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Study of γ-H2AX as DNA double strand break biomarker in resident living in high natural radiation area of Mamuju, West Sulawesi

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ABSTRACT

High expression of phospho histone γ-H2AX, a sensitive marker of double stranded DNA damage, is believed to be an indication of defective DNA repair pathway or genomic instability that may cause mutations and ultimately cancer. DNA damage can be caused by ionizing radiation exposure. Beside in medical treatment/diagnosis or industry, ionizing radiation exposure can also be found in naturally in regions of high natural back ground radiation. In this study we collect the blood from 45 volunteers living in Mamuju, a region with highest natural radiation in Indonesia (dose of ~7 mSv/year). Subjects were grouped as high natural background area (HNBA) ($n = 37$) and control area ($n = 8$). The expression γ-H2AX foci were evaluated by one of researcher fluorescence microscope examination. Our results show that the average foci numbers per cell were in the normal range. While not statistical different, the average of γ-H2AX foci in exposed area higher in the exposed compared to the control area, 0.31 versus 0.13 ($p > 0.05$), respectively. Moreover, there was also no statistical difference of average γ-H2AX foci between man and woman, old and young people in exposed and control area ($p > 0.05$). In this preliminary study we find that γ-H2AX foci (and thus DNA double strand break) frequency in residents living in the HNBA of Mamuju, West Sulawesi, show a trend towards higher (albeit not significant) average values relative to the control area. More research is needed to further scrutinize these observations.

1. Introduction

There are some areas in the world such as in Ramsar (Iran), Yangjiang (China), Guarapari (Brazil) and Kerala (India), where the natural background radiation level is higher (sometimes 10–100 times the normal levels) than others, either due to high levels of radioactivity in soils, rocks and hot springs or due to high levels of indoor radon and its decay products (Mortazavi et al., 2001; Wei and Sugahara, 2000; Paschoa, 2000; Paul et al., 1998). Indonesia also has a region with high natural ionizing radiation. Mamuju, a village in the state of West Sulawesi in the Sulawesi Sea, has a background radiation around 13 times higher than normal. This place has a highest average dose rate compared to other regions in Sulawesi island and even Indonesia, which can achieved up to 2.8 μSv/h (Iskandar et al., 2010; Syaeful et al., 2014). This radiation is the result of high natural uranium content (Radium-226 and Radon gas, both of which are highly water soluble) in rock and soil. Major concern is due to its location, which is near a densely inhabitated area.

DNA double-strand breaks (DSBs) are one of the most critical events affecting DNA when a cell or an organism is exposed to ionizing radiation, chemical or environmental stress. If not adequately repaired, DSBs can have severe consequences on, cellular senescence cells leading to cell death or the induction of genomic instability, genomic rearrangements which in turn may trigger carcinogenesis (Rothkamm and Horn, 2009; Redon et al., 2010; Vandersickel et al., 2010).

DSBs can be identified and quantified in situ by detecting the γ-H2AX foci that forme around DNA break sites utilizing immunostaining techniques (Moroni et al., 2013). Counting γ-H2AX foci is the most sensitive of current assays for irradiation-induced DSBs (Bonner et al., 2008) with a ratio of DSBs to visible γ-H2AX foci

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close to 1:1 (Sedelnokova et al., 2002; Rothkamm and Lobrich, 2003).

Previous studies on the high natural background radiation area (HBRA) of Yangjiang in China have not shown any evidence of increased cancer mortality (Tao et al., 2000), while cytogenetic analysis has shown higher incidence of stable and unstable chromosomal aberrations in the HBRA group compared to the control group (Ghiassi et al., 2004). Limited studies on the effects of high level natural radiation on some cytogenetic parameters in the inhabitants of HBRA of Ramsar have been reported (Mohammadi et al., 2006). High background natural radiation also has potential to induce DSBs, especially for the residents permanently living at such areas. Distinct cytologically visible foci of γ-H2AX represent DSBs and correlate with the dose deposited both in vitro and in vivo (Löbrich et al., 2005; Kuefner et al., 2010). Hence, the purpose of this investigation was to study the expression of γ-H2AX foci in lymphocytes of residents of Mamuju as one of region with high natural background radiation in Indonesia.

2. Materials and methods

2.1. Sampling and subjects

In this paper we have collected blood samples from 37 residents of one of eight sub villages, Te'bong, Taludu, Taludu Barat, Kassa, Kurasalimbo, Ratte, Kombiling and Tangnga and Botteng Village, with high background radiation (exposed group) (dose of 7 mSv/year). Individuals ($n = 8$) of three sub-villages (Keang, Tabelalang and Lomali of Keang Village) with normal background radiation (dose of 2 mSv/year) served as control group. The ages of all volunteers ranged from 14 to 70 years, we classified for $\leq$40 as young and $\geq$40 as old (7 old and 1 young in control area, 16 old and 21 young in exposed area), and 24 man and 21 woman (4 man and 4 woman in control area, 20 man and 17 woman in exposed area).

