development of HCC, especially in patients with an SVR to the therapy.

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Chernobyl Thyroid Cancer 25 years after: in search of a molecular radiation signature

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ABSTRACT

Chernobyl accident, the worst technogenic catastrophe involving massive radiation release into the environment, will soon reach the 25th anniversary. Its major internationally recognized health consequence is thyroid cancer among the individuals affected by radioiodines at early ages. The largest in the world and unique series of radiation-induced thyroid malignancies has been a subject of investigations in many different aspects of sciences for decades. Here we review the results of investigations aimed at the elucidation of the "radiation signature", a molecular classifier that could help discriminating between radiation-induced and sporadic tumors. The attempts to determine such employ a large variety of techniques, including measurements of DNA copy number variation on microarrays, differential gene expression profiling, proteomics, immunohistochemistry and genotyping of selected target genes or of the whole genome. From the point of view of study design and result interpretation, they could be broadly subdivided into those exploring molecular differences occurring after exposure to different etiological factors (i.e. radiation or other), thus looking for the damage pattern, and the ones seeking the markers of susceptibility to different etiological forms of thyroid cancer. There have been certain advances in both lines of investigations suggestive that establishment of the discriminative molecular signature is plausible. However, studies are far of being accomplished and require further efforts in following-up and investigating the Chernobyl cohort. Possible solutions to create comprehensive molecular concept will likely be integrative approaches combing clinico-pathological and extensive molecular data, and in-depth bioinformatic analyses.

Key-words: Chernobyl accident, thyroid cancer, molecular marker, genomics, gene expression, genetic association study

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Introduction

Ionizing radiation is a well known genotoxic agent that induces a variety of DNA lesions including nucleotide base modifications, abasic sites, strand cross-linking, DNA adducts, and single- and double-strand DNA breaks (DSBs) (1-3). Although all these types of lesions may potentially result in gene mutations, DSBs are considered to be the most significant for chromosomal aberrations, mutagenesis, genetic instability and carcinogenesis (2, 4-7). The multiplicity of DNA damages produced by radiation is thought to be one of the reasons for the diversity in biological consequences of exposure.

Human thyroid is an organ particularly vulnerable to ionizing radiation as was initially seen in the series of patients subjected to external beam therapy of the head and neck area for medical indications who then developed thyroid cancer (8). The Chernobyl accident, which occurred nearly 25 years ago on April 26, 1986, provided evidence of carcinogenic effect of environmental exposure to radioiodine isotopes, especially to ^{131}I. A significant increase in thyroid cancer incidence was documented since early 1990-ies in Belarus, Ukraine and southwestern regions of Russia (9-12) (Fig. 1).

By 2002, the number of thyroid cancer cases registered in the individuals aged less than 18 years at the moment of exposure in the three most affected countries approached to 5,000 (13). Epidemiological studies have established qualitative and quantitative characteristics of causative association of thyroid cancer risk with internal exposure to radioiodine demonstrating that it is comparable to that after external irradiation (11, 14-20).

The outbreak of thyroid cancer in young patients suffered from the radioactive Chernobyl fallouts led to a great number of medical, epidemiological, dosimetric, sociological and laboratory investigations all aimed at evaluation of health impact, short and long-term consequences of the catastrophe for individuals, society and the environment as well as at elucidating the distinctive features of radiation-induced tumors. They resulted in important evidence-based conclusions which may be called lessons from Chernobyl; some of them could be drawn only after decades of observations. Applicably to the thyroid, the most important would be that ingestion of ^{131}I at childhood may later cause thyroid cancer, that period of latency after exposure may be as short as only 4 years, that the use of stable iodine as a dietary supplement or as a thyroid-blocking agent may have a protective effect against cancer. From the molecular and pathological point of view, it has been recognized that radiation excess in thyroid cancer incidence is due to the papillary thyroid carcinoma (PTC) whose morphology and molecular characteristics, such as histological architecture and mutational pattern, appear to be changing with increasing latency or correlate with patient's age (see reff. 20, 22, 23 for extensive reviews). The relative prevalence of *RET/PTC3*, *RET/PTC1* and *BRAF* mutations implicated in molecular carcinogenesis of PTC has been proposed to tentatively parallel the dynamics of thyroid cancer incidence in children, adolescents and adults, respectively, shown in Fig. 1 (20).

