

## Detection of let-7a microRNA by real-time PCR in gastric carcinoma

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### Abstract

**AIM:** To establish an accurate and rapid stem-loop reverse transcriptional real-time PCR (RT-PCR) method to quantify human let-7a miRNA in gastric cancer.

**METHODS:** According to the sequence of let-7a miRNA, the stem-loop reverse transcriptional primer, the primers and quantitative MGB probes of real-time PCR were designed and synthesized. The dynamic range and the sensitivity of quantitative reverse transcriptional real-time PCR were determined. The levels of let-7a miRNA were examined in 32 gastric carcinoma samples by stem-loop RT-PCR method.

**RESULTS:** The dynamic range and sensitivity of the let-7a miRNA quantification scheme were evaluated, the result showed the assay could precisely detect 10 copies of mature let-7a miRNA in as few as 0.05 ng of total RNA of gastric mucosa. The results of specificity analysis showed no fluorescence signal occurred even though 50 ng of human genomic DNA was added to the reverse transcription (RT) reaction. The expression level of let-7a miRNA in gastric tumor tissues was significantly lower compared to normal tissues in 14 samples from 32 patients.

**CONCLUSION:** The stem-loop RT-PCR is a reliable method to detect let-7a miRNA which may play an important role in the development of gastric carcinoma.

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**Key words:** MicroRNA; Let-7a; Real-time PCR; Gastric carcinoma

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### INTRODUCTION

Mature microRNAs (miRNAs) were a recently discovered class of endogenous, small non-encoding RNAs with the length of 21-25 nucleotides<sup>[1]</sup>. Their primary function was believed as translational repression of protein coding mRNAs at post-transcriptional level<sup>[2-4]</sup>. miRNAs were found in the genomes of animals<sup>[5,6]</sup> and plants<sup>[7,8]</sup>. There are about 4000 unique transcripts, including 462 human miRNAs in the Sanger Center miRNA registry<sup>[9]</sup>. Otherwise, it is estimated that human genome has more than 1000 miRNAs<sup>[10]</sup>.

Although the biological functions and the target genes of miRNAs are poorly understood, it has been confirmed that they regulate complicated biological behaviors, such as cell differentiation, proliferation and death, *etc*<sup>[11]</sup>. Recent studies have shown that miRNA is closely related to tumor genesis and differentiation<sup>[12]</sup>, especially, the expression level of let-7a miRNA was reduced in human lung cancer and colon cancer<sup>[13,14]</sup>, and it was shown to be anticancer miRNA that repressed Ras and c-myc expression at translational level<sup>[15]</sup>. As Ras and c-myc are universal pathways of several tumors, let-7a miRNA may be the target of tumor genesis.

Since miRNAs are very different from other traditional RNAs, different methods are needed to quantify their expression. We, therefore, established an accurate and rapid real-time PCR fluorescence quantitative method to quantify human let-7a miRNA in human gastric carcinoma and normal tissue. Based on this method, we preliminarily discussed the relationship between let-7a expression level and gastric carcinoma genesis.

### MATERIALS AND METHODS

#### Patients and specimens

All human tissue samples were obtained from surgical specimens of 32 patients with gastric carcinoma from 2005 to 2006 at Hangzhou First People's Hospital, China. All tissues, including gastric carcinoma and corresponding adjacent normal tissue, were divided into two parts and preserved in liquid nitrogen for 30 min after removing

**Table 1** Oligonucleotides used in this study

let-7aP <sub>RT</sub> : GTCGTATCCAGTGCAGGGTCCGAGGTATTCCGACTGGAT ACGACAACCTA
let-7aPf: GCCGCTGAGGTAGTAGTTGTGA
let-7aPr: GTGCAGGGTCCGAGGT
let-7aT: (6-FAM) TGGATACGACAACCTATAC (MGB)
let-7aSeq: 6-ugagguaguagguuaguauuu-27 (MIMAT0000062)

from the body. Informed consent was taken from all subjects.

