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Transcriptome–Wide Analysis Identifies Cancer–Associated Signalling Pathways Regulated by *Justicia adhatoda*–Derived Silver Nanoparticles in Triple–Negative Breast Cancer

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ABSTRACT

The lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) makes triple-negative breast cancer (TNBC) one of the most aggressive molecular subtypes of breast cancer and one that have poor prognosis, high metastatic rate, and limited treatment options. Therefore, new treatment strategies can now be developed that involve several oncogenic pathways that, from a therapeutic perspective, are very relevant. The present study aimed to investigate the anticancer activities along with the underlying mechanisms of the silver nanoparticles extracted from Justicia adhatoda (JA-AgNPs) on transcriptome profiling and pathway enrichment analyses in human TNBC cell line MDA-MB-231. Morphological examination showed that treatment with JA-AgNPs led to rounded shrunk and decreased adherence of the cells to the plate and detachment of cells from the plate significantly more in JA-AgNPs concentration dependent than the untreated cells, indicating that the viability of the cells was strongly inhibitory after treatment with JA-AgNPs. Whole-transcriptome RNA sequencing was performed to afford knowledge of the underlying molecular mechanisms, which were then analyzed using differential gene expression analysis or Kyoto Encyclopedia of genes and genomes (KEGG) pathways analysis. Transcriptome analyses indicated that many changes in transcripts were associated to a number of cancer-associated cell signalling pathways. Only pathways related with Transcriptional Misregulation in Cancer, MicroRNAs in Cancer, Natural Killer Cell-Mediated Cytotoxicity and Thermogenesis were found to be significantly enriched at large scale. JA-AgNP treatment is linked to wide-spread transcription reprogramming of cellular proliferation, apoptosis, immune-relevant genes, metabolic regulation and tumour growth, as evidenced by changes in the expression levels of important regulatory genes such as HMGA2, HLA-A3, CSF1R, RUNX1 (AML1), CEBPA, SPI1 (PU.1), ERG, ETV1, ETV4, ETV5, DDIT3, FOXO1, MYCN, NR4A3, and TP53. Taken together, these data show that JA-AgNPs have an anti-cancer effect by targeting various pathways involved in cancer signalling pathways simultaneously in a coordinated manner instead of targeting a single pathway or specific protein. This study provides a holistic transcriptomic evidence towards the therapeutic application of silver nanoparticles developed from J. adhatoda as a multifunctional anticancer drug (MCD) against triple negative breast cancer (TNBC) and set a molecular base for future preclinical and translational studies.

Keywords: *Justicia adhatoda; silver nanoparticles; triple-negative breast cancer; MDA-MB-231; RNA sequencing; transcriptomics; KEGG pathway analysis; HMGA2; HLA-A3; anticancer activity.*

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Introduction

Worldwide, breast cancer is the most commonly occurring form of cancer and one of the top three leading causes of cancer-related death in women. Although there are huge steps being taken in the field of cancer detection and management, breast cancer still affects significant populations worldwide and is a significant health issue[1, 2]. The Molecular profile of triple - negative breast cancer (TNBC) is characterized by the absence of expression of ER (estrogen receptor) PR (progesterone receptor) and HER2 (human epidermal growth factor receptor 2) and is the most aggressive as well as clinically the most challenging breast cancer subtype[3, 4].

Without these therapeutic targets, patients with TNBC are only treated with conventional chemotherapy, which is often very toxic, leads to the development of resistance, to the re-appearance of the tumour and has a low long-term survival. Also, TNBC tends to spread to different parts of the body quite early, for instance to the lungs, liver, brain and bone, making it a very bad prognosis[5]. This makes it imperative to find more effective and safe therapeutic approaches allowing the targeting of the complex molecular mechanisms involved in TNBC progression.

The progression of cancer cells is accompanied by pervasive gene expression deregulation, which affects many aspects of cell proliferation, apoptosis, metabolism, migration, invasion and immune escape and therefore plays a role in the control of cancer cell progression. High- throughput transcriptome sequencing, often shortened to RNA sequencing (RNA-seq), has proven to be a powerful method for the genome -wide investigation of these changes in recent years. Traditional gene expression analysis measures the expression of one specific gene while RNA-seq can be used to find genes that expressed at different levels (differentially expressed genes (DEGs)), pathways, transcription regulators and biological process that is important in disease phases and in response to treatment[6-8]. Analysis of combination - transcriptomic profiling and functional pathway enrichment - generates helpful insights into the molecular pathways underpinning the biological effects of novel therapeutic agents.

Natural products have emerged as a major source of bioactive molecules for drug discovery due to their structural diversity and biological activities as well as presumably low toxicity. Medicinal plants contain different types of secondary metabolites such as - alkaloids, flavonoids, phenolic acid, terpenoids, glycosides, tannins, many of which possess antioxidant, anti inflammatory, antimicrobial and anticancer activity. Some more studies have been done in recent years on the plant based nanomaterials because of their pharmacological properties of medicinal plants and unique physico-chemical properties of the nanoparticles[9, 10]. The green synthesis method using plant material to synthesize metallic nanoparticles offers several advantages over the chemical methods for synthesis, including being environmental friendly, cost-effective, less toxic, more biocompatible etc.

