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Research Article

Results of Modern Molecular-Genetic Research for Children Gliomas

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Abstract

Introduction: Glioblastomas are the most frequent malignant neoplasm among primary brain tumors in the childhood. Despite the advances in neurosurgery, radiotherapy and chemotherapy, with combination in multimodal approach, overall survival of such patients remains poor and rarely exceeds 10 to 14 months. Several attempts in use of targeted agents such as gefitinib in unselected patient population showed insufficient efficacy. In general perspective the most interesting approach is the selection of patients for targeted therapy based on predictive markers of response.

Methods: We performed the comprehensive analysis of mutational pattern of 30 child high grade brain tumors, due to WHO-classification 2016. Our research was based on the results of mass spectrometry method (OncoCarta, Sequenom, USA), which estimates 298 known mutations in 19 genes.

Results: We revealed 10 mutations in 9 tumors (30%). Mutations were found in BRAF, CDK, HRAS, EGFR, FGFR, MET and PI3K. The most mutated pathway was EGFR - in 20% of the samples (6/30).

Conclusions: Derived results seem to be very promising in usage of new targeted agents, including BRAF-inhibitors, on the final stage of clinical evaluation.

Keywords: Children Glial Tumors; Mutations; Targeted Therapy; Mass Spectrometry Sequencing

Introduction

Intracranial tumors are a group of the most frequent solid neoplasms in children. Annually, primary brain tumors (UGM) in children are detected in 4.3 cases per 100,000 child and more than 40% neoplasm are represented by gliomas with low degree of malignancy [1-3]. The standard treatment of children with glial tumors includes the most radical surgical removal of the neoplasm, as well as adjuvant radiation (LT) and drug therapy (HT) [4]. Despite the use of modern methods of diagnosis and treatment, the 5-year survival rate of patients does not exceed 5-10%, and recurrence of the tumor within a short time after specialized treatment is in most cases recognized as inevitable [5,6]. One of the reasons for the low results of therapy of those patients is the low possibility for surgical removal of microscopic tumor foci, not visualized with modern diagnostic techniques [4,7,8].

Currently, the most promising therapeutic approach with UGM is the combined use of targeted drugs blocking various signal cascades, which plays a determining pathogenetic role in cessation of tumor development [5]. Today it is well-known fact that the pathogenesis of gliomas, as well as other solid tumors, due to various biological processes, based on the activation process in various signaling cascades. The receptors of growth factors, as well as the bases of angiogenesis, are most studied in this group. However, the results of numerous studies using drugs with a targeted mechanism of action, taking into account a specific target, but without prior selection of patients, did not show significant results [9].

Complex assessment of the profile of molecular genetic disorders is considered to be an extremely promising direction for the selection of optimal therapy [10]. The results of studies conducted in adults with UGM revealed numerous violations of signal cascade activity with significant number of potential targets for target drugs [11,12].

It seems relevant to evaluate the profile of molecular genetic disorders in children with UGM in order to identify potential candidates for target therapy with a directed mechanism of action.

Materials and Methods

The study included 30 patients (20 (66%) boys and 10 (34%) girls, average age 9.9 yrs, from 1 to 17 years). Repeated analysis of all removed tumors was performed by an independent certified pathologist and classified according to WHO-classification 2016 [13]. The characteristics of patients are presented in table 1.

About 70% of tumors had high grade: G_{III} in 47.0% of cases, G_{IV} - 23.0% (Table 1). The remaining 9 tumors are regarded as low-grade neoplasms: G_{I} - in 20.0% and G_{II} - in 10.0% of the samples. From the point of view, the variant with the histological structure, the most frequently were pleyomorphic and anaplastic astrocytomas (30%). We founded 6 picolitre astrocytoma's (20%) and 2 epithelioid glioblastomas. Most tumors (74.0%) localized supratentorial, 3 - in the brainstem and 5 - in the sub tentorial structures. Regardless of the location, 15 (50,0%) neoplasms were removed sub totally and 10 (34.0%) resected radically, in 5 (16%) cases the tumor was removed partially (Table 1).

