Chapter 5

IGF-I Antisense and Triple-Helix Gene Therapy of Glioblastoma

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1. Introduction

1.1. General concept

There is a convergence between ontogenesis and carcinogenesis. In theory all antigens correspond to a specific stage of embryo/fetal development. These antigens – oncodevelopmental proteins - are also present in homologous neoplatic tissues during cancer growth. The first illustration of this theory is alpha-fetoprotein (AFP), a common tumor marker, which is present during the normal development of central nervous system of the rat. Immunohistochemical analysis of the localization of AFP during normal development of the central nervous system and in pathological homologous nervous tissues of teratocarcinoma, shows a remarkable parallelis [1]. A similar demonstration was demonstrated for serum albumin antigen and blood group H antigen [2]. As AFP was a specific marker for neuronal cells, a search for a specific marker of glial cells has constituted the next step in the nervous system research to distinguish glial and neuronal cells, because the known markers were not totally specific. It was demonstrated that the growth factor IGF-I (Insulin like Grow Factor I) [3-6] is present only in glial cells, and absent in neuronal cells in normal and neoplastic developmen [7]. IGF-I is, therefore, a specific marker of glioblastoma. The use of IGF-I for the study of malignant tumors, including the gliomas, became the best choice as a marker [8-10].

1.2. Anti — Gene strategies

1.2.1. Antisense

For nearly twenty years various treatments of tumors, based on the injection of antibodies directed against specific antigens and stem cells have been tested. The results were not

successful because of non-selective affinity for the tumor. Moreover, direct injection of protein coupled to an isotope such as iodine-125, has also posed the problem of the limited specificity of the distribution of the protein [11]. After that, the researchers have approached the study in a radically different way, trying to stop the production of IGF-I, acting directly on the messenger RNA [7,12].

Since 1978 it is known that antisense messengers are naturally produced and destroyed in the process of DNA replicatio [13-17]. The authors successfully demonstrated that this phenomenon is possible in twenty different species. Fortunately, the researchers could produce artificial antisense messengers in a significant number [12,17-19]. Moreover, using antisense oligonucleotides associated with the photoluminescent amino-terminated poly amido amine dendrimer, they can be directly analyzed by fluorescence microscopy and flow cytometry [20]. The antisense sequences are capable of blocking the messenger RNA translation conducting to block of specific protein synthesis.

1.2.2. Triple helix

Since the 90s, another approach in parallel with the strategy of antisense RNA has become successful in gene therapy and clinical trials: the triple helix strategy [21-23]. The triple helix technology (HT) is the newest approach, which belongs to the antisense approach and *sensu lato* anti gene strategies. TH technology was discovered by PB Derwan and C. Helene [24,25] and its action was defined as inhibition of gene expression at the level of transcription. In short, specific oligonucleotide sequences (also called triple helix-forming oligonucleotides, TFOs) are introduced into cells by transfection using chemical carriers, such as plasmid vectors which can direct synthesis TFOs. The TFOs are linked to the genomic DNA forming the triple helix structure with the target gene and inhibiting its transcription. The TFOs usually are directed against sequences located in the promoter region of genes of interest [24]. Examples of the inhibitory activity of TFOs on target genes involved in tumorigenesis are currently available. This approach, based on the triple helix of DNA has been used for the inhibition of IGF-I, which plays a major role in tumorigenesis [26]. Triplex strategy has also been applied to the inhibition of Ras oncogenes which are the most frequently activated in human cancer. Transcription was inhibited by human Hras TFOs targeting sequences recognized by the transcription factor Sp I [27]. Moreover, synthesis of Human Tumor Necrosis Factor (TNF), which acts as an autocrine growth factor in various tumor cell lines including neuroblastoma and glioblastoma has been blocked by treatment with TFOs [28,29].

2. Experimental results

2.1. *In vitro* and *in vivo* experiments on glioma tumors

The vector of antisense type was transfected into established glioma C6 cell line. All transfected cells have changed phenotype. Morphologically these cells had an elongated appearance. Transfected glial cells have produced a large amount of antisense RNA [7,30].

