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Phospho-SMC1 in-Cell ELISA based Detection of Ataxia Telangiectasia

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Abstract

Background

Ataxia telangiectasia (A-T) is a common genetically inherited cause of early childhood-onset ataxia. The infrequency of this disease, vast phenotype variation, disorders with features similar to those of A-T, and lack of definite laboratory test, make diagnosis difficult. In addition, there is no rapid reliable laboratory method for identifying A-T heterozygotes, who susceptible to ionizing radiation (IR), atherosclerosis, diabetes, and cancers. We used SMC1pSer966 (pSMC1) in-cell colorimetric ELISA to diagnosis and screen in A-T families.

Materials and Methods: With informed consent, 2cc peripheral blood was collected from the 15 A-T patients, their parents, and 24 healthy controls with no family history of malignancy, diabetes, and atherosclerosis. Extracted peripheral blood mononuclear cells (PBMCs) were cultured in poly-L-Lysine treated 96-well plate with density of 70,000 cells per well. SMC1 phosphorylation was evaluated with cell-based ELISA kit 1 hour after 5 Gy IR and the pSMC1data normalized with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Results: SMC1 phosphorylation was significantly low in A-T`s PBMC (mean ± standard deviation [SD]: 0.075 ± 0.034) in comparison to carriers (mean ± SD: 0.190 ± 0.060) and healthy controls (mean ± SD: 0.312 ±0.081), but unluckily could only discriminate A-T patients (Area Under the Curve -receiver operating characteristic [AUC-ROC]: 1.00, 1.00-1.00). This method in spite of rapidness and simplicity showed poor imprecision (22.49% coefficient of variation [CV] for intraday imprecision).

Conclusion: It seems pSMC1 assessment by in-cell ELISA can be used for detection of A-T patients, but it may not sensitive enough for identification of carriers. This ELISA test is very simple, rapid, and requires less than 2cc blood. Thus it may be proposed for the early differential diagnosis of A-T as an alternative method.

Key Words: Ataxia telangiectasia, Children, ELISA, PBMC, SMC1.

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1- INTRODUCTION

Ataxia-telangiectasia (A-T) is a rare autosomal recessive multisystemic syndrome characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia, variable immunodeficiency, chromosomal instability, radiosensitivity, and high risk for malignancy, predominantly leukemia and lymphoma in children and epithelial cancers in surviving adults (1, 2). Onset of neurological changes in A-T patients usually occurs between 1 and 4 years of age. A-T is occurred worldwide, with an incidence of 1 in 40,000 to 100,000 newborns (3). This frequency varies from country to country and it is relatively prevalent in Iran because of high consanguinity marriage rate and ethnic components (4, 5). Importantly, the A-T carrier (heterozygote) frequency is estimated to be approximately 1-2% of the general population (3). Although, A-T heterozygotes are usually asymptomatic and mostly considered healthy, but in comparison to the general population, they are more susceptible to ionizing radiation (IR) and have a higher risk of heart disease, diabetes, and cancers in particular breast cancer (6, 7). Early diagnosis of A-T patients and carriers is helpful for prognosis and genetic counseling of families (8).

A-T is caused by germline mutations in the ataxia-telangiectasia mutated (ATM) gene, which is located at 11q22.3 (9). ATM protein is a ubiquitous serine/threonine kinase mainly involved in the maintenance of genomic stability (10). Mutations of ATM occurs across the full-length transcript without any hotspots. Over 600 different A-T mutations have been described and most of the patients are compound heterozygotes (2, 11). Diagnosis of A-T is difficult and based on the combination of clinical features with laboratory tests including high levels of serum alpha-fetoprotein, cell sensitivity to IR, acquired 7- and 14-chromosome translocations in the blood karyotype and reduced or absent levels of ATM protein (1, 12). Mutation analysis can be used for confirmation of diagnosis in A-T patients and identification of carriers; however, currently DNA sequencing is relatively expensive, also ATM gene is large and the causality of many missense DNA changes is often unclear (13-17). Therefore, the use of a functional test might be useful for the identification of A-T patients and carriers.

