let-7 MicroRNA Functions as a Potential Growth Suppressor in Human Colon Cancer Cells

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MicroRNAs (miRNAs) are endogenously expressed RNAs, 18—25 nucleotides in length, that repress protein translation through binding to target mRNAs. miRNAs have been implicated in many cellular processes including cell proliferation, differentiation, and death. Recently, *let-7* miRNAs were found to regulate human *RAS* oncogene expression and to be often down-regulated in human lung tumors. In this study, we examined the expression of *let-7* miRNAs in human colon cancer tumors and cell lines, with the result that 2 of 6 cases and 1 of 3 cell lines showed reduced expression of *let-7*. When *let-7* low-expressing DLD-1 human colon cancer cells were transfected with let-7a-1 precursor miRNA, which is located at chromosome 9q22.3, the cells underwent significant growth suppression. At that time, the levels of RAS and c-myc proteins were lowered after the transfection, whereas the levels of both of their mRNAs remained almost unchanged. These findings suggest the involvement of *let-7* miRNA in the growth of colon cancer cells. Thus, miRNAs might provide a basis for novel RNA anti-cancer agents.

Key words microRNA; *let-7*; colon cancer; RAS; growth inhibition

The microRNAs (miRNAs) are an extensive class of small noncoding RNAs, 18 to 25 nucleotides in length, with the role of regulating gene expression through translational repression by base-pairing with partially complementary mRNAs.[1)] Currently, more than 3000 miRNAs have been annotated from a wide range of organisms including plants, worms, flies, mammals, and viruses.[2,3)] The expression of miRNAs is regulated developmentally and spatially, and is involved in cell differentiation, proliferation, and death.[2)] Recently several reports have indicated the involvement of miRNAs in human cancer and further that miRNAs might be used in future therapeutic and diagnostic applications.[4)] Especially, the expression level of *let-7* miRNA was reduced in human lung cancer,[5)] and it was shown to be the anticancer miRNA that represses *RAS* and/or *c-MYC* expression at the translational level.[6)] On the other hand, it has been established that genetic aberration of *RAS* and over-expression of c-myc are frequently involved in colon cancer.[7—9)] Such findings led us to speculate that *let-7* may also be reduced in its expression in colon cancer cells.

In the current study, we focused on the expression of *let-7* miRNA in colon cancer and its expression profile. Our functional analysis indicates that *let-7* may function as one of the growth suppressive miRNAs in human colon cancer cells.

MATERIALS AND METHODS

Patients and Tissue Preparation All human tissue samples were specimens from patients who had undergone biopsy for histological diagnosis of colon cancer at Saiseikai Ibaraki hospital, Ibaraki, Osaka, between 2000 and 2003. Informed consent in writing was obtained from each patient. The tumor and its adjacent normal tissues were also obtained. Each specimen was divided into 2 parts. One of them was subjected to Western blot analysis, and the other was used for extraction of RNA and DNA.

Cell Culture and Cell Viability Human malignant cell lines SW480, DLD-1, and COLO101 (colon cancer); HL60 (myeloid leukemia); U937 (monocytic leukemia); Jurkat (T-cell leukemia); PC3 and LNCaP (prostate cancer); SH-SY5Y (neuroblastoma); HeLa (cervical cancer); and HuH7 (hepatic cancer) were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS (Sigma, St. Louis, MO, U.S.A.) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO_2 at 37 °C. The number of viable cells was determined by the trypan-blue dye exclusion test. Plating efficiencies (the average number of colonies per 60-mm dish divided by the number of cells seeded per dish ($\times$100) were determined 14 d following inoculation of 1000 cells. The ability of the cells to grow as colonies in or on the soft agar was assessed. Briefly, 10^3 individual trypsinized cells were suspended in 5 ml RPMI 1640 medium/10% FBS/0.35% liquid Bacto-agar (Difco Laboratories. Inc.) at 40 °C, immediately poured onto a 4-ml basal layer of 0.5% Bacto-agar/RPMI 1640 medium/10% FBS in a 60-mm dish, and incubated at 37.5 °C. The dishes were examined for the presence of colonies growing in the 0.35% agar layer or on the soft agar by phase-contrast light microscopy at 7, 14, and 21 d after seeding. In all procedures, neomycin selection was continued.

