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Abstract

The etiology and pathogenesis of tumors of the central nervous system are still inadequately explained. This study analyses tumor suppressor gene—adenomatous polyposis coli (APC) in 28 patients with glioblastoma, the most aggressive form of glial tumors. APC protein has structural role in adherens junctions, but also plays a signaling role as a negative regulator of the wnt pathway. Our interest in APC gene stemmed principally from the findings that the wild-type APC protein is highly expressed in the central nervous system, and upon the finding that it is critically involved in particular syndromes, among which brain tumors play a significant role. Glioblastoma samples were tested for gene instability by PCR/loss of heterozygosity using RFLP method. Two polymorphic markers were used: an Rsa I polymorphic site in exon 11, and an Msp I polymorphic site in exon 15. The results of our analysis for both markers showed allelic loss of the APC gene in 40% of our sample out of 25 heterozygous patients (informativeness 89%). Another 20% of samples demonstrated allelic imbalance of the APC allele in tumor tissue. Altogether, there were 15 samples (60%) demonstrating instability of this tumor suppressor gene. Despite increasing knowledge on glioma biology and genetics, the prognostic tools for glioblastoma still need improvement. Our findings on genomic instability of APC gene may contribute to better understanding of glioblastoma genetic profile and could be used as prognostic marker of disease evolution and progression.

Key words: glioblastoma, adenomatous polyposis coli gene (APC), loss of heterozygosity, wnt signaling pathway

Introduction

The genome of tumor cells is affected by two crucial types of changes: the accumulation of somatic mutations and the acquisition of genomic instability. The usual incidence rate of spontaneous somatic mutations is much lower to the rates of genetic changes observed in tumor cells and this increased frequency is the result of genomic instability that characterizes such cells. It is now believed that the increase of frequency of genetic changes is to be at least 100 times. Although it is usually assumed that the genetic instability is a later event in tumor progression, the question whether it may be an early causative event [1] in the formation of a specific tumor type is still speculative.

The well known two-hit hypothesis for tumor suppressor genes in hereditary cancer, that has nowadays transcedented to the multi-hit hypothesis, describes that as many as seven distinct and sequential mutations are needed for the development of malignant tumor, whereas fewer could give rise to benign precursors. Kinzler and Vogelstein [2] investigating colo-rectal carcinoma provided evidence for this hypothesis and showed that the mutations could accumulate over a span of years to decades. On the other hand body of evidence indicates that the mutation rate of normal human cells is much too low to account for the accumulation of this number of genetic alterations over a single individual's life time [3]. We know that multiple genetic changes are required for carcinogenesis and the inadequate incidence rate observed during human life time seeks explanation. The answer to this discrepancy is given in the so called mutator hypothesis. A mutator gene is a gene whose mutation increases the level of genetic changes of the individual's genome [4]. The mutator gene can be any gene, but usually comes from the group of genes whose products are involved in DNA repair mechanisms or genes whose products control the fidelity of DNA replication. Mutations of those genes are referred to as mutator mutations, and cancer cells are said to exhibit a mutator phenotype with the increased rate of genomic instability.

There are two categories of genomic instability observed in human cancers: microsatellite instability (MIN) and chromosomal instability (CIN). MIN involves the defects in the repair of short mismatches in the replication/repair machinery [5] resulting from the slippage of DNA polymerase as it synthesizes segments of short repeats. The consequential instability observed in tumor is sometimes termed as RER+ tumor phenotype from

Replication Error Positive phenotype. RER-positive tumor samples can be visualized on high resolution gels after electrophoresis techniques as samples with bands on different positions in comparison to bands of autologous normal tissue. CIN occurs at the chromosome level and is caused by chromosomal mis-segregation due to improper alignment of chromosomes in mitosis. The resulting daughter cells comprise aneuploidy and allelic imbalance. Besides instability at the whole chromosomal level, there are other forms of CIN. Translocations, segmental duplications and deletions, as well as gene amplifications, can also be considered a form of CIN, although mechanisms that cause such alterations are distinct from partitioning at mitosis [1, 6].

Our study analyzed genomic instability in the brain tumor glioblastoma, with regard to the role of the tumor suppressor gene—adenomatous polyposis coli (APC). Protein product of this gene is a component of the adherens junction. Its central part binds to E-cadherin, the most important molecule of cell-cell adhesion, via beta-catenin molecule [6, 7]. APC is illustrative of multiple roles that certain tumor suppressors play in a cell. Besides its structural role in cellular architecture, the protein product of the APC gene is also one of the key players of the wnt signaling pathway. The wnt/wingless pathway was first discovered in mouse and *Drosophila* and is one of the most interesting signal transductions, in which key components have multiple functions. In vertebrate cells, it is named after Wnt proteins, a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis. Insights into the mechanisms of Wnt action have emerged from several systems: genetics in *Drosophila* and *Caenorhabditis elegans*; biochemistry in cell culture; and ectopic gene expression in *Xenopus* embryos. Many Wnt genes in the mouse have been mutated, leading to very specific developmental defects. As currently understood, Wnt proteins bind to receptors of the Frizzled family on the cell surface. Through several cytoplasmic relay components, the signal is transduced to beta-catenin, which then enters the nucleus to activate transcription of Wnt target genes [9, 10]. Although the main signaling molecule of the pathway is beta-catenin, APC is a critical component of the beta-catenin destruction machinery heading to the proteasome, and acts as a negative regulator of the wnt pathway. When wnt ligand is absent, beta-catenin binds to APC protein and is being destroyed. Another tantalizing compartment of the wnt signaling pathway lies in downstream transcriptional activation. In response to wnt signaling, or under the circumstances of mutated APC, beta-catenin is stabilized, accumulates in the cytoplasm and enters the nucleus, where it finds a partner, a member of the DNA binding protein family LEF/TCF (lymphoid enhancer

factor-T cell factor). Together they activate new gene expression programs. Target genes for beta-catenin/TCF encode c-MYC and cyclin D1 proteins [11], explaining why constitutive activation of the wnt pathway can lead to cancer. The schematic illustration of key components of the wnt signaling is shown in figure 1.

