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Revolutionary Nanocarrier Strategies for Harnessing Phytochemicals in Breast Cancer Therapy

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ABSTRACT:

Early detection programs and various treatments have led to improved survival rates in some countries, the current chemotherapy approach faces challenges linked to side effects and drug resistance. Consequently, there is a growing need for alternative therapeutics that offer improved efficacy and safety. Phytochemicals have emerged as attractive alternatives due to their biocompatibility and ability to combat cancer cells effectively through various mechanisms. Moreover, using nanocarriers loaded with phytochemicals has shown promise in treating breast cancer, either alone or in combination with other therapies.

This review highlights the anticancer activity of nanocarriers instigated by phytochemicals and phytochemical-loaded nanocarriers in both in vitro and in vivo studies. The review emphasizes the underlying anticancer pathways of phytochemical action, considering the diverse mechanisms involved. The selective targeted delivery of phytofabricated nanocarriers to cancer cells is discussed, along with recent developments, research gaps, and the potential of phytoceuticals. The integration of nanotechnology with phytochemical and chemotherapeutic agents holds promising prospects for the future of breast cancer treatment.

Keywords: phytochemicals; nanocarriers; breast cancer; chemotherapy; drug resistance.

1. INTRODUCTION:

Cancer remains a major global health issue, and breast cancer is one of the most common malignancies affecting women across the world. The risk of developing breast cancer increases with age, and in 2020, there were 2.3 million reported cases of breast cancer, resulting in 685,000 deaths [1]. Despite significant improvements in survival rates through early detection and various treatments, breast cancer continues to pose a significant burden on society, exacerbated by the rising global ageing population [2]. Projections suggest that the number of new breast cancer cases annually will rise by 40% to over 3 million by 2040, with a

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corresponding 50% increase in annual deaths [3], exceeding 1 million. Every 14 seconds, a woman is diagnosed with breast cancer somewhere in the world. To address this growing challenge, researchers are actively exploring potential solutions, including phytomedicines and nanotechnology-based interventions [4].

Breast cancer is a highly diverse and heterogeneous form of cancer that originates from the epithelium of milk ducts. It exhibits variations within individual tumors (intratumor heterogeneity) and significant differences between patients (intertumor heterogeneity). The classification of breast cancer is based on these variations [5], with invasive ductal carcinoma being the most common type (40-75%), and there are 21 more specific subtypes, including invasive lobular carcinoma (5-15%). Immunohistochemistry assessments reveal varying expression levels of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) in invasive breast carcinomas [6]. Breast cancer is categorized into four main molecular subgroups based on gene expression analysis: luminal A, luminal B, HER2-enriched, and basal-like [7]. The luminal A and B subtypes, which are ER-positive, exhibit tumor heterogeneity and generally have better survival rates compared to the HER2-enriched and basal-like subtypes [8]. The HER2-enriched subtype shows elevated levels of HER2 and proliferating genes, while the basal-like subtype is triple-negative and enriched for genes expressed in basal epithelial cells. Breast cancer diagnosis, treatment [9] , and theranostics (therapeutics and diagnostics) have advanced significantly [10], with breast imaging playing a crucial role in detection and clinical treatment [11]. Traditional treatments like chemotherapy, surgery, radiotherapy, and immunotherapy are still commonly used, but they face challenges such as systemic toxicity [12], limited selectivity, and the development of resistance. Preoperative systemic therapy may be necessary based on the diagnosis and assessment of breast cancer extent [13]. Effective and targeted medicines with fewer side effects are essential for the successful management of breast cancer.

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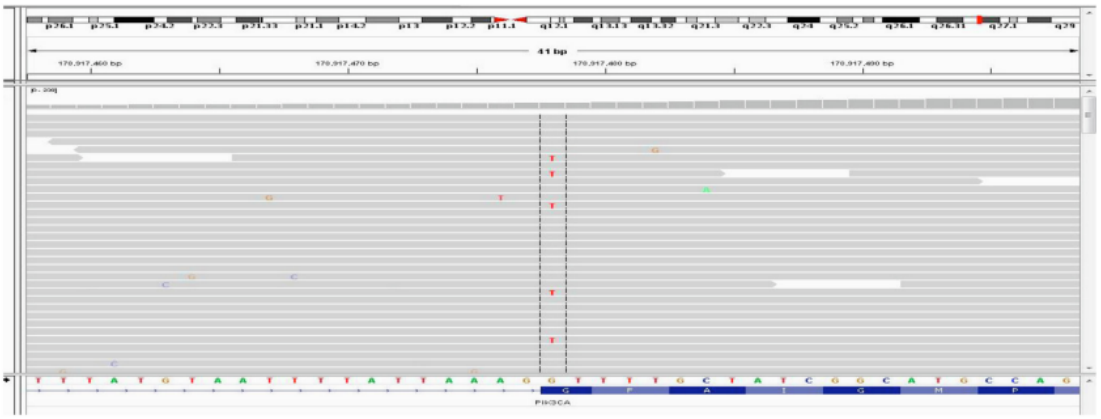

Fig. 1. Integrated Genomics Viewer showing PIK3CA D118D mutation detected in the breast cancer patient.

