UNIVERSA MEDICINA

September-December, 2014 *Vol.33 - No.3*

Enhancer of zeste homolog 2 regulates cell differentiation and proliferation in neuroblastoma

Amallia N. Setyawati*, and Takehiko Kamijo****

ABSTRACT

BACKGROUND
Neuroblastoma (NB) is one of the most common extracranial solid tumors occurring in infancy and childhood with highly variable outcomes. Polycomb group (PcG) proteins are epigenetic gene silencers. Enhancer of zeste homolog 2 (EZH2) is a member of the polycomb repressor complex 2 (PRC2) group, with the main function to catalyze the polycomb repressor complex by methylating lysine 9 and 27 of histone H3. This study aimed to investigate the biological functionality of EZH2 in NB.

METHODS
This was an experimental study with an analysis of correlation initially of the known prognostic factors of NB patients' outcomes, by comparing the expression of v-myc avian myelocytomatosis viral oncogene neuroblastoma (MYCN) with that of EZH2, on the basis of the patients' overall and relapse free survival rates. This was followed with a biological functional study to assess the role of EZH2 expression in NB.

RESULTS
EZH2 knockdown induces neurite extension and differentiation marker growth associated protein 43 (GAP43) in NB cells, although it does not affect cell cycle. By ectopic expression of EZH2, all-trans retinoic acid (ATRA) induced neurite extension was suppressed and GAP43 was decreased. Overall, EZH2 seems to have an important role in NB cell differentiation. Although EZH2 did not alter cell proliferation, in the soft agar colony formation assay there was a significant increase in total colony number and number of large colonies.

CONCLUSION
Our result clarified the potential role of EZH2 in the regulation of cell differentiation and proliferation, which subsequently may play an important role in the poor prognosis of NB patients.

Keywords: Neuroblastoma, EZH2, MYCN

*Department of Biochemistry, Faculty of Medicine, Diponegoro University, Semarang, Indonesia
**Department of Biochemistry and Molecular Carcinogenesis, Chiba Cancer Center Research Institute, Japan

Correspondence
dr. Amallia N. Setyawati, MSi.Med.
Department of Biochemistry, Faculty of Medicine, Diponegoro University
Jl. Prof. Soedharto, SH, Tembalang, Semarang
P.O.Box.: 1269
Email:
amallia.setyawati@fk.undip.ac.id

Univ Med 2014;33:153-62

Enhancer of zeste homolog 2 berperan pada diferensiasi dan proliferasi sel neuroblastoma

ABSTRAK

PENDAHULUAN

Neuroblastoma (NB) merupakan salah satu penyakit keganasan tumor solid ekstrakranial yang terjadi pada bayi dan masa kanak-kanak dengan luaran yang beragam. Protein kelompok gen polycomb *(PcG) merupakan* silencer gen epigenetic. Enhancer of zeste homolog 2 *(EZH2) diketahui merupakan anggota kelompok* polycomb repressor complex *2 (PRC2) dengan fungsi utamanya yaitu katalisasi subunit kompleks repressor* polycomb *dengan cara metilasi lisin 9 dan 27 dari histon H3. Beragam jenis kanker menunjukkan ekspresi tinggi EZH2. Penelitian ini bertujuan untuk menyelidiki fungsi biologis EZH2 pada NB.*

METODE

Penelitian ini merupakan penelitian experimental dengan melakukan analisis korelasi menghubungkan faktor prognostik luaran pasien NB, dengan membandingkan ekspresi v-myc avian myelocytomatosis viral oncogene *neuroblastoma (MYCN) dibandingkan dengan EZH2 berdasarkan tingkat kesintasan dan tingkat bebas penyakit pasien NB. Selain itu dilakukan pemeriksaan regulasi diferensiasi NB cell oleh EZH2 dan studi fungsional biologis untuk mengetahui peranan ekspresi EZH2 pada neuroblastoma.*

HASIL

Knockdown EZH2 menginduksi ekstensi neurit dan marker diferensiasi growth associated protein *43 (GAP43) pada sel NB, walaupun tidak berpengaruh terhadap siklus sel. Pada ekspresi ektopik EZH2, terjadi penekanan ekstensi neurit yang diinduksi* all-trans retinoic acid *(ATRA) dan GAP43 mengalami penurunan. Secara keseluruhan, EZH2 tampak berperan terhadap diferensiasi sel NB. Walaupun EZH2 tidak memiliki efek pada proliferasi sel, namun demikian pada* study soft agar colony formation assay *didapatkan peningkatan jumlah koloni dan sejumlah koloni berukuran besar. Hal ini menunjukkan bahwa EZH2 berperanan di dalam tumorigenesis NB.*

KESIMPULAN

EZH2 memiliki peranan di dalam pengaturan diferensiasi dan proliferasi sel neuroblastoma, sehingga dapat berpengaruh terhadap luaran buruk pasien NB.

