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CASE REPORT

Mitochondrial control region and GSTP1 polymorphism associated with familial urinary bladder cancer in Karbi-Anglong tribe of Assam, Northeast India

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KEYWORDS

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Abstract *Background:* Multiple studies have suggested that subjects with glutathione S-transferase P1 (GSTP1)-mutations are at high risk for urinary bladder cancer (UBC).

Methods: In the present study, we evaluated the mutations in GSTP1 and mitochondrial D-loop genes in two unrelated familial bladder cancer patients belonging to Karbi Anglong Assam tribe. Mitochondrial D-loop and nuclear GSTP1 polymorphic region were amplified and sequenced for all the family members and patients. Two SNPs in the GSTP1 gene for amino acid substitutions at codons 105 (Ile→Val) and 114 (Val→Ala) were genotyped by PCR-RFLP-based methods.

Results: mtDNA D-loop sequence variations were found and there were A and C insertions common at positions 235 and 309, respectively for both the families. Two sequence differences were identified in urinary bladder cancer samples in GSTP1 gene. These two heteroplasmic mutations were found at positions 11qG3037G/A and 11qC3038C/A in patient, father, mother, brother and son, but not in the sister and wife samples in family 2. The GSTP1, 105Ile > Val is most susceptible to inherited UBC risk for these ethnic families. The samples from families 1 and 2, including healthy subjects were placed in macrohaplogroup L3e, except the wife (macrohaplogroup F1c1a) of patient in family 1, and the wife and son (haplogroup M) of the patient in family-2.

Conclusion: A strong familial nuclear GSTP1 sequence variation and mitochondrial control region was observed in this study for familial urinary bladder cancer. This could afford early

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recognition of patients at risk of developing micro- or macroscopic, pathological lesions as well as the introduction of preventive measures for familial bladder cancer.

1. Introduction

Bladder cancer is more common in men and women from industrial areas and the incidence increases with exposure to cigarette smoke and arylamines [1]. Men are affected more often than women (3–4:1) and in most cases patients are over the age of 50 years [2]. As per Indian cancer registry data, in men, it is the ninth most common cancer accounting for 3.9% of all cancer [3]. Urothelial carcinoma comprises about 90% of all primary tumors of the urinary bladder [4]. Glutathione-S-transferases (GSTs) constitute a superfamily of phase II enzymes that are involved in the detoxification of various compounds such as xenobiotic, environmental substances, and potentially carcinogenic compounds [5,6]. Most studies concentrate on the impact of GST, mainly GSTM1 and GSTT1, or on other polymorphic candidate genes for cancer detection [7]. An amino acid variation of isoleucine/valine at codon position 105 in the protein was found due to the GSTP1313 A/G polymorphism at the nucleotide level and valine amino acid which leads to a reduced enzyme activity [8].

Fliss et al. [9] demonstrated somatic mutations of mtDNA in bladder, head and neck, and lung cancers, mainly observed in the D-loop region of mtDNA [10]. The mtDNA transcription and replication are mainly regulated by the D-loop, and alterations in this region might definitely change the rate of mtDNA replication. This may be why a range of cancers commonly have mutations in the D-loop region. Therefore, we predict that variation in the mitochondrial control region might also develop into a genetic marker of hereditary urinary bladder cancer. Jakupciak et al. demonstrated that mtDNA mutations in the D-loop were also detected in urine precipitation from bladder cancer patients [11]. Twenty-three of twenty-six bladder cancer [88%] patients were found to have mtDNA mutations in either the tumor or body fluid or in both [11].

The aim of the present study is to evaluate the presence of mutations in GSTP1 and mitochondrial D-loop sequence of all the family members of the two unrelated bladder cancer patients from Karbi-Anglong tribes of Assam, India

2. Subjects and methods

2.1. Sample collection and processing

Ethnically, the Karbis belong to the Mongoloid tribe and linguistically to the Tibeto-Burman group. In the current study, a Karbi-Anglong family in which at least two first-degree relatives had urinary bladder cancer and another family in which two cases were pathologically diagnosed in second-degree relatives were identified from District Hospital, Dimapur, Nagaland, India (Fig. 1A). Ten members were recruited from family 1 to 9 members from family 2 including the urinary bladder cancer patients. Two healthy subjects from the same ethnic tribes were also included as controls. The protocol of our study was approved by the Institutional Review Board of all institutes involved in the study. The work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for doing the research work with human samples.

