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Research Article

Functional Characterization of a Novel Variant of the Constitutive Androstane Receptor (CAR, NR1I3)

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Abstract. The nuclear receptor constitutive androstane receptor (CAR; NR1I3) controls the inducible expression of many enzymes and transporters involved in drug metabolism and transport, energy metabolism and toxicity. Single nucleotide variants of CAR are quite rare and usually associated with changes in pharmacokinetics of therapeutic drugs. Recently, a non-synonymous variant (F243S in the wild-type CAR) has been linked to the Kleefstra syndrome (MIM 610253) affecting neurological development. We identified another, previously unknown CAR variant (I281T) in a patient suffering from Kleefstra-like symptoms. Detailed reporter gene assays and molecular modelling indicated that the I281T mutation decreases the ability of CAR to recruit co-activators, likely by interfering with the assembly of functional CAR/retinoid X receptor (RXR) heterodimers. Although the I281T variant does not seem to cause the features of the patient, the present study adds to our knowledge about CAR function.

Keywords: NR1I3, single nucleotide variant, Kleefstra syndrome, co-activator, recruitment

1. Introduction

The constitutive androstane receptor CAR (NR1I3) is a ligand-activated transcription factor that regulates the inducible expression of many enzymes and transporters involved in drug metabolism and transport [1–3]. In addition to its crucial role in defence against xenobiotics, CAR is also modulating chemically-induced carcinogenesis [4, 5] and glucose metabolism [6, 7]. CAR can be activated either indirectly via signalling pathways involving *e.g.* epidermal growth factor and insulin receptors [8, 9], extracellular signal-regulated kinase [10] and

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protein phosphatase 2A [11], or by direct ligand binding [1, 12, 13]. These signals dephospho-rylate cytoplasmic CAR and translocate it into the nucleus, eliciting binding of CAR/retinoid X receptor-α (CAR/RXRα) heterodimer to its cognate DNA elements, followed by recruitment of NR coactivators that culminates in transcriptional activation of CAR target genes [14, 15].

CAR has many splicing variants that encode truncated and inactive proteins [16-18]. The abundantly expressed isoform CAR1 (wild-type CAR; 348 residues; Uniprot variant Q14994-2) has been best characterized. It is transcriptionally more active and responsive to more ligands than the two other relatively well-expressed isoforms CAR2 (352 residues; Uniprot reference sequence Q14994; insertion of SPTV after V231 in CAR1) and CAR3 (357 residues; Uniprot variant Q14994-8; additional insertion of APYLT after P270 in CAR1) [16-19]. In contrast, non-synonymous single nucleotide variations (SNV) in CAR are quite rare [20, 21], much below 0.1% allele frequency in the ExAC database [22, 23]. Information on the phenotypic effects of these SNVs is limited. Cell-based assays have identified two very rare non-synonymous SNVs in the ligand-binding domain (LBD) that decrease constitutional or ligand-elicited transcriptional activity [3, 20]. These mutations (H246R, L308P in wild-type CAR) are not present in ExAC. More common intronic SNVs (*e.g.* *rs*3003596; *rs*2501873; *rs*4073054) or synonymous SNVs (e.g. Pro180Pro; *rs*2307424) are associated with changes in efavirenz pharmacokinetics and side effects [24–26], outcome of sunitinib therapy [27] or warfarin dosages [28], presumably via an effect on CAR expression and subsequent changes in levels of enzymes metabolizing these drugs.

Recently, a novel variation in CAR [29] has been associated with the developmental Kleef-stra syndrome (MIM 610253), which is distinguished in early childhood by intellectual dis-ability, hypotonia and facial abnormalities [30]. In one of the affected patients (KS220), a *de novo* mutation of the *NR1I3* gene (NM_001077482.2; c.740T>C) was identified by next-generation sequencing [29]. This variant codes for F247S in the ExAC reference sequence (*i.e.*, the longest CAR3 isoform) and corresponds to F243S in the wild-type CAR1 [19, 20]. The authors suggested that CAR could contribute to the Kleefstra syndrome due to its interaction with chromatin-modifying complexes including the histone methyltransferase EHMT. They supported this view by detecting genetic interactions between EHMT and the ecdysone receptor (EcR), a candidate for a human CAR homolog, in a *Drosophila* model [29]. Here, we identi-fied another novel non-synonymous CAR variant in a patient with Kleefstra-like symptoms by exome sequencing and characterized the effects of this SNV on CAR function in cell-based assays.

