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Nuclear translocation of telomerase reverse transcriptase: a critical process in chemical induced hepatocellular carcinogenesis

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Telomerase, a ribonuclearprotein complex, functions not only in cancer development but also in apoptosis, and senescence. As a catalytic subunit of telomerase, telomerase reverse transcriptase (TERT) has been confirmed to regulate telomerase activity in a rate-limiting manner. Although a lot of work has been done, the dynamic state of TERT protein and the relationship with telomerase have not been delineated systematically in cancer development. The purpose of this study was to do such an exploration. To investigate the role of TERT in the *in vivo* carcinogenesis, we performed immunofluorescence and Western blot analysis, respectively, to detect the alteration of TERT status as well as telomeric repeat amplification protocol (TRAP) assay to detect telomerase activity in diethyl nitrosoamine (DENA) induced rat hepatocellular carcinoma (HCC). The course of cancer development was divided into three main stages, which were inflammation (<12 weeks), hepatocirrhosis (15 weeks), and hepatocarcinoma (18-21 weeks). In normal liver and its early inflammatory stage, concomitant with a weak positive TERT signal, which was detected exclusively in the cytoplasm, telomerase activity was very low at this stage. However, in late hepatocirrhosis and particularly cancer stage, high levels of TERT expression ($P = 0.044$ and $P = 0.001$, respectively) and telomerase activity ($P = 0.02$ and $P = 0.01$, respectively) were observed following TERT nuclear translocation. Our data suggest that TERT protein might regulate telomerase activity. TERT translocation from cytoplasm to the nucleus might be a turning point in cancer development. Therefore, TERT translocation might be more useful than TERT expression level and telomerase activity in predicting the progression of HCC.

Key words: hepatocellular carcinoma; telomerase; telomerase reverse transcriptase; TERT translocation

Telomerase, as a ribonucleoprotein complex, adds small tandem hexanucleotide repeats (TTAGGG) to the end of the chromosomes, therefore compensating for the telomere loss that accompanies cell division and chromosome replication, and thus prolonging telomere length-restricted replicative lifespan of cells [1–3]. In contrast to most normal human somatic cells that do not express telomerase and eventually enter into senescence when telomeres shorten to a crucial point, over 80% of human cancers have a high level of telomerase activity that maintains telomere length. Recently, telomerase has been confirmed to function not only in cancer development but also in apoptosis, and senescence [3–5]. As a result, more and more attention has been given to telomerase and its components [2]. There is growing more evidence that telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC), the core components of telomerase, are emerging to be the potential players of this enzyme, although the relations thereof remain unclear. TERC is ubiquitously expressed in all normal and cancer cells, whereas TERT, being involved in cellular immortalization and in carcinogenesis, acts as a rate-limiting factor of the activation of telomerase [6–8].

TERT expression, which undergoes a very complicated regulation process, mainly involving transcriptional and translational control, correlates positively with cellular immortalization and carcinogenesis [9–14]. Although a lot of work has been done in TERT mRNA expression pattern, TERT translational control has been evidenced to play a critical role in its functional regulations due to its subcellular location [13]. The dynamic subcellular location of TERT is dependent on the cell cycle, DNA damage or cellular transformation [15]. Noteworthily, few reports have shown that TERT, being long regarded as a nuclear protein, has been found to be located not only in nucleus but also occasionally in cytoplasm [13, 16, 17]. Unfortunately, most, if not all, of researches have dismissed the cytoplasmic TERT as non-specific staining and non-functional protein, although cytoplasmic TERT was once

reported to play important roles in maintaining normal cell and/or tissue functions especially when mitochondrial TERT has been found to be involved in apoptosis pathways [18, 19]. But even now, the biological significance of TERT subcellular location in vivo carcinogenesis remains an enigma.

The role of prolonged cellular damage such as viral or bacterial infection-related chronic inflammations has become widely recognized in human carcinogenesis [20], particularly in hepatocellular carcinomas. Chronic liver damage by DENA can be more effective than acute in experimental carcinogenesis and simulate the major factors affecting human cancers. With this in mind, we tried to investigate the relations between telomerase activity and TERT expression, and the subcellular localization in the chronic tissue damage induced tumor based on the Solt-Farber hepatocarcinogenesis protocol [21]. The aim of this study was to improve our understanding of the dynamic state of TERT and telomerase activity in the in vivo carcinogenesis from normal hepatocytes to cancer cells and investigate whether TERT cytoplasm-to-nuclear translocation is true or not and the relationship between TERT subcellular location and telomerase upregulation as well.

