RESEARCH ARTICLE

Editorial Process: Submission:11/15/2023 Acceptance:08/06/2024

G2/M Checkpoint Modulation: Insights from miRNA Profiles in FAM and Breast Cancer

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Abstract

Objective: The aim of this research is to understand the role of microRNA in cell cycle regulation especially on G2M Checkpoint from Luminal A samples Indonesian population. The profile results are used as biomarkers and therapeutic targets for breast cancer. For this reason, analysis was carried out on the comparison of miRNA expression between Luminal A and Fibroadenoma mamae (FAM) using Nanostring nCounter. **Methods:** In this study, 5 (Formalin-Fixed Paraffin-Embedded) FFPE Luminal A tissues and 4 FFPE FAM samples were used. RNA was isolated from cancer tissue samples. Differential expression analysis of miRNA was conducted using Nanostring nCounter technology, subsequently followed by the expression analysis between FAM and Luminal A using nSolver softwere. Elevated expression levels of miRNAs were subjected to pathway and gene regulation analysis using KEGG and GSEA MsigDB databases. Data visualization was performed utilizing Cytoscape, NetworkAnalyst, and SRplot tools. **Result:** Based on 792 miRNAs detected on Nanostring nCounter, it was found that 60 miRNAs were upregulated and 6 miRNAs were downregulated. The 15 upregulated miRNAs analyzed show their role in the G2M Checkpoint through several pathways. The five miRNAs that significantly regulate the G2M Checkpoint are hsa-miR-196b-5p, hsa-miR-218-5p, hsa-miR-7-5p, hsa-miR-19a-5p, and hsa-miR-18a-5p Where each of these miRNAs regulates the CDKN1B gene. **Conclusion:** Significant differences in the expression of multiple miRNAs between Luminal A and FAM samples were observed. Furthermore, several of these miRNAs were found to modulate the G2M Checkpoint in Luminal A cancer by suppressing tumor suppressor genes.

Keywords: Luminal A- G2M Checkpoint- MicroRNA Profiling- Nanostring nCounter

Asian Pac J Cancer Prev, **25 (8)**, 2661-2668

Introduction

Breast cancer, a prevalent malignancy affecting women globally, is characterized by its frequent occurrence across diverse geographical regions. According to Globocan data from 2020, the worldwide incidence of breast cancer accounted for 11.7%, totaling 2,261,419 cases, with a corresponding mortality rate of 6.9%. This underscores the significant burden and impact of breast cancer on public health worldwide. In general, there are four subtypes of breast cancer: Luminal A, Luminal B, human epidermal growth factor receptor 2 (*HER2*)-enriched and basal-like, where each subtype responds to different receptors and treatments. The luminal subtype accounted for 62.7% of the total number of diagnosed cases carried out [1]. Luminal A is one of the subtypes of breast cancer that is characterized by increased expression of positive Estrogen and Progesterone receptors but no expression of *HER2*. In addition, luminal A is also characterized by decreased cell proliferation in the Ki-67 protein which has a low severity and has a good prognosis [2].

In the development of cancer, cell cycle dysregulation frequently occurs, leading to unrestricted cell division. Moreover, this dysregulation contributes to genome instability, resulting in heterogeneity that impacts chemotherapy resistance. In the cell cycle genome instability is examined at G2 checkpoint and mitosis [3]. In addition, G2 checkpoints also play a role in stopping cyclin activity and CDKs (Cyclin-dependent kinases) in cell cycle regulation [4]. Cell cycle regulation plays a crucial role in orchestrating the precise replication and division of cells, thereby mitigating aberrant cell regulation implicated in cancer progression [5]. Therefore, the regulation of the G2 checkpoint phase is one of the potential regulations in cancer proliferation.

Research related to potential biomarkers based

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on microRNAs is widely conducted. MiRNA plays a role in the transformation of cancer cells and regulates gene expression so that dysregulation does not occur. when upregulated miRNA acts as oncomiR and when downregulated as a tumor suppressor [6, 7]. The application of miRNA as a biomarker will contribute to the development of diagnostics and treatment in precision medicine and be able to improve the hope and quality of life of breast cancer patients [8] and knowing the miRNA involved in cancer malignancy and its role will help in the diagnosis, prognosis, and development of therapeutic agents [9].

