

Disseminated tumor cells homing into rats' liver: A new possible mechanism of HCC recurrence

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Abstract

AIM: To detect the origin of hepatocellular carcinoma (HCC) recurring and attempt to propose a new recurrent mechanism.

METHODS: Orthotopic liver allotransplantation was performed on male rats with HCC- induced by diethylnitrosamine using female donors. Metastatic tumors in transplanted livers were obtained. A DNA probe that exhibits specificity for the rat Y chromosome was generated by using a set of primers specific to murine *sry* gene. *In situ* hybridization (ISH) for Y chromosome was used to detect the origin of HCC recurring. Male HCC tissue was designed to be positive control. ISH on female tissue and using non-labeled with DIG probe was thought to be negative control.

RESULTS: Positive marks were seen through ISH for Y chromosome in recurrent tumor tissue and positive control. No signal was detected in both negative controls.

CONCLUSION: Recurrent HCC after liver transplantation originated from disseminated tumor cells in recipients. Extrahepatic cells homing into liver may be a new HCC recurrence mechanism. Likewise, it implicates that this mechanism is responsible for HCC recurring after hepatectomy.

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INTRODUCTION

The poor outcomes of patients with hepatocellular carcinoma (HCC) are mainly resulted from high postoperative recurrence rate with 65% after radical resection versus 58% after liver transplantation^[1,2]. Over the past decades, many investigations using clinicopathological and molecular biological methods have showed that there existed two HCC recurrent mechanisms of intrahepatic metastasis (IM) and multicentric occurrence (MO)^[3-5]. However, the both mechanisms are very reluctant to elucidate the HCC recurrence following liver transplantation in rigidly selected patients. Therefore, we refer a hypothesis that recurrent HCC in transplanted liver is likely to originate from extrahepatic tumor cells in recipient if de novo

carcinogenesis is excluded. Likewise, disseminated tumor cells possibly go back to remnant liver after hepatectomy.

We have established an animal model of liver transplantation for HCC in rats. Male rat liver with HCC induced by diethylnitrosamine (DENa) was replaced by allograft from normal syngenic female animal. Recurrent tumors from male recipient of female liver were analyzed using *in situ* hybridization (ISH) for the Y chromosome to indicate cells origins. On the basis of our investigation and other supportive literatures, we make an attempt to propose a new possible mechanism of HCC recurrence.

MATERIALS AND METHODS

Orthotopic liver transplantation (OLT) for HCC-induced in gender discordant rats

Ninety-eight inbred seven-week-old male SD rats, weight ranged from 100 g to 120 g, were purchased from PEAK Company in Shanghai with the approval of Shanghai Animal Committee. HCC was induced by oral administration of 100 ppm DENa (Sigma Company, USA) water solution. OLT for HCC was performed according to Kamada cuff techniques^[6] on male rats with HCC and donor livers were from normal syngenic female SD rats with weight ranged from 250 g to 300 g. No immunosuppressant was postoperatively administered. Explanted livers were examined pathologically. The recurrent tumors in the transplants may be explored through laparotomy before the recipients' death. Harvested specimens were preserved at -70 °C.

Using ISH to detect the Y chromosomes in recurrent HCC cells^[7,8]

Male rat genomic DNA was purified from 300 µL blood sample using Wizard Genomic DNA Purification Kit (Promega). *sry* gene specific primer, of which the sequences are 5' -CAGAGATCAGCAAGCAGCTG-3' and 5' -TGCAGCTCTACTCCAGTCTTG-3', was synthesized by Shengong Biochemical Incorporation in Shanghai. 0.1 µg of the genomic DNA as template, *sry* gene was amplified by PCR. The reaction was comprised of 35 cycles of 5 min at 94 °C, 0.5 min at 60 °C, 1 min at 72 °C. PCR products were analyzed on 15 g/L agarose gel and then purified with QIAquick Gel Extraction Kit (Qiagen). Target gene was labeled according to the instruction of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche).