2. Materials and Methods

2.1. Clinical description

The proband is a first-born male out of three children to non-consanguineous Jewish Moroc-can parents. The family history is negative for similarly affected children. The pregnancy and delivery were normal but in the first few weeks of life, irritability and feeding problems were noted. The development was slow and behavioral problems persisted. At age 13 years, the patient does not speak or walk independently, he is not toilet-trained and persistent self-mutilation and physical aggression prevents any meaningful efforts in habilitation. The eyesight and hearing are

normal except for strabismus. No syndromatic stigmata or focal neurological deficits are present on examination. The patient is otherwise healthy except for recurrent vomiting that ceased after fundoplication. Numerous investigations including brain MRI, plasma levels of acylcarnitines, very long chain fatty acids, amino acids, uric acid, urinary organic acids, isoelectric focusing of transferrin, biopsy of the sural nerve and video-EEG have all yielded normal results. During the newborn period, elevated levels of serum alkaline phosphatase up to 4777 IU (normal range 40 – 200 IU) were present for several months before normalizing. Informed consent was granted by the parents, and experiments involving biological material of the patient were approved by the Hadassah Institutional Review Board (Hebrew University) and the Israeli Ministry of Health.

2.2. Exome analysis

Exome analysis was performed at a CLIA-certified laboratory using the HiSeq 2000 machine (Illumina, San Diego, CA) after enrichment up to 50 MB using the Agilent V4 platform (Agilent, Santa Clara, CA). In total, 53.5 million pair-ended reads were produced. Following alignment of the reads to the human genome (GRCh37 *Hg19*) and variant calling, we filtered the variants according to their frequency and predicted pathogenicity using MutationTaster2 [31]. Two hundred and thirty-nine variants remained that had minor allele frequency <0.5% in the ExAC database [22] and <4% in the Hadassah in-house database at the Hebrew University and were not predicted benign. Two of these were a hemizygous non-synonymous SNV for gene *TENM1* (chrX:g.123518249G>A, NM_001163278.1:c.6532C>T, p.R2178C) and a homozygous non-synonymous SNV for gene *NR1I3* (chr1:g.161200678A>G, NM_001077482.2 c.869T>C, p.I290T; corresponding to I281T in the wild-type CAR1 isoform). We then noted an individual hemizygous for this very rare *TENM1* variant in the ExAC database with an estimated allele frequency of 6.6×10^{-6} and with no homozygotes detected among more than 60, 000 individuals. Eight individuals were heterozygous for the *NR1I3* variant but no homozygotes were present in the ExAC database. In view of this and the previously identified *NR1I3* gene variant in the Kleefstra syndrome [29], we excluded the *TENM1* variant as a disease-causing SNV and focused on characterization of the CAR I281T variant.

2.3. Chemicals

The CAR agonist 6-(4-chlorophenyl)imidazo[2,1-*b*,1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) [32] was from Sigma (St. Louis, MO). Other CAR agonists FL81 and clotrimazole (CLOTR) have been previously reported [33]. Synthesis and characterization of the CAR inverse agonist 1-[(2-methyl-benzofuran-3-yl)methyl]-3-(thiophen-2-ylmethyl)urea (S07662) has been described [34]. All other chemicals were at least of analytical grade from major vendors.

2.4. CAR expression vectors and mutagenesis

The full-length human CAR and GAL4-driven CAR ligand-binding domain (CAR LBD) constructs have been described [35]. Mutation of the CAR residue isoleucine-281 to threonine

(I281T) was done with the QuickChange kit (Stratagene, La Jolla, CA) and verified by dideoxy sequencing as before [19].

