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Research paper

Exploring the deleterious SNPs in XRCC4 gene using computational approach and studying their association with breast cancer in the population of West India

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ABSTRACT

Non-homologous end joining (NHEJ) pathway has pivotal role in repair of double-strand DNA breaks that may lead to carcinogenesis. XRCC4 is one of the essential proteins of this pathway and single-nucleotide polymorphisms (SNPs) of this gene are reported to be associated with cancer risks. In our study, we first used computational approaches to predict the damaging variants of *XRCC4* gene. Tools predicted rs79561451 (S110P) nsSNP as the most deleterious SNP. Along with this SNP, we analysed other two SNPs (rs3734091 and rs6869366) to study their association with breast cancer in population of West India. Variant rs3734091 was found to be significantly associated with breast cancer while rs6869366 variant did not show any association. These SNPs may influence the susceptibility of individuals to breast cancer in this population.

1. Introduction

Breast cancer is the most frequently occurring cancer among women worldwide and it is the leading female cancer and cause of death in India, with approximately 75,000 deaths annually (Parkin et al., 2005; Indian Council of Medical Research, 2010). The DNA repair system maintains genomic integrity of mammalian cells. Decrease in DNA repair capacity leads to high oxidative stress, which can further initiate carcinogenesis (Oldenburg et al., 2007). Many high penetrant genes of DNA repair pathways (*e.g.*, BRCA1 and BRCA2 *etc.*) are reported to be involved in inherited predisposition to breast cancer. The high penetrant genes only explain 5% of breast cancer cases, thus, other low penetrant genes that have essential role in maintaining genomic integrity may cause predisposition to breast cancer (Bau et al., 2007). Genomic variants of these genes significantly increase breast cancer risk and depict a major role of the DNA repair pathway in breast carcinogenesis (Kennedy et al., 2005). Several studies have investigated the role of single nucleotide polymorphisms (SNPs) in DNA repair genes in relation to breast cancer and have reported associations with breast cancer risk (Goode et al., 2002; Kuschel et al., 2002; ´a-Closas et al., 2006; Haiman et al., 2008). Based on these reports, it is suggested that SNPs in genes involved in DNA repair may influence DNA repair capacity and, in turn, alter susceptibility to develop breast cancer. The *XRCC4* gene is an important protein of non-homologous end-joining (NHEJ) repair pathway, that repairs DNA double-strand break (Li et al., 1995). The XRCC4 protein interacts directly with Ku70/Ku80 dimer, and its associated protein, ligase 4 (Lig4) (Mari et al., 2006). One study on gene-targeting mutation mouse model, found that *XRCC4* gene inactivation led to late embryonic lethality, defective lymphogenesis and neurogenesis because of severe apoptotic death of newly generated neuronal cells (Gao et al., 1998). XRCC4 was also found to play a role in the age at diagnosis and risk of breast cancer in non-heritable cases

Abbreviations: ARMS, amplification-refractory mutation system; BER, base excision repair; CI, confidence interval; DDG, Gibbs free energy change; HWE, Hardy–Weinberg equilibrium; HMM, Hidden Markov Model; NCBI, National Centre for Biological Information; NHEJ, non-homologous end-joining; nsSNPs, non-synonymous single nucleotide polymorphisms; OR, odds ratio; PANTHER, Protein ANalysis THrough Evolutionary Relationships; PDB, Protein Data Bank; POLYPHEN, polymorphism phenotyping; PSIC, profile extraction from sequence alignments with position-specific counts; RI, reliability index; SIFT, sorting intolerant from tolerant; SNPs, single nucleotide polymorphisms; subPSEC, substitution position-specific evolutionary conservation

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Table 1
Case-control studies of polymorphisms in *XRCC4* gene.

SNP	Population	No. of cases	No. of controls	Result	Reference
rs2075685 rs1805377 rs2075686	Taiwanese	254	379	S[a] NS[b] NS	(Fu et al., 2003)
rs1056503	Korean	872	671	NS	(Lee et al., 2005)
rs1805377	Caucasian (Poland and USA)	10,979	10,423	NS	(Garcia-Closas et al., 2006)
rs1478485 rs13180316 rs963248 rs1056503	North European	457 458 455 459	576 576 574 576	S S NS NS	(Allen-Brady et al., 2006)

[a] S-significant.
[b] NS-nonsignificant.

(Gao et al., 2000).

Many studies were reported which investigated the association of polymorphisms of XRCC4 and cancer risk (Table 1). The studies of different SNPs were reported in different populations which showed variable results. SNP (rs2075685) which located in the intron of *XRCC4* gene was found to be significantly associated with breast cancer in a Northern Taiwan population (OR = 0.583, $P = 0.02$) while SNPs rs1805377 and rs2075686 were not associated with breast cancer risk (Fu et al., 2003).

In this study, we hypothesized that genetic variants of *XRCC4* gene may modify susceptibility to breast cancer in population of West India. We screened genetic variants of *XRCC4* gene by computational analysis and then selected the most damaging SNP. We also selected two SNPs rs3734091 and rs6869366 which have been investigated earlier and found to be associated with oral cancer and breast cancer respectively in Taiwan population (Chiu et al., 2008a; HCWe et al., 2008). That is why these three SNPs were selected to assess their association with breast cancer in a case-control study of women from West India.

