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Analysis of the oncogenic cooperation between mutp53 and HER2 to propose mutp53 as a biomarker for HER2-positive breast cancer

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Abstract of the Thesis

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Breast cancer is the second deadliest cancer in the United States and is classified into four main subtypes including HER2-positive breast cancer. There have been a number of HER2-targeted therapeutic treatments that were discovered, but they are expensive and patients frequently exhibit a resistance for these treatments. To alleviate patients undergoing expensive therapy with the possibility of not benefitting from the treatment, it is important to understand the cooperation between HER2 and mutp53, a tumor suppressor gene that is mutated in the majority of breast cancers. Analysis of scientific articles illustrate that HER2 and mutp53 cooperate to promote tumorigenesis via the master transcriptional regulator of the heat shock response, HSF1 in HER2-positive breast cancer. Therefore, this HSFS1-mediated oncogenic cooperation between mutp53 and HER2 may amplify HER2 signaling and sensitize breast cancer

cells to HER2 targeted therapies. Thus, mutp53 can be used as a potential biomarker for successful treatment of HER2-positive breast cancer.

Dedication Page

I dedicate this thesis to the women in my family who have battled breast cancer.

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List of Abbreviations

mutp53—mutant p53

wtp53—wild type p53

DNA- deoxyribonucleic acid

ER—estrogen receptor

PR—progesterone receptor

HER2—human epidermal growth receptor 2

EGFR/ErbB—Epidermal growth factor receptor

PI3K—phosphoinositide-3-kinase

Akt—serine/threonine specific protein kinase

TKI—tyrosine kinase inhibitor

FDA—U.S. Food and Drug Administration

HSP—heat shock protein

HS—heat shock

HSE—heat shock element

HSF1—heat shock transcription factor 1

HSP90/HSP90α—heat shock protein 90

HSP70—heat shock protein 70

HSP110—heat shock protein 110

HSP27—heat shock protein 27

Chapter 1: Introduction

Breast cancer, according to the National Cancer Institute, is the second leading cause of cancer related deaths in the United States following closely behind lung cancer [1]. The estimated amount of women who are to be diagnosed with breast cancer in 2014 was estimated to be at 14%, which is equivalent to roughly 232,670 females [1]. It is apparent that since this disease is so prevalent and will impact numerous lives, it is essential that we conduct research to gain a better understanding of the molecular mechanism underlying the disease progression that will help to develop a novel treatment strategy to win the war on cancer. Previous research has been done to illustrate that a protein known as p53 is an important tumor suppressor that plays an essential role in breast cancer.

The p53 protein, infamously known as the "guardian of the genome," was identified in 1979 as a transcriptional activator for numerous downstream proteins that act to suppress tumorigenesis [2]. The most intriguing aspect of the p53 gene is that it is mutated in at least 50% of all cancers [2], demonstrating that not only is it a tumor suppressor, but that it is immensely vital to ensure proper cell cycle progression. This important protein is activated by numerous conditions, including, but not limited to DNA damage or any other damage involved in genetic handling, hypoxia and oncogenic signaling [2]. Some of p53 functions include regulating cell cycle checkpoints, induction of cellular senescence, autophagy and apoptosis [3]. Some of the downstream signaling that is activated by p53 is presented in Figure 1, which was illustrated in *Classic and novel roles of p53: prospects for anticancer therapy* [2].

Since previous research has identified p53 as a tumor suppressor, the next proposed idea was that p53-null cells would disrupt the blockade of tumorigenesis in the majority of cancers.

According to Rotter and Oren, the expectation is that mutant p53 (mutp53) proteins will generate a loss of p53 phenotype through the formation of truncated mutant proteins [4]. However, it was found that the majority of cancer cells have a full-length protein with one amino acid substituted, thus creating a form of p53 that is equivalent to null p53 and also generates a new function [4]. This information is evident by evaluating the data emphasized by Brosh and Rotter in Figure 2, which illustrates that the majority of mutations found in p53 are missense mutations that occur in the DNA-binding domain [5].

In general, mutp53 has three common outcomes including reducing the normal p53 cellular response, exerting a "dominant-negative" effect, which allows mutp53 to render wild type p53 (wtp53) virtually inactive, and additionally seems to acquire new "functions" [4]. This so called "gain-of-function" that mutp53 obtains has prompted numerous research studies, which have demonstrated that mutp53 seems to lead to a more aggressive cancer via various effects that include, but are not limited to enhancing genomic instability which enhances tumor progression, resisting apoptotic features, promoting cell migration and invasion, and cancer metastasis [4]. More specifically in breast cancer, mutp53 promotes similar aggressive oncogenic properties found in cancers in general and is found to be the principal molecular causes of tumor progression [6]. Furthermore, with the idea that mutp53 is so prevalent in breast cancer as well as other cancers, it is evident that targeting mutp53 as therapeutic agent is a growing area of research. There are some drugs that have been developed to target mutp53 and/or restore wtp53 function, but these drugs have not been released yet because they are still waiting to undergo clinical testing or some of the drugs are not specifically being tested for breast cancer [6].

Breast cancer can be divided into four subtypes: ER-PR-positive, which includes Luminal A and Luminal B, triple negative and HER2 positive. The ER-PR subtype represents a high level

of hormonal receptors such as estrogen receptor (ER) and progesterone receptor (PR). The ER-PR is divided to include two specific types, Luminal A and Luminal B. Specifically, Luminal A is either positive for ER or PR with negative HER2 and normally has the best prognosis [7]. Luminal B is similar to Luminal A in that it is ER/PR positive, however it is found that it usually a poorer prognosis compared to Luminal A [8]. Triple negative type breast cancer is also sometimes referred to as "basal-like" and is negative for ER, PR and HER2 receptors. Due to its "triple negativity," this type of breast cancer does not respond to hormone therapy or HER2-type therapy, but has been found to respond to chemotherapeutic agents [8]. The HER2-positive breast cancer has a high level of HER2 receptor and has found to be cured with a drug called Herceptin, but usually reveals poor prognosis [8]. According to a study done by The Cancer Genome Atlas Network, samples of HER2 subtype had a high frequency of p53 gene mutations, which illustrates a need for understanding the mechanism between the two proteins and will be the prevalent basis for this thesis [9].

In more detail, HER2-positive breast cancer, as previously stated, has a high expression of the HER2 receptor. This receptor is part of the Epidermal Growth Factor Receptor (EGFR or ErbB in rodents) family, which is a collection of receptor tyrosine kinases that trigger downstream signaling to induce cell growth. The EGFRs undergo dimerization once a ligand binds causing autophosphorylation of tyrosine residues on the cytoplasmic domain, which causes further activation of downstream pathways such as PI3K/Akt and ERK, thus promoting cell proliferation [10]. With this knowledge, it is evident that since the discovery of the role of HER2 in breast cancer, numerous studies have been conducted to develop a treatment that would block this pathway. The most notable clinical approach for treating HER2-positive breast cancer is Herceptin (Trastuzumab), which was implemented in the 1990s and is now the most standard use

of clinical treatment for HER2-positive breast cancer [11, 12]. Herceptin is a monoclonal antibody that binds and interferes with the HER2/neu receptor. As a result, Herceptin induces upregulation of p27 and a decrease in cylcin D1 and cdk-2, which will help to inhibit cell growth. Additionally, it is believed that Herceptin is involved in antibody-dependent cell-mediated cytotoxicity via T-cells to increase cell death [11]. Furthermore, numerous tyrosine kinase inhibitors (TKIs) were and are being developed to block the autophosphorylation of the HER2 receptor or inhibit the downstream signaling pathways, which are illustrated in Figure 3, adapted from Roy and Perez 2009 [10]. The most infamous of the TKIs is the FDA approved drug, Tykerb or chemically and more commonly known as Lapatinib.

