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RESEARCH ARTICLE

Genetic variant of *CCND1*: Association with HPV-mediated cervical cancer in Indian population

Nisha Thakur[1], Showket Hussain[2], Indu Kohaar[1], Rubina Tabassum[3], Vilas Nasare[1], Pratibha Tiwari[1], Swaraj Batra[4], Suresh Bhambhani[5], Bhudev C. Das[2], Seemi Farhat Basir[6], Dwaipayan Bharadwaj[3], and Mausumi Bharadwaj[1]

[1]Divisions of Molecular Genetics & Biochemistry,[2]Molecular Oncology, Institute of Cytology & Preventive Oncology (ICMR), Noida, India,[3]Functional Genomics Unit, Institute of Genomics & Integrative Biology (CSIR), Delhi, India, [4]Department of Obstetrics & Gynaecology, LNJP Hospital, New Delhi, India,[5]Cytopathology, Institute of Cytology & Preventive Oncology (ICMR), Noida, India, and [6]Department of Biosciences, Jamia Millia Islamia, New Delhi, India

Abstract

The potential association of single nucleotide polymorphisms (SNPs) (G870A and G1722C) of *CCND1* with susceptibility to cervical cancer was investigated. The study included 200 cervical cancer cases along with an equal number of healthy controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and direct sequencing were employed for genotyping. We found that women carrying the 870AA genotype have a 2.49-fold increased risk for the development of cervical cancer (odds ratio (OR) 2.49; 95% confidence interval (CI) 1.51–4.09; $p = 0.0004$) compared with GG+GA genotypes. For the 1722 locus, the frequency of the polymorphic 'C' allele was strongly associated with a reduced risk of cervical cancer ($p = 0.019$; OR 0.71; 95% CI 0.54–0.94). Our data suggest that *CCND1* G870A polymorphism could act as a risk factor for the development of cervical cancer. And G1722C polymorphism may play a protective role against the development of human papillomavirus-associated cervical cancer among Indian women.

Keywords: *Cyclin D1; cervical cancer; human papillomavirus; single nucleotide polymorphism; G870A; G1722C*

Introduction

Cancer of the uterine cervix is the second most common gynecological cancer worldwide but it is the most common cancer among Indian women and it forms a major public health problem (Haverkos et al. 2000). In India, it has been estimated that more than 1 20 000 women develop this cancer every year with about 75 000 annual deaths, constituting about 16% of the world's annual incidence (Cohen 2005). Several risk factors are reported to be associated with the development of this cancer. But the infection with specific types of human papillomaviruses (HPVs) has emerged as the major etiological factor for the development of cervical cancer (Gissmann et al. 1977). HPV type 16 is the most prevalent HPV accounting for more than 70% of cervical cancer cases in India, followed by HPV type 18 and other high-risk types (Franceschi et al. 2003). However, an infection with HPV is essential but it is not sufficient for the development of cervical cancer, which implies the involvement of host genetic factors (Kohaar et al. 2007, Das et al. 2008).

The primary cause in the development and progression of cervical neoplasia has been shown to be dependent on a series of cellular genetic and epigenetic events including mutation, deletion, polymorphism and/or methylation on various checkpoints of cell cycle machinery. Integration of HPV DNA into the host genome results in the expression of viral oncoproteins E6 and E7, which deregulate the cell cycle by interacting with oncogenes and tumour suppressor gene products

Address for Correspondence: Mausumi Bharadwaj, Division of Molecular Genetics & Biochemistry, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida 201301, India. Tel.: 91-120-2579471 (home 91-120-2486763). Fax: 91-120-2579473. E-mail: bharadwajm@icmr.org.in; mausumi02@yahoo.com

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thereby initiating tumorigenesis. The E7 oncoprotein binds with retinoblastoma protein (RB) and related pocket proteins p107, p130, leading to the deregulation of the G1–S phase which is regulated by cyclins, cyclin-dependent kinases (CDKs) and their inhibitors (Dyson et al. 1989).

