PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• GASTRIC CANCER •

Inhibition of human telomerase in MKN-45 cell line by antisense hTR expression vector induces cell apoptosis and growth arrest

Run-Hua Feng, Zheng-Gang Zhu, Jian-Fang Li, Bin-Ya Liu, Min Yan, Hao-Ran Yin, Yan-Zhen Lin

Run-Hua Feng, Zheng-Gang Zhu, Jian-Fang Li, Bin-Ya Liu, Min Yan, Hao-Ran Yin, Yan-Zhen Lin, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China

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Correspondence to: Dr. Zheng-Gang Zhu, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Second Medical University, Shanghai 200025, China. digsurg@online.sh.cn
Telephone: +86-21-64373909 Fax: +86-21-64373909

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Abstract

AIM: **To investigate the effects of antisense human telomerase RNA (hTR) on the biologic behavior of human gastric cancer cell line: MKN-45 by gene transfection and its potential role in the gene therapy of gastric cancer.**

METHODS: **The hTR cDNA fragment was cloned from MKN-45 through RT-PCR and subcloned into eukaryotic expression vector (pEF6/V5-His-TOPO) in cis-direction or trans-direction by DNA recombinant methods. The constructed sense, antisense and empty vectors were transfected into MKN-45 cell lines separately by lipofectin-mediated DNA transfection technology. After drug selection, the expression of antisense hTR gene in stable transfectants and normal MKN-45 cells was detected by RT-PCR, the telomerase activity by TRAP, the apoptotic features by PI and Hoechst 33258 staining, the cell cycle distribution by flow cytometry and the population doubling time by cell counting. Comparison among the stable transfectants and normal MKN-45 cells was made.**

RESULTS: **The sense, antisense hTR eukaryotic expression vectors and empty vector were successfully constructed and proved to be the same as original design by restriction endonuclease analysis and sequencing. Then, they were successfully transfected into MKN-45 cell lines separately with lipofectin. The expression of antisense hTR gene was only detected in MKN-45 cells stably transfected with antisense hTR vector (named as MKN-45-ahTR) but not in the control cells. In MKN-45-ahTR, the telomerase activity was inhibited by 75%, the apoptotic rate was increased to 25.3%, the percentage of cells in the G0/G1 phase was increased to 65%, the proliferation index was decreased to 35% and the population doubling time was prolonged to 35.3hours. However, the telomerase activity, the apoptotic rate, the distribution of cell cycle, the proliferation index and the population doubling time were not different among the control cells.**

CONCLUSION: **Antisense hTR can significantly inhibit telomerase activity and proliferation of MKN-45 cells and induce cell apoptosis. Antisense gene therapy based on telomerase inhibition can be a potential therapeutic approach to the treatment of gastric cancer.**

