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Genetic variations of microRNAs in human cancer and their effects on the expression of miRNAs

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the posttranscriptional level to lead to mRNA degradation or repressed protein production. The expression of miRNA is deregulated in many types of cancers. To determine whether genetic alterations in miRNA genes are associated with cancers, we have systematically screened sequence variations in several hundred human miRNAs from >100 human tumor tissues and 20 cancer cell lines. We identified 8 new single-nucleotide polymorphisms (SNPs) and 14 novel mutations (or very rare SNPs) that specifically present in human cancers. These mutations/SNPs are distributed in the regions of pri-, pre- and even mature miRNAs, respectively. Importantly, whereas most of the mutations did not exert detectable effects on miRNA function, a G → A mutation at 19 nt downstream of miRNA *let-7e* led to a significant reduction of its expression *in vivo*, indicating that miRNA mutation could contribute to tumorigenesis. These data suggest that further screening for genetic variations in miRNA genes from a wide variety of human cancers should increase the discovery and identification of molecular diagnostic and therapeutic targets and complement the mutation analysis of consensus coding sequences in human cancers.

Introduction

MicroRNAs (miRNAs) represent an abundant class of small non-coding RNAs that regulate gene expression by targeting messenger RNAs through sequence-specific base pairing with the 3′-untranslated regions of messenger RNAs, resulting in RNA degradation and/or translational repression (1–3). MiRNAs are initially transcribed from genomic DNA as long primary transcripts (pri-miRNAs) that are then processed in the nucleus (4,5) into hairpin-shaped precursor RNAs (pre-miRNAs). Pre-miRNAs are exported to the cytoplasm through Exportin-5 (6) and further processed into 18–25 nt long mature miRNAs by RNase Dicer (7,8). To date, 533 human miRNA sequences have been deposited at the Sanger Institute miRBase (http://microrna.sanger.ac.uk/sequences/index.shtml). Emerging evidence has indicated that miRNAs are involved in the oncogenesis of many human cancers. Over half of all known human miRNA genes are located at fragile sites and genomic regions involved in cancers (9). Similarly, mouse miRNA genes are also frequently located near mouse cancer susceptibility loci (10). High-resolution array-based comparative genomic hybridization has revealed that miRNA copy numbers are quite abnormal in human cancers (11). More over, global repression of miRNA maturation promotes cellular transformation and tumorigenesis, suggesting that some miRNAs are potential tumor suppressors (12,13). On the other hand, miRNAs might also function in an oncogenic manner (14–16). Indeed, miRNA profiling has revealed that most of miRNAs are significantly downregulated in human cancers (17,18).

The mechanism through which miRNA expression is deregulated in cancers is not well characterized. Among others, the deregulation of miRNA expression in cancers could be caused by sequence variations in miRNA, such as mutation or single-nucleotide polymorphism (SNP). A mutation or a SNP at a miRNA gene region might affect the transcription of miRNA primary transcripts, the processing of miRNA precursors to mature miRNAs or miRNA–target interactions (19). Accordingly, efforts have been directed toward the identification of mutation or SNP in miRNAs. Whereas most of the identified mutations and SNPs in miRNAs do not affect miRNA expression and function (20–23), a germ line mutation (C to T) located at 7 nt downstream of the pre-miR-16-1 sequence identified in B-cell chronic lymphocytic leukemia nearly abolished the expression of both *miR-15a* and *miR-16-1* (24). Similarly, a single-nucleotide mutation located at 6 nt downstream of the mouse pre-miR-16-1 sequence also led to reduced *miR-16* expression in lymphoid tissues and the NZB-derived malignant B-cell line, LNC (25). An SNP located at nucleotide 8 (+8) of mature miR-125a in a normal subject (26) blocks pri-miR-125a processing to pre-miRNA and thus reduces the miRNA-mediated translational suppression of its target. Consistently, editing specific adenosine residues to inosine in pri-miR-142, the precursor of *miRNA-142*, results in the suppression of its processing by Drosha (19). Hence, systematic genetic analysis of miRNA genes in human cancers could lead to the identification of additional functional mutations for evaluating the role of miRNA in tumorigenesis. Here, we screened sequence variations in 288 conserved miRNA genes deposited at the Sanger Institute miRBase and 48 human-specific miRNA genes (27) and identified 14 novel sequence variations in the four types of human tumor tissues from ∼150 patients and in 20 human tumor cell lines. Nine of 14 sequence variations were located in the pri-miRNA regions, 4 in the pre-miRNA regions and 1 in the mature miRNA. Interestingly, we found that a germ line mutation (G to A) located at 19 nt downstream of the pre-*let-7e* miRNA led to a significant reduction of its expression, suggesting that screening for genetic variations in miRNA genes in human cancers has potential for identification of molecular diagnostic and therapeutic targets.

