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. The malignant development of epidermal squamous cells causes HNSCC. HNSCC displays a benign clinical course but is linked with a high risk of metastasis.

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). 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. Alterations in the level of NOTCH1 expression and the RAS oncogene were also reported in HNSCC. Moreover, stimulation of PI3K/AKT/mTOR, MAPK, and NF-kB pathways and over-expression of EGFR were also detected in HNSCC tissues.

PBRM1 was implicated as a tumor suppressor gene in many other cancer types. PBRM1 is listed among the genes in which their mutations were detected in cutaneous squamous cell carcinoma (cSCC); 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. Some studies showed that PLAU gene was involved in the initiation of different tumor types including colorectal, breast, and esophageal. CLEC3B gene was also reported to play a role in the hepatocellular carcinoma, lung, ovarian, breast, lymph node, oral, and colon cancers. Altered expression levels of PLAU and CLEC3B genes were detected in HNSCC tissues. The mechanism of PLAU and CLEC3B actions in the development of HNSCC remains uncertain and requires further investigation.
The high mutation rate in the signaling pathway component genes implicated in HNSCC development makes medical treatment problematic\cite{26, 27}. Radiation therapy, cryosurgery, and topical medicines like Imiquimod and 5-FU are among the nonsurgical treatment options for HNSCC\cite{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 \textit{PBRM1}, \textit{PLAU}, and \textit{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 RNAAlater 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

\textit{PBRM1}, \textit{PLAU}, and \textit{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; \textit{PBRM1} (Transcript ID: ENST0000394830.7); \textit{PLAU} (Transcript ID: ENST0000372664.4); \textit{CLEC3B} (Transcript ID: ENST0000428034.1). 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 \textit{PBRM1}, \textit{PLAU}, and \textit{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 (\textit{GAPDH} forward: 5'-GAAGGTGAAAGTCCGAGTCA-3'; reverse: 5'-AATGAAAGGCTTCTGAGTGG-3'; \textit{PBRM1} forward: 5'-ACCTGAGGAGATGAGGAC-3'; and reverse: 5'-GTGGGATCCACAGCAGGAA-3'; \textit{PLAU} forward: 5'-CCGCTTCTTGTGGTGTTC-3' and reverse: 5'-AGGCCTATGCCTGAGGTTAA-3'; \textit{CLEC3B} forward: 5'-TTTTAGGAGACTCAAGAGCG-3'; and reverse: 5'-GGTCTCTGCTGGTGAAGG-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 15 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing (\textit{PBRM1} 60°C; \textit{PLAU} 58°C; \textit{CLEC3B} 59°C) for 30 sec, and extension 72°C for 30 sec. 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, \textit{GAPDH}, was used for normalization of the expression levels of the target genes. The delta-delta-CT (\textit{ΔΔCT}) method was utilized to determine the transcript levels of each gene\cite{30}.

2.5 Analysis of diagnostic performance of \textit{PBRM1}, \textit{PLAU}, and \textit{CLEC3B} mRNA expression in HNSCC

To study the potential diagnostic values of \textit{PBRM1}, \textit{PLAU}, and \textit{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\cite{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-expression, 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).

The RT-qPCR analysis showed that the relative fold change of gene expression (log2) 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 (log2) 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).

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.

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, TCHHII1, SMARCC2, SMARCD1, and GTF3C5 were the five most significantly interacting genes with **PBRM1** (Figure 4).

**PLAU** has multiple interactions with SERPINB2, HRG, PLAU, 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, PLAU, SERPINE1, PLAT, MMP2, HGF, and ITGA2. **PLAU** has genetic interactions with HRAS and MAP2K1; shared pathway with SERPINB2, HRG, PLAU, SERPINE1, PLG, PDGFD, MMP2, HGF, and TRIp6; and shared protein domains with PLG, PLAT, MMP2, and HGF (Figure 5).

**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 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.

**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.

**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\(^\text{33}\). Patients with HNSCC frequently develop local and/or distant metastasis, which adds to the under diagnosis of the malignancy\(^\text{36}\). HNSCC is usually diagnosed at a very developed stages because of absent of symptoms in the early stage\(^\text{37}\). 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, TCHH1L1, SMARC2, 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, PLAIR, SERPINE1, and MRC2 (Figure 5). CLEC3B mainly interacted with PLG, HGF, PLAT, FGF13, and THRPA3 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[25]. 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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**References**