2.5. CAR co-transfection and reporter assays

C3A hepatocellular carcinoma cells (ATCC CRL-10741) were seeded on 48-well plates (183 000 cells/cm^2) and transfected with pCMVβ (900 ng/well), expression vector alone or with wild-type or I281T CAR (100 ng/well) and human PBREM-tk-luc reporter [35] (450 ng/well) using the calcium phosphate method essentially as described [36]. After transfection for 4 hours, medium was removed and fresh DMEM complemented with 5% delipidated serum (HyClone, Logan, UT) and including either vehicle control (DMSO, 0.1% v/v) or CAR ligands (final concentrations 1 μM CITCO, 10 μM S07662, 10 μM FL81 or 4 μM CLOTR) was added to wells. After ligand exposure for 24 hours, the cells were lysed and assayed for luciferase (LUC) and β-galactosidase activities as before [36].

2.6. Mammalian 1-hybrid assay

C3A cells were seeded as above and transfected with wild-type or I281T GAL4-CAR LBD construct (450 ng/well) together with GAL4-responsive UAS4-tk-luciferase reporter (300 ng/well) and control pCMVβ plasmid (600 ng/well). Transfected cells were exposed to ligands and assayed for reporter activities as described above.

2.7. Mammalian 2-hybrid assay

The expression vectors for human SRC1 and NCoR nuclear receptor interaction domains [37] have been described. The human GAL4-RXRα construct was cloned as before [35]. The wild-type or I281T VP16-CAR LBD constructs (250 ng/well) and one of the interaction partners (SRC1, NCoR or RXRα; 250 ng/well) were co-transfected with the luciferase reporter pG5-luc (Promega, Madison, WI; 300 ng/well) and control pCMVβ (600 ng/well) as reported [33], treated with DMSO, CITCO or S07662 and assayed as described above.

2.8. Molecular modeling

The human RXRα-CAR LBD complex (1XVP) with agonist CITCO [38] was used as the template. The two threonine rotamers at the position of 281 were generated using the Mutagenesis module in PyMOL (The PyMOL Molecular Graphics System, Version 1.8.0.3 Schrödinger, LLC, New York, NY). The generated structures were imported into Maestro Small-Molecule Drug Discovery Suite (Schrödinger Release 2015-1: Maestro, Schrödinger, LLC, New York, NY). The structures were preprocessed using the Protein preparation wizard module. After minimization, the structures were visualised and analysed in PyMOL.

2.9. Western blotting

C3A cells were transfected with equal amounts of wild-type and I281T CAR expression vectors on duplicate 35 mm dishes. After 24 hours, whole-cell extracts were prepared as described [39]. Protein samples (50 μg) were separated on 4-20% SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred onto PVDF membranes. Mouse antibodies to human CAR (PP-N4111-00; Perseus Proteomics, Tokyo, Japan) and α-tubulin (loading control; sc-23948; Santa Cruz Biotechnology, Dallas, TX) were used at 1:1000 dilution to assess the protein expression levels as described before [39].

2.10. Statistics

The data presented are means $\pm$ SEM of two or three independent experiments, each performed in triplicate. Student's t-test with appropriate Bonferroni's correction for conservative group comparisons (nominal p value for statistical significance = 0.05/N of groups) was employed.

3. Results

The full-length human CAR had high basal activity of the PBREM enhancer-driven reporter (Figure 1A) which was more than 30-fold over the empty vector, as expected [36, 40]. In contrast, the I281T mutation caused a statistically significant decrease (by 45–78%) in reporter activity in three independent experiments. The constructs containing only the CAR LBD region (Figure 1B) showed a similar 47–72% decrease in GAL4-responsive reporter activity by the I281T mutation. This indicates that the I281T mutation may affect binding of either ligands or proteins interacting with the LBD. In other experiments, co-transfection of the PGC1α coactivator cDNA enhanced the PBREM reporter activity only by 25–40% for both wild-type and mutant CAR (Figure 1C). As often found with full-length CAR [3, 37], addition of agonist CITCO did not further enhance the basal activity of either receptor (Figure 1D). The inverse agonist S07662 attenuated LUC activity to similar levels, with a decrease of ∼90% for the wild-type CAR and ∼60% for the I281T mutant. These findings suggest that decreased I281T activity was not caused by insufficient availability of cellular coactivators.