2.2. Selection criteria

Clinical Practice Points about the cases are as follows:

- Family 1
  Patient having hematuria: Tumor was removed from the bladder through the urethra by transurethral resection (TURBT). Tumor was non-invasive type. A low-grade superficial papillary carcinoma (Ta) was found in the bladder. Tumor was found on a small section of tissue that was easily removed with TURBT. Patient has a cigarette smoking habit.
- Family 2
  Patient having hematuria: Tumor was removed from the bladder through the urethra by transurethral resection (TURBT). Tumor was invasive type. High-grade superficial T1 tumors are found in the bladder (lamina propria carcinoma). The tumor has spread to the subepithelial connective tissue but does not involve the bladder wall muscle. Patient has a cigarette smoking habit.

2.3. DNA extraction and PCR amplification

Genomic DNA was isolated from 200 µl of each blood sample using the protocol of Ghatak et al. [12]. D-loop region of mitochondrial DNA was amplified by PCR using primers HMt-F (5′-CACCATTAGCACCCAAAGCT-3′) and HMt-R (5′-CT GTTAAAAGTGCATACCGCCA-3′) for HVI region [13]. PCR (vapo.protect; Eppendorf) was carried out in 25 µl total reaction volume, each containing 100 ng template DNA, 0.2 pM of each primer, 2.5 µl 10× PCR buffer (final 1× PCR buffer), 1.5 mM $MgCl_2$, 200 mM dNTP, and 1 unit Taq DNA polymerase. The reaction mixture was heated to 94 °C for 5 min, followed by 40 cycles, each consisting of 1 min denaturation at 94 °C, 1 min annealing at 63 °C, 90 s extension at 72 °C, and a final 10-min extension at 72 °C. GSTP1 polymorphism study was performed with the primers (F-5′-ACAGGATTTGGTACTAGCCT-3′; R-5′-AGTGCCT TCACATAGTCATCCTTG-3′) for intron 5–6 and exon 6 amplification [14]. PCR cycling conditions were 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s with a final elongation step at 72 °C for 7 min. The PCR amplification products (10 µl) were subjected to electrophoresis (Bio-Rad) on 1.2% agarose gel in Trisacetate-EDTA buffer at 80 V for 30 min and stained with ethidium bromide (Himedia), and images were obtained in gel documentation (G-Box; Syngene, Cambridge, UK) systems. PCR products were sequenced by Sanger sequencing method in Scigenom, Cochin, India.

