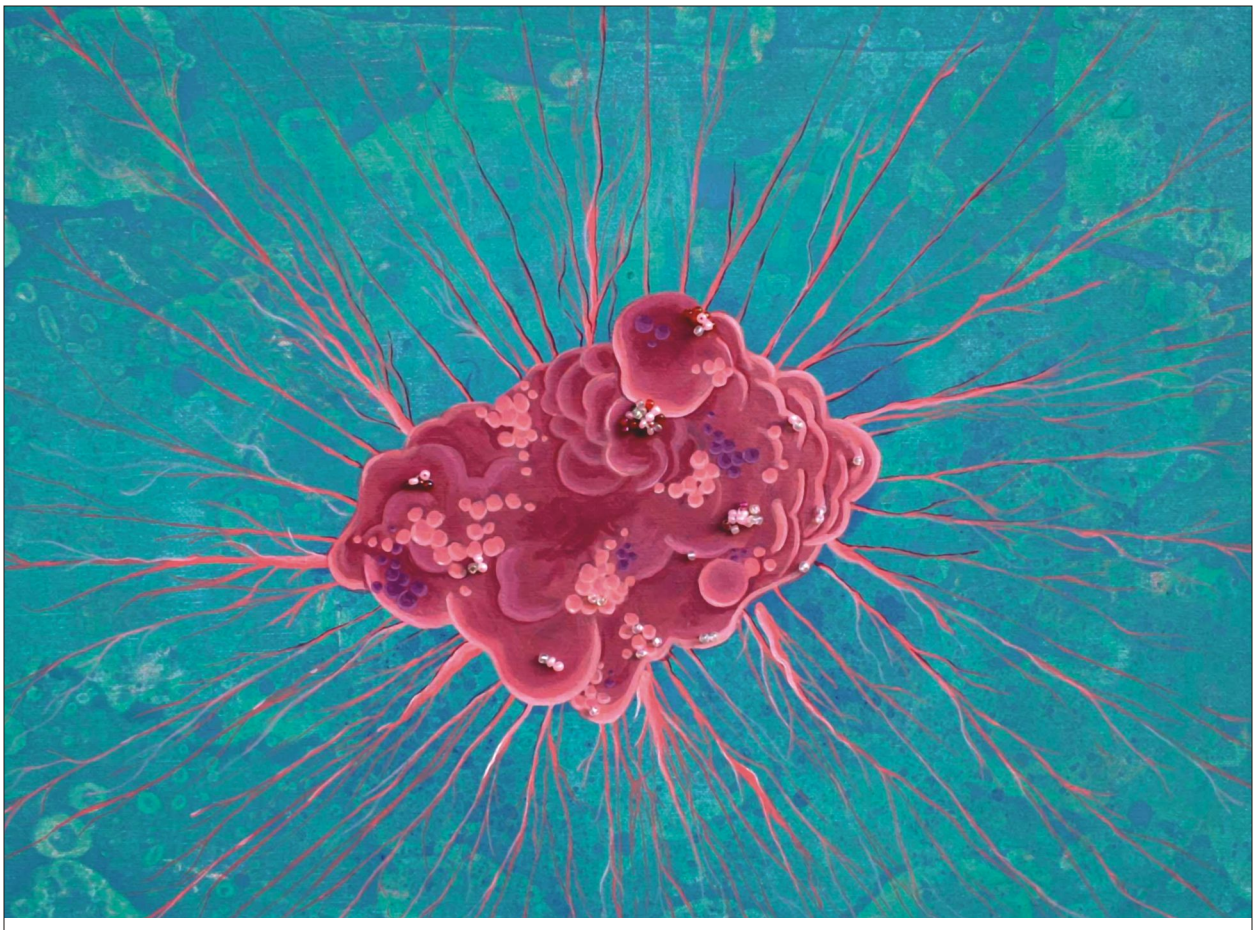

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Research Article
CYP2E1 and L-myc EcoRI Gene Polymorphisms and Heavy Metals Exposure in Hepatocellular Carcinoma Patients

