**Cross-species hybridization of woodchuck hepatitis virus-induced hepatocellular carcinoma using human oligonucleotide microarrays**

Paul W Anderson, Bud C Tennant, Zhenghong Lee

**Abstract**

**AIM:** To demonstrate the feasibility of using woodchuck samples on human microarrays, to provide insight into pathways involving positron emission tomography (PET) imaging tracers and to identify genes that could be potential molecular imaging targets for woodchuck hepatocellular carcinoma.

**METHODS:** Labeled cRNA from woodchuck tissue samples were hybridized to Affymetrix U133 plus 2.0 GeneChips®. Ten genes were selected for validation using quantitative RT-PCR and literature review was made.

**RESULTS:** Testis enhanced gene transcript (BAX Inhibitor 1), alpha-fetoprotein, isocitrate dehydrogenase 3 (NAD+) beta, acetyl-CoA synthetase 2, carnitine palmitoyltransferase 2, and N-myc2 were up-regulated and spermidine/spermine N1-acetyltransferase was down-regulated in the woodchuck HCC. We also found previously published results supporting 8 of the 10 most up-regulated genes and all 10 of the 10 most down-regulated genes.

**CONCLUSION:** Many of our microarray results were validated using RT-PCR or literature search. Hence, we believe that woodchuck HCC and non-cancerous liver samples can be used on human microarrays to yield meaningful results.

**Key words:** Cross-species hybridization; Gene expression; Woodchuck hepatitis virus; Hepatocellular carcinoma; Woodchuck; Marmota monax

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**INTRODUCTION**

Patients with chronic hepatitis originating from infection with hepatitis B virus (HBV) are among those with the highest risk for hepatocellular carcinoma (HCC). The role of HBV in hepatocarcinogenesis has been studied by many investigators, but still not entirely understood. The first case of hepatocellular adenomas in a woodchuck was reported by Fox in 1912[1]. The model of hepatitis-induced HCC was later described by Summers in 1978[2] and is now an accepted animal model for studying HBV-induced HCC[3]. We have performed PET imaging with various positron-emitting radionuclide-labeled imaging tracers involved in the woodchuck hepatitis virus (WHV)-induced HCC animal model (details of the imaging study will be reported separately). Early studies in this project include investigating the genetic basis of pathways involving the tracers and looking for new genetic targets for molecular imaging.

In the past decade, microarray experiments have paved the way for thorough studies of gene expression. To date there has been no commercially available microarray to study gene expression in the woodchuck largely because the genome of this species has not been sequenced and it is not among the common animal models. Cross-species hybridization analysis is one of the methods to investigate genetic regulation of unusual species. Some authors have succeeded in using porcine samples on human Affymetrix GeneChips®[4,5]. One group has demonstrated the feasibility of using normal woodchuck samples on commercially available human nylon membrane arrays that have since been discontinued[6].

To the best of our knowledge, there have been no studies that used woodchuck samples on human...
oligonucleotide arrays to explore differential gene expression in non-cancerous woodchuck liver versus HCC. In this study, we analyzed the woodchuck gene expression using human Affymetrix GeneChips® with validation of differential expression of selected genes with quantitative RT-PCR and literature search.

**MATERIALS AND METHODS**

**RNA isolation**

All animals received humane care and the study protocol complies with the institutional guidelines. Woodchucks were euthanized after PET imaging and the livers were immediately removed from the animal. A pathologist separated non-cancerous liver and HCC. Tissues were snap-frozen in liquid nitrogen and stored at -80 °C. RNA was extracted with RNeasy Midi Kit from Qiagen (Valencia, CA) according to the recommended protocol. An RNA integrity number (RIN) was found using a Bioanalyzer 2100 from Agilent (Palo Alto, CA).

**Preparation of cRNA and microarray hybridization**

RNA analyses were performed in the Gene Expression Array Core Facility at Case Western Reserve University. cRNA was prepared and hybridized to Affymetrix Human U133 plus 2.0 GeneChips® (Santa Clara, CA) according to the manufacturer’s instructions. Microarray results were delivered in the form of cab files.

