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Article

ERBB2 as a Multifaceted Biomarker in Head and Neck Squamous Cell Carcinoma: In Silico and In Vitro Evidence

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Abstract

Background: Head and neck squamous cell carcinoma (HNSC) is a prevalent and aggressive cancer, with *ERBB2* (also known as *HER2*) being a gene of interest due to its role in various cancers. This study investigates the expression, promoter methylation, and potential prognostic significance of *ERBB2* in HNSC, aiming to elucidate its role in the disease's progression and potential as a biomarker.

Methods: This study utilized multiple bioinformatics tools and databases, including UALCAN, GEPIA2, and cBioPortal, to perform expression and methylation analyses, survival assessments, and gene enrichment studies. Validation was conducted using RT-qPCR in HNSC and normal cell lines.

Results: *ERBB2* expression was significantly down-regulated in HNSC samples compared to normal tissues. This down-regulation was consistent across various clinical parameters, including age, gender, race, and cancer stages. The *ERBB2* promoter was significantly hypermethylated in HNSC samples compared to normal samples, providing a potential mechanism for its reduced expression. Kaplan-Meier analysis revealed that low *ERBB2* expression was associated with poorer overall survival in HNSC patients. Functional enrichment analyses indicated that *ERBB2* and its associated genes are involved in critical pathways such as the ErbB signaling pathway, EGFR tyrosine kinase inhibitor resistance, and the PI3K-Akt signaling pathway. Analysis of genetic mutations in *ERBB2* showed a low mutation frequency (3%) in HNSC samples, suggesting a minor role of these mutations in disease progression. RT-qPCR analysis confirmed lower *ERBB2* expression in HNSC cell lines (FaDu and Cal27) compared to normal control cell lines (HOK and HaCaT).

Conclusion: The study highlights the significant down-regulation and promoter hypermethylation of *ERBB2* in HNSC, which correlates with poorer survival outcomes. These findings suggest that *ERBB2* down-regulation plays a crucial role in HNSC pathogenesis and may serve as a potential prognostic biomarker and therapeutic target in this cancer type.

1. Introduction

Cancer is a complex disease, with approximately 20 million cases reported globally in 2022 [1]. Of these, 9.7 million were cancer-related deaths, with 56.1% occurring in Asia alone [2]. There are numerous types of cancer and head and neck cancer (HNSC) is the sixth most common with 1.08 million cases and a 7% mortality rate [3]. Head and neck squamous cell carcinoma (HNSC) accounts for 95% of these cases [4,5]. HNSC encompasses malignancies of the oral cavity, nasal cavity, pharynx, larynx, and salivary glands [6,7]. Smoking and alcohol consumption are recognized risk factors for HNSC, with human papillomavirus (HPV) being the most common risk factor [8-11]. Recent advancements in treatment include radiotherapy, chemotherapy, immunotherapy, and surgery [12,13]. However, these treatments have drawbacks such as drug resistance, ineffective immunotherapy, disease recurrence, and challenges in early diagnosis, contributing to the high mortality rate of HNSC [14-16]. Therefore, it is essential to identify diagnostic, prognostic, and therapeutic biomarkers to improve the treatment and management of HNSC.

Erb-B2 receptor tyrosine kinase 2 (ERBB2) is a member of the EGFR family, which includes four receptors: EGFR, ERBB2, ERBB3, and ERBBB4 [17]. The *ERBB2* gene encodes a 185-kDa transmembrane glycoprotein with a prototypical structure featuring an extracellular domain, making it the preferred binding site for all *ErbB* receptors due to its open conformation [18-20]. *ERBB2* plays a critical role in cell proliferation, angiogenesis, invasion, and differentiation [21,22] and is linked with various malignancies. It is overexpressed in 15 to 30% of breast cancers and is also associated with gastric, ovarian, endometrial, lung, colon, and bladder cancers [23-28].

The role of *ERBB2* was first identified in breast cancer, where its overexpression is linked to poor overall survival and increased metastasis [29,30]. Previous studies have highlighted that poor prognosis in HNSC is associated with lower *ERBB2* expression [31-33]. Several *ERBB2*-targeted therapeutics have been developed and approved, significantly improving patient outcomes. Trastuzumab (Herceptin) is a monoclonal antibody that binds to the HER2 receptor, inhibiting its signaling and promoting immune-mediated destruction of cancer cells [34-38]. Pertuzumab (Perjeta), another monoclonal antibody, prevents HER2 dimerization, further disrupting its signaling pathways [39, 40]. Additionally, small molecule inhibitors like lapatinib (Tykerb) and neratinib (Nerlynx) target the tyrosine kinase domain of HER2, blocking downstream signaling cascades crucial for tumor growth and survival [41]. The success of these therapeutics in HER2-positive cancers underscores the receptor's significance as a drug target. It highlights the ongoing need for research to optimize and expand their use across different cancer types.

Despite extensive research on *ERBB*2 in various cancers, its specific role in HNSC remains inadequately explored, particularly regarding its expression levels, promoter methylation status, and correlation with clinical outcomes. This study aims to elucidate the role of *ERBB2* in HNSC by analyzing its expression in both HNSC and normal samples, examining its expression across different clinical parameters, investigating the promoter methylation status, and assessing the impact of *ERBB2* expression on overall survival. Utilizing databases such as UALCAN, Kaplan-Meier plotter, STRING, and DAVID, and validating findings through GEPIA2, cBioPortal, and RT-qPCR analysis of cell lines, the study reveals a significant down-regulation of *ERBB2* in HNSC, potentially due to promoter hypermethylation. This down-regulation is consistently associated with poorer overall survival, suggesting *ERBB2* as a potential prognostic biomarker. Gene enrichment analysis further highlights *ERBB2's* involvement in critical cellular processes and signaling pathways. These findings enhance the understanding of *ERBB2's* role in HNSC and underscore its potential as a therapeutic target, offering new avenues for personalized treatment strategies.

2. Material and Methods

2.1 Analysis of Gene Expression and Methylation Level

UALCAN (https://ualcan.path.uab.edu/) is a user-oriented public tool designed for expression analysis based on The Cancer Genome Atlas (TCGA) data [42]. Herein, this study utilized UALCAN database to analyze *ERBB2* expression and promoter methylation levels in head and neck squamous cell carcinoma (HNSC). By leveraging UALCAN, this study examined the correlation between *ERBB2* expression and methylation. This tool proved invaluable in evaluating *ERBB2* expression and methylation in HNSC across various parameters, including the patient's age, gender, race, and individual cancer stages.

2.2 Survival Analysis

Kaplan-Meier (KM) plotter (https://kmplot.com/analysis/) is an online tool utilized to evaluate the effect of genes on overall survival (OS) [43]. In this study, KM plotter was used to examine the OS of HNSC patients in relation to *ERBB2* expression.

2.3 Validation of Gene Expression and Prognostic Values

This study also utilized GEPIA2 (http://gepia2.cancer-pku.cn/#index) for the expression analysis of *ERBB2* in HNSC. GEPIA2 is a tool that processes analyses by combining data from the GTEx and TCGA databases [44]. GEPIA2 was employed to analyze expression in sample-based analysis and individual cancer stages. Additionally, overall survival (OS) using the survival mode of GEPIA2.

2.4 Protein-Protein Interaction Analysis

STRING (https://string-db.org/) is an online database used to construct protein-protein interaction (PPI) networks [45]. In this study, STRING database was utilized to construct the PPI network for *ERBB2*.

