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Evaluation of centrosome abnormalities and p53 inactivation in chemical induced hepatocellular carcinogenesis

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Abnormal centrosome frequently found in human cancer is a major cause of mitotic defects and chromosome instability in cancer cells. Centrosome duplication is controlled in a cell cycle-specific manner, whereas cancer cells with dysregulation of centrosome duplication can survive and reenter the cell cycle through defective cell cycle checkpoint systems. Although numerous studies showed that centrosome amplification can be readily induced by loss or mutational inactivation of p53, however, the role of centrosomally localized p53 in the regulation of centrosome duplication had been enigma. To investigate the role of centrosome and p53 in the in vivo carcinogenesis, we performed immunofluorescence and Western blot analysis, respectively, to detect the alteration of centrosome and p53 status as well as immunohistochemical assay to detect cell proliferation in diethyl nitrosoamine (DENA) induced rat hepatocellular carcinoma (HCC). The frequencies of the centrosome abnormalities in HCC lesions were significantly higher than that of in their preneoplasitc counterparts as well as cell proliferation expression profile. Intriguingly, there was no correlation between centrosome abnormalities and cell proliferation. As for p53, the level of p53 increased in inflammation lesion, but decreased in hepatocirrhosis lesion, even undetectable in HCC lesion. These findings may imply that in inflammatory lesions aberration centrosome occurred irrespective of p53 background. However, the significantly increased percentage of cells with abnormal centrosome in hepatocirrhosis, particularly in HCC lesion concomitant with p53 inactivation and increased cell proliferation rate might synergistically contribute to carcinogenesis. Taken together, centrosome abnormalities were an early event prior to p53 inactivation in the time course of carcinogenesis, suggesting that p53 inactivation may not be the cause of centrosome aberration and centrosome may be a susceptible organelle responding to cellular insults.

Keywords: centrosome, p53, hepatocellular carcinoma, cell proliferation

In most animal cells, centrosomes are structurally complex organelles that comprise a pair of centrioles surrounded by matrix-pericentriolar material (PCM), and function as a microtubule-organizing center (MTOC) during interphase and mitosis determined a cell's polarity, morphology, mobility, and so on [1–3]. Recently, centrosome has been confirmed to be involved in some important cellular processes such as cell cycle, DNA synthesis, DNA repair, apoptosis regulation and signal transduction [4, 5]. The increasing important novel roles that centrosome plays in cell control, particularly that in maintaining genetic stability, have led us to rememorize Theoder Boveri who had proposed the role of centrosome in carcinogenesis [6]. Abnormal centrosome is believed to cause abnormal mitotic processes, thereby failing to separate chromosomes equally may result in aneuploid cells and cancerous transformation, which has been confirmed in almost all tested tumors including cancers in brain, breast, bile duct, colon, head and neck, lung, pancreas, bladder, prostate as well as HCC [7–10]. Currently, among several mechanisms related to centrosome amplification, over-duplication of centrosome during one cell cycle has been given much more attraction through which supernumerary centrosome has been produced [11]. Just like strictly regulated DNA replication, in normal cells centrosome duplicates once and only once in every cell cycle and initiates at G1/S phase and completes at early M phase, thus ensuring the faithful segregation of chromosomes. Recent studies have shown that loss of centrosome

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integrity could arrest cell in G1 phase via p53-p21 pathway [12].

As a centrosome located protein, the tumor suppressor protein p53 is a critical regulator of the G1/S checkpoint. p53 protein binds DNA, which in turn leads to G1 arrest via induction of p21 that interacts with a cell division-stimulating protein (cdk 2), a key initiator of centrosome duplicaton [13]. In contrast, multiple copies of centrosome duplication may occur in one cell cycle with an uncontrolled cell cycle-specific manner in a cell context of absence and/or lost of function of p53 which no longer binds DNA in an effective way and acts as the negative signal for cell division, thus DNA damaged cells could reenter into cell cycle through loosely surveillance of G1/S and G2/M checkpoints, thereby leading to series of catastrophic cascade, such as uncontrolled cell growth, pro-oncogenes activation, and tumors formation.