**RT-PCR**

Available sequence information for woodchuck genes was found in the nucleotide database in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). These sequences were put into Applied Biosystems (Foster City, CA) for determination and production of optimal Custom Taqman® Gene Expression Assays. The Taqman® primer and probe sequences for our genes of interest are listed in Table 1. The Taqman® Assays and total RNA were given to the Gene Expression Array Core Facility. Assays were run in triplicate in a 15 µL reaction volume in a 384-well plate using a PRISM® 7900HT Sequence Detection System from Applied Biosystems (Foster City, CA). The RT-PCR assay consisted of a 2-minute incubation at 50 °C, a 10-minute incubation at 95 °C followed by 40 cycles of a 15-second incubation at 95 °C, and then a 1-minute incubation at 60 °C. Results were provided to us as values relative to a baseline sample. HCC was compared with the non-cancerous baseline sample from each animal, and > 2.5 or < 2.5 fold gene expression was considered up- or down-regulated, respectively.

**RESULTS**

**Quality control**

The first step in determining the usefulness of the results from a microarray study is to examine the quality control data. Table 2 shows some of RNA and microarray quality control data that we examined in this study. Two sample t tests were performed to see if there were differences between normal liver samples and HCC samples in any of the categories listed in Table 2. The “Percentage of

**Table 1  RT-PCR primer and probe sequences for select genes**

| Target gene                  | Forward primer sequence | Reverse primer sequence | Reporter probe sequence |
|------------------------------|-------------------------|-------------------------|-------------------------|
| Testis enhanced gene transcript (BAX inhibitor 1) (TGT) | GCACCTGTAATTGTGCGAACAG | ACTTCCCATGGACTCTCCTCCTC | CTCCACCCCTCAGTGCC |
| Acetyl-CoA synthetase 2 (thiokinase) (ACAS2) | TGCTGAGGACCCTCTCCTCAT | AGCATGGAACCCCTCAAGG | CCACAGGCAACCCCA |
| Alpha-fetoprotein (AFP) | AGCTGAGGACTCCGGAAGGTAAC | AGGCTGTCATTGCAGATTTCTCT | CTCAGGACCTAGTCTCC |
| C-myc (MYC) | TGCTGAGGACTCCGGAAGGTAAC | AGGCTGTCATTGCAGATTTCTCT | CTCAGGACCTAGTCTCC |
| Hexokinase (glucokinase regulatory protein) (GCKR) | ATGCTGACCGGGTCTTCT | CTGGCAGCATGTCTCC | CTCAGGACCTAGTCTCC |
| Isocitratedehydrogenase 3 (NAD+) beta (IDH3B) | TCTCACCCGATTGCAAATGTTG | CTCAGGACCTAGTCTCC | CTCAGGACCTAGTCTCC |
| Fatty-acid-Coenzyme A ligase, very long-chain 1 (FACVL1) | GCGGGATGACACAGCAAA | TCTTTCTGGAATGTTACCTTCTCCTC | TCTTAAAAATGAACTTCCC |
| Spermidine/spermine N1-acetyltransferase (SSAT) | TTTATGCAACCATCGGCTTCT | CACTGGACCTCCGCAAGTG | CCACTACCAACTCAG |
| Carnitine palmitoyltransferase II (CPT2) | TGGCTCTCTCCAGGGGTCCT | TCTTTCTGGAATGTTACCTTCTCCTC | TCTTAAAAATGAACTTCCC |
| N-my c 2 (NMYC2) | GAGGCCGCTGAGTGGAT | GCCACCTGTAATTGTGCGAACAG | CTCCACCCCTCAGTGCC |

rt tests were performed to see if there were differences between normal liver samples and HCC samples in any of the categories listed in Table 2.
Genes Called Present” and the “Scaling Factor” showed a statistically significant difference ($P < 0.05$) between the normal and the tumor samples. Housekeeping control and spiked control genes were qualitatively examined to have similar signal values and similar 3’-to-5’ ratios across all of the samples.

