Heat Shock Factor 1 (HSF1) controls chemoresistance and autophagy through transcriptional regulation of Autophagy-related Protein 7 (ATG7)

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Running Title: HSF1 controls autophagy via regulation of ATG7

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Background: HSF1 influences chemoresistance in cancer.

Result: Chemotherapy activates HSF1, leading to direct transcriptional regulation of autophagy related gene-ATG7. In-vitro findings are supported by patient sample study.

Conclusion: HSF1 regulates cytoprotective, heat-shock independent-autophagy by directly regulating ATG7, which plays an important role in chemoresistance.

Significance: Identification of novel HSF1/ATG7 axis in chemoresistance strongly supports development of robust combination therapies targeting it in cancer.

SUMMARY

HSF1, a master regulator of heat shock responses, plays an important role in tumorigenesis. In this study we demonstrated that HSF1 is required for chemotherapeutic agents -induced cytoprotective autophagy through transcriptional upregulation of autophagy related gene ATG7. Interestingly, this is independent of HSF1’s heat shock response function.

Treatment of cancer cells with FDA approved chemotherapeutic agent Carboplatin induced autophagy and growth inhibition, which was significantly increased upon knockdown of HSF1. Mechanistic studies revealed that HSF1 regulates autophagy by directly binding to ATG7 promoter and transcriptionally upregulating its expression. Significantly, breast cancer patient sample study revealed that higher ATG7 expression level is associated with poor patient survival. This novel finding was further confirmed by analysis of two independent patient databases, demonstrating a prognostic value of ATG7. Furthermore, a strong positive correlation was observed between levels of HSF1 and ATG7 in triple negative breast cancer patient samples, thus validating our in-vitro findings.

This is the first study identifying a critical role for HSF1 in controlling cytoprotective autophagy through regulation of ATG7, which is distinct from HSF1’s function in heat shock response. This is also the first study demonstrating a prognostic value of ATG7 in breast cancer patients. These findings strongly argue that combining chemotherapeutic agents with autophagy inhibition by repressing HSF1/ATG7 axis represents a promising strategy for future cancer treatment.
Breast cancer is the most common type of cancer as well as second leading cause of cancer-related deaths in women. The increasing emergence of resistance to commonly used chemotherapeutic agents, leading to low disease free survival rates for women with advanced and highly metastatic cancer, has warranted the development of novel robust treatment options for these patients. Carboplatin, a second generation platinum agent has been approved by FDA for the treatment of non-small cell lung cancers and ovarian cancers. Its reduced side-effects compared to Cisplatin have led to its increased use in combination with taxanes to treat highly metastatic breast cancers. Moreover, Carboplatin has shown promising results as a single-agent in previously untreated metastatic breast cancer with response rates of 20%-35% (1). Currently there are around 52 clinical trials undergoing to examine the expanded use of Carboplatin in breast cancer as a combination treatment with taxanes and other drugs (clinicaltrials.gov). Hence, an important step in improving clinical management of these patients is to identify the mechanism of Carboplatin sensitivity in these tumors, to develop more robust combination therapy to overcome drug resistance that may develop.

HSF1 is a transcription factor, that functions both as an activator and a repressor of its target genes, depending on spacing of the heat shock elements (HSEs) it binds to and the co-factors it interacts with (2-7). Exposure to a variety of stress leads to oligomerization of HSF1 into active trimers, followed by translocation into the nucleus and binding to the HSEs located within the promoter region of the target genes such as Hsp90, Hsp25 and Hsp70 (8-10). Overall HSF1 mediates cellular recovery under a variety of stress conditions such as thermal injury, ischemia and age related neurodegeneration by orchestrating a gamut of cellular processes (11,12). Although less well understood, HSF1 has also been shown to regulate non-heat shock response genes to support highly malignant human cancers (7,13,14). Emerging evidence suggests an important role for HSF1 in cellular transformation and in cancer development (14-17). HSF1 supports malignant transformation by modulating multiple pathways regulating proliferation, survival, protein synthesis and cellular metabolism (14). We and others have shown that HSF1 regulates glucose metabolism, mediates tumorigenesis and cell growth in cancer cells (14,15). Besides association of spontaneous mutations in HSF1 with tumorigenesis, a recent study using a large breast cancer patient sample cohort showed that high levels of HSF1 is associated with poor patient prognosis (18). HSF1 has also been reported to regulate drug resistance. However, the mechanism for HSF1 mediated drug resistance is not well understood.

Autophagy, an evolutionary conserved catabolic process, is induced in response to metabolic stress such as nutrient deprivation and hypoxia (19-21). During autophagy, autophagosomes are formed as double-membrane vesicles engulfing cellular organelles and cytoplasm. These autophagosomes fuse with lysosomes, where the sequestered cytoplasmic contents are degraded and recycled for synthesis of protein and ATP (22). Autophagy is genetically regulated by a family of autophagy-related genes (ATGs), which coordinate specific steps in autophagy induction and cytoplasmic sequestration (23). Autophagy has been recognized as a cytoprotective process against environmental stress as well as stress induced by certain chemotherapeutic agents (24-27). Recent studies have demonstrated that inhibition of autophagy sensitizes the cancer cells to DNA damaging agents, hormonal therapies, radiation therapy and chemotherapeutics (24,25,27-29). Therefore, there has been an increased interest in targeting this process to reduce drug resistance in several different cancers.

