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Polymorphisms in DNA repair genes increase the risk for type 2 diabetes mellitus and hypertension

DNA repair gene polymorphisms in risk for T2DM and HT

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Abstract: We investigated the effect of polymorphisms in four DNA repair genes, *viz*. *RAD18* Arg302Gln (G>A) (rs373572), *XPD* Asp312Asn (G>A) (rs1799793), *APE1* Asp148Glu (T>G) (rs3136820), and *OGG1* Ser326Cys (C>G) (rs1052133) on the risk for type 2 diabetes mellitus (T2DM) and hypertension (HT) in association with smoking, tobacco chewing, and alcohol consumption in a population from Northeast India. The study subjects were comprised of 70 patients suffering from both T2DM and HT and 83 healthy controls. Genotyping was performed using ARMS-PCR for *XPD* Asp312Asn (G>A) and PCR-CTPP for *RAD18* Arg302Gln (G>A), *APE1* Asp148Glu (T>G) and *OGG1* Ser326Cys (C>G). The *RAD18* Gln/Gln genotype was found to significantly increase the risk for T2DM and HT by 30 fold. Significant high risk was observed for individuals with *XPD* Asn/Asn-*RAD18* Arg/Gln genotypes. Smoking was found to be the single most important independent risk factor for T2DM and HT. This study concludes that *RAD18* Arg302Gln and *XPD* Asp312Asn polymorphisms might increase the risk for T2DM and HT in association with smoking, tobacco chewing, and/or alcohol consumption, while *APE1* Asp148Glu (T>G) and *OGG1* Ser326Cys (C>G) polymorphisms do not contribute to such risk.

Keywords: *RAD18*; *XPD*; *APE1*; *OGG1*; T2DM; HT.

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Introduction

Hypertension (HT) and type 2 diabetes mellitus (T2DM) are two leading causes of human mortality worldwide. Both develop due to polygenic defects, in association with various environmental factors like smoking, use of chewing tobacco, and alcohol consumption [1,2]. Individuals with T2DM are often found to develop hypertension, thus reflecting a significant overlap in the etiology and mechanisms of these two diseases.

Various tobacco products, smoking, and alcohol consumption may lead to the increased production of reactive oxygen species (ROS) [3,4]. Previous evidence demonstrates that an increase in ROS production may be involved in diabetes and its complications [5]. ROS have also been reported to be associated with other diabetes related metabolic abnormalities like hypertension [6]. Accumulation of ROS may damage biological macromolecules, including DNA. DNA damage can also be due to various endogenous or exogenous stresses including telomere erosion, genotoxic stress, and metabolic stress. DNA repair genes play a vital role in maintaining genomic integrity by eliciting the repair of damaged DNA through various mechanisms which include nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) [7]. Genetic variations including single nucleotide polymorphisms (SNPs), which may hamper the DNA repair activity leading to genetic instability, are reported to be present in more than 100 DNA repair genes [8].

Although there are 20 validated sequence variants of *OGG1* gene, the Ser326Cys polymorphism is the most studied functional polymorphism. The human 8-oxoguanine DNA glycosylase (OGG1) helps in the cleavage of the glycoside bond between a modified base and the corresponding sugar in a DNA molecule. The C/G n polymorphism in codon 326 (rs1052133) of *OGG1* resulting in a substitution of serine with cysteine is a loss-

of-function SNP reported to lower the activity of the OGG1 enzyme, thus playing a major role in the pathogenesis of various diseases [9].

The apurinic/apyrimidinic (AP) endonuclease (*APE1*) gene functions in preventing base transversion resulting from oxidized or reduced bases [10-12]. APE1 acts as a rate limiting enzyme in the BER process. APE1 cleaves the abasic site to form a 5′-dioxiribose phosphate residue, which is then removed by DNA polymerase β (Pol β) by its AP-lyase activity, followed by insertion of a correct nucleotide by Pol β. The DNA is then sealed by DNA ligase III [13-15]. Although a total of 18 polymorphisms have been reported in *APE1*, the polymorphism Asp148Glu has been studied most extensively due to its role in altered DNA repair activity [16]. The polymorphism in *APE1* is the result of T>G transition at codon 148 of exon 5 (rs3136820) which leads to the substitution of aspartic acid with glutamic acid, resulting in loss-of-function of APE1, with altered DNA binding and endonuclease activity, decreased interaction with other BER proteins and reduced oxidative damage repair [17].

The RAD18 DNA repair enzyme has a key role in the post-replication repair process in many organisms from yeast to human [18]. RAD18 functions in DNA damage bypass and post-replication repair (PRR) at stalled replication forks. Emerging reports have also indicated the role of RAD18 in homologous recombination (HR) in mammalian cells, which mediates the repair of double-strand breaks (DSBs). The *RAD18* gene G>A polymorphism at codon 302, (rs373572) results in an amino acid substitution from arginine to glutamine [19]. However, neither the proper function nor the molecular mechanism of this SNP have been clarified [18].

The NER gene Xeroderma Pigmentosum Group D (*XPD*) is involved in the removal of bulky DNA lesions from DNA, including those that may be formed by tobacco products. XPD is important in transcription coupled repair, cell cycle regulation, and apoptosis by being an important part of the BTF2/TFIIH complex [20]. *XPD* Asp312Asn and *XPD* Lys751Gln are the most two most commonly studied polymorphisms of *XPD*. However, the Asp312Asn polymorphism was reported to be functionally more relevant in comparison to the Lys751Gln polymorphism. The G>A transition polymorphism at codon 312 of exon 10 (rs1799793) of *XPD* results in the amino acid substitution aspartic acid to asparagine which leads to reduced DNA repair, and is thus a loss-of-function of SNP [21-24].

Over the last three decades, the prevalence of T2DM in India has risen from 1.2% to 11% and is projected to house one-third of all the world's diabetic patients [25]. India has therefore been christened the "diabetic capital of the world" [26]. Similarly, the prevalence of HT has been found to be rapidly increasing in developing countries, and accounts for a large number of deaths as well as disability specially among the elderly [27]. Various genetic and environmental factors interplay in determining the susceptibility to T2DM and HT. However, the role of DNA repair genes in the pathogenesis of T2DM and HT is not well documented. We hypothesised that elevated levels of ROS due to smoking, chewing tobacco, and/or alcohol use may lead to increased DNA damage, which, if left unrepaired due to a polymorphism in DNA repair genes, may contribute to an increased risk for T2DM and HT. This case-control study was therefore undertaken to assess the contribution of loss-of-function polymorphisms in the DNA repair genes *RAD18*, *XPD*, *APE1*, and *OGG1* alone or in combination, and also the interaction of these genes with smoking, use of chewing tobacco, and alcohol use toward the risk for T2DM and HT in a northeastern Indian population. No previous studies related to the association of DNA repair gene polymorphisms with the risk for T2DM and HT have been performed on the Indian population as a whole, or the analyzed sub-population of northeast India, to the best of our knowledge. Therefore, the expected SNP distribution in the study population cannot be determined or compared with previous studies.

