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Identification of CAR/RXRα Heterodimer Binding Sites in the Human Genome by a Modified Yeast One-Hybrid Assay

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Abstract

The constitutive androstane receptor (CAR) is a transcription factor that belongs to the nuclear receptor superfamily. CAR binds as a heterodimer with the retinoid X receptor α (RXRα) to CAR response elements (CAREs) and regulates the expression of various drug metabolizing enzymes and transporters. To identify CAR/RXRα binding sites in the human genome, we performed a modified yeast one-hybrid assay that enables rapid and efficient identification of genomic targets for DNA-binding proteins. DNA fragments were recovered from positive yeast colonies by PCR and sequenced. A motif enrichment analysis revealed that the most frequent motif was a direct repeat (DR) of RGKTCA-like core sequence spaced by 4 bp. Next, we predicted 149 putative CAR/RXRα binding sites from 414 unique clones, by searching for DRs, everted repeats (ERs) and inverted repeats (IRs) of the RGKTCA-like core motif. Based on gel mobility shift assays, the CAR/RXRα heterodimer could directly interact with the 108 predicted sequences, which included not only classical CAREs but also a wide variety of arrangements. Furthermore, we identified 17 regulatory polymorphisms on the CAR/RXRα-binding sites that may influence individual variation in the expression of CAR-regulated genes. These results provide insights into the molecular mechanisms underlying the physiological and pathological actions of CAR/RXRα heterodimers.

Keywords

Constitutive Androstane Receptor, Retinoid X Receptor, Transcription, SNP, Polymorphism, Nuclear Receptor

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1. Introduction

Constitutive androstane receptor (CAR), a DNA-binding and ligand-regulated transcription factor that belongs to the superfamily of nuclear receptors [1], is mainly expressed in the liver and acts as a chemical sensor of xenobiotics [2]. In addition, it is expressed in a wide range of organs such as the small intestine, kidney, adrenals, testis, and brain [3]. Generally, it is localized in the cytoplasm in a complex with heat shock protein 90 and the CAR cytoplasmic retention protein and is translocated to the nucleus in response to the stimulation of cells by phenobarbital (PB) and other CAR activators [4] [5]. In the nucleus, CAR binds as a heterodimer with the retinoid X receptor *α* (RXR*α*) to CAR response element (CARE) and regulates the expression of various drug metabolizing enzymes and transporters including CYP2B6 [6], CYP3A4 [7], CYP2C9 [8], UDP-glucuronosyltransferase type 1A1 [9], sulfotransferase 2A1 [10], and ABCG2 [11].

Initial studies indicated that CAR/RXR*α* bound 5 bp-spaced direct repeats (DR5) [2]. Later studies found that CAR/RXR*α* bound DR4 motif within phenobarbital response enhancer modules (PBREMs) in a proximal promoter region of the human CYP2B6 gene and a variety of DRs [6] [12]. Moreover, CAR can also bind to everted repeat (ER) and inverted repeat (IR) arrangements [7] [10] [13] [14]. Because most known CAREs have been identified within promoter regions, CAREs at distal regions, *i.e.*, more than 10 kb upstream and downstream of transcription start sites (TSS) of target genes, remain unknown. Although DNA microarray experiments have been performed to study the expression profiles of genes regulated specifically by CAR/RXR*α*, microarray approaches cannot determine whether the regulated genes are primary or secondary target genes [13] [15]-[16]. Recently, genome-wide screening to identify transcription factor-binding sites, using methods such as ChIP-on-chip and ChIP-seq, has been performed against the many nuclear receptors [17]-[23]. Such a whole genome-approach has not been adopted previously for CAR/RXR*α*.

We previously developed a modified yeast one-hybrid (MY1H) system that enabled rapid and efficient identification of genomic targets for DNA-binding proteins [24]. Here, using this system, we reported functional screening for CAR/RXR*α* binding sites in the human genome. We demonstrated that 108 human genomic fragments could directly interact with the CAR/RXR*α* heterodimer by electrophoretic mobility shift assays (EMSAs). Moreover, we identified 17 regulatory single nucleotide polymorphisms (rSNPs) within the identified CAR/RXR*α* binding sites.

2. Materials and Methods

2.1. Plasmid Constructions

Human RXR*α* was amplified by the polymerase chain reaction (PCR) from uterus cDNA (PCR Ready-cDNA, Maxim Biotech, Inc., San Francisco, CA, USA). This cDNA fragments were cloned into pGADT7 (CLONTECH, Mountain View, CA, USA) and reamplified by PCR with primers (**Table 1**, RXR*α*_F and RXR*α*_R) to generate the restriction sites for subcloning. pGADT7 was cleaved with *Hind*III and ligated with linker DNA to remove the nuclear localization signal (NLS) and GAL4 activation domain (GAL4AD). The resulting plasmid was designated as pADH1. Then, RXR*α* cDNA was inserted into pADH1 and the resulting plasmid was named pADH1_RXR*α*. A foot-and-mouth disease virus (FMDV) 2A sequence was amplified by annealing synthetic oligonucleotides (**Table 1**, FMDV 2A_F1, and FMDV 2A_R1) and reamplified by PCR with primers (**Table 1**, FMDV 2A_F2, and FMDV 2A_R2) to generate the restriction sites. FMDV 2A fragment was inserted at the C-terminal end of RXR in pADH1_RXR*α* and the resulting plasmid was named pADH1_RXR*α*_2A. The fragment including NLS-GAL4AD was amplified by PCR from pGADT7 with primers (**Table 1**, NLS_GAL4AD_F and NLS_GAL4AD_R) and inserted into pADH1. The resulting plasmid was named pADH1_NLS_GAL4AD. Similarly, three tandem copies of NLS_GAL4AD were inserted at pADH1 and the resulting plasmid was designated pADH1_NLS_GAL4AD × 3. Human CAR cDNA was cloned by PCR with primers (**Table 1**, NLS_GAL4AD_F and NLS_GAL4AD_R) from pcDNA-CAR [25] and reamplified by PCR with primers (**Table 1**, pADH1-hCAR_F and pADH1-hCAR_R) to generate the restriction sites for subcloning. The cDNA was inserted into pADH1_NLS_GAL4AD × 3 and the resulting plasmid was named pADH1_NLS_GAL4AD × 3_ CAR. The NLS_GAL4AD × 3_CAR fragment obtained from pADH1_NLS_GAL4AD × 3_CAR was inserted at the C-terminal end of RXR*α*_2A in pADH1_RXR*α*_2A and the resulting plasmid was named pADH1_RXR *α*_2A_NLS_GAL4AD × 3_CAR. pSUR (GeneBank AB425277) was constructed as previously described [24] and used as a reporter in yeast one-hybrid assays. pSUR-DR4 and pSUR-CYP2B6-NR3 were constructed

Table 1. Primer sequences.

