RESEARCH ARTICLE

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Association of Interactions between Metabolic 'Caretaker' Genes, *p53*, *MDM2*, and Tobacco Use with the Risk of Oral Cancer: A Multifactor Dimensionality Reduction Approach

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Abstract

Background: The present study investigated the association of interactions between gene polymorphisms in metabolic 'caretaker' genes (Phase I: *CYP1A1*, *CYP2E1*; Phase II: *GSTM1*, *GSTT1*), the cell cycle regulatory gene, *p53*, along with its negative controller, *MDM-2*, and the environment variable (tobacco). A nonparametric model, multifactor dimensionality reduction (MDR), was applied to analyse these interactions. **Materials and Methods:** This case-control study was carried out on 242 subjects. Genomic DNA was extracted from peripheral blood lymphocytes.11 gene variants with an exposure variable (tobacco use) were analysed using MDR to identify the best locus model for gene-gene and gene-environment interactions. Statistical significance was evaluated using a 1000-fold permutation test using MDR permutation testing software (version 1.0 beta 2). The value of $p<0.05$ was considered statistically significant. **Results:** The best three-locus model for gene-gene interaction included two of the *p53* gene polymorphisms; rs17878362 (intron 3) and rs1042522 (exon 4) and rs6413432 in the Phase I gene, *CYP2E1*(DraI). The three-locus model to evaluate the gene-environment interaction included two intronic polymorphisms of the *p53* gene, that is, rs17878362 (intron 3) and rs1625895 (intron 6), and rs4646903 in the Phase I gene *CYP1A1**2C. The interaction graphs revealed independent main effects of the tobacco and *p53* polymorphism, rs1042522 (exon 4), and a significant additive interaction effect between rs17878362 (intron 3) and rs1042522 (exon 4). **Conclusions:** The nonparametric approach highlighted the potential role of tobacco use and variations in the *p53* gene as significant contributors to oral cancer risk. The findings of the present study will help implement preventive strategies in both tobacco use and screening using a molecular pathology approach.

Keywords: Oral cancer- *CYP1A1*- *CYP2E1*- GST- *p53*- *MDM-2*- multifactor dimensionality reduction

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Introduction

In India, oral cancer is the most common malignancy among men and the fifth most common among women (Saini, 2021). Approximately 77,000 new cases are added annually, contributing to 26% of the global cancer burden (Borse et al., 2020). It has become a major health concern due to an alarming trend of an increase in oral cancer cases in <40-45 years of age in recent years (Hussein et al., 2017). The malignancy is also associated with high mortality and disfigurement. This can be attributed to the increased use of various tobacco-chewing products such as gutkha and pan masala. 60% of tobacco users in India use only smokeless tobacco (Sankhla et al., 2018). The Gujarat region (western part of India) is showing a serious growing trend in the use of areca nut-based tobacco products, according to the Global Adult Tobacco Survey (GATS) conducted in India (Sharma et al., 2018).

Well-established risk factors for oral cancer include tobacco use in various forms, alcohol, and HPV16/18 infection. However, all tobacco users do not acquire cancer, implying the role of genetic predisposition. In the past two decades, the role of the genomic constitution/ genetic makeup of individuals has emerged in oral cancer susceptibility (Damani et al., 2020), suggesting the existence of differences in risk between individuals and between populations (Xie et al., 2016). Single-nucleotide polymorphisms (SNPs), the most abundant variations in the human genome, represent individual inherited differences. These SNPs affect DNA stability, transcriptional factor binding stability, DNA processivity, and nucleosome assembly functions. Consequently, these SNPs in a number of genes affect a number of processes, including cell proliferation, immunological function, inflammation, transcription, DNA repair, and xenobiotic metabolism. Therefore, genetic variations represent significant risk

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factors for the growth and transformation of a normal cell to a malignant phenotype. These SNPs may be synonymous or non-synonymous, directly or indirectly affecting gene expression and phenotype (Zeng et al., 2019).

