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DETECTION OF ATAXIA TELANGIECTASIA USING PHOSPHO-SMC1 IN-CELL ELISA IN CHILDREN

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ABSTRACT

Ataxia-telangiectasia (A-T) is a rare autosomal recessive multisystemic syndrome marked by progressive cerebellar ataxia, oculocutaneous telangiectasia, variable immunodeficiency, chromosomal instability, radiosensitivity, and a high risk of malignancy, primarily leukemia and lymphoma in children and epithelial cancers in surviving adults. The onset of neurological abnormalities in A-T patients usually happens between the ages of 1 and 4. A-T affects 1 in 40,000 to 100,000 births worldwide. Because of the high consanguinity marriage rate and ethnic components in Iran, this frequency differs from country to country. Importantly, the frequency of A-T carriers (heterozygotes) is estimated to be around 1-2 percent of the overall population. The aim of this study is to integrate them in order to create a quick, simple, and low-cost approach to assess SMC1 phosphorylation. To identify A-T patients, we used an in-cell ELISA colorimetric detection technique to evaluate ATM-dependent phosphorylation of the SMC1 protein after DNA damage as well as carriers. Flow cytometry can also be used in addition to ELISA on protein lysate. Although ELISA is a simple, inexpensive, and quick test that can be found in any laboratory, flow cytometry is more repeatable than ELISA because it uses a direct fluorescence detection system rather than the more indirect detection method used by ELISA, and it is a less error-prone and more precise method. As a result, more research is needed to provide a quick and easy method for identifying A-T carriers and patients with high sensitivity and specificity.

Key words Ataxia Telangiectasia, Phospho-SMC1, ELISA.

INTRODUCTION

Ataxia-telangiectasia (A-T) is a rare autosomal recessive multisystemic syndrome marked by progressive cerebellar ataxia, oculocutaneous telangiectasia, variable immunodeficiency, chromosomal instability, radiosensitivity, and a high risk of malignancy, primarily leukemia and lymphoma in children and epithelial cancers in surviving adults [1, 2]. The onset of neurological abnormalities in A-T patients usually happens between the ages of 1 and 4. A-T affects 1 in 40,000 to 100,000 births worldwide. Because of the high consanguinity marriage rate and ethnic components in Iran, this frequency differs from country to country [3, 4]. Importantly, the frequency of A-T carriers (heterozygotes) is estimated to be around 1-2 percent of the overall population [5, 6]. Although A-T heterozygotes are frequently asymptomatic and generally considered healthy, they are more vulnerable to ionizing radiation (IR) and have a higher risk of heart disease, diabetes, and malignancies, particularly breast cancer, than the general population [7, 8]. Early detection of A-T patients and carriers is beneficial for prognosis and family genetic counselling. Germline mutations in the ataxia-telangiectasia mutated (ATM) gene, which is found, cause A-T. The ATM protein is a ubiquitous serine/threonine kinase that is primarily involved in maintaining genomic integrity. ATM mutations are found throughout the whole transcript, with no hotspots. Over 600 A-T mutations have been identified, with the majority of patients being compound heterozygotes [9, 10].

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A-T is diagnosed using a combination of clinical and laboratory testing, including high levels of serum alpha-fetoprotein, cell sensitivity to IR, acquired 7- and 14-chromosome translocations in the blood karyotype, and low or missing amounts of ATM protein. However, DNA sequencing is currently relatively expensive, the ATM gene is large, and the causality of many missense DNA changes is often unclear. Mutation analysis can be used for confirmation of diagnosis in A-T patients and identification of carriers; however, currently DNA sequencing is relatively expensive, the ATM gene is large, and the causality of many missense DNA changes is often unclear. As a result, a functional test could be effective in identifying A-T patients and carriers [11]. R.A. Gaetti recently developed a functional flow cytometry technique to diagnose A-T patients and carriers. They measured ATM-dependent phosphorylation of structural maintenance of chromosomes 1 (SMC1) following deoxyribonucleic acid (DNA) damage using nuclear labelling of Lymphoblastoid cell lines and transformed peripheral blood mononuclear cells (PBMCs). Later, a blood-based pSMC1 enzyme-linked immunosorbent test (ELISA) was utilized to identify radiation exposure in people. After DNA damage, ATM kinase phosphorylates SMC1, a cohesion binding protein [12].

Aim and Objective:

The aim of this study is to integrate them in order to create a quick, simple, and low-cost approach to assess SMC1 phosphorylation. To identify A-T patients, we used an in-cell ELISA colorimetric detection technique to evaluate ATM-dependent phosphorylation of the SMC1 protein after DNA damage as well as carriers.

