Endocrine (2011) 40:14–20
DOI 10.1007/s12020-011-9489-7

ORIGINAL ARTICLE

Mutation prediction by PolyPhen or functional assay, a detailed comparison of *CYP27B1* missense mutations

Minjing Zou · Essa Y. Baitei · Ali S. Alzahrani · Ranjit S. Parhar · Futwan A. Al-Mohanna · Brian F. Meyer · Yufei Shi

Received: 7 February 2011 / Accepted: 5 May 2011 / Published online: 21 May 2011

Abstract Vitamin D-dependent rickets type 1 (VDDR-I) is caused by mutation in *CYP27B1*. The glycine residue at codon 102 is not conserved between human (G^{102}) and rodent (S^{102}). G102E mutation results in 80% reduction in its enzymatic activity but PolyPhen predicts benign change. It is not known whether G102S has any damaging effect on 1α-hydroxylase activity. We investigated the effect of $CYP27B1^{G102S}$ on its enzymatic activity and compared mutation prediction accuracy for all known *CYP27B1* mutations among three free online protein prediction programs: PolyPhen, PolyPhen-2, and PSIPRED. G102S has no damaging effect on 1α-hydroxylase activity. G102D retained 30% enzymatic activity. All three programs correctly predicted damaging change for G102D. PolyPhen predicted benign change for G102S, whereas PolyPhen-2 and PSIPRED indicated possible damaging effect. Among 24 reported damaging mutations, PSIPRED, PolyPhen-2, and PolyPhen achieved 100%, 91.7% (22/24), and 75% (18/24) accuracy rate, respectively. The residues of incorrectly predicted mutations were not conserved. We conclude that G102D resulted in a significant reduction in 1α-hydroxylase activity, whereas G102S did not. PSIPRED and PolyPhen-2 are superior to PolyPhen in predicting damaging mutations.

Keywords *CYP27B1* mutation · 1α-hydroxylase · Vitamin D · Rickets · P450c1α

M. Zou · E. Y. Baitei · B. F. Meyer · Y. Shi (✉)
Department of Genetics (MBC-03), King Faisal Specialist Hospital and Research Centre, P.O. Box 3354, Riyadh 11211, Saudi Arabia
e-mail: yufei@kfshrc.edu.sa

A. S. Alzahrani
Department of Medicine, King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia

R. S. Parhar · F. A. Al-Mohanna
Department of Biological and Medical Research, King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia

Introduction

Vitamin D plays an important role in calcium homeostasis and exists in two major forms: ergocalciferol (vitamin D2), and cholecalciferol (vitamin D3) [1]. Both forms need two-step hydroxylation at carbons 25 and 1 for activation. The first step occurs in the liver where vitamin D is hydroxylated to 25-hydroxyvitamin D [25(OH)D] by the hepatic 25-hydroxylase [1]. At least three enzymes have 25-hydroxylase activity: mitochondrial CYP27A1 [2], microsomal CYP3A4 [3], and CYP2R1 [4]. The second step occurs mainly in the kidney where 25(OH)D is hydroxylated by the mitochondrial vitamin D1α-hydroxylase to biologically active hormone $1,25(OH)_2D$, which binds to its nuclear receptor and achieves its biological activities [1, 5, 6].

The human *CYP27B1* gene is located in chromosome 12q14 and encodes vitamin D 1α-hydroxylase [7–10]. It is around 5 Kb and composed of 9 exons and 8 introns [11]. Enzymatic deficiency of 1α-hydroxylase as a result of mutation in the *CYP27B1* gene causes vitamin D-dependent rickets type 1 (VDDR-I) [7, 12]. In our previous study, we reported a novel G102E mutation, which caused 80% reduction of 1α-hydroxylase activity [13]. However, PolyPhen program predicted a benign change, whereas PSIPRED program correctly predicted protein secondary structure change. In the present study, we created two additional mutations at the codon 102 (G102D and G102S) and investigated the correlation between mutation

prediction and the effect of the mutation on vitamin D1α-hydroxylase activity. We further compared the prediction accuracy rate for all known *CYP27B1* gene mutations among three protein structure prediction programs: PolyPhen, PolyPhen-2, and PSIPRED.

