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HSP27 AND CHEMOTHERAPY-INDUCED AUTOPHAGY AS BIOMARKERS IN OSTEOSARCOMA

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HSP27 AND CHEMOTHERAPY-INDUCED AUTOPHAGY AS BIOMARKERS IN OSTEOSARCOMA

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John Andrew Livingston, M.D.

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Survival for patients with osteosarcoma has not improved for > 30 years. Despite aggressive multi-agent chemotherapy combined with surgical resection, a significant fraction of patients with localized disease relapse after optimal treatment. We evaluated the occurrence of cytoplasmic LC3B (light chain 3B)-positive puncta (a marker of autophagy) and presence of HSP27 (heat shock protein 27) in cancer cells within pre-treatment biopsy, post-treatment surgical resection, and metastatic osteosarcoma specimens by immunohistochemistry in 260 patients. LCB3+ puncta expression was seen in 34% of pre-treatment. 50% of resection, and 67% of metastasis samples. Sixty-six percent of all specimens were scored positive for HSP27 (85% of pre-treatment. 52% of resection, and 50% of metastasis samples). Among 215 patients with localized disease, pre-treatment HSP27 expression was associated with inferior overall survival (adjusted HR 26.7, p=0.0263) as well as at resection following chemotherapy (adjusted HR 1.85, p=0.039). Lack of LC3B-puncta expression was an independent poor prognostic marker at resection (adjusted HR 1.75, p=0.045). Patients with LC3B+/HSP27- tumors at resection had the best prognosis whereas patients with LC3B-/HSP27+ osteosarcoma had the worst long-term survival. Neither HSP27 nor LC3B expression correlated with tumor necrosis. These findings indicate that HSP27 expression is a negative prognostic

biomarker in osteosarcoma. Conversely, presence of autophagy following neoadjuvant chemotherapy, as measured by LC3B-puncta, predicts longer overall survival in osteosarcoma patients with localized disease.

We additionally evaluated the significance of chemotherapy-induced autophagy in 2 human osteosarcoma cell lines: LM7 and CCH-OS-D. Both doxorubicin (DOX) and cisplatin (CDDP) were found to induce autophagy. In LM7 cells, autophagy inhibition with hydroxychloroquine (HCQ) prior to chemotherapy resulted in a trend towards decreased viability consistent with a cytoprotective role of autophagy. In CCH-OS-D cells, autophagy inhibition prior to DOX significantly decreased chemosensitivity suggesting a cytotoxic role of autophagy in this setting. The post-treatment expression of phosphorylated HSP27 was increased in LM7 and decreased in CCH-OS-D following DOX or CDDP. These findings support a dual role of chemotherapy-induced autophagy and potential application of pHSP27 as a predictive biomarker of autophagy inhibitors in osteosarcoma.

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I. Background:

Current Therapies for Osteosarcoma

Bone sarcomas are rare, making up 0.2% of all cancers.[1] Osteosarcoma is the most common bone sarcoma with approximately 1000 new cases per year in the U.S. and predominantly effects adolescents and young adults.[2] Prior to the introduction of multi-agent chemotherapy, survival for patients with osteosarcoma was extremely poor with long-term survival <20%.[3] Up to 90% of patients developed metastatic disease following surgery alone, leading to the hypothesis that the majority of patients have subclinical micrometastatic disease at presentation. With the addition of perioperative chemotherapy beginning in the 1960s and 1970s, survival for patients with localized osteosarcoma has now improved to 60-65%.[4] While chemotherapy plays an integral role in curative treatment for patients with osteosarcoma, chemotherapy alone is inadequate. Patients who do not undergo surgical resection of their disease will eventually relapse.[5] These observations have informed the current treatment paradigm for osteosarcoma which consists of preoperative combination chemotherapy with MAP (cisplatin, doxorubicin, and high-dose methotrexate) followed by limb-sparing surgery (when feasible) with pathologic response assessment and subsequent MAP chemotherapy (Figure 1). Despite these advances, there is still a significant fraction of patients who relapse and die of pulmonary metastasis,[6] and there have been no significant improvements in overall survival for >30 years.[4, 7]

