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Expression of MMP-14 and CD44 associated with proliferation of retinoblastoma cells

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Abstract

Retinoblastoma is a the most common primary intraocular malignant tumor in childhood. The eexpressions of CD44 and MMP-14 and Their role in proliferation and migration cells appear crucial for retinoblastoma invasion. Thirty-five paraffin blocks from retinoblastoma patients with tumor material were compared with eight paraffin blocks of normal retina. Retinoblastoma patients were selected from patients diagnosed with retinoblastoma at Dr. Soetomo General Hospital, Surabaya. Immunohistochemical method was used to evaluate the expression of CD44, MMP-14, and cell proliferation (Ki-67). The expression of CD44, MMP-14, and Ki-67 protein was positive in all retinoblastoma samples. In retinoblastoma samples, 54.3% (19/35) of CD44 showed high expression (score 3). The majority (40%) of MMP-14 expression was low (score 1) in retinoblastoma samples. The majority (60%) of Ki-67 (21/35) showed high expression (score 2) in retinoblastoma samples. In normal retina, 100% CD44 was moderately expressed (score 1), Ki-67 and MMP-14 were not expressed. Their expression in retinoblastoma was significant when compared with normal retina. In retinoblastoma sample, positive staining of CD44 showed high expression in the cytoplasm of the cell. This suggests that hyaluronic acid, as a ligand to CD44 in cell's cytoplasm, has low molecular weight, expresses angiogenesis properties, and promotes tumor cell proliferation. MMP-14 in this study was reported to be expressed mostly on low level group. The expression of CD44 and MMP-14 is thought to play a role in inducing cell proliferation and invasion of retinoblastoma cells.

Keywords: CD44, MMP-14, proliferation, retinoblastoma

Introduction

Retinoblastoma is a the most common primary intraocular malignant tumor in childhood. It occurs in younger children and it is both hereditary and non-hereditary. Unilateral (non-hereditary) retinoblastoma comprises 60% cases and the median age at diagnosis is two years. Bilateral (hereditary) form comprises about 40% of the cases with median age at diagnosis is one year old. Retinoblastoma is reported to occur 1 in every 15,000–20,000 live birth in the world [1-5].

Retinoblastoma is a tumor in which the initial genetic mutation is known. Knudson hypothesis was able to establish a correlation between a mutation in the first identified tumor-suppressor gene (RB1, chromosome 13q14) and the development of the tumor. Mutation in the RB1 gene is inherited via the germline and the second mutation occurs in somatic cells [6]. Tumors grow because the homeostatic control mechanisms that maintain the appropriate number of cells in normal tissues are defective, leading to an imbalance between cell proliferation and cell death as there is a disturbance in the immune system and some cancer cells have the ability to destroy host's immune system[7,8].

Cells are surrounded either by other cells or by extracellular matrix (ECM that interact tightly and specifically with each other. This interaction is mediated by cell adhesion molecules (CAMs), which enable the cells to adhere tightly and specifically with cells of the same or similar type. CAMs are transmembrane receptor proteins that can be divided into four major families: immunoglobulin superfamily, integrins, cadherins, and selectins[9-10]. Extracelullar matrix (ECM) has a crucial role during the invasion of tumor cells. The invasion and migration of tumor cells involves coordinated adhesion as well as proteolytic interaction with the ECM substrate,

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resulting in the degradation and remodeling of interstitial tissue barriers [11]. Tumor cells produce a variety of lytic enzymes and cytokines that degrade and modify the ECM. The degradation and modification of the ECM allow the invasion of the tumor through tissue barriers, blood vessel and lymph channel walls, with the possible further metastatic development [12-14].

CD44 is a ubiquitous multistructural and multifunctional cell surface adhesion molecule from the family of transmembrane glycoproteins, which is the principal cell surface receptor for hyaluronan, a component of extracellular matrix (ECM) [15,16]. It was first identified as having cell adhesion and cell homing functions, but it has already been shown to have multiplefunctions [17]. Matrix metalloproteinases (MMPs), also called matrixins, function in the extracellular environment of cells and degrade both matrix and non-matrix proteins. Membrane-type 1 matrix metalloproteinase (MT1-MMP) or called MMP-14 is a zinc-dependent type-I transmembrane metalloproteinase involved in pericellular proteolysis, migration and invasion. Numerous substrates and binding partners have been identified for MT1-MMP, and its role in collagenolysis appears crucial for tumor invasion [18,19]. Therefore, we evaluated the expression of CD44 and MMP-14 and its role in cell proliferation and migration that appeared crucial for retinoblastoma invasion.