Recently a functional flow cytometry assay has been developed to diagnose patients and carriers of A-T by R.A. Gatti and his colleagues. They used nuclear staining of Lymphoblastoid cell lines and transformed peripheral blood mononuclear cells (PBMCs) to measure ATM-dependent phosphorylation of structural maintenance of chromosomes 1 (SMC1) following deoxyribonucleic acid (DNA) damage (18). Later, phosphorylation of SMC1 was used to detect radiation exposure in humans by blood-based pSMC1 enzyme-linked immunosorbent assay (ELISA) (19). SMC1 is a cohesin binding protein that is phosphorylated by ATM kinase after DNA damage (20). The results of previous studies were encouraged us to combine their findings to seek a rapid, easy and not expensive way to asses SMC1 phosphorylation. So, we used in-cell ELISA colorimetric detection system to measures ATM-dependent phosphorylation of the SMC1 protein after DNA damage, for identifying A-T patients and carriers.

2- MATERIALS AND METHODS

2-1. Blood sample and culture

This study was conducted in Children's Medical Center, Tehran University of Medical Sciences from September 2015 through May 2016 after obtaining approval from research ethics committee of university. With informed consent, we collected 2cc peripheral blood from 15 patients with A-T, 30 obligate carrier

parents, and 24 healthy controls unrelated to A-T families with no family history of malignancy, diabetes, and atherosclerosis. Sample size was calculated according to pervious study (18).

In-cell ELISA assay was performed according to the SMC1 (phospho-Ser966) colorimetric cell-based ELISA kit (CytoGlow™, assaybiotech) with some modifications. Briefly, PBMCs were isolated by Ficoll (Biosera, East Sussex, UK) and 70,000 cells were cultured for at least 15 hours in 96-well plates treated with poly-L-lysine (sigma, US) in 200ul of Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Germany) medium supplemented with 5% fetal bovine serum (Gibco, Germany), 1% L-glutamine, 1% penicillin/streptomycin, and in a humidified incubator with 5% Carbon dioxide [CO2]. Gamma irradiation was carried out in Theratron 780E (Canada) at room temperature and at a dose rate of 0.9 Gy/min. One hour after 5 Gy irradiation, 100 µL of medium was replaced by 100 µL of 8% formaldehyde solution (sigma, Germany) to fix and crosslink the cells to the microplate. After 15 minutes incubation, the plate was washed 3 times with wash buffer and the cells permeabilized in Triton™ X-100 (Merck, Germany) for 20 minutes. After quenching and blocking, the cells incubated with primary antibody for overnight. Substrate was added to wells, after secondary antibody incubation, for 20 minutes. The optical density (OD) data were measured with micro-plate reader 4 Plus (Hyperion. Inc., USA) at 450nm wavelength.

Each condition was performed in duplicate and anti-GAPDH antibody was used as a positive control, and SMC1 and GAPDH secondary antibodies (without primary antibody) were used as negative control. Both positive and negative controls were performed in the same plate with the pSMC1 target experiments.

2-2. Precision studies

We determined the ELISA intraday assay (within-run) precision, expressed as a coefficient of variation (CV), by testing PBMCs isolated from one healthy donor and one A-T patient donor samples, in 7 measurements on the same day. The interday assay (between-day) variability calculated in 4 measurements on 4 different days for 1 healthy donor.

2-3. Data normalization and statistical analysis

The OD data of pSMC1 protein were normalized with OD values of GAPDH and these quantitative values were presented as the means ± standard deviations (SD). The Kruskal–Wallis test and Dunn's multiple comparisons test as post hoc were used to compare the groups and the Receiver operates characteristic (ROC) curves were used to determine relevant sensitivity and specificity of pSMC1 ELISA values. All Data were analyzed using GraphPad Prism version 6 (San Diego, CA, USA) and SPSS 22 (IBM SPSS Statistics, IBM Corporation, Chicago, IL) softwares. P values <0.05 were considered significant.

