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Novel missense mutation in *FHIT* gene: interpreting the effect in HPV-mediated cervical cancer in Indian women

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Abstract Human papillomavirus (HPV) is considered to be a major etiological factor but is not sufficient for the development of cervical cancer. Other host factors including altered tumor suppressor gene activities might contribute to the carcinogenic process. Fragile Histidine Triad (FHIT) has been shown to play a pivotal role in carcinogenesis. Therefore, we made an attempt to find out point mutation of *FHIT* gene in HPV mediated cervical cancer in Indian women. 112 cases of cervical carcinoma tissue biopsies and 38 cervical scrapes samples of normal cytology were employed for this study. Herein, we report a novel mutation identified at nucleotide position 655, at codon 98 from CAT → CGT with ultimate replacement of amino acid Histidine by Arginine in cervical cancer cases. Molecular modeling was performed to predict the effect of this mutation in disease pathology. We predict that this change, His to Arg substitution in substrate-binding domain may generate catalytically inactive protein with loss of tumor suppressor activity.

Keywords Cervical cancer · Human papillomavirus · HPV · FHIT · Mutation

Introduction

Cancer of the uterine cervix is the second most common gynecological cancers worldwide; but is the most common cancer among women in India [1] and it comprises about 16% of the world's annual incidence and is the major public health problem [2]. Of the several risk factors associated with this cancer, infection with high-risk human papillomaviruses (HR-HPVs) has emerged as the major etiological factor for the development of cervical cancer. The presence of HR-HPV DNA has been found in more than 90% of cervical cancer cases. In India, HPV type 16 is the most prevalent type, accounting for more than 70% of cervical cancer cases followed by HPV type 18 and other high-risk types [1, 3]. However, infection of HPV is essential but is not sufficient for the development of cervical cancer, which implies the involvement of genetic factors [4, 5].

In cancer, the functional loss of tumor suppressor genes is one of the most common genetic alterations. A number of studies revealed frequent genetic deletion especially at chromosome 3p locus, which harbors many tumor suppressor genes including Fragile Histidine Triad (*FHIT*)

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gene [6]. This site is also reported to be the site for HPV integration [7]. Therefore, *FHIT* gene inactivation due to mutation, methylation, and LOH at several loci on chomosome 3p have been reported in both invasive and high-grade preinvasive cervical lesions [8, 9]. Inactivation of this gene has also been reported in various other cancers including colon, lung, breast, head and neck, and skin [10–12]. *FHIT* gene spans 1.8 Mb of genome region and is composed of 10 exons. It encodes a small mRNA transcript of 1.1 Kb and a protein of 16.8 kDa [13]. The reported point mutations of *FHIT* gene is very rare and vary from 0–5% in oral and cervical cancers [14].

Various mechanisms of tumor suppressor activity of *FHIT* protein have been proposed based on its crystal structure [15], but the most important one is the formation of enzyme substrate complex and the different residues in substrate-binding domain of *FHIT* that plays a major role for its active function [16]. However, no comprehensive study has been done to investigate its inactivation in cervical cancer. Therefore, the present study was designed to investigate a detailed evaluation of *FHIT* gene mutation in HPV mediated cervical cancer in North Indian population. In addition, we have done molecular modeling studies to predict the probable effect of this mutation in disease pathology.

Materials and methods

Patients and specimens

A total of 112 cases of cervical carcinoma tissue biopsies and 38 cervical scrapes samples of normal cytology of Indo-Aryan ethnicity were employed for this study. The patients were recruited from Jawaharlal Nehru Medical College & Hospital, Aligarh and other hospitals of Delhi with histopathologically confirmed cases. The patients had a mean age of 47 ± 12 years, written informed consent was obtained from all the participants and the study was carried out in accordance with the principles of Helsinki Declaration. The study was approved by the Ethics Committee of the Institute.

DNA extraction and HPV detection

Genomic DNA was extracted from fresh cervical tissue biopsy samples (patients) and cervical scrapes (control) by standard method using Proteinase K followed by Phenol/chloroform/isopropanol treatment [17].

HPV diagnosis was performed by PCR amplification using consensus primers MY09 and MY11 and further typing was done by PCR using type specific primers for HPV 16 and HPV18 [18, 19].