Materials and Methods

Subjects

A total of 35 (thirty five) paraffin blocks from retinoblastoma patients with tumor material were compared with 8 (eight) paraffin blocks of normal retina from patients with eye cancer whose retina had not been invaded with cancer. Retinoblastoma patients were selected from patients diagnosed with retinoblastoma at Dr. Soetomo General Hospital, Surabaya, between 2010 – 2013. Immunohistochemical method was used to evaluate the expression of CD44, MMP-14, and proliferation cells (Ki-67).

Immunohistochemical Staining

Paraffin-embedded tissues of all sample sections were retrieved for sectioning and immunohistochemical staining. Deparaffinized sections were immersed in methanol containing 3% diluted hydrogen peroxide. For blocking non-specific binding, Dakocytomation (peroxidase blocking reagent S200/30-2) was applied to the sections and then they were incubated at room temperature, with primary Rabbit Monoclonal Antibody Anti-CD44/HCAM/PGP1, Primary Polyclonal Antibody (bs-0521R) Bioss Inc. (1:150), Anti MMP-14 Polyclonal Antibody (NB100-91872) Novus Biological (1:300), and Ki-67 (CRM325 AK,BK)-monoclonal antibody Biocare Medical (1:75). All samples were stained by using Labelled Streptavidin Biotin II (LSAB II) method, then incubated with biotin-labeled secondary antibody (Trekkie Universal Link) and peroxidase-conjugated streptavidin (Trekavidin-HRP Label) overnight. DAB was used as the chromogen and counterstained with Mayer`s hematoxylin.

The numerical value for percent stained is determined by a geometric rather than linear division. Result of CD44 staining was scored on the percentage proportion of positive and negative staining with categories: 0= negative (no stained), 1= low (<35% cells stained), 2= moderate (35-75% cells stained), and 3= high (≥75% cells stained) [20]. The MMP-14 staining wasdefined as: 0= negative (no stained), 1= low (1-20% cells stained), 2= moderate (21-40% cells stained), and 3= high (>40% cells stained) [21]. Ki-67 expressions were categorized as 0= negative (no cells stained), 1= low (≤40% cells stained), and 3= high (≥40% cells stained) on nuclear cell [22]. Positive control tissues were used to compare with positive expression of the samples, including tonsil cancer (CD44), ovarian cancer (MMP-14), and prostate cancer (Ki-67).

Statistical analysis was conducted by using Chi Square Test to assess the differences between retinoblastoma and normal retina. Spearmann correlation test was used to analyze correlation of the expression variables score. A p-value of < 0.05 was considered statistically significant.

Results

The immunohistochemical expression of CD44, MMP-14, and Ki-67 was assessed in 35 retinoblastoma samples and 8 normal retina samples. Positive CD44, MMP-14, and Ki-67 protein expression was obtained in all retinoblastoma samples (35/35). In retinoblastoma samples, 54,3% (19/35) of CD44 showed high expression (score 3). The majority (40%) of MMP-14 expression was low (score 1) in retinoblastoma samples. The majority (60%) of Ki-67 (21/35) showed high expression (score 2) in retinoblastoma samples. In normal retina, 100% CD44 was moderately expressed (score 1), Ki-67 and MMP-14 were not expressed (Table 1).

Table 1. Result of Immunohistochemical Expression of Variables

Variable	CD44		MMP-14		KI-67	
Score	Cell	%	Cell	%	Cell	%
Retinoblastoma Sample (35 cells)						
3	19	54.29	10	28.57	-	-
2	10	28.57	11	31.43	21	60.00
1	6	17.14	14	40.00	11	31.43
0	-	-	-	-	3	8.57
Normal Sample (8 cells)						
1	8	100,00	-	-	-	-
0	-	-	8	100.00	8	100.00
	p: 0.000*		p: 0.007*		p: 0.000*	

* statistically significant (p: < 0.05)

CD44 expression was shown by positive staining expressed in the cytoplasm and cell membrane. From the retinoblastoma sample, positive staining showed expression in the cytoplasm of the cell, but in normal retinal samples the expression was seen on the cell membrane. MMP-14 was expressed both in the cytoplasm and in the cell membrane. Ki-67 expressed positive staining in the cell nucleus (Figures 1 and 2).