Responses to CAR ligands can be efficiently analyzed using the mammalian 1-hybrid assay, which employs a fusion protein between the yeast GAL4 DNA-binding domain and CAR LBD to activate a GAL4-responsive reporter [19]. Figure 2 indicates that the reporter activity driven by wild-type CAR LBD increased 2.5- to 6-fold by agonists and decreased to 30% by the inverse agonist S07662.

For the I281T mutant, the lower basal activity increased almost 9-fold by CITCO but only ∼40–80% by FL81 or CLOTR while exposure to S07662 did not change the reporter activity. These results suggest that the I281T mutant can still respond to potent CAR agonists such as CITCO but dampened transcriptional activation with weaker agonists.

One potential cause for the reduced activity is impaired interaction of CAR LBD with its interactors such as coactivators such as SRC1, corepressors such as NCoR, or the heterodimerization partner RXRα. We tested these options with our established mammalian 2-hybrid assay [33] where agonist binding can recruit coactivators while inverse agonists/antagonists attract
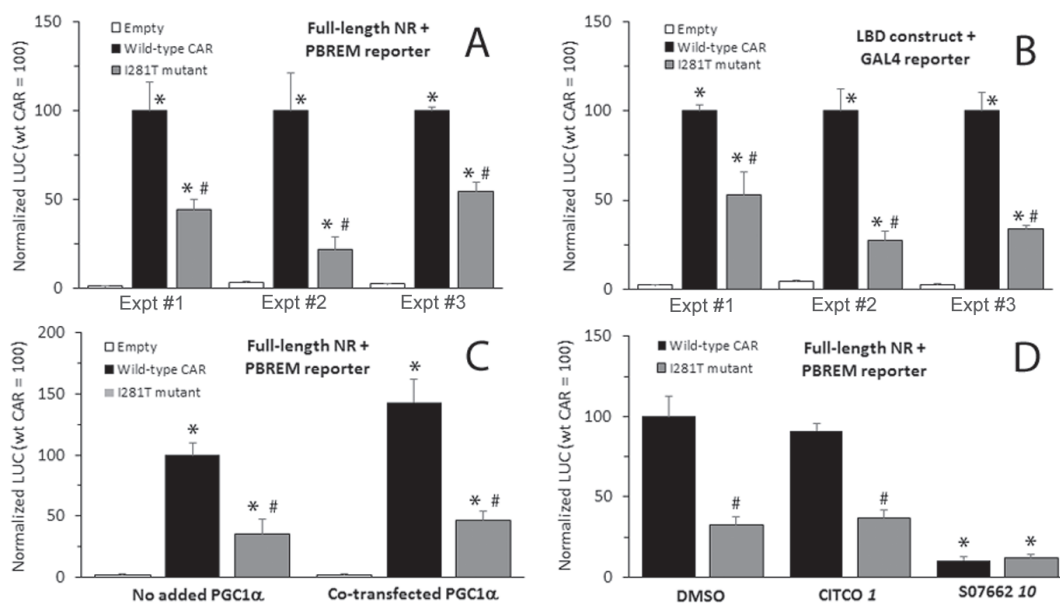
