Polymorphism Evaluation of TLR2 Gene Associated with Endometritis Infection in Buffalo Reared in Egypt

ABSTRACT

Background and Aim: Toll-like receptors (TLRs) are important for the recognition of pathogen-associated molecular patterns. Single nucleotide polymorphisms (SNPs) within TLRs have a potential impact on the alteration of susceptibility or resistance to inflammatory diseases. This work focused on a case-control study for the distribution of SNPs in TLR2 gene to test their role for endometritis occurrence in river buffalo.

Materials and Methods: Egyptian buffaloes from the slaughterhouse were tested, where forty samples divided into apparently healthy uteri (20n) and clinically infected ones (20n) were used to detect the genetic association between TLR2 gene SNPs and endometritis disease

Results: Nineteen novel polymorphic sites were identified. Three SNPs were found to be statistically significant. AA genotype at 5`UTR and CC genotype at the coding region had significant association with susceptibility to endometritis, while GG genotype at 5`UTR had significant association with resistance to endometritis. Polyphen 2 analysis revealed three amino acids substitutions in TLR2 protein having potential functional significance. Haplotype reconstruction revealed the statistical significance of haplotype frequencies between cases and controls indicating its association with the occurrence of endometritis.

Conclusions: It is to be concluded that the innate immune activation response may be interfered by the effect of polymorphisms and mutations of TLRs due to the reduction of the protein ability to recognize Pathogen associated Molecular pattern (PAMPs).

Keywords:*Bubalus bubalis*, TLR2, endometritis, SNPs

1. INTRODUCTION

“Water Buffalo has been for long playing a pivotal role in livestock and agriculture economy of many developing countries across the globe. Domesticated species of buffalo are river and swamp buffalo which are typically found in tropical and subtropical forests, wet grasslands,

marshes and swamps regions of the world. The current water buffalo population is 195 million head including both river and swamp buffaloes, 77% of which are river buffalo. Egyptian buffaloes are of the river type with a population of 4 million head, it ranks the third after Indian (over 115 million) and Pakistani buffalo (31.7 million)"[1] and it is a representative of the Mediterranean buffalo.

"Uterine diseases related to microbial infection represent the main cause of reduced fertility in bovine; the economic losses related to these diseases are double to those caused by ovarian disorders with a great impact on the economy" [2]. "The incidence of uterine infections in buffaloes is higher than cows which might be related to poor hygiene, vaginal stimulation for milk let down and possibly wallowing. Buffaloes are culled and sent to slaughterhouses either because they are uneconomic to maintain or because they have some disease problems. Hence, abattoirs are a good source for studying pathological conditions of buffalo reproductive organs that are severe enough to cause infertility and even sterility" [3].

"Endometritis is the most common uterine disease observed in buffaloes slaughtered at abattoirs and is one of the main causes of infertility in both cattle and buffalo" [4]. "Endometritis disease is highly prevalent, and lead to economic losses due to decreased milk yield, medication as well as prolonged calving intervals" [5]. "Numerous bacteria in a variety of combinations have been isolated from infected uteri of buffaloes, where *Arcanobacterium pyogenes*, *E.coli* , *streptococcus spp.*, and *Staphylococcus spp.* are responsible for toxic metritis and endometritis in buffaloes" [6]. "*E.coli* are usually associated with uterine infection with other bacteria in buffaloes" [7]. "Furthermore, *Escherichia coli* and *Arcanobacterium pyogenes* are the most prevalent bacteria isolated from the uterine lumen of bovine with uterine disease" [8].

"Toll-like receptors (TLRs) are part of pattern recognition receptors (PRRs) that play an essential role in recognizing molecules that are produced by pathogens, inflammatory initiation, and stimulation of adaptive immune responses. PAMPs include various components of pathogens such as lipopolysaccharides (LPS), peptidoglycans, flagellin, bacterial DNA and viral double stranded RNA" [9]. "Polymorphisms within TLR genes are associated with the occurrence of wide range of diseases in human and mice. Furthermore, polymorphism in TLRs genes is also associated with variations in disease resistance traits in livestock. TLR2 and TLR4 are the most

two studied TLRs due to their essential role in the recognition of bacterial pathogens. Several studies have focused on the functional consequences of TLR2 and TLR4 SNPs where it was found that polymorphisms in TLR2 and TLR4 genes have potential effect on the binding of these TLRs to its ligands and disease resistance" [10]. "TLR2 gene in buffalo consists of two exons with 2355 bp open reading frame encoding 784 amino acids. The gene plays an essential role in the innate immune response to a variety of pathogens, making TLR2 polymorphism to have a potential impact on diseaseresistance in livestock" [11].

