ABSTRACT

Background: Oral cancer accounts for 2 to 4% of all cancer and 90% of oral cancer cases are squamous cell carcinoma. Apart from etiological factors genetic factors also play an important role in oncogenesis. The common type of sequence variations is single nucleotide polymorphisms (SNPs), described as a change in the coding and amino acids sequence in the related proteins which could confer protective or lethal effects on the organism.

Objective: To analyze the genetic variation in the CA9 gene (rs2071676) and to compare allele frequencies in different populations worldwide. The rs2071676 variant of the CA9 gene is a missense variant that results in the substitution of the amino acid methionine in place of valine.

Methods: Genotype frequencies of the SNP rs2071676 were collected from the Ensembl database for different populations and the deviations were analyzed. Furthermore, the expression profile of the CA9 gene in HNSCC was assessed using in-silico tools. The survival of patients based on the expression of the CA9 gene was also assessed.

Results: The present study identified deviations in allele frequencies for rs2071676 polymorphism between different populations. The minor allele frequency in the ancestral population or the African population was found to be much lower than the other populations such as American, East Asian, and South Asian, where both the alleles showed similar frequencies.

Conclusion: The significant deviation between allele frequencies in different groups provides clues about the positive selection of these alleles in certain populations. This led us to further investigate the plausibility of association of the CA9 gene with HNSCC. The intriguing facts observed from the study could aid in revealing the association between the CA9 gene and the progression of oral cancer.

Key Words: CA9 gene, Polymorphism, SNP, Oral cancer, Genetic variation, In silico study

INTRODUCTION

Oral cancer accounts for 2 to 4% of all cancer cases in the world. 90% of oral cancer cases are squamous cell carcinoma (OSCC).1-3 Globocan in 2012 stated that the worldwide oral cancer incidence is 2.1%.4 In India, the prevalence of oral cancer is high and it accounts for 45% of all the cancers reported in India.5 In Asian countries, the major etiological factors for developing OSCC include tobacco, areca nut chewing, and smoking.7,8 Genotoxic substances present in tobacco and areca nuts such as nitrosamines, polycyclic aromatic hydrocarbons, volatile aldehydes, and hydroquinone are converted to carcinogens. Nicotine derived nitrosamine, n-nitrosonornicotine, NNK, areca nut alkaloids, arecoline, arecaidine can result in the formation of oral submucous fibrosis, oral precancerous lesions, and oral cancer.7,9-11

Apart from the etiological factors, genetic factors also play a major
role in oncogenesis.\textsuperscript{12-15} It is well known that the tumor cell during its course of development undergoes molecular alterations in several cellular molecules including DNA, RNA, and proteins. This could be attributed to the inherent biological properties of the cancer cell.\textsuperscript{16,17} The up-regulation and down-regulation of certain genes were found to be involved in extracellular matrix degradation (ECM), epithelial to mesenchymal transition (EMT), and detoxification pathways.\textsuperscript{18,19}

The most common type of DNA sequence variation is single nucleotide polymorphisms. This results in errors in the coding sequence, which leads to alterations in the amino acid being coded or incorporated into the protein chain.\textsuperscript{2} SNP directly or indirectly affect the gene expression pattern and functions.\textsuperscript{12} SNPs at intronic regions of genes may result in three-dimensional changes in the DNA molecules which result in Gibbs free energy change. The possible consequence of it may impact DNA polymerases enzyme activity and transcription factor binding. SNPs can be present as homozygous and heterozygous. An ancestral allele is referred to as the wild type allele and the altered allele is referred to as SNP or variant allele.\textsuperscript{7}