Material and Methods:

After receiving approval from the university's research ethics committee, the study was carried out at Children's Medical Center from September 2014 to May 2015. We collected 2cc peripheral blood from 15 patients with A-T, 30 obligate carrier parents, and two healthy controls who were not linked to A-T families and had no family history of cancer, diabetes, or atherosclerosis, with informed consent. According to a previous study, sample size was estimated. The SMC1 (phospho-Ser966) colorimetric cell-based ELISA kit (CytoGlowTM, assaybiotech) was used for the in-cell ELISA assay, with some modifications. Ficoll isolated PBMCs, and 70,000 cells were cultured for at least 15 hours in 96-well plates treated with poly-L-lysine in 200ul of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, and a humidified incubator with 5% carbon dioxide [CO2]. At room temperature and a dose rate of 0.9 Gy/min, gamma irradiation was carried out in the Thermatron 780E. To fix and crosslink the cells to the microplate one hour after 5 Gy irradiation, 100 L of media was replaced with 100 L of 8% formaldehyde solution. The plate was rinsed three times with wash buffer after 15 minutes of incubation, and the cells were permeabilized for 20 minutes in Tritons X-100. The cells were treated with the primary antibody overnight after quenching and blocking. After incubating the secondary antibody for 20 minutes, the substrate was applied to the wells. Micro-plate reader 4 Plus at 450nm wavelength was used to quantify the optical density (OD). Anti-GAPDH antibody was utilized as a positive control, and SMC1 and GAPDH secondary antibodies (without primary antibody) were employed as negative controls for each condition. In the same plate as the pSMC1 target studies, both positive and negative controls were performed. In-depth research by evaluating PBMCs obtained from one healthy donor and one A-T patient donor sample on the same day, we were able to calculate the ELISA intraday assay (within-run) precision, which was reported as a coefficient of variation (CV). For one healthy donor, the interlay assay (between-day) variability was estimated using four measurements taken over four days. The OD values of the pSMC1 protein were normalized with GAPDH OD values, and the quantitative values were provided as means standard deviations (SD). To compare the groups, the Kruskal–Wallis test and Dunn's multiple comparisons test were employed as post hoc tests, and the Receiver operates characteristic (ROC) curves were utilized to establish the pSMC1 ELISA values' relevant sensitivity and specificity. GraphPad Prism version 6 and SPSS 22 (IBM SPSS Statistics, IBM Corporation) were used to analyses all data. The significance of P values less than 0.05 was determined.

Results and Discussion:

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 to investigate SMC1 phosphorylation by in-cell ELISA. For A-T patients, carriers, and healthy controls, the mean (lowest-highest) ages were 10 (4-22), 37(26-55), and 32 (19-46) years, respectively. Using serially diluted cells (0-280,000 cells) per well in 96-well plates, the best cell seeding density was identified. 70,000 cells were chosen after irradiation, fixation, and staining based on color intensity without saturation and confluency (At two levels, we assessed the precision of the PBMC-based test: intraday (within-run) and interlay (between-run) (between-run). PBMCs from 1 healthy donor and 1 A-T patient donor were irradiated, fixed, and stained in duplicates on the same day for intraday examination (one time, 7 aliquots). For a healthy donor and an A-T patient, respectively, the intraday assay imprecision (CV) was 13.16 percent and 14.33 percent (Table.1).

Table 1: Precision of ELISA assay				
Inter-day	**Measurements**	**Mean**	**Standard deviation**	**CV (%)**
Healthy Control	6	0.296	0.038	13.18
A-T control	7	0.092	0.012	14.32
Inter-day	5	0.301	0.068	21.48

PBMCs were assayed 7 times in the same day

PBMCs were assayed one time on 5 different days

PBMCs were taken from a single healthy donor on five different days for the second stage. The CV of 5 measurements in the inter-day assay (between runs) was 22.49 percent. It shows that after 5 Gy IR, SMC1 phosphorylation is absent or severely reduced in A-T patients (mean+ SD: 0.075 + 0.034), while it is present in A-T carriers (mean+ SD: 0.190 + 0.060) and healthy controls (mean + SD: 0.312 + 0.081). Although there is a statistically significant difference between A-T carriers and healthy controls (Kruskal–Wallis test, P = 0.0007), there is overlap between them that prevents us from setting a detection cut-off. As demonstrated in Figure.3, ROC curve analysis of pSMC1 values revealed a good sensitivity and specificity for detecting A-T patients, with an AUC–ROC of 1.00 (95 percent CI 1.00–1.00).

Despite the fact that the pSMC1 in-cell ELISA assay is a promising approach for AT diagnosis, the assay's precision, particularly inter-assay precision, was inadequate. The observed variances could be attributable to operator error, but it's more likely that they're due to procedural difficulties. All procedures (particularly the IR component) should be carried out in the same lab, and the assay should be carried out on protein lysate rather than PBMCs. Antibody coating should be done with caution when working with protein lysates, as lack of consistency in the antibody coating on the walls of the plate wells has been found to be the major contributor to total assay imprecision. Flow cytometry can also be used in addition to ELISA on protein lysate. Although ELISA is a simple, inexpensive, and quick test that can be found in any laboratory, flow cytometry is more repeatable than ELISA because it uses a direct fluorescence detection system rather than the more indirect detection method used by ELISA, and it is a less error-prone and more precise method. As a result, more research is needed to provide a quick and easy method for identifying A-T carriers and patients with high sensitivity and specificity.

Conclusion:

In conclusion, our findings indicated that within 1-2 days, in-cell ELISA on PBMCs may differentiate A-T patients but not carriers. ATM heterozygosity can be used to provide genetic counselling to AT families, as well as to estimate cancer and treatment risks. pSMC1- ELISA can also be used to identify radiation exposure in humans via blood. Despite its speed, simplicity, and accessibility, this approach should be used with caution in routine practice due to its poor precision and risk for false-positive results for other genomic instability disorders.

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