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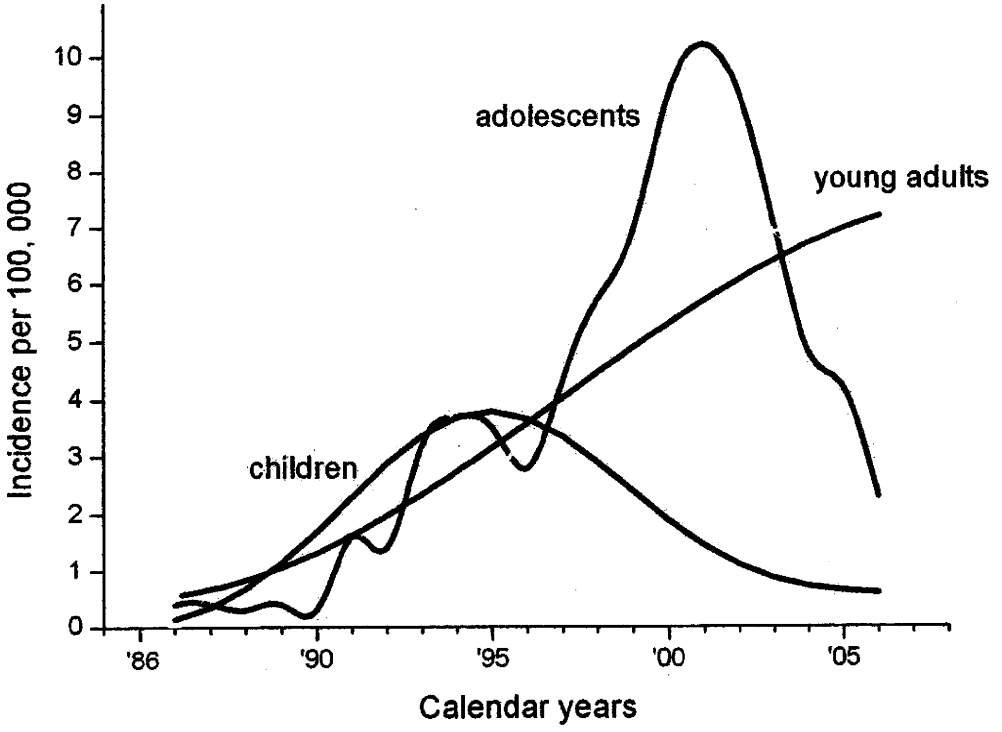

Fig. 1. Incidence of thyroid cancer in Belarus among the residents of radiocontaminated territories by age groups. This graph is inferred from the original one published earlier (21).

Molecular studies in Chernobyl thyroid cancer, depending on design, could be broadly classified into those attempting to determine a "damage signature" or "susceptibility signature" (24-26). The first type of investigations explores frequencies and distribution of various mutations, in a comparative manner, between radiation-induced and sporadic thyroid cancers. Initial works on Chernobyl series were mostly mutational studies. As a whole, they demonstrated that none of oncogenes such as gene rearrangements (*RET/PTC*, *NTRK*, *AKAP9-BRAF*) or point mutations (*BRAF*, *RAS* family genes) could have been identified as radiation-specific.

Studies of the second type investigate if gene expression patterns or genetic factors may modify or serve as markers of inherited predisposition for developing cancer after radiation exposure. They generally require more advanced techniques because of the need to cover a large number of targets, ideally the whole genome. So far, several factors have been established to affect risk for developing thyroid cancer following internal exposure: radiation dose for the thyroid, younger age at exposure and iodine deficiency. Whether or not the genetics particularities of the individuals who developed thyroid cancer after Chernobyl remains largely unknown, but some facts, such as inter-

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patient variations in the clinical course and latency as well as development of cancer only in a small proportion of the exposed victims, may be indicative of such a possibility.

In this review we focus on the works performed to establish molecular classifiers capable of distinguishing radiation-induced Chernobyl cancers form sporadic PTCs. The importance and a need of a classifier is determined by the necessity to improve radiation risk assessment and risk communication, as well as to better manage and justify occupational and medical exposures tending to be expanding in the modern era of nuclear technologies.

