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RTC GENE DELETION LEADS TO INCREASED SUSCEPTIBILITY TO HEPATOCELLULAR CARCINOGENESIS

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***Objective*: to study the relationship between retuculon C (RTC) gene deletion and susceptibility to hepatocellular carcinoma (HCC) *in vivo*. *Methods and Results*: Western blot analysis for cell cycle-associated proteins showed that the levels of expression of cyclin D, Cdk 2 and 4, and cdcA were not altered in the hepatocytes of RTC heterozygous (HG) mice when compared with those of wild-genotype (WG) littermates. On the other hand, p27 and c-myc expression levels were significantly decreased in liver tissue of 20 days-old HG mice and unaffected — in 10 months-old animals; cyclin A expression was unaffected in liver tissue of 20 days-old HG mice but increased — in 10 months-old ones. The level of RTC protein expression was significantly lower in HG tumors in comparison with surrounding normal hepatocytes. HG mice had significantly increased incidence of spontaneous and chemically induced HCC compared to WG littermates. *Conclusions*: our data suggest that the decreased level of RTC expression results in higher susceptibility to hepatocellular carcinoma.**
***Key Words*: heterozygous mice, *RTC* gene, tumor susceptibility, hepatocellular carcinogenesis.**

Hepatocellular carcinoma (HCC) is the second most common cause of death from cancer in China with the mortality rate of 18 per 100 000. The mechanism of HCC is still unclear, although some genes are known to play a role in the malignant transformation of liver cells, and a variety of studies have described differences in gene expression which distinguished tumor from non–tumor tissue. The new genes, especially the functional genes directly related with tumor are still worth to be found. Reticulon C (RTC) gene is a member of a gene family encoding reticulons [1] and some researchers suggested that it is associated with cancerogenesis, but there is no reports about the relationship between RTC and HCC.

RTC heterozygous mice. RTC heterozygous mice (HG) have been generated by gene targeting. The RTC gene was targeted using a genomic fragment derived from a mouse genomic library. RTC was disrupted using a phosphoglycerate kinase–neocassette, and the resulting construct was electroporated into mouse ES cells. After successful selection with neomycin, the resulting ES cells were then analyzed using PCR and Southern analysis, and ES clones containing a successfully targeted RTC gene were injected into C57BL/7 mouse blastocysts and implanted into pseudopregnant carrier mice. The resulting chimeric mice were then analyzed, and HG mice were bred. For the carcinogenesis study, wild–genotype mice (WG) and HG littermates were generated by mating the HG. At the age of 20 days, male mice of both genotypes were given either a single i.p. injection of 5.5 mg/kg N–nitrosodiethylamine (DEN) in 0.1 ml of saline or saline alone. Animals were euthanized after 10 months. Tissues were analyzed microscopically and histologically for evidence of tumors by a board certified veterinary pathologist using established diagnostic criteria. All animal procedures were performed according to the rules of Ethic Committee.

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Abbreviations used: DEN — N–nitrosodiethylamine; HCC — hepatocellular carcinoma; HG — RTC heterozygous mice; RTC — reticulon C; WG — wild–genotype mice.

RNA isolation and Northern blot analysis. The harvested mouse liver tissues were immediately frozen in liquid nitrogen and subsequently processed for RNA isolation using the TRIzol reagent (Shenggong, Shanghai, China) according to the protocol recommended by manufacturer. Once total RNA was isolated, 20 μg were separated by electrophoresis and transferred onto nitrocellulose blotting membrane (Bio–Rad, USA). Prehybridization, hybridization, and washing of the filters was done by standard protocol. The RTC and β–actin probes were labeled using the PRIME–IT random primer labeling kit (Shenggong, Shanghai, China).