2. MATERIALS AND METHODS

2.1 Sampling and study design:

Egyptian buffaloes at the slaughterhouse were tested, where forty samples were divided into apparently healthy uteri (20n) and clinically infected ones (20n), according to physical examination and presence of abnormal secretions and inflammation signs in uterine tissues . All samples were used to detect thegenetic association between TLR2 gene SNPs and endometritis disease.Therefore, microbiological processing was performed on all uterine samples to identify bacterial pathogens in clinically infected samples.

2.2 TLR2 sequencing:

Overlapped primers were designed to amplify two exons of TLR2 gene in healthy and diseased uterine samples. DNA extraction was performed for single nucleotide polymorphisms (SNPs) analysis of TLR2, using DNeasy Blood &Tissue kit (Qiagen) according to Manufacturer's instructions. In this investigation, primers for TLR2 gene were designed using known DNA sequences of *Bubalus bubalis* published in database with accession No. HM756161 and the web interface primer3 as shown in Table (1). The TLR2 coding sequence was amplified using five overlapped primers according to the following thermal profile: initial denaturation at 95°C for 1-3 min followed by 40 cycles of 30s at 95°C ,30s at ™ann(C) which is the annealing temperature specific for each primer in and 1 min at 72°C, with a final extension at 72°C for 1 min.PCR reactions were performed with PCR Master Mix (2X) kit (Thermo Fisher Scientific) which included 50ng of genomic DNA and 10 µM of each primer (forward and reverse) in a final

volume of 50 µl. Amplicons were purified (QIAquick PCR Purification Kit ,Qiagen) and bi-directionally sequenced by Laragen company (USA).

2.3 Genotyping and SNP selection:

CLUSTAL - W program [12] was used to analyze the forty river buffalo Egyptian breed sequences of TLR2 gene with CDs of publicly available GenBank: HM756161. A consensus sequence for each sample was generated from alignment of each forward and reverse sequence of a single DNA sample to identify polymorphisms among the samples.

2-4 Genetic association and protein analysis:

Genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) by using Fisher's Exact test, where the allelic and genotypic frequencies were carried out for each SNP independently.

"Univariate logistic regression model was performed for the SNPs that were significantly associated with the disease. Odds Ratio test (OR) was calculated with 95% confidence interval (CI)[13]. All statistical analyses were performed using R statistical programand P-value was corrected using Bonferroni method" [14]. "Haplotype reconstruction was performed using PHASE software, version 2.1"[15]. "Moreover, the potential impact on protein structure and function due to amino acid substitutionswas performed by PolyPhen-2 software (Polymorphism Phenotyping V2)with default parameters [16] and the colored figures are available at http://genetics.bwh.harvard.edu/cgi-bin/ggi/ggi2.cgi". "Protein domain has been predicted from the CD sequence by using the Conserved Domain Database available at: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)" [17].

3. RESULTS AND DISCUSSION

3.1 Bacterial results:

"Microbial infections of the genital tract cause infertility by disrupting uterine and ovarian function" [18]. Uterine lumen contamination can be categorized depending on the bacterial pathogenicity [19] which is also correlated in part with the severity of postpartum endometritis; although, the establishment and persistence of uterine infection is also influenced by thegenetic factors, uterine environmentand the animal's innate and acquired immunity. Uterine pathogens including *Escherichia coli*, *Proteus* species, *Arcanobacterium pyogenes* and

Prevotellamelaninogenicus are associated with greater endometrial inflammation and more severe clinical uterine disease [20].

Examination of the collected uterine swab samples was performed involving colony characters, cellular morphology and the purity of the culture and the suspected colonies were identified.The most highly isolated bacterium from diseased sampleswas found to be*Escherichia coli*. Other potential bacterial pathogens were also detected with different percentages including *Streptococcus pyogenes* and *S. aureus*. Opportunist contaminants like *Klebsiella spp., Micrococcus species, Proteus spp.* and *S. epidermidis* were also identified.