Cyclin D1 (*CCND1*), located on 11q13 is a proto-oncogene and acts as a key regulator of the G1/S phase of the cell cycle. Alteration of this gene has been found to be associated with several types of cancers (Motokura et al. 1991). The activity of *CCND1* reaches a maximum during the G1 phase and regulates the cell cycle progression by activating the CDK4 and CDK6, which in turn phosphorylate RB and inactivate it. Overexpression of cyclin D1 is found to be associated with the disruption of normal cell cycle control with possible promotion and development of cancer growth (Sherr 1996).

Single nucleotide polymorphism (SNP) G870A in the splice donor region of exon 4 of the *CCND1* gene (Betticher et al. 1995) has been studied with several cancers including urinary bladder (Wang et al. 2002), endometrial (Kang et al. 2005), breast (Grieu et al. 2003), head and neck (Zheng et al. 2001), prostate (Koike et al. 2003), colorectal (Kong et al. 2000) and cervical cancer (Catarino et al. 2005, Jeon et al. 2005, Kaur et al. 2008). Several reports have also shown that overexpression of this proto-oncogene is associated with carcinogenesis and clinical outcome of the disease (Zheng et al. 2001).

A second common polymorphism of the cyclin D1 gene at nucleotide 1722(G1722C) within *CCND1* 3'UTR has also been described (Heighway 1991). Holley et al. (2001) studied this polymorphism in patients with squamous cell carcinoma of head and neck (SCCHN) but not with the risk of cancer. To date, only one study has been conducted to investigate the effect of *CCND1* (G1722C) polymorphism on cancer risks (Sathyan et al. 2005).

To the best of our knowledge, no report is available addressing the influence of the G1722C polymorphism of the *CCND1* gene on the susceptibility to HPV-associated cervical cancer. Therefore, the present study has been designed to investigate the potential association of *CCND1* gene polymorphisms (G870A/G1722C) with the susceptibility of cervical cancer in Indian women, who have the largest incidence of this cancer in the world.

Materials and methods

Study subjects

Cervical tissue biopsies samples from 200 patients (cases) of Indo-Aryan ethnicity comprising cervical precancer (46) and invasive carcinoma (154) were used for the study. The patients were recruited from Lok Nayak Jai Prakash (LNJP) Hospital, New Delhi, with histopathologically confirmed precancer (cervical intraepithelial neoplasia (CIN) I, II, III)/invasive carcinoma (grade IV) of the uterine cervix. The World Health Organization and CIN classification systems were used for classification and grading of the precancerous and cancerous lesions. The patients had a mean age of 49 ± 11.3 years.

The 200 age- and ethnicity-matched control samples (cervical scrapes/tissue biopsies) were obtained from women with normal cervical cytology and no clinical history of cancer who attended the clinic for other gynecological reasons. Written consent was obtained from all the participants and the study was carried out in accordance with the principles of Helsinki Declaration and was approved by the Ethics Committee of the Institute.

DNA extraction and HPV detection

Genomic DNA was extracted from fresh cervical tissue biopsy/cervical scrape samples from patients and controls, respectively, by a standard method using proteinase K followed by phenol/chloroform/isopropanol treatment (Sambrook et al. 1989).

HPV diagnosis was performed by polymerase chain reaction (PCR) amplification using consensus primers MY09 and MY11 (Manos et al. 1989) and further typing was done by PCR using type specific primers for HPV 16 and HPV 18 (Saiki et al. 1988).

Determination of CCND1 genotype by PCR-RFLP

We employed the PCR-restriction fragment length polymorphism (PCR-RFLP) approach to genotype the G870A locus as described elsewhere (Wang et al. 2002). Briefly, the 167 bp fragment encompassing G to A polymorphic site in the *CCND1* exon 4 terminal region was amplified. After confirmation of successful PCR amplification by 2% agarose gel electrophoresis, each PCR product was digested overnight with 2 units of *Scr*FI enzyme (New England Biolabs Inc., Beverly, MA, USA) at 37°C and was electrophoresed on 10% native polyacrylamide gel. The 167 bp PCR fragment was digested into 145 bp and 22 bp fragments when the *Scr*FI site was present. The genotype was designated as G or A when the *Scr*FI restriction site was present or absent, respectively.

