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Cytoplasmic and Nuclear HER4 Expression in HER2 Negative Breast Cancer Cell Lines

(Sitoplasma dan Pengekspresan Nuklear HER4 pada Titisan Sel Kanser Payudara HER2 Negatif)

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ABSTRACT

HER4 cleavage and its subcellular localisations have been previously proven to mediate anti-HER2 resistance. The description of the HER4 subcellular localisations in HER2 negative breast cancer, on the other hand, is incomplete. The objective of this study was to determine the cytoplasmic and nuclear expression of HER4 in HER2 negative breast cancer cell lines in order to gain a better understanding of the key features of HER4 signalling in the context of anti-HER2 resistance. MCF-7 and MDA-MB-231 cells were cultured for 48 h at 37 °C and 5% CO_2 in fresh DMEM medium with 10% foetal bovine serum and 1% penicillin-streptomycin. The western blot analysis for HER4 protein was done on cytoplasmic and nuclear extracts that had been obtained previously using the NE-PER Nuclear and Cytoplasmic Extraction Kit. Cytoplasmic and nuclear HER4 proteins were expressed in a variety of sizes, including 120 kDa, 55 kDa, 50 kDa, and 43 kDa. MCF-7 cells expressed significantly more cytoplasmic $HER4_{120kDa}$ than MDA-MB-231 cells. MCF-7 exhibited a single nuclear HER4 variant with a molecular weight of 50 kDa, whereas MDA-MB-231 expressed two nuclear HER4 variants with molecular weights of 50 kDa and 43 kDa. In comparison to MDA-MB-231 cells, MCF-7 cells exhibit higher level of cytoplasmic $HER4_{120kDa}$ with positive nuclear $HER4_{50kDa}$ expression. Due to the presence of ER and PR in MCF-7, it is important to investigate if the interaction of these HER4 variants with ER and PR confers resistance to anti-HER2 treatment on breast cancer. Meanwhile, results from MDA-MB-231 cells indicate that nuclear HER4 contributes to the development of TNBC.

Keywords: Breast cancer; cytoplasmic HER4; HER2 negative; nuclear HER4

ABSTRAK

Pembelahan HER4 dan pensetempatan subselnya telah dibuktikan mempengaruhi kerintangan anti-HER2. Namun, penerangan mengenai pensetempatan subsel HER4 dalam barah payudara negatif HER2 masih tidak lengkap. Tujuan kajian ini adalah untuk menentukan pengekspresan sitoplasma dan nuklear HER4 dalam barisan sel barah payudara HER2 negatif untuk mendapatkan pemahaman yang lebih baik mengenai ciri utama isyarat HER4 dalam konteks ketahanan anti-HER2. Sel MCF-7 dan MDA-MB-231 dikultur selama 48 jam pada suhu 37 °C dan 5% CO_2 dalam media DMEM segar dengan 10% serum anak lembu dan 1% penisilin-streptomisin. Analisis pemblotan western untuk protein HER4 dilakukan pada ekstrak sitoplasma dan nuklear yang telah diperoleh sebelumnya menggunakan Kit Ekstraksi Nuklear NE-PER dan Sitoplasma. Protein sitoplasma dan nuklear HER4 diekspreskan dalam pelbagai ukuran, termasuk 120 kDa, 55 kDa, 50 kDa dan 43 kDa. Sel-sel MCF-7 mengekspreskan lebih banyak sitoplasma $HER4_{120kDa}$ daripada sel MDA-MB-231. MCF-7 mempamerkan varian HER4 nuklear tunggal dengan berat molekul 50 kDa, sedangkan MDA-MB-231 menyatakan dua varian HER4 nuklear dengan berat molekul 50 kDa dan 43 kDa. Dibandingkan dengan sel MDA-MB-231, sel MCF-7 menunjukkan tahap sitoplasma $HER4_{120kDa}$ yang lebih tinggi dengan mengekspreskan $HER4_{50kDa}$ nuklear yang positif. Oleh kerana kehadiran ER dan PR dalam MCF-7, penting untuk mengkaji apakah interaksi varian HER4 ini dengan ER dan PR dalam menyebabkan kerintangan terhadap rawatan anti-HER2 pada barah payudara. Sementara itu, keputusan pada kajian sel-sel MDA-MB-231 menunjukkan bahawa HER4 nuklear menyumbang kepada perkembangan TNBC.

