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Analysis of Exon 19 and 39 of ATM Gene in Brain Tumors; Considering the P53 Accumulation in Patients with ATM Alteration

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Abstract

Many studies have been focused on cytogenetic and molecular genetic defects in brain tumors; therefore the role of ATM as a tumor suppressor gene in these tumors is poorly considered. In this study mutation analysis of exon 19 and 39 of ATM gene and P53 accumulation were investigated by PCR-SSCP, sequencing, and flow cytometry . Four polymorphisms including D1853N, IVS 38-8 T→C, F858L, P872T were reported for the first time in brain tumors other than medulloblastoma. Expression of P53 could be detected in more than 10% of cells in patients affected with meningioma and 4.08% and 3.46% of cells inastrocytoma and chordoma respectively. The present findings could confirm the importance of ATM gene alterations in tumor genesis of brain tumors and further investigation is essential.

Keywords: Brain tumor; ATM gene; P53 expression

Introduction

The ATM gene was cloned on chromosome 11q22-23 [1], consisting of 66 exons and extends over 160 kb of genomic DNA [2]. Its protein product with serin/threonin kinase activity has 350.6 kD weight; belongs to PI3-kinase like (PIKK) family [3].

This large serin/threonin kinase protein has an initial and crucial role during the repair of DNA double strand breaks caused by radiation [4,5].

Mutation in ATM gene is a hallmark of Ataxia Telangiectasia disease (A-T) [1] with increased in cancer incidence in affected patients. Common neoplasms in these patients include tumors of immune system [6,7] and solid tumors. The main type of these solid tumors are central nervous system tumors that includes gliomas [8] and medulloblastomas [9].

In this regard a few studies have investigated the role of ATM gene in development of brain tumors in patients without A-T disease. In one study Liberzon et al. reported two well-known polymorphisms, D1853N and F858L in medulloblastoma tumors [10].

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ATM as a serin/threonin kinase is targeted many substrates including P53 during its role in repairing DNA break damage. ATM can phosphorylate P53 stabilizing this protein [11]. However, some studies have reported the correlation between P53 accumulation and prognosis of patients affected with brain tumors [12-15].

Considering these findings we have designed the study to analyze exon 19 and 39 which have shown polymorphisms in medulloblastoma [10] as well as breast tumors [3] and normal population [15] in different types of brain tumors. In the other part of this research and To investigate the role of ATM gene alterations found in these two exons in our study we investigate P53 expression in samples with ATM gene alteration by flow cytometry.

Materials and Methods

The study included tumors obtained from 50 patients affected with brain tumors. Genomic DNA samples were extracted from paraffin-embedded tissues using Phenol-chloroform method. PCR and single-strand conformational polymorphism (SSCP) were used to detect the ATM gene alterations**.**

PCR

PCR amplification was carried out using oligonucleotide primers with following sequences: Exon 19: 5′-CTCCTGCAAGAAGCCATCT-3′, 5′-AAGAAA TCCCAAGTAGTAAAT-3′,

Exon 39: 5'-GCAGTATGTTGAGTTTATGCCA-3', 5′-GCAACTGTTGGCAACTTTTAT-3′ [15]. PCR fragments were generated from100ng of genomic DNA in 23 µl of reaction mixture consisting 1.5 µl 10 x buffer (10 mM Tris HCL, PH 8.3, 50 Mm KCL, 10 µg/ml gelatin), 0.43 µl of 10x dNTP, 0.5 µl of MgCl2 10 Mm, 18.56 µl of deionized water, 3 U Taq DNA polymerase and 1 µl (10 pm) from forward and reverse for each exon separately.

PCR reaction were performed for an initial 3 min at 94° C , followed by 35 cycles of 94° C for 30 sec, 55° C for 30 sec and 72° C for 30 sec.

