miR-195, miR-455-3p and miR-10a* are implicated in acquired temozolomide resistance in glioblastoma multiforme cells

Kenta Ujifuku[a, c], Norisato Mitsutake[a, *], Shu Takakura[a], Michiko Matsuse[a], Vladimir Saenko[b], Keiji Suzuki[a], Kentaro Hayashi[c], Takayuki Matsuo[c], Kensaku Kamada[c], Izumi Nagata[c] and Shunichi Yamashita[a, b]

[a]Department of Molecular Medicine, [b]Department of International Health and Radiation Research, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan; [c]Department of Neurosurgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Running title: miRNAs and temozolomide resistance in glioblastoma

Key words: glioblastoma, resistance, microRNA, miR-195, miR-455-3p, miR-10a*, temozolomide.

*Correspondence: Norisato Mitsutake, MD PhD

Department of Molecular Medicine, Atomic Bomb Disease Institute,

Nagasaki University Graduate School of Biomedical Sciences

1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

Tel: +81-95-819-7116

Fax: +81-95-819-7117

E-mail: mitsu@nagasaki-u.ac.jp

ABSTRACT

To identify microRNAs (miRNAs) specifically involved in the acquisition of temozolomide (TMZ) resistance in glioblastoma multiforme (GBM), we first established a resistant variant, U251R cells from TMZ-sensitive GBM cell line, U251MG. We then performed a comprehensive analysis of miRNA expressions in U251R and parental cells using miRNA microarrays. miR-195, miR-455-3p and miR-10a* were the three most up-regulated miRNAs in the resistant cells. To investigate the functional role of these miRNAs in TMZ resistance, U251R cells were transfected with miRNA inhibitors consisting of DNA/LNA hybrid oligonucleotides. Suppression of miR-455-3p or miR-10a* had no effect on cell growth, but showed modest cell killing effect in the presence of TMZ. On the other hand, knockdown of miR-195 alone displayed moderate cell killing effect, and combination with TMZ strongly enhanced the effect. In addition, using *in silico* analysis combined with cDNA microarray experiment, we present possible mRNA targets of these miRNAs. In conclusion, our findings suggest that those miRNAs may play a role in acquired TMZ resistance and could be a novel target for recurrent GBM treatment.

1. Introduction

Glioblastoma multiforme (GBM) is one of the most incurable forms of human cancers. Its five-year survival rate is less than 3% [1]. Because of its highly invasive nature, not only surgical therapy but also adjuvant chemoradiotherapy is indispensable. In 1990s, a new drug temozolomide (TMZ) emerged [2]. TMZ chemotherapy concomitant with radiotherapy has been reported to make statistically significant prognostic progress in GBM patients, and thus it has become the first line chemoradiotherapy regimen for treatment of GBM [3]. TMZ is an alkylating agent that methylates the O^6 position of guanines. The formation of O^6-methyl guanine leads to mismatch with thymine during DNA replication, eventually resulting in cell death. These methyl adducts are removed by a DNA repair enzyme, O^6-methyl guanine methyltransferase (MGMT) [4]. High level of MGMT activity in cancer cells creates a resistant phenotype by blunting the effect of TMZ. Accordingly, epigenetic silencing of the *MGMT* gene by promoter methylation compromises DNA repair in cancer cells and is associated with better prognosis [5]. However, little is known about acquired TMZ resistance, which is a serious impediment in the treatment of GBM.

Micro RNAs (miRNAs) are approximately 22 bases long, non-coding and single-stranded regulatory RNAs that are found in both plants and animals [6]. Mature miRNA binds to target

messenger RNA (mRNA) and induces its cleavage or translational repression depending on the degree of complementarity [7]. Although hundreds of miRNAs have been already cloned, only a part of them has been characterized. Several miRNAs have been shown to participate in cell proliferation or apoptosis in various types of cancers. miR-15a and miR-16 have been reported to induce apoptosis by targeting *BCL2* [8], and they are frequently deleted or underexpressed in chronic lymphocytic leukemia, prostate cancer and pituitary adenoma [9-11]. Reduced let-7 expression has been identified in lung cancer with poor prognosis [12] and inversely correlated with expression of RAS protein [13]. Compared to these underexpressed miRNAs, miR-17-92 cluster has an anti-apoptotic function and is overexpressed in lung cancer, B-cell lymphoma and anaplastic thyroid cancer [14-16]. Knockdown of miR-17-3p in anaplastic thyroid cancer cells induced caspase activation, resulting in apoptotic cell death [16]. As mentioned above, miRNAs can act as both tumor suppressor and oncogene.

Distinct miRNA expression patterns have also been reported in GBM. The functional significance of some of these up- or down-regulated miRNAs has been identified so far. For example, miR-21, one of the best characterized cancer-associated miRNAs, is highly expressed in GBM and has multi-faced functions such as inhibition of apoptosis and growth promotion [17, 18]. miR-221 and miR-222 are other up-regulated miRNAs in GBM [19-21].

These miRNAs have been thought to affect cell cycle by targeting *CDKN1B* and *CDKN1C*. miR-128 has been identified as one of the most frequently down-regulated miRNAs in GBM. It has been demonstrated that miR-128 targets *Bmi-1* and then reduces cellular proliferation and also self-renewal of glioma stem cells [22]. As described above, miRNAs play a variety of oncogenic roles in GBM; however, there is no report regarding the relationship between drug resistance and miRNAs in GBM. It is likely that miRNAs can also modulate sensitivity to anti-cancer drug and induce resistance. In fact, there were significant correlations between miRNA expression pattern and compound potency using the NCI-60 cancer cell panel [23]. In the present study, therefore, we used TMZ-sensitive GBM cell lines to generate TMZ-resistant variants by continuous exposure to the drug. We then performed comprehensive analysis of miRNA expression using miRNA microarray to explore the mechanisms of acquired resistance against TMZ.

2. Materials and Methods

2.1. Cell culture and reagent

We used GBM cell lines of human origin: U251MG, U87MG, M059K and M059J, which are sensitive to TMZ. These were purchased from American Type Culture Collection (Manassas,

VA, USA). All cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin at 37ºC in a humidified atmosphere with 5% CO_2. TMZ was purchased from WAKO Pure Chemical Industries (Osaka, Japan).

2.2. Cell survival assay

Cells (5×10^2 cells) were plated in each well of 96-well plates and treated with or without TMZ for 7 days. After incubation, a water-soluble tetrazolium salt (WST)-based assay was performed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction. Optical densities were measured at 450 nm in a microplate reader ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan).