Polymerase chain reaction

The coding region of FHIT gene, which comprises exon 5–9, was screened for detection of mutations/polymorphism. Five sets of primers were used for this analysis, the sequences of which were adopted from the published article [20]. These primers were designed to amplify all the exonic regions as well as intron–exon boundaries. A typical PCR amplification was performed in 25 µl reaction volume containing 10 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 µM of each dNTPs (dATP, dCTP, dGTP, and dTTP) (125 mM for SSCP-PCR for *FHIT* exons), 5 pmoles of each oligonucleotide primer, 100–500 ng of tumor DNA, and 0.5 U Taq DNA polymerase. The cycling conditions used were 95°C (5 min) initial denaturation, 94°C (0.5) min, 53°C (0.5) min, and 72°C (0.5) min for exon 5–9 with 35 cycles of amplification.

PCR-SSCP for detection of *FHIT* gene mutations

Single strand conformation polymorphism (SSCP) analysis was performed according to the method described by Orita et al. [21] with certain modifications [22]. The PCR products were radiolabelled for an additional 15 cycles by using 1 mCi of (α-^{32}P) dCTP (BARC, Mumbai, India) following amplification of the FHIT exons by standard procedure. One microliter of labelled PCR product was diluted 10 times with SSCP gel loading buffer/dye containing 95% formamide, 20 mM EDTA (pH 8.0), 0.05% each of xylene cyanol, and bromophenol blue. The diluted products were denatured for 5 min at 95°C and then chilled on ice for 5 min. Three microliters of this product were subjected to non-denaturing electrophoresis in a 6% polyacrylamide sequencing gel run in 0.5 × TBE (Tris–borate EDTA) buffer. The electrophoresis was carried out at 200 V for 12 h at 17 ± 1°C in Base Ace sequencing gel apparatus (Stratagene GmbH, Heidelberg, Germany). The gel was removed on a Whatman paper and subjected to vacuum drying in gel dryer (BioRad,USA) and exposed to X-ray film at −70°C. Alteration in electrophoretic mobility of single strand DNA bands was analyzed in comparison to that of normal controls.

DNA sequencing

Direct DNA sequencing was performed on those PCR products that showed altered band mobility in SSCP along with normal controls. Fresh PCR products were prepared by PCR amplification of original tumor DNA and purified by using Qiagen PCR purification columns (Qiagen GmbH, Hilden,Germany). The mobility-shifted mutant and polymorphic bands samples were further subjected to sequencing by Macrogen Sequencing Company, Korea.

Modeling of the putative substrate-binding site

Molecular modeling was performed on silicon Graphics System using the graphics program packages PyMOL, 2002 (DeLano Scientific, Palo Alto, CA, USA.) using the atomic coordinates of Fragile Histidine Triad protein (PDB ID: 2FHI) [16]. The interaction between the substrate and substrate-binding pocket was determined by using contact/act program from CCP4 package (Collaborative Computational Project, Number 4, 1994). The mutant model (His (35) â†' Arg (35) was generated by using COOT package from CCP4 suite [23]. The generated coordinates were subjected to energy minimization on Swiss-PdbViewer software for correcting its stereochemical features [24]. The final model was validated on online server (http://deposit.pdb.org/validate/) which provides validation report from PROCHEK [25] and Ramachandaran Plot [26]. Energetically best-fitted model was used for structure analysis and comparison with that of wild-type protein. The ribbon diagram was plotted by using Pymol software.

Results

Detection of HPV type 16/18 in cervical cancer cases

In the studied cohort, about 83% (93/112) of the cases showed positivity for HPV infection and out of HPV positive cases, 97% (90/93) of cases were infected with HPV type16 while only 3.2% (3/93) of cases were found to be positive for HPV type 18 and 5.2% (2/38) in normal control.

FHIT gene alteration in cervical tumors

In this study, 112 cervical cancer DNA samples were analyzed for mutations in the coding region (exons 5–9) of the *FHIT* gene. Each exon was amplified and analyzed separately for detection of mutations. 5 tumor DNAs (4.4%) showed the altered mobility shift in PCR-SSCP for exon 6 (Fig. 1a). Figure 1b showed the partial electrophoregram of *FHIT* exon 6 in normal tissues. Sequencing of the DNAs with shifted band indicated a single mutation in Exon 6 (Fig. 1c). The single missense mutation occurred due to A → G transition at nucleotide position 655, at codon 98 from CAT → CGT with ultimate replacement of amino acid Histidine by Arginine. By using direct sequencing, we found evidence for the presence of another mutation, a frame shift mutation in intron 8. The frame shift mutation was due to insertion of T in intron–exon boundary (data not shown).