The developed clinical approaches have been found to be successful in most patients, but they do have their own caveats. Foremost, the prices for receiving the medication are extremely expensive and are not realistic for the average United States citizen. According to an article in *Medical News Today,* Herceptin is ideally used for a 12-month span and roughly costs about $70,000 U.S. dollars [13]. Additionally, the use of Lapatinib costs roughly about $3,625 U.S. dollars a month, which would correlate to $43,500 U.S. dollars if also used for a 12-month period [14]. Regardless of the enormous amount of money that is required to use these clinical treatments, most patients who are diagnosed with HER2-positive breast cancer find that the treatments are helpful, but there have been cases where the patients are resistant to the treatment, specifically Herceptin. There is no confirmed reason as to why some patients have a resistance to Herceptin, but there have been numerous studies done to isolate a hypothesis, one including the idea that the HER2 is truncated in the extracellular domain. Due to the fact that Herceptin binds to the extracellular domain to inhibit HER2 function, Herceptin would be ineffective in this case [11]. Equally important is the known side effect of cardiac toxicity, which is found

predominantly in patients who use chemotherapy, however a small percentage of patients using Herceptin as well as Lapatinib were found to have a form of cardiac dysfunction [10]. Although overall, these drugs have been approved and certified to be effective in treating patients diagnosed with HER2-positive breast cancer, a significant amount of patients do not benefit from the medication due to primary or acquired resistance. This points toward the idea that it is essential to understand the mechanism of HER2-positive breast cancer and whether or not we can develop predictive biomarkers to ensure that patients who will benefit the most from these drugs receive the medication so that they do not waste their money as well as develop possible unwanted side effects such as cardiac toxicity.

The basis for this thesis is the investigation of oncogenic cooperation between mutp53 and HER2, which we propose is mediated by universal pro-survival heat shock response machinery. In 1962, the heat shock response was discovered by Ferruccio Ritossa after observing the chromosome of a fruit fly and since then has been the integral topic of numerous scientific studies [15]. Currently, the mechanism for the heat shock response entails that proteotoxic stress induces an increase of gene expression for heat shock proteins (HSP), which in turn activates a survival response and protects cells from protein aggregation and proteotoxic stress [15]. Furthermore, the expression of the HSPs is regulated by heat shock transcription factors (HSF) that bind heat shock elements (HSE) to induce transcription of HSPs [15]. An HSF of particular interest for eukaryotes is HSF1, which has a universal regulatory mechanism for multiple HSPs, including HSP70, HSP27, and inducible form of HSP90. A diagram of the regulatory negative feedback loop is represented in Figure 4, adapted from a journal article written by Dörthe M. Katchinski [16].

In understanding that the role of HSP is to contribute to cell survival, it is indisputable that these proteins contribute to the progression of tumorigenesis. In January of 2000, Hanahan and Weinberg proposed six characteristics that classified a cancer cell as cancerous including self-sufficiency in growth signals, insensitivity to anti-growth signals, invasion/metastasis, a limitless replicative potential, angiogenesis, and the evasion of apoptosis [17]. In 2006, Calderwood and group at Boston University School of Medicine outlined each characteristic and how HSP played a role. For example, in regards to self-sufficiency in growth signals, it was shown that HSP90 was needed for the stability of HER2 and it's downstream signaling, thus promoting cell growth and proliferation [18]. An additional example shows that HSP90 as well as HSP70 have been shown to bind tumor suppressors such as p53, thus preventing the cell from activating apoptotic pathways [18]. Lastly, increased expression of HSF1 and thus increased expression of HSPs have a suggestive role of resisting senescence [18]. Consequently, the role of the heat shock response and its components is vital to understanding cancer on a more mechanistic view.

Overall, the amount of women who are diagnosed with breast cancer each year is astounding and therapeutic treatment is not 100% successful due to issues such as resistance. Therefore, if we were able to better comprehend the mechanism in which cell proliferation pathways are enhanced while tumor suppressive pathways are nullified, it would be easier for doctors to analyze which clinical treatment would be best for patients. By analyzing the way in which HER2 and mutp53 cooperate within breast cancer cells, the effects of clinical treatment for HER2-positive breast cancer could be more beneficial. For this thesis, I have analyzed three papers that illustrate the mechanistic interaction between HER2 and mutp53 as well as the cooperation between both HER2 and mutp53 with heat shock response transcription factor

HSF1. I propose that there is an oncogenic cooperation between mutp53 and HER2 via master transcriptional regulator of heat shock response, HSF1, which may amplify HER2 signaling and sensitize breast cancer cells to HER2-targeted therapies. This suggests that mutp53 can be used as a potential predictive biomarker for successful treatment of HER2-positive breast cancer.

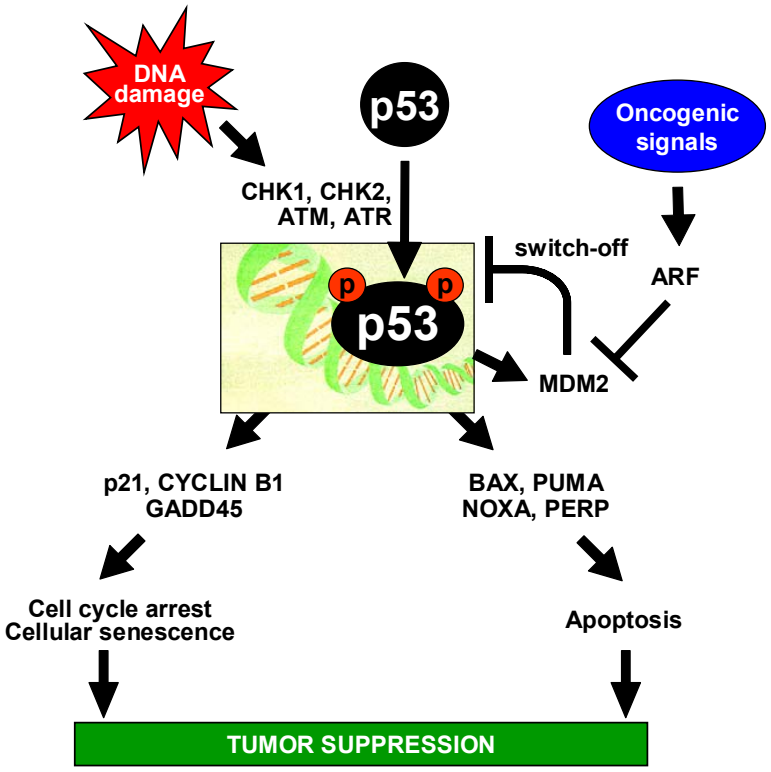

Figure 1. Summary of downstream proteins and functions of activated p53. Image adapted from Fuster *et al.* 2007 as Figure 1b [2].

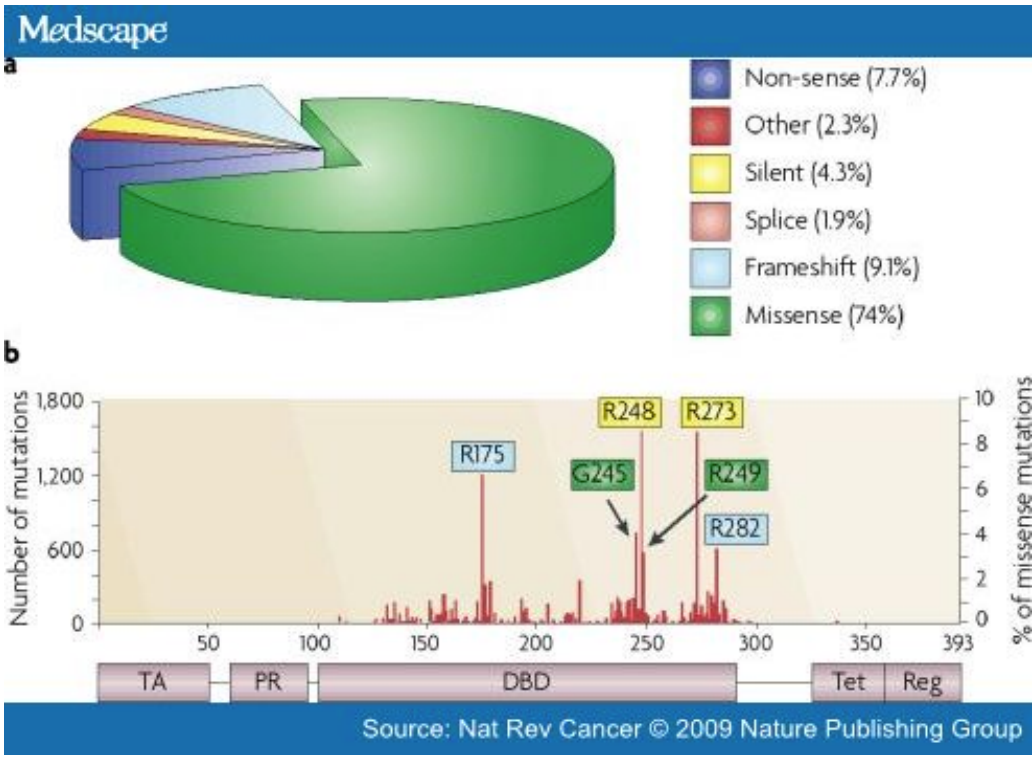

Figure 2. Percent measurements of varying types of p53 mutations. (**a**) Percentage of different types of mutations of p53. (**b**) Number of missense mutations in particular regions of p53 protein. Image adapted from Brosh and Rotter 2009 as Figure 2 [5].