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Regarding *in vivo* experiment, subcutaneous injection of C6 glioma cells into DBX rats have produced a glioma tumor after ten days. When transfected glioma cells were injected (with vector carrying the cDNA antisense against IGF-I) the tumors did not develop.

The experience was reproducible in 200 rats and the results were confirmatory. It was demonstrated that if injected initially native C6 glioma cells were followed a week later by injection into another point of subcutaneously transfected glial cells, the tumor developed, but disappeared completely after 2 to 3 weeks [12].

The tumor examined histologically shortly before the death of animals, have shown an important lymphocytes / plasmocytes infiltration. Histological analysis of the tumor during its disappearance suggests that the essential mechanism is related to the presence of specifically CD8 + cytotoxic T lymphocytes. Systematic analysis of all tissues showed that these lymphocytes were of splenic origin, and were observed in very large quantities in the spleen of experimental animals (10 to15%) [12,31,32].

2.2. Mechanism of glioma tumor destruction

The cytotoxic T CD8+ cell can exert its effect, if a bridge between CD8 and the antigen of class I major histocompatibility complex (MHC) occurs [33-36]. Following transfection of glial cells with the antisense cDNA of IGF-I, the expression of MHC-I in transfected glioma cells and in vivo, is greatly enhanced (5 times). This mechanism may play a role in the cytotoxic response, although not the only one involve [37]. The bearing tumor rats receiving injections of transfected cells, have revealed a very high rate of CD8 + cells. This anti-tumor immune response has stopped the tumor development [12,31,38-40].

3. Application in clinical trial

3.1. Ethical committees

Approval for the clinical trial of gene therapy (based on the NIH clinical study No 1602, Bethesda, Maryland, 24. 11. 1993) [41] was administered by the Commission of Bioethics at the University of Medicine, Bromberg (No KB / 176/2001, 28. 06. 2002) and registered by Wiley International Gene Therapy Clinical Trial database No. 635 and 636 (J Gene Med, updated 2002), and by NATO Science program 2003/2007 - USA, France, Poland, Germany (LST 980 517).

3.2. Conditions

The NIH Committee raised a number of conditions. Initially, for all patients, tumor cells must be isolated from biopsies and cells clones to be used should be only IGF-I and GFAP positive. NIH also requires that all cells are irradiated with a dose of 5000 cGy before reinjection, to avoid the possibility of subsequent cell divisions. After irradiation, the cells must be maintained in cell culture for 24 hours.

The elimination of the plasmid in the cells before injection is ensured by the possibility of a deletion in vitro of the hygromicine for 2 to 3 weeks. (The safety of the method was guaranteed by the use of an episomal vector without the risk of integration of the DNA vector).

Gene therapy will be preceded by a lymphocyte transformation test (TTL): in vitro, the lymphocytes of patients with glioblastoma are brought into contact with their own tumor cells transfected; the increase in tritiated thymidine incorporation will allow to demonstrate the stimulation of immune cells.

The first stage is the biopsy, to harvest the cells for transfection in vitro. This step requires 4 to 6 weeks. Then, cells are frozen after verification that all cells are positive for IGF-I and GFA. In case of cellular heterogeneity after cloning, only IGF-I positive population will be frozen and preserved. The transfected cells should not produce IGF-I. The cells before injection should be irradiated. The first authorization was granted in 1994 to 12 patients [30,41].

3.3. Methodology

Primary cultures of glioma cells were etablished from biopsies of human glioblastoma patients [40]. The human glioma cell lines were transfected with plasmid vector of the "triple helix" pMT-AG TH type [26]. Clones of transfected cells, no expressing IGF-I but expressing MHC-I and B7 molecules, were selected one month after transfection. Prior to injection the transfected cells were irradiated with cGy. The first injection was performed using only cell membranes [42,43] derived from 100 000 cells, followed by two cell injection of 1 – 2 million cells, with interval of one month each (subcutaneous injection in the left arm of patients). Blood was collected before the first “membrane” vaccination, and then 3 weeks after the first and second injection. The labeling of peripheral blood lymphocytes (PBL) was performed using mouse monoclonal antibodies directed against cell surface antigens. The samples of monoclonal antibodies were used for flow cytometric analysis as follows: FITC conjugated - (a) CD45, (b) of CD4, (c) CD3, (d) CD25, (e) CD45RO, (f) CD19 (g) CD8, (h) CD8CD11b +, (i) IgG1 control antibody, and these conjugated with PE - (a) CD14, (b) CD8, (c) CD16 + CD6, (d) CD4 (CD8), e) CD4 (CD8), (f) CD5, (g) CD8CD11b-, (h) CD8CD28, (i) IgG2 (18,27) [43].

