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# ACCURATE DIAGNOSTICS OF ATAXIA-TELANGIECTASIA CELLULAR PHENOTYPE BY EMPLOYING *IN VITRO* LYMPHOCYTE RADIOSENSITIVITY TESTING

by

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**In this paper we present the data of lymphocyte radiosensitivity testing used for characterization of radiosensitive cellular phenotype and diagnostics of ataxia-telangiectasia disease. We point out the advantage of lymphocyte micronucleus test (CBMN) over other cellular tests for assessment of radiosensitivity: the first advantage of CBMN is that primary patient cells are used (less than 1 ml), the second one is that the results of testing are obtained within 3 days and there is no need for establishing a patient-derived cell line, which requires additional time and application of more expensive methods. The third advantage of CBMN method is that it gives information about proliferative ability of cells, which can recognize dysfunctional ataxia-telangiectasia mutated protein. The results are fast and accurate in diagnostics of ataxia-telagiectasia diseases.**

*Key words: ataxia-telagiectasia, radiosensitity testing, diagnostics*

## INTRODUCTION

The ataxia-telangiectasia (A-T) is inherited progressive neurodegenerative disorder causing severe disability. The ataxia refers to poor co-ordination, whereas telangiectasia indicates small dilated blood vessels, both of which are hallmarks of the disease. Other characteristics of this disease are immunodeficiency, spontaneous chromosomal instability, hypersensitivity to ionizing radiation, predisposition to cancer, and premature aging. Most often, the symptoms first appear in early childhood. The A-T is caused by a defect in the A-T mutated gene (ATM). The mutation in ATM gene can happen across entire gene which is the main reason for the phenotypic difference of the disease [1]. Among mutations that are found in ATM gene the most frequent are truncating and splicing mutations. Because not all children develop disease in the same manner or at the same rate, it may take some years before A-T is properly diagnosed. Laboratory testing is an important part of diagnostics of the disease. It includes the assessment of the ATM protein function, the induction of the tumor suppressor protein p53, the level of serum alpha-fetoprotein (AFP) and the intrinsic cellular radiosensitivity test. In A-T patients, the ATM protein is dysfunctional consequently leading to defective damage-induced activation of the cell cycle checkpoints, delayed expression of the tumor suppressor protein p53, high level of serum AFP and increased intrinsic radiosensitivity [2]. An early investigation of radiosensitivity demonstrated that A-T patients exhibit hypersensitivity to ionizing radiation. The latter research showed that the dose-response to low linear energy transfer (LET) radiation for A-T cells shows a linear dependence instead of a linear-quadratic one which is found for normal cells [3], as a consequence of repair deficiency of double-strand breaks of the DNA [4]. These observations further suggest suitability of radiosensitivity testing as diagnostic tool for this disease. Current methods of testing consider mostly colony survival assay, which requires establishing a patient-derived cell line via transformation by Epstein-Barr virus and assessment of cell survival after *in vitro* irradiation.

In this paper we present more simple way to estimate intrinsic radiosenstitivity *in vitro* using primary lymphocytes and lymphocyte micronuclei assay. We show that this method can be used as a fast and accurate method for diagnostics of AT cellular phenotype, that it does not require establishing a patient-derived

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cell line, and that it provides accurate results of testing within 3 days.

## METHODS AND SAMPLES

Two children of the same family, both girls, aged 2 and 3 years, were clinically diagnosed as A-T. To confirm the diagnosis, the cellular sensitivity to ionizing radiation was in parallel investigated in Italy employing colony survival assay and in our Laboratory using lymphocyte micronuclei test. The peripheral blood samples (1 ml) were collected by vein puncture in heparinized vacutainers in accordance with current Health and Ethical Regulations in Serbia [5]. The baseline level of micronuclei (MN), *in vitro* radiosensitivity and proliferative ability of lymphocytes were examined. The incidence of radiation-induced lymphocyte micronuclei, in response to radiation dose of 2 Gy ($^{Co}60$ $\gamma$-rays) was used as the indicator of radiation sensitivity *in vitro*.