Semi-quantitative RT-PCR Total RNA was isolated from the cells and tissues by the phenol/guanidium thiocyanate method with DNase I treatment. To determine the expression of miRNAs, we measured their levels by using a mirVana™ qRT-PCR miRNA Detection Kit (Ambion Inc. Austin, Texas). Briefly, after reverse transcription of 50 ng of total RNA, cDNA was generated. The PCR reaction consisted of 22 cycles (95 °C for 15 s, 60 °C for 30 s) after an initial denaturation step (95 °C for 3 min). The PCR products were analyzed by electrophoresis on 2% agarose gels. U6 was used as an internal control. The PCR primer pair used was for let-7a-1 (Ambion). This PCR primer detects the mature miRNAs of the let-7 family. The intensity of bands for PCR products was measured by densitometry.

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Table 1. Summary of Clinicopathologic Features in Human Colorectal Tumors

Case No.	Age	Sex	Size (mm)	Site	Cancer differentiation status	Dukes' system
1	44	Female	55×25×15	Sigmoid	Well	C
2	62	Female	67×55×20	Ascending	Well	C
3	50	Male	120×74×25	Rectum	Well	C
4	65	Male	50×40×15	Rectum	Moderate	C
5	62	Female	60×60×25	Caecum	Moderate	C
6	70	Male	70×60×28	Sigmoid	Well	C

To determine the levels of mRNAs, we purified prepared cDNA samples by use of a PCR Purification kit (Qiagen, Hilden, Germany) and used them for PCR (Takara, Ohtsu, Japan). The PCR was performed according to the manufacturer's instruction. The primers for *RAS* and *c-myc* were as follow: kRas-sense-207, 5′-ACTTGTGGTAGTTGGAGC-3′, and kRas-antisense-648, 5′-AGACAAGACAGGGTGTTG-3′; cMyc-sense-139, 5′-GGCTTTATCTAACTCGCTGT-3′, and cMyc-antisense-449, 5′-GCTATTCTGCCCATTTGGGG-3′.

Transfection of DLD-1 and SW480 Cells with let-7a-1 miRNA DLD-1 and SW480 cells were seeded in 6-well plates at a concentration of 1—2×10^5/well (30—40% confluence) on the day before the transfection. The *let-7a-1* precursor miRNAs (UGAGGUAGUAGGUUGUAUAGU; Ambion) were used for the transfection of the cells (20—80 nM/ml) by using cationic liposomes TransIT-TKO (Mirus Bio Co., Madison, WI, U.S.A.) according to the manufacturer's Lipofection protocol. The transfection efficiency was evaluated by the transfection of the cells with a duplex siRNA-FITC (Dharmacon, Lafayette, CO, U.S.A.). Non-specific control miRNA (NS, 57% GC content; Ambion) was used as a control for non-specific effects. The effects caused by the introduction of let-7a-1 precursor miRNA into the cells were assayed at 48 h after the transfection by Western blot and qRT-PCR analyses for mRNA and miRNA.

Western Blotting The cells were homogenized in chilled lysis buffer comprising 10 mM Tris–HCl (pH 7.4), 1% NP-40, 0.1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1% Protease Inhibitor Cocktail (Sigma) and stood for 30 min on ice. After centrifugation at 14000 rpm for 20 min at 4 °C, the supernatants were collected as protein samples. Protein contents were measured with a DC Protein assay kit (Biorad, Hercules, CA, U.S.A.). Ten micrograms of lysate protein for Western blotting of RAS and c-myc was separated by SDS-PAGE using a 10% polyacrylamide gel and electroblotted onto a PVDF membrane (Du Pont, Boston, MA, U.S.A.). After blockage of nonspecific binding sites for 1 h with 5% nonfat milk in PBS containing 0.1% Tween 20, the membrane was incubated overnight at 4 °C with anti-human RAS antibody, clone RAS10 (Upstate, Charlottesville, VA, U.S.A.), which detects mutated RAS[10] or with anti-human c-myc antibody (Santa Cruz, Santa Cruz, CA, U.S.A.). The membranes were then washed 3 times with PBS containing 0.1% Tween 20, incubated further with HRP-conjugated sheep anti-mouse or donkey anti-rabbit Ig antibody (Amersham) at room temperature, and then washed 3 times with PBS containing 0.1% Tween 20. The immunoblots were visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA, U.S.A.).

