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RESEARCH ARTICLE

Lymphocyte Proliferation Kinetics in Inhabitant of Takandeang Village, Mamuju: A High Background Radiation Areas in Indonesia

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Abstract

BACKGROUND: Mamuju area in West Sulawesi considered as the high natural background radiation area in Indonesia. Our previous study showed that the mean mitotic index (MI) and nuclear division index (NDI) in lymphocytes of Botteng Village, Mamuju inhabitants was lower compared to control samples. To validate our previous study results, here in this study the evaluation of cell proliferation markers which were MI and NDI in peripheral blood lymphocytes (PBL) of Takandeang Village inhabitants was conducted.

METHODS: A total 60 people were enrolled in this study, consisted of 35 samples from Takandeang Sub-Village and 25 from normal background radiation area. MI was calculated manually and automatically using Metafer 3.11.2 imaging system. The NDI defined as proportion of mononucleated, binucleated, trinucleated and tetranucleated cells were conducted using cytokinesis block micronucleus (CBMN) assay.

RESULTS: The results of this study showed that the mean manual MI in Takandeang Sub-Village inhabitants was lower compared to control group (4.96 ± 2.25 vs. 5.93 ± 2.14). In contrast, the mean automatic MI (20.37 ± 10.49 vs. 18.87 ± 7.49) and NDI (1.555 ± 0.174 vs. 1.523 ± 0.112) in Takandeang Sub-Village inhabitants was higher compared to the control group. Statistical analysis revealed that the difference of mean manual MI, automatic MI and NDI in Takandeang Sub-Village inhabitants was not significantly different compared to the control group ($p > 0.05$).

CONCLUSION: It can be concluded that based on this study the chronic low radiation dose exposure in Takandeang Sub-Village, Mamuju has no significant effect on the lymphocytes proliferation.

KEYWORDS: lymphocytes, mitotic index, nuclear division index, high background radiation

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Introduction

Until now the natural radiation exposure is considered as the major sources of ionizing radiation exposure in human life.(1,2) The worldwide average annual exposure to natural radiation sources is 2.4 mSv.(2,3) However, there are several areas in the world that have a high level of natural background radiation like Ramsar in Iran and Kerala in India. People living in high natural background radiation areas (HBRA) received a higher natural radiation exposure

than the worldwide average annual exposure for a human being.(2) The HBRA also can be found in Indonesia, and it was located in Mamuju, West Sulawesi. This area was known for the high radiation dose rates due to the natural uranium contents in the soil surface that can be reached to 25 ppm eU and it is eight times higher than the average in the Earth that was only about 3 ppm eU.(4) Several areas in Mamuju have a radiation dose rate more than 400 nSv/h, which were Ahu, Takandeang, Botteng, Pengasaan, Tande-Tande and Mamunyu. Three areas (Tande-Tande, Takandeang and Botteng) were used for settlement, while

Ahu, Pengasaan and Mamunyu only consisted of forest and weeds.(4)

In our previous study, we found that the mean of mitotic index (MI) and nuclear division index (NDI) values in Botteng Village inhabitants was lower than the control samples.(5) The possible explanation for our previous study results was the high natural radiation exposure cause a delay in the cell cycle and inhibits the mitosis of peripheral blood lymphocytes (PBL). MI represented the proportion of metaphases among harvested lymphocytes and also can be used to evaluate the proliferation cells rate include identify the compounds that can inhibit it. There are two factors that can affect the MI. First is the numbers of the cells that participate in the interphase leading to division, and second the relative lengths of interphase and recognizable mitotic stages.(6) The decrease of the MI value is usually a consequence of a reduced rate of cell proliferation (mitotic delay). Another cell proliferation marker to measure the general cytotoxicity in blood cultures is the NDI. NDI that represents the relative frequencies of the cells may be used to define cell cycles progression of the lymphocyte after mitogenic stimulation and how this has been affected by the exposure. Cells with higher numbers of chromosomal damage can possibly die before enter the cell division.(7,8)

The aim of this study was to validate our previous study results and considered as the preliminary study to find out the association between MI and NDI in PBL with the leukocyte and lymphocyte numbers of people living in HBRA. Since fewer leukocytes and lymphocytes mean a slower mechanism of mitosis, thus we predicted that the leukocyte and lymphocyte numbers of Takandeang Village inhabitants should be lowered compare to control sample.

