Research in Molecular Medicine

Original Article

2016, Vol: 4, Issue: 2, Pages: 9-14

DOI: 10.18869/acadpub.rmm.4.2.9

Investigation of *fimH* Single Nucleotide Polymorphisms (C640T and T591A) in Uropathogenic *E. coli* Isolated from Patients with Urinary Tract Infections

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Received: 21 Feb 2016

Revised : 18 Mar 2016

Accepted: 30 Mar 2016

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Abstract

Background: Urinary tract infections (UTIs) are one of the most frequent health problems and uropathogenic *Escherichia coli* is the major pathogen resulting in UTIs. Sever forms of UTIs are caused by expression of a large range of virulence factors. In this study, we evaluated the allelic frequency *fimH* gene, in uropathogenic strains of Escherichia coli (UPECs) isolated from patients with UTIs. This study also aimed at determining the roles of C640T and T591A SNPs of the *fimH* gene in the ability of UPEC to cause UTIs.

Materials and Methods: A total of 140 UPEC strains isolated from patients with UTIs were screened by PCR-RFLP to determine the prevalence of the *fimH* gene C640T and T591A SNPs in UPEC strains isolated from patients attending educational hospitals in Shahrekord. The genotyping of C640T and T591A SNPs was performed using *Bme*1390I and *BseN*I restriction enzymes, respectively through PCR-RFLP method.

Results: There were no significant association between C640T and T591A SNPs of *fimH* gene and the ability of UPEC fimH variants to cause UTIs in the studied *E.coli* isolates.

Conclusion: FimH is one of the major virulence factors among UPECs which is confirmed in most *E.coli* isolates. Further studies are required to determine the association between different *fimH* gene SNPs of isolated UPECs from patients with UTIs and the ability of UPEC fimH variants to cause UTIs.

Keywords: UTIs; fimH; C640T and T591A SNPs; PCR-RFLP

Please cite this article as: Molaie R, Dehghanian F, Hojati Z, Gholipour A. Investigation of fimH Single Nucleotide Polymorphisms (C640T and T591A) in Uropathogenic *E. coli* Isolated from Patients with Urinary Tract Infections. Res Mol Med. 2016; 4 (2): 9-14

Introduction

Urinary tract infection (UTI) is one of the most widespread infections in humans which causes severe morbidity and significant expenditures (1). UTIs affect different parts of the urinary tract and are classified into various disease groups based on infection sites. UTIs are categorized into cystitis (the bladder), pyelonephritis (the kidney) and bacteriuria (the urine) (2-4). Uropathogenic *Escherichia coli* (UPEC) strains are considered as a primary pathogen in about 80% of patients with UTIs (5, 6). Successful establishment of infection by UPECs needs different cascade of events including adhesion to host cells colonization of tissues and in certain cases, cellular invasion. These events include different cellular and molecular pathways which depend on the presence of multiple virulence factors (7). In fact, various range of virulence factors including siderophore receptors encoding genes, iron intake system, type 1 fimbriae encoding genes and hemolysin encoding gene amplify the pathogenicity of UPEC strains and the severity of UTIs (8, 9).

Among all cellular pathways relating to UPECs infection, adherence is an essential step. Adherence of bacteria to the surface of the host cell is a preliminary stage in colonization and central step relating to infection (10). This step is mediated by

structures called adhesions which are cell-surface components or appendages of bacteria that make possible adherence to other cells or surfaces. Type 1 fimbriae adhesion is the best described bacterial adhesions which are very important in the mechanism of UPECs adhesion to the uroepithelium. Type 1 fimbriae involves a number of subunits, the most significant of which is an adhesion protein identified as FimH (11,12). FimH, which is located at the tip of type 1 fimbriae plays a major role in the pathogenesis mechanism of UPECs at urinary tract. It mediates binding to α-D-mannosylated glycoproteins, which are located on epithelial cells of the urinary tract.

Recently, a variety of single nucleotide polymorphisms (SNPs) have been identified in bacterial pathogens which belong to pathoadaptive mutations (13). These mutations defined as genetic alterations in regulatory or structural genes that lead to discriminative benefits during infection. Different Phenotypic variants of FimH subunit provide important advantages to the bacteria in the colonization of the urinary tract and its pathogenicity (14, 15). In this study, we attempt to investigate the association between C640T and T591A SNPs of *fimH* gene and its effect on the severity of pathogenicity of UPECs to cause UTIs. In addition, possible interactions between the polymorphisms, age and gender status were also analyzed.

