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Mutation Analysis of PRD-domain of ROR2 and Ig-domain of FLNA in Breast Cancer Development: A Case Study in Malang

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ABSTRACT
ROR2 is a *WNT* receptor involved in non-canonical Wnt signaling pathways. *PRD* and *IgFLNA* domains are functional domains for binding to downstream proteins that lead to cancer development. This study aimed to analyze mutations in the *PRD-IgFLNA-ROR2* domain also the role of the *WNT5A-ROR2* pathway involved in the development of breast cancer. The research sample was gDNA of breast cancer tissue provided by Saiful Anwar Regional Public Hospital, East Java, Indonesia. Mutation analysis was conducted using Sanger sequencing. The results of the analysis showed that there were no mutations in the *PRD-ROR2* and Ig*FLNA-ROR2* domains. Based on this study, we suggest that the *WNT5A-ROR2* pathway may involve in the incidence of breast cancer in the cases studied. Further analysis is required to give a clear picture of this finding.

Keywords: *Breast Cancer, ROR2, WNT5A.*

1. INTRODUCTION

WNT5A is currently widely known to be involved not only in various processes of normal development but also in cancer development regulation, both either as a tumor suppressor or as a cancer inducer since it is also reported to be overexpressed in the advanced development of certain cancers [1]. More than a decade ago this gene was reported as others' WNT family members inhibitor, especially those of canonical WNTs [2,3]. Currently, breast cancer development takes its concerning path. In many cases, this type of cancer is even found in young age women yet has not experience in pregnancy or breastfeeding [4]. The usage of reproduction control pills is suspected to be a culprit besides an unhealthy lifestyle shared by teenagers [5].

In Indonesia, by 2020 as many as 65,858 cases of breast cancer were diagnosed and contributed to the second cause of death after lung cancer with 22,430 cases [6]. The has risk factors come from both non-genetic and genetic factors including mutation in some cancer regulators genes. Non-genetic factors include the usage of hormonal contraception [5], chest radiation therapy [7], alcohol consumption [8], obesity [9], and in adult women including either having no children or never giving breastfeeding, and a history of long menstrual cycles [4]. Genetic factors include mutations in the *BRCA1* and *BRCA2* genes [10,11], *TP53* [12], *PALB2* [13], *ATM* [14], and overexpression or repression of *WNT5A* [15]. *WNT5A* overexpression was found in stage IV breast cancer cases in Malang [16]. It was associated with lymph node metastasis and lymphatic invasion [17], and this is contrary to the finding that low *WNT5A* expression triggers lymph node metastasis and proliferation in breast cancer [18]. This gene is one of WNT gene family members as briefly described above which bind to different membrane receptors, including Frizzled (FZD) family receptors, in corporation with low-density lipoprotein receptor-related protein (LRP) 5/6 co-receptors or not, and many

other receptors, such as the tyrosine kinase-like receptors including Orphan receptors (ROR) 1 and 2, receptor-like tyrosine kinase (RTK), and protein tyrosine kinase-7 (PTK7), as well. WNT proteins play a role in intracellular and physiological processes including regulating cell proliferation, differentiation, survival, migration and polarity, embryogenesis, and tissue homeostasis by triggering different signaling pathways [19]. The inhibition or activation of the Wnt/β-catenin pathway by this gene is depending on the receptor *WNT5A* binds to [20]. The binding of *WNT5A* to the FZD4/LRP6 receptor complex activates ADP-ribosylation factor 6 (ARF6), which triggers the nuclear transfer of β-catenin to activate the transcription factor of melanoma development regulator genes [21]. The binding of *WNT5A* to *ROR2*, on the other hand, inhibits the canonical WNT-β-catenin pathway via the WNT non-canonical Ca^{2+} pathway [22].

In normal development, *ROR2* plays a role in many morphogenesis processes, including bones, cartilage plates, and fingers growth [23]. Inhibition of *ROR2* in osteoblast precursor mesenchymal cells causes dwarfism [24]. ROR1 and *ROR2* are highly expressed in embryonic development, and their expression levels decrease after birth [25]. In later development, overexpression of *ROR2* was reported in non-small cell lung cancer (NSCLC) which had been metastasized [26], triggered cell invasion [27,28], reduced cell adhesion [29], as well as metastasis in basal subtype breast cancer [28]. In contrast, knock-down of *ROR2* can increase cell adhesion [29], suppress proliferation, and induce apoptosis in breast cancer [30].

