Effectiveness of Natural Killer (NK) Cells in Peripheral Blood Stem-Cell towards Expression of EZH2, Ki-67 and Apoptosis in Retinoblastoma (RB) Cells Culture

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Abstract

A natural killer (NK) cell is a large granular lymphocyte having a cytotoxic activity against malignant tumor cells in the cellular immune response. NK cells play an important role to inhibit the metastasis of tumor and express glikoprotein molecule antigen (IgG) and variable cytotoxic factors like perforin, granzyme, cytolysin such as TNF-α against retinoblastoma cells. The aim of the study was to investigate the role of Natural Killer Cells (NK cells) as an alternative therapy, especially in cases of retinoblastoma. It is highly expected that the study can be used as a starting point in determining the strategic therapy, e.g. the cell targeted therapy to inhibit EZH2 and retinoblastoma cell proliferation. There were 2 groups of retinoblastoma cell cultures in this study, the groups of control (n=10) and exposure (n=10). The control group was a group of retinoblastoma cell cultures that did not get any therapy, while the exposure group was a group of retinoblastoma cell cultures with NK Cell exposure in vitro for 3 days. The data obtained were analyzed using the T- test and Pearson Correlation. In T-test, the results of the two groups indicated a significant difference in the positive expression of cell count of EZH2, Ki-67 and Apoptotic cells. The Pearson Correlation test showed that the expression of EZH2 was statistically negative significant (α: -0.930; p<0.05) towards the apoptotic cells expression. While in the proliferation cell expression (Ki-67) towards the apoptotic cell expression, the correlation was significantly negative (α: -0,868; p<0.05). The study showed that NK cells played an important role in the aggressiveness of RB cell cultures by changing the expression of EZH2, Ki-67 and apoptosis in RB cells. It is expected that the research can be used as a reference to determine the strategic therapy in retinoblastoma.

Keywords: NK cells, retinoblastoma, EZH2, proliferation, apoptosis

(Rec.Date: Nov 25, 2014 Accept Date: Dec 29, 2014)

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Introduction

A natural Killer (NK) cell is a large granular lymphocyte secreting IFN-Ɣ and one of the most essential cell types. It ranks with Cytotoxic T Lymphocytes (CTLs) as regards strong cytotoxic activity against malignant tumor cells in the cellular immune response [1,2]. NK cells consisting of approximately 10% of the lymphocytes and 2% of the peripheral blood are the mediators of anti-viral and anti-tumor immunity [3,4].

NK cells play an important role in inhibiting the metastasis of the tumor and express glikoprotein molecule antigen (IgG) and a variable repertoire of receptors on their surfaces. The activating and inhibitory receptors are both represented on their surfaces. They express Killer Inhibitory Receptors (KIRs) which can recognize MHC class I expressed on the surface of healthy but infected cells and cancerous cells down regulated MHC molecules [5,6]. They prevent the activation of NK cells because without the inhibitory signal, NK cells are free to empoy their cytotoxic system [7]. Pradeu *et al.* said that the theory of immunity is not discontinuity. The chronic presence of activating ligands reprogrammes NK cells to become hyporesponsive although they are activated by modified cells (infected and cancerous cells) expressing ligands for activating receptors [1,8].

NK cells release cytotoxic factors such as perforin, granzyme, cytolysin as TNF-α, serin, protease and proteoglican into a tumor cell through tumor cell membranes pore formed by perforin after the process of cell adhesion and finally due to the apoptosis, tumor cells die [1]. NK cells for adoptive transfer have been used with autologous or allogenic methods for treating patients with glioma [2], glioblastoma [9], gastric cancer [10] and breast cancer [11].

Primary intraocular retinoblastoma is a highly metastatic cancer in children. It is usually aggressive and often leads to blindness, disability and death. Retinoblastoma mass grows in the retina and spreads to the posterior chamber of the eye and if it is not immediately treated, then goes to invade and metastasize through the sclera and choroid until optic nerve with poor prognosis [12].

Cancer cell aggressiveness is determined based on the proliferation, cell nucleus, angiogenesis, necrosis and tumor cell apoptosis [13,14]. The balance in proliferation and apoptosis cell (cell turnover) was reported in various cancers, such as astrositoma [14], liver

cancer [15], prostate cancer [16], breast cancer [17] and retinoblastoma [18]. The level of cell proliferation in various kinds of cancer cells generally increases and express Ki-67 except in G0 phase [19]. While apoptosis is used to measure the cell death that has been programmed [20].

In addition, the aggressiveness of cancer cells, especially in retinoblastoma, is affected by catalytic protein in the nucleus in a group of polycomb proteins that is a transcription repressor and involved in controlling cellular memory, the invasion of and the development of cancer cells that is Enhancer Zeste of Homolog 2 (EZH2). EZH2 is a protein with the molecular weight of 85 kDa that is a component of PRC2 mediating the process of a transcription genes through a process of methylation lysine 27 in histon H3 [21-23]. This complex played an important role in regulating the expression of gen formation. Expression of this protein increased in both invasive carcinoma and metastatic tumors [24,25]. EZH2 was expressed in some cancers, including breast cancer [24,26], prostate cancer [27,28], colorectal cancer [29], soft tissue sarcoma [30] and liver cancer [31].

Collection of samples from intratumoral retinoblastoma tissues were cultured and exposed with NK cells. Then it occurred a reaction in which the NK cells could reduce the spread of retinoblastoma cells *in vitro*.

Materials and Methods

Samples

Samples of fresh intratumoral retinoblastoma tissues were collected from an RB patient in Dr.Soetomo Hospital-Faculty of Medicine Airlangga University Surabaya. The sample was taken from the tumor fresh tissues in exenteration surgery after inform consent was given to the patient's parents. The fresh tumor tissue was sent to the laboratory, evaluated and separated from the other soft tissues. Then they were cultured and frozen at a temperature of -80℃. The growth of RB cell cultures were confirmed through some histological evaluations and homogenizations carried out by a pathologist. NK cells then were taken from peripheral blood stem-cell of the retinoblastoma patients.