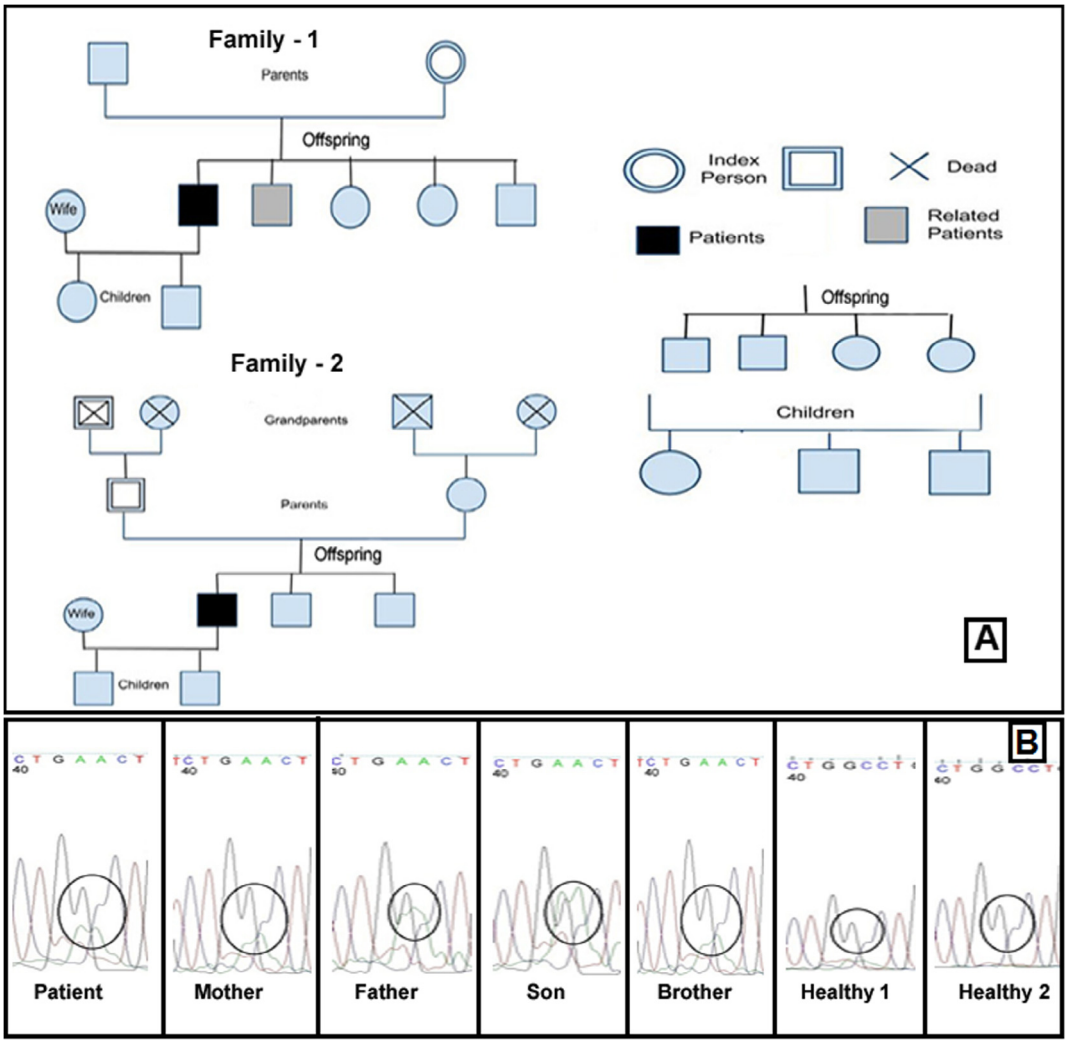

Figure 1 (A) Pedigree chart for family-1 and family-2, (B) GSTP1 heteroplasmic mutation in the familial urinary bladder cancer (11qG3037G/A and 11qC3038C/A) in family 2.

2.4. GSTP1 polymorphic site identification by RFLP

In addition, two SNPs in the GSTP1 gene for amino acid substitutions at codons 105 (Ile→Val) [8] and 114 (Val→Ala) [14] were genotyped by PCR-RFLP-based methods. The fragment containing the GSTP1 Ile105Val (rs1695) polymorphic site was amplified: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and a final elongation at 72 °C for 7 min. About 10 µl of each PCR product was digested with 1 unit of *BsmAI* (New England Biolabs, Spain) for 6 h at 55 °C. Digests were then electrophoresed on 8% polyacrylamide gel resulting in three fragments of 305, 135 and 128 bp (for allele A) or in four fragments of 222, 135, 128 and 83 bp (for allele G). For studying the GSTP1 Val114Ala (rs1138272) polymorphism, PCR cycling conditions were 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s with a final elongation step at 72 °C for 7 min. 10 µl of PCR product was digested with 1 unit of *AciI* (New England Biolabs, Spain) for 6 h at 37 °C and electrophoresed on a 8% polyacrylamide gel. The T allele was defined by the presence of an intact fragment of 170 bp and the C allele by the presence of two fragments of 143 and 27 bp.