2.3. Methylation-specific PCR

DNA methylation patterns in the promoter region of the *MGMT* gene were analyzed by methylation-specific PCR as previously described [24, 25] with minor modifications. Briefly, genomic DNA was isolated from each cell line using QIAamp DNA Mini kit (QIAGEN, Valencia, CA, USA). Bisulfite conversion was then performed using EpiTect Bisulfite kit (QIAGEN). Following PCR was done using ExTaq HS (TaKaRa Bio, Ohtsu, Japan). The sequences of used primers were: for methylated template

5’-TTTCGACGTTCGTAGGTTTTCGC-3’ (forward),

5’-GCACTCTTCCGAAAACGAAACG -3’ (reverse); for unmethylated template,

5’-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3’ (forward),

5’-AACTCCACACTCTTCCAAAAACAAAACA-3’ (reverse). The thermal profile was as follows: 95ºC for three minutes; 35 cycles of 94ºC for 30 seconds, 59ºC for 30 seconds and 72ºC for 10 seconds; and then stored at 4ºC. DNA from U138MG was treated with SssI methyltransferase (New England Biolabs, Beverly, MA, USA) and used as a methylated control. DNA from U251MG was amplified by GenomiPhi V2 kit (GE Healthcare Bio-sciences, Piscataway, NJ, USA) and used as an unmethylated control. PCR product was loaded onto a 15% polyacrylamide gel, stained with ethidium bromide and visualized under ultraviolet illumination with a BioDoc-It (UVP, Cambridge, UK).

2.4. miRNA microarray and cDNA microarray

Total RNAs were extracted from U251MG and U251 TMZ-resistant (U251R) cells using mirVana miRNA Isolation kit (Applied Biosystems/Ambion, Austin, TX, USA). Three hundred nanograms of the total RNA were subjected to Agilent miRNA microarray analysis service (Bio Matrix Research, Nagareyama, Japan). The array contained 556 probes for mature miRNAs. Specificity of this array is excellent and basically is capable of discriminating single base difference. Probes with “1” flag score in both samples were used for data analyses (264

probes). One microgram of the total RNAs was also subjected to Affymetrix GeneChip Human Genome U133 Plus 2.0 microarray analysis service (Bio Matrix Research). The array contained 54,675 probes for mRNAs. Probes with “present call” flag score in both samples were used for data analyses (22,075 probes).

2.5. Real-time reverse transcription-polymerase chain reaction (RT-PCR) for miRNA

The quantitative real-time RT-PCR for miRNA was performed using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA). Briefly, 10 ng of total RNA was reverse transcribed using a specific looped RT primer for each miRNA using a corresponding TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). The following amplification was performed using a corresponding TaqMan MicroRNA Assay Mix, TaqMan Universal PCR Master Mix and No AmpErase UNG (Applied Biosystems) in a Thermal Cycler Dice Real-time System (TaKaRa Bio). The cycle threshold value, which was determined using second derivative, was used to calculate the normalized expression of the indicated miRNAs using Q-Gene software [26].

2.6. miRNA loss of functional analysis using LNA/DNA oligonucleotides

Locked nucleic acid (LNA) and deoxyribonucleic acid (DNA) hybrid (LNA/DNA) oligonucleotides (miR inhibitors) were designed using LNA design web tool, IDT SciTools

(http://test.idtdna.com/analyzer/Applications/lna/) and chemically synthesized by Gene Design Inc. (Osaka, Japan). LNA bases were inserted to increase specificity for the target as previously described [27]. The sequences of the miR inhibitors were as follows: LNA-195: 5'-gCcaaTaTttCTgTgCtgcTa-3', LNA 455-3p: 5'-gTgtaTatGcCcaTggaCtgc-3', LNA-10a*: 5'-tatTcCcCtagaTACgaatTtg-3' and LNA control: 5'-cgTcAgTaTgCgAaTc-3'. Capital letters indicate LNAs and lower-case letters indicate DNAs in the above sequences. Cells ($1.5x10^4$ cells) were plated in each well of 24-well plate, treated with TMZ for 24 hours. Then the cells were transfected with miR inhibitors using Lipofectamine 2000 reagent (Invitrogen). After 96 hours, the cells were detached by trypsinization, and the cell number was counted using a Coulter counter (Beckman Coulter, Fullerton, CA, USA) as the manufacturer's instruction.

2.7. Statistical analysis

Differences between groups were examined for statistical significance using ANOVA followed by Tukey's post test. P-value not exceeding 0.05 was considered statistically significant. Data were analyzed with PRISM version 4 software (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Generation of TMZ-resistant cell lines

First of all, U251MG wild type (U251Wt) cells were exposed to 100 μM TMZ for two weeks to generate TMZ-resistant variant. The majority of the cells died, but a small population survived and propagated. We then selected surviving colonies and established U251MG TMZ-resistant cells (U251R). Their chemosensitivity was evaluated by water-soluble tetrazolium salt (WST)-based assay, and U251R cells displayed apparent resistance to TMZ as compared to U251Wt cells (Fig. 1A). Similarly, we established U87R, M059KR and M059JR cells from U87MG, M059K and M059J cells, respectively (data not shown).

3.2. MGMT *promoter methylation status in U251Wt and U251R cells*

To determine whether *MGMT* expression is responsible for the acquired resistance in U251R cells, methylation status of the *MGMT* promoter was assessed by methylation-specific PCR. As shown in Fig. 1B, the promoter region was fully methylated in both U251Wt and U251R cells. In addition, we also examined *MGMT* mRNA expression by real-time RT-PCR. The *MGMT* expression in both U251Wt and U251R cells was hardly detectable, compared to that in U138 cells (positive control). These data suggest that *MGMT* expression is not involved in the acquisition of TMZ resistance in U251MG cells.

3.3. Distinct miRNA expression pattern in GBM cells

To identify miRNAs specifically deregulated in U251R cells, we performed a comprehensive analysis of miRNA expression in U251Wt and U251R cells using miRNA microarrays. Thirteen miRNAs were overexpressed (>2.0-fold) and two were underexpressed (<0.5-fold) in U251R cells compared to U251Wt cells. Fold changes of representative miRNAs expression are listed in Table 1. To validate the microarray data, we utilized TaqMan real-time RT-PCR assay for miR-455-3p, miR-195 and miR-10a*, the three most up-regulated miRNAs. As shown in Fig. 2, these miRNAs were certainly upregulated in U251R cells. We also investigated expression of those miRNAs in other three established resistant variants: U87R, M059KR and M059JR cells. Similar trend was observed in all tested cells except miR-195 in M059JR cells (Fig. 2), suggesting that up-regulation of those miRNAs is involved in the acquisition of TMZ resistance in GBM cells.

