RESEARCH ARTICLE

Lack of any Association of the CTLA-4 +49 G/A Polymorphism with Breast Cancer Risk in a North Indian Population

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Abstract

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an important protein involved in the regulation of the immune system. The +49 G/A polymorphism is the only genetic variation in the CTLA-4 gene that causes an amino acid change in the resulting protein. It is therefore the most extensively studied polymorphism among all CTLA-4 genetic variants and contributions to increasing the likelihood of developing cancer are well known in various populations, especially Asians. However, there have hiterto been no data with respect to the effect of this polymorphism on breast cancer susceptibility in our North Indian population. We therefore assayed genomic DNA of 250 breast cancer subjects and an equal number of age-, sex- and ethnicity-matched healthy controls for the CTLA-4 +49 G/A polymorphism but no significant differences in either the gene or allele frequency were found. Thus the CTLA-4 +49 G/A polymorphism may be associated with breast cancer in other Asians, but it appears to have no such effect in North Indians. The study also highlights the importance of conducting genetic association studies in different ethnic populations.

Keywords: CTLA-4 - immunology - breast cancer - single nucleotide polymorphisms - ethnic groups - North Indians

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Introduction

Immunobiology is an exciting area of life sciences with respect to the cancer biology. One of the reasons why cancer develops and spreads so rapidly is because of the weak immunosurveillance (Reiman et al., 2007). Proper function of cytotoxic T Lymphocytes (CTLs) is critical for immunosurveillance but cancer cells develop multiple mechanisms to evade the immune response. In other words the body's immune system fails to recognize and destroy the rapidly growing cancer cells. This immunosurveillance or policing action by the immune system is regulated by numerous proteins; some of which upregulate it while some downregulate it.

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an interesting protein involved in the downregulation of immune system. On one hand it is known to increase the susceptibility of various autoimmune diseases and on the other hand it is also known to be associated with susceptibility and aggressiveness of various cancers (Ghaderi et al., 2011; Geng et al., 2013). Its role in skin cancer is well documented and we now even have a drug (Ipilimumab) against this important molecular target (Blank, 2014). CTLA4 downregulates the immune system response as it reduces the T-cell response to foreign antigens as well as to autoantigens.

The gene CTLA-4 is located on chromosome 2q33 and several single nucleotide polymorphisms (SNP) in this gene are known to have an effect on its genetic expression and protein activity (Ligers et al., 2001; Ueda et al., 2003). SNPs in CTLA-4 gene can have a profound effect on immune system and how it responds towards certain diseases. Genetic alterations which upregulate the immune system activity decrease the risk of developing cancer, but at the same time, increase the risk of developing certain autoimmune diseases (Ghaderi et al., 2011). Conversely genetic alterations which downregulate the immune system activity increase cancer susceptibility but decrease the risk of developing autoimmune diseases.

The +49 G/A polymorphism is the only genetic variation in CTLA-4 gene that causes an amino acid change in the resulting protein due to which it is the most extensively studied polymorphism among all the CTLA-4 genetic variants. It causes a 17Thr to 17Ala substitution in the leading peptide of CTLA-4 receptor. Its role in increasing cancer susceptibility is well known in various populations across the world, especially Asians (Geng et al., 2013). The effect of SNPs on cancer susceptibility differs in different ethnic populations and there is no data till date with respect to the effect of this SNP on breast cancer susceptibility in our North Indian population. Breast cancer risk and pathogenesis can be influenced by

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single nucleotide polymorphisms and several studies in the past have identified different genetic variants in the human genome that showed strong or moderate evidence of associations with breast cancer (Zhang et al., 2011). Besides, breast cancer is the leading type of cancer among women of North India and its rate is expected to rise in the years to come (Takiar et al., 2010). We therefore decided to study the association of CTLA-4 +49 G/A polymorphism with breast cancer susceptibility in a North Indian population.

Materials and Methods

Subjects

A total of 250 North Indian breast cancer subjects and an equal number of age, sex and ethnicity matched healthy subjects (controls) were recruited for this study. All subjects gave written informed consent. The study was approved by the ethics committee of Sir Ganga Ram Hospital.

DNA extraction and genotyping

Four mL of venous blood was obtained from each subject. DNA was subsequently isolated using the QIAamp DNA Blood Mini Kit (QIAGEN, Germany).

Genotyping of CTLA-4 +49 G/A polymorphism was performed using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) based assays, with Taq polymerase (Genetix Biotech, India) and a GeneAmp PCR System 9700 (Applied Biosystems, USA). Genotypes were analysed with minor modifications in methods previously described by Sun et al. (Sun et al., 2008). PCR reaction and genotyping conditions used are summarized in Table 1. The gel picture (2.5% agarose) is shown in Figure 1.

Table 1. PCR Reaction and Genotyping Conditions

Forward primer	5'-AAGGCTCAGCTGAACCTGGT-3'
Reverse primer	5'-CTGCTGAAACAAATGAAACCC-3'
Annealing temperature	61°C
PCR product fragment length	152 bp
Restriction enzyme	Eco91I (Fermentas)
Fragment length produced after RFLP	152 bp for G allele
	130 bp and 22 bp for A allele

Table 2. Distribution and Comparison of Genotypes of CTLA-4 +49 G/A Polymorphism in Breast Cancer Patients and Controls

	AA	AG	GG
Patients (n=250)	111	113	26
Controls (n=250)	105	121	24
Statistical analysis		X^2=0.520, p=0.771	
		OR (GG *vs* AA)=1.0248 (95% CI=0.5537-1.8967)	
		OR (GG *vs* AG)=1.1600 (95% CI=0.6295-2.1375)	

Statistical analysis

Hardy-Weinberg equilibrium (HWE) software (Rodriguez et al., 2009) was used to calculate the expected genotype frequencies and, subsequently, the X^2 test was used to compare the observed and expected genotype frequencies. The distribution of genotypes in patients was compared with that of controls, also with the X^2 test. Values with $p<0.05$ were considered significant. Odds ratio (OR) was estimated to calculate the relative risk of breast cancer susceptibility for each genotype and allele.