There were 2 groups of retinoblastoma cell cultures in this study, the groups of control (n=10) and exposure (n=10). The control group was a group of retinoblastoma cell cultures that did

not get any therapy, while the exposure group was a group of retinoblastoma cell cultures with NK Cell exposure in vitro for 3 days. Based on the design of the experimental case control study, the total samples used were 10 wells per group (control, n=10; exposure, n=10) [32,33].

The Peripheral Blood Collection

The isolation process of peripheral blood mononuclear cells (PBMC) was based on Boyum method (1968) with some modifications. The blood collecting for PBMC was conducted in the patient with retinoblastoma. The separation of the cell components was done through the centrifugation of blood samples. The blood was poured into a 15 cc sterile disposable tube and10 cc PBS was added. After that resuspension was performed. Five (5) cc of Ficoll solution was put slowly into a 15 cc sterile disposable tube through the wall, then a mixture of blood and PBS was poured into the Ficoll solution. The solution was then centrifuged at 20°C for 25 minutes with the speed of 1600 rpm. The results of the centrifugation were several layers consisting of red blood cell layer, Ficol, buffy coat and plasma. A layer of buffy coat mostly contained of lymphocytic cells was taken and then PBS 10 cc was added to wash buffy coat and centrifugation was performed again at 20°C for 10 minutes with 1600 rpm. The results of centrifugation were supernatant and pellet. The Supernatant was thrown out and the pellet was added to MEM (Modified Eagles Medium) as much as 2 cc and then resuspension was done. The resuspension results were moved into petridish and 10 cc of MEM was added for the process oof incubatation at 37°C for 3 days in an incubator. On the 4^{th} day it was added to retinoblastoma cell culture [11,34-36].

Retinoblastoma Cell Culture Samples

The retinoblastoma isolat was added to the MEM medium, then it was centrifuged at 3000 rpm for 10 minutes twice. After that, the sediment was cultured in MEM medium and added with antibiotic, anti fungal and 15% of Fetal Calf Serum. The pasase was repeated until the cell line was formed. The tumor tissue was put into a culture plate and incubated at 37°C, under the air humidity of 95% and 5% of CO_2 for 24 hours. Characterization of the growth of cell culture was checked on the 3^{rd} and 4^{th} day in the both groups. NK cells were mixed with the retinoblastoma cell culture with the ratio of 1:1 on the 4^{th} day [11,36].

Immunohistochemical Assay

RB cell culture was fixated on a coverslip, then it was follwed by rehydration process, washing and finally it was fixated with methanol. After that H_2O_2 was added on the cells cultured, then blocked with 5% FBS serum, containing 50 µl Cytonin and combinations Cytonin + Proteinase-K, for apoptosis cells assay. Then primary antibody EZH2 was washed and coated with *Rabbit Anti-KMT6 Polyclonal Antibody / EZH2 (bs-3521R) Bioss Inc.* (1:100). The cell proliferation was measured by using primary antibody Ki-67 *(CRM 325 AK, BK) - Biocare Medical Polyclonal Antibody* (1:75). While cell apoptosis used *apoptag kit primary antibody TACS® 2TdT DAB In Situ Apoptosis (4810-30-K) Trevigen, Inc.,* then it was incubated overnight. A cell culture was coated with secondary antibody labeled biotin *(Trekkie Universal Link (STU700H)) Biocare Medical.* After that cell cultures was labeled with StrepHRP *(Trekavidin-HRP Label (STHRP700 L10, Streptavidin Horseradish Peroxidase) Biocare Medical*. DAB chromogen was given to all cell cultures to mark the positive cell expressions. Counterstain using Meyer hematoxylen and green methylen were used to count the apoptosis cells. Observation with an Olympus CX-31 microscope with magnification 400x and counting 10 per high power field was done. The positive cell precentage was counted with Axio Vision software, Carl Zeiss Microscopy [37-40].

Data Analysis

The counting results of the expression of EZH2, Ki-67 and apoptosis cells were proceeded by using SPSS 15.0 for Windows. T-test for parametric data and Mann-Whitney U-test for non-parametric data and Pearson's correlation test for relationship variables analysis were applied for Statistical analysis. A p-value <0.05 was considered statistically significant.

Results

Retinoblastoma Culture Cells Characterization

The pictures showed the control group compared to the exposure group with the NK cells after being incubated for 3 days and characterized on the 4^{th} day. In Control group the RB cells showed the confluent layer growth (**A**) and in exposure group, the growth of RB cells was rare and NK cells (yellow arrows) showed in distinct clusters around the upper layer of dead cells (apoptosis) (B).

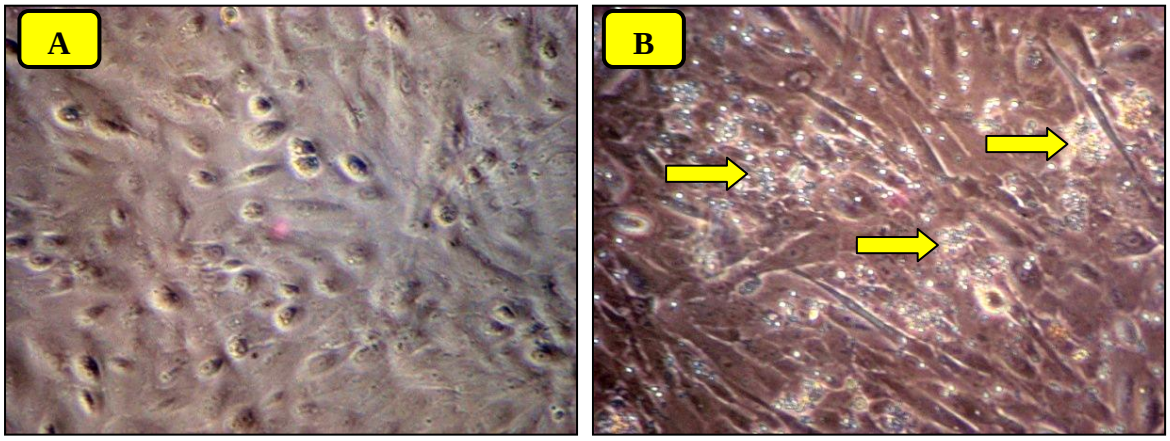

Figure 1. Retinoblastoma Cells Culture. On the 4th day in Control group the RB cells showed the confluent layer growth (**A**). On the 4th day in exposure group the growth of RB cells was rare and NK cells (yellow arrows) showed in distinct clusters around the upper layer of dead cells (apoptosis) (**B**).