It has been well documented that wnt genes, together with other components of wnt signaling pathway, are implicated in cancer [10], especially in neoplasms of epithelial origin. Mutations in the adenomatous polyposis coli gene are responsible for familial adenomatous polyposis and the majority of sporadic colorectal cancers. The APC gene (chromosome 5q21) [12] is organized in 16 translated exons and encodes a 2843 amino acid protein that is expressed in specific (frequently post-mitotic) epithelial and mesenchymal cells of several fetal and adult human tissues. The final protein product of APC gene with a mass of 311.8 kDa shows little similarity to other known proteins. There are three known APC protein isoforms that arise through a complicated pattern of alternative splicing. The conventional APC splice isoform is present in many cell types, while other splice forms are present only in differentiated cells [13]. These alternative splice forms exclude exon 1 but include alternate exons 5 ’of the missing exon 1 [14]. Two of the splice variants, BS (brain-specific) and 0.3 (exon 0.3) contain in-frame translation initiation codons, and the protein isoforms lack exon 1 encoded homodimerization domain. It is interesting that these protein isoforms are expressed in neuronal cells and other tissues with nondividing cell populations. Carson et al. [15] demonstrated that all isoforms cause similar reduction in beta-catenin activity and show no difference in the subcellular localization, and concluded that different isoforms are not trancriptionally different but rather involve unidentified protein functions of the specific domains. The majority of identified mutations of the APC gene are frameshift mutations [16] that almost always lead to a truncated protein product, devoid of C-terminal peptide sequences. A number of tumors, including colorectal, gastric, lung, hepatocellular, renal and laringeal tumors demonstrate loss of heterozygosity (LOH) of this gene [2, 17-19]. It has been shown that tumor suppressors are differentially expressed during normal embryonic development [20] and that specific developmental pathways are often inappropriately regulated during tumorigenesis [21, 22].

Our interest in elucidating the role of tumor suppressor gene—adenomatous polyposis coli (APC) stemmed principally from the findings that wild-type APC protein is expressed in the central nervous system [23, 24] and that there are strong indications that wild-type APC

protein is critically involved in initiation of neuronal differentiation [25]. Moreover, new knowledge on wnt signaling shows that wnt proteins regulate critical developmental processes of normal brain development [26-30]. Processes that include cellular adhesion and synaptic rearrangements require the expression of molecular components of the wnt pathway [31]. As we have already pointed out, the wnt pathway has repeatedly been implicated in tumorigenesis and lately in brain tumorigenesis as well [32]. These findings inspired us to focus our interest on the key player of the wnt signaling, APC tumor suppressor gene. The majority of brain tumors arise sporadically except those that are part of defined clinical syndromes. APC protein is critically involved in particular syndromes, like the Tourcot's syndrome, which includes the development of primary brain tumors such as medulloblastomas and gliomas [33, 34]. Cytoplasmically associated to E-cadherin is beta-catenin, yet another important molecule in brain tumorigenesis. Activating mutations of beta-catenin gene are found in a variety of tumors suggesting that beta-catenin gene acts as an oncogene. In 2003. beta-catenin was identified as a critical factor for dendritic morphogenesis [35]. Mutations of this gene have been reported in sporadic medulloblastoma [36] and recently this molecule was proposed prognostic factor for medulloblastoma [37].

The etiology and pathogenesis of the tumors of the central nervous system are still inadequately explained. Our knowledge on genetic background of specific histopathological type of brain tumor still needs to be elucidated although great progress has been achieved along with the increasing advances in molecular genetics. Glioblastoma is the most aggressive form of glial brain tumor. The majority of glioblastomas arise without clinical or histological evidence of less malignant lesion and this kind of glioblastoma is categorized as primary or *de novo* glioblastoma. They manifest in older patients (mean age 55 years) after a short clinical history. In contrast, secondary glioblastomas develop more slowly by malignant progression from diffuse or anaplastic astrocytoma and they manifest in younger patients (mean age 40 years) [38]. At present specific genetic alterations found to be associated with glioblastoma are emerging [39-41]. Both types of glioblastoma have disrupted p53 and Rb1 pathways, although in different ways. Primary glioblastomas have amplification of the 12q14 region encompassing the CDK4 and MDM2 genes which results in their over-expression and the dysfunction of both pathways. In addition to the genetic abnormalities involving p53 and Rb1 pathways, glioblastomas exhibit loss of alleles from 10q in over 90% of cases. This genetic region encompasses PTEN tumor suppressor gene. Moreover, amplification of the epidermal

growth factor receptor (EGFR) gene has been reported in about 35% of glioblastoma [40, 42, 43]. Secondary glioblastomas generally have no wild type p53 due to loss of one allele and mutation of the other allele and also loss of functional Rb1 pathway in a similar manner. Nevertheless, the genetic basis of primary as well as reoccurring glioblastoma is unclear, and much work is still required to determine the final list of genes involved, as well as the point in time of their activation/inactivation. In this chapter we offered a new candidate to fill in the puzzle of genetic basis of glioblastoma. Novel genes and molecular candidates involved in mechanisms of brain tumor formation will offer improvement in comprehension, diagnosis and treatment of this disease.

