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The Infrequent Simultaneous Genetic Alterations in Glioblastoma Multiforme (LOH 10, 17, 19q, TP53 mutation and EGFR amplification) with Short Clinical Course

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We described the case of an unusual, complex genetic alteration in 57 year-old male patient with glioblastoma multiforme (GBM) with short survival (6 and half months). Alterations consisted of p53 mutation, LOH 10, LOH 17, LOH 19q and EGFR amplification. LOH1p, LOH 9 and LOH 13 were negative. Immunohistochemical study did not correlate with molecular results. The overexpression of TP53 protein and RB protein was detected only in small percentage of cells and interestingly the overexpression of EGFR was present only focally. Immnunostainings for PTEN, P16, PI3-K were negative. Additionally, we observed an overexpression of IGFB2 protein. This case indicates the accumulation of molecular changes in glioblastoma multiforme in patient with short survival.

Introduction

Glioblastoma multiforme (GBM, WHO GIV) is the most common and most aggressive primary tumors of the central nervous system in adults. Median survival of GBM patients is 10–12 months [5, 34]. According to the classical genetic pathway theory glioblastomas can be subdivided into primary (de novo) and secondary tumors, reflecting the progression of low grade astrocytomas during the multistep carcinogenesis [18]. The main molecular hallmarks of primary glioblastoma is EGFR amplification with loss of heterozygosity (LOH) on chromosome 10q, and MDM2 amplification, p16 deletion and PTEN mutation [22], further overexpression of IGFBP2 [11]. In secondary glioblastoma the main molecular hallmark are TP53 mutations, LOH on 17p, 10q and 19q [3, 18]. Because in GBMs, TP53 mutation and/or LOH 17p and EGFR gene amplification occur in a mutually exclusive fashion, thereby two genetic subsets were defined [42, 45]. The clinical relevance of the existence of the coexpression of TP53 mutations and EGFR amplification is still unclear.

LOH 10q is the most common alteration in GBMs and was detected in over 80% of tumours [12, 25]. Amplification of EGFR is described in over 60% of primary GBMs and 10% in secondary GBM cases [44]. TP53 mutations were identified in 27%–31% of unselected GBM [2, 28] and is more frequent in secondary glioblatomas (63%) [28].

PTEN gene, identified on chromosome 10q23.3 is a suppressor gene inactivated in multiple cancer including glioblastoma multiforme (20–44% of cases) [48]. The expression of wild-type (normal) PTEN leads to cell cycle arrest in G1 phase, mainly in the P27/Kip1 related pathway. It negatively regulates the phosphoinositide 3-kinase (PI3-K) signaling pathway by dephosphorylating the 3' position of phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) [26] and inactivation of kinase PKB/Akt [23]. This pathway is presumably unrelated to TP53 [13,24]. P27 and P21 belong with P57 to Cip/Kip families of suppressor genes, and are cyclin dependent kinase inhibitors [39].There is some evidence, that dysregulation of PTEN/PI3K/p27 pathway in gliomas and glioblastomas is associated with aggressive clinical behavior [9]. Moreover, PI3-K target therapy seems to be one of the most promising in gliomas.

Overexpression of insulin-like growth factor binding protein 2 (IGFBP2) was recently found as a most frequent

alteration in advanced stage of gliomas [11, 47]. IGFBP2 is crucial in determining the phenotypes of advanced stage of tumors, such as increased cell proliferation and invasion [44]. However, in our knowledge, the significance of immunohistochemical overexpression of this protein is not well established in glioblastomas.

The significance of histopathological findings and molecular changes in GBM is still controversial regarding the influence on the prognosis and further on the modern therapy. Therefore, we evaluated the immunoexpression of the proteins involved in molecular pathways such as PTEN, PI3-K, IGFB2, P16, P21, P27, RB in glioblastoma case with complex molecular alteration and short survival.

We discuss this case with context of histological and immunohistochemical study and clinical history, and the possible prognostic factors that may influence the clinical outcome.

