

P53 codon 72 polymorphism and its association with cervical cancer

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Abstract

Cervical cancer frequently associated with multiple HPV types. Recent report suggests that a polymorphism of the p53 tumor suppressor gene that results in the substitution of a proline residue with an arginine at position 72 protein, it act as a risk factor in HPV associated malignancies. The present study, we examined the genotypic frequency of this polymorphisms in 24 patients with cervical cancer using allele-specific polymerase chain reaction to detected P53 polymorphism at 72 codon. The frequency distribution of genotypes in cervical cancer patients as 29% (7tumors) for the arginine/arginine allele, 63% (15 tumors) for the heterozygous arginine/proline status, and 8% (2 tumors) for the proline/proline allele respectively. Allele frequencies of proline and arginine at 72 codon of P53 gene in proline (0.400) and arginine (0.600) in cervcal cancer patients. Statistically significant difference was observed between the Proline/proline, arginine/arginine and proline/arginine genotypes in cervical cancer tumors ($\chi^2=16.125$; $p=0.003$). In our result p53 codon72 Arg homozygosites has been associated with increased risk developing cervical cancer. The present study reports P53 codon 72 arginine homozygosity constitutes a risk factor for the development of invasive cervical carcinoma.

In conclusion, the P53 arginine represents a potential risk for cervical cancer development.

Key words: cervical cancer , p53,codon 72 polymorphism, HPV

Introduction

Among the different types of cancers cervix cancer, the second most common cancer in women worldwide. Cervical cancer starts in the cells on the surface of the cervix. Infection with human papilloma virus (HPV) is an important etiological factor in the development of cervical cancer. The HPV family has been implicated in the development of cervical cancer. HIV 16 and HPV 18 are mostly associated with cancers. HPV genome consists of 7200-8000 bp and encodes six early (E1-E7) and two late proteins (L1-L2). The early proteins E6-and E7 especially of high – risk HPV, The development of cervical cancer due to HPV infection is now believed to be associated with host genes such as tumour suppressor gene(p53) of the host. The single nucleotide polymorphism (SNP) is the variation in a single nucleotide (C or G) at codon 72 (CCC or CGC) of the p53 gene that results in the translation to either proline or arginine aminoacid residue. The arginine polymorphic variant of p53 could represent a risk factor for cervical carcinoma, p53 codon 72 polymorphism, encoding either arginine or proline has been proposed to affect the susceptibility of p53 protein to HPV E6 –mediated degradation in vitro. Moreover, the frequency of the Arg/arg genotype was found to be significantly higher in cervical cancer patients compared to the general Population, indicating that individuals homozygous for Arg are seven times more susceptible to HPV-associated tumor genesis than heterozygotes and it has been proposed that individuals homozygous for Arg/Arg at codon-72 of p53 are seven times more susceptible to HPV-mediated cancer. The scope of the study is that the detection of these risk factors (p53 polymorphism and HPV infection) may lead to early diagnosis there by better management of the disease and survival.

2. Methodology

Sample collection

The present study includes 24 cases of invasive cervical cancer patients. The age of the patients was between 20-80 years. The age-group 20-80 years was chosen because that is the group with the highest number of cervical cancer cases in Sample collection. All cases were histopathologically diagnosed, graded and staged.

2 ml of peripheral blood samples were collected from patients and controls with prior consent. Samples were collected into a sterile tubes containing EDTA. Samples were stored at -20 °C till DNA isolation.

DNA isolation

DNA was isolated by simple, rapid, non-enzymatic method (Lahiri and Nurnberger, 1991). In this all solutions are used with double distilled water and autoclaved. All solutions should be filtered and autoclaved except NaCl and SDS. The Blood sample washed with the reagent A solution (10m M Tris-HCL PH 8.0, 10mM KCL, 10mM MgCl₂. 2mM EDTA) and centrifuged. The residual was washed with the reagent B solution (10mM Tris -HCL PH8.0, 10mM KCL, 10mM MgCl₂. 0.4mM NaCl,2mM EDTA and 10% Sodium dodecyl sulphate(SDS)). Tissue sample was lysised with reagent B solution adding proteinase k 50 ug and incubated for 5 h at 65°. All samples are then extracted with phenol/chloroform and chloroform.DNA was precipitated with absolute ethanol, washed with 70% ethanol and re suspended in 100ul TE buffer (ph: 8.0 10mM Tris HCl 1mM EDTA) Store the DNA solution at -20°C.

