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Deciphering the Genetic Alterations in *SPARC* Gene Family and Its Association with HNSCC

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Authors' contributions

This work was carried out in collaboration among all authors. Author JVP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ASSG and AP managed the analyses of the study. Author SV managed the literature searches and performed certain computational analysis. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study is to identify the genetic alteration in *SPARC* gene family and its association with head and neck squamous cell carcinoma (HNSCC). Head and neck cancer is a set of cancerous lesions arising from the squamous cell of the mucous membrane of the oral cavity, nose throat, larynx and pharynx. *SPARC* gene encodes for cysteine rich acid matrix metalloprotein, osteonectin whose expression in metastatic OSCC (Oral squamous cell carcinoma) was found to be higher. This expression pattern also correlated with the worst pattern of invasion and differentiation of OSCC tumors. In line with the above facts, the present study was carried out to

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ascertain the gene alterations and their consequences. Also the putative association of gene alterations with HNSCC was analyzed using computational tools. The Cancer Gene Atlas (TCGA, Firehose Legacy) dataset hosted by the cBioportal server was used in the present study. The non-synonymous variants identified were further assessed for protein stability and pathogenicity employing IMutant and PROVEAN tools. Gene amplification was observed in the *FSTL1* gene, which was also shown to present with the highest frequency of gene alterations (5%) among eight genes. Furthermore, the expression of the *FSTL1* gene was found to differ significantly among different grades of HNSCC. In conclusion, the study throws light on the possible association of the *FSTL1* gene of the *SPARC* family with HNSCC.

Keywords: HNSCC; SPARC; matrix metalloproteinases; metastasis; osteonectin.

1. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the most common type of cancer which accounts for more than 6,50,000 cases and 3,30,000 deaths annually. HNSCC represents the cancerous lesions arising from the squamous cell of the mucous membrane of the oral cavity, nose throat, larynx and pharynx [1]. According to the GLOBOCAN survey, 2018, the incidence of HNSCC was found to be clustered in specific regions in the world with a high incidence rate recorded in the south Asian countries [2]. While the total number of incidences of HNSCC has reduced since 1975 there can also be unusual bleeding, facial swelling, or trouble breathing. The most common etiology of head and neck cancer are tobacco smoking and alcohol consumption. It has been shown that the frequency of occurrence is more in men than women [3]. The human papilloma virus (HPV) has also emerged as a major cause of HNSCC among non-smokers and light drinkers [4].

SPARC gene encodes for osteonectin also known as basement membrane protein 40 or secretary protein acidic and rich in cysteine. It is a 32000 molecular weight glycoprotein and has high affinity for hydroxyapatite and type 1 collagen and regulates bone remineralisation [5]. It binds to the structural matrix protein such as vitronectin and collagen and as a consequence regulates the cellular interaction with the extracellular protein [6]. It is generally transient and expressed during embryogenesis, tissue repair and remodelling. Each SPARC member of the family possesses a characteristic conserved EC (E–F hand calcium binding) area with an E–F hand motif [7]. Based on collection homologies of the EC domain names, the gene family can be of 4 types- SPARC and Helvin, SMOCK1 & 2, Testican 1, 2 &3 and FSTL-1(Follistatin - like protein) [8,9] The most prominent proteases associated with tumourigenesis are matrix metalloproteinases which are produced due to proteolysis of the extracellular matrix. Due to the studies based on their association with multiple forms of cancer, they are considered as targets for drugs in anticancer therapies [8]. SPARC has also been proven to regulate the activity of matrix metalloproteinases and it's NH2 terminal regulates the activation of MMP2 at the cell surface and aids in tumour progression [10]. MMPs are a family of enzymes taken into consideration to be the primary mediators of ECM proteolysis and turnover. SPARC is also involved in extracellular matrix formation, remodelling and is associated with the initiation of changes in cell's shape.