The role of prolonged cellular damage such as viral- or bacterial infection-related chronic inflammations has become widely recognized in human carcinogenesis [14], particularly in hepatocellular carcinomas, which are usually associated with viral hepatitis B, C and cirrhosis resulting from chronic hepatitis [15]. 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 detect the roles of centrosome and p53 in the chronic tissue damage induced tumor based on the Solt-Farber hepatocarcinogenesis protocol [16]. Although p53 deficiency/mutation has been found associated with centrosome amplifications, the discrepancy has remained. Some demonstrated that centrosome abnormalities induced improper p53 phosphorylation, thus leading to p53 inactivation, while others found that centrosome amplification was a downstream consequence of p53 inactivation [17–22]. Although there have been few reports of centrosome abnormalities in HCC and their relationship with p53 in human HCC, the alteration of centrosome and p53 in the time course of carcinogenesis is largely unknown. The aim of our study was to improve our understanding of the centrosome and p53 in the in vivo carcinogenesis from normal hepatocytes to cancer cells to investigate whether abnormal centrosome is a pre-cancerous event or not and centrosome amplification is the cause of p53 inactivation, or vice verse.

Materials and methods

Animal models and treatments. Three-month-old Male Sprague-Dawley (SD) rats (purchased from Zhengzhou University, Henan Province, China) weighing 115-125 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 ^{o}C), humidity (40-70%) and a 12 h light/dark alternation. Based on the previous report, 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, with the control animals (n=42) being fed with physiological salt solution for the same period. Throughout the experiment all animals were fed with food and water *ad libitum*, 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 third week (week 3, 6, 9, 12, 15, 18, 21 respectively) to evaluate the development of liver tumors and their changes. The freshly removed liver was cut into two pieces. The small part was fixed in 10% poraformaldehyde for 24 h, 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 ^{o}C until use for other analysis.

Immunofluorescence assay of centrosome abnormalities. Four μm-thick sections were immersed, post de-paraffinization and re-hydration, in PBS for 30 min, and were then subjected to heat antigen retrieval in EDTA buffer with 0.1% Tween 20 (pH 9.0) for 30 min at 95^{o}C. After turning back to room temperature, these sections were incubated in 20% normal goat serum at 37 ^{o}C for at least 1 h. An anti-γ tubulin monoclonal rabbit antibody diluted at 1: 4000 (T3559, Sigma, St. Louis, M) was applied to the sections at 4 ^{o}C for overnight. Fluorescin isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin at a dilution of 1: 400 was used as a secondary antibody for γ- tubulin detection. The nuclei were counterstained with Propidium iodide (PI). The cover-slips were then sealed with n-Prophyl gallate mount medium before having the images captured with leica confocal fluorescence microscope. To determine centrosome abnormalities in each section, at least 1000 cells were examined with respect to the number and diameter of γ-tubulin-staining dots. The value was the average of three independent experiments.

Western blot analysis of p53 protein level. Total tissue lysate was performed by standard protocol. 75 μg tissue extracts were resolved in 12% SDS-PAGE and transferred to nitrocellulose membrane. Blocked membranes were incubated with anti-p53 (sc-6243; Santa Cruz Biotech) and anti-actin (sc-1616; Santa Cruz Biotech) primary antibodies at a 1:50 and 1:1000 dilution, respectively. Enhanced chemiluminescence kit (sc-2048, Biotechnology, Santa Cruz, CA) was used to detect p53 immunoreactive bands. Quantitative evaluation was measured using Labworks 4.5 software (UVP, Inc. USA). The value was the average of three independent experiments.

Immunohistochemistry for Ki-67 antigen. Deparaffinized sections were treated with 0.3% hydrogen peroxide in methanol for 15 min at room temperature (RT) to block endogenous peroxidase activity. Then the sections were incubated in 0.01 M, pH 6.5 sodium citrate buffer for 10 min at 121^{o}C and cooled to RT. After blocking with 10% normal goat serum for 1 h at RT, the slides were subsequently incubated overnight with anti-Ki-67 (RM-9106, NeoMarkers, USA) at a dilution of 1:80. After extensive washing with PBS, the slides were incubated with PV™ kit (PV-6001, Zhongshan golden bridge, Beijing). The sections were then counterstained with DAB (ZLI-9032, Zhongshan golden bridge, China). Quantitative evaluation was measured using IDA-2000 software (Beijing konghai tech-