Materials and methods

Animal models and treatments. Three-month-old Male Sprague-Dawley (SD) rats (purchased from Zhengzhou University, Henan province, China) weighing ranging from 115 to 125 grams were employed. All animals were required to undergo institutional quarantine for 7 days prior to use. The environment for animal housing was equipped with controlled temperature (22±3 °C), humidity (40-70%) and a 12h-light/dark alternation. A group of animals (n=42) was fed with water containing DENA (N-0756, Sigma, St. Louis, MO) (2‰, w/w) at a dose of 5mg/kg/day for 15 weeks, and the animals of control group (n=42) fed with physiological salt solution for the same period. Throughout the experiment, all animals were fed with food and water *ad libitum* and were inspected every day and measured twice a week for the body weight.

Histologic examination. After DENA treatment was initiated, 6 randomly chosen rats were sacrificed under anesthesia every three weeks (week 3, 6, 9, 12, 15, 18, 21 respectively). The freshly removed liver was cut into two pieces. The small part was fixed in 10% poraformaldehyde for 24 hrs, embedded in paraffin and attained with hematoxylin-eosin (H&E). The big part was frozen in liquid nitrogen immediately after removal and was then kept at -80 °C until use for other analysis.

Immunofluorescence assay of TERT expression. The anti-TERT monoclonal rabbit antibody (sc-7212, Biotechnology, Santa Cruz, CA) was applied to the 4 μm-thick sections at 4 °C overnight at a dilution of 1: 600, after heat antigen retrieval in 10 mM citrate buffer with 0.1% Tween 20 (pH 6.0) for 30 min at 95°C. Fluorescin isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (ZF-0311, Zhongshan Golden Bridge Biotechnology Co., LTD, Beijing.) at a dilution of 1: 400 was used as a secondary antibody, and then propidium iodide (P-4170, Sigma, St. Louis, MO) was used to counterstain nuclei. The images were captured with Zeiss confocal microscopy (Zeiss LSM510). Ex/Em (excitation wavelength/measurement wavelength): 488nm/(505-550)nm and 536/(580-650) nm, respectively. objective: ×40, ocular: ×10, pinhole: 150, scan zoom: 1.2, pixel: 1024×1024. Based on the previously described protocol for semiquantitative evaluation of proteins using the immunofluorescence technique, TERT protein was shown to be relative fluorescence staining intensity per cell [22, 23]. At least 10 visual fields were captured and more than 1000 cells were counted. The value was the average of three independent experiments.

Western blot assay of TERT expression. Total tissue lysate was performed by standard protocol. Cytosol and nuclear extracts were prepared as previously described. 75 μg tissue extracts were resolved in 6% SDS-PAGE and transferred to nitrocellulose membrane. Blocked membranes were incubated with anti-TERT (sc-7212, Biotechnology, Santa Cruz, CA), anti-actin (I-19; Santa Cruz Biotech, CA) primary antibodies at a 1:300 and 1:1000 dilution, respectively. NBT/BCIP kit (Sino-American Biotechnology Co, China) was used to detect immunoreactive bands. Quantitative evaluation was measured using Labworks 4.5 software (UVP, Inc. USA). The value was the average of three independent experiments.

TRAP assay of telomerase activity. TRAP assays were performed using Telo TAGGG Telomerase PCR ELISA Kit (Roche, Germany) according to the manufacturer's protocol. The relative telomerase activity was calculated by the formula: [sample A_{450nm}-A_{690nm} unit / positive control A_{450nm}-A_{690nm} unit] × 100. The mean value in this paper is the three independent experiments.

Statistical analysis. All of the statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) program. All data were analyzed by ANOVA. A P<0.05 was considered statistically significant.