Osteonectin (ON) are generally produced by fibroblasts [11]. ON has also been shown to play a major role in wound healing and is secreted by macrophages in such cases. Proliferation of specific cells, most commonly endothelial cells are mediated by osteonectin [12]. Overproduction of osteonectin is seen in scleroderma fibroblasts. This indicates the presence of osteonectin in tissue remodelling [13]. The human *SPARC* gene is located on chromosome 5q31-q33. Low expression of SPARC leads to osteogenesis imperfecta and osteoporosis while upregulation is seen in pulmonary and cardiac fibrosis, cardiac disease, and cancer [14].

2. MATERIALS AND METHODS

2.1 Data Source

The Cancer Genome Atlas, TCGA dataset consists of a total of 528 cases of head and neck squamous cell carcinoma, of which 512 tumor samples had sequencing and copy number alteration data. There is a full profile of mutated, amplified, deleted genes for each sample. Table 1 contains the demographic data of the patients

analysed in the study. Oncoprint data was obtained on submitting user-defined queries based on the eight crucial genes of the *SPARC* family *viz., FSTL1, SMOC1, SMOC2, SPARC, SPARCL1, SPOCK1, SPOCK2* and *SPOCK3* which were analysed for further study [15,16].

Table 1. Demographic details of patients analysed in the present study (as obtained from the cBioportal site)

Gender	Male (n = 386) Female (n = 142)
Mutation count	6-3181
Diagnosis age	19-90 years
Smoking status	Smokers: 515 Data not available: 12 Unknown: 1
Alcohol history	Yes – 352 No – 165 Data not available: 11
Neoplasm Histologic grade*	Grade 1: 63 Grade 2: 311 Grade 3: 125 Grade 4: 7 Grade GX: 18 Data not available: 4
Race category	White: 452 African: 48 Asian: 11 American Indian or Alaska native: 2 Data not available: 15

2.2 Oncoprint Data Analysis

Oncoprint analysis is the shortened and concise summation of the genetic alterations in graphical form. It provides data on multiple genes across a set of tumor samples. The details on the frequency distribution of variations in each of the genes, the variant allele frequency, gene deletions, amplifications, insertions, frameshift etc., were recorded [15,16].

2.3 Protein Stability Analysis

I-Mutant v3.0 is a support vector machine (SVM)based method for automated detection of improvements in protein stability through single point mutations. The change in the free energy change values (DDG) was used for interpreting the protein stability. A value > 0 was found to increase and < 0 was found to decrease the stability of the protein [17].

2.4 Pathogenicity Analysis

PROVEAN (Protein Variation Effect Analyzer) predicts the impact on the biological function of a protein upon substitution with an amino acid (Table 3). The present analysis employs a user-defined query of missense variants entered along with the reference sequence obtained from the NCBI database with a default cut-off value of -2.5. A score less than -2.5 or greater than -2.5 was considered to be deleterious and neutral respectively (Table 3) [18].

2.5 gnomAD Analysis

Dataset gnomAD v2.1.1 consists of an array of 125,748 exomes and 15,708 individual sequencing genomes. This database was used to check whether the variants identified in the present study were previously reported in the population. If the variant details are not available it was designated as novel [19].

2.6 UALCAN Analysis

The expression of the gene in HNSCC was analysed using the UALCAN (http://ualcan.path.uab.edu/cgi-bin/TCGA-survival1.pl?genenam=SPARC&ctype=HNSC) database. Survival curve analysis based on the tumor grade and expression profile was performed to demonstrate the putative role of *SPARC* gene with HNSC. Gene expression data are expressed as transcripts per million (TPM). The survival effect analysis based on the gene expression pattern was assessed using Kaplan-Meier survival analysis [20].

3. RESULTS AND DISCUSSION

Head and neck cancers have globally affected more than 5.5 million people and have caused about 379,000 deaths [21]**.** The majority of the squamous cell carcinomas occur in the head and neck region [22]. Genetic factors have also been recognised as an important predisposing factor of squamous cell carcinoma [23]. Head and neck cancer includes oral, pharyngeal, laryngeal and throat cancer [24]**.** The primary database used was cBioportal which hosts several datasets of which the dataset (TCGA, Firehose Legacy) was selected for the present study. The TCGA dataset had information on 528 HNSCC patients (530 samples). The male:female ratio was found to be 2.7:1, with the diagnostic age groups ranging from 19 - 90 years. The number of individuals with the history of smoking and alcohol were roughly around 98% (515 individuals) and 67% (352 individuals). The dataset had samples from patients of American (85.6%), African (9.1%), Asian (2.1%) and