Materials and Methods

Bacterial isolates

A total of 140 *E. coli* isolates was collected from the urine samples of patients with UTIs during inpatient and outpatient treatment in Educational Hospitals of Shahrekord, Iran, during April to July 2012. Patients who stayed in a ward for clinical treatment were considered as inpatients. Outpatients were defined as patients who were not hospitalized for 24 hours or more, but who visited a hospital or clinic for diagnosis and treatment. All the isolates originated from inpatients and outpatients with significant *E. coli* bacteriuria (defined as $\geq 10^5$ CFU ml^{-1} in clean-voided urine). The microbiological characteristics of all bacterial isolates were identified using standard biochemical identification methods (16, 17). *E. coli* reference strains K-12 (Pasteur Institute, Iran) was also studied as a positive control for PCR reaction.

DNA isolation

A sweep of each sample growth on a nutrient agar was boiled in 500 µl of distilled water for 10 minutes in order to extract DNA. After thermal lysis, centrifugation was done at 12000 rpm for 7 minutes to pellet the cell debris. The supernatant was transferred to a new vial, kept at 20 °C and used as the template for PCR amplification.

Genotyping analyses

The genotyping of C640T and T591A SNPs was carried out using PCR-RFLP method. The primers for SNPs were designed using the primer design software Oligo 7 and were listed as follows:

Forward primer (C640T): 5′ GTGCCAATTCC TCTTACCGTT 3′

Reverse primer (C640T): 5′ TGG AAT AAT CGT ACC GTT GCG 3′

Forward primer (T591A): 5′ CCG TTA CTC TGC CGG ACT ACA C 3′

Reverse primer (T591A): 5′ CCC AGG TTT TGG CTT TTC GCA CAA T 3′

The Touchdown PCR reaction for C640T genotyping was performed in 25 µl reaction mixture containing 0.5 µl of each forward and reverse primers (10 pM), 2.5 µl of 10× solution buffer (20 mM Tris-HCl pH 8.6, 50 mM KCl, Cinnagen Inc, Iran), 1 µl of four mixed dNTPs (10 mM, Cinnagen Inc, Iran), 1µl of Mgcl2 (50 mM, Cinnagen Inc, Iran), 0.1 µl of 5u/µl Taq DNA polymerase (Cinnagene, Co., Iran), 2 µl (100 ng/µl) of template DNA.

The Touchdown PCR program for C640T genotyping was as follows: initial denaturation for 3 min at 96 °C followed by 10 cycles of 30 sec at 96 °C, 30 sec at 64 ºC, 72 °C for 30 sec; 24 cycles of 30 sec at 96°C, 30 sec at 54 ºC, 72 °C for 30 sec and a final extension step of 7 min at 72 °C. The Touchdown PCR reaction mixture for genotyping of T591A SNP was as follows: 0.5 µl of each forward and reverse primers, 2.5 µl of 10× solution buffer, 1 µl of four mixed dNTPs (10 mM, Cinnagen Inc, Iran), 1.5 µl of Mgcl2, 0.1 µl of Taq DNA polymerase and 2 µl of template DNA. The Touch-down PCR program for T591A genotyping was carried out with the following temperature profile: an initial step at 96 °C for 3 min followed by 7 cycles of 30 sec at 96 °C, 30 sec at 69 ºC, 72 °C for 20 sec; 30 cycles of 30 sec at 96 °C, 30 sec at 62 ºC, 72 °C for 20 sec and a final extension step of 5 min at 72 °C. The PCR products were run on 8% polyacrylamide gel and were visualized by silver staining. Moreover, restriction analyses of C640T and T591A SNPs were performed using *Bme*1390I and *Bse*NI enzymes (Fermentas, Vilnius, Lithuania), respectively (Table 1). Then the restriction fragments were analyzed on 8% polyacrylamide gel. Finally, dideoxy termination sequencing with the ABI automated sequencer was performed for three amplified products of each SNPs to confirm the authenticity of the SNPs genotyping analyses.