Medicinal plant-mediated silver nanoparticles (AgNPs) are showing great potential as anticancer agents for multiple human malignancies. They are used due to their small size and high surface area that leads to efficient cellular uptake and thus the interaction with biomolecules and signalling pathways of the cell. There are several studies reported, which confirm that the AgNPs produced by plants has the ability to cause and produce excessive generation of Reactive oxygen species (ROS) in cancer cells, which causes mitochondrial dysfunction, oxidative stress, damage to DNA, cell cycle arrest, autophagy and apoptosis. In addition, AgNPs have been found to interact with a few oncogenic signalling pathways which include PI3K/AKT, MAPK, NF-κB, Wnt/β-catenin, JAK/STAT, TGF-β and p53 signalling. In particular, AgNPs are potential therapeutic drugs for TNBC, being able to affect several pathways in the cell[10, 11].

Justicia adhatoda L. Backbone or Vasaka (S. reciprocata), commonly known as Malabar nut is an important medicinal plant utilized in traditional medical system Ayurveda. It has been found rich in various pharmacologically active phytochemicals like the quinazoline alkaloid, vasicin and vasicinone, flavonoids, phenolic compounds, tannins and essential oils. J. adhatoda is historically used for the treatment of respiratory disorders, inflammatory disorders, microbial infections, wound healing and other chronic diseases. Pharmacological studies have provided more and more evidence of the antioxidant, anti-microbial, anti-inflammatory, immunomodulatory and anti-cancer activities of the extracts of J. adhatoda[12-14].

The bioactive compounds produced from plant might be help in synthesis of the silver nanoparticles as a natural reducing and stabilizing agent and also might be helpful to enhance the biological activity of synthesized silver nanoparticles to different level through synergetic activity.

The J. adhatoda (JA-AgNPs) has been recently demonstrated to be a potent source for the synthesis of silver nanoparticles with increased killing effect on various cancer cell lines as well as with higher biocompatibility with normal cells as compared to nanoparticles synthesized by chemical method. Advances in understanding the mechanisms include induction of oxidative stress, damage to mitochondria, possible initiation of intrinsic apoptotic pathways, inhibition of cellular proliferation, and inhibition of metastatic behaviour. However, despite the increasing evidence of the effectiveness of JA-AgNPs in cancer treatment, the exact anticancer mechanism(s) and molecular pathways in global remain poorly understood. In particular, there is a scarcity of information regarding the changes of the transcriptomes of breast cancer cells associated with JA-AgNPs[15-17].

The genome-wide transcriptomic profile could be used to discover new therapeutic targets and to visualize new signalling pathways induced by the treatment with nanoparticles. An interpretation of the transcriptomic changes can be shown systematically through functional enrichments such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, which can pinpoint biological processes and signalling networks affected by a treatment. These analyses enable the identification of genes involved in processes of tumour progression, metabolic adaptation, immune control, cell death and response to treatment, which further enhances knowledge of the ways in which nanoparticles inhibit cancer[18-21].

The present study aimed to investigate the transcriptomic response of the MDA-MB-231 triple-negative breast cancer cells to the treatment with synthesized. The cytotoxic effects of JA-AgNPs were for the first time carried out through phase-contrast microscopy where morphological change was found out. Whole-transcriptome RNA sequencing was then done to identify differentially expressed genes that relate to the exposure to nanoparticles. Functional enrichment was accomplished by KEGG pathway mapping to find any pathway(s) most affected by treatment. Transcriptomic analysis revealed modulation in various pathways related to cancer including Transcriptional Misregulation in Cancer, MicroRNAs in Cancer, Natural Killer Cell-Mediated Cytotoxicity and Thermogenesis indicating that JA-AgNPs have a multi-regulatory ability on the molecular networks responsible for cancer tumour growth, immunological pathways, cellular metabolism and progression. These in total provide an elaborate molecular picture of the anticancer efficacy of JA-AgNPs, and testify the future use of these multi-purpose therapeutics for TNBC.

2. Materials and Methods

2.1. Preparation of *Justicia adhatoda*-derived silver nanoparticles (JA-AgNPs)

The fresh leaves of Justicia adhatoda were harvested, washed with distilled water and dried in the shade at room temperature. The finely powdered dried leaves were extracted in distilled water by heating the leaves at 60-70 C for 30 min and filtered. The extract was filtered through whatman No.1 filter paper and kept in the refrigerator at 4C for further analysis.

The aqueous leaf extract was used to prepare synthesis of silver nanoparticles. The short story is that the plant extract solution was prepared by adding 1mM around the corner or indistinguishable of silver nitrate ($AgNO_3$) solution and it was stirred for one hour at room temperature using magnetic stir. The reaction mixture was left in dark for a period of time until they changed their color to brown indicating the formation of silver nanoparticles. The collected nanoparticles were centrifuged at 12,000 rpm for 20 min and washed thrice with sterile distilled water and was dried under vacuum conditions. The prepared synthetic JA-AgNPs were tested beforehand for biological evaluation[22, 23].

2.2. Cell culture

MDA-MB-231 cells, a human triple-negative breast cancer cell line, were purchased from a certified cell repository and cultured using Dulbecco's Modified Eagle Medium (DMEM), which contained 10% fetal bovine serum (FBS), 1% penicillin–streptomycin and 2 mM L-glutamine. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 and subcultured every 2-3 days[24].

