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The Role of ATG16L1 (Thr300Ala) Genetic Variants and Autophagy in Development of Acute and Chronic Urinary Tract Infection Caused by Uropathogenic *Escherichia coli*

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Abstract

Background Uropathogenic *Escherichia coli* (UPEC) is the major cause of urinary tract infection (UTI), establish quiescent intracellular bacterial reservoirs (QIRs). These latent reservoirs, which persist indefinitely, are resistance to antibiotic therapies and can induce recurrence. Autophagy related 16 like 1 gene (ATG16L1) Thr300Ala genetic variant confer an increased risk for the development of urinary tract infection caused by UPEC. This study aimed.

Objective To determine the possible relationship between the ATG16L1 Thr300Ala genetic variant and UPEC for the development of acute and chronic urinary tract infection.

Methods A total of 100 urine and blood samples were collected from patients complain from UTI, 20 blood samples of apparently healthy during the period between (November 2014 to May 2015) from two hospitals in Baghdad; Al-Imamein Al-Kadhimein Medical City and Al-Yarmauk Teaching Hospital. The age range exactly with mean ± SD or SE. *E. coli* were isolated by ordinary methods and the identification of non entero-pathogenic *E. coli* was performed at a group level by slide agglutination test with specific antisera. UPEC isolates were tested for their susceptibility to 12 antimicrobial agents by disc-diffusion method. ATG16L1 T300A genotyping was done by Species Specific Primer – Polymerase Chain Reaction (SSP-PCR), after genomic DNA extraction from each blood sample.

Results A total of sixty *E. coli* were isolated from 20 acute UTI (16 females and 4 males) and 40 chronic UTI (32 females and 8 males). There is a high rate of acute UTI among the age range exactly groups (≤10-20 years) and chronic UTI among age groups (21-40 years). Overall isolates had a complete resistance to Ampicillin and Gentamycin (100%), high resistance to Nalidixic acid (88%), Piperacillin (86%), Trimethoprime+Sulfa (84%), Cefotriaxon (80%), Ciprofloxacin (78%) and Cephalosporin (66%). Moderate resistance to Azithromycin (51%) and Cephalothin (50%) were seen. Whereas these isolates were highly susceptible to Imipenem and Nitrofuranton with the resistance rate 8% & 27% respectively. Ninety percent of the isolates were resistant to three or more antibiotics. The SSP-PCR result showed that a (89%) and (92%) of acute positive *E. coli* infection (P=0.009) and chronic positive *E. coli* infection (P=0.006) respectively were carried allele-G. While the occurrence of allele-G was (62%) in acute negative *E. coli* infection, and (30%) in chronic negative *E. coli* infection.

Conclusion There is a relationship between allelic variants of ATG16L1 gene with acute and chronic UTI. In the other hands, the risk of the present allele G was associated with increased susceptibility to infection by UPEC, which developing chronic and acute UTI.

Keywords *Escherichia coli*, autophagy, Thr300Ala genetic variant, PCR

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List of abbreviations: QIRs = Quiescent intracellular bacterial reservoirs, SSP-PCR = Sequence Specific Primer-Polymerase Chain Reaction, UPEC = Uropathogenic Escherichia coli, UTI = Urinary tract infection

Introduction

A urinary tract infection (UTI), one of the most common infection and difficult health problem in many different countries around the world [(1)]. Uropathogenic *Escherichia coli* (UPEC) is the major cause of UTI, establish quiescent intracellular bacterial reservoirs (QIRs). These latent reservoirs, which persist indefinitely, are resistance to antibiotic therapies and can induce recurrence [(2)].

Autophagy is a cellular degradation process that can eliminate intracellular pathogens by utilizing them to lysosomes for destruction [(3)]. In the other hands, ATG16L1 (autophagy-

related 16-like 1), its product plays an important role in the innate immune response and in the resistance to intracellular pathogens [4]. UPEC depend on a function of autophagy program [2].

The objective of this study was to determine the possible relationship between the ATG16L1 Thr300Ala genetic variant and UPEC for the development of acute and chronic urinary tract infection.