Table 1. Selective tyrosine kinase inhibitors approved or in development for HER-2^+ breast cancer

Agent	Principal targets	Comments	Stage of clinical development
Lapatinib	EGFR, HER-2	Reversible	Approved
Neratinib	EGFR, HER-2	Irreversible	Phase III
Canertinib	Pan-HER	Irreversible	Phase II
Vandetanib	EGFR, VEGF		Phase II
BIBW 2992	EGFR, HER-2	Irreversible	Phase II
TAK-285	EGFR, HER-2		Phase I
BMS 599626	EGFR, HER-2	Inhibits heterodimerization	Phase I
CP 724714	HER-2	Highly selective	Phase I

Abbreviations: EGFR, epidermal growth factor receptor; HER-2, human epidermal growth factor receptor 2; VEGF, vascular endothelial growth factor.

Figure 3. Table of types of tyrosine kinase inhibitors for HER2-positive breast cancer. Image adapted from Roy and Perez 2009 as Table 1. Selective tyrosine kinase inhibitors approved or in development for HER-2+ breast cancer [10].

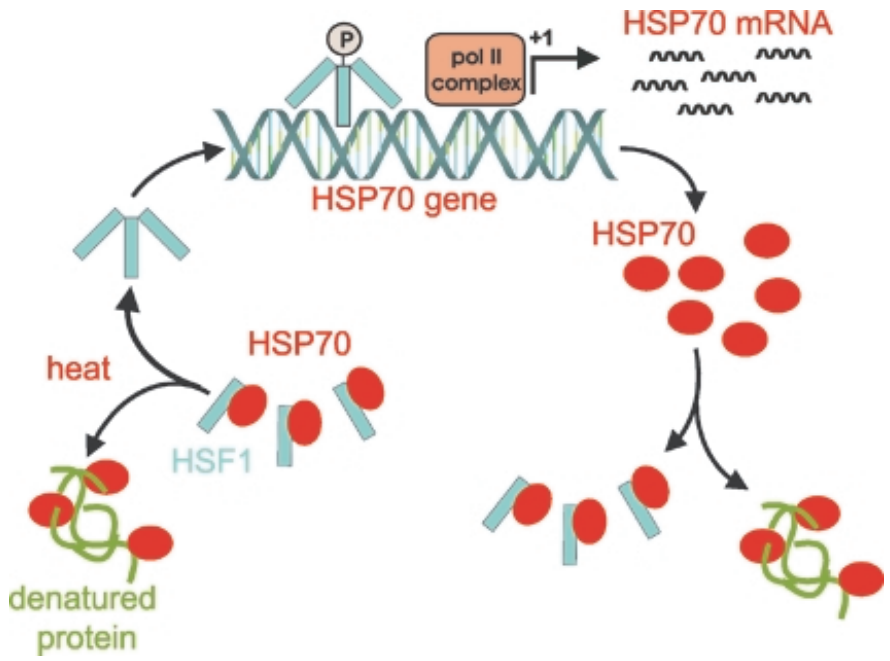

Figure 4. A diagram of negative feedback loop of HSF1 and Hsp70. Adapted from Katchinski 2004 as Figure 2 [16].

Chapter 2: Methods

In order to prove that there is an oncogenic cooperation between mutp53 and HER2, to justify mutp53 as a biomarker in HER2-positive breast cancer, I have analyzed three papers that illustrate a relationship between mutp53 and HER2 in reference to breast cancer. The methods are discussed below in regards to each paper that was used.

2.1 *Role of p53 in HER2-induced Proliferation or Apoptosis*

2.1.1 Cell Lines and Cell Culture

In 2001, Casalini *et al.* published a paper emphasizing the role of p53 as an influencer of HER2 to promote tumorigenesis. Following the experimental procedures written out in their journal article, Casalini *et al.* obtained IGROV1 cell line harboring wtp53 from the Institute Gustave Roussy in France that was a moderately differentiated ovarian carcinoma from an untreated patient. They also obtained IGROV1/Pt1 from the Istituo Nazionale Tumori, which is a variant that contains mutations of amino acids 270 and 282 in p53, thus representing a cancerous cell with mutp53. The cell lines were cultured at 37˚C in humidified atmosphere in RPMI 1640 medium with 10% heat-inactivated fetal calf serum and 2μM L-glutamine [19].

2.1.2 Testing for Growth Inhibition in IGROV1 and IGROV1/Pt1 Cells

In order to test wtp53 or mutp53 with HER2, the cell lines were transfected with pcDNA/HER2 and pcDNA/neo, which was used as the control and is represented as “mock” throughout the experiment. These plasmids were excised from full-length HER2 cDNA using *Xho*I restriction enzymes and transfected into the cells with Lipfectin from Life Technologies,

Inc. and selected for via G-418 resistance. Both IGROV1 and IGROV1/Pt1 were transfected and after three weeks were counted [19].

2.1.3 Testing for Apoptotic Cells in IGROV1 and IGROV1/Pt1 Cells

Casalini *et al.* continued their experiment by analyzing the percentage of cells that illustrated apoptosis. Both IGROV1 and IGROV1/Pt1 were transfected with pEGFP/HER2 that contained HER2 cDNA that was fused to the N-terminus of green fluorescent protein (GFP) or was transfected with pEGFP-C2, representing the control ("mock" transfection). Transfection followed similar procedure as discussed in the previous experiment. The amount of cells undergoing apoptosis was determined by counting the number of apoptotic nuclei relative to the total number of cells that were fluorescing green [19].

2.2 *HER2/ErB2 activates HSF1 and thereby controls HSP90 clients including MIF in HER2-overexpressing breast cancer*

2.2.1 Cell Lines and Cell Culture

Schulz *et al.* documented the relationship between HER2 and HSF1 in HER2-overexpressing cells. The cells used were human breast cancer cells, mutp53 HER2 expressing SK-BR-3, and were cultured in RPMI with 10% FBS [20].

2.2.2 Testing the Effect of HER2 Inhibition on HSF1 and Downstream Protein Levels

To demonstrate the effect of HER2 inhibition on HSF1 and its transcriptionally activated downstream proteins, HER2 was inhibited by specific HER2 inhibitor, CP724.714 (Selleck Biochem in Munich, Germany). The SK-BR-3 cells were treated for 48 hours with 2 μM of CP724.714. To test the effect of the HER2 inhibition, the authors used immunoblot assay. They

lysed the cells using RIPA buffer, centrifuged and through BCA protein assay measured the amounts of proteins so that they could load equal amounts of protein to be loaded onto SDS gel for electrophoresis. The gel was transferred to nitrocellulose membrane, was blocked and was probed with antibodies to test for specific proteins. Schulz *et al.* used Gapdh as a loading control [20].

