Identifying HER2 Positive Cells Using Herceptin

1. Abstract

Breast cancer remains a leading cause of cancer in women, with HER2-positive subtypes presenting distinct challenges due to their aggressive nature and poor prognosis without targeted therapies. This study evaluated an immunofluorescence-based approach to detect HER2 expression using trastuzumab (Herceptin) in four cell lines with varying HER2 levels. The hypothesis was that HER2-positive cells would show significantly higher fluorescence compared to HER2-negative ones. Fluorescence microscopy revealed strong HER2 expression in SKBR3 and MDA-MB-435 cells, while MCF7 and HeLa cells displayed minimal fluorescence. Despite limitations such as small sample sizes and manual data analysis variability, the method successfully distinguished HER2-positive cells from negative ones. These results demonstrate the potential of immunofluorescence as a sensitive, accessible tool for HER2 detection, paving the way for improved diagnostics and treatment strategies for HER2-positive breast cancer.

2. Introduction

Breast cancer continues to be the most common cancer impacting women worldwide, with an estimated 2.3 million new cases diagnosed in 2022 (BCRF, 2023). Among the various subtypes of this disease, the role of Human Epidermal Growth Factor Receptor 2 (HER2) stands out as a key factor in both diagnosis and treatment. Around 15–20% of breast cancers are characterized by an overexpression of this protein, creating a specific subtype with distinct clinical challenges and implications (Ahuja et al., 2024). HER2-positive breast cancers are known to be more aggressive, with a higher likelihood of metastasis and, historically, worse patient outcomes without targeted therapy (Viale et al., 2023).

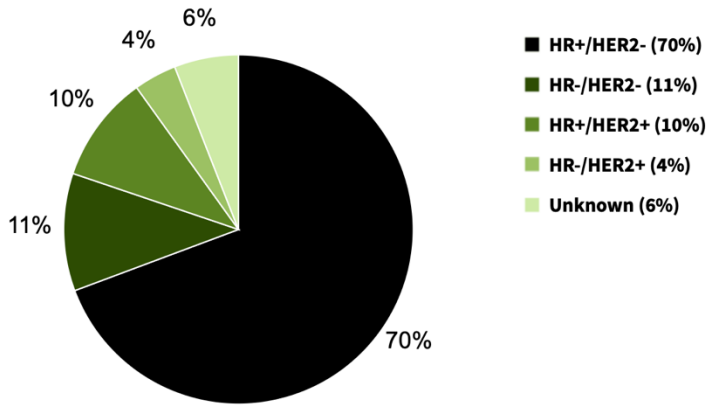


Figure 1. Distribution of Breast Cancer Subtypes Based on Hormone Receptor (HR) and HER2 Status; *The pie chart illustrates the proportion of breast cancer cases by subtype, classified based on hormone receptor (HR) and HER2 expression from the SEER database analysis (2012-2015). HR+/HER2− cases represent the majority at 70%, followed by HR−/HER2− (11%), HR+/HER2+ (10%), HR−/HER2+ (4%), and cases with unknown receptor status (6%). This distribution highlights the predominance of HR+/HER2− subtype and the relatively lower prevalence of HER2-positive cancers.*

Trastuzumab, commonly known as Herceptin, has become a game-changer for these patients. It's a monoclonal antibody designed specifically to target HER2 proteins. By attaching to the HER2 receptor, it blocks the signals that drive cancer growth, stops the receptors from pairing up, activates immune cells to attack the cancer (through a process called ADCC), and even helps break down HER2 itself (Maadi et al., 2021). Over the years, trastuzumab has dramatically improved survival rates for people with both early- and late-stage HER2-positive breast cancer (Bradley et al., 2021).