Statistical Analyses

The genotypes and allele frequencies of *fimH* C640T and T591A SNPs were analyzed, using the $\chi 2$ test. A probability of P value less than 0.05 was considered

to be statistically significant. The t-test was also used to analyze the quantitative data. Statistical analyses were performed using SPSS version 19.0 software (SPSS, Inc., Chicago, IL, USA).

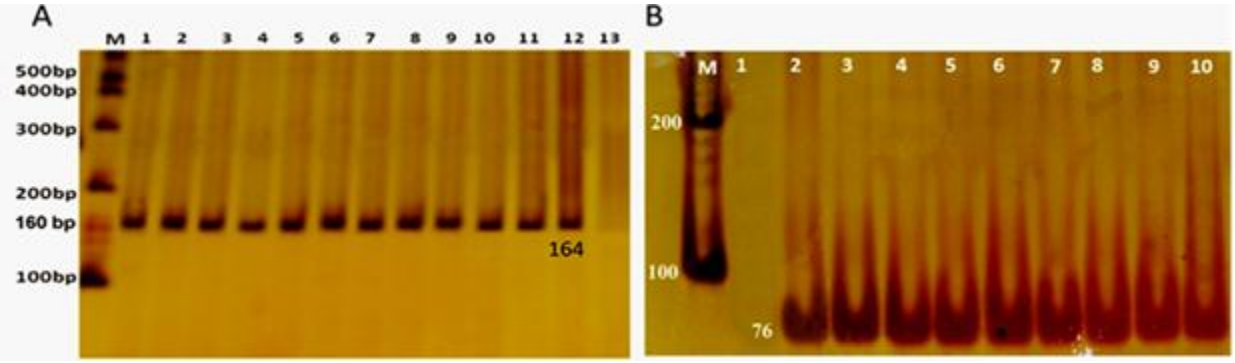

Figure1. Gel Electrophoresis of PCR products containing the C640T and T591A polymorphic sites. Regional amplification of *fimH* gene, which consist of C640T and T591A polymorphisms results in 164 bp (A) and 76 bp (B) PCR product, respectively. 13 A and 1B: negative controls in two PCR reactions; M: Marker (100 bp); 8% polyacrylamide gel.

Results

Forty-five inpatients at the hospital were females and 25 patients were males (36 patients younger than 35 years and 35 patients older than 35 years). Forty-two and 28 isolates were recovered from urine samples of female and male outpatients, respectively (32 patients younger than 35 years and 38 patients older than 35 years). The patients aged 1 to 90 years (mean age: 59.5 years). The $\chi 2$ test indicated no significant association between the *fimH gene* frequency; and gender, age and inpatients or outpatients status (data not shown).