Clinical History

The 57 years old man patient was admitted to the Department of Neurosurgery; Copernicus Hospital, Łódź, Poland in March 2003 due to epileptic seizures and psychomotoric retardation. The CT scan revealed the tumor mass in the right frontal lobe. The patient underwent total tumorectomy 13.03.2003 and the clinical status was improved. The patient was treated by radiotherapy therapy along with chemotherapy (Temodal). After the period of 6 months patient had a relapse of the disease and was reoperated in 12.09.2003. The patient died 3.10.2003.

Material and Methods

PCR analysis

DNA for molecular study was isolated from frozen tumor tissue (at temp. –80^{o}C) and from blood samples (serving as normal control) by using the conventional proteinase K digestion and phenol/chloroform extraction method. (Approval of Bioethical Committee Nº RNN/192/03/KE).

PCR was performed in a 20 μl reaction mixture containing 50ng DNA, 1×PCR buffer, 50 μM dNTPs, 1.5 mM Mg Cl2, 0.5U Taq DNA polymerase, 0.5 μM each primer

PCR was carried as follows. An initial denaturation of 10 minutes at 95°C was followed by 35 cycles consisting of denaturation at 95°C for 1 minute, annealing at 51–62°C for 1 minute, extension at 72°C for 1 minute and final extension cycle at 72°C for 10 minutes.

Thermocycling conditions were individually established for each pair of primers.

PCR products were mixed with formamide-loading buffer and denaturated by heating for 10 minutes at 95°C and cooled on ice.

Exon 5–8 and 4 of the p53 gene were PCR amplified individually with oligonucleotide primers localized in entire coding region and in flanking intronic sequences.

LOH analysis

Approximately 0.3 μl of each sample were loaded on an 8% denaturing polyacrylamide gel and visualized with LiCor automatic sequencer. Paired blood and tumor DNA samples were analyzed for LOH using microsatellite markers on chromosomes 10q, 9p, 17p 13q using a microsatelites markers D10S587, D9S156, D17S675, D13S256, D9S162.

Amplification of EGFR

EGFR amplification was analyzed by PCR and visual examination of ethidium bromide-stained gels after gel electrophoresis.

Immunohistochemistry

Immunohistochemical study was performed using antibodies against p16 (clone F-12, dilution 1:100, Santa Cruz, Biotechnology), p53 (clone DO-7, 1:20, DAKO), Rb (NCL-RB-358, 1:50, Novocastra), EGFR vIII (clone H11, 1:200, DAKO), p21 (clone 4D10, 1:10, Novocastra), π27 (clone 1B4, 1:10, Novocastra) , PI3-Kinase p85á (clone B-9, 1:100, Santa Cruz), IGFBP2 (clone c-18, 1:100, Santa Cruz), PTEN (clone.1:200, Novocastra).

Paraffin-embedded sections of tumor were deparaffinized, rehydrated and heat-treated for citrate microwave antigen retrieval in 10 mM citrate buffer pH 6.0 at 350 WAT for 15 min and then cooled to room temperature. Sections were then blocked for peroxidases in 0.3% H_2O_2 in methanol for 30 min, and incubated with primary antibody for 30 min at room temperature in a humidity chamber. For detection, a DAKO ENVISION System HRP with DAB staining and hematoxylin counterstaining was used.

Results

Histopathological findings

The typical tissue pattern with the presence of highly anaplastic glial cells (Fig. 1a) with mitotic activity, microvascular proliferation with formation a so called glomeroid tuft vessels, necrosis in the form of large ischemic necrosis and pseudpalisading necrosis were seen (Fig. 1b). The recurrent tumor showed the more dense, cellular and anaplastic areas with focal presence of multinucleated giant cells (Fig. 1c).