All volunteers were informed about the nature, aims, and intention of the study and signed a consent form and questionnaire before providing blood samples. Any individuals suffering from an illness or taking medication were excluded.

2.2. Site of sampling

2.2.1. Isolation of lymphocyte

Isolation procedure was done, with some modifications, as previously published (Chua et al., 2011; Rothkamm et al., 2013). Heparinized whole blood sample was collected from local donors around Mamuju and transported to our laboratory. Histopague separation was used to isolate white blood cells by layering 2.5 mL of whole blood mixed with an equal volume of Phosphate Buffered Saline (PBS) pH 7.4 onto 2 vol of lymphocyte-separating medium (Histopaque 1077) in a centrifugation tube followed by centrifugation for 30 min at 1500 rpm. The lymphocyte cells that appeared as a whitish/gray layer between the blood plasma and the Histopaque were then carefully transferred to a new 15 mL centrifuge tube containing 5 mL of PBS (pH 7.4) and centrifuged for 15 min at 1000 rpm. The leukocytes were washed three times and resuspended in PBS at a density of $(5–6) \times 10^4$/mL. The cell viability was determined to be 98% by Trypan blue staining.

2.2.2. Gamma-H2AX assay and observation

The procedure for γ-H2AX foci assay was done according to published papers with some modification (Nakamura et al., 2006). Medium (RPMI) containing the isolated lymphocyte from blood of residents was put on hydrophobic slides and left for 15 min (minutes). Cells were then fixed in 2% paraformaldehyde for 5 min, washed in PBS for 3×10 min, permeabilized for 5 min on ice in 0.25% Triton X-100, and blocked in PBS with 0.25% BSA for 3×10 min at room temperature. After removing BSA the primary anti-gamma H2AX (mouse anti-Phospho-Ser139 gamma H2AX Antibody, ThermoFisher) and 53BPI antibodies (ThermoFisher; used for control staining) were mixed together at 1: 500 dilution in PBS. The Abs were dropped on the slides and incubated in a dark moist chamber for 45 min at 30 °C. To remove the first antibodies the slides were washed with PBS, 0,25% BSA 3×5 min. After that, the second antibodies (Goat Anti-mouse IgG Dylight 488 and anti-rabbit-Dylight 594 nm, both from Thermo Scientific) diluted in 1: 500 in PBS/BSA and with DAPI (diluted 1: 500) was added and incubated for 30 min. After 2–3 washes with PBS, slides were dried for 10 min with a fan. The mounting medium Entellan was dropped and mounting with cover slip and let 15 min in fridge. Observation was done by an experienced investigator (IK) using a fluorescence microscope (Nikon) equipped with red, green and blue fluorescence filters and a 100x lens under immersion oil. Generally, 50 γ-H2AX foci were counted per individual (Rothkamm et al., 2013; Eberlein et al., 2016).

2.3. Statistical analysis

Student T- Test was used to analyze the difference of average of γ-H2AX foci between exposed area and control area. The Analysis of Variance Test was used to analyze the associate γ-H2AX foci with ages and sexes in control and exposed area. All the data were analyzed with MedCalc. Software 12.7.00 (see Fig. 1).

3. Results

Our preliminary investigation could only be done with a limited number of sample/volunteers. The expression of γ-H2AX was detected as bright green foci. The bright foci were the result of bonding of antibody γ-H2AX with day light 488 secondary antibody. The average foci number in exposed was 0.31 (0.00–2.24) and in control 0.13 (0.00–0.50) per lymphocyte cell as shown in Fig. 2 (a and b), even though there were no statistical significant ($p = 0.07 > 0.05$) the average of γ-H2AX from resident in exposed area seem higher than in control area. There is no statistical significant of γ-H2AX foci with sexes and ages of resident both in control and exposed area as seen in Figs. 3 and 4.

4. Discussion

The effort to predict the genetic consequences of ionizing radiation on humans has been one of the most important issues of human genetics in the past 60 years (UNSCEAR, 2001). Some previous experimental studies have not yet completely explained radiation-induced germline mutation in humans a topic that still remains highly controversial. All current estimates of genetic hazard of radiation exposure for humans are largely derived by extrapolation of animal study data that have their own limitation (Sankaranayanan and Chakraborty, 2000). The residents of the HLNRA (High Level Natural Radiation Area) receive low-level chronic radiation and are therefore very important source of information to understand the effects of chronic low-level radiation. The studies in these areas may guide in solving the enigma among the scientific community concerning the effects of low dose radiation.

γ-H2AX foci can be observed with fluorescence microscopy by immunostaining cells with primary γ-H2AX antibodies coupled with fluorescent labeled secondary antibodies. The discernible hallmark of γ-H2AX foci counting is the ability to detect a single DSB in an individual cell (Rothkamm and Lobrich, 2003). The use of fluorescence measurements can be extended to the detection of