Nat. Volatiles & Essent. Oils, 2021; 8(4): 13414-13422

NVEO
Natural Volatiles &
Essential Oils

Molecular Genetic Analysis- Invivo Model Of Annonareticulata Leaves Against Den Induced Hepatocellular Carcinoma

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ABSTRACT

Cytochrome P450 2E1 (CYP2E1) plays an important role in both alcohol-induced and immune-mediated liver injury. However, the mechanism underlying CYP2E1 transcriptional regulation has not been clarified. In the present study, the immune gene expression profile was determined in rat liver samples and surrounding tissues by the synthesis of mRNA and cDNA synthesis. The gene and protein expression profile of CYP2E1 in response to treatment with AqAr and AgNPs-AqAr in the liver of experimental rats are discussed. However, the specific changes to CYP2E1 following immune-mediated and alcohol-induced liver injuries, the role and underlying mechanism of CYP2E1, and whether the injury process can be influenced by the selective regulation of CYP2E1 or CYP2E1-dependent oxidative stress have not yet been determined (1).

Keywords: Hepatocellular carcinoma, CYP2E1 gene, Annona reticulata, silver nanoparticles, gene expression

INTRODUCTION

Liver is the largest and most important organ in the human body, which plays a pivotal role in the regulation of physiological process. The internal structure is made up of nearly one lakh small hexagonal functional units known as lobule. Each lobule consists of central vein surrounded by 6 hepatic portal vein and 6 hepatic arteries, and these vessels are connected by capillary like tubes called sinusoids (2).

Over the past 20 years, cancer research has identified molecular abnormalities leading to a better knowledge of the oncogenic process. A new era is upon us with recent advances in powerful

molecular technologies, such as DNA microarrays and PCR-based arrays or proteomics, which have generated an explosion of studies aiming to translate the molecular information into the clinical practice in oncology. These studies have identified molecular markers of early diagnosis, predictors of aggressive biological behavior leading to metastases, and even molecular signatures defining prognosis, measured as survival or recurrence after 'curative' treatments (3). Based on these advances, new molecular classifications have been proposed for acute myeloid leukemia, large-B-cell lymphoma, lung cancer and breast cancer, amongst others. These studies generally include a cohort of patients followed for years after treatment, in which fresh frozen tissue enables researchers to analyze RNA expression profiles, which might link gene clusters with good or poor prognosis. After an initial attempt to identify the predictive gene signature, validation studies are conducted (4). Ideally, these studies are performed in a new cohort of patients to reproduce the predictive power of the gene signature when analyzed together with other well-known clinical variables. This has been the case for breast cancer and large-B-cell lymphoma. In breast cancer, a gene signature of 70 genes was identified by using DNA microarrays and was prospectively validated as an independent predictor of distant metastases, along with other known clinical variables including age, lymph node involvement, vascular invasion, tumor diameter and chemotherapy. In another landmark study of large-B-cell lymphoma, genes previously recognized by microarray studies were further tested by quantitative real-time PCR in a new cohort of patients with the disease, resulting in a selection of six genes that were independently associated with survival, along with the known International Prognostic Index of this neoplasm. Thus, new molecular classifications in all types of cancer are awaited (5).

CYP2E1 mRNA, protein, enzyme activity and induction by ethanol have been found in neural and glial cells of the CNS. In a study of human brains obtained from smoking and non-smoking alcoholics, increased expression of CYP2E1 protein was found in granular cells of the dentate gyrus and pyramidal cells of the hippocampus. Animal studies have found a higher concentration of CYP2E1 protein in astrocytes and glial cells following inflammatory induction. The identification of CYP2E1 in dopaminergic neurons of the substantia nigra suggests that it may be involved in dopamine regulation and have potential implications in Parkinson disease (6).