2.5. Sequence analysis

All PCR products were sequenced from the opposite direction to ensure reading accuracy. Sequences and chromatograms obtained were examined using chromas software version 2.13 and aligned by BLAST (http://www.ncbi.nlm.nih.gov/blast). All sequences were compared with the latest version of Revised Cambridge Reference Sequence (rCRS) of the human mitochondrial DNA (NC_012920) and subsequently analyzed for the variation in sequences using Mito Tool Programming. The results of the DNA sequence analysis were compared with the published Cambridge Sequence using Mutation Surveyor version 1.4 DNA mutation analysis software (Softgenetics, State College, PA). Sequence differences between patients and healthy blood samples were recorded as mtDNA polymorphisms. Each polymorphism was then verified against the Mitomap database (http://www.mitomap.org/) and further classified as novel or reported, depending on whether or not

it is recorded in the database. Mitochondrial D-loop sequences were used for the mitochondrial haplotypes estimation using Haplogrep server to detect mitochondrial DNA profiles based on Phylotree build 16. The estimated haplogroups were verified using manual checking of Phylotree build 16 from www.Phylotree.org database. The GSTP1 gene sequence were analyzed by the mutation surveyor and ensemble genome browser for polymorphism identification.

3. Results

PCR resulted in a successful amplification of 1030 and 190 bp bands from the D-loop and GSTP1 genes, respectively. All the familial samples with healthy Karbi tribe were placed in macrohaplogroup L3e, except for the wife of patient who was placed in macro-haplogroup F1c1a for family-1 and wife and son (major-haplogroups M) for family-2 (Table 1).

A total of 5 mtDNA D-loop sequence variations at 5 distinct nucleotide positions were found in patient, mother and sister samples in family 1, and 3 distinct variations in family 2. Insertions were found in positions 235 (intA) and 309 (intC) in both the families (Table 2). Among these variations, 2 insertions, 1 small deletion, 1 short sequence repeat and 1 base substitution were observed in the case of family-1. The sequence variations were mostly transitional substitutions except for a cytosine copy number change (7C/8C) at np 303–309 (Table 2). The mutations were mostly not reported in the literature or the public mtDNA mutation databases (Table 2).

Two nucleotide base differences were identified at GSTP1-intron 5–6 position in all the members of family 2 in contrast to the healthy sample (Fig. 1B). This two heteroplasmic mutations were found at positions 11qG3037G/A and 11qC3038C/A in patient, father, mother, brother and son samples, but absent in the case of sister and wife samples for family 2. Significant differences in genotype or allele frequencies of the GSTP1 IIe105Val polymorphisms were observed in family 2 (Fig. 1A) but not in family 1. The presence of heterozygous allele A/G in rs1695 was found in maternal DNA as an index person, and homozygous allele G/G in rs1995 was found in the patient in family 2 (Fig. 1A). In case of rs1138272, C/C homozygous was observed for both the family members.

4. Discussion

Our sequence analysis focused on the mtDNA D-loop region which is highly polymorphic and contains two hypervariable regions, HV1 (16024–16383) and HV2 (57–333), that are considered as somatic mutation "hot spot" regions in many types of cancer [15]. In this study, no deletions were seen in the mitochondrial genome. A total of 5 mtDNA D-loop sequence variations at 5 distinct nucleotide positions were found in patient, mother and sister samples out of the all samples examined for family 1, which were not previously reported. Fliss et al. screened 14 urinary bladder cancers for somatic mutations in the D-loop region and found mutations in 4 (29%) samples [9].

The mononucleotide repeats at nucleotide position 303–309 represent a well known mutational hotspot prone to instability; the instability at this position were found in mother and patient samples for family 1. Marchington et al. [16] first used the term D310 to describe a highly polymorphic mononucleotide tract of

Table 1 Haplogroup information based on the mitochondrial D-loop gene.

