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REVIEW ARTICLE

Analysis of miRNAs: Biomarkers for HER2-Targeted Breast Cancer Therapy

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ABSTRACT

Breast cancer remains a leading lethal cancer in women globally, with the HER2+ positive subtype associated with a higher chance of therapeutic resistance. Treatments tailored specifically to this subtype, such as targeted therapies, have significantly developed over the past decade. However, the issue of drug resistance to targeted drugs underlines the necessity of better treatment approaches. Recent studies have focused on microRNAs (miRNAs), single-stranded non-coding RNAs, as critical regulators in drug resistance mechanisms. These miRNAs, capable of influencing various cellular processes, have emerged as critical modulators in numerous diseases, including different cancer types, particularly breast cancer. This article reviews current methodologies in the study of miRNAs within the context of HER2-positive breast cancer, from the selection of study models and sample extraction to comprehensive analysis methods. Our objective is to highlight the potential that miRNAs hold as biomarkers capable of regulating drug resistance in this specific cancer subtype. Moreover, we also discuss the importance of integrating advanced study models alongside the latest bioinformatics tools to enrich this research domain. Furthermore, this article evaluates the use of clinical samples and cell line models for studying miRNAs in this field, outlining the advantages and limitations of each method and proposing a refined approach to research design. The main contribution of this work is the establishment of a detailed taxonomy of research strategies that address current challenges while also outlining promising future directions, particularly focused on elucidating the regulatory mechanisms of miRNAs in therapeutic resistance in breast cancer. In addition, by underscoring the necessity of employing a diverse array of study models and capitalizing on bioinformatics advancements, this article seeks to uncover the complex interactions between miRNAs and drug resistance mechanisms in breast cancer. Ultimately, our goal is to pave the way toward overcoming therapeutic resistance, thereby improving the prognosis for patients afflicted with HER2-positive breast cancer.

Keywords: HER2-positive breast cancer, miRNAs, Treatment response, Targeted-drugs

1 Introduction

Breast cancer is among the common types of cancer that affects many women around the globe. Among other molecular subtypes, HER2-positive breast cancer, which accounts for 20-25% of all cases, is given increased attention due to its aggressive nature [1, 2]. This subtype is characterized by the excessive expression of the epidermal growth factor receptor 2 (HER2) gene [3]. Recent years have seen significant advancements in the therapeutic landscape of HER2+ breast cancer, with a significant concentration on HER2-targeted therapy [4-8]. These targeted drugs, even though have demonstrated impressive efficacy, are still associated with the challenge of drug resistance.

MicroRNAs (miRNAs) are small, non-coding RNA molecules, typically composed of 20-25 nucleotides. As regulators of gene expression, they modulate various cellular processes by binding to specific mRNA targets. Since the initial discovery of Lin-4 in *Caenorhabditis elegans* in 1993 [9], followed by the identification of the conserved miRNA let-7, in 2000 [10], the significance of miRNAs in many pathological and biological processes has been underscored. These processes encompass development, differentiation, proliferation, apoptosis, and immune responses [11].

Given the widespread involvement of miRNA in influencing extensive cellular pathways by targeting a broad spectrum of genes, their implications in many diseases, including cancer, have garnered immense interest. In the context of cancer biology, various facets of diseases from tumor initiation to metastasis mediated by miRNA regulations have been exploited [12, 13]. Many studies have discovered intriguing regulatory roles of miRNAs in breast cancer [14-17]. Particularly in HER2-positive breast cancer [18, 19], miRNAs have been uncovered to play pivotal roles, with some directly influencing drug resistance [20-22]. Considering the leading role of breast cancer in cancer-related deaths and the potential modulating role of molecular factors like miRNA, understanding miRNA dynamics in this context holds significant therapeutic value. However, despite the availability of various methods to study miRNAs, a significant gap remains in applying these findings to overcome drug resistance in breast cancer, particularly within the HER2-positive subtype. This gap is largely due to the limitations associated with traditional study models, such as 2D cell cultures, which fail to accurately replicate the complex tumor microenvironment where miRNA-mediated drug resistance mechanisms unfold. These conventional models often oversimplify the intricate interactions between cancer cells and their surrounding environment, hindering our ability to fully uncover the mechanisms through which miRNAs regulate drug resistance in breast cancer. This paper will delineate the methods and challenges in studying miRNAs as biomarkers in the responsiveness of target treatment in breast cancer, from sample selection and miRNA extraction to advanced data analysis techniques. By diving into these nuances, we aim to highlight the increasing importance of miRNAs as potential biomarkers in breast cancer, particularly focusing on their relevance in anti-HER2 therapy. Additionally, we strive to address current gap in this field by advocating for the adoption of more advanced study models that better mimic the in vivo conditions of tumor biology.