Kata kunci: HER4 nuklear; HER4 sitoplasma; kanser payudara; negatif HER2

Introduction

Human epidermal growth factor receptor 4 (HER4) is the fourth member of the human epidermal growth factor receptor (hEGFR) family, which signals through classical receptor tyrosine kinase-activated signalling cascades. In comparison to other members of the hEGFR family, the involvement of HER4 in breast cancer is elusive (Brockhoff 2019). A two-step proteolytic cleavage is required for receptor signalling. When a disintegrin and metalloproteinase (ADAM) 17 cleaves HER4, the extra and intradomains are split, and γ-secretase subsequently degrades the HER4 intracellular domain to produce 80-kDa HER4 intracellular domain (Miller et al. 2017; Strunk et al. 2007). To determine if HER4 can contribute as a significant diagnostic biomarker for breast cancer patients, further research is necessary to gain a better understanding of the receptor's properties, including its subcellular localization.

Moreover, it has been demonstrated earlier that HER4 cleavage and its subcellular fractions play a role in mediating anti-HER2 resistance. Herceptin, a monoclonal antibody directed against HER2, triggers the release of ADAM 17, the enzyme responsible for HER4 cleavage (Feldinger et al. 2014). It was also shown that preventing nuclear translocation with a γ-secretase inhibitor (GSi) improves Herceptin responsiveness (Mohd Nafi et al. 2014). However, the involvement of various HER4 subcellular fractions should be studied further to lay a greater emphasis on HER4's role in developing anti-HER2 resistance. Therefore, this study examined the protein expression of cytoplasmic and nuclear HER4 in HER2 negative breast cancer subtypes. We hope that this discovery will pave the way for a better understanding of certain fundamental features of HER4 signalling in the context of drug resistance.

Materials and Methods

Cell Culture

MCF-7 and MDA-MB-231 cells were employed in this study as HER2-negative breast cancer cell lines. These cells were kindly provided by Dr Noor Fatmawati of INFORMM Laboratory (USM). Cell culture was carried out for six weeks in accordance with a previously published procedure (Mohd Nafi et al. 2014). Initially, cells were cultured for 48 hours at 37 °C and 5% CO_2 in fresh Dulbecco's Modified Eagle Medium (DMEM; Gibco, cat. no. 11965-118) containing 10% foetal bovine serum (FBS; Gibco, cat. no. 10270-098) and 1% penicillin-streptomycin (Pen Strep; Gibco, cat. no. 15140-122). Following that, cells were passaged using 0.25 percent trypsin/EDTA (Gibco, cat. no. 25200-056) at 70-80% confluency. A western blot analysis was performed on both kinds of cells at passages 4-8.

Subcellular Protein Extraction and Quantification

The NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific™, cat. no. 78833) was used to separate subcellular proteins from MCF-7 and MDA-MB-231 cell lines. To maintain the target protein's integrity, a protease inhibitor was added to cytoplasmic extraction reagent I (CER I) and nuclear extraction reagent (NER). Prior to centrifugation at 4 °C, cells in a petri dish were detached using 0.25% trypsin-EDTA. To extract the cell pellet, the supernatant was discarded. Following that, 200l of CER I was added and vortexed rapidly to suspend the cell pellet. After 10 min on ice, 11 µL cytoplasmic extraction reagent II (CER II) was added. For 5 min, the mixture was centrifuged at 14,000 × g. The supernatant containing cytoplasmic protein was transferred to a new 1.5 mL tube. The pellet was then treated with 100 µL of NER reagent to extract nuclear protein.

For a total of 40 min, the mixture was incubated on ice and vortexed every 10 min. After 40 min, the tube was centrifuged for 10 min at 14,000 × g. The supernatant containing nuclear protein was discarded and the pellet was transferred to fresh tubes. Protein concentrations were determined in cytoplasmic and nuclear extracts using the Quick Start™ Bradford 1 × Dye Reagent (Bio-Rad, cat. no.5000202). Varioskan Flash Multimode Reader (Thermo Scientific™, cat. no. 5250030) was used to determine the protein absorbance at 595 nm. Standardisation of the proteins was performed using the absorbance value obtained. The absorbance with the lowest value was used to normalise the protein. A RIPA lysis buffer (Thermo Scientific™, cat. no. 89900) was added to modify the volume of the protein. The nuclear and cytoplasmic proteins were stored at -80 °C until further usage.