3.4. Loss of functional analysis using LNA/DNA oligonucleotides

To determine the biological function of those up-regulated miRNAs, we knocked down each miRNA using specific miR inhibitors (LNA/DNA hybrid oligonucleotides, see Materials and Methods section) and investigated cell killing effect in U251R cells. Knockdown of miR-455-3p or miR-10a* did not show difference from LNA control (transfection control) or

TMZ treatment alone; however, combined treatment with both LNA-455-3p or LNA-10a* and TMZ showed modest cell killing effect (Fig. 3A). On the other hand, miR-195 knockdown alone showed moderate growth inhibition, and, moreover, combination with TMZ strongly enhanced cell death (Fig. 3A).

Since miR-195 up-regulation in M059JR cells was not clear (Fig. 2), we also performed knockdown experiments in M059JR cells. As shown in Fig. 3B, knockdown of miR-455-3p and miR-10a* showed similar results. However, knockdown of miR-195 alone strongly induced cell killing effect, and combination with TMZ did not make significant differences (Fig. 3B) (although there was a tendency of enhancement). These data suggest that miR-195 is not involved in the acquisition of TMZ resistant in M059J cells. Taken together, inhibition of those miRNAs, at least in part, successfully reversed TMZ resistance in GBM cells.

3.5. In silico *identification of possible targets and cDNA microarray analysis*

To explore the mechanisms by which those miRNAs regulate TMZ resistance, three web-based databases, miRanda [28], TargetScan [29] and PicTar [30], were used to find possible target mRNAs of those miRNAs. Regarding targets of miR-195, 962 genes in miRanda, 967 genes in TargetScan and 746 genes in PicTar are predicted. Among the three databases, 62 genes are commonly shared. Correspondingly, 47 genes are shared as targets of miR-455-3p in miRanda and TargetScan (but there is no data in PicTar). Regarding miR-10a*,

only miRanda shows predicted target genes. Of the 796 possible targets, we selected the top 100 scored genes (Fig. 4).

miRNAs were initially proposed to mediate translational repression of their target mRNAs. However, it has been recently demonstrated that this is often accompanied by decrease in mRNA abundance itself [31, 32]. Therefore, transcriptional profiling is a useful means to identify potential miRNA targets. We therefore performed a cDNA microarray analysis using total RNA extracted from U251Wt and U251R cells. A comprehensive analysis of this microarray data will be reported elsewhere. Here we attempted to identify target genes which were actually down-regulated. Among possible targets selected using the above-mentioned databases, genes that were down-regulated (<0.8-fold) in the cDNA microarray data are shown in Table 2. Ten genes for miR-195, 15 genes for miR-455-3p and 15 genes for miR-10a* were selected. We further performed a literature search using PubMed and Online Mendelian Inheritance in Man (OMIM) and selected promising candidate targets (Table 3).

4. Discussion

TMZ has improved the prognosis of GBM patients [3, 5]; however, almost all of them die of recurrence after chemoradiotherapy. We first assessed whether the TMZ resistance in U251R

cells depends on epigenetic silencing of the *MGMT* promoter, the most well-known drug resistant mechanism in GBM. In our data, methylation status of the promoter region was not changed (fully methylated in both U251Wt and U251R cells), suggesting that MGMT expression is not involved in the acquired TMZ resistance in GBM. This is consistent with a previous report by Auger et al. [33] in which SNB-19 glioma cells and their resistant variants were used. Other mechanism may be involved in the step of obtaining the resistance.

In the present study, miR-455-3p, miR-195 and miR-10a* were up-regulated in U251R cells compared to parental U251Wt cells. There seems to be only one published paper about miR-455-3p. Guled et al. [34] showed that miR-455-3p was up-regulated in smoker group of malignant mesothelioma patients compared to non-smoker group. Its function, however, remains to be further examined. Regarding miR-10*, there is no report at the present time.

Compared to the two above-mentioned miRNAs, a greater number of reports about miR-195 have been published so far. miR-195 was reported to be up-regulated in cardiac hypertrophy, and transgenic mice overexpressing miR-195 resulted in cardiac growth with disorganization of cardiomyocytes [35]. Ji et al. [36] reported that miR-195 was highly expressed in rat carotid artery and down-regulated in the process of wound healing caused by balloon injury (see its supplemental data). The *Neurogenin3* transcript is targeted by miR-195, which

contributes to islet cell regeneration in pancreas [37]. These data indicate that miR-195 plays a variety of roles in different diseases. In oncology reports, miR-195 has been considered as tumor suppressor and/or inhibitor of cell proliferation. miR-195 is down-regulated in tongue squamous cell carcinoma [38], primary peritoneal carcinoma [39], bladder cancer [40], gastric cancer [41], and hepatocellular carcinoma cells [42]. However, in our present study, inhibition of miR-195 itself significantly decreased the cell number, suggesting that the function of this miRNA might depend on the type of cancer cells. In addition, miR-195 has been reported to drive cell cycle progression in embryonic stem cells through WEE1 [43]. The function of WEE1 is further discussed below. Since drug resistance may be involved in cancer stem cells which have become a very important concept in glioma field, miR-195 might facilitate growth of cancer stem cells. However, to our knowledge, there is no paper addressing the relationship between miR-195 and drug resistance in cancer cells.

Since the recent evidences have suggested that mRNA abundance is also affected by miRNA [31, 32], we combined *in silico* analysis of predicted targets of those miRNAs with cDNA microarray data to select the possible target genes (Table 3). Among these candidates, three genes (*SIAH1*, *WEE1* and *RANBP3*), which are possible targets of miR-195, seem to be promising. SIAH1, an E3 ubiquitin ligase, was underexpressed in breast cancer compared to normal tissue, and low level of its expression correlated with decreased probability of

disease-free survival [44]. *SIAH1* is a p53 target gene and contributes to p53-mediated cell cycle arrest through β-catenin pathway [45]. Moreover, SIAH1 down-regulates MDR/P-glycoprotein expression and sensitizes the multidrug-resistant cells to chemotherapeutic agents [46]. Hara et al. [47] demonstrated that SIAH1 mediates apoptotic cell death by its binding to GAPDH. WEE1, a cell cycle regulator, inhibits G_2/M transition and blocks cell division. Knockdown of WEE1 promotes G2/M progression and sensitizes stathmin-overexpressing breast cancer cells to paclitaxel and vinblastine [48]. RANBP3 ablation causes overactivation of Wnt signaling pathway, and the chromosome region 19p13.3 containing the *RANBP3* gene is deleted in various types of cancers and may contain tumor suppressor gene [49]. Three promising genes (*LTBR*, *EI24* and *SMAD2*) were identified as targets of miR-455-3p. *LTBR*, a proapoptotic gene, was down-regulated in busulfan-resistant human myeloid leukemia cells compared to sensitive cells [50]. *EI24*, a p53-target gene, was lost in invasive breast cancers, and knockdown of EI24 conferred resistance to etoposide treatment in breast cancer cells [51]. SMAD2 expression is lower in glioma cells than in normal astrocytes [52]. Similarly, two genes (*EPHX1* and *BRD7*) are promising targets of miR-10a*. *EPHX1* expression was higher in BCNU-resistant L1210 leukemia cells than in sensitive cells. BRD7, a putative tumor suppressor, interacted with BLOS2 and selectively suppressed transcription of *E2F3* and *cyclin A*.

