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Potential of Anti-Cancer Therapy Based on Anti-miR-155 Oligonucleotides in Glioma and Brain Tumours

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MicroRNAs are aberrantly expressed in many cancers and can exert tumour-suppressive or oncogenic functions. As oncomirs promote growth of cancer cells and support survival during chemotherapy, thus microRNA-silencing therapies could be a valuable approach to be associated with anticancer drugs and chemotherapy treatments. miR-155 microRNA was found overexpressed in different types of cancer, such as leukaemias (PML, B-cell lymphomas), lung cancer and glioblastoma. GABA-A receptor downregulation was found correlated with glioma grading, with decreasing levels associated with higher grade of malignancies. A relationship between knock-down of miR-155 and re-expression of GABRA 1 protein *in vivo* was recently individuated. This finding has implication on the effectiveness of RNA-silencing approaches against miR-155 with the scope to control proliferation and signalling pathways regulated by GABA-A receptor. Applying microRNAs for treatment of brain tumours poses several problems, and fields to be solved are mainly the passage of the brain–blood barrier and the targeted delivery to specific cell types. Glioblastoma multiforme cells bud off microvesicles that deliver cytoplasmic contents to nearby cells. Thus, the exploitation of these mechanisms to deliver antagomir therapeutics targeting microvescicles in the brain could take the lead in the near future in the treatment for brain cancers in substitution of invasive surgical intervention.

Key words: biological screening, carbohydrate, gene expression, lipid, nanotechnology (drug discovery), nucleic acid, RNAi and antisense, techniques

Cancer is a multistep genetic and epigenetic disease with a complex aetiology. Several defects such as mutations, downregulation, over-expression and deletions in oncogenes and tumour suppressor protein-coding genes have been extensively described in cancer cells. It has recently become evident that cancer is subject to translational control and to epigenetic reprogramming, because of mutations in several proteins in chromatin complexes and histones modifying enzymes, and deregulated chromatin states (1,2). As oncogenes promote survival during chemotherapy, thus RNA silencing has been developed to inactivate overexpressed genes.

Antisense therapies targeting clusterin and survivin, an anti-apoptotic protein, have been shown to be valuable approaches in association with conventional chemotherapy. Currently, survivin-antisense (LY2181308) and clusterin-antisense (OGX-011) oligonucleotides are in phase II/III clinical trials for the treatment of several cancers.

RNA interference (RNAi) has become a widely used technique that permits the knock-down, and hence functional analysis, of individual genes in vertebrate cells. Experimental steps and expression vectors have been optimized to facilitate the effective knock-down of almost any vertebrate gene product in cultured cells or in experimental animals.

Several non-protein-coding RNAs have been implicated in cancer development, and among these microRNAs (miRNAs) (3). miRNAs are a class of highly conserved small non-coding RNAs duplicated, approximately 22 nucleotides in length, involved in the post-transcriptional control of gene expression of a large number of genes.

miRNAs and small-interfering RNAs (siRNAs) originating from the overlapping region of natural antisense transcript pairs (NAT) (nat-siRNAs) are the output of this maturation process. These RNAs act as single-strand filaments incorporated into the effector complexes (RISC) containing one of the Argonaute enzymes degrading the mRNAs possessing sequences complementary to these microRNA (4).

miRNAs bind to the seed sequence at the 3′-UTR (untranslated region) of target mRNAs, resulting in translational repression (low levels of expressed protein with nor-

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mal levels of mRNA) or mRNA degradation (no protein expressed and low mRNA levels).

Because miRNAs target a large number of mRNAs of genes associated with cancer, they regulate all aspects of cancer biology (5). Lee and Dutta reviewed microRNAs able to regulate cell cycle and cell proliferation (such as p27/kip deregulated by miR-222), cell death and apoptosis (such as Bcl-2 targeted by miR-16), cell migration, invasion and metastasis (such as PTEN and Pdcd4 targeted by miR-21), angiogenesis (such as Tsp-1 targeted by KHSV miRNAs), tumour microenvironment (such as miR-26, miR-107 and miR-210, able to decrease proapoptotic signalling in hypoxia), tumour immunology as well as many aspects of cancer stem cell biology. Individual cases of microRNA with specific functions and roles are discussed in the next paragraphs.