**Significantly changed genes**

A number of the genes was found to be significantly changed in the HCC tissues compared with the normal portion of the liver from the same animal (Table 3). Sixty-six genes were found significantly changed in all three animals and 286 genes changed in 2 out of the 3 animals. Some genes with multiple probes on the microarray were also markedly changed. Of the 18 gene probes that were down-regulated in all 3 tumors, 5 presented metallothioneins, 3 for early growth response 1, and 2 for spermidine/spermine N1-acetyltransferase. Five genes were up-regulated in 2 probes in all 3 tumors: H2A histone family member Z, F-box protein 9, a disintegrin and metalloproteinase domain 10, butyrate-induced transcript 1, and ribophorin II.

We studied further into the 10 most up- and down-regulated genes that were common to all three HCC versus normal liver sample pairs (Table 4). Eight of the top 10 up-regulated genes found previously in literature are in agreement with our findings[7-14]. We were unable to find any publication describing the up-regulation in any cancer for 2 of these genes, one of which was an open reading frame, but found published data supporting all of the top most down-regulated genes[15-21].

One group used normal human and normal woodchuck liver samples on human nylon membrane arrays[6]. To compare our results to theirs, we looked into 48 genes which were found to be commonly expressed in both normal human and normal woodchuck livers. We used 2 normal woodchuck liver samples from uninfected woodchucks on the Affymetrix human U133 plus 2.0 GeneChip® and found 21 of the 48 previously reported genes to be expressed. We were unable to find any probes on the GeneChip® for 3 of the 48 genes. We also explored the normal portion of the liver from 3 woodchucks with HCC and found 22 of the 48 genes were present in all 3 animals, 26 were present in 2 animals and 34 were present in at least 1 animal. Eighteen genes were present in both the normal liver from an uninfected woodchuck and the non-cancerous liver samples from all 3 woodchucks with HCC.

### Table 2  Quality control data for cross-species hybridization

| Samples | RNA integrity number | Average background | Noise | Percentage of genes called present | Scaling factor (scaled to 15) |
|---------|----------------------|-------------------|-------|-----------------------------------|-------------------------------|
| W904Nb  | 7.5                  | 33.750            | 1.00  | 9.0                               | 2.959                         |
| W361N   | 7.8                  | 34.170            | 0.97  | 9.5                               | 2.970                         |
| W380N   | 9.7                  | 32.089            | 0.93  | 9.9                               | 2.955                         |
| W904T   | 9.8                  | 36.880            | 1.07  | 10.3                              | 2.206                         |
| W361T   | 9.2                  | 35.720            | 1.05  | 10.5                              | 2.355                         |
| W380T   | 9.9                  | 31.610            | 0.94  | 10.8                              | 2.463                         |

The data is presented as HCC vs normal portion of the liver sample where the normal sample is the baseline.

### Table 3  Significantly changed genes

| Sample pair                  | Number of up-regulated genes | Number of down-regulated genes | Total number of changed genes |
|------------------------------|-------------------------------|--------------------------------|------------------------------|
| W904 vs normal               | 596                           | 205                            | 801                          |
| W361                         | 359                           | 242                            | 601                          |
| W380                         | 215                           | 84                             | 296                          |
| W904, W361                   | 142                           | 47                             | 189                          |
| W904, W380                   | 66                            | 3                              | 69                           |
| W361, W380                   | 19                            | 9                              | 28                           |
| W904, W361, W380             | 48                            | 18                             | 66                           |

RT-PCR

Ten genes were selected for RT-PCR analysis. Two genes were selected to validate the microarray analysis because the microarray analyses demonstrated they were significantly changed (TEGT and SSAT). RT-PCR was also performed on other genes due to their role in metabolic pathways and hepatocellular carcinogenesis. The RT-PCR results along with the corresponding microarray results are shown in Table 5. It should be noted that the terms “no change” and “undetermined” are not the same. A sample with no change shows similar expression in the normal and tumor samples, whereas the undetermined means that expression could not be measured in either the normal or tumor sample using RT-PCR.