2.2.3 Testing the Effect of HER2 Inhibition on HSF1 Transcriptional Program

The authors used quantitative real time polymerase chain reaction (qRT-PCR) to analyze the quantity of mRNA of HSF1 targets, Hsp70, Hsp110, and Hsp90α. Firstly, SK-BR-3 breast cancer cells were treated with 2 μM of HER2 inhibitor CP724.714 or DMSO (control) for 48 hours. RNA was isolated from cells using Trizol reagent. Equal amounts of RNA were used and were quantified using the qPCR Master-Mix. The authors used the following primers [20]:

Hsp70: 5'-TCAAGGGCAAGATCAGCGAG-3' and 5' TGATGGGGTTACACACCTGC-3'

Hsp90: 5'-GCCCAGAGTGCTGAATACCC-3' and 5'-GTGGAAGGGCTGTTTCCAGA-3'

Hsp110: 5'-ACTGCTTGTTCAAGAGGGCTGTGA-3' and 5'-AACATCCACACCCACACACATGCT-3'

2.2.4 Correlation Between Activated pSer326-HSF1 and HER2 in HER2-positive Breast Cancer

The authors used immunohistochemical staining to demonstrate correlation between the activated pSer326 of HSF1 (transcriptionally active form) and HER2 in human cell lines and murine and human breast cancer tissues. The immunohistochemistry was done on a BenchMark XT Autostainer using 4B5 antibody for HER2 and EP17137 antibody for pSer326-HSF1. The

samples were classified by a board-certified pathologist using X400 magnification and ImageJ analysis software for quantification [20].

2.3 *A gain-of-function mutant p53-HSF1 feed forward circuit governs adaptation of cancer cells to proteotoxic stress*

2.3.1 Cell Lines and Cell Culture

Li *et al.* used human breast cancer cell lines with mutations in p53 including MDA231 (mutation R280K), SK-BR-3 (mutation R175H) and H1299, which is p53 null. The cells were cultured in 10% FCS/DMEM and viability was determined using CellTiter-Blue Assay [21].

2.3.2 Testing if mutp53 Upregulates HSF1

The authors used RNAi-mediated depletion in SK-BR-3 breast cancer cells by using siRNA for scrambled control (siScr), siRNA for p53 (sip53 and siRNA for HSF1 (siHSF1). The siRNAs were transfected into cells using Lipofectamine and harvested 48 hours later where they were analyzed using immunoblotting to determine protein levels. The immunoblotting was done with equal protein from cell lysates and detected with specific antibodies. Actin was used as a loading control [21].

Li *et al.* also used H1299, null p53 cells, to test upregulation of HSF1 by ectopically expressing mutp53R175H and testing the levels of HSF1 as well as HSF1 targets, HSP70 and HSP27. The authors also tested whether heat shock (42˚C for 30 minutes) would cause a change in the HSPs protein levels. A vector was used as a control for the ectopic expression and Hsc70 was used as a loading control for the immunoblots [21].

They further studied this phenomenon by comparing the levels of HSF1 and its downstream targets by ectopically expressing different mutant p53s compared to a vector as the control. Actin was used as a loading control for this procedure [21].

2.3.3 Testing mutp53 Activation of HSF1

Li *et al.* used MDA231-R280K (MDA231 with ectopically overexpressed matching R280K mutp53) and MDA231 control cells to determine the effects on the activation of HSF1. The cells were lysed and used immunoblotting, in a similar manner as previously discussed by the authors, to determine protein levels. Actin was used as a loading control. The experiment was repeated using heat shock and taking into consideration the nuclear and cytoplasmic fraction of protein levels. For this procedure, GAPDH was used as a cytoplasmic fraction loading control and HDAC1 as the nuclear fraction loading control [21].

2.3.4 Testing effect of mutp53 on HSF1 Binding to HSE

Li *et al.* used chromatin immunoprecipitation (ChIP) analysis to determine if mutp53 has an effect on HSF1 binding to the HSE. The authors used the same steps as Denissov *et al.* 2007. The MDA231 cells were used in this experiment [21].

2.3.5 Immunohistochemical Staining of Human Breast Cancers

The authors used tissue microarray of 150 breast cancer biopsies with known molecular status and analyzed the correlation between mutp53 and nuclear phosphor-activated pSer316-HSF1. Antibody for p53 was used from Santa Cruz Biotechnology and antibody for pSer326-HSF1 was used from Epitomics. Staining intensities were blindly scored [21].

Chapter 3: Results

After analysis of the scientific literature, I have found three relevant papers that illustrate a clear connection for understanding the relationship between HER2 and mutant p53 in regards to HER2-positive breast cancer. As described in Chapter 2: Methods, the following are a list of the selected papers: *Role of p53 in HER2-induced Proliferation or Apoptosis*, *HER2/ErB2 activates HSF1 and thereby controls HSP90 clients including MIF in HER2-overexpressing breast cancer,* and *A gain-of-function mutant p53-HSF1 feed forward circuit governs adaptation of cancer cells to proteotoxic stress*. Each paper includes figures that represent the data that was collected as described in Chapter 2. The data described in this chapter is an analysis of the data presented in each select paper.

Role of p53 in HER2-induced Proliferation or Apoptosis

In this paper, Casalini *et al.* performed an experiment demonstrating a clear connection between HER2 and mutp53. In their first experiment, they transfected IGROV1 and IGROV1/Pt1 cells with HER2 to test the effect on cell growth. IGROV1 cells represent wt p53 cells where as IGROV1/Pt1 cells represent mutp53 in that they have a mutation at amino acids 270 and 282. After incubation for three weeks, colonies were counted revealing a small percentage of cell growth in IGROV1 transfected cells compared to a large growth in IGROV1/Pt1 transfected cells (Figure 5A) [19].

Casalini *et al.* next determined the percentage of cells undergoing apoptosis comparing wtp53 and mutp53 relevant to HER2. To illustrate the effect of mutp53 on HER2 inhibition of apoptosis, the authors transfected IGROV1 (wtp53) and IGROV1/Pt1 (mutp53) cells with HER2 using the pEGFP/HER2 plasmid, which was fused to the N-terminus of GFP. Additionally, they

transfected each cell type with pEGFP-C2 as a control (Mock). The authors used 4,6-diamidino-2-phenylindole (DAPI) stain as a means to count the number of apoptotic cells within the green fluorescing cells. According to the results (Figure 5B), IGROV1 with transfected HER2 had about 30% apoptotic cells compared to roughly 10% of apoptotic cells in Mock IGROV1 cells [19]. On the other hand, IGROV1/Pt1 cells transfected with HER2 had about 10% apoptotic cells, which was similar to the Mock IGROV1/Pt1 cells [19]. Therefore, in contrast to mutp53 cells, HER2 expression induced apoptosis in cells carrying wtp53 [19].

HER2/ErB2 activates HSF1 and thereby controls HSP90 clients including MIF in HER2-overexpressing breast cancer

The first relevant experiment that was conducted by the authors investigated the effect of HER2 inhibition on HSF1 and its downstream effectors. Schulz *et al*. inhibited SK-BR-3 cells for 48 hours with 2 μM of HER2 inhibitor, CP724.714 and immunoblotted the cells to detect protein levels. As seen in Figure 6a, the levels of pHSF1, HSF1, Hsp90α, Hsp70 and Hsp27 decreased after 48 hours compared to the 0 hour time mark [20]. Furthermore, as time progressed, there is a clear decrease in the protein levels [20]. Additionally, the other HSP90 clients MIF, AKT, Bcl-xl and mutp53 decreased as well (Figure 6b) [20].

The authors continued to show correlation between HER2 and HSF1 by analyzing the mRNA expression levels of Hsp70, Hsp110 and Hsp90α using qRT-PCR. The SK-BR-3 breast cancer cells contained DMSO, a vehicle control, or HER2 inhibitor CP724.714. As a control, the relative level of DMSO mRNA expression was 1, while Hsp70 had a level of about 0.8, Hsp110 had a level of about 0.7 and Hsp90α had a level of about 0.4 (Figure 7) [20]. In essence, the

levels of mRNA expression of the HSF1 downstream targets Hsp70, Hsp110 and Hsp90α decreased when HER2 was inhibited [20].

Lastly, the authors evaluated the relationship between activated pSer326-HSF1 and HER2 in human cell lines and murine and human breast cancer tissues. The samples were studied and classified by a board-certified pathologist and stated that "there was a strong correlation between HER2 and pSer326-HSF1 specifically in HER2-positive breast cancer," which can be found in Figure 8 [20].