Methods

A total of 100 urine and blood samples were collected from patients suffering from UTI and 20 blood samples from apparently healthy controls. The differentiation between acute and chronic UTI according to clinical symptoms may be absent or include urinary frequency, urgency, dysuria, lower abdominal pain, and flank pain. Systemic symptoms and even sepsis may occur with kidney infection. Diagnosis of patients with UTI is based on analysis and culture of urine. Also, the diagnosis of control groups is depending on analysis and negative culture of urine so which considered as normal urinary tract(s) (apparently healthy control group) All urine samples were cultured on blood agar and MacConkey agar for identification of the bacteria. Api20E system was used for the final confirmation of the bacteria. For the identification of the bacteria at a group level, a slide agglutination test with specific antisera was done.

Antimicrobial susceptibility tests

Disk diffusion test was performed to determine the resistance patterns of isolated *E. coli* against twelve antibiotics includes Gentamicin (10 µg), Imipenem (10 µg), Ceftriaxone (30 µg), Ampicillin (10 µg), Nalidixic acid (30 µg), Ciprofloxacin (5 µg), Nitrofurantoin (50 µg), Trimethoprim + Sulfa (25 µg), Azithromycin (15 µg), Cephalothin (30 µg) and Piperacillin (30 µg).

DNA extraction

DNA was extracted from 300 µL peripheral blood EDTA containing tubes using DNA isolation kit (Wizard® Promega & QIAamp® Qiagen, USA). following manufacturer informations.

Sequence Specific Primer-Polymerase Chain Reaction (SSP-PCR)

Allelic discrimination was checked by SSP-PCR, for study groups using two sequences (Table 1). Three µl of extracted blood DNA was added to 12.5 µl GoTaq® Green Master Mix PCR master mix reaction with a 1.5 µl for each forward and reverse of two specific primers (Promega, USA) of ATG16L1 (Thr300Ala) in addition to internal control in a separated reaction mixture. The volume complete to 25 µl with a 1.5 µl ddH_2O. PCR products was 201bp for both allele A and allele G, allowing the discrimination of homozygous or heterozygous alleles. PCR program includes initial denaturation 94 °C for 3 min; denaturation 94 °C for 1 min, annealing 58 °C for 1 min, elongation 72 °C 1 min for 30 cycles the final extension at 72 °C for 10 min and hold at 4 °C using thermal cycler (Eppendroff-thermal cycler, Germany) and separated PCR-runs-for each allele.

Table 1. Sequence specific primers (SSP) and internal control primers (IC)

Gene	Primer	Sequence	Genomic position	Amplicon size (bp)
ATG16L1 (T300A)	F allele A	5´CCCCAGGACAATGTGGATA´3	2q37.1	201
	F allele G	5´CCCCAGGACAATGTGGATG´3		
	R**	5´AGGTGGAAAGGCTTGATATAAG´3		
β-globin	F	5`ACACAACTGTGTTCACTAGC´3	11p15.5	157
	R	5`GAAAATAGACCAATAGGCAG´3		

* F = Forward; ** R = Reverse

Gel-electrophoresis

PCR products were resolved in 1.5% agarose gel at 7 v/cm^2, 1 hr visualized by UV transilluminator and photographed by digital camera (Sony-Japan).

Statistical analysis

The statistical analysis performed with (SPSS) 19.0 and Microsoft Excel 2013.

Results

Patients and bacterial isolates

This study involved 100 Iraqi patients suffering from UTI, 34 with acute UTI and 66 with chronic – recurrent UTI in addition to 20 cases with normal urinary tract, which considered as apparently healthy group (HG). The age group ranging from ≤10 years to >50 years as shown in table (2).

Table 2. UTI patients and apparently healthy control were classified according to the age groups

Age groups		Study groups		Total
		Healthy	UTI	
≤10 years	Count	1	9	10
	%	5.0%	9.0%	8.3%
11-20 years	Count	3	19	22
	%	15.0%	19.0%	18.3%
21-30 years	Count	6	26	32
	%	30.0%	26.0%	26.7%
31-40 years	Count	5	24	29
	%	25.0%	24.0%	24.2%
41-50 years	Count	4	14	18
	%	20.0%	14.0%	15.0%
>50 years	Count	1	8	9
	%	5.0%	8.0%	7.5%
Total	Count	20	100	120
	%	100.0%	100.0%	100.0%
p value		0.948		

Classifications of UTI according to UPEC infection

There were 40 chronic UTI and 20 acute UTI showed positive bacteriuria, whereas 26 chronic and 14 acute UTI showed negative urine culture.