Materials and methods

Site-directed mutagenesis, cDNA cloning, and expression

The wild-type (wt) *CYP27B1* cDNA was used as a template for the creation of G102S and G102D mutants by site-directed mutagenesis [14]. The mutants were cloned into pcDNA3.1 expression vector (Invitrogen Co., CA) and stably expressed in CHO cells. The stable clones were pooled for gene expression as described previously [15]. T321R was reported to have no enzymatic activity and was used as a negative control [16].

Western blot analysis

60 μg of protein were loaded into a 12% SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed with CYP27B1 antibody (Santa Cruz Biotechnology, CA).

Analysis of 1α-hydroxylase activity

CHO cells stably transfected with wt or the different mutants were seeded in 6-well plates overnight in growth medium and incubated in 2 ml serum-free medium with 0.1 and 1 μM 25(OH)D3 (Sigma, MO) for 1 and 4 h, respectively. The concentration of $1,25(OH)_2D3$ in the medium was measured by enzymeimmunoassay (EIA) according to the manufacturer's procedure (Immunodiagnostic Systems, AZ).

Mutation prediction

Three free online protein structure prediction programs: PolyPhen [17, 18], PolyPhen-2 [19], and PSIPRED [20, 21] were used to predict mutational consequence of CYP27B1 (protein identifier # O15528). PolyPhen or PolyPhen-2 (an updated version) (http://genetics.bwh.harvard.edu/pph/) is a tool for prediction of possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations [18, 19]. For PolyPhen and PolyPhen-2, three empirically derived outcomes were used: probably damaging (it is with high confidence supposed to affect protein function or structure), possibly damaging (it is supposed to affect protein function or structure), and benign (most likely lacking any phenotypic effect). PSIPRED (http://www.psipred.net) is a simple and accurate secondary structure prediction method, incorporating two feed-forward neural networks which perform an analysis on output obtained from PSI-BLAST (Position Specific Iterated-BLAST) [20, 21]. For PSIPRED analysis, any predicted changes in protein secondary structure were considered to be damaging mutations.

Statistical analysis

Fisher's exact test was used to determine whether there is any statistical significance among different prediction programs.

Results

Partial protein alignment surrounding codon 102 of *CYP27B1* from different species

As shown in Fig. 1, the Glycine (G) residue at the position 102 is conserved across different species, even though mouse and rat have serine (S) residue at this position. The two rodents may represent an experiment of evolution. PolyPhen predicted benign change for G102S whereas PolyPhen-2 and PSIPRED indicated possible damaging effect. All the three programs predicted damaging change for G102D.

Expression of wild-type and CYP27B1 mutants in CHO cells

Both wt and different mutants were transfected into CHO cells for stable expression. Figure 2a shows that wt and mutant CYP27B1 proteins were expressed in CHO cells.

Effect of different CYP27B1 mutants on 1α-hydroxylase activity in CHO cells

1α-hydroxylase activity was measured by its ability to convert 25(OH)D3 into1, $25(OH)_2D3$ in CHO^{G102S}, CHO^{G102D}, CHO^{G102E}, CHO^{T321R}, and CHO^{wt} cells. CHO^{T321R} was used as a negative control. As shown in Fig. 2b, during 4 h incubation with 1 μM 25(OH)D3, 1, $25(OH)_2D3$ produced by CHO^{vector} cells was similar to that in culture medium (20 ± 3 fmol/10^5 cells vs. 18 ± 2), indicating that CHO cells have no 1α-hydroxylase activity (the background value was thus subtracted for calculating enzymatic activity). CHO^{WT}, CHO^{G102E}, CHO^{G102D}, and CHO^{G102S} produced 405 ± 58, 69 ± 11, 115 ± 12, 417 ± 72 fmol/10^5 cells of $1,25(OH)_2D3$ during 1 h