A gain-of-function mutant p53-HSF1 feed forward circuit governs adaptation of cancer cells to proteotoxic stress

Li *et al.* began their study with an analysis of the effect of mutp53 on regulation of HSF1. They first used siRNA-mediated depletion to test protein level of HSF1 and downstream target Hsp70 when mutp53 was removed. According to Figure 9a, when mutp53 was deleted, HSF1 levels as well as Hsp70 levels decreased compare to the control siScr (scrambled controls) [21]. Additionally, the authors tested effects on HSF1 and its targets in the presence of mutp53 by ectopically expressing mutp53 as R175H in H1299 null p53 cells. Levels of HSF1, Hsp70 (short and long) and Hsp27 were evaluated in immunoblots both with and without heat shock. Compared to the control, levels of HSF1, Hsp70 and Hsp27 increased when R175H mutp53 was expressed in cells, more evidently in cells that underwent heat shock (Figure 9b) [21]. Lastly, to confirm mutp53 upregulation on HSF1 and downstream targets, a similar experiment was repeated using varying p53 mutations. Compared to the vector control, all p53 mutations upregulated HSF1, Hsp27, Hsp70 and Hsp90 as evident in Figure 9c [21].

Li *et al.* continued their study by testing the relationship between mutp53 and the activation of HSF1, represented as pSer326-HSF1. Levels of p326-HSF1 were detected on immunoblots in both MDA231 control cells and MDA231-R280K mutant p53 cells. The MDA231-R280K cells were a line of mutp53 breast cancer cells that ectopically expressed excess mutp53 R280K protein [21]. The experiment was done with cells undergoing heat shock and without heat shock for comparison. Without heat shock, there is an increase in presence of p326-HSF1 in MDA231-R280K cells compared to the control cells [21]. Comparatively, for the cells that underwent heat shock, there is a significant increase in p326-HSF1 in MDA231-R280K cells compared to control cells (Figure 10a) [21]. The authors then conducted a similar experiment that illustrated protein levels of p326-HSF1, HFS1 and p53 in both nuclear and cytoplasmic fractions. Depicted in Figure 10b, p326-HSF1 had a greater presence in the nuclear fraction of MDA231-R280K cells compared to the MDA231 control cells when underwent heat shock [21].

Using chromatin immunoprecipitation assay (ChIP), Li *et al.* analyzed the relationship of mutp53 to HSF1 binding to heat shock element (HSE) [21]. MDA231 and MDA231-R280K cells were used and the antibody was for either irrelevant GST (antibody control) or p53, analyzing HSE levels. Cells that did not undergo heat shock, showed an increase in HSE for mutp53 in MDA231-R280K cells compared to control MDA231 cells (Lines 3 and 4 of Figure 12) [21]. Comparing lines 7 and 8 of Figure 11, illustrate that cells that underwent heat shock, have an evident increase in HSE levels for MDA231-R280K cells compared to control cells [21].

The last relevant experiment done by Li *et al.* was to show a correlation between mutp53 and activated pSer326-HSF1 in breast cancer cells. The authors used immunohistochemical staining of known molecular status of 150 breast cancer biopsies. Figure 12 shows select samples

that demonstrate that scoring was absent, moderate or strong and from a scale of 0 to 4. According to the journal article, the authors noted a “clear correlation between mutp53 and pSer326-HSF1 proteins specifically only in HER2-positive breast cancer samples and not in HER2-negative or ER/PR+ tumors” [21].

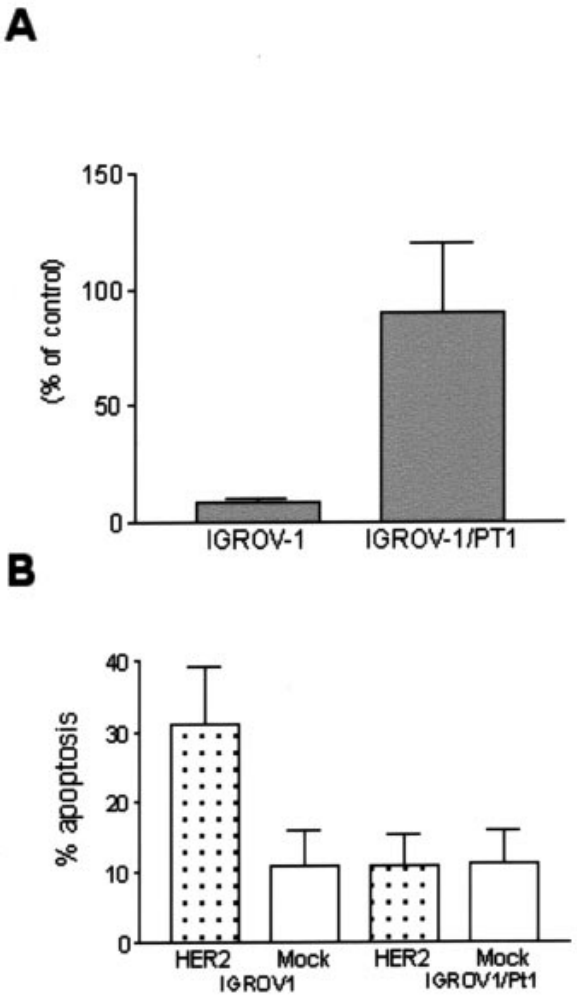

Figure 5. Analysis of p53 on HER2 (**A**) **Measure of cell growth with transfected HER2.** Measure of the growth of IGROV1 and IGROV1/Pt1 cells with transfected HER2. (**B**) **Measure of inhibition of apoptosis with transfected HER2.** Measure of the amount of IGROV1 and IGROV1/Pt1 cells that underwent apoptosis when transfected with HER2.

Image adapted from Casalini *et al.* 2000 as Figure 1A and 1B [19].

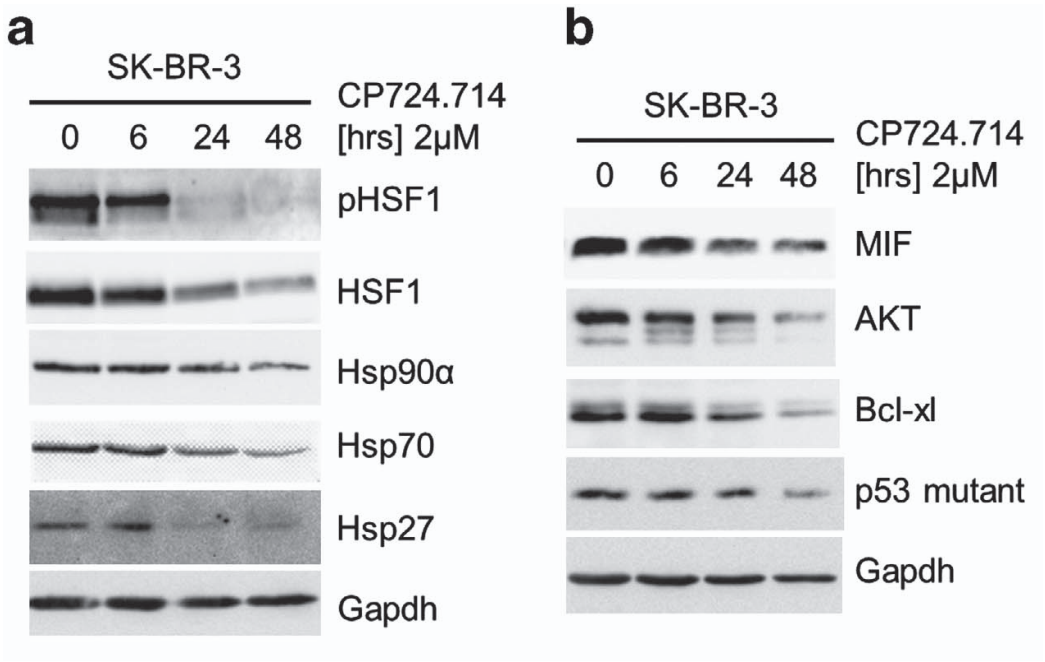

Figure 6. Inhibition of HER2 and the effects on HSF1 protein and downstream targets. (a) Western blot analysis of inhibition of HER2 on HSF1 and downstream targets. HER2 receptor in SK-BR-3 cells was inhibited with 2 μM of CP724.714 for 48 hours and protein levels of pHSF1, HSF1, Hsp90α, Hsp70 and Hsp27 were analyzed using immunoblots. Gapdh was used as a loading control. **(b) Western blot analysis of inhibition of HER2 on MIF, AKT, Bcl-xl and mutp53.** HER2 receptor in SK-BR-3 cells was inhibited with 2 μM of CP724.714 for 48 hours and protein levels of MIF, AKT, Bcl-xl and mutant p53 were analyzed using immunoblots. Gapdh was used as a loading control.