Materials and Methods

Twenty-eight glioblastoma samples together with 28 autologous blood tissues were collected from the Department of Neurosurgery, University Hospital "Sisters of Charity", Zagreb, Croatia. Using the magnetic resonance imaging (MRI) tumor lesions were found in different cerebral regions (predominantly temporal and parietal region), with the surrounding zone of perifocal oedema (table 1). During the operative procedure the tumor was maximally reduced using a microneurosurgical technique. The patients had no family history of brain tumors and did not undergo chemotherapy or radiotherapy prior to surgery. Collected tumor tissues were frozen in liquid nitrogen and transported to the laboratory, where they were immediately transferred at -75°C. The peripheral blood samples were processed immediately. The histopathological classification was glioblastoma. The local Ethical Committee approved our study and patients gave their informed consent.

DNA extraction. Tumor sample for DNA isolation was the part of obvious tumor mass evaluated by the neurosurgeon and based on macroscopic appearance and tissue color, density, and consistency on gross section. The sample was also evaluated for the percentage of tumor cells by pathologist and consisted of more than 85% of tumor cells.

Approximately 0.5 g of tumor tissue was homogenized with 1 ml extraction buffer (10 mM Tris HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) and incubated with proteinase K (100 μg/ml; Sigma, USA; overnight at 37°C). Phenol chloroform extraction and ethanol precipitation followed.

Blood sample was used to extract lymphocyte DNA. Five ml of blood was lysed with 7 ml distilled water and centrifuged (15 min/5000 *g*). The pellet was then processed as for DNA extraction from the tissue samples.

Polymerase chain reaction. The optimal reaction mixture (25 µl) for APC's exon 11 amplification was: 20 pmol of each primer (5'-GGACTACAGGCCATTGCAGAA-3' and 5'-GGCTACATCTCCAAAAGTCAA-3'), 200 µM of each dNTP, 200-400 ng template DNA, 1 µl (0.5 U) of Taq polymerase, 1 mM $MgCl_2$, 5 µl 10 X reaction buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3). Polymerase chain reaction (PCR) conditions were: initial denaturation, 4 min/95°C; denaturation, 1 min/94°C; annealing, 2 min/58°C; extension, 1.5 min/72°C; 35 cycles.

To amplify fragment of the exon 15 of the *APC* gene we used in a volume of 25 µl: 5 pmol of each primer (5-ATGATGTTGACCTTTCCAGGG-3 and 5-CTTTTTTGGCATTGCGGAGCT-3), 200 ng template DNA, 2.5 µl 10X buffer II, 1.5 mM MgCl2, 25 µM of each dNTP, 0.2 µl (5U/µl) of Taq polymerase. PCR conditions were: initial denaturation, 5 min/95°C; denaturation, 30 s/95°C; annealing, 30 s/57°C; extension, 45 s/72°C; final extension, 72°C/7 min; 30 cycles. The PCR products for both markers were analyzed on 2% agarose gels.

Loss of heterozygosity. Loss of heterozygosity (LOH) of the APC gene was detected on the basis of restriction fragment length polymorphism (RFLP) of the PCR products. Two different polymorphisms were investigated. One is an Rsa I polymorphic site in exon 11, and the other is an Msp I polymorphic site in exon 15. PCR amplification of exon 11 generated a 133- bp fragment that is cleaved to 85- and 48- bp fragments by Rsa I restriction if the polymorphic site is present, and remains uncleaved if the site is absent [44]. The amplified fragment of exon 15 is 550 bp long and is cleaved with the Msp I restriction endonuclease to two 250 bp fragments if the restriction site is present. LOH/Rsa I was demonstrated only in informative (heterozygous) persons when the tumor DNA showed loss of either the single uncut band (133 bp) or of the two cut bands (85+48 bp) compared to autologous blood tissue. For Msp I polymorphism heterozygous patients demonstrated two bands (550+250 bp), while LOH was shown when either band was missing in comparison to the autologous blood tissue. Samples that demonstrated quantitatively weaker allelic band in tumor tissue than in normal blood DNA were described as samples with allelic imbalance (A.I.).

PCR aliquots (10-15 µl) were digested with 6 U Rsa I (Gibco, USA; 12 h at 37°C) and with 6 U Msp I (AGS, Germany, overnight at 37°C) and were electrophoresed on Spreadex gels EL 300 in the SEA 2000 submarine electrophoresis apparatus (Elchrom scientific, Switzerland) at 120V. Temperature of the running buffer was kept constant at 55°C. The

samples with LOHs were additionaly electrophoresed on 15% polyacrylamide gels stained with silver.

Results

From 28 glioblastoma samples 79% appeared to have developed *de novo* , while six samples developed from previously diagnosed tumors of lower malignancy grade, two from diffuse astrocytoma (GII), one from anaplastic astrocytoma (GIII), two from oligoastrocytoma and one from oligodendroglioma. The histopathological diagnosis of glioblastoma (malignancy grade IV) was based on recognition of poorly differentiated astrocytic tumor cells with brisk mytotic activity and marked nuclear atypia. Differentiated elements were intermingled with bizzare multinucleated tumor giant cells. Prominent microvascular hyperplasia, in which proliferating blood vessels come to be lined by cells heaped up in disorderly fashion and transformed into glomeruloid or solid tufts (figure 2), and necrosis were also recognized as diagnostic features. The age of patients varied from 31 to 77 years (mean age = 59.1), and there were 15 females and 13 males. Tumor localization and the duration of symptoms are shown in table 1.