## IRRADIATION

Two hours after blood collection, an aliquot of heparinized whole blood from each subject was positioned in a 15 cm 15 cm plexiglas container and irradiated using a $^{60}$Co $\gamma$-ray source. The radiation dose employed was 2 Gy, the dose-rate was 0.45 Gy/min, the dimensions of the radiation field were 20 cm 20 cm, and the distance from the source was 74 cm. Blood samples were irradiated at room temperature.

## LYMPHOCYTE MICRONUCLEUS TEST

Baseline levels of micronuclei and radiosensitivity were estimated employing lymphocyte micronuclei test of Fenech *et al.*, [6]. A minimum of 1000 binucleated cells was scored with an AxioImager A1 microscope (Carl Zeiss, Jena, Germany) using a magnification of 400× or 1000 .

## RESULTS AND DISCUSSION

The results obtained in this work are presented in tabs. 1 and 2 and figs. 1 and 2. The results obtained using colony survival and lymphocyte micronuclei assay in both patients are presented in tab.1. Both tests showed hypersensitivity to irradiation. By using colony survival assay, the hypersensitivity is seen as low number of cells able to form colonies after irradiation. By using lymphocyte micronuclei assay hypersensitivity is seen as increased yield of radiation-induced micronuclei in binucleated cells – the population of cells who conceded first division after irradiation. Furthermore, the lymphocyte micronuclei assay enable measurement of cell proliferation potential, called cytochalasin B proliferation index (CBPI), which was also scored in those samples. The proliferative index is calculated by means of the number of mononucleated, bi, and polinucleated cells [7]. In both patient samples, a high proliferation rate of lymphocytes is observed after irradiation which is a consequence of dysfunctional ATM protein. The proliferation index distinguishes checkpoint dysfunction caused by truncating ATM protein. The ATM protein is the first molecule in signaling cascade in DNA damage response. The ATM is being phosphorylated when DNA damaged occurs. That recruits DNA repair proteins to the break site. At the same time the ATM protein makes a puzzle with other proteins of cell cycle checkpoints and stops the cell division until the repair of the DNA is complete. When truncating or splicing mutations in ATM gene happen, consequently dysfunctional ATM protein formes, powerless for puzzling with other cell cycle checkpoint proteins and the time for the DNA repair is not allowed. In the first division after irradiation, the unrepaired DNA damages are seen as complex chro-

**Table 1. *In vitro* radiosensitivity testing of ataxia-telangiectasia patients (colony survival and CBMN test)**

| | Colony survival assay SF SD [%] | | Incidence of micronuclei (MN/1000 BN cells) | | Proliferation index (CBPI) | | MN/mBN cell (irradiated) |
|---|---|---|---|---|---|---|---|
| | Base line | Irradiated | Base line | Irradiated | Base line | Irradiated | |
| $AT_1$ patient | 89 2 | 12.8 6.8 | 21 | 990 | 1.99 | 2.2 | 1.81 |
| $AT_2$ patient | 82 6 | 11.5 7.1 | 19 | 1030 | 1.91 | 2.3 | 1.89 0.12 |

**Table 2. *In vitro* radiosensitivity testing of healthy persons and cancer patients (CBMN test)**

| | Incidence of MN/1000 BN cells | % of micronucleated cells (mBN cells) | MN/mBN cell (irradiated) |
|---|---|---|---|
| Normal sensitivity | 200 29 | 16 2 | 1.26 0.18 |
| Radiosensitive | 322 31 | 23.8 6.7 | 1.33 0.12 |
| Radioresistant | 136 23 | 11.4 3 | 1.19 0.17 |
| Radiosensitive cancer patients | 366 21 | 12.1 4 | 1.31 0.12 |

