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 (RXRα) 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/RXRα bound 5 bp-spaced direct repeats (DR5) [2]. Later studies found that CAR/RXRα 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/RXRα, 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/RXRα.

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/RXRα binding sites in the human genome. We demonstrated that 108 human genomic fragments could directly interact with the CAR/RXRα heterodimer by electrophoretic mobility shift assays (EMSAs). Moreover, we identified 17 regulatory single nucleotide polymorphisms (rSNPs) within the identified CAR/RXRα binding sites.

2. Materials and Methods

2.1. Plasmid Constructions

Human RXRα 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, RXRα_F and RXRα_R) to generate the restriction sites for subcloning. pGADT7 was cleaved with HindIII 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, RXRα cDNA was inserted into pADH1 and the resulting plasmid was named pADH1_RXRα. 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_RXRα and the resulting plasmid was named pADH1_RXRα_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 RXRα 2A in pADH1_RXRα_2A and the resulting plasmid was named pADH1_RXRα_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.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
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<tr>
<td>hRXRα_F</td>
<td>AAAAGCTTACCGGTGCCACCATGGACACC</td>
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<tr>
<td>hRXRα_R</td>
<td>TTTAAGCTTCTAGAAGATTTGACGAGTCATTTTGGGTGCCGC</td>
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<tr>
<td>FMDV-2A_F1</td>
<td>AAAAGATCTTTTACTAGTGGACTGCCTGGATGGTCCTTACATCCCAGCCAGTTTGAAGTAAATCA</td>
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<td>FMDV-2A_R1</td>
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<td>AAAAGATCTTATGGATAAAGCGGAATTTCCTGGAGCAGCGG</td>
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<td>FMDV-2A_R2</td>
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<td>NLS-GAL4AD_F</td>
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<td>NLS-GAL4AD_R</td>
<td>TTTTCTAGAAGATTTGACGAGTCAGCTTCCTGGAGCAGCGG</td>
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<td>hCAR_R</td>
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<td>dpSUR_F</td>
<td>AAAAGATCTATGGATAAAGCGGAATTTCCTGGAGCAGCGG</td>
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<td>dpSUR_R</td>
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<td>SPO13-S</td>
<td>CAGGCTATTTCTCAATATTCAGCAGGGAGAGATG</td>
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<td>Consensus DR4_F</td>
<td>GATCAGTTCATGGCGATGGTCGAGAGATG</td>
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<td>Consensus DR4_R</td>
<td>GATCAGTTCATGGCGATGGTCGAGAGATG</td>
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<tr>
<td>random_F</td>
<td>CAGGCTATTTCTCAATATTCAGCAGGGAGAGATG</td>
</tr>
<tr>
<td>random_R</td>
<td>CAGGCTATTTCTCAATATTCAGCAGGGAGAGATG</td>
</tr>
</tbody>
</table>

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 HindIII/XhoI site and the SalI 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 MgCl2, 1 mM EDTA, 12% glycerol, and 0.5% Nonidet P-40. The reaction mixtures were directly...
loaded onto 4% non-denaturing polyacrylamide gels made in 0.5 × TBE. In the supershift experiments, CAR and RXRα proteins were incubated with antibodies against CAR (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-13065) or RXRα (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/RXRα Heterodimer and CARE in Yeast

To simultaneously express CAR and RXRα proteins in yeast, we constructed effector plasmids by placing FMDV 2A peptide between CAR and RXRα [32]. Although CAR is expressed as a fusion to the NLS_GAL4AD, RXRα was expressed as the native protein to minimize the effect of RXRα homodimer on reporter activations (Figure 1(a)). To evaluate the function of CAR/RXRα heterodimer, yeast cells were transformed with these effectors and the indicated reporters (Figure 1(b)). The transformants expressing either CAR or RXRα alone were unable to grow, whereas the transformants expressing both CAR and RXRα 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/RXRα heterodimer could activate the reporter gene via CAREs.