Kata kunci: *Neuroblastoma, EZH2, MYCN*

INTRODUCTION

Neuroblastoma is one of the most common extracranial solid tumors occurring in infancy and childhood, derived from sympathetic neuroblasts. Clinical outcomes are highly variable, from spontaneous regression, caused by neuronal differentiation and/or apoptotic cell death, to malignant progression.[1] Cytogenetic and molecular genetic studies identified that genetic abnormalities such as loss of the short arm of chromosome 1 (1p), amplification of v-myc avian myelocytomatosis viral oncogene neuroblastoma (MYCN) and 17q gain are frequently observed and often associated with poor clinical outcome.[2] Epigenetic alterations such as altered DNA methylation, misregulation of chromatin remodeling by histone modifications, and aberrant expression of polycomb group (PcG) genes proteins, have emerged as common hallmarks of many NBs,[3,4] other than genetic changes that occur in cancer

such as the deletion of tumor suppressor genes (TSGs), amplification/activation of oncogenes, and loss of heterozygosity or gene mutations in tumor associated genes.[5]

Polycomb group gene proteins are epigenetic gene silencers and are involved in the maintenance of embryonic and adult stem cells, which are implicated in neoplastic development.[6] Polycomb group gene proteins construct multiprotein complexes known as polycomb repressive complexes (PRCs). Polycomb group gene proteins are classified into two groups. One group, PRC2, contains enhancer of zeste homolog 2 (EZH2), embryonic ectoderm development (EED), Suz12, and RbAp48. While the other, PRC1, consists of >10 subunits including the oncoprotein B lymphoma Mo-MLV insertion region 1 homolog (BMI1), and the HPC proteins, namely HPH1-3, RING1-2 and SCML.[3] Previously, there were reports that PRC1 BMI1 has an important role in NB tumorigenesis and that BMI1 transcription was induced by MYCN.[7,8]

Enhancer of zeste homolog 2, also called histone lysine methyltransferase (HKMT), was cloned as one of the polycomb group genes. The function of EZH2 is to catalyze the polycomb repressor complex by methylating lysine 9 and 27 of histone H3. Enhancer of zeste homolog 2 has recently been identified to be involved in essential celullar processes, namely cell fate decision, cell cycle regulation, senescence, cell differentiation and cancer. Various cancer types also highly express EZH2.[9]

Cancer initiation and progression has also been associated with overexpression of EZH2. Overexpression of EZH2 contributes not only to cancer stem cell formation but also to expansion of an aggressive cancer stem cell population that promotes cancer progression.[10] EZH2 plays a crucial role in stem cell maintenance and tumor development. It was identified that reducing EZH2 expression using siRNA or treatment with a small molecule, S-adenosylhomocysteine hydrolase inhibitor 3-deazaneplanocin (DZNep), inhibits methyltransferases and induces degradation of EZH2, which subsequently result in cell growth inhibition and reduced tumor formation in various cancers.[11]

Although previous studies indicate involvement of EZH2 in various cancers, however, specifically in NB, the biological molecular mechanisms underlying this remain poorly understood. In view of the above mentioned background, this study aimed to identify the association of EZH2 expression with the classical poor prognosis outcome of NB and its biological translation.

METHODS

Research design

The present study using an experimental design was conducted from January to December 2012 at the Laboratory of Biochemistry and Molecular Carcinogenesis, Chiba Cancer Center Research Institute.

