RESEARCH PAPER

VIRTUAL SCREENING FOR MUTATIONS IN NOS2 GENE AND ITS PUTATIVE ASSOCIATION WITH HNSCC

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Type of study: *Original research*

Running title: *Genetic association of NOS2 gene and their putative association with HNSCC*

ABSTRACT

Introduction

Head and neck squamous cell carcinoma (HNSCC) develops in the mucous membranes of the mouth, nose, and throat. Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities.

Objective

The primary objective of the study is to identify the high risk mutations in the NOS2 gene and to evaluate their potential association with the pathogenesis of HNSCC.

Methodology

The mutation data of the NOS2 gene was obtained from the Ensembl database. Since, the consequences of missense mutations vary based on the type of amino acid that is getting replaced, several computations tools such as SIFT, PolyPhen, PROVEAN, MutPred, IMutant were used to assess the pathogenicity of these mutations.

Results

Out of the initial pool of SNPs, virtual screening identified a subset of high-confidence deleterious mutations (e.g., specific residues in the oxygenase domain) that significantly destabilized the protein structure.

Conclusion

This study highlights several critical mutations in the NOS2 gene that likely contribute to the molecular etiology of HNSCC. The identified variants might serve as theranostic leads used for disease susceptibility and may offer targets for personalized therapeutic interventions. These in silico findings provide a robust foundation for subsequent in vitro validation.

Keywords: Cancer, Polymorphism, Nitric Oxide, Biomarkers, Computational.

How to cite this article: Deepasakthi J, Namira Fateen K, Vijayshree priyadharshini J, Abilasha R. Virtual Screening for Mutations in NOS2 Gene and Its Putative Association with HNSCC. Int J Drug Deliv Technol. 2026;16(54s): 1416-1422. DOI: 10.25258/ijddt.16.54s.126

Source of support: Nil.

Conflict of interest: None.

Introduction:

Squamous cell carcinoma is a cancer that arises from particular cells called squamous cells. Squamous cells are found in the outer layer of skin and in the mucous membranes, which are the moist tissues that line body cavities such as the airways and intestines(1). Head and neck squamous cell carcinoma (HNSCC) develops in the mucous membranes of the mouth, nose, and throat(2). HNSCC is classified by its location: it can occur in the oral cavity, the middle part of the throat near the mouth (oropharynx), the space behind the nose, the upper part of the throat near the nasal cavity (nasopharynx), the larynx, or the lower part of the throat near the larynx(3). Depending on the location, the cancer can cause abnormal patches or open sores in the mouth and throat, unusual bleeding or pain in the mouth, sinus congestion that does not clear, sore throat, earache, pain when swallowing or difficulty swallowing, a hoarse voice, difficulty breathing, or enlarged lymph nodes(4). HNSCC can spread to other parts of the body, such as the lymph nodes or lungs. If it spreads, the cancer has a worse prognosis and can be fatal. About half of affected individuals survive more than five years after diagnosis(5)

Nitric oxide is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities. This gene encodes a nitric oxide synthase which is expressed in the liver and is inducible by a combination of lipopolysaccharide and certain cytokines(6). Three related pseudogenes are located within the Smith-Magenis syndrome region on chromosome 17. The NOS2 gene, also known as the inducible nitric oxide synthase gene, is responsible for producing an enzyme called inducible nitric oxide synthase (iNOS)(7). This enzyme plays a role in the production of nitric oxide (NO) in our bodies. Nitric oxide is a molecule that has various functions in the body. The NOS2 gene is usually activated in response to certain stimuli, such as inflammation, infection, or injury. When the NOS2 gene is activated, it leads to the production of iNOS, which then produces nitric oxide. Nitric oxide can have both beneficial and harmful effects, depending on the context. It can help fight off pathogens and regulate immune responses, but excessive nitric oxide production can also contribute to tissue damage and inflammation(8).