Primer name	Sequence (5'→3')
hRXRα_F	AAAAAGCTTACGCGTGCCGCCACCATGGACACC
hRXRα_R	TTTAAGCTTTCTAGACTACTCGAGAGTCATTTGGTGCGGCGC
FMDV-2A_F1	AAAAGATCTTAAAATTGTCGCTCCTGTCAAACAAACTCTTAACTTTGATTTACTCAAACTGGCTG
FMDV-2A_R1	AAATCTAGAGGATCCTTTACTAGTTGGACCTGGATTGCTTTCTACATCCCCAGCCAGTTTGAGTAAATCA
FMDV-2A_F2	AAAGTCGACAAAATTGTCGCTCCTGTCAA
FMDV-2A_R2	TTTTCTAGAGAATTCCCGCGGCTCGAGACGCGTTGGACCTGGATTGCTTTC
NLS-GAL4AD_F	AAAAGATCTATGGATAAAGCGGAATTAATTCCCGAGC
NLS-GAL4AD_R	TTTCTCGAGTTTGGATCCCTCTTTTTTTGGGTTTGGTGG
pADH1-hCAR_F	CTTGTCGACAGATCTGCCGCCACCATGGCCAGTAGGGAAGATGA
pADH1-hCAR_R	CTTTCTAGACTACTCGAGTCAGCTGCAGATCTCCTGGAGCAGCGG
hCAR_F	AAAGTCGACGGATCCGCCGCCACCATGGCCAGT
hCAR_R	TTTTCTAGACTACTCGAGTCAGCTGCAGATCTCCTGGA
dpSUR_F	AAAAAGTTATCAAGAGACTGC
dpSUR_R	CTAATGCTTCAACTAACTCCA
SPO13-S	CGGCTATTTCTCAATATACTCC
Consensus DR4_F	GATCAGTTCATGGCAGTTCATGGCAGTTCAGATC
Consensus DR4_R	GATCTGAACTGCCATGAACTGCCATGAACTGATC
random_F	CGCGTTGTGTGTGTTTTATTCC
random_R	GGAATAAAACACACACAACGCG

by inserting 3 copies of consensus DR4 [26] and 4 copies of CYP2B6 XREM-NR3 [27] upstream of the SPO13 promoter of pSUR, respectively. For *in vitro* transcription/translation, the cDNAs for human RXRα and CAR were inserted into the *Hind*III/*Xho* I site and the *Sal* I site of pSP64 Poly (A) (Promega, Madison, WI, USA), respectively.

2.2. A modified Yeast One-Hybrid Assays

The human genomic library for a modified yeast one-hybrid assay was generated as previously described [24]. The effector plasmid, pADH1_RXRα_2A_NLS_GAL4AD × 3_CAR, was transformed into the 5FOA-selected yeast containing the human genomic library using polyethylene glycol/lithium acetate. The obtained transformants were grown on synthetic complete media lacking leucine, tryptophan, and uracil but containing 25 µg/ml 6-azauracil for 3 weeks at 30 ˚C. Human genomic fragments were recovered from the positive colonies by colony-direct PCR with primers corresponding to the vector sequences (**Table 1**, dpSUR_F and dpSUR_R). The PCR fragments were directly sequenced with the primers (**Table 1**, SPO13-S) and used for further experiments.

2.3 EMSAs

The TNT SP6 High Yield system from Promega was used to prepare the human CAR and RXRα proteins. Double-stranded DNA probes (**Table 1**, consensus DR4_F and consensus DR4_R) were used with both ends labeled with Cy5. Proteins were incubated with 200 ng of calf thymus DNA (Invitrogen, Carlsbad, CA, USA) and 1 pmol of the labeled oligonucleotide at 4˚C in the presence or absence of the unlabeled oligonucleotides. The binding reaction was carried out in the EMSA binding buffer containing 12 mM HEPES (pH 7.9), 60 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 12% glycerol, and 0.5% Nonidet P-40. The reaction mixtures were directly

loaded onto 4% nondenaturing polyacrylamide gels made in 0.5 × TBE. In the supershift experiments, CAR and RXR*α* proteins were incubated with antibodies against CAR (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-13065) or RXR*α* (Santa Cruz Biotechnology, sc-553X) overnight before incubation with the probe. After electrophoresis was performed at 4˚C, the gels were analyzed using a bio-imaging analyzer (FLA-7000 FUJIFILM). The consensus DR4 and random sequences (**Table 1**, random_F and random_R) were used as positive and negative controls, respectively.

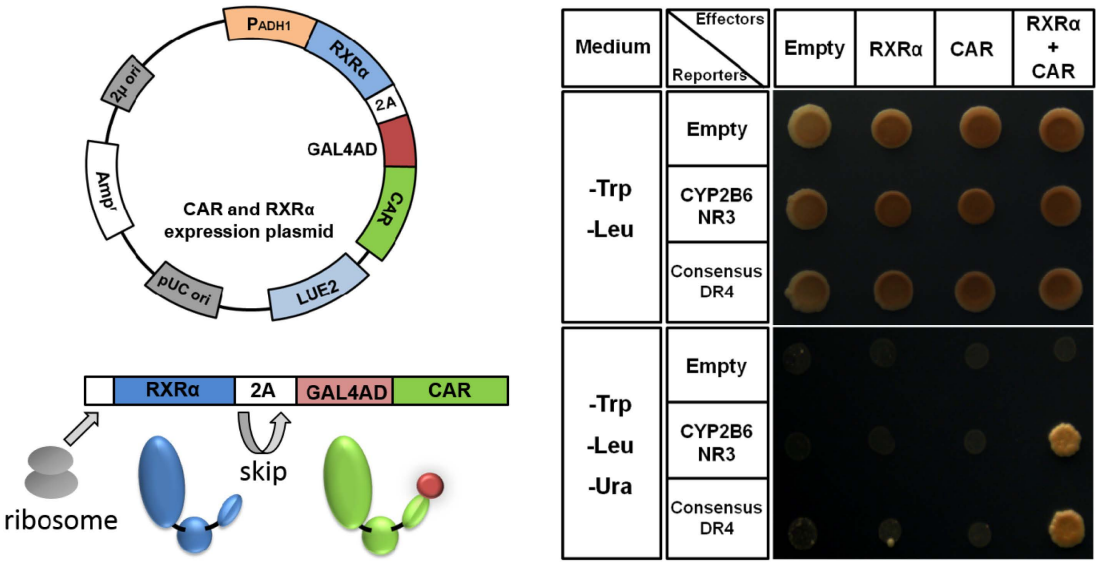
2.4. Bioinformatics

To map the obtained sequences on the human genome assembly (GRCh37), the cloned sequences were analyzed using NCBI's BLAST and the RGKTCA motif was searched using EMBOSS fuzznuc of Galaxy (https://usegalaxy.org/) [28]-[30]. For stringency of the search, we allowed up to 2-bp mismatches in the DR4 motif and 1-bp mismatches in the other motifs. The nearest gene and the distance from the center of the binding site to the transcriptional start site of the gene within 1000 kb were identified with GREAT (http://bejerano.stanford.edu/great/public/html/) [31].