Expression of EZH2, Ki-67 and Apoptosis Cell with Immunohistochemical Assay

EZH2 expression percentage in cells culture for the control and exposure groups was measured based on the positive cell percentage. The calculation of the positive EZH2 expression was based on the positive immunological staining reactions indicated by brown colour in the cell nuclei. The average number of cells expressing EZH2 in the control group was 2.81±0.64% while in the exposure group was 0.16±0.05%.

The positive cell expression of Ki-67 was indicated by brown to blackish brown colour in the cell nuclei while the positive cell expression of apoptosis cell was indicated by the dark colour in the cell nuclei. The average number of the cells expressing Ki-67 in the control group was 0.344±0.114% while in the exposure group was 0.05±0.02%. The number of cells expressing the cell apoptosis in the control group was 0.298±0.08% and 3.07±0.49% in the exposure group.

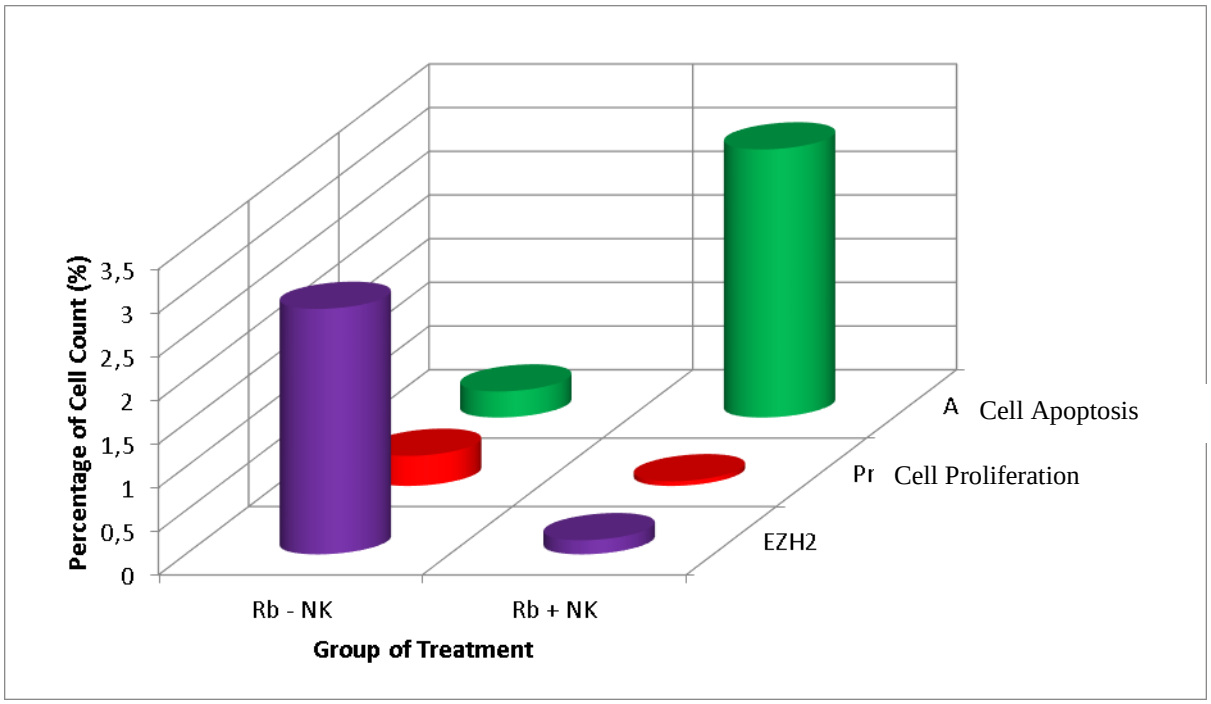

Figure 2. Graphic of EZH2, Ki-67 (Cell Proliferation) and Cell Apoptosis Expression. Percentage of cell count in the the control group (**Rb-NK**) and in the exposure group (**Rb+NK**).

The statistical analysis expression of EZH2 in the control and exposure groups using a *Levene's Test* on a *T-Test analysis* showed the statistically significant result (α: 0.000 and $p<0.05$). It indicated that EZH2 expression in the control and exposure groups given NK cells was a statistically different. The expression of Ki-67 also showed significant result using *Levene's Test* on a *T-Test analysis* (α: 0.007 and $p<0.05$). This showed that between control and exposure group there was a statistically difference of cell proliferation. While cells apoptosis in both groups revealed significant result also (α: 0.001 and $p<0.05$). It means between control and exposure groups there were differences in cells apoptosis expression.

Expression of EZH2 were determined according to the criteria of Eskander *et al.*, (2013): a score consisting of 0% (negative expression at nuclei or expressions in the cytoplasm), a score of 1+ (nuclei expression<25%), 2+ (50%≤ nuclei expression <25%) and 3+ (nuclei expression >50% /*strong staining*).