Male SD rat HCC tissue confirmed by pathology was designed to be positive control. ISH on female SD rat liver tissue and using non-labeled with DIG probe was thought to be negative control. ISH efficacy would be verified by the both controls.

Frozen tissue sections (5 µm in thickness) were fixed on the slides treated by 40g/L polyformaldehyde. Slides were washed in PBS (PH7.4) for 5 min (2 times), 3g/L Triton X-100/PBS for 10 min, and PBS (PH7.4) for 5 min (2 times). Tissue was digested with pepsin 2 µg/mL for 15 min at 37 °C, and then washed in PBS for 5 min and rinsed in 4×sodium saline citrate (SSC) for 2 min at room temperature. Slides were

denatured with 50% formamide in 2×SSC for 15 min at room temperature, then dehydrated and air dried. Probe for Y chromosome (10 ng/μL) was denatured for 5 min at 75 °C, added to the denatured tissue, coverslipped and incubated in a humid chamber overnight at 42 °C. Slides were then washed in 2×SSC for 10 min (2 times), 1×SSC for 10 min (2 times), 0.1×SSC 10 for min (3 times), and in buffer1 (Tris-HCl, PH7.5, 100 mmol/L; NaCl 150 mmol/L) for 5 min. Anti-DiG-Ap (1:500 dilution) was added to tissues and incubated for 2 h at 43 °C. Slides were washed in buffer1 for 5 min (2 times) and buffer 2 (Tris-HCl, PH9.5, 100 mmol/L; NaCl 100 mmol/L, MgCl₂ 50 mmol/L) for 10 min. And then slides were transferred to NBT/BCIP solution to be stained for more than 2 h, and rinsed with distilled water. Tissue sections were mounted and evaluated by light microscopy.

RESULTS

HCC recurrence was found in 6 transplants from 98 transplanted rats. Of them, 3 grafts were discarded because the recipients have been dead when laparotomy. Thirteen lesions were obtained in other three available transplants, confirmed to HCC by pathological examination. These specimens were preserved at -70 °C as a bank for further utilization.

HCC cells carrying a positive reaction product (blue staining) were seen in control male rats. No signal was detected in the controls of female liver and system using non-labeled probe. Control trials showed that the *sry* gene specific probe was efficient.

Positive staining was seen in frozen sections of the 3 recurrent transplants. Typically, the probe was successfully hybridized with target gene on tumor cells, whereas failed to on para-tumor tissues. The capsule, aimed at by arrow, definitely parts the recurrent tumor from the recipient liver parenchyma under 10×10 magnification (Figure 1).

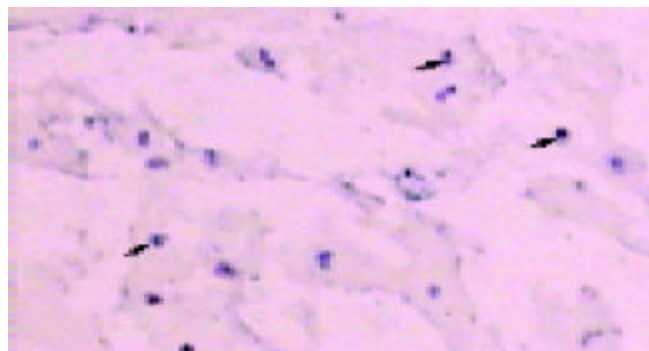


Figure 1 The blue blots under the purple background indicated Y chromosomes in these nuclei of recurrent tumor cells with 10×10 magnification microscope. The capsule definitely parted the recurrent tumor tissue from the recipient liver parenchyma. No positive signals were found out in the para-tumor tissue.

DISCUSSION

Liver transplantation for HCC in rats provides an excellent animal model to carcinogenesis investigation^[9]. Intrahepatic tumors and underlying lesions are removed completely, and then some interventional trials could be executed etiologically. In our experiments, no immunosuppressants were administered because rejective reaction was weak in allotransplantation on syngenic SD rats. The promotion of immunosuppressants to tumor growth can be excluded. However, it remains indefinite that induction of HCC existed in recipients after the withdrawal of DENA^[10]. There were two possible mechanisms of tumor recurring with the inclusion of disseminated tumor cells homing

into implanted liver and sequential contribution of DENA. Therefore, a marker is a key to discriminate between the two possibilities.

