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Hypoxia responsiveness linked variant in *EGLN1* gene is enriched in oral cancer patients

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ABSTRACT

Objective: The aim of this study was to determine the association of *EGLN1* gene variant SNP rs479200 (T > C) with the risk of oral cancer.
Materials and methods: A case-control study was conducted by involving 103 oral cancer patients and 206 age and gender-matched healthy controls. SNP rs479200 was genotyped by polymerase chain reaction-restriction fragment length polymorphism method.
Results: Minor allele frequency was 47 % in oral cancer patients and 35 % in healthy individual ($P = 3.0 \times 10^{-3}$, Odds ratio = 1.61). The association was highest under the additive genetic model (0.0005).
Conclusions: Our results show that the *EGLN1* gene variant SNP rs479200 is associated with the risk of developing oral cancer. This relationship highlights the significance of oxygen sensing in the pathophysiology of oral cancer.

1. Introduction

Oral cancer is a common cancer worldwide and an important cause of mortality in the developing world (Sankaranarayanan et al., 2005; Subapriya, Thangavelu, Mathavan, Ramachandran, & Nagini, 2007). It is one of the most common cancer in India with a mortality rate of about 50 % (Kumar, Nanavati, Modi, & Dobariya, 2016). The major risk factors of oral cancer are tobacco use, alcohol use, high-risk strains of human papillomavirus (HPV), and genetic factors (Chocolatewala & Chaturvedi, 2009). More than 100 different strains of HPV are oncogenic in nature (Shukla et al., 2009). Genetic risk factors involve gene polymorphisms in critical pathways such as cell cycle regulation, DNA repair, immunity and inflammation (Wu, Zhao, Suk, & Christiani, 2004). Knowledge of oral cancer pathogenesis will help in developing biomarkers and drug targets.

Neo-angiogenesis is a cardinal event in the pathogenesis of solid tumors. Tumor expansion results in the buildup of hypoxia in the core (Carmeliet, 2005). This activates the hypoxia response pathway and the upregulation of angiogenic factors. (Krock, Skuli, & Simon, 2011). The new vessels formed as a result support nutrient and gas exchange in the tumor core.

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that plays a key role in the hypoxia response pathway. HIF-1 protein comprises of α and β subunits that are expressed in a differential manner. The expression of the β subunit is constitutive while that of the α subunit is inducible by hypoxia. Hypoxia promotes the accumulation of HIF-1α subunit while normoxia promotes its rapid degradation. The degradation proceeds through the ubiquitin-proteasome pathway (Tekin, Dursun, & Xi, 2010). HIF-1α subunit undergoes degradation on hydroxylation. This reaction is catalysed by an enzyme called prolyl hydroxylase domain-containing protein 2 (PHD2). Therefore, PHD2 plays a key role in regulating the cellular levels of the HIF-1α subunit. Its functional integrity would thus be important for hypoxia-responsiveness of a cell. PHD2 enzyme is coded by the *EGLN1* gene that is located on chromosome 1. A common *EGLN1* gene variation viz., single nucleotide polymorphism (SNP) rs479200 T > C is associated with hypoxia-responsiveness. The C allele is linked to the reduced expression of the PHD2 enzyme. The activities of PHD2 and HIF-1 show a reciprocal relationship. As a result, individuals with the C allele show poor response to hypoxia (Aggarwal et al., 2010).

Hypoxia-induced neo-angiogenesis is an important feature of carcinogenesis. Thus, factors promoting hypoxia-responsiveness would be common in cancer patients. *EGLN1* gene polymorphism rs479200 affects the functioning of the hypoxia response pathway. Due to this, hypoxia-responsiveness supporting allele should be enriched in cancer patients. There is no information on the status of this polymorphism in cancer patients. Therefore, this study was undertaken. The aim of this study was to evaluate the association between the *EGLN1* SNP rs479200

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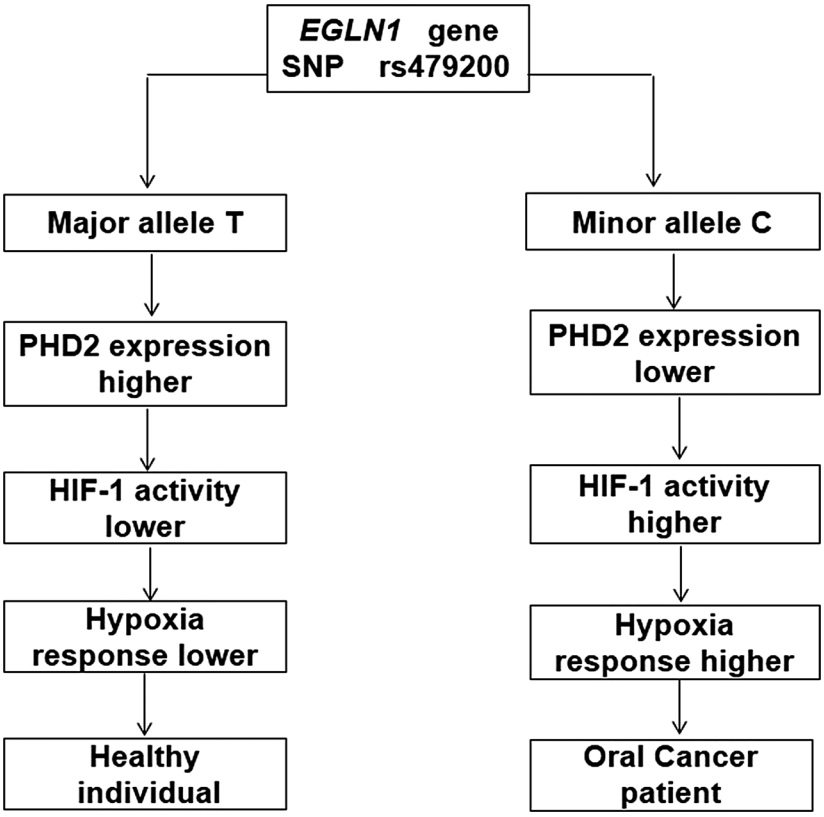