Researched indicators	Number of pts abs. (%)
Gender	
Men	20 (66,0)
Women	10 (34,0)
Variants of histological structure	
Anaplastic astrocytoma	9 (30,0)
Pleyomorphic astrocytoma	2 (6,6)
Pilomixoid astrocytoma	2 (6,6)
Pilocitar astrocytomas	6 (20,0)

Anaplastic oligoastrocytomas	9 (30,0)
Epithelioid glioblastoma	2 (6,6)
Degree of malignancy	
I	6 (20,0)
II	3 (10,0)
III	14 (47,0)
IV	7 (23,0)
Nature of surgical treatment	
Radical	10 (34,0)
Subtotal	15 (50,0)
Partial	5 (16,0)
Localization in the brain structures	
Sub tentorial	5 (16,0)
The trunk	3 (10,0)
Supratentorial	22 (74,0)

Table 1: Clinical characteristics of patients (n = 30).

DNA extraction was carried out from the tumor material obtained after surgical treatment and passed the standard procedure of histological examination in the pathomorphological laboratory. After histological verification of tumor, 2 - 4 sections of tumors from paraffin blocks, 15 μm thick, were applied to slide glasses. The selection of regions with the maximum content of tumor cells was performed by the method of stereotactic dissection, under the control of light microscopy. At this stage included samples of neoplasms, containing more than 40% of tumor cells. To obtain DNA from sections of paraffin blocks, we used a standard technique based on tissue cleavage with proteinase K, followed by the isolation of nucleic acids by the phenol-formalin method [14]. The volume of the obtained DNA evaluated with spectrophotometry. Subsequently, the nucleic acid solutions were diluted with distilled water to a concentration of 10 mg/ml.

Molecular genetic analysis was performed on the basis of a platform developed by Sequenom. In this work we used the mutation panel OncoCarta v1.0, which included 24 pools of pairs of primers and their corresponding 24 pools of "expanding" primers [15], which allowed to determine 298 mutations in 19 genes (Table 2).

Gene	Verified mutations
ABL-1	G250E, Q252H, Y253H, Y253F, E255K, E255V, D276G, F311L, T315I, F317L, M351T, E355G, F359V, H396R
AKT-1	V461L, P388T, L357T, E319G, V167A, Q43X, E17del
AKT-2	S302G, R371H
BRAF	G464R, G464V/E, G466R, F468C, G469S, G469E, G469A, G469V, G469R, G469R, D594V/G, F595L, G596R, L597S, L597R, L597Q, L597V, T599I, V600E, V600K, V600R, V600L, K601N, K601E
CDK-4	R24C, R24H
EGFR	R108K, T263P, A289V, G598V, E709K/H, E709A/G/V, G719S/C, G719A, M766_A767insAI, S768I, V769_D770insASV, V769_D770insCV, D770_N771 > AGG/V769_D770insASV/V769_D770insASV, D770_N771insG, N771_P772 > SVDNR, P772_H773insV, H773 > NPY, H773_V774insNPH/PH/H, V774_C775insHV, T790M, L858R, L861Q, E746_T751del, E746_A750del, E746_T751del, E746_T751del, S752D, L747_E749del, L747_T750del, L747_S752del, L747_T751del, L747_S752del, P753S, A750P, T751A, T751P, T751I, S752I/F, S752_I759del, L747_Q ins, E746_T751del, I ins (combined), E746_A750del, T751A (combined), L747_E749del, A750P (combined), L747_T750del, P ins (combined), L747_S752del, Q ins (combined)
ERBB2	L755P, G776S/LC, G776VC/VC, A775_G776insYVMA, P780_Y781insGSP, P780_Y781insGSP, S779_P780insVGS
FGFR-1	S125L, P252T
FGFR-3	G370C, Y373C, A391E, K650Q/E, K650T/M
FLT-3	I836del, D835H/Y
JAK-2	V617F
KIT	D52N, Y503_F504insAY, W557R/R/G, V559D/A/G, V559I, V560D/G, K550_K558del, K558_V560del, K558_E562del, V559del, V559_V560del, V560del, Y570_L576del, E561K, L576P, P585P, D579del, K642E, D816V, D816H/Y, V825A, E839K, M552L, Y568D, F584S, P551_V555del, Y553_Q556del
MET	R970C, T992I, Y1230C, Y1235D, M1250T
PDGFRa	V561D, T674I, F808L, D846Y, N870S, D1071N, D842_H845del, I843_D846del, S566_E571 > K, I843_S847 > T, D842V
PIK3CA	R88Q, N345K, C420R, P539R, E542K, E545K, Q546K, H701P, H1047R/L, H1047Y, R38H, C901F, M1043I
H-RAS	G12V/D, G13C/R/S, Q61H/H, Q61L/R/P, Q61K
K-RAS	G12C, G12R, G12S, G12V, G12D, G12A, G12F, G13V/D, A59T, Q61E/K, Q61L/R/P, Q61H/H
N-RAS	G12V/A/D, G12C/R/S, G13V/A/D, G13C/R/S, A18T, Q61L/R/P, Q61H, Q61E/K
RET	C634R, C634W, C634Y, E632_L633del, M918T, A664D