MiRNA profiling enables the differential expression analysis crucial for utilizing miRNAs as biomarkers in diagnosis and prognosis. MiRNA profiling is performed to classify subtypes of breast cancer. Currently, various technologies that can be used for differential profiling of microRNAs are nanostring analysis. Nanostring analysis can create 800 miRNA profiles quickly, sensitively and detect specifically [10]. In this study, we performed a nCounter Nanostring miRNA test to profile miRNAs on Luminal Subtype A with FAM. The data were subsequently analyzed to assess the differential expression of miRNA fold change, as well as to investigate the pathways and genes regulated during cell cycle progression at the G2M Checkpoint.

Materials and Methods

Samples

The tissues used are FFPE luminal A and FFPE fibroadenoma mammary tumors. Tissue in the form of FFPE (tissue fixed in formaldehyde and embedded in a paraffin block) was obtained from the Anatomical Pathology Department of FKKMK UGM. The samples taken had inclusion criteria including: 1) Tissue resulting from surgery to remove tumors from breast cancer patients with NST Grade III characteristics. 2) Tissue resulting from surgery to remove tumors from mammary fibroadenoma patients.

RNA isolation and RNA concentration measurement for Nanostring nCounter Profiling

The RNA extraction protocol utilized the RNeasy DSP FFPE Kit (Cat. No. / ID: 73604). Following extraction, total RNA concentration was assessed using a Nanodrop Spectrophotometer, with scrutiny of the 280/260 and 260/230 ratios. Acceptable concentrations of total RNA were established at a minimum of 100 ng, with preferred values of 1.6 for the 280/260 ratio and 1.8 for the 260/230 ratio. The extracted RNA samples were subsequently forwarded to a third-party genetic science facility, Nanostring nCounter, for further analysis. Data collection was performed on an nCounter Digital Analyzer (NanoString Technologies, Seattle, WA, USA) following the manufacturer's instructions.

Data analysis

Analysis of miRNA expression profiles

MiRNA expression profiles in breast cancer and FAM tissue samples were analyzed using nSolver 4.0 software to obtain comprehensive data.

Functional Analysis

Functional analysis was carried out to determine the cellular pathways affected by micro RNA deregulation in breast cancer tissue samples compared to FAM tissue. This analysis uses DIANA miRPath version 4.0 (http://microrna.gr/miRPathv4) which is integrated with KEGG, GO, and MSigDB GSEA. then the data obtained was visualized using SRplot (https://www.bioinformatics.com.cn/en), Cytoscape (Cytoscape 3.10.1), and networkanalyst (https://www.networkanalyst.ca/).

Results

MiRNA Expression Profiling in Breast Cancer and Mammary Fibroadenoma with Nanostring

Mapping using Nanostring identified 792 miRNAs in Luminal A and FAM. This data was then analyzed using nSolver 4.0. The results of the analysis produce a foldchange value for each miRNA along with p(p-value) which indicates the significance of differences in the expression of the miRNA. Based on the fold change value, upregulated Luminal A miRNAs can be distinguished from downregulated miRNAs. A positive fold change value indicates that miRNA expression is higher in Luminal A compared to FAM, so it can be called upregulated miRNA. On the other hand, the fold change value is negative (indicating that miRNA expression is lower in Luminal A, so it can be called downregulated miRNA. 60 of them are upregulated and 6 are downregulated. There is a fold change with a range between 2.97 to 0.27 for upregulated miRNA Downregulated miRNAs have a fold change range between -0.28 to -0.37 (Tabel 1). In this study, further analysis was carried out on the 15 top upregulated miRNAs, where each miRNA had a different Foldchange value (Figure 1).

Hamam et al. [11] research on breast cancer patients in Riyadh, showed a profile of 18 upregulated miRNAs that was different from the results of this study. Examples of these miRNAs include hsa-miR-4270, hsa-miR-1225-5p, hsa-miR-188-5p, hsa-miR-1202, and hsa-miR-4281. These five are the miRNAs with the highest fold change in the study. This shows the heterogeneity of genes in each sample. The high level of miRNA expression in a cancer sample indicates its important role in the cancer growth process, which means it plays a role as an oncomir. Increased expression of oncogenic miRNAs in cancer cells inhibits tumor suppressor genes [12].