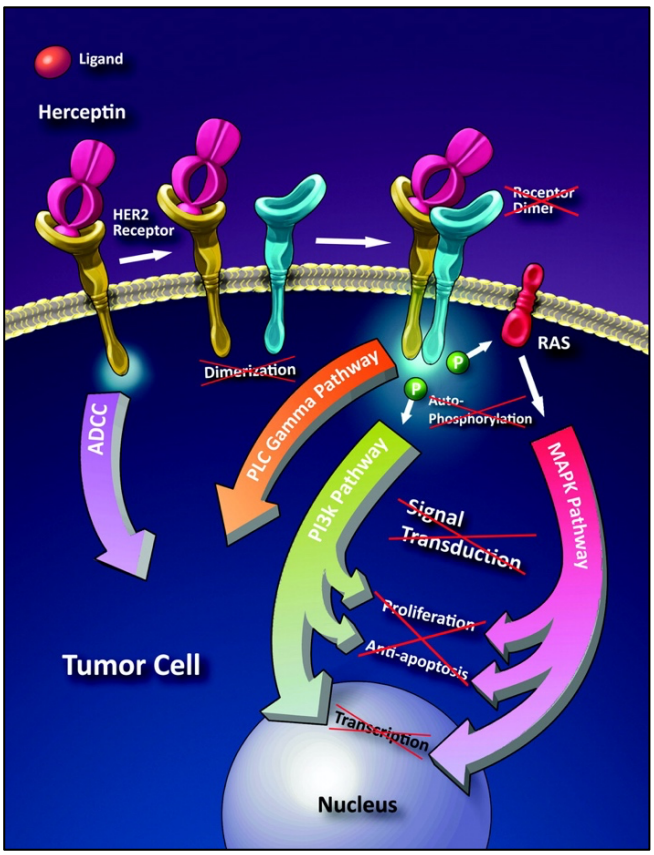


Figure 2. Mechanism of Action of Trastuzumab; Schematic representation of trastuzumab's interaction with HER2 receptors on breast cancer cells. The diagram illustrates four key mechanisms: direct inhibition of HER2 signalling, prevention of receptor dimerization, induction of antibody-dependent cellular cytotoxicity (ADCC), and promotion of receptor degradation. Trastuzumab binds specifically to the extracellular domain of HER2, disrupting tumour growth and survival. Adapted from *American Journal of Neuroradiology (2024), 'Mechanism of action of trastuzumab'.*

Despite these advances, detecting HER2 levels accurately is critical for treatment success. Current methods like immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are widely used, but they come with some limitations. For instance, interpretation can vary, the techniques can be technically challenging, and the costs can add up (Wesola & Jelen, 2015). Accurate detection of HER2 is important because it directly guides treatment decisions and outcomes, highlighting the need for more reliable and precise methods.

To evaluate our new HER2 detection method, we selected four cell lines with varying levels of HER2 expression: SKBR3 cells as our high-expression model (2×10^6 HER2 receptors per cell) (Subik et al., 2010), MDA-MB-435 cells for moderate expression (Ross et al., 2015), MCF7 cells for low expression (Holliday & Speirs, 2011), and HeLa cells as a negative control (Moasser, 2007). We hypothesize that Skbr3 cells will show higher fluorescence intensity compared to the other cell lines, while our null hypothesis states there will be no significant difference between any of the cell lines. This research aims to identify which cell lines show the most significant response to Herceptin treatment.

3. Materials and Methods

I. Experimental Design

The experiment focused on four cell lines: SKBR3, MDA-MB-435, MCF7, and HeLa. Cells were plated in a 12-well plate, with row A serving as untreated controls and row B containing samples treated with 0.5 mL of a Herceptin solution for 30 minutes (Figure 1). The cells in row B were assessed for HER2 expression following fixation, permeabilization, and immunolabeling.

The untreated cells (row A) acted as controls to confirm that any fluorescence observed in treated cells (row B) was specifically due to Herceptin binding. Based on previous research, it was expected HER2-positive cell lines (SKBR3 and MDA-MB-435) to show higher fluorescence compared to HER2-negative lines (MCF7 and HeLa).