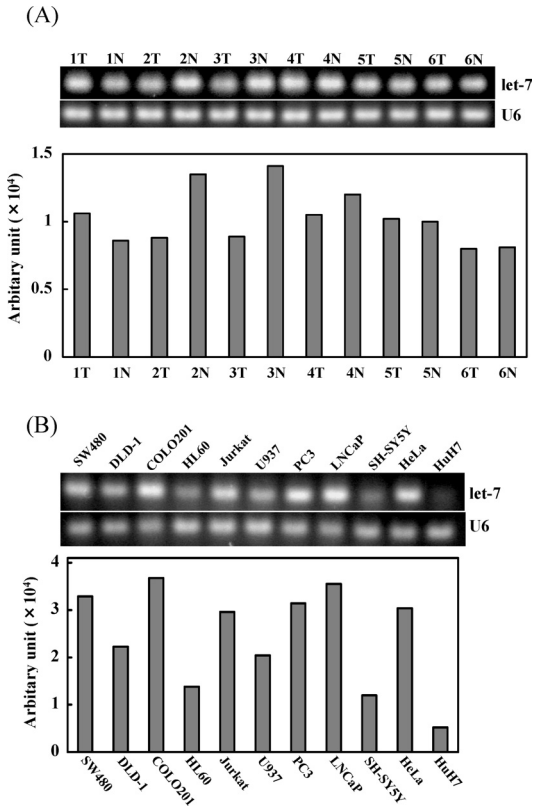

Fig. 1. Expression of *let-7* miRNA in Human Colon Tumor Tissues and Cancer Cell Lines Examined by qRT-PCR

(A) The two pairs of lanes T and N contained samples from tumor tissues (T) and the adjacent non-tumor tissues (N), respectively. The patient numbers correspond to those in Table 1. The band intensity of the PCR products was expressed as arbitary densitometric units (lower panel). (B) Expression level of *let-7* miRNA in human colon cancer cell lines SW480, DLD-1, and COLO201 and in the other cell lines. U6 was used as an internal standard. The band intensity of the PCR products was expressed as arbitary densitometric units (lower panel).

RESULTS AND DISCUSSION

Expression of *let-7* in Human Colon Cancer Cells In order to confirm whether the levels of *let-7* miRNA were reduced in human colon cancer cells, we examined the expression of mature *let-7* miRNA in the samples from 6 patients with colon cancer (Table 1), and in human colon cancer cell lines DLD-1, SW480, and COLO201 and other cell lines by quantitative RT-PCR (qRT-PCR, Fig. 1). As shown in Fig. 1A, the expression level of *let-7* miRNA in tumors was significantly decreased in 2 of the 6 patients tested (cases 2 and 3) compared with that in adjacent non-cancerous tissue from the same patient. Clinical difference between the 2 groups, patients with *let-7* low-expression and the others, was not observed. Furthermore, among the colon cancer cell lines (DLD-1, SW480, and COLO201), DLD-1 cells showed the