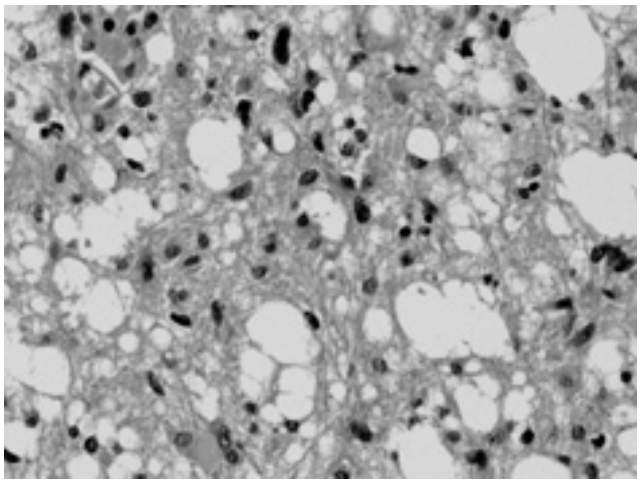

Fig. 1a. The histopathological pattern of primary glioblastoma multiforme with atypical astrocytic cells HE, Magn. 200 ×.

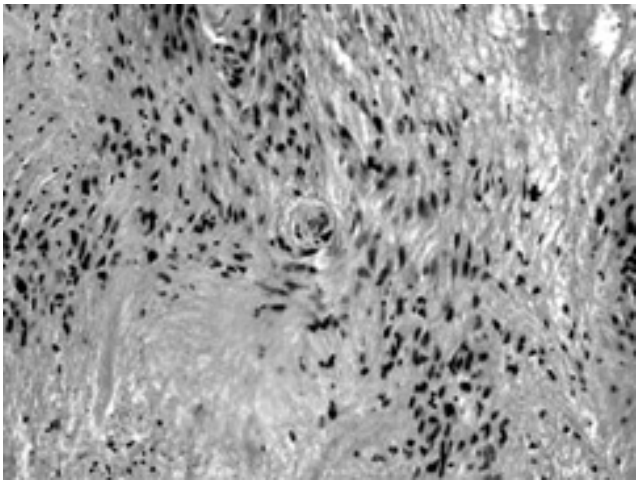

Fig. 1b. The primary tumour exhibits typical areas of palisading necrosis. HE, Magn. 100×.

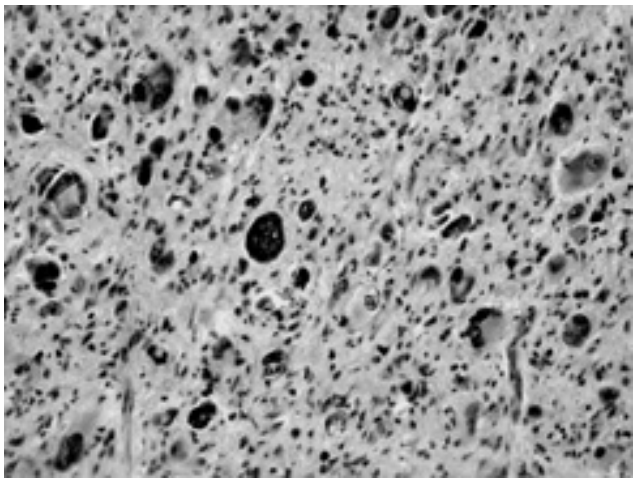

Fig. 1c. The recurrent tumour shows the most cellular and atypical areas with accumulation of neoplastic giant cells HE, Magn. 200 ×.

Molecular study

The TP53 missence mutation was detected in exon 8 codon 286 (arginin›tryptofan) (Fig. 2) along with the EGFR amplification, LOH 10 and LOH 17. LOH 9 and 13 was absent.