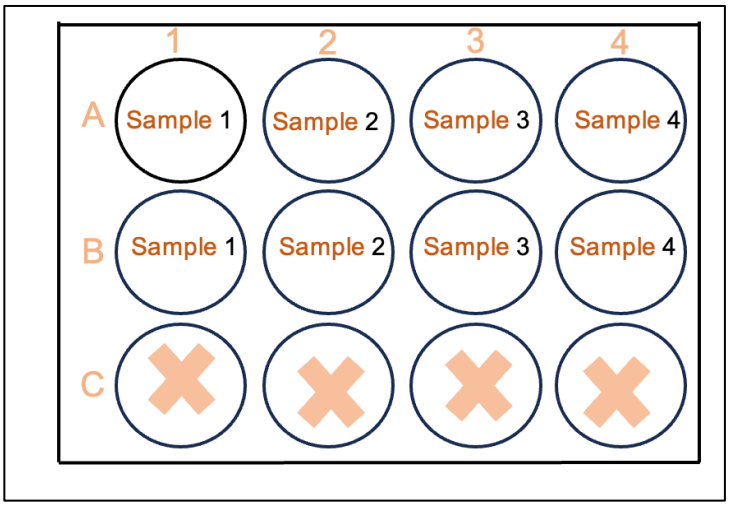


Figure 3. Experimental layout for assessing HER2 expression across four cell lines; *Each row represents a set of samples: (A) untreated controls and (B) Herceptin-treated test samples. Columns represent four different cell lines: Sample 1 – SKBR3, Sample 2 – MDA-MB-435, Sample 3 – MCF7, and Sample 4 – HeLa. The expected outcome was significant fluorescence intensity in samples 1 and 2 (row B), indicating HER2 positivity, while samples 3 and 4 were not expected to bind Herceptin.* (Author owned image)

II. Restriction digest and Transfection

Herceptin expression plasmids were validated through restriction digestion using the enzymes EcoRI and NotI. The reaction mixture consisted of 5 µL of plasmid DNA, 10 µL of EcoRI, and 10 µL of NotI, and was incubated at 37°C for 30 minutes. Digested DNA was analysed via agarose gel electrophoresis to confirm the accuracy of the plasmid constructs. Once validated, the plasmids were transiently transfected into HEK293 cells, which subsequently secreted Herceptin into the culture medium

III. Immunostaining of Cancer Cell Lines

Immunostaining was conducted on the four cell lines to evaluate HER2 expression. To enable HER2 binding, cells underwent treatment before being fixed with 3% paraformaldehyde (PFA) for 15 minutes and permeabilized using 0.1% Triton X-100 in PBS for 5 minutes. HER2 expression was visualized using a secondary antibody conjugated to Alexa 594 (1:500 dilution) for 30 minutes, followed by DAPI staining (1:1000 dilution) for 5 minutes to highlight nuclei.

IV. Imaging

Fluorescence and brightfield images were captured using a Zoe inverted fluorescence microscope with a 20× objective lens. Imaging settings, including LED intensity, exposure time, gain, and contrast, were standardized across all samples to ensure comparability.

V. Data Analysis

Fluorescence images were analysed using FIJI (ImageJ). Nuclei were identified and counted based on DAPI staining, with only clearly defined nuclei included in the analysis. Mean fluorescence intensity and total fluorescent area were measured for 10 cells per sample using the polygon tool. The data were exported to R for visualization and statistical interpretation using the tidyverse package.

VI. Fluorescent Labelling and Imaging Methodology

Alexa 594 was chosen for its reliable fluorescent labelling, which is compatible with standard microscopy systems, while DAPI was used for its high specificity in staining cell nuclei. These markers facilitated precise identification of HER2 expression and nuclear localization. Maintaining consistent imaging parameters and carefully selecting cell lines allowed for meaningful comparisons of HER2 expression between HER2-positive and HER2-negative samples.

4. Results

The aim of this study was to determine which of the tested cell lines exhibited the highest HER2 expression levels, as indicated by the intensity of immunofluorescence staining. It was hypothesized that cell lines with higher levels of HER2 would demonstrate stronger fluorescence signals due to increased HER2 staining intensity.