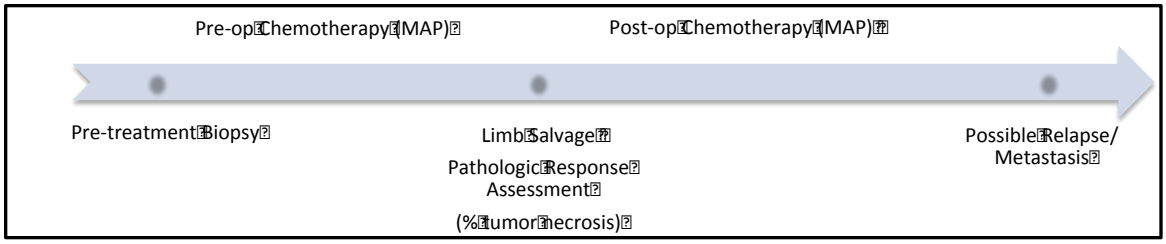

Figure 1. Standard treatment schema for localized osteosarcoma. Tumor tissue is obtained prior to treatment, at resection, and at relapse (blue dots).

The prognosis for patients with metastatic disease is particularly poor and treatment of recurrent/metastatic disease remains a challenge. Patients with primary metastatic disease (those presenting with overtly metastatic disease at diagnosis) have a 5-year survival of approximately 30-50%[8, 9] and those with metastatic recurrence have even lower 5-year survival of 15-20%.[10] Pulmonary metastasectomy can be curative in patients with limited/resectable pulmonary disease whereas unresectable metastatic disease (such as multiple osseous metastases or extensive pulmonary disease) is likely incurable. While a number of chemotherapeutic regimens have been evaluated either retrospectively or in the context of clinical trials, response rates range from 10-40% with 4-month progression-free survival rates ranging from 25 to > 45% (selected studies in Table 1). The pooled outcomes for patients with recurrent osteosarcoma enrolled on 7 phase 2 clinical trials through the Children's Oncology Group (COG) and its predecessor cooperative groups are even more discouraging.[11] In each individual trial, the drugs tested were deemed to be inactive. The event-free survival (EFS) for patients with measurable disease was 12% at 4 months (95% CI, 6% - 19%) with no significant difference in EFS based upon patient demographics or the number of prior treatment regimens. For patients with pulmonary metastasis who were rendered disease free by surgery and were treated

on study AOST0221 (inhaled GM-CSF), the EFS was significantly better than those with unresectable disease but remained low, 20% at 12 months (95% CI, 10-34%). Another recent study of advanced bone sarcoma patients treated in phase I clinical trials at MD Anderson Cancer Center re-demonstrated the overall poor outcomes and lack of effective therapies for these patients. This included pediatric and adult osteosarcoma patients treated across 14 unique protocols and showed no response to any of the cytotoxic, targeted, or biologic agents used.[12] The 4-month progression-free survival (PFS) was 26% for patients with recurrent osteosarcoma.

Chemotherapy Regimen	Outcome	Study
Gemcitabine + docetaxel	PR: 3/10; SD 1/10	Navid F, et al. Cancer. 2008.[13]
Cyclophosphamide + etoposide	PFS at 4 months: 42%	Massimo B, et all. Cancer. 2009.[14]
Cyclophosphamide + topotecan	PR: 2/18	Saylors RL, et al. JCO. 2001.[15]
Ifosfamide + etoposide	PR or CR: 3/8	Miser JS, et al. JCO. 1987[16]
Ifosfamide + carboplatin + etoposide	ORR: 51% (all sarcomas)	Van Winkle P, et al. Pediatr Blood Cancer. 2005.[17]
High-dose ifosfamide	ORR 20% PFS at 4mo: 25%	Palmarini E, et al. ASCO, 2015.[18]
Sorafenib + everolimus	PFS at 6 mo: 45%	Grignani G, et al Lancet Onc. 2015.[19]

Table 1. Treatment outcomes for relapsed/metastatic osteosarcoma. Complete response (CR), objective response rate (ORR), progression-free survival (PFS), partial response (PR), stable disease (SD).

Given the adverse outcomes and limited efficacy of treatment in the recurrent/metastatic setting as well as the lack of promising agents in early-phase development, it is important to identify patients with a high-likelihood of metastatic relapse and death for consideration of clinical trial participation in studies that combine

molecular biomarkers and targeted therapies, either in the neoadjuvant or adjuvant setting, to improve the efficacy of standard chemotherapy for osteosarcoma. As such, the identification of novel prognostic biomarkers either at diagnosis or resection following neoadjuvant chemotherapy is an unmet need in osteosarcoma.