Fig. 1. Schematic representation of the pathophysiological mechanism for the link between the *EGLN1* gene variant (SNP rs479200) and oral cancer.

and oral cancer. The hypothesis of the study is schematically represented in Fig. 1.

2. Materials and methods

2.1. Study design and participants

A 1:2 case-control design was used to conduct this study. The case group comprised of 103 oral cancer patients while the control groups comprised of 206 healthy individuals. The sample size was selected based on a pilot study of the frequency of the minor allele in the control group and the odds ratio for its association with squamous cell carcinoma. All participants gave informed consent in writing before enrolment in the study. The study was conducted in accordane with the Declaration of Helsinki. The inclusion criteria for patient recruitment were: (i) patients diagnosed with oral squamous cell carcinoma (OSCC), (ii) both male and female patients and (iii) age ≥18 years. The exclusion criteria for patient recruitment were (i) patients who had undergone radiotherapy. OSCC was diagnosed based on clinical and histopathological evaluation. Age and gender-matched individuals without a history of any cancer were recruited as controls. Genomic DNA was prepared from the tumor or blood samples of the patients and controls respectively. The genotype of the *EGLN1* gene variant was the main outcome of the study. The differences in the allele frequencies in the study groups was assessed by statistical methods.

2.2. Preparation of genomic DNA

The salting-out method was used to prepare the genomic DNA (Miller, Dykes, & Polesky, 1998). UV spectrophotometric method was used to determine the purity and the concentration of the genomic DNA preparation (Perkin Elmer model Lambda 35, Waltham, USA).

2.3. Genotyping of EGLN1 gene variant

PCR was carried out using the following primer pair: 5′ CTC CCA GCA CAT CTG TGA AT 3′ and 5′ CAT GCT GAC CTG GGC TAT T 3′. The PCR comprised of assay buffer (1X), genomic DNA (100 ng), dNTP (0.2 mM), primer (10 pmol each), $MgCl_2$ (1.5 mM), and *Taq* DNA polymerase (1 unit; Bangalore Genei, Bengaluru, India). The following thermal program was used: initial denaturation at 95 °C for 3 min., 28 repetitive cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min; final extension at 72 °C for 5 min. The PCR amplicon was analyzed by electrophoresis in 1 % agarose gel. The PCR amplicon was subjected to restriction digestion using 5 units of *BsrGI* enzyme (New England Bio Labs, Ipswich, USA) in a final volume of 10 μL for 8 h at 37 °C. The digestion product was profiled by electrophoresis on 1.5 % agarose gel. The size of the PCR amplicon was 510 bp. T allele was represented by a band of size 501 bp. The C allele was represented by two bands of sizes 270bp and 231bp fragments. A positive control was used in every set of restriction digestion reactions. A Sanger sequenced sample with the genotype homozygous for the minor allele (CC) was used as the positive control.

2.4. Statistical analysis

OpenEpi web-tool was used for all the statistical analyses (Dean, Sullivan, & Soe, 2013). P-value, as determined by Fisher's exact test, was used to determine the differences in the distribution of alleles and genotypes between the study groups. A P-value of less than 0.05 was considered to be significant. 'Simple Hardy-Weinberg Calculator' was used to determine the conformity of the control group with the Hardy-Weinberg Equilibrium (Rodriguez, Gaunt, & Day, 2009).

3. Results

The demographic and clinicopathological characteristics of the

Table 1
Demographic and clinicopathological details of the study participants.

Parameters	Patients (n = 103)	Controls (n = 206)
Male	21	39
Female	82	167
Age (Mean ± standard deviation; years)	54.1 ± 10.8yrs	54.8 ± 10.9yrs
Site of primary tumor		
Buccal mucosa	78	NA
Floor of mouth	1	NA
Anterior 2/3rd of the tongue	8	NA
Lower alveolus	14	NA
Retromolar trigone	2	NA
Grade		
Grade 1	72	NA
Grade 2	28	NA
Grade 3	3	NA
TNM classification		
T_2	8	NA
T_3	21	NA
T_4	74	NA
Stage		
II	20	NA
III	22	NA
IV_a	61	NA

NA – Not applicable.

patients are given in Table 1. Patients' age was in the range of 21–80 years (Mean, 54.1 ± 10.8 years). The patients were were predominantly female (79.6 %). The cancer stage was advanced in most of the patients (stage IV_a, 59 %; stage III, 21 %, and stage II, 20 %). The carcinoma was well-differentiated in most of the patients (well-differentiated, 80 %; moderately differentiated, 18 %, and poorly differentiated, 2%). The lesion size was commonly around 2–4 cm^2 (85 %). The tumor was frequently from the buccal mucosa (65 %) followed by the lower alveolus, anterior 2/3rd of the tongue, retromolar trigone, and floor of the mouth.