4. Clinical results

The promising results were obtained in six patients at University Hospitals of Cleveland, in two patients in Bangkok and in four patients at the University Hospital of Bromberg. Admitting that the group of glioblastoma patients treated with antisense/triple helix cell injection has given the significant results, comparatively studied two cases of colon cancer and two cases of prostate cancer patients were treated, after surgery and radiotherapy with this type of “cellular therapy (Hospital of Bromberg).

Significant changes were observed mainly after the first cell vaccination. Phenotypic changes in peripheral blood lymphocytes were as follows: an increase in the percentage of CD8 + T cells accompanied in parallel by an increase of CD8 + CD11b- and CD8CD28 + molecules, after each

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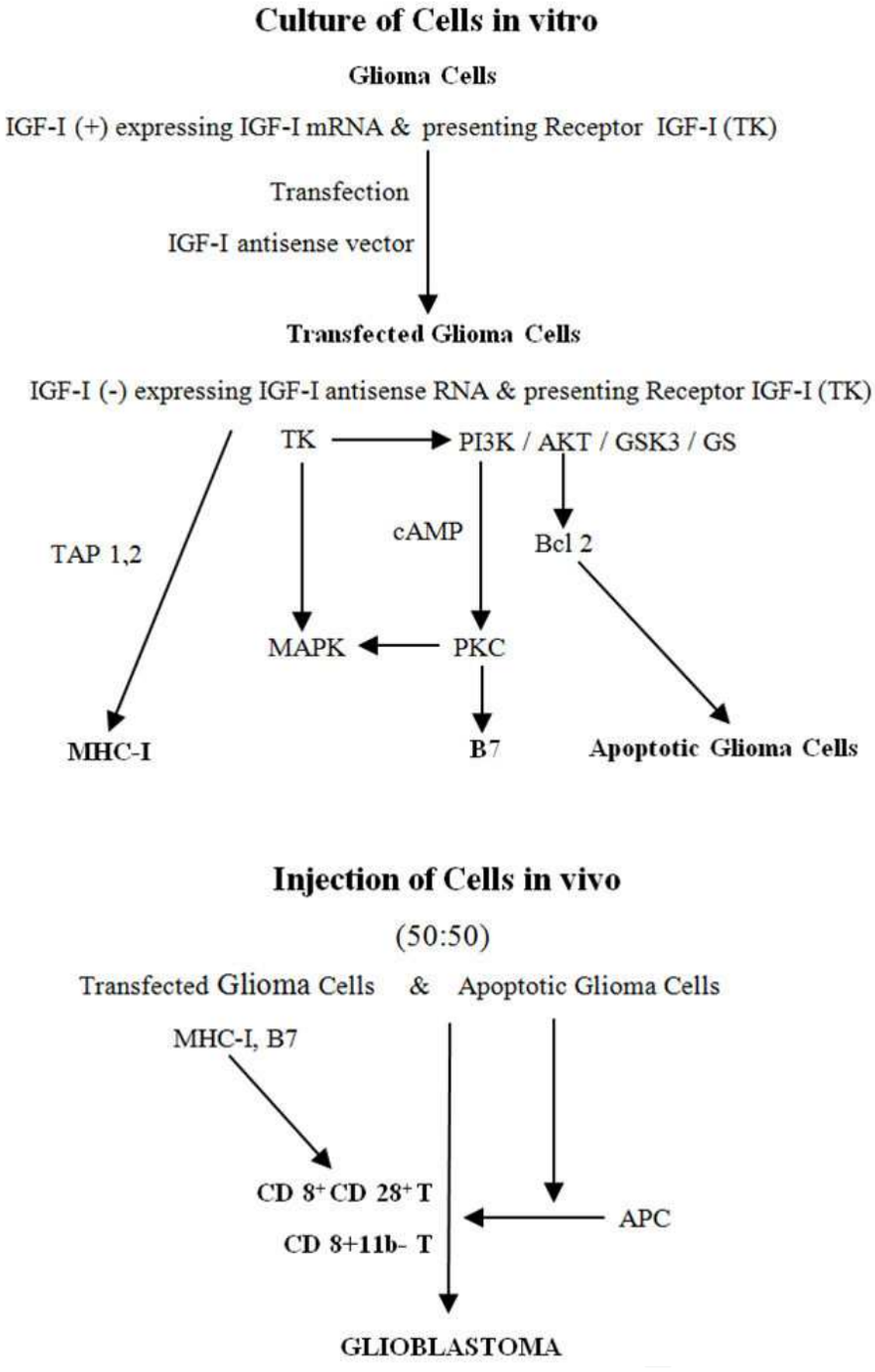