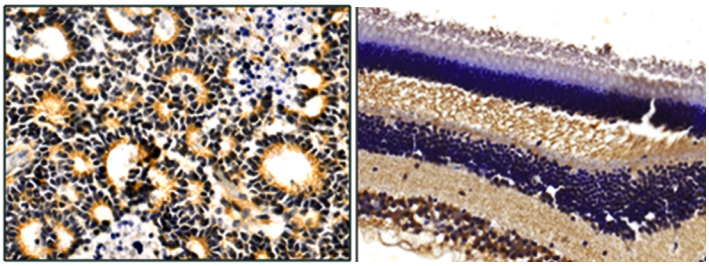

Figure 1. Immunohistochemical staining pattern of CD44 in retinoblastoma and normal retina sample. (A) Expression of CD44 at cytoplasm in retinoblastoma sample. Original magnification x400; (B) CD44 expressed at cell membrane in normal retina. Original magnification x200.

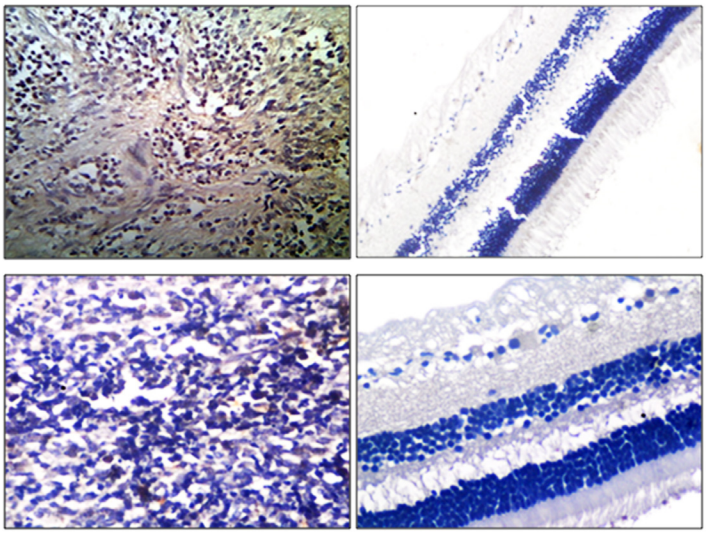

Figure 2. Immunohistochemical staining pattern of MMP-14 and Ki-67 in retinoblastoma and normal retina sample. (A) Expression of MMP-14 at cytoplasm and membrane cells in retinoblastoma sample and (B) negative in normal retina. (C) Retinoblastoma cells overexpressing Ki-67 with nuclear staining. (D) Normal retina cells do not express Ki-67. Original magnification x400

The expression of CD44, MMP-14, and proliferation (Ki-67) in retinoblastoma was significant when compared with normal retina(Chi Square Test, (CD44=p:40,140^{c};α: 0,000);(MMP-14=p:29,930^{b};α:0.007); (Ki-67=p:40,930^{d};α:0.000)) (Table 1).

A significant correlation was found between the expression of CD44, MMP-14, and Ki-67. Spearmann correlation test analysis showed significant correlation between CD44 with MMP-14 (r=0.383, p=0.011) and CD44 with Ki-67 (r=0.561, p=0.000). However, the association between CD44 with MMP-14 (B=0.220, p=0.100) in terms of cell proliferations was not significant. Significant correlation was found between the expression of MMP-14 with Ki-67 (r=0.567, p=0.000). Then, the correlation of these expression was tested with pathway analysis to identify the association and role of CD44 and MMP-14 expression in proliferative cells. The expression of CD44 was associated with the expression of Ki-67 (B=0.442, p=0.007) and MMP-14 was also associated with the expression of Ki-67(B=0.545, p=0.004).

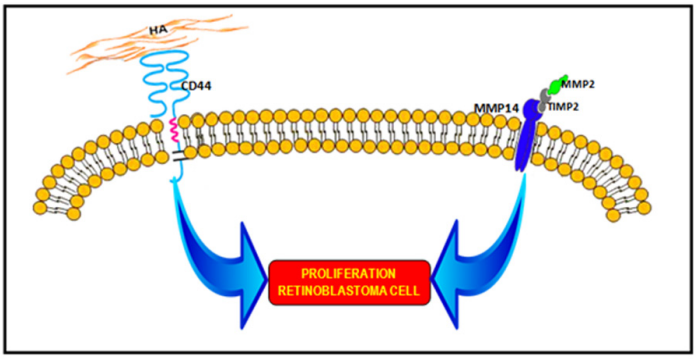

Figure 3. A schematic representation of CD44 and MMP-14 signaling in the regulation of proliferation retinoblastoma cell.

Discussion

Retinoblastoma is the most common form of ocular cancer in children. Retinoblastoma is a malignant tumor in retina originating from the neuroectodermal primitive tissue. This tumor is caused by mutation on 13q14 chromosome. Several malignant properties retinoblastoma possess include tumor cell proliferation and progressive metastatic process.