Several studies demonstrated that *Escherichia coli* bacteria is one of the most common isolates in endometritis cases. Furthermore, it has been widely reported that*Escherichia coli* presence was correlated with increased endometrial inflammation and more severe clinical diseases [20 and 4]. In this respect, *E. Coli* was isolated with high incidence from Egyptian buffalo with endometritis [21]. Moreover, *E. coli* (36.66%), *Klebsiella spp (30%),Proteus* (13.33%) were isolated from endometritis cases of bovine [22].

3.2 SNPs identification:

Buffalo TLRs can be used as positional markers in the genome and polymorphism reported in their genes may be related to differences in resistance and susceptibility to various bacterial infections [23]. “Single nucleotide polymorphisms (SNPs) within TLRs genes have a potential impact on the response of animals to TLR ligands which lead to the alteration of susceptibility or resistance to infectious and inflammatory diseases” [24]. “On the other hand, polymorphisms, and mutations of TLRs genes were reported to interfere with the innate immune activation due to the reduction of the protein ability to recognize PAMPs” [25].

Sequence analysis of TLR2 exons in healthy samples yielded twenty-six SNPs which were bi-allelic (two in 5` UTR, 22 in coding region and two in 3` UTR), in addition to the identification of a dinucleotide SNP at genomic positions 482 and 483 bp of coding region.Sequence analysis of TLR2 exons in diseased samples yielded nineteen SNPs which were bi-allelic (two in 5` UTR, 15 in coding region and two in 3` UTR), in addition to the identification of a dinucleotide SNP at genomic positions 482 and 483 bp of coding region.

This study focused on the mutual SNPs found in healthy and diseased samples to identify SNPs of TLR2 gene associated with endometritis occurrence in Egyptian buffalo. Nineteen novel polymorphic sites were found in both samples (two in 5` UTR, 15 in coding region and two in 3` UTR).Fourteen SNPs (73.7 %) showed transitions while five SNPs (26.3 %) showed tranversions. Seven mutual SNPs, located at the coding region were non-synonymous (nsSNPs) with five showing transitions and two transversions, while eight mutual SNPs were synonymous(sSNPs) with seven transitions and one transversions.There was a mutational bias between transitions and transversions with nsSNPsand sSNPs ($p=0.017$) by using Fisher`s two tailed exact test. The *TLR2* CD sequences generated from healthy and diseased samples were submitted to GenBank with the accession numbers KU984438 and KU984439, respectively.

3.3 Allele and Genotype frequencies of TLR2 mutual SNPs:

Genotype and allele frequency of each mutual SNP of TLR2 gene were calculated in healthy and diseased samples as the first step of genetic association study using Fisher`s exact test[26].All TLR2 mutual SNPs conformed to Hardy-Weinberg equilibrium ($P>0.05$) [27]. Three mutual SNPs were found to be statistically significant in allele and genotype frequency after applying Bonferroni correction to reduce the false positive results, one of those SNPs was located at coding region(374bp), while the other two SNPs were located at 5` UTR (90&124bp).On the other hand,there was no relationship in allele and genotype frequencies of 14,1034,1375 mutual SNPs ($P=1$).Moreover, there was no genetic association between other mutual SNPs (Table2).

3.4 Odds Ratio test analysis of statistically significant TLR2 mutual SNPs:

Only three SNPs (90bp, 124bp located at 5`UTR and 374bp at CD) showed significant P values ($p<0.01$) in allele and genotype frequency (Table 2) were subjected for Odds Ratio test analysis after logistic regression model performance to determine the association between these SNPs and endometritis occurrence in river buffalo Egyptian breed. Three mutual SNPs in TLR2 gene, associated with endometritis disease, were found to be located at 5`UTR and coding region, wherethedominant homozygous genotypes AA and CC of 90bp and 374bp SNPs had a significant P-value(0.0015) with OR value more than one (2.88), indicating a significant association with susceptibility to endometritis. Whereas OR value of recessive homozygous genotypes of those SNPs (CC &TT) were less than one (0.297) with significant P-value (0.011) revealed association of those genotypes with resistance to endometritis.On the

other hand, SNP at 124 bp showed OR value less than one (0.320) for dominant homozygous genotype (GG) with P-value (0.0002) indicating a significant association with resistance to endometritis while showed OR value more than one (3.833) with P-value (0.002) for recessive homozygous genotype (AA) indicated the significant association with susceptibility to endometritis (Table 3).