3- RESULTS

For analyzing SMC1 phosphorylation by in-cell ELISA, 15 patients with A-T (male: female, 0.87), 30 obligate carrier parents, and 24 healthy controls (male: female, 1.4) were enrolled in this study. The mean (lowest-highest) age was 10 (4-22), 37(26-55), and 32 (19-46) years old for A-T patients, carriers, and healthy controls respectively.

We determined optimal cell seeding density by using serially diluted cells (0-280,000 cells) per well in 96-well plates. After irradiation, fixing and staining, 70,000 cells were chosen based on sufficient color intensity without saturation and confluency (**Figure.1**).

3-1. Precision studies

We evaluated the precision of the test based on PBMCs at two levels: intraday assay (within-run), and interday assay (between-run). For intraday evaluation, PBMCs from 1 healthy donor and 1 A-T patient donor samples were irradiated, fixed, and stained in replicates the same day (one time, 7 aliquots). The intraday assay imprecision (CV) was 13.16% and 14.33% for healthy donor and A-T patient, respectfully (**Table.1**). For the second level, PBMCs collected from 1 healthy donor on 5 different day. Interday assay (between-run) imprecision (CV) of 5 measurement was 22.49%.

3-2. Detection of ATM kinase activity by SMC1 phosphorylation

Figure.2 demonstrates that phosphorylation of SMC1 after 5 Gy IR is absent or significantly reduced in A-T patients (mean± SD: 0.075 ± 0.034), but it is present in A-T carriers (mean± SD: 0.190 ± 0.060) and healthy controls (mean ± SD: 0.312 ± 0.081). Although the difference between A-T carriers and healthy controls is statistically significant (Kruskal–Wallis test, P = 0.0007), there is overlap between them that prevent us to set a detection cut-off. ROC curve analysis of pSMC1values has provided a high sensitivity and specificity for detection of A-T patients with AUC–ROC of 1.00 (95% CI 1.00–1.00) as shown in **Figure.3**.