Figure 1: The effect of I281T mutation on CAR activity. (**A**) Activation of PBREM-driven reporter by empty vector (*white columns*), wild-type CAR (*black columns*) or I281T mutant (*grey columns*) full-length receptors. (**B**) Activation of GAL4-driven reporter by empty vector (*white*), wild-type CAR (*black*) or I281T mutant (*grey*) LBD constructs. (**C**) Activation of PBREM-driven reporter by empty vector (*white*), wild-type CAR (*black*) or I281T mutant (*grey*) full-length receptors in the absence or presence of co-transfected coactivator PGC1α. (**D**) Activation of PBREM-driven reporter wild-type CAR (*black*) or I281T mutant (*grey*) full-length receptors after a 24-hour exposure to either vehicle DMSO, agonist CITCO (1 μM) or inverse agonist S07662 (10 μM). The data shown in A - D are means $\pm$ SEM from three independent experiments, each with three biological replicates and the results were normalized to the activity of wild-type CAR (set at 100). A and B shows each independent experiment (Expt #1 - #3) while C and D show combined results. * denotes a statistically significant difference (p < 0.017) to empty vector (A – C) or wild-type CAR exposed to DMSO (D). # denotes a statistically significant difference (p < 0.017) to wild-type CAR (A – D).
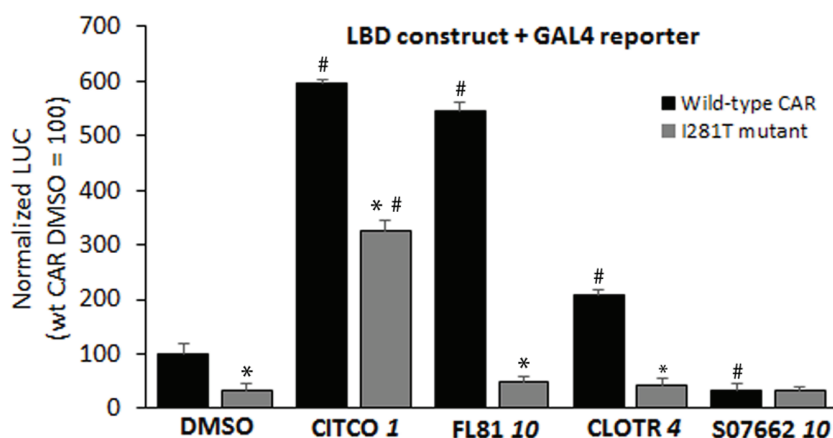