Figure 1. Antisense anti – IGF-I therapy. After *in vitro* transfection of tumor cells with a vector containing IGF-I cDNA in antisense orientation, the cells express IGF-I antisense RNA; the cells become negatively labeled with anti IGF-I antibodies, and positively with anti MHC-I and B7 antibodies. On the other hand, about half of these transfected cells become apoptotic. Both immune and apoptotic phenomena are related to signal transduction pathway. The injected transfected cells including apoptotic cells, together with induced *in vivo* APC cells, activat T lymphocytes (CTLCD8 + CD28 +). Activated CTL produce an antitumor immune response [40,44,47,61,79,80-83]. Abreviations: TAP 1,2 (transporter associated with antigen processing antigen); TK (tyrosine kinase); PI3K (phosphatidyinositol 3 kinase); PDK1 (phosphoinositide-dependent kinase 1); AKT (PKB, protein kinase B); Bcl 2 (key molecule of apoptose); GSK3 (glycogene synthetase kinase 3); GS (glycogene synthetase); MAPK (MAP kinase – mitogen activated protein kinase); PKC (protein kinaseC C).

of the three vaccinations. This alteration may reflect the increased activation of T cells cytotoxic blood (Figure 1.). Additionally, it was observed an increased percentage of lymphocytes positive for surface receptor of interleukin-2 (CD25). No changes were demonstrated in other CD molecules [44]. In our ongoing work (new protocol), 4 and 5 injections of IGF-I TH cells were introduced in patients with glioblastoma. After the fourth injection, the samples of the blood obtained from treated patients have shown a progressive increase in CD8 and NK cells, which highlights the effect of treatment on immune response. Also an increase of CD25 molecules was observed after the second and third injection. There was no difference before or after vaccination in the levels of CD3, CD16 + CD56, CD19, CD5, CD45 and CD14. The only side effect was a fever of 38°C noted after cell vaccinations. That probably corresponds to a cellular immune response (T cell induction). These changes may reflect the increased activation of cytotoxic T cells [36,45-47].

One glioblastoma patient who was treated at University Hospital of Cleveland, had lived 24 months from the time of diagnosis. Among other five glioblastoma patients treated in U.S.A. (University Hospital of Cleveland), two of the patients have survived 19 months. The treatment in U.S.A. has shown that the number of cell vaccines (between two and four) was not related to median survival of the patients. Another group of three patients treated in the U.S.A. have not responded so positively to therapy. These patients had an advanced disease with cerebral edema before gene therapy and were also treated with high doses of decadron or related steroids to reduce the effect of edema in CNS. Of course, this additional treatment has produced a negative effect on immune response and survival of patients. In two of the four patients with glioblastoma multiforme treated in Bromberg (NATO Science Program - USA / France / Poland / Germany), the average survival has ranged from 19 to 24 months, whereas the two patients in the control group survived 9.5 and 10 months respectively. Histopathological examination of removed glioblastoma tumors has shown that subjects had developed a necrosis around the tumor. Moreover the necrotic tissue surrounding the tumor has shown an infiltration of CD4 + and CD8 + T lymphocytes [32]. As to colon and prostate cancer patients the period of 19 months was also chosen as the end of clinical observations in all treated cancer patients. At 19 months, all these cancer patients were alive and the treatments were well tolerated. The PBL labelling results were similar to those obtained with glioblastoma patients (Figure 2. A,B,C) [48].