Materials And Methods

Subjects

Ethics approval for the study was obtained from the Institutional Ethics Committee (IEC) of Assam University, Silchar (IEC/AUS/2015-009 dt-4/9/15). The study has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Venous blood (2 mL) was collected in EDTA vials from both patients and controls drawn from the Northeast Indian population, and stored at 4°C until further use. The group of patients was comprised of 70 males (mean age = 47.2, median age= 46) and females (mean age = 46.9, median age = 51) suffering from both T2DM and HT. The controls were comprised of 83 healthy males (mean age = 48.9, median age = 51) and females (mean age = 46.9, median age = 46.5) with no symptoms or history of T2DM or HT. The samples were collected in 2015 from a hospital in northeast India with written informed consent from the study subjects. The patients were categorised as diabetic

if either their fasting plasma glucose (FPG) ≥ 126 mg/dL or their two-hour plasma glucose ≥ 200 mg/dL [28]. The patients with either systolic blood pressure (SBP) ≥ 140 mm Hg or diastolic blood pressure (DBP) ≥ 90 mm Hg were categorised as hypertensive [29]. Information pertaining to etiological habits like smoking, chewing tobacco, and alcohol use of the subjects was also collected with the help of a standard questionnaire. Since the study population of northeast India is not ethnically homogenous, such heterogeneity may reduce the statistical power to detect genetic association and greatly decrease the estimates of risk attributed to genetic variation [30], which is a limitation of the current study.

DNA isolation and genotype analysis

Genomic DNA from venous blood was isolated using phenol:choloroform extraction method as proposed by Albarino et al. [31] with some modifications. DNA purity and concentration were determined by spectrophotometric readings (BioRad SmartSpec, USA).

Genotyping of SNP for *XPD* Asp312Asn (rs1799793) was performed using a recent amplification refractory mutation system-PCR (ARMS-PCR) as described by Hussien et al. [8]. An internal control primer pair (ARMSA 5′-CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG; ARMSB: 5′-GGG CCT CAG TCC CAA CAT GGC TAA GAG GTG) at a ratio of 1:5 was used with the allele specific primer (Reverse primer 5′-CAG GAT CAA AGA GAC AGA CGA GCA GCG C; G allele forward 5′-GTC GGG GCT CAC CCT GCA GCA CTT CGG C; A allele forward 5′-GTC GGG GCT CAC CCT GCA GCA CTT CGA T). The product size of the common band and the allele specific bands were 360 bp and 150 bp band respectively (Fig.1a). The cycling condition used were initial denaturation of 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 61°C, and 50 s at 72°C, and finally 7 min at 72°C. The PCR amplified products were resolved in a 2% agarose gel.

Genotyping of SNP for *OGG1* Ser326Cys and *APE1* Asp148Glu was performed using PCR confronting two-pair primers (PCR-CTPP) as described by Kanzaki et al. [18] and Li et al. [14], respectively. For genotyping *RAD18* Arg302Gln polymorphism (rs373572), the primers used were: F1: 5'-ATA CCC ATC ACC CAT CTT C and R1, 5'-GTC TTC TCT ATA TTT TCG ATT TCT T for the Gln allele producing a 146-bp band while F2, 5'-TTA ACA GCT GCT GAA ATA GTT CG and R2, 5'-CTG AAA TAG CCC ATT AAC ATA CA amplified a 106 bp band for the Arg allele. A 206 bp band was amplified using the F1 and the R2 primers (Fig.1b). The cycling conditions used were initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 64°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were resolved in a 3% agarose gel.

For typing *APE1* Asp148Glu (rs3136820) polymorphism, the primers used were as follows: F1: 5′-CCT ACG GCA TAG GTG AGA CC; R1:5′-TCC TGA TCA TGC TCC TCC-3'; F2: 5′-TCT GTT TCA TTT CTA TAG GCG AT; R2: 5′-GTC AAT TTC TTC ATG TGC CA. Three bands amplifying a 236 bp, 167 bp and a 360 bp band corresponded to the T allele, G allele and a common band respectively (Fig.1c). The PCR conditions used were initial denaturation at 95°C for 5 min followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min with a 5 min extension at 72°C. The PCR products were resolved in a 2% agarose gel.

The primers used for genotyping *OGG1* Ser326Cys (rs1052133) polymorphism were F1: 5′-CAG CCC AGA CCC AGT GGA CTC and R1: 5′-TGG CTC CTG AGC ATG GCG GG amplifying a 252 bp band corresponding to C allele while F2: 5′-CAG TGC CGA CCT GCG CCA ATG and R2: 5′-GGT AGT CAC AGG GAG GCC CC amplifying a 194 bp band corresponding for G allele (Fig.1d). The cycling conditions were initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 64°C for 1 min, and 72°C for 1 min followed by a final extension at 72°C for 5 min. The PCR products were resolved in a 2.5% agarose gel.

All the PCR reactions were performed in a total volume of 20 μL containing 100 ng of DNA, 1 mM dNTP mix, 1.5 mM $MgCl_2$, 1X GoTaq Green buffer, and 1 U *Taq* polymerase (GoTaq, Promega, USA).

Statistical analyses

Fisher's Exact test was performed in order to evaluate the differences in the demographic data and genotypic frequencies among the cases and the controls. The Hardy-Weinberg equilibrium between the observed and expected frequency of the genotypes for our control population was performed using the Pearson's two-sided chi-square test. In order to evaluate the association of the genotypes of the DNA repair genes along with the etiological habits of smoking, tobacco chewing, and/or alcohol consumption in determining the risk for T2DM and HT, the Odds Ratio (OR) at 95% confidence interval (CI) was calculated using unconditional logistic regression (LR) method, performed using the Statistical Package for Social Sciences version 16.0 (SPSS Inc, Chicago, USA). Multinomial logistic regression method was used to calculate Adjusted Odds Ratio (AOR) with an adjustment for age, gender, smoking, tobacco chewing, and alcohol habits. All the tests with a p-value of < 0.05 were considered to be statistically significant.