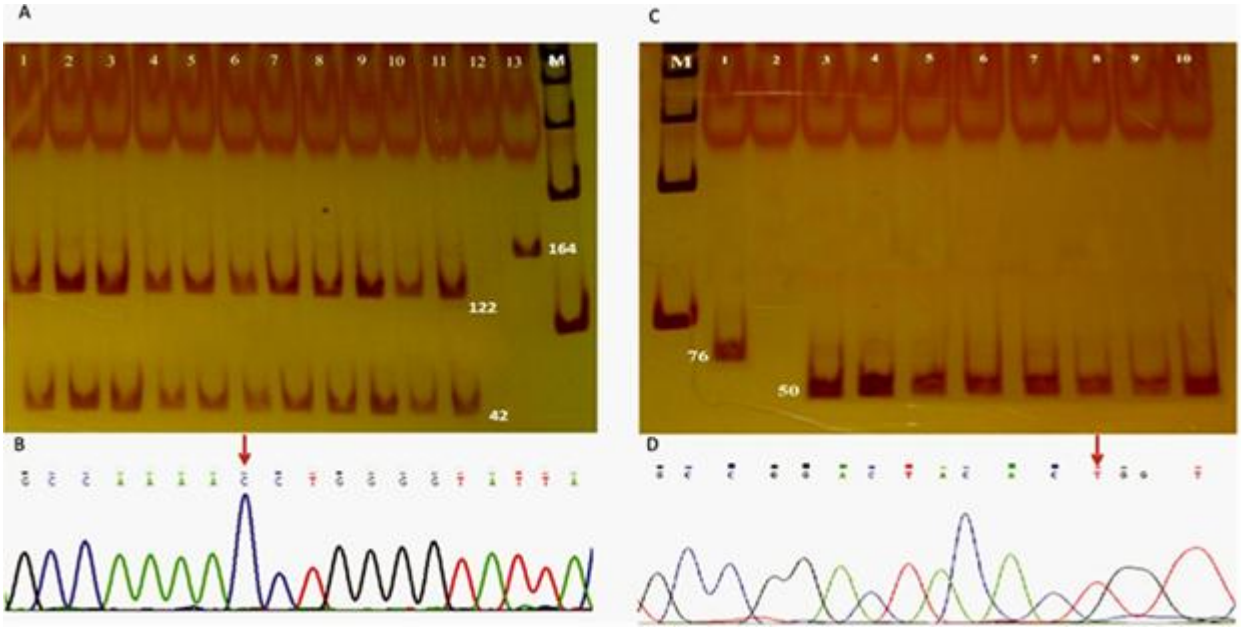

Figure 2. Genotyping analysis of C640T and T591A SNPs. *Bme*1390I enzyme digested the 164bp fragment PCR product in C location and led to the creation of fragments with 122 and 42 bp lengths (A). T allele of T591A SNP was affected by *BseN*I restriction enzymes and resulted in creation of 50 and 26 bp fragments. The 26 bp fragment was removed from gel because of the small size of fragment (C). 12A and 2C: negative control; 13A and 1C: un-digested PCR products; M: Marker (100 bp); 8% polyacrylamide gel.
Examples of direct DNA sequencing analysis for one sample of C640T (B) and T591A (D) polymorphisms.

Distribution of fimH SNPs

Regional amplification of *fimH* gene, which consist of C640T and T591A polymorphic site results in a 164 bp and a 76 bp PCR products, respectively (Figure. 1). Genotyping was accomplished using the Bme1390I and BseNI restriction enzymes for C640T

and T591A SNPs, respectively. Bme1390I enzyme only affect the C allele sequence and it does not have any influence on the T sequence. This enzyme digested the 164bp fragment PCR product in C location and led to the creation of fragments with 122 and 42 bp lengths (Figure. 2 A). Relating to T591A SNP, the T allele was affected by BseNI restriction enzymes and led to the creation of 50 and 26 bp fragments (Figure. 2C). Therefore, discrimination of two alleles was possible and the restricted and non-restricted fragments were simply recognizable through polyacrylamide gel electrophoresis.

Table 1. PCR-RFLP analyses for detection of C640T and T591A SNPs in *fimH* gene.

Polymorphism	Restriction enzyme	PCR Product (bp)	RFLP Products (bp)	The mixture of restriction analyses				
				Enzyme	Buffer	ddH2O	PCR Product	Total volume
C640T	(10U/µl) Bme1390I	164	122, 42 * 164**	0.5µl	1µl	9µl	5µl	15/5µl
T591A	(10U/µl) BseNI	76	50, 26 * 76*	0.5µl	1µl	9µl	5µl	15.5µl

Allelic frequencies of *fimH gene* C640T and T591A polymorphisms of 130 *E. coli* isolates which contain *fimH gene* are summarized in Table 3. All *E. coli* isolates contain the normal allele (C and T allele) of *fimH gene* C640T and T591A polymorphisms. The comparison of the allelic frequency of C640T and T591A polymorphisms among inpatients and outpatients groups showed similar frequency (Table 2). The association of C640T and T591A polymorphisms and gender status was also analyzed between these two groups (Table 3). Finally, the direct DNA sequencing analyses for three different samples of each SNP approved the presence of normal allele and the results of genotyping analyses (Figure 2B, 2D).

Table 2. Allelic frequency of C640T and T591A SNPs among outpatients and inpatients groups.

