1.2. Three-hit hypothesis in astrocytoma

Parvin Mehdipour

Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran 1417613151, Iran

Abstract. Astrocytoma as a primary tumour is the core item in this section in which, by considering a pedigree-based research approach, the involvement of Ataxia-Telangiectasia (AT) gene is investigated. The two-hit -hypothesis of Knudson’s model is given to clarify the process of tumourigenesis in retinoblastoma. Lack of any available data for any hit hypothesis in brain tumours was the reason why the three-hit hypothesis in astrocytoma was formulated and included in this section.

ATM alterations were reported in medulloblastomas, gliomas, but not in astrocytoma. The polymorphism D1853N was only reported in healthy individuals and medulloblastomas. This polymorphism was detected in a 28 year-old female- proband affected with astrocytoma. As the triangle initiator of the three-hit hypothesis, D1853N is the first germ line hit, IVS 38- 63T→A is the second hit, and IVS38- 30 A→G is the third hit. In addition, the D1853N polymorphism was on a different allele than from IVS 38-63T→A and IVS 38- 30 A→G. However, these three triggers could be considered as triangle initiators for the course of evolution in astrocytoma. However, the present data could highlight the crucial role of specific intronic region of the ATM gene involved in cancer and also the importance of pedigree analysis. This polymorphism might be useful as a marker in astrocytoma.

Correspondence/Reprint request: Professor Dr. Parvin Mehdipour, Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, P.O.Box 14155-6447, Zip code 14176-13151, Tehran Iran. E-mail: mehdipor@tums.ac.ir
Introduction

The brain, as a remarkable organ, governs the whole human body, world, edits the faith of the globe, and can coordinate harmony. Brain tumour is characterized by abnormal and uncontrolled growth of cells within the brain or located outside the brain within the skull classified as cancerous or non-cancerous. But, still there are pitfalls in resolving the real picture of its nature.

Different aspects in tumours of the central nervous system (CNS) were previously published [1]. They have stated that the general classification of astrocytic tumours relied on tumour location within the CNS system, age, gender, growth potential, spectrum of invasiveness, morphological features, progression and clinical outcome. In addition, genetic events play a critical role in tumour development. Primary tumours originating from glial cells, are called gliomas, and include astrocytoma, glioblastoma, and oligodendroglioma. Approximately three quarters of all gliomas are astrocytomas which are classified into four grades, including Grade 1 (Pilocytic Astrocytoma), Grade 2 (Low-grade) Astrocytoma, Grade 3 (Anaplastic Astrocytoma), and Grade 4 (Glioblastoma). In spite of its lengthy medical history and unlimited scientific documentation, still, its entire biology is not totally understood and is not well defined.

Cancer, basically, reflects the regulation of cell growth and differentiation. Oncogenes with their novel properties are inappropriately expressed, and the nature of tumour suppressor genes (TSGs) was reported as recessive trait, containing loss-of-functional mutations [2]. It might follow the 'two-hit hypothesis', in which both alleles of a particular gene must be altered before the specific phenotype is manifested. This is due to the fact that when only one allele of a gene is damaged, the second is capable of producing the normal protein. However, mutant TSG usually acts as a recessive trait whereas mutant oncogene alleles are dominant and characterized by gain-of-functional mutations. The germline mutations of TSGs are passed on to the offspring of affected probands which increase the likelihood of cancer diagnosis in subsequent generations. This might increase the incidence of cancer for the family members. However, the tumour types depend on the nature of TSG mutation as well [3].

There are pitfalls in diagnosis and treatment of brain complications regarding the medical management of patients affected with brain tumours. Considering a significant estimation of new cases and deaths per year as a result of brain tumours, cooperation of medical and basic Sciences is essential for appropriate planning. This important process could try to
provide the diagnostic tests, prognostic and predictive factors within a frame of biomarkers, towards screening of populations, genetic counselling, and early detection. All these steps could facilitate selection of appropriate protocol(s) for effective treatment and planning for pre-malignant lesions by considering the patients' benefit.

There are many peculiarities in the central nervous system including the following questions in brain tumours (B.T.):

1. Do the brain tumour cells really have the same genomic origin?
2. What is the spectrum of complexity of organization and functions in B.T.?
3. How could we define the cellular diversity and cellular signaling in B.T.?
4. Is there any cooperation between exonic and intronic domains of specific genes in B.T. which possibly could lead to a successful outcome through the medical follow-up period of the patients’ life affected with B.T.?