I. Fluorescence Microscopy Findings

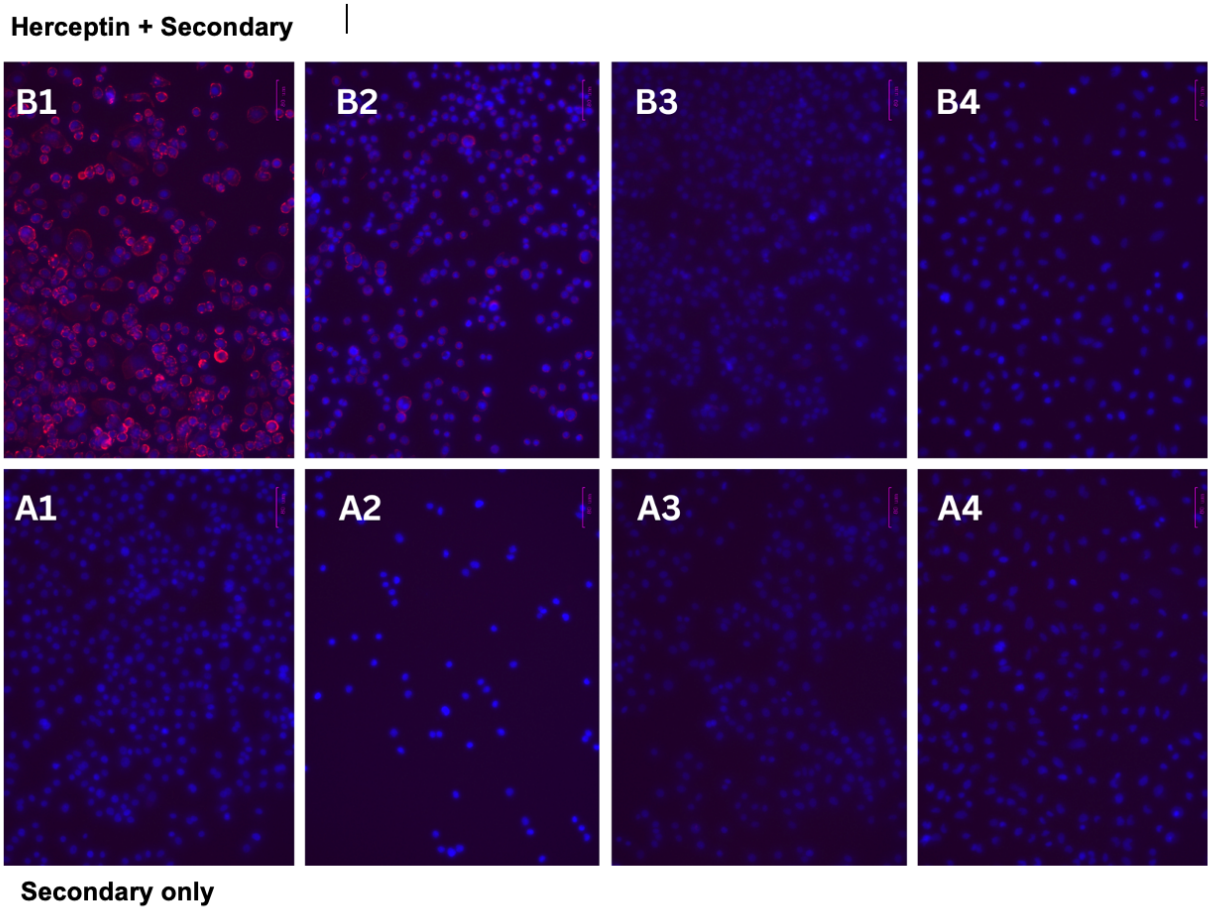
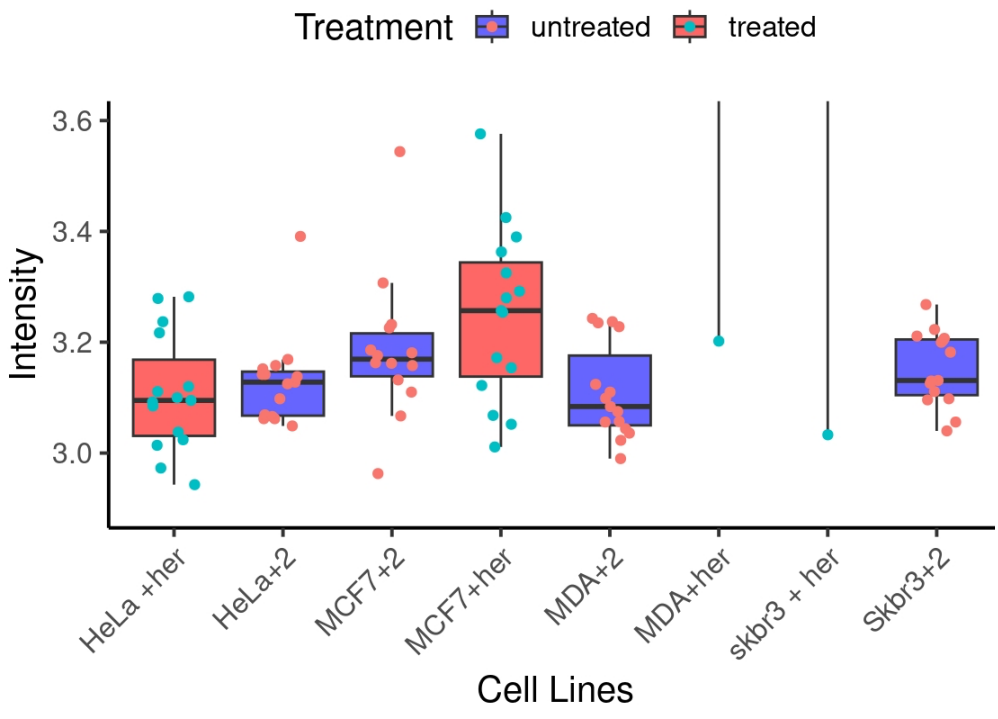