Image adapted from Schulz *et al.* 2014 as Figure 3a and 3b [20].

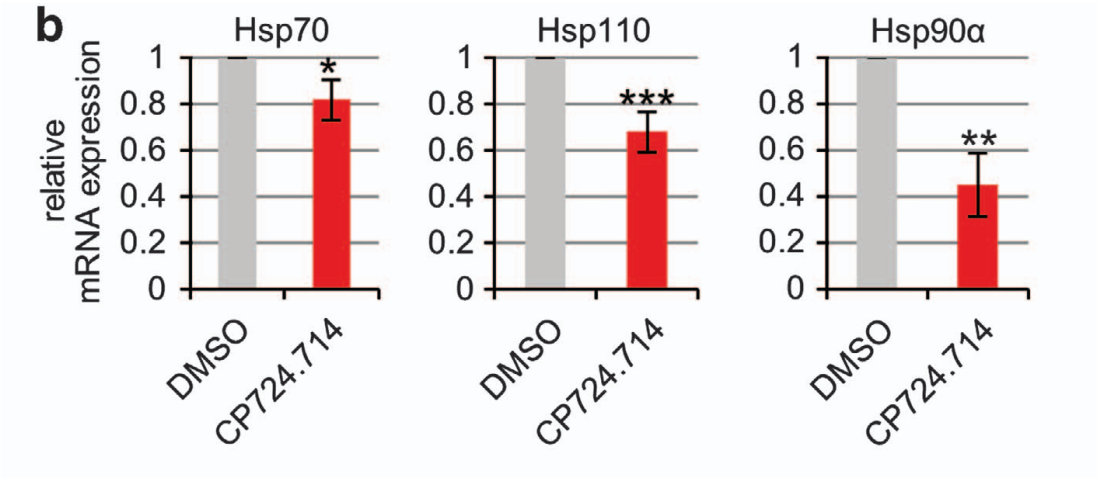

Figure 7. The effects of HSF1-regulated gene expression when HER2 is inhibited. SK-BR-3 cells were inhibited with 2 μM of Cp724.714 for 48 hours and cells were analyzed using qRT-PCR to determine the mRNA expression of Hsp70, Hsp110 and Hsp90α. DMSO was used as a vehicle control.

Image adapted from Schulz *et al.* as Figure 4b [20].

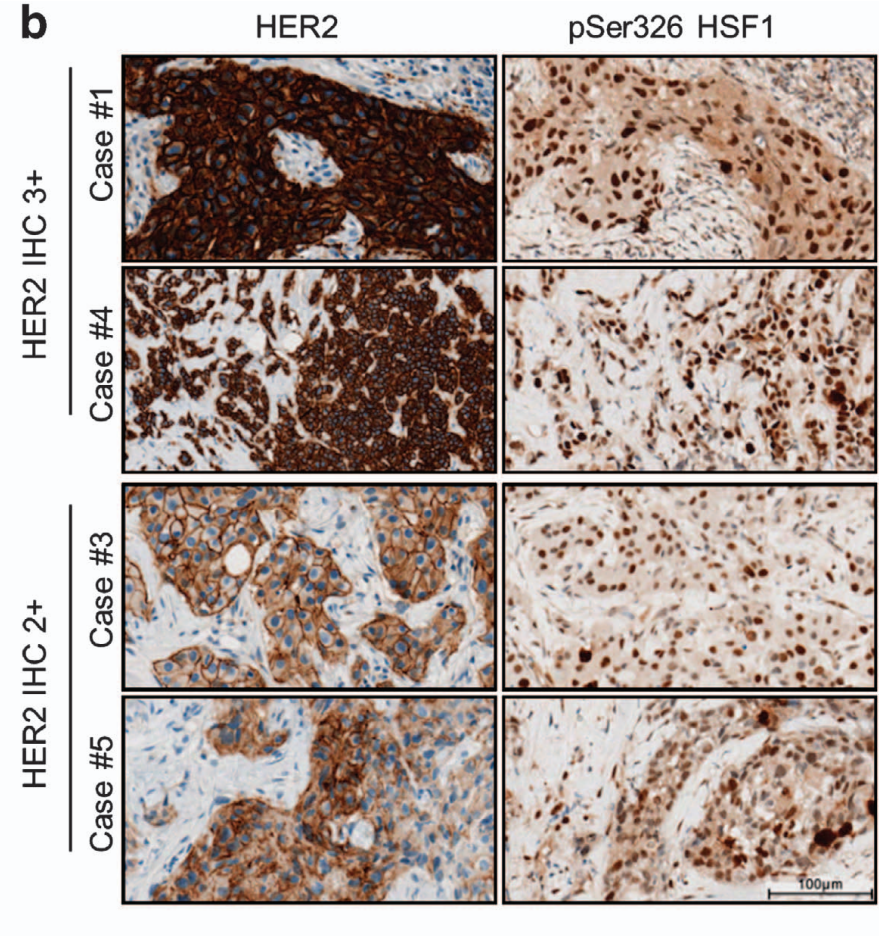

Figure 8. Immunohistochemical staining of the relationship between pSer326-HSF1 and HER2 in HER2-positive breast cancer cells. Immunohistochemical staining of selected HER2 positive breast cancer samples viewed at X400 magnification.

Image adapted from Schulz *et al.* as Figure 6b [20].