This report shows that in cancer cells, HSF1 is required for chemotherapy induced cytoprotective autophagy and this is independent of HSF1’s function in heat shock response. This is the first study to demonstrate that HSF1, a multifunctional transcription factor, is activated by Carboplatin treatment and leads to up-regulation of a novel HSF1 target gene ATG7. This regulation plays a critical role in cancer cell resistance to chemotherapy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Antibodies**

MDA-MB-231 and MDA-MB-436 obtained from ATCC, cultured in DMEM/F-12 (Mediatech Inc.) with 10% FBS. Antibodies to HSF1, PARP, cPARP, cleaved caspase 3, p62 and LC3 were purchased from Cell Signaling Technology. Anti-ATG7, Millipore; anti-β-actin, Sigma; anti-pS326
HSF1, Abcam. HRP-conjugated secondary anti-mouse IgG and anti-rabbit IgG were purchased from Bio-rad; ECL western blotting substrate, Pierce.

**Flowcytometry Analysis**

Control and Carboplatin treated cells were stained with Annexin V- FITC and 7AAD as per the manufacturer’s protocol (BD Pharmingen). Acridine orange staining was analyzed using flowcytometry. Control, Carboplatin and Carboplatin +3-MA treated stable HSF1 knockdown and scramble MDA-MB-231 cells were stained with 1µg/ml of Acridine Orange for 15 minutes at 37°C. Flowcytometry was done on FACSCanto and analyzed using Diva software.

**Stable HSF1- knockdown cells**

Five different seed shRNA sequences specific to HSF1 were obtained from Sigma. Clone NM_005526.1-331s1c1 generated maximum knockdown of HSF1 expression and was used to generate Lentiviral particles according to manufacturer’s protocol. MDA-MB-231 and MDA-MB-436 were transduced with shRNA against HSF1 and scramble shRNA. After primary puromycin selection (3µg/ml) cell pools of MDA-MB-231-shHSF1, MDA-MB-231-shscramble, MDA-MB-436-shHSF1 and MDA-MB-436-shscramble were maintained for multiple passages in 1.5µg/ml of puromycin.

**siRNA Experiments**

Downregulation of HSF1 and ATG7 confirmed with two siRNA sequences to avoid off-target effects. HSF1 siRNA were Sigma [0050] SASL_Hs02_00339745 and invitrogen HSF1 stealth siRNA. ATG7 siRNA were [0020] SASL_Hs01_00077648 and [0050] SASL_Hs01_000341471. siRNA [0020] showed significantly more knockdown and was used for all experiments described below. Transfection performed using Lipofectamine2000 (Invitrogen) according to manufacturer’s instructions. 24 hours after siRNA transfection, cells were treated with Carboplatin (75mg/ml) for 24 hours. Forty-eight hours after transfection, cell lysates were prepared for Western Blot analysis.

**Cell counting**

MDA-MB-231 control and stable HSF1 knockdown cells (4 x10^5 cells/well) were seeded in 6-well plates overnight. Cells were treated with Carboplatin for 24 hours. Cell viability measured by Trypan Blue staining and direct cell counting using hematocytometer.

**Western Blot**

Western blots were performed as described (30). Immunoblot analysis for HSF1 trimers was performed as described earlier. Cell extracts were treated with cross-linker ethylene glycol bis (succinimidyl succinate) (EGS) at a final concentration of 1mM followed by glycin at a final concentration of 75mM (9). The reaction mixture was run on 8% SDS-PAGE gel and immunoblotted with HSF1 antibody.

**Fluorescent Microscopy**

Transient transfection with EGFP-LC3B (Addgene plasmid 11546) (31), carried out in scramble and stable HSF1 knockdown breast cancer cells using Lipofectamine2000. 24 hours after transfection the cells were treated with 75 µg/ml of Carboplatin or Rapamycin for 24 hours. Punctate EGFP-LC3 was captured using Automated Nikon TE2000E at 60X magnification. NIS- Elements software used to calculate punctate structures.