Methods

Indoor and Outdoor Gamma Dose Rates Measurement

Takandeang Village consisted of 9 Sub-Village which were Salumatti, Taloba, Limbeng, Salubiru, Takandeang, Palada, Rantedunia, Tabanga-banga and Bettengkata. The Takandeang Sub-Village was chosen in this study as the representative of Takandeang Village inhabitants. The indoor and outdoor background gamma radiation measurements were performed by gamma spectrometer (Exploranium GR-135 Plus) calibrated in the secondary standard dosimetry laboratory under the National Nuclear Energy Agency of Indonesia. In the outdoor gamma dose rate measurements, the detector was placed at least six meters away from the walls of any building nearby one meter higher than the

ground level. For the indoor measurements inside the houses, the detector was placed one meter higher than ground level with the total exposure time of 10 minutes. The average of all measurements in 10 houses were calculated and considered as the outdoor and indoor gamma dose rates of Takandeang Sub-Village in Takandeang Village.

Annual Effective Dose Calculation

The annual effective dose from indoor and outdoor background gamma dose rate was estimated using this equation.(9)

$$E = (D_{out} \times OF_{out} + D_{in} \times OF_{in}) \times T \times CC$$

Where E (mSv/y) is annual effective dose, D_{out} and D_{in} (nSv/h) are average outdoor and indoor gamma dose rates, T (hour) is time to convert from year to hour (8760 hours), OF_{out} and OF_{in} are outdoor and indoor occupancy factors (30% and 70% for outdoor and indoor, respectively) and CC is conversion coefficient (0.7 for adults) reported by United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) to convert absorbed dose in air to the effective dose in human.(9) The occupancy factor for indoor and outdoor were calculated based on the observation of Takandeang Sub-Village inhabitants that spent almost 8 hours in outdoor and 16 hours in indoor.

Blood Sampling

Thirty-five healthy adult subjects from Takandeang Sub-Village in Takandeang Village, Mamuju and 25 healthy adult subjects from normal background radiation area were included in this study. Blood samples were obtained by phlebotomy in sterile heparinized vacutainer tubes. The leukocyte and lymphocyte numbers per unit volume were determined using the haemogram device type ABX Micros 60 (ABX Diagnostics, Montpellier, France). This study was perform with the approval of the Ethics Committee of the National Institute of Health Research and Development, Indonesian Ministry of Health, number LB.02.01/5.2.KE.051/2015. The procedures used in this study also were in accordance with the Helsinki declaration in 1964 and its later amendments or comparable ethical standards. Informed consent was obtained from all samples. The inclusion criteria used in this study were having complete information on gender, age, smoking status, no intake of alcohol, no family history of genetic disorder and no exposure to ionizing radiation except for natural background radiation. The exclusion criteria were having a medical treatment especially radiation exposure.

Blood Culture

Blood cultures were set up according to the International Atomic Energy Agency (IAEA) standard procedures with minor modifications.(10) Two cultures were set up from each blood sample. One mL of heparinized blood was mixed with 7.5 mL of Rosewell Park Memorial Institute (RPMI) 1640, 1 mL heat inactivated fetal bovine serum (FBS), 0.25 streptomycin/penicillin, and 0.1 mL of phytohemagglutinin (PHA). Blood culture was incubated for 48 hours in the incubator at 37°C containing 5% CO₂. Colchicine then was added to the culture for the last 3 hours of culture at a final concentration of 0.05 µg/mL. After 48 hours the blood culture flasks were centrifuged for 10 minutes at 1500 rpm and cells were re-suspended in 10 mL of 0.075 M KCl (pre-warmed to 37°C) for 25 minutes. The flasks then were centrifuged for 10 minutes at 1500 rpm and cells were fixed in 2 mL of methanol and acetic acid (3:1). After the white sediment was obtained then it was stored in -20°C for one night. Fixed cells then were dropped onto clean, wet slides, dried and stained with 4% Giemsa solution (pH 6.8) for 10 minutes. Three slides were obtained from each sample.