3. Results

3.1. Interaction between the CAR/RXR*α* Heterodimer and CARE in Yeast

To simultaneously express CAR and RXR*α* proteins in yeast, we constructed effector plasmids by placing FMDV 2A peptide between CAR and RXR*α* [32]. Although CAR is expressed as a fusion to the NLS_GAL4AD, RXR*α* was expressed as the native protein to minimize the effect of RXR*α* homodimer on reporter activations (**Figure 1(a)**). To evaluate the function of CAR/RXR*α* heterodimer, yeast cells were transformed with these effectors and the indicated reporters (**Figure 1(b)**). The transformants expressing either CAR or RXR*α* alone were unable to grow, whereas the transformants expressing both CAR and RXR*α* were able to grow in a CARE-dependent manner (**Figure 1(b)**). The known CAR activators, CITCO [14] and PB [33] had no effect on the yeast growth in our assay conditions (data not shown). These results indicated that CAR/RXR*α* heterodimer could activate the reporter gene via CAREs.



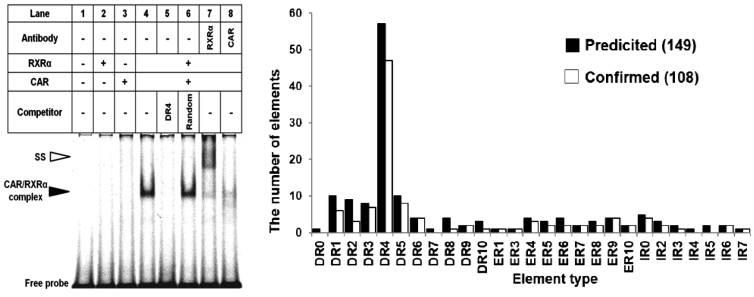
(a) (b)

Figure 1. Yeast one-hybrid assay of the CAR/RXR*α* heterodimer. (a) Schematic diagrams of the effector plasmid. FMDV 2A peptide sequence was placed between CAR and RXR*α* under the control of the ADH1 promoter. CAR and RAR*α* were expressed via a 2A-mediated translational skip mechanism; (b) An examination of the CAR/RXR*α* heterodimer in yeast cells. Yeast cells were transformed with each effector and reporter plasmids and were grown on synthetic complete media lacking leucine and tryptophan (-Trp -Leu) or and lacking leucine, tryptophan, and uracil (-Trp -Leu -Ura). The plates were photographed after 4 days of growth at 30˚C.

3.2. Identification of CAR/RXR*α* Heterodimer-Binding Sites in the Human Genome

The human genomic library was constructed by inserting approximately 300 bp fragments in reporter plasmids and treating with 5FOA to efficiently eliminate false-positive interactions in the yeast one-hybrid assay [24]. The CAR/RXR*α* expression plasmids were transformed into the library and more than 2.8×10^6 were selected on synthetic complete media lacking leucine, tryptophan, and uracil but containing 25 μg/ml 6-azauracil. After 3 weeks, 421 positive colonies were picked from the selection plates and colony-direct PCR was performed to recover human genomic fragments. The PCR fragments were directly sequenced and 414 unique sequences were obtained. As a result of genome mapping, the majority of the 414 unique sequences were located at distal sites, far from the TSS, or in introns. Next, we performed motif enrichment among the 414 unique clones using MEME-ChIP [34]. The most frequently observed motif was a DR4 motif which is known as a classical CARE (**Figure 2**). Then, to obtain putative CAR/RXR*α* binding sites from the 414 unique clones, we selected these sequences based on the half-site core motif (RGKTCA). As a result, a total of 149 putative CAR/RXR*α* binding sites were obtained (**Figure 3(b)**). Interestingly, these sequences contained various types of elements such as DRs, ERs and IRs.

Figure 2. Motif enrichment. The most significant motifs found by MEME-ChIP (http://meme.nbcr.net/meme/doc/meme-chip.html) in the clones obtained from the modified yeast one-hybrid assay. E value: 9.7×10^{-34}. R = A or G, K = T or G, W = A or T.



(a) (b)

Figure 3. Validation assays for direct interactions between the CAR/RXR*α* heterodimer and the predicted elements. (a) EMSAs were performed with the *in vitro*-synthesized human CAR and RXR*α* proteins. The proteins were incubated with 1 pmol of the Cy5-labeled consensus DR4. In a competition assay, 100-fold molar excess of the unlabeled oligonucleotides (DR4 or Random) were added to the reaction mixture. In a supershift experiment, the indicated antibodies were incubated in the reaction mixture. Closed and open arrowheads indicated the CAR/RXR*α* protein-DNA complexes and the super shifted band, respectively. (b) The number of the predicted CAR/RXR*α* binding motif and the confirmed. The predicted CAR/RXR*α* binding sites were obtained by EMBOSS fuzznuc of Galaxy based on RGKTCA motif. For the stringency of the search, motif substitution allowed up to 1-bp mismatches. Motif substitution allowed up to 2-bp mismatches only for the DR4 motif. The direct interaction was confirmed by EMSA competition assays.

3.3. Experimental Validation of the Putative CAR/RXR*α* Binding Sites

To examine the direct interaction of putative CAR/RXR*α* binding sites with the CAR/RXR*α* heterodimer, we performed EMSA. Incubation of the Cy5-labeled consensus DR4 with the combination of CAR and RXR*α*, but not either receptor alone, produced retarded complexes (**Figure 3(a)**, lanes 2 - 4). The complexes represented a sequence-specific interaction between consensus DR4 and the human CAR/RXR*α* proteins, since the formation of this complex was specifically reduced with molar excess of unlabeled competitors (**Figure 3(a)**, lanes 5 and 6). Moreover, the addition of anti-RXR*α* antibody created a slower-migrating complex and the addition of anti-CAR antibody resulted in the disappearance of the band (**Figure 3(a)**, lanes 7 and 8). No supershifted bands were observed with anti-HNF4 antibodies (data not shown). These results indicated that the sequence-specific binding complex contained both CAR and RXR*α*, presumably as a heterodimer.