Since it has been demonstrated that each miRNA can have hundreds of mRNA targets, the mechanism of acquisition of the resistance might be the consequence of deregulation of several hundreds of mRNAs/proteins even though the change in each expression is less prominent. Presumably, such an integral change in mRNA/protein may cooperate to create a TMZ-resistant phenotype in our setting. In four established resistant variants, those miRNAs were consistently up-regulated (except miR-195 in M059JR cells), suggesting that this common mechanism may be shared, at least in part, in GBM cells. Importantly, in the present study, our miR inhibitors (LNA/DNA hybrid oligonucleotides) successfully reversed TMZ resistance in U251R cells, likely due to change of many target gene expressions including the above mentioned. Further study is needed to identify definite target gene sets.

In conclusion, our results demonstrate that miR-195, miR-455-3p and miR-10a* , at least in part, may play a role in acquired TMZ resistance in GBM cells, and miR inhibitors against these miRNAs can reverse the resistance. Corresponding miR inhibitors could be a novel therapeutic approach to recurrent GBM after treatment with TMZ.

Acknowledgements

We thank Hiroko Kawasaki and Kazuto Miyazaki, students of Nagasaki University School of Medicine, for their contributions to the analysis of *MGMT* expression. This work was supported in part by Global COE Program and in part by Grant-in-Aid for Scientific Research (#21890192) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We have nothing to disclose in terms of financial support or relationships that may pose a conflict of interest.

References

[1] H. Ohgaki, P. Kleihues, Epidemiology and etiology of gliomas, Acta Neuropathol 109 (2005) 93-108.

[2] J. Plowman, W.R. Waud, A.D. Koutsoukos, L.V. Rubinstein, T.D. Moore, M.R. Grever, Preclinical antitumor activity of temozolomide in mice: efficacy against human brain tumor xenografts and synergism with 1,3-bis(2-chloroethyl)-1-nitrosourea, Cancer Res. 54 (1994) 3793-3799.

[3] R. Stupp, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R.O. Mirimanoff, Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, N. Engl. J. Med. 352 (2005) 987-996.

[4] R. Stupp, M.E. Hegi, M.R. Gilbert, A. Chakravarti, Chemoradiotherapy in malignant glioma: standard of care and future directions, J. Clin. Oncol. 25 (2007) 4127-4136.

[5] M.E. Hegi, A.C. Diserens, T. Gorlia, M.F. Hamou, N. de Tribolet, M. Weller, J.M. Kros, J.A. Hainfellner, W. Mason, L. Mariani, J.E. Bromberg, P. Hau, R.O. Mirimanoff, J.G. Cairncross, R.C. Janzer, R. Stupp, MGMT gene silencing and benefit from temozolomide in glioblastoma, N. Engl. J. Med. 352 (2005) 997-1003.

[6] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281-297.

[7] V. Ambros, MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing, Cell 113 (2003) 673-676.

[8] A. Cimmino, G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, S.E. Wojcik, R.I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C.G. Liu, T.J. Kipps, M. Negrini, C.M. Croce, miR-15 and miR-16 induce apoptosis by targeting BCL2, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 13944-13949.

[9] D. Bonci, V. Coppola, M. Musumeci, A. Addario, R. Giuffrida, L. Memeo, L. D'Urso, A. Pagliuca, M. Biffoni, C. Labbaye, M. Bartucci, G. Muto, C. Peschle, R. De Maria, The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities, Nat. Med. 14 (2008) 1271-1277.

[10] A. Bottoni, D. Piccin, F. Tagliati, A. Luchin, M.C. Zatelli, E.C. degli Uberti, miR-15a and miR-16-1 down-regulation in pituitary adenomas, J. Cell. Physiol. 204 (2005) 280-285.

[11] G.A. Calin, C.D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich, C.M. Croce, Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15524-15529.

[12] J. Takamizawa, H. Konishi, K. Yanagisawa, S. Tomida, H. Osada, H. Endoh, T. Harano, Y. Yatabe, M. Nagino, Y. Nimura, T. Mitsudomi, T. Takahashi, Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival, Cancer Res. 64 (2004) 3753-3756.

[13] S.M. Johnson, H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K.L. Reinert, D. Brown, F.J. Slack, RAS is regulated by the let-7 microRNA family, Cell 120 (2005) 635-647.

[14] Y. Hayashita, H. Osada, Y. Tatematsu, H. Yamada, K. Yanagisawa, S. Tomida, Y. Yatabe, K. Kawahara, Y. Sekido, T. Takahashi, A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation, Cancer Res. 65 (2005) 9628-9632.

[15] L. He, J.M. Thomson, M.T. Hemann, E. Hernando-Monge, D. Mu, S. Goodson, S. Powers, C. Cordon-Cardo, S.W. Lowe, G.J. Hannon, S.M. Hammond, A microRNA polycistron as a potential human oncogene, Nature 435 (2005) 828-833.

[16] S. Takakura, N. Mitsutake, M. Nakashima, H. Namba, V.A. Saenko, T.I. Rogounovitch, Y. Nakazawa, T. Hayashi, A. Ohtsuru, S. Yamashita, Oncogenic role of miR-17-92 cluster in anaplastic thyroid cancer cells, Cancer Sci 99 (2008) 1147-1154.

[17] J.A. Chan, A.M. Krichevsky, K.S. Kosik, MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells, Cancer Res. 65 (2005) 6029-6033.

[18] A.M. Krichevsky, G. Gabriely, miR-21: a small multi-faceted RNA, J Cell Mol Med 13 (2009) 39-53.

[19] J.K. Gillies, I.A. Lorimer, Regulation of p27Kip1 by miRNA 221/222 in glioblastoma, Cell Cycle 6 (2007) 2005-2009.

[20] W.J. Lukiw, J.G. Cui, Y.Y. Li, F. Culicchia, Up-regulation of micro-RNA-221 (miRNA-221; chr Xp11.3) and caspase-3 accompanies down-regulation of the survivin-1 homolog BIRC1 (NAIP) in glioblastoma multiforme (GBM), J. Neurooncol. 91 (2009) 27-32.