2.3. Treatment with JA-AgNPs

MDA-MB-231 human breast cancer cells were seeded in culture plates, and given time to adhere overnight. Cells were treated with increasing concentrations of JA-AgNPs (25, 50, 75, 100, 150, and 200 μM) for 24 h. Untreated cells were used as negative control while camptothecin (CPT) was used as positive control for induction of cytotoxicity. Each experiment was repeated thrice[25, 26].

2.4. Morphological assessment

Morphological changes were investigated after treatment by JA-AgNP by the use of an inverted phase-contrast microscope. After treatment, representative micro images were taken of the same magnifications. Cellular morphology, such as change in cell shape, integrity of cell membrane, cell shrinking, rounding, detachment and cell confluency was assessed qualitatively.

2.5. RNA isolation

Total RNA was extracted from untreated and JA-AgNP treated MDA-MB-231 cells using TRIzol reagent as per manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop spectrophotometer and the integrity was verified by using an Agilent Bioanalyzer. Transcriptome sequencing was performed with samples with an RNA Integrity Number (RIN) > 7.0.

2.6. RNA sequencing

Libraries for RNA sequencing were prepared with a high quality RNA sample with a kit for mRNA library preparation with poly(A) enrichment, according to the manufacturer's instructions. To obtain paired-end reads, sequencing was carried out on an Illumina high throughput sequencing platform.

Reads from both samples were processed through the QC/QA pipeline for adaptor trimming and quality control. Clean reads with high quality were mapped to the reference genome of H. sapiens (GRCh38) by a splice-aware aligner. All annotated genes were quantified, with read counts being generated [27, 28].

2.7. Differential gene expression analysis

Standard RNA-seq statistical pipelines were used to identify the genes that were differently expressed in untreated samples compared to JA-AgNP treated samples. Differential genes with adjusted P value < 0.05 and absolute value of $\log_2$ fold change ≥ 1 were considered to be significantly different[29, 30].

2.8. KEGG pathway enrichment analysis was used to determine potential signaling pathways.

Significantly differentiated expressed genes were further tested with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis in order to obtain the biological pathways found to be affected by the JA-AgNP treatment. An adapted p-value<0.05 was used to identify enriched pathways. KEGG Mapper was then used to map the differentially expressed genes onto KEGG reference pathways to obtain pathway-specific gene changes.

2.9. Functional enrichment analysis

Differentially expressed genes were classified according to Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) by using Gene Ontology (GO) enrichment analysis. Protein domains functional enrichment analysis was performed to determine which biological function(s) were enriched significantly by JA-AgNP treatment by using well-established bioinformatics resources.

2.10. Statistical analysis

Data of the experiments are reported as the mean with SD (Standard Error) from a minimum of three independent experiments. Data were analysed with GraphPad Prism software. One-way analysis of variance (ANOVA) with appropriate post hoc test was performed for comparison of experimental groups. P values < 0.05 were deemed significant.

Morphological alterations induced by *Justicia adhatoda* silver nanoparticles in MDA-MB-231 cells

Observation of the morphological changes in MDA-MB-231 cells was performed with regards to varying concentrations after exposure to silver nanoparticles (AgNPs) synthesized from Justicia adhatoda.After treating with silver nanoparticles (JA-AgNPs) synthesized using the extracts of J. adhatoda, concentration-dependent morphology change in MDA-MB-231 cells was seen compared to non-treated control cells. The untreated cells appeared as elongated spindle-shaped with well attachment with high confluency. However, cells slowly lost their normal morphology and rounded up, decreased in size, lost contact with each other and became detached from culture plate, in responding to JA-AgNPs. The modifications increased in extent as a function of the concentration of nanoparticles. Untreated cells were not visible at highest concentration and there were many rounded or detached cells indicating high cell cytotoxicity. The same morphological changes were observed in cells treated with camptothecin, thus providing the evidence of cell killing. These results show that the JA-AgNPs are highly concentration dependent cytotoxic in triple negative breast cancer cells (MDA-MB-231 cells).