Abbreviations: miRNA, microRNA; PCR, polymerase chain reaction; RT, reverse transcription; SNP, single-nucleotide polymorphism; ssRNA, single-stranded RNA.

[†]These authors contributed equally to this work.

Materials and methods

Materials

Freshly frozen cancer tissue specimens from Caucasian patients with various cancers were provided by the Cooperative Human Tissue Network in the USA. Mammalian cell lines were obtained from American Type Culture Collection and National Cancer Institute and cultured in the media as recommended. Genomic DNAs of normal subjects (Caucasian) were obtained from the British 1958 birth cohort that is based on all persons born in Britain during 1 week in 1958 and Coriell Institute for Medical Research. Collection and use of the tissue and genomic DNA samples were approved by the National Research Council Canada. DNeasy® Tissue kit used for genomic DNA extraction was purchased from Qiagen (Hilden, Germany). TRIZOL® Reagent used for RNA extraction was from Invitrogen (Carlsbad, CA). MinElute 96 UF plates for DNA purification were purchased from Qiagen. High-capacity cDNA reverse transcription (RT) kit and TaqMan MicroRNA assay kit were purchased from Applied Biosystems (Foster City, CA, USA).

Identification of miRNA mutations

Sequence and genomic location of human miRNAs were obtained from the Sanger Institute miRBase (http://microrna.sanger.ac.uk/sequences/index.shtml). A computer program was developed to download the surrounding genomic DNA sequences of miRNA genes and design primers for polymerase chain reaction (PCR). Genomic DNA was extracted from freshly frozen tissue specimens and cultured cell lines using DNeasy® Tissue kit following the

manufacturer's protocol. An ~400 bp genomic DNA fragment of each miRNA gene, including 100–200 bp at each end of the mature miRNA, was amplified by nested PCR. The DNA products from the first PCR were diluted 50 times and used as templates for the second PCR. The DNAs amplified from the second PCR were purified using MinElute 96 UF plates (Qiagen) and used for sequencing. The sequences of the primers used for genomic DNA amplification and sequencing of the miRNA genes studied are available upon request. The mutations were first confirmed by sequencing the same sample from the other direction. A computer program based on PolyPhred (28) was designed and used to identify heterozygous sites for single-nucleotide substitutions. Then, the mutations and/or SNPs were further confirmed by re-PCR of the sample and sequencing again to avoid any artificial mutation that could be introduced by PCR amplification. In the initial screening, we sequenced ~20 samples for each miRNA. When a mutation was identified, we extended to screen ~150 cancer patients for the mutation. If a mutation destroys or creates a restriction enzyme site, then enzyme digestion method was also used (29) in the extended screening. To determine whether a mutation is significantly enriched in cancer samples, for some interesting mutations, screens were also performed using ~200 normal subjects.

Plasmid construction and expression of miRNAs in mammalian cells

An ~1.5 kb genomic DNA fragment containing the respective miRNA gene at the middle was amplified by PCR and inserted into pcDNA3.1 hygro vector (Invitrogen). Heterozygous genomic DNA containing both the wild-type allele and the mutant allele of the interesting miRNA gene was used as template for PCR. The clones that contain the wild-type or mutant miRNA gene were identified by sequencing. HeLa cells were respectively transfected with the wild-type and mutant constructs along with empty vector, using Lipofectamine 2000 reagent following the manufacturer's protocol (Invitrogen). The transfection efficiency for each pair of wild-type and mutant constructs was normalized by cotransfected luciferase expression vector (30). Three independent transfections with comparable transfection efficiencies for both the wild-type and mutant constructs were used for northern blot and quantitative RT–PCR (see below). Forty-eight hours after transfection, cells were lysed and total RNA was extracted.