Prognostic Markers in Osteosarcoma

Due to the potential for relapse amongst patients treated with standard therapy, several studies have sought to identify prognostic markers in patients with osteosarcoma. To date, stage at presentation (localized vs metastatic) and pathologic response to neoadjuvant chemotherapy (assessed by percent necrosis at the time of surgical resection) remain the most widely accepted and clinically utilized prognostic factors. Other clinical and pathologic factors have been explored as they relate to metastasis-free and overall survival including age at diagnosis, sex, stage at presentation, tumor location, tumor size, histologic subtype, surgical margins, and serum markers such as lactate dehydrogenase (LDH) level or alkaline phosphatase levels among others.[20-23] For example, in one large retrospective cohort of 1,702 patients with high-grade osteosarcoma treated on neoadjuvant cooperative group protocols, older patient age at diagnosis (≥ 40 years), axial tumor site, larger tumor size (for extremity tumors), and presence of primary metastasis were associated with inferior overall survival.[21] Treatment-related factors including response to chemotherapy and the extent of surgery (incomplete vs macroscopically complete) were also shown to be significant. In multivariate analysis, residual tumor (HR 4.01, $p < 0.0001$) and poor response to chemotherapy (HR 2.44, $p < 0.0001$) were identified as key prognostic factors for overall survival.

Identifying patients with localized (i.e. non-metastatic) disease that are at high-risk for relapse is clinically relevant and has informed the design of adjuvant therapy trials in osteosarcoma. Pathologic response to chemotherapy, as assessed by percent tumor necrosis on the primary resection specimen, is a well-established prognostic factor in osteosarcoma.[24] Poor response has been defined as <90% tumor necrosis in most studies, however this cutoff may vary. In early studies of preoperative chemotherapy, patients with a poor response to MAP chemotherapy had significantly worse outcomes.[25, 26] These findings were confirmed in an initial literature review of studies that included non-metastatic high-grade osteosarcoma patients published between 1973-1992 where only tumor necrosis following preoperative chemotherapy was found to have independent prognostic significance.[27] In a subsequent systematic review including studies from 1992-2006, poor response to chemotherapy (pooled relative risk (RR) of death or recurrence 2.37, 95%CI 2.07-2.70), larger tumor volume (pooled RR 1.36, 95%CI 1.18-1.58) and ablative surgery as compared to limb salvage (pooled RR 2.18, 1.58-3.00) were all identified as predictors of adverse outcomes.[28] However, the authors noted that a pooled analysis was challenging due the heterogeneity of the prognostic factors analyzed across studies.

With the identification of tumor necrosis as a prognostic biomarker in osteosarcoma, multiple clinical trials have sought to intensify either preoperative chemotherapy to improve pathologic response rates or modify postoperative therapy to improve outcomes among poor responders. Intensification of preoperative therapy can result in higher rates of "good necrosis," but this strategy results in greater toxicity without clear survival benefit. In a phase II study evaluating intensification of

preoperative chemotherapy, the addition of ifosfamide to a standard doxorubicin/cisplatin backbone with autologous stem cell rescue resulted in an improvement in the objective rate of tumor necrosis but resulted in unacceptable toxicity and no significant improvement in survival.[29]

The role of tailored or modified post-operative therapy for patients with poor tumor necrosis remains controverted. The initial rationale for modification of postoperative chemotherapy amongst poor responders was established in the 1970s when primary (preoperative) therapy consisted of a less active drug combination than current MAP chemotherapy.[26] In subsequent studies from the Rizzoli Institute, intensification of postoperative chemotherapy amongst poor responders improved survival: patients received neoadjuvant chemotherapy with 2 cycles of high-dose methotrexate (HD MTX), intra-arterial cisplatin, and intravenous doxorubicin followed by surgical resection; poor responders (<90% tumor necrosis) went on to receive additional chemotherapy with ifosfamide and etoposide (I/E) in addition to MAP.[30] The 5-year continuous disease-free survival (CDFS) was 63%, with no significant difference between good and poor responders. These findings have informed the subsequent treatment paradigm for patients with poor response to MAP chemotherapy. In one large retrospective series of adult patients with osteosarcoma treated at MD Anderson Cancer Center, the addition of high-dose ifosfamide to postoperative therapy increased the CDFS amongst poor responders to 67%.[31, 32] These findings were validated in an updated series of adult patients treated within the same center.[33] A recent large international randomized clinical trial has called the utility of this approach into question, however. In the EURAMOS-01 study, 618 patients with poor tumor necrosis following preoperative MAP chemotherapy were

