expression of several cell cycle and apoptosis-associated proteins, comprising cyclin D1, B-cell lymphoma 2 (Bcl-2) and Bcl-2-like protein 4 (12). Thus, although HOXA1 was not differentially expressed in LSCC, the prognostic value of HOXA1 has been highlighted in various human cancers, including in LSCC. Exploration of the HOXA1-related mechanisms is still required.

In hepatocellular carcinoma cells, HOXA10 knockdown induced cell cycle arrest at the G0/G1 phase and apoptosis by reducing the expression of Cyclin D1 and Survivin (26). Decreased expression of HOXA10 accelerated the acetylation
FIGURE 5 | Correlations between tumor infiltrating immune cells (TIICs; B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) and HOXA members (including HOXA7, HOXA10, and HOXA13) in LSCC. Tumor purity is shown in the panels on the left.

FIGURE 6 | Cancer-related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with HOXA10 (A), HOXA11 (B), and HOXA13 (C) based on a gene set enrichment analysis (GSEA).

of p53 (Lys382) and suppressed the transcription of histone deacetylase 1 (HDAC1; a potential deacetylase for p53) to activate p53 transcription (26). Additionally, HOXA10 might promote cell proliferation by elevating Bcl-2 expression and inhibiting apoptosis in gastric cancer, and high expression of HOXA10 predicted poor overall survival in gastric cancer patients (27). In this study, we found high expression of HOXA10 in LSCC tissues. Both univariate and multivariate Cox proportional hazards regression analyses affirmed the prognostic value of HOXA10 in the prediction of poor outcome in LSCC patients.

Overexpression of HOXA11 has been observed in ovarian cancer (28), bladder cancer (29), renal cell carcinoma (29) and lung cancer (30), while downregulation of HOXA11 has been observed in gastric cancer (31) and glioblastoma (32). In glioblastoma, overexpression of HOXA11 confers a tumor suppressive effect, reduces treatment resistance and contributes to a favorable prognosis (32). However, overexpression of HOXA11 showed a poor association with overall survival in lung cancer (33). HOXA11 was significantly downregulated in cisplatin-resistant lung adenocarcinoma cell lines compared with parent cell lines, and in vitro experiments showed that overexpression of HOXA11 increased cisplatin sensitivity by inhibiting Akt/β-catenin signaling (34). Our results showed high expression of HOXA11 in LSCC, which was associated with unfavorable outcomes in LSCC patients. However, given that there is little relevant research on the topic, the biological and prognostic values of HOXA11 warrant further intensive investigation. It may be useful to systematically explore the prognostic value of HOXA11 using meta-analysis.

HOXA13 is expressed more in normal colons than in malignant colons. Additionally, HOXA13 was differentially expressed based on location, with higher expression on the left side of the normal colon compared to the right side (10). Differential expression of HOXA13 was also reported in breast
This in silico study demonstrated the expression profile of HOXA family members in LSCC and the biological and prognostic values of the HOXA family in LSCC, providing insights for further investigation of HOXA members as potential targets in LSCC.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are openly available in The Cancer Genome Atlas (TCGA) program at https://portal.gdc.cancer.gov/.

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**AUTHOR CONTRIBUTIONS**

JL and CZ designed the research study and analyzed the data from public database. JL, MY, and CZ were involved in data analysis. CZ was responsible for writing of manuscript. JL and MY contributed to the revised manuscript. All authors reviewed the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/onc.2020.00368/full#supplementary-material

Figure S1 | Pearson’s correlation between methylation levels and expression of all differentially expressed HOXA members in LSCC: (A) HOXA2, (B) HOXA4, (C) HOXA7, (D) HOXA9, (E) HOXA10, (F) HOXA11, and (G) HOXA13.

Figure S2 | Correlations between tumor infiltrating immune cells (TIICs; B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) and all HOXA members in LSCC.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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