Northern blot

Total RNA was extracted from HeLa cells with TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions. Ten micrograms of total RNA was separated on 15% (wt/vol) polyacrylamide gel containing 8 M urea, and then electrotransferred to a positively charged nylon membrane (PerkinElmer). The membrane was ultraviolet cross-linked followed by prehybridization for 30 min with ULTRAhyb-Oligo hybridization buffer (Ambion, Austin, TX) and hybridization with γ-32p-ATP-labeled probe at 37°C for overnight. The membrane was washed once with 2× SCC–0.5% sodium dodecyl sulfate for 5 min and twice for 25 min at 37°C. The ratio of the miRNA processing level between samples was estimated by using Typhoon Trio+ (Amersham Biosciences).

Quantitative RT–PCR

Total RNA was treated with DNA*free*™ kit to remove possible contaminating DNA from RNA preparations following the manufacturer's instruction (Ambion). cDNA was synthesized from 10 ng of total RNA and quantitative PCR was carried out with the gene-specific primers using Power SYBR Green PCR Master Mix and RT–PCR kit (Applied Biosystems). The primers for hsa-*let-7e* were 5′-TGTCTGTCTGTCTGTCGGGTCTGT-3′ (F) and 5′-GAAAGCTAGGAGGCCGTATAGTGA-3′ (R). The expression levels of mature miRNA were evaluated with TaqMan MicroRNA Assays from Applied Biosystems. Relative quantities of miRNA were calculated using the ΔΔCt method after normalization with reference to the expression of RNU48 as endogenous control. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Table I. New SNPs identified in miRNA genes from human cancer and normal subjects

Human miRNA	Location[a]	Cancer patients (no./total no.)	Normal subjects (no./total no.)
miR-138-1	G+40A	1/252	3/124
miR-30a	A−48G	1/156	8/120
miR-216	T105A	9/48	19/202
HS_177	C−10T	2/134	6/196
HS_77	A25G	1/134	1/196
HS_116	C+2T	8/130	6/90
miR-453	T30C	1/42	8/220
miR-1-2	G−15A	4/62	6/95

[a]The locations of SNPs are numbered according to their upstream position 5′ (negative base count, '−') to the start of the pre-miRNA or downstream 3′ (positive base count, '+') to the end of the pre-miRNA.

Results

MiRNA mutations and/or SNPs identified from human tumor tissues and cancer cell lines

To investigate the potential oncogenic role of mutations/SNPs in miRNA genes, we screened ~150 tumor tissues obtained mainly from human colon and prostate cancer patients and 20 human cancer cell lines (supplementary Table S1 is available at *Carcinogenesis*Online) for analysis of 288 human miRNAs that were available at that time when the project was started (http://microrna.sanger.ac.uk/sequences/index.shtml) (see the list in Supplementary Table S2 is available at

Table II. Genetic variation of miRNAs in cancer patients and cancer cell lines

miRNA	Variation location[a]	Type of mutation (germ line/somatic)	Patients or cell lines	Ratio of mutants (no./total no.)	Normal subjects (no./total no.)
miR-155	T+38C	Unknown[b]	P193[c]	1/157	0/216
miR-206	C+35T	Germ line	C163[c]	1/158	0/192
let-7e	G+19A	Germ line	P254	1/114	0/215
let-7e	G+14A	Germ line	P192	1/156	0/215
miR-199a-2	G−75T	Somatic	P142	1/172[d]	0/213
miR-520e	A77C	Unknown	C17	1/155	0/225
miR-181b-2	G80A	Unknown	P245	1/114	0/192
HS_303_PT_124	G−20A	Germ line	C270	1/134	0/192
HS_164	T36C	Germ line	P32	1/134	0/192
HS_176	T+38C	Germ line Unknown	P35, P218, P347, C112, C148, C368, B89[c], L40[c] C114, P187	10/134	1/192
HS_115	G27T	Germ line	C98, C167	2/133	0/192
miR-323	G+39A	Unknown	Colon cancer cell line DLD-1	1/20	0/192
miR-208	C12T	Unknown	Prostate cancer cell line DU-145	1/20	0/192
miR-362	C+74T	Unknown	Lymphoblastic leukemia cell line CCRF-CEM	1/20	0/192

[a]The locations of sequence variations are numbered as in Table I.
[b]Unknown, the adjacent unaffected tissue was not available.
[c]P, prostate cancer patient; C, colon cancer patient; B, breast cancer patient and L, lung cancer patient.
[d]Seventy-two of 172 patients were prostate cancer, 62 were colon cancer, 23 were lung cancer and 16 were breast cancer.