Annona reticulata is a small deciduous or semi-evergreen tree in the plant family Annonaceae and part of the Annona's group. It is best known for its fruit, called custard apple, small deciduous or semi-evergreen tree reaching 8 meters to 10 meters tall with an open, irregular crown. The slender leaves are hairless, straight and pointed at the apex (in some varieties wrinkled), 10 centimeters to 20 centimeters long and 2 centimeters to 7 centimetres wide. Traditionally the plant has been employed for the treatment of epilepsy, dysentery, cardiac problem, parasite and

worm infestations, constipation, hemorrhage, bacterial infection, dysuria, fever, ulcer and as insecticide. Bark is a powerful astringent and used as a tonic whereas leaves used for helminthiasis treatment. The different phytoconstituents have been identified from various part of this plant; the leaves of Annona reticulata contain a wide range of chemicals like amino acids, proteins, carbohydrates, alkaloids, steroids, tannins, glycosides and phenolics (7).

MATERIALS AND METHODS

Gene Expression Analysis

Isolation of mRNA

The total RNA was isolated from liver and mammary tissues and homogenized with 1 mL of TRIZOL reagent per 50 to 100 mg of tissue using a glass-Teflon or power homogenizer. The sample volume should not exceed 10% of the volume of TRIZOL reagent used for the homogenization. To all the tubes 0.2 mL of chloroform per 1 mL of TRIZOL reagent was added. Vortex the samples vigorously for 15 sec and incubate them at room temperature for 2 to 3 min. Centrifuge the samples at 12,000 x g for 15 min. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase (8). Carefully transfer the upper aqueous phase to another fresh tube without disturbing the interphase layer. To precipitate the RNA from the aqueous phase, 0.5 mL of isopropyl alcohol was added and the sample was incubated samples at 15 to 30ºC for 10 min and centrifuge at 12,000 x g for 10 min at 2 to 4^{o}C. After centrifugation, a gel- like RNA precipitate was formed on the side and bottom of the tube. Then remove the supernatant completely and wash the RNA pellet with 75% ethanol. Mix the samples by vortexing and the tubes were centrifuged at 7,500 x g for 5 min at 2 to 8^{o}C. Repeat the above washing procedure and remove all leftover ethanol. The tubes were kept under air-dry or vacuum dry for 5-10 min and re-suspend the RNA pellet in 200 –300 µL DEPC –TE which was stored at -4ºC for gene expression studies (9).

Synthesis of cDNA from mRNA

First Strand cDNA Synthesis

The complementary DNA was synthesized from total ribonucleic acid by first strand complementary DNA synthesis kit, and then ribonucleic acid was degraded with the assistance of Ribonuclease H treatment of reaction mixture. The amount of the reaction mixture was evaluated through the nano-drop1000 (Thermo fisher, Germany) before loading into the agarose gel electrophoresis (10). Just about 10 pmol to 1ng of messenger RNA was taken to synthesize the complementary DNA. The oligo

dT (Thymine) primer set was used, that is particular to M-MuLV protein reverse transcriptase enzyme for synthesis from the poly A tail of mRNA accomplished by the primary strand complementary DNA synthesis kit (New England Bio labs, England) (11).

First Strand cDNA Synthesis was performed by adding the components followed.

TABLE: 1

Total RNA	1–6µl (10pg–1µg)
d (T) 23VN	(50µM) 2µL
Nuclease-free H_2O	Variable
Total Volume	8µL

From the above table 1 it clearly explains that total RNA value contributing can be determined as 1–6µl (10pg–1µg), for d (T) 23VN the value can be obtained as (50µM) 2µL, it also has nuclease-free H_2O as variable and finally total volume as 8µL (12).

TABLE: 2

M-MuLV Reaction Mix	10µL
M-MuLV Enzyme Mix	2µL
M-MuLV Reaction Mix	10µL
H_2O	2µL

From the above table 2 it consists of three types of mixes/ M-MuLV Reaction Mix have a contribution of 10µL, Enzyme Mix contributed as 2µL, another reaction Mix as 10µL and finally H_2O as 2µL (13).

Mix well ribonucleic acid sample and primer into clean ribonuclease free micro centrifuge tubes. The RNA was denatured for six min at 70°C and spins for a short time, then place duly on ice. This step was optional; however, but, it improves the DNA yield for long messenger RNAs and GC - rich RNA regions (14).