5. Discussion

The significant clinical findings were published in 2006/2012; it was demonstrated that due to the use of AS anti - IGF-I therapy following radiotherapy, the median survival of glioblastoma patients has reached 21 months. In 2010 we have reported that this survival could be explained by the immune anti-tumor response related to the increase of CD28 molecules in PBL cells analyzed after every of two "vaccinations". Moreover, this phenomenon was also observed in other tumors studied (four cases of liver cancer, colon, ovary, uterus and prostate). Phenotypic changes in peripheral blood lymphocytes (PBL) were clearly observed in all types of cancer treated with "cell therapy" - after every vaccination, an increase of CD8 + T lymphocytes,

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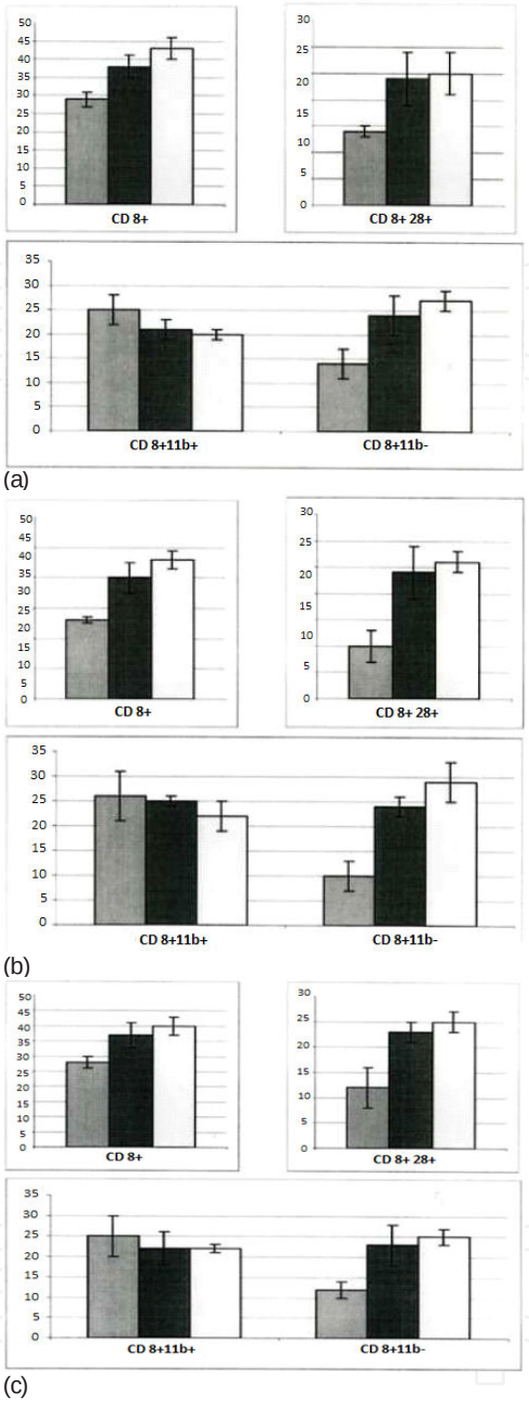