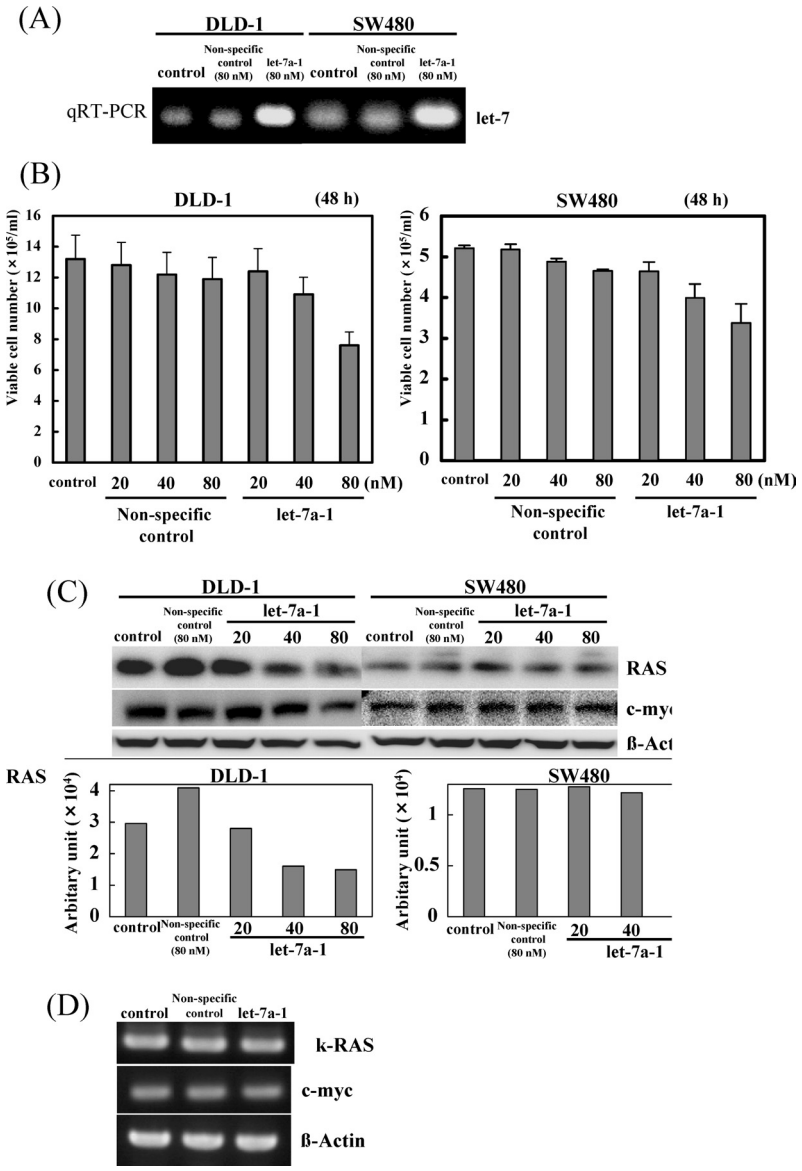

Fig. 2. Effect of Transfection with *let-7a-1* Precursor miRNAs on Human Colon Cancer Cells

(A) Levels of *let-7* miRNA in DLD-1 and SW480 cells after transfection of the cells with let-7a-1 at 80 nM. (B) Number of viable transfected or control cells at 48 h after transfection, which was performed at 24 h after inoculating cells into the culture dishes (1×10^5/ml). Data represent the mean±S.D. of 3 different experiments, each carried out in duplicate. (C) Expression levels of RAS and c-myc proteins at 48 h after the transfection of DLD-1 and SW480 cells with let-7a-1, as evaluated by Western blot analysis. The intensity of the RAS band for DLD-1 and SW480 cells was determined by densitometry (lower panel). (D) mRNA levels of *RAS* and *c-myc* evaluated by qRT-PCR at 48 h after the transfection of the cells. Control cells were incubated in medium containing transfection reagent only.

lowest expression level (Fig. 1B). Importantly, differences in the levels of *let-7* miRNA also existed among the other human cancer cell lines (Fig. 1B).

Growth Inhibitory Effect of let-7a-1 Precursor miRNA in DLD-1 Cells The *let-7* family of miRNAs includes 14 isomers, and each isomer is usually located on a different chromosome. Among them, we focused on *let-7a-1*, which is located at chromosome 9q22.3; because this locus is frequently deleted in colon cancer.[11] In order to examine the growth inhibitory effect of *let-7*, we used *let-7a-1* precursors to transfect *let-7* low-expressing DLD-1 cells and normal-expressing SW480 cells, both of which showed good transfection efficiency by the use of FITC-siRNAs (data not shown). qRT-PCR demonstrated that the level of mature *let-7* was increased in both cell lines after the transfection at 80 nM (Fig. 2A), which indicated that the increased *let-7* expression was due to the introduction of let-7a-1 miRNA precursor into the cells. Notably, the growth of DLD-1 cells was inhibited in a dose-dependent manner; and at 80 nM the viable cell number was reduced to approximately 60% of that in the control, whereas only slight suppression was found in the normal-expressing SW480 cells (Fig. 2B). The result of the colony formation assay indicated a lower plating efficiency for the let-7a-1-transfected DLD-1 cells, compared with that for the non-specific (NS) miRNA-transfected cells (Table 2). It was already reported that *let-7* represses the expression of *RAS* and *c-myc* translationally.[6] Therefore, by Western blot analysis we examined the expression of both of these proteins after the transfection. The results indicated that the levels of RAS protein in DLD-1 cells were significantly reduced by the transfection with let-7a-1 in a dose-dependent manner, whereas such effect was only slight in SW480 cells (Fig. 2C).

Table 2. Growth of DLD-1 Cells in Soft Agar

DLD-1 cells	Plating efficiency[a] (%)[b]	Colony formation[c]
Control	48.6±8.2	+
Non-specific miRNA	42.8±5.2	+
let-7a-1	28.6±13.8	+

a) Average number of colonies per dish obtained 10 d after seeding of 2×10^3 cells/dish. *b*) Mean±S.D. is given ($n=8$). *c*) Formation of 1 or more colonies in or on agar.