Chromosomal imbalances

In an early study, chromosomal imbalances were examined using conventional comparative genomic hybridization (CGH) in a group of 60 Chernobyl childhood and adolescent PTCs (27). About 30% tumors were found to carry copy number variation (CNV). Both DNA gains (chromosomes 2, 7q11.2-21, 13q21-22, 21) and losses (16p/q, 20q, 22q) were found. Interestingly, deletions or loss of heterozygosity (LOH) on chromosomes 22q and 16p/q have been reported previously in PTC, FTC or ATC and associated with an aggressive tumor behavior (28-30). This study did not reveal correlations between the *RET/PTC* status of a tumor and specific DNA imbalance, yet the observation of a deletion at 22q in both *RET/PTC*-positive and *RET/PTC*-negative tumors was suggestive of the existence of alternate routes contributing to carcinogenesis, genetic heterogeneity or oligoclonal tumor development. The latter suggestion is supported by the observation of non-homogenous distribution of *RET/PTC*-harboring nuclei across tumor tissues (31). In a later work of the same group, employing an BAC-based array CGH, it was shown that *RET/PTC*-positive and *RET/PTC*-negative cases could be discriminated by the alteration pattern of chromosomes 1p, 3q, 4p, 7p, 9p/q, 10q, 12q, 13q and 21q (32). Furthermore, there was a significant difference between *RET/PTC*-positive childhood and adult PTCs: deletions on 1p35–36 were more frequent in adult cases. Regardless of *RET/PTC* rearrangement, chromosomal losses were more common than gains. In line with the previous study, the existence of additional, sometimes multiple, DNA alterations in both *RET/PTC*-positive and in *RET/PTC*-negative tumors could be interpreted as pointing at alternative paths of tumor development.

Another CGH study of 23 Chernobyl and 20 sporadic PTCs demonstrated that the overall prevalence of DNA gains was 2-4 higher in exposed patients as compared to non-exposed, and even more frequent (up to 10-fold) for recurrent gains (33). It was possible to determine the alteration pattern that discriminated radiation-related PTCs from sporadic (chromosomes 1p36.32-.33, 2p23.2-.3, 3p21.1-.31, 6p22.1-.2, 7q36.1, 8q24.3, 9q34.11, 9q34.3, 11p15.5, 11q13.2-12.3, 14q32.33, 16p13.3, 16p11.2, 16q21-q12.2, 17q25.1,19p13.31-qter, 22q11.21, 22q13.2) but because of limited sample size and non-uniform distribution of individual thyroid doses in the investigation the assessment of dose-response relationship has proved difficult. It was concluded that CNV, in addition

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to carcinogenesis-related alterations, also depend on radiation exposure and patient's age at exposure.

Using a 50K Mapping array, 10 childhood Chernobyl PTCs were recently analyzed to demonstrate that DNA gains were more consistently observed at chromosome 1p, 5p, 9q, 12q, 13q, 16p, 21q, and 22q, while losses were found at 1q, 6q, 9q, 10q, 13q, 14q, 21q, and 22q (34). CNV amplifications were more frequent than deletions in line with the study by Kimmel et al. (33); no significant LOH was registered. This study is interesting because an overlay analysis was done to evaluate the concordance between CNV and gene expression. As a result, none of genes mapped to deleted regions was found to be downregulated. On the contrary, 87 genes that were amplified on CGH also displayed overexpression. After filtering gene expression profiles in Chernobyl PTCs against those reported previously for sporadic tumors and available from Gene Expression Omnibus, a radiation-related PTC identifier was established that included 113 messages among which 24 were downregulated and 41 were upregulated at least 3-fold. Six genes, *CAMK2N1*, *AK1*, *DHRS3*, *FBXO2*, *ECE1* and *PDE9A* were unique to childhood radiation-induced PTC.

As a whole, the results of CGH analyses performed to date are not yet comprehensive enough to derive a CNV-based radiation signature. Usually the studies deal with small sample size, do not report validation experiments on independent specimens and employ platforms that are quite different in their resolution cumulatively making cross-analysis difficult. They, however, provide insights into the genomic regions, candidate genes and functional pathways involved in radiation-related thyroid carcinogenesis.

Gene expression profiles

Several studies have been undertaken to elucidate characteristic expressiosome features of Chernobyl thyroid cancers. The earliest one analyzed 12 Ukrainian and 8 sporadic PTCs from French patients, and 13 thyroid adenomas using Micromax microarrays with a set of 2400 known human cDNA probes (24). Neither unsupervised nor supervised classification algorithms could distinguish radiation-related from sporadic PTCs, perhaps in part due to the relatively small number of tested genes. However, separation from benign thyroid neoplasia was effective: based on a 36-gene signature a 3% misclassification rate was achieved. The importance of this investigation was in obtaining molecular evidence of similarity between PTCs of different etiology which confirmed previous observations of their morphological resemblance once again proving that radiation-induced and sporadic PTCs are closely related diseases presumably having much in common pathogenetically.

The whole genome study used Human Genome Survey Microarray V2.0 platform that combines >29000 genes (35). Screening was done on pooled RNA samples from 11 Chernobyl patients aged 15-22 years at diagnosis and 41 patients from southeastern Germany aged 15-83 years and the results were confirmed on an RTQ-PCR low-density array for selected genes.