[21] R. Medina, S.K. Zaidi, C.G. Liu, J.L. Stein, A.J. van Wijnen, C.M. Croce, G.S. Stein, MicroRNAs 221 and 222 bypass quiescence and compromise cell survival, Cancer Res. 68 (2008) 2773-2780.

[22] J. Godlewski, M.O. Nowicki, A. Bronisz, S. Williams, A. Otsuki, G. Nuovo, A. Raychaudhury, H.B. Newton, E.A. Chiocca, S. Lawler, Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal, Cancer Res. 68 (2008) 9125-9130.

[23] P.E. Blower, J.S. Verducci, S. Lin, J. Zhou, J.H. Chung, Z. Dai, C.G. Liu, W. Reinhold, P.L. Lorenzi, E.P. Kaldjian, C.M. Croce, J.N. Weinstein, W. Sadee, MicroRNA expression profiles for the NCI-60 cancer cell panel, Mol Cancer Ther 6 (2007) 1483-1491.

[24] M. Esteller, S.R. Hamilton, P.C. Burger, S.B. Baylin, J.G. Herman, Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia, Cancer Res. 59 (1999) 793-797.

[25] N. Umetani, M.F. de Maat, T. Mori, H. Takeuchi, D.S. Hoon, Synthesis of universal unmethylated control DNA by nested whole genome amplification with phi29 DNA polymerase, Biochem. Biophys. Res. Commun. 329 (2005) 219-223.

[26] P.Y. Muller, H. Janovjak, A.R. Miserez, Z. Dobbie, Processing of gene expression data generated by quantitative real-time RT-PCR, Biotechniques 32 (2002) 1372-1374, 1376, 1378-1379.

[27] S. Karkare, D. Bhatnagar, Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino, Appl. Microbiol. Biotechnol. 71 (2006) 575-586.

[28] D. Betel, M. Wilson, A. Gabow, D.S. Marks, C. Sander, The microRNA.org resource: targets and expression, Nucleic Acids Res 36 (2008) D149-153.

[29] B.P. Lewis, C.B. Burge, D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, Cell 120 (2005) 15-20.

[30] A. Krek, D. Grun, M.N. Poy, R. Wolf, L. Rosenberg, E.J. Epstein, P. MacMenamin, I. da Piedade, K.C. Gunsalus, M. Stoffel, N. Rajewsky, Combinatorial microRNA target

predictions, Nat. Genet. 37 (2005) 495-500.

[31] D. Baek, J. Villen, C. Shin, F.D. Camargo, S.P. Gygi, D.P. Bartel, The impact of microRNAs on protein output, Nature 455 (2008) 64-71.

[32] M. Selbach, B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin, N. Rajewsky, Widespread changes in protein synthesis induced by microRNAs, Nature 455 (2008) 58-63.

[33] N. Auger, J. Thillet, K. Wanherdrick, A. Idbaih, M.E. Legrier, B. Dutrillaux, M. Sanson, M.F. Poupon, Genetic alterations associated with acquired temozolomide resistance in SNB-19, a human glioma cell line, Mol Cancer Ther 5 (2006) 2182-2192.

[34] M. Guled, L. Lahti, P.M. Lindholm, K. Salmenkivi, I. Bagwan, A.G. Nicholson, S. Knuutila, CDKN2A, NF2, and JUN are dysregulated among other genes by miRNAs in malignant mesothelioma -A miRNA microarray analysis, Genes Chromosomes Cancer 48 (2009) 615-623.

[35] E. van Rooij, L.B. Sutherland, N. Liu, A.H. Williams, J. McAnally, R.D. Gerard, J.A. Richardson, E.N. Olson, A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 18255-18260.

[36] R. Ji, Y. Cheng, J. Yue, J. Yang, X. Liu, H. Chen, D.B. Dean, C. Zhang, MicroRNA expression signature and antisense-mediated depletion reveal an essential role of

MicroRNA in vascular neointimal lesion formation, Circ. Res. 100 (2007) 1579-1588.

[37] M.V. Joglekar, V.S. Parekh, S. Mehta, R.R. Bhonde, A.A. Hardikar, MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3, Dev. Biol. 311 (2007) 603-612.

[38] T.S. Wong, X.B. Liu, B.Y. Wong, R.W. Ng, A.P. Yuen, W.I. Wei, Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue, Clin. Cancer Res. 14 (2008) 2588-2592.

[39] R.J. Flavin, P.C. Smyth, A. Laios, S.A. O'Toole, C. Barrett, S.P. Finn, S. Russell, M. Ring, K.M. Denning, J. Li, S.T. Aherne, D.A. Sammarae, N.A. Aziz, A. Alhadi, B.L. Sheppard, K. Lao, O.M. Sheils, J.J. O'Leary, Potentially important microRNA cluster on chromosome 17p13.1 in primary peritoneal carcinoma, Mod. Pathol. 22 (2009) 197-205.

[40] T. Ichimi, H. Enokida, Y. Okuno, R. Kunimoto, T. Chiyomaru, K. Kawamoto, K. Kawahara, K. Toki, K. Kawakami, K. Nishiyama, G. Tsujimoto, M. Nakagawa, N. Seki, Identification of novel microRNA targets based on microRNA signatures in bladder cancer, Int. J. Cancer 125 (2009) 345-352.

[41] J. Guo, Y. Miao, B. Xiao, R. Huan, Z. Jiang, D. Meng, Y. Wang, Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues, J. Gastroenterol. Hepatol. 24 (2009) 652-657.

[42] T. Xu, Y. Zhu, Y. Xiong, Y.Y. Ge, J.P. Yun, S.M. Zhuang, MicroRNA-195 suppresses tumorigenicity and regulates G(1)/S transition of human hepatocellular carcinoma cells, Hepatology (2009).

[43] J. Qi, J.Y. Yu, H.R. Shcherbata, J. Mathieu, A.J. Wang, S. Seal, W. Zhou, B.M. Stadler, D. Bourgin, L. Wang, A. Nelson, C. Ware, C. Raymond, L.P. Lim, J. Magnus, I. Ivanovska, R. Diaz, A. Ball, M.A. Cleary, H. Ruohola-Baker, microRNAs regulate human embryonic stem cell division, Cell Cycle 8 (2009) 3729-3741.

[44] S. Confalonieri, M. Quarto, G. Goisis, P. Nuciforo, M. Donzelli, G. Jodice, G. Pelosi, G. Viale, S. Pece, P.P. Di Fiore, Alterations of ubiquitin ligases in human cancer and their association with the natural history of the tumor, Oncogene (2009).

[45] S.I. Matsuzawa, J.C. Reed, Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses, Mol. Cell 7 (2001) 915-926.