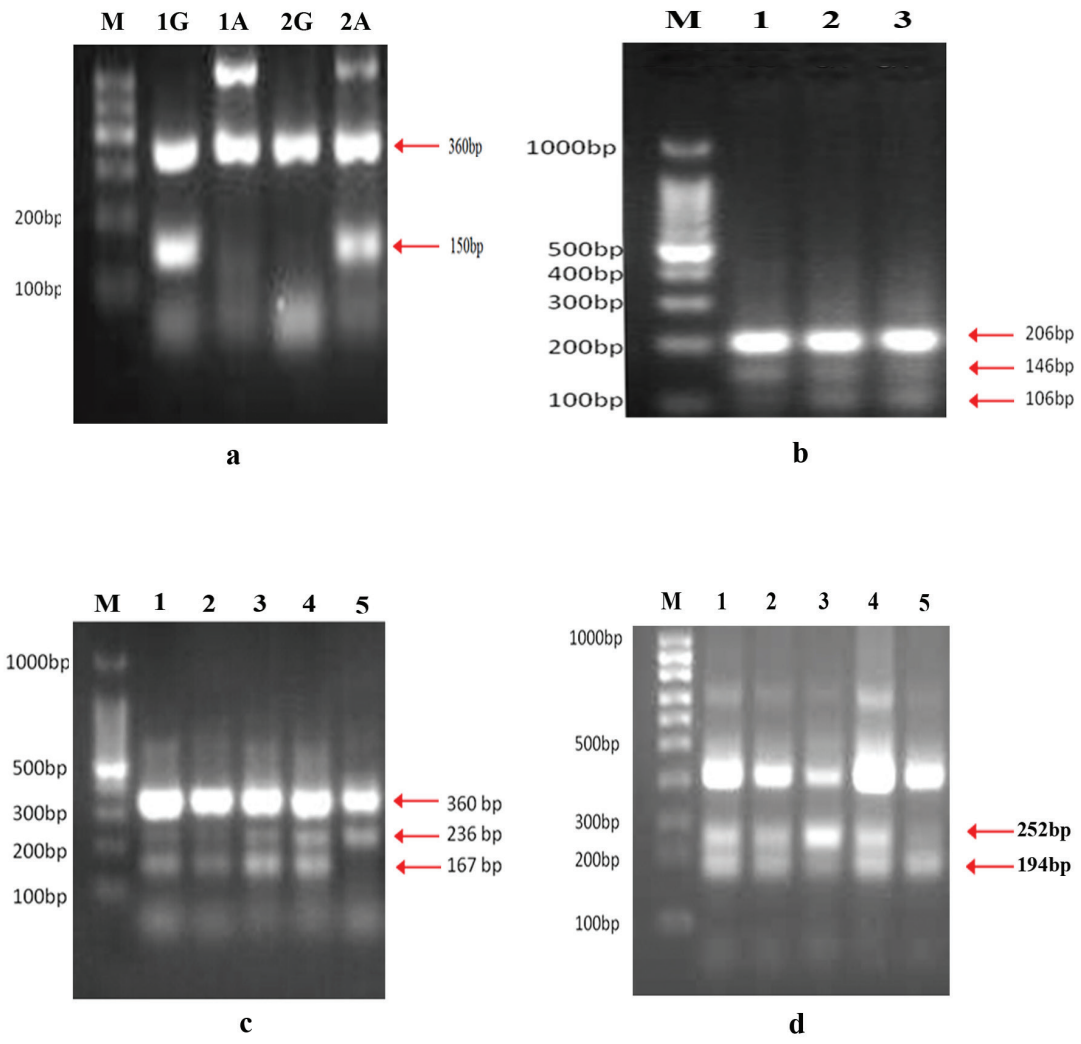

Figure 1: Representative electropherogram depicting genotyping for **a)** *XPD* Asp312Asn (G>A) (rs1799793) polymorphism using the ARMS-PCR method. Genotypes were typed based on the development of a 150 bp band when primers specific to the G or A allele were used for the same samples in two different reactions: lanes 1G and 2G contain the amplicons obtained using the G allele specific primers, while lanes 1A and 2A contain the amplicons obtained using the A allele specific primers, respectively; lane M = 100 bp marker, lanes 1G and 1A = GG genotype, lanes 2G and 2A = AA genotype. **b)** Genotyping for *RAD18* Arg302Gln (rs373572) polymorphism was performed using allele specific PCR. The 146 bp band corresponds to the A allele while the 106 bp band corresponds to the G allele. Lane M = 100 bp marker, lane 1 = AA genotype, lanes 2, 3 = GA genotype. **c)** Genotyping for *APE1* Asp148Glu (T>G) (rs3136820) polymorphism was performed by the PCR-CTPP method. The 236 bp and the 167 bp bands correspond to T and G alleles respectively. Lane M = 100 bp marker, lanes 1, 2, 3, 4 = TG genotype, lane 5 = TT genotype. **d)** Genotyping for *OGG1* Ser326Cys (C>G) (rs1052133) polymorphism was performed by the PCR-CTPP method. The 252 bp band corresponds to the C allele while the 194 bp band corresponds to the G allele. Lane M = 100 bp marker, lanes 1, 2, 4 = CG genotype, lane 3 = CC genotype, lane 5 = GG genotype.

Multifactor dimensionality reduction (MDR) analysis

MDR analysis was performed to study the gene-gene and gene-environment effects on the risk for T2DM and HT using MDR software package (MDR 3.0.2). MDR utilizes a nonparametric, model-free statistical approach in order to create the best one-dimensional model to predict T2DM and HT susceptibility. It has an advantage over parametric methods like LR, which tends to produce low statistical power of test due to a small sample size. Data were generated using 10-fold cross-validation procedure as well as 10 times random seed number in order to reduce the chance of false positives. The best model was selected considering the best testing balance accuracy (TBA) and cross-validation consistency (CVC). The results were considered to be significant at $p<0.05$.

Interaction entropy graphs

For better visualization of the gene-gene and gene-environment interactions, an interaction graph was generated using MDR. A node for each variable was generated along with pairwise connections between them. For each node, the percentage of entropy removed by each variable was displayed. The percentage of entropy calculated were used to understand the independent effect of each variable, interaction effect of the variables as well as to find out if the interactions were synergists.

