The Role of Estradiol-17β and Type IV Collagen on Gpr55 Gene Expression that Contributed to Increase Skov-3 Ovarian Cancer Cell Line Proliferation

Merry Meryam Martgrita, I Ketut Gunarta, and Marselina Irasonia Tan

Abstract**—**Ovarian cancer is the third cause of death from cancer among women and is the second cause of death due to gynecological malignancies, after cervical carcinoma. Ovarian cancer that overexpress c-erbB2 receptor along with the expression of estrogen receptor is an aggresive cancer. In cancer development, extracellular matrix components can play a role in increasing cancer cells proliferation, migration and invasion. Both components can affect cancer development by regulating the signal transduction pathways in cancer cells. To reveal the role of estradiol-17β and type IV collagen on ovarian cancer cells, SKOV-3 ovarian cancer cell line, that overexpress c-erbB2 receptor, was cultured on type IV collagen and treated with estradiol-17β. In this research, estradiol-17β and type IV collagen proved to affect RNA and protein expression of GPR55 (GI: 115345344) in SKOV-3 ovarian cancer cells. Signal transduction pathways induced by the activity of GPR55 presumably contribute to increase SKOV-3 ovarian cancer cells proliferation.

Keywords**—**Estradiol-17β, GPR55, SKOV-3, type IV collagen.

I. Introduction

OVARIAN epithelial cancer is the third cause of death from cancer in Indonesian women, after endometrial and cervical cancer [1]. One of the factors that could increase cancer potential is the increasing synthesis of steroid hormone in ovary. Hormone that is expressed in high level in ovary is estradiol. Estradiol has a mitogenic characteristic that can increase cell proliferation in ovarian cancer cells [2]. The development of ovarian cancer is also affected by basal membrane components. Basal membrane acts as substrate for the development of ovarian cancer cells and induces cell proliferation. Metastasis and invasion of cancer cell can be caused by the decreasing response level of cells to basal membrane [3]. Type IV collagen and laminin are the main components of basal membrane. Type IV collagen and laminin, afer binding their reseptors, can induce cytoskeleton remodelling and signal transduction pathway that have roles in cell proliferation [4].

Merry Meryam Martgrita is with the Faculty of Biotechnology, Del Institut of Technology, Toba Samosir, Indonesia (e-mail: merry.martgrita @del.ac.id, merrymartgrita@gmail.com).

I Ketut Gunarta is with Cancer Research Institute, Kanazawa University, Kanazawa, Japan (e-mail: ketut1988@stu.kanazawa-u.ac.jp).

Marselina Irasonia Tan is with the School of Life Science and Technology, Institut Teknologi Bandung, Bandung, Indonesia (e-mail: marsel@sith.itb.ac.id).

GPR55 and its ligand, LPI (L-α-lysophosphatidylinositol), have a closed partnership in cancer cell proliferation and migration [5]. GPR55 expression in mammalian tissues has already found in some region of central nervous system, adiposal tissue, testes, myometrium, tonsil, spleen, osteoclast, adrenal glands, and gastrointestinal tract [6,7,8,9,]. The expression of GPR55 in cancer cell lines has reported in IM-9 lymphoblastoid cells [7], OVCAR3 ovarian cancer cell line, PC-3 and DU-145 prostate cancer cell line [10].

In this study, we identified a cooperation of type IV collagen, as main basal membrane component, and 17β-estradiol to increase the expression of GPR55.

II. Material And Method

A. Cell Culture

The SKOV-3 cell line was cultured in medium DMEM/F-12 (Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 10 U/ml penicillin G and 10 µg/ml streptomycin (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2/95% air, and when confluent, was passaged with 0.02% EDTA and 0.25% trypsin in phosphate buffer saline (PBS). For monolayer culture, cell line was maintained on Nunclon tissue culture flask.

B. Treatment

After 70-80% confluent, cell line was prepared for treatment. Treatment was divided into four groups. In the first group, cell was grown in six-well dish (P). In the second group, cell was grown in six-well dish and treated with 10^{-6} M estradiol-17β (E). In the third group, cell was grown in six-well dish that was previously layered with collagen-IV (40 µl/well, 0.5 µg/µl collagen-IV in 0.5M acetic acid) (K). And in the forth group, cell was grown in six-well dish that was previously layered with collagen-IV and then treated with 10^{-6} M estradiol-17β (KE). Each well was plated with $5x10^5$ cells, and grown overnight in DMEM/F-12 medium containing 5% FBS. In the next morning (stated as 0 hour), medium in each well was changed with 1998 µl serum-free defined medium (SFDM; 20 µg/ml insulin, 20 µg/ml transferin, 10 ng/ml selenite, 5 µg/ml hydrocortison, 16.11 µg/ml putrescin, and DMEM/F-12), into the first and third group were added 2 µl ethanol absolute (solvent of estradiol-17β), and into the second

and forth group were added 2 µl 10^{-6}M estradiol-17β. Replacement of SFDM and 10^{-6} M estradiol-17β was obtained every 24 hours, and the treatment was lasted at 72 hour.

