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Expression Profile of *PBRM1*, *PLAU* and *CLEC3B* Genes in Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is a frequent type of cancer in human which is capable of metastasis and is the second most common reason of skin cancer related death. Various molecular mechanisms have a role in the progress of HNSCC. This study investigates expression levels of *PBRM1*, *PLAU*, and *CLEC3B* genes in HNSCC compared to normal tissue to identify their potential use as molecular biomarkers for HNSCC diagnosis; and uses bioinformatics analysis to detect the functional association of the target genes. HNSCC skin and matched normal tissue were obtained from patients underwent surgical removal treatment. RNA extraction, cDNA synthesis, and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed to detect the relative expression levels of *PBRM1*, *PLAU*, and *CLEC3B* genes. The potential use of *PBRM1*, *PLAU*, and *CLEC3B* expression as diagnostic biomarkers was studied using Receiver Operator Characteristic (ROC) and Area Under the Curve (AUC). GeneMANIA software was used to analyze the functionally relevant genes. RT-qPCR showed that expression of *PLAU* and *PBRM1* genes were significantly up-regulated by 2.4 and 1.8 fold, respectively, in the HNSCC tissues compared to normal control tissues. In comparison, the level of *CLEC3B* expression was decreased by z-1.4-fold in the HNSCC versus the control tissues. The diagnostic performance criteria of *PBRM1*, *PLAU*, and *CLEC3B* assessed with the ROC curve and AUC analysis demonstrated that they could be used as potential molecular biomarkers for the HNSCC diagnosis. The network of interaction between the genes generated by GeneMANIA showed that our target genes had multiple interactions with several other genes and the most significant interactions were physical interactions and co-expression. The outcomes of this study suggest that *PBRM1*, *PLAU*, and *CLEC3B* genes play roles in the development of HNSCC, and they can be used as potential molecular biomarkers for the diagnosis of HNSCC.

Keywords: Biomarkers, Head and neck squamous cell carcinoma, Gene expression, CLEC3B, PBRM1, PLAU.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the second most prevalent cancer in human globally[1-3]. The malignant development of epidermal squamous cells causes HNSCC[4]. HNSCC displays a benign clinical course but is linked with a high risk of metastasis[5].

The exact causes of HNSCC are unknown, but its development is generally connected with tobacco consumption, excessive alcohol use, and infection with human papillomavirus (HPV)[6]. At the molecular level, several signaling pathways and their component genes have been connected to the development of HNSCC, especially mutations in the *CDKN2A* gene[7, 8]. Alterations in the level of *NOTCH1* expression and the *RAS* oncogene were also reported in HNSCC[9, 10]. Moreover, stimulation of PI3K/AKT/mTOR, MAPK, and NF-kB pathways and over-expression of EGFR were also detected in HNSCC tissues[11, 12]. The *ITGA3*, *SMAD4* and *SIRT7* genes have been demonstrated to have a role in the development of HNSCC[13-16].

PBRM1 was implicated as a tumor suppressor gene in many other cancer types[17]. *PBRM1* is listed among the genes in which their mutations were detected in cutaneous squamous cell carcinoma (cSCC)[18]; however, its relative expression in HNSCC compared to normal tissue has not been investigated yet. The level of plasminogen activator urokinase (*PLAU*) gene expression has been linked to tumor cell metastasis in mice[19]. Some studies showed that *PLAU* gene was involved in the initiation of different tumor types including colorectal, breast, and esophageal[20, 21]. *CLEC3B* gene was also reported to play a role in the hepatocellular carcinoma, lung, ovarian, breast, lymph node, oral, and colon cancers[22-24]. Altered expression levels of *PLAU* and *CLEC3B* genes were detected in HNSCC tissues[25]. The mechanism of *PLAU* and *CLEC3B* actions in the development of HNSCC remains uncertain and requires further investigation.

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The high mutation rate in the signaling pathway component genes implicated in HNSCC development makes medical treatment problematic[26, 27]. Radiation therapy, cryosurgery, and topical medicines like Imiquimod and 5-FU are among the nonsurgical treatment options for HNSCC[28, 29]. Surgical removal is currently the most effective treatment for HNSCC.