**Luciferase Reporter Assay**

pGL3-promoter-ATG7 luciferase vector (ATG7) was constructed by amplification of HSF1 binding site within ATG7 promoter region in the human genomic DNA using forward primer: ACTGGTACCTGTGGCTCCTCGCTTTAC and reverse primer: ACTAGATCTCCATCATCCCCAGTGACC. This amplified region was cloned within BglII and KpnI sites in pGL3 promoter. Mutant luciferase constructs were generated using site-directed mutagenesis approach. Deletion mutants generated using pGL3-promoter-ATG7 and forward primer: GGTGAGAATTACAAAATTAAGGGTGGGGGA GACT, reverse primer: AGTCTCCCCACCCCTAATTGTAATTTCTC ACC for mut1 and forward primer GGTGAGAATACAAAATTAAGGGTGGGGGA GATT and reverse primer: AATCTCCCCACCCCTAATTGTAATTTCTC ACC for mut2. Cells were transfected with 1µg of luciferase reporters and 8ng of internal control.
plasmid pRL-SV40 vector (Promega) using Lipofectamine2000. 24 hours later cells were treated with 75µg/ml Carboplatin. 48 hours after transfection, cells were harvested and lysed with passive lysis buffer (Promega). Luciferase activity was measured using Dual-Luciferase Reporter Assay System protocol (Promega) and 20/20n luminometer (Turner Biosystems).

**Quantitative real-time PCR**

Total RNA isolated from cultured cells using Trizol reagent (Invitrogen). cDNA generated using cDNA synthesis kit (ABI), followed by amplification using SYBRgreen PCR master mix (Roche) and primers specific for ATG7 (26) and GAPDH (FWD- ATCATCCTGCCTCTACTGG; REV- CTGCTTCACCACCTTCTGGA) All reactions were performed in duplicates. Relative mRNA levels were calculated using 3 independent experiments. Data normalized to GAPDH; control groups set to 1.

**Chromatin Immunoprecipitation (ChIP) assay**

ChIP assay was performed as described earlier (32). Chromatin was sonicated using the Sonicator Ultrasonic processor with microtip. Antibodies; anti-HSF1, Rabbit IgG (control), anti-H3 acetylation (Millipore).

**Immunofluorescence staining**

Staining performed as described previously (32). Nucleus stained by DAPI (1:10,000); HSF1 (1:500). Images captured using Automated Nikon TE2000E at 60X magnification. Nuclear staining intensity was quantified using NIS – Elements software.

**Immunohistochemical staining and analysis**

Randomly selected 142 invasive breast carcinoma cases were surgically resected and archived at M.D. Anderson Cancer Center. Tissue microarray (TMA) blocks were constructed by taking core samples from representative areas of paraffin-embedded breast tumor tissues and assembling them on a recipient paraffin block. Two 1-mm tissue cores were obtained from each specimen. Paraffin sections of recipient blocks were obtained and stained with hematoxylin-and-eosin to confirm the presence of neoplastic tissues. Patient information was delinked from the specimens, and study has been approved by Institutional Review Board (IRB) of M.D. Anderson Cancer Center. ER/PR/HER2 results were obtained from the original pathology reports for each case in the TMA. Standard immunohistochemistry was performed on TMA samples for HSF-1(1:250 dilution, 4356, Cell Signaling Technology) and ATG7 (1:250 dilution, Millipore). Nuclear expression of HSF1 and cytoplasmic expression of ATG7 was assessed semi-quantitatively using staining intensity and percentage by two independent pathological investigators. H score was determined by multiplying staining intensity by percentage of positive tumor cells (33). The Kaplan-Meier method was used to analyze breast cancer patient overall survival. Two-tailed P values ≤.05 were considered statistically significant. SPSS software and XLSTAT were used for statistical analysis. Tetrachoric correlation analysis was used to analyze correlation between HSF1 and ATG7 (34).

**Survival analysis using external breast cancer expression datasets:**

Microarray gene expression and clinical data (GSE1456 and GSE7390) were downloaded from NCBI GEO website (http://www.ncbi.nlm.nih.gov/geo/). Analyses were performed using the R software (http://www.r-project.org). Gene expression levels were normalized using the “rma” package from R and patients were automatically assigned into ATG7- low and –high groups using the kmean function performed on ATG7 expression. Kaplan-Meier analysis was performed using the “survival” package from R.

**Statistical Analysis:**

Statistical evaluation was determined by Student’s t-test. All data shown as mean + standard error (SE). p value <0.05 was considered significant.

**RESULTS**

**HSF1 expression protects cancer cell from chemotherapeutic agent induced cell death.**

To study the role of HSF1 in cancer cell survival, we first analyzed a published breast cancer patient database. The analysis revealed that HSF1 expression is higher in breast tumors compared to normal tissue (Figure 1A). Moreover, survival analysis using patient database indicated an association between high HSF1 expression and...
poor patient survival (Figure 1B). This is consistent with the results of a recent study (18). These data indicate that HSF1 expression plays a protective role in cancer cell survival. Furthermore, published study from our laboratory has shown that HSF1 expression is elevated and contributes to trastuzumab resistance (15,35), as well as reduces sensitivity to taxol and doxorubicin treatments (Figure S1A and S1B). Next, to study the role of HSF1 in sensitivity to Carboplatin treatment, we used stable HSF1 knockdown cell lines which were generated using a lentiviral construct targeting HSF1. Treatment with 75µg/ml Carboplatin, a second generation DNA alkylating agent increasingly used in the clinic and in combination with taxanes in several breast cancer clinical trials (1), showed an increase in apoptosis in HSF1 knockdown cells as measured by cleaved PARP and cleaved caspase 3 in MDA-MB-231 and MDA-MB-436 cells (Figure 1C and S1C). Increase in Annexin V-FITC and 7-AAD staining further confirmed that knockdown of HSF1 increased sensitivity to Carboplatin (Figure 1D). Increase in Annexin V-FITC and 7-AAD staining further confirmed that knockdown of HSF1 expression also enhanced growth inhibition in MDA-MB-231 cells treated with Carboplatin (Figure 1E). To further confirm that HSF1 increases resistance to Carboplatin, we transiently overexpressed HSF1 in stable HSF1 knockdown cells. Restoring HSF1 expression led to decreased apoptosis as measured by cleaved PARP, in presence of Carboplatin (Figure 1F). Collectively, these data indicate that HSF1 plays an important role in protecting cancer cells from Carboplatin-induced cell death.