C. RNA Extraction and Differential Display Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was prepared from cultured cells using High Pure RNA Isolation Kit (Roche) according to the manufacturer's suggested procedure. Total RNA isolation was performed 72 h after treatment. RNA integrity was confirmed by using 1% agarose gel electrophoresis and ethidium bromide staining. The total RNA concentration was determined from spectrophotometric analysis at $A_{260/280}$. Complementary DNA was synthesized from 1 µg of total RNA by reverse transcriptase (RT) at 37°C for 1 h using Reverse Transcriptase M-MuLV (Roche). The synthesized cDNA was used as a template for PCR amplification. PCR was carried out for 40 cycles, with denaturing for 1 min at 94°C, annealing for 1 min at 34.5°C, extension for 2 min at 72°C, and final extension for 5 min at 72°C using a (PCR machine). Five 10-mer random primers have been previously selected from 20 random primers, and the primers are A15, 5'-ATCCGAACCC-3', A16, 5'-AGCCAGCGAA-3', A17, 5'- GACCGCTTGT -3', A18, 5'- AGCTGACCGT -3', A19, 5'- CAAACGTCGG -3'. PCR reactions were performed in 25 µl of PCR mixture containing 1.5X PCR buffer, 0.2 mM of each dNTP, 0.5 µM of random primer, 0.6% cDNA template, and 0.25 units of Taq polymerase (Roche). Five microliters of PCR products was denatured at 92°C for 3 min, mixed with 5 µl loading buffer (10 mM NaOH, 95% Formamide, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol), analyzed by TBE-urea (7%) gel electrophoresis for 3 h, and visualized with silver staining (Benbouza *et al*., 2006). Each cDNA band expressed differentialy among treated groups was recovered using the following procedure. The cDNA band was excised with a new razor blade and the gel slice was placed into a PCR tube. A gel slice added with 5 µl deionized water can be directly used as template. PCR reactions were performed in 20 µl of PCR mixture containing 1X PCR buffer, 0.2 mM of each dNTP, 3 µM of random primer, 0.3 units Taq polymerase/µl PCR volume. The mixture was denatured at 95°C for at least 5 min prior to PCR amplification. PCR was 40 cycles of 1 min at 94°C, 1 min at 34.5°C, 2 min at 72°C, and final extension for 5 min at 72°C. Five microliters of PCR products was denatured at 92°C for 3 min, mixed with 5 µl loading buffer, analyzed by TBE-urea (7%) gel electrophoresis for 1.5 h, and visualized with silver staining. Single band obtained from the PCR product was purified and sent to Macrogen Inc. (Korea) for direct sequencing. If the PCR product generated multiple bands, each cDNA band has to be recovered again using the above procedure, until a single band is obtained from the PCR product before it is sent for direct sequencing. After verified using Blast program in NCBI, one of the sequence resulted from the sequencing procedure matched with human GPR55 gene.