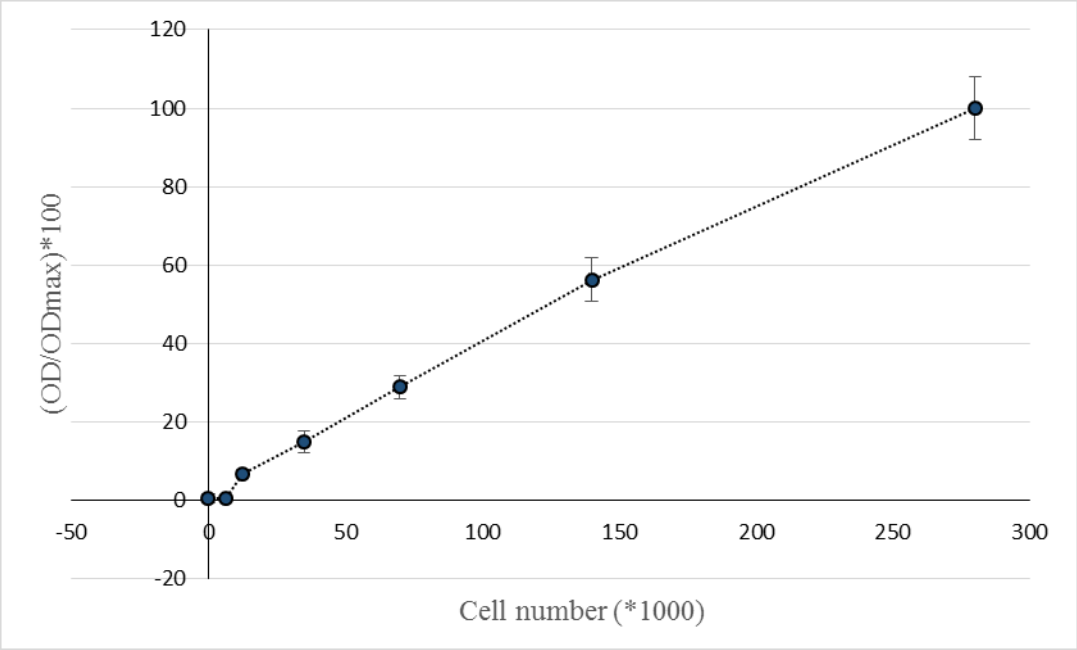

Fig.1: Determination of the optimal cell number for seeding. Serially diluted PBMCs (0-280000 cells) from one healthy donors were seeded in poly-L-lysine treated 96-well plate. After irradiation, cells were fixed and stained with HRP-conjugated antibody. For all densities, experiment was performed in triplicate. The solid circles indicate mean and the error bars represent SDs.

Table-1: Precision of ELISA assay

Intra-day[a]	Measurements (n)	Mean	Standard deviation	CV (%)
Healthy control	7	0.296	0.039	13.16
A-T patient	7	0.091	0.013	14.33
Inter-day[b]	5	0.302	0.067	22.49

[a] PBMCs were assayed 7 times in the same day.
[b] PBMCs were assayed one time on 5 different days.

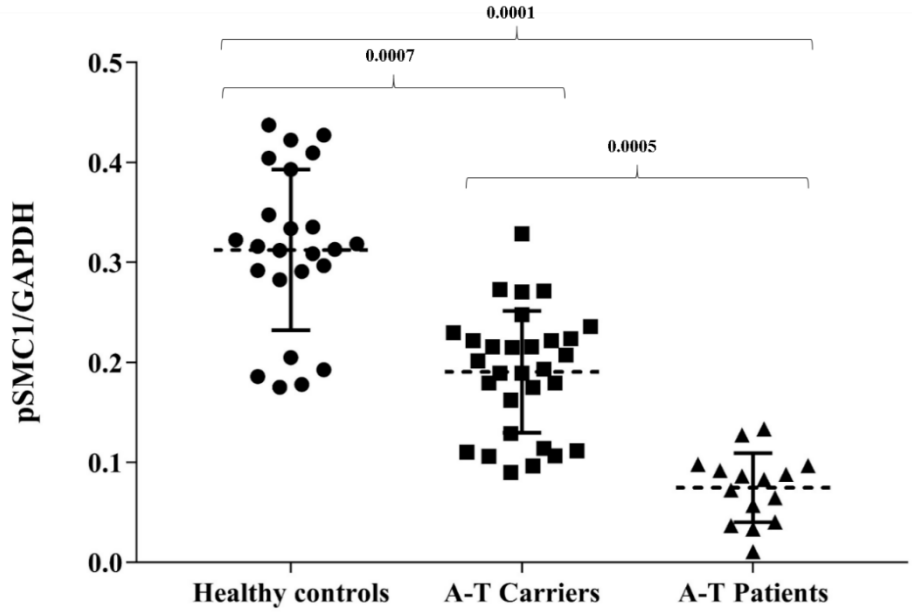

Fig.2: In-cell ELISA analysis of SMC1 phosphorylation on cells after 5 Gy irradiation in PBMCs from healthy controls, A-T carriers and patients. Dot plot of normalized OD data performed on PBMCs from 24 healthy controls [0.312 mean (0.081 SD)], 30 A-T carriers [0.190 mean (0.060 SD)], and 15 A-T patients [0.075 mean (0.034 SD)]. P values between groups are indicated above each panel. The dashed lines indicate mean and the error bars represent SDs.