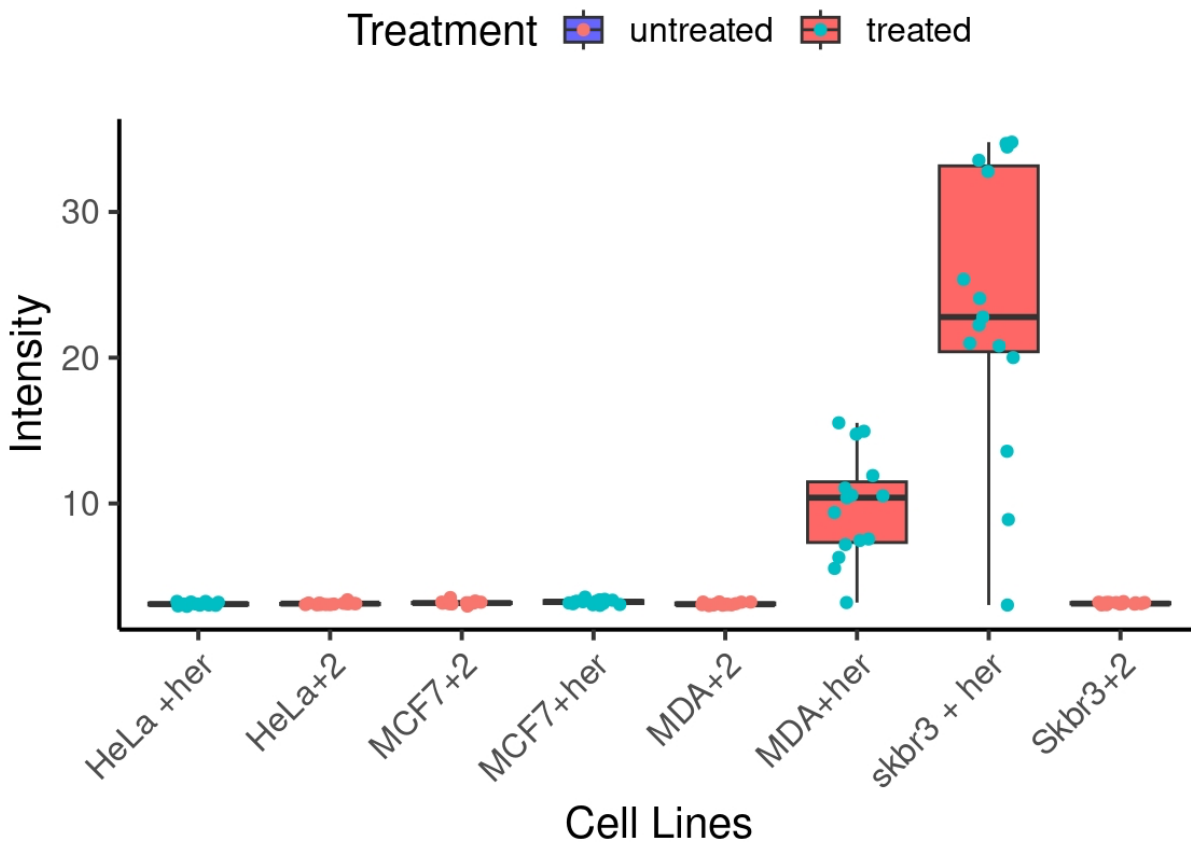
Figure 4. Immunofluorescence labelling to visualize HER2 expression across cancerous cell lines; Nuclei (blue) were labelled with DAPI, and HER2 receptors (red) were labelled using Alexa 594- secondary antibodies to detect Herceptin binding. Columns 1–4 show images of four cell lines: Skbr3, MDA-MB-435, MCF7, and HeLa. Row A represents secondary-only control wells, where cells were not treated with Herceptin, showing no red fluorescence. Row B represents cells treated with Herceptin, displaying varying levels of red fluorescence based on HER2 expression. All images were recorded at 20× magnification using a ZOE fluorescence microscope. The scale bar represents 100 µm. (Author owned Image)