Heatmap analysis was performed on the top 15 upregulated and 6 downregulated miRNAs using the SRplot heatmap cluster. Heatmap visualization shows that the miRNA expression in the FAM group tends to be more stable than the miRNA expression in the Luminal A group. The miRNA expression between Luminal A patients can be different as shown in Luminal A-1 and Luminal A-2, whose expression is different from other Luminal A (Figure 1).

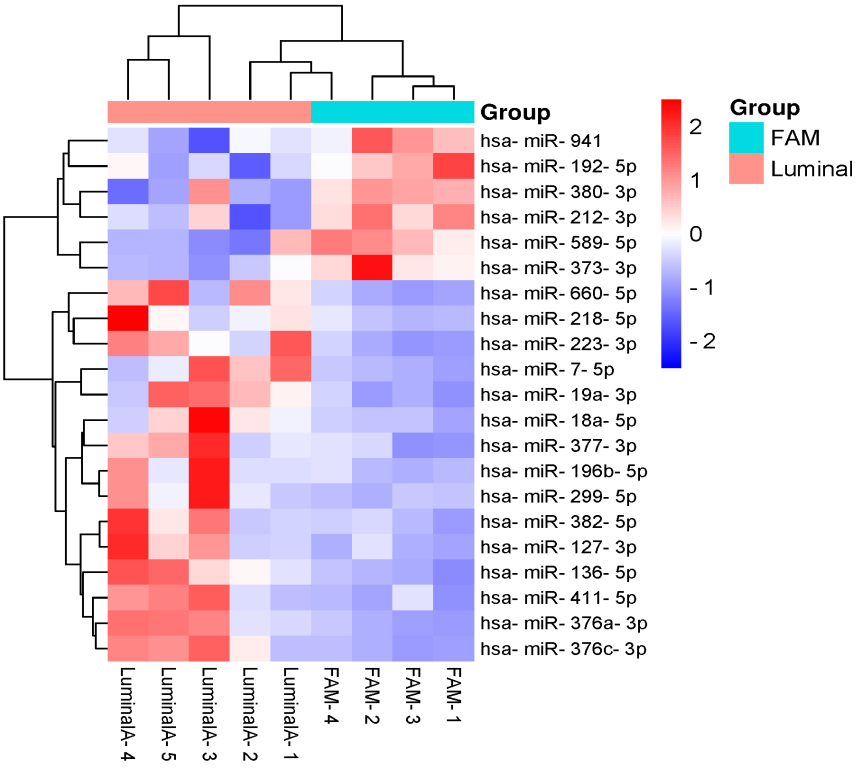

Figure 1. Cluster Heatmap between Luminal A and FAM Groups. Red indicates upregulation and blue indicates downregulation. red indicates high expression, blue indicates low expression.

Molecular Pathways Based on KEGG Analysis of Top 15 Upregulated miRNAs

The top 15 upregulated miRNAs were analyzed using DIANA miRPath v4.0 software by referring to Tarbase 8.0 as the miRNA and mRNA interaction dataset and KEGG as the molecular pathway data provider. The results of this analysis are represented in the form of a heatmap, which groups pathways based on their level of