**HSF1 is required for induction of autophagy.**

Autophagy is a cellular process that is activated to mitigate cellular damage to allow cells to survive in stressful conditions (36). Cisplatin, a first generation platinum agent, has been reported to induce autophagy in cancer cells leading to decreased chemo-sensitivity (36). Similarly, we wanted to test whether Carboplatin treatment also induced autophagy in cancer cells. As observed in figure 2A, treatment with Carboplatin robustly induced autophagy, similar to Rapamycin, an mTOR inhibitor and a well known inducer of autophagy (Figure 2A and 2B). Interestingly, knockdown of HSF1 expression in these cells completely blocked the induction of autophagosomes by both Rapamycin and Carboplatin as indicated by the EGFP-LC3 punctate structures (Figure 2A, 2B). Similarly, treatment with Carboplatin in MDA-MB-436 cells also increased formation of EGFP-LC3 punctate structures, which was blocked upon knockdown of HSF1 (Figure 2C, 2D). These data indicate that HSF1 is required for induction of autophagy. Moreover, the requirement of HSF1 for induction of autophagy was further confirmed by flowcytometry using acridine orange to stain for acidic vesicular organelles (AVOs) in the cells. Carboplatin treatment increased formation of AVOs in cells expressing HSF1, but failed to increase accumulation of AVOs in HSF1 knockdown cells (Figure 2E and 2F). Since AVOs are a measure of all acidic vesicular organelles including but not limited to autophagolysosome, we treated cells with 3-Methyladine (3-MA), an autophagy inhibitor, to inhibit formation of AVOs because of autophagy. Inhibition of autophagy reduced AVOs in scramble cell population (Figure 2E and 2F), indicating that the increase in AVOs in Carboplatin treated cells is mainly due to the induction of autophagy. Taken together, these results demonstrate that HSF1 expression is essential for induction of autophagy in cancer cells.

**HSF1 transcriptionally upregulates ATG7**

HSF1 is a transcription factor that regulates expression of its target genes under conditions of cellular stress, specifically heat shock. Besides its ability to regulate heat shock proteins, HSF1 has been shown to regulate the expression of a distinct set of genes in cancer cells (7). A genome-wide analysis of human HSF1 using chromatin Immunoprecipitation microarray studies revealed several gene promoters that can be bound by HSF1 regulating cellular adaptation and survival (13). Of these possible HSF1 targets only two autophagy related genes –ATG7 and ATG4b were identified (13). Upon further promoter sequence analysis using TFSearch database, we identified two potential HSF1 binding sites with adjacent inverted repeats of 5´-nGAAn- 3´at positions -1570 and -1602 within the ATG7 promoter. Next, to determine whether HSF1 regulates autophagy by targeting the expression of ATG7, we assessed the expression of ATG7 in cancer cells that express different levels of HSF1. Carboplatin treatment increased expression of ATG7 protein (Figure 3A), but knockdown of HSF1 reduced
ATG7 protein expression in presence of Carboplatin (Figure 3A). To further confirm this, we overexpressed wild type HSF1 and dominant negative (DN) HSF1 in stable HSF1 knockdown cells. Re-expression of wild type HSF1 restored the expression of ATG7 in these cells, whereas DN-HSF1 did not (Figure 3B). The regulation of ATG7 at the protein level upon Carboplatin treatment was a direct effect of regulation of ATG7 mRNA expression (Figure 3C). These results further confirmed that HSF1 regulates the expression of ATG7 and may subsequently regulate autophagy.