SSCP

PCR products were mixed with SSCP loading buffer consisting of 95% formamide, NaOH 10mM, bromophenol blue 0.05%, xylene cynaol with 1:1 proportion. The samples were electrophoresed on11% polyacrylamide (29:1) gel containing 25.6 ml acrylamide solution , 4.6 ml TBE 10x buffer, 39.6 ml d H2O , 300 µl APS 10x and 30 µl of TEMED. Gel was run overnight at 150 voltage for 26 hours then stained using Silver-Nitrate staining procedure (0.25 g of Silver nitrate was dissolved in 250 ml d H2O shaking for 15 min then 25 grain of NaOH dissolving in 250 d H2O mixing with 1 ml formaldehyde was added after removing silver nitrate solution till the bands be appeared).

The bands and shifts were analyzed compared with negative control and undenatured samples. Direct sequencing was performed for shifted-band samples.

Cell Preparation

Tissue sections, 5 µm thick, were cut from Four paraffin-embedded blocks brain tumors (Two meningioma, 1 astrocytom, and 1 chordoma a) showing ATM alterations in their DNA sequencing analysis were used to study the P53 accumulation. The sections dewaxed by two times adding xylol (Merck- Germany). To remove the xylol we washed the tissues two times with phosphate buffer salin 1x (PBS; 8 gr NaCl, 0.2 gr KCl , 1.15 gr Na2HPO4, 0.2 gr KH2PO4 dissolve in 1litler dH2O). Dewaxed tissues were incubated in mixture of cell culture, PH 7.2, (MaCoy; Grand Island biological company- USA) and 500 µl trypsin 0.01x (Sigma- Aldrich, St Louis, MO, USA) for 45 min at 37˚C. To remove trypsin preventing further activity in the mixture we wash the cells two times with KCl 0.075 M. Then we add cell fix solution containing Methanol (Merck, Germany) and Acetic Acid (Merck- Germany) with proportion of 3:1, respectively. The last stage of procedure causes the cells to be swollen and permeablised to antibody using for flow cytometry.

Staining

Extracted cells were stained using monoclonal antibody IgG2b mouse antihuman P53 (clone DO-7, 1:25; DakoCytomation). The average of 500 cells were washed two times with PBS 1x. After adding 5µl of P53 antibody the mixture of cells were incubated at 4˚C for 25 min. Then PBS 1x was used to wash the solution for two times. The antibody was detected using goat anti-mouse IgG2b-FITC (0.5 ml, 1:4; M32501, GE/ Amersham Biosciences) as flurochrome conjugate. The mixtures were incubated for 25 min at 4˚C. To remove further antibodies, we wash the cells three times with PBS 1x.

To prepare negative control for flow cytometry, we used the cells and repeated the above procedures using goat anti-mouse IgG2b-FITC antibody.

Flow Cytometry

To perform flow cytometry using instrument (Partec-Denmark); firstly, we insert the negative control cell mixture to adjust the test samples. Then test samples were analyzed obtaining graphs and data which showed the number of cells expressed P53 protein.

U87- $P53^{-/-}$ cell lines were used to check the specificity of the antibody used in our study.

Results

The 50 brain tumor samples analyzed in our study were consisted of 38% (19/50) Meningioma, 12% (6/50) GBM, 18% (9/50) Astrocytoma, 8% (4/50) Schwannoma, 4% (2/50) Oligodendroglioma, 2% (1/50) Hemangioblastoma, 4% (2/50) Medulloblastoma, 2% (1/50) chordoma, 2% (1/50) Optic glioma, 2% (1/50) Neurofibroma, 2% (1/50) Craniopharyngioma and 2% (1/50) Esthesioneuroblastoma. The patients included 46% (23/50) female and 54% (27/50) male, with mean age of 42. 20%. Ten out of 50 patients participated in our study had a family history of other cancers and 80% (40/50) did not have any other malignancies in their family members. Parent consanguinity was positive for 18% (9/50) of patients. 4% (2/50) of brain tumors were the metastatic form of other tumors and 14% (7/50) of patients showed recurrence (Table 1).