Results

Hepatocarcinogenesis. Paraffin-embedded sections were submitted anonymously to two histologists for histologic classification of hepatocellular lesions in rats based on the published criterion [24]. Histopathology assay showed that the pathological changes in rat liver might be roughly divided into three stages, inflammation (<12 weeks), hepatocirrhosis (15 weeks), and cancer (18–21 weeks) stages (Fig. 1).

TERT protein expression increased in the development of HCC in rats. To investigate the alteration of TERT expression level in cancer development, TERT protein was measured at different stages of cancer by immunofluorescence and Western blot. As shown in Fig. 2A, total cell extracts from normal liver and that in early inflammatory stage as well as in normal tissue adjacent to tumor mass exhibited TERT expression at a low stable level whereas in hepatocirrhosis and in HCC stages TERT protein expression was significantly increased. When quantified, the figures were 1±0.17 and 2.59±0.07 in non-cancerous tissue, which was compared to 8.57±0.06, 8.84±0.08, and 6.08±0.08 in hepatocirrhosis and in HCC stages. The increase of TERT in

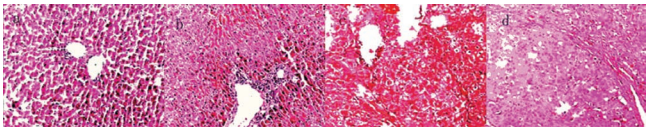

Figure 1 Histopathological examination to evaluation the changes of liver tissue in cancer development (×400). a: Control; b: Inflammation (<12 weeks); c: Hepatocirrhosis (15 weeks); d: Cancer stages (18-21 weeks).

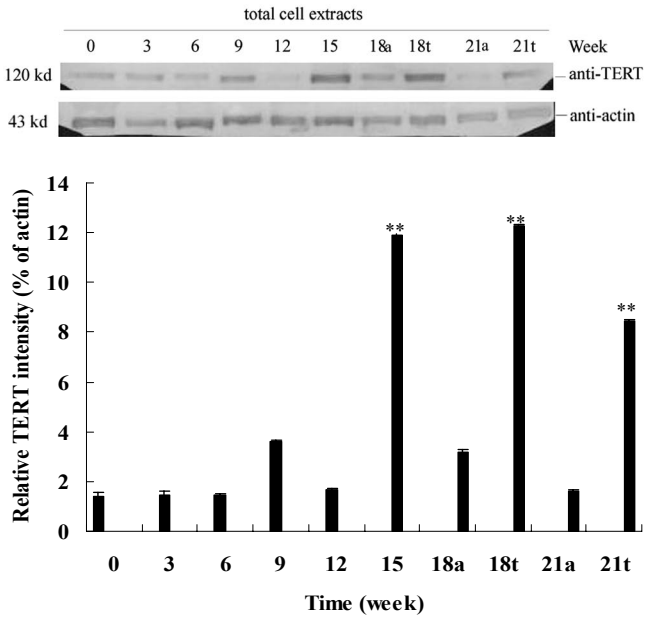

Figure 2 TERT expression level identified by Western blot. (A) TERT expression in the development of HCC. Total cell extracts were assayed for TERT expression level at different timepoints (week) by Western blot using an anti-TERT antibody. Anti-actin was loaded as positive control to ensure the equal loading amount. (B) The relative TERT protein levels in HCC development are shown. The TERT level shown in A was quantified by densitometer and Labworks 4.5 software (UVP, Inc. USA) and relative TERT level was normalized on the basis of loaded protein equivalents. Data were the mean of three independent experiments. *and ** statistically significant differences (P<0.05 and P<0.01 respectively) compared with the control group. "a" was for normal adjacent tissue to tumor mass; "t" was for tumor.