### Total RNA and genomic DNA preparation

Total RNA and genomic DNA were extracted from the gastric carcinoma tissues and normal gastric mucous tissues. Total RNA was extracted by using Trizol (Invitrogen, Carlsbad CA) and genomic DNA was extracted by using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The total RNA and genomic DNA were quantified by ultraviolet spectrophotometer (UVPC2401, SHIMADZU) at a wavelength of 260 nm. Total RNA from normal gastric mucosa was quantified to 5 µg, and then diluted to 500 ng, 50 ng, 5 ng, 0.5 ng, 0.05 ng, 0.005 ng, respectively.

### Stem-loop RT primer and TaqMan MGB probe

All of the oligonucleotides described are displayed in Table 1. The stem-loop RT primer, real-time PCR primers and TaqMan MGB probe were designed as previously described by Chen *et al*<sup>[6]</sup>. The let-7a miRNA template sequence was accessed from [http://microrna.sanger.ac.uk/cgi-bin/sequences/mirna\\_entry.pl?acc=MI0000060](http://microrna.sanger.ac.uk/cgi-bin/sequences/mirna_entry.pl?acc=MI0000060). All primers, and probes were synthesized by Shanghai GeneCore Biotechnologies Co., Ltd.

### Reverse transcription

The miRNAs were reverse transcribed into cDNAs by SuperScript III reverse transcription kits (Invitrogen). Ten microliters of the total RNA or artificially synthesized let-7a miRNA template, 1.0 µL of 1.0 µmol/µL stem-loop RT primer (let-7aP<sub>RT</sub>), 1.0 µL of 10 mmol/L dNTPs (Promega) and 1.0 µL of H<sub>2</sub>O were mixed and heated at 65°C for 5 min. The mixture was immediately placed on ice for 2 min. Then 4.0 µL of 5 × First-Strand buffer, 1.0 µL of 0.1 mol/L DTT, 1.0 µL of RNase inhibitor (Invitrogen) and 1.0 µL of SuperScript III reverse transcriptase were added and mixed. The 20 µL of reaction volume was incubated for 60 min at 55°C, 15 min at 70°C and then held at 4°C. All reverse transcriptions as well as no-template controls were run at the same time.

### Real-time PCR

Real-time PCR was performed using a standard TaqMan PCR protocol on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems). The 50 µL of PCR mixture included 5.0 µL of RT product, 5.0 µL of 10 × PCR buffer (Takara), 1.0 µL of 10 mmol/L dNTPs (Takara), 7.0 µL of 25 mmol/L Mg<sup>2+</sup> (Takara), 0.6 µL of AmpliTaq DNA polymerase (5 U/µL, Takara),

0.2 µL of TaqMan probe (let-7aT, 10 µmol/µL), 1.5 µL of amplification primer I (let-7aPf, 10 µmol/µL), 0.7 µL of amplification primer II (let-7aPr, 10 µmol/µL) and 29 µL of autoclaved distilled water. The reaction mixtures were incubated at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. TaqMan CT values were converted into absolute copy numbers using a standard curve from synthetic let-7a miRNA. We also quantified transcripts of beta-actin as the endogenous RNA control, and each sample was normalized on the basis of its beta-actin content<sup>[17]</sup>.

## RESULTS

### Sensitivity and dynamic range

The dynamic range and the sensitivity of the let-7a miRNA quantification scheme were evaluated using synthesized let-7a miRNA. The synthesized let-7a miRNA was quantified at the ultraviolet (UV) wavelength of 260 nm ( $A_{260}$ ) and diluted by ten orders of magnitude, including 10<sup>10</sup>, 10<sup>9</sup>, 10<sup>8</sup>, 10<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, 10<sup>1</sup> and 1 copies/µL. The real-time PCR assay showed excellent linearity between the log of target input and CT value, suggesting that the assay has a dynamic range of at least 8 logs and is capable of detecting as few as ten copies let-7a in the reaction (Figure 1), and the correlation coefficient was 0.996. The total RNA input ranged from 0.005 ng to 5 µg, and the result showed the method could detect the let-7a miRNA as few as 0.05 ng of total RNA from gastric mucosa (Figure 2).