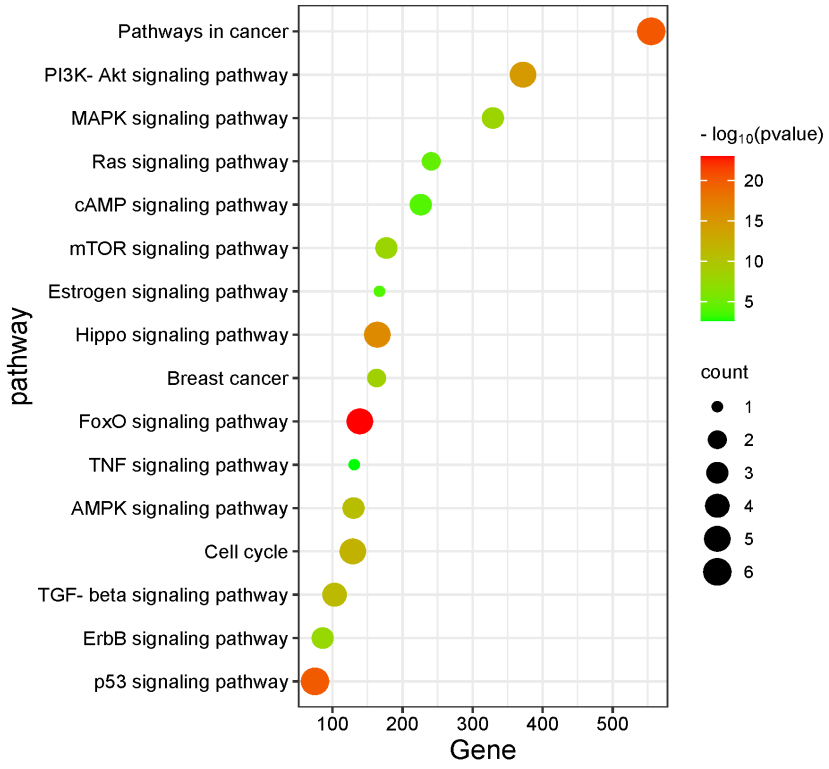

Figure 2. Pathways that Influence the G2M Checkpoint. bubble plot shows the number of miRNAs. Red indicates a high –log10(pvalue) value, green indicates the opposite.

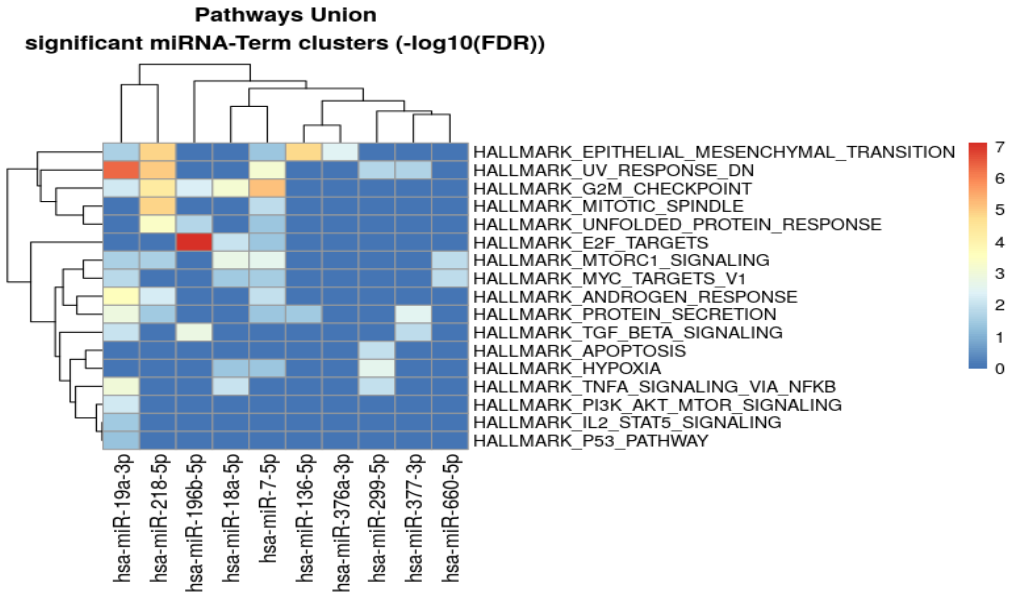

Figure 3. Top 15 miRNA Regulated Hallmark Gene Sets based on the MSigDB Dataset

significance. Then the heatmap data was analyzed again and obtained 5 miRNAs that can regulate many pathways, namely hsa-miR-218-5p, hsa-miR-7-5p, hsa-miR-19a-3p, hsa-miR-196b-5p, hsa-miR -18a-5p. (Figure 2). To determine the relationship between miRNAs and the G2M Checkpoint process in luminal A, several pathways were selected that are related to this process (Figure 2, Table 1).