MMP-14 is important in the cancer differentiation process, tumor invasion, and metastasis process. MMP-14 is also expressed in gliablastoma and medulloblastoma brain tumor, pleural mesothelioma, and breast cancer [18,21,23-25].

Samples taken from retinoblastoma group displayed MMP-14 expression with the percentage of 40%, 31,43%, and 28,57% for group Score 1, 2, and 3, respectively. Normal retina specimen showed no MMP-14 expression. This finding is comparable to a study on mestohelioma cancer conducted by Crispi et al. (2009) which reported a low rate (score 1) MMP-14 expression as the most frequent expression group. They also described the group with a high rate of MMP-14 expression had a short survival time [21].

The expression of MMP-14 in retinoblastoma group was found significant compared to normal retina (Chi Square Test, (MMP-14=p:29,930^{b};α:0.007). MMP plays a role in the process of cancer cell invasion and metastasis. MMP-14 plays a role in the migration and metastasis of tumor cells by diffusing into the basement membrane and in cell invasion through interstitial collagen type-I tissue. MMP-14 accumulates in invadopodia ECM, which specifically degrades invasive cell membrane protrusions. MMP-14 degrades some type I, II, III collagen, laminin-1 and -5, fibronectin, vitronectin, fibrin, and aggrecan which are the constituent components of ECM. In the metastatic provess of cancer cells, these collagen components are the most important substrate. Collagen is the most ECM component found in the body and forms a tissue frame structure. Therefore, collagen is a component that needs to be degraded so that cancer cells can mirate. During metastasis, cancer cells break down the extracellular matrix to clear the path for movement, and then enter the blood vessels or the lymphatics. Those results indicate the presence of MMP-14 expression in retinoblastoma, whereas it was absent on normal retina. [18,24,26-28].

In retinoblastoma samples, 54,3% of CD44 showed high expression (score 3). As much as 28,57% expressed score 2 CD44, and the remainder expressed score 1 CD44. Normal retina expressed score 1 CD44, too. From these study results, authors concluded that retinoblastoma expressed CD44. CD44 is a hyaluronic acid principal receptor. CD44 is a transmembrane glycoprotein. CD44 is encoded by chromosome 11p13 and pre-mRNA CD44 is encoded by 20 exons. This CD44 extracellular domain is connected and binds the ligand, the hyaluronic acid [17,29,30].

In retinoblastoma sample, positive staining showed expression in the cytoplasm of the cell. Hyaluronic acid in cell's cytoplasm is observed to have particular properties such as low molecular weight, 2-25 pM polymer length, oligosaccharide fragmented shape, possessing angiogenesis capabilities, suppressing cellular apoptotic process, inducing CD44 proliferation and FasL regulation, adhesion, migration by activating several signals pathway such as kinase adhesion, mitogen activated protein (MAP) kinase and tirosin kinase cascade, and also stimulating inflammation cytokine production, suspending anchorage-independent to affect several tumor cells types growth [31-35].

Retina in normal condition expressed score 1 CD44. The expression of CD44 in retinoblastoma was significant when compared to normal retina (Chi Square Test, (CD44=p:40,140^{C};α: 0,000). CD44 expression was shown by positive staining expressed in the cell membrane normal retina. In this study, CD44 expression is associated with hyaluronic acid's role in normal retina. Expression in cell membrane as receptor, hyaluronic acid has a heavy molecular weight with its common properties including progressive tumor inhibitor, antiangiogenesis, resisting endothelial cellular growth, antiinflammatory and immnosuppresive properties, decreasing monocyte and macrophage, phagocytosis process, suppressing hyaluronic acid synthesis and keeping intercellular balance. Hyaluronic acid with its 2 x 10^{6} Da to 2 x 10^{7} Da or 2500-25.000 disaccharide molecular weight on cell membrane holds physiologic, biologic, and physicochemical characteristics [30,31-32,36-37].

On retinoblastoma cells, majority of Ki-67 expression as much as 60% was found in Score 2 category, with the remainder made up Score 1 and 0 group. However, examination on normal retina confirmed there was no Ki-67 expression. Normal half time of Ki-67 was believed to be one to one and a half hour. Cell expressed Ki-67 during G1, S, G2, and M phase but did not during its resting (G0) phase. In cancerous cell, proliferation increases as more cells enter S phase causing neoplastic transformation. The increasing number of cells entering S phase leads to Ki-67 rising up to its highest level during mitotic phase. This phenomena, however, does not occur on normal mature retina as it is a differentiated neuronal cell and stays on G0 phase. [38,39].