Oral cancer is preceded by well-established premalignant disorders that undergo several molecular changes on the path to invasive oral cancer (Speight et al., 2018). The interaction between an individual's genetic predisposition and environmental variables (i.e., tobacco and alcohol use) substantially impacts these molecular changes (Batta et al., 2019). Accumulating published evidence indicates the association of several SNPs that involve numerous pathways with the risk and progression of oral cancer. However, there are inconsistencies in the results, even in studies that analyze similar SNPs and their association with this complex multifactorial disease. Most of these studies involve a single SNP analysis, which does not provide a clear picture of the association. Comprehensive analysis of gene-gene interaction is necessary to characterize multigenic and multifactorial diseases, as interactions between gene polymorphisms can show a synergistic or nonadditive effect on the pathogenesis of oral cancer (Ritchie et al., 2018). Furthermore, recent meta-data have suggested that variants of Phase I: *CYP1A1*, *CYP2E1*; Phase II: *GSTT1*, *GSTM1*, *p53*, and *MDM-2* show regional, ethnic, and geographical differences in distribution and can modify the risk of oral cancer. These studies have particularly emphasized the inclusion of tobacco in analyses (Tang et al., 2010; Guo et al., 2015; Yang et al., 2015; Zhuo et al., 2016; Li et al., 2018; Zeng et al., 2019).

The tobacco components undergo biotransformation by Phase I and Phase II xenobiotic metabolising enzymes. The generated metabolites lead to the formation of DNA adducts and cause DNA damage. These damages are detected by the guardian of the genome, *p53*, which in turn is regulated by its negative regulator, *MDM-2*. Each individual has inherited differences in their ability to metabolise these carcinogens and effectively repair the damage caused by them. However, it is challenging to create and identify the susceptibility and predictive biomarker panel that may be used to identify high-risk individuals for this complicated disease. Robust estimation of gene-gene and gene-environment interaction effects requires a large number of samples, thereby incurring a prohibitive expense. Additionally, large sample collection and analysis yield high-dimensional data. Analysing these high-dimensional data using parametric statistical methods, such as logistic regression, has limitations (Ritchie et al., 2018).

Taking into account this background, we investigated the role of SNPs in genes associated with tobacco metabolism (*CYP1A1*, *CYP2E1*, *GSTM1* and *GSTT1*) and cell cycle regulation (p53 and MDM2) using a nonparametric statistical tool; Multifactor Dimensionality Reduction method (MDR). This tool has been effectively used to characterize gene-gene and gene-environment interactions in various other diseases in a relatively small sample size (Fu et al., 2017).

Materials and Methods

This study was approved by the Institutional Ethical Committee, (no. EC/35/2012). Written informed consent was obtained from all subjects (cases and controls) after a proper explanation of the blood collection procedure and the purpose of the study in the local language.

Subjects

We included 121 cases of histopathologically confirmed oral squamous cell carcinoma that attended the outpatient department of the institute. Genetically unrelated healthy subjects from the same region with similar socioeconomic status (n=121) were also included as controls. Controls were blood donors who attended the institute's blood bank. The inclusion criterion for controls was the absence of a history of cancer, precancer, or other significant health hazards. A standard questionnaire was designed to collect epidemiological data, including details on age, sex, occupation, ethnicity, tobacco consumption habits, and family history of the study population. 92.3% and 81.2% were males in the control and case/patient groups, respectively. 47.9% were tobacco users in the control group, while 85.1% were in the patient group. The mean age of the subjects was 40.2±0.62 years in controls and 42.6±0.42 years in cancer cases.

DNA Isolation and Genotyping

Genomic DNA was isolated from peripheral blood lymphocytes using commercially available DNA isolation kits (Qiagen, CA) according to the manufacturer's instructions and stored at -20°C until analysis. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) (Eppendorf master cycler gradient, Germany) to determine the polymorphic genotypes of the genes mentioned in Table 1 according to the procedures previously reported by our laboratory (Singh et al., 2014; Patel et al., 2013).

Multifactor-dimensionality reduction (MDR)

MDR is a statistical tool that uses a non-parametric approach (Fu et al., 2017) to generate the best locus model for gene-gene and gene-environment interactions (Moore et al., 2002). The entire case-control data set for the SNPs studied is divided into ten equal portions, or subsets, by default, with one subset for testing and the rest for training. The algorithm uses the training part for all combinations of factors/locus to create contingency tables based on cases and controls. The multilocus genotypes are then pooled into high- and low-risk groups. Subjects in cells with a case/control ratio greater than 1 are labeled high risk, while the other cells are labeled a low risk. Based on this new categorization of the training part (9/10), the training error is calculated. With the testing part (1/10), the prediction error is calculated. Therefore, with the algorithm, genotype predictors are effectively reduced from dimensions 'n' (high-dimensional genotype data) to one dimension, that is, a single character with two levels of risk: high and low. These steps are repeated for each cross-validation fold (by default, 10-fold cross-validation).