![Figure 1. Yeast one-hybrid assay of the CAR/RXRα heterodimer. (a) Schematic diagrams of the effector plasmid. FMDV 2A peptide sequence was placed between CAR and RXRα under the control of the ADH1 promoter. CAR and RXRα were expressed via a 2A-mediated translational skip mechanism; (b) An examination of the CAR/RXRα 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.](image-url)
3.2. Identification of CAR/RXRα 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/RXRα expression plasmids were transformed into the library and more than $2.8 \times 10^6$ 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/RXRα 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/RXRα binding sites were obtained (Figure 3(b)). Interestingly, these sequences contained various types of elements such as DRs, ERs and IRs.
3.3. Experimental Validation of the Putative CAR/RXRα Binding Sites

To examine the direct interaction of putative CAR/RXRα binding sites with the CAR/RXRα heterodimer, we performed EMSA. Incubation of the Cy5-labeled consensus DR4 with the combination of CAR and RXRα, 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/RXRα 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-RXRα 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 RXRα, presumably as a heterodimer.

To examine the direct interaction of CAR/RXRα with the 149 putative CAR/RXRα 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/RXRα 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/RXRα binding sites and confirmed that at least 108 CAR/RXRα binding sites could directly interact with the CAR/RXRα 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/RXRα 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/RXRα binding sequences using the NCBI SNP database. Thirty five rSNPs were identified and the effect on the binding affinity between CAR/RXRα 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/RXRα-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/RXRα 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/RXRα binding sites were identified by analyzing differences in DNA-binding affinity.
Table 2. CAR/RXRα-binding sites.

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<th>#</th>
<th>Position (bp)</th>
<th>Neighboring Gene (Distance to TSS)</th>
<th>CAR/RXRα binding sequence</th>
<th>Motif</th>
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<td>Chr18: 55201286-55201390</td>
<td>FECH (+52,631), ONECUT2 (+98,421)</td>
<td>tggAGTTCaAGGTCAcat DR1</td>
<td>14.6 ± 1.6</td>
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<td></td>
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<td></td>
<td>95.2 ± 0.4</td>
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<tr>
<td>2</td>
<td>Chr17: 1918186-191819861</td>
<td>RTN4RL1 (+9,094), RPA1 (+185,001)</td>
<td>tggAGCTCAgAGTTCAaag DR1</td>
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<td>90.3 ± 1.1</td>
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<td>3</td>
<td>Chr13: 59071533-59071644</td>
<td>PCDH17 (+865,800)</td>
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<td>110.3 ± 9.6</td>
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<td>ttaAGGTCAactgAGGTCAtat DR4</td>
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<td>107.6 ± 0.7</td>
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<td>Chr1: 120575966-120576060</td>
<td>STXBPS1 (+51,037), GTF2E1 (+114,455)</td>
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<td>62.8 ± 1.3</td>
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<td>62.8 ± 1.3</td>
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<td>ttaAGGTCAaCTgAGGTCTAat</td>
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<tr>
<td>53</td>
<td>Chr12: 80739933-80740212</td>
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<td>PTPRQ (−98,053), OTOGL (+136,840)</td>
<td>caaGGTTGAttagAGTTCAgcc</td>
<td>48.2 ± 1.3</td>
<td>93.5 ± 0.3</td>
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<td>63</td>
<td>Chr1: 48570878-48570955</td>
<td>69</td>
<td>CETN3 (−117,374), LOC388630 (+108,421)</td>
<td>ggtGGATCAcctgAGGTCAttc</td>
<td>27.2 ± 0.8</td>
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<td>64</td>
<td>Chr3: 23048624-23048748</td>
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<td>gaaAGGTCActcaAGTTCAttc</td>
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<td>66</td>
<td>Chr4: 80819846-80819929</td>
<td>84</td>
<td>G2K (−490,516), ANTXR2 (+174,589)</td>
<td>gaaAGGTCActcaAGTTCAttc</td>
<td>37.2 ± 0.7</td>
<td>86.4 ± 1.0</td>
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<td>67</td>
<td>Chr6: 33407228-33407375</td>
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<td>RASGRP3 (−331,640), LTBP1 (+234,933)</td>
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<td>ER5</td>
<td>78.2 ± 0.5</td>
<td>115.0 ± 0.4</td>
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<td>ER5</td>
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<td>26.9 ± 2.5</td>
<td>87.2 ± 1.4</td>
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### Table 3. rSNPs in the identified CAR/RXRα binding sites.