The high incidence rate of HNSCC and absence of symptoms in the initial stages of its development calls for attention on discovering a better diagnostic and therapeutic approach. Therefore, investigating potential biomarkers and networks of functionally related genes may contribute in further uncovering the molecular basis of HNSCC and may lead to developing new strategies for diagnosing and treating this common malignancy. To achieve this, this research was planned to investigate the levels of *PBRM1*, *PLAU*, and *CLEC3B* gene expression in HNSCC versus normal control and assess their potential use as molecular biomarkers for HNSCC diagnosis; and also create a network of functionally associated genes.

2. Materials and methods

2.1 Samples

HPV-negative HNSCC tissue samples of about 0.5-1 cm^2, with pre-operation pathological confirmation, and adjacent non-tumoral tissue from the tumor margin were collected from 18 local patients who had a surgical operation between February and May 2022, after obtaining appropriate informed consent. This study was approved by the Human Ethics Committee of the College of Science, University of Sulaimani. The samples were placed in RNA*later* stabilizing solution (Sigma-Aldrich, Gillingham, UK) immediately to prevent RNA degradation, kept at 4°C for 24 hours, and then stored at -18°C till utilized.

2.2 Extraction of RNA and cDNA synthesis

Homogenization of the collected HNSCC and adjacent non-tumoral tissue (50 mg) was performed using the Qiagen Tissue Lyser system. Extraction of RNA was carried out using RNeasy Plus Universal Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Gel electrophoresis was used to check the quality and integrity of the whole RNA samples through the appearance of two clear bands. RNA concentration was measured by spectrophotometric NanoDrop instrument (Thermo Fisher Scientific Inc., Massachusetts, USA), and samples of RNA with an A260/280 ratio bigger than 1.8 and an A260/230 near 2.0 were selected for the gene expression investigation. The mRNA samples were treated with DNase I (Invitrogen, Paisley, UK) to remove possible genomic DNA, before complementary DNA (cDNA) been synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Crawley, UK). Reverse-transcription of 600 ng of mRNA from each sample was carried out in a final volume of 50 µl following the manufacturer's instructions, and the generated cDNA was used as a template for RT-qPCR analysis.

2.3 RT-qPCR primers

PBRM1, *PLAU*, and *CLEC3B* primer sets for RT-qPCR were designed newly from the human gene sequence. Ensembl genome browser (http://asia.ensembl.org/index.html) was used to find the specific genomic sequence for each gene, and the exon nucleotide data, from two successive exons crossing the intron, was used for the primer design; *PBRM1* (Transcript ID: ENST00000394830.7); *PLAU* (Transcript ID: ENST00000372764.4); *CLEC3B* (Transcript ID: ENST0000042-8034.1). The primers were designed using the NCBI Primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The designed primers were synthesized by Qiagen (Crawley, UK).