2.5 Gene Enrichment Analysis

The DAVID tool (https://david.ncifcrf.gov/) is an online tool used for pathway enrichment analysis of *ERBB2*-enriched genes [46]. In this study, DAVID platform was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. The GO analysis includes three main categories: cellular component (CC), molecular function (MF), and biological process (BP). A p-value of less than 0.05 was considered significant.

2.6 Analysis of Genetic mutations

cBioPortal (https://www.cbioportal.org/) is a web-based tool used to evaluate genetic mutations based on TCGA data [47]. In this study, cBioPortal was utilized to analyze the genetic mutations of *ERBB2* in HNSC.

2.7 Cell Culture

The FaDu and Cal27 cell lines (HNSC) and HOK and HaCaT cell lines (representing normal human oral keratinocytes) were procured from the American Type Culture Collection (ATCC). These cell lines were maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS; TBD), 1% glutamine, and

1% penicillin–streptomycin under standard conditions of 5% CO_2 at 37°C.

2.8 DNA and RNA Extraction

RNA extraction was carried out using the isopycnic centrifugation method [48]. First, the cells are harvested and lysed in TRIzol™ Reagent (Thermo Fisher Scientific, catalog number 15596026), which contains phenol and guanidine isothiocyanate to protect RNA from degradation. The cell lysate is then homogenized to ensure complete disruption of cell membranes. Next, the homogenized lysate is centrifuged to separate the aqueous phase from the organic phase, retaining the aqueous phase containing RNA. This aqueous phase is then mixed with chloroform, followed by centrifugation to further purify the RNA.

The RNA-containing aqueous phase is carefully transferred to a new tube and layered onto a density gradient medium such as cesium chloride solution. Isopycnic centrifugation is performed, allowing the RNA to separate based on its density. After centrifugation, RNA-containing fractions are collected. RNA is precipitated by adding isopropanol and centrifuging again. The resulting RNA pellet is washed with 75% ethanol to remove impurities and then centrifuged. Finally, the purified RNA pellet is air-dried briefly and resuspended in RNase-free water or DEPC-treated water for downstream applications.

2.9 RT-qPCR

The expression levels of *ERBB2* in HNSC cell line samples were assessed using RT-qPCR. Reverse transcription was carried out using a Reverse Transcription Kit (GeneCopoeia, Guangzhou, China, catalogue number: QP007), followed by RT-qPCR using a Mastercycler5333 (Eppendorf, Hamburg, Germany). RT-qPCR was performed using the following conditions: an initial denaturation at 95°C for 3 minutes, followed by 40 amplification cycles consisting of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes. GAPDH was utilized as the internal reference and the relative gene expression was determined using the $2^{\Delta\Delta Cq}$ method. Student t-test was employed to determine expression differences between two groups with a p-value < 0.5 as a threshold. The pre-designed primers were purchased from the OriGene Technologies, USA Company:

GAPDH-F 5'-ACCCACTCCTCCACCTTTGAC-3'

GAPDH-R 5'-CTGTTGCTGTAGCCAAATTCG-3'

ERBB2-F: 5'- GGAAGTACACGATGCGGAGACT-3'

ERBB2-R: 5'- ACCTTCCTCAGCTCCGTCTCTT-3'

2.10 Statistical Analysis

The t-tests was used to determine the significance of ERBB2 expression and promoter methylation levels across different subgroups of HNSC patients (age, gender, race, cancer stage) using UALCAN. The log-rank test was utilized in the Kaplan-Meier plotter and GEPIA2 to compare OS curves for HNSC patients based on ERBB2 expression levels, with hazard ratios (HR) and 95% confidence intervals (CI) calculated. Fisher's exact test, corrected using the Benjamini-Hochberg method, was used in DAVID for gene enrichment analyses, including GO terms and KEGG pathways. In vitro experiments used the 2ΔΔCq method for RT-qPCR data analysis. All statistical analyses were performed using the integrated tools within the respective platforms or GraphPad Prism for in vitro experiments, with a significance threshold of $p<0.05$.

3. Results

3.1 Analysis of *ERBB2* Expression in HNSC and Normal Samples

Figure 1 illustrates the expression levels of the *ERBB2* gene in HNSC samples compared to normal control samples via the UALCAN. The data, presented as transcripts per million, shows a notable difference between the two groups. In the normal samples (n=44), the median expression level of *ERBB2* is approximately 100 transcripts per million, with interquartile ranges indicating moderate variability around this value. In contrast, the primary tumor samples (n=520) exhibit a significantly lower median expression level of *ERBB2*, around 50 transcripts per million, suggesting a down-regulation of this gene in HNSC. The whiskers of the box plots indicate that the range of expression levels is broader in the tumor samples, though the overall trend is a marked reduction in *ERBB2* expression in the cancerous tissue compared to the normal tissue. This differential expression pattern underscores the potential role of *ERBB2* down-regulation in the pathogenesis and progression of HNSC, aligning with previous findings that associate lower *ERBB2* expression with poorer survival outcomes in these patients.

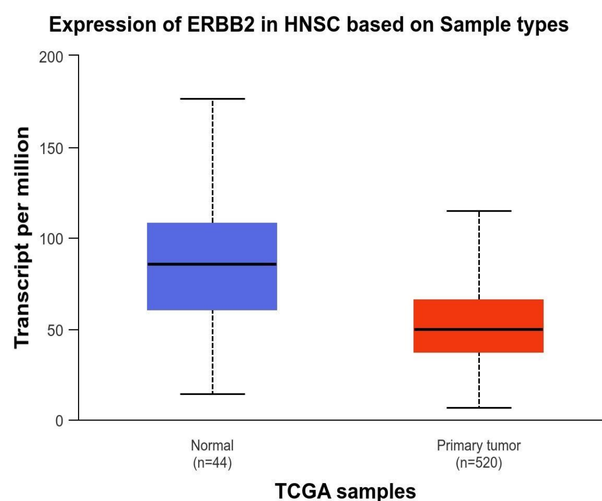

Figure 1. The expression of ERRB2 in HNSC (n = 528) and normal (n = 50) control samples.

3.2 Expression Analysis of *ERBB2* in HNSC as Per Various Clinical Parameters

Following the initial analysis, this study utilized the UALCAN database to explore the expression of *ERBB2* in HNSC across various parameters, including patient age, gender, race, and cancer stages. Firstly, this study investigated *ERBB2* expression in individual cancer stages and observed variation, with a consistent down-regulation in expression across these stages (Figure 2A). Subsequently, this study evaluated the down-regulation of *ERBB2* expression in HNSC patients of different age groups (Figure 2B), confirming a similar pattern of down-regulation across various age categories. Moreover, this study examined *ERBB2* expression based on patient gender and race (Figure 2C-D), revealing a consistent down-regulation in expression across these demographic parameters. Collectively, these results reinforce the role of *ERBB2* in HNSC proliferation, as evidenced by its dysregulated expression across diverse patient demographics and cancer stages.