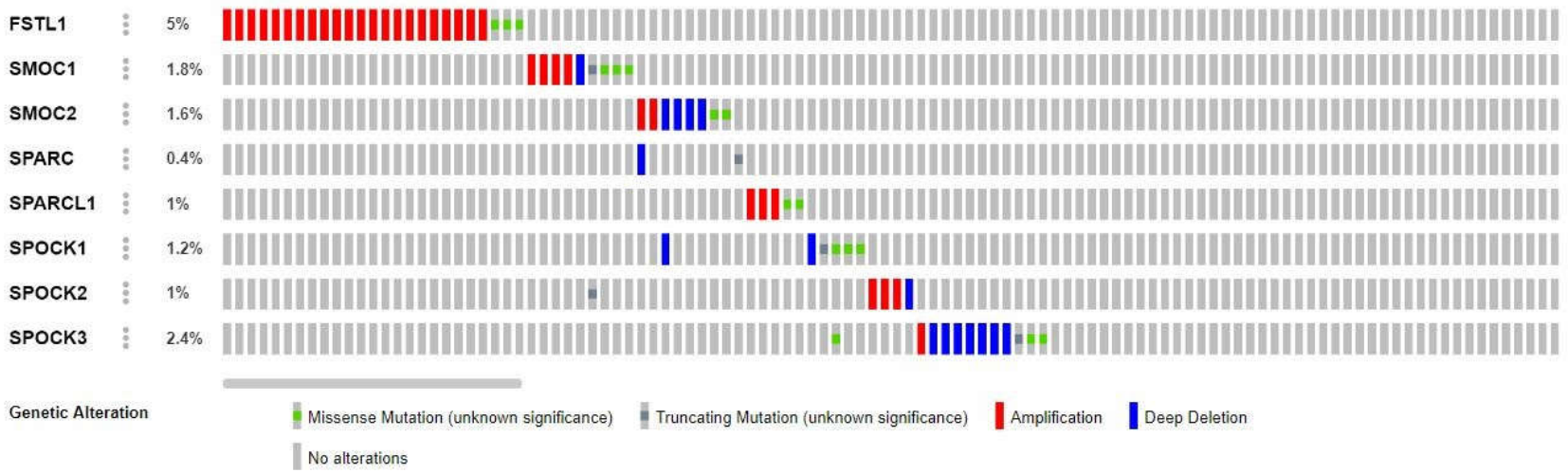

Fig. 1. Oncoprint analysis depicting multiple types of alterations in the *SPARC* gene family. Each of the grey bars represent patients with HNSC

American Indian (0.4%) descent. The distribution of patients based on the histologic grade of neoplasm is given in Table 1, of which 59% of patients had grade 2 tumor.

Oncoprint data analysis was performed to analyse the genetic alterations or variations seen in the gene family. Here, the *FSTL1* gene has been observed to have a high level of variation (Fig. 1). The oncoprint data analysis revealed gene amplification in 8 genes, of which *FSTL1* (5%) harboured the highest frequency of gene amplification. The genes *SMOC1, SMOC2, SPARC, SPOCK1, SPOCK2* and *SPOCK3* demonstrated deep deletions. The *SMOC1* and *SPOCK3* genes harboured the highest number of variations/mutations from among all the genes identified with alterations (Table 2). Several truncating and mis-sense variants of unknown significance have been documented (Fig. 1). Genetic alterations in the genes associated with the SPARC family have been documented. The alteration of each gene has been noted and seen whether the changes noted were new and novel or already reported in previous studies. Only missense mutation has been taken into consideration here. In the *FSTL1* gene, which shows the most variance, the changes noted are, amplification, replacement of glutamic acid by lysine in the 280th position, replacement of aspartic acid by asparagine in the 242nd position and replacement of asparagine by lysine in the 69th position of the amino acid chain.