[46] M. Liu, R. Aneja, H. Wang, L. Sun, X. Dong, L. Huo, H. Joshi, J. Zhou, Modulation of multidrug resistance in cancer cells by the E3 ubiquitin ligase seven-in-absentia homologue 1, J. Pathol. 214 (2008) 508-514.

[47] M.R. Hara, N. Agrawal, S.F. Kim, M.B. Cascio, M. Fujimuro, Y. Ozeki, M. Takahashi, J.H. Cheah, S.K. Tankou, L.D. Hester, C.D. Ferris, S.D. Hayward, S.H. Snyder, A. Sawa, S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding, Nat Cell Biol 7 (2005) 665-674.

[48] E. Alli, J.M. Yang, J.M. Ford, W.N. Hait, Reversal of stathmin-mediated resistance to paclitaxel and vinblastine in human breast carcinoma cells, Mol. Pharmacol. 71 (2007) 1233-1240.

[49] J. Hendriksen, F. Fagotto, H. van der Velde, M. van Schie, J. Noordermeer, M. Fornerod, RanBP3 enhances nuclear export of active (beta)-catenin independently of CRM1, J. Cell Biol. 171 (2005) 785-797.

[50] B.C. Valdez, D. Murray, L. Ramdas, M. de Lima, R. Jones, S. Kornblau, D. Betancourt, Y. Li, R.E. Champlin, B.S. Andersson, Altered gene expression in busulfan-resistant human myeloid leukemia, Leuk. Res. 32 (2008) 1684-1697.

[51] C.N. Mork, D.V. Faller, R.A. Spanjaard, Loss of putative tumor suppressor EI24/PIG8 confers resistance to etoposide, FEBS Lett. 581 (2007) 5440-5444.

[52] L. Zhang, E. Sato, K. Amagasaki, A. Nakao, H. Naganuma, Participation of an abnormality in the transforming growth factor-beta signaling pathway in resistance of malignant glioma cells to growth inhibition induced by that factor, J. Neurosurg. 105 (2006) 119-128.

Table 1. Differentially expressed miRNAs between U251Wt and U251R cells

miRNA	Fold change (R/Wt)
hsa-miR-455-3p	5.477
hsa-miR-195	4.281
hsa-miR-10a*	3.597
hsa-miR-502-3p	3.564
hsa-miR-193b*	3.447
hsa-miR-584	3.253
hcmv-miR-US25-2-5p	3.078
hsa-miR-500*	2.906
hsa-miR-193a-5p	2.824
hsa-miR-452	2.755
hsa-miR-132	2.334
hsa-miR-503	2.062
hsa-miR-106b*	0.3765
hsa-miR-210	0.3715

Table 2A. Predicted targets of miR-455-3p and their expressions in cDNA microarray

Gene symbol	Gene description	Probe set	Fold change
LHX2	LIM homeobox 2	206140_at	0.503
ALS2CR8	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 8	219834_at	0.523
DIP2A	DIP2 disco-interacting protein 2 homolog A (Drosophila)	1561286_a_at	0.561
DYNLL2	dynein, light chain, LC8-type 2	229106_at	0.675
C6orf145	chromosome 6 open reading frame 145	212923_s_at	0.697
FBXL15	F-box and leucine-rich repeat protein 15	218938_at	0.699
LTBR	lymphotoxin beta receptor (TNFR superfamily, member 3)	203005_at	0.715
ASB1	ankyrin repeat and SOCS box-containing 1	212819_at	0.721
PNPLA6	patatin-like phospholipase domain containing 6	203718_at	0.736
RTN4	reticulon 4	214629_x_at	0.741
RUSC1	RUN and SH3 domain containing 1	206949_s_at	0.7592
EI24	etoposide induced 2.4 mRNA	216396_s_at	0.763
HSF1	heat shock transcription factor 1	202344_at	0.775
SMAD2	SMAD family member 2	226563_at	0.776
GNL1	guanine nucleotide binding protein-like 1	203307_at	0.779

Table 2B. Predicted targets of miR-195 and their expressions in cDNA microarray

Gene symbol	Gene description	Probe set	Fold change
PPP2R1A	Protein phosphatase 2 (formerly 2A), regulatory subunit A , alpha isoform	200695_at	0.431
AP2A1	adaptor-related protein complex 2, alpha 1 subunit	234068_s_at	0.496
SIAH1	seven in absentia homolog 1 (Drosophila)	202980_s_at	0.589
HAS2	hyaluronan synthase 2	206432_at	0.640
ALS2CR2	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 2	223266_at	0.657
CCNE1	cyclin E1	213523_at	0.657
SESN1	sestrin 1	218346_s_at	0.686
WEE1	WEE1 homolog (S. pombe)	215711_s_at	0.735
RANBP3	RAN binding protein 3	202640_s_at	0.756
VAT1	vesicle amine transport protein 1 homolog (T. californica)	208626_s_at	0.768

Table 2C. Predicted targets of miR-10a* and their expressions in cDNA microarray

Gene symbol	Gene description	Probe set	Fold change
CCL2	chemokine (C-C motif) ligand 2	216598_s_at	0.214
SULT1A3	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3	233334_x_at	0.298
EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	202017_at	0.429
CDK5	cyclin-dependent kinase 5	204247_s_at	0.476
GRTP1	growth hormone regulated TBC protein 1	229377_at	0.510
BRD7	bromodomain containing 7	221776_s_at	0.556
PDIA5	protein disulfide isomerase family A, member 5	203857_s_at	0.579
IDS	iduronate 2-sulfatase (Hunter syndrome)	212223_at	0.610
PSPC1	paraspeckle component 1	226574_at	0.613
RWDD2B	RWD domain containing 2B	218377_s_at	0.632
COL6A1	collagen, type VI, alpha 1	212091_s_at	0.711
GIYD2	GIY-YIG domain containing 2	218317_x_at	0.725
HSPA1B	heat shock 70kDa protein 1B	202581_at	0.752
GRM6	glutamate receptor, metabotropic 6	208035_at	0.763
MRPL19	Mitochondrial ribosomal protein L19	232071_at	0.784

Table 3. Potential TMZ resistance-related candidates of target genes

Gene symbol	Function	Reference
SIAH1	SIAH1 is a significant prognostic factor in breast cancer.	Confalonieri S, 2009.
	SIAH1 down-regulates MDR1/P-glycoprotein expression through the JNK pathway.	Liu M, 2008.
	SIAH1-GAPDH mediates apoptotic signal	Hara MR, 2005.
WEE1	WEE1 sensitizes resistant breast cancer cells to paclitaxel and vinblastine.	Alli E, 2007
	WEE1 blocks cell division when overexpressed in HeLa cells.	McGowan, 1993.
RANBP3	RANBP3 ablation causes overactivation of Wnt signaling.	Hendriksen, 2005.
LTBR	proapoptotic gene, down-regulated in busulfan-resistant human myeloid leukemia.	Valdez BC, 2008.
EI24	Loss of EI24 confers etoposide resistance in invasive breast cancer.	Mork CN, 2007
SMAD2	down-regulated in malignant glioma cells.	Zhang L, 2006.
EPHX1	EPHX1 is overexpressed in BCNU-resistant leukemia cells compared to sensitive cells.	Ribrag V, 1994.
BRD7	Putative tumor suppressor, negatively regulates cell proliferation.	Sun J, 2008.