Genomic instabilities. The Rsa I polymorphic site in APC's exon 11 was analyzed first. From 28 glioblastoma samples analyzed, 18 (64.3%) were informative for this polymorphism. For Msp I polymorphism in APC's exon 15, we found 20 heterozygous patients (71.4%). The results of our analysis showed 7 samples with LOH/exon 11 of the APC gene (39%) when tumor DNA was compared to autologous constitutive blood DNA. Another three samples demonstrated allelic imbalance (quantitatively weaker allelic band in tumor tissue than in normal blood DNA). Genetic changes of exon 15 demonstrated 3 samples with LOHs (15%) and another two samples with allelic imbalance.

When summing the informativeness provided by both markers, our sample showed (25/28) 89% of heterozygosity. The results of our analysis for both markers showed allelic loss of the APC gene in 10 glioblastoma samples out of 25 heterozygous patients (40%). Another 20% of samples demonstrated allelic imbalance of the APC allele in tumor tissue. Altogether, there were 15 samples (60%) demonstrating instability of this tumor suppressor gene. Three glioblastoma samples had both LOH at exon 11 and 15, while four demonstrated LOH exclusively on exon 11, and another three A.I. were detected in this genetic area. Two A.I. found on exon 15 did not show losses on exon 11. Genomic instabilities of tumor suppressor gene investigated and the polymorphic status for both markers are summarized in

table 1. LOHs of the APC gene that both markers revealed are shown in Figure 1 A (exon 11) and B (exon 15).

When we correlated our molecular findings with the demographic variables we observed that genomic instability of the APC gene was confined to younger patients.

Discussion

The mechanisms of brain tumor initiation and progression have not yet been completely investigated and elucidated. As with other tumors, the formation of brain tumors is the result of multiple consecutive genetic changes that represent a critical factor in tumor evolution. No consistent genetic abnormalities have been detected that would indicate the genetic profile of glioblastoma [40]. Histopathologically, an unambiguous distinction of glioma subtypes has remained elusive, but they clearly evolve through different gentic pathways. It also remains to be shown whether these subtypes differ significantly with respect to the molecular genetic alterations underlying the oncogenesis and progression of glioblastoma. With this in mind, we investigated a new candidate gene, tumor suppressor gene APC, in a set of twenty eight glioblastomas. Although APC has been thought of primarily as a colon-specific tumor suppressor gene, its association with certain brain tumors and its expression in the CNS suggests that it performs important functions in these tissues also.

Genes involved in formation and acquisition of the full metastatic potential of specific tumor are not only those responsible for cell proliferation and survival, but also genes responsible for the control of cell adhesion and cell motility [45]. It is now apparent that tumor malignancy can, in certain aspects, be explained by alterations in the adhesive properties of neoplastic cells. One of the most important hallmarks of malignant gliomas is their invasive behavior. Despite modern diagnostics and treatment the median survival time does not exceed 15 months which is in accordance to extreme invasive characteristics of glioblastoma. Demuth and Berens in their review paper [46] state that glioblastoma recur predominantly within 1 cm of the resection cavity mainly due to the fact that at the time of surgery, cells from the bulk tumor have already invaded normal brain tissue. The problem of new rise of the malignant cells left behind the surgical resection of diffuse gliomas is one of the main disadvantages of therapeutic decision-making. The culprit of the highly invasive phenotype of human gliomas is thought to be associated to the cadherin group of adhesion molecules. Classical cadherins such as E-type and N-type are involved in forming both

adherens and synaptic junctions in the nervous system. Shimamura and Takeichi [47] found that E-cadherin is transiently expressed in restricted regions of the mouse embryonic and adult brain. Although E-cadherin molecule is a well known and almost universal suppressor of invasion, little is known on the role of cell-cell adhesion in astrocytes and its alteration in migrating and invasive glioblastomas. In 2002. [48] Perego and co-workers demonstrated that disorganization of cadherin mediated junction, in which APC is included, is required to promote migration and invasiveness in glioblastoma cell lines.

The results of the analysis reported in this chapter suggested that APC plays an important role in the mechanisms of development and progression of this specific tumor of the central nervous system. Our previous investigations are also in favor of this presumption. We showed that neuroendocrine tumor pheochromocytoma exhibits microsatellite instabilities of the E-cadherin gene, which is the main adherens junction member molecule and an indirect member of the wnt signaling [49]. Preliminary analysis on oligoastocytoma showed gross deletion of the APC gene [50]. which is also indicative of involvement of this tumor suppressor gene in the formation and development of central nervous system tumors.