When it comes to squamous carcinoma, the NOS2 gene has been found to have some associations(9). The NOS2 gene may play a role in the development and progression of squamous cell carcinoma(10). Specifically, increased expression of the NOS2 gene and elevated levels of nitric oxide have been observed in squamous cell carcinoma cells(11). However, it's important to note that the relationship between the NOS2 gene and squamous cell carcinoma is complex and still being studied(12). It's just one of many factors involved in the development of this type of cancer. The purpose of the study is to determine the genotype and allele frequencies of NOS2 gene polymorphism and to derive an association between the genotypes with susceptibility to head and neck squamous cell carcinoma

Materials and methods:

Data collection:

The rationale behind choosing the NOS2 gene for the present study is that it plays a significant role in inflammation and tumor progression and has been implicated in the development of HNSCC. Nitric oxide produced by inducible nitric oxide synthase (iNOS), encoded by the NOS2 gene, is known to influence cellular processes such as apoptosis, angiogenesis and immune response which are critical in cancer pathogenesis. Hence, it is imperative to identify pathogenic mutations in the NOS2 gene to obtain a clearer understanding of its role in the progression of HNSCC. The information on missense mutations of the human NOS2 gene was collected from the Ensembl database. The reported missense mutations were screened using three different computational tools, namely SIFT, PolyPhen, and PROVEAN. The curated data obtained from these tools were further analyzed using I-Mutant 3.0 and MutPred to determine the stability of protein variants and to predict their pathogenic effects, respectively. The description of the individual software tools used in the study is discussed below.

SIFT analysis:

The SIFT (Sorting Intolerant From Tolerant) tool utilizes information derived from multiple sequence alignments to predict whether amino acid substitutions at specific positions in a protein sequence are tolerated or deleterious. Variants with a normalized probability score lower than the tolerance threshold of 0.05 are predicted to be deleterious or intolerant, whereas substitutions with scores higher than 0.05 are considered tolerated (13).

PolyPhen analysis:

PolyPhen-2 (Polymorphism Phenotyping v2) analysis predicts the potential impact of amino acid substitutions on the structure, stability, and function of human proteins by integrating structural information and comparative evolutionary analysis. The tool evaluates the likelihood of a missense mutation being damaging by combining several parameters, including functional annotation of single-nucleotide polymorphisms (SNPs), mapping coding SNPs to gene transcripts, extracting protein sequence annotations and structural features, and generating conservation profiles (14).

I-Mutant analysis:

I-Mutant v3.0 is a support vector machine (SVM)–based computational tool used to predict changes in protein stability resulting from single point mutations. The predictions are primarily derived from the protein sequence. The outcomes are categorized into three groups: neutral mutations ($-0.5 \leq DDG \geq 0.5$ kcal/mol), large decrease in stability (<-0.5 kcal/mol), and large increase in stability (> 0.5 kcal/mol). The predicted free energy change (DDG) represents the difference between the unfolding Gibbs free energy of the mutant protein and that of the native protein, expressed in kcal/mol (15).

MutPred analysis:

MutPred v2 is a standalone and web application developed to classify amino acid substitutions as pathogenic or benign in humans. The wild-type protein sequence in FASTA format is used for the purpose and the substitution sites identified. The probability of the mutation being deleterious is reported (16).

Gene expression analysis:

UALCAN is an interactive web portal for analyzing cancer OMICS data, primarily from TCGA and

CPTAC. It facilitates *in silico* validation of genes and identifies biomarkers by correlating expression with clinicopathological features. In HNSCC, researchers use UALCAN to identify prognostic markers by linking high-risk gene expression to significantly lower patient survival rates (17).

RESULTS:

The NOS2 is located on chromosome 17 at the cytogenetic band 17q11.2 (Figure 1). This location is consistently reported across genomic databases including HUGO Gene Nomenclature Committee, NCBI, and Ensembl. The UCSC Genome Browser shows the NOS2 gene positioned on the long arm (q arm) of chromosome 17 within band q11.2, along with neighboring genes in the surrounding genomic region. The cytogenetic mapping helps in identifying the precise chromosomal position and genomic context of NOS2 which is important for studying its role in genetic alterations associated with HNSCC.

The OncoPrint analysis obtained from cBioPortal illustrates the spectrum of genetic alterations identified in the NOS2 among samples of HNSCC. The data indicate that approximately 2.4% of the analyzed cases exhibited alterations in the NOS2 gene. The detected alterations include missense mutations, truncating mutations, and gene amplifications while the majority of samples showed no detectable genetic alterations.

The lollipop plot (Figure 2) obtained from the The Cancer Genome Atlas dataset illustrates the distribution of mutations across the protein domains of the NOS2 in patients with HNSCC. The mutations are mapped along the NOS2 protein structure highlighting their positions within important functional domains such as the NO synthase domain, Flavodoxin domain, FAD-binding domain, and NAD-binding region. A notable mutation R633W is observed within the flavodoxin domain, which plays a role in electron transfer during nitric oxide synthesis.