The occurrence of *E. coli* according to gender type

In UTI, there were 87/100 (87%) females and 13/100 (13%) males, while in apparently healthy control groups (HG) there were 11/20 (55%) female and 9/20 (45%) males. Sixteen out of twenty females (80%) and 4 out of 20 males (20%) were positive urine culture of *E. coli* in acute UTI, while 14/14 (100 %) female and there is no male were negative urine culture. In chronic – recurrent UTI with positive culture of *E. coli*, there were 32 out of 40 females (80%) and 8/40 males (20%). While in negative urine culture, there were 25/26 (96.15 %) females and 1/25 (3.85%) males.

Antibiotic resistance of Escherichia coli isolates

By the disc-diffusion method, *E. coli* isolates showed complete resistance to Ampicillin and Gentamycin (100%). High rate of resistance to

Nalidixic acid, Pepracillin, Trimethoprime + Sulfa, Ceftriaxone and Ciprofloxacin. Moderate to low resistance to Cephalosporin, Cephalothin, Azithromycin, Nitrofuranton and Imipenem as shown in figure (1).

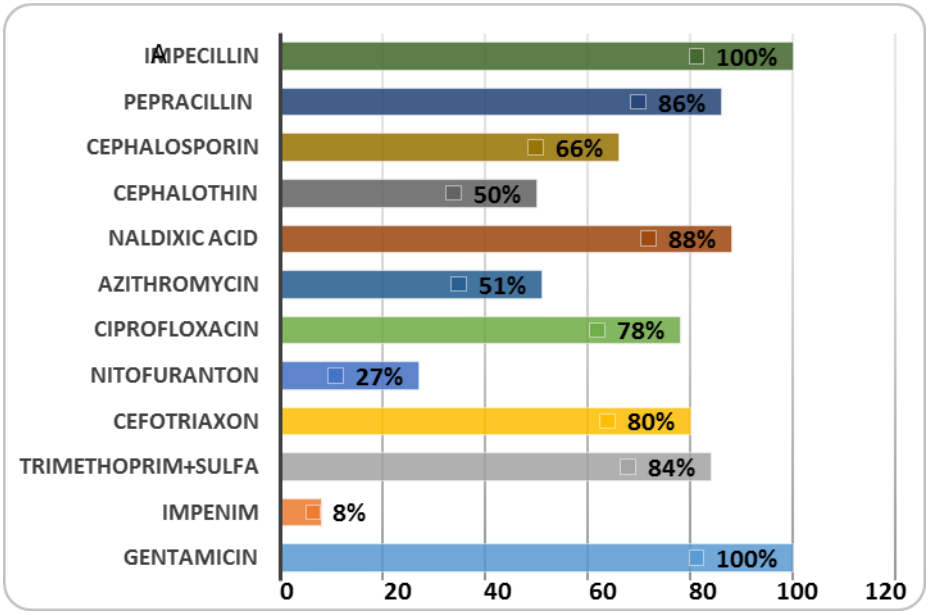

Figure 1. Antimicrobial susceptibility results of *Escherichia coli* isolates by the disc-diffusion methods

The association of ATG16L1 Thr300Ala genotypic variants with disease susceptibility

Most samples were investigated for the presence of rs2241880 ATG16L1 Thr300Ala polymorphism by SSP-PCR. This study showed that, higher percentages are of the homozygous mutant genotype (GG) 77.55% and 68.42%, and lower percentage for each heterozygous genotype (GA) 8.16% and 10.53% and homozygous wild type genotype (AA) 14.29% and 21.05% in chronic and acute UTI patients respectively. In the presence of allele G, the risk of incidence of acute and chronic UTI is more 10 times and 14.4 times than control respectively (Table 3).