To examine the direct interaction of CAR/RXR*α* with the 149 putative CAR/RXR*α* binding sites, we performed a semi-quantitative EMSA competition assay. In this method, it is possible to examine binding intensity using a 10- and 100-fold molar excess of unlabeled competitors. Typical examples are shown in **Figure 4**. Sequence I (lanes 15 - 20) was equivalent in binding intensity to the positive control (Consensus DR4, lanes 9 - 14). That is, sequence I had strong binding affinity with the CAR/RXR*α* heterodimer. In the same way, sequence II (lanes 21 - 26) had moderate binding affinity and sequence III (lanes 27 - 32) had low binding affinity. Thus, we evaluated 149 putative CAR/RXR*α* binding sites and confirmed that at least 108 CAR/RXR*α* binding sites could directly interact with the CAR/RXR*α* heterodimer by EMSA (**Table 2**). These sequences contained not only classical CAREs but also a wide variety of additional arrangements.

3.4. rSNPs in the Identified CAR/RXR*α* Binding Sites

rSNPs in transcription factor-binding sites, which alter the ability of a transcription factor to interact with DNA, may lead to predictable differences in gene expression and may be associated with disease susceptibility. Therefore, we tried to identify rSNPs in each of the CAR/RXR*α* binding sequences using the NCBI SNP database. Thirty five rSNPs were identified and the effect on the binding affinity between CAR/RXR*α* and 35 rSNPs was examined by EMSA. As a result, 17 rSNPs were identified based on differences in the DNA-binding affinities (**Table 3**)

4. Discussion

In the present study, we identified CAR/RXR*α*-binding sites in the human genome by a modified yeast one-hybrid assay. Next, we demonstrated that the 108 obtained sequences could directly interact with the CAR/RXR*α* heterodimer by EMSAs. These sequences contained not only classical CAREs but also a wide variety of arrangements (e.g., DRs, ERs, and IRs). Moreover, 17 functional rSNPs on the CAR/RXR*α* binding sites were identified by analyzing differences in DNA-binding affinity.

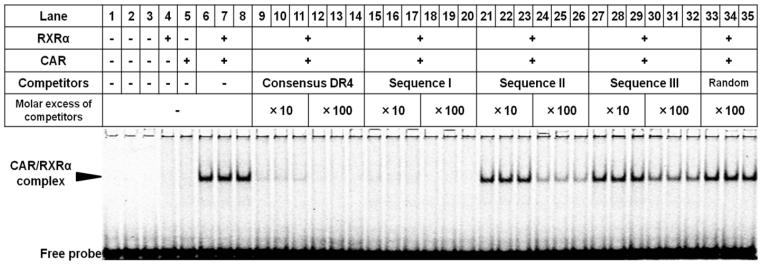


Figure 4. Typical examples of evaluation assays for the putative CAR/RXR*α* binding sites. EMSAs were performed with the *in vitro*-synthesized human CAR and RXR*α* proteins. The proteins were incubated with 1 pmol of the Cy5-labeled consensus DR4. In a competition assay, 10 or 100-fold molar excess of the unlabeled oligonucleotides were added to the reaction mixture. The closed arrowhead indicates the CAR/RXR*α* protein-DNA interaction. Sequence I is a strong binding sequence (**Table 2**, #30). Sequence II is a moderate binding sequence **Table 2**, #79). Sequence III is a week binding sequence (**Table 2**, #89).

Table 2. CAR/RXRα-binding sites.

#	Position	Length (bp)	Neighboring Gene (Distance to TSS)	CAR/RXRα bindnig sequence	Motif	Competition (%)	
						×10	×100
1	Chr18: 55201286-55201390	105	FECH (+52,631), ONECUT2 (+98,421)	tggAGTTCAaAGGTCAcat	DR1	14.6 ± 1.6	95.2 ± 0.4
2	Chr7: 138292264-138292495	232	SVOPL (+71,410), TRIM24 (+147,301)	cgcAGGTCAaAGGTCAggt	DR1	10.7 ± 2.2	78.7 ± 2.9
3	Chr5: 76841961-76842018	58	OTP (+92,532), PDE8B (+335,284)	cagAGGTCGgAGGTCAagt	DR1	17.9 ± 0.5	43.5 ± 2.1
4	Chr20: 23387626-23387738	113	NAPB (+14,474), GZF1 (+42,682)	caaAGTTCAaAGGTCActg	DR1	52.0 ± 1.3	115.8 ± 0.9
5	Chr13: 59071533-59071644	112	PCDH17 (+865,800)	ttaAGTGCAgGGGTCAgga	DR1	25.2 ± 0.9	44.6 ± 0.8
6	Chr17: 1918186-1918361	176	RTN4RL1 (+9,904), RPA1 (+185,001)	tggAGCTCAgAGTTCAaag	DR1	41.4 ± 1.8	90.3 ± 1.1
7	Chr4: 121700806-121701088	283	MAD2L1 (−712,934), PRDM5 (+143,066)	ttgAGGTCAagAGTTCAagg	DR2	30.2 ± 2.6	101.9 ± 1.4
8	ChrX: 40929970-40930061	92	MED14 (−335,212), USP9X (−14,872)	tgtGGGTGAacAGGTCAatg	DR2	71.5 ± 3.0	102.7 ± 0.3
9	Chr13: 49397697-49397876	180	FNDC3A (−152,261), CYSLTR2 (+116,834)	ggcGGATCAtgAGGTCAaga	DR2	22.2 ± 0.8	50.2 ± 2.1
10	Chr17: 8796437-8796604	168	PIK3R6 (−25,527), PIK3R5 (+19,313)	tggAGGTCAgagAGGTCAagc	DR3	35.3 ± 1.1	110.3 ± 9.6
11	Chr2: 103266921-103267061	141	SLC9A2 (+30,825), MFSD9 (+86,346)	tgaAGGTCAaagAGTTTAaca	DR3	56.8 ± 0.5	102.8 ± 1.1
12	Chr5: 142827845-142828030	186	NR3C1 (−43,893), YIPF5 (+722,340)	cagAGGTCAcagAGGTCAtaa	DR3	60.0 ± 0.6	111.6 ± 2.1
13	Chr7: 33720609-33720751	143	BMPER (−223,843), BBS9 (+551,528)	ccaAGTTCAcatAGTTCAtaa	DR3	65.5 ± 0.4	111.3 ± 1.3
14	ChrX: 45732008-45732304	297	CXorf36 (−672,010), ZNF673 (−574,468)	ctgAGGCCAgggAGTTCAcca	DR3	35.9 ± 2.1	91.5 ± 2.9
15	Chr17: 54747517-54747691	175	NOG (+76,544), C17orf67 (+145,767)	cacAGGTCAggcAGTTGAtgc	DR3	13.1 ± 0.9	46.9 ± 1.9
16	Chr6: 4942951-4943334	384	RPP40 (+61,128), CDYL (+166,463)	cggAGGACAgtgGGGTCAggc	DR3	34.4 ± 5.8	89.6 ± 2.6
17	Chr3: 120575966-120576060	95	STXBP5L (−51,037), GTF2E1 (+114,455)	taaAGGTCAaggaAGTTCAatt	DR4	62.8 ± 1.3	107.8 ± 1.4
18	Chr12: 14672183-14672322	140	PLBD1 (+48,538), ATF7IP (+153,642)	ttaAGGTCAactgAGGTCAtat	DR4	51.5 ± 1.2	107.6 ± 0.7
19	Chr1: 220849174-220849356	183	C1orf115 (−14,363), MARK1 (+147,697)	gcaAGGGCAggggAGGTCAata	DR4	30.6 ± 1.1	77.2 ± 0.6
20	Chr10: 30521090-30521250	161	SVIL (−597,269), MTPAP (+117,097)	agtGGATCActtgAGGTCAgga	DR4	20.6 ± 0.8	69.0 ± 1.1
21	Chr2: 55562474-55562589	116	MTIF2 (−66,148), CCDC88A (+84,525)	gtgAGGTCAttaaAGTTCAtgt	DR4	70.8 ± 0.8	112.6 ± 0.4
22	Chr8: 1165688-1165800	113	C8orf42 (−670,413), DLGAP2 (−283,825)	atgGGTTCAtttgGGTGCAtca	DR4	50.1 ± 0.7	104.2 ± 0.0
23	Chr13: 91370194-91370452	259	GPC5 (−680,612)	ctgGGGTCAtatgATTTCAtag	DR4	41.2 ± 1.2	93.4 ± 0.8
24	Chr3: 177287827-177287945	119	KCNMB2 (−966,338), TBL1XR1 (−372,838)	ttaGGGTCAgctgAGTTCAtat	DR4	61.5 ± 1.9	100.8 ± 1.3
25	Chr6: 42700810-42701034	225	PRPH2 (−10,564), TBCC (+12,962)	tcaAGGTTAcaagAGTTCAaga	DR4	75.4 ± 0.2	127.9 ± 4.5