Western Blot

Ten microlitres of sample buffer containing β-mercaptoethanol (Sigma Aldrich, cat. no.M6250) was added to 30 µL of protein sample. The mixture was heated at 70 °C for 10 min. Proteins were resolved using precast NuPAGE™ 4-12% Bis-Tris gel (Invitrogen™, cat. no. NP0321). Electrophoresis on gels was performed at a constant voltage of 120 V. The iBlot Dry Blotting System

(Invitrogen™, cat. no. IB21001) was used to transfer proteins onto the PVDF membrane. Immunoblotting was carried out utilising a sequential lateral flow technique, namely the iBind western device (Invitrogen™, cat. no. SLF10004PK). The membrane and the iBind card were put together and placed on the platform of the device. Membranes were blocked using a 1X solution of iBind (Invitrogen™, cat. no. SLF1020).

Primary antibodies were incubated at room temperature for 1 h at the manufacturer's recommended concentration (Table 1). The membranes were then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Table 1). Proteins were detected by using an enhanced chemiluminescence method with Amersham ECL (GE Healthcare, cat. no. RPN2232) and visualized using the Fusion-FX7 imaging system (Vilber, cat. no. 151102001). The membranes were exposed at various times and the scans of shorter exposures were captured. Images were analyzed using ImageJ to quantify band intensities by volume/area integration.

STATISTICAL ANALYSIS

The amount of HER4 protein in cells was normalized to their β-actin and histone levels. Results were presented as mean ± standard error (SE) protein expression. We performed one-way statistical analysis ANOVA by GraphPad Prism to compare the relative protein expression of HER4 in MCF-7 and MDA-MB-231 cells. All p values of 0.05 or below were considered statistically significant.

TABLE 1. The dilution factor for primary and secondary antibodies

Antibody	Manufacturer, catalogue number	Dilution (in a 1X iBind solution)
Rabbit polyclonal HER4	(Thermo Fisher Scientific; PA5-32347)	1:25
Rabbit polyclonal β Actin	(Invitrogen™; PA1-183)	1:1000
Rabbit polyclonal Histone	(Abcam; Ab171870)	1:1000
Goat anti-Rabbit IgG Secondary Antibody, HRP (Horseradish Peroxidase) conjugate	(Invitrogen™; 65-6120)	1:4000

RESULTS AND DISCUSSION

MCF-7 cells were described as being negative for HER2, but positive for ER and PR, whilst MDA-MB-231 cells were described as being negative for HER2, ER, and PR (Flodrova et al. 2016). Moreover, MCF-7 and MDA-MB-231 cells are often used in breast cancer research as non-invasive and aggressive metastasis models, respectively (Gomes et al. 2015). It has been demonstrated that variations in the expression of breast cancer receptors have an effect on medication sensitivity (Lovitt et al. 2018). The purpose of this work was to examine HER4 subcellular expression in two HER2-negative breast cancer cell lines, MCF-7 and MDA-MB-231, in order to gain a better understanding of the key features of HER4 signaling in the setting of anti-HER2 resistance.

It appears that HER4, which is located in the nucleus or cytoplasm, has a substantial impact on drug effectiveness for breast cancer (Brockhoff 2019). Tamoxifen appears to be partly dependent on the intracellular cytoplasmic HER4 for its anti-estrogen activity (Gothlin Eremo et al. 2015), whereas Herceptin's efficacy in HER2 positive breast tumours appears to be dependent on nuclear HER4 localisation (Mohd Nafi et al. 2014). It was found that heregulin, a HER4 ligand, induces HER4 nuclear translocation in breast cancer cells treated with Herceptin and that inhibiting γ-secretase, an enzyme involved in HER4 cleavage, reduces HER4 nuclear translocation and enhances Herceptin sensitivity (Mohd Nafi et al. 2014). Though HER4 has been implicated in an anti-HER2 resistance mechanism (Mohd Nafi et al. 2014), the study did not examine HER4 subcellular expression in breast cancer expressing HER2 in a negative manner.