Manual and Automatic MI Evaluation

Two slides were analyzed from each donor for the manual MI evaluation. The slides were analyzed based on the protocol in IAEA publication.(10) The number of metaphase cells per 1000 total metaphase and blast cells was counted at 400x magnification using a light microscope (Figure 1). The number of metaphase cells then was converted to a percentage in order to calculate the MI. Automatic MI calculation for the whole slide was conducted using metaphases finder module from Metafer 3.11.2 imaging system (MetaSystems, Altlussheim, Germany) with a Zeiss Axioplan 2 Imaging epifluorescent microscope connected to a Cool Cube (MetaSystems, Altlussheim, Germany).

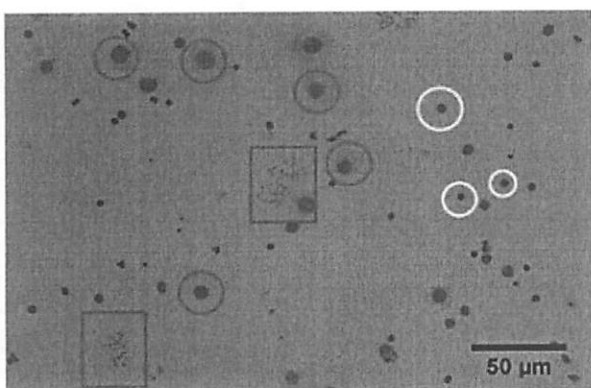


Figure 1. A microscopic view of Giemsa stained slide. It contains metaphase chromosome spread (boxes), blast cells (red circles) and nuclei which are not counted (white circles) for the manual evaluation of MI (magnification of 400x).

Micronucleus Assay

Micronucleus assay was conducted based on protocol in IAEA publication.(10) Two cultures were set up from each blood sample. Briefly, 0.5 mL blood samples were mixed with 4.5 mL of Rosewell Park Memorial Institute (RPMI) 1640, 1 mL heat inactivated fetal bovine serum (FBS), 0.2 mL streptomycin/penicillin, and 0.1 mL of phytohemagglutinin (PHA). Blood culture was incubated for 72 hours at 37°C containing 5% CO₂. Cytochalasin-B (4.5 µg/mL) then was added to the culture at 44 hours after PHA stimulation. The culture tubes then were centrifuged for 10 minutes at 1000 rpm and the cells were fixed in 7 mL of 0.075 M cold (4°C) KCl. The tubes then were centrifuged again for 8 minutes at 1000 rpm and cells were resuspended in freshly made fixative consisting of methanol:acetic acid (10:1) diluted 1:1 with Ringer's solution. The cells then were washed with two to three further changes of freshly prepared fixative consisting of methanol:acetic acid (10:1) without Ringer's solution, until the cell suspension is clear. The cell suspension then was stored for one night in -20°C. Fixed cells then were dropped onto clean, wet slides, dried and stained with 4% Giemsa solution (pH 6.8) for 12 minutes. Three slides were obtained from each sample.

NDI Evaluation

From two slides in each sample, the proportion of mononucleated, binucleated, trinucleated and tetranucleated cells per 500 cells scored was assessed. NDI was calculated based on formula in IAEA publication where M1, M2, M3 and M4 indicate the number of cells with one, two, three and four nuclei and n is the total number of cells analyzed (n=500 minimally).(10)

$$NDI = \frac{(M1+2M2+3M3+4M4)}{n}$$

Statistical Analysis

The statistical difference of categorical variables (sex and smoking habits) in Takandeang Sub-Village inhabitants and control samples using χ^2 -test, whilst for the continuous variable (ages) was using t-test analysis. Unpaired t-test also used to compare the mean of MI and NDI values in Takandeang Sub-Village inhabitants and control samples, if the data have a normal distribution. The Kolmogorov-Smirnov test was applied to know the distribution of data. The statistical difference of MI and NDI in each age, gender and smoking habit groups were tested using One-way ANOVA and t-tests analysis. All tests were conducted using SPSS for Windows version 22.0 (IBM, New York, USA) and the significance value was set at $p < 0.05$.

Result

Indoor and Outdoor Gamma Dose Rate

The minimum and maximum gamma dose rates for indoor measurements were 320 nSv/h and 500 nSv/h, whilst for outdoor measurements were 340 nSv/h and 560 nSv/h. The average indoor and outdoor gamma dose rates were 398 ± 17.68 nSv/h and 450 ± 25.21 nSv/h, respectively.