MicroRNAs are aberrantly expressed in many cancers and can exert tumour-suppressive or oncogenic functions by regulating the expression of target mRNAs. By targeting the mRNA of oncogenes or tumour suppressors, miRNAs can act as tumour suppressors or oncogenes. Deleted microRNAs have been classified as tumour suppressors, while over-expressed microRNAs have been named oncomiRs. A high interest has been directed towards the potential use of microRNAs as biomarkers. A consistent number of expressed miRNAs are shared by several cancers and cluster in seven genomic regions susceptible to genetic and epigenetic alterations. These clusters encompass the miR-17 family, miR-183-182, and stem cell–specific clusters, miR-367-302 and miR-371-373, which are upregulated in gliomas, embryonic stem cells (ESCs) and neuronal precursor cells (NPCs) (6). Clinically, miRNAs could serve as therapeutic targets and as diagnostic, prognostic and therapeutic tools (7).

There is increasing evidence that microRNAs could be clinically useful as biomarkers for brain tumours (5) and possibly will become even more useful as targets for antisense therapies (8). Among various anti-microRNA oligonucleotides, there are locked nucleic acids (LNA)-containing oligonucleotides (short but with high binding stringency), phosphorothioate backbone sequences that prevent their cleavage, the 2′-*O*-methoxyethyl modified sequences and the antagomirs, miRNA inhibitors conjugated to cholesterol groups (9).

Antisense therapy targeting specific microRNAs was proposed: the silencing was effective especially in rodents (10), but it was shown to work also in primates (11). The effectiveness of knock-down of microRNAs using locked nucleic acids (LNA) has recently been demonstrated in non-human primates (12). Up-to-date, miravirsen successfully inhibited miR-122, a liver-expressed microRNA important for Hepatitis C virus replication (13), in primates with chronic hepatitis C virus infection. The Danish company Santaris was founded by Exiqon, one of the leading companies in the microRNA field. Other companies, such as Isis Therapeutics/Regulus, have started trials on anti-miRNAs, of which miR-21 and miR-122 are in advanced testing phases.

The delivery of miRNAs/miRNA inhibitors may control cancer growth more strongly than siRNAs. siRNAs allow specific knockdown of individual gene targets. Using similar concentrations, microRNAs affect the expression of several genes and of hundreds of mRNAs of one gene target. Hence, the ability of individual miRNAs to target multiple genes and pathways is potentially a major advantage. Several methods have been developed to produce a high copy number of expressed antagomirs. One is based on the encapsulation of RNA molecules inside a delivery device. A new method relies on siRNA sponges, in which long RNA strands containing hundreds of thousands to millions of nucleotides are designed to be cleaved by cell's RNA processing machinery into siRNAs inside the cells (14).

miR-155

A long ncRNA, BIC, exerts multiple functions according to the cellular type in which it is expressed. The BIC transcript originates miR-155, important in the hematopoietic function as well as in the homeostasis and function of the immune system (15,16).

The expression of miRNA-155 was found to be elevated in children with Burkitt's lymphoma (3). BIC cooperates with the oncogene c-Myc in lymphomagenesis and erythroleukemogenesis (17). miR-155 microRNA has been found overexpressed in different types of cancer, such as leukaemias (PML, B-cell lymphomas), lung cancer, glioblastoma (18), and also in non-neoplastic diseases such as multiple sclerosis (19).

It is estimated that 20% of all cancers are linked to infectious agents. Studies of oncogenic DNA viruses have contributed to the understanding of key molecular mechanisms of tumorigenesis and viral oncogenicity. Possible mechanisms by which viruses survive inside cells are either inactivation of the cellular antiviral machinery, inactivation of RNA interference response or reprogramming of RNA interference systems. The family of human herpesvirus includes human pathogens such as herpes simplex virus, Epstein–Barr virus (EBV), human cytomegalovirus (HCMV) and Kaposi's sarcoma herpesvirus (KSHV). These viruses share the ability to establish latency in the host after an early replication phase.