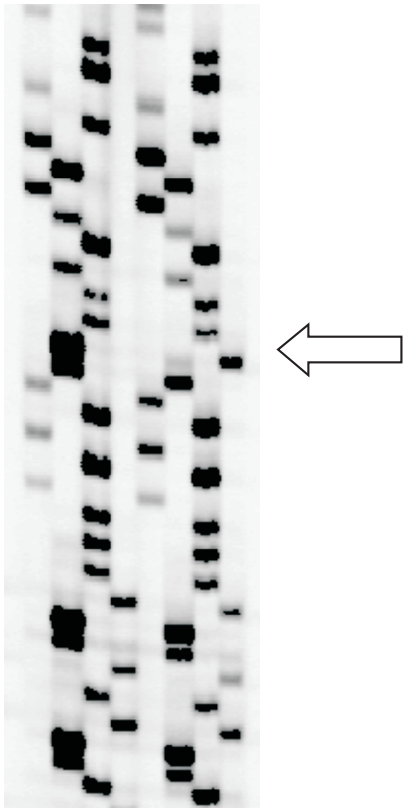

Fig. 2. The arrow indicates the point of missence mutation in codon 282 (CGG›TGG) of exon 8 of TP53 suppressor gene.

Immunohistochemical study

The p53 protein expression was detected in the nuclei of 10% of cells (Fig. 3a), RB in 20 % of tumor cells, EGFR showed only the focal positivity expression with a typical membrane staining pattern, p16 was positive in small percentage of tumour cells (below 5%), p27 was positive in 90% of cells (Fig. 3b) and p21 was negative, PI3K and PTEN was totally negative, IGFR was positive as a cytoplasmic reaction mostly in large neoplastic cells (Fig. 3 c). The Ki67 expression (indicated the proliferating index of neoplastic cells) was detected in only 10% of cells. The results of immunohistochemical study were identical in a paraffin tumor section from the recurrent tumour.

Discussion

The clinical significance of morphologic and genotypic heterogeneity in glioblastoma multiforme (GBM) is still unclear. The case presented here shows the infrequent simulta-

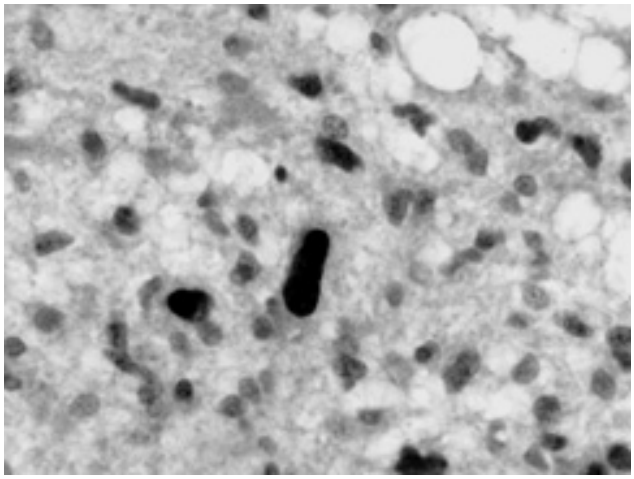

Fig. 3a. Nuclear accumulation of TP53 is marked mainly in large tumour cells, atni-TP53, DakoCytomation, Magn. 400 ×.

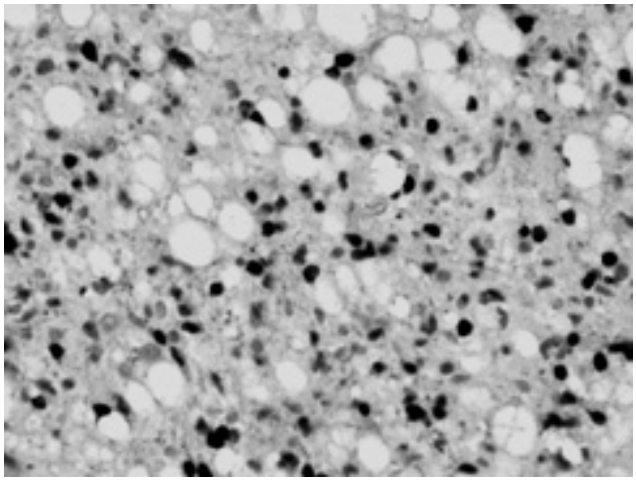

Fig. 3b. The wide distribution of nuclear staining with antibody against P27 in glioblastoma cells, Novocastra, Magn. 200 ×.