Annual Effective Dose

The average annual effective dose for Takandeang Sub-Village was 2.52 mSv/y, with range from 1.99 to 3.17 mSv/y. This values were 2.2 to 3.6 times higher than the values of effective environmental gamma dose rate due to cosmic rays and terrestrial gamma radiation estimated for the world average report by UNSCEAR which was 0.87 mSv/y.(11) However, it should be note that the average annual effective dose calculation in Takandeang Sub-Village did not concerning the ingestion and inhalation exposures from radon gas.

Samples Characteristics

The mean ages of Takandeang Sub-Village inhabitants used in this study were 42.89 ± 15.58 with range 16 to 72 years. The mean ages in controls ranged from 16 to 68 years with a mean of 39.92 ± 14.58 . Statistical analysis revealed that sex, age and smoking habits status between Takandeang Sub-Village inhabitants and control samples were not significantly different ($p > 0.05$).

Manual and Automatic MI Evaluation

The mean of manual MI value in Takandeang Sub-Village inhabitants (4.96 ± 2.25) was slightly lower compared to

the mean MI value in control group (5.93 ± 2.14). A non-parametric test (Mann-Whitney) analysis was applied since the distribution of manual MI value data in Takandeang Sub-Village inhabitants was not normal. The Mann-Whitney test revealed that the difference was not statistically significant ($p > 0.05$) (Figure 2). In contrast the mean of automatic MI in Takandeang Sub-Village inhabitants (20.37 ± 10.49) was higher compared to the mean automatic MI in control group (18.87 ± 7.49), even though the difference was not statistically significant ($p > 0.05$) (Figure 2).

NDI, Lymphocyte, Leukocyte Numbers and Lymphocyte/Leukocyte Ratio

The distribution of NDI, lymphocyte and leukocyte data were normal, thus t-test analysis was performed to find out the significant difference of NDI, lymphocyte and leukocyte between Takandeang Sub-Village inhabitants and control samples. The mean of NDI values in Takandeang Sub-Village inhabitants (1.555 ± 0.174) was slightly higher compared to the mean NDI values in control group (1.523 ± 0.112). T-test analysis revealed that the difference was not statistically significant ($p > 0.05$) (Figure 3). Similar to NDI value, the mean of lymphocyte and leukocyte numbers in Takandeang Sub-Village inhabitants (7213 ± 213 ; 2420 ± 73) was also slightly higher compared to control group (7004 ± 114 ; 2156 ± 54) (Figure 3).

Confounding Factors Effects on Automatic MI and NDI

Table 1 shows the mean of automatic MI and NDI values with respect to age, smoking habit and sex in Takandeang Sub-Village inhabitants and control samples. There was non-significant difference of automatic MI and NDI between smokers and non-smokers in both of Takandeang Sub-

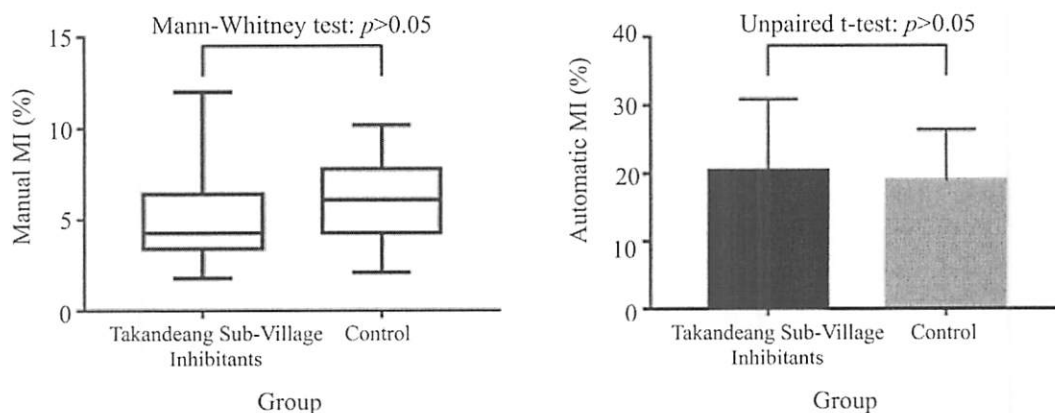


Figure 2. The mean of manual and automatic MI of Takandeang Sub-Village inhabitants and control samples.