Immunoblot analysis. Frozen liver tissues from the mice were homogenized with PBS at 4 °C, and the protein concentration was determined. Samples were placed in sample buffer (0.0625 M Trizma base, 2% SDS, and 5% 2–mercaptoethanol) and boiled for 5 min. Electrophoresis was carried out under reducing conditions using 50 μg of total protein in each lane. After electrophoresis, protein was transferred to a 0.2–μm nitrocellulose membrane and incubated for 1 h in blocking buffer (5% non fat dry milk, Tris–buffered saline, and 1% Tween). Subsequently, the membrane was incubated with the appropriate antibody overnight at room temperature. After washing with 0.1% Tween–Tris–buffered saline, the membrane was incubated in the presence of appropriate horseradish peroxidase–labeled secondary antibody (Boshde, Wuhan, China) at a dilution of 1 : 5000 for 1 h at room temperature. After washing three times, immunoreactive bands were visualized by enhanced chemiluminescence (Boshde, Wuhan, China). Primary antibodies used were as follows: anti–p27, anti–cdc A, anticyclin D, anti–cdk 2, and anti–cdk 4, anti–c–myc from Oncogene Research Products (Cambridge, MA).

Immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin overnight, processed and embedded in paraffin blocks. Immunochemical staining was performed using an indirect immunoperoxidase protocol (Boshde, Wuhan, China). In paraffin blocks, the tissues were sectioned at a thickness of 5 μm, deparaffinized in Hemo–de Boshde (Wuhan, China), and rehydrated in PBS (pH 7.2). The endogenous peroxidase activity was inactivated by incubation in 0.3% H_2O_2

for 10 min, and normal goat serum was used to block nonspecific sites. Next, the sections were incubated with primary antibodies for 12 h at 4 °C in humidified chambers. RTC was detected by polyclonal antihuman RTC (prepared by ourselves). Antigenic binding sites were visualized by incubation with biotinylated secondary antibody, avidin–biotin–horseradish peroxidase complex and diaminobenzidine tetrachloride before counterstaining with Gills' hematoxylin. Negative control sections were processed in an identical manner after substituting the primary antibodies with rabbit IgG fraction.

RTC mRNA expression. In the liver tissues of WG and HG mice, the relative levels of expression of RTC mRNA were investigated by Northern blot analysis. In both 20–days–old and 10–month–old mice, the levels of expression of RTC mRNA are significantly different between WG and HG animals; RTC mRNA expression wasn't detectable in HG mice and was reduced in the liver tissues from 10–month–old mice compared with that of the 20–days–old animals (Fig. 1).

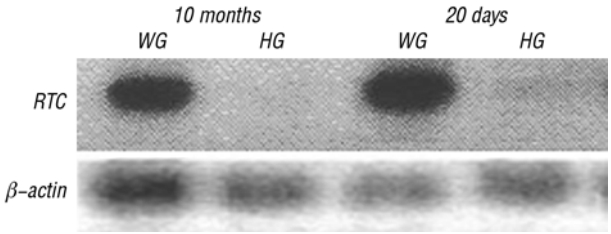

Fig. 1. Northern blot analysis of RTC expression in liver tissues of RTC +/– mice

Western blot analysis for cell cycle–associated proteins. As shown in Fig. 2, the levels of expression of cyclin D, Cdk 2 and 4, and cdcA were not altered in HG mice when compared with the WG littermates. On the other hand, p27 and c–myc expression was significantly decreased in the 20–days–old HG mice and unaffected — in 10 months–old animals whilst cyclin A expression was unaffected at 20 days–old animals but increased at 10 months–old mice.

Hepatocarcinogenesis in the HG mice. Among the 98 untreated mice (49 WG and 49 HG) observed during a 1.5–year period, liver tumors were registered in 10 HG mice and none — in WG mice. These results suggest that the RTC genotype may affect spontaneous tumorigenesis in mice. Among 88 mice (44 WG and 44 HG) treated with single–initiating dose of DEN