The result of statistical analysis on the expressions was strengthened with the analysis of Pearson correlation. Pearson correlation test analysized the correlation between EZH2

expression and apoptosis cell expression. The expression of EZH2 was significantly negative towards apoptosis cell (α: -0.930 and $p<0.05$). The higher expression of EZH2, the lower expression of apoptosis cells. The expression of cell proliferation (Ki-67) was also significantly negative correlated to apoptosis cell (α: -0.868 and $p<0.05$). It means that the higher expression of Ki-67, the lower expression of apoptosis cells.

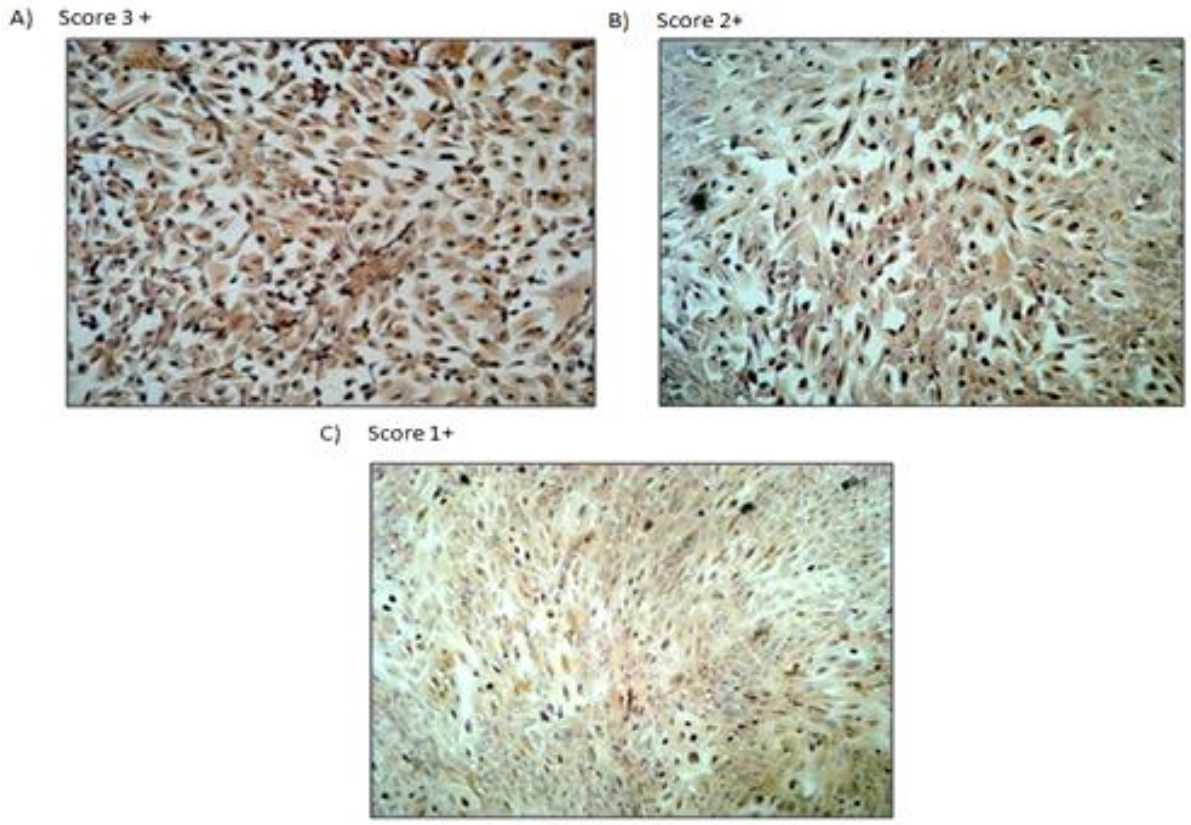

Figure 3. Expression EZH2 on Retinoblastoma Cells Culture. Expression strong of EZH2 on the score 3 + and 2 + **(A and B).** Expression weak of EZH2 on the score 1+ magnification of 400x **(C)**.

Discussion

The results of characterization on the exposure group with the NK cells showed the distinct layers around apoptotic retinoblastoma cells. The death of retinoblastoma cells in the exposure group because of the capaility of NK cells to induce apoptosis of retinoblastoma cells. Therefore, characteristic of retinoblastoma cells in the exposure group tend to grow rare and most die. NK cells were showed in distinct clusters around of the dead cells. In contrast,

the control group characteristic showed that the growth of retinoblastoma cells was faster and the percentage of the apoptosis cells in the control group was lower than that of the exposure group.

Statistical analyses *Levene's Test* on a *T-Test analysis* to mediate apoptosis cells in both groups indicated the existence of a significant difference (α: 0.001; $p<0.05$). These results showed that NK cells had a particularly important role on the tumor cell apoptosis process. Imai *et al.* said that NK cells had a role in inhibiting the growth and the metastasis of cancer cell. The role of NK cells was in accordance with the role of NK cells in ovary tumor, breast cancer, gastric cancer and glioblastoma [2,9,11,41,42].

While an expression of EZH2 in both groups of retinoblastoma cell culture was still expressed in strong EZH2 (strong staining) by Mann Whitney U-Test analysis. It was in accordance with the roles of EZH2 as a marker of proliferation and cancer aggressiveness [30], in cancer progressiveness and a bad prognosis [43] and on the aggressiveness of cancer stem cells but not in the process of cell and tissue differentiation [44].

On the exposure group, an expression of EZH2 was lower because apoptosis cell was higher in the expression and the cell proliferation was inhibited. P*earson correlation test* showed that an expression of EZH2 was negatively correlated with apoptosis cell (α: -0.930; p: 0.05) which was explained by biomolecular role of EZH2 as inhibitor of tumor suppresor gen. EZH2 had the role to inhibit genes protein synthesis $p14^{Arf}$ and p53 [44,45] and p16INKa [46] leading to the process of proliferation and metastasis of tumor cells increased. However, it did not occur in the exposure group with NK cells inducing an increase of apoptosis in retinoblastoma cells via NKG2D ligand and TNF-*apoptosis-inducing ligand* (TRAIL) [47].

