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Full Length Research Paper

The use of TERT and CK20 as a trial for early diagnosis of HCC in animal model

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Abstract

To evaluate the use of TERT and CK20 gene expression as a diagnosis of HCC in animal model. Thirty adult albino male rats were divided into 3 groups of 10 animals each. Animals in group A underwent a sham experiment, group B was injected by CCL4 for 4 weeks (early) and group C was injected by CCL4 for 8 weeks (late). At end of experiments, specimens from liver were all stained by H and E dye. TERT and CK20 gene expression were done by Real-Time PCR in blood and tissue samples. The relative quantification of target genes and statistical analysis were done. A histopathological study of the rat liver of early group showed mild fibrosis and the late group showed formation of cirrhotic nodule with dysplasia of hepatocytes. TERT mRNA was increased significantly in early and late tissue by about 17 and 23 folds and in blood samples, the expression was increased about 3 and 11 folds, respectively. The gene expression of CK20 was not observed in blood stream but was expressed significantly in tissues with about 4 and 44 folds. P value was <0.001 between control and early or late HCC. Human TERT gene expression was observed to increase according to HCC in both tissue and blood samples but CK20 was regulated in tissue samples.

Keywords: TERT, CK20, HCC, Gene expression, CCl4, H and E staining, Real-Time PCR.

INTRODUCTION

Hepatocellular Carcinoma (HCC) represents the fifth most common form of neoplastic diseases worldwide (accounting for > 5% of all human cancers) and the third most common cause of cancer-related death. The latest GLOBOCAN survey in 2012 found about 782000 new HCC cases and 746000 patients died within this year. In USA, Cancer statistics 2012 estimated new cancer cases in liver 28,720 and estimated death 20,550 cases (Torre et al., 2015; Hung et al., 2016). The continuous high incidence of HCC suggests that they may continue to a global health problem now and in the future (Bruix et al., 2016).

Egypt studies revealed liver cancer (23. 8%), breast (15.4%) and bladder (6.9%) in both sexes. The incidence of cancer was in Liver (33.6%) and bladder (10.7%) among men. While the cancer cases were in breast (32.0%) and liver (13.5%) in women. (Ibrahim et al., 2014).

In the case of experimental animal models, Carbon tetrachloride (CCL4) is a widely used hepatotoxic agent in rodents and its trichloromethyl radical (CCL3) induced toxicity which closely resembles human cirrhosis. Hence, it is an acceptable animal model for characterizing hepatoprotective activity (Al-Shabanah et al., 2000) .

The early diagnosis for HCC will increase the potential for curative treatment and improves survival rate (Hirko et al., 2013). For early diagnosis of HCC, blood is the best source for revelation of cancer related biomarkers (Bruix et al., 2016).

Telomerase reverse transcriptase (TERT) is a ribonucleoprotein which maintains telomere length and blocks inconvenient repair of natural ends of linear chromosomes. Dysfunction of telomerase is responsible for failure of tissue repair or regeneration (Madonna et al., 2011). High telomerase activity was found in a high

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percentage of several cancer cells, so the level of this enzyme may represent a type of cancer marker, the importance of quantification of telomerase activity in the diagnosis of small HCC and in the assessment of treatment has been reported (Al-Shabanah et al., 1999) (Hady et al., 2010). Although normal cells have some active telomerase, increased expression of telomerase produce probability of developing cancer cells (Blackburn, 2005). Tumors require maintenance of telomere stability for their long term proliferation, so escape from cellular senescence by activating telomerase and this represents an additional step in oncogenesis that most tumors require for proliferation (Bryan et al., 1995; Shay and Wright, 1996; Shay et al., 2001)

Cytokeratin 20 (CK20) is a low molecular weight cytokeratin distributed in epithelia and their neoplasm (Chu and Weiss, 2000). CK 20 is an established tumor marker (Cerna et al., 2006).