Figure 2: The response of I281T mutant to CAR ligands. Activation of GAL4-driven reporter gene by the wild-type CAR (*black columns*) and I281T mutant (*grey columns*) LBD constructs after a 24-hour exposure to either DMSO vehicle, CAR agonists CITCO (1 μM), FL81 (10 μM), clotrimazole (CLOTR, 4 μM) or the inverse agonist S07662 (10 μM). The results were normalized to the activity of wild-type CAR in the presence of DMSO vehicle (set at 100). The data shown are means $\pm$ SEM of nine replicates except for FL81 (N = 6). The symbols * and # indicate a statistically significant difference (p < 0.01) to wild-type CAR or to the relevant DMSO control, respectively.

corepressors to the NR LBD [41]. The agonist CITCO significantly increased the association of SRC1 with the wild-type CAR (3.5-fold) but much less with the I281T mutant (1.5-fold)
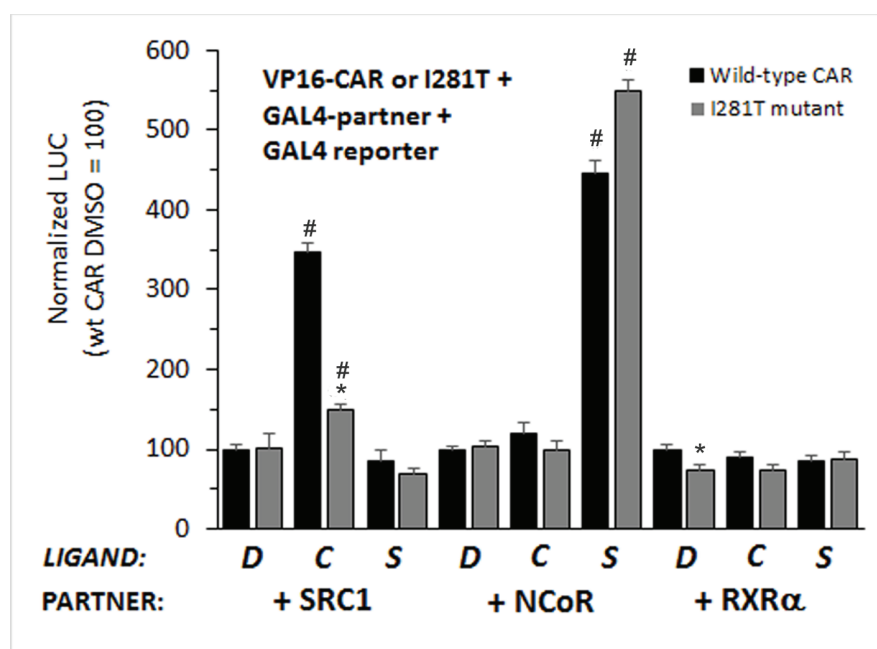
Figure 3: **The effect of I281T mutation on interactions with CAR partners.** Activation of the mammalian two-hybrid GAL4 reporter by wild-type CAR (*black columns*) and I281T mutant (*grey columns*) co-transfected with fusion proteins of either NR coactivator **SRC1**, NR corepressor **NCoR** or the heterodimer partner **RXRα** and after exposure to either vehicle DMSO (**D**), 1 μM CITCO (**C**) or 10 μM S07662 (**S**). The results were normalized to the activity of wild-type CAR in the presence of DMSO (set at 100). The data shown are means ± SEM of six biological replicates. The symbols * and # indicate a statistically significant difference (p < 0.017) to wild-type CAR or to the relevant DMSO control, respectively.

(Figure 3, *left*). The inverse agonist S07662 enhanced the interaction of NCoR with wild-type and mutant CAR to similar extent (4.5- and 5.5-fold, respectively) (Figure 3, *middle*). Finally, the I281T mutation reduced CAR/RXRα heterodimerization modestly but significantly by 25% (Figure 3, *right*).

Collectively, our findings show that the I281T mutation causes reduced activation of CAR, which is due to insufficient recruitment of coactivators to the LBD. However, this mutation is not located on helices H3, H4 and H12 that form the binding cleft for coactivators such as SRC1 in all NRs including CAR [19, 38]. Our molecular modelling studies (Figure 4) indicate that the residue 281 is not lining the ligand-binding pocket either but closer to the surface of the LBD and oriented outward from the ligand-binding cavity.