Cross-validation consistency (CVC) and permutation tests are used to assess the potential of this unique one-dimensional multilocus genotype to classify and predict the disease status. CVC is defined as the number of times a particular SNP combination is identified across the k-fold cross-validation (CV) or the number of times MDR found the same model when it divided the data into different segments. Significant models will have CVC≥9 based on the fact that the data have been cross-validated 10 times by MDR. Testing balanced accuracy (TBA) is the measure of the average of sensitivity and specificity. The CVC and TBA can then be used to choose the best SNP combination (best-model), which should have the highest CVC and/or TBA. If two or more models have similar TBA, then CVC can be used to decide on the best model (Figure 1).

A 1,000-fold permutation test is used to compare observed testing accuracies with those expected under the null hypothesis of no connection to determine the statistical significance of the best model's testing accuracy. By repeating the entire analysis on 1,000 data sets that are compatible with the null hypothesis, the permutation testing corrects for multiple testing. In permutation testing, the case-control labels of a data set are randomly generated thousands of times, and the MDR method is repeated on each permuted data set.

The MDR algorithm also creates interaction graphs/models/dendrograms, which can be used to visualize the nature of the dependencies or interactions effect between polymorphic genes (additive and non-additive) (Pattin et al., 2009). The interaction model describes the percentage of entropy (information gain or IG) by each factor or two-way interaction. In the interaction graphs, a node (point) represents a gene/SNP, and a line represents the interaction between two nodes. The percentage in the nodes expresses the amount of uncertainty in the label eliminated by the node attribute. The IG in the case-control status is eliminated by each variable (independent main effect), and each pairwise combination of characteristics (interaction effect) is indicated by nodes and connections, respectively (Moore et al., 2002). Positive entropy values indicate additive/synergistic interaction, while negative entropy values indicate redundancy between polymorphic genes.

This study used MDR software v.3.0.2 and MDR permutation testing software (version 1.0 beta 2) (www.epistasis.org). High- and low-risk genotypic combinations were determined based on the threshold value of 1 (121/121) for the present data. A statistically significant difference is indicated by a $p<0.05$.

Results

Gene-Gene Interaction

The best two-way gene-gene interaction model generated by the MDR algorithm involved two SNPs in the *p53* gene, that is, rs17878362 (intron 3) and rs1042522 (exon 4). This model exhibited a CVC of 6/10 (p=0.68) (Table 2). However, for the best three-way gene-gene interaction model, the SNP in the *CYP2E1* gene (DraI), rs6413432, the SNPs in exon 4 (rs1042522), and intron 3 (rs17878362) of the *p53* gene had the highest balanced test precision (0.61) and training (0.66) and a high CVC (9/10) (Table 2) (Figure 2a).

For the gene-gene interaction, the network plot (dendrogram) revealed a significant additive interaction between intron 3, rs17878362, and exon 4, rs1042522 of the *p53* gene (IG=1.46%) (indicated by the entropy values marked on the line, red, connecting the intron 3 and exon 4 nodes). Additionally, an interactive/additive effect was observed between the nodes, *CYP2E1* (DraI), and SNP in exon 4. The entropy values in cells (Figure 3a) show a significant independent effect (positive IG) of the *p53* exon 4 polymorphism (IG=2.04%) followed by the intron 3 polymorphism (IG=0.22%) (Figure 3a).