MATERIALS AND METHODS

Experimental animals and study groups

Thirty adult rats (3-month-old) inbred albino male rats weighing an average of 150 g were divided into 3 experimental groups of 10 animals each. The required approvals for all the procedures in this study were obtained from the ethical committee of the University of Mansoura. Animals in group A "normal control" underwent a sham experiment, group B rats were injected by 0.5 mg CCl4/Kg body weight twice a week intraperitoneal for 4 weeks (early) and group C rats were injected CCl4 for 8 weeks (late) by the same procedure. Blood samples were withdrawn from animals before and after the end of experiment and stored in liquid nitrogen (~-160 ^{O}C) until RNA extraction.

Liver rats were eradicated and the samples were divided into 2 parts for each sample, one part was preserved in liquid nitrogen and the other part preserved in formalin solution for pathologic analysis.

Histopathological studies

Specimens from liver of rats were preserved in 10% neutral buffered formalin. Sections of 5 micron thickness were prepared from all specimens, stained by hematoxyline and eyosin dye (H and E) (Sigma Aldrich, USA) and examined microscopically according to *Bancroft and Gamble* 2008.

Extraction of RNA

Total RNA was extracted according to the instruction of purification kit (GF-1 RNA extraction kit, Vivantis, Malaysia) for both blood and tissue samples. 50 µl of the eluted RNA was collected immediately, placed in ice or stored at -80°C for further processing. Spectrophotometric quantification of RNA was done using spectrophotometer (NanoPhotometer P330, Implen, Germany). Samples only have A260/280 greater than 2 and A260/230 more than 1.8 were converted to cDNA which also yielded 2 distinctive bands in agarose gel electrophoresis (agarose 1.5 %). 2 µg of total RNA for each intact sample was reverse transcripted into cDNA using RevertAid First Strand kit (Thermo Scientific, Lithuania).

Relative Quantitation of mRNA of the respective genes by real time PCR

The relative expression ratio is calculated only from the real time PCR efficiencies and the crossing point deviation of an unknown sample versus a control group. TERT and CK20 gene expression were performed by using the primer sequences as in table 1. A single plexus reaction has been done in 96 well plate in triplicate as recommended using Maxima SYBR Green Master Mix (Thermo Scientific, Lithuania). Two µl of the cDNA sample was mixed with 10 Pico mole of each primer and 10 µl of Sybr green mix. Distilled water was added to a volume of 20 µl, and the resulting mixture was subjected to real-time PCR amplification system (CFX96, BioRad, USA). The cycling parameters were as follows: initial denaturation at 95 ^{O}C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds, annealing and extension at 60 ^{O}C for 1 minute and the final melting curve was done to show the specificity of hybridization for primers. A mathematical model introduced by Pfaffl 2001 was used for the relative quantification of target genes.

Statistical Analysis

Analysis was performed using SPSS11 (statistical package for social science) software. Using correlation coefficient to measure the closeness of a linear relationship between relative gene expression values and one way ANOVA to analyze the differences among group means and their associated procedures in which the observed variance in a particular variable is partitioned into components attributable to different sources of variation. Nonparametric data were evaluated by Friedman's test. Post hoc testing was performed by Wilcoxon's Signed Ranking and the P values were corrected by Bonferroni adjustments. A P value of < 0.05 was considered significant.

RESULTS

Histopathological examination

A histopathological study of rat liver sections control rats

Table 1. The primer sequences for gene expression by Real-Time PCR

Gene Name	Primer Sequance	Accesion no.
TERT	Forward GGTCTTCCGCACGTTGGTTG Reverse CAGGGATGACACCTGGTGGA	NM_053423.1
CK20	Forward GCAAGGTCGTGTCCTCTGAA Reverse AGCTCCCCAGAGTGAAAACG	NM_173128.1
GAPDH	Forward CCAGGGCTGCCTTCTCTTGT Reverse CTGTGCCGTTGAACTTGCCG	NM_017008.4