“Despite the relatively small number of samples used, the results give a rather good idea of the association between TLR2 genotypes and resistance / susceptibility to endometritis disease. Those results are in good agreement with previous results reporting that TLRs polymorphisms are associated with resistance and susceptibility to diseasesin many species” [28; 29 and 24].

“Previous studies have demonstrated the influence of polymorphisms on susceptibility to several bacterial diseases, where *TLR2* polymorphisms were found to increase the risk of infections like tuberculosis [30], urinary tract infections [31], Mastitis [32] and other disease conditions” [33]. On the other hand, the association between genetic polymorphisms and susceptibility to COVID-19 disease and severity was revealed[34]. “The relation between endometritis occurrence and genetic polymorphism of TLRs in human and cattle was previously reported” [35]. It is also to be mentioned that the polymorphism in the 5`UTR of TLRs genes and its role in diseases susceptibility was previously detected in bovine [36]. “In this respect, several polymorphisms have been found in the *TLR2* gene, with a study demonstrating theassociation between*TLR-2*and the increased type 2 diabetes(T2DM) risk, sincecarrying the D allele of the *TLR-2* del -196-174 variant may be related as a risk factor for T2DM” [37]. “Another study performed a meta-analysis suggesting that TLR2 Arg753Gln polymorphism is significantly associated with high TB risk, where in the subgroup analysis based on ethnicity it was found that TLR2 Arg753Gln polymorphism elevates the risk of TB in Asian and Caucasian populations, but not in African or mixed populations”[38].

3.5 Protein analysis of TLR2 CD of healthy and diseased samples:

Translation of nucleotide sequences (2352 bp) of TLR2 coding regions of healthy and diseased samples was carried out by Open Reading Frame software (ORF). Eight non-synonymous (nsSNPs) were detected in coding region of healthy samples which, leads to seven amino acids

substitution, while Seven non-synonymous (nsSNPs) were detected in coding region of TLR2 of diseased samples, which leads to six amino acids substitution (Table 4).

"For protein analysis, PolyPhen-2 software was used to predict the potential impact on the protein structure and function due to amino acid substitutions in TLR2 coding region of healthy and diseased samples.The majority of amino acid substitutions were detected to be benign. Three substitutions (L 5 W, T 18 M and N 345 S), which were found within *TLR2* coding region, were found to be possibly damaging and may have a potential impact on the alteration of protein structure and function" [39]. "It is to be noted that the change of polarity from polar Threonine to non-polar Methionine (T 18 M) might also affect the protein structure and function" [40]. "This effect of change in protein function related to that of amino acid polarity was also reported in cattle *TLR* SNPs"[41]. "It was also previously reported that non-synonymous SNPs that modify amino acids polarity in TLRs may affect ligand binding and recognition" [42]. "Furthermore, amino acid substitutions that alter the amino acid polarity may also have a potential impact on host immune responses and resistance to diseases" [43]. On the other hand there was no effect on protein function due to change the amino acid (583)from serine to threonine in coding region of healthy samples (Table 4).

"Tables 5A & 5B show all the SNPs in diseased and healthy samples, respectively, their positions in TLR2 CDs, the protein domain and PolyPhen-2 analysis of amino acid substitution effects of these SNPs.Four non-synonymous SNPs were detected in Leucine-rich repeats (LRR) domains of *TLR2*of diseased samples in addition to one other in healthy samples and might have a role in altering its ability to identify extracellular pathogens" [44]. "These results are in accordance with other studies, which reported that LRR domains are rich in non-synonymous SNPs in canine, human, porcine and bovine TLRs" [41], [45] and [46]. "Furthermore, polymorphisms that occur in LRR domains may cause changes in responsiveness towards pathogenic microorganisms" [47].