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INTRODUCTION

Gastric cancer is a very common tumor in China. More and more patients with gastric cancer can now be found in early stage because of the improvement of the technology of diagnosis[1]. Although surgery and chemotherapy are effective for these patients with localized tumors, the prognosis of patients having advanced or metastatic tumors is not ideal[2-6]. As a result, it is absolutely necessary to explore a novel modality of treatment. Fortunately, with the development of molecular biology, medicine is on the brink of a new era-that of molecular genetic medicine. People are now equipped with a new and powerful weapon: gene therapy which was previously only the stuff of dreams and scientific fantasy to fight against disease. Just like other kinds of cancer, the gastric cancer is now recognized as a genetic disease. The gastric cancer cells contain many genetic alterations (caused by some pathogenic agents such as Helicobacter pylori) which accumulate as tumor develop[7-28]. This makes it possible to treat cancer with gene therapy[29,30]. Because the target aimed by the gene therapy is undoubtedly the abnormal gene, thus, the task to find an effective target gene directed against by the gene therapy is becoming increasingly important and urgent. Human telomerase is a ribonucleoprotein which can add the telomeric repeats (TTAGGG) to the ends of the chromosome to maintain the telomere length using its integral RNA component (hTR) as a template[31,32]. Initially identified in HeLa cell extracts, human telomerase has been detected in immortalised cell lines and more than 85% of tumors while normally quiescent in normal somatic cells (except for proliferative cells of renewable tissues such as activated lymphocytes)[33-35]. It is suggested that cancer cells maybe achieve cellular immortality, an important characteristic of cancer cell, through the reactivation of telomerase[36,37]. The seemingly essential roles of telomerase in maintaining telemere length, ensuring chromosome integrity and its nearly ubiquitious reactivation in human cancers have made telomerase a new therapeutic target for anticancer therapy[38,39]. It was reported that HeLa cells transfected with an antisense hTR lost telomeric DNA and began to die after 23 to 26 doublings. Accordiog to a recent review, telomerase activity was also detected in 85-88% of gastric carcinomatous tissues. To gastric cancer, hTR was expressed at a higher level in the tumor than that in the corresponding mucosa and tumors with telomerase activity were generally large in size with a high frequency of lymph node metastasis. Moreover, the patients with telomerase-positive tumors shared poorer prognosis than those with telomerase-negative tumors[40-45]. However, whether antisense gene therapy directed against telomerase will be useful in gastric cancer is so far unknown. We describe here the biologic behavior changes in MKN-45 cell line, a human gastric cell line, after transfected with antisense hTR expression vector and investigate the potential value of telomerase as a target fos antisense gene therapy in gastric cancer.

MATERIALS AND METHODS

Cell Culture

MKN-45 cell, a human gastric cancer cell line, was obtained from Shanghai institute of Cell Biology, Chinese Academy of Sciences. The cells were routinely cultured in RPMI-1640 media (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100u/ml penicillin and 100u/ml streptomycin in an atmosphere consisting of 5% CO_2 in air at 37℃ in a humidified incubator.

Construction of sense and antisense hTR eukaryotic expression vector

The hTR cDNA fragment was cloned from MKN-45 cell line through RT-PCR and subcloned into eukaryotic expression vector: pEF6/V5-His-TOPO vector (Invitrogen) in cis-direction or trans-direction by using DNA recombinant methods as described previously[46]. They were all proved to be the same as original design by restriction endonuclease analysis and sequencing. The sense, antisense and empty vectors were named as pEF-hTR, pEF-ahTR and pEF-empty correspondingly.

Transfection of eukaryotic expression vector

Stable transfection of pEF-hTR, pEF-ahTR and pEF-empty was carried out by standard lipofection mediated DNA transfection method. In brief, approximately 1.5×10^5 MKN-45 cells were transfected with 2μg vector DNA that had been complexed with 20μl lipofectin reagent (Gibco BRL). Two days after the transfection, the stable transfectants were selected by 2μg/ml blasticidin (Invitrogen) in the culture media. They were named as MKN-45-hTR, MKN-45-ahTR and MKN-45-empty correspondingly.

In addition, the pEF6/V5-His-TOPO/lacZ vector (pEF6/V5-His-TOPO vector carrying the reporter gene: lacZ gene in its multi cloning sites, provided by Invitrogen) was transfected into MKN-45 cells and selected by the same method as described above. It was named as MKN-45-lac correspondingly.

RT-PCR for detecting antisense hTR expression

Total RNA was extracted from the transfectants and normal MKN-45 cells using Trizol reagent (Gibco BRL). One microgram of total RNA was reversetranscribed with ahTR specific primer1 (5’-gaacgggccagcagctgacat-3’) using THERMOSCRIPT RT-PCR system for first-strand cDNA synthesis (Gibco BRL). The RT condition was set for 65℃, 30min→85℃, 5min. Then, the cDNA was amplified with the PCR using PLATINUM Taq DNA polymerase (Gibco BRL) and employing ahTR specific primer1 and primer2 (5’-gggttgcggagggtgggcct-3’). The PCR conditions were set for 94℃, 2min→94℃, 20s;69℃, 20s;72℃,20s;30 cycles→72℃,2min. The product length is 196bp. The G6PDH was co-reversetranscribed empolying G6PDH specific primer1 (5’-cgcccctttcctccccctcgtc-3’) and co-amplified with PCR using G6PDH specific primer1 and primer2 (5’-cccgcccctcgctgctgctactac-3’) as internal control. The product length is 247bp. Each RT-PCR product was electrophoretically separated in 2% agarose with EtBr.