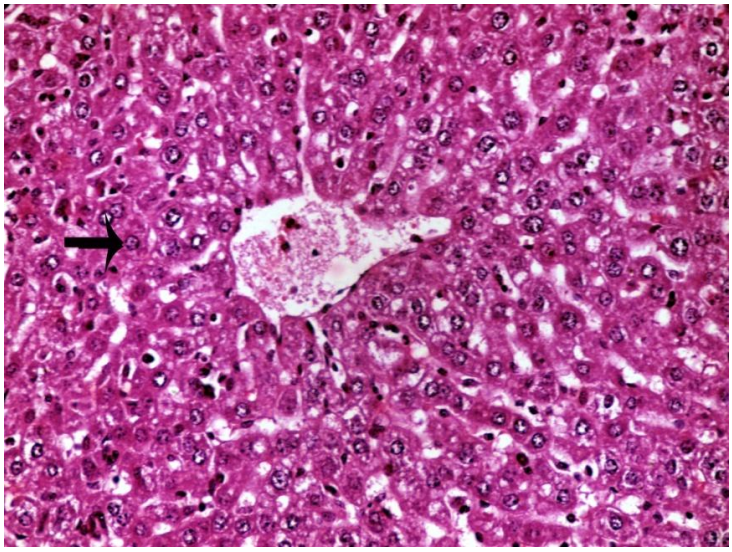

Figure 1. Specimen was taken from the liver of control group is showing normal hepatocytes (arrow) with normal radial arrangement around central vein (HE, 400x).

revealed normal hepatocytes with normal radial arrangement around central vein. This liver was normal architecture hepatocytes as shown in Figure (1). Liver of CCl4 rats after 4 weeks injecting was marked fatty change with vacuolization of hepatocytes and scattered inflammatory cells with mild fibrosis. It was observed in figure 2 the thick intralobular septa originating from portal area in arborizing pattern with marked hydropy of hepatocytes. Microscopically, examination of liver sections of rats after 8 weeks with CCl4 the intralobular fibrous septa forming bridging fibrosis, and formation of cirrhotic nodule (CN), with severe degeneration and dysplasia of hepatocytes (figure 3).

Relative quantitation of Telomerase activity and CK20 mRNA:

The expression of genes was done relative to that of normal rats. TERT gene was expressed in rat liver by the mean 2.28 (±1.4) fold more than blood specimens among the control rat. TERT mRNA was increased significantly in early and late tissue by 17.33 (±7.4) fold and 23.44 (±10.52) fold change, respectively. In the case of their blood samples, the expression of TERT was increased significantly with 3.19 (±1.95) fold and 11.33 (±8.65) fold change in early and late blood groups correspondingly (figure 4).

The expression of Cytokeratin 20 was tested relative to rat tissue. The gene expression was not observed clearly in blood stream. This gene was expressed significantly in tissues with 4.1 (±1.5) fold and 44.13 (±12.57) fold changes in the early and late tissue groups, respectively (figure 5).

DISCUSSION

For determination of specific mRNA, real time PCR has become the most sensitive method in the last decades. It was found that quantification of mRNA of catalytic subunit of the enzyme TERT is more accurate than quantification of the whole gene encoding both TERT and telomerase RNA component (TERC) because the regulatory units which regulate TERC expression in tumor cells aren't well defined (Hady et al., 2010).

Some studies found that telomerase activity assay is very useful method for HCC diagnosis and it is superior to other tumor markers since they found that mean telomerase activity, prothrombin induced by vitamin K

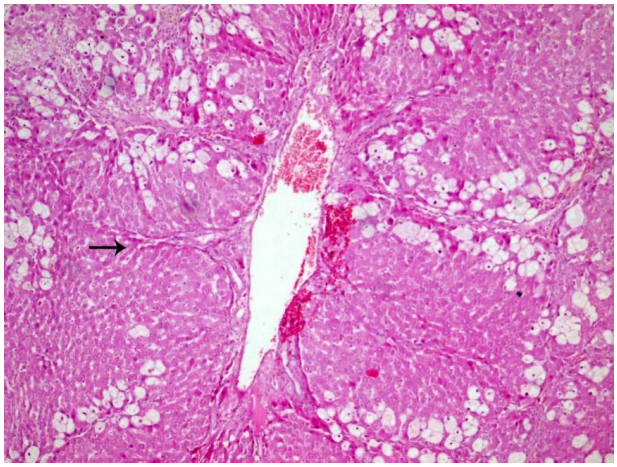

Figure 2. The specimen was taken from one month group showed thick intralobular septa originating from portal area in arborizing pattern (arrow) with marked hydropy of hepatocytes (HE, 100x).