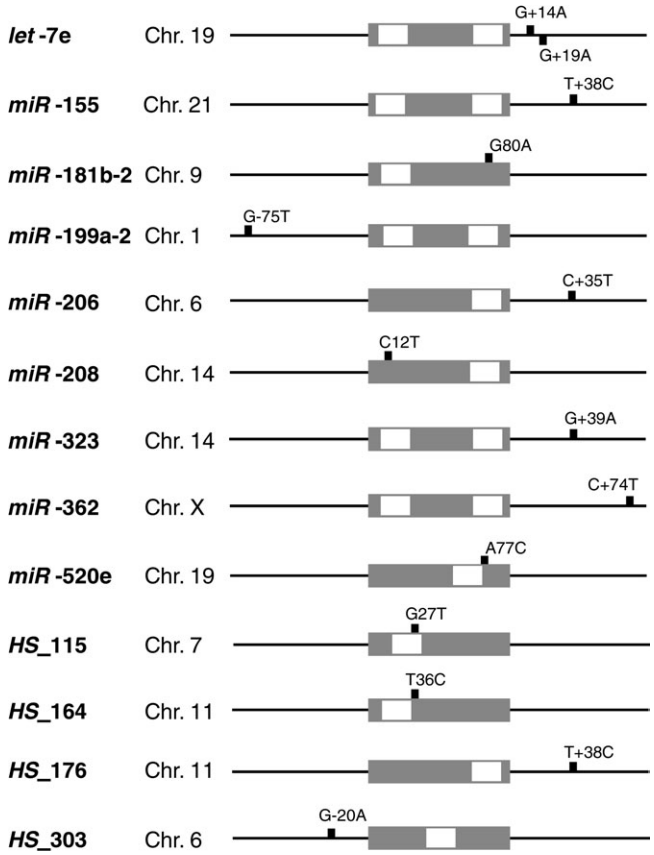

Fig. 1. The locations of 14 novel sequence variations in the pri-miRNA transcripts. The locations of mutations in pri-miRNAs are depicted on black line at the upstream (negative base count, '–') or downstream (positive base count, '+') of the precursor hairpin (pre-miRNA). The locations of mutations in the pre- and the mature miRNAs are marked on filled box and unfilled box, respectively. The figure is not drawn to scale.

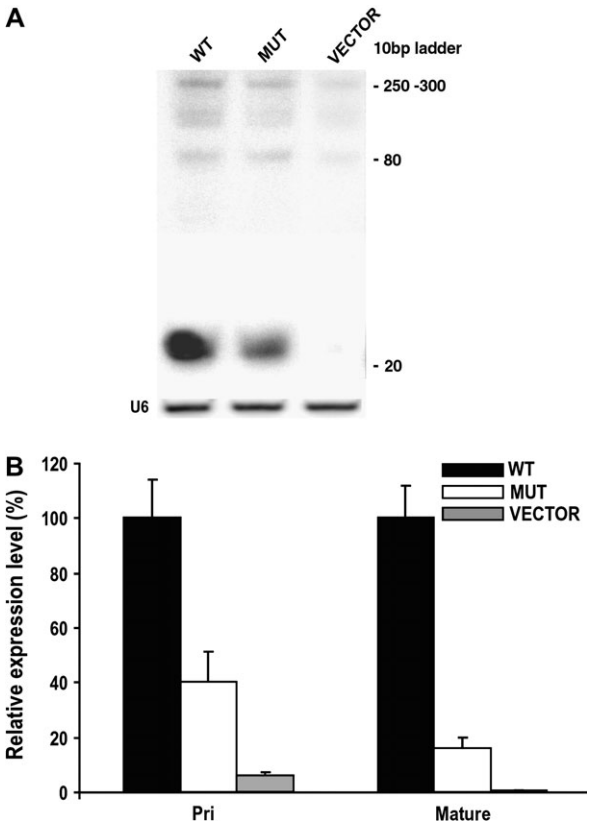

Fig. 2. A G → A mutation located at 19 nt downstream of the *let-7e* precursor led to a remarkable reduction in the expression of the mature miRNA. HeLa cells were transfected with the vectors expressing the wild-type and variant miRNA *let-7-e*, along with the control vector. Luciferase activities for the normalization of the transfection efficiencies were 1.47×10^4 for mutant *let-7e* and 1.42×10^4 for wild-type *let-7e*, respectively. U6 RNA was used as a loading control. (**A**) The total RNAs isolated from transfected cells were used for northern blots as shown. (**B**) Quantitative RT–PCR was used to measure levels of the primary and mature forms of miRNA *let-7e* and its variant *let-7e* (G+19A) in transfected HeLa cells. The relative expression levels as determined by ΔΔCt analyses are shown. Three independent transfectants were performed and each transfection was in triplicates for quantitative RT–PCR.