**Ataxia-Telangiectasia (AT)**

AT is reported by different investigators as a multisystem, autosomal recessive disorder with progressive neuronal degeneration. AT is caused by mutations in the ATM gene which encodes the nuclear protein kinase ATM. The reported mutations were correlated with variant phenotypes in AT-patients. AT and related AT-diseases are among the best studied and defined of the human neurological syndromes due to DNA damage response defects [4].

The typical AT phenotype could be due to homozygosity or composite heterozygosity for null ATM alleles. These situations would lead to truncation of ATM or inactivation of gene by missense mutations. Truncated ATM and other inactive forms of ATM could be unstable, therefore as it was also stated elsewhere, detection of protein expression in the cells of classical AT is not successful and they are mostly lacking [5,6]. It is also important to consider the pattern of accumulation on cell cycle, proliferation, differentiation and maturation resulting in diversity of cell functions.

There are many other factors playing an important role in the medical complications in which ATM gene is involved. The occurrence of events, including translocations and telomeric fusions, and an increased rate of telomeric shortening which could be found in AT-patients, were also reported [7]. Regarding ATM mutations, it could occur throughout the entire ATM gene, and according to the previous reports, there are no hotspots or high frequency of mutations [8].
Cancer might develop in approximately one-third of AT patients. Children might be affected with Acute Lymphocytic Leukemia (ALL) or B cell lymphoma, or T cell lymphomas and T cell Prolymphocytic Leukemia (T-PLL). Non-lymphoid malignancies are seen predominantly in older AT patients.

Telomeric stability and instability in human tumours have not been understood well in vivo. However, a recent publication by us [9] on alterations of telomere length in human brain tumours showed highly significant difference in meningioma and astrocytoma. The higher grade meningioma and astrocytoma tumours showed more heterogeneity in telomere length, and it might be concluded that the shortening process of telomeres is an early event in brain tumours.

ATM protein expression was variable in the tumour glioma cell lines and at low level in primary tumours [10, 11]. At the cell line level, tissue culture and several established GBM cell lines from glioma specimens were used and the expression levels of ATM, p53, and p21 proteins were determined by Western blot [11]. There was a relationship between ATM protein expression and radiosensitivity, but with variability in the primary gliomas. This data emphasizes that attenuating ATM gene expression may be a successful strategy in the treatment of GBM tumours.

However, our recent publication focused on the comparison of the mRNA expression of cyclin D2, P53, Rb and ATM, between astrocytoma and meningioma of human tumours with consideration given to different grades [12]. It has been shown that higher grade (III and IV) of astrocytoma tumours had up-regulation for cyclin D2 and ATM genes, and down-regulation of P53 and Rb genes. Considering different templates of up-and down-regulation for these gene interactions in different types of brain tumours, it seems that these genes do not have a unique model of interaction.

Although the application of multiple techniques were previously not available, innovation in tumour development could be based on the discovery of Knudson [13,14] who proposed that cancer development depended on two molecular events in retinoblastoma, including an inherited germ-line mutation in a tumour suppressor gene followed by a complementary mutation within the organism's life by inactivating the other allele of that TSG. This valuable historical phenomenon was not detected in other tumours for thirty-seven years; however, an additional step of tumour development could be proposed by proof as “the three-hit hypothesis” by considering different paradigms including molecular genetics and cell biology [15].
Hypothesis

Knudson’s model (13) has revealed the role of germline mutation in inherited retinoblastoma in which the process of tumour development could be beautifully clarified by a complementary somatic mutation. The Knudson hypothesis is derived from observations of accumulating mutations at DNA level. The multi-mutation theory on cancer was initially proposed by Nordling (16) and later formulated by Knudson whose work led indirectly to the new insight in cancer-discovery of cancer-related genes.

Knudson suggested the requirement of multiple "hits" to DNA for cancer formation. Development of malignancy depends on the activation of proto-oncogenes by stimulating cell proliferation and deactivation of tumour suppressor genes by keeping proliferation in check. In addition, the first "hit" in an oncogene, and a damaged Rb1 gene as a TSG in retinoblastoma would not, as a sole alteration, lead to cancer.