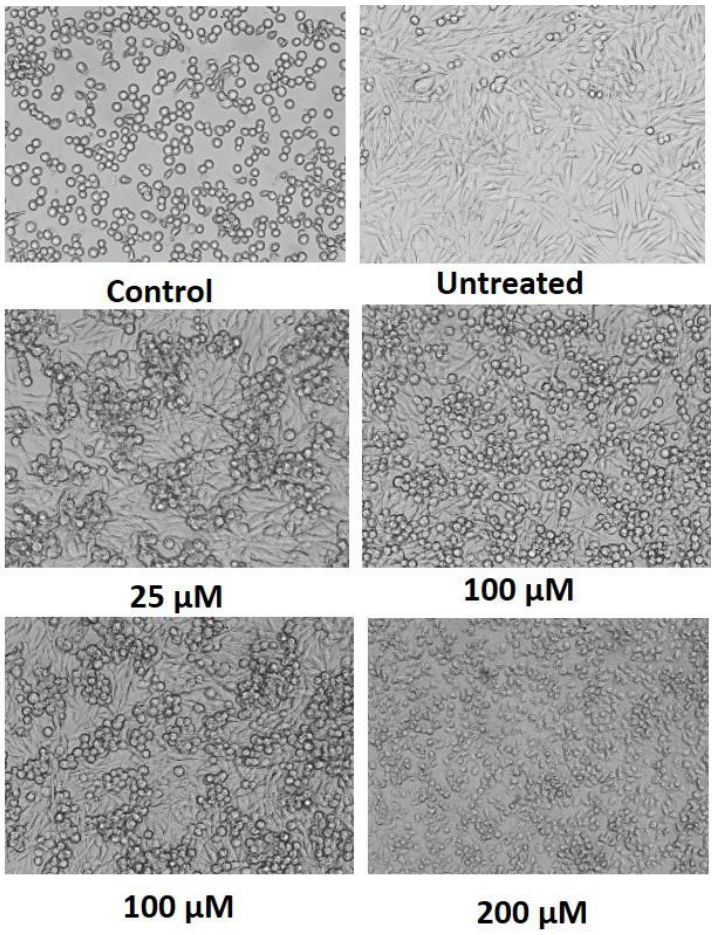

Figure 1. Bright-field microscopic images of the MDA-MB-231 cells treated with G. adhatoda (JA)-extracted silver nanoparticles at a rising dose (25-200microM) demonstrating the representative images. Normal spindle-shaped morphology and good adhesion characteristic were observed in the untreated control cells. Progressive morphological changes of cell rounding, cells shrinkage, losing their adherence, and increased cellular detachment occurred concentration-dependently in JA-AgNPs treatment. The positive control cells treated with Camptothecin showed significant changes in cell morphology that resembled that observed in cells undergoing apoptosis.

JA-AgNPs treatment modulates genes associated with the Natural Killer cell-mediated cytotoxicity pathway

KEGG pathway enrichment analysis was performed in the MDA-MB-231 cells treated with silver nanoparticles of Justicia adhathoda that showed the differential enrichment of Natural Killer (NK) cell-mediated cytotoxicity pathway (hsa04650). One of the differentially expressed genes that mapped to this pathway, HLA-A3, was statistically significant upon treating with JA-AgNPs suggesting a potential effect on molecules related to tumour-immune interactions.

The steps involved in NK cell-mediated killing of target cells involve a number of complex, sequential signaling events, such as cells keeping watch for pathogens, target cell recognition, a signaling mediated by cytokines, activation of intracellular kinases, and triggering of the pathway. Although this study did not directly examine the activity of NK cells, alterations at the genome of tumour cells which are associated with immune recognition mechanisms are known to occur along the transcriptome as well.

HLA-A3 belongs to the major histocompatibility complex class (MHC) class I pathway that functions to present peptides to the immune cells in this pathway. Regulation of its expression of amounts is an important element in the immune surveillance of tumours because the ratio between the inhibitory andactivating signals for the NK cells are modulated.

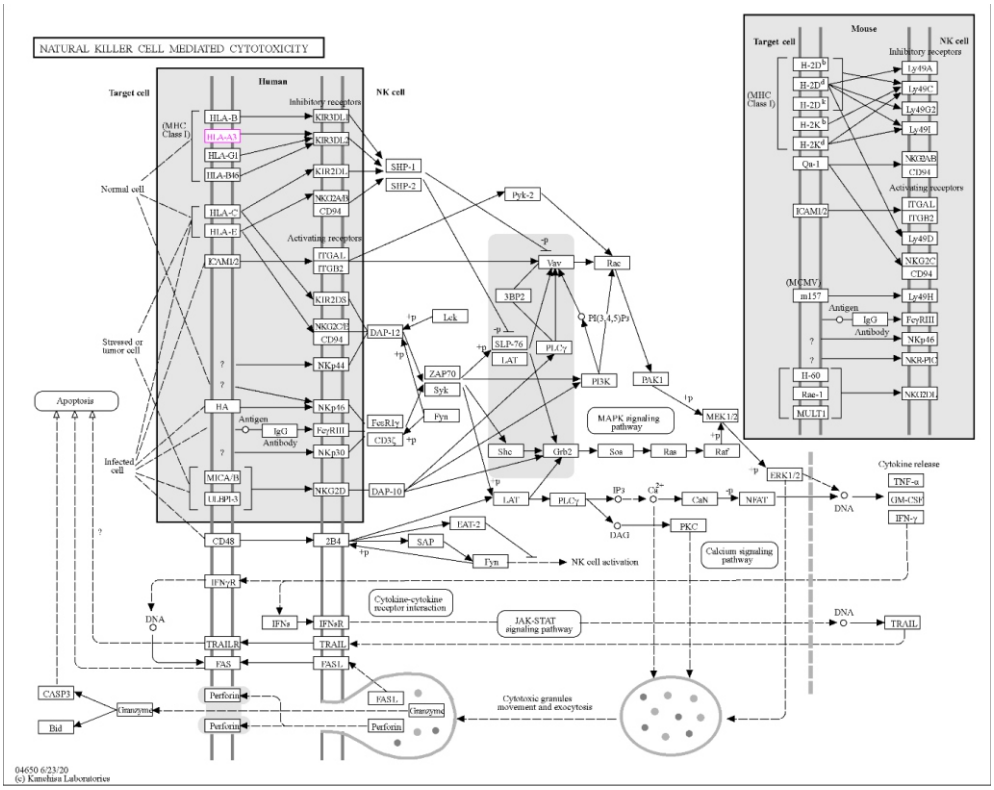

Figure 2. KEGG enrichment map of Natural killer (NK) cell-mediated cytotoxicity pathway (hsa04650) in JA-AgNP treated MDA-MB-231 cells. Differentially expressed genes are highlighted in purple boxes (based on RNA sequencing results). The pathway shows the modulation effect on various immune recognition and cytotoxic signalling including the HLA-A3 (Human Leukocyte Antigen-A3), revealing that genes involved in tumour-immune interactions and anticancer response are regulated when exposed to silver nanoparticles of Justicia adhatoda.