ROR2 binds to Filamin A (*FLNA*) through the Proline-Rich Domain (PRD) of *ROR2* [31], increasing the migration of melanoma and breast cancer cells. *FLNA* overexpression was found in lung cancer associated with metastases [32], melanomas that can induce cell migration [33], and breast cancers that metastasize to lymph nodes and vascular invasion [34]. *FLNA* silencing can inhibit cell invasion and migration in melanoma [35] and breast cancer [35,36]. This is contrary to the finding that low *FLNA* expression can increase metastasis, and inhibition of *FLNA* can increase motility and invasion of breast cancer cells [37]. The PRD domain also binds to the proto-oncogene tyrosine-protein kinase Src (c-Src). So that, it is increasing the invasion of breast cancer cells [38] and osteosarcoma [39–41].

Based on the explanation above, it has known that the contradictory expression of *WNT5A* affects cancer metastasis which mutations in downstream proteins can cause. Several studies reported the binding of the PRD-*ROR2* domain to *FLNA* and c-Src plays a role in cancer development. The genomic conditions of breast cancer in different countries can be different, and there have been no reports of mutations in the PRD-*ROR2* domain involved in the development of breast cancer. This study aimed to analyze the possible involvement of the *WNT5A* pathway through *ROR2* in breast cancer development.

2. METHODOLOGY

2.1. Samples

The DNA samples were obtained from fresh breast cancer tissue provided by Oncology-surgery Team of Saiful Anwar Public Hospital (RSSA) in Malang, East Java. Two samples of stage IIA (sample 1 and 2) and one sample of stage IIIB (sample 6), was determined based on TNM factors. This work was a part of the *WNT5A* breast cancer project under ethics No. 400/209/K.3/302/2018 declared by The RSSA Research Ethics team.

2.2. Target Gene Amplification

The amplification of the PRD domain of *ROR2* and Ig-domain of *FLNA* was performed using a Thermo Scientific™ DreamTaq™ PCR Master Mix with PRD primers were designed based on sequences of both genes provided by the National Center of Biotechnology Information (NCBI) using blast primer (ncbi.nlm.nih.gov). Each pair of primers were: forward 5'-GTA TGC CCT CAT GAT CGA GT-3' and reverse 5'-AGC TCA GTC TCT GGG ACA GA-3', for PRD domain of ROR2 and forward 5’-TGC TGG CCC TTG TAC TTC AC-3’ and reverse 5’-CCG GGT GAA AGA GAG CAT CA-3’ for Ig-*FLNA*. PCR cycle consisted of initial denaturation cycle of 95°C for 3 minutes, 40 cycles of denaturation 95°C for one minute, annealing 52°C for one minute, extension 72°C for 1 minute, and final extension 72°C for 10 minutes. Amplified fragments integrity examinations were carried out in 1% Agarose gel at 50 volts for 60 minutes using Mupid®-Exu horizontal electrophoresis machine. The electrophoresis results were visualized using a UV transilluminator.

2.3. Sequencing and Data Analysis

PCR samples were sent to FirstBase Malaysia for sequencing process. The obtained sequences for both *ROR2*-PRD and *FLNA*-IG domains were analyzed using online BLAST analyzer for fragment confirmation and ClustalX for mutation analysis.

3. RESULTS AND DISCUSSION

3.1. Sample Pathology Profile

The samples used were sample numbers 1, 2, and 6 from RSUD dr. Saiful Anwar Malang. Based on the primary tumor (T), invasiveness towards lymph nodes

(N), and metastases (M) analyzed refer to the American Joint Committee on Cancer, samples 1 and 2 are at stage IIA, while sample #6 is at stage IIA. Stage IIIB (table 1). From this TNM status, it is known that samples 1 and 2 were still in the cancer/tumor growth stage, while sample 6 is in the metastatic stage.

Table 1. Samples pathology profile

No	T	N	M	Metastasis Location	Stadium	Position
1	2	0	0	-	IIA	Sinistral
2	2	0	0	-	IIA	Dextral
3	4c	1	0	-	IIIB	Dextral

3.2. PRD Domain of ROR2 and Ig-FLNA Mutation

The results of the ROR2 PRD domain samples 1, 2, and 6 did not show any mutations (Figure 1). It is indicated that there was no change in the protein structure of ROR2 PRD domain.