Cell cultures

We selected thirteen primary neuroblastoma cell lines (NB-69, SK-N-SH, SH-SY5Y, SK-N-AS, SK-N-BE, SK-N-DZ, NB39-nu, NGP, NB19, NB9, IMR32, TGW, SMS-SAN) which represent MYCN single-copy and MYCN-amplified cells. The cells were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), ~50 μg/mL penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA), L-glutamine, phenol red supplemented with 10% fetal bovine serum (FBS), and 1% antibiotics under humidified 5% CO_2 in air at 37°C.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analyses

The quantitative RT-PCR (qRT-PCR) was performed with a SYBR green real time Quantitative RT-PCR assay kit (Qiagen) on RNA extracts obtained from neuroblastoma cancer stem cell lines using Real time PCR applied

biosystem 7500. The primer set used was 1096F 5'-TATAAGCGGAAGAACACAGAAACA-3' and 1171R 5'-TCCCTCCAAATGCTGGTAAC AC-3'.

As an internal control, the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed. A master mix (50 µl) of the following reaction components was prepared to the indicated end reaction: 2xSYBR green master mix (SYBR DE), 10µl of Dyell (Reff Dye II), 0.4µl of 200 nM Reverse Primer (10µM), 0.4µL of 200 nM Forward Primer (10µM), and H_2O 6.8µl.

Western blot analyses

Cells were lyzed in a buffer containing 120 mM NaCl, 50 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$, 40µg/ml aprotinin, 10 mM β-glycerophosphate, 1mM phenylmethylsulfonyl fluoride, 1 mM NaF, 250 U/ml Benzonase® (Novagen, San Diego, CA, USA), 0.1mM Na_3VO_4, and 0.5% Nonidet P-40.

Western blot analysis was performed as previously reported.[12] After transferring to an Immobilon-P membrane (Millipore, Bedford, MA, USA), proteins were reacted with either anti-Bmi1 mouse monoclonal (229F6; Upstate, Charlottesville, VA, USA), anti-EZH2 (rabbit polyclonal, millipore 07-681), or anti-Ring1B mouse monoclonal antibody as described in a previous report.[13]

Semi quantitative RT-PCR

Semi-quantitative RT-PCR was conducted as previously described.[12] Total cellular RNA to prepare RT-PCR templates was extracted from NB cell lines using Isogen (Nippon Gene K, Tokyo, Japan) and subsequently cDNA was synthesized from 1 µg total RNA template according to the manufacturer's protocol (River Tra Ace-α, RT PCR kit, Toyobo, Osaka, Japan).

Lentiviral infection

The packaging cell line HEK 293T ($4x10^6$) was plated and transfected the next day, when 1.5 µg of the transducing vectors containing the gene or shRNA and 2.0 µg of the packaging vectors (Sigma-Aldrich) were cotransfected by the Fugene6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's protocol. The medium was changed the next day and cells were cultured for another 24 hours. Conditioned medium was collected and cleared from the debris by filtering through a 0.45µm filter (Milipore). Thereafter, $1x10^5$ NB cells were seeded in each well of a 6 well-plate and transduced by lentiviral-conditioned media. Transduced cells were analyzed by Western blot and RT PCR.

Overexpression and knockdown of EZH2

For the overexpression of EZH2, FLAG-tagged hEZH2 was subcloned into lentivirus pHR-SIN-CSGW. For the knockdown of EZH2, the five PLKO.1-puromycin based lentiviral vectors containing shRNA sequences targeting EZH2 (RefSeq NM_004456) were obtained from the MISSION TRC-Hs 1.0 (Human) shRNA library (Sigma Aldrich). Virus production, infection and selection were performed according to the manufacturer's protocol. One week post infection, cells were collected and knockdown efficiency was examined by Western blot. We checked EZH2 knockdown by the five lentiviral shRNAs and used two for experiments.

Cell proliferation assay

NB cells were seeded in 96-well plates at a density of 750 cells per well in a final volume of 100 µl. The culture was maintained under 5% CO_2 and 10 µl WST-8 labeling solution (Cell counting Kit-8; DOJINDO, Kumamoto, Japan) was added, then the cells were returned to the incubator for 2 h. The absorbance of the formazan products formed was detected at 450nm in a 96-well spectrophotometric plate reader, according to the manufacturer's protocol.

Soft agar colony formation assay

IMR32 cells were seeded at 2,000 cells in semi-solid Noble agar medium in a 2-cm dish and evaluated after 2 weeks for colony numbers.

Colony number was automatically counted using WINROOF software.

Statistical analysis

All data were tested statistically using the 2-tailed t test. P <0.05 was considered to indicate statistical significance.

Ethical clearance

Ethical clearance was granted by the institutional review board at Chiba Cancer Center Research Institute after the study protocol had been carefully reviewed.