Hallmarks Analysis

Heatmap based on pathway union hallmark (MsigDB)

Apart from using KEGG, the top 15 upregulated miRNAs were also analyzed using MSigDB, where this analysis looked for links between miRNAs and various hallmarks that regulate cells. The analysis results show that 10 miRNAs influence several hallmarks (Figure 3). Hallmark G2M Checkpoint is one of the Hallmarks that is regulated by 5 miRNAs at once, namely hsa-miR-218-5p, hsa-miR-7-5p, hsa-miR-19a-3p, hsa-miR-196b-5p, hsa-miR- 18a-5p.

MiRNA target genes based on Hallmarks

Apart from heatmap analysis, MSigDB gene union analysis was also carried out on the top 15 upregulated miRNAs, the same as heatmap analysis. There were 17 hallmarks with high significance values. Hallmark G2M Checkpoint shows the highest number of gene targets, 118 genes (Figure 3)

Interaction between miRNA and gene targets related to G2M Checkpoint

To see more clearly the effect that miRNAs have on the G2M Checkpoint process, several miRNAs that regulate genes in the G2M Checkpoint process were taken. There are 5 miRNAs that have high regulatory values based on KEGG and MSigDB analysis, namely hsa-miR-196b-5p, hsa-miR-218-5p, hsa-miR-7-5p, hsa-miR-19a-5p, hsa-miR- 18a-5p, where each miRNA regulates a different number of target genes To see overlapping genes, Venn diagram analysis was carried out. The results obtained show that many genes overlap with each other (Figure 4).

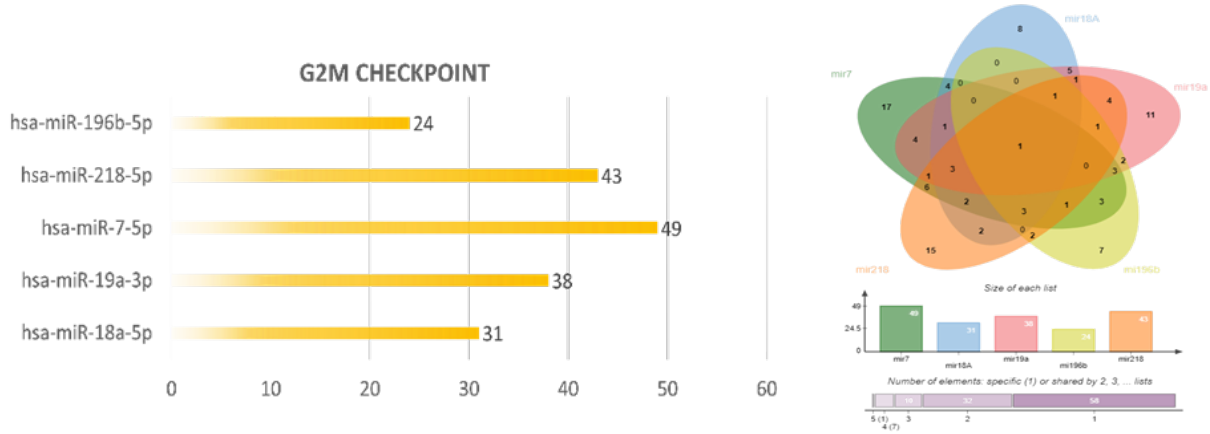

Figure 4. 5 miRNAs that have High Regulatory Values based on KEGG and MSigDB and Venn Diagram of Genes Regulated by miRNA in the G2M Checkpoint Process. green (hsa-miR-7-5p), blue (hsa-miR18a-5p), pink (hsa-miR-19a-5p), yellow (hsa-miR-196b-5p) and orange (hsa-miR-218-5p)