In the exposure group, the lower expression of EZH2 caused the lower expression of cell proliferation and increased the expression of apoptotic cells. It was indicated by the expression of proliferation marker Ki-67 which was higher in the control group. Differential test with *Levene's Test* on *T-Test analysis* showed significant results (α: 0.007 and $p<0.05$). It means the percentage of proliferation cell expression (Ki-67) in the control group was higher than in exposure group. These results indicated that NK cells played a role in inhibiting the proliferation of tumor cells [48].

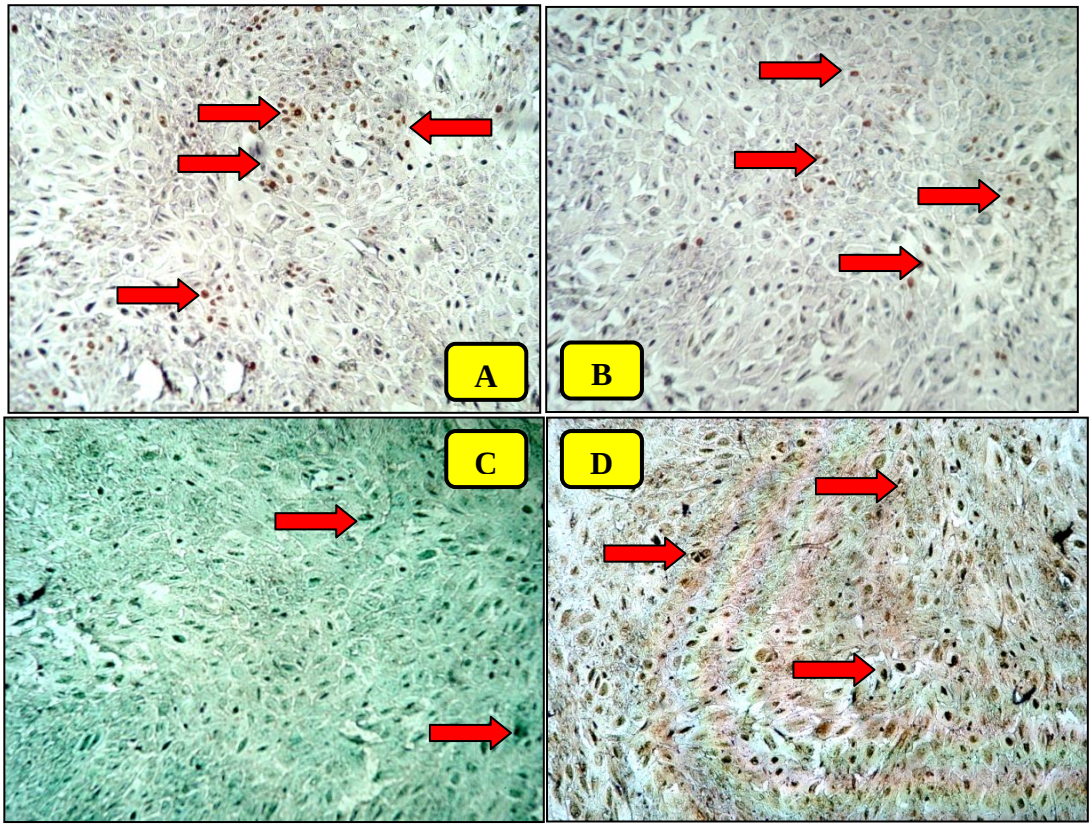

Figure 4. Expression of Proliferation Cell and Apoptosis Cell. Retinoblastoma cells in the control group showed an expression of high cell proliferation (**A**). Retinoblastoma cells in the exposure group showed an expression of low cell proliferation (**B**). Retinoblastoma cells in the control group showed expression of low mediate apoptosis / rarely (**C**). Retinoblastoma cells in the exposure group showed expression of high cell apoptosis (**D**). Expression of positive cell (red arrows) with 400x magnification.

The increase number of Ki-67 proliferation marker indicated the aggressiveness of tumors was characterized by an increase number of mitotic cells [49]. Normal RB cells expressed high number of proliferation cells which was not found in normal retina because it was not experienced cell proliferation or fixed [50,51]. Ki-67 expression on retinoblastoma was in accordance with the expression on breast cancer [19], colorectal cancers [48] and prostate cancer [52].

An increase number in cell proliferation was negatively correlated with mediate apoptosis cells (α: -0.868 and $p<0.05$). It means that the higher expression of Ki-67, the lower expression of apoptosis cells. Trihia *et al.* said that the increase of proliferation characterized

by marker Ki-67 indicated a tumor aggressiveness. Cancer cells have the characteristics of high cell proliferation and lower mediate apoptosis cells (cell turnover) [14,49].

The process of apoptosis cancer cells by the NK cells was through 2 processes: NKG2D ligand and the TNF-apoptosis-inducing ligand (TRAIL) [47,53]. In line with the process of NKG2D ligand, a tumor cell was subjected to mediate apoptosis because NK cells excreted cytotoxic granules, perforin and granzyme. Cellularly, NK cells induced apoptosis tumor cells by expressing killer activation receptors (KARs), for example NKG2D ligand. In a tumor cell, NK cells induced apoptotic signals through NKG2D ligand while the KIR signal was obstructed because tumor cells inhibited an expression of MHC class 1 so that cytotoxic granules could not be excreted into a tumor cell [54]. The exocytosis process of cytotoxic granular could happen because receptors of NK cells, FcɣRIII (CD16) bind to IgG tumor cells [7]. Perforin functions makes pores in the tumor cells and protein granzyme enter a tumor cell to mediate apoptosis [53].