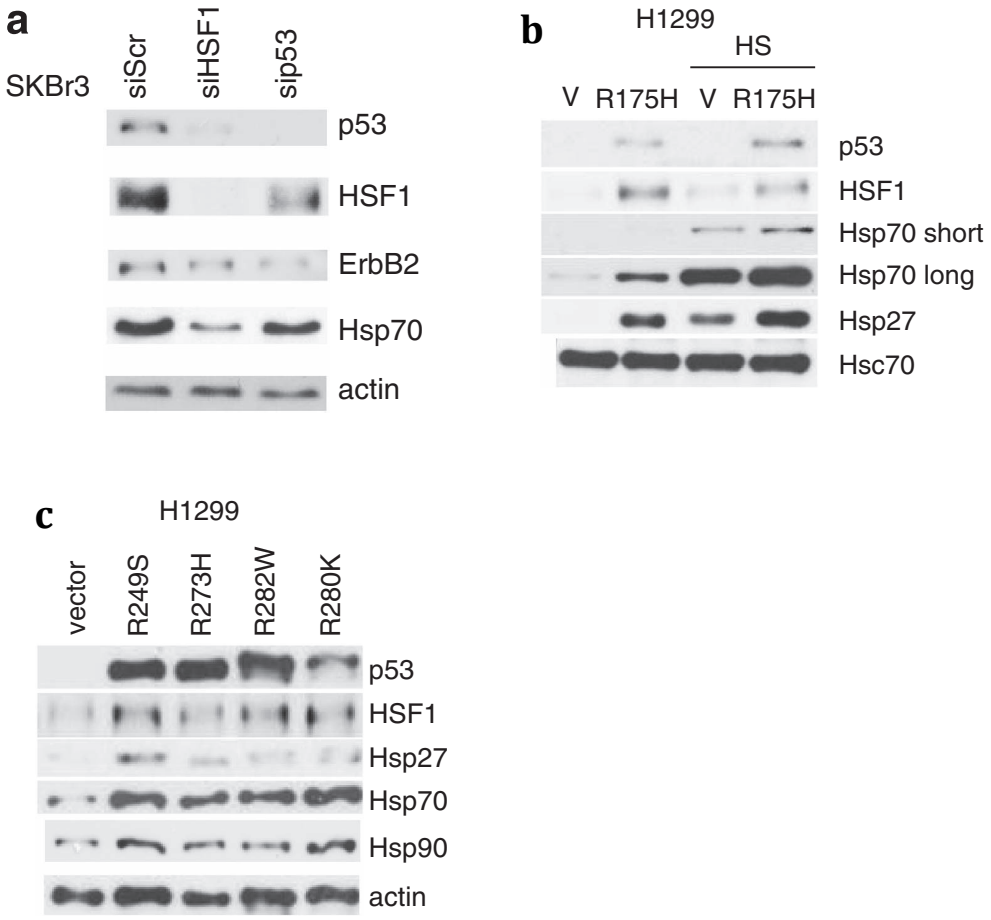

Figure 9. The effect of mutp53 on HSF1 and downstream targets of HSF1. (a) Western blot analysis of HSF1 and Hsp70 after deleting mutp53. The effects of mutant p53 depletion on HSF1 and downstream target, Hsp70, were analyzed using siRNA-mediated depletion of mutp53. siScr and siHSF1 were used as controls for the siRNA-mediated depletion. Protein levels were analyzed using immunoblots. Actin was used as a loading control. **(b) Western blot analysis of HSF1 and downstream targets in mutp53 induced cells with and without heat shock.** Ectopic expression of mutant p53 (R175H) was induced in H1299 null p53 cells and protein levels of HSF1, Hsp70 short, Hsp70 long and Hsp27 were analyzed using immunoblotting. The cells were also heat shocked to enhance effects. V represents vector control and Hsc70 was used as loading control. **(c) Western blot analysis of HSF1 and downstream targets in various forms of mutp53 expression.** The experiment in (b) was repeated using variations of mutant p53 to illustrate similar effects in H1299 null p53 cells. Immunoblotting revealed protein levels of HSF1, Hsp27, Hsp70 and Hsp90. Actin was used as a loading control.

Image adapted from Li *et al.* as Figure 1a, 1d and 1e [21].

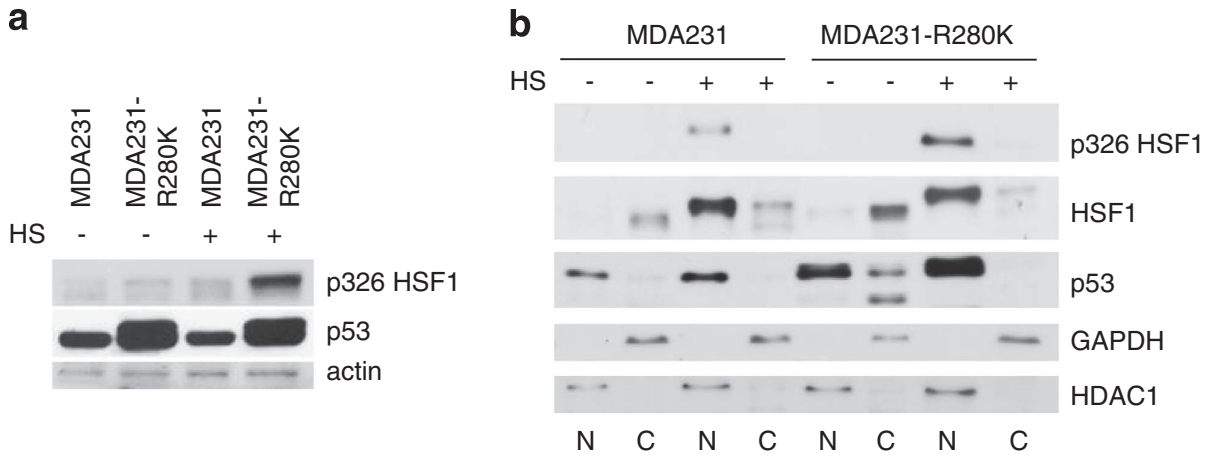

Figure 10. The role of mutp53 in regards to activation of HSF1 as pSer326-HFS1. (a) Western blot analysis of mutp53 effect on activation of HSF1 with and without heat shock. Immunoblotting was used to display effect of mutant p53 on the activation of HSF1 by measuring for p326-HSF1 with or without influence of heat shock (HS). Actin was used as a loading control. **(b) Western blot analysis of mutp53 effect on activated HSF1 in nuclear and cytoplasmic fractions.** The experiment in (a) was repeated this time taking into consideration effects from heat shock (HS). Immunoblotting was done to reveal the protein levels of p326-HSF1, HSF1 and p53. Gapdh was used as cytoplasmic loading control and HDAC1 was used as nuclear loading control.

Image adapted from Li *et al.* as Figure 2a and 2b [21].

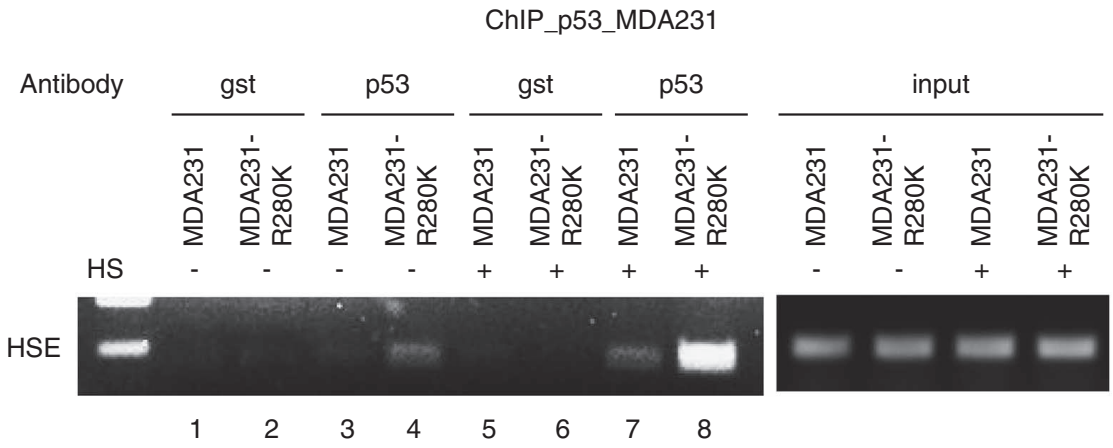

Figure 11. Chromatin immunoprecipitation assay to determine correlation between mutp53 and HSF1 binding to heat shock element. This ChIP assay illustrated the effect of MDA231 (control) versus MDA231-R280K (mutant p53) and the effect of HSF1 binding to its heat shock element (HSE). Heat shock (HS) was induced to test for further effects.

Image adapted from Li *et al.* 2014 as Figure 4g [21].

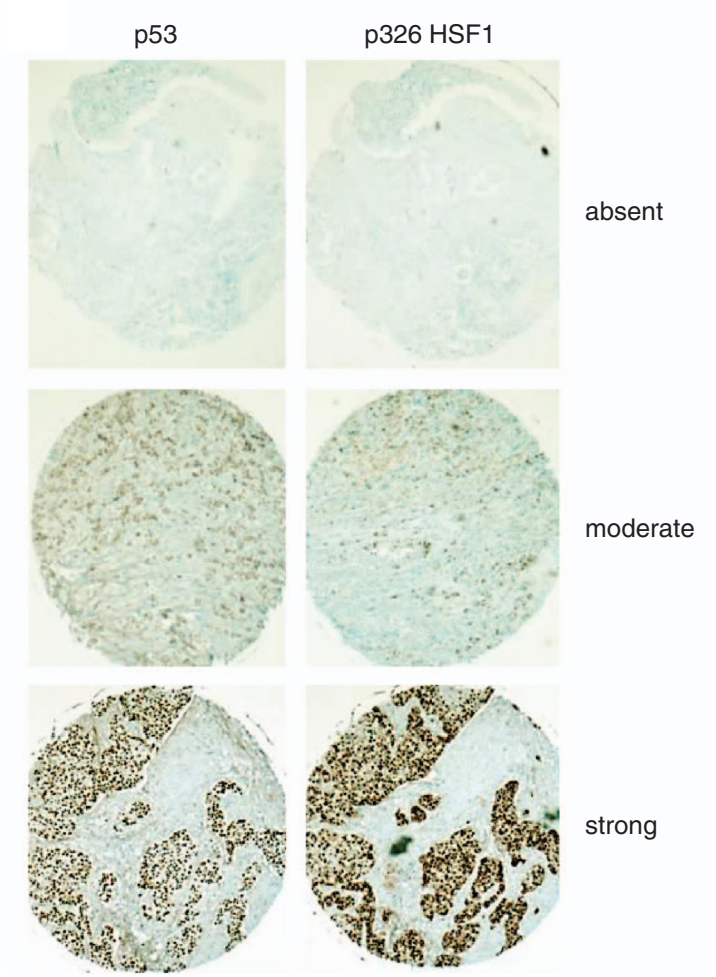

Figure 12. The relationship between mutp53 and activated pSer326-HSF1 in human breast cancer samples. Immunohistochemical staining of tissue microarray was done in 150 samples to demonstrate a correlation between mutantp53 and activated pSer326-HSF1 in cells with known molecular status.