1	refNP_000776.1	human (homo sapiens)	...AAPALVEELLRQE G PRPERCSFSPWTE...
2	ref|XP_509175.2|	chimpanzees [Pan troglodytes]	...AAPALVEELLRQE G PRPERCSFSPWTE...
3	ref|XP_001116450.1|	rhesus monkeys (Macaca mulatta)	...AAPALVEELLRQE G PRPERCSFSPWTE...
4	ref|XP_588481.1|	cattle (Bos taurus)	...AAPTLVEQLLRQE G PRPERCSFSPWTE...
5	ref|NP_999160.1|	pig [Sus scrofa]	...AAPTLVEQLLRQE G PLPERCSFSPWTE...
6	ref|XP_538254.2|	dog (Canis lupus familiaris)	...AAPALVEQLLRQE G PRPERCSFSPWAE...
7	ref|XP_001490036.1|	horse [Equus caballus]	...AAPTLIEQLLRQE G PRPERCSFSSWAE...
8	ref|XP_001380742.1|	opossum (Monodelphis domestica)	...AAPALIEQLLRQE G PHPERCSFSPWVE...
9	dbj|BAA22434.1|	mouse (Mus musculus)	...ADPTLVEQLLRQE S HCPERCSFSSWAE...
10	ref|NP_446215.1|	rat (Rattus norvegicus)	...ADPALVEQLLRQE S HCPERCSFSSWSE...
11	ref|XP_422077.2|	red jungle fowl [Gallus gallus]	...ADRDMVAQVLRSE G RAPQRANMESWQE...
12	gb|AAH77308.1|	frog [Xenopus laevis]	...GDPEALQQLLRQE G KYPMRNKEDIWKA...
13	gb|AAH94536.1|	Xenopus tropicalis	...ASPELLETLLRQE G KYPMRTDMFMWKE...

Fig. 1 Partial protein alignment of *CYP27B1* gene from human, monkey, cattle, dog, rat, mouse, and frog around position 102. The Glycine residue at the 102 is conserved across different species, even though mouse and rat have serine (S) residue at this position. Glycine (G) is a non-polar hydrophobic amino acid whereas serine (S) is a polar neutral amino acid

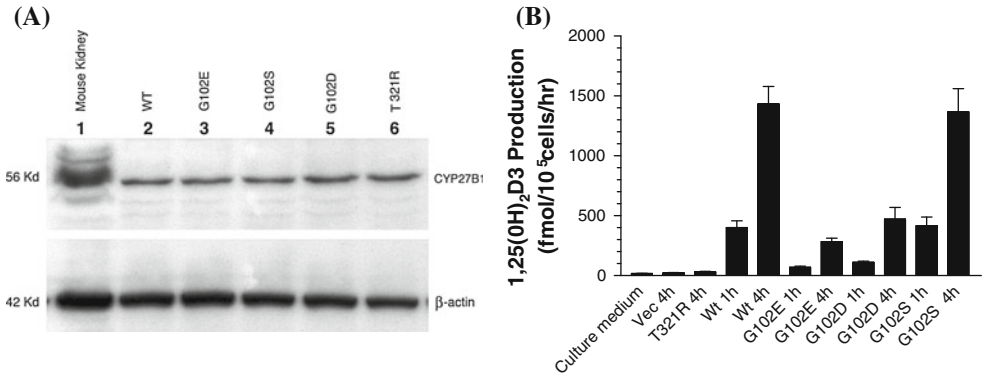

Fig. 2 CYP27B1 protein expression and 1α-hydroxylase activity. **a** Western blot analysis of wild-type and mutant CYP27B1 expression in CHO cells. The CYP27B1 protein (56 Kd) was detected by a rabbit anti-human CYP27B1 antibody in CHO^{G102D}, CHO^{G102S}, CHO^{G102E}, CHO^{T321R}, and CHO^{WT}. The membrane was re-probed with a *β*-actin (42 Kd) antibody to monitor protein loading. **b** 1α-hydroxylase activity in CHO cells expressing G102E, G102S, G102D, T321R, or wt CYP27B1 cDNA. The mutants and wt cDNAs were stably transfected into CHO cells. The cells were incubated with 1 μM 25(OH)D3 in 2 ml serum-free medium for 1 and 4 h, respectively. The concentration of 1,25 $(OH)_2$D3 in the medium was measured by EIA. Data are expressed as means ± SEM of three separate experiments

incubation, and 1432 ± 147, 282 ± 30, 472 ± 97, and 1366 ± 195 fmol/10^5 cells of 1,25$(OH)_2$D3 during 4 h incubation, respectively. T321R did not show any enzymatic activity (Fig. 2b). CHO^{G102D} lost about 70% of wt activity, whereas CHO^{G102S} maintained at least 95% enzymatic activity. These data indicate that G102S did not cause any significant change in the protein structure and function. Similar results were obtained with 0.1 μM 25(OH)D3 during 1 and 4 h incubation (data not shown).