CA9 is a gene located on chromosome 9p13.3.\textsuperscript{12} CA9 gene codes for carbonic anhydrase 9 which is a glycoprotein and a member of a family of metalloenzymes which can catalyze the reversible hydration of carbon dioxide into carbonic acid.\textsuperscript{20} CA9 glycoprotein plays an important role in the stabilization of extracellular pH hydration of carbon dioxide.\textsuperscript{20} Solid tumor have a common feature called cellular hyperoxia.\textsuperscript{21} Tumor hypoxia causes tumor cells to undergo adaptive changes that can enable them to survive and proliferate.\textsuperscript{12} Increased CA9 synthesis is regulated through the binding of hypoxia-inducible factor (HIF) to a hypoxia response element (HRE) within its basal promoter. This contributes to the neutralization of intracelluar pH and acidification of extracellular microenvironment which promotes cell proliferation, adhesion, and invasion.\textsuperscript{21} CA9 expression is highly increased in various cancers and it has been considered as an endogenous marker for tumor hypoxia.\textsuperscript{24,25} Several studies have reported various polymorphisms of the CA9 gene in renal cell carcinoma.\textsuperscript{26} The increased expression of the CA9 gene is also seen in several cases of oral squamous cell carcinoma. Haplotypes of rs2071676, rs3829078, and rs1048638 when combined have potential in predicting significance in oral carcinogenesis and the gene-environment interaction of CA9 polymorphism.\textsuperscript{12} This study was used to determine the consequence of a genetic variation (rs2071676) in the CA9 gene and their putative association with OSCC.

**MATERIALS AND METHODS**

One of the exon variants of the CA9 gene was selected for the study based on the literature mining process. Since oral cancer is a disease that is more predominant in patients with a chronic history of using smoking and smokeless tobacco such as pan, gutka, etc., genetic variants that were more closely related to environmental factors were identified in the gene and selected. A recent study by Chien et al., 2012, demonstrated that patients who are heterozygous for rs2071676 polymorphism had a higher risk of developing lymph-node metastasis when compared to patients with homozygous AA allele.\textsuperscript{12} The Ensembl database was used to acquire the frequency data of the polymorphism variant in different populations (https://asia.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=20:56387216-56388216;v=rs2064863;vdb=variation;vf=52924966).\textsuperscript{27} IMutant suite version 2.0 was used to identify the stability of the protein.\textsuperscript{28} PROVEAN and MutPred were the tools used to predict the pathogenicity of the variant.\textsuperscript{29} Furthermore, the expression of the CA9 gene in HNSCC was analyzed using the UALCAN (http://ualcan.path.uab.edu/cgi-bin/TGCA-survival1.pl?genenam=CA9&ctype=HNSC) database.\textsuperscript{30} Survival curve analysis based on the tumor grade and expression profile was performed to demonstrate the putative role of the CA9 gene with HNSCC. Furthermore, the expression of the CA9 gene in HNSC was analyzed using the UALCAN database. Expression in different grades of tumors were assessed viz., grade 1- well-differentiated, grade 2- moderately differentiated, grade 3- poorly differentiated, grade 4- undifferentiated. Transcript per million (TPM) is a normalization method for RNA-seq data. The TPM values used for the generation of box whisker plots were also used to determine the significant difference between the groups. The t-test was performed using a PERL script with the Comprehensive Perl Archive Network (CPAN) module. Combined survival effect analysis of gene expression and other clinical parameters such as race, gender, tumor grade, cancer subtypes were assessed using multivariate Kalpan-Meier survival analysis.

