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FULL PAPERS

A MULTIVARIATE ANALYSIS OF PATIENTS WITH BRAIN TUMORS TREATED WITH ATN-RNA

ELIZA WYSZKO[1], KATARZYNA ROLLE[1], STANISLAW NOWAK[2], RYSZARD ZUKIEL[2], MONIKA NOWAK[1], RAFAŁ PIESTRZENIEWICZ[2], IWONA GAWROŃSKA[1], MIROSŁAWA Z. BARCISZEWSKA[1] AND JAN BARCISZEWSKI[1*]

[1] Institute of Bioorganic Chemistry of the Polish Academy of Sciences, 12 Noskowskiego St., 61-704 Poznań, Poland
[2] Department of Neurosurgery and Neurotraumatology, University of Medical Sciences, 49 Przybyszewskiego St., 60-355 Poznań, Poland

Abstract: Glioblastoma multiforme (GBM) is the most common form of malignant glioma, characterized by genetic instability, intratumoral histopathological variability, and unpredictable clinical behavior. Malignant gliomas express preferentially a number of surface markers that may be exploited as therapeutic targets, such as tenascin-C, an extracellular matrix glycoprotein contributes to tumor cell adhesion, invasion, migration and proliferation. Disappointing results in the treatment of gliomas with surgery, radiation and chemotherapy have fuelled a search for new treatment modalities. Here we present the data for 46 patients suffering from brain tumor. They were resected and treated with dsRNA (ATN-RNA) complementary to the sequence of tenascin-C mRNA. MRI and CT follow up studies showed growth tumor delay or lack of its recurrence symptoms, due to inhibition of TN-C synthesis. A significant improvement in overall survival (OS) was observed without loosing of the quality of life (QOL) of patients. This novel therapy based on RNA interference shows a big therapeutical potential. To our knowledge intervention with RNAi (iRNAi) is the first protocol of application of RNAi in human disease treatment.

Keywords: brain tumor, GBM, RNAi, tenascin-C, survival, KPS, OS, QOL

Gliomas have an annual incidence of five to 10 cases per 100 000 in Western populations (1). They are a leading cause of death among children and adults diagnosed with a neoplasia of the brain. The origin of brain tumors still remains unresolved, but they may originate from mature cells or from neuroectodermal stem cells of the brain (2).

This is a heterogenous group of tumors clasiffied by the World Health Organization (WHO) into pilocytic astrocytomas (WHO I) with slow growth and rarely undergoing malignant transformation and three groups of diffusely infiltrative: astrocytomas comprising diffuse astrocytomas (WHO II), anaplastic astrocytomas (WHO III) and glioblastoma (WHO IV). Accurate classification of astrocytic gliomas is often, however, difficult due to their heterogeneous nature. There is a strong piece of evidence suggesting that those clinicopathological differences reflect the type of genetic alteration aquired during the process of transformation (3-6). Glioblastoma multiforme (GBM), the most devastating primary human brain tumor, is a rapidly growing malignant astrocytic tumor with a high morbidity and mortality (7). The current management of GBM is based on cytoreduction through surgery, radiotherapy and chemotherapy. Patients with glioblastoma invariably fail to achieve long-term survival, which is though to result of acquired resistance to chemotherapeutics or radiation (8, 9). Despite a broad approach to treatment, the median patient survival is approximately 6-8 months although about 40% of patients with GBM die within 6 months after diagnosis (10, 11). A general mechanism of glioma cell invasion is based on the attachment of tumor cells to extracellular matrix, its degradation and subsequent penetration into adjacent brain structures (12, 13). GBM represents an excellent target for focused therapy because GBM cells are among a few rapidly proliferating ones in CNS (central nervous system) (8, 12). It is known that tenascin-C (TN-C), the extracellular matrix protein is highly expressed in tumor tissue of the major-

* Corresponding author: Prof. dr hab. Jan Barciszewski, e-mail: Jan.Barciszewski@ibch.poznan.pl; phone: (48061) 8528503; fax: (48061) 8520532

ity of malignant tumors including brain (14-16). TN-C commonly elevated in high-grade gliomas increases the invasiveness of glioma cells (17, 18).