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<td>rs115089792</td>
<td>NONE</td>
<td>atgGGGTCAacaaAGTTTAaga</td>
<td>DR4</td>
<td>62.8 ± 0.6</td>
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<td>rs371021620</td>
<td>FAM188A (−543,247), PTER (−33,201)</td>
<td>taaAGTTCAtgtaaAGTTCAaggg</td>
<td>DR6</td>
<td>64.0 ± 1.1</td>
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<td>rs62097972</td>
<td>TMX3 (+168,639)</td>
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<td>ER9</td>
<td>74.4 ± 0.4</td>
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<td>CSMD1 (+5915)</td>
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<td>DR4</td>
<td>62.9 ± 1.1</td>
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<tr>
<td>rs117662912</td>
<td>CETN3 (+144,942)</td>
<td>aggAGGTCAaggaggAGTTCActg</td>
<td>DR5</td>
<td>56.9 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>rs113398776</td>
<td>SLC9A2 (+30,825), MFSD9 (+86,346)</td>
<td>tgaAGGTCAaagAGTTTAaca</td>
<td>DR3</td>
<td>95.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>rs190307664</td>
<td>ESM1 (+232,659), SNX18 (+235,162)</td>
<td>agaTGACCTctgcccgctagAGTTCAacctg</td>
<td>ER10</td>
<td>101.8 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>rs115070434</td>
<td>MPDZ (−24,841)</td>
<td>taaAGGTAAatgAGGTCAacta</td>
<td>DR4</td>
<td>102.8 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>rs148735323</td>
<td>NOX5 (−76,449), SPESP1 (+7746)</td>
<td>ggtGGGTCAacactaAGGTCAagga</td>
<td>DR4</td>
<td>97.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>rs145145243</td>
<td>EYA4 (−231,989), RPS12 (+194,798)</td>
<td>agtGGTTCAtcactaAGGTCActg</td>
<td>DR5</td>
<td>60.7 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>rs369662801</td>
<td>RTN4RL1 (+9904), RPA1 (+185,001)</td>
<td>tggAGCTCAtgAGGTCAaag</td>
<td>DR1</td>
<td>82.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>rs79536015</td>
<td>RPIA (+385,204)</td>
<td>caaAGGTTCacttAGGTCAattt</td>
<td>DR4</td>
<td>96.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>rs55676645</td>
<td>MTRNR2L1 (−228,816)</td>
<td>accTGAAACTtcaaggaagGTTCAtca</td>
<td>ER10</td>
<td>94.5 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>rs138716773</td>
<td>PRL (−745,604)</td>
<td>ttgAGCTCAattcAGGTCAaacc</td>
<td>DR6</td>
<td>62.6 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>rs11914034</td>
<td>ACR (+12,179), RABL2B (+33,256)</td>
<td>ccaaGGACATGAACAtcctt</td>
<td>IR0</td>
<td>46.9 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

It has been reported that CAR/RXRα 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/RXRα 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\(\alpha\) would compete for binding to these elements and cross talk with other nuclear receptors [35] [38] [41]. On the other hand, CAR/RXR\(\alpha\) 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\(\alpha\)-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\(\alpha\) 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\(\alpha\) binding sites in the human genome was fundamentally different and was based on the direct interaction of CAR/RXR\(\alpha\) 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\(\alpha\) heterodimers.

5. Conclusion

In this paper, we identified CAR/RXR\(\alpha\)-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\(\alpha\) heterodimer by EMSAs. Furthermore, we identified 17 regulatory polymorphisms on the CAR/RXR\(\alpha\)-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\(\alpha\) heterodimers.

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Declaration of Interest

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