The two-hit hypothesis was initially published by Knudson in patients affected with retinoblastoma. He emphasized the requirement of two independent genetic events. The Retinoblastoma protein (pRb) was the first tumour-suppressor protein discovered in human retinoblastoma which was also considered as a tumour-survival factor [13,3]. Knudson has beautifully clarified tumourigenesis by his two hit hypothesis which included the first hit being a point mutation inactivating one copy of Rb1 (asTSG), and the second hit being a large deletion leading to loss of functioning of the TSG allele. These events lead to a non-functioning copy of the TSG and finally the development of cancer. However, the developmental role of ATM gene in cancer was not previously proposed in tumours.

To varnish and highlight our knowledge in cancer, the following paradigms could be considered:

1. Cancer cell biology, in which the researchers would be able to define the wide spectrum characteristics of normal-and malignant-tissues. By considering the required timing from initial cancer development and progression, specific biological behavior of the pre- and post-malignant cells gradually alter the original nature of tissue from normal to cancerous.
2. Clonal evolution might occur in an unchecked population of cells which is also called somatic evolution. This process shows how cancer originated and becomes more malignant [17, 18]. This avenue could pave the way to direct cells to choose their response to drugs, i.e., to be resistant or sensitive, which is the matter of pharmacogenetics.
3. Natural selection which considers the alterations in the cellular metabolism priority of the few cells with new genetic makeup that enhance their survival or reproduction, continue to multiply, and act in a dominant manner through which tumours could rapidly grow [19]. This may lead to genetic heterogeneity in cancer as well. However, the system instability was considered as a major contributing factor for genetic heterogeneity. Genetic instability could facilitate the acquisition of other mutations which have occurred due to defects in DNA repair [20].

4. Regarding stem cells, biological behaviors vary in different types of cancers and also depend on the cancer stem cells which have the capacity of replication and could generate differentiated cells.

**Three-hit hypothesis**

Available papers within the avenue of the molecular-hit hypotheses seem to be very limited and, so far, only include two papers; one on colorectal cancer and another on neurofibromatosis. The first study was performed on colorectal cancer (CRC) cell lines and primary CRCs [21]. They have shown that the two-hit model requires modification of the APC–tumour suppressor gene, leading to an optimal level of Wnt activation. They stated that “some had acquired third hits at APC which mostly was copy number gains or deletions and could be protein-truncating mutations”. They have also noticed that the “third hit was significantly less common when the second hit at APC had originated by copy-neutral loss of heterozygosity”. The second paper reported patients affected with Neurofibromatosis 2 (NF2) who developed bilateral tumours of Schwann cells [22]. They revealed the occurrence of two mutations in these patients and have suggested that “more than two mutations may be necessary for NF2 development”. However, no available publication could be found on astrocytomas. Many studies are available at cytogenetic and descriptive molecular level. However, the initial finding of the three-hit hypothesis in brain tumour was published on astrocytomas by us [15].

**Three-hit hypothesis in astrocytoma**

The ataxia telangiectasia (AT) gene was cloned at chromosome band 11q22–q23. It is mutated in AT patients and plays an important role in the double-stranded break DNA repair pathway and cell-cycle checkpoints. The ATM gene gives rise to a ubiquitously expressed transcript of ~13 kb which
encodes a nuclear protein of 350 kDa with homology to PI3 kinases [23]. It was reported that patients with AT have a predisposition to cancer including lymphocytic, chronic and B-cell origin leukemias, mucinous adenocarcinoma of the stomach, medulloblastomas and gliomas [24-26]. In this regard, two polymorphisms including D1853N and F858L were reported in medulloblastoma tumours of 19 children not affected with AT [27]. The available reports reveal around 479 mutations in this gene but there was no available report on brain tumours [28]. The main aim of our investigation was to trace the molecular alteration in exon 39 of the ATM gene in an astrocytic tumour of a proband, and through her pedigree generate a patterned model in direction of tumour development which is summarized from our previous publication [15].

**Materials and methods**

Genetic counselling was performed to draw the pedigree. All relatives in this study were informed, in detail, regarding the nature and purpose of the study, and they, voluntarily, decided to participate in this investigation. The pathologic diagnosis in a 28 year-old female proband was found to be differentiated astrocytoma, cystic grade I (in four grade classification) and sized 2 x 3 x 4 cm.

Sampling included peripheral blood (PB) and brain tissue of proband for detecting germline and somatic events respectively. The proband's relatives included parents, 1 sister, 3 brothers, 1 nephew, 4 first cousins and 2 second cousins (Figure 1).

Fourteen PBs were also sampled from healthy family members, as the familial controls within the pedigree (group I), and fourteen age-matched controls were also sampled (group II) without any family history of cancer or other medical complications.