The residue I281 is located in the N-terminal part of the helix H9, probably influencing CAR residues that make hydrophobic contacts with the RXRα. The decrease in transcriptional activity is not due to poor stability of the CAR protein, because the expression of wild-type and I281T mutant CAR was comparable (Figure 5). In support of similar expression, responses of both wild-type and I281T mutant CAR to the inverse agonist S07662 in the mammalian 2-hybrid assay (Figure 3) were similar, and in small-scale bacterial expression systems, no major differences in the yield of receptor proteins were seen (F.M., *unpublished data*).
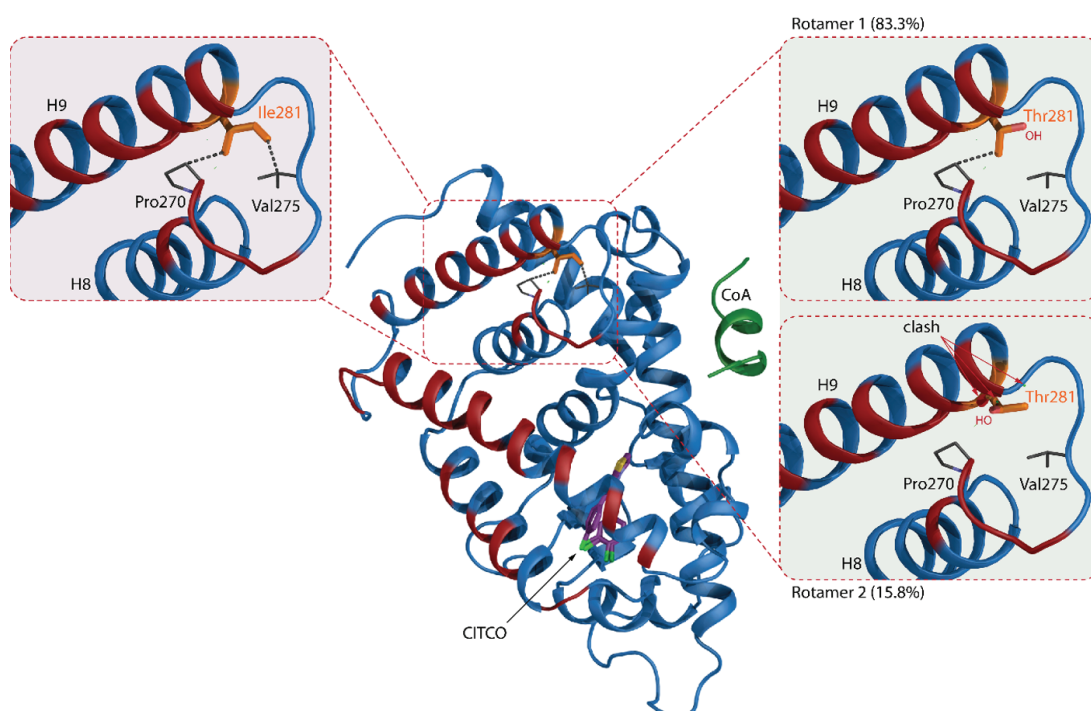
Figure 4: **Molecular modelling of the CAR I281T mutation.** The residue 281 (*orange*) is located in the N-terminal part of helix H9 pointing to the loop between helices H8 and H9. I281 is separated by >17Å from the SRC1 coactivator peptide (CoA; *green*) and >22Å from the agonist CITCO, thus having no direct involvement in coactivator or ligand binding. The N-terminal section of the loop forms a part of the heterodimerization interface (*red*) with RXRα. Although I281 does not interact directly with RXRα, it stabilizes the loop through hydrophobic interactions (*dashed lines*) with P270 and V275 (*grey*). The I281T mutation introduces a shorter hydrophilic residue, which may disrupt the interaction with V275 in the first rotamer (*rotamer probabilities shown in percentages*), or disabling both hydrophobic interactions in the second rotamer. The latter creates a possible clash with the backbone of R278 that destabilizes the N-terminal part of H9. Both possibilities are likely to produce an allosteric effect that may decrease heterodimerization of CAR with RXRα.
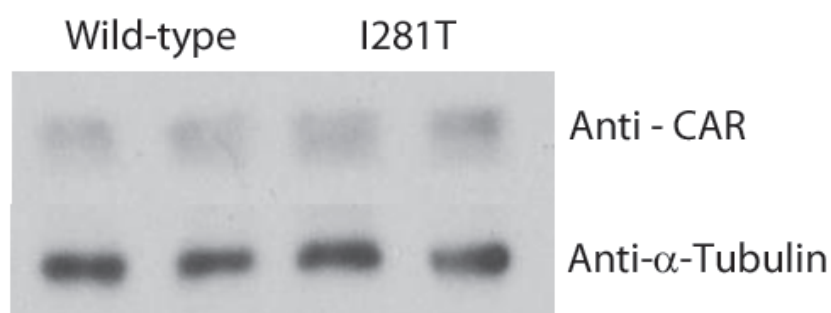

Figure 5: **The I281T mutation does not affect protein expression.** C3A cells were transfected with either wild-type of I281T CAR mutant expression vectors, protein extracts were prepared in duplicate and 50-μg aliquots analyzed by Western blotting using anti-CAR and anti-α-tubulin primary antibodies.