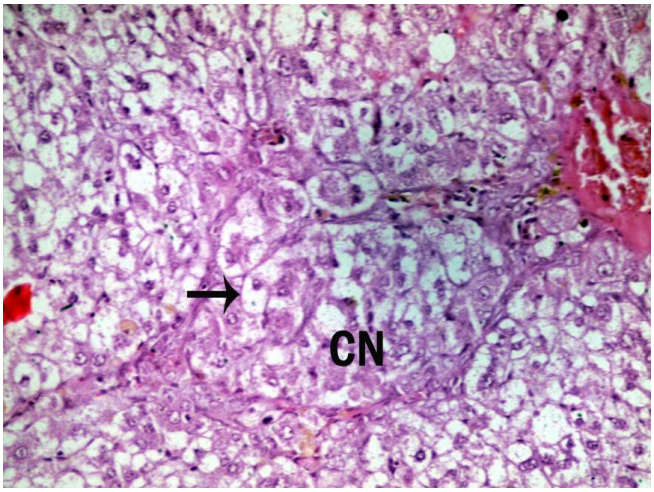

Figure 3. Specimen was taken from two moths group found the intralobular fibrous septa forming bridging fibrosis and formation of cirrhotic nodule (CN) with severe degeneration and dysplasia of hepatocytes (arrow) (HE, 100x).

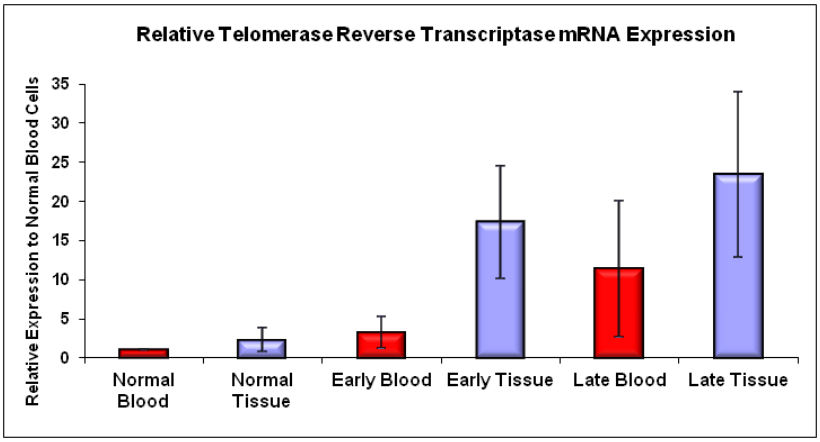

Figure 4. TERT gene expression was performed relative to normal blood in tested rats. This figure was showed qPCR of gene by the mean and standard deviation of gene expression.

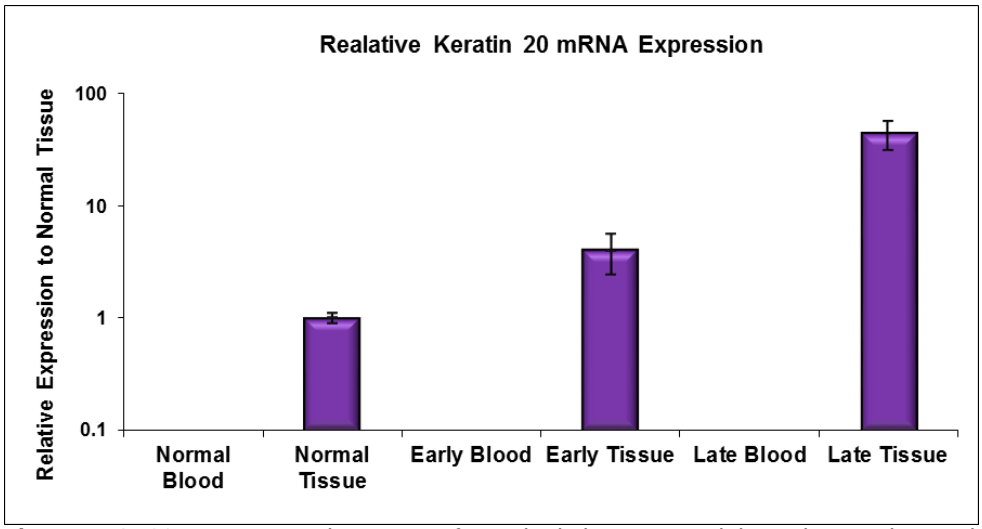

Figure 5. CK20 gene expression was performed relative to normal tissue in experimented rats. This figure showed qPCR of gene by the mean and standard deviation of gene expression.