2.4 RT-qPCR analysis

RT-qPCR was performed to determine the relative expression levels of *PBRM1*, *PLAU*, and *CLEC3B* genes in HNSCC and normal tissue using the QuantiTect SYBR Green PCR kit (Qiagen, Crawley, UK; Cat. No. 204143) and real-time cycler instrument (Applied Biosystems Fast 7500; CA, USA). All reactions were carried out in triplicate in 96-well plates. Each reaction mix was prepared in a 25 µl reaction volume containing 0.3 µM primers (*GAPDH* forward: 5'-GAAGGTGAAGGTCGGAGTCA-3', reverse: 5'-AATGAAGGGGTCATTGA-TGG-3'; *PBRM1* forward: 5'-ACCTGGAGGGAGATGAGGAC-3' and reverse: 5'-GTTGG-GATCCACAGCAGGAA-3'; *PLAU* forward: 5'-CCGCTTTCTTGCTGGTTGTC -3' and reverse: 5'-AGGCCTATGCCTGAGGGTAA-3'; *CLEC3B* forward: 5'-TTTGAGGAGC-TCAAGAGCCG -3' and reverse: 5'-GGTCTTCGTCTGGGTGAAGG -3'; 12.5 µl of 2x QuantiTect SYBR Green Master Mix (1x final concentration); cDNA (<500 ng/reaction; cDNA solutions were diluted 5-fold before RT-qPCR). cDNA was substituted with nuclease-free water for the negative controls. The PCR cycling conditions were as follow: one initial activation cycle at 95°C for 15min, followed by 40 cycles of denaturation at 94°C for 15sec, annealing (*PBRM1* 60°C; *PLAU* 58°C; *CLEC3B* 59°C) for 30sec, and extension 72°C for 30sec. After each PCR cycle, fluorescence data were collected to generate an amplification plot and then determine the Ct value. The melting curves for each amplification product were assessed by determining the decline in fluorescence from 95°C to 60°C. The reference gene, *GAPDH*, was used for normalization of the expression levels of the target genes. The delta-delta-CT (ΔΔCT) method was utilized to determine the transcript levels of each gene[30].

2.5 Analysis of diagnostic performance of PBRM1, PLAU, and CLEC3B mRNA expression in HNSCC

To study the potential diagnostic values of *PBRM1*, *PLAU*, and *CLEC3B* mRNA expression in HNSCC, ROC analysis was carried out. A ROC curve was developed for each gene by plotting sensitivity versus (1-specificity), and the optimal diagnostic cut-off point was determined. The Hanley and McNeil method was used to analyze the area under the curve (AUC), and an AUC value close to 1 was regarded as a potent diagnostic test[31].

2.6 Gene interaction analysis

GeneMANIA (http://www.genemania.org) server was used to predict gene interactions and to analyze co-expression, co-

localization, physical interactions, shared pathways, and protein domain of the target genes with the rest of the human genome.

The top 20 genes closely related to each of the *PBRM1*, *PLAU*, and *CLEC3B* for co-coexpression, co-localization, physical interactions, shared pathways, and protein domain was visualized and correlated using GeneMANIA. The GeneMANIA serves as a biological network integrator for predicting gene interactions and functional association[32, 33].

2.7 Statistical analysis

GraphPad Prism version 8 (Graphpad, California, USA) was utilized to analyze data of the experimental results expressed as mean ± standard error of the mean (SEM). Groups were compared by grouped t-test. Relative quantity (RQ) was used to represent the relative mRNA expression level of the target genes measured by RT-qPCR. The RQ represents the level of expression of *PBRM1*, *PLAU*, and *CLEC3B* genes relative to a reference gene *GAPDH*. ROC curves were constructed for all the target genes to determine their potential diagnostic values by plotting the true-positive rate (sensitivity) versus true negative rate (1-specificity), and the optimal diagnostic cut-off point was determined via the Youden's J Index. The analysis of AUC was carried out using Hanley and McNeil method. A *p*-value less than 0.05 was considered as statistically significant[34].

3. Results

3.1 Relative expression of *PBRM1*, *PLAU* and *CLEC3B* genes

RT-qPCR analysis revealed significant statistical differences in the relative expression of *PBRM1*, *PLAU*, and *CLEC3B* genes between the HNSCC tissue group and matched normal tissue from the tumor-free margin. The relative expression of *PBRM1* and *PLAU* genes were significantly up-regulated in the HNSCC tissue compared to the normal tissue from the tumor-free margin ($p < 0.05$; Figure 1). The relative expression of *CLEC3B* gene was significantly down-regulated in the HNSCC tissue compared to control group ($p < 0.05$; Figure 1).