Transcriptional misregulation in cancer pathway analysis

The RNA sequencing performed in this study yielded the identification of a set of differentially expressed genes (DEGs) which were uploaded for KEGG pathway enrichment analysis to identify the molecular mechanisms underlying the anticancer activity of the silver nanoparticles (AgNPs) prepared using Justicia adhatoda. The Transcriptional Misregulation in Cancer (hsa05202) was one of the most significant pathways that was enriched, and the largest of the significantly affected pathways following treatment with JA-AgNP (Figure 3).

The DEGs were mapped onto KEGG pathways and revealed that the expression of many transcription factors, oncogenes, tumour suppressor genes and chromatin-associated regulatory proteins involved in cancer initiation and its progression were reportedly changed after the JA-AgNP treatment. JA-AgNP exposure caused widespread transcriptional reprogramming as reflected by changes in the expression of several cancer associated genes such as CSF1R, RUNX1 (AML1), CEBPA, SPI1 (PU.1), PAX3, ERG, ETV1, ETV4, ETV5, DDIT3, NR4A3, FOXO1, MYCN, MLL, ENL, TLX1, TLX3, LMO2 and TP53.

Many of these genes are known to regulate cell growth and the differentiation, apoptosis, cellular mobility, invasion, angiogenesis and survival of tumour cells. In particular, modulation of transcription factors with members of the ETS family (ERG, ETV1, ETV4, ETV5) indicates alterations of the transcriptional programs pertaining to tumour progression and metastatic behaviour. Likewise, modulation of stress and cell death pathways, and modulation of cell differentiation, are hinted at with the altered expression of PAX3, FOXO1 and DDIT3.

Additionally, genes on the pathway also included genes involved in tumour suppressor signalling (TP53) which is one of the most important genes controlling cell-cycle arrest, DNA damage response and apoptosis. Moreover, changes related to CSF1R, RUNX1, CEBPA and SPI1 suggest that this JA-AgNP message might interfere with pathways of transcript expression related to cell growth and cell differentiation.

In general, the results indicate that, following JA-AgNP treatment, major adjustments in the gene regulatory networks of the cancer path are produced. These changes in the transcriptomic data give rise to a sense of the observed cytotoxic and antiproliferative effects of JA-AgNPs that could be indicative of a mode of action of modulation of cancer associated transcriptional programs being a component of their therapeutic activity in MDA-MB-231 cells.