D. Amplification of cDNA using GPR55 Specific Primer

The primer was designed to amplify GPR55 mRNA based on the published sequence of human GPR55 in NCBI. The forward primer is 5'-GTGAAGAGAGACAGTGAGAGAC-3' (22-mer) and the reverse primer is 5'-CCCAGATCCTACAA GTAGAC-3' (20-mer) that generated approximately 555 base pair of amplified cDNA. LightCycler FastStart DNA Master SYBR Green I kit (Roche) was using for cDNA amplification. Realtime PCR was performed using the following condition: Step I is 1 cycle of preincubation, for 10 min at 95°C; Step II is 40 cycles of amplification: denaturation for 10 sec at 95°C, annealing for 7 sec at 61°C, extension for 22 sec at 72°C (single mode); Step III is 1 cycle of melting curve: denaturation for 0 sec at 95°C, annealing for 15 sec at 56°C, extension for 0 sec at 95°C (slope = 0.1°C/sec; continuous mode); and Step IV is 1 cycle of cooling, for 30 sec at 40°C. PCR reaction was performed in 20 µl of PCR mixture containing 2 µl 10x LightCycler FastStart DNA Master SYBR Green I, 6.4 µl H_2O PCR grade, 1.6 µl $MgCl_2$ stock solution, 4 µl each of forward and reverse GPR55 primer (2,5 µM), and 2 µl cDNA. PCR product was analyzed in 1% agarose gel electrophoresis, visualized with ethidium bromide staining, and the size and concentration of amplified cDNA was estimated by comparison to DNA molecular weight marker (Promega). The PCR product was isolated from gel (Qiagen) and sent to Macrogen Inc. (Korea) for direct sequencing to verify the sequence of GPR55. Amplification of cDNA was also performed for glyceraldehyde-6-phosphate dehydrogenase (GAPDH), as housekeeping gene. The forward primer is 5'-GGTCATCATCTCTGCCCC CTCTGC-3' (24-mer) and the reverse primer is 5'-CGACGCCTGCTTCACCACCTTCTT-3' (24-mer) that generated approximately 500 bp of cDNA. Realtime PCR was performed using the following condition: Step I is 1 cycle of preincubation, for 10 min at 95°C; Step II is 35 cycles of amplification: denaturation for 10 sec at 95°C, annealing for 7 sec at 70°C, extension for 20 sec at 72°C (single mode); Step III is 1 cycle of melting curve: denaturation for 0 sec at 95°C, annealing for 15 sec at 65°C, extension for 0 sec at 95°C (slope = 0.1°C/sec; continuous mode); and Step IV is 1 cycle of cooling, for 30 sec at 40°C. PCR reaction was performed in 20 µl of PCR mixture containing 2 µl 10x LightCycler FastStart DNA Master SYBR Green I, 6.4 µl H_2O PCR grade, 1.6 µl $MgCl_2$ stock solution, 4 µl each of forward and reverse GPR55 primer (2,5 µM), and 2 µl cDNA. PCR product was analyzed in 1% agarose gel electrophoresis, visualized with ethidium bromide staining, and the size of amplified cDNA was estimated by comparison to DNA molecular weight marker (Promega). The photo gel was analyzed by measuring the thickness of each band using Scion Image computer program, and the band thickness ratio of GPR55 was determined compared to GAPDH.

E. Western blotting

Protein isolation was performed 72 h after treatment. Cells were washed three times with ice-cold PBS and lysed using 100 µl lysis buffer (consists of 10 µl MOPS 20 mM, 20 µl β-

glucerophosphate 50 mM, 50 µl NaF 50 mM, 5 µl Na-vanadate 1 mM, 50 µl EGTA 5 mM, 6.06 µl EDTA 2 mM, 10 µl NP_4O 1%, 10 µl DTT 1 mM, 1 µl Benzamidine 1 mM, 825.44 µl sterile deionized water; the following components were added right before using, 10 µl PMSF 1 mM, 0.5 µl Leupeptin 4110 U/mg (10 µg/ml), and 2 µl Aprotinin 7 TIU/mg (10 µg/ml)). Cells from each well were collected using cell scraper and homogenized by repeated pippetting. Homogenates were centrifuged 10,000 rpm for 15 min at 4°C. Supernatan was transfered into 1.5 ml tube and stored in -80°C refrigerator. Twenty µg protein was loaded per lane and electrophoresed on 10% SDS PAGE, transferred to PVDF membrane. Blots were blocked before incubated overnight with GPR55 or β-actin primary antibody at 4°C. Then blots were treated with reagens and secondary antibody from BM Chemiluminescence Western Blotting Kit (Roche), exposed to film, scanned and quantified using Scion Image Programe.

III. Results And Discussion

Expression of GPR55 mRNA and protein in SKOV-3 cell line

The mRNA expression of GPR55 in SKOV-3 ovarian cancer cell line was investigated by differential display RT-PCR. A predicted PCR product of GPR55 was obtained at approximately 555 base pairs (bp) and confirmed by sequence analysis with 99% similarity to GPR55 mRNA in NCBI genome library (Fig. 1). All of the cDNA samples were amplified using GPR55 primers (Fig. 2A) and GAPDH primers as internal control gene (Fig. 2B). Western blotting result of GPR55 and β-actin protein as internal control are showed in Fig. 2C & 2D. And the ratio expression of GPR55 gene and protein is showed in Fig. 3.

