Research Article

The Methylation Status of DNA in Hepatocellular Carcinoma

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Introduction

The most common cause of cancer liver *is* hepatocellular carcinoma (HCC) and it is an important cause of cancer-related death all over the world (Crissien and Frenette, 2014). The pathogenesis of hepatocellular carcinoma is a multifactorial process involving different molecular and cellular events that lead to the progressive accumulation of molecular alterations at both genetic and epigenetic levels (Ho et al., 2016). Epigenetic modification means the presence of heritable states of gene expression without alteration in DNA sequences. Deregulated epigenetics modifications lead to affection of gene transcription, chromosomal stability, and cell differentiation participating in induction of carcinogenesis. Epigenetic alterations may be changes in particular DNA regions or of the histone proteins around which DNA is organized including the methylation, hydroxymethylation, or acetylation (or a combination of these), as well as non-coding RNAs regulation of gene expression (Wang et al., 2015).

P16 is a cell cycle regulator and a tumor suppressor protein; hence, its suppression promotes tumor progression (Narimatsu et al., 2004). P16 binds to Cyclin Dependent Kinases 4 and 6 (CDK4/6), this leads to inhibition of its kinase activity and so preventing the phosphorylation of Retinoblastoma tumor suppressor gene (Rb) (Rayess et al., 2012). Silencing of P16 gene occurs by hyper methylation of its promoter that is a commonly observed event in HCC (Narimatsu et al., 2004).

So the aim of this study was to determine the methylation status of P16 in HCC induced by DEN and phenobarbital in rat model.

Methods

1. Animals

This study was carried out on 50 male Wistar albino rats. Rats were kept in animal house under standard conditions of boarding and feeding with free access to water.

The rats were divided into 3 groups:
1- Control group (10 rats) received single intraperitoneal injection of phosphate buffered saline (PBS).
2- DEN induced HCC group (15 rats) received single intraperioneal injection of DEN (200 mg/kg).
3- Phenobarbital induced HCC group (15 rats) had received phenobarbital only through drinking water (0.05%) for 12 successive weeks after 2 weeks interval from single injection with PBS (Yoshiji et al., 1991). During the injection period, animals were kept in their cages well ventilated, in 12 h day/night cycle. Ethical approval was obtained for the study from Research Ethics Committee, Faculty of Medicine, Minia University.

2. Experimental procedures

Animals were sacrificed at the end of week fourteen. The liver tissue was removed and weighed. The liver tissue was immediately frozen in liquid nitrogen, stored at -80 °C for methylation specific PCR.

3. Methylation-specific PCR (MSP):

Tissue samples were subjected to DNA extraction by digestion with proteinase K and RNase followed by phenol/choloroform extraction and ethanol precipitation (Sambrook and Russell, 2001). The

methylation status of 5' CPG islands of P16 gene was assessed by bisulfate modification of DNA and methylation specific PCR (MSP) according to the method of Herman

et al., (1996). MSP was performed on the sodium bisulfite-treated DNA samples to amplify the promoter region of the p16 gene. Two pairs of PCR primers were used in the amplification, one for methylated sequence and one for unmethylated sequence. PCR was performed according to the manufacture instructions using MyTaq Red Mix (BIOLINE). The forward and reverse primers for unmethylated p16 sequence were: 5'-AGT ATT GTA TTA GGT AGG GGT GTG G-3' and 5'-ACC TAT CAA TAA CCC AAA AAA CAT T-3' respectively. Forward and reverse primers for methylated P16 sequence were: 5'-TAG TAT TGT ATT AGG TAG GGG CGC-3' and 5'-TATCGA TAA CCC GAA AAA CGT T-3' respectively. The PCR conditions were as follows: one cycle of initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, an annealing step for 30 s (at 62.0°C for methylated p16 or 60.0°C for unmethylated p16), and extension at 72°C for 30 s. Final extension step at 72°C for 10 min. Final PCR products were separated by electrophoresis in a 2% agarose gel and visualized under UV illumination. PCR amplification of methylated and unmethylated products was carried out separately in two tubes with the same PCR conditions and reagents (except the primers and the annealing temperature).