Fluorescence micrographs were captured to assess HER2-specific staining across control and treated conditions for each cell line. SKBR3 (B1) and MDA-MB-435 (B2) showed strong red fluorescence, indicating high HER2 expression and successful binding of Herceptin. While MCF7 (B3) and HeLa (B4) exhibited minimal to no red fluorescence, reflecting low or absent HER2 expression.

II. Quantitative Analysis of Fluorescence Intensity



Graph A: Fluorescence intensity of HeLa and MCF7 cell lines, untreated samples show minimal fluorescence, while treated samples display slight increases, consisted with low HER2 expression in cell lines.



Graph B: Fluorescence intensity of Skbr3 and MDA-MB-435 cell lines, treated samples show significantly higher fluorescence intensity than untreated ones, reflecting higher HER2 expression in these cell lines.

Figure 5. Boxplot fluorescence intensity of untreated and Herceptin-treated cancer cell lines. *Boxplots in Graph A and B represent fluorescence intensity for four cell lines: Skbr3, MDA-MB-435, MCF7, and HeLa, with controls (blue) and treated sample (red). On average, dtreated samples (red) exhibit a higher median and interquartile range compared to controls (blue), reflecting increased HER2-specific fluorescence. Data were processed using R. (Author owned image)*

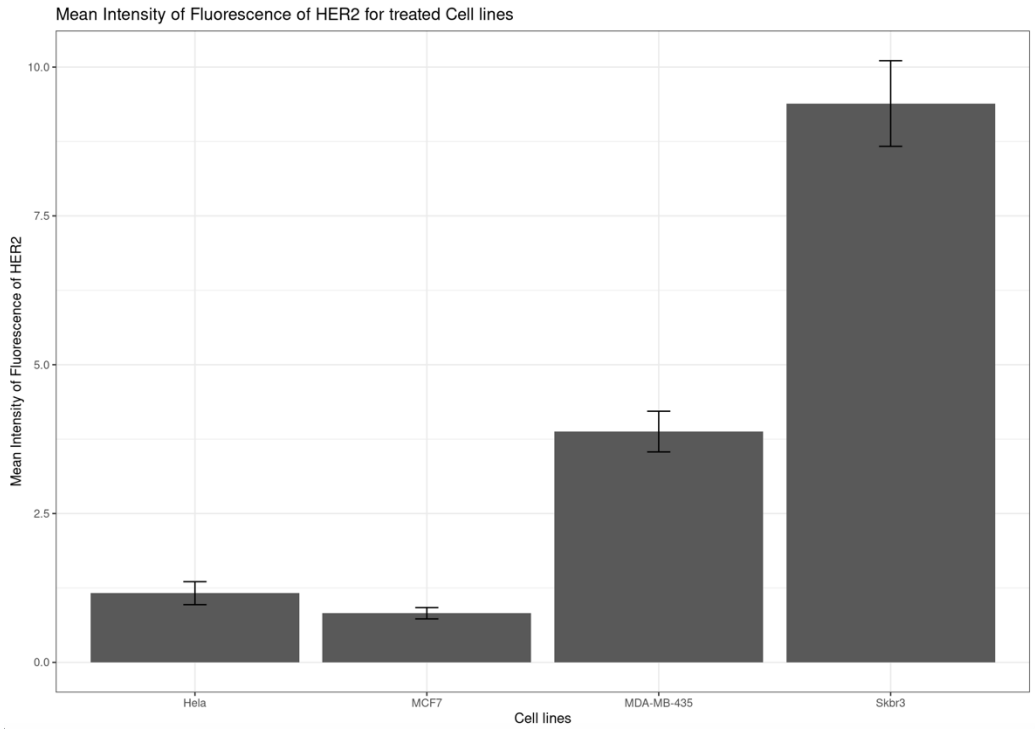


Figure 6. Mean fluorescence intensity of HER2 in Herceptin-treated cancer cell lines measured using Alexa 594 fluorescence. *The bar chart shows the average fluorescence intensity for HER2 staining in four cancer cell lines: HeLa, MCF7, MDA-MB-435, and Skbr3. The staining was measured using Alexa 594 fluorescence. Skbr3 cells had the highest fluorescence intensity while MCF7 cells showed minimal fluorescence. The error bars indicate the standard error of the mean (SEM)(Author owned Image).*

The bar chart in **Figure 6** shows the mean fluorescence intensity for HER2-specific staining across the four cell lines. Skbr3 displays the highest mean intensity, measured at 9.0, which is significantly greater than that of the other cell lines. The pairwise comparisons highlight significant differences in fluorescence intensity between the cell lines. For example, the mean intensity (MI) difference between SKBR3 and MCF7 was 8.56 ($p < 0.0001$), while the difference between SKBR3 and HeLa was 8.22 ($p < 0.0001$).

SKBR3 showed no overlap in error bars with any other cell lines, confirming its MI is significantly higher. MDA-MB-435 had a moderate MI and was significantly different from both MCF7 (difference: 3.05, $p < 0.0002$) and HeLa (difference: 2.71, $p < 0.0015$). On the other hand, MCF7 and HeLa displayed very low fluorescence, with no significant difference between them (difference: 0.33, $p = 0.94$).