Gene-Environment Interaction

The probable gene-environment interactions in patients were also analyzed using the MDR approach, taking tobacco as a factor/variable (Figure 2b and Figure 3b). Tobacco exposure showed the most significant univariate effect, with the highest TBA (68.2%) and CVC (10/10). The two-way interactions between *CYP1A1**2C (Ile/Val) and tobacco exposure exhibited the highest TBA (66.9%) and CVC (7/10). The best-three-way model had interactions between the SNPs present in the intronic

Table 1. Gene Variants Included in the Present Study (MDR Model)

Gene	Function	Polymorphism
Metabolic pathway		
CYP1A1 (Xenobiotic metabolising enzyme)	Phase I oxidative and reductive	*CYP1A1**2A (MspI), rs1048943 *CYP1A1**2C(Ile/Val), rs4646903
CYP2E1 (Xenobiotic metabolising enzyme)	Phase I oxidative and reductive	*CYP2E1**5B (c2) (PstI restriction, position: −1019), rs3813867 *CYP2E1**5A (c1) (RsaI restriction, position: −1259), rs2031920 *CYP2E1**6 (DraI restriction, intron 6), rs6413432
GSTT1(Xenobiotic metabolising enzyme)	Phase II	Null polymorphism
GSTM1(Xenobiotic metabolising enzyme)	Phase II	Null polymorphism
p53	Maintenance of genomic integrity, Regulation of cell cycle progression, apoptosis, autophagy, differentiation, senescence, DNA repair and oxidative metabolism	Intron 3 duplication, rs17878362 Exon 4 Pro72Arg, rs1042522 Intron 6, rs1625895
MDM2	*p53* negative regulator	rs2279744

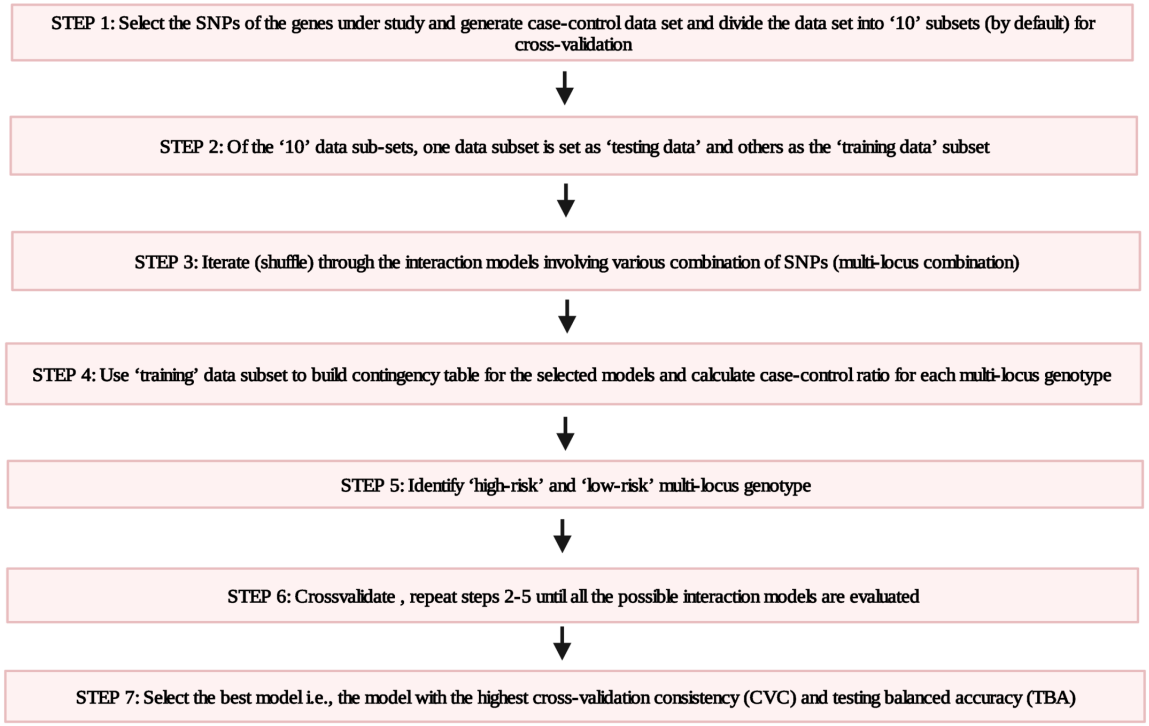
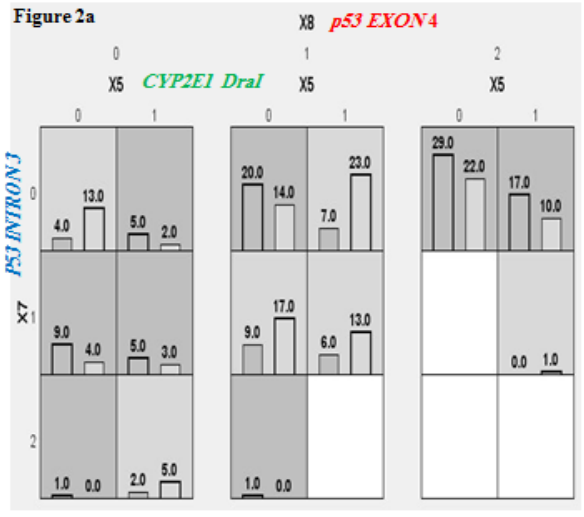