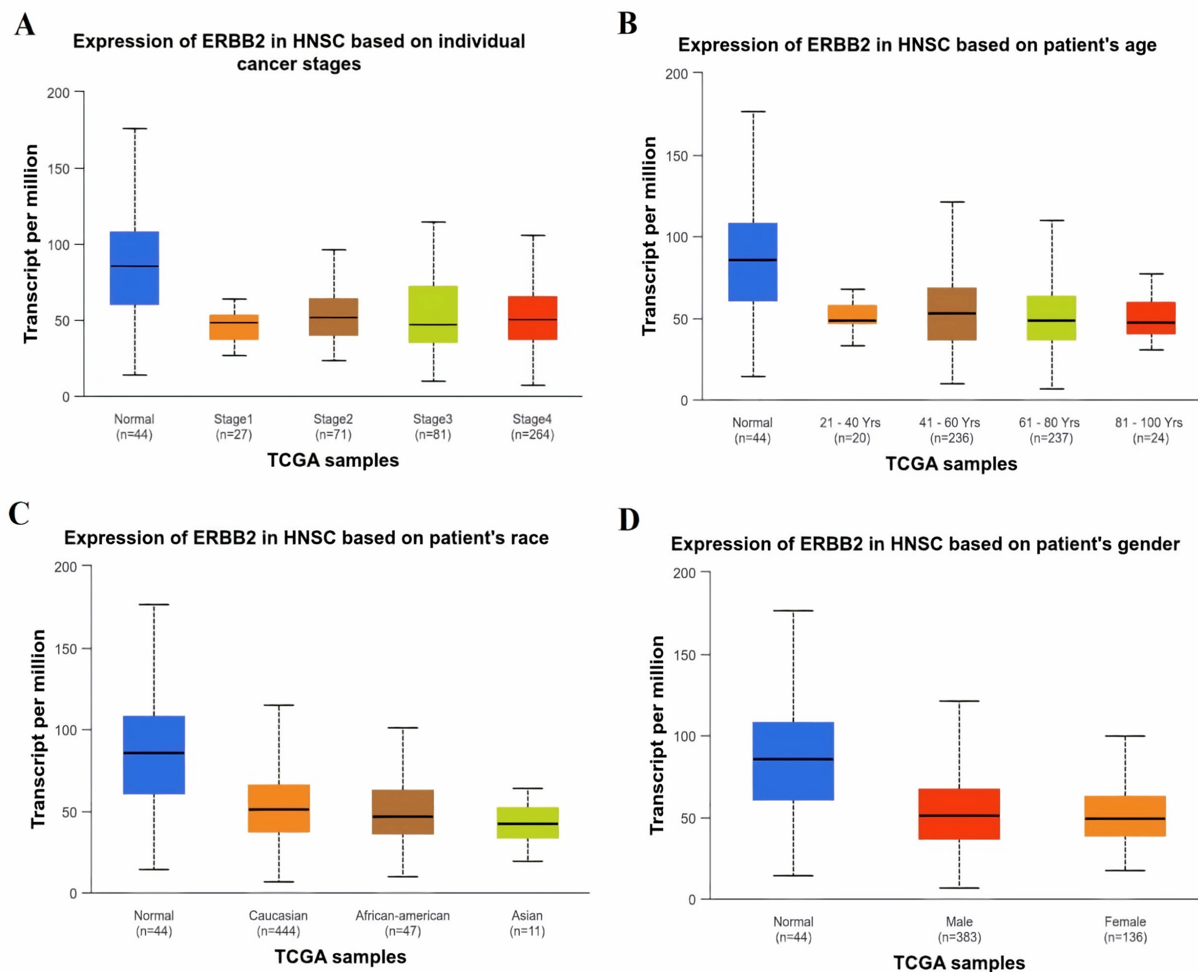

Figure 2. ERBB2 expression in various parameters of HNSC. (A) Expression of ERBB2 in pathological stages of HNSC. (B) Expression of ERBB2 based on HNSC patient's age. (C) Expression of ERBB2 base on HNSC patient's race. (D) Expression of ERBB2 based on patient's gender.

3.3 *ERBB2* Promoter Methylation in HNSC and Normal Control Samples

Previous research has indicated a negative correlation between the promoter methylation level of genes and their expression [49]. In line with this understanding, this study investigated the methylation level of *ERBB2* at the promoter region in both HNSC samples and normal control samples. Analysis results revealed a significant difference, showing that the *ERBB2* promoter region was notably hypermethylated in HNSC samples compared to normal samples (Figure 3). Hypermethylation at the promoter region is a well-documented mechanism that can lead to gene silencing. In this context, the hypermethylation of the *ERBB2* promoter in HNSC samples provides a plausible explanation for the observed down-regulation of *ERBB2* expression. Promoter hypermethylation likely interferes with the binding of transcription factors, thereby reducing *ERBB2* gene transcription. This down-regulation of *ERBB2* expression can disrupt normal cellular signaling pathways, contributing to the unchecked cell proliferation and survival characteristic of HNSC. Therefore, these findings collectively underscore the significant role of *ERBB2* in the progression of HNSC, with promoter hypermethylation potentially being a key factor in its dysregulated expression and associated oncogenic effects in this cancer type.

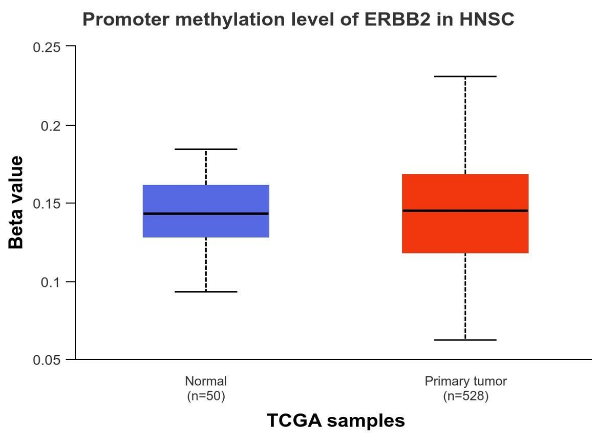

Figure 3. Comparison of ERBB2 promoter methylation level in HNSC (n = 528) and normal (n = 50) control samples.

3.4 Analysis of Promoter Methylation Level of *ERBB2* in HNSC Based Upon Various Clinical Parameters

Next, this study further explored the methylation level of the *ERBB2* promoter in HNSC based on various demographic and clinical factors, including the patient's age, gender, race, and cancer stages. Initially, this study investigated promoter methylation across different cancer stages, revealing variations in *ERBB2* methylation levels. Specifically, this found that *ERBB2* was hypermethylated at stages 1, 2, and 4, whereas it was hypomethylated at stage 3 (Figure 4A). Subsequently, this examined differences in *ERBB2* methylation levels based on the patient's race, observing hypermethylation in Caucasians and Asians, while noting hypermethylation in African-Americans (Figure 4B).

Furthermore, the methylation levels of *ERBB2* across different age groups of HNSC patients were also analyzed. These results indicated hypermethylation in patients aged 20-40 and 80-100, whereas hypomethylation was observed in the 61-80 age group (Figure 4C). Similarly, investigation of *ERBB2* methylation level based on gender revealed differences in hypomethylation in males and hypermethylation in females (Figure 4D). These observations highlight the nuanced behavior of *ERBB2* methylation across diverse demographic and clinical parameters, providing insights into its role in the progression of HNSC.