3.6 Haplotype prediction:

"The determination of haplotype reconstruction for several SNPs in one gene is a powerful tool to provide more information about genotype-phenotype associations than individual SNPs" [48]. PHASE v2.1 was used to detect haplotypes for TLR2 coding region SNPs of healthy and

diseased samples. Unphased diploid data was used. The best pairs for each sample were determined as well as frequencies of each determined haplotype.

It was found that 5% of healthy samples had the potential to transmit GTTACCAGCCGCACT haplotype, while 8% of diseased samples had the potential to transmit TTTACCAGCGACGTT haplotype. Moreover, there was a statistical significance (P=0.03) of haplotype frequencies of healthy and diseased samples by performing a permutation test, included in PHASE v2.1, which indicated the presence of a significant association between haplotype frequency of TLR2 gene in healthy and diseased samples with endometritis occurrence in Egyptian buffalo. This result was previously found to apply also for TLR4 gene [49]. “Haplotype reconstruction of TLRs and its association with disease occurrence has been reported in human ,cattle [50] and buffalo” [24].

4. CONCLUSION

The innate immune activation response may be interfered by the effect of polymorphisms and mutations of TLRs due to the reduction of the protein ability to recognize PAMPs. The present study describes novel polymorphic sites in the river buffalo Egyptian breed *TLR2* gene and their association with endometritis disease occurrence. We can improve our ability to understand complex diseases by studying the genetic factors involved,which may help identifying their underlying physio-pathological pathways and therefore improving our ability to understand the disease in its entirety and to determine the risk of developing it. This will pave the way to both improvement of disease resistance in herds by selective breeding and also to identification and synthesis of innovative drugs.

Ethical Approval:

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and

producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table (1): PCR primers , Annealing temperature and amplicon length of amplified bubaline TLR2

Region of TLR2 gene	Sequence 5`-3`	Annealing Temp. ™ann(C)	Size (bp)
5`UTR(Exon1)	F: GGAAATGCTTAGGTTCAAATCG R:TTACCCTGAAACACTCGGTCT	54 °C	382
CD (Exon2)	F: TGGAATTAAGCCATGATGTCAA R:TTCTGTCCAAACTCAGTGCTC	54 °C	980
CD(Exon2)	F:TCAATCCAGAACATTAGCCATCT R:GGAAATATAAAGTTCTTTGAGTTGTGG	53 °C	870
CD(Exon2)	F:GGCAGAATCGTTTGAAATCAC R:AATGGGAGAAGTCCAGCTCA	54 °C	981
CD(Exon2)	F:AGGGACATCTGCTACGATGC R:CGTCGCTAAATTCTAACTCTTTGC	55 °C	812

Table (2): Allele and genotype frequencies of TLR2 mutual SNPs

SNPs position	P value of Allelic comparison	P value of Genotypic comparison
5` UTR 90M(A/C)	0.043*	0.005*
5` UTR 124R(G/A)	0.044*	0.003*
CD 14K(T/G)	1	1
CD 53 Y(T/C)	0.36	0.88
CD 108 Y(T/C)	0.36	0.95
CD 153 R(A/G)	0.68	0.99
CD 156 Y(C/T)	0.08	0. 43
CD 374 Y(C/T)	0.043*	0.005*
CD 381 R(A/G)	0.08	0. 43
CD 482 S(G/C)	0.08	0.43
CD 483 Y(C/T)	0.08	0.43
CD 519 S(G/C)	0.68	0.98
CD 1034 S(G/C)	1	1
CD 1375 Y(C/T)	1	1
CD 1678 R(G/A)	0.68	0.97
CD 1707 Y(T/C)	0.68	0.96
CD 2064 Y(C/T)	0.078	0.19

3` UTR 158 S(G/C)	0.07	0.27
3` UTR 231 Y(C/T)	0.68	0.95

-*Statistically significant values (*P value* <0.01).

-Fisher Exact test was used because of the small number of samples.

-Bonferroni was used for p value correction.

Table (3): Odds Ratio test results of statistically significant TLR2 mutual SNPs.

SNPs	Genotype	P-value	OR	95% Cl
90M(A/C)	AA	0.0015*	2.88	1.58-5.25
	AC	0.771	0.724	0.415-1.265
	CC	0.011*	0.297	0.131-0.674
124R(G/A)	GG	0.0002*	0.320	0.176-0.583
	GA	0.202	1.439	0.823-2.517
	AA	0.002*	3.833	1.634-8.992
374 Y(C/T)	CC	0.0015*	2.88	1.58-5.25
	CT	0.771	0.724	0.415-1.265
	TT	0.011*	0.297	0.131-0.674

-*Statistically significant p values (p <0.05) and ORs.