### Specificity analysis

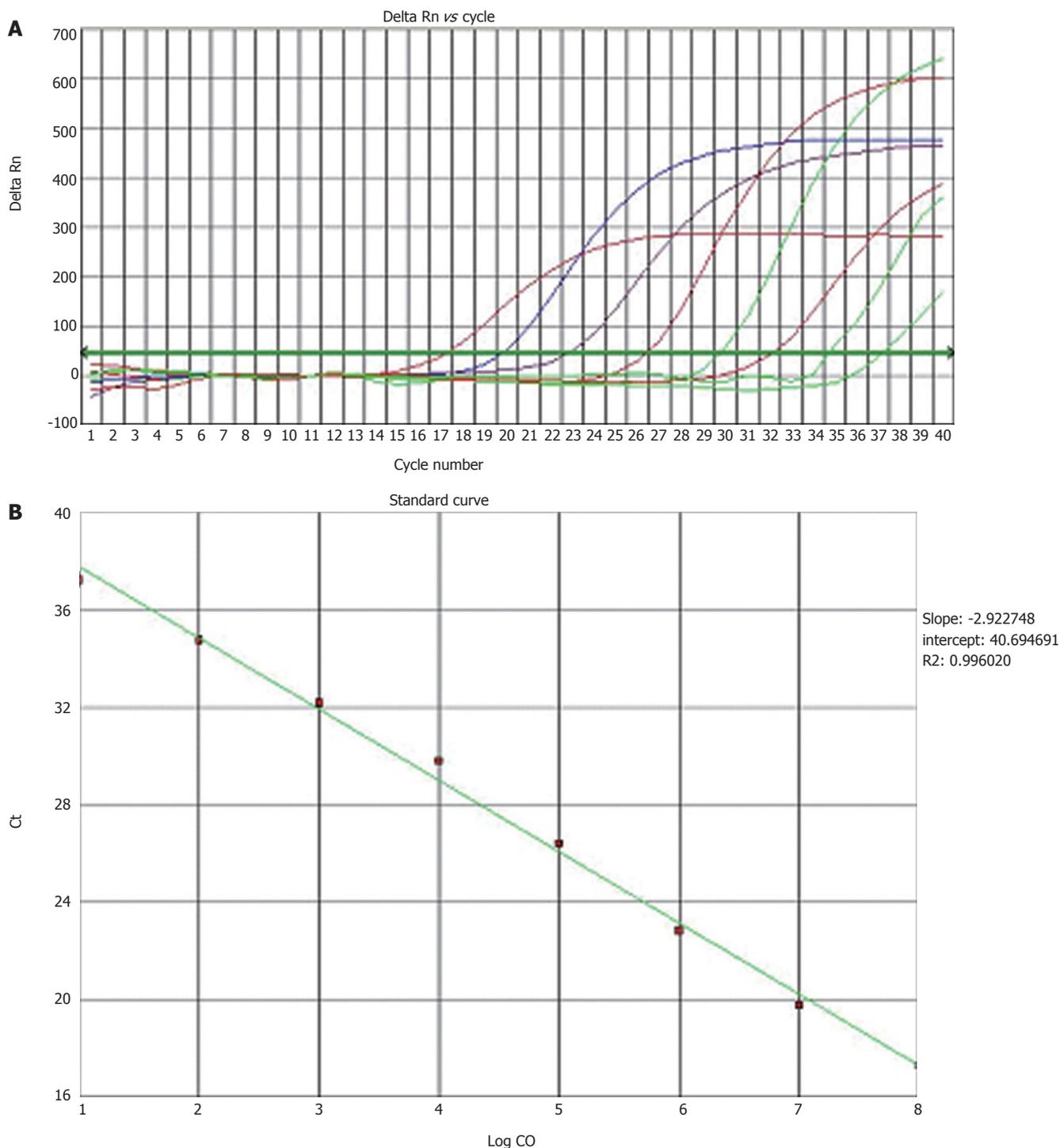
The effect of non-specific genomic DNA on let-7a miRNA assay was tested. The results showed that no fluorescence signal occurred even though 50 ng of human genomic DNA was added to the RT reaction, indicating that the method has a good specificity without disturbance of genome.