Mutation prediction

Among 24 missense mutations known to cause reduction in1α-hydroxylase activity (Table 1), PolyPhen, PolyPhen-2, and PSIPRED correctly predicted damaging effect or protein secondary structure change in 18 (75%), 22 (91.7%), and 24 (100%) mutations, respectively. Eighteen mutations (18/24, 75%) were correctly predicted by all three programs. All misidentified mutations were located in amino acid residues not well conserved across different species (Table 1). We next analyzed 21 additional mutations in three different genes: *SRD5A2, PHEX,* and *CYP27A1*. Similar results were obtained as shown in Table 1. PolyPhen, PolyPhen-2, and PSIPRED correctly predicted damaging effect or protein secondary structure change in 15 (71.4%), 18 (85.7%), and 21(100%) mutations, respectively. There is no statistically significant difference between PolyPhen-2 and PolyPhen ($P = 0.059$, Chi-Square test), probably due to small sample size. These data suggest that PolyPhen-2 and PSIPRED are more accurate than PolyPhen in mutation prediction, especially in predicting mutations located in non-conserved residues. There are more structural changes in G102E and G102D as compared to G102S (Fig. 3). Interestingly, PSIPRED predicted structural change for G102S at two locations: 9 amino acid α-helix (residues 133–141, equivalent to α-helix C [22]) was shortened by one amino acid and C-terminal 19 amino acid α-helix (residues 456–474) was increased by one amino acid. The change is identical to that caused by G125E (Fig. 3).