It was discovered years ago that the full-length HER4 receptor is 180 kDa in size, while HER4 cleaved

fragments are 120 kDa and 80 kDa in size (Rio et al. 2000). Additional experiments indicated that HER4 products had molecular weights of 146 kDa, 110 kDa, and 43 kDa (Gothlin Eremo et al. 2015; Mohd Nafi et al. 2014). Based on the findings, the range of HER4 molecular sizes observed was characterised by different antibodies and cell types used. In our study, two proteins at 120 kDa and 55 kDa were detected in the cytoplasmic region of HER4 in MDA-MB-231 and MCF-7 (Figure 1(A)). Previously, HER4 protein at 120kDa was identified in two HER2 positive breast cancer cell lines, SKBR3 and BT474, and it was shown to be specific for HER4 by the siRNA experiment (Mohd Nafi et al. 2014). Additionally, 180 and 80 kDa HER4 proteins were previously identified in SKBR3 and BT474 cells (Mohd Nafi et al. 2014), but these variants were not found in MCF7 or MDA-MB-231 cells in our investigation (Figure 1(A)).

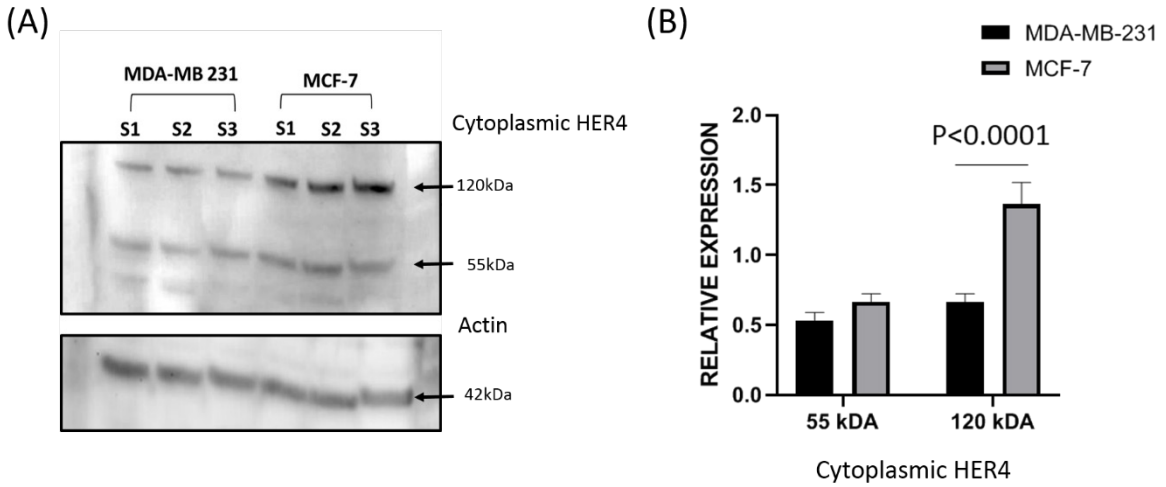

FIGURE 1. Western blot analysis of cytoplasmic HER4 in MDA-MB-231 and MCF-7 cells (A) Representative western blot of cytoplasmic expression that was performed from three independent samples, labelled as S1-S3. Cytoplasmic extracts were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific™; 78833). The upper blot was incubated for one hour with HER4 rabbit polyclonal antibody (1:25; Thermo Fisher Scientific; PA5-32347). In the lower blot, β-actin (1:1000; Invitrogen™; PA1-183) was used as for normalization of protein loading. (B) Comparative densitometric analysis of the average expression of cytoplasmic HER4 relative to the intensity of β-actin. Results are presented as mean ± SE protein expression