Continued

26	ChrX: 66743819-66744135	317	EDA2R (−908,105), AR (−19,897)	gagAGGTCAatgaGGTTCAcag	DR4	49.7 ± 0.8	99.8 ± 0.7
27	Chr9: 13275072-13275351	280	MPDZ (−24,841)	taaAGGTTAaatgAGGTCActa	DR4	53.2 ± 0.8	104.7 ± 1.6
28	Chr19: 5982545-5982685	141	RANBP3 (−4295)	tcaAGTTCAtgaaAGGTCAcac	DR4	61.7 ± 1.3	114.6 ± 0.6
29	Chr5: 168554759-168554923	165	PANK3 (−548,227), SLIT3 (+173,292)	caaAGTTCActtaAGTTCAgag	DR4	57.6 ± 0.7	116.7 ± 0.7
30	Chr8: 4846264-4846562	299	CSMD1 (+5915)	caaAGGTCActtaAGGTCAttt	DR4	71.4 ± 1.5	125.1 ± 1.1
31	Chr1: 88626432-88626556	125	PKN2 (−523,428), LMO4 (+832,343)	tttAGGTCAttgaAGTTCAagt	DR4	55.4 ± 0.7	106.2 ± 3.5
32	Chr8: 95584473-95584707	235	ESRP1 (−68,774), KIAA1429 (−18,844)	aagAGGTCAgttgGGGTCAgat	DR4	45.0 ± 1.3	99.8 ± 1.3
33	Chr7: 15597442-15597713	272	DGKB (−716,503), AGMO (+4062)	caaAGGGCAccagAGTTAAcag	DR4	23.9 ± 1.6	67.6 ± 0.8
34	Chr14: 54345870-54346006	137	DDHD1 (−725,892), BMP4 (+75,332)	ctgAGGTCAagtgAGTTCAcct	DR4	70.2 ± 0.9	118.5 ± 1.4
35	Chr15: 49543989-49544276	288	FGF7 (−171,242), GALK2 (+81,711)	ggtGGATCActtgAGGTCAgga	DR4	26.0 ± 2.4	65.0 ± 0.7
36	Chr13: 55084028-55084114	87	NONE	atgGGGTCAacaaAGTTTAaga	DR4	83.3 ± 4.9	119.2 ± 5.2
37	Chr8: 112300235-112300383	149	NONE	aagAGGTTAcaagAGTTCAtat	DR4	69.2 ± 1.1	112.9 ± 0.3
38	Chr3: 28008615-28008785	171	CMC1 (−274,424), EOMES (−244,915)	gtgGGGTCAcatgAGGGCAcat	DR4	51.9 ± 1.8	105.3 ± 1.0
39	Chr15: 87420670-87420730	61	AGBL1 (+735,458)	aagAGGTCAactgAGTTCAtcc	DR4	69.2 ± 0.6	114.4 ± 0.3
40	Chr9: 81575106-81575356	251	TLE4 (−611,647), PSAT1 (+663,172)	aggAGGTGAtaaaGGTTAAatg	DR4	21.0 ± 1.5	64.6 ± 0.5
41	Chr15: 65168224-65168305	82	PIF1 (−50,427), ANKDD1A (−35,836)	atgGTGTCAgaagAGTTCAtca	DR4	64.7 ± 0.6	107.5 ± 0.2
42	Chr10: 31605586-31605754	169	ZEB1 (−2431)	aaaAGGTGAacagAGTTCAttg	DR4	73.5 ± 1.4	113.6 ± 1.0
43	Chr4: 4003949-4004240	292	OTOP1 (+224,526), ADRA2C (+235,799)	atgGGGTCAgctaGGTTTActt	DR4	43.3 ± 0.8	100.1 ± 2.4
44	Chr5: 154114732-154114879	148	LARP1 (+22,344), C5orf4 (+115,407)	ggcAGGTCAcctgAGGTCAgga	DR4	34.2 ± 1.1	97.2 ± 0.3
45	Chr15: 69230539-69230630	92	NOX5 (−76,449), SPESP1 (+7746)	ggtGGGTCAactaAGGTCAgga	DR4	50.2 ± 3.9	100.7 ± 0.9
46	Chr8: 140124212-140124570	359	COL22A1 (−198,155), KCNK9 (+590,908)	gccAGTGCAgaagAGGTCAcac	DR4	27.7 ± 2.0	78.3 ± 0.9
47	Chr8: 107263662-107263790	129	OXR1 (−196,426), ZFPM2 (+932,579)	gtcGGGTCActtaAGGTCAgga	DR4	40.7 ± 0.4	95.7 ± 0.9
48	Chr15: 78475549-78475775	227	IDH3A (+33,943), ACSBG1 (+51,387)	ggaGGGTCAtttgAGGTCAgga	DR4	39.3 ± 0.8	98.1 ± 2.7
49	Chr14: 65099982-65100056	75	PLEKHG3 (−94,292), HSPA2 (+92,833)	attAGTTAAgatgAGGTCAtac	DR4	19.3 ± 5.4	62.2 ± 5.6
50	Chr6: 31790280-31790663	384	HSPA1B (−5040), HSPA1A (+7181)	caaGGATCAaagaAGTTCAgtg	DR4	57.1 ± 1.0	88.6 ± 0.2
51	Chr13: 90222946-90223063	118	NONE	gtaAGGTCAtaaaAGTTCAggt	DR4	63.1 ± 1.8	110.6 ± 0.4
52	ChrX: 132353555-132353667	113	TFDP3 (−1235)	ttaAGGTTAaatgAGGTCAtaa	DR4	55.1 ± 0.2	97.8 ± 0.4