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Microarray analysis detected 646 differentially upregulated and 677 downregulated genes (>5-fold difference) between the groups. Interestingly, the genes predominantly overexpressed in Chernobyl tumors included G-proteins (RAS family genes), growth factors and receptors (*VEGFA*, *EGFL9*, *PDGFC*, *PDGFRB*, *IGF1R*, *IGBP1*) and some of oxidoreductases (cyclooxygenase 2 (*PTGS2*), superoxide dismutase (*SOD1*)) which were associated with tumor aggressiveness and poorer prognosis in previous studies (36-42). Such overexpression was interpreted as supportive to the notion that Chernobyl PTC manifested particularly high aggressiveness with frequent lymph node metastases and extrathyroidal invasion. This work also identified a molecular classifier consisting of 7 genes (*SFRP1, MMP1, ESM1, KRTAP2-1, COL13A1, BAALC* and *PAGE1*) that enabled a confident classification into radiation-related and sporadic PTCs.

One more investigation explored transcriptomes in 12 Chernobyl and 14 French patients using Human 1 cDNA Microarray slides covering 8000 genes (43). Similarly to the previous report from this group (24), unsupervised classification did not provide distinction between the two groups of cancers on a global scale. A supervised analysis, however, using four different algorithms, succeeded to determine classifiers that included from one to several thousands genes (median 256) with overall error rates ranging 12-27%. This study is noteworthy because the effects of possible etiological agents, which are presumably gamma radiation in Chernobyl tumors and hydrogen peroxide in sporadic tumors, were taken into account. Hydrogen peroxide is produced during thyroid hormone synthesis (44) and may play a role in thyroid tumorigenesis (45). Furthermore, it is a potent DNA-damaging substance which produces not only single-strand DNA breaks and base modifications but also double-strand breaks and, as recently shown, is capable to generate *RET/PTC1* rearrangement in a human thyroid cell line (46). Using previously available data (47), the authors found that in a B-lymphocyte cell line treated with 10 different genotoxic agents, *in vitro* gene expression responses to 200 μM of hydrogen peroxide and 2.5 Gy of gamma-rays were the most resembling. There were however 293 genes whose expression levels differed >1.5-fold between the two types of treatment of which, after removing genes related to immune reactions, 118 were present on the arrays used to profile PTCs. These genes were tested as a molecular classifier and, as a result, led to the separation of Chernobyl and sporadic PTC with the error rates 15-27%. In addition, whether the genes whose products are involved in five major DNA repair mechanisms, i.e. base-excision repair, mismatch-excision repair, nucleotide-excision repair, homologous recombination and nonhomologous end joining, may constitute a classifier was explored. Thirteen genes of homologous recombination pathway were found to make a classifier that distinguished radiation-induced and sporadic PTCs with error rates of 15-31%. It was proposed that, given DNA repair is largely accomplished within hours after damage while differential gene expression in the tumors persisted for many years, such profile may be a signature of susceptibility to different etiological forms of thyroid cancer. If these results find further support in independent PTC series, they may well be considered as a piece of evidence suggesting the existence of inherited predisposition to radiation-induced PTC.

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Similarly to the results obtained in CGH studies, gene expression data provide valuable information for the attempts of elucidating molecular radiation signature, but they are not completed yet. So far reported works, being generally encouraging, have been done using relatively small series of cancers and produce the results that do not converge to yield a reliable set of markers. This points at the need to expand the number of analyzed cancers of both etiologies with better matching in terms of clinico-pathological and molecular characteristics to achieve the desired reproducibility and avoid biases.

Proteomic investigation

To date only one proteomic study involving Chernobyl thyroid cancers has been reported to the best of our knowledge. Boltze et al. analyzed protein extracts from 86 Chernobyl and 91 sporadic PTCs from patients of southeastern Germany (48). On 2-D electrophoresis, around 2000 spots were identified on the reference gels and among them 18 candidates upregulated in radiation-induced PTCs were determined. Immunohistochemistry was performed for all these candidates and in addition for two other proteins, potential markers for PTC. The results were evaluated semiquantitatively eventually leaving 6 proteins (NTRK1, MMP-1, MMP-13, MMP-9, Cathepsin W and Cathepsin X) that allowed most efficient separation between the groups. When adjusted for patients' age, NTRK1, MMP-1 and MMP-13 staining resulted in a complete separation of the two etiological groups. Without age adjustment, NRTK1 alone and a combination of either two MMPs or of two Cathepsins also worked well with no false positive and false negative test results. Note that *MMP1* gene upregulation in Chernobyl PTCs was reported previously (35). Interestingly, NTRK1 overexpression in radiation-induced PTCs may indicate structural mutation-independent role of this receptor tyrosine kinase as chromosomal rearrangements involving the *NTRK1* gene are observed in less than 10% of Chernobyl cancers (49).