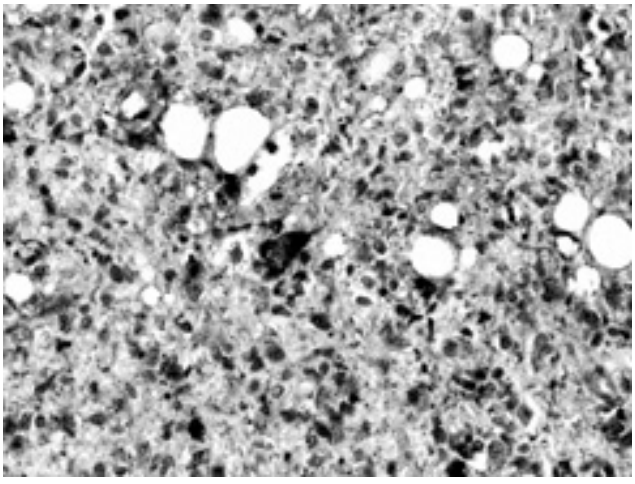

Fig. 3c. IGFB2 immunostaining in cytoplasm of a few cells in GBM. Santa Cruz, Magn. 400 ×.

neous genetic alterations such as mutation of TP53 along with the EGFR amplification, LOH 10 and LOH 17. This molecular complexity is not common but may be presented in a various configurations. According to the Ohgaki's et al. population study the copresentation of LOH 10q occurs typically with other genetic alterations such as TP53 mutations in 23,7%, EGFR amplification in 25,8%, p16 in 23,3%, PTEN in 16,2% [28]. This is in agreement with other findings, in which most GBM cases with EGFR amplification (9/10) had LOH 10q [40, 41, 42, 48]. The inverse association was observed between EGFR amplification , which is a hallmark of primary GBM, and TP53 mutations (7,5%), which is a hallmark of secondary GBM and PTEN mutations (5,8%) [28]. This finding is consistent with findings of other study [48]. The copresentation of EGFR amplification and P53 mutations was noticed sporadically in single cases of a usual type of glioblastoma [2, 29, 40, 45]. In addition, in glioblastoma developed in patients with Li-Fraumeni syndrome, TP53 mutation was accompanied with EGFR amplification along with 1q LOH (which is more frequently associated oligodendroglial component [32]).

Okada et al. demonstrated in FISH study that EGFR amplification occurs in the majority of glioblastomas with TP53 mutations in isolated cells, that remain below the level of detection of tumor lysate-based techniques such as Southern blotting and PCR [29]. Higher copy numbers of EGFR found at the periphery of the glioblastoma presumably provide a tumorigenic advantage for infiltrating the surrounding brain tissue [29]. Such heterogeneity may be explained by the presence of different amplified clone lines [33].

The existence of molecular heterogeneity is not manifested on the morphological level

and only in a few histopathological subtypes determines genetic alterations. Interestingly, the alterations may vary in different microdissected areas of GBM tumour tissues and they are increased in the high-grade pattern of tumour [8]. The correlation between frequent amplification of EGFR with a small cell phenotype or mixed cell was found. EGFR amplification was detected in 67% of exclusively small cell neoplasms, in 32% GBMs with both small cell and non-small cell areas, and only in 9% of non-small cell GBMs [8].

The giant cell type of glioblastoma with extremely bizarre monstrous multinucleated tumour cells exhibited the frequent TP53 mutations (75–90%) and PTEN mutations but the amplification and overexpression of EGFR is very rare (only 5%) [30]. In our case, the recurrent tumour had a focal accumulation of large, more anaplastic cells than in primary tumour, but didn't fit the criteria of giant cell glioblastoma (Fig. 1c). Further, glioblastomas with oligodendroglial component carry LOH on 1p and 19q in 40% and 60% of cases. These alter-

ations has recently been linked to chemosensitivity and good prognosis in anaplastic oligodendrogliomas and glioblastomas [15, 21].