*Carcinogenesis*Online) and 48 human miRNA candidates (27). We amplified the genomic DNA containing the miRNA precursor for each miRNA gene, and its flanking sequences at both ends, and used the amplified PCR products for sequence analysis. When a mutation or a new SNP was identified in a cancer cell line, the human tumor tissues were also further screened. A total of 22 novel mutations/SNPs were identified in the screening that were neither listed in the human SNP database (http://www.ncbi.nlm.nih.gov/SNP/) nor previously reported (20,21,23,24). These mutations/SNPs were confirmed by repeated PCR amplification and sequencing of the reamplified products. We further analyzed genomic DNA from ∼200 normal individuals to determine whether the identified mutations/SNPs are associated with human cancer. We found 8 of 22 mutations/SNPs in the normal individuals. However, these are new sequence variations or SNPs (Table I) and have been not reported in the databases. Of the other 14 variants (Table II), 13 were absent in the normal individuals, and one substitution (T+38C) in miRNA *HS_176* occurred in 10 of 134 patients, but in only one of 192 normal individuals ($P = 0.001$), suggesting that this sequence variation is also potentially associated with human cancer (Table II). Further studies of the adjacent unaffected tissues from the same patients suggested that, except for one somatic mutation (G-75T in *miR-199a-2*), most of them were germ line mutations (Table II). Among these 14 mutations or rare SNPs, only one was located in the mature region of miRNA *HS_115*. Four of them were in the pre-miRNA regions, namely G80A in *miR-181b-2*, C12T in *miR-208*, A77C in *miR-520e* and T36C in *HS_164*. The other eight sequence variations were in the pri-miRNA regions (Figure 1).

Effect of mutations on miRNA expression

We performed functional analysis to determine whether the identified mutations of miRNA genes could affect the expression (transcription) or processing of the mutated miRNA *in vivo*. We cloned both wild-type and mutated alleles of the miRNA genes into a mammalian expression vector. HeLa cells, which express undetectable or very low levels of the endogenous miRNAs tested, were transfected with the constructed expression vectors. Total RNAs were extracted from the transfected cells, and the expression levels of miRNAs were evaluated by northern blotting. The results revealed that whereas most of the identified mutations did not apparently affect the expression of the mature miRNAs (data not shown), a G → A mutation located at 19 nt downstream of the *let-7e* precursor led to a significant reduction in the expression of the mature miRNA when compared with its wild-type transfectant (Figure 2A). The expression of pri-miRNAs for mutated *let-7e* was also partially inhibited (Figure 2A). We further quantified the relative expression levels of both the pri- and mature miRNAs for the wild-type and mutated *let-7e*, respectively, in the transfected cells using quantitative real-time PCRs. Consistently, the results showed that the primary transcript of mutated *let-7e* was partially inhibited (∼40% that of the wild-type), and the expression of the mature miRNA of the mutated *let-7e* was further inhibited by ∼84% when compared with wild-type *let-7e* (Figure 2B). These data indicated that the