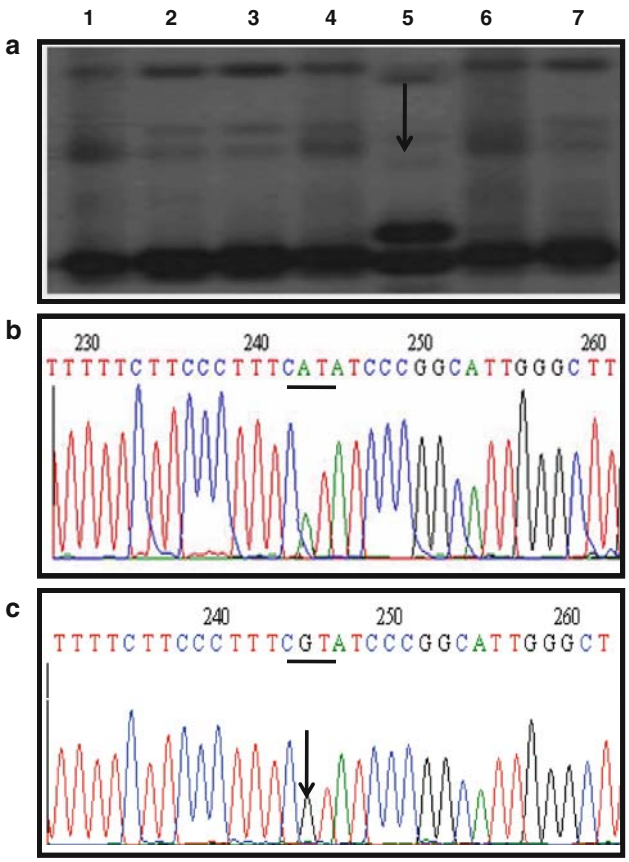

Fig. 1 **a–c** Analysis of FHIT gene mutation in cervical cancer cases: **a** Representative photograph of PCR-SSCP analysis of FHIT gene in cervical tissue samples showing altered band mobility in lane 5, is the indication of mutation shown by an *arrow*. Lane 1, normal control and lane 2–7 cervical cancer tissue samples. (**b** and **c**), partial electropherogram of FHIT exon 6 representing the normal and mutant in cervical cancer as indicated by *arrows*

Structural analysis

The mutation of *FHIT* gene in exon 6 at nucleotide position 655 was modeled computationally on three dimensional structure in order to investigate the physiological changes occurred in substrate-binding site due to change in His35 to Arg35 in substrate-binding domain. As evident from Fig. 2a, b, the substrate-binding pocket of wild-type *FHIT* is sufficient to accommodate substrate in the substrate-binding pocket. The substrate-binding pocket comprises evolutionary conserved residues like Phe5, His8, Ile10, Leu25, Asn27, Val31, His35, leu37, Thr79, Gln83, Thr91, Val92, His96, His98, Arg102 and Met135. It appears that any change in these residues may lead to inactivation or decrease substrate binding of *FHIT*. Interestingly, we observed a significant change in one of the substrate-binding residues that is His35 which has been mutated into Arginine. The substrate-binding pocket of