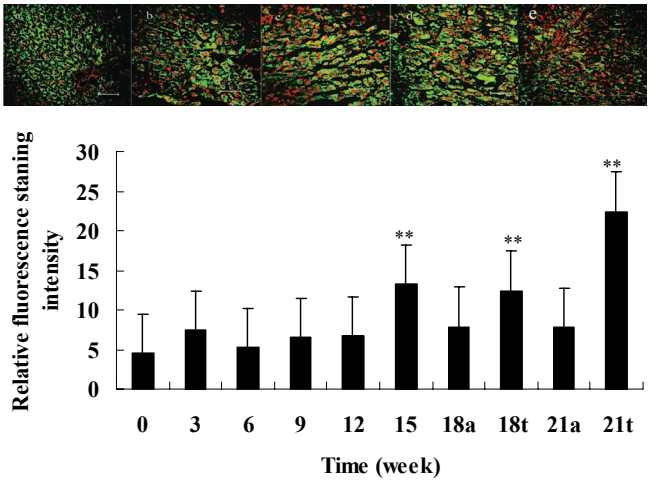

Figure 3 TERT expression level identified by immunofluorescence. (A) Immunofluorencence staining of TERT was performed with FITC-conjugated IgM (green), with the nucleus being stained with PI (red). (a) Control; (b) early inflammation stage (6 weeks); (c) Hepatocirrhosis lesion (15 weeks); (d) Hepatocellular carcinoma (21 weeks); (e) adjacent tissue to tumor mass. The bar represents 50 μm. (B) Simiquantitative evaluation of TERT expression level per cell was performed using immunofluorescence. All the experiments were performed as described in Materials and Methods section. Data were the mean of three independent experiments. *and ** statistically significant differences (P<0.05 and P<0.01 respectively) compared with the control group. "a" was for normal adjacent tissue to tumor mass; "t" was for tumor.

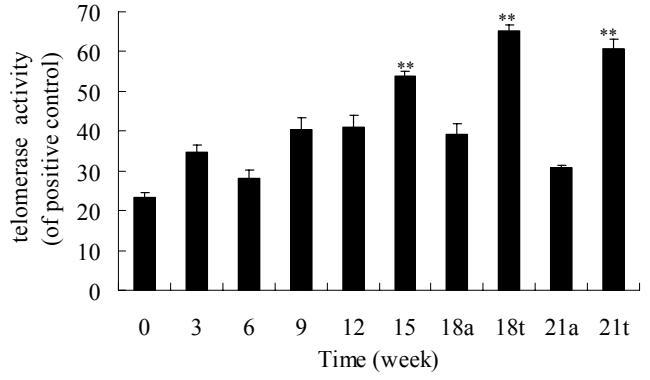

Figure 4 Telomerase activity in the development of HCC. Telomerase was measured by the TRAP assay. The value was the average of three independent experiments. *and ** statistically significant differences (P<0.05 and P<0.01 respectively) compared with the control group. "a" was for normal adjacent tissue to tumor mass; "t" was for tumor.

HCC stage was about three-fold higher than that in the normal (Fig. 2B). To further confirm the Western blot assay, an anti-TERT antibody was used to evaluate the TERT expression level per cell. In line with the Western blot findings, compare with weak TERT protein expression in control group and in inflammation group, there was significant difference in quantity of TERT protein in hepatocirrhosis and in HCC stages (P = 0.044 and P = 0.001, respectively) (Fig. 3A and B). Thus, the regulation of telomerase activity in the process carcinogenesis is controlled at the level of total cellular TERT protein.

Increased telomerase activity in the development of HCC. Telomerase activity in control group and DENA treated group were assayed using TRAP (Fig. 4). Similar to the change seen with TERT protein, telomerase activity was at a low level in control group and slightly increased in the liver of early inflammatory stage and normal tissue adjacent to tumor. Along with the progression of the lesion, telomerase activity retained at a high level and reached a level of significant difference in hepatocirrhosis and HCC stages (P = 0.02 and P = 0.01, respectively).