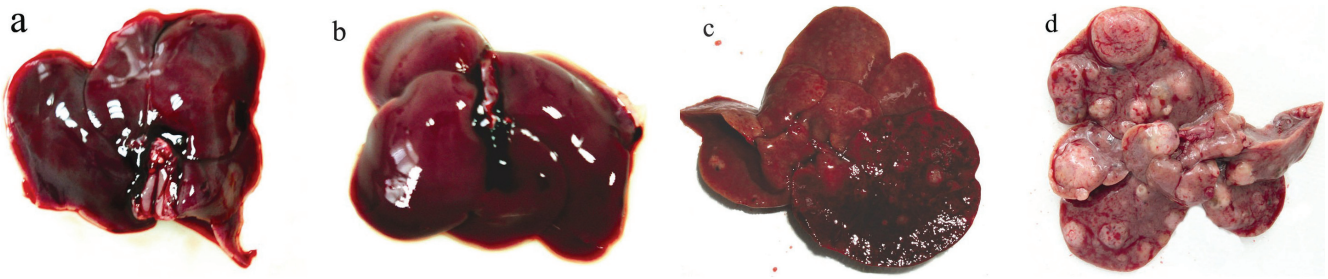

Figure 1. Gross changes of rat livers in the different stages of HCC. a: Control; b: liver in inflammatory phase (<12 weeks); c: liver in hepatocirrhosis stage (15 weeks); d: liver in HCC (18-21 weeks).

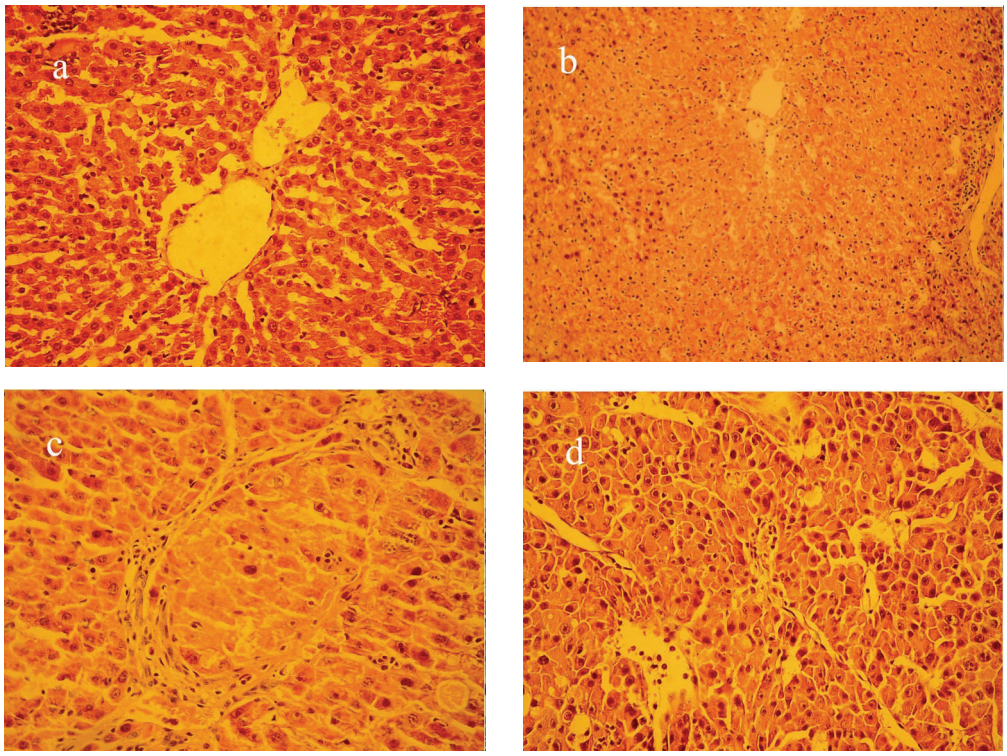

Figure 2. Histopathological examination to evaluation the changes of liver tissue in cancer development (×400). a: Control; b: inflammation (<12 weeks); c: hepatocirrhosis (15 weeks); d: cancer stages (18-21 weeks).

nology com, China). At least 10 visual fields were captured and more than 500 cells were counted. The median value was calculated from the three individual samples.