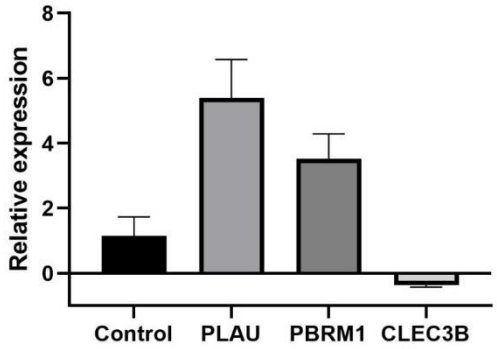

Figure 1: Mean relative mRNA expression of *PBRM1*, *PLAU* and *CLEC3B*. The relative mRNA expression levels of *PBRM1*, *PLAU*, and CLEC3B in the HNSCC and matched normal control skin tissue from the tumor-free margin were determined with RT-qPCR. Error bars represent SEM.

3.2 Fold change of gene expression (log2) of PBRM1, PLAU and CLEC3B

The RT-qPCR analysis showed that the relative fold change of gene expression (log_2) of the *PBRM1* and *PLAU* genes were up-regulated in HNSCC tissue versus the normal tissue from the tumor-free margin (*PBRM1* by 1.8 fold; *PLAU* by 2.4 fold; Figure 2)($p < 0.05$). The relative fold change of gene expression (log_2) of the *CLEC3B* gene was down-regulated in the HNSCC tissue compared to the control group by -1.4 fold (Figure 2)($p < 0.05$).

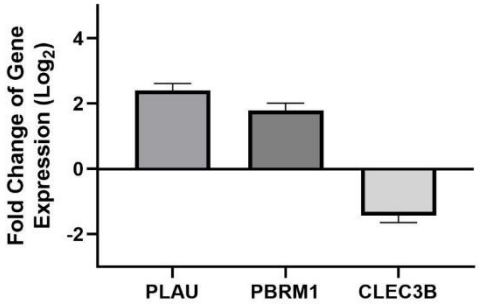

Figure 2: Fold change of gene expression (log_2) of *PBRM1*, *PLAU*, and *CLEC3B*. Data represent the mean ± SEM of the fold change in gene expression. Error bars represent SEM.

3.3 Diagnostic performance criteria of PBRM1, PLAU, and CLEC3B mRNA expression in HNSCC

The diagnostic performance criteria of *PBRM1*, *PLAU*, and *CLEC3B* were investigated using the ROC and AUC analysis to determine their potential use as molecular biomarkers for the diagnosis of HNSCC. The ROC and AUC illustrated that *PBRM1*, *PLAU*, and *CLEC3B* mRNA expression efficiently discriminate HNSCC from normal tissue (*PBRM1* AUC=0.92; *PLAU* AUC=0.96; *CLEC3B* AUC=0.81; $p<0.001$) (Figure 3). The optimal diagnostic cut-off values for *PBRM1*=0.66 RQU, *PLAU*=0.78 RQU, *CLEC3B*=0.61 RQU. The sensitivity & specificity of *PBRM1* cut-off values were 93% and 72%; for *PLAU* were 94% and 83%; and for *CLEC3B* were 78% and 82%, respectively (Figure 3). The optimal diagnostic cut-off values and their sensitivity and specificity indicate that *PBRM1*, *PLAU*, and *CLEC3B* mRNA expression can be used as potential biomarkers for distinguishing HNSCC from normal tissue.

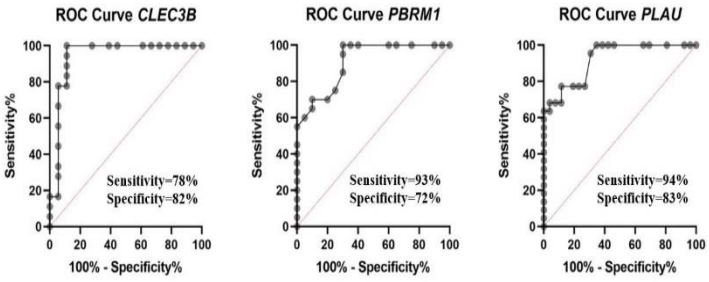

Figure 3: The ROC curve analysis of *PBRM1*, *PLAU*, and *CLEC3B* mRNA expression. The *PBRM1* AUC=0.92; *PLAU* AUC=0.96; *CLEC3B* AUC=0.81 ($p<0.001$). The optimal diagnostic cut-off values for *PBRM1*=0.66 RQU, *PLAU*=0.78 RQU, and *CLEC3B*=0.61 RQU. AUC: Area Under Curve; RQU: relative quantification unit.