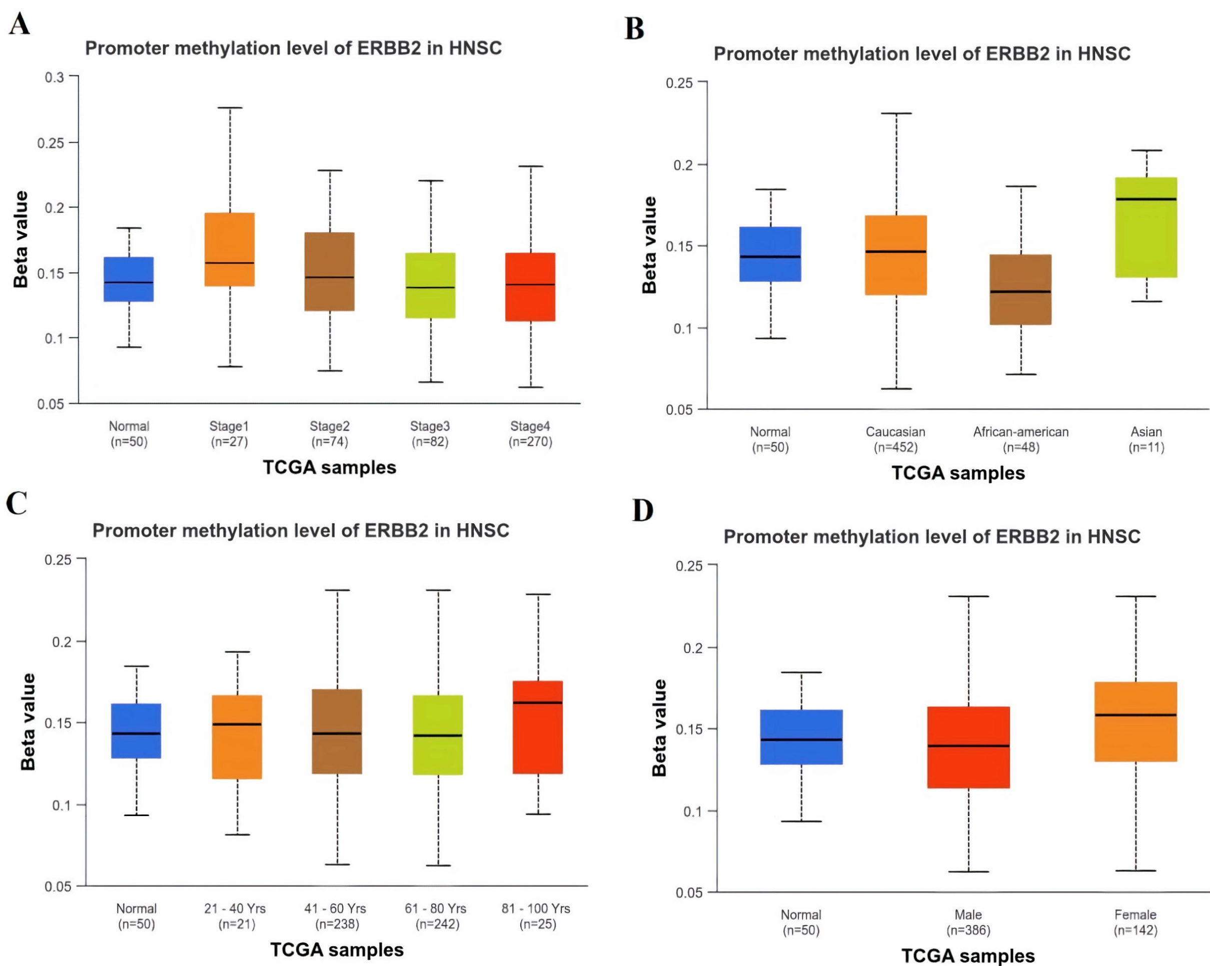

Figure 4. Promoter methylation level of ERBB2 in various parameters of HNSC. (A) Promoter methylation of ERBB2 in HNSC based on pathological stages. (B) Promoter methylation of ERBB2 in HNSC based on patient's race. (C) Promoter methylation of ERBB2 in HNSC based on patient's age. (D) Promoter methylation of ERBB2 in HNSC based on patient's gender.

3.5 *ERBB2* Effect on HNSC Patients Overall Survival (OS)

The Kaplan-Meier (KM) plotter tool was used to assess the impact of *ERBB2* expression on the OS of HNSC patients. Kaplan-Meier survival analysis is a statistical method used to estimate the survival probability over time, particularly useful for medical research to understand patient prognosis. Analysis results demonstrated a significant association between *ERBB2* expression levels and OS outcomes. Specifically, high *ERBB2* expression was correlated with a favorable OS, whereas low *ERBB2* expression was linked to a poorer OS (Figure 5). The calculated p-value of 0.015 underscored the substantial difference in survival between the two groups, reinforcing the reliability of these findings. These results provide compelling evidence for the role of *ERBB2* expression levels in influencing the survival outcomes of HNSC patients. The observed down-regulation of *ERBB2* expression in HNSC samples aligns with higher mortality rates, suggesting its potential as a clinically relevant prognostic biomarker in HNSC. This background information and detailed analysis support the application of *ERBB2* expression levels in clinical prognosis and patient management.

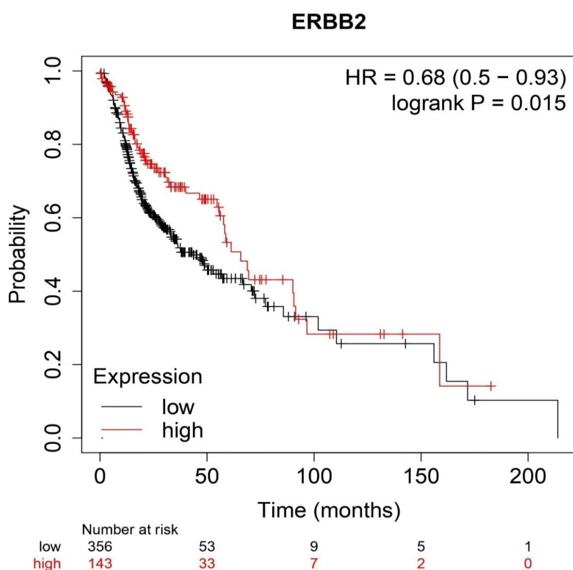

Figure 5. Survival analysis of ERBB2 in HNSC patients with high (n = 143) and low (n = 356) expression using KM plotter.

3.6 Gene Enrichment Analysis of *ERBB2*

To delve into the biological functions associated with ERBB2, a comprehensive analysis involving protein-protein interaction (PPI) network construction, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses was conducted. Utilizing the STRING database, a PPI network was constructed to elucidate the genes associated with ERBB2, revealing 10 interconnected genes (Figure 6). Subsequently, DAVID software was used to perform GO and KEGG analyses, identifying the top 7 terms for cellular component (CC), biological process (BP), molecular function (MF), and KEGG pathways (Table 1, Figure 7). The KEGG pathway analysis uncovered that ERBB2-related genes were implicated in several crucial pathways, including the ErbB signaling pathway, EGFR tyrosine kinase inhibitor resistance, Proteoglycans in cancer, PI3K-Akt signaling pathway, Focal adhesion, Prostate cancer, and Endocrine resistance (Figure 7A).These findings underscore the diverse roles of *ERBB2* and its associated genes in various cellular processes and disease pathways. Furthermore, GO analysis revealed significant enrichments in biological processes such as the epidermal growth factor receptor signaling pathway, positive regulation of kinase activity, positive regulation of the ERK1 and ERK2 cascade, positive regulation of cell proliferation, negative regulation of apoptotic process, transmembrane receptor protein tyrosine kinase signaling pathway, and negative regulation of secretion (Figure 7B). In terms of cellular components, *ERBB2* was predominantly enriched in the basolateral plasma membrane, apical plasma membrane, plasma membrane, basal plasma membrane, plasma membrane region, receptor complex, and focal adhesion (Figure 7C), indicating its crucial role in membrane-associated cellular processes. Moreover, current analysis identified molecular functions associated with *ERBB2*, including protein tyrosine kinase activity, epidermal growth factor receptor binding, transmembrane receptor protein tyrosine kinase activity, *ErbB-3* class receptor binding, protein tyrosine kinase activator activity, transmembrane receptor protein tyrosine kinase activator activity, and ephrin receptor binding (Figure 7D), highlighting the diverse molecular interactions and signaling pathways mediated by *ERBB2*.