RESULTS

To identify the association of EZH2 expression and patient's outcome, we analyzed the correlation of the known prognostic factor of NB patients' outcome, MYCN, with EZH2 expression (Figure 1A). Subsequently Kaplan-Meier analysis was used to determine overall and relapse free (event free) survival rate of NB patients, who were divided into high and low EZH2 expression groups. (Figure 1B and 1C). R2: microarray analysis and visualization platform openware database was used for these analyses.

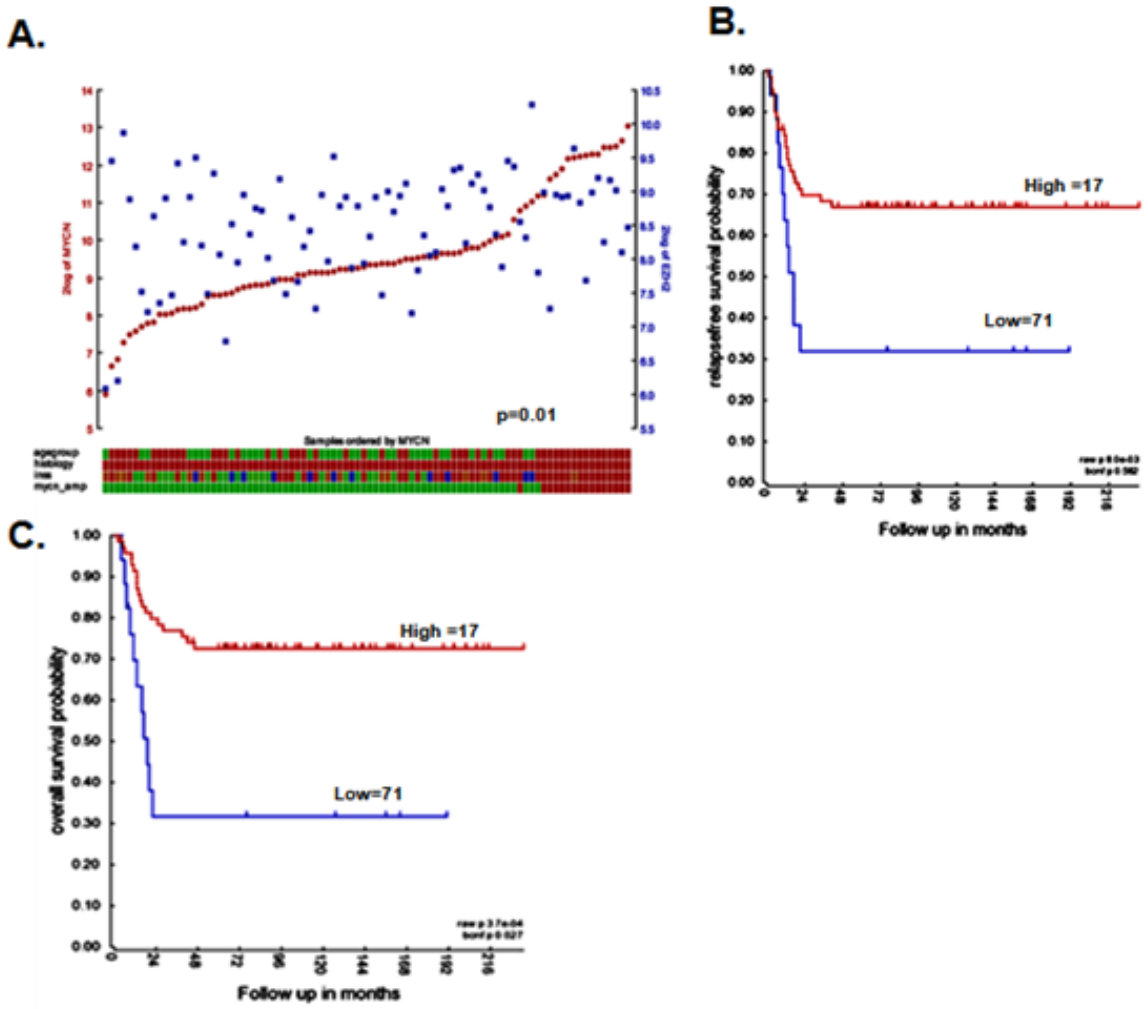

Figure 1. Clinical relevance of EZH2 expression in NB patients.
EZH2 is associated with a poor prognosis in neuroblastoma. (A) Average EZH2 expression in 88 neuroblastoma tumors according to their INSS stage and correlation with MYCN expression (p=0.01). (B,C) Kaplan-Meier analysis for event free and overall survival of neuroblastoma patients, divided into high and low EZH2 expression groups for all neuroblastoma patients (n=88).