A total of 5 reported variants were identified using gnomAD analysis viz., *SMOC1* (*rs899963298, rs766160933*), *SPARCL1* (4-88415749-GCCTT-G), *SPOCK2* (*rs1291754865*) and *SPOCK3* (*rs1400335404*). All the variants identified in the present study had a minor allele frequency < 0.01, implying the fact that these are rare variants that may be associated with the risk of a particular disease.

The stability of the protein largely affects the biological function of the protein. Hence, protein stability was assessed for all the non-synonymous variants identified in the study. The I-Mutant analysis produced a score which was used for interpretation of results. The protein stability for each of the non-synonymous variants were assessed and tabulated (Table 3). The majority of missense variants observed were found to decrease the stability of the protein product, thereby giving away a chance for influencing the catalysis process. Although presented with decreased stability all the variants were not found to lead to a deleterious phenotype. Interestingly, the majority of the variants produced neutral effect with the exception in a few gene variants exhibiting deleterious outcomes viz., *FSTL1, SMOC1, SMOC2* and *SPOCK1*.

Considering the *FSTL1* gene, as it exhibits the highest frequency of alterations among the eight genes its expression has been documented across various grades of HNSC tumour. The comparison of gene expression patterns between different grades of HNSC returned significant values between grade 1 vs grade 2 ($p=6.2 \times 10^{-3}$), grade 1 vs grade 3 ($p=1.13 \times 10^{-2}$) and grade 3 vs grade 4 ($p=3.8 \times 10^{-2}$). A p value less than 0.05 is considered to be significant (Fig. 2). The effect of differential expression pattern of FSTL1 gene upon the survival probability of HNSC patients was also recorded. A significant difference (p value = 0.011) was found between low/medium level expression in African American patients and low/medium level expression in Caucasian patients. A low/medium level expression presented with a low survival rate in African American patients. Furthermore, high-level expression in Caucasian patients and low/medium level expression of the *FSTL1* gene in African American patients also returned a significant result (p value = 0.04). Here, a low/medium level expression was related to a low survival rate in African American patients (A p value less than 0.05 is considered to be significant) (Figs 3a and 3b).

Based on a previous study done on oral cancer, it was observed that the OSCC cells expressed more SPARC expression than normal cells. Also, a higher level of SPARC expression correlated with lesser differentiation of the cells and thus, a higher grade of cancer [25,26]. Increased number of SPARC positive cells are seen in leukoplakia, carcinoma in situ and early SCC. It was also found that the malignancy or the migration of the tumor cells were not brought about or regulated by the *SPARC* gene. In the early stage of neoplasia, the dysplasia cells tend to induce SPARC which may improve the survival characteristics but it is not involved with metastasis which occurs in the final stages of cancer [27]. SPARC expression in different tumours is variable. SPARC is expressed in high levels in breast [28], neuroblastoma, rectal and brain cancers [29-32] and in low levels in pancreatic cancer [33,34], bladder cancer [35] and acute leukemia [36] It was also seen experimentally that there is the presence of SPARC localised in the stroma adjacent to the tumor in OSCC patients. Also

Table 2. The frequency of genetic alterations, cytogenetic location of the gene, protein encoded by genes, variant allele frequency in tumor sample and population data as obtained from gnomAD for the *SPARC* gene family