The *CCND1* G1722C locus was also genotyped using PCR-RFLP as previously described (Betticher et al. 1995). A 159bp fragment was amplified including the polymorphic site at position G1722C in the *CCND1* gene. The PCR products were visualized by 2% agarose gel electrophoresis. Ten microlitres of PCR products were digested for 16 h at 37°C in a 30 µl reaction volume containing 0.2 µl of *Hae*III (10 U μl^{-1}, MBI Fermentas Life Science Inc., Burlington, Canada) to detect G1722C polymorphism. *Hae*III cleaves the amplified fragments

containing the 'G' allele into 111 bp, 26 bp and 22 bp while the 'C' allele is cleaved into 137 bp and 22 bp fragment sizes. The cleaved fragments were resolved on 10% native polyacrylamide gel electrophoresis.

DNA sequencing

We randomly sequenced 15% of the samples to validate the data generated by the PCR-RFLP method. Sequencing reactions were performed according to the conventional dideoxy chain termination method using CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

The data analysis was performed using the computer software Statistical Package for the Social Sciences (SPSS) for Windows (version 12.0). The χ^2 test/Fisher's exact test (for smaller numbers on subgroup analysis) was used to compare the distributions and linkage disequilibrium (LD) of *CCND1* gene polymorphisms between cancer patients and healthy controls.

The odds ratio (OR) and its 95% confidence intervals (CI) were also calculated as a measure of the association between the *CCND1* genotype and cervical cancer risk. The significance of statistical test χ^2/Fisher's exact was considered as two-tailed. Genotypes were further checked for the conformance of Hardy–Weinberg equilibrium.

Attributable proportion was calculated by using formula AP = PRF × (1-1/OR), where, AP = fraction of disease attributable to the risk factor, PRF = percentage of the risk factor in case subjects and OR = odds ratio.

Results

HPV prevalence

In this case–control study about 85% (170/200) of cases were found to be positive for the HPV DNA sequence. Out of the HPV-positive cases, 98.2% (167/170) were positive for HPV 16 and 1.8% (3/170) was infected with HPV 18. Out of the total HPV-positive cases 76.47% (130/170) and 23.53% (40/170) were cancer and precancer cases, respectively.

While only 4.5% (9/200) healthy controls found to be HPV positive and all of them were infected with HPV type16.

CCND1 G870A polymorphism

The genotype distributions and allelic frequencies for the G870A locus of the *CCND1* gene in controls and cases with cervical precancerous and cancerous lesions are presented in Table 1. PCR-RFLP was performed to analyze this locus, which was further confirmed by DNA sequencing for 15% of the samples (Figure 1). Both the techniques revealed similar results. We used different types of genetic models (dominant, co-dominant, recessive) to evaluate the association of *CCND1* genotypes with the cervical cancer susceptibility.

We observed that there was a difference in *CCND1* 870 carrier A (GA+AA) genotype distribution between