4. Discussion

Based on the findings presented, the basal transcriptional activity of the CAR I281T variant is decreased due to its reduced interactions with NR coactivators, and addition of a CAR-interacting coactivator PGC1α did not restore the activity. Studies with CAR LBD constructs

indicated that the mutant receptor was still activated by CAR agonists to a variable degree but fully responsive to the inverse agonist S07662. This was consistent with the protein interaction experiments where the I281T variant showed reduced interaction with the coactivator SRC1 but recruitment of the corepressor NCoR was comparable to that of the wild-type CAR. Based on molecular modeling, the residue I281 does not map to the ligand-binding pocket or to the established interaction surface for coactivators. Instead, it is located near the site for the common NR heterodimerization partner RXRα. Because optimal RXRα binding is essential for the activation of CAR target genes, it is possible that the reduced transcriptional activity of the I281T mutant is caused by defective RXRα interaction. Indeed, mammalian two-hybrid assays showed a modest decrease in RXRα recruitment.

The hypothesis of reduced CAR I281T interaction with RXRα gains further support from properties of the CAR3 splicing isoform. This variant contains an in-frame insertion of five residues (APYLT) just after P270, a residue involved in stabilizing the binding surface for RXRα. The five-residue insertion extends the loop between helices H8 and H9, pushing the other loop-stabilizing residue V275 farther and likely interfering with CAR-RXRα interaction. Indeed, this CAR3 isoform shows negligible binding to CAR DNA response elements and very weak *trans*-activation of CAR-driven reporter genes [17, 18].

After these functional studies had been conducted, repeated exome analysis of the patient revealed a genomic 28-bp deletion in the gene *KIAA2022* (Chr X:73961458-73961485del, NM_001008537: c.2907_2934del, p.F969*fs*). Unfortunately, our in-house algorithm used in the initial alignment missed this deletion. We found it only by outsourcing the analysis using another program at DNAnexus (https://www.dnanexus.com). This deletion was not present in parents' leukocyte DNA, suggesting that a *de novo* mutation in the *KIAA2022* gene had occurred in the proband. Other deleterious mutations in *KIAA2022* have been reported in patients with similar phenotypes consisting of profound intellectual disability, self-mutilation and strabismus [42-44], making this novel deletion a more likely candidate than the CAR I281T mutation for the patient's condition.

However, the present findings may also help illuminate the proposed role of CAR F243S mutation in the Kleefstra syndrome [29]. As positive evidence for the role of CAR in this syndrome, its mRNA is expressed at low levels in several human brain regions including pituitary and *nucleus accumbens* [18, 45]. Thus, the F243S mutation may have gene- or cell type-specific effects in some neuronal tissues. In addition, the F243S mutation is rare as it is not present in the homozygous form in the ExAC database, suggesting that it might be of damaging nature. It might also be possible that this variant modulates, e.g. via CAR-dependent expression of hepatic CYP enzymes, levels of a metabolite that can accumulate into and damage neuronal tissues.

As negative evidence for the role of CAR, our laboratory has found that a similar mutation from phenylalanine to a smaller amino acid (F243A) decreases CAR basal activity by ~40% but does not affect the activation by many agonists at all [46]. Another mutation (F243L) did not affect the basal activity [19]. This suggests that the position 243 is quite permissive of amino acid changes and the F243 mutations might be benign. In addition, mice with a disrupted *Car* gene are phenotypically normal and do not show neurological symptoms [47]. Finally, the selection of *Drosophila* EcR as the homolog to CAR and a proxy for the *Drosophila* genetic interactions studies [29] may not be accurate, because our sequence comparison with the NCBI BLAST algorithm preferably aligns EcR with the human *NR1H2* instead of the *NR1I3* gene. The

NR1H2 gene codes for the liver X receptor-*beta* (LXRβ) that has $\sim$42% amino acid identity to EcR [48] and regulates brain development [49]. Collectively, it seems more likely that CAR does not underlie the Kleefstra syndrome. However, the current unavailability of patient-specific cells precludes attempts in testing hypotheses of the CAR F243S or I281T variants affecting hepatic or neuronal processes.

5. Conclusion

We have identified via exome sequencing a previously unreported non-synonymous SNV that reduces the trans-activation capability of CAR by reducing recruitment of coactivators. Although no clear link of the CAR I281T variant to developmental problems exists, the present studies add to our knowledge on CAR function.

Acknowledgments

Competing Interests

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