DISCUSSION

PET imaging allows clinicians to view in vivo distribution and uptake of a radiolabeled compound, or imaging tracer. It is a useful diagnostic tool when a tracer is used that is highly specific to its target. We investigated the performance of various tracers and their metabolism regulated at multiple levels in the woodchuck model of hepatitis virus-induced HCC. We conducted a microarray study to explore the transcriptional regulation of interesting genes.

There is a logical rationale to explain the disparity in quality control data between normal and tumor samples. In the normal tissue, there were fewer genes called “Present” and a larger scaling factor as shown in Table 2. More genes were up-regulated in the tumors than were down-regulated. Therefore, more genes were expressed in the tumors compared with the non-cancerous portion of the liver, hence the larger ‘Percentage of Genes Called Present’. The fact that fewer genes were called ‘Present’ in the non-cancerous samples is the likely reason for the lower overall average signal intensity, which would also mean that a larger scaling factor is needed to reach a target value compared with the tumor samples.

To determine if our microarray results were consistent with other studies, we compared our results using normal woodchuck liver samples from 2 uninfected animals on Affymetrix U133 plus 2.0 GeneChips® to the results of another group that used similar uninfected woodchuck
liver samples on human nylon membrane arrays. Using the standard microarray protocol according to the manufacturer’s instructions, we have demonstrated a large overlap of results from the two studies as well as a high degree of similarity between the normal liver tissue from uninfected woodchucks and those of woodchucks with liver samples on human nylon membrane arrays.

### Table 4 Top 10 up- and down-regulated genes in common on all three tumor/normal sample pairs

| Fold change | Gene title                                                                 | Gene symbol | Location          | Process                                                      | Agreement |
|-------------|----------------------------------------------------------------------------|-------------|-------------------|--------------------------------------------------------------|-----------|
| 14.3294     | Stearoyl-CoA desaturase (delta-9-desaturase)                               | SCD         | 10q23-q24         | Fatty Acid Biosynthesis                                      | [7]       |
| 11.6297     | ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast) | ELOVL6      | 4q25              | Fatty Acid Biosynthesis                                      | [8]       |
| 7.7381      | Chromosome 9 open reading frame 41                                         | C0enf41     | 9q21.13           |                                                               | none      |
| 5.1036      | Cytochrome P450, family 51, subfamily A, polypeptide 1                      | CYP51A1     | 7q21.2-q21.3      | Electron Transport/ Cholesterol Biosynthesis                 | [9]       |
| 4.1746      | H2A histone family, member Z                                               | H2AFZ       | 4q24              | DNA Replication                                              | [10]      |
| 3.4227      | H2A histone family, member Z                                               | H2AFZ       | 4q24              | DNA Replication                                              | [10]      |
| 3.3977      | TATA box-binding protein-like protein 1, TBP-like 1                        | TBPL1       | 6q22.1-q22.3      | Transcription                                                | none      |
| 2.9307      | Diacylglycerol O-acyltransferase homolog 2 (mouse)                         | DGAT2       | 11q13.3           | Triacylglycerol biosynthesis                                 | [11]      |
| 2.8134      | ARP2 actin-related protein 2 homolog (yeast)                              | ACTR2       | 2p14              | Protein Binding/Structural Molecule Activity                 | [12,13]   |
| 2.8134      | Calcium binding protein P22                                                | CHP         | 15q13.3           | Potassium Ion Transport/Small GTPase Mediated Signal Transduction | [14] |
| -3.6151     | Metallothionein 1H                                                         | MT1H        | 16q13             | Metal Ion Binding                                            | [15,16]   |
| -3.6353     | Metallothionein 2A                                                         | MT2A        | 16q13             | Metal Ion Binding                                            | [15,16]   |
| -3.9071     | Metallothionein 1X                                                         | MT1X        | 16q13             | Metal Ion Binding                                            | [15,17]   |
| -4.1870     | EST similar to early growth response 1                                    |             |                   |                                                               | [18,19]   |
| -4.5774     | Metallothionein 1F (functional)                                            | MT1F        | 16q13             | Metal Ion Binding                                            | [15,16]   |
| -4.9625     | Early growth response 1                                                    | EGR1        | 5q31.1            | Regulation of Transcription                                  | [18,19]   |
| -5.5956     | Metallothionein 1F (functional)                                            | MT1F        | 16q13             | Metal Ion Binding                                            | [15,16]   |
| -5.7266     | Sprouty homolog 2 (Drosophila)                                             | SPRY2       | 13q31.1           | Cell-Cell Signaling/ Development/ Organogenesis/ Regulation of Signal Transduction | [20] |
| -10.3842    | Early growth response 1                                                    | EGR1        | 5q31.1            | Regulation of Transcription                                  | [18,19]   |
| -24.2139    | Insulin-like growth factor binding protein 2, 36 kDa                      | IGFBP2      | 2q33-q34          | Regulation of Cell Growth                                    | [21]      |