The key therapeutic advantage of RNA interference lies in its ability to specifically and efficiently knock down the expression of disease-causing genes of known sequence (19-24).

RNAi phenomenon relies on a multistep intracellular pathway which can be roughly divided into two phases. Firstly, endogenous or exogenous dsRNA molecules present within the cell are processed through the cleavage activity of RNase III-type (Dicer) into short 20-30 nucleotide fragments called siRNAs. Next, siRNAs as well as many proteins including nucleases and helicase form RNA-induced silencing complex (RISC). Through unwinding of double stranded siRNA, the complex becomes activated with single–stranded, non coding siRNA which guides the RISC complex to its complementary target RNA causing its endonucleolytic cleavage (21, 24).

Although standard treatment approaches to brain tumor can prolong life, they have a little impact on survival (10, 11). At the time of appearance and recurrence of the tumor, standard options are limited by the tumor location, size and the overall condition of the patient. A minority of patients may be candidates for reoperation, but prolonged survival is rare with this approach alone (10, 11).

In our brain tumor experimental therapy we use double-stranded interferering RNAs (dsRNAs) to reduce tenascin-C expression in brain tumor cells. ATN-RNA was injected into postoperative area of 46 patients. The technology we called intervention with RNAi (iRNAi). The follow up study with MRI and CT clearly showed an increased survival at better quality of life. The results of the treatment of first 11 patients with ATN-RNA have been recently published (25).

Here we present a results of the follow up study. We analyzed here Karnofsky Performance Scale (KPS), necrosis level, extent of resection, tumor location and patients age.

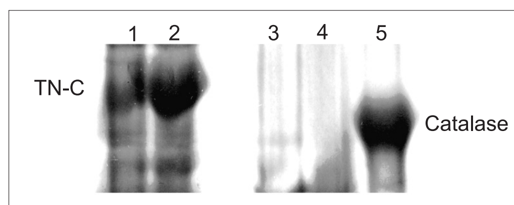

Figure 1. Expression level of TN-C in GBM cells. Lanes: 1 and 2 – 10 μg soluble, 3 and 4 – 10 μg insoluble fraction of extract from a tumor and border part of tumor, respectively; (5) molecular mass standard catalase (C) with molecular mass 230 kDa.

EXPERIMENTAL

Materials and Methods

Patients

Patients suffering from brain tumor qualified for surgery and molecular intervention (iRNAi) were managed at the Department of Neurosurgery and Neurotraumatology, University of Medical Sciences in Poznań. Precise tumor localization was determined with computerized tomography (CT) or magnetic resonance imaging (MRI), before every surgical procedure. 46 patients with brain tumor diagnosed according to WHO criteria for 25 primary WHO IV, 7 WHO III and 14 WHO II were resected and ATN-RNA was injected into postoperative area.

Efficacy of ATN-RNA treatment was estimated on the basis of the follow-up studies including general and neurological condition as well as neuroimaging studies with the CT and MRI measured

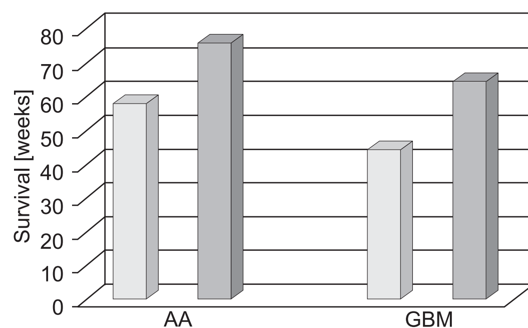

Figure 2. Comparison in overall survival of patients with GBM and III Grade tumors: literature data (gray bars) and data from present analysis (black bars).

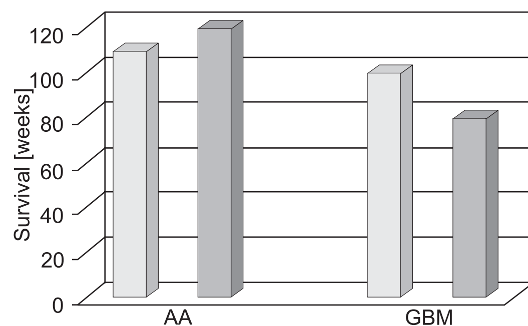

Figure 3. Differences in overall survival for patients with GBM and AA treated with ATN-RNA in primary (gray bars) and recurrent tumor (black bars).