2 Study Models and Sampling Method

Studies of miRNA biomarkers in anti-HER2 drug resistance are based on both clinical samples and cell line models. Each model offers unique insights and advantages tailored to specific research objectives.

2.1 Biofluids: An Emerging Trend in miRNA Sampling

Identified miRNAs in circulation could serve as potential biomarkers for monitoring cancer treatment responses [23]. Due to their potential, a significant number of studies have turned to biofluids for miRNA research in HER2-positive (HER2+) breast cancer drug resistance [24-26]. The rapid and minimally invasive nature of biofluid sampling, coupled with the stability of miRNAs in these samples, offers an efficient alternative to traditional tissue biopsies [27]. However, external factors such as diet, gender, age, alcohol, or medication can influence the miRNA spectrum in biofluids, which can introduce variability [28-30]. This variability necessitates strict controlling for these factors and understating their implications in findings. Such complexities make tissue biopsies, despite their invasive nature, a valuable source for consistent and representative data.

2.2 Tissue Biopsies: An Invaluable Resource in Unravelling Drug Resistance Markers

Despite a myriad of advantages of using biofluids, tissue biopsies remain essential in breast cancer research. They offer a direct snapshot of the tumor's molecular landscape, which is crucial for understanding drug resistance mechanisms and advancing translational research [21, 31, 32]. Their value stems from the specificity, sensitivity, and reliability they provide. Direct sampling from tumor tissues typically yields a higher concentration of specific miRNAs, offering profound insights into molecular alterations propelling

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resistance [33]. Conversely, biofluids, with their diluted miRNA concentration, might challenge the sensitivity of detection. Nevertheless, factors like biopsy size, location, and tumor heterogeneity can introduce variability if not carefully considered [34]. Despite potential variabilities in biofluids due to external factors, tissue biopsies often yield more consistent data. However, their invasiveness and potential inability to capture the entire miRNA landscape due to tumor heterogeneity underscore the challenges in repeated sampling for comprehensive insights into HER2 breast cancer drug resistance.

A recent study has elucidated the interplay between miRNAs in body fluids and specific human tissues. Notably, plasma and serum miRNAs correlate with miRNAs in the liver, adipose, and spleen, while urinary miRNAs mirror renal-specific miRNAs [35]. This suggests a potential origin of certain biofluid miRNAs from specific tissue sites, possibly impacting drug resistance mechanisms in HER2-positive breast cancer. Therefore, leveraging miRNA profiles from both tissue and biofluid sources might yield a more holistic understanding of miRNA-mediated pathways in drug resistance.

Nevertheless, the inherent challenges in tissue sampling and potential dilution effects in biofluids necessitate exploring additional alternative models for consistent and in-depth studies on HER2-positive breast cancer drug resistance. Cell line models, which offer reproducibility and ease of manipulation, emerge as an essential complement, providing insights that might be challenging to obtain from patient-derived samples alone.

2.3 Cell Line Models in Breast Cancer Drug Resistance Research

Complementing clinical samples, cell line models have significantly enriched our understanding of breast cancer research. Such models are extensively employed in various studies, particularly for elucidating the mechanisms through which target miRNAs modulate HER2 drug resistance [36-38]. Common HER2+ breast cancer cell lines used for targeted drug resistance research include BT-474, SK-BR-3, and HCC1954 [39-41]. Each of these lines exhibits unique characteristics in terms of growth rates, molecular markers, and therapeutic agent responses, making them favorable choices for acquired resistance studies. Leveraging these models, researchers have identified specific miRNA-driven pathways that influence drug sensitivity or resistance [40, 42].