The details of standard techniques for DNA extraction, PCR, sequencing cloning, and immunofluorescence are available in our publication [15].

**Results and discussion**

Pedigree could be laddered to screen the classic D1853N polymorphism through different generations (Fig. 1). Two novel intronic alterations i.e, IVS 35- 63 T→A and IVS35- 30 A→G within splicing sites could be found in proband’s peripheral blood and tumour respectively (Fig. 2). This polymorphism together with IVS 35- 15 G→C could be detected in proband’s mother (IV/10 (Fig. 1 and 2).
Figure 1. Pedigree of proband affected with astrocytoma and her relatives. Arrow indicates the proband affected with astrocytoma. Left main box illustrates information on peripheral blood sample. Right main box illustrates information on tumour sample for proband. Column left/top of each main box presents alterations of the 3' splicing site of intron 38. Column left/bottom of each main box presents alterations of exon 39. Left – side numbers of each individual presents age of healthy relatives at the time of sampling and age of deceased for two affected persons in the pedigree. Right-side numbers of each individual presents systematic reference number of individuals through each generation modified (from: ref.15).
It was stated in our previous paper that “genes are efficient and characterized by the programmed commitments. Cancer genes are cooperative and contribute their efforts in a harmonic manner through the tumourigenic process”[15]. Some facts were also highlighted which included specific organic targeting for each cancer gene, sharing by different genes, i.e., oncogenes, tumour suppressor- and predisposing-genes.

The core reason for malignant behavior is due to the manner and timing of initiation, promotion, and progression of tumours through which we could find the roots in the function of genes and proteins. Complementary information on tumour genesis in brain tumours is not fully known which could be due to heterogeneity and lack of data on the involved genes in brain tumours.

Two novel heterozygously intronic changes including IVS 35- 63 T→A and IVS35-30 A→G, found in a proband within 3' regions of splicing site, highlights the importance of this alteration during tumour genesis of our proband (Fig. 2). As a matter of fact, the splicing needs some intronic DNA sequences in which the 5' and 3' regions of intron are involved and play an important role supporting our finding [28].

As a matter of fact there is interaction between gene mutation and protein expression. In this regard, there are limited reports, even at brain tumour cell line level, on the protein expression of ATM. It was reported that ATM participates in controlling the S-phase checkpoint through CDK2-dependent phosphorylation leading to degradation of Cdc25A [11, 29]. In order to confirm the diversity of ATM-protein expression, we analyzed individual cells by immunofluorescence which revealed low levels of ATM protein (Fig.3).

The hit model proposed by Knudson (13) for the development of Retinoblastoma was based on family history, bilateral/ multifocal cases and young age at onset. However, in our investigation the heterozygous alterations in ATM could be confirmed by cloning results revealing the involvement of two alleles.

Three categorical events for creation of the three-hit could be summarized:

1. The first hit (D1853N) is a germline inherited trait from proband’s mother. This was the required fundamental change for initiation of an evolutionary process in astrocytoma.
2. The second hit is IVS 35- 63T→A as a result of the 1st somatic evolution in the proband’s peripheral blood and tumour. This trigger has apparently occurred at a very early moment in the zygotic stage of the
proband before differentiation of peripheral blood and brain tissues (Fig. 1: generation V/14).

3. The third hit (IVS35-30 A→G) is a somatic alteration which might have occurred in an astrocyte or during the development of an astrocytoma. These intronic triggers were required for promoting tumour genesis.

4. These facts explain the catalytic and complementary role of events considered as the key triggers at genomic and somatic levels, respectively.

5. There is correlation between severity of cancer and the number of hits in a specific gene.

6. The role of environmental factors.

7. The specific pattern at molecular- and expression- level in different populations.

A sad scenario in nature is played. The initiation and termination of two evolutionary events in a proband affected with astrocytoma has occurred which could have been passed on to her offspring, but the proband was deceased, and there was no chance and sign of inheritance any more. This might be called “genetic block” or “natural prevention”.