Complex of perforin-granzyme or commonly called lytic granules produced by NK cells from within the cytoplasmic actin filaments move towards NK cells penetrating the microtubules and polarization of the contact area of the target cell [55,56]. Lytic granules binding into SNARE receptors (*soluble N-ethylmaleimide-sensitive factor attachment protein*) move to the plasma and the walls of the lytic granule membrane connected by a plasma membrane. It was in line with binding of SNARE receptors with Qa-SNARE syntaxin-11 (STX-11) receptors protein into the plasma membrane. The next process was the excretion of the perforin-granzyme into the targeted cells. This process occured through 2 mechanisms, *internalization model* and *the plasma membrane pore formation model* [57].

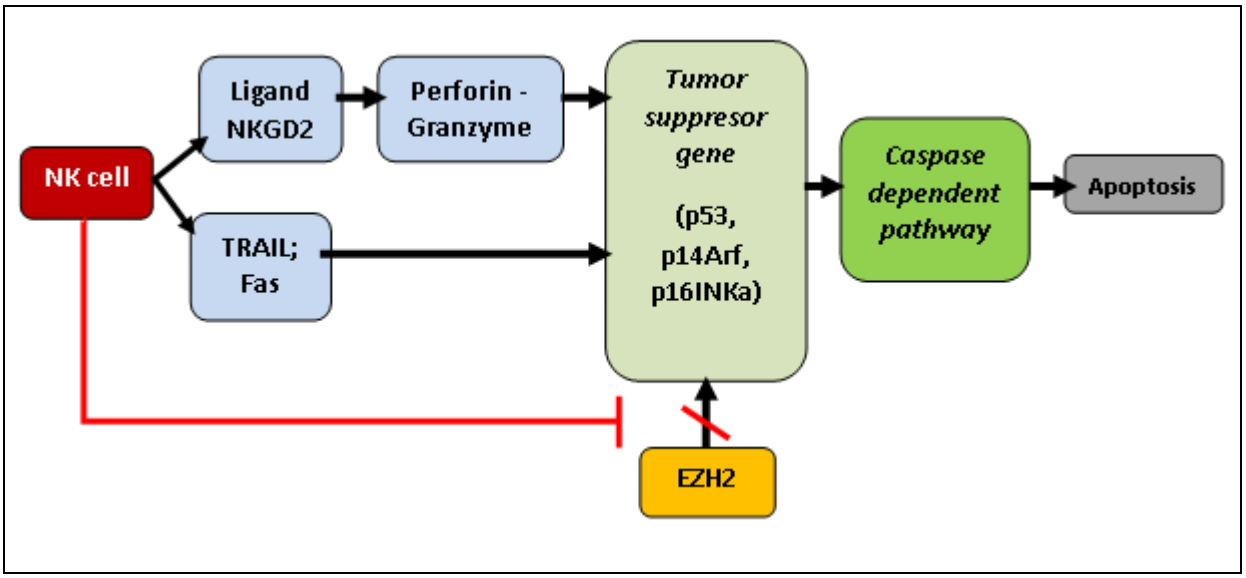

Figure 5. A schematic possible view of the induction of apoptosis retinoblastoma cell and EZH2 by the NK cell.

Internalization model happened when perforin-granzyme entered the cytoplasm cancer of the targeted cells through the endocytosis process and shaping enlargosome formation. Perforin formed the pores on the enlargosome wall and granzyme was secreted toward the cytoplasm of cancer cells for apoptosis [58,59]. In the plasma membrane pore formation model, the pore was formed by perforin in plasma membranes of cancer cell without the endocytosis process, so that granzyme was capable to enter the cytoplasm cancer cells directly after excreted from plasma membranes of NK cell. The pH neutral and high concentration of calcium on a synapse between space NK cells and cancer cells target supported perforin oligomerization on this model [60]. In both of the models, apoptosis induction on cancer cells could happen via three lines: activation of cascade caspase, damage of mitocondria, or damage of DNA cancer cells [57].

NK cells could also induce apoptosis signal through a TNF system or FasL that correlated with TRAIL in which the system mediate apoptosis extrinsic by cascade caspase [53,57,61]. Apoptosis signals was sent by FasL to Fas and formed FADD protein. FADD protein was bound to death receptor and activated caspase-8 toward caspase-3 as executor of apoptosis [61]. Fas/FasL interaction had a role against an onset of apoptosis in of cytotoxic reaction by involving the role cellular immunologis against a tumor cell [62,63].

Increase of apoptosis occured through various pathways so that the role of EZH2 was less in supressing the tumor supressor gene. EZH2 had a capabality to suppress p14Arf, p53 and p16INKa protein. However, apoptosis signal due to induction of NK cells was capable of passing through the cytotoxic granules, binding NK cell receptors FcɣRIII (CD16) with IgG tumor cells, through a cytokine IFN-ɣ, the TRAIL and FasL pathway. All pathways caused apoptosis increase and the role of EZH2 inhibited [7,53].

Conclusion

The study showed that NK cells played an important role in the aggressiveness of RB cell cultures by changing the expression of EZH2, Ki-67 and apoptosis in RB cells. It is expected that the research can be used as a reference to determine the strategic therapy in retinoblastoma.

References

1. Abbas AK, Lichtman AH. Immunity to Tumors. In: Celluler and Molecular Immunology. 5th edition. Philadelphia; WB Saunders Co. 2003:391-410.
2. Ishikawa E, Tsuboi K, Saijo K, Harada H, Takano S, Nose T, Ohno T. Autologous natural killer cell therapy for human recurrent malignant glioma. Anticancer Res. 2004; 24(3b):1861-71.
3. Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, Caligiuri M. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. Blood. 2000;101(8):3052-7.
4. Marcenaro E, Carlomagno S, Pesce S, Chiesa MD, Parolini S, Moretta A, Sivori S. NK Cells and Their Receptors During Viral Infections. Immunotherapy. 2011;3(9):1075-86.
5. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med. 2002;195(3):327-33.
6. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact dependent stimulation and inhibition of dendritic cells by natural killer cells. J Exp Med. 2002;195(3):335-341.
7. Abe H, Akiyama S, Okamoto M. Clinical Cancer Immunotherapy: Molecular Targeting Immunotherapy. J Integ Med. 2008;1(1):38-46.