The regulation of ATG7 expression by HSF1 was only observed after treatment with Carboplatin. To function as an active transcription factor cytoplasmic HSF1 translocates into the nucleus, phosphorylates and trimersizes allowing it to bind to its target genes and subsequently regulating their expression (8). To test if Carboplatin can activate HSF1, which could explain why Carboplatin induces upregulation of ATG7 in cancer cells (Figure 3A), we examined HSF1 trimer formation in HSF1 expressing cells upon Carboplatin treatment. As seen in Figure 4, treatment with Carboplatin increased HSF1 trimer formation along with phosphorylation at residue S326 (Figure 4A) and enhanced its nuclear translocation (Figure 4B), indicating activation of HSF1. These data indicate that Carboplatin treatment increased the transcription activity of HSF1. Next, ChIP analysis was used to confirm that activated HSF1 regulates ATG7 expression at the level of transcription by directly binding to the ATG7 promoter region. ChIP assay followed by quantitative PCR analysis using primers flanking the two potential HSF1 binding sites (ATG7 (1)), shows a significant increase in HSF1 binding upon Carboplatin treatment which was lost upon knockdown of HSF1 (Figure 4C). Furthermore, no HSF1 binding was detected using ATG7 primer set flanking region approximately 780bp upstream of the HSF1 binding sites (ATG7 (4)) (Figure 4C). This indicates that HSF1 binding was specific to ATG7 promoter region containing potential HSF1 binding sites. The increased binding of HSF1 at the ATG7 promoter was accompanied by an increase in the histone H3 acetylation mark (Figure 4D), which is an indication of active gene transcription. This change in acetylation was specific to recruitment of HSF1 at the ATG7 promoter, since no change in histone H3 acetylation was observed in HSF1 knockdown cells. The beta-globin gene is a negative control which shows no binding of HSF1 as well as no H3 acetylation (Figure 4C, 4D). To further validate that the two potential HSF1 binding sites are required for regulation of ATG7, we cloned HSF1 binding region within the human ATG7 promoter region upstream of the luciferase gene and assessed the luciferase activity. Two ATG7 promoter deletion constructs were also generated. The HSF1 binding site at -1602 was deleted in ATG7 mutant 1 (ATG7 mut1), while both the HSF1 binding sites were deleted in ATG7 mutant 2 (ATG7 mut2). Carboplatin treatment significantly increased the ATG7 promoter luciferase activity only in presence of HSF1 (Figure 4E). Deletion of HSF1 binding sites reduced the promoter activity by approximately 50% in presence of Carboplatin (Figure 4E). A similar degree of inhibition of luciferase activity for the two deletion constructs suggests that HSF1 binding site at -1602 is more important for the ATG7 promoter activity. Furthermore, downregulation of HSF1 by shRNA dramatically decreased the luciferase activity of the wild-type promoter by 70% and to a lesser extent in the HSF1 binding site mutants (Figure 4E). Since HSF1 is a key regulator of heat shock response, we next wanted to identify whether ATG7 was also regulated under heat shock conditions. As shown in supplemental figure S2A heat shock at 42°C induced expression of Hsp70, a classical heat shock response gene, but failed to increase the expression of ATG7. Furthermore, chemotherapeutic treatment activated HSF1 (Figure S2B) and increased ATG7 expression (Figure S2A) but did not induce a heat shock response as shown by no change in Hsp70 expression (Figure S2B). These data indicate that regulation of ATG7 by HSF1 is independent of heat shock. Taken together, these results strongly indicate that Carboplatin activates HSF1 which directly binds to the ATG7 promoter and transcriptionally activates ATG7 expression independent of heat shock.

Autophagy confers HSF1-mediated resistance to chemotherapy –induced cell death

Next, to examine whether regulation of ATG7 by HSF1 plays a role in HSF1-mediated resistance to chemotherapy –induced cell death, we transiently
knockdown ATG7 expression in MDA-MB-231 cells (Figure 5A). Reduction of ATG7 expression reduced autophagy as indicated by the accumulation of p62 and also increased sensitivity to Carboplatin treatment as shown by the increase in cleaved PARP (Figure 5B). The transient knockdown efficiency of ATG7 was significantly higher in the HSF1 stable knockdown cells (Figure 5A). This may explain for the significantly higher apoptosis in the double knockdown cells. To further confirm the role of ATG7 in chemo-sensitivity, ATG7 was overexpressed in HSF1 knockdown cells (Figure 5C). Overexpression of ATG7 reduced Carboplatin sensitivity, as indicated by the reduction of apoptosis in these cells detected by decrease in cleaved PARP and cleaved caspase3 expression (Figure 5D). These data indicate that autophagy regulated by ATG7 plays an important cytoprotective role in Carboplatin treated cancer cells.

**HSF1 expression is correlated with ATG7 and ATG7 expression is associated with poor patient survival in breast cancer patients**