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 and spearman. A $P<0.05$ was considered statistically significant.

Results

Hepatocarcinogenesis. Paraffin-embedded liver sections of rats were submitted anonymously to two histologists for histological classification of hepatocellular lesions in rats based on the published criterion [23] (Fig. 1). Histopathology observation showed no apparent changes in the control group at all the checked time points. In the DENA treated groups, however, progressive pathological changes were observed in the livers, which were roughly divided into three stages, inflammation (<12 weeks), hepatocirrhosis (15 weeks), and cancer stages (18–21 weeks) (Fig. 2).

Cell proliferation in the development of HCC. One of the typical features of cancerous cells is unlimited proliferation. In this report, Ki-67 was used as a biomarker to evaluate the cell proliferation property in the development of HCC using

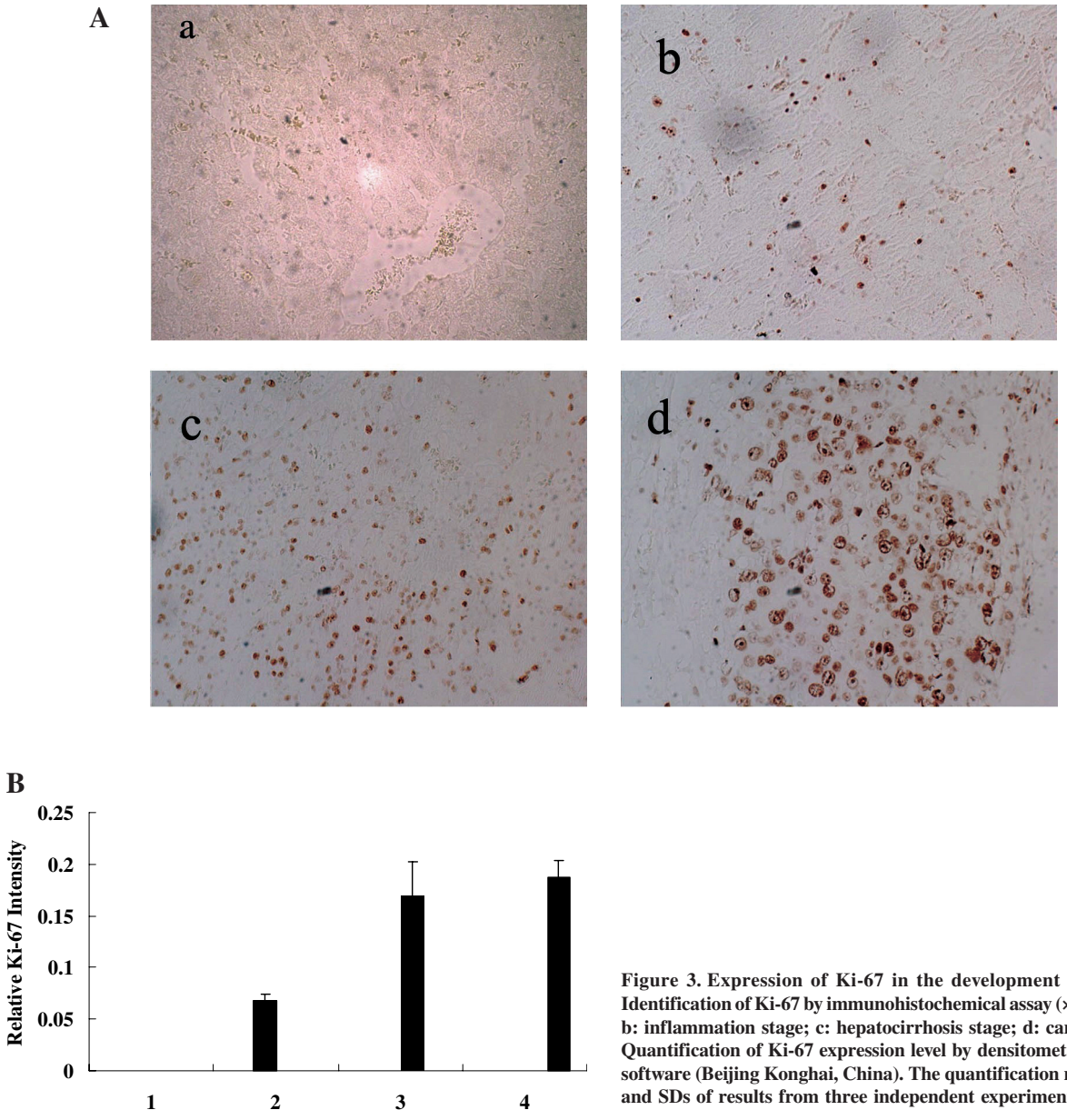

Figure 3. Expression of Ki-67 in the development of rat HCC. A: Identification of Ki-67 by immunohistochemical assay (×400). a: Control; b: inflammation stage; c: hepatocirrhosis stage; d: cancerous stage. B: Quantification of Ki-67 expression level by densitometry and IDA-2000 software (Beijing Konghai, China). The quantification represents means and SDs of results from three independent experiments.