Cell line models offer several benefits. They are cost-effective, easy to maintain, and due to their availability, they facilitate the replication of experiments [43]. Moreover, they allow for quicker turnarounds compared to *in vivo* studies. However, there are also several associated challenges. The inherent tumor heterogeneity is often a concern since a single cell line might not capture the extensive molecular diversity present in patient tumors [44]. Furthermore, over prolonged periods, *in vitro* adaptation can cause cell lines to drift from their initial *in vivo* behavior, potentially influencing research outcomes [45]. Therefore, cells should be handled properly to represent a viable model, and proper knowledge of cell characteristics is required to interpret results.

While cell line models are indispensable, recognizing their limitations is crucial. Employing a multifaceted approach by combining cell lines with other models will aid in the comprehension of HER2 drug resistance in breast cancer.

3 Sample Handling and miRNA Analysis

3.1 Ensuring Validity in miRNA Studies of Breast Cancer Drug Resistance

Optimal experimental design and sampling technique are essential to guarantee the accuracy of the acquired data and their potential application in clinical settings. Due to issues with poor reproducibility and confounding variations, miRNA studies frequently face challenges in achieving consistency, with some reported discrepant findings even when they are regarded as stable molecules [46, 47], proper sample collection techniques and measurements are required. The inability to do so may cause contamination and introduce bias to the results. Specifically, since miRNA contamination in biofluids most often comes from erythrocyte leakage [48], hemolysis should be routinely inspected in plasma and serum samples. In terms of tissue samples, liquid nitrogen can be used to snap freeze the fresh tumor [31], or fixative like RNAlater can be used to preserve tissue samples [49], especially for RNA extraction. Also, in cases of histological analysis, FFPE is often used to preserve the architecture of the tissue [32].

3.2 The Criticality of miRNA Extraction in Breast Cancer Research

Accurate and efficient extraction of miRNAs is a crucial step in ensuring the reliability and precision of results, especially in studies focused on breast cancer drug resistance. The extraction process serves as the foundation upon which subsequent analyses are built, hence, the extraction workflow should be conducted with careful selection and optimization to reduce variability. To ensure the preservation of miRNA integrity post-extraction, rigorous adherence to established standard operating procedures during sample handling and storage is vital. This approach minimizes the risk of potential sample degradation. To ensure consistency and reliability of the results, it is essential to employ the same miRNA extraction procedure throughout the entire study.

Additionally, for qualitative assessment of the miRNA extraction workflow, the implementation of spike-in controls is recommended to identify severe losses in extraction procedures. This method involves the incorporation of an exogenous sequence, either synthetically designed or derived from an alternate species. This sequence, though distinct in its sequence homology, parallels the length of endogenous miRNAs. The spike-in is introduced to the sample before extraction. By monitoring the recovery of this introduced sequence at various points across the extraction continuum, severe losses in extraction procedures can be identified [50, 51].

3.3 Common Techniques for miRNA Quantification in Breast Cancer Drug Resistance Studies

Mature miRNA presents inherent characteristics that pose challenges in its quantification [33]. Specifically, their small size and lack of a poly(A) tail can hinder selective capturing and priming during reverse transcription. Furthermore, miRNAs can exist in variant forms known as isomiRs, originating from physiological process affecting the canonical miRNA biogenesis. While their presence adds complexity to the miRNA landscape of cancer study, isomiRs have both cooperative and functional roles distinct from canonical miRNAs [52]. The terminal sequence heterogeneity observed in isomiRs can introduce significant bias, particularly if the quantification method lacks the sensitivity to discern between closely related miRNAs that differ by only a single nucleotide. In addition, the typically low abundance of circulating miRNAs in body fluids can contribute to the difficulty in effective quantification. Three predominant methods have been adopted for quantifying miRNAs in studies focused on resistance to HER2 therapy: RT-PCR [22, 24, 53, 54], microarrays [38, 55], and RNA-seq [36].