Table 1. Patients with brain tumors (Grade II-IV) after resection and treatment with ATN-RNA.
Patients 23, 27, 34 and 41 (marked with *) were reoperated and treated with ATN-RNA after surgery.

Patient no.	Patient name	Sex	Age (years)	Glioma grade	Brain tumor location	Tumor area (mm)
1	DM	F	25	II	frontal	45 x 27
2	WJ	F	62	IV	fronto-parietal	46 x 40
3	WF	M	53	III	frontal	45 x 49
4	ŚM	M	53	IV	temporal	55 x 40
5	GE	F	48	II	fronto-parieto-temporal	81 x 43
6	OA	M	49	IV	parietal	43 x 35
7	GL	M	47	IV	frontal	60 x 50
8	KM	M	32	II	fronto-parieto-temporal	89 x 72
9	CZ	F	46	II	bifrontal	55 x 40
10	JR	M	36	II	fronto-parieto-temporal	69 x 38
11	TK	F	52	II	frontal	23 x 37
12	RJ	M	59	IV	frontal	50 x 40
13	ZM	M	47	III	parietal	43 x 27
14	MJ	M	67	IV	fronto-temporal	47 x 36
15	KM	F	48	III	frontal	45 x 27
16	BZ	M	52	II	bifrontal	60 x 50
17	WK	F	69	III	parietal	40 x 40
18	WG	M	46	IV	parieto-temporal	57 x 50
19	WD	F	48	IV	temporal	49 x 40
20	PS	F	43	II	fronto-temporal	34 x 42
21	KJ	F	70	IV	parietal	60 x 40
22	JB	F	48	IV	fronto-parietal	52 x 62
23*	CW	M	48	III	parieto-temporal	50 x 60
24	CR	M	48	II	fronto-parieto-temporal	72 x 52
25	BM	M	75	IV	fronto-parietal	71 x 64
26	DM	F	55	IV	occipital	50 x 60
27*	SM	M	54	IV	frontal	60 x 50
28	RK	M	45	IV	temporal	70 x 50
29	TZ	M	49	IV	fronto-temporal	40 x 65
30	WK	M	54	IV	bifrontal	60 x 70
31	KB	F	44	III	frontal	60 x 55
32	JS	M	35	II	frontal	55 x 50
33	HJ	M	65	IV	bifrontal	60 x 37
34*	KJ	M	66	IV	fronto-temporal	50 x 46
35	ST	F	63	II	bifrontal	60 x 50
36	LD	F	40	II	temporal	49 x 43
37	JT	F	59	IV	bifrontal	40 x 50
38	BK	F	54	III	parietal	55 x 55
39	PH	F	35	II	parietal	38 x 45
40	LB	M	64	IV	frontal	85 x 65
41*	KA	F	59	IV	occipital	50 x 40
42	KE	F	54	IV	temporal	60 x 50
43	ND	F	50	IV	parietal	40 x 35
44	TT	M	58	IV	frontal	55 x 50
45	GL	M	51	IV	temporal	50 x 55
46	ŁL	M	19	II	parietal	60 x 50

Table 2. Classification of the patients cohort with primary (20) and recurrent (26) brain tumors treated with ATN RNA based on WHO grade.

Tumor histological diagnosis	**WHO tumor grade**	**Primary tumors (No. of patients)**	**Recurrent tumors (No. of patients)**
Glioblastoma muliforme	IV	15	10
Anaplastic astrocytoma	III	3	3
Oligodendroglioma	II	-	3
Mixed oligo-astrocytoma	II	-	3
Anaplastic oligo-astrocytoma	III	-	1
Astrocytoma	II	2	6

Table 3. Analysis of the level of brain tumor resection in 46 patients treated with ATN RNA in primary and recurrent brain tumors.

Resection (%)	**Patients**			
	Primary tumors		**Recurrent tumors**	
	Number	**percent**	**Number**	**percent**
Gross total (> 98%)	5	25	6	23,1
Subtotal (> 75% & < 98%)	11	55	14	53,8
Partial (< 75%)	4	20	6	23,1

for each two month periods after the treatment (manuscript submitted). The consent for the treatment was obtained from patients.