Cellular markers have been obtained through varieties of strategies such as transgenic animal, retroviral transduction. But these strategies are very complicated and low efficient. Discordant gender transplants offer the benefit of having 100% of the cells marked, as opposed to retroviral transduction, where at best only 5-10% of the cells are marked^[11]. In our experience, Y chromosome, uniquely existed in male cell, was initially acted as a marker with which male and female tissues were differentiated. Y chromosome-specific probe was successfully hybridized with *sry* gene of which multiple copies facilitated this hybridization^[7]. This *in situ* hybridization system was proved to be reliable by positive and negative control we designed. Our results therefore indicated that the origin of recurrent HCC in female liver was from disseminated tumor cells in male recipient. We personally define this phenomenon that extrahepatic tumor cells go back to liver as "homing".

Other studies could support the homing hypothesis we proposed. (1). Alpha-fetoprotein (AFP) messenger RNA (mRNA) has been proposed as a marker of HCC cells disseminated into the circulation. Multiple molecular methods, such as nested and semi-quantitative retro-transcription polymerase chains reaction (RT-PCR), have been utilized to detect AFP mRNA in order to confirm the presence of hematogenous HCC cells. This marker expression in peripheral blood of patients with HCC indicates the existence of tumor cells, although it remains controversial that AFP mRNA is taken as an evidence of HCC recurrence^[12-14]. (2). The homing of both lymphocytes and malignant hematic cells has been acknowledged^[15,16]. Involved adhesion molecules that mediate their migration also contribute to invasiveness of liver cancer^[16]. (3). Metastasis is the result of multiple sequential steps and is a highly organized, nonrandom, and organ-selective process^[17]. A group of biological molecules is collectively responsible for determining whether tumor cells can progress from a single malignant cell to a metastatic disease^[17]. But metastatic cells eventually colonize a particular organ that provides an optimal microenvironment^[18,19]. It therefore is warranted that transplanted liver or regenerating liver after resection may be a particularly fertile ground for extrahepatic HCC cells to proliferate.

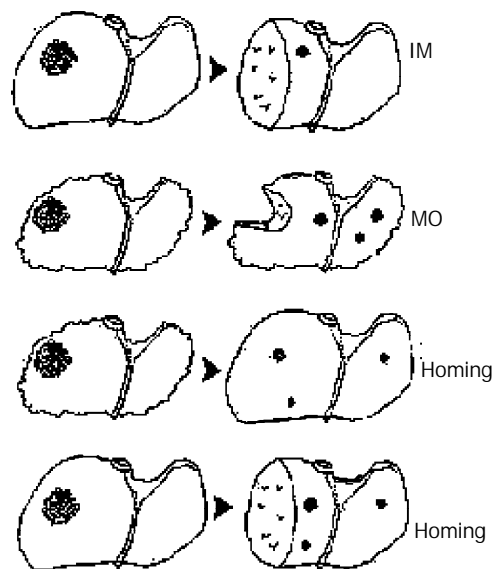


Figure 2 It is acknowledged that there exist two HCC recurring mechanisms of intrahepatic metastasis through portal venous system and multicentric occurrence from cirrhotic nodules. These extrahepatic cells homing into liver may be a

new mechanism after liver transplantation. Likewise, it implicates that this mechanism is responsible for HCC recurrence after hepatectomy.

On the basis of this experiment, we concluded that recurrent HCC after liver transplantation originated from the disseminated tumor cells. These extrahepatic cells homing into liver may be a new HCC recurrence mechanism. Likewise, it implicates that this new mechanism is responsible for HCC recurrence after hepatectomy besides MO and IM (Figure 2). But our experiment, to some extent, was to describe a homing phenomenon. Its real mechanism needs to be further investigated by molecular biology.

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