Family-1				Family-2			
Sample ID	Sample	Age (yrs)	Haplogroup	Sample ID	Sample	Age (yrs)	Haplogroup
NMD1	Mother (index person)	67	L3e	NGF1	Grandfather (index person)	Deceased at 74	L3e
NFD1	Father	71	L3e	NMD1	Mother	65	L3e
NPD1	Son (target patient)	46	L3e	NFD1	Father (index person)	67	L3e
NBD1 (2 no.)	Brother (1 is patient)	49 44	L3e	NPD1	Son (target Patient)	42	L3e
NSD1 (2 no.)	Sister	41	L3e	NBD1 (2 no.)	Brother	46 38	L3e
NWD1	Wife	41	F1c1a	NWD1	Wife	39	M
NDD1	Daughter	12	L3e	NDS1 (2 no.)	Son	14 10	M
NDS1	Son	14	L3e				
NN1D1	Healthy control	52	L3e	NN1D1	Healthy control	52	L3e
NN1D1	Healthy control	49	L3e	NN1D1	Healthy control	49	L3e

Table 2 Mitochondrial D-loop mutations in the cancer patients.

Family-1				Family-2			
Position	Sample	Reference nucleotide	Nomenclature of the mutation	Position	Sample	Reference nucleotide	Nomenclature of the mutation
202†	Sister	–	202A-Int	235†	Patient	–	235A-Int
235†	Patient	–	235A-Int				
309	Mother and patient	–	310C-Int	309	Father and patient	–	310C-Int
337†	Patient	A	337A-Del	384†	Patients	A	384A > C
384†	Patients	A	384A > C				

† Indicates novel mutations (not reported in the Mitomap database).

poly (C) that varies from 12 to 18 Cs, located between nucleotide positions 303 and 318 in CSB II, that forms a RNA–DNA hybrid known as an R-loop. This poly (C) region is interrupted at nucleotide position 310 by a T (CCCCCCCTCCCCC), in which the number of Cs before the T can varies between 7 and 9 in normal polymorphic variants [17]. The sequence variations in D-loop region were mostly transitional substitutions except for a cytosine copy number change (7C/8C) at np 303–309 (Table 2) which was found in both the families. Wada et al. [18] also reported that the majority of somatic mutations were homoplasmic suggesting that the mutant mtDNA became dominant in tumor cells. Hence, a strong maternal mutational inheritance was observed in this study.

A strong familial nuclear GSTP1 sequence variation was observed in this study for urinary bladder cancer in family 2. Although the risk is modest, the high prevalence of deficiency is likely to make a significant impact on bladder cancer incidence [19]. Törüner et al. [20] reported that GSTP1Ile/Val or Val/Val genotype enhanced the vulnerability to bladder cancer by 1.75-folds. In a different study, Srivastava et al. [21] reported that the Val/Val genotype of the GSTP1 gene polymorphism increases the susceptibility by 7.1-fold. In the GSTpi-1 gene, GSTP1 105 (rs1695) polymorphism evaluated was related to familial urinary UBC susceptibility or phenotype. The homozygous status of GSTP1 105 (rs1695) (A > 105G) is most susceptible to UBC risk in Karbi-Anglong tribe of Assam, Northeast India, but familial heterozygous (A/G) is also a risk factor for UBC. Two heteroplasmic mutations were found at positions 11qG3037G/A and 11qC3038C/A in family-1. These two heteroplasmic mutations might be the potential factors for inherited familial UBC. In Caucasians, complete lack of GSTM1 activity was observed in 50% of the population. About 1.47-fold increase in bladder cancer risk is associated with GSTM1 deficiency and GSTT1 mutation in one fifth of cases [22]. Inheritance of the GSTT1 null genotype was found to be associated with several types of carcinogenesis including bladder cancer [23]. Indeed, the GSTP1codon 105Val allele was associated with a significantly increased risk of lung, bladder, and testicular cancers [23].

5. Conclusion

To our knowledge, the present study is a novel finding in-terms of the possible role of mtDNA D-loop and nuclear GSTP1 gene mutations in familial urinary bladder cancer in Assam Karbi tribe population. The impact of this novel finding for clinical practice in the foreseeable future is that the mitochondrial D-loop gene and nuclear GST alterations may attribute to familial urinary bladder cancer in Assam Karbi population.

Conflict of interest

The authors declare no conflict of interest.

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