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Abstract

Background and Objective: The liver is the main metabolic organ, carcinogen-metabolizing liver enzymes are the most important candidates that may influence hepatocarcinogenesis associated with heavy metal overload. This study was aimed at analyzing the genetic pattern regarding the proto-oncogene (L-myc) and the cytochrome (CYP2E1), which are involved in detoxification and oncogenesis, in both HCC patients and controls and to study the likelihood of each genetic pattern to develop HCC in the presence of heavy metals load. **Materials and Methods:** Total 171 hepatocellular carcinoma patients were included and 143 matched healthy controls in this study. Routine laboratory tests and heavy metals (lead, cadmium, arsenic and mercury) concentration were measured. Genetic polymorphisms was detected by a PCR-RFLP method. Student's t-test and SPSS software used for statistical analysis. **Results:** C2 allele and C2 containing genotypes of CYP2E1 showed a significant higher percentage in HCC group ($p = <0.001$). Distribution of different L-myc genotypes in HCC and control groups showed nonsignificant difference ($p = 0.140$). As regards the heavy metals concentration in carriers of different genotypes of both CYP2E1 and L-myc revealed no significant difference except for mercury which reported higher levels with the mutant homozygote genotype of L-myc ($p = 0.015$). **Conclusion:** There was no significant association between gene polymorphisms and heavy metal burden within HCC patients. It is also noted that CYP2E1 polymorphisms may increase HCC susceptibility while L-myc gene polymorphism showed non significant difference between HCC patients group and control group.

Key words: CYP2E1, L-myc EcoRI, gene polymorphisms, heavy metal, HCC, hepatocarcinogenesis, homozygote

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatocellular carcinoma is the most prevalent primary cancer of the liver. It is the fifth most frequent cancer in men and the seventh most common in women worldwide[1]. Liver cancer is the fourth most common cancer in Egypt and the second cause of cancer mortality in both sexes among the Egyptian population[2]. Much effort is now being paid to the field of prevention, screening and treatment programs[3].

Heavy metals as catalysts are involved in the oxidative deterioration of biological macromolecules via induction of Reactive Oxygen Species (ROS) and the development of free radicals. Accumulation of ROS can induce epigenetic mechanisms which are increasingly linked with exposure to heavy metals[4].

As the heavy metals are widespread environmental contaminants, they have been associated with many diseases, including cancer[5,6] . In a previous study held by the same team, a strong correlation was found between the development of HCC and the heavy metals burden in blood[7].

As the liver is the main metabolic organ, the SNPs related to genes encoding carcinogen-metabolizing liver enzymes should be the most important candidates for association analyses. As Cytochrome P-450 (CYP), the superfamily of mono-oxygenases is responsible for phase I enzyme detoxification reactions, it may be also responsible for the metabolic activation of toxic or carcinogenic compounds. Cytochrome P450 2E1 (CYP2E1) is a key microsomal enzyme that induces the generation of Reactive Oxygen Species (ROS)[8].

MYC gene family of transcription factors controls the key cellular processes such as cell proliferation, apoptosis, differentiation and tumor development and its activation is the most frequent molecular alteration observed in human cancer, others have recently observed that the transcription of tumor suppressor genes are inhibited by MYCs[9].

*Eco*RI polymorphism resulting from a T/G variation located in the second intron of the L-myc gene produces Short (S) and Long (L) fragments after enzymatic digestion. This was the first genetic variation found to be associated with the outcome in certain cancer patients. Since then, contradictory data have been reported in different tumor types[10].

To the best of our knowledge, no studies were carried out involving both polymorphisms and their influence on hepatocarcinogenesis associated with heavy metal overload. The current study was designed aiming to assess whether the selected SNPs of CYP and L-myc (EcoR1) genes influence individual susceptibility to HCC, considering their combination and interaction with blood heavy metal load.

MATERIALS AND METHODS

Study area: The study was carried out at Mansoura University hospitals and laboratories, Egypt and the Department of Plant Pathology, Faculty of Agriculture, Mansoura University, Mansoura, Egypt from 2012-2016 .

Subjects: The study was conducted on 171 primary Hepatocellular Carcinomas (HCC) patients recruited from inhabitants of the North Delta region in Egypt attending the out-patients and in-patients clinics of the Internal Medicine Department, Mansoura University Hospital. The diagnosis was based on clinical examination, positive imaging studies ,the α-fetoprotein concentration of at least 500 ηg mL^{-1} and sometimes confirmed by fine-needle aspiration cytology and histological examination. Cases were compared to 143 matched healthy individuals resident in a relatively non-heavy metal-contaminated area serving as controls. They were proven healthy by clinical examination, laboratory and imaging tests.