Classification and regression tree analysis (CART)

The CART analysis was performed to build a decision tree in R (version 3.3.0) using "rpart" and "rpart.plot" packages. The tree was created with a root node, followed by splitting the nodes into sub nodes until a full grown tree was generated. The CART analysis was performed in order to determine the combination/s of DNA repair genes, age, and gender along with various etiological habits in predicting the risk for T2DM and HT.

Result

Study subjects

Samples from 70 T2DM and HT cases and 83 controls were included in this study. The demographic characteristics of the subjects are shown in Table 1. Individuals with the combined habits of smoking + tobacco chewing and smoking + alcohol consumption were found to have higher rates of T2DM and HT than the controls. Similarly, the percentage of study subjects who had the combined habits of smoking, tobacco chewing, and alcohol use was found to be higher among cases (31.43%) than in the control (2.41%) group.

Table 1: General information pertaining of controls and T2DM and HT patients included in the study.

Characteristic	Controls (%)	Cases (%)	p value
Sex			
Male	55 (66.3)	49 (70)	a 0.728
Female	28 (33.7)	21 (30)	
Age			
Below 30	10 (12)	6 (8.6)	b 0.489
30–60	53 (63.9)	51 (72.8)	
Above 60	20 (24.1)	13 (18.6)	
Mean age ± SD	48 ± 14.20	47.14 ± 13.47	
Smoking, tobacco, and Alcohol exposure			
Only smoking	6 (7.23)	6 (8.57)	b <0.001
Only tobacco	35 (42.17)	11(15.71)	
Only alcohol	7 (8.43)	1 (1.43)	
Smoking + Tobacco	4 (4.82)	9 (12.86)	
Smoking + Alcohol	2 (2.41)	9 (12.86)	
Tobacco + Alcohol	5 (6.02)	2 (2.86)	
All	2 (2.41)	22 (31.43)	
None	22 (26.51)	10 (14.28)	

a- Fisher's p
b- Chi square

Genotype distribution and Hardy-Weinberg Equilibrium (HWE)

The distribution of *XPD* (Asp312Asn) (rs1799793), *OGG1* (Ser326Cys) (rs1052133), *RAD18* (Arg302Gln) (rs373572), and *APE1* (Asp148Glu) (rs3136820) were found to be on par with those expected under HWE (P>0.05).

Genotype distribution in association with T2DM and HT risk

The genotype distribution and allele frequency of the DNA repair gene polymorphisms of both the cases and the controls are depicted in Table 2. The adjusted odds ratio for age, sex, smoking, and tobacco habits of all the genotypes were determined by taking Asp/Asp, Ser/Ser, Arg/Arg, and Asp/Asp genotypes as reference for *XPD*, *OGG1*, *RAD18*, and *APE1* genes respectively. The *RAD18* Gln/Gln (OR=30.46; 95% CI: 3.23–287.16; p <0.01) genotype was found to be significantly associated with increased risk for T2DM and HT by 30.46-fold, followed by the *RAD18* Arg/Gln (OR=8.02; 95% CI: 2.85–22.58; p<0.01) genotype by 8-fold. The *XPD* Asp/Asn (OR=2.62; 95% CI: 0.99–6.94; p=0.052), *APE1* Asp/Glu (OR=2.67; 95% CI: 0.99–7.22; p=0.052), and *APE1* Glu/Glu (OR=1.86; 95% CI: 0.28–12.03; p=0.512) genotypes also contributed to a risk for T2DM and HT by 2.62-fold, 2.67-fold, and 1.86-fold respectively, albeit non-significantly. Similarly, the *OGG1* polymorphisms

Table 2: *RAD18* (rs373572), *XPD* (rs1799793), *APE1* (rs3136820), and *OGG1* (rs1052133) genotype frequencies and Odds Ratio (OR) in controls and T2DM and HT patients.

Genotype	Control	Case	OR (95% CI)	P value[a]
RAD18 (rs373572)				
Arg/Arg	54	21	1 (Ref)	-
Arg/Gln	25	43	8.02 (2.85–22.58)	**<0.01***
Gln/Gln	4	6	30.46 (3.23–287.16)	**<0.01***
XPD (rs1799793)				
Asp/Asp	48	22	1 (Ref)	-
Asp/Asn	32	39	2.62 (0.99–6.94)	0.052
Asn/Asn	3	9	0.69 (0.109–4.41)	0.699
APE1 (rs3136820)				
Asp/Asp	48	20	1 (Ref)	-
Asp/Glu	31	44	2.67 (0.99–7.22)	0.052
Glu/Glu	4	6	1.86 (0.28–2.03)	0.512
OGG1 (rs1052133)				
Ser/Ser	42	33	1 (Ref)	-
Ser/Cys	36	27	1.33 (0.50–3.54)	0.567
Cys/Cys	5	10	1.63 (0.30–8.88)	0.567

Adjusted odds ratio was calculated by adjusting to age, gender, smoking, tobacco-chewing, and alcohol consumption habits.
*p<0.05
[a]Fisher's two tailed

Ser/Cys (OR=1.33; 95% CI: 0.50–3.54; p=0.567) and Cys/Cys (OR=1.63; 95% CI: 0.30–8.88; p=0.567) contributed only to a slight and non-significant risk for T2DM and HT.

Effects of gene-gene interactions between *XPD* (rs1799793), *OGG1* (rs1052133), *RAD18* (rs373572), and *APE1* (rs3136820) polymorphisms on T2DM and HT risk

Stratified analyses based on effect of gene-gene interaction on T2DM and HT risk was performed (Table 3). *RAD18* Arg/Gln-*APE1* Glu/Glu (OR=33; 95% CI: 3.77–288.62; p<0.01) and *RAD18* Gln/Gln-*APE1* Asp/Glu (OR=16.50; 95% CI: 2.78–97.78; p<0.01) genotypic interactions were found to significantly increase the risk for T2DM and HT by 33-fold and 16.50-fold respectively. Interactions between the *APE1* Glu/Glu-*XPD* Asp/Asn (OR=22.14; 95% CI: 2.66–184.56; p<0.01) and *XPD* Asn/Asn-*RAD18* Arg/Gln (OR=36.17; 95% CI: 4.25–307.78; p<0.01) genotypes increased the risk for T2DM and HT by 22.14-fold and 36.17-fold respectively. Interaction between *RAD18* Arg/Gln-*OGG1* Cys/Cys genotypes (OR=24; 95% CI: 2.86–201.51; p<0.01) increased the risk for T2DM and HT by 24-fold. Finally, a significant increase in risk for T2DM and HT by 9.67-fold was observed for the interaction between *XPD* Asn/Asn-*OGG1* Ser/Ser genotypes (OR=9.67; 95% CI: 1.22–76.57; p<0.05).