The allele and genotype distribution is summarised in Table 2. The genotype frequencies in the control group conformed with the Hardy-Weinberg equilibrium ($\chi^2 = 7.36$). Next, we compared the distribution of the genotypes in the patient and the control groups. The C allele was under homozygous condition commonly in the patient (22 %) than in the control group (13 %). In contrast, the homozygosity of the T allele was higher in the control group (42 %) than in the patient group (29 %). The genotype and allele frequencies were significantly different in the two groups ($P < 0.05$). The genotype distribution was also compared by using genetic models (Table 3). The association was highest with the additive genetic model.

4. Discussion

The purpose of this study was to evaluate the association between the *EGLN1* gene variant (SNP rs479200 T > C) and oral cancer. The frequency of the C allele was found to be significantly higher in oral cancer patients than in healthy individuals. This indicates that the C allele is a risk factor for oral carcinogenesis. This allele is linked to the downregulation of PHD2 and therefore to the increased expression of HIF-1 and the corresponding hypoxia-responsive genes. Therefore, the C allele enables tolerance to hypoxia. Tumor formation requires an aggressive capacity for tolerating hypoxia. Consequently, the C allele would be more common in oral cancer patients than controls. In contrast, the T allele would be relatively uncommon in oral cancer patients (Fig.1). The distribution of the allele frequencies in the study groups agrees with their biological effect. To the best of our knowledge, this is the first report that links *EGLN1* gene polymorphism with cancer predisposition.

Table 3
Evaluation of association between SNP rs479200 polymorphism and oral cancer risk under different genetic model.

Model	Genotype	P-value
Dominant	TT vs.TC + CC	0.0013*
Recessive	TT +TC vs. CC	0.012*
Additive	TT > TC > CC	0.0005**
Multiplicative	T vs. C	0.017*

* Chi-square, two-tailed (Fisher's exact test).
** Mantel Haenszel Chi Square for linear trend.

Recent studies have linked SNP rs479200 with several hypoxia conditions. It plays an important role in the adaptation of the Indian population to high altitude (Aggarwal et al., 2010). The C allele frequency in the Tibetan sub-population depends on their geographical location; the frequency is higher in the subgroup residing at an altitude of 3500 m above the sea level but lower in the subgroup that resides at lower altitudes. Furthermore, the T allele has been associated with the risk of high altitude pulmonary edema and preeclampsia (Aggarwal et al., 2010; Kumar, Arcot, Munisamaiah, & Balakrishna, 2019). Preeclampsia is a common complication of pregnancy involving placental hypoxia (Kumar et al., 2019). Also, physiological studies have shown that SNP479200 is associated with arterial oxygen saturation (Mishra, Mohammad, Thinlas, & Pasha, 2013). This study expands the spectrum of phenotypic manifestations of SNP rs479200. The association of SNP rs479200 with oral cancer and preeclampsia are contrastingly different. The T allele is a protective factor for oral cancer but a risk factor for preeclampsia. The involvement of both the alleles in disease predisposition indicates that the genetic landscape that regulates hypoxia responsiveness is under tight selective pressure.

Till now, only the *HIF1A* gene in the hypoxia-responsive pathway was linked with the risk of oral cancer (Prasad et al., 2018; Munoz-Guerra et al., 2009). This study adds *EGLN1* to the list of oral cancer-associated genes in the hypoxia-responsive pathway.

In conclusion, this study shows that the *EGLN1* gene variation SNP rs479200 is associated with oral cancer. This relationship underlines the importance of the oxygen-sensing mechanism in oral cancer pathogenesis. This study was undertaken as an exploratory proof of principle pilot study. The positive results encourage replication is other ethnicities and their meta-analysis thereof.

Declaration of conflicting interests

The authors declare that they have no conflict of interest.

Table 2
Profile of SNP rs479200 in the study groups.

Genotype/ Allele	Patients (n = 103)	Controls (n = 206)	P –value*	Odds ratio (0.95 confidence interval)
TT	30 (29.1 %)	87(42.2 %)	0.025	0.56 (0.34 − 0.93)
TC	50 (48.5 %)	93 (45.1 %)	0.57	1.14 (0.71–1.80)
CC	23 (22.3 %)	26 (12.6 %)	0.032	1.99 (1.07–3.69)
T	110 (53.3 %)	267 (64.8 %)	3.0×10^{-3}	1.61 (1.14–2.25)
C	96 (46.6 %)	145(35.1 %)		

* Chi-square,two-tailed (Fisher's exact test).

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