Whether a relatively simple immunostaining approach can be universally used to discriminate radiation-induced from sporadic PTCs remains to be established. Concerns are related first of all to patients' age (and/or duration of latent period) and associated changes in tumor morphology as well as underlying mutational events all potentially leading to the shifts in the spectrum of expressed proteins. This direction certainly needs further investigation.

Genetic association studies

The purpose of this type of investigations is to determine genetic factors associated with disease thus addressing issue of inherited susceptibility. In general, there are two methodologies of selecting gene polymorphisms, usually SNPs, to be analyzed. The first one, termed candidate gene approach, is based on a hypothesis that genetic variations in one or in a limited number of genes may affect risk for or the phenotype of a given disease. A more comprehensive way is initially hypothesis-free and employs analysis throughout the genome; it is termed genome-wide association study

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(GWAS). While a substantial number of studies has been done in sporadic thyroid cancers, only few explored radiation-induced thyroid malignancies.

Candidate gene approach

In a study by Stephens et al. (50) no evidence for LOH in the *RET* gene was found in 28 of 46 PTCs from Ukraine heterozygous for at least one of three SNPs of interest (G691S, S904S and L769L); this observation is in line with the later microarray findings (33). Investigation of the additional 68 cases demonstrated that the rare S allele of G691S was significantly overrepresented in patients aged more than 30 years (30-72 years old, range and exposed 10-14 years before operation) as compared to the younger ones. Since excess radiation risks for PTC in the individuals exposed at the age older than 20 years old is very low and further declines with age at exposure, it was proposed that *RET* polymorphisms may influence carcinogenesis in sporadic but not in radiation-induced PTCs.

The Arg72Pro polymorphism of the *TP53* gene (encodes tumor suppressor protein p53) was assessed in 48 pediatric/adolescent and 68 adult Ukrainian and Russian patients with PTC, residents of radiocontaminated territories in Chernobyl areas (51), and 53 adult patients with sporadic PTC and 313 healthy controls from Russia. The Arg/Arg homozyotes were found to be significantly underrepresented in adult patients, but not in children and adolescents. In tumor tissues, no LOH or imbalanced *TP53* allele expression in heterozygous individuals was found. These findings suggested that germline *TP53* allele combinations other than Arg/Arg may contribute to the risk of development of PTC in individuals exposed to radiation during their late childhood, adolescence or in young adulthood, particularly females aged between 18 and 30. Of note, elevated risk for thyroid cancer was reported in females exposed to Chernobyl radiation at the age below 30 years in an epidemiological investigation (52).

A recent study of 9 SNPs in 5 genes (*ATM*, *XRCC1*, *TP53*, *XRCC3* and *MTF1*) involved in DNA damage response in 255 PTC patients (123 from Chernobyl areas and 132 sporadic) and 596 healthy controls (198 residents of Chernobyl areas and 398 subjects without history of radiation exposure) showed that the *ATM* G5557A and *XRCC1* Arg399Gln polymorphisms, regardless of radiation exposure, were associated with a decreased risk of cancer (53). Interestingly, the *ATM* IVS22-77 T>C and *TP53* Arg72Pro SNPs interacted with radiation exposure: the *ATM* IVS22-77 associated with the increased risk of sporadic PTC whereas *TP53* Arg72Pro correlated with the higher risk of radiation-induced PTC in adult patients, in support to the previous report (51). A possibility of gene-gene and gene-environment interactions was demonstrated. Some particular *ATM*/*TP53* genotypes strongly associated with either sporadic or radiation-induced cancer indicating that variability of these genes may be potential risk modifiers for developing PTC of different etiology.

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Molecular epidemiology based on whole genome association data

To date only one investigation of Chernobyl PTCs employing GWAS has been published (54). A total of 667 patients from Belarus diagnosed for PTC in 1989–2009 and 1275 controls from Belarus and Russia were studied, of which 408 cases and 627 controls were genotyped using Illumina Human610-Quad BeadChips (>500,000 SNPs) and the remaining samples were used for validation study. Statistical meta-analysis identified 4 SNPs at chromosome 9q22.33 showing significant association with disease. For one of them, rs965513, used for validation, a *P*-value of 4.8×10^{-12} was obtained which far surpasses the threshold of genome-wide significance of 5×10^{-8} (55). This SNP is located within a linkage disequilibrium (LD) block centromeric to the *FOXE1* gene which encodes a thyroid-specific transcription factor TTF2 playing pivotal roles in thyroid morphogenesis. In addition, two candidates SNPs on chromosomes 9p and 12p that strongly tended to associate with disease risk were identified but genotyping of additional samples would be necessary to validate the significance of those.