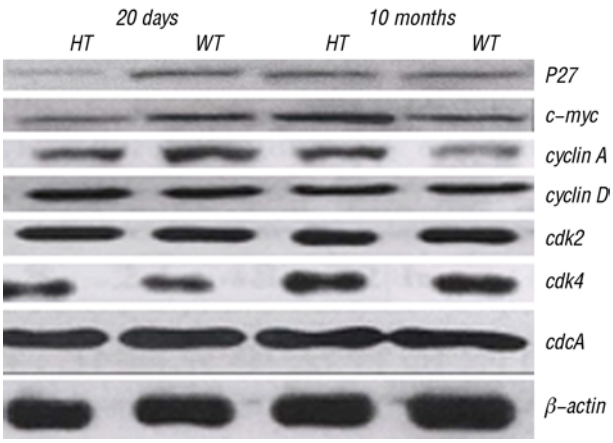

Fig. 2. Western blot analysis of cell cycle–associated proteins (p27, c–myc, cyclin A)

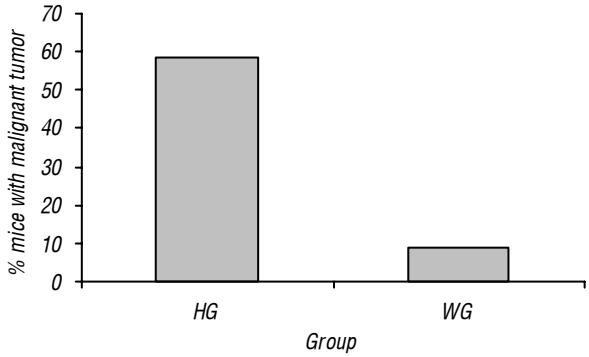

Fig.3. Incidence of liver tumors in HG and WG mice after treatment with DEN

at the age of 20 days, HG mice had significantly increased incidence of liver tumors (both adenoma and carcinoma) compared with WG littermates (all animals were sacrificed after 10 months of study) (Fig. 3). In HG group, HCC was detected in 20 mice, and in WG group — in none of studied animals.

Immunohistochemical analysis of RTC protein expression in transplanted tumors. As shown in Fig. 4, the level of expression of RTC protein was significantly lower in HG tumors in comparison with surrounding normal hepatocytes. These observations allows us to suggest that the increased incidence of tumors in HG mice is likely to be attributable to the decreased levels of RTC expression.

Results of the present study demonstrated that the decreased levels of RTC expression in HG mice result in a subtly altered cellular phenotype of hepatocytes. HG mice exhibited increased susceptibility to hepatocarcinogenesis after carcinogenic challenge. These results suggest that RTC is a suppressor of liver tumorigenesis with gene dosage effect.

Some studies have demonstrated that RTC–induced growth arrest is associated with the changes in the expression and/or activity of various cell cycle–associated genes [2]. In particular, RTC induces the expression of p15, p21, p27 and suppresses the expression of c–myc, cyclins A, D1, and E, cdks 2 and 4, and the cyclin–dependent kinase activator cdcA. To determine the mechanism underlying the decreased sensitivity to the growth–inhibitory effect in the HG mice, the levels of expression of cell cycle–associated proteins were investigated using Western blot analysis. Our results showed that although the decreased level of expression of p27 in the HG mice is consistent with the results of previously published *in vitro* studies [3], the change in c–myc expression is in the opposite direction to that predicted one according to [4]. As proposed previously, these results suggest that the action of RTC on target genes may differ significantly between *in vivo* and *in vitro* environments.

Our results demonstrated that RTC genotype may affect spontaneous tumorigenesis in mice. Upon chemically induced carcinogenesis, the rate of liver tumorigenesis was significantly higher and developed tumors were significantly larger in the HG mice compared to WG littermates. These results indicate that RTC genotype is a significant factor during progression of the HCC, and endogenous RTC has a tumor suppressor activity in the liver tissue. Such conclusion is consistent with the reports [5, 6]. The mechanism responsible for the in–