As seen in Figure 1(B), MCF-7 produced more cytoplasmic HER4 proteins at 120 kDa and 55 kDa than MDA-MB-231. The expression of MCF-7 cytoplasmic $HER4_{55kDa}$ (0.67 ± 0.13) increased slightly in comparison to the MDA-MB 231 cytoplasmic $HER4_{55kDa}$ (0.53 ± 0.39), but the difference was not statistically significant (Figure 1(B)). Remarkably, the expression of cytoplasmic $HER4_{120kDa}$ (1.36 ± 0.44) in the MCF7 cells was significantly higher compared to cytoplasmic $HER4_{120kDa}$ (0.66 ± 0.95) in the MDA-MB-231 cells ($p<0.001$, Figure 1(B)). It is interesting to note that cytoplasmic $HER4_{120kDa}$ was shown to be higher in MCF-7 cells, which cells have been demonstrated to be less sensitive to Herceptin treatment (Kong et al. 2019). We therefore suggest that a future study could be carried out to characterize the role of the cytoplasmic HER4 variant at 120 kDa in the anti-HER2 resistance mechanism.

Nuclear HER4 protein was expressed in two sizes in MDA-MB-231 cell lines, 50 kDa and 43 kDa (Figure 2(A)). However, in MCF-7 cell lines, only one size of nuclear HER4 was detected at 50 kDa (Figure 2(A)). Despite the blots being exposed for a longer amount of time, no nuclear HER4 protein larger than 50kDa was produced (Figure 2(A)). The expression of nuclear HER4 at 50 kDa was 0.43 ± 0.51 in MDA-MB-231 cells, which was lower than the nuclear $HER4_{50\,kDa}$ (0.53 ± 0.38) found in MCF-7 cells (Figure 2(B)). Given that nuclear $HER4_{43kDa}$ was expressed positively in MDA-MB-231 cells (0.53 ± 0.29) but negative in MCF-7 cells, a further test should be performed to determine if this 43 kDa protein represents the nuclear HER4 fragment.

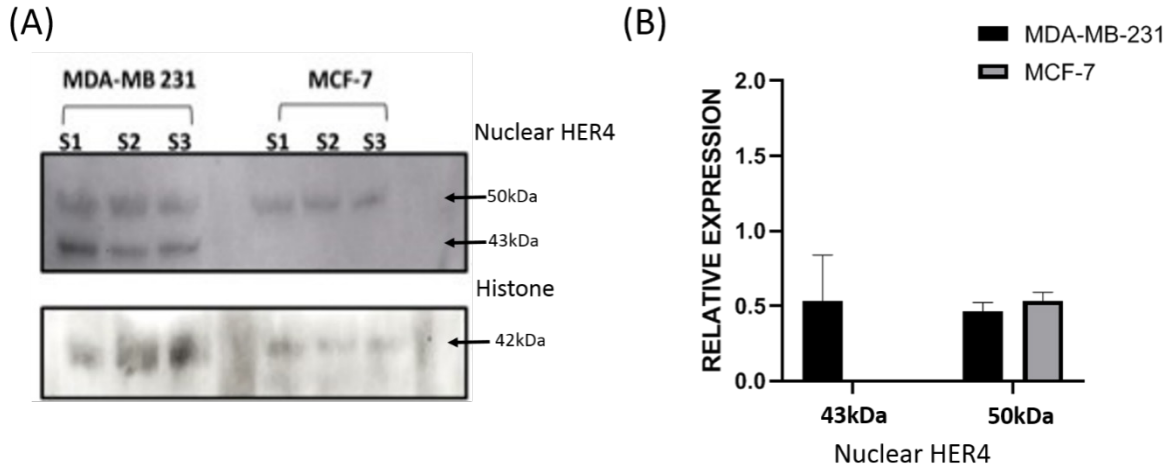

FIGURE 2. Western blot analysis of nuclear HER4 in MDA-MB-231 and MCF-7 cells (A) Representative western blot of cytoplasmic expression that was performed from three independent samples, labelled as S1-S3. Nuclear extracts were obtained using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific™; 78833). The upper blot was incubated for one hour with HER4 rabbit polyclonal antibody (1:25; Thermo Fisher Scientific; PA5-32347). In the lower blot, Histone (1:1000; Abcam; Ab171870) was used as for normalization of protein loading. (B) Comparative densitometric analysis of the average expression of nuclear HER4 relative to the intensity of Histone. Results are presented as mean ± SEM protein expression