**RESULTS AND DISCUSSION**

The CA9 gene can be considered as a potentially important biomarker for the evaluation of tissues. The CA9 gene codes for carbonic anhydrase-IX protein. This protein is a tumor-associated cell-surface glycoprotein that is induced during hypoxia. This protein is also involved in adaptation to acidosisis and implicates in cancer progression via its catalytic activity and/or non-catalytic functions.\textsuperscript{31} CA9 gene expression in non-cancerous tissues is rare and confined to epithelia of the stomach, gallbladder, pancreas, and intestine.\textsuperscript{30} CA9 expression is significantly up-regulated in several cancers like lung, colon, colorectal, gastric, pancreatic, breast, cervix, bladder, ovaries, brain, head and neck, and oral cancers.\textsuperscript{31,32} Lin et al., identified polymorphic variants in the CA9 gene which was a predictor of lymph node metastasis of prostate cancer. Their study was the first to demonstrate the correlation between CA9 gene polymorphism and expres-
These various findings suggest that modifier genes...may contribute to the disruption of cell adhesion structures leading to tissue invasion and metastasis. CA9 expression was also found to be indicative of aggressive and resistant tumors. In this study, the global allelic frequency of rs2071676 polymorphism in the CA9 gene was found to be 65% for G and 35% for A allele (Figure 1). The global distribution among different populations like African, American, East Asian, European, and South Asian were also included (Figure 2). To derive a more vivid picture about the deviations in allele frequencies for the selected polymorphism, the allele frequency was computed from the data available from the reports of Chien et al., 2012. Since the population described in the report was of Taiwanese population, the East Asian data from the database was selected for comparison and is presented in figures 3 and 4. The results showed a slight deviation in the altered and reference allele frequencies. But when the same data was compared with the ancestral population i.e., the African population [G allele - 92%; A allele - 8%] the allele frequency was found to deviate dramatically. This result showed that the A allele was positively selected in certain populations. Generally, an allele tends to be positively selected when it is beneficial to the individual or a group. Interestingly, we observed that the variant allele frequency to be markedly elevated. The reason for the same could provide clues on the putative association of the CA9 gene with the progress of HNSCC.

The haplotype of rs2071676, rs3829078, rs1048638 combined have a potential predictive value in oral carcinogenesis. Although a single variation in the gene might not always affect the gene expression profile, the cumulative effect of several genes might influence the process of gene expression. The placement of the variant in the gene and the type of alteration will determine the consequences of the variant. The lymph-node metastasis tendencies in oral cancer were highly in heterozygous GA allele combination rather than AA allele combination. The missense mutation of the CA9 gene can cause altered CA9 gene expression and protein production. The prognostic value of CA9 expression has been proven for other cancers such as renal clear cell carcinoma, cervical, colorectal, esophageal, lung, and breast cancer. These various findings suggest that mutations in the CA9 gene play an important role in all OSCC progression.

In the present study, the I-Mutant analysis (DDG value: -1.02) revealed that the variation from valine to methionine decreased the stability of the protein (data not shown). Furthermore, the pathogenicity analysis of the resultant protein after substitution with variant amino acid was assessed using Mutpred and PROVEAN tools which returned scores that predicted it to be neutral or non-pathogenic (data not shown). Despite the contrasting results, experimental evidence emphasizing the functional role of the CA9 gene would aid in deriving the association between the candidate gene and the disease phenotype. Gene expression analysis revealed an increase in the transcript levels with increasing grades of tumor which differed significantly in most of the groups (Figure 5). In addition, the Kaplan-Meier method was used to assess the survival period of head and neck squamous cell carcinoma (HNSCC) patients based on the expression of the selected gene. The results showed a marginal significance on the survival profile of patients with high and medium/low-level expression of CA9 gene [p value = 0.073]. High expression of CA9 gene in grade 2 HNSCC patients correlated with poor prognosis compared to grade 1 patient with HNSCC. In other words, there was a sharp decline in the survival of patients upon down-regulation of the CA9 gene in HNSCC patients (Figure 6a). Furthermore, we observed that the low/medium level expression among African-American and Caucasian population exerted a significant effect on the survival of patients. African-American patients with a low/medium expression of the CA9 gene exhibited low survival probability when compared to Caucasian patients (Figure 6b). The cumulative effect of single nucleotide variants might play a vital role in the development and progression of a disease. Since cancer is a multifactorial disease precipitated by the interaction of several candidate genes, a thorough knowledge of the consequences of individual variants and their collective effect would aid in deducing the possible mechanisms underlying the phenotype analyzed. The association of genetic variants of the CA9 gene with HNSCC is worth studying since it might play a direct role in tumor metastasis and resistance. Identifying significant genetic markers will aid in creating an SNP panel which can be correlated with progressing grades of tumor. Various etiological factors like consumption of tobacco and its products can also induce the aberrant expression of the CA9 gene via the expression of nuclear hypoxia-inducible factor. Consumption of tobacco is one of the main etiological factors of oral cancers in the South Asian population. Hence a detailed study to analyze the CA9 gene expression on oral cancer susceptibility in the South Asian population needs to be done to get a more comprehensive picture. Computational analysis has been used to identify potential targets related to the cancer phenotype. Hence such studies would aid in solving population-based queries related to polymorphic markers associated with cancer.
CONCLUSION