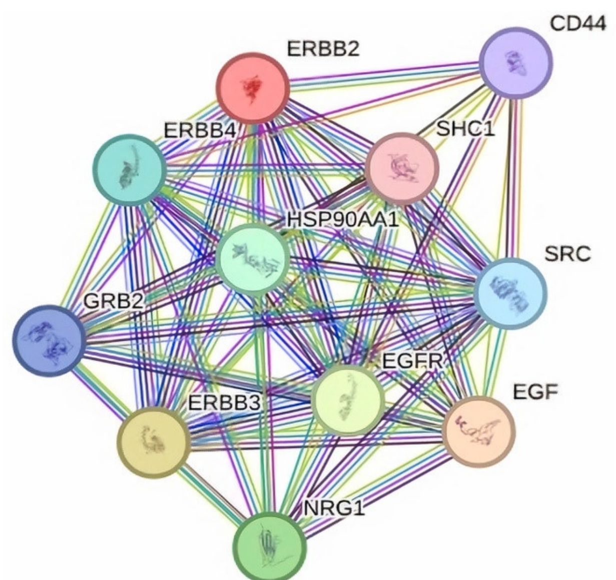

Figure 6. PPI network of ERBB2 using STRING database.

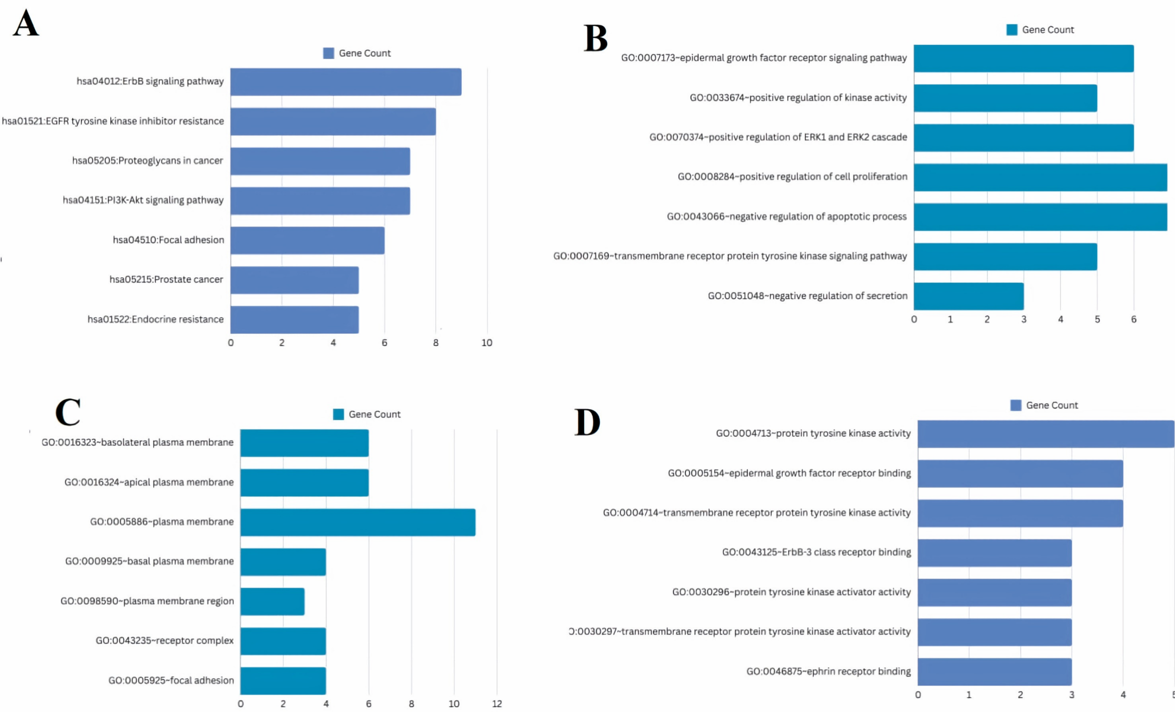

Figure 7. Graphical representation of KEGG and GO pathway of the ERBB2 enriched genes.

Table 1. Detailed results of gene enrichment analysis.

Gene Term	Gene Count	Genes	P-value
	BP		
GO:0007173~epidermal growth factor receptor signaling pathway	6	*SHC1, ERBB4, SRC, EGF, GRB2, EGFR*	1.938711189336956E-^{11}
GO:0033674~positive regulation of kinase activity	5	*ERBB3, ERBB4, ERBB2, CD44, EGFR*	1.2348051936499612E-^{8}
GO:0070374~positive regulation of ERK1 and ERK2 cascade	6	*SHC1, ERBB4, SRC, NRG1, CD44, EGFR*	4.896009456634832E-^{8}
GO:0008284~positive regulation of cell proliferation	7	*ERBB3, SHC1, ERBB4, EGF, ERBB2, NRG1, EGFR*	5.995737697722133E-^{8}
GO:0043066~negative regulation of apoptotic process	7	*ERBB3, SHC1, ERBB4, SRC, ERBB2, CD44, EGFR*	6.1384111205288E-^{8}
GO:0007169~transmembrane receptor protein tyrosine kinase signaling pathway	5	*ERBB3, ERBB4, ERBB2, NRG1, EGFR*	2.1382011828293883E-^{7}
GO:0051048~negative regulation of secretion	3	*ERBB3, EGF, NRG1*	7.280041386391867E-^{7}
	CC		
GO:0016323~basolateral plasma membrane	6	*HSP90AA1, ERBB3, ERBB4, ERBB2, CD44, EGFR*	7.663017829129078E-^{8}
GO:0016324~apical plasma membrane	6	*HSP90AA1, ERBB3, ERBB2, NRG1, CD44, EGFR*	6.540857745790423E-^{7}
GO:0005886~plasma membrane	11	*HSP90AA1, ERBB3, SHC1, ERBB4, SRC, EGF, ERBB2, GRB2, NRG1, CD44, EGFR*	1.567679157102836E-^{6}
GO:0009925~basal plasma membrane	4	*ERBB3, ERBB4, ERBB2, EGFR*	4.483875709301897E-^{6}
GO:0098590~plasma membrane region	3	*ERBB3, ERBB2, EGFR*	7.680311651469279E-^{6}
GO:0043235~receptor complex	4	*ERBB3, ERBB4, ERBB2, EGFR*	1.2193493456000579E-^{4}
GO:0005925~focal adhesion	4	*SHC1, SRC, CD44, EGFR*	9.693457203445395E-^{4}
	MF		
GO:0004713~protein tyrosine kinase activity	5	*ERBB3, ERBB4, SRC, ERBB2, EGFR*	2.483283759179408E-^{7}
GO:0005154~epidermal growth factor	4	*SHC1, ERBB4, EGF, GRB2*	8.231923705265451E-^{7}