### Expression of let-7a miRNA in gastric carcinoma

In order to confirm whether the level of let-7a miRNA was reduced in human gastric cancer, we examined the expression of mature let-7a miRNA and beta-actin in the samples from 32 patients with gastric cancer. As shown in Table 2 and Figure 3, the expression level of let-7a miRNA in tumors was significantly lower (down-regulation rate over 2-fold) in 14 patients tested. However, in the other 18 patients, no significant difference in let-7a miRNA expression was observed between tumors and normal tissues, and the up- or down-regulation rates were within two-fold.

## DISCUSSION

MicroRNAs are endogenous short non-coding RNA molecules that regulate cell differentiation, proliferation, and apoptosis through post-transcriptional suppression of gene expression by binding to the complementary sequence in the 3'-untranslated region (3'-UTR) of target messenger

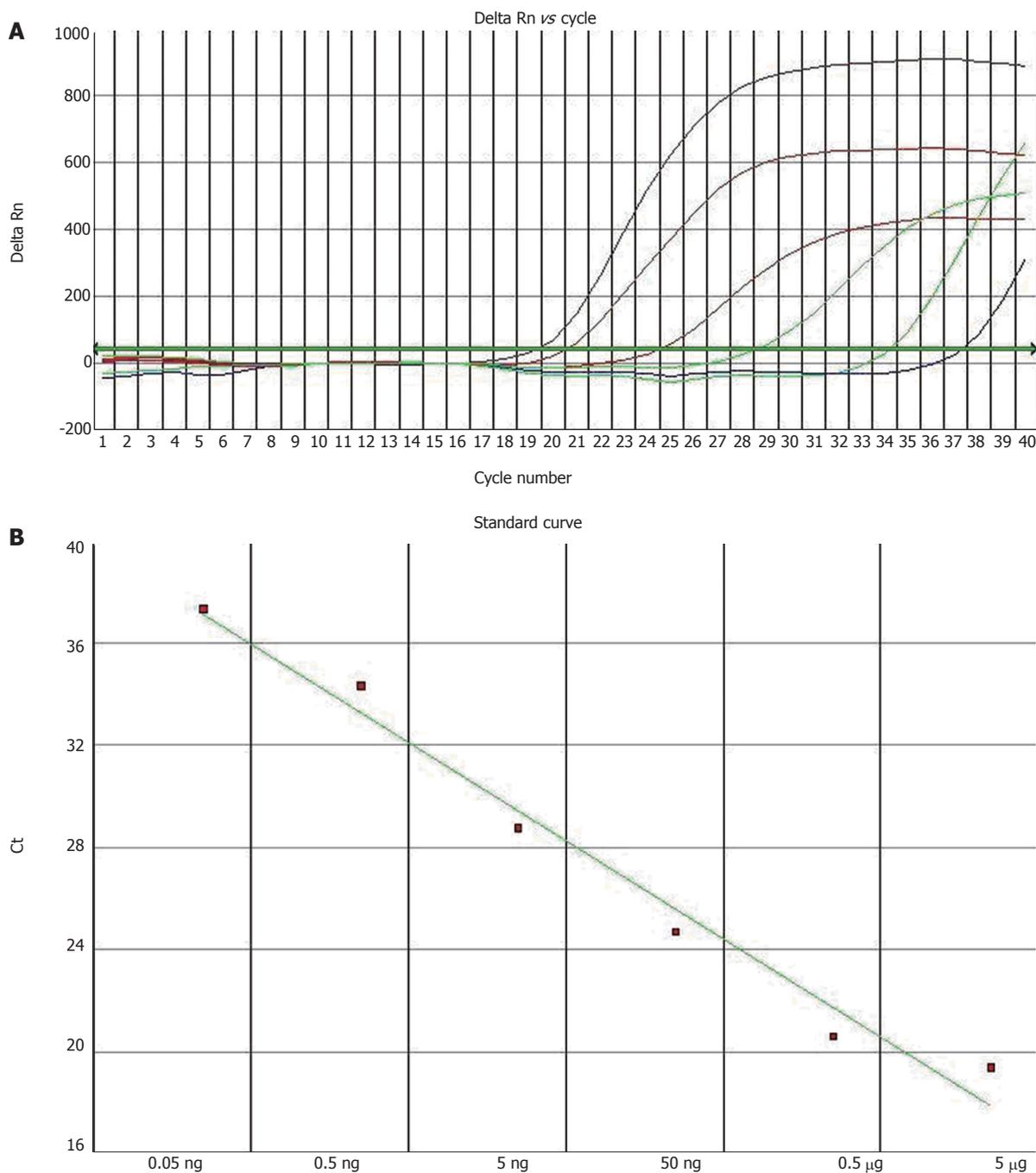


**Figure 1** Sensitivity and dynamic range of let-7a miRNA assay. **A:** Amplification curve of the synthesized let-7a miRNA, the well-behaved assay exhibited high sensitivity and broad dynamic range between the signal of 10 copies and  $10^8$  copies input template and the no-template-control background; **B:** Standard curve of the let-7a miRNA ( $r^2 = 0.996$ , slope = -2.92).

RNAs (mRNAs)<sup>[9]</sup>. Recently, it has been revealed that the change of miRNA expressions contributes to the initiation and progression of carcinoma. More than 50% of miRNAs are located in cancer-associated genomic regions or in fragile sites<sup>[18]</sup>. The relationship between miRNA and tumor has currently become the focus of many scientists. Current methods for detection and quantification of let-7a miRNA are largely based on cloning, Northern blotting<sup>[19]</sup>, or primer extension<sup>[20]</sup>. Although microarrays could improve the throughput of miRNA profiling, the