For all subjects enrolled in the study, an explanation of the procedures and an informed consent was obtained. Approval of the Research Ethics Committee of Mansoura University was also obtained.

Sample collection: After overnight fasting, 8 mL of venous blood was collected from each subject and were divided into aliquots, 4 mL was transferred to a plain vacutainer test tube and were left for 20 min then clear serum was separated and kept at -20°C to be used for analysis of routine laboratory tests (liver, kidney function tests) and α-fetoprotein (for patients only). About 2 mL was delivered to EDTA metal- free vacutainer tube, kept at -20°C till analysis of heavy metal concentration by atomic absorption spectrophotometry. Total 2 mL was delivered to an EDTA metal- free vacutainer tube kept at -80°C for DNA extraction to be used in molecular studies.

Blood heavy metal assay: The analysis was conducted by atomic absorption spectrometry "Buck Scientific Accusys "214" atomic absorption spectrophotometer, USA". The results were reported in mg L^{-1} (ppm)[11]. A mixture of HNO_3:H_2SO_4:$HClO_4$:blood in the ratio 5:1:1:1 was left for digestion at a temperature of 180-190°C for 3-4 hrs. After cooling, the solution was completed up to 50 mL total volume using acidified distilled water.

DNA extraction and analysis: DNA was extracted from leucocytes using the Whole Blood Genomic DNA Extraction Kit (Fermentas, USA, #K0781).

RsaI mutation at the 5' flanking region of CYP2E1 and EcoRI L-myc proto-oncogene polymorphisms were studied using PCR-RFLP technique as described by Ladero *et al.*[12] and Taylor *et al.*[13], respectively .

The RsaI genotype was estimated by the presence of digests of 421 and 144 bp (allele Cl) or 565 bp (allele C2), where the RsaI genotype digests of 421 and 144 bp (allele Cl) or 565 bp (allele C2).

The LL genotype shows a single uncut band (145 bp DNA). The SS homozygote undergoes complete digestion of the PCR fragment because the EcoR1 site is present in the S variant (104 and 41 bp). LS heterozygotes combine the features of both L and S variants (145-104-41 bp).

Statistical analysis: Statistical analysis was performed using SPSS software (Chicago, USA). Percentage distribution of the genotypes of CYP2E1 and L-myc EcoRI gene polymorphism was determined and the differences in genotype and allele frequencies between the specified groups were compared using the Pearson's chi-square (χ^2). The risk estimates for genotype contrasts were obtained by computing Odds Ratio (OR) and 95% Confidence Interval (CI). The genotype distribution patterns were assessed using the Hardy-Weinberg equation system and all control groups were in equilibrium Student's t-test was used to compare the means of the two groups when both of them are normally distributed. Mann Whitney test was used to compare the variables across two groups when one or both of them were abnormally distributed. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

The distribution of CYP2E1 genotypes and alleles in HCC patients and control subjects revealed that all control subjects are of C1C1 genotype. The frequency of C1C1, C1C2 and C2C2 in HCC patients were 90, 6 and 5%, respectively. A significantly higher frequency of C2 allele carriers (26) in HCC cases when compared to control subjects (0) as shown in Table 1. While, comparing the distribution of L-myc genotypes and alleles in HCC patients versus the control subjects revealed a non-significant difference (Table 2).

No significant difference presents regarding the Heavy metal levels in different subjects with different CYP2E1 genotypes in the studied HCC group as shown in Table 3. Also, no significant difference is found in Heavy metal levels in different subjects with different L-myc genotypes in the studied HCC subjects except for mercury (p = 0.015) on comparing homozygote wild and mutant genotypes (LL vs SS) (Table 4).