We performed a mutation analysis of genomic PCR products amplified from 50 brain tumor DNA samples of non A-T patients by SSCP analysis of two polymorphic exons, 19 and 39, published elsewhere (liberzon et al. 2003).

4 Patients showed shifted band in SSCP (Fig. 1).

In this regard three patients showed alteration in both exons and one in exon 39 (Table 2).

We could detect D1853N and F858L polymorphism in four and two patients respectively. The alteration P872T was found in three patients. Interonic polymorphism, IVS 38-8 T→C, exhibited in one patient (Fig. 2) this is the first report of this intronic alteration in brain tumor. Among tumors with affected ATM gene were two meningioma, one chordoma and one astrocytoma grade II. Three patients had family history of other malignancies. The grand daughter of person who showed IVS 38-8 T→C polymorphism had a benign breast cancer, regarding to the point that this alteration may be related to the age of onset of the ones affected with breast cancer [17].

Considering P53 expression in those tumors showing alteration in exon 19 and 39; we could detect its expression in more than 10% of cells in patients affected with meningioma (Fig. 3). The two other tumors including astrocytoma and chordoma showed P53 expression in just 4.08% and 3.46% of cells, respectively (Table 2).

Discussion

ATM gene product is a large highly conserved protein [18] belonging to PIK-3 like kinase family [1].

Regarding its pivotal role as a safe guard molecule during DNA double strand breaks [18]; any mutation in the ATM gene which results to defective protein can be harmful.

Mutation in ATM gene results in ataxia telangiectasia as a cancer prone disease [18]. The A-T patients are face to many malignancies including tumors of immune system and nervous system tumors such as glioma and medollublastoma [8,9].

The ATM gene alterations in A-T families are fully investigated by many scientists, but the question is raised about alterations of this gene in tumors occurred in non A-T patients.

In deed the alteration of ATM gene in many malignancies of immune system in patients without family history of A-T has been discovered [6,7].

Among solid tumors, the involvement of ATM mutations and polymorphisms in breast cancer is reported in many studies [3,19].

However the influence of ATM gene in non A-T patients is poorly understood. Therefore Liberzon et al. reported D1853N and F858L polymorphisms in 5 children with medulloblastoma [10].

These two polymorphisms have been identified in general and healthy population [15], patients affected with A-T [20] and breast cancer [3].

Here we report these two polymorphisms in addition of two other alterations including IVS 38-8 T→C and P872T in 4 out 50 patients involved in our study.

This is the first report of these alterations in types of brain tumors rather than medulloblastoma and glioma. As just 4 out of 50 patients have showed the polymorphisms and not any mutations of these two polymorphic exons in our study we concluded the poor involvement of this gene in brain tumor development. However further investigation on other exons of this gene is needed.

In order to find whether these amino acid substitutions in ATM gene are actually neutral and don't have any influence in function of protein products; in the other part of this study we have investigated the P53 expression in patients with ATM gene alteration in these two exons; considering P53 as a direct substrate of ATM protein.