β-Gal staining

The MKN-45 cells stably transfected with pEF6/V5-His-TOPO/lacZ vector were subjected to β-Gal staining by using β-Gal Staining Kit (Invitrogen) to detect whether the vector could effectively express lacZ gene.

Apoptotic features

To determine whether MKN-45-ahTR transfected with the pEF-ahTR vector displayed an apoptotic morphology, it was stained with the DNA binding fluorochrome bis (benzimide) trihydro-chloride, Hoechst 33258 (provided by Shanghai institute of Immunology) and observed under UV florescence microscope.

To determine the apoptotic rate and cell cycle distribution, the MKN-45-ahTR and control cells were stained with PI and analysed by flow cytometry.

Telomerase activity assay

Telomerase activity was measured using the commercially available $TRAP_{EZE}$ Telomerase Detection Kit (Intergen). In brief, the cell extract was made according to the protocol provided, then 2μl of the cell extract was added to 48μl of the reaction mixture containing 10× TRAP Reaction Buffer, 50× dNTP Mix, TS Primer, TRAP Primer Mix, Taq Polymerase and dH_2O in amounts and types specified by the $TRAP_{EZE}$ Telomerase Detection Kit. After centrifuged briefly, this mixture was incubated at 30℃ for 30min to allow telomerase elongation of the TS primer and then subjected to PCR amplification in a thermal cycler for 35 cycles: 94℃ for 30s;59℃ for 30s and 72℃ for 30s. The product was then electrophoresed on a 12.5% non-denaturing PAGE (without urea). After electrophoresis, the gel was stained with SYBR Green I (Molecular Probes) according to the manufacturer’s instructions.

Quantification of telomerase product was calculated using BIO-RAD Fluor-S™ MultiImager and the formula (discussed in detail in the $TRAP_{EZE}$ Telomerase Detection Kit instruction booklet).

$$\text{TPG(units)}=\frac{(\chi-\chi_o)/c}{(\gamma-\gamma_0)/c_R}\times100$$

Abbreviation in the above formular is as follows: TPG, total product generated; χ, sample signal; χ_0, heat-inactivated control; γ, 0.1 amole quantitation TSR8 control; γ_0, 1× CHAPS Lysis Buffer only control; c, sample internal standard band; c_R, 0.1 amole quantitation TSR8 internal standard band.

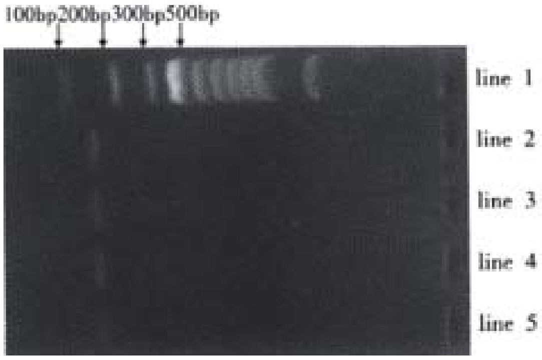
Cell growth curve and population doubling time

Cells were seeded at a density of 2×10^5 per 25ml flask in 1.5ml of cell culture media. The number of cells per flask was counted every day for 6 days. The population doubling time of cells transfected with pEF-hTR, pEF-ahTR and pEF-empty and normal MKN-45 cells were calculated and the cell growth curve was drawn.