The expression of Ki-67 proliferation in retinoblastoma was found significant when compared with normal retina(Chi Square Test, Ki-67=p:40,930^{d};α:0.000). Ki-67 expression was reported to increase when retinoblastoma's differentiation got worse. The increase of Ki-67 expression was also found in breast cancer, colorectal carcinoma, prostate cancer, oral squamous carcinoma, and gastric cancer. The growing level of Ki-67 as one of cell proliferation markers indicates an aggresiveness of a tumor shown as an increasing mitotic cell numbers [38,40-44].

Pathway analysis demonstrated that The expression of CD44 was associated with theexpression of Ki-67 (B=0.442, p=0.007). The binding of hyaluronic acid to CD44 on plasma extracellular cell membranes triggers the signaling process of tyrosine kinase receptors (ErbB2 and EGFR), other signal receptors (TGFßR1), and non-receptor kinases (Src families) that control the pathway of oncogenes, such as MAP kinase and proliferation pathways of PI3 kinase/Akt cells. A variety of adapter proteins such as Vav2, Grb2, and Gab-1 mediate interaction between CD44 and effectors, such as RhoA, Rac1, and Ras. On the other hand, CD44 also interacts with another carbohydrate, the heparin sulfate, to regulate and activate other tyrosine kinase receptors, the c-Met receptor. The interaction of hyaluronic acid-CD44 also induces changes in cell motility and cell invasion. The actin filaments are bound to the CD44 cytoplasmic tail by the ERM family (ezrin-radixin-moesin) or ankyrin for the process of cancer cell motility. In the interaction process of hyaluronic acid-CD44 which increases motility invasion and tumor growth, CD44 binds hyaluronic acid which has a low molecular weight. In addition, the hyaluronic acid-CD44 complex is able to induce interleukin-6 to suppress the function of p27 (kip1) CDK2-inhibitor and hyperphosphorylation of Rb protein which have a role in the proliferation process in myeloma cells. CD44 has a role to induce the E-CDK2 cyclin complex in cancer through its inhibitor, p27 (kip1), in myeloid leukemia. CD44 also induces CDK2 p21 inhibitors. These factors induce the process of tumor cell proliferation, especially in retinoblastoma. [45-47].

MMP-14 was also associated with theexpression of Ki-67(B=0.545, p=0.004).MMP-14 has a role of increasing the expression of K-Ras and EGFR and inhibiting Ink4a/Arf which is a CDK4 inhibitor. The inhibited p14Arf protein activates Mdm2 to suppress the apoptotic pathway and suppressed p16Ink4a activates the D1-CDK-4 cyclin complex. MMP-14 also degrades ECM and induces cells for migration and invasion, activates proMMP2 and proMMP13, induces syndecan, activates ERK, and induces laminin-5 cleavage for the invasion of tumor cells [27,48,49].

In retinoblastoma cells, the association between CD44 and MMP-14 was found not significant 14 (B=0.220, p=0.100). Ali et al (2013) reported an increase in MMP-14 expression on prostate cancer. This increasing expression was thought to correlate with the increase of EGFR and K-Ras expression which was affected by m-RNA and resulting in Ink4a/Arf expression being suppressed. Downregulated Ink4a/Arf would activate cell proliferation pathway through CDK4. Suppressed $p14^{Arf}$ protein would trigger Mdm2 to halt p53 and cell apoptoticpathway while downregulated $p16^{Ink4a}$ would activate D1-CDK4 cyclic complex which in turn would suppress Rb protein so that E2F activates proliferation pathway. MMP-14 degrades ECM and induces cell to migrate and invade, activates proMMP2 and proMMP13, induces syndecan, activates ERK, induces laminin-5 replication during tumor cell invasion process. This study described that MMP-14 induced proliferation process without CD44, while on the contrary, Itoh (2006) reported that MMP-14 was able to cut off CD44 cleavage in cell metastasis. [27,48,49].

Conclusion

Retinoblastoma is the most common primary intraocular malignant tumor in childhood.This study reported expression of MMP-14, CD44, and Ki-67 in retinoblastoma samples group. Their

expression in retinoblastoma was found to be significant when compared to normal retina. In retinoblastoma samples, positive staining of CD44 showed high expression in cell cytoplasm. This suggests that hyaluronic acid, as a ligand to CD44 in cell's cytoplasm, has low molecular weight, expresses angiogenesis properties, and promotes tumor cell proliferation. MMP-14 in this study was reported to be expressed mostly on low level group. The expression of CD44 and MMP-14 is thought to play a role in inducing cell proliferation and invasion of retinoblastoma cells.

Competing interests
The authors declare that they have no competing interest.

Financial Disclosure
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Ethical approval
This study was ethical cleareance certified at July 9th, 2013 in RSUD Dr. Soetomo, Surabaya.

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