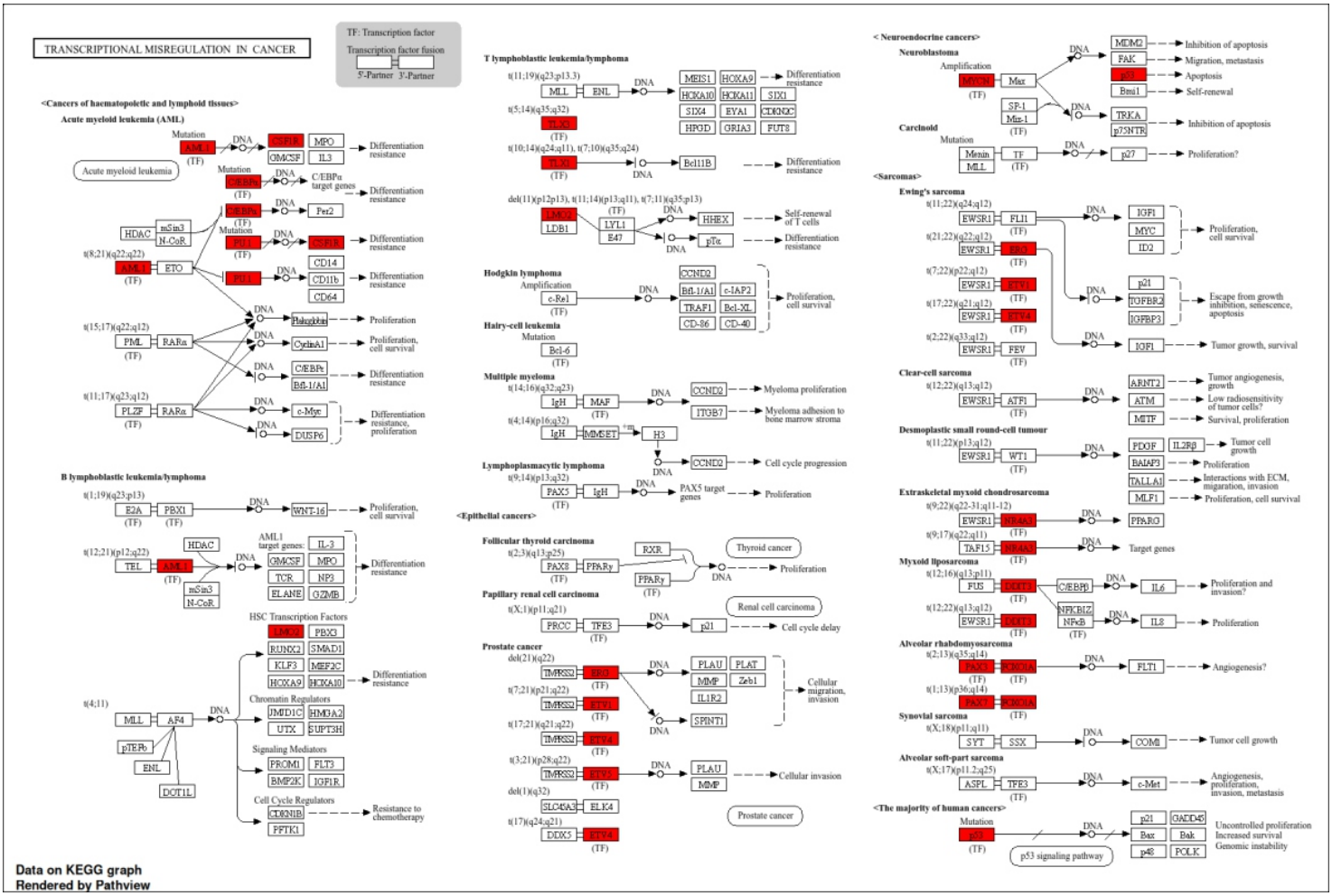

Figure 3. Up-regulation of genes in the Transcriptional Misregulation in Cancer pathway (hsa05202) in MDA-MB-231 treated with Justicia adhatoda-derived silver nanoparticles (JA-AgNPs). Using the KEGG mapper, the Differentially expressed genes (DEGs) derived from the RNA sequencing results were annotated on the KEGG pathways. The red-highlighted rectangle means the gene is significantly altered after JA-AgNP treatment, while the white rectangle means it is not significantly altered. The pathway highlights the role of different transcription factors, oncogenes, tumour suppressor genes and chromatin regulators in cell proliferation, differentiation, migration, invasion, apoptosis and tumour progression.

KEGG enrichment analysis of the MicroRNAs in Cancer pathway

This silver nanoparticles (JA-AgNPs) derived from Justicia adhatoda had significantly enriched MicroRNAs in Cancer (hsa05206) pathway, in MDA-MB-231 cancerous cells, as depicted in figure X. By mapping the DEGs onto the KEGG pathway, it was noted that gene expression of large number of genes changed following the JA-AgNP exposure and importantly, the expression of HMGA2 (High Mobility Group AT-hook 2) was significantly changed.

HMGA2 is a chromatin associated, architectural type, transcription regulator which has been involved in various oncogenic events, such as tumour initiation, epithelial–mesenchymal transition (EMT), cellular proliferation, invasion, metastasis and therapeutic resistance. The HMGA2 has been identified as a common downstream target in several types of cancer in the KEGG pathway, suggesting its overall role in a malignant progression.

It was seen that genes regulated by treatment with JA-AgNP affect networks that are usually shared by microRNAs that are thought to be cancer causal.

The differential expression of HMGA2 suggests deregulation of transcriptional programmes related to tumour progression and a metastatic behaviour and, although not measured in the current study, expression of microRNAs is related to translocation. Several tumour suppressor microRNAs, including let-7 family, have been reported as being downstream of HMGA2, the activity of which negatively regulates the expression of HMGA2 under physiological conditions. The changes in expression of HMGA2 alone suggest that the treatment with JA-AgNPs affects the molecular pathways related to the growth, differentiation and aggressiveness of the tumour.

To conclude, enrichment of the MicroRNAs in Cancer pathway shows that the genes that become mutated by JA-AgNPs have significant changes in transcription upon enrichment of cell culture with the same nanoparticles. The modulation of HMGA2, in addition to the observed cytotoxic and antiproliferative properties in MDA-MB-231 cells, indicates that the treatment of these cells with JA-AgNPs seems to impact oncogenic regulatory networks involved with the progression of triple-negative breast cancer.