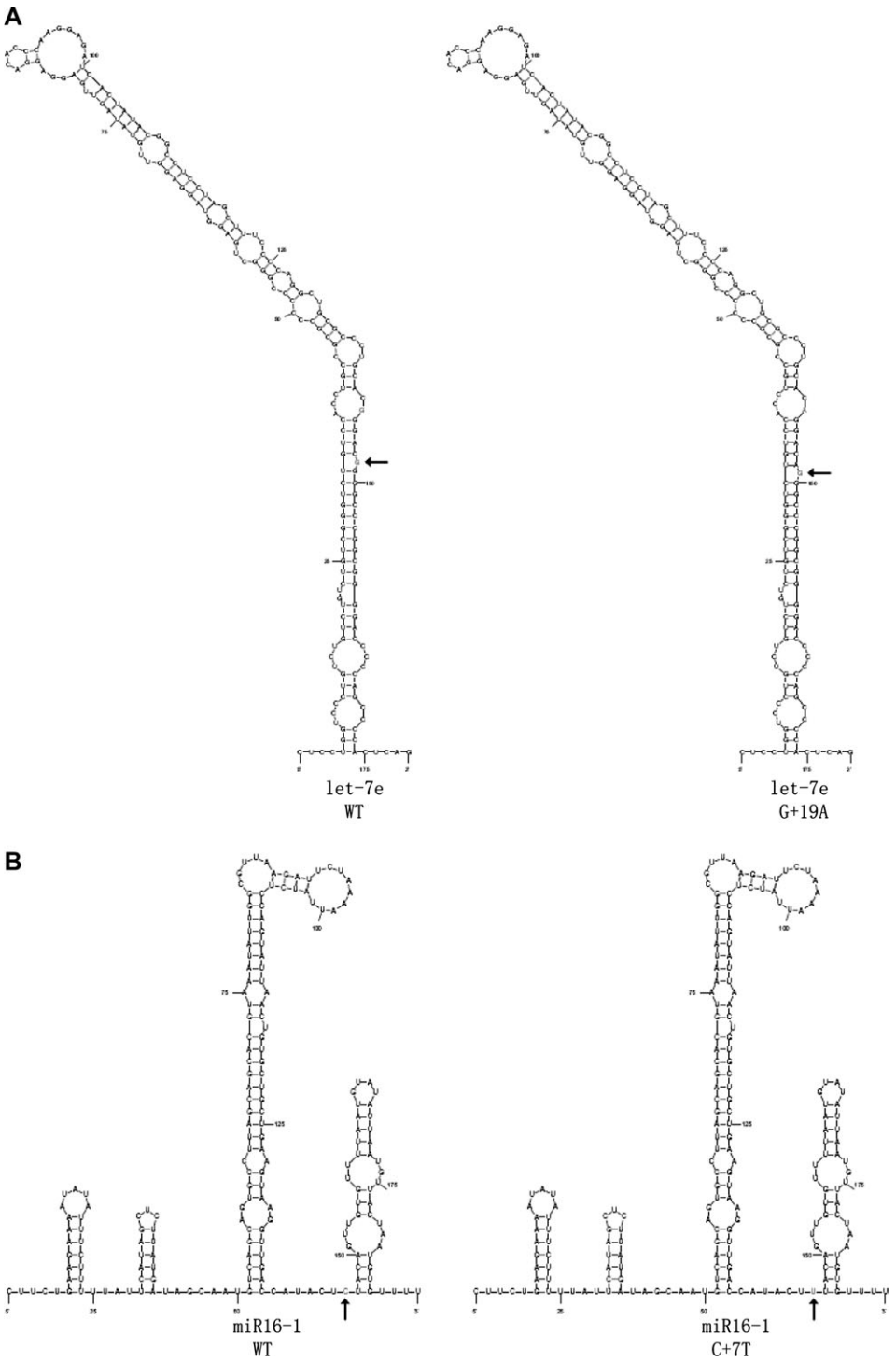

Fig. 3. The RNAfold program (34) predicts no secondary structure changes for both the mutated *let-7e* and *miR-16-1*, the expression of which were remarkably reduced. Depicted are the most stable secondary structures with the lowest free energy as predicted by the RNAfold program (34). The arrows indicate mutation positions. (**A**) *let-7e* and (**B**) *miR-16-1*.

sequence located at 19 nt downstream of *let-7e* precursor is critical for the efficient expression and/or processing of miRNA *let-7e in vivo*.

Effects of sequence variations on the secondary structures of miRNA precursors

The RNaseIII-mediated processing and maturation of a miRNA precursor *in vivo* lead to the final product, namely a functional mature miRNA. Thus, it is expected that the processing and maturation of a miRNA precursor require appropriate secondary structures and specific sequence elements within the miRNA precursor or pri-miRNA (26,31–33). Considering the significant reduction in the expression of the *let-7e* variant (G+19A) (Figure 2A and B), we investigated whether this sequence change could alter the secondary structure of the mutated miRNA primary transcript. We used the RNAfold program (34) to predict the most stable secondary structure of both the wild-type and variant sequences. However, except for an A:U base pair upper shift, no other differences were evident (Figure 3A). Since the reported G+7C substitution in human *miR-16-1* also dramatically