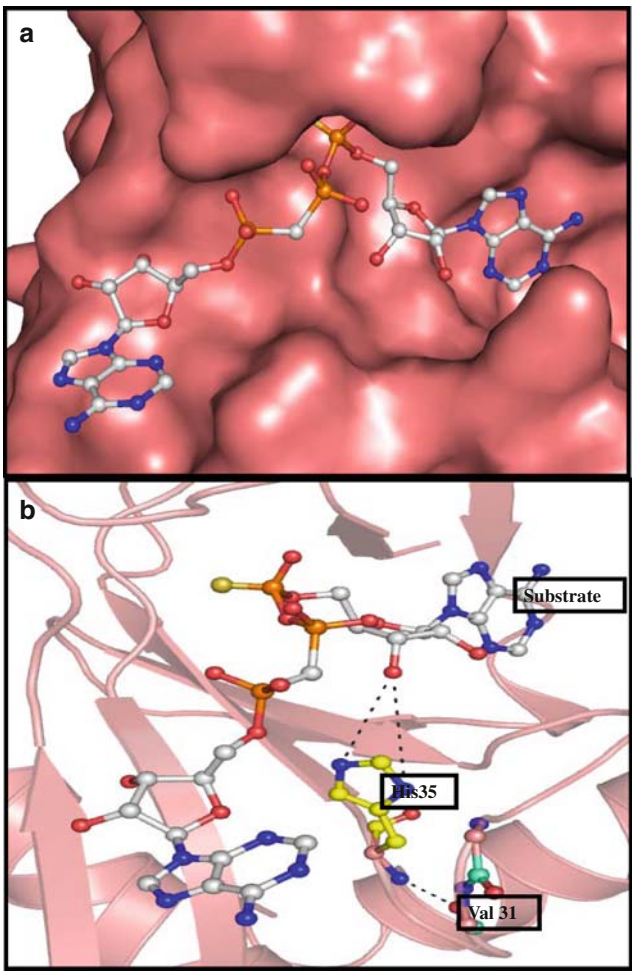

Fig. 2 **a** and **b**: Molecular modeling of FHIT gene in cervical cancer. **a** substrate-binding pocket of wild-type FHIT is shown in surface diagram. The substrate analog, P1-P2-Methylene-P3-Thio-Diadenosine Triphosphate is present in the substrate-binding groove is shown in ball and stick. **b** structure of wild type FHIT-substrate complex. The substrate analog P1-P2-Methylene-P3-Thio-Diadenosine Triphosphate (*White*) and His35 are shown in ball and stick model (*Yellow*). Vall31 has close interaction with His35 is shown in green (*ball and stick model*). The hydrogen bonds are shown in *dotted line*

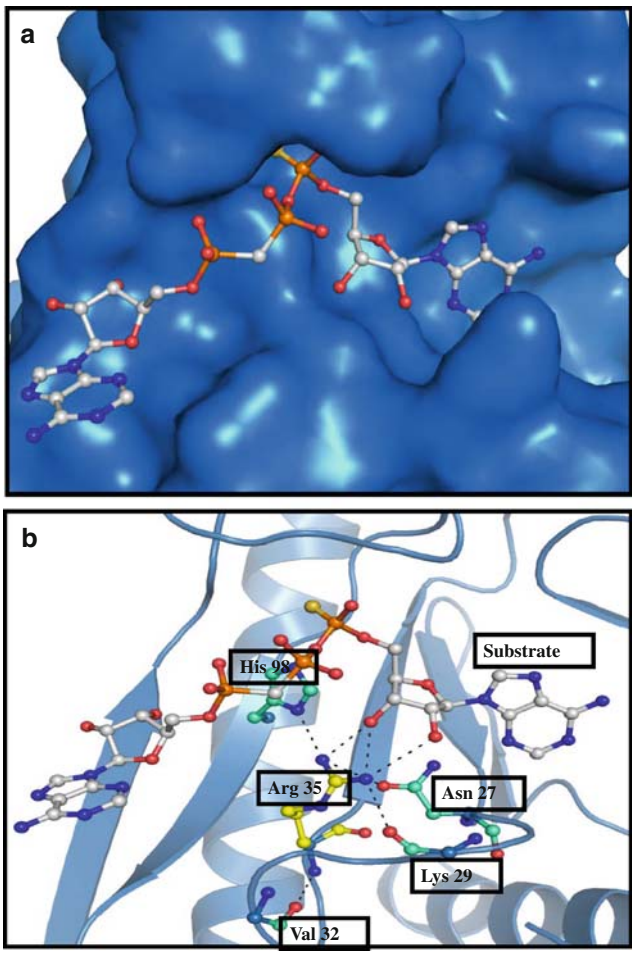

Fig. 3 **a** and **b**: Substrate-binding pocket of FHIT His35Arg model is shown in surface diagram. **a** the substrate analog, P1-P2-Methylene-P3-Thio-Diadenosine Triphosphate is present in the substrate-binding groove is shown in ball and stick. **b** Structure of FHIT His35Arg model-substrate complex. The substrate analog P1-P2-Methylene-P3-Thio-Diadenosine Triphosphate (*White*) and Arg35 (*Yellow*) are shown in ball and stick model. Residues showing close interaction with Arg35 is shown in green (*ball and stick model*). The hydrogen bonds are shown in *dotted line*

His35Arg is substantially narrower than native (Fig. 3a). The reason is evident from the structure of substrate-binding residues in wild-type (Fig. 2b) as well as His35Arg (Fig. 3b). ND2 and NE1 of His35 form van derwaals interaction with oxygen atoms of substrate. In addition, the main chain nitrogen forms hydrogen bond with the main chain oxygen of Val 31 (Fig. 2b). On the other hand, in His35Arg a numerous hydrogen bonded interactions have been observed for Arg35 with either substrate atoms or protein atoms which significantly narrowed the size pocket. Moreover, the side chain of Arg is much larger as compared to His which further supports the inability of substrate to bind the *FHIT*.