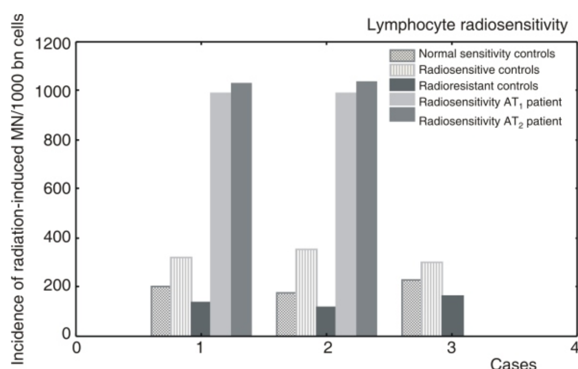


**Figure 1. Radiosensitivity of ataxia-telangiectasia cells *versus* normal cells**

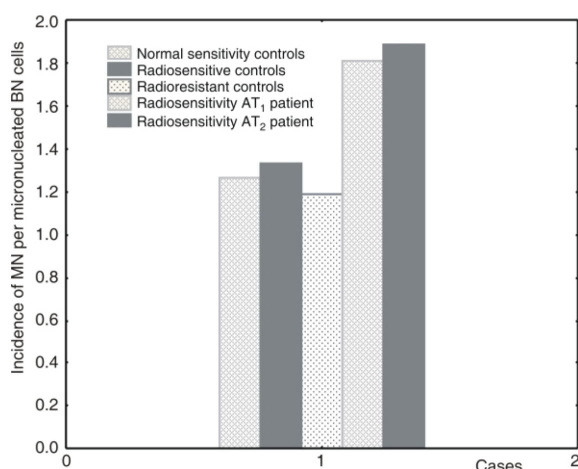


**Figure 2. Incidence of micronuclei per micronucleated binuclei cells**

mosomal aberration, high incidence of micronuclei or very low percent of colonies that survive irradiation. The results of colony survival testing showed that the mean survival fraction (SF) of irradiated patient cells was 12.8% 6.8% and 11.5 7.1, respectively. SF value for the control healthy cell lines was 50.1% 13.5%. Our previous study of intrinsic radiosensitivity among healthy individuals [8] and cancer patients revealed that radiosensitive cellular phenotype maximally display 363 and 389 micronuclei per 1000 binucleated cells, respectively (tab. 2). Similar findings were observed by West *et al* [9]. The ataxia patients exhibit almost 3 times more micronuclei than radiosensitive individuals (tab. 1, fig. 1) which is accompanied with higher incidence of MN per micronucleated binuclei cells (tab. 2, fig. 2) and high proliferation of irradiated cells. The percentage of micronucleated binuclei cells is significantly higher than in all other study groups, which additionally support radiosensitivity findings.

According to results of radiosensitivity testing, presented in this paper we point out advantages of lymphocyte micronuclei assay *vs*. other tests for assessment of intrinsic radiosensitivity. Threefold higher incidence of micronuclei in AT cells accompanied with enhanced cell proliferation after irradiation provides accurate recognition of AT cellular phenotype and helps in early diagnosis of A-T disease.

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## AUTHOR CONTRIBUTIONS

Theoretical analysis was carried out by D. S. Vujić, S. Ž. Petrović, A. R. Leskovac, and I. D. Joksić. Experiments were organized and carried out by D. S. Vujić, S. Ž. Petrović, A. R. Leskovac, I. D. Joksić, J. G. Filipović, and A. P. Valenta Šobot. All authors analyzed and discussed the results. The manuscript was written by D. S. Vujić, S. Ž. Petrović, A. R. Leskovac, and I. D. Joksić and the figures were prepared by J. G. Filipović and A. P. Valenta Šobot.

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## ПОУЗДАНА ДИЈАГНОСТИКА ATAXIA TELANGIECTASIA ЋЕЛИЈСКОГ ФЕНОТИПА ПРИМЕНОМ ЛИМФОЦИТНОГ ТЕСТА РАДИОСЕНЗИТИВНОСТИ *IN VITRO*

У овом раду приказани су резултати испитивања радиосензитивности који су коришћени за клиничку дијагностику обољења Ataxia-telangiectasia (AT). Истакнуте су предности лимфоцитног микронуклеусног теста у односу на остале тестове који се користе за процену геномске радиосензитивности: прва предност CBMN теста је да користи веома малу количину примарних ћелија пацијента (мање од 1 ml крви), не захтева трансформацију примарних ћелија у ћелијске линије, што иначе захтева додатно време и примену специјалних метода. Трећа предност CBMN теста је што истовремено даје податке о пролиферативном капацитету ћелија, на основу чега се може препознати дисфункционални ATM протеин. Резултати радисензитивности CBMN тестом су брзи и поуздани те се са сигурношћу могу користити за дијагностику обољења Ataksia telangiectasia.

*Кључне речи: ataxia-telangiectasia, тест радиосензитивности, дијагностика*