The correlation between allelic frequency of rs2241880 ATG16L1 polymorphism and UPEC in UTI

Upon comparing the genotypic possibility (Ala300Ala, Thr300Ala and Thr300Thr) for 19 acute UTI and 49 chronic UTI, there were 14 acute UTI and 32 chronic UTI positive for urine culture with higher percentage of homozygous mutant genotype (GG) 85.71% and lower percentage of heterozygous genotype (GA) 7.14% and homozygous wild type genotype (AA) 7.14% in acute UTI. Simultaneously, there is a higher percentage in homozygous mutant genotype (GG) 87.50% and lower percentage of heterozygous genotype (GA) 9.38% and homozygous wild type genotype (AA) 3.13% in chronic UTI.

Regarding allelic frequencies, 25 out of 28 (89%) acute UTI were carrying allele G give *E. coli* positive compared with 3/10 (30%) of acute UTI negative for *E. coli*, whereas the percentage of chronic UTI carrying allele G with positive *E. coli* were 59/64 (92%) compared with 21/34 (62%) negative for *E. coli*. In the other hand, the risk of UPEC, which develop acute and chronic UTI was increased 19.44 times and 7.3 times respectively than those of *E. coli* negative (Table 4, Figures 2,3).

Table 3. Genotypic and allelic frequencies of rs2241880 ATG16L1 polymorphism in Iraqi acute, chronic UTI patients and apparently healthy controls

		Acute UTI	Chronic UTI	Healthy
ATG16L1 Genotype	**AA**	4 (21.05%)	7 (14.29%)	12 (70.59%)
	GA	2 (10.53%)	4 (8.16%)	1 (5.88%)
	GG	13 (68.42%)	38 (77.55%)	4 (23.53%)
	Total	19 (100.00%)	49 (100.00%)	17 (100%)
***p* value**	**vs healthy**	0.011	<0.001**	
ATG16L1 Allele	**G**	32 (80%)	84 (86%)	10 (29.0%)
	A	8 (20%)	14 (14%)	29 (71.0%)
	Total	40	98	34
Odd ratio (Confidence interval)	**vs healthy**	10 (2.114 to 43.60)	14.4 (3.866 to 53.64)	-
***p* value**	**vs healthy**	0.003*	<0.001**	-

*Statistical significant difference (p≤0.05), **= highly significant difference (p≤0.001)

Table 4. The association between the presence of uropathogenic *Escherichia coli* and ATG16L1 genotypic variants in UTI patients

		Acute UTI *E. coli*		Chronic UTI *E. coli*	
		Positive	**Negative**	**Positive**	**Negative**
ATG16L1 Genotype	**AA**	1 (7.14%)	3 (60.0%)	1 (3.13%)	6 (35.29%)
	AG	1 (7.14%)	1 (20.0%)	3 (9.38%)	1 (5.88%)
	GG	12 (85.71%)	1 (20.0%)	28 (87.5%)	10 (58.82%)
	Total	14 (100%)	5 (100%)	32 (100%)	17 (100%)
***p* value**	**vs no growth**	0.020*		0.009*	
ATG16L1 allele	**A**	3 (11.0%)	7 (70.0%)	5 (8.0%)	17 (38.0%)
	G	25 (89.0%)	3 (30.0%)	59 (92.0%)	21 (62.0%)
	Total	28	10	64	34
Odd ratio (Confidence interval)	**vs no growth**	19.44 (3.192 -118.5)	-	7.3 (2.323 -22.97)	-
***p* value**	**vs no growth**	0.009*	-	0.0006*	-

*Statistical significant difference (p≤0.05), **= highly significant difference (p≤0.001)