Table 1. Upregulasi miRNA dan Downregulasi miRNA

No	Mikro RNA	Fold Change	p-value	No	Mikro RNA	Fold Change	P-value
1	hsa-miR-136-5p	3.97	0.01	34	hsa-miR-3151-5p	1.43	0.02
2	hsa-miR-223-3p	3.46	0	35	hsa-miR-548l	1.43	0.02
3	hsa-miR-218-5p	3.37	0.03	36	hsa-miR-802	1.43	0.02
4	hsa-miR-127-3p	3.14	0.02	37	hsa-miR-890	1.40	0.03
5	hsa-miR-7-5p	3.01	0.02	38	hsa-miR-1255a	1.39	0.03
6	hsa-miR-376a-3p	2.95	0.01	39	hsa-miR-3614-3p	1.39	0.03
7	hsa-miR-376c-3p	2.89	0.01	40	hsa-miR-520e	1.39	0.03
8	hsa-miR-660-5p	2.72	0.01	41	hsa-miR-552-3p	1.39	0.03
9	hsa-miR-19a-3p	2.52	0.01	42	hsa-miR-1295a	1.39	0.04
10	hsa-miR-299-5p	2.42	0.03	43	hsa-miR-514a-5p	1.38	0.03
11	hsa-miR-377-3p	2.42	0.04	44	hsa-miR-548k	1.37	0.03
12	hsa-miR-382-5p	2.32	0.05	45	hsa-miR-1249-3p	1.37	0.05
13	hsa-miR-196b-5p	2.31	0.04	46	hsa-miR-1306-5p	1.36	0.04
14	hsa-miR-18a-5p	2.28	0.03	47	hsa-miR-3127-5p	1.36	0.04
15	hsa-miR-411-5p	2.26	0.03	48	hsa-miR-506-5p	1.36	0.04
16	hsa-miR-154-5p	2.26	0.03	49	hsa-miR-515-5p	1.36	0.04
17	hsa-miR-146b-5p	2.23	0.04	50	hsa-miR-567	1.36	0.04
18	hsa-miR-374b-5p	2.2	0.04	51	hsa-miR-219b-3p	1.33	0.04
19	hsa-miR-379-5p	2.08	0	52	hsa-miR-302a-3p	1.33	0.04
20	hsa-miR-409-3p	2.05	0.01	53	hsa-miR-3168	1.33	0.04
21	hsa-miR-574-5p	1.68	0.01	54	hsa-miR-3918	1.33	0.04
22	hsa-miR-421	1.62	0.05	55	hsa-miR-483-5p	1.33	0.04
23	hsa-miR-487b-3p	1.60	0.05	56	hsa-miR-5010-3p	1.33	0.04
24	hsa-miR-513b-5p	1.59	0.01	57	hsa-miR-892b	1.33	0.04
25	hsa-miR-1260b	1.57	0.01	58	hsa-miR-378d	1.32	0.05
26	hsa-miR-378f	1.55	0.02	59	hsa-miR-329-3p	1.29	0.03
27	hsa-miR-593-3p	1.55	0.05	60	hsa-miR-937-3p	1.27	0.04
28	hsa-miR-532-5p	1.5	0.02	61	hsa-miR-373-3p	-1.28	0.04
29	hsa-miR-486-3p	1.47	0.02	62	hsa-miR-589-5p	-1.29	0.01
30	hsa-miR-638	1.45	0.02	63	hsa-miR-380-3p	-1.29	0.03
31	hsa-miR-760	1.45	0.04	64	hsa-miR-941	-1.31	0.02
32	hsa-miR-1185-5p	1.45	0.04	65	hsa-miR-212-3p	-1.34	0.02
33	hsa-miR-410-3p	1.43	0.02	66	hsa-miR-192-5p	-1.37	0.02

to see the interaction between each miRNA and the target genes that regulate the G2M Checkpoint, gene network analysis was carried out using the networkanalyst program. There are a large number of genes that are regulated by more than one miRNA (Figure 4).

Discussion

Profiling miRNA

Based on the results of miRNA profiling in luminal A, it is known that 15 miRNAs are upregulated so they can be considered as oncomirs. However, from other research it is known that some miRNAs act as tumor suppressor genes. Hsa-miR-136-5p is higher in normal breast tissue than in breast carcinoma. Hsa-miR-136-5p plays a role in the regulation of cell adhesion molecules, focal adhesions, the complement cascade and blood coagulation. Low expression levels of miR-127-3p were associated with lymph node metastasis, clinical stage, and shorter OS [13]. Hsa-miR-223-3p suppresses tumors by inhibiting NLRP3 [14]. It is known that has-miR-299-5p acts as a tumor suppressor by directly targeting serine/threonine kinase 39 (STK39) thereby suppressing epithelial-mesenchymal transition [15]. Hsa-miR-411-5p inhibits the proliferation and metastasis of breast cancer cells through targeting GRB2 [16].