The negative control tube was incubating with 20µl of complementary DNA synthesis reaction (without primer) mixer at 42°C for one hour. Specific primer combination was used and incubated at 25°C for 5 min, before the 42°C incubation. The enzyme was inactivated at 80°C for 5 min. The reaction mixture was diluted to 50µL with addition of 30µL Milli-Q for PCR. The DNA product was kept at –20°C for downstream PCR amplification (15).

RESULTS AND DISCUSSION

Based on the necessity of the study the samples were segregated into following groups -

Groups
Group I –Control
Group II – DEN induced
Group III - DEN + AqAr
Group IV - DEN + AgNPs-AqAr
Group V - DEN + Cyclophosphamide

All the animals were sacrificed under mild ethyl ether anesthesia by cervical decapitation after the experimental stage. To confirm the protect effect, a molecular level genetic analysis was carried out.

MOLECULAR GENETIC ANALYSIS OF LIVER OF EXPERIMENTAL RATS

Gene and protein expression of liver samples of only one animal of each group was carried out.

EFFECT OF AqAr AND AgNPs–AqAr ON CYP2E1 GENE AND PROTEIN EXPRESSION PROFILE OF THE LIVER OF EXPERIMENTAL RATS

The gene and protein expression profile of CYP2E1 in response to treatment with AqAr and AgNPs-AqAr in the liver of experimental rats are presented in **Plate 1 and Figure 1.**

PLATE 1

Gene expression profile of CYP2E1

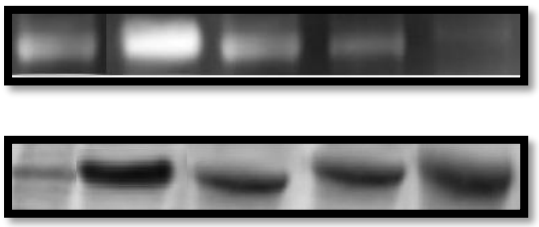
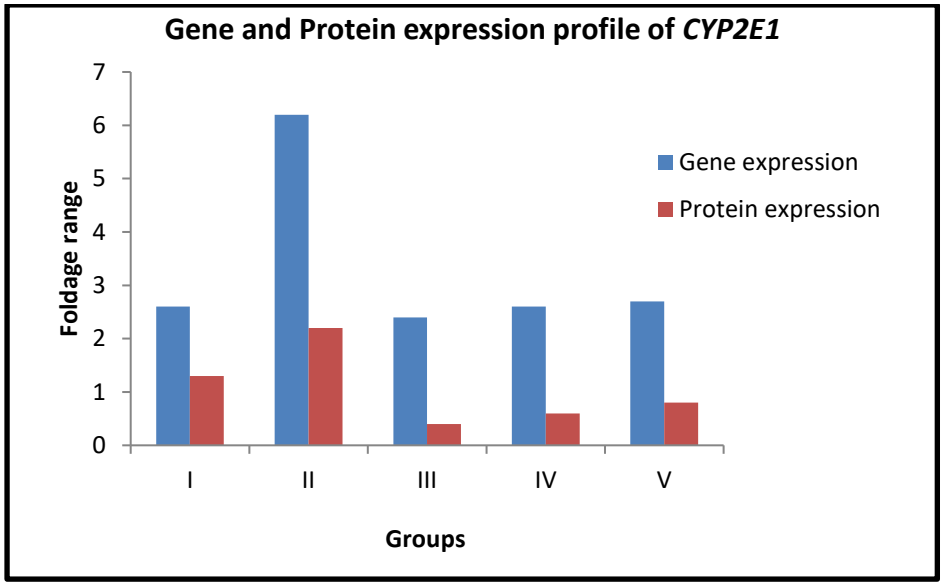