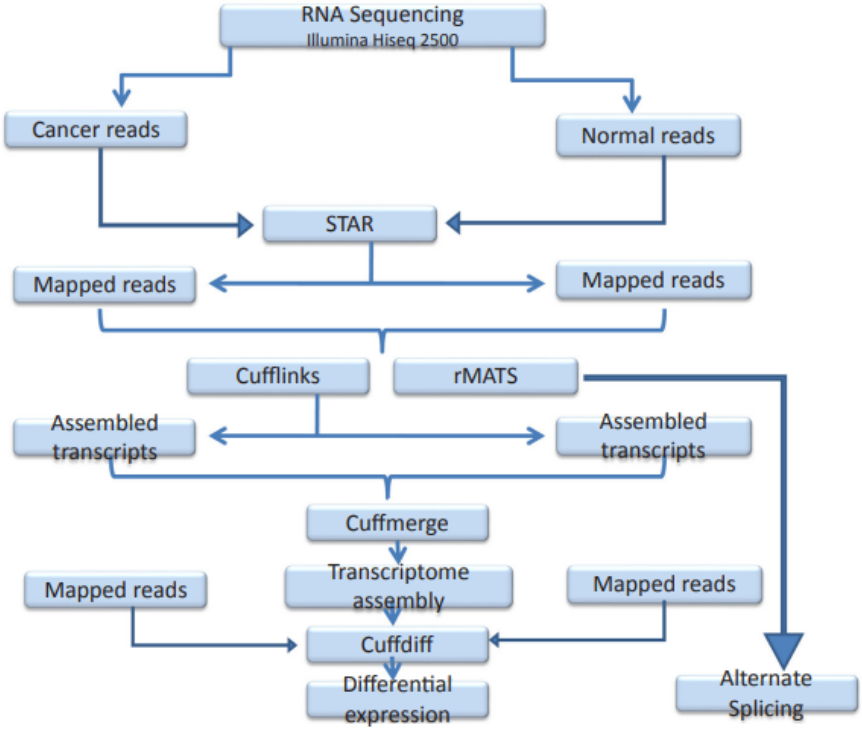

Fig. 2. Flowchart showing different steps for differential expression and alternative splicing of genes.

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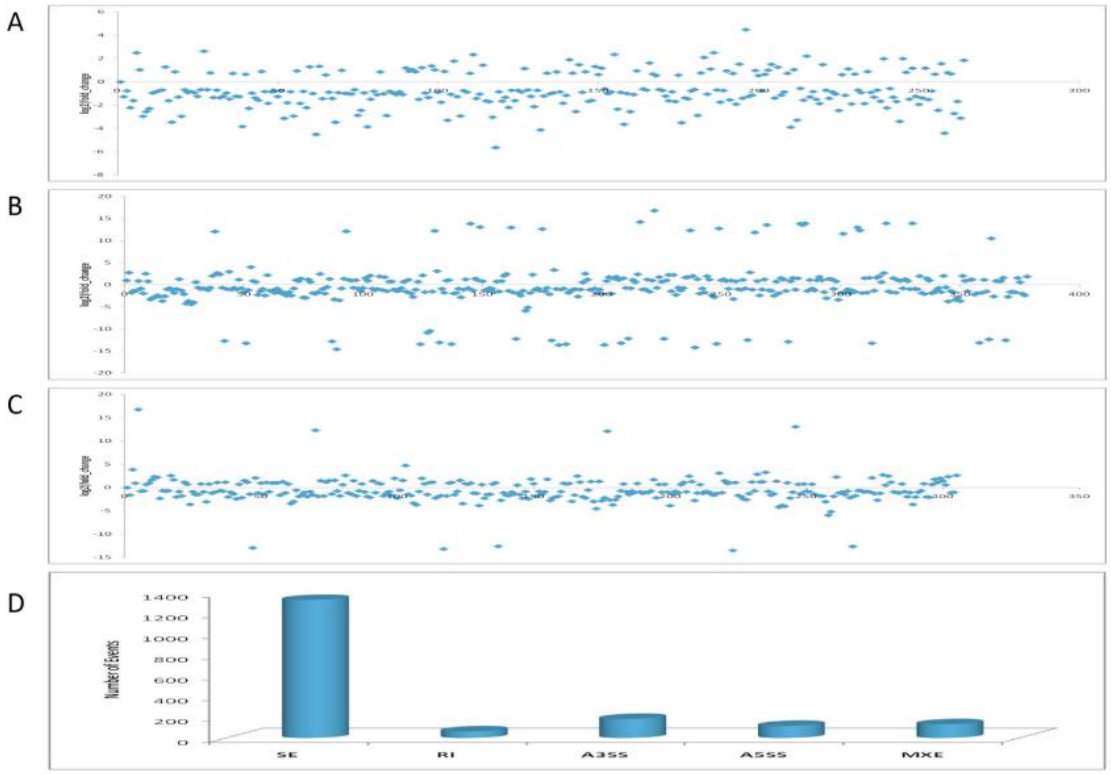