Gene	Protein	Alteration	Cytogenetic location	% of alteration	Variant allele frequency in tumor sample	gnomAD frequency data
FSTL1	Follistatin like 1	Amplification E280K D242N N69K	3q13.33	5	0.35 0.29 0.24	Novel Novel Novel
SMOC1	SPARC related modular calcium binding 1	Amplification Deep deletion R382Q D164G Q42* E302D	14q24.1	1.8	0.25 0.10 0.22 0.13	rs899963298 rs766160933 Novel Novel
SMOC2	SPARC related modular calcium binding 2	Amplification Deep deletion E371K S438F	6q27	1.6	0.25 0.25	Novel Novel
SPARC	Secreted protein acidic and cysteine rich	Deep deletion V41=	5q33.1	0.4	0.34	Novel
SPARC1	SPARC like 1	Amplification D659N A68V	4q22.1	1	0.20 0.08	Novel 4-88415749-GCCTT-G
SPOCK1	SPARC (osteonectin), cwcv and kazal like domains proteoglycan 1	Deep deletion V96G F237L X62_splice G362E	5q31.2	1.2	0.29 0.27 0.26 0.40	Novel Novel Novel Novel
SPOCK2	SPARC (osteonectin), cwcv and kazal like domains proteoglycan 2	Amplification Deep deletion Y278Hfs*9	10q22.1	1	0.14	rs1291754865
SPOCK3	SPARC (osteonectin), cwcv and kazal like domains proteoglycan 3	Amplification Deep deletion Q350S V434I S252* K332N	4q32.3	2.4	0.23 0.47 0.20 0.36	Novel Novel rs1400335404 Novel

higher SPARC expression was noted in tumours of the tongue rather than tumors of the lips. The SPARC expression also changes according to the grade of the tumor [37].

In a study by Tai et al., the SPARC gene is shown to play a multi-faceted role in various types of tumors. It is seen that, while in cancers such as gliomas and melanomas SPARC isCorrelated with a highly aggressive tumor phenotype, in other cases like colorectal cancers and neuroblastomas, it functions as a cancer suppressor [38]. This can be attributed to the fact that the latter types of cancers have a lesser metastatic property when compared to gliomas and melanomas [39]. It has been noticed by Yiu et al., that the down regulation of SPARC is essential for ovarian carcinogenesis as the SPARC tends to induce apoptosis in the ovarian cancer cells and thus sensitising the cells to the apoptotic activity of SPARC [40]. In a research for novel markers for poor prognosis in HNSCC by Chin et al., SPARC was found to be highly expressed in transition from normal mucosa to

Table 3. Protein stability and pathogenicity analysis of non-synonymous variants as predicted using IMutant and PROVEAN tools

Gene	Variation	I-Mutant prediction	Score	PROVEAN score	Prediction
FSTL1	E280K	Decrease	-0.06	-1.494	Neutral
	D242N	Increase	0.55	-2.951	Deleterious
	N69K	Decrease	-0.74	-3.905	Deleterious
SMOC1	R382Q	Decrease	-2.13	-1.598	Neutral
	D164G	Decrease	-2.02	-2.758	Deleterious
	E302D	Decrease	-1.38	-0.584	Neutral
SMOC2	E371K	Decrease	-2.05	-3.094	Deleterious
	S438F	Increase	1.17	-1.365	Neutral
SPARCL1	D659N	Decrease	-0.15	-2.118	Neutral
	A68V	Decrease	-1.35	-0.825	Neutral
SPOCK1	F237L	Decrease	-1.94	-1.571	Neutral
	G362E	Increase	0.12	-6.517	Deleterious
SPOCK3	Q350S	Decrease	-0.80	-1.564	Neutral
	V434I	Decrease	-1.21	-0.254	Neutral
	K332N	Increase	0.48	-1.527	Neutral