antagonist and α-fetoprotein levels were significantly higher in HCC patients when compared to both cirrhotic patients and controls. In addition, they found a positive correlation between size of hepatic focal lesions and telomerase activity (Hady et al., 2010). In our experiments we have selected to study telomersae gene activity and CK20 to evaluate its expression in early and late HCC models compared to the normal healthy controls.

TERT and CK20 mRNA expression was measured using real time PCR and mean level in HCC patients was significantly higher than both the group of chronic liver disease patients and the control group. TERT was expressed in early tissue about 17 folds and in late tissue about 23 folds relative to that of normal cells while in blood samples the expression increased in early about 3 times and in late about 11 folds so real time PCR is a satisfactory molecular marker for diagnosis of HCC and TERT is superior to AFP in early diagnosis of HCC as illustrated by other authors (Raouf et al., 2013). Statistical analysis showed a highly significant between normal, early and late the P value was less than 0.001 as using TERT to diagnose the HCC in liver tissue and blood samples. In the case of CK20 expression was significant only in tissue samples, but wasn't found in blood samples. The level of expression relative to that of normal tissue was regulated in early tissue about 9 folds while in late tissue showed highly expressed reach to 87 fold changes. This observation is in agreement with (Cerna et al., 2006) who found number of correlations between relative values of CK20 and CEA in liver tumor group and no significant difference in CK20 or CEA expression between the stage group, so CK20 is only expressed in tissues with certain degree of differentiation. So, CK20 mRNA is expressed only in circulating blood as epithelial tumor cells when circulating tumor cells appear in rich in blood samples as mentioned by many authors (Moll et al., 1993; Molnar et al., 2008). Early and late HCC may not be observe in any circulating tumor cells in our experiment. Also, CK20 is a marker of circulating tumor cells in peripheral blood (Pantel and Alix-Panabieres, 2010). There is a good significance between normal, early and late tissues as indicated in P value.

Nault and his parteners found that the earliest genetic disorder found in the multistep process of carcinogenesis is the mutation of TERT promoter (Nault et al., 2013). In the recent study we found significant differences in TERT mRNA expression among normal group, cirrhosis group and fibrosis group in agreement with other authors (Raouf et al., 2013), (Miura et al., 2007) ,(Miura et al., 2010) and (Tatsuma et al., 2000).

The activation of telomerase contributes to progression of tumors, premalignant tumors show low telomerase activity, there was slight increase in expression of TERT mRNA level in cirrhosis patients as compared to controls but doesn't reach a significant level (Miura et al., 2010) who reported that normal hepatocytes may express a negligible amount of TERT mRNA and that inflamed hepatocytes may still express more weakly than HCC cells.

Some HCC cases may show low level of telomerase activity and (Nittis et al., 2008) suggested that this may be because immortal hepatocytes may acquire an "Alternative Lengthening of Telomerase" ALT mechanism for maintenance of their chromosomal stability. El-Fadle et al. 2011 suggest that this may be due to their samples were HCC tissues and the low telomerase enzymatic activity in tissue samples taken from necrotic malignant tissue possibly due to variability in the collected sample and the used technique.

Over 80% of human cancers show an activation of telomerase which stabilizes telomeres thus facilitating

immortal proliferation of cells. The activation of telomerase is a general phenomenon in different types of human cancer (El-Fadle et al., 2011). In conclusion, mRNA of hTERT is detected early as a diagnosis of HCC in both blood and tissue samples and contributes to progression of tumors. On the other hand, CK20 is only up regulated in tissue samples.

CONCLUSION

The present study demonstrates that the human TERT mRNA was up regulated in tissue as well as in blood samples due to formation of HCC model and CK20 was observed in tissue only. So, TERT gene expression could be use as a diagnosis of HCC.

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