3.4 Gene interaction analysis of PBRM1, PLAU, and CLEC3B

The gene interaction network analysis of the *PBRM1*, *PLAU*, and *CLEC3B* was carried out via GeneMANIA. The GeneMANIA results showed that the function of *PBRM1*, *PLAU*, and *CLEC3B* genes have physical interactions, co-expression, genetic interactions, shared pathways, and protein domains with several human genes.

PBRM1 has physical interactions with most of the genes displayed except *HDAC6*, *TOP1*, *KDMA4*, *GEMIN5*, *KDM4B*, and *TAF8*; and has genetic interactions with *HDAC6*, *TOP1*, *CHD7*, and *GEMIN5*. *PBRM1* shared protein domains with *SMARCA4* and *BRD7*. *ARID2*, *TCHHL1*, *SMARCC2*, *SMARCD1*, and *GTF3C5* were the five most significantly interacting genes with *PBRM1* (Figure 4).

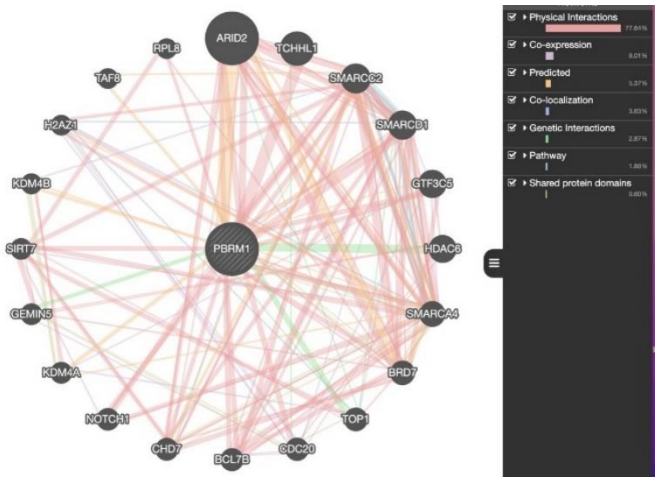

Figure 4: Network of gene interactions associated with *PBRM1* function. The network of functional analyses shows the PBRM1 gene's most close interactions with other genes in humans using GeneMANIA. The different line colors represent the different bioinformatics.

PLAU has multiple interactions with *SERPINB2*, *HRG*, *PLAUR*, *SERPINE1*, and *MRC2;* and the most significant interactions were physical interactions and co-expression. *PLAU* has physical interactions with most of the genes displayed except *HRAS*, *MAP2K1*, *PDGFD*, *MMP2*, *HGF*, *TRIP6*, and *ITGA2*; and shows co-expression with *HRG*, *PLAUR*, *SERPINE1*, *PLAT*, *MMP2*, *HGF*, and *ITGA2*. *PLAU* has genetic interactions with *HRAS* and *MAP2K1*; shared pathway with *SERPINB2*, *HRG*, *PLAUR*, *SERPINE1*, *PLG*, *PDGFD*, *MMP2*, *HGF*, and *TRIP6*; and shared protein domains with *PLG*, *PLAT*, *MMP2*, and *HGF* (Figure 5).