Table 1 Mutation prediction by PolyPhen and PSIPRED for missense mutations

Mutation	Residue conservation	PolyPhen	PolyPhen-2	PSIPRED (protein secondary structure change)	Enzymatic activity (% of wt)	Reference
CYP27B1^{Q65H}	Not conserved	Benign	Probably damaging	Yes	0	[27]
CYP27B1^{G102E}	Conserved	Benign	Possibly damaging	Yes	20	[13]
CYP27B1^{G102D}	Conserved	Probably damaging	Possibly damaging	Yes	33	Current study
CYP27B1^{G102S}	Conserved	Benign	Possibly damaging	Yes	95	Current study
CYP27B1^{R107H}	Conserved	Probably damaging	Probably damaging	Yes	0	[12]
CYP27B1^{P112L}	Not conserved	Benign	Benign	Yes	0[a]	[25]
CYP27B1^{G125E}	Conserved	Probably damaging	Probably damaging	Yes	0	[12]
CYP27B1^{P143L}	Not conserved	Possibly damaging	Possibly damaging	Yes	0	[16]
CYP27B1^{D164N}	Conserved	Probably damaging	Probably damaging	Yes	0	[16]
CYP27B1^{E189G}	Not conserved	Probably damaging	Probably damaging	Yes	22	[33]
CYP27B1^{E189K}	Not conserved	Benign	Possibly damaging	Yes	11	[33]
CYP27B1^{E189L}	Not conserved	Benign	Possibly damaging	Yes	1.6	[27]
CYP27B1^{T321R}	Conserved	Probably damaging	Probably damaging	Yes	0	[16]
CYP27B1^{S323Y}	Not conserved	Probably damaging	Probably damaging	Yes	0	[34]
CYP27B1^{R335P}	Not conserved	Probably damaging	Probably damaging	Yes	0	[12]
CYP27B1^{L343F}	Not conserved	Possibly damaging	Probably damaging	Yes	2.3	[33]
CYP27B1^{P382S}	Conserved	Probably damaging	Probably damaging	Yes	0	[12]
CYP27B1^{R389C}	Conserved	Probably damaging	Probably damaging	Yes	0	[16]
CYP27B1^{R389G}	Conserved	Probably damaging	Probably damaging	Yes	0	[33]
CYP27B1^{R389H}	Conserved	Probably damaging	Probably damaging	Yes	0	[27]
CYP27B1^{T409I}	Not conserved	Benign	Benign	Yes	0	[27]
CYP27B1^{R429P}	Not conserved	Possibly damaging	Possibly damaging	Yes	0	[27]
CYP27B1^{R453C}	Conserved	Probably damaging	Probably damaging	Yes	0	[27]
CYP27B1^{V478G}	Not conserved	Probably damaging	Probably damaging	Yes	0	[34]
CYP27B1^{P497R}	Conserved	Probably damaging	Probably damaging	Yes	0	[27]
SRD5A2^{L55Q}	Not conserved	Probably damaging	Probably damaging	Yes	[b]	[35]
SRD5A2^{G85D}	Not conserved	Possibly damaging	Possibly damaging	Yes	[b]	[36]
SRD5A2^{Q126R}	Conserved	Probably damaging	Probably damaging	Yes	[b]	[35]
SRD5A2^{G158R}	Not conserved	Possibly damaging	Benign	Yes	[b]	[35]
SRD5A2^{G183S}	Conserved	Possibly damaging	Probably damaging	Yes	[b]	[35]
SRD5A2^{G196S}	Conserved	Probably damaging	Probably damaging	Yes	[b]	[35]
SRD5A2^{A207D}	Not conserved	Probably damaging	Possibly damaging	Yes	[b]	[35]
SRD5A2^{S245Y}	Not conserved	Benign	Possibly damaging	Yes	[b]	[36]
SRD5A2^{R246W}	Conserved	Probably damaging	Probably damaging	Yes	[b]	[35]
PHEX^{Y317F}	Not conserved	Benign	Benign	Yes	[b]	[37]
PHEX^{P534L}	Conserved	Probably damaging	Probably damaging	Yes	[b]	[37]
PHEX^{G579R}	Conserved	Probably damaging	Probably damaging	Yes	[b]	[37]
PHEX^{Q621R}	Conserved	Probably damaging	Probably damaging	Yes	[b]	[37]
PHEX^{A720T}	Not conserved	Benign	Possibly damaging	Yes	[b]	[37]
PHEX^{F731Y}	Conserved	Benign	Probably damaging	Yes	[b]	[37]
PHEX^{W749R}	Conserved	Probably damaging	Probably damaging	Yes	[b]	[37]
CYP27A1^{R127W}	Conserved	Probably damaging	Probably damaging	Yes	0	[38]
CYP27A1^{G145R}	Conserved	Probably damaging	Probably damaging	Yes	0	[38]
CYP27A1^{A216P}	Not conserved	Benign	Probably damaging	Yes	0	[38]

Table 1 continued

Mutation	Residue conservation	PolyPhen	PolyPhen-2	PSIPRED (protein secondary structure change)	Enzymatic activity (% of wt)	Reference
CYP27A1^{K259R}	Not conserved	Benign	Benign	Yes	0	[38]
CYP27A1^{T339M}	Conserved	Probably damaging	Probably damaging	Yes	0	[38]

[a] Functional assay was not performed but patient had compound heterozygous mutations of P112L and R389H and severe rickets with seizures. The enzymatic activity is predicted to be severely compromised

[b] Functional assay was not performed but are disease-causing mutations

Defects in *CYP27A1* cause cerebrotendinous xanthomatosis, a rare autosomal recessive lipid storage disease due to steroid 27-hydroxylase deficiency

Defects in SRD5A2 (protein identifier #: P31213) cause pseudovaginal perineoscrotal hypospadias also known as male pseudohermaphroditism due to 5-alpha-reductase deficiency

Defects in PHEX (protein identifier #: P78562) cause X-linked hypophosphatemic rickets due to phosphate-regulating neutral endopeptidase deficiency