1This is the average fold change for all 3 HCC vs normal sample pairs.

### Table 5 RT-PCR and microarray results

| Gene                        | Process                        | W904 RT-PCR | W904 Microarray | W361 RT-PCR | W361 Microarray | W380 RT-PCR | W380 Microarray |
|-----------------------------|--------------------------------|-------------|-----------------|-------------|-----------------|-------------|-----------------|
| Testis enhanced gene transcript (BAX inhibitor 1) (TEGT) | Negative regulation of apoptosis | No change   | Up              | Up           | Up              | Up           | Up              |
| Spermidine/spermine N1-acetyltransferase (SAT) | Polyamine metabolism | Down        | Down            | Down         | Down            | No change   | Down            |
| Acetyl-CoA synthase 2 (thiokinase) (ACAS2) | Metabolism, lipid biosynthesis | Up          | Up              | Up           | Up              | Up           | No change       |
| Alpha-fetoprotein (AFP) | Immune response, transport      | Up           | No change       | Up           | No change       | Up           | Up              |
| Isocitrate dehydrogenase 3 (NAD+) beta (IDH3B) | Tricarboxylic acid cycle       | Up           | Up              | Undetermined | No change       | Up           | Up              |
| Carnitine palmitoyltransferase II (CPT2) | Lipid metabolism, fatty acid metabolism, transport | Up           | No change       | Up           | No change       | No change   | No change       |
| N-myc 2 (NMYC2) | Unknown                        | Undetermined | N/A             | Up           | N/A             | Undetermined | N/A             |
| C-myc (CMYC) | Regulation of transcription, DNA-dependent | Up           | Up              | Down         | No change       | No change   | No change       |
| Fatty-acid-Coenzyme A ligase, very long-chain 1 (FACVL1) | Lipid metabolism, fatty acid metabolism | Down        | Down            | Undetermined | Down            | No change   | No change       |
| Hexokinase (GCKR) | Carbohydrate metabolism        | Undetermined | No change       | Undetermined | Up              | No change   | No change       |

1RT-PCR results are for ACAS2, microarray results are for a hypothetical protein similar to ACAS2. 2RT-PCR results are for c-myc, microarray results are for a c-myc binding protein and a downstream target. 3RT-PCR results are for glucokinase regulatory protein, microarray results are for an EST similar to hexokinase domain containing 1.
HCC.

We found supporting data for 8 of the top 10 up-regulated genes and all 10 of the top 10 down-regulated genes (Table 4). The top two up-regulated genes (steroyl-CoA desaturase and elongation of long chain fatty acids, family member 6) are involved in fatty acid biosynthesis, which suggests that fatty acid synthesis is up-regulated in HCC compared with the surrounding non-cancerous liver tissues in woodchucks.