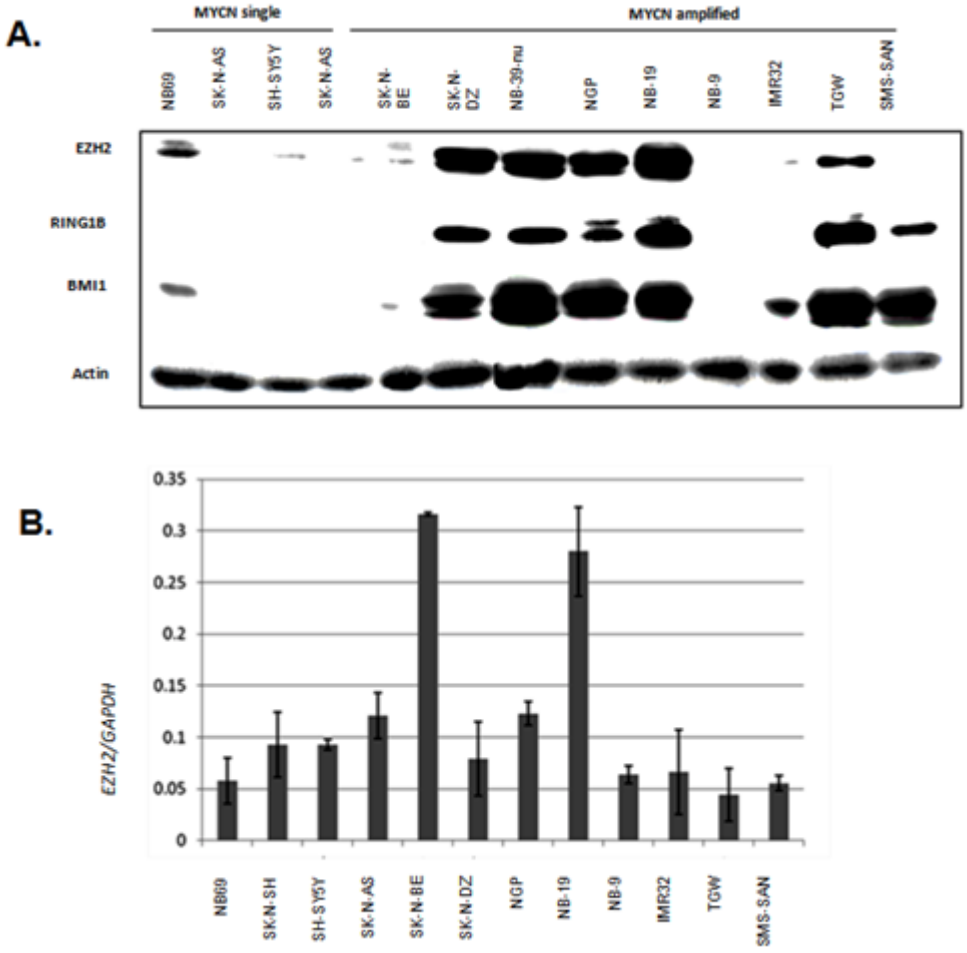

Figure 2. Endogenous EZH2 expression in NB cell lines
(A) Western blot analysis and (B) qRT-PCR of EZH2 in neuroblastoma cell lines

The study was initiated by comparing EZH2 expression among NB cell lines both at protein and transcriptional level (Figure 2). The result suggested that there is an enhancement of EZH2 expression in MYCN-amplified NB cell lines at protein level.

To identify the association of PRC1 group member with EZH2, we overexpressed Ring1b in SK-N-BE and NB69 cells and examined the molecules as indicated (Figure 3). The results suggested that PRC1 group Ring1b expression does not induce EZH2 expression, but induces BMI1, another PRC1 molecule. Next, we overexpressed EZH2 in these NB cell lines. EZH2 expression did not induce either BMI1 or RING1B proteins.

A significant increase in total colony number and number of large colonies, was also observed in soft agar colony formation assay of ectopically expressed EZH2 (Figure 4).