Discussion

Both human and mouse CYP27B1 share significant protein sequence homology [7, 9, 23, 24]. Among 24 damaging missense mutations found in human *CYP27B1* [25], 19 occurred in amino acid residues common to both human and mouse, suggesting that these residues are important for enzymatic activity. However, there are five mutations located in residues that are not conserved: P112 in human versus S112 in mouse, G102 versus S102, T409 versus S408 (the mouse is one amino acid short as compared to human), and R429 versus N428. The changes from hydrophobic amino acids proline (P) or glycine (G) to neutral amino acid serine (S), neutral amino acid threonine (T) to serine, and basic hydrophilic amino acid arginine (R) to neutral amino acid asparagine (N) may not significantly affect protein structure and function. The current study has confirmed that the substitution of hydrophobic amino acid glycine (G) to neutral amino acid serine (S) at codon 102 would not cause any damaging effect, whereas the substitution of G to either hydrophilic amino acid aspartic acid (D) or glutamic acid (E) would result in detrimental effect on vitamin D 1α-hydroxylase activity. S408 of mouse CYP27B1 (corresponding to T409 of human CYP27B1) is involved in substrate binding by forming a hydrogen bond with 25-hydroxyl group of 25(OH)D3 [26]. It has been demonstrated that S408T has least effect on vitamin D 1α hydroxylase activity among S408T, S408A, S408V, and S408I mutants; and S408I has the most detrimental effect [26]. The human T409I, which cause VDDR-I, has no1α-hydroxylase activity [27]. Human T321 is involved in oxygen activation [22]. T321S shows a 1α-hydroxylase activity similar to wt. However, T321N, T321A, and T321R exhibit no activity [28] even though threonine (T) and asparagine (N) are both polar neutral amino acids. Structurally, the threonine ($C_4H_9NO_3$, side group –CH (CH_3)–OH) is more resemble to S ($C_3N_7NO_3$, –CH_2–OH) than N ($C_4H_8N_2O_3$, –CH_2–$CONH_2$), which may explain no damaging effect for T321S substitution. Among 21 randomly selected mutations in *SRD5A2, PHEX,* and *CYP27A1*, G to S substitution is only present in *SRD5A2*^{G183S} and ^{G196S} (Table 1), which causes pseudovaginal perineoscrotal hypospadias (male pseudohermaphroditism) due to 5-alpha-reductase deficiency. G183 and G196 residues are highly conserved across different species. PSIPRED shows multiple protein secondary structure changes caused by G183S and G196S (data not shown). Therefore, the overall structural and functional context unique to each mutation should be considered to predict function.

PolyPhen mispredicted six mutations (Q65H, G102E, P112L, E189K, E189L, and T409I) and PolyPhen-2 missed two mutations (P112L, T409I). These amino acid residues are not conserved (sharing low amino acid sequence identity) among mitochondrial p450 enzymes or across different species but show remarkable conservation of their topology and their tertiary structures [29]. Q65 is in α-helix A′ (αA′) and T409 is in strand 3 of β-sheet 1 (β1-3). Both of them are involved in substrate binding [26]. G102 and R107 are very close to strand 5 of β-sheet 1 (β1-5). R107 has been described as a heme binding residue [30]. Homology modeling of human *CYP27B1* based on the crystal structure of rabbit *CYP2C5* suggests that R107H could disrupt protein folding [31]. G102E or G102D may disrupt heme incorporation or folding. P112 is located in the substrate recognition site 1 (SRS1) [26, 32] and is very close to α-helix B′ (αB′). E189 lies in the α-helix E (αE′). The mutants P112L, E189K, and E189L would disrupt protein folding as well. Given that all these residues are not well conserved and their mutations result in either substrate