Due to the limited number of woodchuck genes that have been sequenced (fewer than 600), we could not perform RT-PCR on all of the genes of interest, and in some cases the sequence was not available for the exact gene. FACVL1, AFP, IDH3B, CPT2, and SSAT had specific probes on the microarray; ACAS2, CMYC, GCKR, and TEGT were similar to what we had microarray probes for; and there was no probe on the microarray for NMYC2. The microarray results showed expression of a hypothetical protein similar to the 5’ region of ACAS2, a c-myc binding protein, a c-myc downstream target, an EST similar to hexokinase domain containing 1, and an EST similar to TEGT. Of the 66 probes on the microarrays that significantly changed in all 3 pairs of samples, we validated 2 genes with available sequence information. TEGT was up-regulated and SSAT was down-regulated in the tumors as shown by both the microarray and RT-PCR analysis. TEGT is a negative regulator of apoptosis and has been shown to be up-regulated in cancer cells and SSAT, which is the rate limiting step in polyamine catabolism, has been shown to be induced at the transcriptional level to sensitize tumors for adjuvant treatment.

We also investigated genes involved in carcinogenesis and various metabolic pathways. IDH3B, the rate-limiting enzyme in the tricarboxylic acid cycle, was up-regulated in 2 probes in the same 2 tumors and expression validated with RT-PCR. A hypothetical protein similar to ACAS2, which catalyzes the conversion of acetate to acetyl-CoA for use in many metabolic and biosynthetic pathways, was up-regulated in 2 tumors according to the microarray results and shown to be up-regulated in all 3 tumors with RT-PCR. ACAS2 is of particular relevance because of the usage of the PET imaging tracer [11C]-Acetate. Currently, it is not entirely known what pathways are responsible for the high uptake of acetate which can be seen in HCC compared with surrounding normal liver tissues in the PET images. We have demonstrated high up-regulation of the enzyme that catalyzes the formation of acetyl-CoA from acetate, so we hypothesize that this may be the first step involving [11C]-Acetate.

An EST similar to hexokinase was up-regulated in 1 tumor according to the microarray results, and glucokinase regulatory protein (GCKR) was up-regulated in a different tumor based on RT-PCR. Sequence information for the woodchuck glucokinase gene was not available, so RT-PCR was performed on a GCKR, a negative regulator of glucokinase. Glucokinase is important for its role in PET imaging of [18F]-fluorodeoxyglucose (FDG). Glucokinase catalyzes the phosphorylation of FDG, thereby trapping it in the cell according to previous kinetic modeling data. Up-regulation of glucokinase or down-regulation of GCKR would be expected to result in higher uptake of FDG in HCC compared with the non-cancerous liver tissues. However, our results were not conclusive and future work will require sequencing of the woodchuck hexokinase gene or examination of glucokinase regulation at the protein level.

CPT2, a protein that facilitates transport of long-chain fatty acids to the inner mitochondrial membrane for fatty acid metabolism, did not show any change according to the microarray results, but was found to be up-regulated in 2 of the 3 HCCs using RT-PCR. In the target sequence used to create the probes on the human microarray, there is a 436 residue overlap with 40.4% homology between the microarray CPT2 target sequence and the woodchuck CPT2 sequence used for RT-PCR. The lack of homology between the sequences might be the reason for not obtaining significant microarray results.

AFP, a serum marker for HCC in humans, was up-regulated in one tumor and showed no change in the other two on the microarrays, while it was up-regulated in all three tumors as measured by using RT-PCR. NMYC2, which was found highly expressed in 60% of woodchuck HCCs, was up-regulated in one tumor by using RT-PCR.

These results from using human Affymetrix GeneChips® provide a part of the foundation for our imaging work to explore the woodchuck model of virus-induced HCC. Future work will include more investigations of the regulation of pathways involving PET imaging tracers using enzyme assays and immunohistochemistry. Additional efforts will be focused on developing new radiolabeled antibodies and/or ligands for potential imaging targets found to be up-regulated in woodchuck HCC compared with surrounding non-neoplastic woodchuck hepatic tissues.

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