RESULTS

Expression of antisense hTR gene in MKN-45 cells

We transfected MKN-45 cells with pEF-hTR, pEF-ahTR and pEF-empty vector respectively, following the blasticidin selection, the drug resistant cells were collected and RT-PCR was performed with antisense hTR specific primers. We detected the ahTR specific product only in the pEF-ahTR vector transfected cells but not in the pEF-hTR, pEF-empty vector transfected cells and parental cells. However, steady state expression of G6PDH (as internal control) was observed in all cells.



Line 1: 100bp DNA Ladder (Promega), Lane 2: normal MKN-45, Lane 3: MKN-45-hTR, Lane 4: MKN-45-empty, Lane 5: MKN-45-ahTR

Figure 1 The expression of antisense hTR gene.

Only the cells transfected with pEF-ahTR expressed the antisense hTR, however, the G6PDH was detected in all samples that indicated an appropriate RT-PCR reaction.

β-*Gal staining*

After drug selection, nearly all MKN-45 cells stably transfected with pEF6/V5-His-TOPO/lacZ vector were stained blue by β-Gal Staining Kit while parental cells were not.

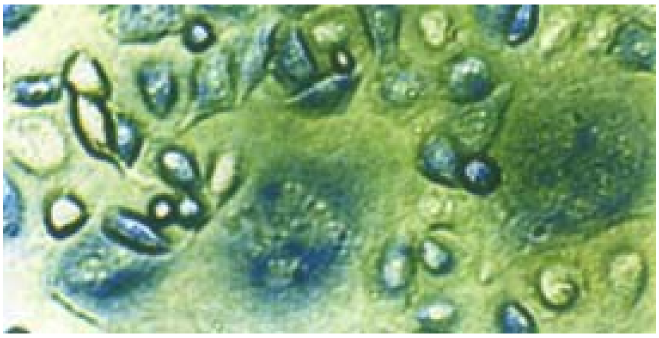
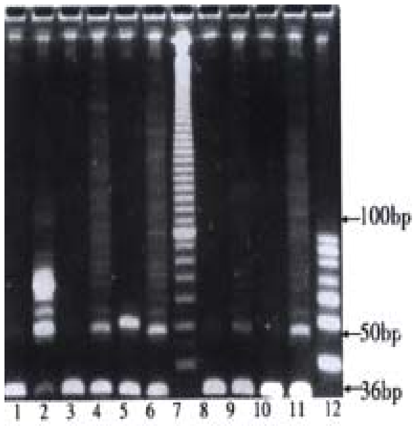


Figure 2 The result of β-Gal staining of MKN-45-lac. (×600)

Telomerase activity

We measured telomerase activity of the pEF-hTR, pEF-ahTR and pEF-empty transfected MKN-45 cells and parental cells through TRAP method described above. It was found that the level of telomerase activity in the pEF-ahTR transfected MKN-45 cells was greatly inhibited, compared with that in the parental MKN-45 cells. However, there was no difference between the level of telomerase activity in the pEF-hTR, pEF-empty transfected MKN-45 cells and parental MKN-45 cells.



Line 1: 1× CHAPS Lysis Buffer only control; Line 2: 0.1 amole quantitation TSR8 control; Line 3: MKN-45-empty heat-inactivated control; Line 4: MKN-45-empty; Line 5: MKN-45 heat-inactivated control; Line 6: MKN-45; Line 7: 10bp DNA Ladder (Gibco BRL); Line 8: MKN-45-ahTR heat-inactivated control; Line 9: MKN-45-ahTR; Line 10: MKN-45-hTR heat-inactivated control; Line 11: MKN-45-hTR; Line 12: 10bp DNA Step Ladder (Promega). **Figure 3A** Detection of telomerase activity with the telomeric repeat amplification protocol (TRAP) assay. A 36bp internal control band present in all samples indicated an appropriate polymerase chain reaction (PCR).