RNA preparation

ATN-RNA was synthesized *in vitro* with T7 RNA polymerase (25). Plasmid harboring ATN DNA was cleaved with HindIII or EcoRI. ATN-RNA was synthesized *in vitro* with T7 and T3 RNA polimerases. Two RNA strands were prepared separately and then hybridized in buffer containing 20 mM Tris-HCl, pH 7,5 and 50 mM NaCl. Reaction was carried out at 95°C for 3 min, then at 75°C for 30 min, and after that slowly cooled down to room temperature.

Protein extraction from human tumor tissue

Tumor and border tissues were sonicated (2 × 10 s with 30 s intervals) in 10 mM Tris-HCl, pH 7.4, centrifuged at 10,000 rpm for 15 min and supernatant (soluble fraction) was collected.

SDS-polyacrylamide gel electrophoresis

Appropriate amounts of protein extract were supplemented with buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 6 M urea, 0.1 M DTE, 0.05% bromophenol blue). Prior to electrophoresis, the protein fractions were denatured for 10 min at 95°C and then cooled on ice. The electrophoresis was run on 10% SDS-PAGE with 4% stacking gel at 120 V. Staining was performed with Coomassie brilliant blue R-250.

ATN-RNA delivery

80 μg of dsRNA in calcium chloride were injected into surgically created cavity in the brain of patients with brain tumor in a few sites approximately 200 μL each.

Legal issue

These studies and the experiments were approved by Bioethics Council of the University of Medical Sciences in Poznań.

RESULTS AND DISCUSSION

Altered expression patterns of certain genes play a pivotal role in several pathological conditions (26). Malignant gliomas selectively express proteins that are not present in normal CNS tissue, among them is tenascin-C (TN-C) (14-16). TN-C is expressed ubiquitously in high-grade gliomas but not in normal brain, what we confirmed for glioblastoma tumor tissue in this study (Fig.1). We observed TN-C overexpresion in soluble fraction isolated directly from the tumor, whereas the level of TN-C from the

border of GBM was undetectable. The level of TN-C correlates well with enhanced tumorigenesis and supports malignant transformation, uncontrolled proliferation, metastasis, angiogenesis and escaping from tumor immunosurveillance (14-16).

Management of malignant gliomas currently consists of a maximal safe resection followed by adjunctive therapies with radiation treatment and chemotherapy (8, 9). As a failure of brain tumor treatment is usually due to insufficient of surgical resection, there is a necessity for novel therapies to improve the survival of patients suffered from these tumors. As most malignant gliomas are well known to be chemo- and radio-therapy resistant due to the inhibition of an apoptotic pathway, recently discovered RNA interference (RNAi) phenomenon may be the method of choice suitable for such treatment (19, 24, 27). RNA-based therapeutics hold promise of tremendously expanding the number of "druggable" targets by overcoming the major limitation of existing medicines, which are able to target only a limited number of proteins involved in disease pathways. RNAi is a sequence specific, conserved pathway in which dsRNA have been identified as a mediator of functional gene silencing of specific genes in variety eukaryotic organisms (28). dsRNAs are processed into small RNAs of 20-49 nucleotides in length by

Table 4. Brain tumor location sites in 46 patients treated with ATN RNA.

Location of brain tumor	No. of patients	Patients (%)
frontal	11	23.9
parietal	8	17.4
temporal	6	13.0
occipital	2	4.4
fronto-temporal	4	8.7
fronto-parietal	3	6.5
parieto-temporal	2	4.4
fronto-parieto-temporal	4	8.7
bifrontal	6	13.0

Table 5. Changes of Karnofsky Performance Scale (KPS) scores as a result of primary operation (**A**) or reoperation (**B**) and ATN-RNA application.