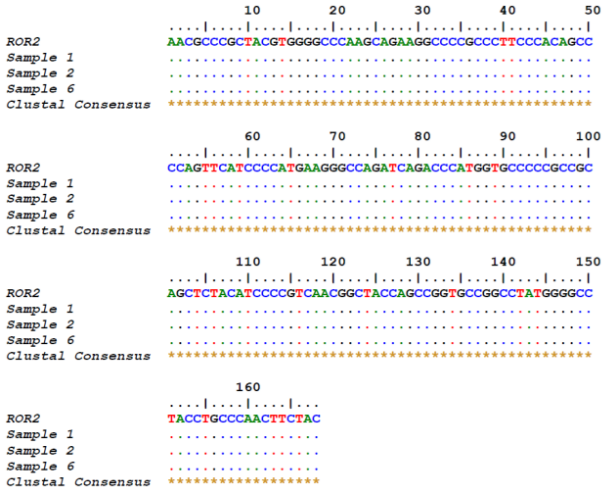

Figure 1 Multiple alignment samples 1,2 and 6 with PRD domain ROR2 from NCBI BC130522.1

The binding between PRD and FLNA mediated by Src homology 3 (SH3) [42], plays a role in the formation of filopodia, lamellipodia, and microtubule-organizing center (MTOC) through activation of c-Jun N-terminal kinase (JNK) [43] triggering breast cancer cell migration [31]. This is in accordance with the condition in sample 6 (stage IIIB) in the other case that the cancer cells have spread to the lymph nodes [44]. The PRD domain also binds to c-Src, activating phosphatidylinositol 3-kinase (PI3K)-phosphoinositide-dependent kinase-1 (PD1K)-serine/threonine-protein kinase (AKT) increasing breast cancer cell proliferation [30]. This pathway is very likely to work in stage IIA where the tumor size is enlarged to 2-5 cm as in samples #1 and #2 [44]. In the case of osteosarcoma, c-Src activates the dishevelled (Dvl2)–Ras-related C3 botulinum toxin substrate 1 (RAC1)–JNK pathway and induces MMP-13 transcription via binding of c-Jun/ATF-2 to the AP-1 binding site in the MMP-13 promoter [41] plays a role in the formation of invadopodia [39] and the development of osteosarcoma [40]. Both signaling pathways can trigger the expression of transforming growth factor-β, matrix metalloproteinase-2 (MMP-2), and MMP-9 leading to breast cancer cell invasion [45]. The normal PRD domain plays a role in the development of breast cancer. On the extracellular side, the role of ROR2 in cancer development is activated by binding to WNT ligands through the cysteine-rich domain (CRD) [46]. WNT5A is one of the ligands that activate the non-canonical pathway [47].

ROR2 was activated by WNT5A on SK-BR-3 cells, WNT6 on T-47D cells, and WNT11 in MCF-7 and BT-474 cells. This proves that in different types of breast cancer, ROR2 is activated by different ligands [48]. WNT5A-ROR2 increases the invasion of Esophageal squamous cell carcinoma [49] and breast cancer through the MAPK/p28 pathway [27,31] and triggers epithelial-mesenchymal transition (EMT) in osteosarcoma by inducing expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-13 (MMP-13) [39,50]. WNT5A-ROR2 also triggers an increase in Ca2+ to activate the calpain protease to induce FLNA cleavage in motility and migration of melanoma cells WNT5B-ROR2 enhances osteosarcoma cell migration [51].

Analysis of FLNA in repeat-20 of FLNA-Ig binding domain of all three samples showing no mutation was occurred (Figure 2). It seems that WNT5A-ROR2 plays a role in the development of cancer we studied. Since there are many pathways had known to be play roles in breast cancer development, the WNT5A-ROR2 pathway downstream c-Src gene is also required to be analyzed to confirm whether this signaling pathways was involved. ROR2 is also well known to facilitate another member of the WNT family genes in activating many cellular activities, including tumorigenesis and cancer development. As an example, the binding of ROR2 to the WNT11 ligand was also reported increasing breast cancer cells invasion through the rhodopsin (RHO) or Rho-associated Coiled-Coil Containing Protein (ROCK) pathway [48].