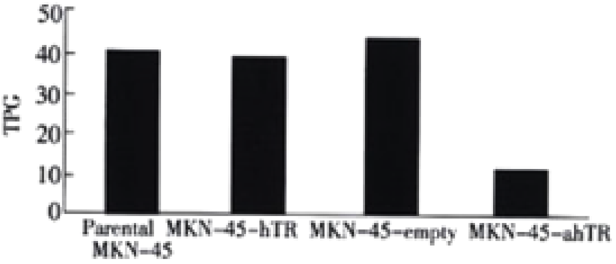


Figure 3B Comparison of telomerase activity in MKN-45-ahTR and control cells. Compared with control cells, the telomerase activity in MKN-45-ahTR was inhibited by about 75%.

Cellular effects of telomerase inhibition

As Figures 4, 5 and Table 1 showed, compared with controls cells, the MKN-45-ahTR cell displayed a longer population doubling time, an increased percentage of cells in the G0/G1 phase, a lower cell proliferation index and a higher apoptotic rate, which demonstrated that, through inhibiting telomerase activity, antisense hTR gene transfection could inhibit the proliferative capacity of NKN-45 and induce cell apoptosis.

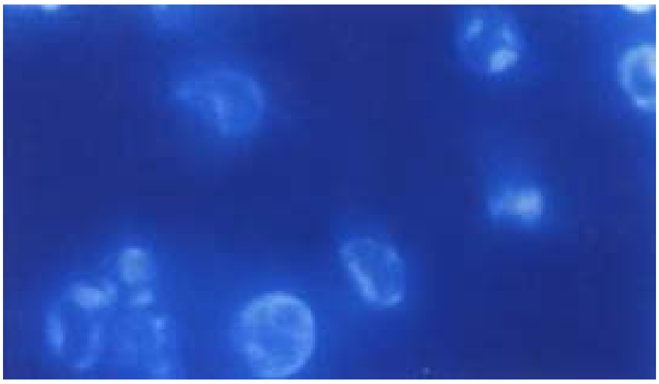


Figure 4 Hoechst 33258 staining of MKN-45-ahTR. (×600)
The cells undergoing apoptosis demonstrated apoptotic chromatin changes: blebbing, fragmentation and condensation under fluorescence microscope.

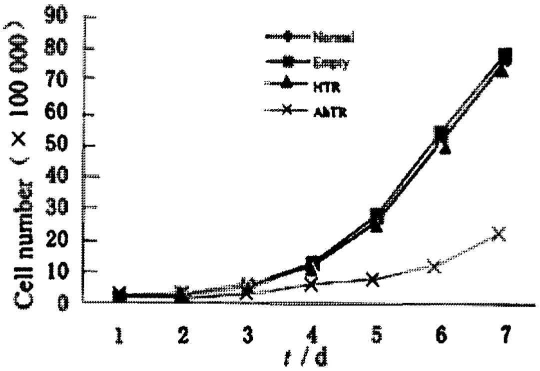


Figure 5 Cell growth curve.

Table 1 Comparison of distribution of cell cycle, cellular proliferation index, apoptotic rate,population doubling time of MKN-45-ahTR and those of control cells

	Distribution of cell cycle (%)			cell proliferation index (%)	Apoptotic rate (%)	population doubling time (hours)
	G0 / G1	S	G2 / M			
MKN-45-ahTR	65	34.2	0.8	35	25.3	35.3
MKN-45-hTR	55.7	37.5	6.8	44.3	0	23.2
MKN-45-empty	54.1	38.4	7.5	45.9	0	22.7
normal MKN-45	54.7	37.8	7.5	45.3	0	22.9

DISCUSSION

Compared with normal somatic cells, which reach the end of their replicative capacity after a limited number of population doubling and enter a senescence phase, the cancer cells have an unlimited replicative capacity. This important characteristic of cancer, named immortality, is gaining more and more attention, seeing that cancer cells may achieve cellular immortality through only a major pathway: activation of the telomerase[47].