Other miRNAs are in accordance with this study where high expression levels increase the growth rate of luminal A breast cancer. Research by Cui et al. [17] on several cancer cells shows that the level of has-miR-7-5p is increased in luminal cancer but decreased in other cell types. other breast cancers. upregulation of hsa-miR-218-5p increases metastasis to osteoblasts in TNBC cancer, but overexpression of has-miR-218-5p also occurs in luminal breast cancer cells by targeting the SLIT3 gene [18]. Has-miR-660-5p had increased expression in cultured

MCF7 breast cancer cells and showed downregulation of miR-660-5p significantly suppressed cell proliferation, migration and invasion by targeting CDKN1A. In addition, miR-660-5p can bind to TET2 and activate PI3K/AKT/mTOR signaling [19]. Then miR-19a-3p inhibits the expression of PMEPA1 and PTEN [20]. Has-miR-377-5p overexpression will increase recurrence of estrogen-positive breast cancer [21]. miR-382-5p targets RERG to attenuate its inhibitory effect on the oncogenic Ras/ERK pathway thereby promoting breast cancer cell viability, clonogenicity, survival, migration, invasion and tumorigenesis/metastasis [22]. Overexpression of miR-18a-5p leads to increased proliferation ability accompanied by decreased expression of ESR1 and downstream targets such as GREB1 and TFF1 [23].

Based on the fold change, a heatmap of miRNA expression is obtained, red indicates upregulation and blue indicates downregulation. there are variations in luminal A compared to FAM, this is related to the epigenetic effects received by the patient such as the type of therapy received, living habits, ethnicity and the effects of gene mutations. These epigenetic differences are also related to the heterogeneity of the role of different miRNAs in each patient [24].

G2M checkpoint analysis based on Pathway KEGG and Hallmark MsigDB

Analysis of the role of miRNAs in the KEGG pathway revealed that 10 miRNAs regulate the G2M Checkpoint. hsa-miR-18a-5p plays a role in the FoxO signaling pathway through inhibiting E2F1. FoxO plays a role in checking replication stress that occurs in the S/G2 phase, where FoxO collaborates with the ATR/CHK1 and p53/p21 proteins. Changes in FoxO regulation will increase cellular transformation towards mitosis or back to G0/G1. This is related to the inhibition of the E2F1 transcription factor which regulates APC/CCDH1 EMI1/FBXO5. Inhibition of E2F1 can trigger the senescence process in cells [25]. Then, Has-miR-7-5p apparently regulates the BIRC5 gene which plays a role in the TGF-beta signaling pathway. Survivin or BIRC5 protein acts as a TGF-beta regulatory factor where a decrease in BIRC5 will increase G2M arrest, whereas if BIRC5 increases it will trigger cells to experience EMT [26].

Activation of the p53 transcription factor directly induces the expression of the p21 protein known as a cyclin-dependent protein kinase (CDK) inhibitor whose activity can induce cell cycle arrest. Then induction of p21 by p53 suppresses Mad2 expression thereby affecting the mitotic checkpoint complex [27]. It is known that miR-19a-3p plays a role in inhibiting p53 by suppressing its regulatory factor, namely PTEN [28]. Apart from the p53 transcription factor, PTEN is also known to regulate several pathways such as the AMPK signaling pathway, MAPK signaling pathway, mTOR signaling pathway and PI3K-Akt signaling pathway. Apart from inhibiting the G2M checkpoint, inhibition of PI3K-Akt signaling is also known to inhibit the G1 to S phase, resulting in accumulation of the G1 phase [29].

The cell cycle process, especially in the M phase, is also influenced by the Hippo signaling pathway, which is a series of MST1/2(Hpo)/SAV1)/MOB1A/B(Mats)/LATS1or2/(Wts)/YAP/TAZ protein phosphorylation. YAP that has been phosphorylated will become a cell transcription regulator [30]. In addition, active YAP will increase B-MYC regulation at the promoter location to increase G2/M [31]. In other studies apart from breast cancer, it is known that hsa-miR-18a-5p plays a role in inhibiting HIF1a which regulates Hippo-YAP [32]. Apart from that, hsa-miR-18a-5p also targets the MST1 protein which is upstream of the Hippo signaling pathway in cervical cancer so that cell proliferation decreases [33].