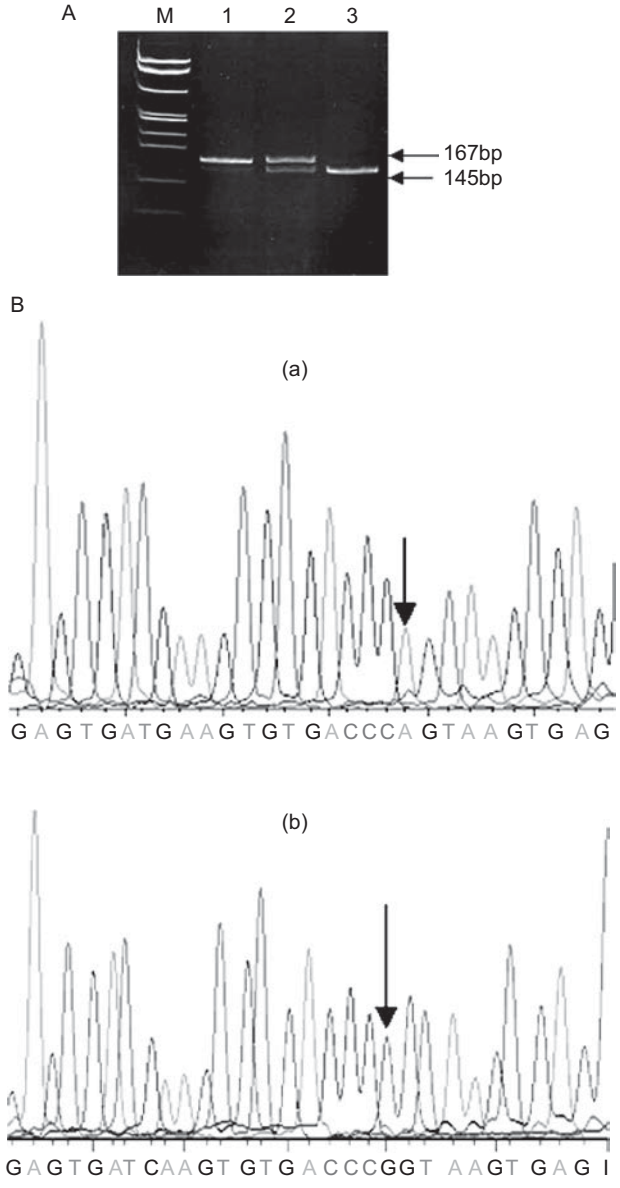

Figure 1. (A) Restriction fragment length polymorphism analysis of the *CCND1* G870A genotypes. M, Φ*X174* DNA/*Hae*III digest marker; lane 1, *CCND1* homozygous GG; lane 2, heterozygous GA; lane 3, homozygous AA. (B) Electropherogram of *CCND1* G870A polymorphism (polymorphic site is shown with an arrow), (a) homozygous A and (b) homozygous G.

Table 1. Distribution of *CCND1* genotypes among cervical precancerous and cancerous patients and healthy control subjects.

				Calculation model													
				Dominant model		Recessive model		Co-dominant model				Allelic association					
SNP (rsID)	Genotype *n* (%)			(GG vs GA+AA)		(GG+GA vs AA)		(GG vs GA)		(GG vs AA)							
G870A (rs603965)	GG	GA	AA	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)		*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	G	A	*p*-Value	OR (95% CI)	
Control (*n*=200)	47 (23.5)	119 (59.5)	34 (17.0)	Reference		Reference		Reference	Reference		Reference		213 (53.3)	187 (46.7)	Reference		
Cases (*n*=200)	39 (19.5)	94 (47.0)	67 (33.5)	0.394	1.27 (0.79-2.05)	**0.0002**	**2.46 (1.54-3.94)**	1	0.949	0.95 (0.58-1.58)	**0.006**	**2.38 (1.31-4.29)**	172 (43.0)	228 (57.0)	**0.005**	**1.51 (1.42-2.0)**	
Precancer (*n*=46)	13 (28.3)	18 (39.1)	15 (32.6)	0.568	0.78 (0.38-1.60)	**0.0289**	**2.36 (1.15-4.85)**	1	0.141	0.55 (0.25-1.20)	0.399	1.59 (0.67-3.79)	44 (47.8)	48 (52.2)	0.41	1.24 (0.79-1.96)	
Cancer (*n*=154)	26 (16.9)	76 (49.4)	52 (33.7)	0.164	1.51 (0.89-2.58)	**0.0004**	**2.49 (1.51-4.09)**	1	0.717	1.15 (0.66-2.02)	**0.003**	**2.77 (1.45-5.27)**	128 (41.6)	180 (58.4)	**0.003**	**1.6 (1.19-2.16)**	
								GG	GC		CC						
G1722C (rs678653)	GG	GC	CC	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)		*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	G	C	*p*-Value	OR (95% CI)	
Control (*n*=200)	43 (21.5)	111 (55.5)	46 (23.0)	Reference		Reference		Reference	Reference		Reference		197 (49.3)	203 (50.7)	Reference		
Cases (*n*=200)	71 (35.5)	89 (44.5)	40 (20.0)	**0.0028**	0.49 (0.32-0.78)	0.543	0.84 (0.52-1.35)	1	**0.004**	0.49 (0.30-0.78)	**0.038**	0.53 (0.30-0.93)	231 (57.7)	169 (42.3)	**0.019**	0.71 (0.54-0.94)	
Precancer (*n*=46)	23 (50.0)	17 (37.0)	41 (20.0)	**0.0002**	0.27 (0.14-0.53)	0.163	0.50 (0.20-1.26)	1	**0.001**	0.29 (0.14-0.59)	**0.005**	0.24 (0.09-0.66)	63 (68.5)	29 (31.5)	**0.001**	0.45 (0.28-0.72)	
Cancer (*n*=154)	48 (31.2)	72 (46.8)	42 (20.0)	0.0523	0.61 (0.37-0.98)	0.938	0.95 (0.57-1.57)	1	**0.048**	0.58 (0.35-0.97)	0.236	0.66 (0.36-1.21)	168 (54.5)	140 (45.5)	0.186	0.81 (0.60-1.09)	