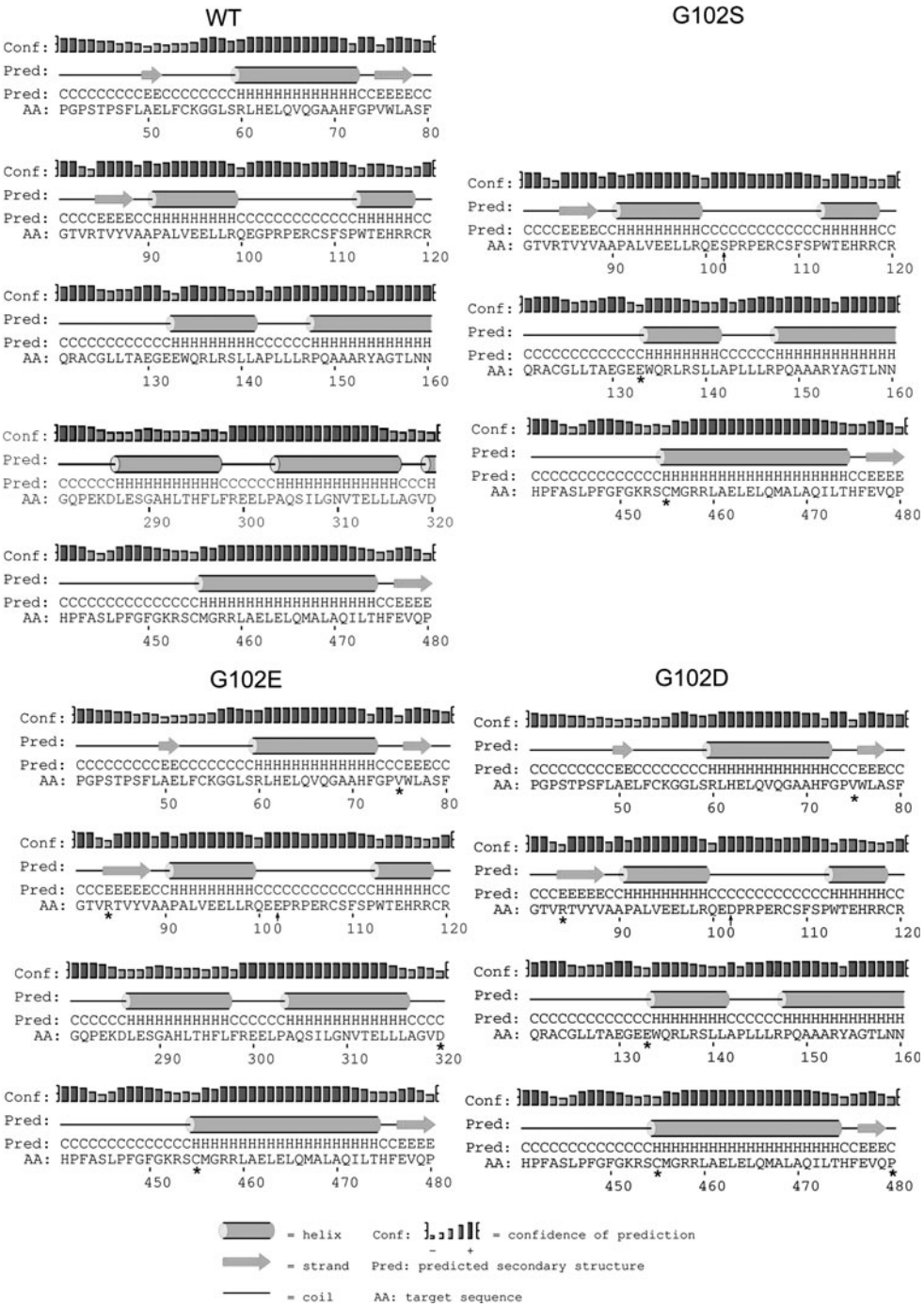

Fig. 3 Protein secondary structure prediction by PSIPRED. The predicted protein structural changes caused by G102S, G102E, and G102D are presented. The location of the changes is indicated by *asteisk*. The amino acids are indicated by the *single letter code*, and the location of the missense mutation is indicated by an *arrow*. *C* coil, *H* α-helix, *E* β-sheet

binding or folding defects, PolyPhen or PolyPhen-2 may have a higher rate of inaccuracy for prediction of non-conserved residues.

PSIPRED achieved the highest accuracy among three programs. The drawback for PSIPRED is that the protein structure change caused by a mutation is not highlighted by the program and one has to manually compare mutant structure prediction with that of wild-type. Subtle changes may be missed. For example, E189K and E189L caused only one subtle change: 4 amino acid β-strand (residues 477–480) was shortened by one amino acid in E189K and 19 amino acid α-helix was increased by one amino acid in E189L (residues 456–474); T321R resulted in two subtle changes: N-terminal α-helix (residues 2–32) was shortened by one amino acid and C-terminal α-helix (residues 456–474) was increased by one amino acid. However, the

predicted subtle changes may not reflect the real change in protein structure or function and should be used only as a guide for prediction of damaging mutations.

Acknowledgments This study was funded by a grant from National Comprehensive Plan for Science & Technology.

Conflicts of interest All authors have nothing to disclose.

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