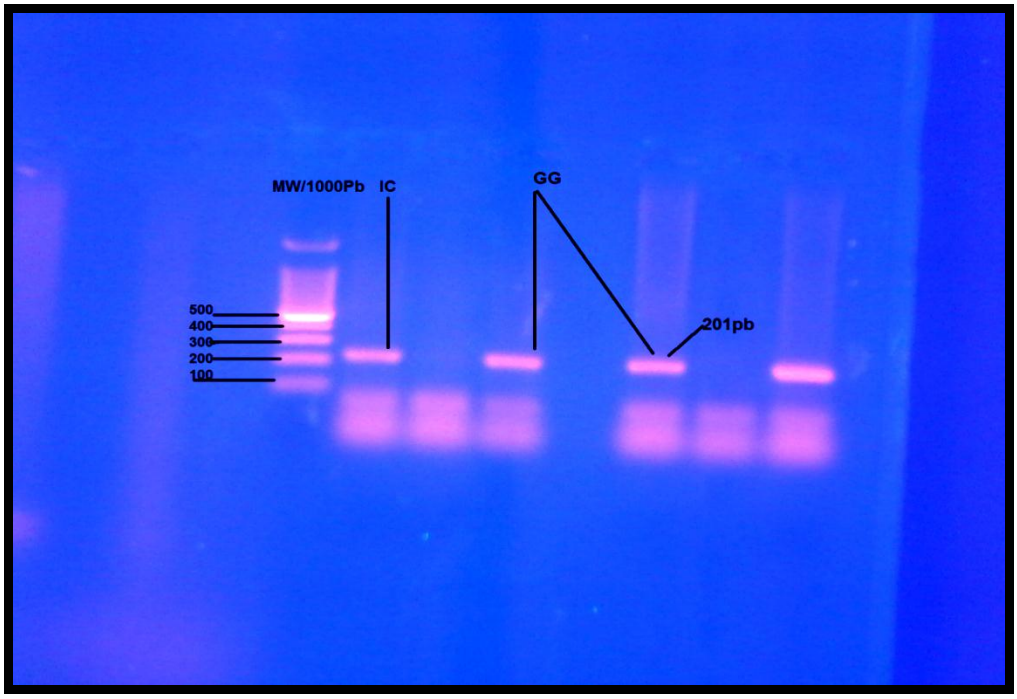

Figure 2. Electrophoretic profiles corresponding to homozyges mutant genotypes (GG) of the SNP T300A of the gene ATG16L1. M: molecular size standard (100 bp ladder), IC: Internal control (negative control or Housekeeping gene) with molecular size 157 bp and (GG)=201bp

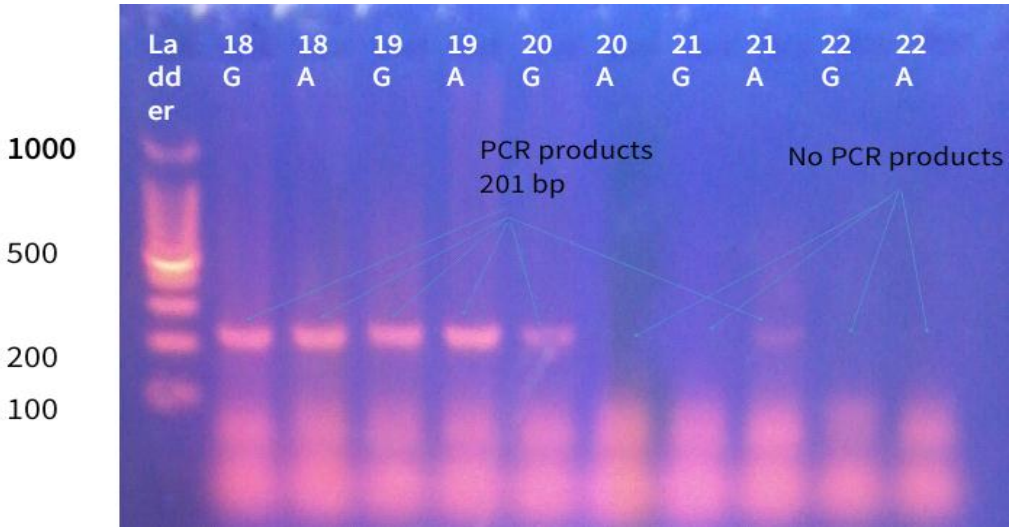

Figure 3. Electrophoretic profiles corresponding to PCR products, patients 20 homozyges mutant genotypes (GG). Patients 21 homozygous for allele A, patients 18 and 19 were heterozygous for allele A and G. patients, Molecular size standard (100 bp ladder, with molecular size of G-G=201bp

Discussion

Uropathogenic Escherichia coli (UPEC) cause 90% of UTI in human. These infections are highly prevalent in developing countries and are usually difficult to eradicate because these pathogens have acquired drug resistance [5,6].

In the current study, the prevalence of UTI with positive urine culture was in female (80%) more than in male (20%) in both acute and chronic UTI patients. The spread of infection was easy in females due to shorter urethra. This result was agreed with study conducted in Kurdistan region-Iraq that found 296 of UPEC strains were isolated from female accounting (81%) and male accounting (19%) [7].