reduced its expression *in vivo* (24), we also used the RNAfold to calculate its most stable secondary RNA structure. Again, the structure was not affected as the substitution occurred in a single-stranded region (Figure 3B). Interestingly, whereas most of the mutations identified in this study did not cause major conformational changes (data not shown), the C+35T variant of *miR-206* appeared to alter the distance (∼11 nt) between the cleavage site and the stem-single-stranded RNA (ssRNA) junction because the original large bulge in the ssRNA–double-stranded RNA junction region located in the wild-type for DCGR8 function (32) was changed to a very small bulge by the mutation, and a new, relatively large bulge was created nearby, which could serve as an ssRNA to shift the cleavage site for DGCR8 in the variant (Figure 4A) (32). If this would be the case, a much shorter mature miRNA, a 17 nt rather than a 22 nt in length, might be produced by DGCR8-mediated processing in the variant. However, northern blotting revealed no differences in the expression level or the length of the mature miRNAs between the wild-type and variant (Figure 4B). Together, these data indicate that the requirements of sequence features and elements in miRNA for its processing and maturation remain obscure.

Discussion

The expression profiles of miRNAs in human cancers have been extensively investigated over the past few years. Many miRNAs are up- or downregulated in various human cancers, indicating that miRNAs play a role in tumorigenesis. However, the mechanism through which miRNA expression in cancer deteriorates is largely unknown. To further explore the potential link of miRNA with cancer, we screened sequence variations in 288 human miRNAs available at the time and 48 human candidate miRNAs in ∼150 human tumor tissues and 20 human cancer cell lines. In addition to eight SNPs that were not listed in the SNP database, we identified 14 mutations (or very rare SNPs) in cancers. An analysis of mutation patterns showed that no mutation was conserved in any individual tumor and that most of the identified mutations occurred in only one tumor tissue specimen among >100 specimens analyzed, suggesting that most of these sequence variations in miRNA might not be cancer specific. Of 14 mutations, the T+38C mutation in miRNA *HS_176* was found in 10 of 134 cancer patients with a *P* value of 0.001, indicating a potential association with cancer.