OR, odds ratio; CI, confidence interval; *p*-value, probability from χ^2 test comparing the genotype distribution for controls and cases. Significant *p*-values and ORs are shown in bold.

cases and controls with 80.5% (161/200) in cases and 76.5% (153/200) in controls, but this difference did not reach the limits of statistical significance ($p = 0.3942$, OR 0.79) when dominant model (GG vs GA+AA) was studied. However, when we stratified the cases according to the disease severity, we found a trend of increase in frequency of polymorphic homozygous (AA) genotype from precancer (32.6%, 15/46) to cancer (33.8%, 52/154) in comparison with the control group (17%, 34/200), but no significant difference was found. But data from recessive model (GG+GA vs AA) showed that women carrying the AA genotype had a 2.36-fold higher risk for the development of precancerous lesions (OR 2.36; 95% CI 1.15–4.85; $p = 0.0289$) and a 2.49-fold increased risk for the development of invasive cervical cancer (OR 2.49; 95% CI 1.51–4.09; $p = 0.0004$) compared with those with GG+GA genotypes. Therefore, the homozygous mutant (AA) genotype presented a 2.77-fold higher risk for the overall cervical cancer (OR 2.77; 95% CI 1.45–5.27; $p = 0.003$). The risk was also shown to be increased with the heterozygous mutant genotype (OR 1.15; 95% CI 0.66–2.02) but the p-value was not significant ($p = 0.717$), by using a co-dominant model (GG vs AA and GG vs GA).

Furthermore, the increasing trend of the 870 'A' allele frequency and decreasing trend of the 'G' allele was observed with progression of disease from precancer to cancer (Table 1). The 'A' allele frequency was higher in cases (including precancerous and cancer) (57%) than in controls (46.7%), and this difference was statistically highly significant ($p = 0.005$, OR 1.51). A 1.24-fold higher susceptibility was observed for the development of precancerous lesions in women having the 'A' allele (OR 1.24; 95% CI 0.79–1.96) compared with those with the 'G' allele but this difference could not attain the statistical significance ($p = 0.41$). Women carrying the 'A' allele have a 1.6-fold increased risk for the development of cervical cancer (OR 1.6; 95% CI 1.19–2.16; $p = 0.003$) than those with the 'G' allele (Table 1). The proportion of cervical cancer cases attributable to the AA genotype was 19.9%.

The frequencies of the *CCND1* 870 genotypes were found to be in concordance with the Hardy–Weinberg equilibrium in controls as well as in cases.