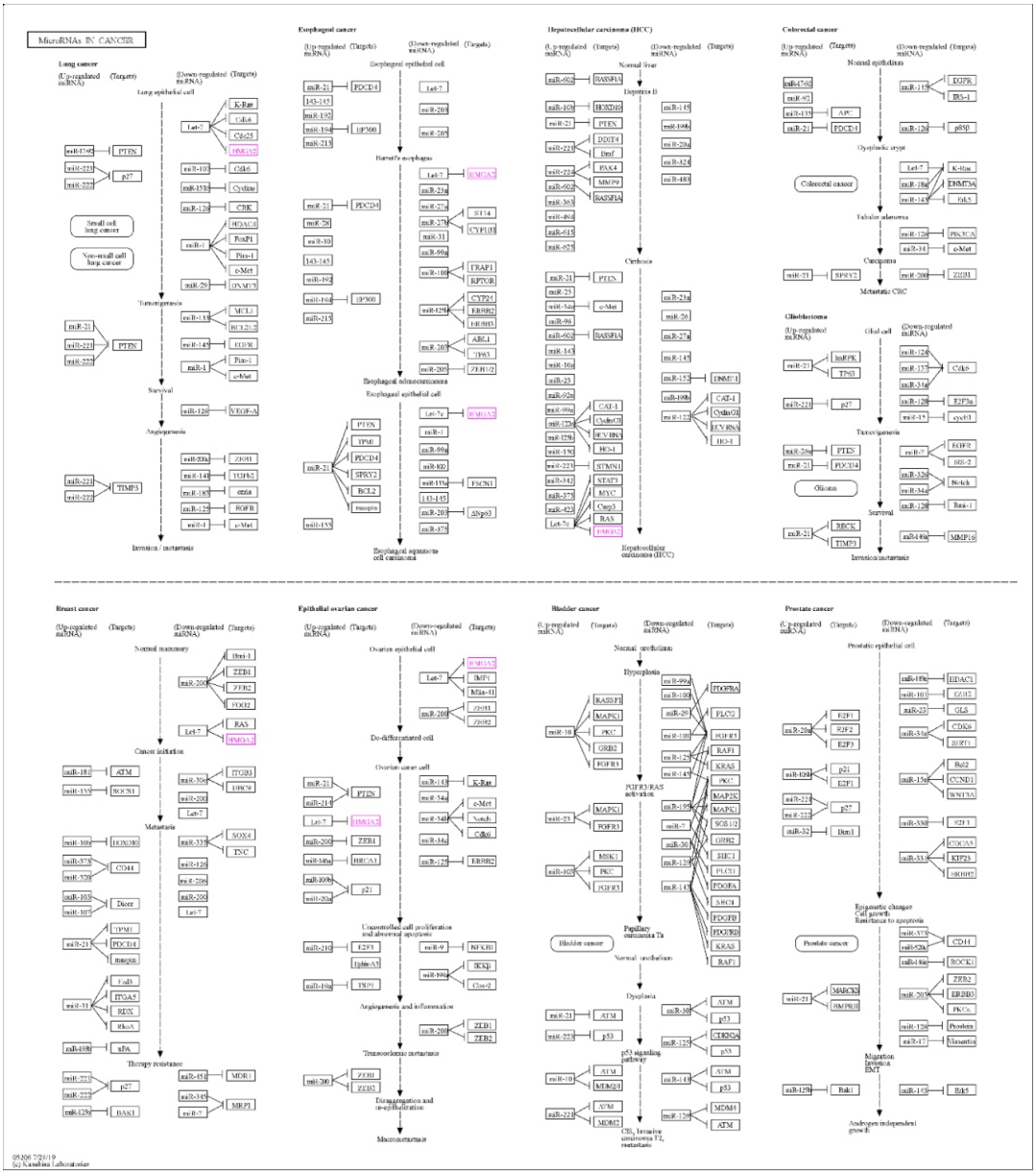

Figure 4. KEGG pathways enrichment for MicroRNAs in cancer (hsa05206) pathway for MDA-MB-231 cell (treated with silver nanoparticles from Justicia adhatoda (JA-AgNPs). The human KEGG MicroRNAs in Cancer pathway (hsa03202) was mapped with the differentially expressed genes (DEGs) obtained by RNA sequencing (RNA-seq) with KEGG Mapper. White boxes represent genes that were not significantly differentially expressed and pink highlighted boxes are those that were significantly differentially expressed after JA-AgNP treatment. The top ranking gene in enriched pathway was HMGA2 indicating the JA-AgNP treatment-modulated transcriptional networks are involved in tumour proliferation, epithelial–mesenchymal transition, invasion, and metastasis, as well as cancer progression.

Discussion

However, Triple-negative breast cancer (TNBC) is one of the most aggressive molecular subtypes of breast cancer, characterized by high metastatic potential, poor prognosis and no targeted therapeutic options. Hence, identifying other therapeutic molecules that inhibit both tumour growth and a number of oncogenic pathways has become a major cancer target area. The objective of the present study was to understand the molecular mechanism behind anticancer activity of JA-AgNPs by studying the change in the transcriptomic level in MDA-MB-231 TNBC cells upon treatment with JA-AgNPs[19, 31, 32].

All the known JA-AgNP concentration-dependent cytotoxicity effects such as cell rounding/shrinking and reduced but not complete cell detachment were confirmed by morphology. Furthermore, the mechanism of action, a process known as apoptosis, was observed in the current study, confirming previous findings which showed that silver nanoparticles remnants from plants have notable anticancer properties by inducing DNA damage, mitochondrial damage, oxidative stress and programmed cell death on breast cancer cells[33, 34].

The global transcriptomic changes mediated by the JA-AgNP treatment were also seen in the whole genome sequencing. As shown in the KEGG pathway enrichment analysis, different cancer-related signalling pathways were significantly changed, suggesting that JA-AgNPs have the potential to impact multiple biological pathways instead of targeting a specific molecular target. The latter could be used for the treatment of TNBC, where the signal transduction mechanisms at work tend to activate other pathways that result in resistance[35, 36].