immunohistochemical method. In normal liver tissue, the expression of Ki-67 was rarely detectable. In the DENA treated group, the sporadic positive cells with nucleus staining were detected in the early inflammation lesion (at the beginning of 3rd week), and a significant increase was found in hyperplastic nodules and cancerous lesions seen in hepatocirrhosis and HCC stages (Fig. 3).

Centrosome amplification in HCC. Centrosomes were detected in tissue sections using γ-tubulin antibody, which is a major component of the centrosome matrix. As previously reported, centrosomes were considered to be abnormal if they met at least one of the following three criteria: (1) abnormal of centrosome in number (more than two stainging spots); (2) the diameter of the centrosomes in the tumor was more than twice the diameter of those in nontumor cells; (3) the centrosomes were greater than 3 μm in length in tumor cells. The frequency of cells with abnormal centrosome was gradually higher in HCC lesion than that of in preneoplastic lesion (Fig. 4A, B). In normal liver tissue from the control group, one centrosome per cell was detected as a round dot, usually in the vicinity of the nuclear membrane (Fig. 5-a). In inflammation and hepatocirrhosis stage, particularly in HCC stage, the number of cells with centrosome abnormalities increased dramatically, and several distinct patterns of centrosome abnormalities were detected, including supernumerary centrosome and centrosome with an abnormal shape and size (Fig. 5-b-d).