3.3.1 Microarrays

Microarrays were one of the first methods applied for high-throughput analysis of miRNAs in breast cancer research. While they have seen limitations such as a low dynamic range and the requirement of annotated miRNAs for detection, their capability to measure a large number of miRNAs, their user-friendliness, and the abundance of available reference data with standardized guidelines still make them attractive in this field, particularly for large-scale screening studies [56].

Chi et al. used this method to analyze miRNAs that were expressed differently between breast cancer and tumor cell lines and found some differentially expressed miRNAs in HER2 resistance treatment [36]. Differentially expressed miRNAs between parental and breast cancer trastuzumab resistance cell lines have been identified via microarray screening in the study conducted by Bai et al. [55]. A common trend observed in many HER2 drug resistance studies involves the use of microarray methods initially to screen and identify miRNAs with altered expression, followed by in-depth analyses on specific miRNAs of interest. Over the years, refinements across multiple stages, including miRNA labeling, hybridization, signal scanning, and quantification, have been integrated to enhance the efficacy and accuracy of microarray analyses [57, 58].

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While significant advantages can be offered by large-scale screening of miRNAs using microarrays, there are still several limitations. First, the issue of specificity causing cross-hybridization between closely related miRNA sequences can happen due to their small size and sequence similarity. Accurate detection of miRNAs in this technology is also limited by its dynamic range since high miRNA concentration can result in saturation and low concentrations may cause insufficient binding. Furthermore, unannotated miRNAs may not be detected and in case of genomes with significant variability, important miRNAs might be missed out when miRNA microarrays are designed based on reference strains [59, 60].

3.3.2 RT-qPCR (Reverse Transcription Quantitative PCR)

Serving as the gold standard in HER2 drug resistance miRNA studies, RT-qPCR brings high sensitivity and specificity coupled with a broad dynamic range [61]. Its primary advantage lies in the need for a minimal input amount [62] and a well-established downstream data analysis workflow [63]. Particularly, this method is ideal when analyzing a specific set of chosen miRNAs to minimize costs and reduce turnover timing. In addition, it is worth mentioning that the attributes of this method make it a helpful approach in clinical settings, especially the application in diagnostics [64, 65].

In the study conducted by Mattos-Arruda et al., miRNAs of functional significance were chosen based on comprehensive literature assessments. Subsequent RT-qPCR evaluations were executed on HER2-positive breast cancer clinical biopsies to determine the expression levels of these selected targets [32]. Moreover, RT-qPCR results can be calibrated for absolute quantification, and it possesses the capability to distinguish miRNAs with single nucleotide disparities [66]. The fundamental workflow for RT-qPCR encompasses the reverse transcription (RT) phase, which yields cDNA, followed by the amplification and quantification of this cDNA through qPCR [67].

In recent years, many qPCR-based methods have been introduced to facilitate the analysis of miRNAs in breast cancer; including methods based on stem-loop RT [68], strategies based on polyadenylation [69], or approaches based on ligation [70].

Given its role as the gold standard in miRNA expression quantification, issues such as variability in miRNA expression, the quality of the template, and operator variability can obfuscate results. Also, due to the high sensitivity of this method, minor contaminations can result in inaccuracies. These drawbacks make achieving both accurate and biologically relevant results challenging. This necessitates the requirement for standardized protocols and validation processes in this approach [71, 72].

3.3.3 RNA-seq

Emerging as a leading high throughput technology in miRNA research, RNA-seq allows the detection of both annotated and unannotated miRNAs with high sensitivity and specificity [73]. One of the notable advantages of this technology is the recent affordability attributed to innovations like multiplexing. By utilizing methods such as PCR barcoding, multiple samples can be uniquely tagged and processed in a single sequencing run, and substantial cost savings can be achieved [74]. Of note, the ability to discover previously unidentified isomiRs make this technique stand at the frontier of miRNA research, especially in HER2+ breast cancer drug resistance studies where only limited number of isomiRs have been identified [75]. However, despite the advantages, RNA-seq remains underused in this research field. Its complex library preparation, the need for advanced bioinformatics skills for data analysis, and the associated costs might be limiting factors [33]. Adding to this, the higher number of samples requires a higher cost. Ludwig et al. have also reported that errors in RNA-seq data analysis can be caused by RNA degradation [76]. Although not as prevalent in HER2 drug resistance miRNA studies, its primary application remains in large-scale screening and discovery endeavors. The benefits and drawbacks of each method are compared in Table 1.