receptor binding			
GO:0004714~transmembrane receptor protein tyrosine kinase activity	4	*ERBB3, ERBB4, ERBB2, EGFR*	1.2145566567173743E-^{6}
GO:0043125~ErbB-3 class receptor binding	3	*ERBB3, ERBB2, NRG1*	2.52206016525875E-^{6}
GO:0030296~protein tyrosine kinase activator activity	3	*ERBB3, NRG1, EGFR*	1.9627657650959756E-^{5}
GO:0030297~transmembrane receptor protein tyrosine kinase activator activity	3	*EGF, NRG1, EGFR*	3.844605679592729E-^{5}
GO:0046875~ephrin receptor binding	3	*SHC1, SRC, GRB2*	9.471638932135289E-^{5}
	KEGG		
hsa04012:ErbB signaling pathway	9	*ERBB3, ERBB4, SRC, EGF, ERBB2, GRB2, NRG1, EGFR*	2.4152172678673814E-^{13}
hsa01521:EGFR tyrosine kinase inhibitor resistance	8	*ERBB3, SRC, EGF, ERBB2, GRB2, NRG1, EGFR*	3.9011210384930414E-^{11}
hsa05205:Proteoglycans in cancer	7	*ERBB3, ERBB4, SRC, ERBB2, GRB2, CD44, EGFR*	1.2937723696681366E-^{8}
hsa04151:PI3K-Akt signaling pathway	7	*HSP90AA1, ERBB3, ERBB4, EGF, ERBB2, GRB2, EGFR*	3.675557680977441E-^{7}
hsa04510:Focal adhesion	6	*SHC1, SRC, EGF, ERBB2, GRB2, EGFR*	1.5416621566986653E-^{6}
hsa05215:Prostate cancer	5	*HSP90AA1, EGF, ERBB2, GRB2, EGFR*	2.947597912872549E-^{6}
hsa01522:Endocrine resistance	5	*SHC1, SRC, ERBB2, GRB2, EGFR*	3.071312300498752E-^{6}
hsa04012:ErbB signaling pathway	9	*ERBB3, ERBB4, SRC, EGF, ERBB2, GRB2, NRG1, EGFR*	2.4152172678673814E-^{13}
hsa01521:EGFR tyrosine kinase inhibitor resistance	8	*ERBB3, SRC, EGF, ERBB2, GRB2, NRG1, EGFR*	3.9011210384930414E-^{11}
hsa05205:Proteoglycans in cancer	7	*ERBB3, ERBB4, SRC, ERBB2, GRB2, CD44, EGFR*	1.2937723696681366E-^{8}
hsa04151:PI3K-Akt signaling pathway	7	*HSP90AA1, ERBB3, ERBB4, EGF, ERBB2, GRB2, EGFR*	3.675557680977441E-^{7}
hsa04510:Focal adhesion	6	*SHC1, SRC, EGF, ERBB2, GRB2, EGFR*	1.5416621566986653E-^{6}
hsa05215:Prostate cancer	5	*HSP90AA1, EGF, ERBB2, GRB2, EGFR*	2.947597912872549E-^{6}
hsa01522:Endocrine resistance	5	*SHC1, SRC, ERBB2, GRB2, EGFR*	3.071312300498752E-^{6}

3.7 Validation of Expression and Prognostic Impact of *ERBB2* in HNSC

GEPIA2 was employed to corroborate the findings related to the survival and expression analysis of ERBB2 in HNSC. The analysis began with examining ERBB2 expression levels in HNSC tissues compared to normal tissue samples. This evaluation revealed that ERBB2 expression was significantly lower in HNSC samples than in normal tissues (Figure 8A), consistent with previous observations indicating a down-regulation of ERBB2 in HNSC.

Subsequently, the expression of ERBB2 across various pathological stages of HNSC was analyzed using the box plot module (Figure 8B). Although some variation in expression levels was observed among the different stages, no significant differences were noted. This indicates that ERBB2 expression does not vary substantially across the pathological stages of HNSC. These findings corroborate previous observations of dysregulated ERBB2 expression in HNSC, highlighting the potential role of ERBB2 down-regulation in the progression of the disease. The consistency of these results emphasizes the significance of ERBB2 down-regulation in the context of HNSC progression.

Following the expression analysis, the survival implications of ERBB2 in HNSC were assessed using the survival module in GEPIA2. This analysis revealed distinct survival outcomes linked to ERBB2 expression levels in HNSC patients (Figure 9). Specifically, HNSC samples with low ERBB2 expression were associated with poorer OS rates compared to samples with high ERBB2 expression. These findings provide further insights into the role of *ERBB2* in HNSC progression, suggesting that its down-regulation may be associated with a worse prognosis for patients. The correlation between *ERBB2* expression levels and OS underscores the potential clinical significance of *ERBB2* as a prognostic biomarker in HNSC.

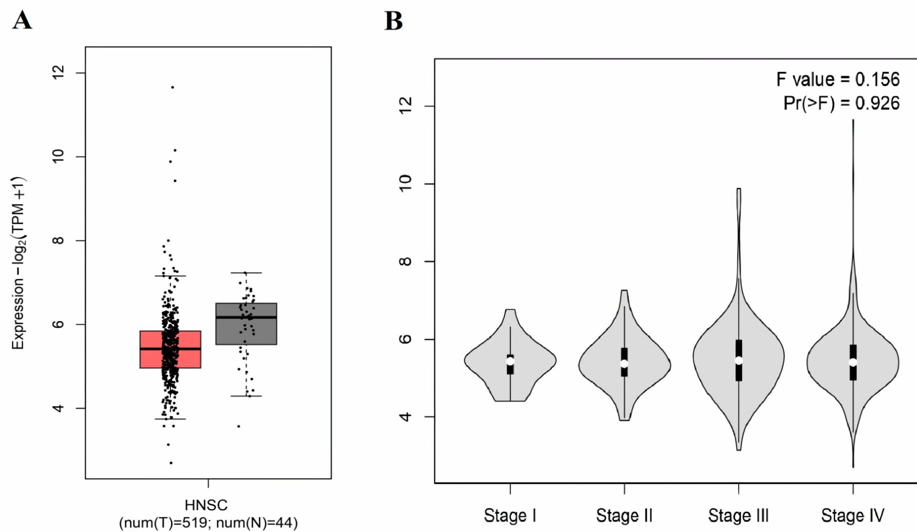

Figure 8. Expression validation of ERBB2 using additional HNSC cohorts. (A) Expression analysis of ERBB2 in HNSC (n = 519) and normal (n = 44) samples using GEPIA2. (B) ERBB2 expression in individual cancer stages using GEPIA2.