Table 1: Distribution of CYP2E1 genotypes and alleles in HCC patients and control subjects

	Control (n = 143)		HCC (n = 171)				
CYP	Number	Frequency	Number	Frequency	p-value	OR	95% CI
C1C1	143	1	153	0.90	<0.001	0.52	0.46-0.58
C1C2	0	0	10	0.06	0.002	1.89	1.70-2.10
C2C2	0	0	8	0.05	0.009	1.88	1.69-2.09
C1	286	1	316	0.92	<0.001	1.91	1.77-2.06
C2	0	0	26	0.08			

CYP: Cytochrome P, HCC: Hepatocellular carcinoma, n: Number, Freq: Frequency, p: Significance value

Table 2: Distribution of L-myc genotypes and alleles in HCC patients and control subjects

	Control (n = 143)		HCC (n = 171)				
L-myc	Number	Frequency	Number	Frequency	p-value	OR	95% CI
SS	26	0.18	33	0.19		Reference	
SL	65	0.46	94	0.55	0.67	1.14	0.62-2.08
LL	52	0.36	44	0.26	0.22	0.67	0.35-1.30
SL+LL	117	0.82	138	0.81	0.80	0.93	0.53-1.64
S	117	0.41	160	0.47	0.14	0.79	0.57-1.08
L	169	0.59	182	0.53			

HCC: Hepatocellular carcinoma, n: Number, Freq: Frequency, p: Significance value

Table 3: Heavy metals level in HCC subjects according to CYP2E1 genotypes

Heavy metals (mg L^{-1})		HCC patients (n = 171) C1C1 (n = 130)	C1C2 (n = 10)	C2C2 (n = 8)	P
Mercury	Mean SD (minimum-maximum)	0.14±0.12 (0.002-1.05)	0.07±0.03 (0.002-0.22)	0.105±0.051 (0.002-0.27)	C1C1 vs. C1C2 = 0.23 C1C1 vs. C2C2 = 0.62 C1C2 vs. C2C2 = 0.71
Arsenic	Mean SD (minimum-maximum)	0.26±0.12 (0.002-1.71)	0.22±0.10 (0.002-0.791)	0.17±0.08 (0.002-0.22)	C1C1 vs. C1C2 = 0.94 C1C1 vs. C2C2 = 0.74 C1C2 vs. C2C2 = 0.83
Cadmium	Mean SD (minimum-maximum)	0.05±0.018 (0.002-0.26)	0.021±0.01 (0.002-0.05)	0.02±0.01 (0.002-0.04)	C1C1 vs. C1C2 = 0.20 C1C1 vs. C2C2 = 0.41 C1C2 vs. C2C2 = 0.90
Lead	Mean SD (minimum-maximum)	0. 70±0.33 (0.01-1.37)	0.53±0.18 (0.35-0.8)	0.81±0.30 (0.35-1.2)	C1C1 vs. C1C2 = 0.11 C1C1 vs. C2C2 = 0.41 C1C2 vs. C2C2 = 0.09

P, HCC: Hepatocellular carcinoma, n: Number, p: Significance value, vs: Versus

Table 4: Heavy metals level in studied HCC subjects according to L-myc genotypes

Heavy metals (mg L^{-1})		HCC patients (n = 171) LL	LS	SS	P
Mercury	Mean SD (minimum-maximum)	0.20±0.10 (0.002-1.02)	0.13±0.10 (0.002-1.05)	0.07±0.03 (0.002-0.23)	LL vs. LS = 0.12 LL vs. SS = 0.015 LS vs. SS = 0.14
Arsenic	Mean SD (minimum-maximum)	0.20±0.11 (0.002-1.71)	0.21±0.11 (0.002-1.65)	0.25±0.12 (0.002-1.65)	LL vs. LS = 0.97 LL vs. SS = 0.72 LS vs. SS = 0.69
Cadmium	Mean SD (minimum-maximum)	0.03±0.03 (0.01-0.11)	0.03±0.02 (0.002-0.15)	0.038±0.01 (0.002-0.26)	LL vs. LS = 0.80 LL vs. SS = 0.62 LS vs. SS = 0.38
Lead	Mean SD (minimum-maximum)	0.67±0.37 (0.09-1.96)	0. 70±0.29 (0.01-1.14)	0.62±0.33 (0.09-1.02)	LL vs. LS = 0.67 LL vs. SS = 0.64 LS vs. SS = 0.32

HCC: Hepatocellular carcinoma, n: Number, p: Significance value, vs: Versus

DISCUSSION

CYP2E1 is a key member of the CYP superfamily that plays an important role in metabolizing chemicals. It can convert molecular oxygen to highly reactive compounds, which may lead to DNA damage and carcinogenesis. Moreover, CYP2E1 inducers can increase the toxicity of xenobiotics when they are added before the exposure to chemicals as it metabolizes and activates many toxic compounds[14] .