Statistical analysis

Statistics were done using graphpad prism (version 7). All data were expressed as mean ± standard error (SE). One way ANOVA followed by Tukey's post-hoc were used for assessment of the statistical significance. Chi Square test and Fisher's exact test were used to compare the incidence of irregular DNA methylation. A p value of 0.05 was considered statistically significant.

Results

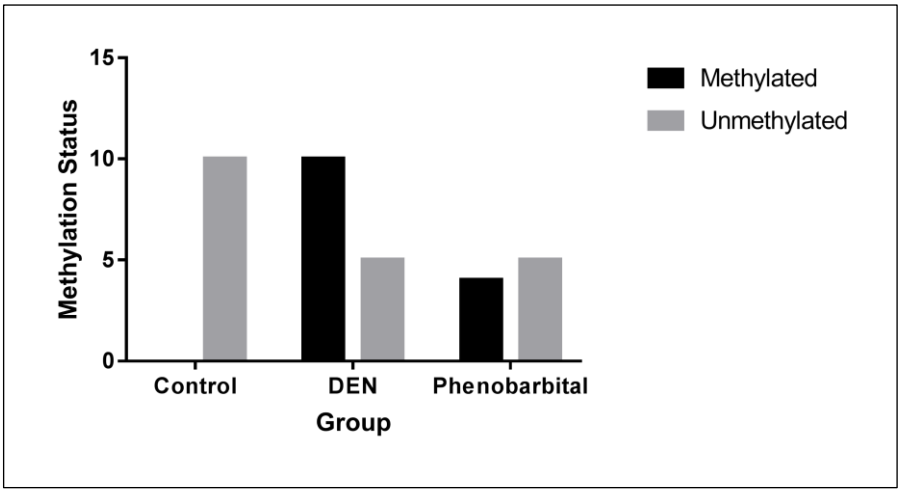
1. General observations

Rats in the control group grew well throughout the entire experiment period while the DEN injection causes death of nearly 40% of the rats of DEN induced HCC group and DEN and phenobarbital induced HCC group.

2. Methylation Status of P16:

Figure 1 shows the results of methylation status of P16 promoter in different groups and there was significant difference between control group and experiment groups ($p \leq 0.05$).

Figure 1: Methylation status of P16 in control and experimental groups.



Discussion

Chemically induced hepatocarcinogenesis in experimental animals by diethylnitrosamine (DEN) or phenobarbital is a good example for studying changes occur through liver cancer as it is a strong hepatocarcinogen (Sreepriya and Bali, 2005) (Björkhem-Bergman et al., 2005). In our study, DEN and phenobarbital induced liver cancer in rats, demonstrating that Wistar albino rat is a suitable model in studying DEN and phenobarbital induced liver cancer. Also it showed that DEN induced HCC produces higher percentage of changes in rat liver (66%) than the phenobarbital group (44%) which indicates that the DEN induced HCC model is more suitable than phenobarbital as an experimental model for induction of hepatocarcinogenesis.

It is known that DEN induces liver damage in many enzymes that are utilized in DNA repair and this will induce liver cancer in experimental model (Santos et al., 2017). Also, DEN causes accumulation of reactive oxygen species which results in DNA oxidative damage that enhances hepatocarcinogenesis (Hanahan and Weinberg, 2000).

Also, Phenobarbital is used to induce liver cancer but it works through a non-genotoxic mechanism of action through a mitogenic/ apoptotic imbalance, which results from both an induction of cell proliferation and the suppression of apoptosis. It induces changes in gene expression and cell cycle signal transduction (Watson and Goodman, 2002).

Our results revealed that P16 promoter methylation is increased in the DEN as compared to the control group which was demonstrated by high incidence of methylation.

We found that the rats of the DEN treated groups showed higher rates of hypermethylation of P16 than phenobarbital treated group as shown in the methylation status figure. This indicates that P16 hypermethylation induces P16 silencing in the rat liver cells, correlating with the development of liver cancer. It is usually occurring at CpG islands in promoters and it is a main example of epigenetic modification that causes silencing of gene expression (Qin et al., 2004).

Finally, we conclude that DNA methylation in the promoter region of P16 gene can be used as a tool for prediction of tumorigenesis.

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