The analysis described in the present chapter showed that 60% of glioblastoma samples demonstrated LOH and allelic imbalance of the APC gene, which indicated that gross deletions of APC are part of genetic profile of these tumors. Allelic imbalances, quantitatively weaker allelic bands in tumor tissue than in normal blood, that we observed in 20% of our sample probably was not the result of normal tissue DNA interference. Because each glioblastoma sample was maximally reduced during the operative procedure using a fine microneurosurgical technique, our samples tested for genomic instabilities consisted of 90% of tumor cells. The explanation for allelic imbalances is that not all tumor cells of the sample underwent this sort of alteration. Behaviorally, gliomas can be viewed as consisting of two discreet subpopulations of cells, the proliferative cells at the tumor core, and cells invading the brain parenchyma. Thus, the gene expression profile of the tumor core may not necessarily depict the profile of genes active in the invading rim. Demuth and Berens [46] identified genes differentially expressed in invasive glioma cells and illustrated the differing biology of the invasive cells in contrast to the tumor cells at the core. Findings on different glioblastoma cellular components could explain for the portion of glioblastoma that did not show instabilities of APC gene. The relatively high number of allelic losses of the APC gene in our sample may be attributed to random variation in tumors, but in our opinion the frequency of APC's genomic instability in our sample was too high to be random. The

observed high frequency led us to conclude that gross deletion of the APC gene that our glioblastoma samples comprised are an important event in the mechanisms of glioblastoma formation. Unfortunately, it was still not clear whether those changes represent an initiation event or come along the path of glioblastoma progression.

In our study genetic alterations were more frequently confined to exon 11 of the APC gene. Three glioblastoma samples had both LOH at exon 11 and 15, which could indicate that the deletion is extensive and goes all the way to exon 15. The alterations that were uniquely found on exon 15 could indicate that this genetic area is affected, and the same applies to the alterations found exclusively on exon 11. Exon 11 is a much smaller exon than exon 15, the biggest exon of the APC gene which encompasses 3/4 of its coding region (6577 bp). We could suspect that losses of exon 11 would not have such severe consequences at the protein level as would losses of exon 15. The alterations of the APC gene found in our sample were not correlated with sex of the patients. Nevertheless, we observed that genomic instability of the APC gene was confined to younger patients. The glioblastomas we considered primary because the diagnosis of glioblastoma was made at the first biopsy, without clinical or histopathologic evidence of a less malignant precursor lesion. Patients with glioblastoma de novo are older at diagnosis, compared to patients with secondary glioblastoma, and the mean age of our patient group (=59.1) was somewhat higher than the mean age reported in literature of 55 years.

The clearest example of wnt signaling involvement in brain tumor formation is the critical role of the tumor suppressor gene APC in the Tourcot's syndrome. Association of polyposis of the colon with a CNS malignancy is represented with this syndrome. Genetically predisposed patients are younger at diagnosis of glioblastoma compared to patients with sporadic disease. The relative risk of brain tumor formation is 23 times greater for Tourcot's syndrome patients with APC-mutation when compared to the general population between the ages of 0 and 29. Hamilton et al, [33] reported 14 families with Turcot’s syndrome in whom patients with medulloblastoma had mutations in the APC gene, whereas patients with glioblastoma multiforme had germline mutations in one of the mismatch-repair genes. Mutation reports by Mori et al. [51] indicated lack of mutations in neuroepithelial tumors they investigated (glioblastomas included), but presence of germ line mutations of APC gene in 3 Turcot’s syndrome patients. Other authors found germline mutations of APC gene in Tourcot's syndrome patients, but failed to detect them in the primary astrocytoma [52], or glioblastoma multiforme [53]. These results suggest that APC gene is associated with

pathogenesis of Turcot's syndrome, but that at least one other gene is responsible for the genesis of neuroepithelial tumors in the CNS.

Somatic mutations of the APC gene were reported predominantly in sporadic medulloblastoma [34], and are much less frequent in glioblastoma. Nevertheless, Steigerwald and co-workers [13] found base change mutations in APC gene in two of 23 sporadic glioblastomas examined and a heterozygous G to A transition at position 148 of exon 0.3 in cell line SW 1088 from a human astrocytoma. This base change affects the amino acid sequence of the APC gene. One of the sporadic glioblastoma they investigated had an additional G following a string of seven Gs located at position 34 of exon 0.1 , but the effect on amino acid sequence could not be predicted. Microarray technology has been applied to the genome of glioblastoma, because it allows a large number of hybridizations to be performed simultaneously and was used for array comparative genomic hybridization by Roversi et al. [54]. Twenty five primary glioma cell lines were investigated for gross and subtle genetic changes involved in glioma initiation and progression at the whole genome level. In this extensive study the authors have found among many other candidate chromosomal regions, recurrent losses of 5q22.2-q23.3 region in 10 glioblastoma cell lines (IV WHO grade) they analysed, which is in accordance to our results.

So far little is known about the events that are involved in tumor initiation and early development of glioblastoma. Different subtypes of gliomas differ significantly in their age distribution, growth potential, tendency for progression and clinical course.

The work that we described in this chapter elucidated the potential contribution of genomic instability of APC tumor suppressor gene, to malignant transformation of the specific tumors of the central nervous system. The functional consequences of the observed instabilities that we found at the genetic level, would need to be confirmed at the protein level by analysing changes of APC's protein expression. We can speculate that wild-type APC protein is going to be absent in glioblastoma cells by inactivation of both alleles of the APC gene. One allele could be deleted, and the remaining one might have suffered another type of mutation, probably point mutation or a small deletion. On the other hand, only one allele may have suffered gross deletion, leaving the other one do the job of wild type protein expression. It is therefore unclear whether the clinical evolution of glioblastoma is due to quantitative reduction or complete loss of APC protein product. Whether genetic losses we found have consequences at the protein level is left for future studies.

Nevertheless, our study offers APC tumor suppressor gene as a potential candidate involved in mechanisms of glioblastoma development. We consider our finding a specific but relevant contribution to understanding the pathophysiological mechanisms of brain tumor formation.