Two linked polymorphisms (CYP2E1 *5B) located in the 5′ regulatory region in the CYP2E1gene, have been studied. They are detectable by RsaI or PstI restriction enzyme digestion [RsaI is 21053C>T (rs2031920) and PstI is 21293G>C (rs3813867)[15] .

Despite the variant CYP2E1*5B allele is associated with the increased transcription of CYP2E1, which leads to the development of HCC by promoting carcinogenesis[16], contradictory results were reported for the relationship between the CYP2E1*5B variant allele and hepatocarcinogenesis.

The current study revealed significant differences in genotype distribution among HCC patients when compared to control with an increased percentage of the mutant allele(c2) among patients. A similar direction was reported in studies showing a higher risk for the development of cancer in association with CYP2E1polymorphism[17,18].

As regards the ethnic variability, a study held on the Spanish population noted a higher risk of hepatocellular carcinoma in association with rare c2 allele[12]. However, no such association between the c2 allele and higher risk of hepatocellular carcinoma was found in Japanese[19,20], Chinese[21], Korean[22] and British[23] populations. On contrary, a correlation was established for CYP2E1 polymorphisms and hepatocellular carcinoma development in the Taiwanese population in whom, the presence of the c1/c1 genotype was associated with a significant increase in hepatic carcinoma risk[24]. Also, a favorable effect of CYP2E1polymorphisms on HCC development was reported despite being inconsistent with the biological action[10]. Another study previously conducted in an Italian population[25] on the CYP2E1*5B c2 allele and HCC did report a similar association[8]. Similarly,

significant associations were observed between RsaI/PstI polymorphism and decreased risk of development of cancer liver in the Chinese population but not the Japanese and the Korean populations[26].

Conflicting results regarding genotype effect on phenotype may be partially owed to the wide variety of contributing factors such as ethanol consumption, dietary and physiological factors including body weight or diabetes that modulate CYP2E1 expression.

Concerning the association between the heavy metals concentration and the incidence of CYP2E1 polymorphism, the current study revealed that no significant association was found. So far, both heavy metals and CYP2E1 mutations might be associated with an increased risk of HCC development without the direct effect of CYP2E1 on mutagenesis. It is worthy to note that many studies have shown that CYP2E1 over expression may synergize and increase the hazardous effect of different chemicals[27].

L-myc EcoRI polymorphism may be one of the possible molecular mechanisms that control hepatocarcinogenesis, an action that may be enhanced by heavy metal burden. To the best of our knowledge, no previous studies have examined the combined effect of L-myc polymorphism and heavy metal exposure , although both of them are closely related to carcinogenesis. We aimed to evaluate them due to their hazardous proximity in carcinogenesis. In the current study no significant difference in L-myc genotype distribution was revealed when comparing the HCC patient group against the control group. Also, it was found that no significant difference in heavy metals level in subjects of different L-myc genotypes.

Since cloning of the L-myc gene (Genbank M19720) in 1985, many studies were held to investigate the possible association between L-myc gene polymorphism and the risk of cancer development. Some studies revealed that individuals carrying the *S* allele tend to have a poor prognosis and an increased risk of several tumor types. Association of the mutant L-myc genotype with tumor susceptibility has been described for non-Hodgkin's lymphoma, hepatocellular carcinoma and sarcoma, thyroid nodule malignancy[13,28-31].

Controversial results have been reported[10,32]. In addition, a previous study was held by Taylor *et al.*[13] and showed that the L-myc EcoRI genotype distribution in patients with other liver tumors and healthy controls did not differ from one another.

CONCLUSION

CYP2E1 polymorphisms may increase HCC susceptibility while L-myc gene polymorphism is nonsignificant. There is no significant association between heavy metal burden and *CYP2E1,* L-myc gene polymorphisms.

SIGNIFICANCE STATEMENT

Hepatocellular carcinoma is the most common primary malignancy of the liver, the fifth most common cancer in men and the seventh most frequent in women worldwide. Therefore much effort is now being paid to the field of screening programs. Carcinogen-metabolizing liver enzymes such as Cytochrome P are the most important regarding influence on hepatocarcinogenesis associated with heavy metal overload. This study discovers that CYP2E1 polymorphisms may increase HCC susceptibility and may be good candidates for HCC screening.

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