In conclusion, the present study is the first of its kind which was designed to assess the frequency of rs2071676 polymorphism in different populations and reveal the deviation which could be associated with positive selection of an allele in specific populations. The intriguing facts observed from the study could aid in revealing the association between the CA9 gene and the progression of oral cancer. Further experimental validation is warranted to confirm the selection of minor alleles in the south Asian population. Effects of the various etiological factors of oral cancers like consumption of tobacco products resulting in hypoxia and aberrant expression of CA9 gene can be better studied in the South Asian population. Genotype analysis of rs2071676 polymorphism of CA9 gene in the South Asian population should be performed to identify the positive selection of A allele and its prevalence. However, more experimental evidence should be accumulated to identify the association between rs2071676 polymorphism of CA9 gene and oral cancer susceptibility in the South Asian population.

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Conflict of Interest

The authors have no potential conflict of interest.

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Figure 1: Pie graph showing Global distribution of allele frequency for rs2071676 polymorphism of CA9 gene [data derived from Ensembl database]. The global reference allele frequency is 65% and represented in blue color and the global altered allele frequency is 35% and represented in orange color.

Figure 2: The bar graph showing Allele frequency of rs2071676 polymorphism of CA9 gene among different populations. X-axis representing the different populations [AFR - African; AMR - American; EAS - East Asian; EUR - European; SAS - South Asian] and Y-axis representing the percentage allele frequency. The reference allele frequency is represented by blue color and altered allele frequency is represented in orange color.
**Figure 3:** The bar graph showing the comparison of allele frequencies between East Asian [Ensembl] and case population [Taiwanese population - East Asian] derived from experimental data [Chien et al, 2012]. X-axis represented the East Asian and case population and Y-axis representing the percentage of allele frequency. The reference allele is represented in blue and altered allele is represented in orange color.

**Figure 4:** The bar graph showing the comparison of allele frequencies between East Asian population [Ensembl] and control [Taiwanese population - East Asian] derived from experimental data [Chien et al, 2012]. X-axis represented the East Asian and case population and Y-axis representing the percentage of allele frequency. The reference allele is represented in blue and an altered allele is represented in orange color.

**Figure 5:** The Boxplot showing expression profile of CA9 gene in different grades of HNSCC tumor. The X-axis denotes grades of tumor relative to normal expression of CA9 and Y-axis denotes mRNA counts expressed as TPM (transcript per million). The comparison of gene expression pattern between different grades of HNSC returned significant values between Normal vs Grade 1 (p=2.8 X 10^{-6}), Normal vs Grade 2 (p=1.6 X 10^{-12}), Normal vs Grade 3 (p=3.6 X 10^{-13}), Normal vs Grade 4 (p=3.7 X 10^{-2}), Grade 1 vs Grade 3 (p=3.5 X 10^{-2}), Grade 1 vs Grade 4 (p=1.14 X 10^{-2}), Grade 2 vs Grade 4 (p=1.44 X 10^{-4}), Grade 3 vs Grade 4 (p=2.3 X 10^{-6}).
Figure 6: Kaplan-Meier curve (a) showing the effect of differential CA9 gene expression on the survival of HNSCC patients with different grades of the tumor (p-value = 0.073). X-axis represented the time in days and Y-axis representing the survival probability. (b) showing the effect of CA9 gene expression level and race (low/medium expression in African-American vs Caucasian population, p value = 0.044) on HNSCC patient survival. X-axis representing the time in days and Y-axis representing the survival probability.