ORIGINAL RESEARCH

Telomerase activity (TMA) in tumour and peritumoural tissues in a rat liver cancer model

Huo-Jun Zhang¹
Ji-Jin Yang¹
Jian-Ming Tian¹
Pei-Jun Wang¹
Cheng-Wei Shao¹
Chang-Jing Zuo¹
Shun-Min Zhang²
Sanjay Gupta³

¹ Department of Radiology, ² Department of Pathology, Changhai Hospital, Second Military Medical University, Shanghai, China

³ Department of Diagnostic Radiology, Section of Interventional Radiology, The University of Texas M D Anderson Cancer Center, Houston, TX, U.S.A

Manuscript submitted 25th September, 2008

Manuscript accepted 15th November, 2008

Clin Invest Med 2009; 32 (1): E8-E12.

Abstract

Purpose: To study the levels of telomerase activity (TMA) in tumour and peritumoural tissues in a liver cancer model in rats, and to study the change in TMA expression over time.

Methods: Using the telomeric repeated amplification protocol (TRAP), TMA was measured in tumour tissue, peritumoural tissue and normal liver tissue of Walker-256 tumour-bearing rats at 4, 6 and 8 days after tumour implantation.

Results: TMA at day 4, 6 and 8 was 0.767 ± 0.117 , 0.768 ± 0.118 and 0.774 ± 0.111 in tumour tissue, 0.389 ± 0.263 , 0.492 ± 0.253 and 0.584 ± 0.239 in peritumoural tissue, and 0.231 ± 0.022 , 0.229 ± 0.022 and 0.233 ± 0.021 in normal liver tissue, respectively. TMA in tumour tissue was higher than that in peritumoural and normal liver tissues at all time points of measurement ($P < 0.05$). The TMA levels in tumour tissue and normal liver tissue did not show any change over time. TMA level in the peritumoural tissue increased with time; TMA level in animals sacrificed at day 8 was higher than that seen in animals sacrificed at day 4 ($P < 0.05$).

Conclusion: TMA in Walker-256 tumour-bearing rats was higher than that in normal and peritumoural tissues. TMA level in the peritumoural tissue increased with time suggesting that TMA activation in peritumoural tissue may be an important factor promoting tumour growth.

Telomerase is a ribonucleoprotein composed of RNA and protein and able to synthesize telomere DNA using intrinsic RNA as the template. Telomere plays an important role in maintaining stability of chromosomes, the length and stability of which determine cell life span, and is closely associated with cell aging and carcinogenesis. A number of recent studies have shown an association between telomerase activity (TMA) and various human cancers, including hepatocellular carcinomas.^{1,2} Some previous clinical and laboratory studies have shown increased telomerase activity in hepatocellular carcinoma with no to minimal activity in normal liver tissue.³ However, the exact relationship between telomerase activation and hepatocarcinogenesis remains unclear, and there is a definite need for further characterizing the timing of telomerase reactivation during hepatocarcinogenesis. Also, the TMA in the liver tissue immediately surrounding a growing liver cancer has not been studied. Recently, there have been many pre-clinical studies of TMA and TMA inhibition in various tumour strains.^{4,5} However, most of these studies were limited to cell strains cultured *in vitro*, and only a few studies report

animal models *in vivo*.⁶⁻⁸ There is no study reporting TMA assay in the Walker-256 tumour strain. The Walker-256 tumour strain is a spontaneous rat mammary carcinosarcoma, commonly used in animal models. The biological behaviour of the tumour growing out of the strain implanted in the rat liver is similar to those of the human primary tumour of the liver, and its blood supply is also similar to that of human liver cancer. Therefore, this model has been widely used in experimental research of early imaging diagnosis, tumour growth patterns and intervention therapy of liver cancer.^{9,10} The aim of the present work was to evaluate TMA expression in implanted liver cancer tissue, peritumoural tissue and normal liver tissue in rat tumour model, and to study the change in TMA expression over time in order to understand the relationship between TMA expression and tumour growth.

Materials and methods

Experimental animals and tumour

Adult male SD rats weighing 160~200g were used. Weaning male Wistar rats weighing 50~70g were used for weekly tumour passage. The Walker 256 tumour strain (Shanghai Institutes of Pharmaceutical Industry, Shanghai, China) was used as the implanted liver cancer strain. All animal studies were conducted in accordance with a protocol approved by the Animal Investigation Committee at Second Military Medical University.