To better understand the importance of this finding, it is necessary to mention two studies of genetic predisposition to sporadic differentiated thyroid cancer published last year just before the study by Takahashi et al. The first one reported rs965513, the same polymorphism described in the Chernobyl series, as the strongest genetic marker associating with thyroid malignancy in individuals of European descent. This study also claimed another SNP, rs944289 on chromosome 14q13.3 in the proximity of the *NKX2-1* gene that encodes the TTF1 transcription factor, to be a marker for thyroid cancer (56) but it was not confirmed in the Chernobyl series. The second study, employing candidate gene approach, initially genotyped 768 SNPs in 97 genes in 615 cases and 525 controls from Spain and used 482 patients and 532 controls from Italy for validation (57). The target genes were selected based on their differential expression in primary thyroid tumours or the involvement in thyrocyte biology, metabolism and/or carcinogenesis such as the MAP kinase, JAK/STAT and TGF-beta pathways. An SNP, rs1867277, within the LD block spanning *FOXE1* and located at the 5'UTR of the gene was identified as associating with PTC. Functional study demonstrated that this SNP affects *FOXE1* expression by recruiting the USF1/USF2 transcription factors. Since forkhead transcription factors have been implicated in several human cancers (58-61) including epithelial-mesenchymal transition in colon cancer (62), it was proposed that FOXE1 may influence thyroid tumor call migration and invasion. While its precise role remains to be elucidated, this was an important clue to the understanding the molecular pathogenesis of PTC.

Thus, the three studies, two of sporadic thyroid cancers and one of radiation-induced tumors, have concordantly identified the *FOXE1* (*TTF2*) locus as a marker of inherited susceptibility for PTC of different etiology. This leads to an important corollary that among the genetic factors affecting risk for radiation-induced Chernobyl PTC the strongest one is the same that confers predisposition to the sporadic form of this type of malignancy. Therefore, it is likely that "radiation-sensitive genotype", whose existence may be expected given the possible existence of putative radiation-associated

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markers on chromosomes 9p and 12p and the absence of sporadic PTC marker on 14q13.3 (i.e. *NKX2-1* or *TTF1*), comes next to and after, in terms of the effect strength, the general susceptibility to thyroid cancer. As outlined in Fig. 2, the results of genetic association studies allow to add genetic predisposition to the list of risk factors for radiation-induced thyroid carcinogenesis known from the earlier experience. Further investigation of etiology-specific marker(s) will probably refine our understanding of radiation-induced carcinogenesis by addressing issues of gene-gene and gene-environment interactions.