Table 1: *Comparison of the advantages and disadvantages of miRNA quantification methods in HER2+ breast cancer drug resistance research.*

Method	Advantages	Disadvantages	Study
Microarrays	Measures many miRNAs User-friendly Abundant standardized reference data Ideal for large-scale screens Enhanced efficacy over time	Low dynamic range Requires annotated miRNAs Cross-hybridization issues Limited detection range Misses unannotated miRNAs Can miss miRNAs with genome variability	[38, 77-79]
RT-qPCR	High sensitivity and specificity Minimal input amount required Established data analysis workflow Ideal for specific miRNA sets Applicable in clinical diagnostics Distinguishes miRNAs with single nucleotide differences Allows absolute quantification	Variability in miRNA expression can affect results Quality of template can be an issue Operator variability can skew outcomes High sensitivity can lead to inaccuracies from minor contaminations Needs standardized protocols and validation processes	[22, 26, 31, 32, 40, 80-93]
RNA-seq	Detects both annotated and unannotated miRNAs High sensitivity and specificity Gets more affordable due to innovations like multiplexing Capable of discovering previously unidentified isomiRs Significant application in large-scale screening and discovery	Complex library preparation Requires advanced bioinformatics skills for data analysis Associated costs can be limiting factors Processing a higher number of samples results in higher costs Potential errors due to RNA degradation Less prevalent in specific studies like HER2 drug resistance miRNA research	N/A

The above Table 1 summarizes the primary benefits and limitations associated with the three key techniques employed in miRNA quantification: Microarrays, RT-qPCR, and RNA-seq. Predominantly, research in this domain leverages the RT-qPCR method, with microarrays also seeing consistent use. However, there's a notable absence of deep sequencing (RNA-seq) studies, highlighting a potential area for future research.

4 Advanced Methodologies for Data Analysis in Breast Cancer Research

Data analysis is a significant step in miRNA biomarker identification in breast cancer research. Ensuring reliability necessitates acknowledging the critical points that differ across various analyzing technologies. While standardized protocols for data analysis are available for traditional methods like microarray and RT-qPCR, RNA-seq frequently demands a deeper grasp of computational skills for accurate interpretation.

Therefore, when opting for a quantification and analysis method, it's imperative to weigh both its benefits and the feasibility of meeting its technical and analytical requisites.

MiRNA studies of drug resistance in HER2+ breast cancer aims to identify and characterize miRNA biomarkers that can predict the response of HER2 patients to targeted therapy. Based on the differently expressed miRNAs, interest candidate miRNAs can be identified. The performance of the miRNA biomarker candidates in predicting HER2 drug resistance can be evaluated using receiver operating characteristic (ROC) analysis [32]. Furthermore, leveraging conventional statistical methodologies for survival analysis has also proven invaluable in identifying miRNA predictors of HER2 drug resistance [36]. However, a challenge often encountered, particularly with RNA-seq and microarray, is the expansive list of dysregulated miRNAs, sometimes running into the hundreds. This makes the characterization of potential miRNA biomarkers in subsequent functional analysis difficult. The essence of such characterization of miRNA networks and pathways is to derive meaningful insights from these extensive lists of potential miRNAs associated with drug resistance in breast cancer. In this context, functional analysis allows the decision on the enriched interested miRNAs in a biological process related to the observed phenotype. The entire process encompasses functional annotation, either directly via specialized databases or indirectly by associating target gene terms sourced from gene-centric databases. Researchers often employ prediction algorithms like TargetScan [32] or validated target databases like mirTarBase [31] to search for predicted and validated target genes. Statistical tests can be applied before defining p-values to each annotation to indicate over-represented and significant annotations. A comprehensive overview of the databases and software tools for miRNA functional analysis can be found in the study by A. Garcia-Moreno and P. Carmona-Saez [94]. Figure 1 illustrates the workflow of miRNA analysis in breast cancer drug resistance studies.