-CL: confidence level.

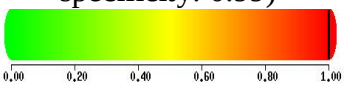
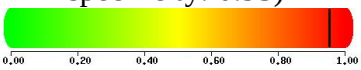
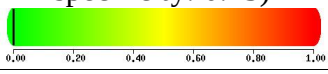
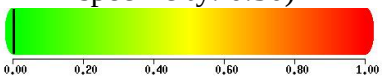
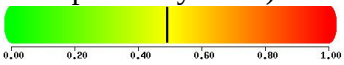
Table (4): Non-synonymous SNPs and amino acids substitution in TLR2 CDs of healthy and diseased uterine samples

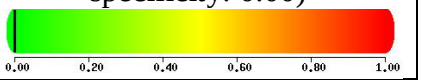
DNA sequence variants	Amino Acid position	Predicted Amino acid
14 K(T/G)	5	Leucine(L)/Tryptophan (W)
53Y(T/C)	18	Methionine(M)/Threonine(T)
374 Y(C/T)	125	Alanine(A)/Valine(V)
482 S (G/C)	161	Serine(S) /Threonine(T)
483 Y(C/T)	161	Serine(S) /Threonine(T)
1034 R(A/G)	345	Asparagine(N)/Serine(S)
1678 R(G/A)	560	Alanine(A)/Threonine(T)
1747 W (T/A)	583	Serine(S) /Threonine(T)

- Bold SNPs indicate mutual SNPs in healthy and diseased samples.

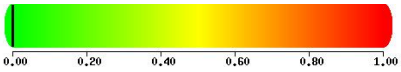
- Italic SNPs indicate SNPs in healthy samples only.

Table (5A): Detected SNPs and their effect on protein function in TLR2 CDs of diseased samples

CDs Exon	SNPs positions	Amino acid substitution	Protein domain	Amino acid substitution effect
Exon(2) CDs	14 K (T/G)	Leu (Non Polar) 5 Trp (Non Polar)	-*	This mutation is predicted to be PROBABLY DAMAGING with a score of 0.999 (sensitivity: 0.14; specificity: 0.99) 
Exon(2) CDs	53Y (T/C)	Thr (Polar) 18 Met (Non Polar)	-*	This mutation is predicted to be POSSIBLY DAMAGING with a score of 0.954 (sensitivity: 0.79; specificity: 0.95) 
Exon(2) CDs	374 Y (C/T)	Ala (Non Polar) 125 Val(Non Polar)	LRR_8 (Vs)	This mutation is predicted to be BENIGN with a score of 0.001 (sensitivity: 0.99; specificity: 0.15) 
Exon(2) CDs	482 S (G/C) 483 Y (C/T)	Ser (Polar) 161 Thr (Polar)	LRR_8 (Vs)	This mutation is predicted to be BENIGN with a score of 0.002 (sensitivity: 0.99; specificity: 0.30) 
Exon(2) CDs	1034 R (A/G)	Asn (Polar) 345 Ser (Polar)	LRR (Vs)	This mutation is predicted to be POSSIBLY DAMAGING with a score of 0.491 (sensitivity: 0.89; specificity: 0.90) 
				This mutation is predicted to

Exon(2) CDs	1678 R (G/A)	Ala (Non Polar) 560 Thr (Polar)	LRR_CT	be BENIGN with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) 

Table(5B): Polyphen-2 analysis of amino acid substitution effects in TLR2 CD of healthy samples only

CDs Exon	SNPs positions	Amino acid substitution	Protein domain	Amino acid substitution effect
Exon(2) CDs	1747 W (T/A)	Ser (Polar) 583 Thr(Polar)	LRR_CT	This mutation is predicted to be BENIGN with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) 

-SNPs positions were calculated by taking the ATG start codon as position 1 on the GenBank sequence: JN786600

- Amino acids substitutions are considered according to the ATG start codon

-* Protein position without known function