Effects of interactions between *XPD* (rs1799793), *OGG1* (rs1052133), *RAD18* (rs373572), and *APE1* (rs3136820) polymorphisms with smoking, tobacco chewing, and alcohol consumption habits on T2DM and HT risk

In Table 4, 5, and 6, the study groups were stratified based on the habits of smoking, tobacco, and alcohol consumption, and their effect on T2DM and HT risk. A high risk for T2DM and HT was observed in smokers with the *RAD18* Arg/Gln (OR=37.29; 95% CI: 10.97–126.70; p<0.01), *APE1* Asp/Glu (OR=13.05; 95% CI: 4.44–38.33; p<0.01), *XPD* Asp/Asp (OR=26.25; 95% CI: 6.34–108.67; p<0.01), and *OGG1* Ser/Cys (OR=7.27; 95% CI: 2.43–21.78; p<0.01) genotypes in comparison to non-smokers. A significant moderate risk was found in tobacco chewers with *RAD18* Arg/Gln (OR=5.20; 95% CI: 1.97–13.76; p<0.01), *APE1* Glu/Glu (OR=6.04; 95% CI: 1.29–28.34; p<0.05), and *XPD* Asn/Asn (OR=6.75; 95% CI: 1.48–30.88; p<0.01) genotypes in comparison to non-tobacco chewers. However, no significant risk was observed among tobacco chewers with any genotype of *OGG1*. Individuals who consumed alcohol and had the genotypes *RAD18* Arg/Gln (OR=17.11; 95% CI: 5.48–53.44; p<0.01), *APE1* Glu/Glu (OR=13; 95% CI: 1.65–102.20; p<0.05), and *XPD* Asp/Asn (OR=12.35; 95% CI: 3.88–39.31; p<0.01) were found to be at significantly higher risk for T2DM and HT. Significant moderate risk was observed among alcohol consumers with the *OGG1* Ser/Ser (OR=3.54; 95% CI: 1.29–9.74; p<0.05) and *OGG1* Ser/Cys (OR=3.31; 95% CI: 1.19–9.17; p<0.05) genotypes. Significant moderate risk was also observed among non-alcohol consumers with *RAD18* Arg/Gln (OR=5.66; 95% CI: 2.22–14.40; p<0.01) and *RAD18* Gln/Gln (OR=6.11; 95% CI: 1.46–25.50; p<0.05) genotypes.

Table 3: Distribution of double-combined genotypes and Odds Ratio (OR) of *RAD18* (rs373572), *XPD* (rs1799793), *APE1* (rs3136820), and *OGG1* (rs1052133) polymorphisms in T2DM and HT patients and controls.

Genotype	Control	Case	OR (95% CI)	P value[a]
RAD18 (rs373572)-*APE1* (rs3136820)				
Arg/Arg-Asp/Asp	33	5	Ref	------
Arg/Arg-Asp/Glu	18	15	5.50 (1.75–17.33)	**<0.01****
Arg/Arg-Glu/Glu	3	1	2.20 (0.25–19.24)	0.474
Arg/Gln-Asp/Asp	13	14	7.11 (2.17–23.31)	**<0.01****
Arg/Gln-Asp/Glu	11	24	14.40 (4.49–46.14)	**<0.01****
Arg/Gln-Glu/Glu	1	5	33 (3.77–288.62)	**<0.01****
Gln/Gln-Asp/Asp	2	1	3.30 (0.38–28.86)	0.386
Gln/Gln-Asp/Glu	2	5	16.50 (2.78–97.78)	**<0.01****
Gln/Gln-Glu/Glu	0	0	------	------
APE1 (rs3136820)-*XPD* (rs1799793)				
Asp/Asp-Asp/Asp	31	7	Ref	------
Asp/Asp-Asp/Asn	17	12	3.13 (1.05–9.27)	0.056
Asp/Asp-Asn/Asn	0	1	------	------
Asp/Glu-Asp/Asp	14	14	4.43 (1.49–13.15)	**<0.01****
Asp/Glu-Asp/Asn	14	22	6.96 (2.45–19.78)	**<0.01****
Asp/Glu-Asn/Asn	3	8	11.81 (2.63–53.03)	**<0.01****
Glu/Glu-Asp/Asp	3	1	1.48 (0.18–12.30)	1
Glu/Glu-Asp/Asn	1	5	22.14 (2.66–184.56)	**<0.01****
Glu/Glu-Asn/Asn	0	0	------	------
XPD (rs1799793)-*RAD18* (rs373572)				
Asp/Asp-Arg/Arg	31	6	Ref	------
Asp/Asp-Arg/Gln	15	15	5.17 (1.70–15.72)	**<0.01****
Asp/Asp-Gln/Gln	2	1	2.58 (0.30–21.98)	0.448
Asp/Asn-Arg/Arg	21	14	3.44 (1.16–10.24)	**<0.05***
Asp/Asn-Arg/Gln	9	21	12.06 (3.80–38.25)	**<0.01****
Asp/Asn-Gln/Gln	2	4	10.33 (1.75–60.86)	**<0.05***
Asn/Asn-Arg/Arg	2	1	2.58 (0.30–21.98)	0.448
Asn/Asn-Arg/Gln	1	7	36.17 (4.25–307.78)	**<0.01****
Asn/Asn-Gln/Gln	0	1	------	------
RAD18 (rs373572)-*OGG1* (rs1052133)				
Arg/Arg-Ser/Ser	24	7	Ref	------
Arg/Arg-Ser/Cys	27	12	1.52 (0.52–4.43)	0.59
Arg/Arg-Cys/Cys	3	2	2.29 (0.38–13.85)	0.581
Arg/Gln-Ser/Ser	16	23	4.93 (1.74–13.95)	**<0.01****
Arg/Gln-Ser/Cys	8	13	5.57 (1.69–18.38)	**<0.01****

Continued **Table 3:** Distribution of double-combined genotypes and Odds Ratio (OR) of *RAD18* (rs373572), *XPD* (rs1799793), *APE1* (rs3136820), and *OGG1* (rs1052133) polymorphisms in T2DM and HT patients and controls.