The decreased expression of c-myc was evident in DLD-1 cells, however, not observed in SW480 cells (Fig. 2C). On the other hand, the levels of *RAS* and *c-myc* mRNAs evaluated by qRT-PCR were almost unchanged in the same samples (Fig. 2D), which indicates that the transfection with exogeneous let-7a-1 caused the decreased expression of mutated ras and c-myc proteins at the translational level, as reported previously.[6] Given the results from the experiments on the high *let-7*-expressing SW480 cells, the efficacy of targeting the mRNAs by let-7a-1 may depend on the level of *let-7* in the cancer cells. Thus, we could conclude that the growth suppression observed in DLD-1 cells was due to enforced expression of exogenous let-7a-1. Several recent reports suggested that the expression levels of *let-7* miRNA are altered in human lung cancers. Takamizawa *et al*. (2004) showed that a reduced *let-7* level was significantly associated with a shortened postoperative survival and that enforced expression of *let-7* by transfection resulted in the inhibition of A549 lung cancer cell growth.[5] On the other hand, no change in *let-7* family expression was reported to occur in colon cancer.[12] However, in the study just cited *let-7* expression was examined in only 4 cases; and these investigators detected reduced levels of miRNA-143 and miRNA-145 in the majority of tumors from 14 patients and in human colon cancer cell lines CoCo-2 and LIM1862. Thus, as far as we know, no other studies except our present one have evaluated whether *let-7* contributes to carcinogenesis in colon tumors. Our present results suggest that *let-7* may function as one of the onco-miRNAs even in human colon cancer. Further study examining cytogenetical deletions or methylated DNA regions located in *let-7* family loci[13] will be needed for better understanding of the processing and function of *let-7* in colon cancer. On the other hand, we also observed growth inhibition after the transfection of SH-SY5Y neuroblastoma cells with the let-7a-1 precursor (data not shown), which showed a decreased *let-7* expression (Fig. 1B). Therefore, it is possible that *let-7* is involved in carcinogenesis in other kinds of cancers as well. Our results demonstrated in this study suggest the possibility of using miRNAs for the development of anti-cancer agents. Further experiments will be required to assess the anti-cancer effect of *let-7* miRNA on various kinds of cancers, and such experiments are currently underway in our laboratory.

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REFERENCES

1) Ambros V., *Cell*, **113**, 673—676 (2003).
2) Ambros V., *Nature* (London), **431**, 350—355 (2004).
3) Bartel D. P., *Cell*, **116**, 281—297 (2004).
4) McManus M. T., *Semin. Cancer Biol.*, **13**, 253—258 (2003).
5) Takamizawa J., Konishi H., Yanagisawa K., Tomida S., Osada H., Endoh H., Harano T., Yatabe Y., Nagino M., Nimura Y., Mitsudomi T., Takahashi T., *Cancer Res.*, **64**, 3753—3756 (2004).
6) Johnson S. M., Grosshans H., Shingara J., Byrom M., Jarvis R., Cheng A., Labourier E., Reinert K. L., Brown D., Slack F. J., *Cell*, **120**, 635—647 (2005).
7) Rigas B., *J. Clin. Gastroenterol.*, **12**, 494—499 (1990).
8) Ahnen D. J., *West J. Med.*, **154**, 700—705 (1991).
9) Malumbres M., Barbacid M., *Nat. Rev. Cancer*, **3**, 459—465 (2003).
10) Allen M. P., Xu M., Linseman D. A., Pawlowski J. E., Bokoch G. M., Heidenreich K. M., Wierman M. E., *J. Biol. Chem.*, **277**, 38133—38140 (2002).
11) Wiesner G. L., Daley D., Lewis S., Ticknor C., Platzer P., Lutterbaugh J., MacMillen M., Baliner B., Willis J., Elston R. C., Markowitz S. D., *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 12961—12965 (2003).
12) Michael M. Z., O'Connor S. M., van Holst Pellekaan N. G., Young G. P., James R. J., *Mol. Cancer Res.*, **1**, 882—891 (2003).
13) Calin G. A., Sevignani C., Dumitru C. D., Hyslop T., Noch E., Yendamuri S., Shimizu M., Rattan S., Bullrich F., Negrini M., Croce C. M., *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 2999—3004 (2004).