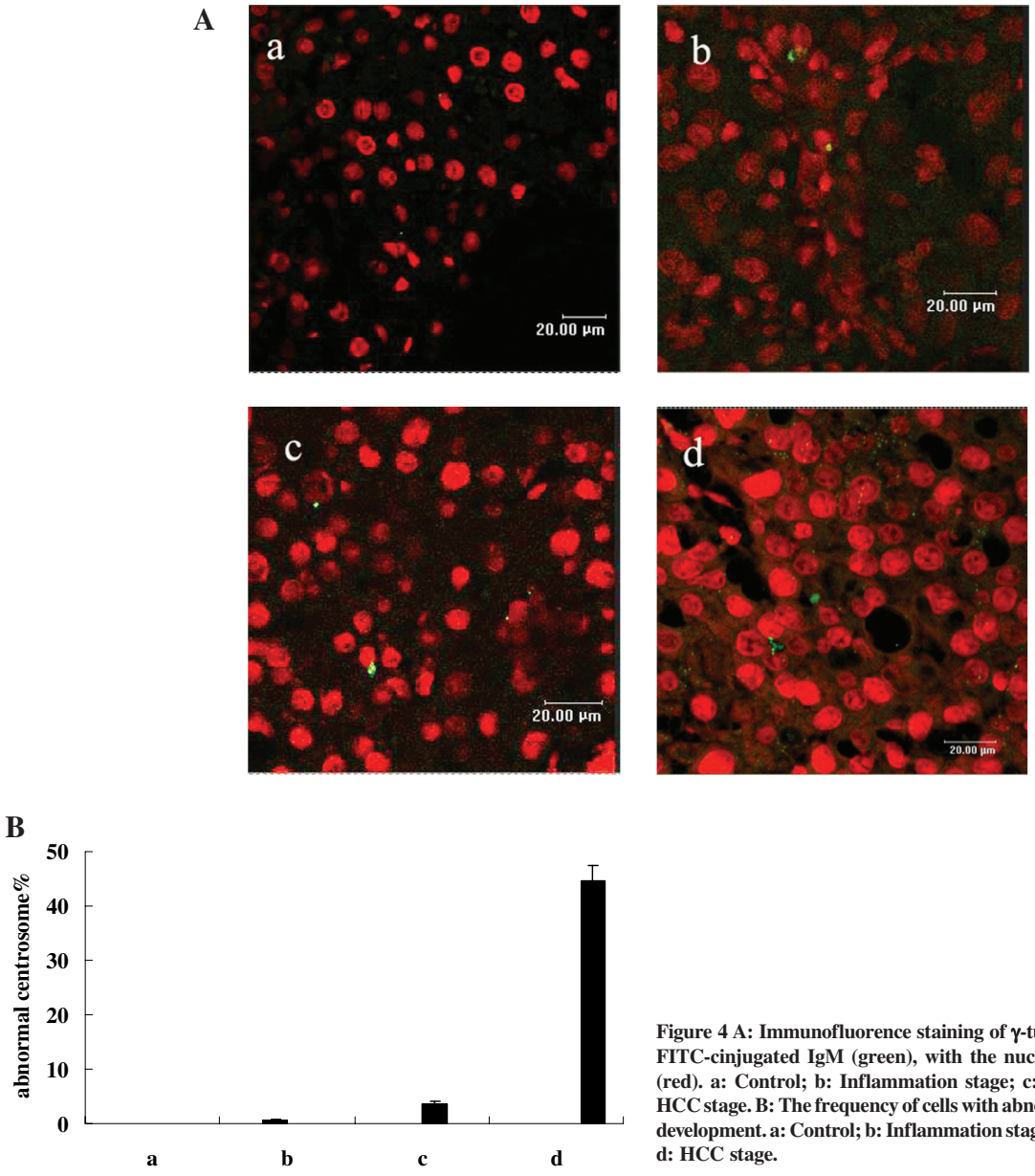

Figure 4 A: Immunofluorence staining of γ-tubulin was performed with FITC-cinjugated IgM (green), with the nucleus being stained with PI (red). a: Control; b: Inflammation stage; c: Hepatocirrhosis stage; d: HCC stage. B: The frequency of cells with abnormal centrosome in cancer development. a: Control; b: Inflammation stage; c: Hepatocirrhosis stage; d: HCC stage.

Effect of p53 in multistage progression to HCC. We analyzed the evolution of the p53 expressions along the multistep progression from the normal liver tissue to hepatocellular carcinoma. p53 expression level was constantly low in normal liver tissue, whereas a significant increase for p53 expression was observed in inflammation stage. However, with the advancement of the lesions, p53 expression got decreased in heparocirrhosis lesions, and even undetectable in hepatocellular carcinoma lesions (Fig. 6).

Centrosome abnormalities have no correlation with cell proliferation in HCC. The percentage of cells with abnormal centrosome was significantly higher in hepatocorrhosis stage and HCC stage than those of in inflammation stage as well as in normal tissue and tended to be higher in the tissues with increased cell proliferation. To establish if abnormal centrosomes became dominant in tumor following frequent cell division, we studied the relationship between the percentage of tumor cells with centrosome aberrations and the cell proliferative rate. However, no significant correlation was detected between these two factors (P=0.3294, r=0.6706).

Dicussion

Centrosome abnormalities, particularly the supernumerary centrosomes, have been observed in a wide range of