Figure 2. Flow cytometric "FACS" peripheral blood lymphocyte CD marker patterns following comparative therapies in cancers of neuroectodermal origin - glioblastoma multiforme (A), entodermal origin – colon adenocarcinoma (B), and mesodermal origin – prostate adenocaarcinoma (C). CD molecules were labelled in peripheral blood lymphocytes (PBLs) obtained from prevaccinated and "vaccinated" patients. Each of the first column corresponds to data obtained before vaccinations; each second and third column corresponds to data obtained after one and two successive cellular vaccinations (IGF-I antisense/triple helix cells). Bar graphs represent the median value of the two cases. Data are expressed as percent of positive cells when compared to the isotype control. Difference in percentage of CD8+ CD11b– and CD8+ CD28+ subpopulations from the relevant patients before and after vaccination was strongly significant with a range of *P* from 0.001 to 0.02 according to the Student's *t*-test. The *P* value for CD8+, CD8+28+, and CD8+11b– (below 0.01) is illustrated in the bar graph for statistical significance.

particularly of CD8+11b-, accompanied by a characteristic shift of CD8+11b+ to CD8+11b- (Figure 2.). This phenomenon was practically not significant in a group of patients treated only with "membrane" injections (as applied to two patients with glioblastoma) [47,48].

Glioblastoma and other malignancies were recently successfully treated with antisense therapy focused on TGF beta [49], using either antisense anti TGF beta expressing vector [50,51] or direct use of antisense oligodeoxynucleotides [52,53]. The use of phosphorothioate antisense oligonucleotides TGF beta2 (AP-12009, trabedersen) was initiated in patients with tumors that showed overexpression of TGF beta, such as high-grade gliomas - anaplastic astrocytoma (AA) or glioblastoma. Treatment was well tolerated. In 2007, the overall survival time was about 24 months, and in the control group, survival was 20 months. Recently, in three phase I/II studies and a randomized, active-controlled dose-finding phase IIb study, trabedersen treatment of high-grade glioma patients with recurrent or refractory tumor disease led to long-lasting tumor responses and so far promising survival data [52,54,55]. The results of clinical trials with other tumors that overexpress TGF beta have also been published recently [52,56]. Recently, the antisense approach, using also antisense oligonucleotides, targeting tumor neovascular trimer protein, laminin-411, was also proposed for clinical trial [57]. The innovation concerns a polymeric nanobioconjugate drug based on biodegradable, nontoxic, and nonimmunogenic polymalic acid as a universal delivery nanoplatform; this platform is applied for synthesis of nanomedicine drug which passes through the blood brain tumor barrier and tumor cell membrane. Other approach of antisense treatment, especially using antisense IGF-I-receptor has been developed [58-60]. It seems that this therapy, could be more efficient if the "cell vaccines" would be prepared after cell cloning for the expression of MHC-I. The different examples of antisense strategy in experimental and clinical trials of gliomas are showed in Table 1.

IGF-I	Antisense vector Clinical trial	Trojan et al. Biomed & Pharmacother 2010; 64(8):
TGF beta & T cell therapy	Antisense oligodeoxynucleotide Clinical trial	Dietrich et al. Curr Opin Oncol 2010; 22(6):604
Laminin-411	Antisense oligodeoxynucleotide Clinical trial	Ding et al. Proc Natl Acad Sci USA 2010; 107(42):18143.
AKT2	Antisense oligonucleotide Experimental therapy	Zhang et al. Oncol Rep 2010;24(1):65.
EGFR	Antisense oligonucleotide Experimental therapy	Li et al. Oncol Rep 2010; 23(6): 1585.
PED/PEA-15 (ERK1/2-interacting protein)	Antisense oligonucleotide Experimental therapy	Botta et al. Hum Gene Ther 2010; 21(9): 1067.
miR-21 & 5FU	Antisense oligonucleotide Experimental therapy	Ren et al. J Biomater Sci Polym Ed 2010; 21(3): 303.
miR-21	Antisense oligonucleotide	Zhou et al. Lab Invest. 2010; 90(2): 144.