Genotype	Control	Case	OR (95% CI)	P value[a]
Arg/Gln-Cys/Cys	1	7	24 (2.86–201.51)	**<0.01****
Gln/Gln-Ser/Ser	2	3	5.14 (0.85–31.17)	0.119
Gln/Gln-Ser/Cys	1	2	6.86 (0.82–57.57)	0.164
Gln/Gln-Cys/Cys	1	1	------	------
XPD (rs1799793)-*OGG1* (rs1052133)				
Asp/Asp-Ser/Ser	29	12	Ref	------
Asp/Asp-Ser/Cys	17	7	1 (0.34–2.95)	1
Asp/Asp-Cys/Cys	2	3	3.62 (0.64–20.50)	0.311
Asp/Asn-Ser/Ser	12	17	3.42 (1.28–9.16)	**<0.05***
Asp/Asn-Ser/Cys	17	17	2.42 (0.95–6.18)	0.096
Asp/Asn-Cys/Cys	3	5	4.03 (0.90–17.96)	0.106
Asn/Asn-Ser/Ser	1	4	9.67 (1.22–76.57)	**<0.05***
Asn/Asn-Ser/Cys	2	3	3.62 (0.64–20.50)	0.311
Asn/Asn-Cys/Cys	0	2	------	------
OGG1 (rs1052133)-*APE1* (rs3136820)				
Ser/Ser-Asp/Asp	23	11	Ref	------
Ser/Ser-Asp/Glu	17	18	2.21 (0.84–5.80)	0.145
Ser/Ser-Glu/Glu	2	4	4.18 (0.76–22.97)	0.174
Ser/Cys-Asp/Asp	23	9	0.82 (0.29–2.31)	0.792
Ser/Cys-Asp/Glu	11	17	3.23 (1.16–9.03)	**<0.05***
Ser/Cys-Glu/Glu	2	1	1.05 (0.13–8.40)	1
Cys/Cys-Asp/Asp	2	0	------	------
Cys/Cys-Asp/Glu	3	9	6.27 (1.49–26.41)	**<0.05***
Cys/Cys-Glu/Glu	0	1	------	------

------ could not be calculated due to absence of particular genotype in the sample
*p<0.05, **p<0.01
[a] Fisher's two-tailed

MDR analysis

The MDR analysis was used to validate our LR based study on gene-gene and gene-environment study by using a model free approach. A four-order interaction model was selected, along with the testing balance accuracy (TBA) and cross validation consistency (CVC). Among the entire dataset, smoking is found to be the best one-factor model found, which was also the best overall model, with a testing accuracy of 0.7442 (p<0.001) and CVC of 10/10. The best model was selected based on the highest TBA value and CVC among all the models. The combination of *XPD* and smoking was found to be the best two-factor model with TBA of 0.7322 (p<0.001) and CVC of 8/10. The combination of *RAD18*, *XPD*, and smoking was found to the best three-factor model, which was also the best overall model with a TBA of 0.6501 (p<0.001) and CVC of 5/10. The best four-factor model consisted of *RAD18*, *XPD*, *APE1*, and smoking with a TBA of 0.5929 (p<0.001) and CVC of 5/10 (Table 7).

Table 4: Distribution of genotypes and odds ratio (OR) of *RAD18* (rs373572), *APE1* (rs3136820), *XPD* (rs1799793), and *OGG1* (rs1052133) for T2DM and HT cases and controls stratified on the basis of smoking.

Genotype	Non-smoker			Smoker		
	Control/Case	OR (95% CI)	P value	Control/Case	OR (95% CI)	P value[a]
RAD18 (rs373572)						
Arg/Arg	45/7	Ref	------	9/14	10 (3.21–31.17)	**<0.01****
Arg/Gln	20/14	4.50 (1.60–12.69)	**<0.01****	5/29	37.29 (10.97–126.70)	**<0.01****
Gln/Gln	4/3	4.82 (0.98–23.75)	0.087	0/3	------	------
APE1 (rs3136820)						
Asp/Asp	40/19	Ref	------	8/10	2.63 (0.92–7.55)	0.097
Asp/Glu	26/13	1.05 (0.45–2.47)	1	5/31	13.05 (4.44–38.33)	**<0.01****
Glu/Glu	3/1	0.70 (0.09–5.36)	1	1/5	10.53 (1.38–80.42)	**<0.01****
XPD (rs1799793)						
Asp/Asp	45/8	Ref	------	3/14	26.25 (6.34–108.67)	**<0.01****
Asp/Asn	24/15	3.52 (1.32–9.37)	**<0.01****	8/24	16.88 (5.71–49.89)	**<0.01****
Asn/Asn	0/1	------	------	3/8	15 (3.46–65.12)	**<0.01****
OGG1 (rs1052133)						
Ser/Ser	35/11	Ref		7/22	10 (3.43–29.19)	**<0.01****
Ser/Cys	29/11	1.21 (0.46–3.15)	0.806	7/16	7.27 (2.43–21.78)	**<0.01****
Cys/Cys	5/2	1.27 (0.24–6.69)	1	0/8	------	------

------ could not be calculated due to absence of particular genotype in the sample
**p<0.01
[a] Fisher's two-tailed

Interaction entropy graph

The interaction entropy graph represented in Fig.2a indicates smoking (18.67%) as the single greatest independent effect in increasing the risk for T2DM and HT among all environmental factors. It is followed by alcohol with entropy of 7.07%. Among all genetic factors, *RAD18* (9.03%) was found to contribute the greatest percentage of entropy. It is followed by *APE1* and *XPD* which were also found to contribute lesser independent entropies of 6.34% and 5.95% respectively.

CART analysis

A CART model was constructed based on all the genetic and environmental factors used in the study. The final tree constructed consisted of seven terminal nodes. The root node was first split on the basis of smoking, clearly indicating that smoking was the single most important independent risk factor for T2DM and HT. Among non–smokers, the subsequent split showed interaction between *RAD18, XPD,* and *APE1* genotypes as important risks for T2DM and HT. Among smokers, further interactions were between alcohol and *RAD18* genotypes (Fig.2b).

Table 5: Distribution of genotypes and odds ratio (OR) of *RAD18* (rs373572), *APE1* (rs3136820), *XPD* (rs1799793), and *OGG1* (rs1052133) for T2DM and HT cases and controls stratified on the basis of tobacco chewing.