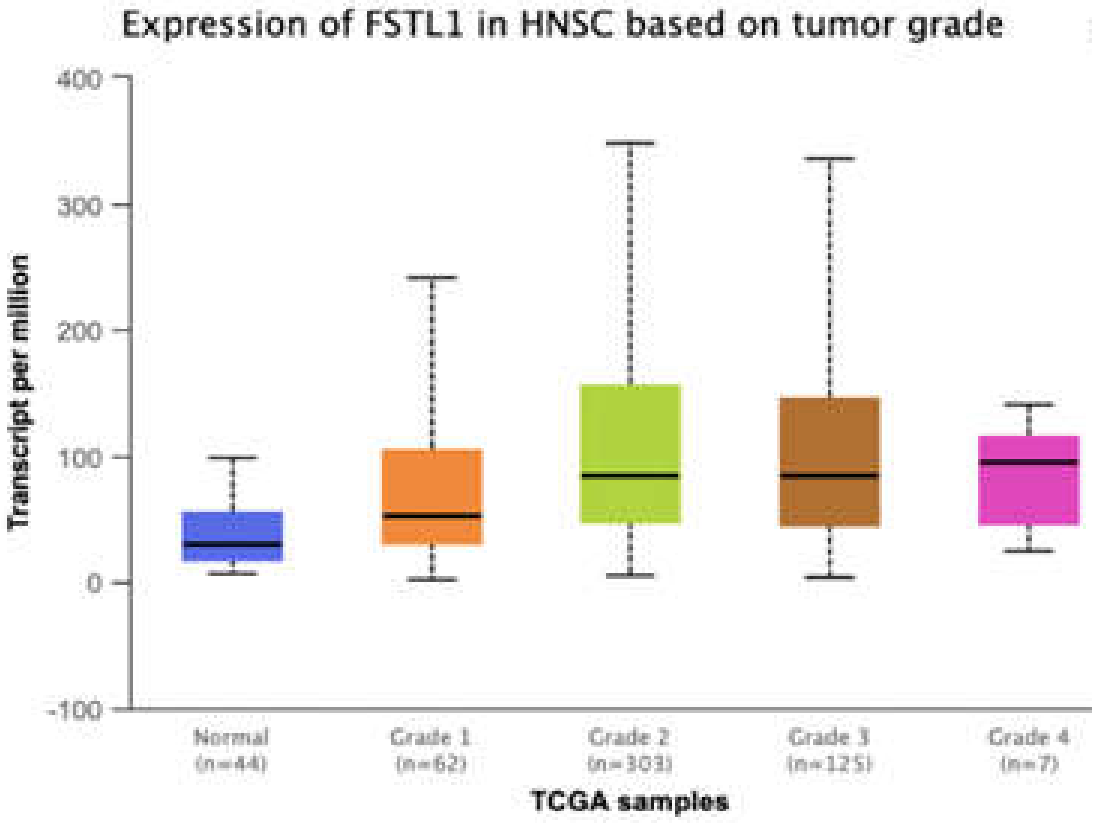

Fig. 2. Box-Whisker plot showing relative expression profile of *FSTL1* gene in different grades of HNSC. The X axis denotes the TCGA samples and Y axis denotes the transcripts per million values. The comparison of gene expression patterns between different grades of HNSC returned significant values between grade 1 vs grade 2 ($p=6.2 \times 10^{-3}$), grade 1 vs grade 3 ($p=1.13 \times 10^{-2}$) and grade 3 vs grade 4 ($p=3.8 \times 10^{-2}$). A p value less than 0.05 is considered to be significant