Fig. 3. (A) Genes showing significant fold change ($p < 0.05$) between normal and cancer samples. (B) Isoforms showing significant fold change ($p < 0.05$) between normal and cancer. (C) TSS showing significant fold change ($p < 0.05$) between normal and cancer. (D) Total number of different significant alternate splicing events.

2. Methods

2.1. Information of the patient:

In continuation of our previous study (Masoodi et al., 2019), we collected a total of 22 tumor samples during the patient's clinical care at the Department of Surgery, Sher-i-Kashmir Institute of Medical Sciences (SKIMS). From this pool, one specific sample was chosen for further investigation due to its PIK3CA G118D mutation (Fig. 1). The patient with this mutation is a 49-year-old individual diagnosed with stage IIIA breast cancer. The mutation is located at the beginning of exon 3. Both normal and tumor samples from the patient were gathered with proper written informed consent. The Institutional Review Board (IRB) of Sher-i-Kashmir Institute of Medical Sciences approved the study under the IRB number IRB00011885. The main focus of this study was to understand the mutation's impact on expression and alternative splicing.

2.2. Construction and sequencing of library:

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Total RNA was isolated from normal and tumor tissues using the TRIzol method as per the manufacturer's instructions (Invitrogen). The purity of the extracted RNA was determined by measuring standard absorbance ratios (260/280 and 260/230), and a 260/280 ratio of approximately 2.0 and a 260/230 ratio within the range of 2.0–2.2 were considered indicative of high-quality RNA. The TruSeq RNA Sample Preparation kit (Illumina) was employed to prepare cDNA libraries. Each sample's total RNA (10 µg) underwent polyA mRNA selection through two purification rounds, followed by thermal mRNA fragmentation. Subsequently, cDNA was synthesized from the fragmented mRNA using reverse transcriptase (SuperScript II) and random primers. The resulting cDNA was converted into double-stranded cDNA, followed by end repair and ligation to Illumina TruSeq adaptors. The cDNA libraries were then size-selected using a 2% agarose gel to obtain the desired sizes. Afterward, 15 cycles of PCR were performed to enrich the cDNA libraries, which were further purified using the PCR purification kit from Qiagen. The final libraries were diluted to the appropriate concentration for sequencing using elution buffer. Both the normal and tumor sample libraries were subjected to Illumina Hiseq 2500 sequencing, generating pair-end reads with a length of 101 bp. Initial RNA-seq reads were filtered using Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) to remove sequencing adaptors, trim bases with a quality score lower than 20, exclude sequencing reads shorter than 50 bp, and discard artificial reads.