Future perspectives

It would be interesting to asses the consequence that the instabilities of the APC gene might have at the protein level. Future studies need to confirm the functional changes of APC molecule by analyzing its protein expression or changes at the APC's mRNA levels.

The question whether microsatellite genetic instabilities, that indicate involvement of mismatch repair, have a role in glioblastoma development and progression is also very tempting and should be considered for future work.

Further investigation regarding this gene on a bigger sample as well as employment of additional markers of the APC gene should be performed in future to confirm our suggestions.

It would also be relevant to obtain data on wnt target genes, such as c-myc, cyclin D1 in future studies.

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Legends to the figures

Figure 1. Wnt signaling pathway demonstrating its key components: frizzled (receptor of WNT proteins; GSK3-beta (glycogen-synthase-kinase 3-beta); Adenomatous polyposis coli (APC); beta-catenin. E-cadherin mediated adhesion is also shown since it modulates wnt signaling by sequestering cytoplasmic beta-catenin.

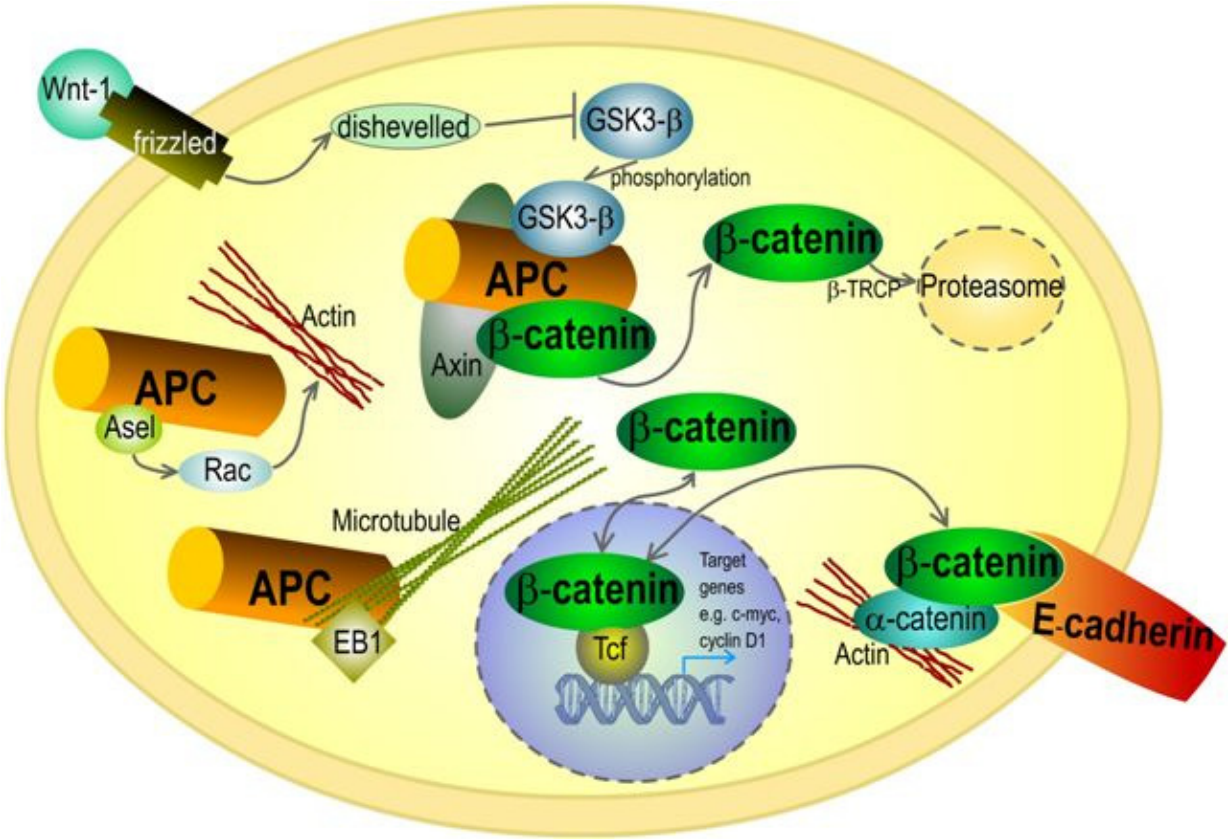


Figure 2. Glioblastoma multiforme (HE, x 200). Dense cellularity, striking pleomorphism, brisk mytotic activity and prominent microvascular proliferation (arrows).