To study the clinical relevance of our findings, we carried out immunohistochemistry analysis for expression of HSF1 and ATG7 in breast cancer patient samples. A total of 142 patient samples were stained for HSF1 and ATG7 using commercially available antibodies (Figure 6A and 6B). Two pathologists scored the HSF1 and ATG7 staining by semiquantitative analysis of nuclear and cytoplasmic staining intensities, respectively. It has been reported that high levels of HSF1 are present in TNBC (7) and are associated with poor prognosis in breast cancer (18). To further explore whether levels of ATG7 and HSF1 were correlated in TNBC we carried out Tetrachoric correlation analysis. As shown in Figure 6C there is a significant positive correlation between HSF1 expression and ATG7 expression levels in TNBC patient samples ($r = 0.461$, $p=0.01$). Since ATG7 and patient survival correlation has never been reported, we next focused our study on ATG7. Interestingly, Kaplan-Meier analysis suggests a significant association between ATG7 status and survival in Triple Negative Breast Cancer (TNBC) which is the more aggressive breast cancer subtype (Figure 6D). Next, survival analysis of all 142 individual scored for ATG7 expression was obtained. The cases were divided into two groups, ATG7 high expressers and ATG7 low expressers. There were 94 (66.19%) cases for high ATG7 expression and 48 (33.80%) cases for low ATG7 expression. The Kaplan-Meier survival curve shows that patients with low ATG7 expression had significantly better survival compared to patients with high ATG7 expression (Figure 6E, $p=0.04$). Survival curves with similar results were obtained from analyzing survival of breast cancer patients using two different cancer patient databases (Figure 6F and 6G). Next, using The Cancer Genome Atlas (TCGA), we analyzed the expression of ATG7 in normal and breast tumors. There is a significant increase in expression level of ATG7 in tumors compared to normal tissues (Figure 6H). We next determined whether ATG7 status correlates with tumor size in the breast cancer patient samples. As seen in Figure 6I high levels of ATG7 were associated with larger tumor size. These results support a possible role for ATG7 in clinical aggressiveness of the breast tumors. Taken together, these data indicate that ATG7 expression is associated with breast cancer patient survival and is significantly correlated with levels of HSF1, thus validating the clinical relevance of our findings.

**DISCUSSION**

HSF1, a master regulator of heat shock response, has been shown to regulate expression of genes involved in cellular adaptation and survival (13). HSF1 has not only been associated with malignant transformation (14,37), but high levels of nuclear HSF1 are also associated with poor prognosis in breast cancer (18). Trimer formation and subsequent nuclear translocation of HSF1 are associated with active form of HSF1 that can bind to promoter of its target genes and regulate their expression (8). Here, for the first time, we show that treatment with a chemotherapeutic agent increases trimer formation and nuclear translocation of HSF1. Moreover, this translocation was accompanied by increased binding of HSF1 and higher H3 acetylation at ATG7 promoter region and enhanced ATG7 promoter activity. HSF1 has been shown to inducibly bind to specific consensus motif that are associated with histone acetylation, H3K4 dimethylation, RNA polymerase II and co-activators, which are markers of activated state of chromatin (38). Using stable HSF1 knockdown cells, we identified that binding of HSF1 was
required to increase the H3 acetylation at the ATG7 promoter region. Since higher acetylation status of the ATG7 promoter region is associated with increased ATG7 expression (39), our data suggest that absence of HSF1 at the ATG7 promoter prevented H3 acetylation leading to repression of ATG7 and thus formation of autophagosomes leading to increased chemosensitivity. Furthermore, we identified that regulation of ATG7 by HSF1 is independent of heat shock. This observation is further supported by a recent report that shows that HSF1 drives a transcriptional program which is distinct from heat shock to support a variety of highly malignant cancers (7).

Defects in autophagy have been found in several human tumors, such as allelic loss of beclin1 observed often in human breast, ovarian and prostate cancers (40-42). These observations suggest that autophagy may play a tumor suppressor role in cancer. However, a large number of recent reports have shown that autophagy can serve as a mechanism of adaptation in cancer cells to induce resistance to apoptosis for survival under conditions of metabolic stress (43). Furthermore, several cancer therapies including radiation therapy, chemotherapy, histone deacetylase inhibitors, anti-estrogen hormonal therapy as well as Herceptin treatment can induce autophagy as a protective and pro-survival mechanism in human cancer cells (26, 28, 29, 36, 44-46). Similarly, our data show that inhibition of autophagy increases sensitivity to Carboplatin. Furthermore, knockdown of HSF1 along with inhibition of autophagy enhances the chemosensitivity of cancer cells. This indicates that inhibition of cytoprotective autophagy can lead to an increase in efficacy of chemotherapy.

This is the first report demonstrating that high ATG7 levels are associated with reduced survival in breast cancer patients. Unbiased bioinformatic study using breast cancer databases along with IHC analysis of 142 breast cancer patients supports the reverse correlation between ATG7 expression and patient survival, suggesting that ATG7 can be used as an independent marker to predict breast cancer disease outcome. A similar association between patient survival and nuclear HSF1 expression has been shown in breast cancer patients (18). Santagata et al. show that higher nuclear HSF1 levels in breast cancer patients are associated with poor patient survival. Similarly, our unbiased bioinformatic analysis of HSF1 expression levels supports this finding. In addition, we also found a significant correlation between ATG7 and HSF1 levels in the TNBC patient samples (r = 0.461). TNBC is a more aggressive and metastatic breast cancer subtype. It is characterized by lack of progesterone receptor, estrogen receptor and HER2/ErbB2 receptor. The absence of these receptors for targeted therapy makes TNBC difficult to treat. We observed that higher ATG7 expression is associated with poor survival in TNBC patients, suggesting that ATG7 may have a strong prognostic value in these patients. Moreover, the positive correlation observed between levels of HSF1 and ATG7 in these patients further corroborates our in-vitro findings.