method is relatively limited in terms of sensitivity and specificity<sup>[21,22]</sup>. Low sensitivity becomes a problem for miRNA quantification because it is difficult to amplify these short RNA targets. Furthermore, low specificity may lead to a false-positive signal. They are not only low-sensitive, but also low-specific, because of the non-specificity of let-7a pre-miRNA and genome.

Real-time PCR is the gold standard for gene expression quantification<sup>[23,24]</sup>. It has been a long challenge for scientists to design a conventional PCR assay from



**Figure 2** Total RNA sensitivity and dynamic range of let-7a miRNA assay. **A:** Amplification plot of total RNA from gastric mucosa at six orders of magnitude. The total RNA input ranged from 50 pg to 5 μg in per stem-loop RT-PCR reaction; **B:** Correlation of total RNA input with the threshold of cycle (Ct) values for let-7a miRNA assays.

miRNAs with 22 nt in length on average. We specifically quantified the let-7a miRNA expression levels in gastric carcinoma using TaqMan PCR assays designed by Chen *et al*<sup>[6]</sup>. This assay can precisely detect 10 copies let-7a in 50 pg of total RNA. Furthermore, the results showed this method could detect let-7a miRNA without disturbance of genome.

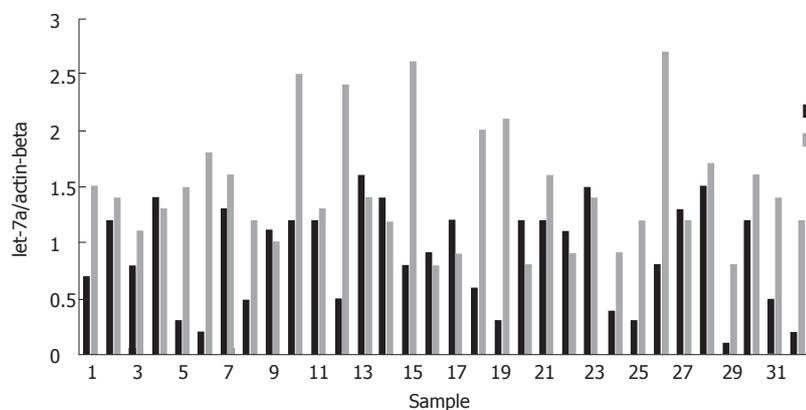
Gastric carcinoma develops through the accumulation of multiple genetic lesions that involve oncogenes, tumor suppressor genes and DNA mismatch repair genes. The relationship between gastric carcinoma genesis and the expression of let-7a miRNA is rarely reported. In the present study, we examined, using real-time PCR, the