Image adapted from Li *et al.* as Figure 7i [21].

Chapter 4: Discussion

The outcomes of the previously discussed journal articles each provide a specific key point that is relevant to understanding the relationship between mutp53 and HER2 in HER2-positive breast cancer. Through an understanding of the relationship, we can suggest mutp53 would be a potential biomarker for HER2-targeted therapies in HER2-positive breast cancer. Casalini *et al.* tests the effects of mut and wtp53 on tumorigensis. They demonstrated mutp53 in contrast to wtp53 does not have growth inhibitory and apoptotic effect upon HER2 overexpression [19]. Schulz *et al.* and Li *et al.* show the interplay of HSF1 with both HER2 and mutp53, suggesting possible translational potential of this liaison for breast cancer treatment.

Firstly, in the results discussed by Casalini *et al.*, it is evident to see that there is in fact a direct relationship between HER2 and mutp53 in cancerous cells [19]. As is apparent in Figure 5A, the amount of growth greatly increases in IGROV1/Pt1 (mutp53 cells) transfected with HER2 than IGROV1 (control cells) transfected with HER2 [19]. This data emphasizes that when HER2 is with a cell that has mutp53, there is a greater increase in growth, which can be correlated to tumorigenesis. Additionally, when testing the amount of apoptotic cells, IGROV1 transfected with HER2 had about a 20% greater amount of cells undergoing apoptosis compared to IGROV1/Pt1 transfected with HER2 (Figure 5B) [19]. In normal cells, wtp53 induces apoptosis to cease uncontrolled cell growth, while mutp53 will have the opposite effect, thus promoting tumorigenesis. Therefore, the relationship between wtp53 and over-expressed HER2 should demonstrate an increased amount of apoptotic cells as compared to mutp53 and over-expressed HER2 cells, which is what is evident in Figure 5B [19]. Together, the increased in cell growth and decrease in apoptosis of mutp53-HER2 cells emphasizes that there is a correlation between HER2 and mutp53 in cancer since uncontrolled growth and inhibition of apoptosis are

known hallmarks of cancer [17]. With this data, it is apparent that a relationship between mutp53 and HER2 does exist in HER2 over-expressed cells (demonstrating HER2-positive breast cancer), which provides an initial idea that mutp53 and HER2 oncogenically cooperate to promote tumorigensis. However, it is vital to demonstrate at first that mutp53 directly regulates HER2 in the HER2-positive breast cancer cells.

Understanding the relationship between mutp53 and HER2 on a molecular level could provide the basics for optimization of therapeutic intervention for HER2-positive breast cancer. Both Schulz *et al.* and Li *et al.* provide evidence that mutp53 and HER2 interact with HSF1, the universal heat shock transcription factor, suggesting relation between mutp53 and HER2 through HSF1 [20, 21]. The article by Schulz *et al.* demonstrates that inhibition of HER2 leads to suppression of HSF1 activity. When Schulz *et al*. inhibited HER2 with CP724.714 in the SK-BR-3 cells, levels of pHSF1, HSF1, Hsp90α, Hsp70 and Hsp27 decreased, emphasizing there is a direct relationship between HER2 and HSF1 [20]. This is clear by the fact that not only did HSF1 levels decrease, but also the downstream targets that it activates (Hsp90α, Hsp70 and Hsp27) decreased (Figure 6a) [20]. Additionally in this experiment, the authors showed that mutp53 decreased as well (Figure 6b), providing an additional point to what Casalini *et al.* had discovered [19, 20]. In a second experiment, the authors proved correlation between HER2 and HSF1 again by inhibiting HER2 with Cp724.714 and measuring mRNA levels of Hsp70, Hsp110 and Hsp90α, which again decreased (Figure 7) [20]. In summary, the data provided by Schulz *et al.* demonstrated that HER2 and its downstream signaling positively regulates HSF1 activity by providing cells superior survival [20].

Furthermore, Li *et al.* provided evidence that mutp53 also regulates HSF1 activity. They first proved this by deleting mutp53 with siRNA and illustrated a decrease in HSF1 levels

(Figure 9a) [21]. In a second experiment, when the authors ectopically expressed mutp53 in H1299 null p53 cells, HSF1 levels as well as downstream targets (Hsp27 and Hsp70) increased, thus displaying a direct relationship between mutp53 and HSF1 [21]. This experiment was repeated in an almost identical procedure to display that various forms of mutp53 have the same effect on HSF1, demonstrating that mutp53 in general has an effect on HSF1 (Figure 9c) [21]. The relationship is further emphasized in the article when the authors measured the effects of mutp53 on the activated form of HSF1, pSer326-HSF1. As displayed in Figure 10a and 10b, the levels of pSer326-HSF1 increased when mutp53 was present compared to null, which could propose the idea that mutp53 not only interacts with HSF1, but has a relation with upregulating the transcription factor [21]. This proposed idea can be evident when the authors performed ChIP assay to show that compared to control MDA231 cells, recruitment of HSF1 to HSE in MDA231-R280K was greatly enhanced as displayed in Figure 11 [21]. Altogether, these data provide the evidence that mutp53 physically interacts and enhances HSF1 activity in cancer cells.

With the combined evidence from Schulz *et al.* and Li *et al.*, it is evident that HER2 and mutp53, respectively, affect HSF1 activity. As Li *et al.* used siRNA-mediated depletion of mutp53 to test HSF1 levels; I think that using the same means to knock out both mutp53 and HER2 would demonstrate to a greater degree whether both proteins are required to promote HSF1 activation. The knowledge that both HER2 and mutp53 have a direct relationship with HSF1 as demonstrated in the data, promote the idea that a direct relationship exists, but using double knock out of mutp53 and HER2 could further establish this proposal.

Furthermore, both groups demonstrated a strong relationship between mutp53 and HER2 with activated HSF1 in specifically HER2-positive breast cancer cells. Both Schulz *et al.* and Li

et al. used immunohistochemical staining to display both a correlation between HER2 and HSF1 as well as mutp53 and HSF1, respectively (Figure 8 and Figure 12) [20, 21]. Both articles demonstrated a clear relationship with their respected studied proteins and activated pSer326-HSF1 in breast cancer cells. More specifically, both articles found that there was a relationship in HER2-positive breast cancer cells. Li *et al.* pointed out "there was a clear correlation between mutp53 and pSer326-HSF1 specifically in HER2-positive breast cancer cells and not in any other breast tumor subtypes" [20]. This data provides supplementary evidence that mutp53 can be used as an additional predictive biomarker in HER2-positive breast cancer due to the fact that the correlation between both mutp53 and HER2 with HSF1 occurs in specifically HER2-positive breast cancer cells.

In summary, understanding of the relationship between mutp53 and HER2 is vital to bringing an end to the war on Her2-positive breast cancer. As previously shown by Casalini *et al.,* Schulz *et al.* and Li *et al.*, mutp53 and HER2 cooperation governs the novel oncogenic function of mutp53 in which constitutive oncogenic stimulation of the HER2 signaling pathway by mutp53 is mediated by a master transcriptional regulator of heat shock response, HSF1. With the idea that mutp53 may be stimulating HER2 signaling via modulation of HSF1 activity, mutp53 can augment HER2 signaling, generating cancer cells addiction to this pathway. Thus, mutp53 can sensitize breast cancer cells to HER2-targeted therapies and can be used as a potential biomarker for efficacy of drugs like Herceptin and Lapatinib. As always, through scientific research we can gain a better understanding of the molecular relationship between proteins that enhance tumorigenesis in breast cancer cells so that we can provide a more promising therapeutic treatment for patients with HER2-positive breast cancer.

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