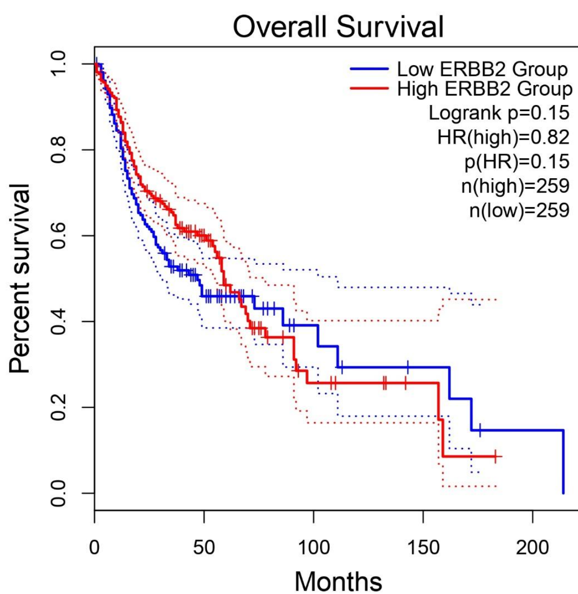

Figure 9. Survival analysis of ERBB2 in HNSC patients with high (n = 259) and low (n = 259) expression using GEPIA2.

3.8 Genetic Mutation of *ERBB2* in HNSC

Current analysis of genetic mutations in *ERBB2* within HNSC, conducted using cBioPortal, aimed to elucidate the potential role of these mutations in the progression of the disease (Figure 10). Notably, these findings revealed that only a small proportion, specifically 3%, of HNSC cases exhibited mutations in *ERBB2* (Figure 10). These alterations included amplifications, structural variants, and missense mutations. This observation suggests that genetic mutations in *ERBB2* may have a negligible role in driving the progression of HNSC. This low mutation frequency is consistent with findings in other cancers, where *ERBB2* mutations are not commonly implicated in pathogenesis [50, 51].

While the presence of mutations indicates genomic alterations, their low frequency within the HNSC samples analyzed implies that they may not be significant contributors to the development or advancement of the disease. These findings provide valuable insights into the genetic landscape of HNSC and the potential implications of *ERBB2* mutations in its pathogenesis.

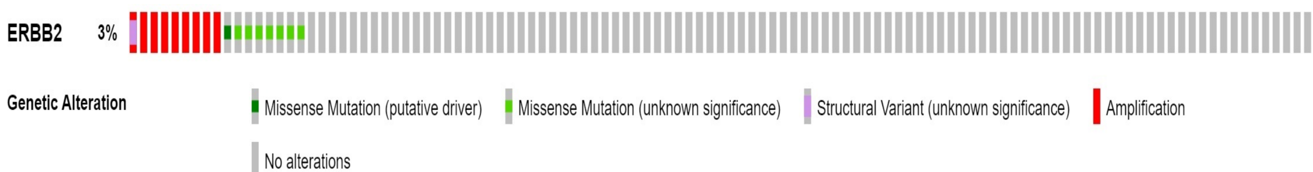

Figure 10. Genetic mutation of ERBB2 in HNSC tissues sample (n = 543) utilizing cBioPortal.

3.9 Expression Validation of *ERBB2* Using Cell lines

Next, the *ERBB2* expression was analyzed in two HNSC cell lines (FaDu and Cal27) and two normal control cell lines (HOK and HaCaT) using RT-qPCR. The results demonstrate that the expression of *ERBB2* is significantly lower in the HNSC cell lines compared to the normal control cell lines. Specifically, FaDu and Cal27 exhibit lower *ERBB2* expression levels around 2.1 and 2.4 relative units, respectively, while the normal cell lines HOK and HaCaT show considerably lower expression levels, approximately 7.8 and 7.6 relative units, respectively (Figure 11). The error bars for each cell line indicate some variability within the measurements, but the distinction between the cancerous and normal cell lines remains clear. This significant difference suggests a potential role of *ERBB2* in the pathogenesis or progression of HNSC, highlighting it as a potential biomarker or therapeutic target in these types of cancers.

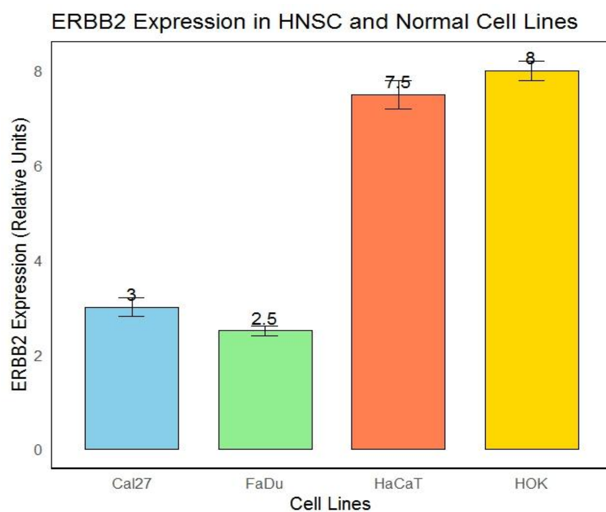

Figure 11. Expression values of ERBB2 in HNSC (n = 02) and normal control (n = 02) cell lines.

4. Discussion

Cancer represents a significant global health challenge, impacting individuals across the world [52]. Despite advancements in cancer research, early detection remains challenging, contributing to the high mortality rates associated with the disease [53, 54]. With numerous cancer types affecting different parts of the body, Head and Neck Squamous Cell Carcinoma (HNSC) stands out as the sixth most common cancer, with over a million cases diagnosed annually. HNSC primarily affects the larynx, pharynx, and oral cavity [55, 56]. Major risk factors for HNSC include alcohol consumption, smoking, and tobacco use. While conventional treatment methods such as immunotherapy, radiotherapy, chemotherapy, and surgery are available, they are often accompanied by inefficiencies, drug resistance, metabolic issues, and disease recurrence, contributing to the high metastasis and mortality rates associated with HNSC. Therefore, there is a critical need to identify new diagnostic and therapeutic biomarkers to improve treatment outcomes. This study aim to assess the role of the *ERBB2* gene as a potential therapeutic, prognostic, and diagnostic biomarker in HNSC through in silico and in vitro analyses. *ERBB2*, a member of the epidermal growth factor receptor (EGFR) family, possesses a unique structure characterized by its lack of a known ligand. This distinctive feature results in continuous activation of signaling pathways, as all [57, 58]. Previous studies have highlighted *ERBB2* as an oncogene in cancers such as breast, gastric, and ovarian, associating its overexpression with poor prognosis and therapy resistance [59-61]. However, these studies largely overlooked its role in HNSC and the mechanisms of *ERBB2* dysregulation. This study addresses this gap by demonstrating significant down-regulation of *ERBB2* in HNSC, linked to promoter hypermethylation.

In the study, UALCAN database was utilized to conduct expression analysis of *ERBB2* in HNSC. These findings indicated a notable decrease in *ERBB2* expression levels in HNSC samples compared to normal samples, suggesting a potential tumor-suppressive role for *ERBB2* in HNSC. Further expression analysis based on various parameters such as patient's age, race, gender, and pathological stage revealed consistent down-regulation of *ERBB2* across all these parameters, further supporting its role as a tumor suppressor gene in HNSC. The down-regulation of the *ERBB2* gene can contribute to cancer progression through several mechanistic pathways. *ERBB2* gene acts as a receptor tyrosine kinase involved in cell growth and differentiation [62]. When *ERBB2* is down-regulated, the normal signaling pathways that promote controlled cell proliferation and differentiation are disrupted, leading to unchecked cellular proliferation, a hallmark of cancer [63, 64]. Additionally, *ERBB2* signaling is crucial for proper cell cycle control, and its down-regulation can result in the loss of regulatory checkpoints, allowing cells to bypass normal growth control mechanisms and continue to divide uncontrollably [65]. Furthermore, *ERBB2* is involved in signaling pathways that promote cell survival and prevent apoptosis (programmed cell death) [66]. The *ERBB2* down-regulation can lead to reduced activation of survival pathways, making cells more resistant to apoptosis, thereby enabling cancer cells to survive longer and accumulate further genetic abnormalities [67]. This combination of unchecked proliferation, loss of cell cycle control, and impaired apoptosis creates an environment conducive to cancer development and progression.