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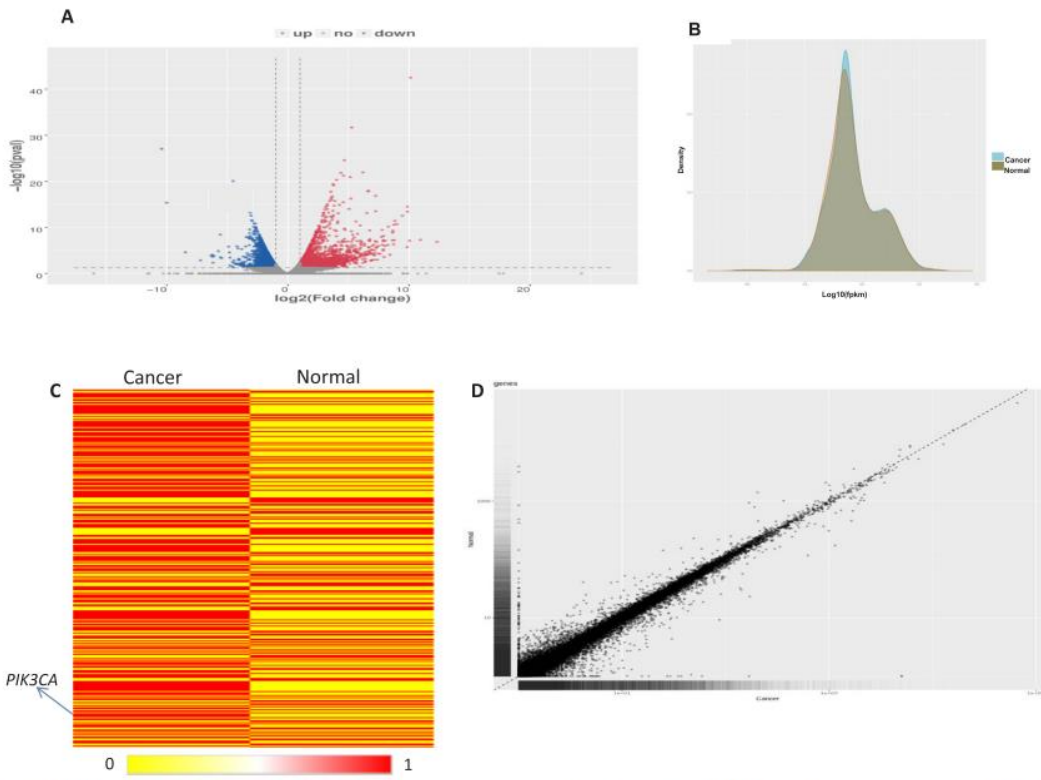

Fig. 4. (A) The plot reveals genes that differ significantly in expression between cancer and normal samples. (B) Plot showing the expression level distribution for all genes in Cancer and normal samples. (C) Differential expression analysis of cancer and normal samples. (D) Scatter plot shows similarities and differences between cancer and normal samples.

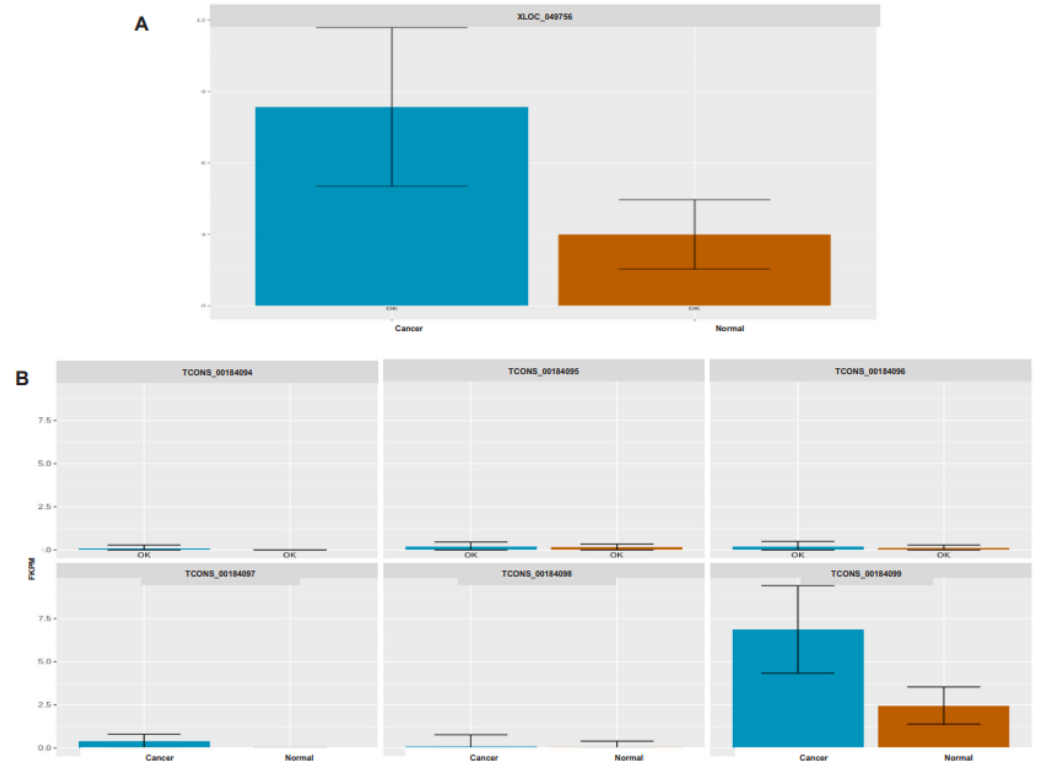

Fig. 5. Differential analysis results for *PIK3CA*. (A) Significantly expressed transcript of *PIK3CA* showing expression levels between cancer and normal samples. (B) Expression levels of different transcripts of *PIK3CA* in cancer and normal samples. Cuffdiff identifies TCONS_00253767 as being significantly differentially expressed.