Figure legends

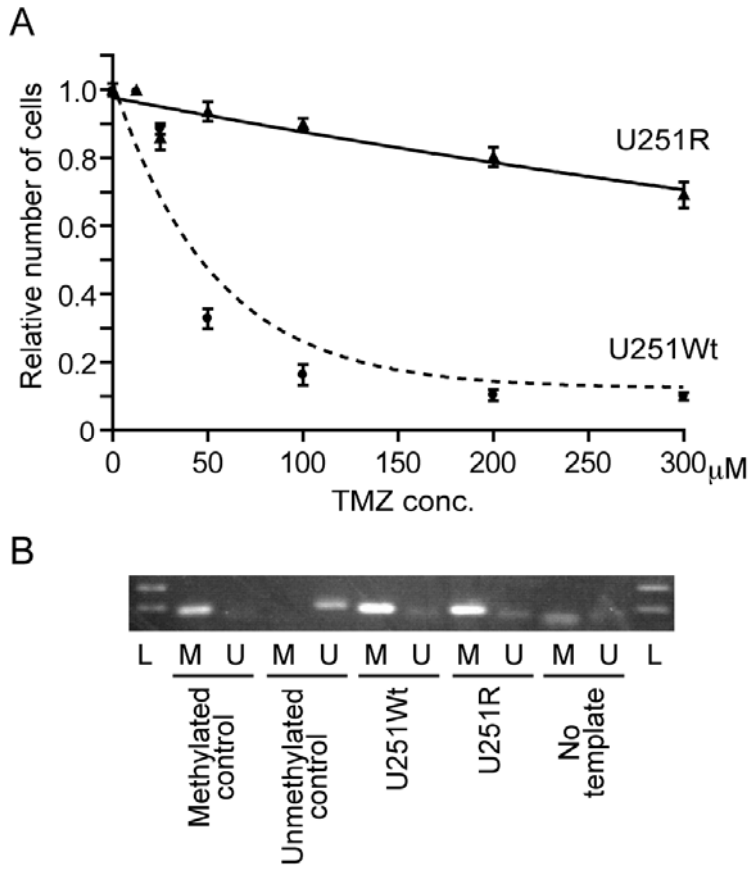

Fig. 1. A, Establishment of TMZ resistant variant: U251R cells. U251R cells were generated from U251MG cells by continuous exposure to TMZ. Indicated cells were treated with indicated concentrations of TMZ for 7 days, and then the relative number of cells was determined using WST-based assay. Each point indicates the mean and standard error of six wells. Similar results were obtained in two independent experiments. B, Methylation status of the *MGMT* promoter in U251Wt and U251R cells. Genomic DNA was extracted and analyzed by methylation-specific PCR. Methylated control, unmethylated control and template-free negative control were also included. The lower bands in the negative control lanes were probably due to primer dimer. L: DNA maker ladder, M: methylated, U: unmethylated.

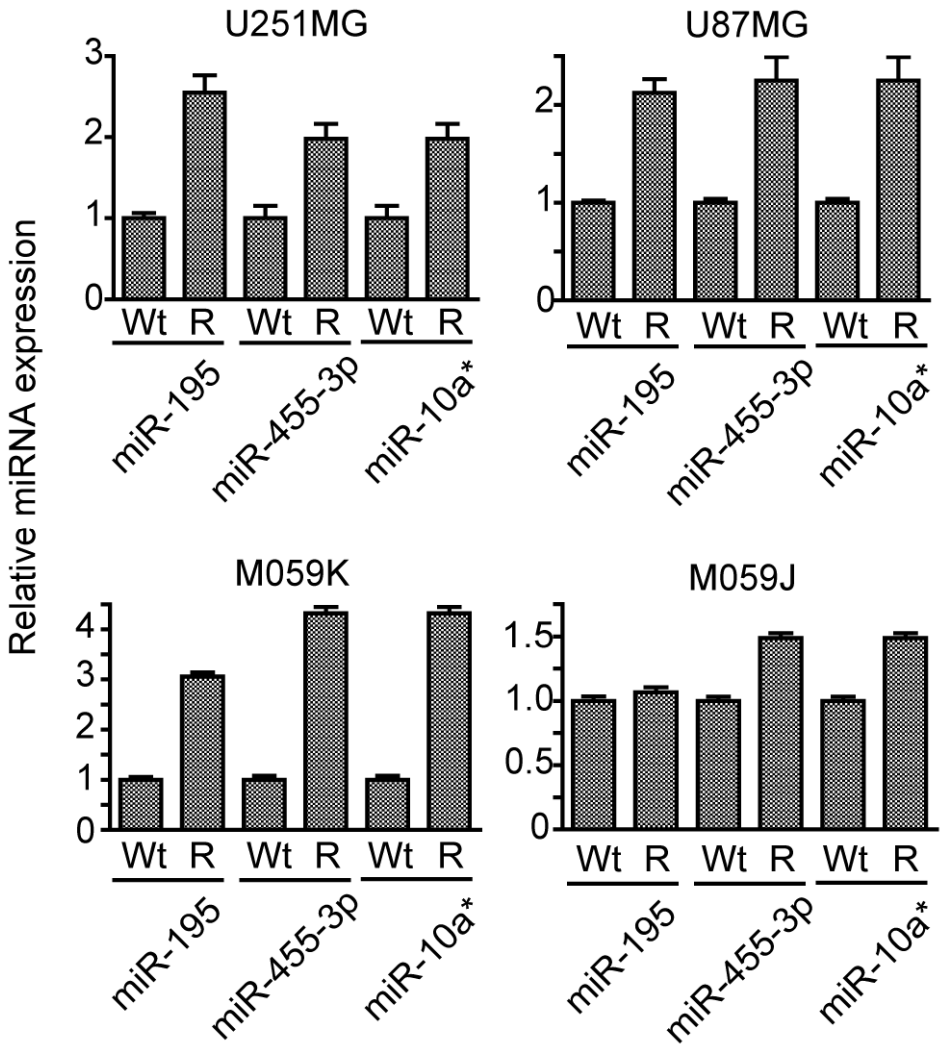

Fig. 2. TaqMan real-time quantitative RT-PCR for miRNAs. In each cell line, the expression level of indicated miRNA was compared between parental (Wt) cells and established resistant variant (R) as described in Materials and Methods section. RNU6B was used as an internal control. Each bar indicates the mean and standard error of the data collected in triplicate. Similar results were obtained in at least two independent experiments.