Figure 1. MDR Algorithm: Stepwise process to identify the best model to predict oral cancer risk for multiple genes

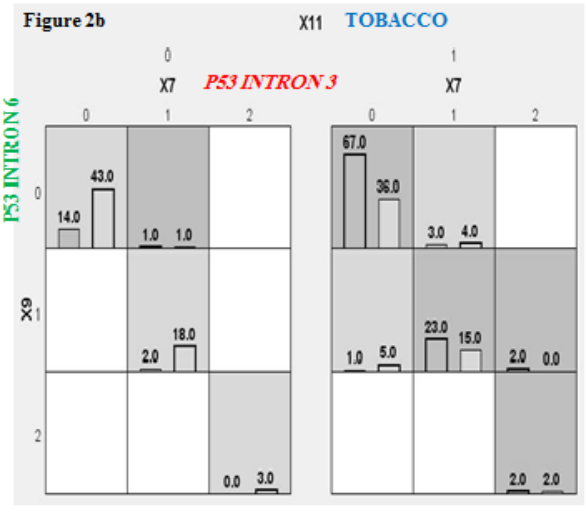
regions of the *p53* gene, i.e. rs 17878362 (intron 3) and rs1625895 (intron 6), Phase I, *CYP1A1**2C (Ile/Val) rs4646903, and tobacco (Table 3). The interaction graph showed a significant independent effect of tobacco (IG=11.19%), less synergy between *CYP1A1* * 2C and the intronic polymorphisms of *p53*. *CYP1A1**2C exhibited an additive effect with tobacco.

Discussion

The balance in the expression and activities of Phase I enzymes such as *CYP1A1* and *CYP2E1* and Phase II enzymes such as GSTs play a critical role in the biotransformation and metabolism of tobacco procarcinogens. This balance, if lost, can potentially cause DNA damage. To deal with the damage caused by



X5: *CYP2E1* DraI; 0=DD, 1= DC genotype
X7: *p53* intron 3; 0= A1/A1, 1= A1/A2, 2= A2/A2 genotype
X8: *p53* exon 4; 0=Arg/Arg, 1= Arg/Pro/ 2- Pro/Pro



X7: *p53* intron 3; ; 0= A1/A1, 1= A1/A2, 2= A2/A2 genotype
X9: *p53* intron 6; 0=G/G, 1=G/A, 2=A/A genotype
X11: Tobacco

Figure 2. Interaction of Attributes: the best 3-locus genotype combinations associated with oral cancer. (a) gene-gene interaction; (b) gene-environment interaction. In each box, the distribution of cases (left bars) and controls (right bars) for each of the genotype combinations are shown. High-risk combinations are depicted as dark-shaded cells and low-risk combinations as light-shaded cells; empty cells are left blank.

Table 2. MDR Interaction Analysis between SNPs (Gene-Gene Interaction)

MDR model	Balanced accuracy CV training	Balanced accuracy CV testing	CV consistency	Permutation test 'p' value
p53 exon 4, rs1042522	0.5786	0.5404	10/10	0.51
p53 intron 3, rs17878362-*p53* exon 4, rs1042522	0.6147	0.5214	6/10	0.68
CYP2E1 DraI, rs6413432- *p53* intron 3, rs17878362 - *p53* exon 4, rs1042522	0.6623	0.612	9/10	0.06