On the other hand, WNT5A was reported to binds the ROR2-protein-tyrosine kinase-7 (PTK7) receptor complex and activate the JNK pathway in planar cell polarity [52]. ROR2 and Frizzled7 (FZD7), via binding to the cysteine-rich domain (CRD), triggers DVL phosphorylation [53,54] and are involved in the proliferation of colorectal cancer cells [55]. In the case studied, WNT5A was expressed at stage IIIB [16], while at stage IIA, there were no reports. At stage IIIB, epithelial-mesenchymal transition (EMT) occurs. This

differentiation of epithelial cells into mesenchyme [56] involves actin cytoskeletal rearrangement, which triggers lamellipodia, filopodia, and invadopodia formations [57–59]. Cytoskeleton rearrangement is regulated by the JNK pathway [31,43] and the MAPK/p28 pathway [45], the downstream of the ROR pathway. Thus, our finding leads to the prediction that breast cancer development in the cases studied was in part regulated by the WNT5A-ROR2 pathway.

Taken together, further studies examining other genes from other WNT5A pathways that might be involved are needed to be done to understand the whole story of our sample's development until it becomes cancer tissues in different stages of development. It is expected to give a contribution to the understanding of WNT5A roles in breast cancer development due to its consistent performance in many cases.

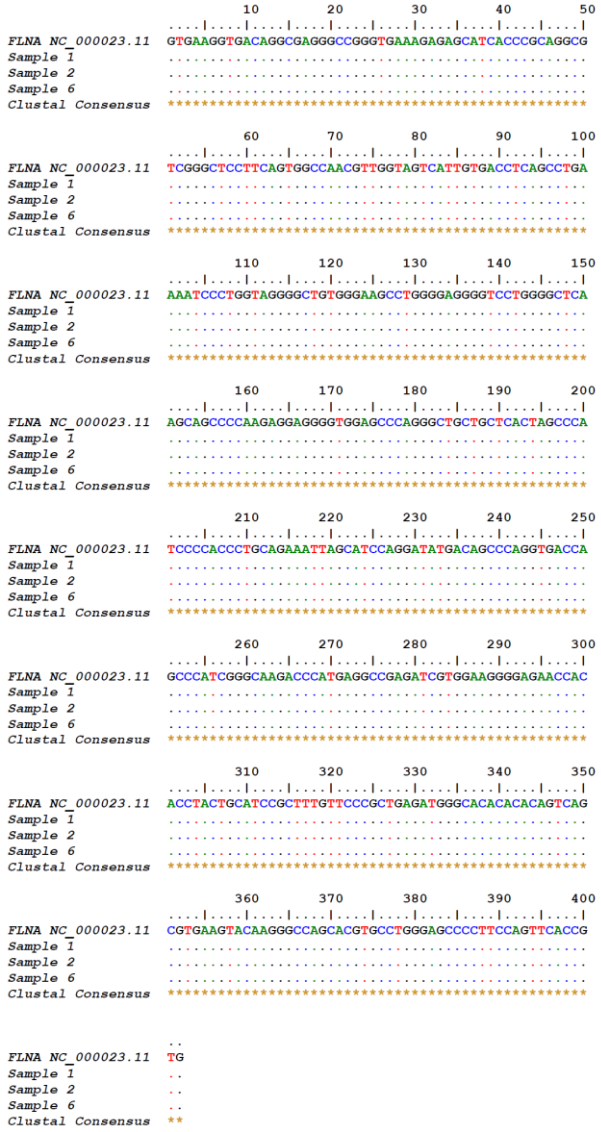

Figure 2 Multiple alignment samples 1,2 and 6 with Ig-FLNA domain ROR2 from NCBI 000023.11

Based on the results of this study, it can be concluded that there is no mutation in the PRD and Ig FLNA ROR2 domain. Further research needs to be done to determine the pathway involved in the development of breast cancer in this case.

AUTHORS' CONTRIBUTIONS

The research is part of Genetic Profiling of Breast Cancer research project. The conceptualization of this project mostly completed by D.L., D.N., D.W.P., R.L.K, and V.R.; methodology development by R.L.K and D.L.; data collection and analysis by R.L.K.; D.L. and R.L.K prepared and wrote the original draft; D.N., DWP., S.K.H.I. prepared the English version of the manuscript; supervision was conducted by D.L; All authors have read and agreed to the published version of the manuscript.

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