The Epstein–Barr virus is a human herpesvirus that can be carried lifelong in asymptomatic subjects. However, EBV is also the causative agent of infectious mononucleosis and is linked to the development of several malignant tumours, including B-cell neoplasms such as Burkitt's lymphoma,

Hodgkin's disease, certain forms of T-cell lymphoma, and epithelial and nasopharyngeal cancers. Epstein–Barr virus is shown to express at least 17 distinct miRNAs in latently infected cells, which potentially regulate both viral and cellular genes. Epstein–Barr virus infection strongly stimulates miR-155 expression, which is critical for the growth of lymphoblastoid cell lines (LCL) *in vitro.*

A viral correspondent of human BIC is expressed in several viruses, such as the avian leukosis virus (17) and Kaposi sarcoma virus (20). A critical role of the virus-encoded microRNA-155 ortholog in the induction of Marek's disease lymphomas has been elucidated (21). The Reticuloendotheliosis virus strain T induces miR-155, which in blood cells targets JARID2. This belongs to a chromatin repressing complex bound to a histone methyltransferase: in absence of JARID2, histone methylation is decreased and the targeted chromatin becomes accessible to RNA polymerase with genes promoting cell survival being unnecessarily transcribed (22).

Upon activation, mir-155 is expressed in several types of human immune cells, including B-cells, T cells, macrophages and dendritic cells. Regulus, a company founded by ISIS therapeutics, has already established a lead program in this field for the treatment for inflammatory diseases. Targeting mir-155 with antisense sequences (antimiRs) is believed to offer a novel therapeutic approach for treating inflammatory diseases, as also approached by competing companies such as Santaris (23).

Brain Tumours, Gliomas and MicroRNAs

Glioblastomas are the most common and deadly malignant primary brain tumours. The origin of gliomas is still unknown. As gliomas have a transformed neural stem cell phenotype, they might arise from glioma stem cells (GSCs). It has been demonstrated that miRNAs are associated with origin, progression and transformation of glioma (7,24). Similar to other cancers, miRNAs have been shown to regulate various cancer-associated genes and oncogenic functions in gliomas. Some evidence also suggests a role for miRNAs in the regulation of GSC biology. Most miRNAs are downregulated in brain cancers, but a number of miRNAs are also upregulated (7). In different studies, several pathways regulated by microRNAs in glioma cells have been identified. A differentiation checkpoint is activated by miR-127 and miR-134 (involved in CDK6 downregulation) and miR-451, while this checkpoint is blocked by miR-17-92.

Gliomas are similar to neural precursor cells in their miRNA expression profile (25). Lavon and co-workers compared the miRNA expression profiles of glial tumours, embryonic stem cells (ESCs), neuronal precursor cells (NPCs) and normal adult brains from both human and mouse tissues. It was found that human and mouse gliomas shared a miRNA expression profile that is reminiscent of NPCs (25).

It has been demonstrated that miR-21 acts as an antiapoptotic factor in glioblastoma cells, suggesting that an aberrantly increased expression of miR-21 may downregulate the translation or stability of mRNAs coding for apoptosis-related genes, although the specific targets of miR-21 remain unknown (26). A role in support of cell proliferation has been assigned to miR-10b (27).

In addition, miR-137 modulates the proliferation and differentiation of adult neural stem cells (aNSC) *in vitro* and *in vivo* (28). Overexpression of miR-137 promotes the proliferation of aNSCs, whereas a reduction in miR-137 enhances aNSC differentiation. It was further shown that miR-137 post-transcriptionally represses the expression of Ezh2, a histone methyltransferase and Polycomb group (PcG) protein (28).

Some reports have shown the effectiveness of *in vitro* suppression of glioblastoma multiforme (GBM) cell viability after delivering tumour-suppressive miRNAs or inhibitors of oncomiRs, such as miR-21- and miR-221-specific antagomiRs or microRNA sponges (7,8). In addition, restoration of depressed or deleted microRNAs could also be a valuable approach. Antioncogenic, downregulated microRNAs have been identified in gliomas, such as miR-451, miR-17-21, miR-124, miR-137 and miR-128 (29–32).

VEGF induces the expression of miR-296 and promotes the formation of blood vessels surrounding the glioma cells. Thus, antagomirs directed to block angiogenic microRNAs, angio-miRs (33), such as miR-296 and miR-132, have been shown effective in blocking neovascularization (34).