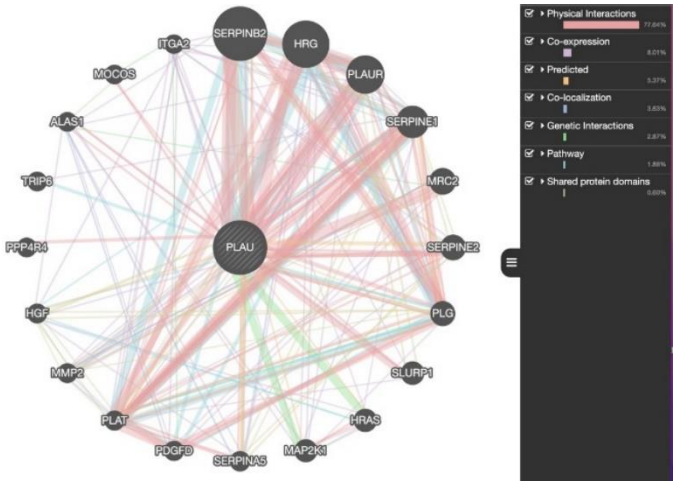

Figure 1: Network of gene interactions associated with *PLAU* function. The network of functional analyses shows the PLAU gene's most closely interactions with other genes in humans using GeneMANIA. The different line colors represent the different bioinformatics.

CLEC3B gene mainly interact with *PLG*, *HGF*, *PLAT*, *FGF13*, and *THRAP3*, and the most relevant interactions were physical interaction and co-expression. *CLEC3B* displays physical interactions with *PLG*, *HGF*, *PLAT*, *FGF13*, *THRAP3*, *OTUD4*, *KRT18*, and *RPS6KB1*. *CLEC3B* has co-expression with *MMRN1*, *ANGPTL1*, *TMEM204*, *GRK5*, *CXCL14*, *ROBO4*, *VIP*, *RNASE1*, *CLEC4M* and *CAV2*; genetic interactions with *DAPK2*, *PLG* and *THRAP3*; and shared protein domains with *CLEC4M* (Figure 6).

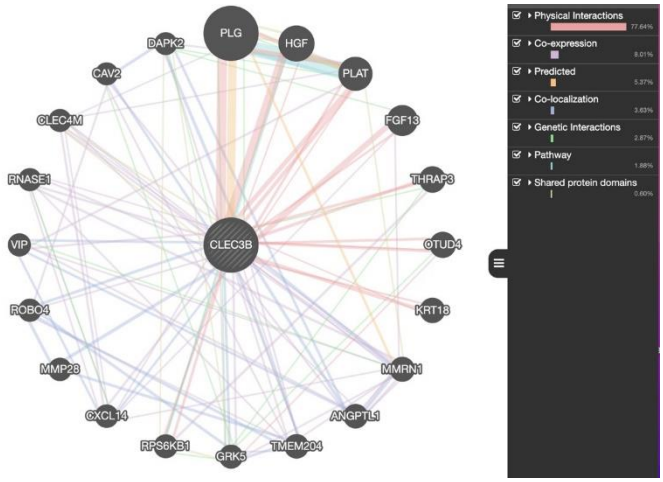

Figure 6: Network of gene interactions associated with *CLEC3B* function. The network of functional analyses shows the *CLEC3B* gene's most closely interactions with other genes in humans using GeneMANIA. The different line colors represent the different bioinformatics analysis methods.

4. Discussion

HNSCC is one of the most prevalent cancers in humans[35]. Patients with HNSCC frequently develop local and/or distant metastasis, which adds to the under diagnosis of the malignancy[36]. HNSCC is usually diagnosed at a very developed stages because of absent of symptoms in the early stage[37]. Therefore, discovering potential molecular biomarkers that can improve the detection of HNSCC through blood tests and solid tissue biopsies and further unveiling the genetics of HNSCC leading to the development of targeted molecular therapy, can improve the clinical outcome of this cancer. Here, we studied the relative expression of *PBRM1*, *PLAU*, and *CLEC3B* in HNSCC compared to normal tissue to identify their potential use as molecular biomarkers for diagnosing HNSCC; and used bioinformatics analysis to detect the functional association of the target genes.