DISCUSSION

In the knockdown study of EZH2, EZH2 knockdown induces neurite extension and differentiation marker GAP43 in NB cells. However, it does not affect cell cycle and this is in contrast to previous results, in which silencing of EZH2 decreases NB cell proliferation and induces neurite extension.[14] By the ectopic expression of EZH2, ATRA-induced neurite extension was suppressed and *GAP43* was decreased. Together, EZH2 seems to have an important role in NB cell differentiation. It is known that methylation of H3K27 by EZH2 in neural stem cells is required to maintain a proper

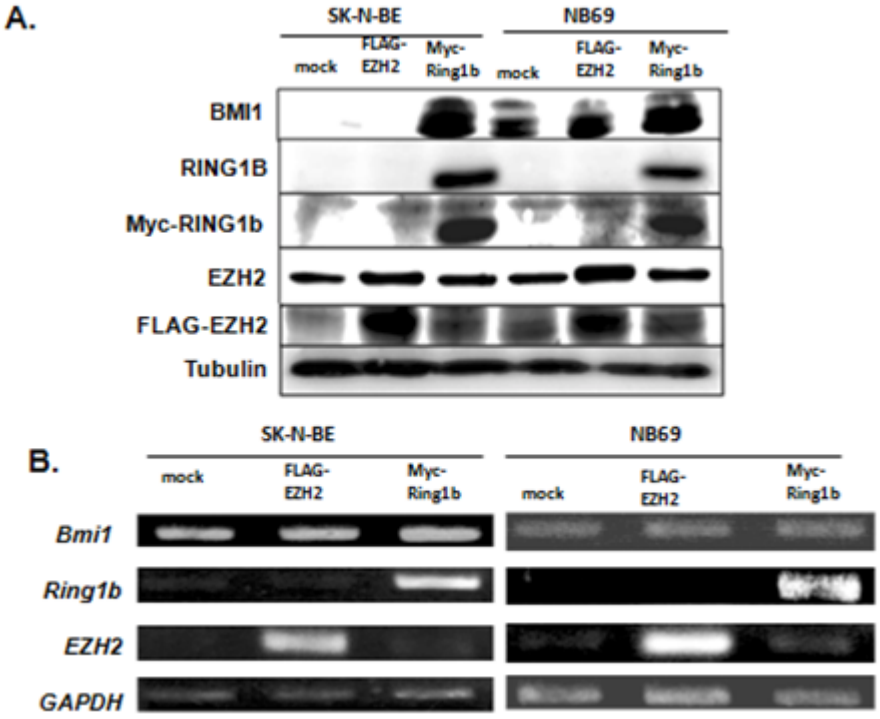

Figure 3. Effects of EZH2 on PRC1 polycomb proteins
FLAG-EZH2 and Myc-Ring1b were expressed by lentivirus as described in Methods
(A) Western blot analysis (B) Semi-quantitative RT-PCR results
Analyzed molecules are shown in the left margin of the panels

balance between self-renewal of neural stem cells and differentiation into neurons and to prevent massive neurogenesis and depletion of the neural stem cell pool that would eventually result in a decreased number of neurons.[15]

Further, we studied the effect of EZH2 on cell proliferation. Although EZH2 did not alter cell proliferation, in the soft agar colony formation assay, which is known to be the hallmark of cellular transformation and uncontrolled cell growth, we identified a significant increase both in total and large colony numbers. These results suggest that EZH2 may have role in tumorigenesis of NB. However we cannot elucidate the exact mechanism on how EZH2 regulates PRC target versus nontarget methylation in neuroblastoma. Our finding reveals that EZH2 has a strong association with tumor progression and cancer survival. The histon methyl transferase (HMT) EZH2 is needed for proper in vitro specification of mouse neural stem cells into oligodendrocytes. While EZH2 is highly expressed in proliferating neural stem cells, its expression is decreased upon differentiation into neurons and abolished during differentiation into astrocytes. However, EZH2