IGF-I	Antisense vector Clinical trial	Trojan et al. Biomed & Pharmacother 2010; 64(8):
	Experimental therapy	
EGFR	Antisense oligonucleotide Experimental therapy	Kang et al. J Biomed Mater Res A 2010; 93(2): 585.
VEGF	Antisense (vector) Experimental therapy	Yang et al. J Neurooncol 2010; Aug 26 Epub
miR-21	Antisense oligonucleotide Experimental therapy	Zhou et al. Oncol Rep 2010; 24(1): 195.
Telomerase and tamoxifen	Antisense oligonucleotide Experimental therapy	Wang et al., Mol Med Report. 2010; 3(6): 935.
c-Met	Antisense oligonucleotide Experimental therapy	Chu et al. Oncol Rep 2010; 24(1): 189.
IGF-I	Antisense vector Clinical trial	Trojan and Anthony, Curr Signal Transd Ther. 2011; 6(3): 411.
TGF beta	Antisense oligodeoxynucleotide Clinical trial	Jashinsky et al., Curr Pharm Biotechnol. 2011; 12(12): 2203.
TGF beta	Antisense oligodeoxynucleotide Clinical trial	Hau et al., Curr Pharm Biotechnol. 2011; 12(12): 2150.
miR-10b	Antisense oligonucleotide Experimental therapy	Sun et al., Brain Res. 2011;1389: 9
micro RNA-7	Antisense oligonucleotide Experimental therapy	Lee et al., Radiother Oncol. 2011; 101(1):171.
uPAR	Antisense vector Experimental therapy	Raghu et al., Mol Cancer. 2011; 10: 130.
IGF-I	Antisense vector Clinical trial	Trojan et al., Chem Res Pract. 2012; doi:10.1155/2012/721873
miR-221/222	Antisense oligonucleotide Experimental therapy	Hao et al., Oncol Rep. 2012; 27(5): 1504.
miR-143 and miR-145	Antisense oligonucleotide Experimental therapy	Koo et al., BMC Cancer. 2012; 12: 143.
miR-1275	Antisense oligonucleotide Experimental therapy	Katsushima et al., J Biol Chem. 2012 Jun 26. [Epub]

Table 1. Examples of experimental and clinical gene therapies of gliomas using antisense technology (selection of articles of last two years).

In the strategies of anti TGF-beta and anti IGF-I and anti IGF-IR antisense techniques, the anti-tumor immune response was designated as a primary mechanism involving growth factors inhibited by antisense technology and its signaling pathway [37,51]. As PI3K/AKT/GWK3/GS pathway is considered in antisense mechanism, it was recently demonstrated that also

antisense anti-glycogen synthase (GS) cells express MHC-I molecules [44,61,62]. The IGF-I, through its binding to IGF-IR, activates the PI3K/AKT transduction cascade related to the pathway of apoptosis (IRS/PI3K/AKT/Bcl or AKT / Ca2 + or GSK3 or caspases). The end result of IGF-I AS approach involves an inhibition of the pathway elements of TK/PI3K/AKT inducing an *in vivo* immune response mediated by CD8 T cells and APC cells (Figure 1.) [44].

6. Conclusions

This review draws attention to the recent studies in cancer gene therapy, particularly of glioblastoma treatment using anti - gene anti IGF-I approach. Although the number of clinical trials of "antisense" type is much lower than those of pre-clinical experimental therapies, we wish to emphasize that any experimental therapy is a potential clinical trial (Table 1.). The current clinical strategies for the treatment of gliomas are usually a combination of chemo-therapy and use of different types of inhibitors (imatinib, gefitinib) including antibodies (i.e. avastin), targeting growth factors and their receptors [63-66]. The new therapies are now focusing on technology of inhibitors and antigene techniques (antisense or triple helix) used alone or combined with drug treatment [67-71]. A pharmacological strategy - the use of temozolomide introduced by R. Stupp, has offered a new hope for treatment of glioblastoma. However, although median survival has reached almost two years, we are still far from victory [72,73]. The new strategies proposing to target different growth factors, especially IGF-I, TGF-beta or VEGF, their receptors and signaling pathway elements, seem to offer a promising solution [54,73-76]. We would like to underline that the research on IGF-I which has resulted in diagnostic application – the IGF-I being considered as one of the principal precancerous markers [10,12], has conducted to experiments on suppression of IGF-I expression in tumors, following directly by immuno-gene therapy of malignant tumors. Gene therapy, particularly cellular imuno-gene therapy, and cellular immunotherapy are currently among the most promising approaches for treatment of cancer diseases [66,68,77,78].

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