8. Pradeu T, Jaeger S, Vivier E. The speed change: towards a discontinuity theory of immunity? Nat Rev Immunol. 2013;13(10):764-9.

9. Navarro, AG. Activated NK cells are potent effectors against glioblastoma cells due to activating KIR2DS2 and KIR2DS4 - HLA ligand interactions-*In vitro* study. Thesis, University of Bergen, Norway, 2013.

10. Saito H, Osaki T, Ikeguchi M. Decreased NKG2D expression on NK cells correlates with impaired NK cell function in patients with gastric cancer. Gastric Cancer. 2012;15(1):27-33.

11. Lim SA, Kim TJ, Lee JE, Sonn CH, Kim K, Kim J, Choi JG, Choi IK, Yun CO, Kim JH, Yee C, Kumar V, Lee KM. *Ex Vivo* Expansion of Highly Cytotoxic human NK Cells by Cocultivation with Irradiated Tumor Cells for Adoptive Immunotherapy. Therapeutics, Targets and Chemical Biology. Cancer Res. 2013;73(8):2598-607.

12. Divan A, Lawry J, Dunsmore IR, Parsons MA, Royds JA. p53 and p21waf-1 expression correlates with apoptosis or cell survival in poorly differentiated, but not well-differentiated, retinoblastomas. Cancer Res. 2001;61(7):3157-63.

13. Catoi C, Baba A. Comparative Oncology. The Publishing House of the Romanian Academy. 2007; Chapter 3.

14. Wahyuhadi J. Ekspresi Sel NK, Sel T Sitotoksik Il-2 dan IFN-Ɣ Intratumoral dan Pengaruh Terhadap Agresivitas Astrositoma. Dissertation, Program Studi Ilmu Kedokteran, Program Pascasarjana Universitas Airlangga, 2010;1-75.

15. Ito Y, Matsuura N, Sakon M, Takeda T, Umeshita K, Nagano H, Nakamori S, Dono K, Tsujimoto M, Nakahara M, Nakao K, Monden M. Both cell proliferation and apoptosis significantly predict shortened disease-free survival in hepatocellular carcinoma. British Journal of Cancer. 1999;81(4):747-51.

16. Xie W, Wong YC, Tsao SW. Correlation of increased apoptosis and proliferation with development of prostatic intraepithelial neoplasia (PIN) in ventral prostate of the Noble rat. Prostate. 2000;44(1):31-9.

17. Liu S, Edgerton SM, Moore II DH, Thor AD. Measures of Cell Turnover (Proliferation and Apoptosis) and Their Association with Survival in Breast cancer. Clin Cancer Res. 2001;7(6):1716-23.

18. Sitorus RS, Gumay S, van der Valk P. The apoptotic paradox in retinoblastoma. Ann N Y Acad Sci. 2009;1171:77-86.

19. Urruticoechea A, Smith IE and Dowsett M. Proliferation Marker Ki-67 in Early Breast cancer. J Clin Oncol. 2005;23(28):7212-20.

20. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.

21. Morey L and Helin K. Polycomb group protein-mediated repression of transcription. Trends Biochem Sci. 2010;35(6):323-32.

22. O'Meara MM, Simon JA. Inner workings and regulatory inputs that control Polycomb repressive complex 2. Chromosoma. 2012;121(3):221-34.

23. Yamaguchi H, Hung MC. Regulation and Role of EZH2 in Cancer. Review Article. Cancer Res Treat. 2014;46(3):209-22.

24. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci USA. 2003;100(20):11606-11.

25. Varambally S, Cao Q, Mani RS, Shankar S, Wang X, Ateeq B. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. Science. 2008;322(5908):1695-9.

26. Chang CJ, Yang JY, Xia W, Chen CT, Xie X, Chao CH, Woodward WA, Hsu JM, Hortobagyi GN, Hung MC. EZH2 Promotes Expansion of Breast Tumor Initiating Cells through Activation of RAF1-b-Catenin Signaling. Cancer Cell. 2011;19(1):86-100.

27. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9.

28. Yang YA and Yu J. EZH2, an epigenetic driver of prostate cancer. Protein Cell. 2013; 4(5):331-41.

29. Kodach LL, Jacobs RJ, Heijmans J, van NoeselCJ, Langers AM, Verspaget HW, Hommes DW, Offerhaus GJ, van den Brink GR and HardwickJC. The role of EZH2 and DNA methylation in the silencing of the tumour suppressor RUNX3 in colorectal cancer. Carcinogenesis. 2010;31(9):1567-75.

30. Ciarapica R, Miele L, Giordano A, Locatelli F, Rota R. Enhancer of zeste homolog 2 (EZH2) in pediatric soft tissue sarcomas: first implications. BMC Med. 2011;9:63.

31. Hajósi-Kalcakosz, S, Dezső K, Bugyik E, Bödör C, Paku S, Pávai Z, Halász J, Schlachter K, Schaff Z, Nagy P. Enhancer of zeste homologue 2 (EZH2) is a reliable immunohistochemical marker to differentiate malignant and benign hepatic tumors. Diagnostic Pathology. 2012;7(86):1-7.

32. Sastroasmoro S and Ismael S. Dasar-Dasar Metodologi Penelitian Klinis, Sagung Seto. Edisi 4. 2011.

33. Setiawan A, Sunyoto D, Buku Ajar: Statistik Kesehatan Parametrik, Non Paramatik, Validitas, dan Reliabilitas, Nuha Medika, Yogyakarta. 2011.

34. Böyum A. Isolation of leucocytes from human blood. Further observations. Methylcellulose, dextran, and ficoll as erythrocyteaggregating agents. Scand J Clin Lab Invest Suppl. 1968;97:31-50.