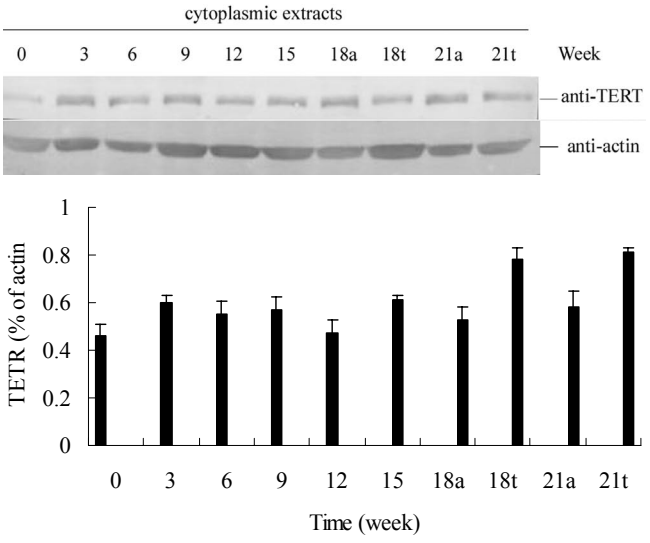

Figure 5 Identification of TERT cytoplasmic expression by Western blot assay. (A) TERT expression in cytoplasm fractions. Anti-actin was loaded as positive control. (B) TERT expression level in cytoplasmic extracts, quantified using Labworks 4.5 software (UVP, Inc. USA). The value was the average of three independent experiments. "a" was for normal adjacent tissue to tumor mass; "t" was for tumor.

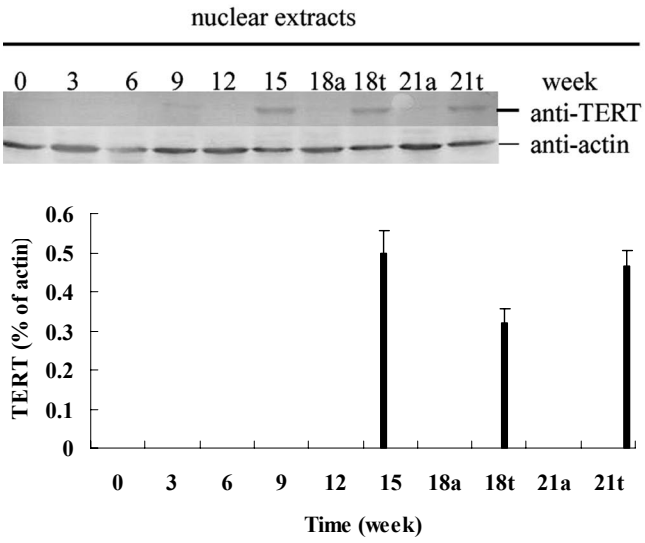

Figure 6 Identification of TERT nuclear expression by Western blot assay. (A) TERT expression detected in nuclear extracts using Western blot. Anti-actin was loaded as positive control to ensure the equal loading amount. (B) TERT expression level in nuclear extracts, quantified using Labworks 4.5 software (UVP, Inc. USA). The result represents three independent experiments. "a" was for normal adjacent tissue to tumor mass; "t" was for tumor.

Nuclear translocation of TERTis associated with telomerae activation in the development of HCC. To address the subcellular localization of TERT protein and to determine whether telomerase activation is accompanied by changes in TERT localization, we assayed the TERT protein expression by immunofluorescence and Western blot assay, respectively. As shown in Figure 4A, positive TERT staining signal was detected in all the tested samples, shown not only in cytoplasmic (green) but also in nucleus (yellow). In normal tissue and in early inflammatory stage, TERT protein was exclusively localized in cytoplasm, with no positive signals being found in nuclei (Fig. 3A a-b). On the contrary, when liver cirrhosis and cancer developed, TERT protein was transported to the nucleus from cytoplasm (Fig. 3A c-d).

To further confirm the subcellular localization of TERT in cancer development, extracts from cytosol and nucleus were used to detect TERT protein by Western blot (Fig. 5 and 6). In line with the confocal microscopy observations, TERT protein was detected in all cytosol extracts and was translocated to the nuclear only in hyperplasia nodules and cancer tissue. The value of relative intensity of TERT from cytosol extracts maintained at a stable level and varied between 1±0.05 and 1.76±0.02 throughout the experiment (Fig. 5B). As for TERT protein from nuclear extracts, the signal intensity was 0.5±0.05, 0.32±0.03, and 0.47±0.04, respectively, and only detected in hepatocirrhosis and cancer stage (Fig. 6B). Thus, nuclear translocation of TERT protein was close correlated with the increased TERT protein and telomerase activity.