Analysis of NOS2 expression in HNSCC samples from the TCGA dataset revealed differences between normal and tumor tissues (Figure 3). The box-whisker plot showed that although the median NOS2 transcript levels were low in both normal (n = 44) and tumor samples (n = 520), tumor tissues exhibited a wider range with several high-expression outliers. Kaplan–Meier survival analysis demonstrated that patients with higher NOS2 expression had significantly better survival compared to those with low or medium expression ($p = 0.028$). Additionally, the jitter plot illustrated heterogeneous NOS2 mRNA expression across HNSCC samples, including both mutated and non-mutated cases.

Table 1 summarizes the analysis of mutations in the NOS2 gene among HNSCC patients revealed predominantly missense variants with varying functional impacts. The P248S mutation showed a SIFT score of 0.00, indicating a deleterious effect supported by a PolyPhen score of 1.00 (probably damaging) and a MutPred score of 0.729, classifying it as a pathogenic variant with decreased protein stability. Similarly, R633W exhibited a low SIFT score (0.01) suggesting functional impact, with PolyPhen predicting it as possibly damaging and MutPred indicating mild deleteriousness, also associated with decreased stability. In contrast, mutations such as S124N, R1140S, Q840K, E156Q, and T11S were largely predicted to be tolerated or benign, with low PolyPhen and MutPred scores indicating minimal functional disruption; however, most of these variants were still associated with decreased protein stability, except Q840K which showed increased stability. Additionally, a nonsense mutation (W295*) was identified, likely resulting in a truncated protein with significant functional consequences. Overall, the findings highlight a mix of deleterious and neutral variants, with certain mutations potentially contributing to altered protein function and stability in HNSCC.

Discussion:

Head and Neck Squamous cell Carcinoma is a heterogeneous malignancy characterized by multiple genetic and molecular alterations that influence tumor initiation and progression(18). Among the genes implicated in tumor biology, the NOS2 gene plays an important role in regulating nitric oxide production, which is involved in inflammation, immune response, and tumor microenvironment modulation. Alterations in NOS2 may therefore influence cancer development and progression(19).

HNSCC is a worldwide public health problem, with an increasing incidence worldwide. In order to improve the diagnosis and treatment of HNSCC, it is necessary to find reliable predictive biomarkers and to understand the molecular mechanisms of the interaction of HNSCC (20). Previous study results suggest that the NOS2 SNP is associated with development of squamous cell carcinoma as a protective SNP, while an association between the NOS2 SNP (21). In another research analysis suggested that NOS2 variant may decrease head and neck squamous cell carcinoma susceptibility, particularly in Asian descendants. Research results suggest that NOS2 polymorphism is associated with SCC and could be a risk marker for predisposition and prognosis of SCC(22). In another research CART analysis gave a wide spectrum of interactive combinations which exhibited a major contribution in modulating head and neck cancer susceptibility. Nitric oxide in NOS2 is a reactive free radical which acts as a biological mediator in neurotransmission(23).

The effect of these mutations on protein stability was evaluated using I-Mutant 3.0. The results indicated that most of the mutations were predicted to decrease protein stability, suggesting that these alterations may disrupt normal NOS2 protein structure and activity(24). Reduced stability may impair nitric oxide regulation and could contribute to tumor progression through alterations in cellular signaling pathways and the tumor microenvironment(25). In this research, by using SIFT and Polyphen score, 88 damaging mutations were found that can affect the NOS2 gene. On comparing this, 7 Missense mutation and 1 truncating mutation was observed. Previous studies found that NOS2 polymorphism was not linked to cancer risk in the Turkish population under any of the above models and in the Japanese population under homozygous, heterozygous, recessive models.

In a different study, breast cancer was linked to NOS2 variants, as well as age, TNM stage, and histologic-molecular subtype ($p = 0.001$). Similarly, there was a significant correlation between HNSCC cancer and NOS2(26). In another study, NOS2 polymorphism was found to be significantly associated with a decreased risk of cancer, particularly lung cancer, in Asians and population-based controls. Thus, NOS2 should be considered as a potential therapeutic target for preventing tumor growth. The involvement of NO in carcinogenesis and cancer progression has been reported in a large body of studies. NO has both positive and negative effects which have been implicated in both promoting and preventing cancer(27). The tumor-promoting effects of NO are through genotoxic mechanisms, antiapoptotic effects, promotion of angiogenesis and metastasis, and limiting immune responses. The role of nitric oxide as a mediator of cancer phenotype has led researchers to investigate strategies for manipulating in vivo production and exogenous delivery of this molecule for therapeutic gain. Transfer of NOS-encoding cDNA sequences into cancer cells for gene therapy purposes was thought to be one of the mechanisms for delivery of NO(28).