35. Rose NR, de Macario EC, Fahey JL, Friedman H, Penn GM. Manual of Clinical Laboratory Immunology. American Society for Microbiology, Washington, D.C.1994.

36. Rantam FA. Metode Imunologi. Surabaya: Airlangga University Press. Hal 83-84. 2003.

37. Bancroft JD and Gamble M. Theory and Practice of Histological Techniques. 6th Edition. Churchill Livingstone Elsevier, 2008;161-86.

38. Chan W, Cheung K, Schorge JO, Huang L, Welch WR, Bell DA, Berkowitz RS, Mok SC. Bcl-2 and p53 Protein Expression, Apoptosis and p53 Mutation in Human Epithelial Ovarian Cancers. Am J Pathol. 2000;156(2):409-17.

39. Eskander RN, Ji T, Huynh B, Wardeh R, Randall LM, Hoang B. Inhibition of Enhancer of Zeste Homolog 2 (EZH2) expression is associated with decreased tumor cell proliferation, migration and invasion in endometrial cancer cell lines. Int J Gynecol Cancer. 2013;(6):997-1005.

40. Jia N, Li Q, Tao X, Wang J, Hua K, Feng W. Enhancer of zeste homolog 2 is involved in the proliferation of endometrial carcinoma. Oncol Lett. 2014;8(5):2049-54.

41. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood. 2005;106(1):376-83.

42. Geller MA, Cooley S, Judson PL, Ghebre R, Carson LF, Argenta PA. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. Cytotherapy. 2011;13(1):98-107.

43. Sauvageau M. and Sauvageau G. Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. J Stem Cell. 2010;7(3):299-313.

44. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J. 2003;22(20)5323-35.

45. Tan D, Tan S, Zhang J, Tang P, Huang J, Zhou W, Wu S. Histone trimethylation of the p53 gene by expression of a constitutively active prolactin receptor in prostate cancer cells. Chin J Physiol. 2013;56(5):282-90.

46. Kotake Y, Cao R, Viatour P, Sage J, Zhang Y, Xiong Y. pRB family proteins are required for H3K27 trimethylation and Polycomb repression complexes binding to and silencing p16INK4alpha tumor suppressor gene. Genes Dev. 2007;21(1):49-54.

47. Mehlen P, Puisieux A. Metastasis: a question of life or death. Nat Rev Cancer. 2006;6(6):449-58.

48. Paholyuk TD, Zacharzeva LM, Koshel KV, Oliynichenko GP, Berezhnaya NM. Stage of differentiation, proliferative index of tumor cells and cytotoxic activity of peripheral blood lymphocytes in colorectal cancer patients. Exp Oncol. 2004;26(2):161-3.

49. Trihia H, Murray S, Price K, Gelber RD, Golouh R, Goldhirsch A, Coates AS, Collins J, Castiglione-Gertsch M, Gusterson BA; International Breast Cancer Study Group. Ki-67 expression in breast carcinoma: its association with grading systems, clinical parameters, and other prognostic factors--a surrogate marker? Cancer. 2003;97(5):1321-31.

50. Dyer MA, Cepko CL. Regulating proliferation during retinal development. Nat Rev Neurosci. 2001;2(5):333-42.

51. Soebagjo HD. Korelasi antara Asam Hialuronat, *Matrix Metalloproteinase-14* (MMP-14), dan *Enhancer of Zeste Homolog 2* (EZH2) dalam Perkembangan Agresivitas Retinoblastoma. Dissertation, Universitas Brawijaya, Malang, 2014.

52. Madani SH, Ameli S, Khazaei S, Kanani M, Izadi B. Frequency of Ki-67 (MIB-1) and P53 expressions among patients with prostate cancer. Indian J Pathol Microbiol. 2011;54(4):688-91.

53. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer. 2004;4(1):11-22.

54. Abbas AK, Lichtman AH. Basic Immunology: Functions and Disorders of the Immune System. 3rd edition. Philadelphia; WB Saunders Co, 2011;32-36.

55. Wood SM, Meeths M, Chiang SC, Bechensteen AG, Boelens JJ, Heilmann C, Horiuchi H, Rosthøj S, Rutynowska O, Winiarski J, Stow JL, Nordenskjöld M, Henter JI, Ljunggren HG, Bryceson YT. Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity. Blood. 2009;114(19):4117-27.

56. Liu D, Meckel T, Long EO. Distinct role of rab27a in granule movement at the plasma membrane and in the cytosol of NK cells. PLoS One. 2010;5(9):e12870.

57. Krzewki K. and Coligan JE. Human NK cell lytic granules and regulation of their exocytosis. Review Article. Front Immunol. 2012;3(335):1-16.

58. Keefe D, Shi L, Feske S, Massol R, Navarro F, Kirchhausen T. Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. Immunity 2005;23(3):249-62.

59. Thiery J, Keefe D, Saffarian S, Martinvalet D, Walch M, Boucrot E, Kirchhausen T, Lieberman J. Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis. Blood. 2010;115(8):1582-93.

60. Praper T, Sonnen A, Viero G, Kladnik A, Froelich CJ, Anderluh G, Dalla Serra M, Gilbert RJ. Human perforin employs different avenues to damage membranes. J Biol Chem. 2011;286(4):2946-55.

61. Lumongga F. Apoptosis. Departemen Patologi Anatomi Fakultas Kedokteran Universitas Sumatera Utara. USU Repository. 2008.

62. Houston A and O'Connell J. The Fas signalling pathway and its role in the pathogenesis of cancer. Current Opinion in Pharmacology 2004;4(4):321-6.

63. Shimizu M, Kondo M, Ito Y, Kume H, Suzuki R, Yamaki K. Soluble Fas and Fas ligand provide new information on metastasis and response to chemotherapy in SCLC patients. Cancer Detect Prev. 2005;29(2):175-80.