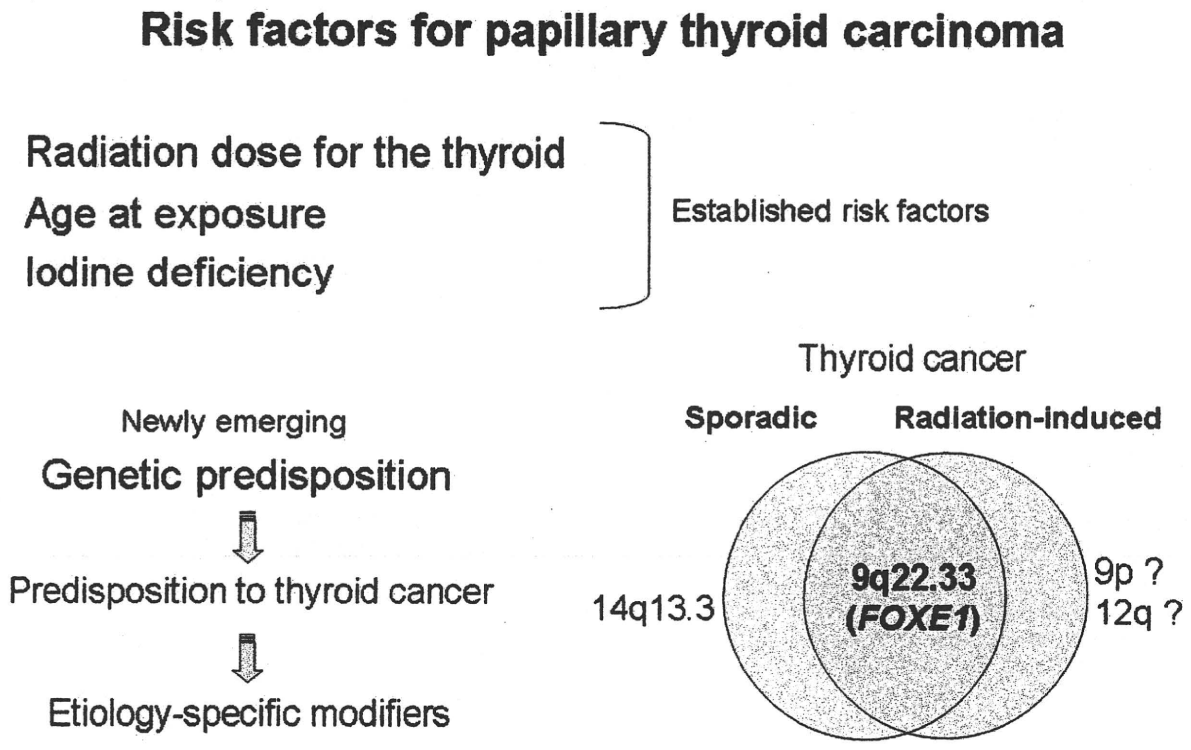

Fig. 2. Genetic predisposition as an emerging risk factor for both sporadic and radiation-induced papillary thyroid carcinoma. Sporadic and radiation-induced PTC share the major genetic determinant of inherited susceptibility to thyroid cancer, *FOXE1* at chromosome 9q22.33, which appears to be stronger than possible etiology-specific genetic markers: on chromosome 14q13.3 (*NKX2-1* or *TTF1*) for sporadic PTC and putative markers on chromosomes 9p and 12q for radiation-induced PTC.

Conclusion

A rapidly growing body of evidence suggests that the identification of molecular "radiation signature" in thyroid cancer is likely to become possible, with certain degree of certainty, in the coming years. The advances in exploring both the damage pattern by genomic microarrays, differential gene expression or immunohistochemically and inherited susceptibility by GWAS and

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expression arrays keep on bringing encouraging results yet they are far of being finalized. At present they rather contribute to work out a proof of principle that radiation-induced and sporadic thyroid cancers could be distinguished using a definite set of validated markers. Perhaps this set will include not only the above-mentioned markers as well as essential clinico-pathological information but also other, such as e.g. miRNA and proteomics, whose integration into the spectrum of potential targets and in-depth analyses may enable better insights into the possible classifiers. Its availability will likely allow future personalized cancer risk prediction which is of a significant importance in view of the growing thyroid cancer incidence in the world and also because of the relevance to occupational and expanding medicinal exposures, and radiation emergency medicine issues.

Undoubtedly, Chernobyl cohort is an inestimable source of knowledge in the area. Continuous observation, follow-up and thorough studies are warranted to yield the higher level of understanding. In this regard, international initiatives, such as the Chernobyl Tissue Bank (http://www.chernobyltissuebank.com/) or EC-coordinated GENRISK-T consortium (http://www.helmholtz-muenchen.de/isb/genrisk-t/index.html), Nagasaki University GCOE Program Global Strategic Center for Radiation Health Risk Control (http://www-sdc.med.nagasaki-u.ac.jp/gcoe/projects/index_e.html) and other cooperative efforts would be the principal roadways to solving the problem.

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

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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 5-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

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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 37 °C 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′-TTTCGACGTTCG-TAGGTTTTCGC-3′ (forward), 5′-GCACTCTTCCGAAAAC-GAAACG-3′ (reverse); for unmethylated template, 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (forward), 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (reverse). The thermal profile was as follows: 95 °C for 3 min; 35 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 10 s; and then stored at 4 °C. 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 Bio-Doc-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).

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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 (1.5×10^4 cells) were plated in each well of 24-well plate, treated with TMZ for 24 h. Then the cells were transfected with miR inhibitors using Lipofectamine 2000 reagent (Invitrogen). After 96 h, 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 2 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).