tobacco procarcinogens, we have *p53*, a well-reported tumor suppressor gene with pleiotropic activities (Ghosh et al., 2022) with the negative regulator of its activity, *MDM-2*. With this as a rationale, research studies on polymorphic variability in xenobiotic metabolizing genes, *CYP1A1*, *CYP2E1*, *GSTT1*, and *GSTM1*, and the cell cycle regulator, *p53* with its negative controller, *MDM-2*, were reported from our laboratory (Patel et al., 2013; Singh et al., 2014). The variability in the candidate genes studied with oral cancer risk was analyzed using a binary logistic regression model. However, no statistically significant risk of oral cancer was observed. For the tumor suppressor gene, *p53*, the proline allele of the SNP rs1042522 in exon 4 conferred protection against oral cancer development (Patel et al., 2013). Furthermore, no association was observed with oral cancer risk for SNPrs2279744 in the *MDM-2* gene (Patel et al., 2013; Singh et al., 2014,). Analysis of gene-gene interaction revealed that of the 'caretaker' genes studied, only *GSTM1* and *GSTT1*, in combination, exhibited a statistically significant higher odds ratio (Singh et al., 2016). In addition to this, a gene-tobacco interaction analysis was also performed on the same population of subjects. The analysis revealed that tobacco use, particularly tobacco chewing, is a significant risk modifier in subjects harboring variant genotypes of *CYP1A1*, *CYP2E1*, *GSTM1*, *GSTT1*, *p53*, and *MDM-2* (Singh et al., 2012; Patel et al., 2015). Unfortunately, due to the limitation of the small sample size in these studies, a combined gene-gene and gene-environment interaction analysis could not be performed (Patel et al., 2013; Singh et al., 2014). However, these results were successful in (i) providing a tentative SNP fingerprint for oral cancer

Table 3. MDR Interaction Analysis between SNPs and Tobacco (Gene-Environment Interaction)

MDR model	Balanced accuracy CV training	Balanced accuracy CV testing	CV consistency	Permutation test 'p' value
Tobacco	0.682	0.682	10/10	<0.001
Tobacco - *CYP1A1**2C, rs4646903	0.6835	0.6694	7/10	<0.001
Tobacco,- *CYP1A1**2C, rs4646903, -p53 Intron 3 rs17878362 - Intron 6 rs1625895	0.7047	0.6333	7/10	0.01

CVC, Cross Validation Consistency; p-values as calculated after 1000 permutations

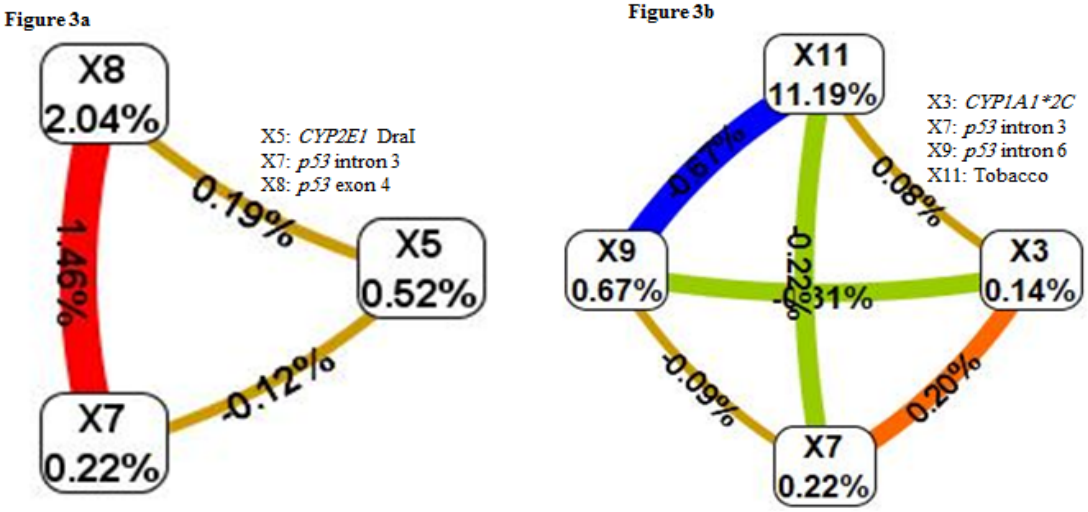


Figure 3. Interaction Entropy Graphs (Network Plots) Highlighting the Amount of Information Gain (IG) in Cells and on Lines as a Percentage: a) gene-gene interaction; (b) gene-environment interaction. Entropy values marked on the lines connecting two SNPs represent the entropy of interaction. Positive percent entropy represents synergy or information gain; negative percent entropy represents redundancy or lack of information gain. . Entropy values in the cells of individual SNPs indicate the main independent effects. Schematic coloration used in graphs represents a continuum from synergy to redundancy. Red represents a high degree of synergy (positive IG); Orange a lesser degree of positive IG; Brown a midway point between synergy and redundancy; Green and blue represent a redundancy