Discussion

The extensive screening program has lowered the global incidences of cervical cancer but still cervical cancer is the second most common cancer among women worldwide [27], and the most common cancer among women in the resource poor countries including India. Early age of marriage, promiscuity, smoking, and the use of contraceptives etc. have been considered as risk factors for the development of cervical cancer but persistent HR-HPV infection has been considered to be the principal etiologic factor. In India, 85–90% cervical cancer cases are squamous cell carcinoma but only 10–15% cases are adenocarcinoma. Interestingly,

in India HPV 16 is the most prevalent type both in squamous cell carcinoma as well as in adenocarcinoma while preferential occurrence of HPV 18 in adenocarcinoma is available globally [28, 29].

Cervical cancer is a suitable model to study genetic and epigenetic changes occurring during its progression because of multiple etiologies as well as prolonged well-differentiated intraepithelial lesions. However, still the role of genetic factors involved in the development of cervical carcinogenesis is poorly understood and only limited reports are available showing alteration of *FHIT* gene in this cancer.

The *FHIT* gene, a human homolog of *S. pombe* hydrolase enzyme prefers dinucleoside 5′, 5‴-p1, and p3-triphosphate (Ap3A) as substrate, in contrast to 5′, 5‴-p1, and p4-tetraphosphate (Ap4A) in case of *S.pombe* [30]. Thus, it is postulated that elevated levels of Ap3A (the substrate for the enzyme) activate signal transduction pathway or impair apoptotic pathway. Ap3A, in some cases can act as ATP analog and inhibit a protein kinase. In the present study, we made an attempt to predict computationally whether the *FHIT* function gets altered by point mutation in substrate-binding domain in cervical cancer cases from North Indian population.

Earlier studies have failed to detect any point mutation in the *FHIT* gene from tumors but later, evidence of such mutations were available from cell lines derived from tumors [20, 31]. Mao et al. [32] reported polymorphisms of *FHIT* at codons 88 and 98, a null mutation without altering the amino acid from head and neck cancer cell lines. In our study, we observed a novel single missense mutation due to A → G transition substitution at nucleotide 665, codon 98, lead to change CAT → CGT with consequent replacement of amino acid Histidine by Arginine in North Indian cervical cancer cases. However in contrast, Kannan et al. could not find any mutation in the coding regions of *FHIT* gene in exon 5–9 but at the ninth nucleotide upstream to the beginning of exon 9 in cervical carcinomas [14]. On the other hand, there are some reports of somatic missense mutations in *FHIT* gene from primary gastric cancer [33].

Earlier studies showed a link between viral integration sites and fragile sites of chromosome 3p [34]. There are two regions of the FRA3B/FHIT fragile site as flanking sites for HPV insertion point that occur within introns 4 and 5, respectively, which surround the frequently deleted exon 5 of *FHIT* [7]. However in contrast, reports were also available showing no apparent correlation between *FHIT* alteration and HPV infection suggesting *FHIT* gene alterations may occur independent of HPV infection [35]. As mutation in *FHIT* is rare event, another mechanism of inactivation of *FHIT* in cervical cancer cases is methylation. Our previous report showed that aberrant promoter hypermethylation of *FHIT* gene was highly significant ($P < 0.01$) for the cervical cancer cases and this late event may be associated with carcinogenesis in Indian population [8].

After determination of crystal structure of FHIT, Pace et al. proposed three different models for its anti-tumor activity. In postulation, it was proposed that the tumor suppressing function of *FHIT* might be due to its catabolic action to Ap3A or related substrates and/or signaling by *FHIT*-bound forms of these compounds which may lead to nucleotide-independent tumor suppressor function of *FHIT* [16]. Our observations are in good agreement with the first mechanism proposed by Pace et al. [16]. The substrate and enzyme binding might be interfered by many factors including mutations. In our study, the change from His to Arg caused an appreciable difference in the size of pocket where *FHIT* was unable to accommodate substrate. A similar observation was reported by Pace et al. [16] where they mutated His98 to Trp which was expected to hinder substrate binding. Formation of an enzyme–substrate complex is essential for tumor suppressor activity and the heavy side chain (Arg) substitution alleles of *FHIT* would be expected to lose function in parallel with a loss of its ability to bind substrate.

To our knowledge, this is the first report showing a novel naturally occurring mutation in the tumor of uterine cervix from Indian population. Our results on the mutations of the *FHIT* gene in squamous cell carcinoma of uterine cervix indicated that *FHIT* gene mutations are very rare. Although the mechanism by which *FHIT* gene mutations play a role in the pathogenesis of cervical cancer is not very clear, however, our mutation based structural analysis indicated that constriction in the substrate-binding pocket due to a close interactions formed by Arginine instead of Histidine in wild type at 35th position in substrate-binding domain may be one of the reason. The bulkiness of side chain of Arg further reduces the size of pocket, which is insufficient to accommodate substrate in the pocket with ultimate loss of protein function.

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