Table 2: Analyzed genetic changes.

Each pool included 5 - 9 pairs of primers for polymerase chain reaction (PCR). Mutations were detected in several stages, according to the OncoCarta Panel v1.0. The reaction volume was 5 μl, included 0.1 U Taq polymerase, 5 ng of genomic DNA, 2.5 pmol of each of the PCR primers, and 2.5 μmol of dNTP (deoxynucleotide triphosphate). The PCR was started with a 10-minute Taq polymerase activation at 95°C; To accumulate the PCR product, 45 cycles of amplification were carried out (denaturation: 15 seconds at 95°C, annealing: 30 seconds at 57°C, synthesis: 30 seconds at 72°C). The primers and probes used for standard and expansion amplification were the part of the work kit. A detailed description of the reagents used has been described in 2007 by RK Thomas., *et al.* [15].

In phase II, all unused dNTPs were degraded by shrimp alkaline phosphatase, 0.3 U of the enzyme was added to each. The solution incubated for 4 hours at 45°C. The primers were expanded by the addition of 5.4 pmol of dilating probes for each of the amplicons, with 50 μmol of the appropriate dNTP/ddNTP combina-

tion and 0.5 U of the thermosequencing DNA polymerase. The cycle of this reaction began with a 2 minute activation of the enzyme at 95°C, followed by 40 amplification cycles (denaturation: 5 seconds at 940C, annealing: 5 seconds at 50°C, synthesis: 5 seconds at 72°C). After removal the residual salts from the solution, by means of a cation-exchange pitch, 7 µl extended amplicons were transferred to the Spectro CHIP chip. The resulting array was analyzed using a mass spectrometer (Spectro READER, Sequenom).

Results

The analysis of the frequency of mutations in sections of paraffin blocks for children's UGM revealed 10 mutations in 9 of 30 samples.

Patient A. (m), 15 y.o. with morphological report: "Tumor (epithelioid glioblastoma GIV) of the left parietal lobe of the brain" carried out a molecular genetic study (CU-22012SQ10-01). Mutation (A289V) in the EGFR gene was detected (Figure 1).

Figure 1: Micro Preparation of tumor of patient A., 15 y.o. Histological report (3578-68): epithelioid glioblastoma.

Patient B. (m), 2,3 y.o. was under observation with the diagnosis: "Giant glioma of chiasma and optic nerves. Occlusive triventricular hydrocephalus". After finalizing the surgical part of the combined treatment the patient was subjected to a molecular genetic study (CU-22015SQ10-01). A mutation (V600E) in the BRAF gene was detected (Figure 2).

The revealed changes at our patients are presented at the table 3.

The changes in 2 genes were simultaneously detected only in one case of the tumor tissue. The following molecular disturbances were detected in EGFR (N771_P772 > SVDNR, A289V), BRAF (V600E), HRAS (G13S) and PI3K (N345K, H1047R). In addition, mutations have been detected in receptors that determine the signaling cascades associated with EGFR: FGFR3 (K650M) and MET (T992I).

Figure 2: Micro Preparation of tumor of patient B., 2,3 y.o. Histological report (3062-67): pleomorphic astrocytoma.

Performing the statistical analysis by Pearson method, we revealed correlations between clinical characteristics (age, sex, tumor size, the nature of surgical treatment) and molecular-genetic changes, no significant patterns in the incidence of mutations in general and their individual species.