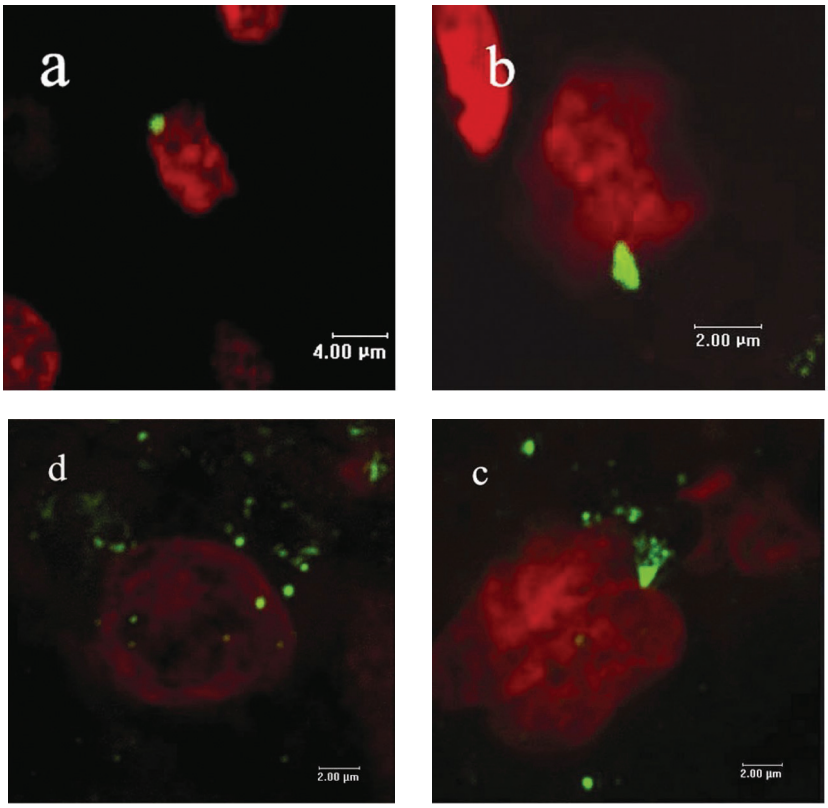

Figure 5 Different forms of abnormal centrosomes. a: Normal centrosome. One centrosome per cell can be identified as a dot, usually in the vicinity of the nuclear membrane. b: Centrosome with abnormal size. c: Centrosome with abnormal shape. d: Supernumerary centrosome.

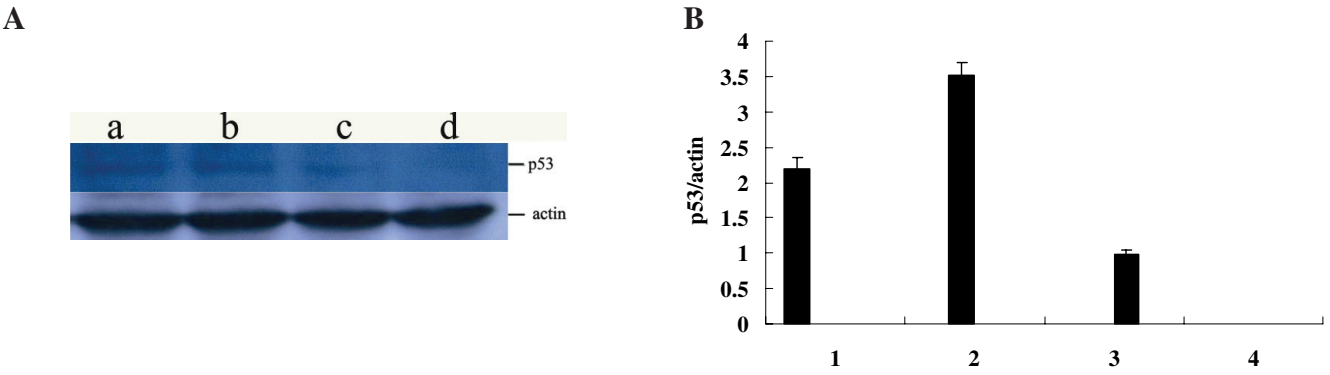

Figure 6 p53 expression levels in HCC development. A: p53 expression detected by Western blot assay. The experiment shown is representative of three independent experiments. Actin was loaded as positive control. a: Control; b: Inflammation stage; c: Hepatocirrhosis stage; d: HCC stage. B: Quantification of p53 expression in HCC development. The relative intensity of p53 was calculated as the intensity of p53 signal normalized to actin as determined by Western blot using Labworks 4.0 software (UVP, Inc. USA). The relative p53 level was normalized on the basis of the equal loading amount. The quantification represents means and SDs of results from three independent experiments.

tumor types as well as in HCC. The main cause of supernumerary centrosome is the dysregulation of centrosome duplication, which has close relation with cell cycle regulation. Here, in this report, we first characterized centrosome and p53 alterations in vivo DENA induced HCC development in a prospectively way in order to study the roles of centrosome and p53 in carcinogenesis and their relationship thereof.