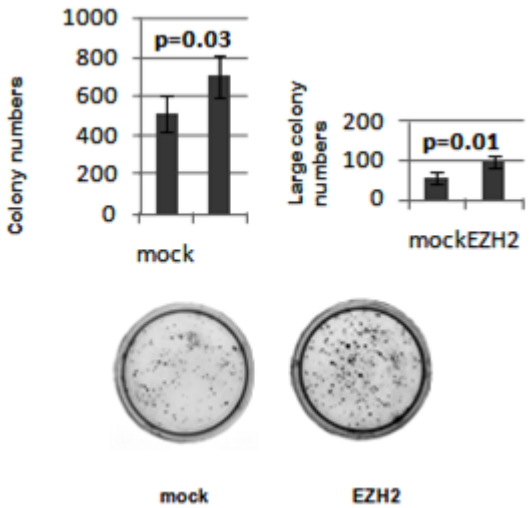

Figure 4. Soft agar colony assay, day 15

expression remains high in oligodendrocytes.[16]

A comparative ChIP-Seq analysis of EZH2 target genes in neural stem cells and in premyelinating oligodendrocytes (pOLs) reports the presence of EZH2 on oligodendrocyte, neuronal and astrocytic lineage specific genes in neural stem cells, whereas in pOLs, EZH2 is not anymore present on the oligodendrocyte lineage specific genes but is still found on astrocytic and neuronal lineage specific genes.[17]

Individual NB tumors can be classified histologically into NB (undifferentiated, Schwannian stroma poor, and high risk), ganglioneuroblastoma, and ganglioneuroma (differentiated ganglion cells). The latter two are Schwannian stroma rich and low risk.[18] Overexpression of EZH2 is found in a number of cancers, including prostate[19] and breast cancers[20] as well as Ewing sarcoma,[21] glioblastoma,[22] and melanoma.[23] The mechanisms responsible for hypermethylation of groups of genes in specific types of human cancers have not yet been identified. There have been researches conducted on the involvement of polycomb repressive complexes (PRCs) in DNA methylation, and particularly on the EZH2 gene, which is the catalytic component of the PRC2 and PRC3 complexes.[24] Previous studies indicate that EZH2 is a prognostic biomarker in breast and prostate cancer. It is known that gene silencing is associated with a well-known histone mark, the methyltransferase EZH2 of which are histone acetylation for gene activation and the trimethylation of histone-3 on lysine-27 (H3K27me3). EZH2 has been shown to control DNA methylation through its ability to produce the nucleosomal histone H3 lysine 27 trimethylation mark (H3K27me3) that provides a platform for recruiting DNA methyltransferases.[25] Polycomb repressive complex (PRC) activation is linked to cell type-specific patterns of gene repression and DNA methylation through the H3K27me3 markings that are produced in stem cells. Thus, PRC gene targets in cancer progenitor cells may be preprogrammed for DNA hypermethylation that is triggered later during cell transformation.[26,27] EZH2 overexpression is associated with aggressive clinical behavior in prostate, breast, and bladder cancers.

In this study we found a relatively high expression of EZH2 at protein level of MYCN-amplified neuroblastoma cells compared to MYCN-single copy cells. However, the MYCN copy status does not show a strong effect on the expression level of EZH2 at mRNA level. Previously, there was a report that MYCN induces BMI1 transcription in NB cells.[7] MYCN-related PRC1 increase may also have some effects on EZH2 protein stabilization.

We could conclude that EZH2 knockdown or ectopically expressed EZH2 caused not only differentiation in neurite extension and significant changes in differentiation markers of GAP43, but also a prominent increment in total colony numbers and the presence of large colonies in our soft agar colony formation assay. However, our study is at an early stage, therefore we still could not identify the mechanism of EZH2 down- regulation and the potential molecular mechanism of NB tumorigenesis.

CONCLUSION

Enhancer of zeste homolog 2 contributes to the maintenance of the differentiation stage of the NB phenotype and may be important in NB tumorigenesis. However, the exact mechanism and targeted pathway remain to be elucidated.

ACKNOWLEDGEMENT

We thank dr. Kamijo for the opportunity to conduct this study, Dr. Takenobu for the supervision, mentoring and helpful discussion, and K Sakurai for technical assistance. Thanks to a Takeda Science Foundation grant for fiscal year 2012, the authors were able to complete this study course.