Discussion

In this perspective study, a double blind assay was used for the first time to investigate TERT protein expression level, subcellular localization and telomerase activity in the development of HCC in rats induced by DENA. We demonstrate that telomerase activity is regulated by the expression of TERT protein and TERT subcellular location. This is supported by the findings that the induction of telomerase activity does require an increase of total TERT protein and TERT nuclear translocation. As was expected, increased TERT protein expression and telomerase activity were observed mainly at hepatocirrhosis and cancer stages, which is consistent with the reported that the higher the telomerase activity in HCC is, the higher the malignant potential will be [25–28]. On the other hand, telomerase activation might be a major player to confer an extended lifespan to replicating hepatocytes [29], which suggests that telomerase activation and TERT over-expression are the early events in large nodule formation in cirrhosis, which may facilitate the action of other factors in the process of carcinogenesis.

Considering the factors that telomerase activity is controlled by TERT and that the relationship between telomerase activities and either TERT expression level or TERT subcellular localization remains unclear, particular attention was given to the cellular localization of TERT in the development of HCC [13]. In the present study, it shows that the significant increase in telomerase activity (Fig. 4) and in TERT protein expression in cirrhotic and cancerous lesions was found to

be closely associated with the nuclear translocation of TERT, which was confirmed by immunofluorescence (Fig. 3A) and Western blot analysis (Fig. 2, 5 and 6). Concomit with the lower expression level of TERT protein and telomerase activity, cytoplasmic TERT, which is solely distributed in normal control group and inflammation stage, plays important roles in maintaining normal liver functions because the low-level expression of telomerase was reported to control replicative lifespan and involved in tissue regeneration [30, 31]. Besides, studies indicate that inactivated cytoplasmic TERT can antagonize p53-dependent apoptosis and sensitizing cells to oxidative stress, therefore it may function to protect cells from malignant transformation through apoptosis pathways in TERT over-expressing cells [19, 32, 33]. However, whether TERT protein cytoplasm-to-nucleus translocation is true or not in vivo carcinogenesis remains unclear. Our results indicate that telomerase reactivation was largely regulated by the nuclear TERT protein and the process nuclear translocation (Fig 2–6). In hepatocirrhosis and cancerous stage, high levels of TERT expression and telomerase activity (Fig 2-4) and significant translocation of TERT into nuclei (Fig 6) were observed. In those stages, the up-regulated TERT and telomerase, particular the nucleus relocated TERT, may be re-characterized to protect the transformed cells from being attacked by apoptosis stimuli [34–37]. In line with normal cells that only phosphorylated TERT could regulate telomerase activity following the cytoplasm-to-nucleus translocation [13, 16, 38], tumor cells that constitutively express high levels of telomerase activity express TERT protein in phosphorylated form that is located predominately in nucleus [30, 39, 40]. Recent studies show that after being phosphorylated by HSP90, Akt and PKCα, full length TERT were transported to the nucleus, playing its roles in maintaining telomerase activity and inhibition of apoptosis. Although there were no approaches available to direct detection of the phosphorylated nuclear TERT in tissue sample and even more tests would be needed to confirm nuclear TERT in this study, at this point we could not rule out the potential roles of phosphorylation that might have taken place in the transformed cells. Therefore, cytoplasmic TERT, if any, may either derive from nucleus TERT because of the homeostasis of phosphorylation-dephosphorylation of TERT protein or to be produced by splicing varieties that can not be transported to nucleus as an active TERT [39, 41]. Accordingly, the roles of cytoplasmic TERT may be different form, or even contradictory to that were seen in the inflammatory stage, since inactive TERT in cancerous stage can inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial cells [29, 41].

In summary, TERT protein expression level and telomerase activity vary with the progression of HCC induced by DENA in rats. Based on the research, three possibilities have been raised: 1) telomerase might be an important target for cancer therapy aiming at controlling cell growth; 2) telomerase activation in the critical step of tumor progression during hepatocellular carcinogenesis might be regulated mainly by nuclear TERT; 3) compared with TERT expression level, the process of TERT nuclear translocation might be more important in the regulation of telomerase activity [40]. As discussed above, TERT nuclear translocation alone or in combination with telomerase activity or TERT expression level might be used as an appropriate biomarker in predicting the progression of HCC. Selective inhibition of the transportation of TERT from cytoplasm to the nucleus or the phosphorylation of TERT would be a potential alternative in the prevention and/or the treatment of cancer including HCC.

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