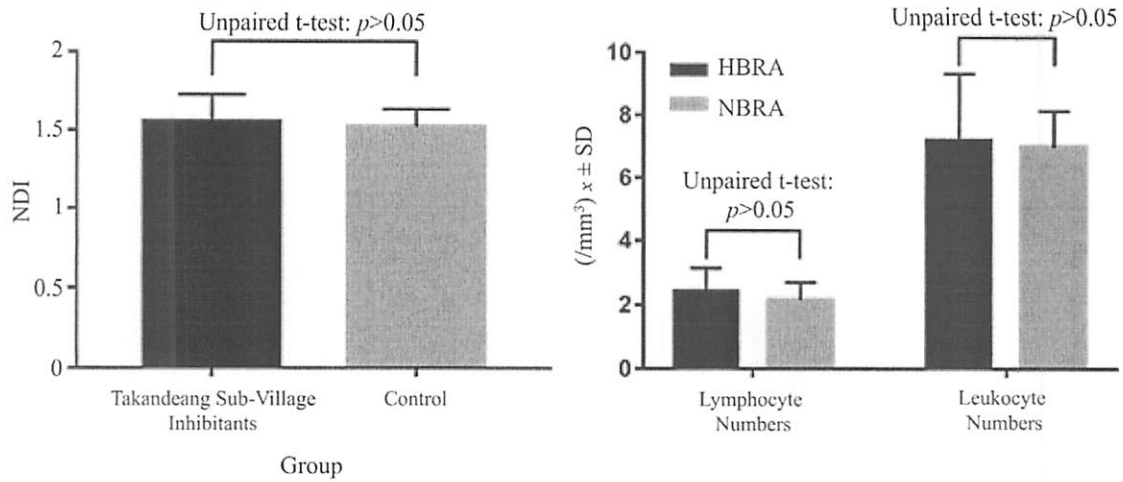


Figure 3. The mean NDI and lymphocyte, leukocytes numbers of Takandeang Sub-Village inhabitants and control samples.

Village inhabitants and control samples ($p > 0.05$). Similar results also found in sex and age variables even when all data were pooled ($p > 0.05$). The negative correlation between automatic MI and NDI data was found when the data was pooled, though the correlation was not significant (Figure 4).

Discussion

Here in this study, we used MI and NDI as the cell proliferation marker for estimates the general toxicity. The proportion of metaphase cells and binucleated cells may be

Table 1. The mean of automatic MI and NDI values with respect to age, sex and smoking habit in Takandeang Sub-Village inhabitants and control groups.

Parameter	n	Mean Automatic MI ± SD (%)	p-value	Mean NDI ± SD (%)	p-value
Age (Takandeang)					
0-30	8	27.66 ± 13.28	0.064	1.653 ± 0.170	0.183
31-50	15	19.29 ± 11.14	(NS)	1.536 ± 0.201	(NS)
>51	12	16.87 ± 4.06		1.513 ± 0.119	
Age (Control)					
0-30	4	12.66 ± 3.65	0.093	1.490 ± 0.112	0.651
31-50	16	21.13 ± 7.40	(NS)	1.520 ± 0.114	(NS)
>51	5	16.60 ± 7.50		1.560 ± 0.113	
Smoking (Takandeang)					
Yes	12	20.36 ± 10.73	0.996	1.577 ± 0.180	0.589
No	23	20.38 ± 10.61	(NS)	1.543 ± 0.173	(NS)
Smoking (Control)					
Yes	11	17.59 ± 6.97	0.46	1.539 ± 0.117	0.539
No	14	19.88 ± 7.98	(NS)	1.511 ± 0.110	(NS)
Sex (Takandeang)					
Male	22	21.10 ± 11.83	0.6	1.572 ± 0.163	0.45
Female	13	19.14 ± 8.03	(NS)	1.525 ± 0.194	(NS)
Sex (Control)					
Male	13	17.32 ± 7.06	0.291	1.542 ± 0.108	0.395
Female	12	20.55 ± 7.88	(NS)	1.428 ± 0.117	(NS)

NS: Not significant ($p > 0.05$)