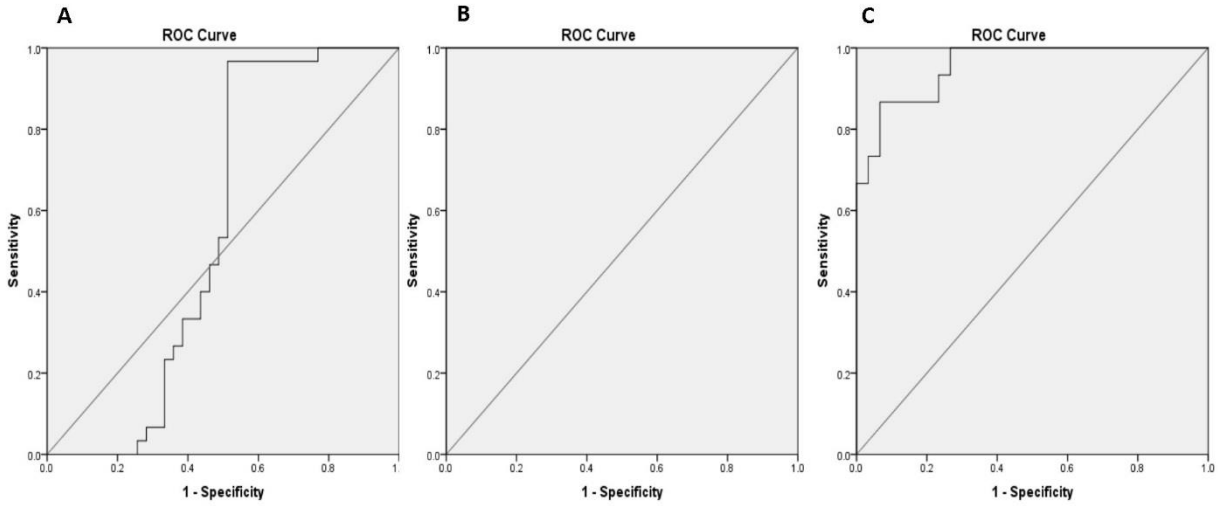

Fig.3: ROC curve analysis of pSMC1 ELISA data. ROC curve comparing sensitivity and specificity of pSMC1 ELISA data for detection of A-T carriers and patients. The value of the AUC–ROC were A: 0.549 (95% confidence interval 0.405–0.693), B: 1.00 (95% confidence interval 1.00–1.00), C: 0.956 (95% confidence interval 0.901–1) for healthy controls vs. A-T carriers, healthy controls vs. A-T patients and A-T carriers vs. A-T patients respectfully. ROC curve analyses suggested that pSMC1 ELISA data had significant diagnostic value for A-T detection.

5- DISCUSSION

We describe here an ELISA based method for the diagnosis of A-T and possibly screening of A-T heterozygotes based on in-cell ELISA analysis of SMC1 phosphorylation after 5 Gy irradiation. We demonstrate that this method is very simple and non-expensive, it needs less than 2 cc of blood and can be implemented directly on PBMCs, thus avoiding the preparation of cell lines and reducing the turnaround time to only1-2 days.

This is the first time that a phosphorylation target of ATM is analyzed by in-cell ELISA for the diagnosis of A-T. Several methods have been developed to measure radiosensitivity (21) in A-T patients and even carriers that summarized in **Table.2**. The colony-survival assay (CSA), the gold standard assay for evaluating 'radiosensitivity', which reflects the efficiency of DNA repair of DNA double-strand breaks (DSBs), identifies radiosensitivity in approximately 90% of AT patients (1). Technical difficulties involved in CSA standardization and optimization significantly interfered with the ability to further study the predictive accuracy of the assay. Some assays have been proposed to distinguish A-T heterozygotes from normal individuals. These assays involve mRNA/miRNA expression profiling, after and also before irradiation of AT cells (22-24), chromosome breakage assay (25, 26), comet assay (27), Gamma- H2AX (28), and pSMC1assay (18). However, due to diverse mutations with different radiosensitivity (29, 30), it needs to be confirmed by other studies.