Telomerase is a unique ribonucleoprotein that can synthesize telomeric DNA onto chromosomal ends using a segment of its RNA component (hTR) as a template to compensate for the lose of telomeric repeats (TTAGGG) caused by the so-called "end-replication" problem. Recent Study demonstrated that 758 of 895 (85 %) of malignant tumors but none of 70 normal somatic tissues expressed telomerase activity. In addition, the level of telomerase activity influences the prognosis of patient to a certain degree. For example, high level of telomerase correlates with poor clinical outcome in neuroblastoma, while patients with metastatic IV-S neuroblastoma without telomerase activity experiences spontaneous regression of tumors. These findings indicate that telomerase plays an important role in carcinogenesis and therefore undoubtedly become the basis of the widely held view of telomerase as a highly selective target for antisense gene therapy of cancer[48].

The RNA component of telomerase (hTR) is crucial to the telomerase activity[49-51]. Human cell lines that expressed hTR mutated in the template region generated the predicted mutant telomerase activity. In addition, recent experiments have shown that antisense gene therapy directed against telomerase RNA component (hTR) could effectively inhibit telomerase activity and induce apoptotic cell death in ovarian cancer, prostate cancer, bladder cancer, malignant gliomas and human breast epithelial cells[52-56]. However, whether such anti-cancer effect can be obtained in human gastric cancer is still unknown. Therefore, we examined the effect of antisense hTR (ahTR) expression on the growth of human gastric cancer cell line:MKN-45 through transfection of an ahTR expression vector.

Given that whether the vector can effectively and stably express the exogenous gene it carries will directly influence the effect of antisense gene therapy. Firstly, we adopted two different methods to detect the expression of exogenous gene by the pEF6/V5-His-TOPO vector before conducting other experimental items. The first one was to detect the expression of reporter gene. pEF6/V5-His-TOPO/lacZ vector is the same as the pEF6/V5-His-TOPO vector except that the former carrying lacZ gene, a kind of widely used reporter gene, in its multi cloning sites. Usually by using â-Gal staining method, the researchers can easily detect the expression of lacZ gene through which they further evaluate the ability of the vector's promoter to express the exogenous gene. We found that nearly all cells stablely transfected with pEF6/V5-His-TOPO/lacZ vector were stained blue while control cells were not. Therefore, we believed that pEF6/V5-His-TOPO/lacZ vector could effectively express the exogenous gene (lacZ gene) it carried, so did the pEF6/V5-His-TOPO vector. The second one was to detect the expression of antisense hTR gene directly through RT-PCR method. Only the cells transfected with pEF-ahTR expressing the antisense hTR gene were found. Both the results proved that pEF6/V5-His-TOPO vector could effectively express the exogenous gene, thus laying the solid fundament for the study of following experimental items.

As the results showed, the most significant conclusions we could draw from this study were that telomerase in human gastric cancer cell line: MKN-45 could be inhibited by a vector expressing mRNA complementary to the template region of hTR and that the growth of cancer cells was retarded and cell apoptosis was induced after telomerase was inhibited by this method. Therefore, our experiment clearly demonstrated that blocking the RNA component of telomerase with antisense hTR expression vector appeared to be a rational approach to the treatment of gastric cancer. The telomerase may become an ideal target for antisense gene therapy in human gastric cancer.

However, it is noticeable that not all cells transfected with antisense hTR gene underwent apoptosis, and underlying mechanism is still unknown. The possible reason is that the carcinogenesis process is complicated and the reactivation of telomerase may play an important but by no means the sole role during this process. Many oncogenes and tumor suppressor genes are also involved in tumorigenesis[57,58]. While believed to be necessary for cancer cells to grow without limit, telomerase is not sufficient to transform a normal cell into a tumor cell. Thus, anti-cancer effect of antisense gene therapy will be more satisfactory if multitargets are aimed. For example, a recent report showed that the combination of 2-5A-anti-hTR and Ad5CMV-p53 had greater anti-tumor efficacy against all p53-mutant glioma cells than each treatment alone[59]. Seeing that telomerase activity and p53 dysfunction are also detected in gastric cancer[60,61], this approach may be effective in treating gastric cancer patients too and the similar investigation is being conducted in our laboratory now.

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