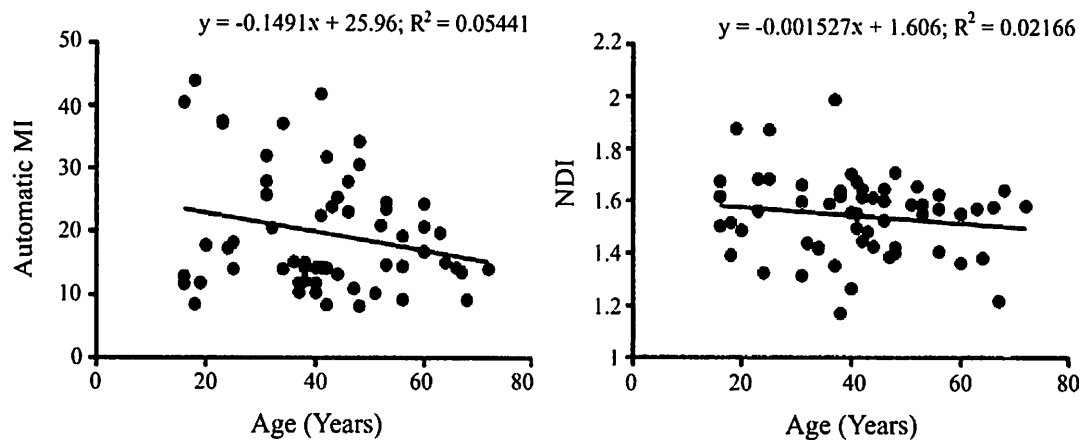


Figure 4. The linier regression analysis between age and automatic MI and also NDI.

used as a biomarker of the lymphocytes mitogen response, immune functions and cytostatic effects of various studied agents included the low radiation dose exposure.(12) The MI and NDI are represented of the number of cells that have correctly completed the cell cycle.(13) Assessment of MI and NDI has a practical meaning in lymphocyte cultures because it interpreted the death or arrest of the cells at any moment in interphase.(14)

We also evaluated both of manual and automatic MI in this study. Interestingly, our study showed that there was a contradictory finding of manual and automatic MI. However, since the data of mean leukocyte and lymphocyte numbers in Takandeang Sub-Village inhabitants were higher compared to control group (Figure 3), thus we hypothesized that automatic calculation of MI was more valid compared to manual calculation. As we already mention that a less leukocytes and lymphocytes numbers mean a slower mechanism of mitosis, thus a more leukocytes and lymphocytes numbers also mean a faster of mitosis process. The possible explanation for inaccuracy of manual calculation is probably because it only assess small area in the slide and cannot represent the real value of MI. Another factor is the misidentification of unstimulated nuclei as the stimulated in manual MI analysis. In the IAEA publication stimulated cells determined as the blast cells with large nuclei and unstimulated cells as the small nuclei.(10) Even though the IAEA publication already explained that the "cut-off" to differentiate the term large and small nuclei should be defined, the observer in manual calculation could false to identify the large nuclei (blast). In contrast, the automatic calculation of MI was performed using the MSearch module on the Metafer 3.11.2 system and its value was obtained during the scan of the slides. The classifier in MSearch module was set to the following criteria. The object threshold set to 50%, Min and Max Area set to 80 and

150 μm^2 , Maximum Relativity Concavity Depth set to 0.150 and Maximum Aspect Ration set to 1. The mean of manual MI values in control group was higher compared to other studies.(6,15,16) Different in MI assay protocol and number of total cells analyzed could be the factors that induced the difference of mean manual MI values.

In our previous study, we hypothesized that the circulating lymphocytes in Botteng Village inhabitants were under the toxic effect of natural radiation exposure as a mutagenic agent and suffer DNA damages thus cannot survive the division cellular cycle that made the MI lower compared to control group.(5) Interestingly, in this study we found that the mean automatic MI values in Takandeang Sub-Village inhabitants was higher compared to control group. Another study by Sinitsky & Druzhinin also found that the proliferation index, which characterizes the rate of cell division, was higher in people living in high radon concentrations area compared to control samples.(17) They proposed that a compensatory mechanism to promote a more rapid renewal of the proliferative pool under the genotoxic effects of radon might cause the higher rate of cell division in people living in high radon concentrations area. Unfortunately, since in this study we did not measure the radon concentration in Takandeang Sub-Village area thus we cannot compare our study results with Sinitsky & Druzhinin study.