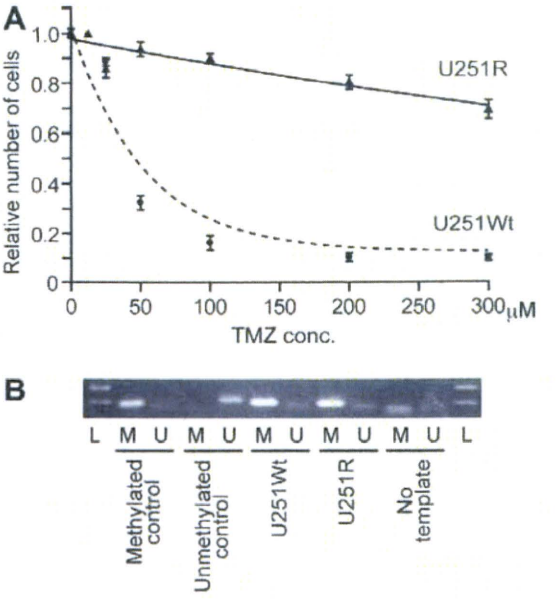

Fig. 1. (A) Establishment of TMZ-resistant variant: U251R cells. U251R cells were generated from U251MG cells by continuous exposure to TMZ. Indicated cells were treated with indicated concentrations of TMZ for 7 days, and then the relative number of cells was determined using WST-based assay. Each point 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.

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 up-regulated 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 Section 2) and investigated cell killing effect in U251R cells. Knockdown of miR-455-3p or miR-10a* did not show difference from

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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

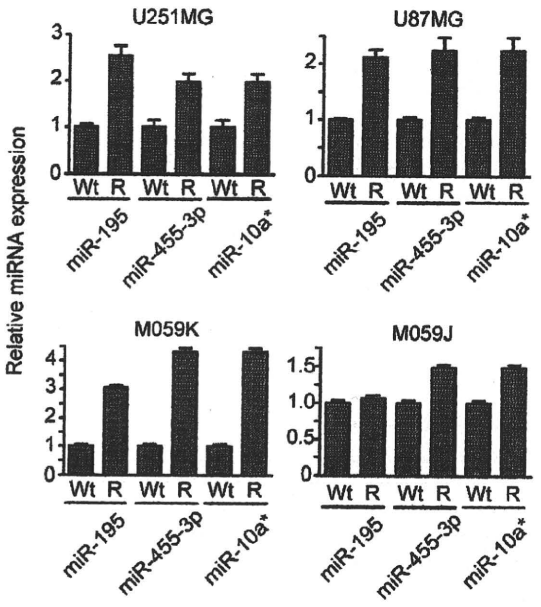

Fig. 2. TaqMan real-time quantitative RT-PCR for miRNAs. In each cell line, the expression level of indicated miRNA was compared between parental (Wt) cells and established resistant variant (*R*) as described in Section 2. RNU6B was used as an internal control. Each bar indicates the mean and standard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

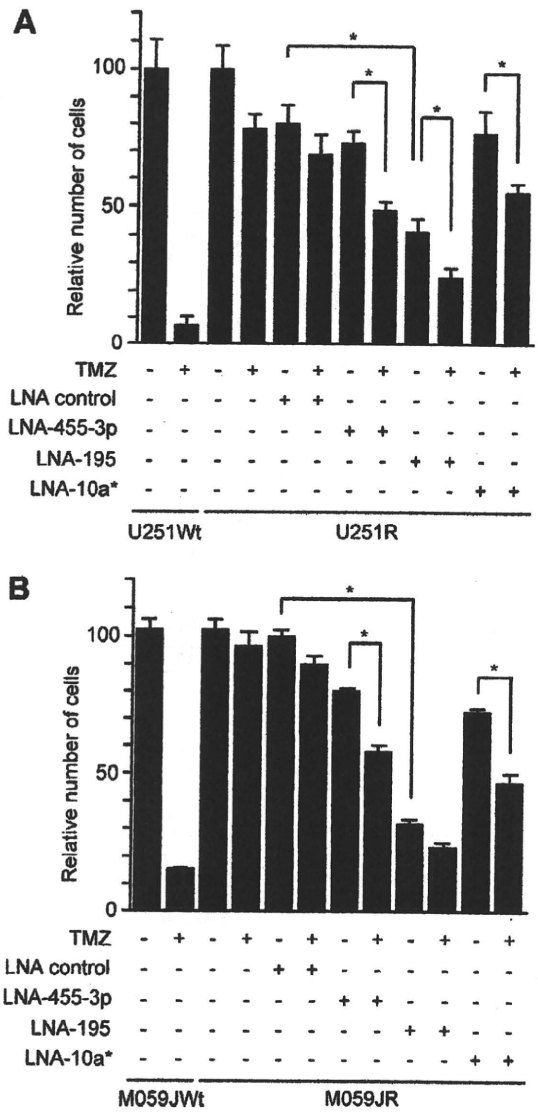

Fig. 3. Effect of miR inhibitors on TMZ resistance. Indicated cells were treated with or without 100 μM (A, for U251 cells) or 25 μM (B, for M059J cells) TMZ for 24 h and then transfected with indicated miR inhibitors. The cells were further cultured with or without TMZ for 96 h, and the number of cells 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.

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,

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