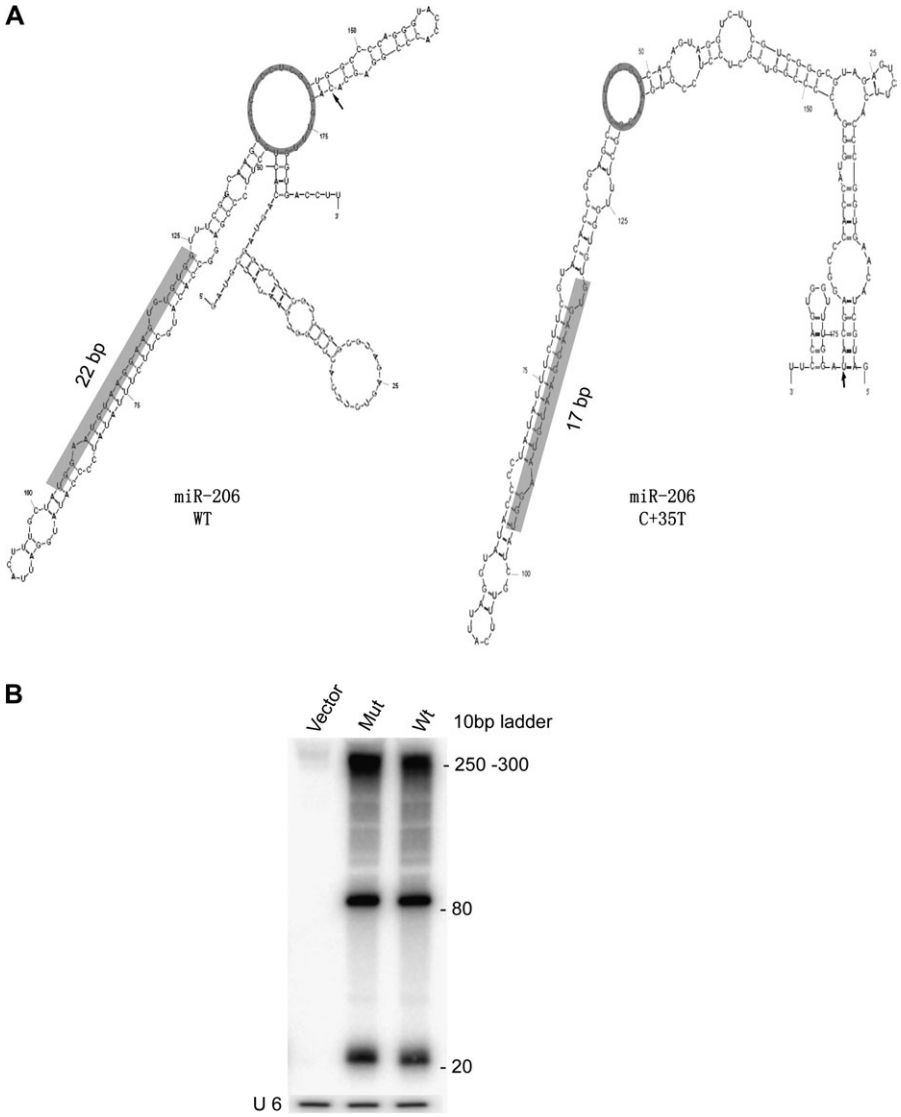

Fig. 4. The predicated secondary structure changes and the detected expression levels of wild-type *miR-206* and its variant. (**A**) The RNA secondary structures of wild-type and mutated *miR-206* were predicated with the lowest free energy by the RNAfold program (34). The original large bulge of wild-type *miR-206* and the new enlarged bulge of the mutated *miR-206* (C+35T), which could serve as the ssRNA–double-stranded RNA junction for DGCR8 (32), are highlighted. The mature sequence of the wild-type *miR-206* and the predicated mature sequence of the mutated *miR-206* are also marked. The arrows indicate mutation positions. (**B**) HeLa cells were transfected with the vectors expressing the wild-type and variant *miR-206*, along with the control vector. Northern blotting as shown was performed as with Figure 2A. U6 RNA was used as a loading control.

Hypothetically, mutations that arise in miRNA genes, including pri- and pre-miRNA regions, could affect processing of the precursor to the mature form of miRNA, resulting in aberrant expression of miRNA. Zeng *et al.* performed RNaseIII Drosha-mediated *in vitro* processing of miRNAs of various sizes and structures. They found that Drosha function requires ssRNA extensions from the stem structure of miRNA precursor and a strong secondary structure within the extension, otherwise a blunt-ended hairpin in the precursor blocks Drosha cleavage (33). In addition, variations in stem structure and sequence around the cleavage site could change the cleavage position (31). An 'ssRNA–double-stranded RNA junction-anchoring' model has recently been proposed to explain the Drosha/DGCR8-mediated processing of pri-miRNA to pre-miRNA *in vitro* (32). The cleavage site of DGCR8 in the model is determined mainly by the distance (∼11 nt) from the stem-ssRNA junction in the miRNA precursor. According to such models *in vitro*, if a mutation causes a structural change in the crucial regions of miRNA, then the mutation could affect the processing and expression of the miRNA. Using the RNAfold program, we found that the C+35T mutation of *miR-206* altered free energy values, resulting in elimination of the original large bulge and the creation of a new enlarged RNA bulge that, as predicated by the model (32), could serve as the ssRNA–double-stranded RNA junction for DGCR8. However, northern blotting (Figure 4B) did not show any change in the cleavage site of the variant. Similarly, some predicated mutations might lead to major conformational changes of the miRNAs that even reach into or close to the stem of the miRNA precursors, but none of these changes affected the processing or expression of the mutated miRNAs (21). In contrast, while the RNAfold program predicted no secondary structural changes for the variant, G+19A mutation in the *let-7e* identified herein, the expression of its mature miRNA was remarkably reduced *in vivo*. Similarly, the reported C+7T substitution in *miR-16-1* also caused the reduced expression of *miR-16-1* (24). Consistently, it was reported that mutation at 40 nt downstream or upstream of the pre-miRNA can influence the transcription of the miRNA (35). These results suggest that the processing and maturation of miRNA *in vivo* is more complex and subtle than that can be currently predicted. Alternatively, the environments surrounding miRNAs and unknown factors that interact with the miRNA might affect its folding *in vivo* or protect its structural conformation against changes caused by some mutations. Nevertheless, the required characteristics and sequence elements residing in the pri-, pre- and mature regions of miRNA for processing need to be defined before the miRNA processing mechanism can be elucidated.

In summary, we presented evidence that miRNA sequences vary in the four different types of human cancers, although the variation rate for the most of the identified individual mutations is relatively low (<1%) in cancer patients. Most of the mutations so far identified in cancer did not exert any apparent effect on miRNA function. However, the expression of one identified sequence variant was remarkably reduced, indicating that miRNA mutation could contribute to tumorigenesis, although the molecular mechanism underlying the particular sequence variations in miRNA that can cause aberrant expression remains to be determined.

Supplementary material

Supplementary Tables S1 and S2 can be found at http://carcin.oxfordjournals.org/

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