In the present study, we found that normal liver tissue typically has only one centrosome per cell largely because most normal hepatocytes rest at G0 phase. Unexpected, a few cells with centrosome abnormalities were observed in preneoplastic lesions, which is consistent with previous reports [24, 25]. Even though the biological significance of centrosome amplification in preneoplastic lesions is not well understood, however, centrosome amplification in these lesions might indicate that they might carry a higher risk of malignant transformation, but may neither be sufficient nor necessary in cancer development. To support this notion, abnormal centrosome numbers have been found in cells exposed to non-specific damaging agents such as chemical toxins, cytokinesis inhibitors, γ-irradiation, or demethylating agents [26–28]. As expected, dramatically increased centrosome abnormalities were found in HCC lesions, indicating that centrosome amplification is a common and fundamental phenomenon in malignancy and might play an important role in the development of malignant tumors (Fig. 3).

Cell proliferation is essential in the genesis of cancer, particularly when chemicals are implicated [29]. Adult rat liver is resistant to carcinogenesis, however, it becomes highly susceptible when hepatocyte proliferation is induced by the carcinogen itself. Some investigators have suggested that there were no centrosome abnormalities in many different types of non-neoplastic cells that have varying degrees of proliferative activity [8]. This is supported by our result where we showed that the percentage of tumor cells with centrosome was not correlated with the Ki-67 positive index.

The tumor suppressor gene *p53* plays a pivotal role in regulation of the cell cycle and in the maintenance of genomic integrity after induction of genetic damage. Here, we found that p53 expression was very low and even undetectable in normal hepatocyte cells, however, after exposure to carcinogen DENA, high levels of p53 expression was observed in preneoplastic lesion, which is consistent with previous report, to protect cells from cell death in order to maintain the turnover of the normal cell number [30, 31]. Interestingly, very low level of p53 has been found in hepatocirrhosis and even undetectable in HCC stage. Inactivation of p53 has long been regarded as the cause of centrosome hyperamplification, leading to aberrant mitosis and chromosomal instability. Despite a wealth of information about centrosome abnormalities and p53 inactivation in increasing types of cancer, there are still major gaps in our understanding of the relationship thereof, particularly in HCC. Considering the alterations of centrosome in the time course of carcinogenesis, p53 inactivation is posterior to centrosome abnormalities, suggesting that p53 inactivation might not be the cause of centrosome alterations.

In order to better understand the functional relevance of centrosome aberrations in the carcinogenesis, it is important to consider the cellular context in which they occur [32, 33]. Centrosome aberrations can be roughly divided into three subgroups based on presumably distinct biological impact: primary centrosome overduplication, transient centrosome accumulation, and permanent centrosome accumulation [34]. The cells of primary centrosome overduplication with normal nuclear morphology and chromosomal content have not been found to be accompanied with p53 inactivation, whereas the latter two categories with abnormal numbers of centrosome were accompanied with p53 inactivity and alterations of nuclear morphology and chromosomal content. In this present study, when considering the role of centrosome abnormalities plays in carcinogenesis, we hypothesize that primary centrosome overduplication in preneoplastic lesions might play a protect role only to eliminate the highest risk cells via cell cycle arrest, whereas abnormal centrosome, including transient centrosome accumulation and permanent centrosome accumulation, and p53 inactivity may synergistically contribute to cancer formation in late carcinogenesis [35], suggesting that the different types of centrosome abnormalities in different cell context, the different lesion of cell would be. Additionally, these findings not only question the significance of p53 for centrosome aberrations, but also underscore that centrosome anomalies are likely to represent a susceptible cellular organelle response to a variety of cellular insults at early time point.

In summary, we propose that the presence of extensive centrosome amplification in preneoplastic lesions prior to p53 inactivation suggests that each alone might not lead to cancer. Centrosome may response to cellular insults resulting in defects in centrosome structure or function in early carcinogenesis irrespective of p53 status and, though this process, accelerated centrosome abnormalities with loss of tumor suppressor p53 might synergistically contribute to carcinogenesis. Further research will be necessary to investigate the interactions between centrosome abnormalities, the complex regulation of the cell cycle, and cell signal.

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