GPR55 gene and protein were differentially expressed in all groups of cells after cultured for 72 hours, but in a similar pattern of expression between GPR55 gene and protein. It seems that GPR55 gene expression has a similar turn over rate with its protein expression.

ATF-2 (activating transcription factor-2) is suggested as transcription factor that is responsible in GPR55 gene expression in SKOV-3 ovarian cancer cells. Allthough the transcription factor of GPR55 gene has not known exactly, ATF-2 can be a strong candidate as transcription factor of GPR55 gene in this research. It is based on the binding site of ATF-2 in the promoter area of GPR55 gene [11]. ATF-2 was also found in ovarian cancer [12]. ATF-2 and GPR55 have an important role in cancer cell proliferation [13,14]. It needs further research to improve the role of ATF-2 to GPR55 gene expression in SKOV-3 cells.

Protein that is suggested to activate transcription factor ATF-2 to express GPR55 gene in all treatment groups of SKOV-3 is p38 MAPK. This suggestion based on research conducted by Ouwens *et al*. [15], stated that transcription factor ATF-2 is phosphorilated by p38 MAPK. Protein p38 MAPK in SKOV-3 cell can be activated through three possible corporated signal transduction pathways to increased GPR55 expression. The first possible pathway is activated by integrin, especially integrin-α2β1. The second possible pathway is activated through the binding of GPR55 and its ligand, LPI (L-α-lysophosphatidylinositol). The third pathway is activated by the binding of LPA (lysophosphatidic acid) and its receptor, GPR26 (LPA1R).

In the first possible pathway, integrin-α2β1 can activate p38 MAPK [16] and is expressed in SKOV-3 cancer cell [17]. Integrin-α2β1 is receptor for type IV collagen [18] that binds polystiren in a weak affinity compared to its strong affinity with type IV collagen. It is seemed that this pathway is activated in all group of treatment in this research, because GPR55 was expressed in all treated groups. With the higher binding affinity between integrin-α2β1 and type IV collagen, it is suggested that type IV collagen can increase integrin clustering efficiency to induce higher signal transduction pathway of integrin-α2β1. It seems this pathway that takes action to increase GPR55 expression in SKOV-3 cells cultured on type IV collagen (group K and KE), compared to SKOV-3 cells cultured on polystiren (P and E).

The second pathway that activates p38 MAPK and induces GPR55 expression is through LPI-GPR55 binding activity. LPI is produced in high level in ovarian cancer cells [19]. In this research, GPR55 regulation expression by LPI is suggested as the factor that caused the high expression of GPR55 in group P, K and KE, but it is inhibited by estradiol-17β in group E. LPI seemed to give positive feedback regulation to GPR55 expression, beside its other function to induce proliferation. Oka *et al*. [7] stated, that LPI-GPR55 binding activity phosphorilates p38 MAPK through RhoA-ROCK pathway.

It is also suggested that the increasing of GPR55 expression in group P, K and KE is regulated by LPA-LPA1R binding activity. Yu *et al*. [20] stated that LPA1R was expressed in SKOV-3 cells. LPA-LPA1R binding activity induces p38 MAPK to activate ATF-2 that initiates GPR55 gene transcription.

It is suggested that the role of estradiol-17β to the expression of GPR55 in SKOV-3 cells is through two signaling pathways, that cause different expression level of GPR55 in group E dan KE. The first pathway that is activated by estradiol-17β causes desensitization of LPA1R. The second pathway that is also activated by estradiol-17β is pathway that induces the expression of GPR55 gene.

In the first pathway, estradiol-17β decreased total expression of GPR55 through desensitization LPA1R. Estradiol-17β is suggested binding with membrane estrogen receptor α (mERα) that will activate PI3K. Activated PI3K will also induce PKCα that will phosphorilates LPA1R. Phosphorilated LPA1R will induce internalization of LPA1R that will degrade LPA1R. LPA1R degradation will inactivate signal transduction pathway and decrease GPR55 expression. Gonzales-Arenas *et al.* [21] states that estrogen can induce phosphorilation and internalization of LPA1R through mERα,

PI3K and PKCα. Interaction PI3K with mERα was proven by Simoncini *et al.* [22] and Kuo *et al*. [23] researches.