Among the pathways greatly enriched was "Transcriptional Misregulation in Cancer", suggesting significant transcriptional factor and oncogenic regulatory networks changes. The data shows that the JA-AgNP treatment has a broad and comprehensive effect on multiple transcription programs associated with the migration and invasion, apoptosis, proliferation and differentiation of tumour cells, including scores that encode CSF1R, RUNX1 (AML1), CEBPA, SPI1 (PU.1), PAX3, ERG, ETV1, ETV4, ETV5, DDIT3, NR4A3, FOXO1, MYCN, MLL, TLX1, TLX3, LMO2 and TP53. Several of these transcription factors are known to have an effect on the plasticity of the cell and the spread of metastasis indicating that the interaction between the JA-AgNPs and the cells in these locations impact basic processes fundamental for the existence of a tumour[37-40].

The other pathway that was significantly enriched was the MicroRNAs in Cancer. Although no direct measurement of the expression of microRNAs was made for this study, the differential expression of downstream target HMGA2 in a set of genes related to transcription and controlled by different regulatory networks in cancer suggest deregulation of expression of genes involved with different transcription programmes such as those associated with the epithelial mesenchymal transition (EMT), dissemination of metastases or tumour aggressiveness. Frequently, the modulation of HMGA2 following the JA-AgNP treatment could be correlated with the decrease of the proliferative phenotype, detected morphologically, and HMGA2 has been associated to cancer development and clinical prognosis in breast cancer.

Interestingly, the genes involved in the process of tumour immune recognition in the Natural Killer Cell-Mediated Cytotoxicity pathway are changed following the JA-AgNP treatment.

The differential expression of HLA-A3 suggests that JA-AgNPs can impact the pathways and mechanisms of antigen presentation process that play a role in immune surveillance process. Based on these transcriptomic results, we can postulate that the treatment with JA-AgNP affects the gene expression pattern of tumour cells, by modifying the expression of genes related to interaction with host immune system, which were not used in the functional immune-cell experiments. In the future, co-culture experiments will be required to determine whether or not such transcriptional differences have a positive effect on the action of NK cells on the clearance of tumours[41, 42].

An enrichment finding that was also a key discovery was that of the Thermogenesis pathway, with genes involved in the regulation of mitochondrial energy metabolism, cAMP signalling, oxidative phosphorylation and cellular bioenergetics. The mitochondrial alterations of genes suggest that treatment with JA-AgNPs could produce the metabolic stress by affecting the ATP production and energy homeostasis[43]. Modulation of bioenergetics may also account for the effect of these bioenergetics perturbations, as perturbation of bioenergetics may require high metabolic activity, which is essential for cells to support rapid proliferation and migration, typical characteristics of TNBC cells[44].

Taken together, all these pathways are enriched, suggesting that the anticancer effect of these pathways may be through concerted regulation of multiple components of transcription regulation, metabolic adaptation, immunity-associated signalling and tumour progression pathways. Unlike a specific signalling pathway, the effect of a JA-AgNP is directed at all the cell transcriptome and hence with regard to their capacity against TNBC, involves general inhibition of cellular functions. The results from the present study will help establish the mechanism of how TNBC cells are responding to JA-AgNP treatment, but there are certain limitations to the study. The result of the transcriptomics needs experimental validation by quantitative RT-PCR and by quantitative analysis of proteins by Western blot or immunofluorescence[45, 46]. Furthermore, functional assay (cell migration, invasion, apoptosis and immune cell-tumor cell interaction) will be used to complement the proposed mechanisms of action. Finally, further in vivo assays to further study the therapeutic activity, biodistribution and safety of JA-AgNPs prior to clinical translation are required.

Finally, the results of this study suggested the utilization of Justicia adhatoda silver nanoparticles as potent multifunctional drugs that can act on the simultaneous multiple oncogenic pathways, which are implicated in TNBC progression.

Conclusion

Based on this research, the massive reprogramming of a lot of the genes has been found for the silver nanoparticles made from Justicia adhatoda (JA-AgNPs) which shows strong anticancer activity against the MDA-MB-231 triple negative breast cancer cells. The JA-AgNP treatment in an extremely remarkable concentration-dependent morphological changes, cytotoxicity features and drastically altered genes involved in different cancer related biological pathways.

Transcriptome profiling with JA-AgNPs identified the key pathways: Transcriptional Misregulation in Cancer, MicroRNAs in Cancer, Natural Killer Cell-Mediated Cytotoxicity and Thermogenesis indicating that JA-AgNPs affect the process of transcription regulation, tumour development, immune associated signalling and metabolic processes involving mitochondria energy.

Additional gene expression comparison of the JA-AgNP regulated genes HMGA2, HLA-A3, CSF1R, RUNX1, ERG and family members (FOXO1 and TP53), suggest that the many regulated networks relate to tumour cell proliferation, differentiation and apoptosis, tumour cell migration and tumour cell survival. These findings indicate that JA-AgNPs target not just a single molecule, but create 'integration' of modulation of multiple signalling pathways that regulate tumour progression. This overall molecular activity makes them good drug candidates for triple negative breast cancer.

Beyond the transcriptomic observations, quantitative gene-expression analysis, protein characterization, functional cellular assays and preclinical animal models will be required to further validate the observations provided by the transcriptome for the potential to treat TNBC using JA-AgNPs.

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