REFERENCES

1. Paffhausen T, Schwab M, Westermann F. Targeted MYCN expression affects cytotoxic potential of chemotherapeutic drugs in neuroblastoma cells. Cancer Lett 2007;250:17-24.
2. Weber A, Starke S, Bergmann E, Christiansen H. The coamplification pattern of the MYCN amplicon is an invariable attribute of most MYCN-amplified human neuroblastomas. Clin Cancer Res 2006;12:7316-21.
3. Rajasekhar VK, Begemann M. Concise review: roles of polycomb group proteins in development and disease: a stem cell perspective. Stem Cells 2007;25:2498–510.
4. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;8:286–98.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-74.
6. Schwartz YB, Pirrotta V. Polycomb complexes and epigenetic states. Curr Opin Cell Biol 2008; 20:266–73
7. Ochiai H, Takenobu H, Nakagawa A, Yamaguchi Y, Kimura M, Ohira M, et al. Bmi1 is a MYCN target gene that regulates tumorigenesis through repression of KIF1Bbeta and TSLC1 in neuroblastoma. Oncogene 2010;6;29:2681-90.
8. Kamijo T. Role of stemness-related molecules in neuroblastoma. Pediatr Res 2012;71:511–5.
9. Sauvageau M, Sauvageau G. Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. Cell Stem Cell 2010;7:299-31.
10. Chang CJ, Yang JY, Xia W, Chen CT, Xie X, Chao CH, et al. EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. Cancer Cell 2011;19:86-100.
11. Piunti A, Pasini D. Epigenetic factors in cancer development: polycomb group proteins. Future Oncol 2011;7:57-75.
12. Kurata K, Yanagisawa R, Ohira M, Kitagawa M, Nakagawara A, Kamijo T. Stress via p53 pathway causes apoptosis by mitochondrial Noxa upregulation in doxorubicin-treated neuroblastoma cells. Oncogene 2008;27:741-54.
13. de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, et al. Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell 2004;7:663-76.
14. Wang C, Liu Z, Woo CW, Li Z, Wang L, Wei JS, et al. EZH2 mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3, and NGFR. Cancer Res 2012;72:315-24.
15. Pereira JD, Sansom SN, Smith J, Dobenecker MW, Tarakhovsky A, Livesey FJ. Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. Proc Natl Acad Sci USA 2010;107:15957–62.
16. Sher F, Rossler R, Brouwer N, Balasubramaniyan V, Boddeke E, Copray S. Differentiation of neural stem cells into oligodendrocytes: involvement of the polycomb group protein Ezh2. Stem Cells 2008;26:2875–83.
17. Sher F, Boddeke E, Olah M, Copray S. Dynamic changes in Ezh2 gene occupancy underlie its involvement in neural stem cell self-renewal and differentiation towards oligodendrocytes. PLoS One 2012;7:e40399.
18. Cohn SL, Pearson AD, London WB, Monclair T, Ambros PF, Brodeur GM, et al. The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. J Clin Oncol 2009;27:289-97.
19. Kidney G, S Baritaki, Marathe H, Feng J, Park S, Beach S, et al. Polycomb protein EZH2 regulates tumor invasion via the transcriptional repression of the metastasis suppressor RKIP in breast and prostate cancer. Cancer Res 2012;72: 309-104.
20. Cao Q, Yu J, Dhanasekaran SM, Kim JH, Mani RS, Tomlins SA, et al. Repression of E-cadherin by the polycomb group protein EZH2 in cancer. Oncogene 2008;27:7274-84.
21. Richter GH, Plehm S, Fasan A, Rossler S, Unland R, Bennani-Baiti IM, et al. EZH2 is a mediator of EWS/FLI1 driven tumor growth and metastasis blocking endothelial and neuro-ectodermal differentiation. Proc Natl Acad Sci USA 2009;06:5324-9.
22. Lee J, Son MJ, Woolard K, Donin NM, Li A, Cheng CH, et al. Epigenetic mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells. Cancer Cell 2008;13:69-80
23. McHugh JB, Fullen RD, Ma L, Kleer CG, Su LD. Expression of polycomb group protein EZH2 in nevi and melanoma. J Cutan Pathol 2007;34: 597-600.
24. Rajasekhar VK, Dalerba P, Passegue E, Lagasse E, Najbauer J. Stem cells, cancer, and context dependence. Stem Cells 2007;26:292-8.
25. Vire E, Brenner C, Deplus R, Blanchon L, Frage M, Didetot C, et al. The Polycomb group protein EZH2 directly controls DNA methylation. Nature 2006;439:871–4.

26. Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, Pikarski E, et al. Evidence for an instructive mechanism of de novo methylation in cancer cells. Nat Genet 2006;38: 149–53.

27. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 2007;39:232–6.