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53	Chr12: 80739933-80740212	280	PTPRQ (−98,053), OTOGL (+136,840)	caaGGTTGAttagAGTTCAgcc	DR4	48.2 ± 1.3	93.5 ± 0.3
54	Chr6: 133030154-133030300	147	TAAR1 (−63,085), VNN1 (+4967)	aaaAGGTCGtctgAGGGCAtca	DR4	37.7 ± 2.3	89.0 ± 0.3
55	Chr20: 38461220-38461351	132	MAFB (+856,590), DHX35 (+870,305)	acaGGGTCAcaaaAGGCCAata	DR4	34.3 ± 0.2	88.9 ± 1.6
56	Chr18: 609485-609841	357	CLUL1 (−7037), CETN1 (+29,294)	ggtGGGTCAtttgAGGTCAgga	DR4	41.9 ± 1.1	96.9 ± 0.6
57	Chr4: 4081417-4081706	290	OTOP1 (+147,059), ADRA2C (+313,266)	ggcGGATCActtgAGGTCAgga	DR4	17.5 ± 1.0	60.6 ± 0.7
58	Chr4: 27697777-27697856	80	STIM2 (+835,504)	gggAGGTCActcaAGTTCActg	DR4	66.1 ± 1.5	114.3 ± 1.4
59	Chr11: 70447681-70447959	279	CTTN (+203,208), SHANK2 (+488,022)	ggtGGATCAcctgAGGTCAgga	DR4	17.5 ± 0.9	55.7 ± 2.6
60	Chr17: 71023806-71024052	247	SLC39A11 (+64,924), SOX9 (+906,768)	caaGGGTCAaatcAGGGCActg	DR4	40.6 ± 1.4	102.2 ± 0.3
61	Chr7: 87875231-87875351	121	SRI (−25,898), STEAP4 (+60,937)	caaAGGTTAcaagAGTTCTctt	DR4	60.4 ± 1.4	96.3 ± 1.8
62	Chr2: 89376298-89376461	164	RPIA (+385,204)	caaAGGTTAccttAGGTCAtat	DR4	36.9 ± 1.5	92.5 ± 1.1
63	Chr2: 95707549-95707691	143	MAL (+16,141), MRPS5 (+80,134)	gggGGGTTAgcagAGTTGAgct	DR4	33.8 ± 3.4	76.2 ± 5.0
64	Chr4: 88898879-88898955	77	PKD2 (−29,882), SPP1 (+2,115)	cagAGTTCAattccAGTTGAaca	DR5	17.6 ± 2.2	73.4 ± 0.3
65	Chr1: 177083927-177084138	212	ASTN1 (+49,991), PAPPA2 (+651,726)	gctGGGTCAggaaaAGTTCAtgg	DR5	69.6 ± 9.1	98.0 ± 0.2
66	Chr6: 133330363-133330649	287	EYA4 (−231,989), RPS12 (+194,798)	agtGGTTCAatcacAGTTCActg	DR5	49.6 ± 0.2	89.9 ± 1.0
67	Chr14: 35253086-35253208	123	CFL2 (−69,118), BAZ1A (+91,706)	aaaAGTTCAaacacAGTTCAttt	DR5	71.6 ± 1.9	109.9 ± 0.9
68	Chr1: 84080031-84080193	163	TTLL7 (+384,721)	aagAGGTCAagtcaGGTACAaac	DR5	37.2 ± 0.7	86.4 ± 1.0
69	Chr5: 89560627-89560695	69	CETN3 (+144,942)	aggAGGTCAggaggAGTTCActg	DR5	67.0 ± 2.5	102.2 ± 0.8
70	ChrX: 36376152-36376213	62	PRRG1 (−832,345), CXorf59 (+311,130)	aggAGGTCAatatgAGGTCAcag	DR5	39.2 ± 0.3	95.8 ± 1.2
71	Chr1: 48570878-48571087	210	SLC5A9 (−117,374), LOC388630 (−108,421)	gaaAGGTCActcagAGTTCAttt	DR5	68.6 ± 9.7	120.4 ± 0.4
72	Chr10: 16445680-16445852	173	FAM188A (−543,247), PTER (−33,201)	taaAGTTCAtggtaaAGTTCAtgg	DR6	74.0 ± 1.2	119.3 ± 0.6
73	Chr4: 80819846-80819929	84	GK2 (−490,516), ANTXR2 (+174,589)	gaaAGTTCAtattgtAGGTCAttc	DR6	64.7 ± 1.0	112.9 ± 1.4
74	Chr2: 33407228-33407375	148	RASGRP3 (−331,640), LTBP1 (+234,933)	ctaAGTTCAtgatagAGTTCAaag	DR6	65.3 ± 1.3	112.5 ± 1.2
75	Chr6: 23048624-23048748	125	PRL (−745,604)	ttgAGCTCAattcctAGTTCAcca	DR6	27.2 ± 0.8	74.3 ± 0.8
76	Chr2: 135675135-135675350	216	CCNT2 (−1149)	ttgAGGTCAggagttcgAGGGCAagg	DR8	11.7 ± 1.0	54.8 ± 2.6
77	Chr20: 36825053-36825172	120	BPI (−107,439), TGM2 (−31,413)	ctgGGGTCAcagggccagAGGTCActg	DR9	18.8 ± 3.0	70.2 ± 3.6
78	Chr21: 34190796-34190941	146	C21orf62 (−4816)	tagGGGTCAcctcggtagGGGTCAggg	DR9	17.9 ± 0.6	58.7 ± 0.7
79	Chr1: 154209880-154209999	120	HAX1 (−35,099), UBAP2L (+16,615)	tcaGGGTCAaacaatgctgAGTTCAaat	DR10	34.4 ± 1.2	90.0 ± 0.6
80	Chr14: 98635538-98635762	225	NONE	gacTGAACTcTGGTCAttt	ER1	13.0 ± 5.0	58.3 ± 3.2
81	Chr12: 34343448-34343791	344	ALG10 (+168,404)	tggTGCCCTcctAGGTCAtgg	ER3	37.6 ± 0.8	41.6 ± 4.5