Checkpoint analysis of miRNA and G2M gene interactions

Based on Figure 4, five miRNAs regulate G2M checkpoint, namely hsa-miR-7-5p, hsa-miR-218-5p, hsa-miR-19a-5p, hsa-miR-18a-5p, and hsa-miR-196b-5p. MiR-7-5p, known as Ancient miRNA, is a tumor suppressor in suppressing proliferation and survival, inhibiting migration, and accelerating apoptosis. Its dysregulation is also found in various types of cancer. miR-7-5p has been shown to suppress epidermal growth factor receptor expression, inhibit the Akt pathway, suppress PI3K and MAPK pathways, and engage directly with p53 such as Cdkn1A (p21) in tumor suppression [34-36].

hsa-miR-218-5p is associated with breast cancer metastasis. The Research by Taipaleenmäki et al. [37] showed the abundance of miR-218-5p in breast cell metastases so that it is aggressive. In addition, it is also related to the signaling of the WNT pathway that promotes increased proliferation by activating the canonical Wnt-responsive gene and CyclinD1. In addition, Qian et al. [38] states that increased regulation of miR-218-5p promotes cell proliferation and migration and inhibits cell apoptosis and cell cycle. so it can be used as a therapeutic target for the treatment of breast cancer.

hsa-miR-196b-5p is an oncogene that can increase the growth and metastasis of breast cancer [39]. In colorectal cancer, miR-196-5p promotes cell proliferation, migration, invasion and inhibits apoptosis. MiR-196b-5p as an oncogene has been found in various cancers so it can be used as a good diagnosis, prognosis, and therapeutic target. hsa-miR-18a-5p has a dual role in the development of various types of cancer and can act as Oncogenes and Tumor suppressors. As an oncogene miR-18a can increase malignancy in cervical cancer, lung cancer, prostate cancer, mesothelioma, and gastric cancer. As a tumor suppressor miR-18a can inhibit proliferation in breast cancer, pancreas, and colorectal [40].

Based on the Venn diagram, several genes overlap with several miRs found in the G2M Pathway. We found one gene that interacts with all five miR that is significant in the G2M pathway, namely CDKN1B Gene. CDKN1B acts as a tumor suppressor that encodes the CDK p27 protein [41] p27 also regulates cell proliferation, motility, and apoptosis. CDKN1B is a hereditary tumor and rarely mutates somatically and its expression level is regulated in post-transcription [42]. CDKN1B also encodes p27 for the regulation and binding of cyclin-dependent kinases (Cdks) activity [43]. CDK is one of the important components in the cell cycle regulation system and kinase deregulation.

This CDK is involved in the proliferation and metastasis of breast cancer so inhibitors with CDK as a target of cancer inhibition can be done because CDK will modulate the cell cycle [44].

CDK-1 is one of the CDK groups involved in cancer development leading to increased proliferation and metastasis of cancer cells. CDK1 has increased expression of both transcriptional and translational factors, leading to a poor prognosis, and inhibition of CDK1 is necessary. Inhibition of CDK1 will reduce the phosphorylation rate of CDK1 so that it will inhibit cell proliferation, invasion and stop the cell cycle in the G2M phase which will make cancer cells will not develop [45]. Decreased CDK1 expression has been shown to decrease proliferation and stop the cell cycle in the G2M phase of breast cancer through increasing P21 expression. So that the utilization of miRNA: hsa-miR-7-5p, hsa-miR-218-5p, hsa-miR-19a-5p, hsa-miR-18a-5p, and hsa-miR-196b-5p with CDKN1B gene targeting as a biomarker for diagnosis, therapeutic target and prognosis of Luminal A can be done.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

This research was made possible through the generous support of a grant from the Ministry of Education, Culture, Research, and Technology under the fundamental research program. We also express our sincere appreciation to the Ethical Commission of the Faculty of Medicine and Public Health, Sardjito General Hospital, for their approval (Approval Number: K/FK/0725/EC/2018). Furthermore, we extend our gratitude to all individuals who participated in this study, including the students of the Biotechnology and Medicine program at Gadjah Mada University, as well as our esteemed faculty members.

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