Figure 2. Partial sequence results of proband affected with astrocytoma; illustrating three- hit hypothesis through an evolutionary process (15). a: Alterations of ATM gene in Blood tissue b: Alterations of ATM gene in brain- tumour tissue. Arrows at N-positions, show the molecular alterations, as heterozygosity.
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![Figure 3. Expression of ATM protein by immunofluorescence. a: illustrates the proband’s tumour cells with DAPI filter. b: illustrates the conjugated ATM protein antibody with FITC in the same tumour cells, showing a low expression of protein.](image)

The present three-hit hypothesis could now be considered as an effective event in the course of tumour genesis in astrocytoma. In addition, to highlight the importance of genetic counselling, the proband’s relatives were informed and warned regarding such data as partial risk factor to avoid consanguineous marriage. The suggestion and performance of further genetic test(s) for the relatives in this pedigree seemed to be helpful.

In addition, four unreported polymorphisms including D1853N, IVS 38-8 T→C, F858L, and P872T were also found by us in brain tumours other than medulloblastoma [30].

This was not the end of the scenario of the ATM gene. Up to the this point the importance of ATM involvement and the three-hit hypothesis were discussed in the brain. Now it is aimed to unfold a continuing story of the ATM gene in breast cancer. Further findings by us involved D1853N which was also reported in breast cancer patients [31]. The case-control study revealed that the frequency of this polymorphism in cases, internal and external controls was 31.0%, 26.9%, and 12.5% respectively. Regarding the Odd Ratio, a significant difference was observed between the patient-carriers and external healthy controls (P=0.001), and the difference between the internal-carrier-control and external controls was statistically significant as well (p=0.004). This finding could be considered as a predisposing marker in our population and specifically in the cancer-prone pedigrees.

Tumour suppressor genes (TSGs) influence the cancer cellular territory, properties and abilities. However, there are various fields including genomic, proteomic and metabolic alterations for generating cancer cells including
mutation, aneuploidy, and expression by the involvement of the promoter. Although the occurrence of mutations in ATM play a vital role in tumour evolution, but the spectrum of protein expression could be considered as an important function and governing ability of this gene in different tumours. This paradigm is included in our current project. This field also requires the evaluation and confirmation of ATM-protein expression according to our previous report [15].

The ATM and p53 genes have functional mechanisms in common, but it seems that in spite of the broad involvement of p53 in variety of tumours, the status of this gene in human tumours cannot predict patients’ outcome [32]. So far the molecular process in cancer development is known to be programmed by mutations through a cascade of events that mediate the following genes and actions respectively:

1. Inactivating tumour suppressor gene leading to cell proliferation.
2. Inactivating DNA repair gene.
3. Alterations in proto-oncogene leading to activation of oncogene.
4. Inactivating several tumour suppressor genes.

As it is obvious, there are two major, a primer and a final, gates which initiate malignant behavior and end up producing the malignant feature of a specific tissue. Inactivation is the key event for these genes except for oncogenes. But, cancer development seems to be beyond such pattern and lists of alterations at biological level are also involved. Some Genetic testing for high-risk individuals for specific cancers are available, but not for brain tumours.

Early detection of inherited predisposing genetic trait for cancer, or a de novo alteration could lead the clinician toward cancer-preventing interventions. This could directly improve quality of life in high-risk relatives of cancer probands within pedigrees. Through tracing genomic-somatic evolution, we would be able to achieve a more complementary definition of the genome and its alterations beyond the previous outcomes; and consider it as the organisms’ measurable genes. This together with cell biology could lead scientists to the right direction in combating cancer by targeted therapy.

Considering the involvement of ATM in healthy individuals in whom the cycling machinery function seems to be apparently normal, and in the cancer population with an abnormal and continuously cycling cell cycle, it could be presumed that this powerful gene has its own regulatory mechanism which may govern cells.
The clinical management for brain tumours relies on imaging, biopsy, tumour grading, and optional treatments including surgery and radiation. There are peculiarities in the genetic-based pattern of cancer development in primary and secondary stages, and there are also problems in clinical outcome for low- and high-grade astrocytoma. However, the fundamental genetic mechanisms and their inflection by environmental factors are important mechanisms through which we might achieve the new avenues for understanding the unknown corners of cancer to prevent or ideally cure it. One possible way would be by re-establishment of tumour-suppressor function. However, to achieve this strategy, a more complementary knowledge of TSGs, including the ATM gene is required.

Conclusion

The three-hit hypothesis includes an inherited D1853N as a first hit at germ line, IVS 38-63T→A as a second hit, and IVS38-30A→G as a third hit. These triggers could be considered as triangle initiators for the course of evolution in astrocytoma. The present data could address the crucial role of the specific intronic region of the ATM gene, and also the importance of pedigree analysis. This polymorphism might be useful as a marker in astrocytoma as well.

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References