This study showed that the incidence of infection increases with age and sexual activity. The rate significantly higher (45%) among 11-20 years in acute UTI in addition to 40 isolates from 66 patients with chronic UTI but less in age group 31-40 years and 41-50 years that gave *E. coli* positive. The National Center for Health Statistics reported that up to 20% of young females with acute cystitis develop recurrent UTI's, this due to the fact that postmenopausal women may undergo bladder or uterine prolapse, loss of estrogen that causes a change in the vaginal flora or loss of lactobacilli, which results in periurethral colonization with gram-negative aerobes (*E. coli*) [8].

This result also observed that 90% of the *E. coli* isolates are very high percentage of resistance was noted against Ampicillin and Gentamycin (100%) followed by Nalidixic acid (88%), Pepracillin (86%), Trimethoprime + Sulfa (84%), Cefotriaxon (80%) and Ciprofloxacin (78%). The current study was agreed with other studies in Iraq [9] and India [10] when they found that very high resistance to trimethoprim Sulfmethaxazol (94.4 %) and Nalidixic acid (92.6%) of *E. coli* in UTI patients respectively.

This study found that the Imipenem and Nitrofuranton were to be the most potent antimicrobial agents with the resistance rate (8%) and (27%) respectively and this was agreed with researches reported find same susceptibility to Imipenem and Ampicillin [7].

This study also conducted to investigate the association of ATG16L1 Thr300Ala genotypic variants. To the best of our knowledge, it is believed that this study is the first study in Iraq concerning the risk of autophagy related gene 16 like 1 protein T300A SNP in urinary tract infection. The current results showed that genotype GG was more prevalent among patients suffering from acute and chronic UTI patients followed by genotypes AA when compared with apparently healthy controls. While other study concluded reported that, the genotype AG was more prevalent among patients and controls, followed by genotypes AA and GG in Crohn's disease [11].

The PCR results showed that the frequency of the allele G polymorphism T300A was higher in the group of patients with acute and chronic UTI (80% & 86% respectively). This leads to the fact that increases susceptibility of acute and chronic urinary tract infections conferred by polymorphism T300A. The results of healthy controls strongly suggest that the active autophagy process was present in the normal human superficial urothelial cells, are the target cells for UPEC invasion and Atg16L1protein expression. In this study, there was also a predominance of the allele A (wild type) among healthy controls (71%). These results agreed with previous study describing the active autophagy process in normal colonic mucosa [12].

The present study found the homozygous mutant genotype GG was more prevalent among acute and chronic UTI patients with positive *E. coli* compared with negative *E. coli*, whereas lower percentage frequencies of (GA) and (AA) from chronic and acute UTI with positive *E. coli*.

In other hand, the risk factor of susceptibility to infection by UPEC was increased on 19.44 times in chronic UTI and 7.3 times in acute UTI on the presence of allele G that made protection to persistence *E. coli* as intracellular bacterial communities. Early invasion and colonization of the bladder were not dramatically altered by the Atg16L1 deficiency-induced urothelial ultrastructural changes. Strikingly, however, in the presence of these

abnormalities, UPEC appears less able to reside in the intracellular niches to persist in the urotheliumas QIRs [11].

The Atg16L1-deficient may elevate proinflammatory cytokine levels this may have had beneficial effect to promote rapid elimination of bacteria, which may prevent additional bacterial invasion into underlying urothelial cells and result in faster clearance of bacterial load leading to restoration of a normal urothelium [13]. While Other study showed that, the invasion of UPEC into epithelial cells was inhibiting proinflammatory cytokine production [14].

In conclusion, there is a relationship between allelic variants of ATG16L1 gene with acute and chronic UTI. In the other hands the risk of the present allele G was associated with increased susceptibility to infection by UPEC, which developing acute and chronic UTI.

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Authors Contribution:

Dr. Kadhim supervise this paper as part from a thesis. Abd and Dr. Abd Al-Rahman prepared, performed and did the tests and sampling. Dr. Ghazi and Dr. Abd Al-Rahman interpreted the results of the research.

Conflict of interest

The authors declare no conflict of interest.

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