However, there is a possibility that the compensatory mechanism also existed in lymphocytes of Takandeang Sub-Village inhabitants. The compensatory cell proliferation by homeostatic mechanisms induced by low dose radiation exposure has been widely investigated until now. The most comprehensive studies explained about the mechanisms of low dose radiation exposure induced lymphocyte stimulation already performed by Liu, *et al.*(18-20) The lymphocyte stimulation induced by low dose radiation exposure is

correlated with the stimulatory effect of low dose radiation on the immune system particularly the T lymphocyte. The stimulation of immune system by low-dose radiation is a complex process concerning intercellular reactions and signal transduction in the immune cells which lead to activation and proliferation of T lymphocyte. In briefly, the activation and proliferation of T lymphocyte involved the directly activation of antigen presenting cell (APC) and T lymphocyte cell (TLC) or via the reactive oxidative species (ROS). Low dose radiation could stimulate the B7-1 (CD80) and B7-2 (CD86) molecules and expression of IL-12 secretion by the APCs. Low dose radiation also up-regulates the expression of CD28, down-regulates of CTLA-4 on the TLCs and suppressed the production of IL-10. The up-regulation in both of B7-1 and B7-2 together with CD28 could up-regulate the TLC activity. The immune surveillance as well as other reactions process such as DNA repair, apoptosis of damaged cells and antioxidants are part of defense and adaptive response activated by low dose radiation. Our previous studies revealed that there is no significant difference of γ -H2AX foci, micronucleus frequencies, comet tail length and binucleate index in Botteng Village, Mamuju inhabitants compared to control samples.(21-24) These finding showed a possibility that the adaptive response already developed in Mamuju inhabitants. Based on the results in this study it is seem that the adaptive response in immune system also probably existed in Mamuju inhabitants. Further investigation should be performed to find out the adaptive response in the immune systems of Mamuju inhabitants.

Here in this study, the significant effect of confounding factors (age, gender and smoking habit) on MI and NDI values in both of Takandeang Sub-Village and control samples was not found. However, there was a trend that the MI and NDI will decrease linearly with ages. The possible explanation for this is the increase of genetic damage in elderly samples that lead to a decrease of lymphocytes proliferation. Several studies found a higher occurrence of genetic damage among more elderly individuals.(25,26) Concerning the effect of nicotine intake to lymphocytes proliferation, our study revealed there is no significant difference of automatic MI and NDI between smokers and non-smokers. Several studies also showed a non-significant difference of lymphocytes proliferation between smokers and non-smokers individuals. A study by Palus, *et al.*, reported no differences in the NDI between a smoker and non-smoker groups.(27) Another study indicated that cellular cycle in smokers was faster than non-smokers samples.(28) In their study, they found that NDI of smokers

was significantly higher compared to non-smokers samples. A deeper investigation should be performed to verify the effect of nicotine intake to lymphocyte proliferation.

Alakoç & Eroğlu stated that the increasing MI rate is a risk for carcinogenicity in welders since they found a significantly higher of MI in people employed in welding. (6) Interestingly, until now studies on the HBRA inhabitants and radiation workers in nuclear industry have not shown the increase in cancer risk.(19) In this study the significantly higher of automatic MI and NDI in Takandeang Sub-Village inhabitants was not found, thus we assumed that there is no increase in cancer risk of Mamuju inhabitants. However, more investigation should be performed to validate whether the HBRA inhabitants were have a higher risk for carcinogenicity than residents of normal background radiation area.

Conclusion

It can be concluded based on this study that chronic low radiation dose exposure in Takandeang Sub-Village inhabitants has no significant effect on the lymphocytes proliferation. Several factors could induce our finding. First, is the number of samples was probably not adequate to obtain meaningful statistical results in our study. Second, we only measured the indoor and outdoor Gamma dose rate from 10 houses in the Takandeang Sub-Village inhabitants. It is possible that the levels of external and internal radiation exposures were not similar and varied from one to another in our samples. Thus, some individuals in Takandeang Sub-Village inhabitants probably received a lower external and internal radiation exposures than the others. Third, there is a possibility that the radio adaptive response (RAR) in the immune system already developed in Takandeang Sub-Village inhabitants. More comprehensive study using a larger sample size should be performed to validate the RAR status in the immune system of Mamuju inhabitants.

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