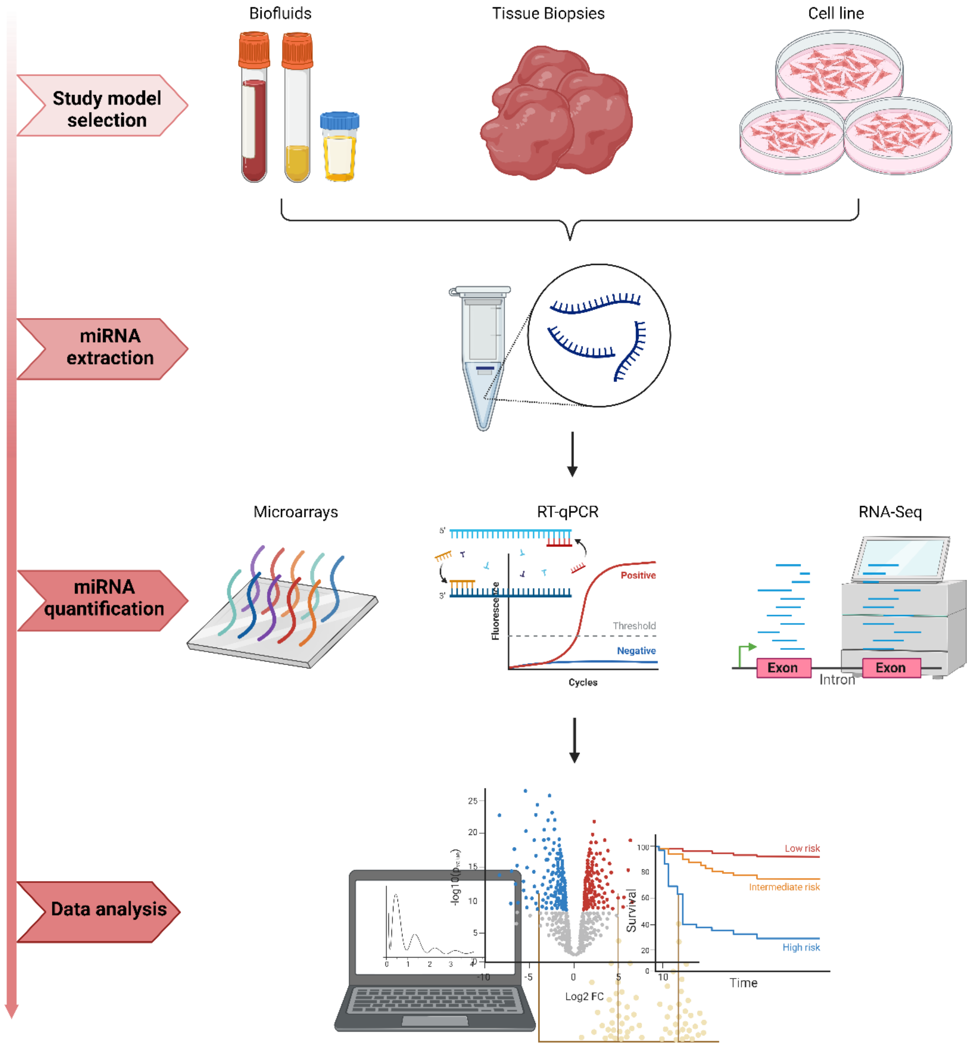

Figure 1: *Workflow of miRNA Analysis in Breast Cancer Drug Resistance Studies*

The workflow begins with selecting the study model, including biofluids, tissue biopsies, or cell lines. The following sample selection is miRNA extraction. After extraction, the miRNAs are quantified using microarray, RT-qPCR, or RNA-seq methodologies. The final step involves data analysis, where the results from the quantification are interpreted and assessed.

5 Discussion and Future Direction

5.1 Sample Selection and Model Validity

Sample selection options and processing procedures are significant when investigating miRNA biomarkers for resistance to HER-targeted therapies. Given the majority of the current studies rely on cell lines for their models [39-41], the advantages of this approach are tempered by its shortcomings. Although cell lines provide advantages in terms of reproducibility and availability for multiple experiments, results derived from them may not directly correlate with clinical outcomes in HER2+ breast cancer patients exhibiting drug resistance.