Genotype	Non-tobacco chewer			Tobacco chewer		
	Control/Case	OR (95% CI)	P value	Control/Case	OR (95% CI)	P value[a]
RAD18 (rs373572)						
Arg/Arg	26/9	Ref	-	28/12	1.24 (0.45–3.37)	0.798
Arg/Gln	10/16	4.62 (1.58–13.56)	**<0.01****	15/27	5.20 (1.97–13.76)	**<0.01****
Gln/Gln	1/1	------	------	3/5	4.81 (1.04–22.31)	0.089
APE1 (rs3136820)						
Asp/Asp	29/8	Ref	------	19/12	2.29 (0.80–6.54)	0.182
Asp/Glu	7/17	8.80 (2.77-28)	**<0.01****	24/27	4.08 (1.59–10.49)	**<0.01***
Glu/Glu	1/1	------	------	3/5	6.04 (1.29–28.34)	**<0.05***
XPD (rs1799793)						
Asp/Asp	27/8	Ref	------	21/12	1.93 (0.68–5.48)	0.29
Asp/Asn	10/13	4.39 (1.43–13.45)	**<0.05***	22/26	3.99 (1.53–10.41)	**<0.01****
Asn/Asn	0/5	------	------	3/6	6.75 (1.48–30.88)	**<0.01****
OGG1 (rs1052133)						
Ser/Ser	19/10	Ref	------	23/23	1.90 (0.74–4.89)	0.235
Ser/Cys	17/13	1.45 (0.52–4.09)	0.596	19/14	1.40 (0.51–3.86)	0.606
Cys/Cys	1/3	5.70 (0.70–46.52)	0.276	4/7	3.32 (0.83–13.38)	0.153

------ could not be calculated due to absence of particular genotype in the sample
*p<0.05, **p<0.01
[a] Fisher's two-tailed

Table 6: Distribution of genotypes and odds ratio (OR) of *RAD18* (rs373572), *APE1* (rs3136820), *XPD* (rs1799793), and *OGG1* (rs1052133) for T2DM and HT cases and controls stratified on the basis of alcohol consumption.

Genotype	Non-alcohol consumer			Alcohol consumer		
	Control/Case	OR (95% CI)	P value	Control/Case	OR (95% CI)	P value[a]
RAD18 (rs373572)						
Arg/Arg	44/9	Ref	------	10/12	5.87 (1.98–17.36)	**<0.01****
Arg/Gln	19/22	5.66 (2.22–14.40)	**<0.01****	6/21	17.11 (5.48–53.44)	**<0.01****
Gln/Gln	4/5	6.11 (1.46–25.50)	**<0.05***	0/1	------	------
APE1 (rs3136820)						
Asp/Asp	39/12	Ref	------	9/8	2.89 (0.94–8.89)	0.122
Asp/Glu	25/22	2.86 (1.22–6.73)	**<0.05***	6/22	11.92 (3.99–35.57)	**<0.01****
Glu/Glu	3/2	2.17 (0.39–12.15)	0.590	1/4	13 (1.65–102.20)	**<0.05***
XPD (rs1799793)						
Asp/Asp	39/12	Ref	------	9/9	3.25 (1.08–9.79)	0.071
Asp/Asn	27/20	2.41 (1.02–5.68)	0.054	5/19	12.35 (3.88–39.31)	**<0.01****
Asn/Asn	1/4	13 (1.65–102.20)	**<0.05***	2/6	9.75 (1.91–49.68)	**<0.01****
OGG1 (rs1052133)						
Ser/Ser	34/18	Ref	------	8/15	3.54 (1.29–9.74)	**<0.05***
Ser/Cys	28/13	0.88 (0.37–2.08)	0.827	8/14	3.31 (1.19–9.17)	**<0.05***
Cys/Cys	5/5	1.89 (0.51–6.97)	0.478	0/5	------	------

------ could not be calculated due to absence of particular genotype in the sample
*p<0.05, **p<0.01
[a] Fisher's two-tailed

Table 7: Summary of multifactor dimensionality reduction analysis for T2DM and HT risk prediction.

Model	TBC	CVC	P value[a]
SMOKING	**0.7442**	**10/10**	**<0.001***
XPD, SMOKING	0.7322	8/10	<0.001*
RAD18, XPD, SMOKING	0.6501	5/10	<0.001*
RAD18, XPD, APE1, SMOKING	0.5929	5/10	<0.001*

Best model predicted for T2DM and HT risk with highest CVC and maximum TBA. Model in bold represents the best model.
TBA test balance accuracy, CVC cross-validation consistency
*p<0.001
[a] Chi-square

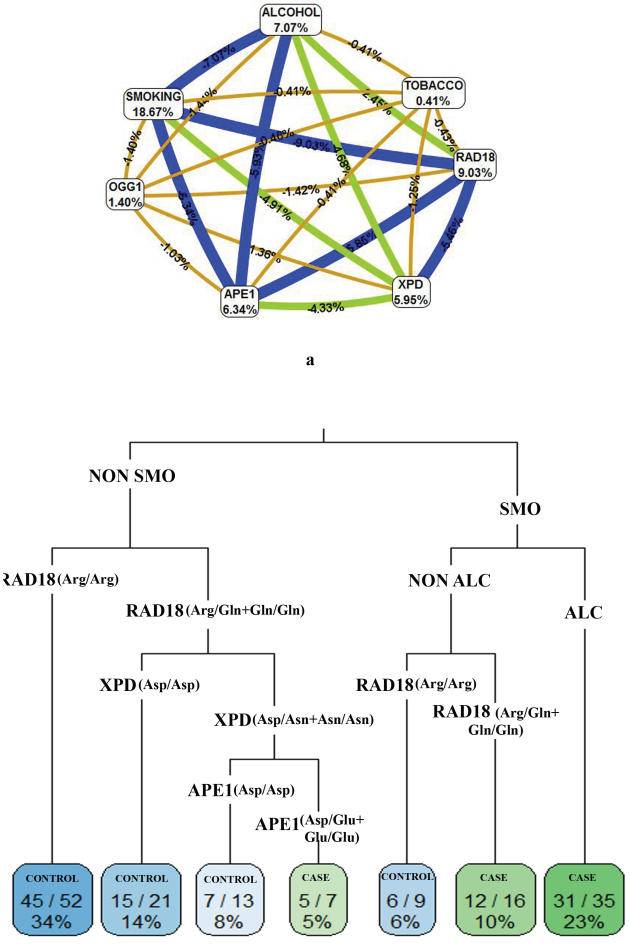

Figure 2: a) Interaction entropy graph to determine the association of gene-gene and gene-environment interaction with T2DM and HT risk. The percent of the entropy for independent factors as well as their interactions are represented in the graph, where positive percentage of entropy denotes synergistic interaction and negative percentage denotes redundancy. Gold denotes the mid-point, while green and blue represent moderate and highest redundancy respectively. **b)** CART analysis for studying the gene-gene and gene-environment risk factors for T2DM and HT. NON SMO and SMO denotes non-smokers and smokers respectively while NON ALC and ALC denotes non-alcohol consumers and alcohol consumers respectively.