susceptibility and emphasizing the role of SNPs in these candidate genes in the development of oral cancer, and (ii) highlighting the importance of the association between genes and the environment in modulating susceptibility to oral cancer in the population of Gujarat. Interestingly, the results indicated a limitation, ie the inability to analyse gene-gene and gene-environment interactions involving multiple genes in the same set of patients.

Considering this limitation, MDR, an alternative statistical tool with a nonparametric approach, was used for gene-gene and gene-environment analysis in our case-control data set. The results of the MDR algorithm successfully generated the best two-way and three-way interactive models for both gene-gene and gene-environment predicting the risk of oral cancer. The best interactive model between genes included SNPs in exon 4 (rs1042522) and intron 3 (rs17878362) of the *p53* gene. These SNPs also exhibited an additive effect. Notably, strong linkage disequilibrium between intron 3 and exon 4 of *p53* has also been reported (Wu et al., 2002).

Interestingly, exon 4 SNP, rs1042522, showed a significant independent effect on oral cancer risk. On the other hand, the results of the best gene-environment interactive model reinforced that tobacco use is the most important environmental factor that affects oral cancer susceptibility. The model also included both intronic polymorphisms of *p53*, ie rs17878362 and rs1625995.

The results for gene-gene and gene-environment interactions share an interesting finding, ie, the putative role of the *p53* gene polymorphisms in oral cancer susceptibility. In the analysis of gene-gene and gene-environment interactions, genomic variations in *p53* were a common factor. These genomic variations allow for the generation of *p53* isoforms through alternate splicing. These isoforms are involved in creating an immunosuppressive environment, which helps to trigger tumour initiation and progression (Eiholzer et al., 2020). Furthermore, at the mechanistic level, there is some evidence that these polymorphisms may have an impact on the structure of the *p53* protein. Therefore, the variants differ in their biochemical and biological activities, such as DNA repair capacity and apoptosis (Wu et al., 2002; Gemignani et al., 2004; Sullivan et al., 2004; Pietsch et al., 2006; Marcel et al., 2011). This makes these loci a potent hotspot region for mutations and increases cancer risk (Sagne et al., 2013). The results of the current analysis also support the findings of our previous study on *p53* mutations and oral cancer risk. We reported that *p53* exon 4 exhibited a maximum cluster of mutations (Singh et al., 2016), and *p53* alterations play an important role in the risk of oral cancer.

In conclusion, the MDR application successfully identified a significant interaction between three polymorphisms of two genes, *CYP1A1**2C (Ile/Val; rs4646903), intron 3 (rs17878362), intron 6 of *p53* (rs1625895), and gene-environment interactions between tobacco and *p53* polymorphisms in the oral cancer case-control data set. Such analysis becomes meaningful in the absence of any statistically significant independent main effects of the candidate genes, mainly when there is a limitation to performing gene-environment interactions owing to a low sample size. Importantly, this analysis assessed SNPs involving critical genes for specific pathways in the same case-control data set. Therefore, these results can be used to design and implement preventive tobacco consumption strategies and screen the 'at risk' population.

Abbreviations

CVC, cross-validation consistency; GATS, Global adult tobacco survey; IG, information gain; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; PCR-SSCP, Polymerase chain reaction-single strand conformation polymorphism; SNP, Single nucleotide polymorphism; TBA, Testing balance accuracy.

Author Contribution Statement

Ragini Singh: conceptualized and planned the experiments, analysis, and interpretation of the results, and writing of the manuscript; Kinjal Patel: Planned and performed the experiments, analysed and interpreted the results, and prepared the manuscript; Jayendra Patel: supervision of experiments; Prabhudas Patel: provided critical feedback and helped shape the research, analysis, and manuscript.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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