Agarose gel:

0.8% agarose (0.320gms of agarose in 40ml TEB 1X buffer) to dissolve in TEB 1X buffer (Stock solution: Tris-54gms, Boric acid-27.5gms, EDTA-3.12gms (PH: 8.0) 1000ml of double distilled water. Cool down the agarose solution to 50°C and add 3µl of Ethidium Bromide to the solution and mix well. Take a gel tray, Seal the free ends and insert the comb. Pour the gel solution into the tray and allow it to solidify for at least 30min. Remove the comb and place the gel in the electrophoresis chamber and add the working solution (1xTEB). The buffer level should be 1-2mm. just above the surface of the gel. Load the samples (5 µl of DNA + 2-3 µl of gel loading buffer (Bromophenol blue : 25mg Xylene cyanol : 25mg 40% sucrose solution) into the wells using micropipette. Set the power and provide constant voltage of 100v for 30min. to one hour. As soon as the blue dye moves 4-5cm. from origin, stop the power supply. Remove the gel and immediately view in the UV transilluminator. Orange colour DNA bands will appear.

Purity of DNA.

Purity of DNA = OD at 260nm /OD at 280nm

The pure preparation of DNA ranges between 1.8 to 2.0.

If DNA is not concentrated, make dilution 1:20 or 1:50.

Quantity assessment: calculate the concentration of DNA the following formula can be used.

Concentration of DNA = $260_{\text{abs}} \times 50 \times \text{dilution factor (at the time of OD taken)}$
 $(\text{OD at } 260_{\text{nm}} \times 50 \times 100)$

DNA analysis

Genomic DNA obtained from cervical cancer patients was analyzed to determine the distribution of polymorphic (proline/ Arginine) variants of P53 gene at codon 72 using allele specific PCR amplification.

PCR analysis

The polymorphic codon 72 of P53 gene was amplified by polymerase chain reaction (PCR) by using allele specific pcr for proline and arginine variants in a 10µl. reaction volume containing 10mM TrisHCl (ph.8.0), 50mM KCl, 0.2mM of each dNTPs, 200µmol of each primer, an optimal concentration of MgCl₂ (1.5mM) and 0.5U of Taq DNA polymerase and 100ng of genomic DNA.

Pcr amplification of the P53 codon 72 polymorphic status

For the determination of polymorphism at codon 72 of P53, an allele specific PCR assay was used as described previously with a little modification. Pcr reaction containing 1x buffer 10mM Tris HCl PH 7.6-8.0 and 50 mM Kcl),1.5mM MgCl₂, 20p mol of DNTPS mix ,200 nm of each primer and 0.025 u/ul Taq polymerage in the final valume of 10 ul .The detection of the two polymorphic variants was done in two different tubes. the amplification was performed as follows :Initial temperature at 94°C for 4 min. Denaturation at 94°C for 40 sec, Annealing at 55°C for 30 sec.30 cycles Extension at 72°C for 30 sec, followed by Final extension at 72°C for 10 min Hold at 4°C . **pcr products were expected to be 177 bp – for proline 141bp – for arginine depending on HPV type.** The product of the Arg allele was 141bp, while the product of the Pro allele was 177bp. Heterozygous specimens had both pcr products, where homozygous

samples exhibited only one of the products. In each pcr reaction two blank samples were employed as negative controls, to ensure that no contaminants were introduced. the determination of p53 codon 72 polymorphism in the corresponding in order to assure that the results were strictly genomic and not due to loss of heterozygosity. Pcr procedures were repeated twice and the results were 100 % reproducible.

PCR protocol (Storay *et al*)

Polymorphism at 72 codon in P53 gene (Proline/Arginine).

P53 gene is located on human chromosome 17.

Template : 100ng of genomic DNA/ reaction volume of 10 μ l.

Proline forward primer : 5'-GCC AGA GGG TGC TCC CC-3'
Proline reverse primer: 5'-CGT GCA AGT CAC AGA CTT T-3'

Arginine forward prime : 5'-TCC CCC TTG CCG TCC CAA -3'
Arginine reverse primer : 5'-CTG GTG CAG GGG CCA CG-3'

10 μ l volume PCR reaction mixture

Double distilled water : 7.25 μ l

1.5mM MgCl₂ PCR mix : 1.00 μ l

Proline/arginine primers : 0.50 μ l
(forward and reverse 0.25 each)

Taq polymerase : 0.25 μ l

DNA sample : 1.00 μ l

Product sizes : 177 bp – for proline : 141bp – for arginine

Pcr products analysis :

The pcr product were analysed by electrophoresis in a 2% agarose gel, stained with Ethidium bromide and photographed on a uv light transilluminator. After PCR, the samples were subjected to 2% agarose gel electrophoresis at 100V for 20-30 minutes. Ethidium Bromide stained gels were visualized under UV light and were documented. Band sizes were compared to molecular weight marker of 100-1000bp for confirmation.