Discussion

The goal of this study was to determine the association between altered activity of the DNA repair genes due to different polymorphisms and its synergistic effect with smoking, chewing tobacco, and/or alcohol use in increasing the risk for T2DM and HT. Based on this goal, we determined the effect of DNA repair gene polymorphisms *RAD18* Arg302Gln (rs373572), *XPD* Asp312Asn (rs1799793), *APE1* Asp148Glu (rs3136820), and *OGG1* Ser326Cys (rs1052133) on increased risk for T2DM and HT in a northeastern Indian population.

The role of *RAD18* Arg302Gln polymorphism has been reported previously to be associated with increased risk for colorectal cancer [18] and non-small-cell lung cancers in humans [19]. However, we did not find any previous studies reporting the effect of this polymorphism in increasing the risk for T2DM and HT together. In this study, we observed a significant 30-fold increase in the risk for T2DM and HT among individuals with *RAD18* homozygous mutant genotype. We also observed a significant 8-fold increase in the risk among individuals with *RAD18* heterozygous genotype, thus rendering the polymorphism to be of great importance in the pathogenesis of T2DM and HT. Interestingly, the combination of *RAD18* Arg/Gln and *APE1* Glu/Glu genotypes as well as *RAD18* Arg/Gln and *XPD* Asn/Asn genotypes further increased the risk for T2DM and HT significantly by 33- and 36-fold respectively, clearly indicating the role of multiple SNPs in adding to the risk of these diseases. Gene-environmental studies revealed that smoking and alcohol further exaggerated the risk for T2DM and HT by a significant 37-fold and 17-fold respectively among individuals with the *RAD18* Arg/Gln genotype, thus indicating that gene-environment interaction is an important factor in the onset of several diseases [32].

However, previous studies have reported no association between the Asp148Glu polymorphism of *APE1* and the risk for T2DM [33]. Although we observed a 2.6- and 1.8-fold risk for T2DM and HT in individuals with *APE1* Asp/Glu and *APE1* Glu/Glu genotype respectively, these results were not significant. Our finding is thus consistent with the previously reported results by Merecz et al. [33]. We observed an increase in the risk for the diseases upon gene-gene interaction of *APE1* Asp/Glu with *XPD* Asn/Asn genotype by a significant 11.8-fold. The habits of smoking, tobacco chewing, and alcohol consumption was

also found to increase the risk for T2DM and HT. Smokers and alcohol consumers with *APE1* Asp/Glu genotype were found to have a significant 13- and 11.9-fold increase in the risk. Among tobacco chewers a significant 6-fold increased risk was observed among individuals with *APE1* Glu/Glu genotype.

XPD Asp312Asn and *XPD* Lys751Gln are among the most commonly studied [8], but have previously been associated only with an increased risk for esophageal cancer in particular [34] and cancer in general [35]. In this study, we observed an increase in the risk for T2DM and HT for the Asp/Asn genotype with a 2.62-fold increase in the risk, indicating the role of this factor in T2DM and HT. The *XPD* polymorphism in combination with *APE1* seems to be a risk factor for T2DM and HT. Combined gene-gene interaction of *APE1* Glu/Glu -*XPD* Asp/Asn genotypes were found to increase the risk of the disease significantly by 22–fold, which was much greater in comparison to the risk conferred by the genotypes alone. A similar exaggerated risk was also observed upon gene-environment interaction where alcohol consumers with *XPD* Asp/Asn genotype were found to increase the risk by a significant 12.3-fold.

A large number of studies have been previously conducted to elucidate the role of *OGG1* Ser326Cys polymorphism on the increased risk for T2DM. In a report by Kasznicki et al., no association of the *OGG1* Ser326Cys polymorphism was reported in increasing the risk for T2DM in a Polish population [36]. However, in another study by Thameem et al., it was suggested that *OGG1* could play an important role in the pathogenesis of T2DM [37]. In this study, we observed a slight increase in the risk for T2DM among individuals with *OGG1* Ser/Cys and Cys/Cys genotype by 1.3- and 1.6-fold respectively. However, these changes were found to be non-significant, thereby making the finding inconclusive. We observed an increase in the risk for T2DM and HT among individuals with both *XPD* Asn/Asn and *OGG1* Ser/Ser genotype by a significant 9.6-fold. A significant 7.2-fold increase in the risk was also observed among smokers with *OGG1* Ser/Cys genotype. Thus, gene-gene and gene-environment interactions may increase the risk for T2DM and HT among individuals with these DNA repair gene polymorphisms. However, in some of the sub group analysis, the sample number was relatively low, which requires a larger study group in future research.

MDR analysis revealed that smoking is the single most important risk factor for T2DM and HT with maximum TBA and 100% CVC, which is in accordance with our LR results. The MDR result was further validated by CART analysis where the root node was divided based on smoking, indicating that smoking is the greatest risk factor for T2DM and HT. MDR analysis also revealed an interaction among smoking and *RAD18* and *XPD* polymorphisms in increasing risk for T2DM and HT. In CART analysis, interaction was shown between non-smoker, *RAD18* GA+AA, *XPD* GA+AA, and *APE1* genotype. Unlike LR, CART and MDR do not presume any specific parametric form for SNP-SNP interaction, so they do not miss any such interactions. MDR also helps in reducing type I errors due to cross validation and permutation-testing procedures [38].

Results from the interaction entropy graph also suggest that smoking is the single most important risk factor among all the other environmental factors, with smoking contributing the maximum entropy. *RAD18* polymorphism was found to contribute the greatest entropy among all genetic factors studied.

To the best of our knowledge, this is the first report on the association of the *RAD18* (rs373572), *XPD* (rs1799793), *APE1* (rs3136820), and *OGG1* (rs1052133) genes with T2DM and HT risk in any population worldwide using both the LR and MDR approach. This study sheds light on the role of various DNA repair gene polymorphisms both individually and in association with each other, and different etiological factors in increasing the risk for T2DM and HT.In conclusion, the *RAD18* Arg302Gln (rs373572) and *XPD* Asp312Asn (rs1799793) polymorphisms play an important role in increasing the risk for T2DM and HT in association with smoking, tobacco chewing, and alcohol consumption in the northeastern Indian population.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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