expression of let-7a mature miRNA in 32 matched pairs of gastric tumoral and non-tumoral tissues from patients. The results showed let-7a miRNA was significantly down-regulated in 14 of 32 patients with gastric cancer. Similarly, the expression of let-7a miRNA was reduced in lung cancer<sup>[13]</sup> and colon cancer<sup>[14]</sup>. The 3'UTRs of the human *Ras* genes contain multiple let-7a complementary sites (LCSs), allowing let-7a to regulate Ras expression. It has been reported that the expression of let-7a is lower in gastric tumors than in normal gastric tissue, while Ras protein is significantly higher in gastric tumors, which provides a possible mechanism for the association of let-7a in gastric cancer. Further studies on relationship between

Table 2 Expression of let-7a miRNA in gastric carcinoma tissue

Sample No.	T (copies/ $\mu$ g)			N (copies/ $\mu$ g)			T/N
	let-7a	$\beta$ -actin	let-7a/ $\beta$ -actin	let-7a	$\beta$ -actin	let-7a/ $\beta$ -actin	
50901	$7.37 \times 10^4$	$1.05 \times 10^5$	0.7	$2.15 \times 10^5$	$1.43 \times 10^5$	1.5	0.47
51005	$1.15 \times 10^4$	$3.83 \times 10^4$	0.3	$4.35 \times 10^4$	$2.90 \times 10^4$	1.5	0.20
51006	$1.03 \times 10^5$	$5.15 \times 10^5$	0.2	$8.57 \times 10^5$	$4.76 \times 10^5$	1.8	0.11
51208	$6.30 \times 10^5$	$1.26 \times 10^6$	0.5	$1.98 \times 10^6$	$1.65 \times 10^6$	1.2	0.42
06031	$1.03 \times 10^3$	$8.58 \times 10^2$	1.2	$2.63 \times 10^3$	$1.05 \times 10^3$	2.5	0.48
60412	$1.35 \times 10^5$	$2.70 \times 10^5$	0.5	$5.88 \times 10^5$	$2.45 \times 10^5$	2.4	0.21
60418	$6.53 \times 10^5$	$1.09 \times 10^6$	0.6	$2.74 \times 10^6$	$1.37 \times 10^6$	2.0	0.30
60519	$6.31 \times 10^4$	$2.10 \times 10^5$	0.3	$4.94 \times 10^5$	$2.35 \times 10^5$	2.1	0.14
60524	$3.05 \times 10^5$	$7.63 \times 10^5$	0.4	$6.22 \times 10^5$	$6.91 \times 10^5$	0.9	0.44
60625	$1.53 \times 10^5$	$5.10 \times 10^5$	0.3	$5.78 \times 10^5$	$4.82 \times 10^5$	1.2	0.25
60726	$8.24 \times 10^4$	$1.03 \times 10^5$	0.8	$2.55 \times 10^5$	$9.46 \times 10^4$	2.7	0.30
60729	$5.61 \times 10^6$	$5.61 \times 10^7$	0.1	$3.49 \times 10^7$	$4.36 \times 10^7$	0.8	0.13
60731	$4.35 \times 10^4$	$8.70 \times 10^4$	0.5	$1.32 \times 10^5$	$9.41 \times 10^4$	1.4	0.36
60832	$7.28 \times 10^5$	$3.64 \times 10^6$	0.2	$3.78 \times 10^6$	$3.15 \times 10^6$	1.2	0.17

T: Gastric carcinoma tissue; N: Normal tissue; T/N: Difference in let-7a expression between tumor and normal tissue.



**Figure 3** Expression of let-7a miRNA in gastric cancer. Difference in let-7a miRNA expression between in tumor tissues (T) and the adjacent non-tumor tissues (N). The patient numbers correspond to those in Table 2. Compared to normal tissues, let-7a miRNAs expression in tumor tissue from 14 patients was significantly down-regulated.

gastric carcinoma and let-7a are currently underway in our laboratory.

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