A

Difference between preoperative and postoperative KPS scores in patients with primary tumors								
Preoperative KPS score	Changes							Patients no.
	-30	-20	-10	0	+10	+20	+30	
>70	2	0	2	0	0	3	1	8
≤70	1	1	1	1	0	5	3	12

B

Difference between preoperative and postoperative KPS scores in patients with recurrent tumors								
Preoperative KPS score	Changes							Patients no.
	-30	-20	-10	0	+10	+20	+30	
>70	1	2	1	2	2	6	5	19
≤70	0	0	0	1	2	4	0	7

Table 6. Degree of necrosis of brain tumor. The MRI and CT image analyses before surgery in scale (0-3); 0 = lack of necrosis; 1 = necrosis less than 25%; 2 = necrosis around 50%; 3 = necrosis over 50%.

GBM (n = 25)			**AA (n = 6)**		
Necrosis	**No. of patients**	**Median survival in (weeks)**	**Necrosis**	**No. of patients**	**Median survival in (weeks)**
3	7	40.5	3	2	57
2	6	58	2	0	0
1	8	48.5	1	1	59
0	4	46	0	3	74

Table 7. Age dependent distribution of patients with brain tumors treated with ATN-RNA.

Patients age (years)	Male No. of patients	Female No. of patients
≤ 20	-	1
21 – 30	1	-
31 – 40	2	3
41 – 50	8	8
51 – 60	5	9
≤ 60	4	5

the ribonuclease III (RNase III) enzyme Dicer (32). The small RNAs are the specificity determinants of the pathway, assembling into the RNA-induced silencing complex (RISC), the multiprotein entity that is guided to complementary RNA, resulting in silencing of the message (28-30). This technology seems to be very suitable for brain tumor treatment.

We have prepared dsRNA (ATN-RNA) ca. 160 nt with the sequence complementarity to TN-C mRNA and applied it for downregulation of TN-C in brain tumor. The method we coined intervention with RNAi (iRNAi) (26).

Overcoming the systemic delivery obstacle remains one of the most crucial challenges on the road of bringing RNAi drugs to the market. Because we applied dsRNA directly for patient treatment, ATN-RNA is a naked molecule without any chemically blocking groups. The processing of that dsRNA to shorter molecules in the human GBM extract was evaluated. To check whether trimming of ATN-RNA was specific or not, we used human Dicer cleavage *in vitro* (30). Among degradation products we observed oligonucleotides of ca. 20-30 nt, which can be acquired for RNA interference pathway *in vivo* by RISC. *In situ* degradation of long dsRNA in tumor is followed by induction of cellular RNAi probably through channeling. That process is defined as a flow of the substrates from extracellular compartment to another. The channeling is very well known in eukaryotic cells and has been identified for example in phosphatidylcholine (PC) synthesis in glioma cells (31, 32). *In situ* degradation of non-protected ATN-RNA has some advantages: provides a set of short interference RNAs which are optimal and the best natural substrates for RISC; hydrolyzed long dsRNA has no interferon induction potential; stabilization of RNA by modification is not required; *in situ* formation of natural short interference RNA can be involved in cellular processes at proper time and space. Recently, it has been shown that dsRNA having a short nucleotide sequence motif of UGUGU can induce interferon response (32, 34). However, this is not the case for ATN-RNA because there is no such sequence.

It has been previously shown that many types of nucleic acids, including siRNAs duplexes can induce type I interferons connected with pattern recognition receptors – Toll-like (TLRs). They are expressed in human and mice, however, e.g. TLR9 and TLR3 expression in human brain is limited only to plasmatocytoid dendritic cells (pCDs) (35).

In this work ATN-RNA was applied locally to 46 patients suffering from brain tumor (Table 1).

Among the patients treated with ATN-RNA we distinguished those with 20 *de novo* tumors and 26 recurrence, which can be further down divided into 15 glioblastoma multiforme WHO IV, 3 gliomas WHO III and 2 WHO II in group of primary tumors and then 10 GBM tumors, 4 gliomas WHO III and 12 gliomas WHO II in group of recurrent tumors (Table 2). The patients in our study were followed up with images of magnetic resonance (MRI) or computer tomography (CT) (manuscript submitted). Our data from CT and MR images proved that patients with gliomas entering clinical trials have variable outcomes evidentioned on clinical characteristics. Here we will discuss an initial histology, KPS (Karnofsky Performance Scale), overall survival, necrosis level, extent of resection and age.
It seems that the critical effect on survival have the extent of resection (Table 3). In general, 24% of patients (including primary and recurrent tumors) had almost totally resected tumor and 54% - subtotally.