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Fig. 6. Skipping of Exon 3. (A) Figure shows the normal distribution of PIK3CA exons. (B) Skipping of exon 3 of PIK3CA due to alternate splicing. (C) Chromotogram showing intron 2 sequence of PIK3CA after the end of intron 2, clearly showing of exon.

Table 1

Post alignment QC metrics of the two samples. Table depicts the total sequenced reads, reads aligned with the reference genome, percentage of reads aligned with the reference genome, unique reads aligned and the percentage of unique reads aligned with the reference genome.

Sample	Total reads	Aligned reads	Mapping percentage (%)	Unique aligned reads	Unique mapped percentage (%)
Cancer	83,748,245	80,352,167	95.94	77,852,167	92.95
Normal	78,563,489	73,673,427	93.77	71,373,427	90.84

3. Results

3.1. Mapping summary and differentially expressed genes:

Sequencing of both normal and cancer samples from the patient was performed using the Illumina Hiseq 2500 platform. The human genome assembly hg19 and Ensembl transcripts were utilized as the reference for alignment. STAR algorithm was employed for aligning the reads to the reference genome. After alignment, quality control (QC) metrics indicated that approximately 80 million reads were mapped to the reference genome in the cancer sample, while 73 million reads were mapped in the normal sample (Table 1). Gene and isoform

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expression analysis was conducted using Cufflinks, revealing the Fragments Per Kilobase of exon per Million (FPKM) values for each gene and isoform (Fig. 3A & B). Subsequently, Cuffdiff analysis was carried out to compare the FPKM values of the genes between the normal and cancer samples.

3.2. Alternative splicing analysis:

To study the variations in transcript isoforms resulting from alternative splicing, we employed the rMATS software algorithm. Alternative splicing of mRNA precursors generates diverse mature mRNAs with different functions. By comparing the tumor sample to the normal sample using rMATS, we identified significant Differential Alternative Splicing (DAS) genes, applying a threshold of FDR < 0.1. The DAS genes were classified into various types of alternative splicing events, including Exon Skipping (ES), Intron Retention (IR), Alternate Acceptor Site (AAS), Alternate 5' Splice Site (A5SS), and Mutually Exclusive Exon (MXE). Among these events, Splicing Exon (SE) was found to be the most prevalent, accounting for 73% of all alternative splicing events. RI accounted for 3.5%, A3SS for 10%, A5SS for 6.4%, and MXE for 7.3% of the total alternative splicing events (Fig. 3D). Additionally, we identified significant cancer-related genes showing alternative splicing, such as PIK3CA, MUTYH, PALB2, NTRK1, and DNMT3A (Table S2). Notably, we observed an exon3-skipping event in the oncogene PIK3CA, which could have implications for its role and regulation in cancer.

4. Discussion and conclusion:

Breast cancer remains a leading cause of cancer-related deaths, and although numerous studies have explored its pathogenesis and potential biomarkers, there is still much to be understood, especially in a large group of patients. In this study, we used RNA-Seq analysis to identify significant differences in gene expression and alternative splicing between tumor and normal samples. The aim was to uncover potential biomarkers for breast cancer patients without known driver markers. RNA sequencing offers a comprehensive and precise approach to studying breast cancer. Alternative splicing, a key mechanism generating functionally diverse proteins from mRNA precursors, was observed to be altered in tumor samples compared to normal ones. In total, 3162 genes exhibited alternative splicing in the tumor sample, while 322 differentially expressed genes (64% downregulated, 36% upregulated) were identified in the tumor sample, potentially influencing patient outcomes. GO and KEGG pathway analyses revealed diverse

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molecular functions, cellular components, and biological processes associated with the differentially expressed genes. Crucial pathways, such as TNF signaling, estrogen signaling, ErbB signaling, and cAMP signaling, were found to play significant roles in breast cancer pathogenesis. Notably, an exon3-skipping event in the PIK3CA oncogene was detected through RNA-Seq, indicating its potential involvement in splicing regulation. Elevated expression of PIK3CA may activate PI3K and downstream AKT pathways, contributing to breast cancer pathogenesis. In conclusion, RNA sequencing is a powerful tool that provides comprehensive insights into breast cancer. It facilitates the discovery of splicing alterations and potential biomarkers for more effective treatment strategies.

5.Acknowledgement:

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