The identification of circulating miRNAs in biofluids is technically challenging due to their low abundance, which complicates detection, especially in platforms like microarrays. Serum is often favored over plasma for miRNA studies to avoid hemolysis-associated artifacts. It has been discovered that interferences like platelet or white blood cells in the preparation of samples can result in lower miRNA levels identified in plasma compared to serum [95]. Given these complexities, it is essential to use a standardized protocol for sample collection and processing techniques to avoid bias during sample processing.

Tissue biopsies, although more invasive than biofluid collection, are indispensable in investigating drug resistance mechanisms in breast cancer due to their ability to allow a more comprehensive investigation of tumor's molecular heterogeneity compared to cell line and biofluid models. However, repeated sampling remains problematic, and thus, combining both models could capture a more comprehensive understanding of the resistance mechanisms of targeted drugs in breast cancer.

5.2 Potential Implications of Advanced Models in HER2+ Breast Cancer Research on Drug Resistance

In recent years, the advancement of oncological research methodologies has signaled potential avenues for delving deeper into drug resistance mechanisms inherent to HER2+ breast cancer. Diverging from traditional two-dimensional (2D) cultures, three-dimensional (3D) cultures offer a more nuanced representation of tumor, which may, in turn, yield a more precise understanding of drug-tumor interactions [96]. Moreover, the advent of patient-derived xenografts (PDX) [97] and tissue-derived organoids [98] into research paradigms provides a closer simulation of in vivo tumor characteristics. This promises a deeper understanding of resistance pathways. Furthermore, "tumor-on-a-chip" technologies [99], which combine microfluidics with cellular biology, have potential for a more dynamic analysis of resistance. Similarly, co-culture systems [100], encompassing a range of cell types, might elucidate the multifaceted interplay contributing to drug resistance.

Collectively, the incorporation of these advanced models could revolutionize our understanding of drug resistance in HER2+ breast cancer, propelling us towards more effective therapeutic strategies.

5.3 MiRNA Quantification Challenges

Predominantly, RT-qPCR has been the method of choice for miRNA quantification in HER2 drug resistance studies. While RT-qPCR surpasses microarray in sensitivity and is less costly than RNA-seq, its associated limitations remain. Notably, the inability to detect novel miRNAs is a significant setback. RT-qPCR demands that miRNA panels are pre-established or that target miRNAs are already identified through screening analyses or literature reviews. Furthermore, reproducibility remains a challenge with minimal overlap in identified miRNA panels across studies. This discrepancy underscores the complex biology of miRNA expression in the circulatory system of HER2+ breast cancer patients.

5.4 Future Directions

Continued advancements in HER2-positive breast cancer research emphasize the need for further standardization of sampling methodologies to achieve reproducible and robust results. Concurrently, advances in bioinformatics, especially the integration of machine learning, offer significant potential. Such advancements promise to enhance the efficiency of data processing and downstream functional analyses. Additionally, the integration of more advanced study models, reflecting the dynamic *in viv*o tumor environment, is essential. This comprehensive approach may facilitate the development of more effective therapeutic strategies for HER2+ breast cancer patients.

6 Conclusion

In this article, we have discussed the complexities of HER2+ breast cancer, with an emphasis on the pressing challenges surrounding drug resistance. A pivotal focus has been on the role of miRNAs, highlighting the associated challenges in their quantification and the need for robust, standardized methodologies. The emerging advances in oncology research, coupled with evolving study models and advancements in bioinformatics, suggest promising avenues that could be harnessed for a deeper understanding of drug resistance mechanisms in HER2+ breast cancer. Current quantification methodologies have demonstrated both progress and existing limitations. As research methodologies in breast cancer undergo refinement, the prospective incorporation of sophisticated models and cutting-edge techniques could further enhance therapeutic strategies. These developments not only hold future potential to refine our understanding of miRNA mechanisms in breast cancer drug resistance but also highlight the continuous need for innovation in research methodologies in this domain. By embracing these advancements, we look forward to the development of more effective, personalized treatments for HER2-positive breast cancer patients.

7 Declarations

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7.2 Competing Interests

The authors declare that they have no competing interests.

7.3 Publisher's Note

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