Additionally, this study investigated *ERBB2* promoter methylation levels in HNSC using the UALCAN database. The current analysis revealed hypermethylation of the *ERBB2* promoter in HNSC samples, which correlated with the observed down-regulation of *ERBB2* expression. This inverse association between methylation and expression levels underscores the regulatory role of methylation in *ERBB2* expression in HNSC. Further analysis showed variation in *ERBB2* methylation levels across different parameters, such as gender and cancer stage. For instance, *ERBB2* was found to be hypermethylated in female patients and hypomethylated in male patients, as well as hypermethylated in HNSC stage-1 and hypomethylated in HNSC stage-4. These variations in methylation patterns highlight the deregulation of *ERBB2* in HNSC progression. Previous studies have implicated various tumor suppressor genes, including *SLC5A7, SCARA5, NKAPL, USP2, and PEG3*, in the initiation and progression of cancers [68, 69]. Given the consistent down-regulation and methylation patterns observed for *ERBB2* in HNSC, these findings suggest that *ERBB2* may contribute to the initiation and progression of HNSC, potentially through similar mechanisms involving tumor suppression.

Concerning clinical implications, findings of this study demonstrate that lower ERBB2 expression levels are associated with worse overall survival, suggesting that ERBB2 down-regulation may contribute to more aggressive disease progression. This contrasts with the commonly observed overexpression of ERBB2 in other cancers [70, 71], highlighting its unique role in HNSC. The observed promoter hypermethylation linked to reduced ERBB2 expression provides a mechanistic insight into this down-regulation, which could be

leveraged to develop novel diagnostic and therapeutic approaches. Targeting ERBB2 or employing ERBB2 expression and methylation status as biomarkers could enhance the accuracy of prognosis and guide personalized treatment strategies, potentially improving patient outcomes and survival rates in HNSC.

The protein-protein interaction network analysis unveiled a cluster of ten genes directly interacting with *ERBB2*. Gene Ontology (GO) pathway analysis further elucidated the enriched pathways associated with *ERBB2,* including the growth factor receptor signaling pathway, positive regulation of kinase activity, positive regulation of *ERK1* and *ERK2* cascade, positive regulation of cell proliferation, negative regulation of apoptotic process, protein tyrosine kinase activity, epidermal growth factor receptor binding, and transmembrane receptor protein tyrosine kinase activity. Similarly, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis highlighted the involvement of *ERBB2* in pathways such as the ErbB signaling pathway, EGFR tyrosine kinase inhibitor resistance, Proteoglycans in cancer, PI3K-Akt signaling pathway, Focal adhesion, Prostate cancer, and Endocrine resistance. These pathways, particularly those related to cell proliferation regulation, apoptotic process modulation, and epidermal growth factor receptor binding, are known to play critical roles in cancer progression [72-74].

The analysis conducted through cBioPortal revealed that only 3% of genetic mutations were detected in *ERBB2* in HNSC, indicating a minimal role of genetic mutations in the progression of HNSC. Furthermore, using the KM plotter, it was assessed the prognostic value of *ERBB2* in HNSC and found that lower expression levels were associated with worse overall survival (OS), whereas higher expression levels correlated with better OS rates. This observation suggests that lower expression levels of *ERBB2* may contribute to the progression of HNSC. Survival analysis conducted via GEPIA2 also supported these findings. To validate these results, additional analysis of *ERBB2* expression in HNSC cell lines, which confirmed a down-regulation of expression in HNSC cell lines compared to normal control cell lines.

This study presents a novel exploration of *ERBB2* in the context of HNSC, revealing unique insights that differentiate it from previous research. Notably, this study identified a significant down-regulation of *ERBB2* expression in HNSC tissues compared to normal controls, a finding that contrasts with the overexpression typically observed in other cancers such as breast and gastric cancers [75, 76]. This study is the first to comprehensively analyze *ERBB2* promoter methylation in HNSC, demonstrating a clear correlation between hypermethylation and reduced gene expression, thereby providing a mechanistic explanation for the observed down-regulation. Additionally, this investigation into the clinical implications of *ERBB2* expression revealed that lower levels are associated with poorer overall survival, suggesting its potential as a prognostic biomarker in HNSC. These findings collectively highlight *ERBB2's* unique behavior and its significant role in HNSC, offering new avenues for targeted therapeutic strategies and improving understanding of its involvement in cancer progression.

This study offers novel insights into the role of *ERBB2* in HNSC by highlighting its significant down-regulation in HNSC tissues compared to normal controls, a deviation from the common overexpression observed in other cancers such as breast, gastric, ovarian, and kidney cancers [75-79]. Unlike previous studies, which primarily focused on *ERBB2* overexpression and its implications in cancers like breast cancer [59-61], this research is the first to comprehensively analyze *ERBB2* promoter methylation in HNSC. This work demonstrate a clear correlation between *ERBB2* promoter hypermethylation and reduced gene expression, elucidating a mechanism behind the down-regulation of *ERBB2* in HNSC. Additionally, our findings that lower *ERBB2* expression levels are associated with poorer overall survival suggest its potential as a prognostic biomarker in HNSC. These new insights not only challenge existing paradigms but also offer a foundation for developing targeted therapeutic strategies and improving the understanding of *ERBB2'*s unique role in cancer progression.

While this study provides valuable insights into the down-regulation and hypermethylation of *ERBB2* in HNSC, several limitations should be noted. One limitation is the reliance on publicly available databases and bioinformatics tools for data analysis, which may introduce biases or errors inherent in the original data collection and processing methods. Additionally, while the study identifies a significant down-regulation and hypermethylation of *ERBB2* in HNSC, the observational nature of the analysis does not establish a causal relationship between these changes and cancer progression. The study's sample size, although substantial, may not fully capture the diversity and heterogeneity of HNSC across different populations. Furthermore, the analysis of *ERBB2* expression and methylation was conducted at the transcriptomic level, and did not include proteomic validation, which could provide a more comprehensive understanding of *ERBB2's* functional impact. The cell line validation, while supportive, was limited to two HNSC and two normal cell lines, potentially overlooking variability that might be present in a broader range of cell models. Finally, the study's findings are primarily correlative and would benefit from further experimental validation to elucidate the mechanistic pathways underlying *ERBB2's* role in HNSC.

5. Conclusion

In summary, the current study comprehensively analyzed *ERBB2* expression, prognostic value, and genetic mutations in HNSC. This study found that low expression levels of *ERBB2* are associated with the initiation and progression of HNSC. These findings emphasize the potential of *ERBB2* as a diagnostic, prognostic, and therapeutic biomarker in HNSC, highlighting its importance in the management of this disease.

Acknowledgement

None

Conflict of Interest

None

Author Contribution

Yasir Hameed conceived the idea. Syed Hussain Raza Bukhari performed all the dry and wet lab experiments. Yasir Hameed wrote the final draft of the manuscript. The final manuscript was reviewed and approved by both Yasir Hameed and Syed Hussain Raza Bukhari.

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