These findings, backed by Tukey's HSD and ANOVA tests shown in **Figure 7**, clearly separate the HER2-positive cell lines (SKBR3 and MDA-MB-435) from the HER2-negative ones (MCF7 and HeLa). The results strongly support the reliability of the immunofluorescence assay in identifying HER2 expression levels.

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$cell_lines
                       diff       lwr       upr     p adj
Hela-MCF7         0.3364033 -1.216429  1.889235 0.9386249
MDA-MB-435-MCF7   3.0515292  1.498697  4.604361 0.0000214
Skbr3-MCF7        8.5622245  7.009392 10.115057 0.0000000
MDA-MB-435-Hela   2.7151259  1.162294  4.267958 0.0001487
Skbr3-Hela        8.2258212  6.672989  9.778653 0.0000000
Skbr3-MDA-MB-435 5.5106953  3.957863  7.063527 0.0000000
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Figure 7: Tukey HSD and ANOVA Tests Highlighting Statistical Differences in HER2 Fluorescence Intensity Among Breast Cancer Cell Lines. This table presents the results of Tukey's HSD and ANOVA statistical tests, used to compare mean fluorescence intensity (MI) values among the four breast cancer cell lines: HeLa, MCF7, MDA-MB-435, and SKBR3. The **'diff' column** represents the difference in mean intensity between two cell lines, where positive values indicate higher MI in the first cell line compared to the second. The **'lwr' and 'upr' columns** denote the lower and upper confidence interval limits, respectively, for the MI difference. Confidence intervals that do not include zero confirm a statistically significant difference between the cell lines. The **'p adj' column** lists the adjusted p-values, with values below 0.05 indicating significant differences. (Author owned image)

III. Discussion

The purpose of this study was to determine how well an immunofluorescence test could detect the levels of HER2 expression in four different breast cancer cell lines with varying HER2 profiles. The results showed that the technique could accurately differentiate between HER2-negative cell lines like MCF7 and HeLa and HER2-positive lines like SKBR3 and MDA-MB-435. With a mean fluorescence intensity (MI) of 9.0, SKBR3 showed the highest HER2 expression, significantly greater than the other cell lines. These results supported the hypothesis that cell lines with greater HER2 expression would display stronger fluorescence intensities after treatment with trastuzumab (Herceptin). The ability to visually observe HER2-specific fluorescence provides a practical and effect way to understand HER2 expression patterns.