FIGURE 1: GENE AND PROTEIN EXPRESSION PROFILE OF CYP2E1



DISCUSSION

The hybridization result showed that CYP2E1 indeed only expressed in non-tumor liver tissues.An ideal support allowed effective immobilization of probe onto its surface, and robust hybridization of target with the probe. Nylon membrane, as a standard support used for making microarrays could hold more DNA. The hybridization and detection of membranes arrays cost much less. Here we used the nylon membranes as the matrix of cDNA arrays. The DNA on the membranes was denatured before cross-linking to the matrix by baking the array at 80°C. In many cases, the cDNA targets were chosen directly from cDNAs library of interest for DNA microarray manufacturing. The collection should include an aggregation of gene clones as many as possible. On the other hand, for certain purpose of an investigation, most genes on a large content array might not be necessary. The method to make cDNA microarray in this study arose from the concept of arrayed library. The cDNA plasmid library was transferred to the microplates to be the arrayed library, which was copied to the nylon membranes to make the DNA arrays after PCR amplification. Because most genes on the arrays did not differently express between samples, the tumor and normal tissues, for example, the sequencing expense of these genes could be saved. It was after the array hybridization, the genes that were detected to express differently would be selected from the arrayed library and were sequenced for further studies. In this study, most genes showed no differential expression between HCC and adjacent liver tissues. Only the differently expressed genes were sequenced for further investigations. CYP2E1 was identified as a liver profound but HCC silent gene.

P450 enzymes were found in almost all eukaryotes and prokaryotes. CYP2E1 enzyme had a molecular weight of 57 KD and the encoding gene was located on chromosome 10 spanning 11413 base pairs. CYP2E1 was found to play an important role in its polymorphism during tumor

occurrence. The significance of this polymorphism currently unclear. To date, the results in possible associations between CYP2E1 genetic polymorphisms and alcoholic liver disease susceptibility have been varied and often contradictory. Because hepatic CYP2E1 has been shown to activate various carcinogens, there has been interest in whether certain CYP2E1 polymorphisms might predispose to liver cancer. It was demonstrated that possession of the less common Rsa I/Pst I allele was associated with increased susceptibility to HCC.

Although CYP2E1 was said to be located in most tissues with the largest concentration in the liver, it was constitutively expressed in the liver and only induced to express in other tissues by treatment with acetone, ethanol, isoniazid and other compounds, many of which are substrates for the enzyme. This study showed that CYP2E1 expressed only in the liver but did not express in the other normal organs in human and rats. So it could be concluded that CYP2E1 is the liver-specific functional gene. Furthermore, CYP2E1 was found to lose expression in HCC. Northern blot analysis was performed to validate the result of chip experiment. CYP2E1 expressed at a high level in normal or cirrhotic tissues but was silent in HCC tissues from 14 cases of HCC. So CYP2E1 did not express in HCC. To further understand the expression of CYP2E1 in the initiation, promotion and progression of HCC, the DENA-induced rat HCC model was made to study the role of CYP2E1 in the process of HCC.

The development of rat HCC underwent the progression of liver lesion and cirrhosis until the tumour occurrence. The results showed that at the early stage of the rat model the expression level of CYP2E1 in the liver lesion tissues was slightly higher than that in normal rat liver in the first to third week. Because CYP2E1 was involved in the metabolism of nitrosamines, DENA might induce CYP2E1 expression. But along with the aggravation of liver lesion and development of cirrhosis, the expression level of CYP2E1 was gradually descended. After the progressing stage of HCC, CYP2E1 did not express as in human HCC. There were very low signals because at the late stages of HCC the tumor were grown sporadically and the samples were intermixed with cirrhotic tissues where CYP2E1 still expressed. The expression pattern of CYP2E1 in the HCC was worth of further studies. It is suggested that these differential expressions might help further understand the molecular genetics and gene regulation mechanism of HCC progression. Because CYP2E1 does not express in HCC cells, there might be a guided biological treatment for HCC with its potential substrate.

CONCLUSION

Plants are the sources of phytochemicals which can be used for treating infectious diseases particularly in most of the developing countries of the world. The phytochemicals serve as natural antibiotics which help the human body to fight infections and microbial invasion. In the present

study, the immune gene expression profile was determined in rat liver samples and surrounding tissues by the synthesis of mRNA and cDNA synthesis. The gene and protein expression profile of CYP2E1 in response to treatment with AqAr and AgNPs-AqAr in the liver of experimental rats are discussed. This approach allows a global characterization of immune function and is particularly suitable for the appraisal of selected gene signatures potentially useful as biomarkers for prognostic classification and therapeutic stratification

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