Table 1. The summery of patients data involved in our study

Ref No	Age	Sex	Tumor Type	Grading	Family History
1	59	m	GBM	IV	N
2	25	f	chordoma		N
3	9	f	medulloblastom		N
4	22	m	astrocytoma	I - II	P
5	34	m	astrocytoma	I	P
6	17	m	astrocytoma	I	N
7	56	f	meningioma		N
8	59	m	meningioma		P
9	49	f	meningioma		N
10	70	f	meningioma		N
11	69	f	meningioma		P
12	23	m	GBM	IV	P
13	69	m	meningioma		N
14	33	m	meningioma		N
15	5	f	medulloblastom		N
16	45	f	meningioma		N
17	43	m	astrocytoma	III	P
18	45	m	astrocytoma LG	II	N
19	38	m	oligodendroglioma		N
20	54	m	GBM	IV	N
21	43	m	astrocytoma LG	I - II	N
22	52	f	lymphomameningioma		P
23	34	m	astrocytoma LG	II	N
24	58	f	meningioma		N
25	42	m	astrocytoma®(A)	III	N
26	52	f	schwannomas		N
27	40	f	meningioma CPA		P
28	62	f	Adenocarcinom colorectal		N
29	55	f	schwannoma		N
30	8	m	schwannoma		N
31	40	f	meningioma		N
32	65	f	meningioma		P
33	42	m	astrocytoma®	II	N
34	55	m	meningioma		N
35	64	m	esthesioneuroblastoma	IV	N
36	66	m	meningioma		N
37	37	f	meningioma		N
38	63	m	schwannoma		N
39	34	f	adenocarcinoma intenstin		N
40	63	f	GBM	IV	N
41	15	m	cnaniopharyngioma		N
42	24	m	meningioma®		N
43	42	m	GBM	IV	P
44	45	m	ODG GBM	IV	N
45	39	f	meningioma		N
46	18	f	optic gliom	I	N
47	27	m	gliom® GBM	IV	N
48	6	m	neurofibrom		N
49	65	f	meningioma		N
50	41	f	hemangioblastom		N

Table 2. Cases showing alteration in exon 19 and 39; with analysis of P53 expression

No	Age	Sex	Tumor type	Family history of other malignancies	Parent consanguinity	ATM Alterations (Exon 19 and 39)	P53 expression (cell percent)
252	34	m	Astrocytoma grade II	+	+	D1853N	4.08%
223	25	f	chordoma	+	−	D1853N F858L P872T	3.46%
234	64	f	meningioma	+	−	IVS 38-8 T→C, D1853N F858L	12.51%
279	65	f	meningioma	−	−	D1853N F858L P872T	25.49%

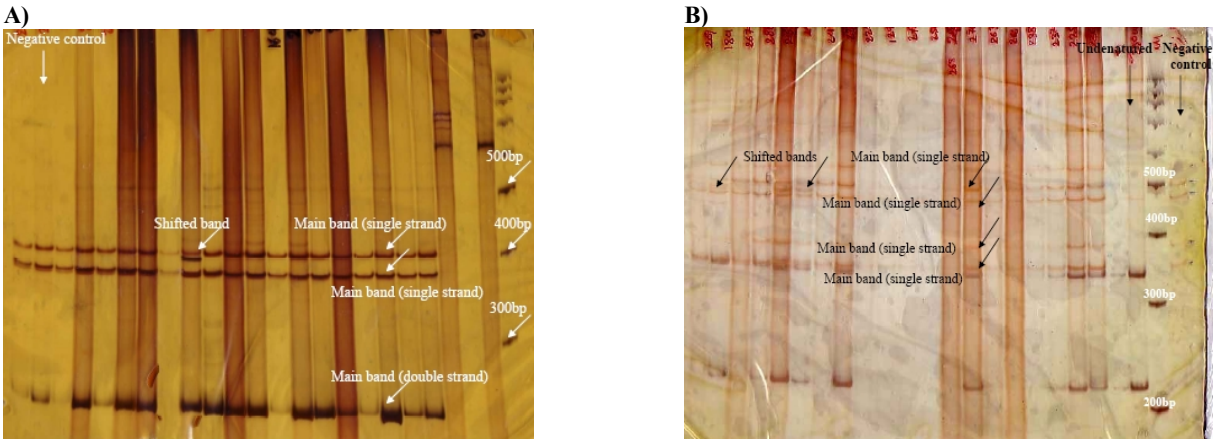

Figure 1. Electrophoregram of ATM gene; Exon 19 (A) and Exon 39 (B).