In conclusion, this is the first study to identify the critical function of HSF1 in regulating drug–mediated autophagy in breast cancer cells. We identified a novel target gene of HSF1, ATG7. We propose a mechanism of action in which stress induced by chemotherapeutic agent Carboplatin activates HSF1, leading to continued expression of ATG7 and induction of autophagy (Figure 7A). This autophagy has a cytoprotective role leading to drug resistance in the cells. Therefore, knockdown of HSF1 inhibits induction of autophagy in chemotherapy treated breast cancer cells, increasing drug sensitivity to Carboplatin in the cells. Our data from in vitro study along with the patient data analyses clearly support the rationale to design novel drugs or combination therapies to target HSF1 as well as its target gene ATG7, leading to inhibition of autophagy (Figure 7B). These findings provide insights into novel mechanisms for HSF1–mediated chemoresistance and have significant implications in the development of strategies to overcome cancer cell resistance to chemotherapy.
References
27. Zhu, K., Dunner, K., Jr., and McConkey, D. J. Oncogene 29, 451-462


38. Guertin, M. J., and Lis, J. T. *PloS Genet* 6


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Abbreviations used are: HSF1, Heat Shock Factor 1; 3-MA, 3-Methyladinine; ATG7, Autophagy related gene 7; cPARP, cleaved Poly-ADP ribose polymerase.

FIGURE LEGENDS

Figure 1: HSF1 confers drug resistance in breast cancer. A) Box plot for expression of HSF1 in normal and tumor tissue using TCGA database B) Survival analysis for breast cancer patient using the GSE7390 database. C) Immunoblot analysis of HSF1 and total and cleaved PARP (cPARP) and cleaved caspase 3 (c Casp3) in MDA-MB-231 after stable HSF1 knockdown and treatment with 75µg/ml Carboplatin for 24 hours. D) Flowcytometry of Annexin V- FITC and 7-AAD staining in Carboplatin treated MDA-MB-231 in presence or absence of HSF1. E) Growth inhibition in presence or absence of HSF1 in MDA-MB-231 treated with 75µg/ml Carboplatin and 3-MA for 24 hours. F) Immunoblot analysis for HSF1 and cPARP in Carboplatin treated control (-), HSF1 knockdown (+) and HSF1 knockdown transiently overexpressing HSF1. Loading control β actin. Vertical gray line in some blots indicates deleted lanes from the blot.

Figure 2: HSF1 influences induction of autophagy in breast cancer cells. A) Microscopy of punctate EGFP-LC3 in scramble and HSF1 knockdown MDA-MB-231 cells in untreated (control) or treated with 10nM Rapamycin or 75µg/ml Carboplatin for 24 hours. Magnification x60. White arrows, punctate EGFP-LC3 structures. B) Bar graph represents average EGFP-LC3 punctate structures per cell in MDA-MB-231 expressing HSF1 (■) or HSF1 knockdowns (■), upon treatment with Rapamycin or Carboplatin. Error bars, SE. Data collected for 2 independent experiments and at least 10 images per slide analyzed for each condition. (**, p < 0.005). C) Microscopy of punctate EGFP-LC3 in presence or absence of HSF1 in MDA-MB-436 untreated (control) or treated with 75µg/ml Carboplatin for 24 hours. Magnification of x60. D) Bar graph, average EGFP-LC3 punctate structures per cell in MDA-MB-436 expressing HSF1 (■) or HSF1 knockdown (■), upon treatment with Carboplatin. Error bars, SE. Data for 3 independent experiments and at least 30 cells for Rapamycin treatment and Carboplatin treatment were analyzed for each condition. (*, p < 0.05). Flowcytometry of acridine orange staining of Acidic Vesicular Organelle (AVO) in scramble and stable HSF1 knockdown MDA-MB-231 cells. Error bars, SE. F) Representative flowcytometric profile of AVO positive cells. FL1 detects green intensity and FL3 detects red intensity. Cells in right quadrants were considered AVO positive.

Figure 3: HSF1 influences expression of autophagy related gene ATG7. Immunoblot analysis for HSF1, ATG7 and LC3-II in A) (Top Panel) MDA-MB-231 scramble (-) and stable HSF1 knockdown (+) in presence of 75µg/ml and 100µg/ml of Carboplatin for 24 hours. (Bottom panel) Bar graph representing quantification of LC3-II expression using imageJ software. LC3-II levels normalized to β-actin. B) Immunoblot analysis for ATG7 upon Carboplatin treatment in stable HSF1 knockdowns (+) transiently overexpressing wild type HSF1 (HSF1) or dominant negative HSF1 (HSF1 DN). Loading control β actin. C) mRNA levels of ATG7 in Scramble (■) and stable HSF1 knockdown (■) MDA-MB-231 cells treated with 75µg/ml Carboplatin for 24 hours. Error bars, SE (N=3), (**,p < 0.02).