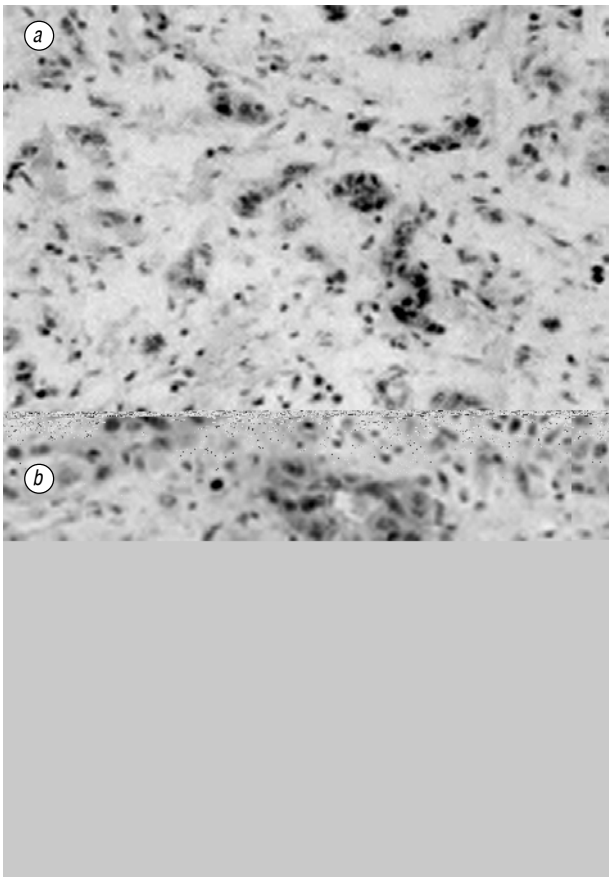

Fig. 4. Immunohistochemical analysis of RTC expression in liver tissues of HG mice: tumor tissue (*a*) and surrounding normal tissue (*b*) (SABC×100)

creased risk of liver tumorigenesis in the HG mice remains to be elucidated. It is likely that the most significant factor is the decreased sensitivity to RTC due to the loss of one RTC allele [7]. However, it is possible that the loss of the remaining WG allele may occur as a result of the exposure to the carcinogens. In the present study, the status of the remaining RTC WG allele in the tumors was not investigated because of the difficulties with separation of malignant and normal cells.

The precise mechanism for the *de novo* inactivation of RTC in tumor tissues is not clear. Similarly to other tumor suppressor genes such as p53 and Rb, RTC is frequently mutated in a subset of liver cancer cells [8, 9]. Alternatively, transcriptional repression is another potential mechanism. In fact, evidence to date suggests that the transcriptional repression of *RTC* gene without mutation or loss of heterozygosity may be the major mechanism of RTC inactivation in breast, prostate, and stomach cancer [10]. Our study demonstrated that HG mice challenged with DEN exhibited increased HCC incidence compared with WG littermates, and point to tumor suppressor role of RTC in hepatocytes.

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ДЕЛЕЦИЯ ГЕНА *RTC* ПРИВОДИТ К ПОВЫШЕННОМУ РИСКУ РАЗВИТИЯ ГЕПАТОЦЕЛЛЮЛЯРНОЙ КАРЦИНОМЫ

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***Цель*: исследовать связь между делецией гена ретикулона С (*RTC*) и риском развития рака печени *in vivo*. *Методы и результаты*: методом Вестерн-блотт-анализа продемонстрировано, что экспрессия циклина D, Cdk 2 и 4, cdcA остается неизменной в гепатоцитах мышей, гетерозиготных по *RTC* (HG), в сравнении с таковой у животных дикого фенотипа (WG). Уровень экспрессии p27 и c-myc значительно снижен в ткани печени у животных HG 20-дневного возраста и не изменены у таковых 10-месячного возраста в отличие от экспрессии циклина D, для которого характерна обратная зависимость. На уровне белка экспрессия *RTC* была значительно снижена в опухолях животных HG в сравнении с таковой в условно-нормальных тканей печени. Частота развития гепатоцеллюлярной карциномы (как спонтанной, так и химически индуцированной) была значительно выше в группе животных HG в сравнении с группой WG. *Выводы*: наши данные свидетельствуют о том, что частота развития рака печени обратно коррелирует с экспрессией *RTC*.**

***Ключевые слова*: гетерозиготные мыши, ген *RTC*, восприимчивость, канцерогенез печени.**