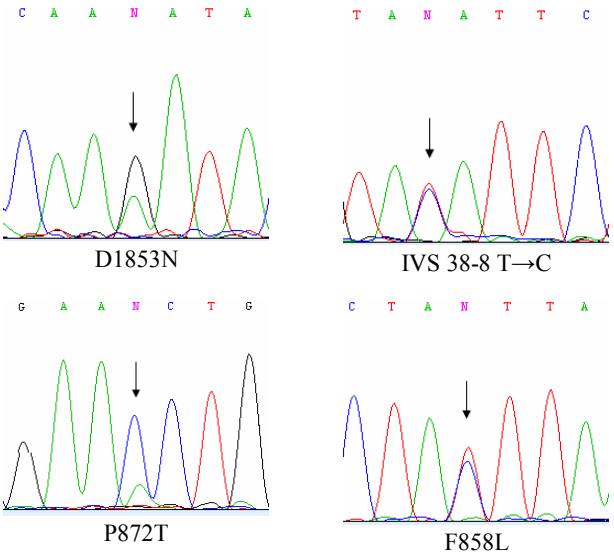

Figure 2. Sequence analysis of samples showed polymorphisms.

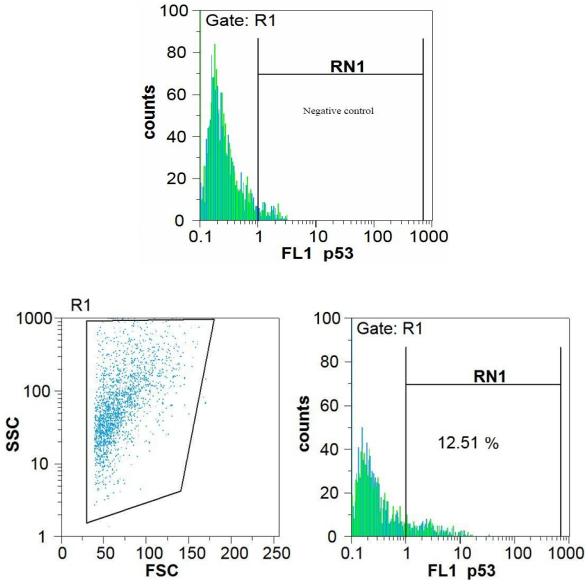

Figure 3. Flow cytometry analysis of samples showing alterations in ATM gene.

Our results show no correlation in the case of ATM polymorphism and P53 expression. In this regard further analysis should have performed on other direct substrates of ATM gene including BRCA1; as the grand daughter of a patient showed IVS 38-8 T→C was affected with breast cancer. This polymorphism is found in breast cancer patients and though to have effective in the age of onset in these patients.

Regarding to unpublished data obtained from one of our studies for searching P53 expression in brain tumors 6 out 7 meninigioma included in the investigation showed P53 expression in more than 5% of cells. This information are consonant to data obtained from this study as two patients affected with meningioma showed the P53 expression in more than 10% of cells. More studies are needed to find the relation of P53 expression and evolution of meningioma.