Figure 4: Chemotherapy activates HSF1 in breast cancer cells. A) Immunoblot analysis of trimer formation of HSF1 and phosphorylation of S326 residue in MDA-MB-231 cells treated with 75µg/ml of Carboplatin. B) Immunofluorescence staining of MDA-MB-231
HSF1 scramble untreated and Carboplatin treated and stable HSF1 knockdown Carboplatin treated. HSF1, Alexa594 (red); nucleus, 4',6-diamidino-2-phenylindole (DAPI). Merged image, nuclear localization of HSF1. Images shown are a representative panel. Magnification of ×60. Columns represent the mean intensity of HSF1 in the nucleus. Bars, SEM. C) ChIP using HSF1 antibody in scramble untreated (○) and Carboplatin treated (■) and stable HSF1 knockdown Carboplatin treated (■). ATG7 (1) primer flank HSF1 binding region, ATG7 (4) primer set and β globin are negative controls. D) ChIP of acetylation of histone H3. The conditions are as described in C, except the antibody used is specific to acetyl H3. Columns, mean of 3 independent experiments; bars, SE, ** p<0.05. E) HSF1 regulates ATG7 luciferase promoter activity. MDA-MB-231 scramble and stable HSF1 knockdown transfected with ATG7, ATG7 mut1, ATG7 mut2. Control (untreated), Carboplatin (75µg/ml, 24 hours). Results normalized to a co-transfected renilla. Columns, mean of 3 independent experiments; bars, SE. (**, p<0.05) β-Actin is the loading control.

Figure 5: Autophagy influences drug sensitivity. Immunoblot analysis of A) ATG7 in presence of siATG7 in MDA-MB-231 scramble control and HSF1 stable knockdown cells in presence and absence of Carboplatin B) cPARP and p62 in transient ATG7 knockdown HSF1 scramble and HSF1 knockdown MDA-MB-231 cells C) ATG7 in scramble control, stable HSF1 knockdown cells and stable HSF1 knockdown cells transiently overexpressing ATG7 in presence of Carboplatin D) Total and cleaved PARP and cCaspase3 levels in Carboplatin untreated and treated scramble controls and in Carboplatin treated stable HSF1 knockdown and stable HSF1 knockdown cells transiently overexpressing ATG7. Vertical gray line in the blots indicates deleted lanes from the same blots.

Figure 6: Atg7–high tumors associated with decreased survival in breast cancer patients. Immunohistochemical staining of breast cancer patient samples for A) HSF1 and B) ATG7. C) Bar graph representing the correlation between expression of HSF1 and ATG7 in the TNBC patient samples. Tetrachoric correlation coefficient is shown along with log p value. D) Kaplan-Meier analysis of TNBC individuals scored for the ATG7 status in the study. E) Kaplan-Meier analysis of all individuals scored for the ATG7 status in the study. F) Survival analysis for breast cancer patient based on ATG7 status using the GSE7390 database and G) using the GSE1456 database. Log–rank P value is shown. H) Box plot for expression of ATG7 in normal and tumor tissue using TCGA database. I) Bar graph for distribution of breast cancer patients with large tumor size (≥ 2) and small tumor size (< 2) expressing different levels of ATG7.

Figure 7: Proposed Model A) Carboplatin treatment activates HSF1 (increasing trimer formation, nuclear translocation and phosphorylation) which allows HSF1 to bind to and upregulate transcription of ATG7 leading to an increase in autophagy. Increase in autophagy leads to an increase in cell survival and a reduction in amount of cell death. B) Inhibition of HSF1 activity or reduction in ATG7 levels may lead to a reduction in autophagy and improve the cellular response to Carboplatin.
Figure 3

A

Carboplatin
-  +  +  ++
shHSF1
-  -  -  +
HSF1
-  -  -  +
ATG7
-  -  -  +
LC3 II
-  -  -  +
β-Actin

B

Carboplatin
-  +  +
HSF1 DN
-  -  -
HSF1
-  +  -
ATG7
-  -  -
β-Actin

C

Relative ATG2 mRNA

-  Scramble  shHSF1

Control  Carboplatin
Figure 4

A

Carboplatin  Trimer  Dimer  Monomer  β actin  pHSF1 (S326)  β actin

B

shHSF1  Carboplatin  DAPI  HSF1  Merge

C

HSF1 Binding

D

H3 Acetylation

E

Relative Luciferase Activity

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Figure 6

A

Case #1
Case #2
Case #3
Case #4
HSF1 Low  HSF1 High

B

Case #1
Case #2
Case #3
Case #4
ATG7 Low  ATG7 High

C

m0.461, p<0.01

ATG7 High
ATG7 Low

HSF1 High  HSF1 Low

D

Survival of TNBC Patients with ATG7 status

E

Survival of Breast Cancer Patients with ATG7 status

F

GSE1481

G

GSE7360

H

ATG7 expression

p=3.3e-16

Normal  Tumor

I

Tumor size >2
Tumor size ≤2

ATG7 High  ATG7 Low