Preparation of the animal model and specimens

Walker-256 cancer cells (at a concentration of 1×10^6 /ml), were injected into the peritoneal space of Wistar rats. After 5~7days, when the rats developed ascites, the animals were euthanized. 5~8ml cancerous ascites (mostly yellow or bloody turbid suspension) were removed from the peritoneal space, and centrifuged at 1200rpm for 2 min. The supernatant was discarded and the remainder was washed with normal saline and

centrifuged twice. Then, 0.4ml of the Walker-256 carcinoma cells suspension were injected subcutaneously (thigh wall) into a donor SD rat for solid tumour development. A 2-week-old subcutaneously growing solid tumour was explanted from a donor rat and cut into small cubes about 1mm³. SD rats were anesthetized with pentobarbital sodium (30 mg/kg injected intraperitoneally). The tumour implantation was performed according to the technique described by Li et al.¹¹ Laparotomy was performed through a midline abdominal incision, the left hepatic lobe was exposed, and a small subcapsular tunnel about 3-5mm depth was made by a fine-point tweezer. Then, the solid tumour fragment was gently inserted into the subcapsular tunnel and fixed with a small piece of gelfoam on the liver surface and the abdominal wall was then closed. The animals were sacrificed using a lethal dose of anesthetic, at three different time points, at 4 (Group A), 6 (Group B), and 8 (Group C) days after the inoculation. Animals in which no tumour was present at autopsy were excluded from the study. The tumour sizes were measured, either by CT performed immediately before sacrificing the animal or at autopsy. Samples were obtained from the tumour tissue, peritumoural tissue (2 mm from the tumour edge), and from normal liver tissue (5mm from the tumour edge). Each group included 18 animals, yielding a total of 162 tissue samples. The samples were cut into small cubes 2 mm³ in volume and stored at -80° for analysis.

TMA assay

TMA test kit was a product of Boehringer Mannheim, Germany. The tissue pieces were pathologically sliced into 10~20µm sections, from which 6~8 sections (about 100~120 mg) were selected, dispersed thoroughly by addition of 200µl detergent, dissolved, frozen-cleaved for 30 min, and centrifuged at 1600rpm at 4° for 15-20 min. 200µl supernatant was pipetted into a tube for analysis.

3-5µl RNA extract solution were taken from each sample and added with 25µl reaction mixture (Tris-buffer containing telomerase substrate, primer, nucleotide and Taq polymerase) in a PCR amplification tube for TRAP: extension at 25⁰ for 30 min; telomerase degeneration at 94⁰ for 10 min; degeneration at 94⁰ for 30 s; annealing at 50⁰ for 30s; polymerization cycling at 72⁰ for 90s for 30 times; and finally extension at 72⁰ for 10 min.

5µl amplification product and 20µl denaturing solution were added to each well of the nuclease-free and un-enveloped micropore plate and then incubated at room temperature for 10 min. Each well was then thoroughly mixed with 225µl hybrid solution (containing PCR DIG probe complementary to telomeric repeated sequences). The antibiotic protein enveloped micropore plate was added with 100µl mixed solution and mounted, kept at 37⁰ in a 300rpm rocking bed, hybridized on oscillation for 2 h, and washed three times for 30s each. After removing the wash solution, each well was added with 250µl DIG-peroxidase compound work solution and mounted. After rocking the plate at 300rpm at room temperature for 30 min and washing three times, each well was added with 100ul peroxidase substrate TMB solution that had been pre-heated to room temperature, mounted, rocked at 300rpm at room temperature, oscillated for color development for 10-20 min, and finally added with stop solution. Absorbance was measured at 450nm and 690nm, and read within 30 min of addition of the stop solution. Relative TMA was calculated according to $A = A_{450} - A_{690}$ and analyzed quantitatively.

Statistical analysis

Data are expressed as $\bar{x} \pm s$, and multi-group data were compared by analysis of variance using SPSS11.0. $p < 0.05$ was considered statistically significant.

Results

The mean tumour size at the time of sacrifice was 3mm in Group A animals, 6mm in Group B animals, and 8mm in Group C animals. The TMA levels are summarized in Table 1. TMA levels in the tumour tissue were significant higher than that in the peritumoural tissue and normal liver tissues at all corresponding time points of measurement ($P < 0.05$). The TMA levels in tumour tissue and normal liver tissue did not show any change over time. TMA level in the peritumoural tissue increased gradually over time; TMA level in animals sacrificed at day 8 was higher than that seen in animals sacrificed at day 4 ($P < 0.05$).

Discussion

The results of the current study showed that TMA was expressed in rat liver Walker-256 tumour tissue and that TMA in tumour tissue was higher than that in peri-tumour and normal liver tissues. Although the TMA levels in tumour tissue and normal liver tissue did not show any change over time, the TMA level in the peritumoural tissue increased with time, indicating that the growth of walker-256 tumour cells is closely associated with TMA.