We have found that tumor functional grade is very important variable for determining the risk of a neurological deficit after surgery. In evaluation of an association between survival and surgical treatment, it is important to take tumor location into account. There was shown that involvement of eloquent brain was associated with shorter duration of survival at the univariate level, but lost its effect when adjusted for other independent predictors (36). Our analysis shows that frontal tumors predominate among a cohort of patients (Table 4), what is also connected withto the level of the tumor resection. It is obvious that such location provides a big probability to remove the tumor totally or subtotally. Only for 22% of patients partial resection was done, because of deep brain tumor location (Table 3).

The optimal resection of brain tumor depends on its size and location, the patient's general and neurological status, and the experience of the surgeon as well. The aggressive resection of 98% or

more of the tumor was a significant and independent predictor of patient survival. So we can conclude that a gross-total resection should be performed whenever possible for patients, although not at the expense of neurological function. That finding brings to light an efficiency of iRNAi technology. We had a patient with tumor recurrent oligodendroglioma infiltrating to corpus callosum which was located in non operative area. In this case we observed a big ATN-RNA effect and decay of tumor. There is an evident superiority of our technology compared to the standard procedure.

In this paper we analyze also: Karnofsky Performance Scale (KPS) before and after surgery, median survival, extent of resection, the degree of necrosis observed on MR images and age of patients (6, 26, 36-38).

Resection and ATN-RNA application changed KPS, which describes functional status of patients with brain tumor. Patients with primary tumors and KPS = 70 after iRNAi gain 20 and 30 points and improved the outcome by 66%. The better results were obtained for patients with recurrent tumors. 68 and 87% of patients with KPS below and above 70, respectively, improved significantly their quality of life (Table 5).

A significant survival advantage was associated with resection of 98% or more of the tumor and high KPS score (6, 36-38).

A comparison with already published data results in 58 weeks of survival for patients with Grade III gliomas and 44.5 weeks for patient with GBM clearly shows that iRNAi extents the time of life for ca. 18 weeks (Grade III) and for 10 weeks (GBM) (26) (Fig. 2). We also observed strong difference between *de novo* and recurrent tumors treated with ATN-RNA. It seems that the *de novo* AA gliomas are the strong predictor for longer survival. We noted median survival for recurrent AA gliomas up to 100 weeks, whereas for patient with primary tumor the survival time is longer than 120 weeks (Fig. 2).

It is already known that the Grade III tumors are associated with a better prognosis than GBM (26, 38). We also observed that there is a little difference between patients with GBM *de novo* and recurrent tumors. Although the survival for patients with primary GBM is longer than for recurrent GBM (110 weeks vs. 80), there is still as much as for Grade III recurrent tumors (Fig. 3).

The absence of necrosis or its presence at the level lower than 25% in our imaging studies was an important prognostically favorable variable (36). For GBM patients the median survival time was 47 weeks, and for AA patients – 66 weeks. For the rest cohort of patients, with necrosis level around and over 50% the meaningful level was around 35 weeks (Table 6). The low-intensity necrotic areas within a glioma are a common imaging feature and are belived to indicate rapid growth and malignant behavior (36).

An advanced age is a strong predictor of shorter survival among patients with GBM who have received modern multimodality treatment with radiotheraphy and chemotheraphy (6, 26, 36-39). The age of the patient is a paramount factor in determining outcome. We observed strong correlation between the age and median survival time for patients treated with ATN-RNA (Table 7). Clinical and laboratory evidence suggests that this difference in survival may result from greater inherent resistance to treatment in gliomas of older patients, rather than from intrinsic differences in tumor growth rate.

In summary, TN-C represents an attractive therapeutic target for malignant glioma because of its widespread expression and physiologic contribution to malignant glioma behavior. It seems that molecular intervention with ATN-RNA is a promising local adjuvant therapy after surgery for patients with malignant gliomas. It involves the direct injection into the tumor tissue resected area. The therapeutical dsRNA induces RNA interference effects downstream of application site and specifically down regulates synthesis of TN-C. In fact, this is the site-directed therapy which may improve local control and overall outcome for malignant glioma patients.

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