Limitations:

One of the main limitations of this study was that the mutation analysis was based solely on data obtained from The Cancer Genome Atlas database for patients with HNSCC, which may not fully represent all populations. The study evaluated only a limited number of NOS2 gene mutations, and the effects on protein stability were predicted using in-silico tools such as I-Mutant 3.0 without experimental validation. Additionally, the research did not assess the functional impact of these mutations or correlate them with clinical parameters such as disease progression, prognosis or treatment response which may limit the overall interpretation of the findings.

Conclusion:

This study demonstrates that NOS2 gene alterations may contribute to the development and progression of head and neck squamous cell carcinoma (HNSCC). In silico analysis identified several mutations affecting protein stability and function, suggesting a potential role in tumor biology. Additionally, variable NOS2 expression, with higher levels associated with improved survival, highlights its possible prognostic value. Although these results underscore the importance of NOS2 as a potential biomarker and therapeutic target, further experimental validation and clinical correlation studies are required to confirm its functional significance and applicability in cancer diagnosis and treatment.

Acknowledgement:

We acknowledge and thank all the participants for their cooperation in the study

Conflict of Interest:

The authors reported no conflict of interest while performing this study.

Funding Agency:

The present project is supported/funded/sponsored by

- Saveetha Institute of Medical and Technical Sciences
- SaveethaDental College and Hospitals.
- Saveetha University

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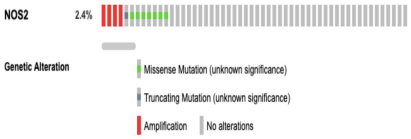

Figure 1: Oncoprint data demonstrating the genetic alterations in *NOS2* gene

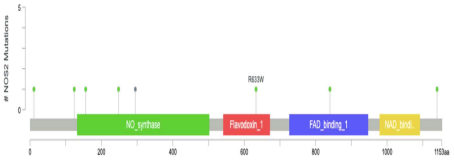

Figure 2: Lollipop plot demonstrating the mutations identified demonstrating the mutations identified in the HNSCC patients of TCGA dataset.

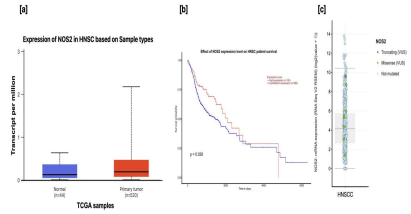

Figure 3: (a) The box-whisker plot comparing NOS2 transcript levels (expressed in transcripts per million) between normal tissue (n=44) and primary tumor samples (n=520). While median expression remains relatively low in both groups, the tumor samples show a broader range of high-expression outliers, (b) A Kaplan-Meier plot showing the effect of NOS2 expression levels on patient survival over time (days). Patients with high expression demonstrate a significantly improved survival probability compared to those with low/medium expression, with a calculated p-value of 0.028, suggesting that NOS2 expression is a statistically significant prognostic marker, (c) Mutational Landscape and mRNA Distribution. A jitter plot showing the distribution of NOS2 mRNA expression (log2 RSEM values) across HNSC samples.

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Table 1: The types of mutations identified in the *NOS2* genes of HNSCC patients (TCGA dataset)

Protein change	Mutation type	SIFT Score	SIFT prediction	Polyphen Score	Polyphen prediction	MutPred	MutPred prediction	IMutant
P248S	Missense	0.000	Affect protein function	1.00	Probably damaging	0.729	Pathogenic variant	Decrease
R633W	Missense	0.001	Affect protein function	0.82	Possibly damaging	0.535	Mildly deleterious	Decrease
S124N	Missense	0.54	Tolerated	0.00	Benign	0.230	Neutral	Decrease
R1140S	Missense	0.63	Tolerated	0.002	Benign	0.114	Neutral	Decrease

Q840K	Missense	1.00	Tolerated	0.240	Benign	0.123	Neutral	Increase
W295*	Nonsense	-	-	-	-	-		-
E156Q	Missense	0.08	Tolerated	0.074	Benign	0.217	Neutral	Decrease
T11S	Missense	0.08	Tolerated	0.028	Benign	0.282	Neutral	Decrease