A close association between TMA and human primary liver cancer has been reported in previous

TABLE 1. Telomerase activity of different groups

Group	Number of samples	Telomerase activity (A value)		
		A(After 4 d)	B(After 6 d)	C(After 8 d)
Tumour tissue	54 (18 at each time point)	0.767±0.117*	0.768±0.118 [△]	0.774±0.111 [□]
Peritumor tissue	54 (18 at each time point)	0.389±0.263**	0.492±0.253 [△]	0.584±0.239 ^{□**}
Normal tissue	54 (18 at each time point)	0.231±0.022*	0.229±0.022 [△]	0.233±0.021 [□]

* $P < 0.05$ vs peritumor tissue group and normal tissue group, [△] $P < 0.05$ vs peritumor tissue group and normal tissue group, [□] $P < 0.05$ vs peritumor tissue group and normal tissue group, ** $p < 0.05$ vs peritumor tissue (after 8d) group. $A = A_{450} - A_{690}$

studies. Shimada et al¹² used TRAP to detect TMA in biopsies of liver cancer tissue and cancer-free liver tissue of 39 liver cancer patients and found that varied degrees of TMA were detectable in all liver cancer tissues. Kojima et al¹³ detected TMA in small liver cancer foci (diameter ≤ 3 cm) and compared that in liver cirrhosis (LC) tissue, tumour-free tissue around the liver cancer, and adenoma-like hyperplastic tissue. The results showed that there was no TMA expression detectable in the normal liver tissue, while TMA of varied degrees was detected in LC tissue, para-cancer chronic hepatitis or LC tissue, and that TMA expression in the liver cancer tissue was higher than that in the LC and para-cancer liver tissue. They pointed out that TMA detection was helpful for the diagnosis of liver cancer. Their results and many other studies suggest that TMA is an important means of maintaining the telomere length of human liver cancer cells¹⁴⁻¹⁷. The present study showed that walker-256 is similar to human primary liver cancer in terms of TMA expression, suggesting that research of walker-256 tumour bearing rats may help understand TMA of human hepatocellular carcinoma (HCC), and this animal model can be used for study of TMA and TMA inhibitors.

None of the previous studies have evaluated the TMA levels at different phases of tumour growth. We found that there was no difference in intratumoural TMA levels detected at different time points of tumour growth suggesting that TMA levels in tumour tissue are independent of tumour size. This is similar to the results of Piao et al,¹⁸ who found no correlation with TMA activity and tumour size. However, we found that TMA in the peritumoural tissue increased with time, along with the growth of the implanted tumour. One report in prostate cancer revealed that telomerase activity is detectable on more than half of the premalignant tissues and benign hypertrophy, indicating that TMA activity may precede the onset of histopathologic changes of overt malignancy and may be an important step in the transformation towards cancer.¹⁹ A number of previous studies have shown

that peritumoural tissue surrounding HCC may show increased TMA activity. Youssef et al,²⁰ using *in situ* technique, found telomerase activity in peritumoural nuclei in 61% of cases. They concluded that telomerase activation is an early mechanism that occurs before conventional histological criteria for malignancy are detected. These reports and the results of the current study indicate that activation of TMA in peritumoural tissue may play an important role in tumour progression. This is in keeping with the results of a study showing that the presence of telomerase activity in adjacent noncancerous tissues in HCC patients who underwent partial hepatectomy was an indicator of tumour recurrence.²¹ Thus, activation of telomerase in peritumoural tissue may be a useful indicator for assessing the malignant potential of liver cancer. Estimation of telomerase activity in peritumoural tissue obtained by image-guided fine-needle aspiration biopsy could be used for assessing growth potential of liver cancer. Previous studies have shown that telomerase activity can be detected in small (21-gauge) needle biopsy specimens.²² Determination of telomerase status in the tumour and peritumoural tissues may also aid in designing more effective anticancer therapy using telomerase as the target.²³

Conclusions

Tumour development and progression is a complex process involving multiple factors and phases. The results of the present study show that telomerase activation is an important factor affecting tumour growth, and increase in telomerase activity with time may be seen in tissues immediately surrounding a growing cancer. Further studies should be done to validate these results and to elucidate the mechanism of TMA activation in tissue around HCC, and its relationship with cancer growth.

Acknowledgments

Supported by the National Natural Science Foundation of China, #39770839.

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Correspondence to:

Dr. Ji-Jin Yang,
Department of Radiology, Changhai Hospital,
No.174, Changhai Rd, Shanghai, 200433,
P. R. China.
E-mail: yangjijin@live.com