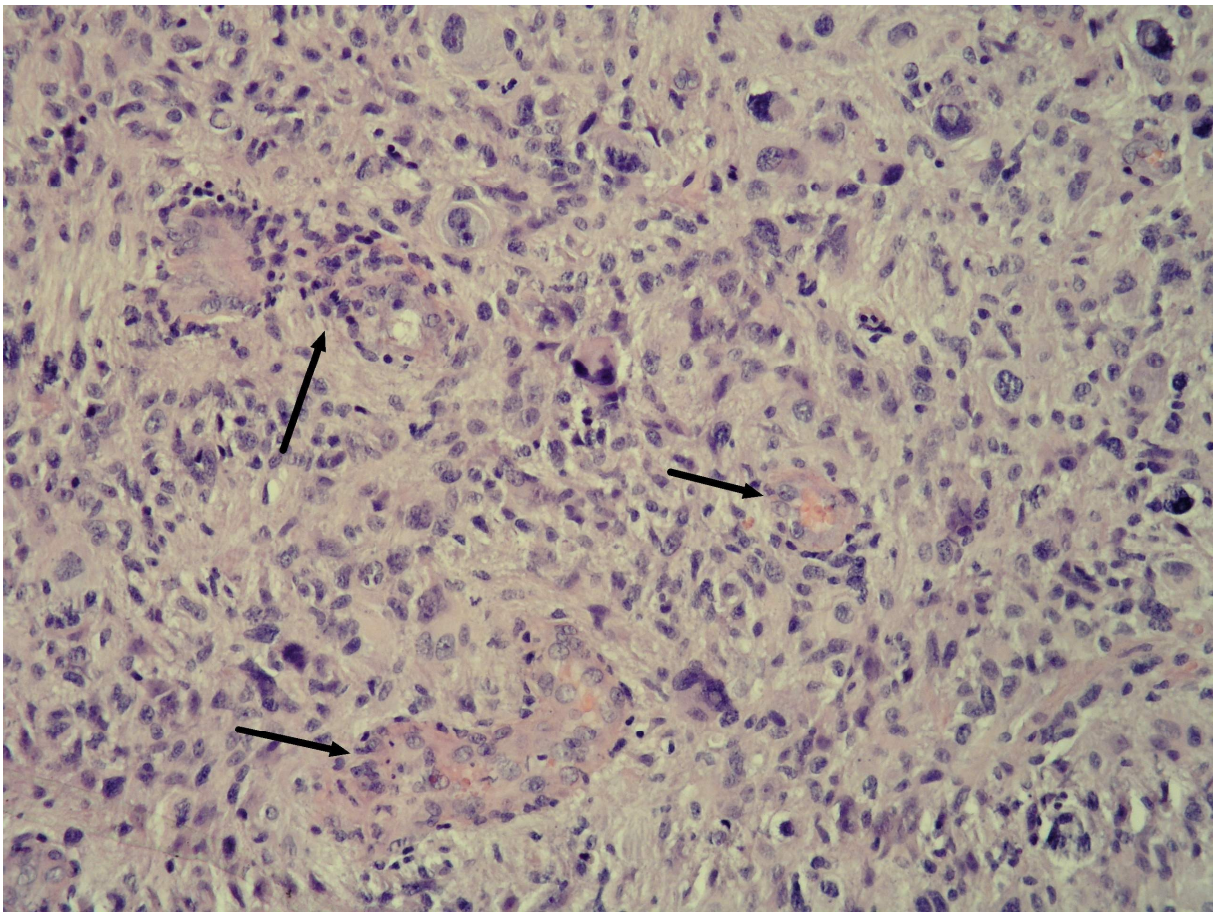
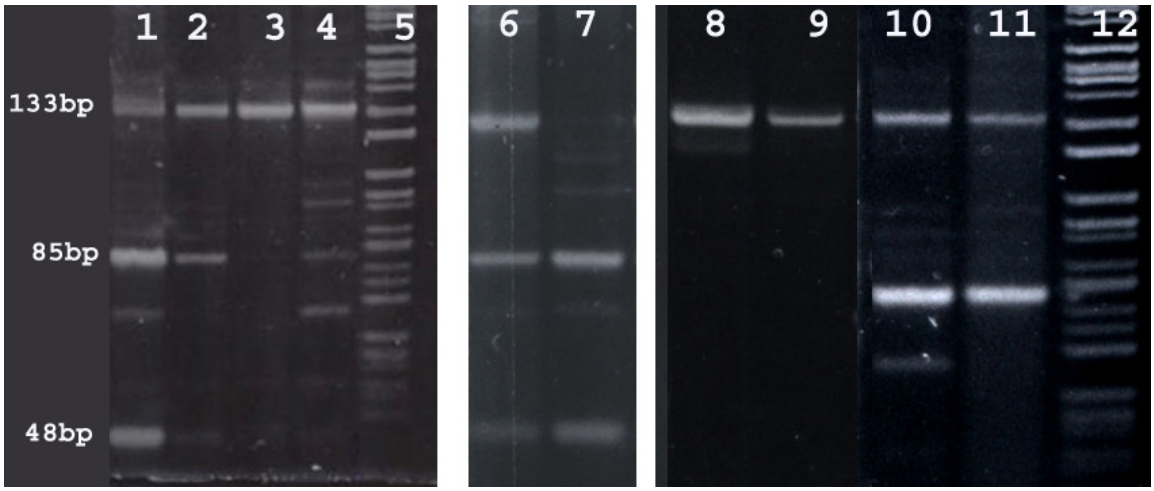


Figure 3. A. Loss of heterozygosity of APC gene in two patients with glioblastoma. Exon 11/RsaI/RFLP is demonstrated. Lanes 1, 2- heterozygous sample (tumor and blood); lane 3- LOH in glioblastoma patient (the digested/cut allele is missing); lane 4- informative blood sample of the same patient showing undigested/uncut allele 133 bp and cut allele (85+48 bp); lanes 5, 12 - standard M3 (Elchrom scientific); lane 6- informative blood sample; lane 7- LOH in the corresponding glioblastoma sample (uncut allele is missing); lanes 8, 9 – uninformative homozygous sample showing uncut allele (tumor and blood), lane 10- informative blood sample; lane 11- allelic imbalance in the corresponding glioblastoma (uncut allele is weaker, 48 bp fragments are not shown).