CCND1 G1722C polymorphism

The genotype distributions and allelic frequencies for the G1722C locus of the *CCND1* gene between controls and cases with cervical precancerous and cancerous lesions are presented in Table 1. Genotyping of the G1722C locus was also performed by PCR-RFLP (Figure 2). The frequency of the wild-type homozygous (GG) genotype was higher in cases 35.5% (71/200) than in controls 21.5% (43/200). It is evident from the dominant model

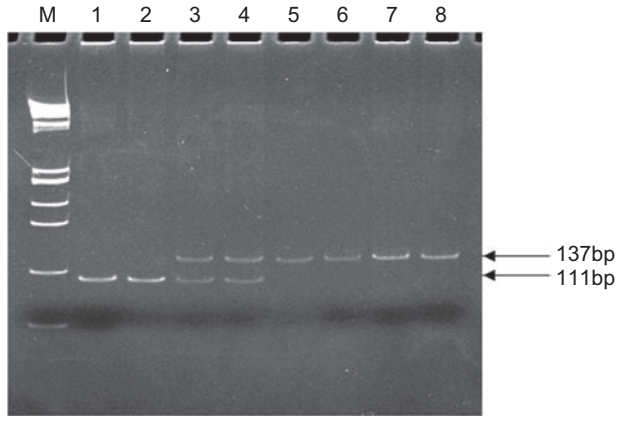

Figure 2. Restriction fragment length polymorphism analysis of the *CCND1* G1722C genotypes. M, Φ*X174* DNA/*Hae*III digest marker; lanes 1 and 2, *CCND1* homozygous GG; lanes 3 and 4, heterozygous GC; and lanes 5–8, homozygous CC.

(GG vs GC+CC) that there was a difference in carrier C genotype (GC+CC) distribution between cases and controls with 64.5% (129/200) in cases and 78.5% (157/200) in controls and this difference was statistically highly significant ($p = 0.0028$, OR 0.49). An extremely significant difference ($p = 0.0002$) was also noticed in carrier C genotype (GC/CC) distribution between precancer 50% (23/46) and controls 78.5% (157/200) ($p = 0.0002$; OR 0.27) (Table 1). When the recessive model (GG+GC vs CC) was used to investigate the association of G1722C genotypes with cervical cancer susceptibility, we observed that the 1722CC genotype was more common in controls (23%) than in total cases (including precancerous as well as cancerous) (20%), but this difference was not statistically significant ($p = 0.543$). These findings show that the 'C' allele has a dominant effect on the development of cervical cancer. Simultaneously, it was also surprising to note that CC genotype was less frequent in the precancerous group (13%) than in the cancer group (22%). The possible reason for this abnormal behaviour may be the small sample size of the precancer group ($n = 46$). The 'C' allele frequency was higher in the control group (50.8%) than in the overall cervical cancer cases (42.3%) and this difference was statistically significant ($p = 0.019$).

The genotype distribution of both the groups (cases and controls) was in agreement with the Hardy–Weinberg equilibrium.

Linkage disequilibrium analysis

The linkage between these two polymorphisms in controls and cervical cancer cases was also analyzed. We observed significant linkage disequilibrium between the G/C1722 and G/A870 genotypes only in the control group ($p = 0.0254$; $\chi^2 = 4.994$) and not in the cancer group ($p = 0.2104$; $\chi^2 = 1.569$) as shown in Table 2. It is

Table 2. Linkage disequilibrium analysis of *CCND1 G870A* and *G1722C* genotypes in cervical cancer cases and healthy controls.

	G870A			
G1722C	GG (%)	GA (%)	AA (%)	*p*-Value
Controls				
GG	4 (9.3)	20 (6.5)	19 (44.2)	
GC	16 (14.4)	84 (75.7)	11 (9.9)	
CC	27 (58.7)	15 (32.6)	4 (8.7)	**0.0254**[a]
Cervical cancer cases				
GG	14 (18.9)	32 (43.2)	28 (37.8)	
GC	13 (15.1)	40 (46.5)	33 (38.4)	
CC	11 (27.5)	22 (55)	7 (17.5)	0.2104[b]

p-Value, probability from χ^2 test comparing the genotype distribution for controls and cases.
[a]Statistically significant; [b]statistically not significant.

also evident from Table 2 that in the control group the majority of 870GG genotypes were linked with the 1722CC genotype (58.7%) but not in the cervical cancer cases (27.5%).