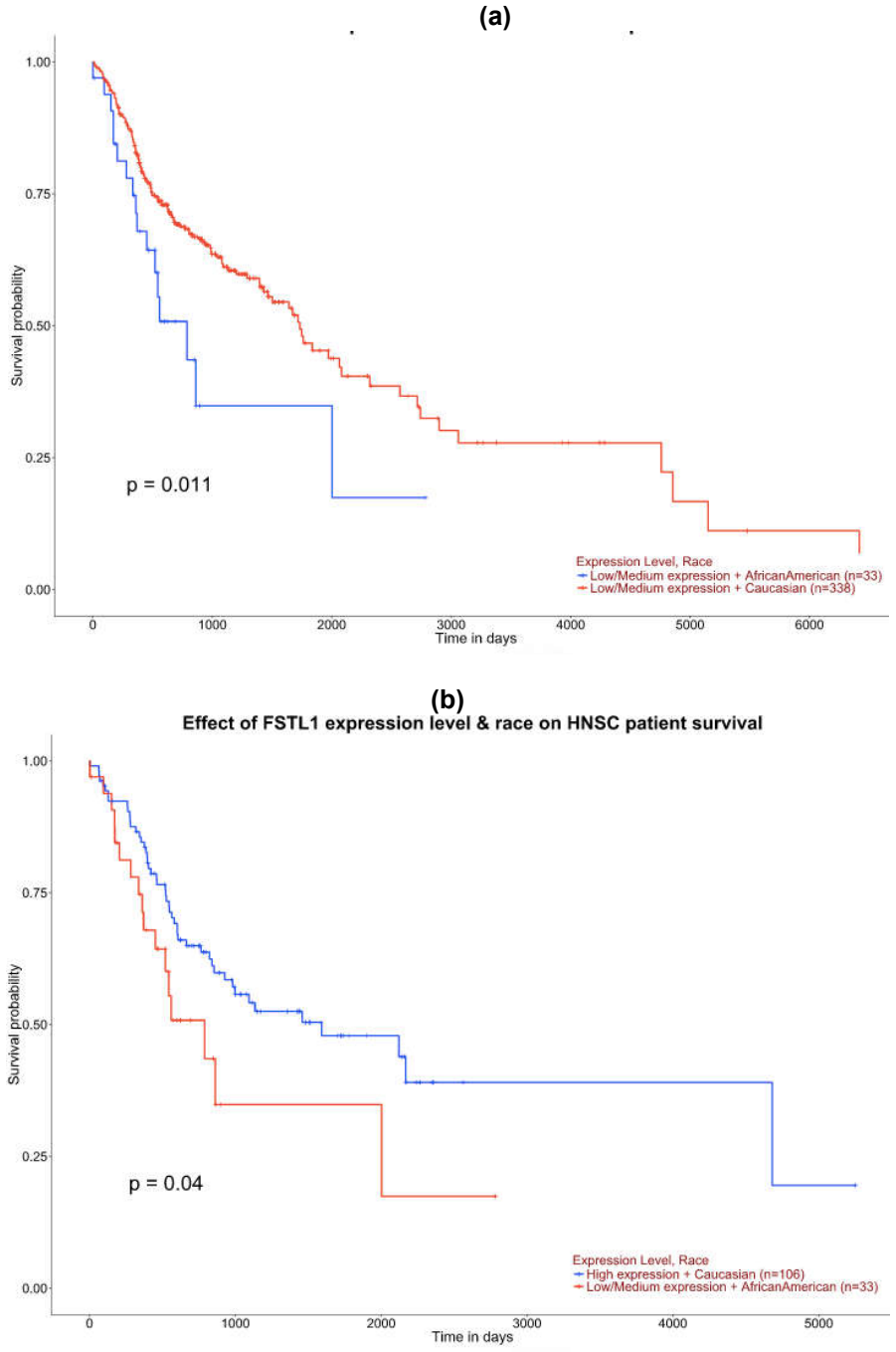

Fig. 3. Kaplan–Meier plots showing the association of *FSTL1 I* gene expression in combination with the race with HNSC patient's survival. The x-axis represents time in days and y-axis shows the survival probability. (a) The blue line indicates low/medium expression in African American patients and the red line indicates low/medium level expression of the *FSTL1* gene in Caucasian patients. A significant difference in the level of gene expression between the two groups was observed (p value = 0.011). A low/medium level expression presented with a low survival rate in African American patients. (b) The blue line indicates high level expression in Caucasian patients and the red line indicates low/medium level expression of the *FSTL1* gene in African American patients. A significant difference in the level of gene expression between the two groups was observed (p value = 0.04). Here, a low/medium level expression presented with a low survival rate in African American patients. A p value less than 0.05 is considered to be significant

tumor tissues [41]. However, in a study by Neil et al., it was found that even though the SPARC was upregulated in head and neck cancer patients, it tended to increase the accumulation of albumin in the tumor, thus assisting in the increase in the effectiveness of albumin bound Paclitaxel, a chemotherapeutic drug and thus aid in better clinical outcomes of SPARC positive patients with poor prognosis [42]. Another research on the association between *SPARC* and anticancer drugs by Trieu et al. yielded similar result [43]. Computational approach has long been used to screen for alterations in candidate genes involved in the crucial biochemical pathways leading to the disease phenotype [44]. Hence the same methodology has been used in this study which shows that the expression of *SPARC* gene variants in patients with HNSC.

4. CONCLUSION

The markers identified in the present study have to be screened in specific populations to derive an association between the genetic markers and HNSCC. This will open new avenues towards identification of potential diagnostic and therapeutic leads. Therefore, results of the computational process require further experimental validation to prove the association factor.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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