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82	Chr12: 10876774-10876871	98	CSDA (−870)	attTGGCCCctagGGTTCAgac	ER4	19.6 ± 0.2	42.6 ± 0.6
83	Chr9: 130011528-130011906	379	ANGPTL2 (−126,673), GARNL3 (−15,039)	tctTGAACTcctgGGCTCAagt	ER4	4.4 ± 2.5	55.2 ± 0.9
84	Chr10: 58646226-58646342	117	ZWINT (−525,250)	ctgTGACCTcttgAGAACTtaa	ER4	19.7 ± 0.5	81.6 ± 1.7
85	Chr20: 7438451-7438525	75	HAO1 (+482,605), BMP2 (+689,743)	acaTGAACTtttggAGGTCAtat	ER5	78.2 ± 0.5	115.0 ± 0.4
86	Chr1: 242533417-242533615	199	MAP1LC3C (−371,131), PLD5 (+154,482)	caaGGAACTaaataAGTTCAttt	ER5	30.9 ± 1.1	75.6 ± 0.9
87	Chr1: 219789689-219789818	130	SLC30A10 (+312,239), LYPLAL1 (+442,562)	atgTGAACTcttctaAGTTCAtgg	ER6	79.0 ± 0.7	111.1 ± 0.7
88	Chr9: 46385533-46385719	187	NONE	gtgTGACCTttttaaAGTTAAatt	ER6	26.9 ± 2.5	87.2 ± 1.4
89	Chr3: 74240550-74240667	118	PDZRN3 (−566,537), CNTN3 (+329,734)	aacTGACCCattttcaGGTTCActg	ER7	30.0 ± 4.9	53.0 ± 2.5
90	Chr8: 41543991-41544261	271	NKX6−3 (−39,251), ANK1 (+210,154)	tgcTGACCTtgctgagAGTTCAtgt	ER7	66.1 ± 0.2	101.5 ± 0.5
91	Chr6: 108964946-108965090	145	ARMC2 (−204,601), FOXO3 (+82,949)	agcTGAACTctgcactaAGGTCAgat	ER8	65.3 ± 1.6	103 ± 0.3
92	Chr14: 91348266-91348370	105	TTC7B (−65,557), RPS6KA5 (+178,675)	gatTAAACTctaatcaaAGGTCAaag	ER8	76.5 ± 1.2	106.7 ± 3.1
93	Chr20: 1565505-1565776	272	SIRPD (−27,298), SIRPB1 (+35,048)	tctTGACCCttttgtcaaAGGTCAatt	ER9	58.9 ± 1.5	101.4 ± 0.6
94	Chr18: 66213526-66213902	377	TMX3 (+168,639)	tttTGAACTctgtctgaaAGGTCActt	ER9	72.2 ± 1.5	105.3 ± 0.5
95	Chr1: 42946546-42946764	219	PPIH (−177,393), PPCS (+24,482)	ggaTGACCTgaggccaggAGTTCAaga	ER9	54.4 ± 0.4	96.9 ± 1.5
96	Chr5: 88641418-88641561	144	MEF2C (−441,568)	accTTAACTctgtctggaAGGTCAaga	ER9	21.9 ± 1.0	79.5 ± 1.9
97	Chr5: 54048617-54048892	276	ESM1 (+232,659), SNX18 (+235,162)	agaTGACCTctgcccgctaAGTTCAagt	ER10	53.4 ± 1.5	111.2 ± 0.8
98	Chr17: 22251179-22251327	149	MTRNR2L1 (+228,816)	accTGAACTttcaaaggaaGGTTCAatt	ER10	31.8 ± 1.0	101.7 ± 1.7
99	Chr21: 42948507-42948610	104	TMPRSS2 (−68,567), RIPK4 (+238,690)	tggAGTTCATGACCTtgt	IR0	50.8 ± 2.9	126.6 ± 2.0
100	Chr22: 51188718-51188943	226	ACR (+12,179), RABL2B (+33,256)	caaAGGACATGAACTctt	IR0	6.4 ± 1.0	52.9 ± 1.1
101	Chr5: 113265138-113265327	190	KCNN2 (−432,783), YTHDC2 (+415,842)	atgAGTTCATGTCCTttg	IR0	19.2 ± 2.8	72.2 ± 3.0
102	Chr19: 27990058-27990456	399	NONE	caaAGGACATGAACTcat	IR0	20.4 ± 2.7	65.6 ± 0.7
103	Chr3: 135695587-135695826	240	PPP2R3A (+11,192), MSL2 (+218,981)	ttaAGTTCAtcTGAAATtta	IR2	15.9 ± 2.3	46.0 ± 1.5
104	Chr16: 27714860-27714998	139	GTF3C1 (−153,678), GSG1L (+359,901)	cttAGTTCAgcTGACCTctg	IR2	48.7 ± 2.4	116.6 ± 1
105	Chr14: 58472137-58472540	404	SLC35F4 (−139,747), C14orf37 (+146,508)	aggAGGTCAaagTGTCCTctg	IR3	8.8 ± 6.8	54.4 ± 6.9
106	Chr7: 152388241-152388469	229	ACTR3B (−68,496), XRCC2 (−15,105)	tggAGTTCAttggcaTGACCTcgg	IR6	12.4 ± 3.7	85.6 ± 1.3
107	Chr5: 162859214-162859417	204	CCNG1 (−5261)	taaAGGTCAttgcctTAACCTagg	IR6	16.1 ± 1.2	70.0 ± 1.1
108	Chr18: 30395764-30395944	181	ASXL3 (−762,687), KLHL14 (−42,880)	aaaAGTTCAacctcccTTAACTatg	IR7	32.9 ± 1.4	95.7 ± 0.8

Table 3. rSNPs in the identified CAR/RXR*α* binding sites.