Data analysis

Estimation of allele frequency

Gene count method was used for estimating allele frequencies. Gene counting method is used for the calculation of allele frequencies of polymorphic genetic markers where all the heterozygotes can be easily identified.

When there are 2 alleles A and B at a locus,

Genotype frequencies: AA - a; BB - b; AB - c

Let the gene frequencies be p and q.

Then, $p = \frac{2a+c}{2n}$

$q = \frac{2b+c}{2n}$

2n

(Where n= total number of individuals).

Association

The association of the genotype with cervical cancer was calculated by using 2 x 3 contingency table (at 5% level of significance and degrees of freedom is calculated as (rows-1) (columns-1). The expected frequencies in any particular cell was calculated by

$$\frac{\text{Row total} \times \text{column total}}{\text{Grand total}}$$

And test for goodness fit by: χ^2 (chi square) = $\sum_{i=1}^n \frac{(O_i - E_i)^2}{E_i}$

Results and Discussion

In an attempt to understand the association of tumor suppressor gene P53 polymorphisms with cervical cancer, genotyping of the polymorphic variants (proline/Arginine) in 72 codon of P53 gene was carried out.

P53 codon 72 polymorphic status of the tumors

Polymorphic status of the P53 codon 72 was analyzed by allele-specific PCR in 24 cervical cancer patients to determine the genotype. The genotype frequencies were determined as 29% (7tumors) for the arginine /arginine allele, 63% (15 tumors) for the heterozygous arginine/proline status, and 8% (2 tumors) for the proline/proline allele respectively. The genotype and percentage frequencies are given in Table 1.

Table 1

Genotype frequencies of arginine/proline polymorphism in P53 gene at codon72.

Table.2

Allele frequencies of proline and arginine at 72 codon of P53 genes in cervical cancer tumors

Table 1**Table 2**

Locus	Genotype	Genotypy frequency
P53 gene	P/P	2 (8%)
	P/A	15 (63%)
	A/A	7 (29%)

Locus	n	Proline	Arginine
P53 gene	24	0.400	0.600
p =			

proline; A= arginine

n=

individuals

Statistically significant difference was observed between the Proline/proline, arginine/arginine and proline/arginine genotypes in cervical cancer tumors ($\chi^2=16.125$; $p=0.003$).

Discussion

During past two decades, studies on HPV and P53 alterations were carried out in cervical cancers. These include P53 expression, mutations, LOH, as well as polymorphism in P53. Howley *et al.* demonstrated that HPV 16 genotype may play an important role in the progress of cervical cancer. It has been suggested that the alteration of mutated P53 leads to malignant phenotype, and the interaction between HPV and P53 pathways may contribute to cervical carcinogenesis (Helland *et al.* 1993 Saranth *et al.* 2002). The correlation between P53 mutation and HPV infection is controversy. Ishikawa *et al.* showed the P53 mutation was associated with HPV infection. In contrast, Helland *et al.* 1998 reported that a significantly higher P53 mutation frequency was found in HPV-negative cervical cancers.

Due to the Polymorphism at codon 72 in P53gene the amino acid proline is replaced by arginine. Homozygous P53Arg found to be associated with cervix cancer compared to heterozygous or homozygous P53Pro, suggesting that individuals homozygous for P53Arg genetically susceptible to cervical cancers.

The other Indian studies reported the association between Human Papilloma Virus and cervical cancer. While most studies reported the association of Arg/Arg polymorphic genotype in P53 with HPV associated cervical cancer, Bhattacharya et al reported a significant association between pro/pro polymorphism and cervical malignancy. Mutation/expression status of P53 in cervical cancer has been reported by Raju et al. 1996, Rajkumar et al. 1998, and Jain et al. 2003.