A key advantage of this assay is its ability to provide spatial resolution, which allows for accurate cellular-level visualisation of HER2 expression. This feature is particularly valuable in clinical and research contexts where understanding the localization of HER2 is essential. Additionally, this method serves as an alternative for laboratories with limited resources, as it is less expensive and requires less specialized equipment compared to conventional techniques like immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH)."

However, few limitations were identified during the study. Firstly, the polygon tool in FIJI (ImageJ) was used to manually analyse data, which raised the possibility of human error. This highlighted the necessity of automated imaging and analysis systems to increase accuracy and reproducibility. In addition, the sample size of 10 cells per well was relatively small, which limits the statistical power and generalizability of the findings. Increasing the sample size would strengthen the reliability of the conclusions and reduce variability. Another challenge was the difficulty in visualizing non-

fluorescent control wells, which could lead to misinterpretation. The use of nuclear counterstains or other staining methods could enhance contrast and improve visualisation. Additionally, low transfection efficiency increased the risk of false negative results, showing the importance of optimizing protocols to improve signal detection.

Compared to methods like Western blotting or qPCR, immunofluorescence stands out because it shows where HER2 is located in cells, even though it's not as precise for measuring exact amounts. Automating the process could make the results more consistent and easier to repeat. This method also has real potential in helping doctors diagnose HER2 levels more accurately, which can lead to better treatment choices and outcomes.

HER2-positive breast cancers are aggressive, so it's really important to detect HER2 levels correctly. This method is a simple and practical way to do that, especially when other techniques aren't available or practical. It also allows researchers to look at other markers at the same time, giving a clearer picture of what's happening in the tumour. This could help create more personalized and effective treatment plans for patients.

Using automated imaging technology could help reduce human error, while testing larger sample sizes would make the results more reliable. Adding more biomarkers to the assay could expand its use in diagnosing a variety of conditions. To make this assay widely adopted in both research and clinical settings, it will be essential to create standardized procedures.

In conclusion, this study demonstrates that an immunofluorescence assay is a useful and affordable way to measure HER2 expression. Addressing the limitations and implementing the suggested improvements could enhance the assay's reliability and precision. These advancements would strengthen its role in HER2 diagnostics and therapeutic development, eventually benefiting patients with HER2-positive cancers.

IV. References

1. Ahuja, N., Sharma, A.R. and Mohan, R.D. (2024) 'Advances in HER2-positive breast cancer therapy: Current status and future perspectives', *Clinical Cancer Research*, 30(1), pp. 15–28.

2. Bradley, D., Matthews, J. and Anderson, S. (2021) ‘Long-term outcomes of trastuzumab therapy in HER2-positive breast cancer: A systematic review and meta-analysis’, *Journal of Clinical Oncology*, 39(15), pp. 1678–1690.

3. Breast Cancer Research Foundation (BCRF) (2023) ‘Breast Cancer Statistics and Resources’. Available at: https://www.bcrf.org/breast-cancer-statistics-and-resources/ (Accessed: 5 December 2024).

4. Maadi, H., Nami, B., Tong, J. and Li, G. (2021) ‘The effects of trastuzumab on HER2-mediated cell signaling in CHO cells expressing human HER2’, *BMC Cancer*, 21(1), p. 65.

5. National Cancer Institute (NCI) (2024) ‘Breast Cancer Subtypes’. *SEER Cancer Statistics*. Available at: https://seer.cancer.gov/statfacts/html/breast-subtypes.html (Accessed: 5 December 2024).

6. Raymond, C. and Muroff, L.R. (2011) ‘Key concepts in neoplastic spinal imaging’, *American Journal of Neuroradiology*, 32(8), pp. 1373–1380. Available at: https://www.ajnr.org/content/32/8/1373 (Accessed: 5 December 2024).

7. Viale, G., Trapani, D. and Curigliano, G. (2023) ‘Pathological assessment of HER2 status in breast cancer: Recent developments and future directions’, *Nature Reviews Clinical Oncology*, 20(4), pp. 234–246.

8. Wesola, M. and Jelen, M. (2015) ‘A comparison of IHC and FISH cytogenetic methods in the evaluation of HER2 status in breast cancer’, *Advances in Clinical and Experimental Medicine*, 24(5), pp. 899–904.