The second pathway was activated by estradiol-17β and type IV collagen, and increase the expression of GPR55, both RNA and protein level. Induction of GPR55 expression by estradiol-17β was suggested through p38 MAPK pathway activated by ATF-2. Activated estradiol-17β bound to membran-bound estrogen receptor (mER), induced p38 MAPK activation. This suggestion is suppoted by Seval *et al.* [24] research, stated that estrogen can induce p38 MAPK pathway through membrane receptor binding. Estrogen can also increase binding afinity of ATF-2 to DNA binding site on GPR55 gene promoter domain, and also cause heterodimerization of ATF-2 and c-Jun [25].

In conclusion, our study identifies a positive interaction between estradiol-17β and type IV collagen for GPR55 gene expression in SKOV-3 ovarian cancer cell line, through several suggested signaling pathways. Therefore, it needs to be conducted further research to improve that the suggested signaling pathway is true for the expression of GPR55 in SKOV-3 ovarian cancer cells and in ovarian cancer cells in general.

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Query  1     TGCTTTCTATCTT-AACAGACTGGGAAGTTGCTCATTACAGCTAGCTTCCTTGAAAACAA  59
             ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2032  TGCTTTCTATCTTAAACAGACTGGGAAGTTGCTCATTACAGCTAGCTTCCTTGAAAACAA  2091

Query  60    TGGCAAAGAATCAGGCCTGCCTTGGTTCCACCATAGGAGGGTGGAGGAGGTGCTGTCTTA  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2092  TGGCAAAGAATCAGGCCTGCCTTGGTTCCACCATAGGAGGGTGGAGGAGGTGCTGTCTTA  2151

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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2152  CTCACCTGCATCCTGGCTGGGCAGCTGGCACCCGTGGCACTGCCCATGGAGCCGTCCCTC  2211

Query  180   CCTGGCAGCTGGGAGGGAGATGAGGCAGCTAGGCACAAGGGTGTGGCCTTGAGAAAGAAG  239
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Query  240   GCAAGGGGGGGTCCCTGCTGGTCCCAGGAAACTGTAAACAATTGAGTCACGCCTGGATGG  299
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Sbjct  2272  GCAAGGGGGGGTCCCTGCTGGTCCCAGGAAACTGTAAACAATTGAGTCACGCCTGGATGG  2331

Query  300   CTTCTCAACGGATCTGGAGGAGGAGTGCAAGTGACAGCGGAAGCCTCCCAATGACTCAGC  359
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Sbjct  2332  CTTCTCAACGGATCTGGAGGAGGAGTGCAAGTGACAGCGGAAGCCTCCCAATGACTCAGC  2391

Query  360   TGCTCCGGAGAGTGAAATGATGAGAAGGATCCATTGTGGGGTGGTTTTGCATGACAGAGC  419
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             |||||||||||||||||||||||||||
Sbjct  2512  AACTGAGGTCTACTTGTAGGATCTGGG  2538
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Fig. 1. Approximately 555 base pairs (bp) of GPR55 cDNA from PCR product, confirmed by sequence analysis with 99% similarity to GPR55 mRNA in NCBI genome library.

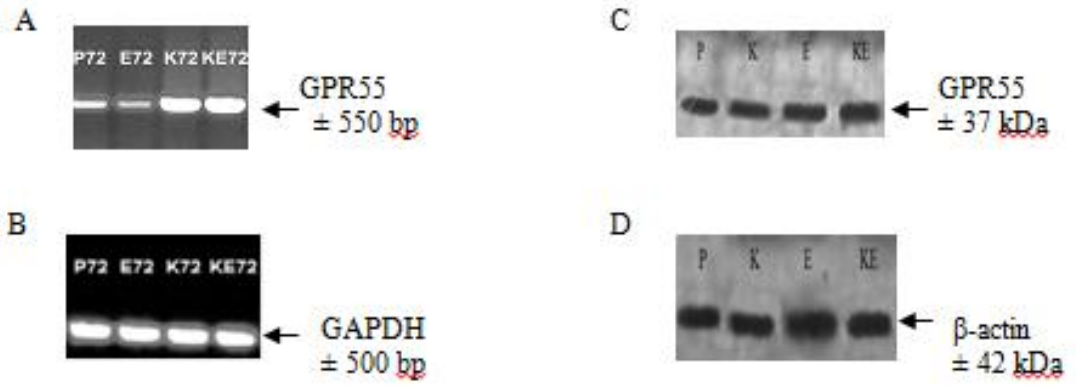

Fig. 2. Amplification result of total cDNA samples using GPR55 primers (A) and GAPDH primers (B), and Western blotting result of GPR55 protein (C) and β-actin protein (D).