Polymorphism	Allelic frequency	Outpatients		Inpatients		P
		(%)	n	(%)	n	
C640T	C	88	62	97	68	0.05
	T	0	0	0	0	
T591A	T	88	62	97	68	
	A	0	0	0	0	

Discussion

Various SNPs in structural and regulatory genes have remarkable effects on the biology of all organisms (18). Analyses of SNPs in human genome are widely performed to investigate the association between specific SNP and individual susceptibility to various diseases (19). These types of studies lead to identification and discovery of different biomarkers for early diagnosis of the potential individuals (20). In recent years, many SNPs have been discovered in different regulatory and structural genes of bacterial pathogens. Scientists have focused on the association between different SNPs of bacterial pathogens and its effect on the severity of pathogenicity of pathogens to cause disease (4, 21). UTI is a major health problem and one of the most frequent hospital acquired infections, with *E. coli* as a predominant pathogen in most cases. Genetic variations in human such as different polymorphisms in Toll-like receptors (TLR) family underlie the susceptibility for UTI. *E. coli* virulence genes are also main factors in the etiology of UTIs, as well as human susceptibility factors (1, 22, 23). It has been reported that the UPEC strains which cause most UTIs form a genetically different group with multiple virulence factors (24). Generally, the combination of various factors in host and pathogen has determined the individual susceptibility to UTIs.

Among all diverse range of UPEC's virulence factors, type 1 fimberia encoding genes are mostly investigated. Type 1 fimberia contains an adhesion at their tip, which is encoded by *fimH gene* as a significant factor in host-pathogen interactions. Scientists particularly targeted the FimH adhesion to prevent from its interaction with host cells using soluble receptors. These investigations suggested FimH receptors as potentially important vaccine candidates (25, 26). Moreover, The FimH phenotypic variants are mainly the result of SNPs in *fimH gene* (27). The naturally occurring of these variants was investigated through different studies. These studies reported that the naturally occurring fimH variations significantly alter the tissue tropism of *E.coli*. Furthermore, the selection of genetic variations in the FimH gene as well as acquisition of virulence genes, results in the shifting from commensal to virulent phenotype (28, 29). Various fimH SNPs were analyzed and reported for *E. coli* clinical isolates by Dias et al. Some fimH polymorphisms such as C640T and T591A SNPs were found only among hospital or community isolates (30). In this study, we investigated the association between C640T and T591A SNPs of *fimH gene* and the ability of UPEC to cause UTIs.

In agreement with other studies, we previously reported a high frequency of *fimH* gene (92.8%) among 140 *E. coli* isolates (31). The incidence of *fimH gene* in *E. coli* strains isolated from Romanian adult with UTI was 86% (32). In addition, Andreu et al. identified the incidence of *fimH* gene in *E. coli* strains isolated from patients with UTI as 90% (33). In another study, Joachim et al. reported that the frequency rate of fimH was 95% among UPEC strains. In further studies by Tarchouna et al. and Daniela et al. which were performed on virulence genes in *E. coli* isolated from patients with UTIs, the prevalence of *fimH gene* was 68% and 86.1%, respectively (28, 32). The high frequency of fimH gene in all studies relating to UTIs indicates the critical roles of this virulence factor in *E. coli* pathogenesis. In the present study, among all *E. coli* isolates which contained *fimH gene* (130 samples), the frequency of C allele in the C640T polymorphic site is 97% and 88% in outpatients and inpatients groups, respectively. In fact, there is no allelic transition in this position and the frequency of the T allele was 0%. Absolutely same results were observed relating to the allelic frequency of the T allele in T591A site. The frequency of the A allele was 0% and no allelic shifting was observed relating to T591A SNP. Our results are in agreement with those of Abdallah et al. in these polymorphic sites (34). Furthermore, the absence of the T and A allele in these positions among the outpatients group is not similar to the results of Dias et al. The differences observed in this study and reports from Dias et al. (29) could be explained by geographic location and the type, source and size of samples. In conclusion, our study indicated that: 1) FimH is one of the most significant virulence factors among UPECs; 2) further studies are required to determine the association between different SNPs of *fimH gene* of isolated UPECs from UTIs patients and the ability of UPECs to cause UTIs.

Acknowledgements

This study was performed at Cell and Molecular Research Center in Shahrekord University of Medical Sciences and was financially supported by the Graduate Office, Shahrekord University of Medical Sciences.

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

MR, HZ and GhA have made substantial contributions to the conception and design of the study and interpretation of data. MR performed the experiments. Drafting the article or revising it critically for important intellectual content have done by GhA and DF. All authors also contributed in final approval of the version of manuscript which to be submitted.

Support/Funding

This work was supported by the Graduate Office, and grant No. 1113 provided by Shahrekord University of Medical Sciences.

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