2. Materials and methods

2.1. In silico analysis

Computational tools were used to analyse the effects of non-synonymous SNPs on XRCC4 structure and function. Sorting intolerant from tolerant (SIFT), Polymorphism Phenotyping (POLYPHEN), I-MUTANT, SNAP, PhD-SNP, nsSNPAnalyzer and Protein ANalysis THrough Evolutionary Relationships (PANTHER) were used to predict the damaging nsSNPs of this gene on the basis of evolutionary, homology and structural properties (Pauline and Steven, 2003; Adzhubei and Sunyaev, 2013; Ramensky et al., 2002; Bao et al., 2005; Brunham et al., 2005; Thomas et al., 2003; Capriotti et al., 2006; Capriotti et al., 2005) (14–22). SwissPDBviewer and PyMOL were used for structure modulation and structural analysis (Bromberg and Rost, 2007; Duhovny et al., 2002; Schneidman-Duhovny et al., 2005). PatchDock and FireDock were used for docking analysis (Mashiach et al., 2008).

2.2. Study participants

Blood samples were collected between 2014 and 2016 from 120 female breast cancer patients between age 30 and 80 years and 130 age-matched healthy controls from Shri Krishna Hospital, Karamsad, Gujarat. Informed consent was obtained from both patients and controls prior to blood collection. This study was approved by Human Research Ethics Committee, H.M. Patel Centre for Medical Care and Education, Karamsad, Gujarat (Ref. No.: HREC/FCT/49/Session 2/8). The clinical characteristics of patients are mentioned in Table 4. BRCA1/2 positive subjects were excluded from the study.

2.3. Genotyping of XRCC4 SNPs (rs3734091, rs6869366 and rs79561451)

Genomic DNA was isolated from collected blood samples using phenol-chloroform method. PCR-RFLP analysis was used to study the DNA samples for two genetic polymorphisms (rs3734091-at codon 247 and rs6869366 (G-1394T) in intronic region. The primer sets used for SNP rs3734091 were: 5′-GCTAATGAGTTGCTGCATTTTA-3′ (forward) and 5′-TTTCTAGGGAAACTGCAATCTGT-3′ (reverse), while for SNP rs6869366 were: 5'-GATGCGAACTCAAAGATACTGA-3′ (forward) and 5′- -TGTAAAGCCAG TACTCAAACTT-3′ (reverse) (Chiu et al., 2008a). The cycling conditions used were: one cycle at 95 °C for 5 min; 35 cycles of 95 °C for 40 s, 55 °C for 40 s, and 72 °C for 45 s; and a final extension at 72 °C for 5 min. The PCR products of rs3734091 and rs6869366 were digested with restriction enzymes *Bbs*I (New England Biolabs, Beverly, MA, USA) and *Hinc*II (New England Biolabs), respectively, in order to detect the variants. There were 308-bp and 300-bp amplified PCR products for rs3734091 and rs6869366, respectively. The BbsI and HincII restriction enzymes were used to detect polymorphisms, which resulted in 204-bp and 104-bp fragments in the presence of A allele (rs3734091); and 200-bp and 100-bp fragments in the presence of G allele (rs6869366).

For SNP rs79561451 (T/C, S110P), we used Amplification-refractory mutation system (ARMS) technique to detect the variant allele. Primers were designed using ARMS primer design software (http://cedar.genetics.soton.ac.uk/public_html/primer1.html) (Ye et al., 2001). Primer sets used were: forward outer- 5′-AAACTTTAGGTAGAA GCCCAGTTTTTTT-3′, reverse outer: 5′-CTTGAACATCATTCCAATCTCT CAGA-3′, forward inner 5′-TTTTTCTTTCATTTTAGTTCAGACTTG TTC-3′ (for C allele) and reverse inner 5′-GGTTTTCAACTTTCTCTAGG TTGAATGA-3′ (for T allele). The cycling conditions used were: one cycle at 95 °C for 5 min; 35 cycles of 95 °C for 40 s, 67 °C for 40 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products of forward outer (FO) and reverse outer (RO) primers is 337 bp, product of forward outer (FO) and reverse inner (RI) is 214 bp (for allele T) and product of forward inner (FI) and reverse outer (RO) is 181 bp (C allele). At 67 °C annealing temperature, we obtained only 337 bp and 214 bp bands that correspond to homozygous wildtype condition (TT). To confirm the results of ARMS method, we designed new primer to introduce restriction site at the end of primer. The new primer sequence is 5′-TTTTTCTTTCATTTTAGTTCAGACTTGAT-3′ (forward inner). The PCR product (180 bp) was analysed for polymorphism using restriction enzyme *Mbo*I which resulted in 151-bp and 29-bp fragments in the presence of C allele. We also used capillary electrophoresis sequencing method to confirm the results. Genetic analyser 3500 of Applied Biosystems with POP7 polymer was used.

2.4. Statistical analysis

The χ^2 test was used to calculate differences between cases and controls for the age. The correlation of genotypes and allele frequency between cases and controls were also obtained. Odds ratio and confidence of interval was obtained using Fisher exact test. Hardy–Weinberg equilibrium (HWE) of rs3734091, rs79561451 and rs6869366 genotypes was tested by performing a Chi square test (χ^2) of control samples. Statistical analysis was carried out using the Prism GraphPad software.

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