All things considered, the rather typical histopathological pattern of this case didn't predict the complex molecular alterations. The rapid recurrence and short survival (6 and half months) of this patient provokes to the discussion about possible prognostic molecular and immunohistochemical factors in glioblastoma multiforme. The median overall survival in patients with glioblastoma multiforme despite the therapeutic efforts is 10–12 months; the better survival is determined mainly by age, better performance status, extensive surgery, and high-dose postoperative radiotherapy [5, 34].

The most of clinical correlations revealed that the prognostic effects of TP53, CDKN2A/ P16 LOH 1p and EGFR alterations depend on the age of patients [1, 10, 19, 28, 36, 49]. Surprisingly, there are no differences in profile of genes alterations involved in cell cycle regulation such PTEN, TP53, CDKN2A, EGFR and MDM2 in patients with short survival <6 months and long survival > 2 years [20]. The prognostic significance of amplification /overexpression of EGFR is still controversial. Mutations of PTEN, EGFR amplification [36, 37], especially with EGFRvIII overexpression were found to be the strongest indicators of a poor overall survival prognosis [35], especially in patients below 60 years-old [10, 19, 49]. Some other studies had an opposite results [27, 28, 43]. The overexpression of EGFRvIII and EGFRwt without amplification didn't have a prognostic relevance [35]. In young patients only EGFR overexpression without P53 mutation (wild type) was negatively associated with survival [36]. In our case we detected the EGFR amplification with only focal overexpression of the protein. The lack of EGFR immunopositivity in amplified EGFR has been already reported [31]. The weak protein overexpression studied by immunohistochemistry in our case suggested the presence of truncated protein with deleted part of the extracellular domain which is not recognized immunohistochemically [31].

Activation of the PI3-K pathway is significantly associated with increasing tumor grade, radiation resistance, and with adverse clinical outcome [7]. Activation of the PI3-K-Akt pathway has been shown to play an important role in EGFR – tyrosine kinase inhibitors sensivity especially in naturally occurring mutant epidermal growth factor receptor known as deltaEGFR. High levels of EGFR expression and low levels of phosporylated Akt /PKB are associated with a better response to the treatment [14]. The expression of PTEN causes a cell cycle arrest in G_0/G_1, which is most closely associated with increased protein levels of the CDK inhibitor p27/ Kip1, but that phosphorylation may be unaltered by PI 3-kinase activity [4]. In our immunohistochemical study, both expression of PTEN and PI–3K were negative, however the strong nuclear accumulation of p27 was found (Fig. 3b) but staining for P21 was negative. The clinical relevance of P27 and P21 overexpression was revealed as a promising prognostic factor. The low level of P27/Kip1 was associated with poor prognosis [17, 38]. This effect was enhanced with decreased levels of P21/P27 [17]. Nevertheless, a decrease in P21 and P27 expression levels was demonstrated in the most radioresistant cell line which correlated with a failure to arrest cell proliferation [46]. The significance of high percentage of immunopositive cells in our case is unclear and didn't reflect the short clinical course of this patient. Presumably, it may be related to downregulation of PI3-K/PTEN. On the other hand, there are results indicating that the regulation of P27/Kip1 didn't correlate with PTEN expression and PI3-K-kinase pathway activity [9].

The strong association between overexpression of IGFBP2 and elevated expression of matrix metalloproteinase-2 (MMP-2) as well as other invasion related genes provide evidence that IGFBP2 contributes to glioma progression and in hence to tumor cell local invasion [44] but there is no data concerning the influences to the prognosis. Immunoexpressions of these proteins should be examined in the larger series of tumors.

The homozygous deletion of P16 was confirmed in 30%–41% [2, 16] and was significantly associated with poor overall survival and progression-free survival in men, but not in women [16]. In our case both LOH 9 and immunostaining for P16 were negative.

The complex and conflicting data of the clinical significance of molecular alterations and proteins overexpression in glioblastomas indicates, that the prediction of a clinical course on the basis of histopathology, immunohistochemistry and even on molecular level is very difficult. The further investigation reflecting the LOH 1p, LOH 19q, metylation status of MGMT gene and status of EGFR receptors is necessary.

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