Results

The distribution and comparison of genotype and allele frequencies of CTLA-4 +49 G/A polymorphism in breast cancer patients and healthy controls are shown in Tables 2 and 3. There was no deviation from HWE in either the cases or controls for this polymorphism ($p>0.05$). Frequencies of the AA, AG, and GG genotypes were 44.4%, 45.2%, and 10.4% in patients, while the frequencies of these genotypes in controls were determined to be 42.0%, 48.4%, and 9.6%, respectively. No significant difference could be found while comparing these values statistically (p=0.771). We also observed no association between any of the genotypes and breast cancer risk (OR for GG *vs* AA=1.0248, 95%CI=0.5537-1.8967; OR for GG *vs* AG=1.1600, 95%CI=0.6295-2.1375). Allele frequencies in patients and control group were 67.0% and 66.0% for A allele, and 33.0% and 34.0% for G allele, respectively. Statistical analysis of these values again did not reveal any significant difference between the two groups or any association with breast cancer risk (OR=0.9647, 95%CI=0.7417-1.2547, p=0.7886).

Discussion

CTLA-4 protein is immunosuppressive in nature and many polymorphisms in its gene are known to have an effect on its immunosuppressive activity. The +49 G/A polymorphism is the one that is most commonly studied

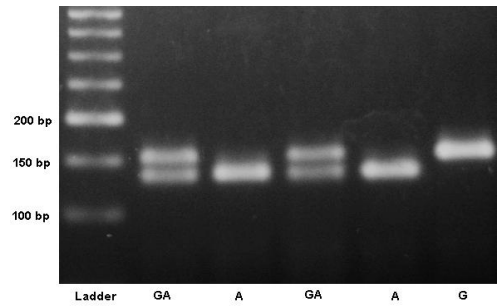

Figure 1. Photograph of Ethidium Bromide Stained Agarose Gel (2.5%)

Table 3. Table 3. Distribution and Comparison of Allele Frequencies of CTLA-4 +49 G/A Polymorphism in Breast Cancer Patients and Controls

	Patients (n=250)		Controls (n=250)			
	No. of alleles	Allele frequency	No. of alleles	Allele frequency	OR (95% CI)	p value
A	335	0.67	331	0.66	1.0 (reference)	0.7886
G	165	0.33	169	0.34	0.9647 (0.7417 - 1.2547)	

SNP in CTLA-4 gene. Previous studies have found that the G allele has lower mRNA efficiency and decreased CTLA-4 protein production than the A allele (Chistiakov et al., 2006). Since CTLA-4 downregulates T-cell production individuals with GG genotype (less CTLA-4 production) have higher T-cell proliferation than those with the AA genotype (Maurer et al., 2002). It is due to this reason that the risk allele for susceptibility to cancer (allele A) is the opposite of that found for susceptibility to autoimmune diseases (allele G) (Sun et al., 2009). The association of this SNP with cancer risks has been studied previously in different ethnic populations. Sun et al. found it to be associated with the cancer of oesophagus and lung among the Chinese (Sun et al., 2008) and an association with gastric cancer was found by Hou et al. (Hou et al., 2010). It has also been associated with non-solid tumours like non-Hodgkin's lymphoma (Monne et al., 2004). Another study found this SNP to be associated with bad lung cancer prognosis (Song et al., 2011).

CTLA-4 +49 G/A polymorphism was not found to be associated with breast cancer risk in our North Indian population. It was found to be positively associated with breast cancer by Sun et al. and Wang et al. in Chinese and by Ghaderi et al. in Iranian breast cancer patients (Ghaderi et al., 2004; Wang et al., 2007; Sun et al., 2008). Ethnic disparities like these do exist with respect to the prevalence of a polymorphism in a particular ethnic population and its association with a disease (Mandal et al., 2010). For example, CTLA-4 +49 G/A polymorphism is known to be associated with the risk of colorectal cancer in Chinese but no such association was seen in a study on Turkish patients (Dilmec et al., 2008; Qi et al., 2010). This SNP was also found to be not associated with colorectal neoplasm among Italian Caucasians (Solerio et al., 2005). According to a recent meta-analysis report CTLA4 +49A/G polymorphism is associated with an increased risk of Hashimoto's thyroiditis in Asian but not Caucasian populations (Feng et al., 2013). Due to different genetic make-up, results of one particular ethnic population may not hold true for some other population of different ethnic background (Taheri et al., 2012; Shaukat et al., 2013).

To the best of our knowledge this is the first report from North India on the association of this polymorphism with breast cancer. In conclusion, our results, combined with results of previous studies in other populations, indicate that CTLA-4 +49 G/A polymorphism is associated with breast cancer in Asians, but it appears to have no such effect in North Indians. This study also highlights the importance of doing such genetic association studies in different ethnic populations and further evaluation of CTLA-4 +49 G/A polymorphism and breast cancer in other ethnic populations is warranted.

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