Our work demonstrated the presence of a range of HER4 variants in the cytoplasmic and nuclear regions of MCF-7 and MDA-MB-231. As seen in Figure 1, MCF-7 cells that were resistant to Herceptin treatment had a significantly higher expression of cytoplasmic $HER4_{120kDa}$ in our study. In addition, nuclear HER4 with a 50kDa molecular weight was the single variant found in MCF7 (Figure 2). Given that MCF-7 expresses ER and PR, it is important to establish if the interaction of cytoplasmic $HER4_{120kDa}$ and nuclear $HER4_{50kDa}$ with the ER and PR contributes to breast cancer resistance to anti-HER2 treatment. It was shown that an interaction between HER4 and the ER impairs tamoxifen's binding to the ER that decreases therapy efficacy (Wege et al. 2018). Estradiol, a kind of oestrogen, was shown to enhance HER4 cleavage via ADAM-17 activation (Hollmén et al. 2012). Therefore, the function of ER and PR in HER4 subcellular translocation and the mechanism by which these interactions promote anti-HER2 resistance require additional elucidation.

MDA-MB-231 cells, in comparison to MCF-7, exhibited a low level of HER4 cytoplasmic expression and generated two distinct sizes of nuclear HER4 protein, 50 kDa and 43 kDa (Figure 2). The positive expression of nuclear HER4 variants in MDA-MB-231 cells with negative HER2, ER, and PR expression shows that nuclear HER4 may potentially play a role in promoting TNBC development. It was demonstrated that the presence of phospho-AKT (pAKT) was highly associated with HER4 expression in TNBC tissues (Hashimoto et al. 2014). In combination with Bcl-2 inhibitor, the HER4 inhibitor (neratinib) induced the death of HER2 + and TNBC breast cancer cells (Booth et al. 2018), highlighting that anti-HER4 therapy may be a viable alternative for TNBC.

Conclusion

To summarise, our findings showed a variety of HER4 variants in the cytoplasmic and nuclear compartments of MCF-7 and MDA-MB-231 cells. MCF-7 cells that were resistant to Herceptin therapy exhibited considerably greater expression of cytoplasmic $HER4_{120kDa}$ with positive expression of nuclear $HER4_{50kDa}$ as compared to MDA-MB-231 cells. Given that MCF-7 expresses ER and PR, it is important to establish if the interaction of these cytoplasmic and nuclear HER4 variants with ER and PR contributes to breast cancer resistance to anti-HER2 treatment. Furthermore, additional research is needed to determine how ER and PR promote HER4 cleavage and subcellular localization, which contribute to the establishment of anti-HER2 resistance. The presence

of two different sizes of nuclear HER4 protein in MDA-MB-231 cells, $HER4_{50kDa}$ and $HER4_{43kDa}$, suggests that nuclear HER4 may play a role in the development of TNBC.

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REFERENCES

Booth, L., Roberts, J.L., Avogadri-Connors, F., Cutler Jr., R.E., Lalani, A.S. Poklepovic, A. & Dent, P. 2018. The irreversible ERBB1/2/4 inhibitor neratinib interacts with the BCL-2 inhibitor venetoclax to kill mammary cancer cells. *Cancer Biol. Ther.* 19(3): 239-247. doi: 10.1080/15384047.2018.1423927.

Brockhoff, G. 2019. Target HER four in breast cancer? *Oncotarget* 10(34): 3147-3150. doi: 10.18632/oncotarget.26867.

Feldinger, K., Generali, D., Kramer-Marek, G., Gijsen, M., Ng, T.B., Wong, J.H., Strina, C., Cappelletti, M., Andreis, D., Li, J.L., Bridges, E., Turley, H., Leek, R., Roxanis, I., Capala, J., Murphy, G., Harris, A.L. & Kong, A. 2014. ADAM10 mediates trastuzumab resistance and is correlated with survival in HER2 positive breast cancer. *Oncotarget* 5(16): 6633-6646. doi: 10.18632/oncotarget.1955.

Flodrova, D., Toporova, L., Macejova, D., Lastovickova, M., Brtko, J. & Bobalova, J. 2016. A comparative study of protein patterns of human estrogen receptor positive (MCF-7) and negative (MDA-MB-231) breast cancer cell lines. *Gen. Physiol. Biophys.* 35(3): 387-392. doi: 10.4149/gpb_2016009.