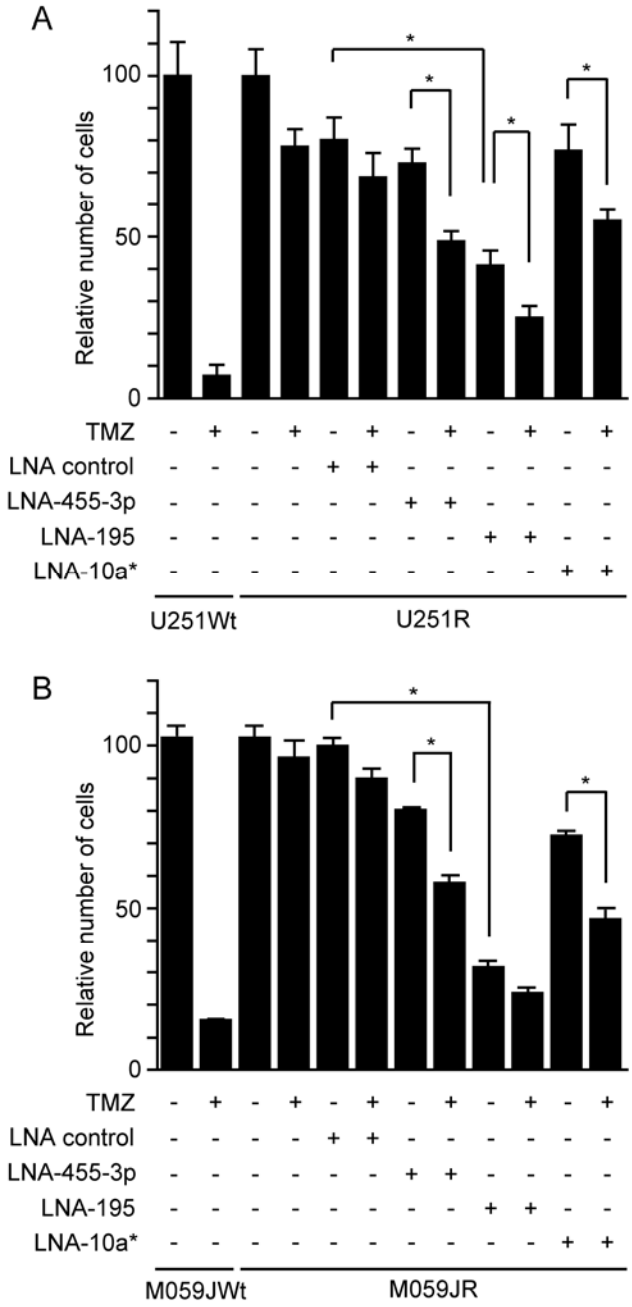

Fig. 3. Effect of miR inhibitors on TMZ resistance. Indicated cells were treated with or without 100 μM (A, for U251 cells) or 25 μM (B, for M059J cells) TMZ for 24 h and then transfected with indicated miR inhibitors. The cells were further cultured with or without TMZ for 96 h, and the number of cells was counted. Each bar represents the mean and standard error of four wells. Similar results were obtained in three independent experiments. *$p<0.05$ between two groups.

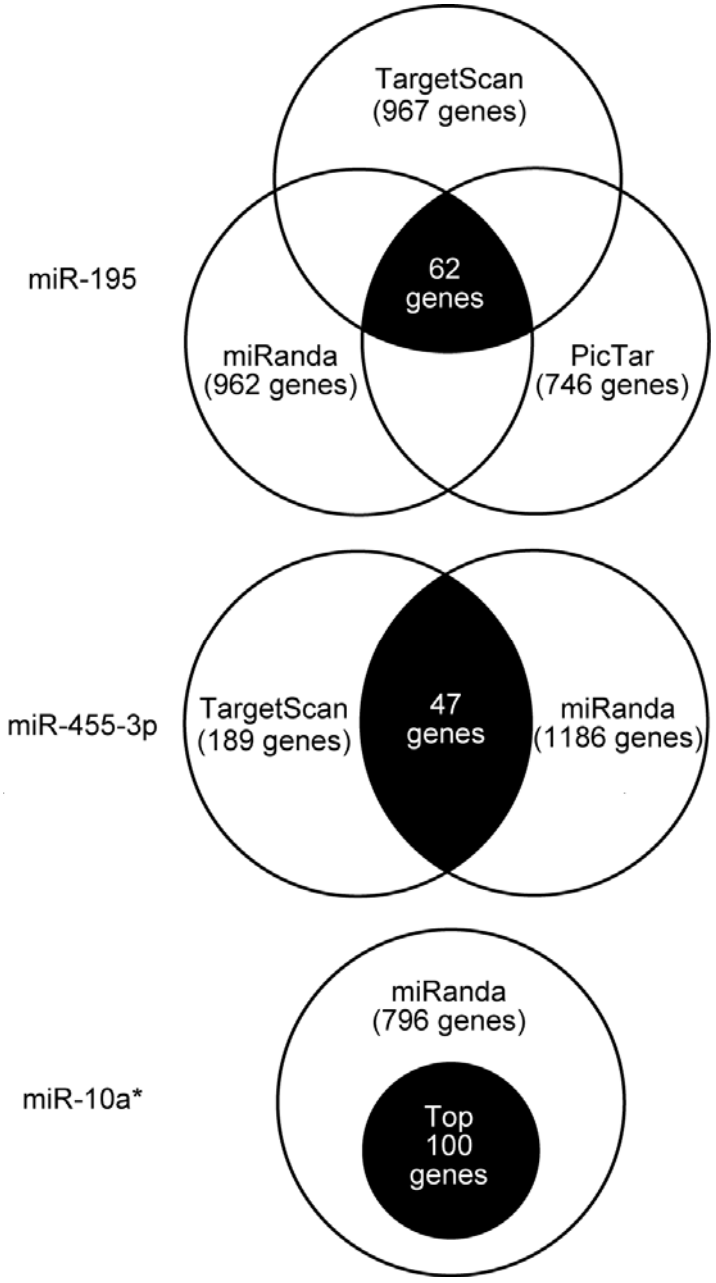

Fig. 4. Possible target mRNAs of each miRNA. Venn diagrams of the number of predicted target mRNAs in indicated web-based database are shown.