Recently, flow cytometry based analysis of Gamma - H2AX and pSMC1 after IR, showed successful strategy to diagnosis of A-T (18, 31) and even to distinguish A-T heterozygotes from healthy controls (18, 28). Both H2AX and SMC1 are phosphorylation target of ATM in DNA damage response and they are involved in controlling cell cycle checkpoints and DNA damage processing (32, 33). Usefulness of H2AX in radiosensitivity analysis has been studied frequently (34), but for A-T disease, SMC1 phosphorylation seems more specific because H2AX is phosphorylated redundantly by several kinases (21, 35) and Gamma-H2AX does not always indicate the presence of DSB (36). ATM directly phosphorylate SMC1 protein on serine residues 957 and 966 in response to DNA damage (20). We assessed phosphorylation of serine 966 and our results showed that in-cell ELISA of pSMC1 be able to diagnosis A-T but cannot distinguish carriers from healthy controls (**Figure.2**).

Previously, it was suggested that SMC1 is phosphorylated specifically by ataxia-telangiectasia mutated (ATM), so it has been seemed promising for A-T heterozygote and homozygotes identification (18). But, recently some studies reported redundant phosphorylation of SMC1 (37-39). In support with that, we saw SMC1 phosphorylation in A-T patients (**Figure.2**), after IR. The study of ataxia telangiectasia and Rad3 related (ATR)-dependent signaling in ATM-deficient cells showed that ATR is also capable of phosphorylating SMC1 by Mre11-Rad50-Nbs1 (MRN) complex dependent manner (40). It seems ATR kinase recruited to the sites of DNA damage in ATM-deficient cells and phosphorylated SMC1 in ATM-independent manner, at low level (41).

In addition to redundant kinases, ATM-dependent phosphorylation of SMC1 may be effected by other DNA damage repair proteins that functionally related to ATM protein. This increase potential of false positive result for other genomic instability disorders (42). Therefore, we should be cautious in using of pSMC1 in screening, because it may have potential false-positive for other genomic instability

disorders (18). Although pSMC1 in-cell ELISA assay is a promising method for A-T diagnosis, the precision of assay especially inter-assay precision was low. Such variability may be due in part to operator error, but it is more likely that observed variations are due to procedural issues. It is recommended that all procedures were performed in the same lab (especially IR part) and assay executed on protein lysate not directly on PBMCs (19).

In working by protein lysate, antibody coating should be performed with caution, because lack of uniformity in the antibody coating on the walls of the plate wells has been shown to be the largest contributor to total assay imprecision (43). In addition to ELISA on protein lysate, flow cytometry technique also can be used. Although ELISA is simple, cheap, and rapid test that can be available in each laboratory, flow cytometry is more reproducible than ELISA, because it has more direct fluorescence detection system versus the more indirect detection method of ELISA, and it is less prone to error and more precise method (44, 45).

Thus, future studies are necessary to introduce the rapid and simple method for A-T carriers and patients identification with high sensitivity and specificity.

Table-2: Different radio-sensitivity assays for ATM defect analysis

	Methods	References
Colony formation		
1	colony survival assay	(46)
Chromosomal damage		
2	G2 chromatid break	(25, 26, 47, 48)
3	Micronucleus assay	(49-51)
4	FISH chromosome painting	(52-54)
DNA damage		
5	Comet assay	(27, 55)
DNA damage pathway proteins		
6	γH2AX	(28, 31, 56)
7	ATM-ELISA	(57, 58)
8	53BP1 foci	(56, 59)
9	pSMC1	(18)
10	p53 centrosomal localization	
Expression Signature		
11	mRNA/miRNA	(22-24)
12	SNP array	(60, 61)

5- CONCLUSION

In conclusion, our results showed that in-cell ELISA on PBMCs can distinguish A-T patients but not carriers within 1-2 days. Assessment of ATM heterozygosity is useful for genetic counselling within A-T families, and possibly for estimation of cancer and therapeutic risks. Also pSMC1-ELISA can be used in blood-based detection of radiation exposure in humans. In spite of rapidness, convenience and accessibility of this method, it needs to be cautious to use it for routine practice due to poor imprecision and possible false-

positive potential for other genomic instability disorders.

6- CONFLICT OF INTEREST

The authors had not any financial or personal relationships with other people or organizations during the study. So there was no conflict of interests in this article.

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