Gomes, L.R., Fujita, A., Mott, J.D., Soares, F.A., Labriola, L. & Sogayar, M.C. 2015. RECK is not an independent prognostic marker for breast cancer. *BMC Cancer* 15: 660. doi: 10.1186/s12885-015-1666-2.

Gothlin Eremo, A., Tina, E., Wegman, P., Stal, O., Fransen, K., Fornander, T. & Wingren, S. 2015. HER4 tumor expression in breast cancer patients randomized to treatment with or without tamoxifen. *Int. J. Oncol.* 47(4): 1311-1320. doi: 10.3892/ijo.2015.3108.

Hashimoto, K., Tsuda, H., Koizumi, F., Shimizu, C., Yonemori, K., Ando, M., Kodaira, M., Yunokawa, M., Fujiwara, Y. & Tamura, K. 2014. Activated PI3K/AKT and MAPK pathways are potential good prognostic markers in node-positive, triple-negative breast cancer. *Ann. Oncol.* 25(10): 1973-1979. doi: 10.1093/annonc/mdu247.

Hollmén, M., Liu, P., Kurppa, K., Wildiers, H., Reinvall, I., Vandorpe, T., Smeets, A., Deraedt, K., Vahlberg, T., Joensuu, H., Leahy, D.J., Schöffski, P. & Elenius, K. 2012. Proteolytic processing of ErbB4 in breast cancer. *PLoS ONE* 7(6): e39413. doi: 10.1371/journal.pone.0039413.

Kong, X., Zhang, K., Wang, X., Yang, X., Li, Y., Zhai, J., Xing, Z., Qi, Y., Gao, R., Feng, X., Wang, J. & Fang, Y. 2019. Mechanism of trastuzumab resistance caused by HER-2 mutation in breast carcinomas. *Cancer Management and Research* 11: 5971-5982. doi: 10.2147/CMAR.S194137.

Lovitt, C.J., Shelper, T.B. & Avery, V.M. 2018. Doxorubicin resistance in breast cancer cells is mediated by extracellular matrix proteins. *BMC Cancer* 18(1): 41. doi: 10.1186/s12885-017-3953-6.

Miller, M.A., Sullivan, R.J. & Lauffenburger, D.A. 2017. Molecular pathways: Receptor ectodomain shedding in treatment, resistance, and monitoring of cancer. *Clin. Cancer Res.* 23(3): 623-629. doi: 10.1158/1078-0432.ccr-16-0869.

Mohd Nafi, S.N., Generali, D., Kramer-Marek, G., Gijsen, M., Strina, C., Cappelletti, M., Andreis, D., Haider, S., Li, J.L., Bridges, E., Capala, J., Ioannis, R., Harris, A.L. & Kong, A. 2014. Nuclear HER4 mediates acquired resistance to trastuzumab and is associated with poor outcome in HER2 positive breast cancer. *Oncotarget* 5(15): 5934-5949. doi: 10.18632/oncotarget.1904.

Rio, C., Buxbaum, J.D., Peschon, J.J. & Corfas, G. 2000. Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. *J. Biol. Chem.* 275(14): 10379-10387. doi: 10.1074/jbc.275.14.10379.

Strunk, K.E., Husted, C., Miraglia, L.C., Sandahl, M., Rearick, W.A., Hunter, D.M., Earp 3rd, H.S. & Muraoka-Cook, R.S. 2007. HER4 D-box sequences regulate mitotic progression and degradation of the nuclear HER4 cleavage product s80HER4. *Cancer Res.* 67(14): 6582-6590. doi: 10.1158/0008-5472.can-06-4145.

Wege, A.K., Chittka, D., Buchholz, S., Klinkhammer-Schalke, M., Diermeier-Daucher, S., Zeman, F., Ortmann, O. & Brockhoff, G. 2018. HER4 expression in estrogen receptor-positive breast cancer is associated with decreased sensitivity to tamoxifen treatment and reduced overall survival of postmenopausal women. *Breast Cancer Research* 20(1): 139. doi: 10.1186/s13058-018-1072-1.

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