RT-qPCR analysis revealed that the relative expression of *PBRM1*, *PLAU*, and *CLEC3B* were significantly different in the HNSCC tissue compared to the control group: *PBRM1* and *PLAU* were higher, while *CLEC3B* was lower in the HNSCC tissue compared to the control group ($p < 0.05$; Figure 1). The relative fold change of expression ($\log_2$) of the *PBRM1* and *PLAU* genes were up-regulated by 1.8 and 2.4 fold, respectively, in HNSCC tissue compared to the control group. In contrast, the relative fold change of expression ($\log_2$) of the *CLEC3B* gene was down-regulated by -1.4 fold in the HNSCC tissue compared to the control group (Figure 2). The ROC curve analysis revealed that

expression of *PBRM1*, *PLAU*, and *CLEC3B* mRNA can efficiently distinguish HNSCC from normal tissue (Figure 3). The optimal diagnostic cut-off values of *PBRM1*, *PLAU*, and *CLEC3B*, as well as their sensitivity and specificity, indicate that they can be utilized as potential molecular biomarkers for diagnosis of HNSCC.

To further unveil the genetics of HNSCC, GeneMANIA was used to detect the genes that are functionally linked with our target genes. GeneMANIA showed that the *PBRM1* gene mainly interacts with *ARID2*, *TCHHL1*, *SMARCC2*, *SMARCD1*, and *GTF3C5* genes and shares protein domains with *SMARCA4* and *BRD7* (Figure 4). *PLAU* had multiple interactions, particularly physical interactions and co-expression, with *SERPINB2*, *HRG*, *PLAUR*, *SERPINE1*, and *MRC2* (Figure 5). *CLEC3B* mainly interacted with *PLG*, *HGF*, *PLAT*, *FGF13*, and *THRAP3* genes (Figure 6).

Very little is known about the expression profile of *PBRM1* in HNSCC tissues versus healthy tissues. Mutation in *PBRM1* has been shown to be linked with tertiary lymphoid structure change in several tumor types[38]. A study of drug-gene associations in HNSCC revealed that the medication had increased the biological activity of the *PBRM1*[39]. In a research, 26 significantly mutated genes (SMGs) including *PBRM1* were detected in ESCC[40]. Prognostic investigations showed that elevated level of *PBRM1* was linked with better outcomes in HNSCC[41].

It has been shown that *PLAU* mRNA was increased significantly in HNSCC tumor tissues and could be used as an indicator for HNSCC prognosis[42]. *PLAU* has been shown to play a leading role in advancement of HNSCC[43]. A different study suggested that *PLAU* activate the epithelial to mesenchymal transition in HNSCC[44]. A study discovered that *PLAU* mRNA and protein levels were abnormally elevated in HNSCC[45].

Eight microarray studies with subsequent quantification by RT-qPCR of HNSCC and normal tissues (n=100) identified 3 overexpressed genes, including *PLAU*, and 3 under expressed genes containing *CLEC3B*[25]. Another study illustrated that *CLEC3B* was significantly down-regulated in lung cancer tissues versus control tissue[22]. It was suggested that *CLEC3B* gene can be used as a potential prognostic biomarker for human hepatocellular carcinoma[23]. *CLEC3B* mRNA, alongside C6 and CLCN1, has been shown to effectively forecast survival of patients with oral SCC[46]. Decreased expression level of *CLEC3B* was connected with bad prognosis[47]. Additionally, significant down-regulation of *CLEC3B* was recorded in metastatic OSCC, indicating that *CLEC3B* may suppress the advancement of OSCC[48]. Patients with high expression level of *CLEC3B* displayed higher survival benefit[49].

These studies support the findings of our current investigation, which show that *PBRM1*, *PLAU*, and *CLEC3B* genes are involved in the development of HNSCC and could be employed as diagnostic biomarkers.

Conclusion

Absent of symptoms in the early stages of HNSCC development and its high mortality rate call for a focus on alternative strategies such as early diagnostic molecular biomarkers and targeted molecular therapy. The findings of this study demonstrate that *PBRM1*, *PLAU*, and *CLEC3B* genes play roles in the development of HNSCC, and they can be used as potential molecular biomarkers for the diagnosis of HNSCC. Further research into the molecular mechanisms involved in developing HNSCC may lead to the development of better diagnostic and therapeutic strategies for this cancer.

Conflict of interests

None

Author contribution

Study design, investigation and methodology including RT-qPCR, biomarker and bioinformatics analysis, as well as original draft preparation and submission were carried out by the author.

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