Discussion

For the first time on the population of Russian patients, we carried out a comprehensive assessment of the molecular changes in signal cascades in glial tumor cells in children. Activating molecular disorders were detected in 9/30 samples (30.0%), which is a clinically significant frequency, determines the expediency of further study for this issue.

In the selected samples we identified several potentially activating genetic disorders. The EGFR gene was changed the most frequently. Thus, in 2 cases, a mutation in exon 20 (N771_P772 > SVDNR) was detected, and in 1 case - in the exon 7 of the extracellular domain (A298V). While the possibility of the existence of the first type of mutation has already been shown for primary UGM, a point-to-point measurement in 771 positions has been revealed for this type of tumors for the first time [16,17]. In addition, violations in underlying signaling molecules involved in the signal transmission from EGFR, in particular PI3K, have been revealed.

As shown earlier, the presence of mutations of PI3K may increase the proliferative activity of tumor cells [18]. Among the molecular disorders already known, the R88Q and H1047R discovered by us are relatively rare [19]. In addition, another type of rare molecular disturbances of PI3K - N345K, which have an activating value for some nonglial malignant tumors, has been identified [20-22].

The shunting EGFR-linked molecular pathway is the hepatocellular growth factor (cMET) signaling cascade. Several mechanisms of changes in the activity of this molecule have been described [23,24]. Examples include amplification, auto- and paracrine activation, as well as mutational disorders. In our study, a mutation of T992I, also known as T1010I [25], was detected in 1 case. The pathogenetic significance of this disorder has not been established yet, since that does not lead to change in the phosphorylation of underlying signaling molecules. Nevertheless, the observation of T992I in glial tumors was performed for the first time, according to available medical literature.

A study using OncoCarta v.1.0 on the genetic material of children's brain tumors of glial nature demonstrated the possibility of determining mutations of BRAF previously described for lung tumors, skin melanoma [26,27]. We found the most frequent variant of violations of this gene - V600E (14.5%), which exceeds the similar index for UGM of low degree of malignancy.

Among other molecular disorders, it is necessary to note the mutation of fibroblast growth factor 3 (FGFR3) K650M, which was previously described for non-muscle-invasive bladder cancer, identified in our study. In addition, there was no mutation in the PDGFR gene, which is confirmed in studies of previous years, with low-grade UGM [28].

In addition to activating disorders of pathogenetically important signaling cascades, we show violations of the fundamental mechanisms of cell division. R24C, the only well-known mutation of the CDK4, which determines the entry of the cell into the G1 phase, is revealed in 1 case. A similar type of impairment was already shown earlier in adults gliomas [29]. The activating mutations revealed in the BRAF, EGFR, FGFR and PI3K genes confirm the need for a thorough study of the molecular genetic features of childhood tumors.

Conclusion

It is highly desirable to implement modern techniques for highly effective screening of brain tumors in childhood patients, using Oncocarta v1.0 (Sequenom, USA). This is relevant for the activities of oncopedics and neurosurgeons, since it will help to widely use the possibilities of adjuvant treatment for patients who have undergone non-radical operations. In turn, the application of the results obtained in clinical practice will help to increase the overall survival rate of children with newly diagnosed malignant brain tumors.

Summary

Glioblastomas are the most frequent malignant neoplasms among primary brain tumors in children. Despite the development of a comprehensive therapeutic approach (inc. surgical, radiation and chemotherapy methods), the overall survival of patients with newly diagnosed tumors remains extremely low, not exceeding 14 months. Using targeted drugs, particularly gefitinib, the general population of patients demonstrated low clinical efficacy. The most promising at the moment is to determine the population of patients potentially susceptible to the action of targeted drugs, taking into account the definition of predictive molecular markers. In this work mutational changes of 30 children's glioblastoma tissues have been determined. Data analysis based on the results of mass spectrometric sequencing (OncoCarta v1.0, Sequenom) made it possible by determination of 298 mutations in 19 genes, while we identified 10 mutations in 9 tumors (30%). The changes were revealed in the genes BRAF, CDK, HRAS, EGFR, FGFR, MET and PI3K. Most often (6/30, 20% of tumors) mutations were detected in participants of the signal cascade EGFR. The obtained results allow us to discuss the potential possibilities of evaluation of the new targeted drugs, including BRAF inhibitors, for treat children with gliomas.

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