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References

1. Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NG, Taylor AM, Arlett CF, Miki T, Weissman SM, Lovett M, Collins FS, Shiloh Y. , A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*, **268**: 1749-1753 (1995).
2. Uziel T., Savitsky.K., Platzer M., Ziv Y., Helbitz T., Nehls M., Boehm T., Rosenthal A., Shiloh Y., Rotman G., Genomic organization of the ATM gene. *Genomics*, **33**: 317-320 (1996).
3. Dork T., Bendix R., Bremer M., Rades D., Klopper K., Nicke M., Skawran B., Hector A., Yamini P., Steinmann D., Weise S., Stuhrmann M., Karstens JH., Spectrum of ATM gene mutations in hospital-based series of unselected breast cancer patients. *Cancer Research*, **61**: 7608-7615 (2001).
4. Lavin M., Shiloh.Y., the genetic defect in ataxia telangiectasia. *Annu. Rev. Immunol.*, **15**: 177-222 (1997).
5. Kastan MB., Lim.D., The many substrates and functions of ATM. *Nat. Rev. Mol. Cell Biol.*, **1**: 179-186(2000).
6. Vorechovsky I., Luo, L., Dyer M. J. S, Catovsky D., Amlot P. L., Yaxley J. C., Foroni L., Hammarstrom L., Webster A. D. B, Yuille M. A. R., Clustering of missense mutations in the ataxia-telangiectasia gene in a sporadic T-cell leukaemia. *Nature Genet*, **17**: 96-99 (1997).
7. Schaffner C., Stilgenbauer.S., Rappold G. A., Dohner H., Lichter P., Somatic ATM mutations indicate a pathogenic role of ATM in B-cell chronic lymphocytic leukemia. *Blood*, **94**: 748-753 (1999).
8. Miyagi K., Mukawa.J., Kinjo N., Horikawa K., Mekaru S., Nakasone S., Koga H., Higa Y., Naito M., Astrocytoma linked to familial ataxia-telangiectasia. *Acta Neurochir (Wein)* **135**: 87-92 (1995).
9. Gatti R. A., Boder.E., Vinters H. V., Sparkes R. S., Norman A., Lange K., Ataxia-telangiectasia: an interdisciplinary approach to pathogenesis. *Medicine*, **70**: 99-117 (1991).
10. Liberzon E., Avigad.S., Cohen I. J., Yaniv I., Michovitz S., Zaizov R., ATM gene mutations are not involved in medulloblastoma in children. *Cancer Genet. Cytogenet*, **146**: 167-169 (2003).
11. Khanna KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, Taya Y, Gabrielli B, Chan D, Lees-Miller SP, Lavin MF. , ATM associates with and phosphorylate p53: mapping the region of interation. *Nat Genet*, **20**: 398-400 (1998).
12. Batussek C. , Naumann.U., Weller M., Accumulationof mutant P53 V143A modulates the growth, clonogenicity and radiochemosensitivity of malignant glioma cells independent of endogenous P53 status. *Experimental cell research*, **253**: 432-439 (1999).
13. Horiguchi H., Sano.T., Hirose T., TP53 deleted cells in de novo glioblastoma using fluorescence in situ hybridization. *Pathology International*, **51**: 187-192 (2001).
14. Peraud A., Kreth.F., Wiestler O.D., Kleihues P., Reulen HJ., Prognostic impact of TP53 mutations and P53 overexpression in supratentoria WHO grade II astrocytoma and oligoastrocytomas. *Clinical Cancer Research*, **8**: 1117-1124 (2002).
15. Thorstenson Y R., Shen P., Tusher V G., Wayne T L., Davis R W., Chu G, and Oefner. PJ. , Global Analysis of ATM Polymorphism Reveals Significant Functional Constraint. *Am J Hum Genet*, **69**: 396-412 (2001).
17. Heikkinen K., Rapako.K., Karppinen SM., Erkko H., Nieminen P., Winqvist R., Association of common ATM polymorphism with bilateral breast cancer. *Int J Cancer*, **116**: 69-72 (2005).
18. Shiloh., Y., ATM and related protein kinases: safeguarding genome integrity. *Nat rev Cancer*, **3**: 155-68 (2003).
19. Swift M, Morrell D,. Chase CL. , Breast and other cancers in families with ataxia-telangiectasia. *N Engl J Med*, **316**: 1289-94 (1987).
20. Sandoval N., Platzer.M., Rosenthal A.,Dork T., Bendix R., Skawran B., Stuhrmann M., Wegner RD., Sperling K., Banin S., Shiloh Y., Baumer A., Bernthaler U., Sennefelder H., Brohm M., Weber B.H., Schindler D., Characterization of ATM gene mutations in 66 ataxia telangiectasia families. *Hum. Mol. Genet,* **8:** 69-79 (1999).