dbSNP	Gene (Distance to TSS)	Sequences	Motif	Concentration of competitor	EMSA Competition (%)
rs115089792	NONE	atgGGGTCAacaaAGTTTAaga	DR4	×10	62.8 ± 0.6
		atgGTGTCAacaaAGTTTAaga			36.0 ± 0.4
rs371021620	FAM188A (−543,247), PTER (−33,201)	taaAGTTCAtggtaaAGTTCAtgg	DR6	×10	64.0 ± 1.1
		taaAATTCAtggtaaAGTTCAtgg			19.4 ± 0.8
rs62097972	TMX3 (+168,639)	tttTGAACTctgtctgaaAGGTCActt	ER9	×10	74.4 ± 0.4
		tttTGAATTctgtctgaaAGGTCActt			Not detected
rs181870712	CSMD1 (+5915)	caaAGGTCActtaAGGTCAttt	DR4	×10	62.9 ± 1.1
		caaAAGTCActtaAGGTCAttt			26.0 ± 1.5
rs117662912	CETN3 (+144,942)	aggAGGTCAggaggAGTTCActg	DR5	×10	56.9 ± 0.9
		aggAAGTCAggaggAGTTCActg			17.9 ± 0.6
rs113398776	SLC9A2 (+30,825), MFSD9 (+86,346)	tgaAGGTCAaagAGTTTAaca	DR3	×100	95.5 ± 0.9
		tgaAGGTCAaagAGATTAaca			9.5 ± 1.1
rs190307664	ESM1 (+232,659), SNX18 (+235,162)	agaTGACCTctgcccgctaAGTTCAagt	ER10	×100	101.8 ± 1.5
		agaTGACCTctgcccgctaACTTCAagt			36.5 ± 0.9
rs115070434	MPDZ (−24,841)	taaAGGTTAaatgAGGTCActa	DR4	×100	102.8 ± 1.1
		taaAGGTTAaatgAGGTCCcta			66.3 ± 5.7
rs148735323	NOX5 (−76,449), SPESP1 (+7746)	ggtGGGTCAactaAGGTCAgga	DR4	×100	97.6 ± 0.4
		ggtGGATCAactaAGGTCAgga			71.1 ± 0.8
rs145145243	EYA4 (−231,989), RPS12 (+194,798)	agtGGTTCAatcacAGTTCActg	DR5	×100	94.4 ± 0.9
		agtGGTTCAatcacAGTTCTctg			60.7 ± 1.5
rs369662801	RTN4RL1 (+9904), RPA1 (+185,001)	tggAGCTCAgAGTTCAaag	DR1	×100	82.1 ± 0.5
		tggAGCTCAgCGTTCAaag			22.1 ± 5.5
rs79536015	RPIA (+385,204)	caaAGGTTAccttAGGTCAtat	DR4	×100	96.5 ± 0.2
		caaAGGTTAccttAGGTCGtat			67.5 ± 0.9
rs4497728 rs4303542 rs4307980	MTRNR2L1 (+228,816)	accTGAACTttcaaaggaaGGTTCAatt	ER10	×100	94.5 ± 2.1
		accTGAAATttcaaaggaaGGTTCAatt			20.2 ± 0.5
		accTGAACAttcaaaggaaGGTTCAatt			23.5 ± 0.6
		accTGAACTttcaaaggaaGTTTCAatt			68.6 ± 0.9
rs138716773	PRL (−745,604)	ttgAGCTCAattcctAGTTCAcca	DR6	×100	62.6 ± 2.4
		ttgAGCTTAattcctAGTTCAcca			12.0 ± 3.9
rs11914034	ACR (+12,179), RABL2B (+33,256)	caaAGGACATGAACTctt	IR0	×100	46.9 ± 0.7
		caaAGGACGTGAACTctt			77.6 ± 0.5

It has been reported that CAR/RXR*α* can bind promiscuously to multiple DNA binding motifs such as DR1 [35], DR3 [7], DR4 [8] [12] [36]-[38], DR5 [2] [11], ER6 [7], ER7 [39], ER8 [40], ER9 [39], ER10 [39], IR2 [10], IR3 [41], and IR6 [42]. In addition to these motif types, our results indicated that CAR/RXR*α* could bind to DR2, DR6, DR8-10, ER1, ER3, ER4, ER5, IR0, and IR7 (**Figure 3(b)** and **Table 2**). As these motif types

overlap with the binding sequences for other nuclear receptors, CAR/RXR*α* would compete for binding to these elements and cross talk with other nuclear receptors [35] [38] [41]. On the other hand, CAR/RXR*α* may preferably bind to DR4 relative to the other motifs, because DR4 motif was enriched by motif enrichment among 414 clones (**Figure 2**).

We could not identify the previously reported CAR/RXR*α*-binding sites in this study. There are some explanations for missed CAR/RXRDNA interactions. First, the quality of a library will affect the efficiency of identification of protein-DNA interactions. Second, some CAREs may exist adjacent to sequences recognized by yeast transcription factors and may be discarded during the negative selection by 5FOA [8]. However, we could identified new CAR/RXR*α* binding sites located in or in the proximity of genes with various functions such as metabolic process, cell cycle, cell proliferation and apoptosis. In particular, Heat shock 70 kDa protein 1A (HSPA1A) is known as a stress inducible gene that promotes liver tumor cell proliferation [43]. Importantly, the HSPA1A gene was reported as a CAR-dependent gene in a microarray analysis of CAR-knockout mice [15]. Furthermore, we confirmed the CAR-dependent activation of human HSPA1A mRNA expression in human hepatoma HepG2 cells by real-time PCR assays (data not shown). CAR is thought to be essential for liver tumor promotion via the direct regulation of the Mdm2 gene [44] [45]. In addition to this mechanism, our results suggested that CAR is associated with liver tumor promotion via direct regulation of HSPA1A.

ChIP-on-chip or ChIP seq techniques are powerful methods for identifying transcription factor-DNA interactions. However, these methods can also identify potential indirect transcription factor-DNA interactions. In contrast, our strategy for the identification of CAR/RXR*α* binding sites in the human genome was fundamentally different and was based on the direct interaction of CAR/RXR*α* with human genomic sequences using yeast genetic selection. We expect that these findings will provide insights into the molecular mechanisms underlying the physiological and pathological actions of CAR/RXR*α* heterodimers.

5. Conclusion

In this paper, we identified CAR/RXR*α*-binding sequences in the human genome by a modified yeast one-hybrid assay. The binding sites were located in the proximity of genes with various functions such as metabolic process, cell cycle, cell proliferation and apoptosis. Motif enrichment analysis revealed that the most frequently observed motif was a DR4, although the identified sequences contained not only classical CAREs but also a wide variety of arrangements. Next, we demonstrated that 108 human genomic fragments could directly interact with the CAR/RXR*α* heterodimer by EMSAs. Furthermore, we identified 17 regulatory polymorphisms on the CAR/RXR*α*-binding sites which may influence individual variation of expression levels of CAR-regulated genes. We expect that these findings will provide insights into the molecular mechanisms underlying the physiological and pathological actions of CAR/RXR*α* heterodimers.

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Declaration of Interest

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