B. Loss of heterozygosity of APC gene in 3 patients with glioblastoma. Exon 15/MspI/RFLP is demonstrated. Lane 1 - standard DNA/50 bp ladder; lanes 2, 3, 16, 17 – uninformative homozygous samples showing uncut alleles of 550 bp (tumors and blood); lanes 4, 5, 6, 7 - heterozygous samples, both alleles, cut and uncut, are visible; lanes 8, 10, 13, 14, 19 -

informative blood samples; lane 9- allelic imbalance in the corresponding glioblastoma (uncut allele is weaker); lane 11, 12- LOHs in the corresponding glioblastomas, uncut alleles are missing; lane 15 – LOH in the corresponding glioblastoma cut allele is missing; lane 18 – allelic imbalance in the glioblastoma corresponding to the blood in lane 19 (uncut allele is weaker).

A



B

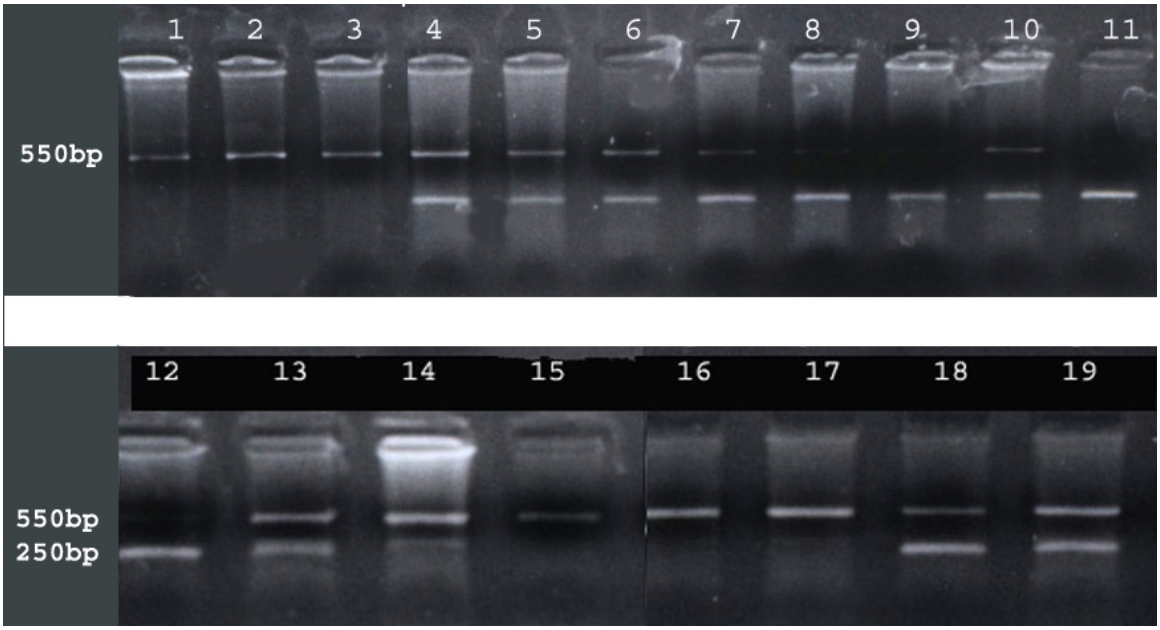


Table 1. Genetic changes of the APC tumor suppressor gene, anatomical location of glioblastoma and the duration of symptoms.

Patient No.	APC gene exon11/Rsa I	APC gene exon15/MspI	Localization	Symptom duration/ months
1[1]	heterozygous	**A.I.**	FTP left	6
2	homozygous b	**A.I.**	FTP left	2
3	**A.I.**	heterozygous	P left	1
4	**A.I.**	heterozygous	TP right	7
5	**A.I.**	heterozygous	T right	0,5
6	heterozygous	heterozygous	FTP right	0,5
7	heterozygous	heterozygous	T left	1
8	heterozygous	heterozygous	FTP left	0,5
9	heterozygous	heterozygous	FT left	0,5
10	heterozygous	heterozygous	P right	1
11[2]	homozygous b	homozygous d	P right	2
12	homozygous a	heterozygous	bifrontal	1
13	homozygous a	heterozygous	FPO right	3
14	homozygous a	heterozygous	FTP left	2
15	homozygous a	heterozygous	T right	3
16	homozygous a	heterozygous	T left	2
17	homozygous b	heterozygous	TO right	2
18[1]	**LOH**	heterozygous	P left	12
19	heterozygous	homozygous c	T left	10
20[3]	heterozygous	homozygous c	T right	18
21	**LOH**	homozygous c	P left	1
22[4]	**LOH**	homozygous c	TO right	3
23	**LOH**	**LOH**	TP left	1
24	**LOH**	**LOH**	T left	3
25[3]	**LOH**	**LOH**	FTleft	2
26	**LOH**	ND	P left	2
27	homozygous b	ND	FP right	2
28	homozygous a	ND	FP left	0,5

Heterozygous = without. LOH or A.I; a = both alleles 133 bp; b = both alleles have restriction sites (85+48 bp); c = both alleles 500 bp; d = both alleles have restriction sites (2 x 250); A.I. = allelic imbalance; [1]from diffuse astrocytoma (GII); [2]from anaplastic astrocytoma (GIII); [3]from oligoastrocytoma; [4]from oligodendroglioma; FTP= frontotemporoparietal region; P = parietal region ; TP = temporoparietal region ; T = temporal region; FPO = frontoparietooccipital region; TO = temporooccipital region; FT = frontotemporal region; FP= frontoparietal region; ND = not determined.