The Role of miR-155 in Glioma Cells

GABA-A receptor downregulation has been found correlated with glioma grading, with reduced levels associated with higher grade of malignancies, while absence of GABA-A receptors has been linked to medulloblastoma growth (35) because of an inverse correlation between GABA-A receptor and cell proliferation. In addition to the role in neurotransmission and regulation of secretion, gamma-amino butyric acid (GABA) through GABA(A) receptors negatively regulates proliferation of pluripotent and neural stem cells, in embryonic and adult tissue (36,37).

Eight different subunits of $GABA_A$ and $GABA_C$ receptors have been identified. The assembly of a heteropentamer, with at least one α-, one β- and one γ-subunit, forms functional $GABA_A$ receptor that functions as a ligand-gated Cl^- channel.

There has also been evidence that GABAergic signalling and its control over proliferation are not only limited to the nervous system but are widespread through peripheral organs containing adult stem cells. GABA has emerged as a tumour signalling molecule in the periphery that controls the proliferation of tumour cells and perhaps tumour stem cells. Thus, GABA has been individuated as a near-universal signal that may be altered in tumour cells resulting in modified mitotic activity (38).

In a recent paper (39), the conserved microRNAs targeting GABA receptors were predicted by TARGETSCAN 5.2 http://www.targetscan.org/. TARGETSCAN software has been updated to version 6.1 (March 2012) because of an updated set of RefSeq genes and UTR sequence. GABA type-A receptor (GABRA1) is the predicted target of miR-155 if using the current version 6.1 of TARGETSCAN software. miRanda software (http://www.microrna.org) was also being able to detect miR-155 seed regions in GABRA1 mRNA.

A relationship between knock-down of miR-155 and expression of GABRA1 protein *in vivo* has been recently described (18). The transient transfection of primary cultures, obtained from surgically resected glioblastomas, with anti-mir-155 oligonucleotides restored the expression of GABRA1 and induced loss of cell viability in few hours, suggesting that GABRA1 could be either a direct target of miR-155 or that its upregulation may be the result of an indirect effect. 3′-UTR assay is mandatory to confirm a direct interaction between miR-155 and GABRA1 upregulation. Nonetheless, this finding has implication on the potential of RNA-silencing approaches against miR-155 to restore the control of proliferation and signalling pathways regulated by GABRA1.

Although these discoveries are of paramount importance for the development of targeted therapies, several problems still remain to deal with, including passage through the brain–blood barrier (BBB) and delivery to specific cell types.

To overcome the difficulties related to the local delivery, methods such as convection-enhanced delivery (CED) (40) may be effective in delivering drugs directly to the brain. However, in case of failure of CED, other strategies could increase the chances of successful systemic delivery (41), such as BBB permeability modulation. Some authors have tested a number of physical methods such as ultrasounds (42,43), and few compounds can disrupt the BBB, such as potassium channel agonists and minoxidil sulphate (44); the intra-arterial infusion of these agents could increase the chances of delivery to the brain (45).

Noteworthy, as most malignant brain tumours manifest with mass effect and surgery is often required, these agents could be directly delivered at the end of neurosurgical procedures.

Other authors have identified peptides that can be conjugated to the structure of a payload thus allowing its transfer across the BBB (46); such peptides could be used for delivery of modified tumour-suppressive microRNAs or inhibitors of oncomiRs.

It has recently been demonstrated that GBM cells bud microvescicles off, delivering their cytoplasmic contents to nearby cells (47). This phenomenon could markedly reduce the threshold efficiency for effective delivery of a miRNA-based therapy, as mRNAs, microRNAs, proteins and other cytoplasmic molecules may be 'shared' by GBM cells with surrounding GBM and normal cells, and this should certainly apply to transfected siRNAs/miRNAs/miRNA inhibitors that are present at high concentrations in the affected cells.

In addition, the development of suitable types of liposomes, nanoparticles or other encapsulation methods could support the delivery of these oligonucleotides to the brain (48–50).

At the present time, the most suitable vectors for miRNA delivery are viruses encoding miRNAs/inhibitors. Adenoviral delivery would yield a relatively short period of high expression, while lentiviral or AAV-based delivery would give incorporation into the genome with long-term lower expression (8).

Further studies and clinical trials are required to step forwards in the treatment for brain tumours, but we foresee that increasing the knowledge in this field will allow improving the survival of patients affected by malignant tumours.

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