Discussion

Cervical cancer is a malignant neoplastic disease for which public health prevention initiatives have had the greatest success. Organized screening of cervical cytology together with high-risk HPVs have substantially reduced the cervical cancer burden by about 75% in developed countries during the past 50 years in contrast to developing countries such as India (Trottier & Franko 2006). It is well established that HPV infection is necessary but not sufficient for the development of cervical cancer. HPV infections are widespread in the general population, but only a small proportion of infected women develop cervical cancer (zur Hausen 2000). Also HPV positivity in healthy Indian women (46+ years age group) with normal cervical cytology is found to be approximately 7% (Das et al. 2008), indicating the role of other environmental and host genetic factors responsible for the progression from HPV infection to cancer. Therefore, it is worthwhile investigating the involvement of SNPs in different stages of cervical cancer to understand the aetiology of the disease.

In this study, we found that women carrying the 870AA genotype are at a remarkable increased risk (2.46-fold) for cervical cancer compared with having GG+AA genotypes. In addition, when the AA+GA genotypes were compared with the GG genotypes no significant association was found, indicating that the 'A' allele has a recessive effect on the development of cervical cancer.

Many investigators have reported an association between cyclin D1 polymorphism and the susceptibility to various carcinomas (Wang et al. 2002, Kang et al. 2005, Grieu et al. 2003, Zheng et al. 2001, Koike et al. 2003, Kong et al. 2000) but only limited reports have been published concerning its relationship with uterine cervical cancer (Catarino et al. 2005, Jeon et al. 2005, Kaur et al. 2008). In contrast to our results, no association was found for the G870A polymorphism in a Korean population in cervical cancer cases (Jeon et al. 2005). Recently, Kaur et al. (2008), also showed no significant association between the *CCND1* 870 genotypes and overall risk of cervical cancer in a North Indian population, but histological stratification of cases showed an association with the risk of development of squamous cell carcinoma. However, Catarino et al. (2005) showed a positive association for this polymorphism with the risk of development of cervical cancer in Portuguese women but in contrast to our findings they showed that the 870GG genotype was associated with a higher risk of cervical cancer. These reports support the fact that this kind of inconsistency may be due to the variations of biological characteristics of each study population.

To the best of our knowledge, this is the first report showing the statistically significant association with protection/resistance against HPV-associated cervical cancer in relation to the G1722C polymorphism of the *CCND1* gene. In the present study the 1722 'C' allele frequency was significantly higher in the control group than in cases ($p = 0.019$), indicating that the 'C' allele has a protective role in the development of cervical cancer.

There are only two reports available showing the influence of G1722C polymorphism of the *CCND1* gene. Holley et al. (2001) demonstrated that this polymorphism in the 3'UTR of *CCND1* (G1722C) is associated with the grade of histological differentiation and clinical outcome in patients with SCCHN but showed no relation with the cancer risk. Sathyan et al (2005) investigated its role in cancer susceptibility and found no significant difference in genotype frequencies between oral cancer cases and controls.

For the first time we have demonstrated a significant LD in the control group between the two polymorphisms of *CCND1* in relation to cervical cancer. However, another two groups also studied LD in *CCND1* polymorphisms but not in cervical cancer. Similar to our findings, Sathyan et al. (2005) also established an association in the control group with reference to oral cancer cases. But in contrast, Holley et al. (2001) showed significant linkage in SCCHN cases.

The main strength of our study is the genetically homogenous study population, as they all belong to the Indo-Aryan ethnic group. But this study also has some weaknesses such as other environmental factors including early age of marriage, promiscuity, smoking and use of contraceptives that were not included in the present study.

In conclusion, our data highlights that G1722C polymorphism probably plays a protective effect against HPV-associated cervical cancer, indicating its role as

a potential biomarker for cervical cancer in the Indian population, and simultaneously suggests that *CCND1* G870A polymorphism seems to have an effect on cervical cancer susceptibility.

Further studies may include the impact of *CCND1* alleles on protein expression besides the analysis of genetic variations of other cell cycle-related proteins such as CDK4, CDK6, pRb and p16 that interact with cyclin D1 and also play key roles in the cell cycle regulation.

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***Declaration of interest*:** We report no potential conflicts of interest. I hereby declare on behalf of all authors that the work has not been published and is not being considered for publication elsewhere.

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