The present study reports P53 codon 72 arginine homozygosity constitutes a risk factor for the development of invasive cervical carcinoma. In conclusion, the P53 arginine represents a potential risk for cervical cancer development. In conclusion, this is the study of p53 codon 72 polymorphism in cervical cancer patients, indicating that p53 Arg homozygous genotype is associated with the development of the disease. The low incidence of HPV infection suggests that it is not a major oncogenic factor but may have a synergistic action with specific genotypes of p53. Further studies are needed in order to elucidate the function of the p53 codon 72 polymorphism, its evolution and ecological presence, as well as its role in the development of cervical and other cancer types, as it is indicated to be one of the strongest susceptibility factors in cancer development and progress.

References

1. Bhattacharya P, Chandralekha Duttgupta, Sharmila Sengupta Proline homozygosity in codon 72 of P53: a risk genotype for human papillomavirus related cervical cancer in Indian women. *Cancer Letters* Volume 188, Issues 1-2, 15 December 2002, Pages 207-211.
2. D P Lane: P53 a guardian of the genome. *Nature* 358: 15-16, 1992.
3. Helland A, Holm R, Kristensen G, Kaern J, Karlsen F, Trope C, Nesland JM, Borresen L. Genetic alterations of the TP53 gene, P53 protein expression and HPV infection in primary cervical carcinomas. *J Pathol.* 1993 Oct; 171(2):105-14. [PubMed].
4. Helland A, Karlsen F, Due EU, Holm R, Kristensen G, Børresen-Dale A Mutations in the TP53 gene and protein expression of P53, MDM 2 and p21/WAF-1 in primary cervical carcinomas with no or low human papillomavirus load. *Br J Cancer.* 1998 Jul;78(1):69-72.
5. Howley PM. Role of the human papillomaviruses in human cancer. *Cancer Res.* 1991 Sep 15; 51(18 Suppl):5019s-5022s. Review. [PubMed].
6. Ingeborg Zehbe, Gianfranco Voglino, Erik Wilander, Hajo Delius, Antonella Marongiu, Lutz Edler, Fritz Klimek, Sonja Andersson, Massimo Tommasino. P53 Codon 72 Polymorphism and Various Human Papillomavirus 16 E6 Genotypes Are Risk Factors for Cervical Cancer Development *Cancer Res* January 1, 2001 61; 608.
7. Ishikawa H, Mitsuhashi N, Sakurai H, Maebayashi K, Niibe H. The effects of P53 status and human papillomavirus infection on the clinical outcome of patients with stage IIIB cervical carcinoma treated with radiation therapy alone. *Cancer.* 2001 Jan 1; 91(1):80-9. [PubMed].
8. J A Mietz, T Unger, J M Huibregtse: The transcriptional transactivation function of wild type P53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J* 11:5013-5020, 1992.
9. Jain D, Srinivasan R, Patel FD, Kumari Gupta S. Evaluation of P53 and Bcl-2 expression as prognostic markers in invasive cervical carcinoma stage IIB/III patients treated by radiotherapy. *Gynecol Oncol.* 2003 Jan; 88(1):22-8. [PubMed].
10. Rajkumar T, Rajan S, Baruah RK, Majhi U, Selvaluxmi G, Vasanthan A. Prognostic significance of Bcl-2 and P53 protein expression in stage IIB and IIIB squamous cell carcinoma of the cervix. *Eur J Gynaecol Oncol.* 1998; 19(6):556-60. [PubMed].
11. Raju GC, Teh M, Wee A. An immunohistochemical study of P53 protein in cervical intraepithelial neoplasia and squamous cell carcinoma. *Pathology.* 1996 Jan; 28(1):17-19. [PubMed]
12. Sankaranarayanan R, Bhagwan M. Nene, Surendra S. Shastri, Kasturi Jayant, Richard Muwonge, Atul M. Budukh, Sanjay Hingmire, Sylla G. Malvi, Ranjit Thorat, Ashok Kothari, Roshan Chinoy, Rohini Kelkar, Shubhada Kane, Sangeetha Desai, Vijay R.

13. Saranath D, Khan Z, Tandle AT, Dedhia P, Sharma B, Contractor R, Shrivastava S, Dinshaw K. HPV16/18 prevalence in cervical lesions/cancers and P53 genotypes in cervical cancer patients from India. *Gynecol Oncol.* 2002. 86(2):157-62.
14. Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I. M., Matlashewski, G., and Banks, L. Role of a P53 polymorphism in the development of human papillomavirus-associated cancer. *Nature (Lond.)*, 393: 229–234, 1998.