randomized to receive either MAP or MAP plus ifosfamide and etoposide (MAPIE).[34] Toxicity was noted to be higher in the MAPIE group and EFS did not differ between treatment groups (HR 0.98, 95% CI 0.78 – 1.23), leading investigators to conclude that the results do not support the addition of I/E to postoperative therapy for patients with poor response and that "new strategies are required to improve outcomes in this setting." Similarly, a retrospective analysis of assessing the prognostic value to histologic response to therapy between a historic regimen (CCG-782[35]) vs increased intensity induction therapy (INT-0133[36]) found that while histologic response predicted outcomes across studies ($p < 0.0001$), there was an inverse relationship between the predictive value of tumor necrosis and the intensity of induction therapy.[37] Stated another way, the difference in outcomes between good and poor responders was less dramatic with modern intensified chemotherapy as compared to prior regimens (10y EFS 70.8% vs 58.4% in good v poor responders respectively in INT-0133 as compared to 75.4% vs 39.9% in CCG-782). Thus the authors concluded that their findings "highlight the need for novel markers" to develop treatment strategies in future trials.

To date, there are no established predictive biomarkers of response to MAP chemotherapy in osteosarcoma. Identifying poor risk patients at diagnosis would allow for a window trial approach with correlative biomarkers. Similarly, additional factors that allow for risk stratification independent of tumor necrosis would be valuable in informing future studies of adjuvant therapy in osteosarcoma. Therefore, research to identify novel biomarkers (both predictive and/or prognostic), potential targets focusing on mechanisms of chemoresistance, and opportunities to enhance efficacy/sensitivity to standard agents

(MAP) is imperative to inform clinical trial design and improve outcomes for patients with osteosarcoma.

Autophagy in Cancer and Cancer Treatment

Autophagy is a conserved physiologic process of cellular catabolism that allows the degradation and recycling of intracellular organelles, proteins, and macromolecules in order to maintain homeostasis during times of nutrient depravation and cellular stress.[38] The process of sequestering and breakdown of these cellular components through lysosoamal degradation is termed macroautophagy (referred to as autophagy henceforth). In this process, autophagy-related proteins sequester macromolecules to be degraded into double-membrane vesicles (termed autophagosomes). Autophagosomes fuse with the lysosome to form the autolysosome in which the macromolecules and organelles are then broken down into amino acids, nucleic acids, and fatty acids which can be used to maintain adenosine triphosphate (ATP) synthesis and support other key anabolic pathways. Macroautophagy has been the primary focus for studies of autophagy and autophagy inhibition in cancer. This differs from microautophagy (a nonselective lysosomal degradation by direct engulfment)[39], chaperone-mediated autophagy (in which chaperones in the cytosol facilitate lysosomal proetolysis)[38], and mitophagy (which refers to the degradation of mitochondria by autophagy).[40]

Autophagy serves a dual role, either as a mechanism of cell survival or of cell death. As a cytoprotective mechanism, autophagy promotes cell survival in the setting of nutrient deprivation or other stress. Alternatively uncontrolled/excess autophagy can lead to cell death through apoptosis or an alternate cell death pathway termed “autophagic cell

death." For example, studies have shown that knockdown of key autophagy (*ATG*) genes can promote both apoptotic and non-apoptotic cell death, suggesting that the role of autophagy is cytoprotective.[39] Conversely, multiple lines of evidence support the rationale for autophagy as an alternate cell death pathway: 1) overexpression of autophagy proteins such as Beclin 1 or Atg1 can lead to cell death,[41, 42] 2) cells that are deficient in apoptosis can undergo *ATG*-dependent cell death,[43, 44] and 3) pharmacologic inhibition of autophagy may prevent cell death in certain settings.[45, 46] This duality is particularly important in cancer and has implications for both carcinogenesis and cancer treatment.

In cancer, the dual role of autophagy is often referred to as the "autophagy paradox." In the early stage of tumorigenesis, autophagy has been shown to act as a tumor suppressor. Inhibition of autophagy through allelic deletion of *Becn1* in genetic mouse models ($BENC^{+/-}$) promotes tumorigenesis and results in a high incidence of spontaneous tumors.[47, 48] Additionally, autophagy inhibition can lead to increased metabolic stress and genomic instability which can promote tumorigenesis and tumor progression.[49] Conversely, activation and up regulation of autophagy is thought to contribute to tumor growth, tumor progression, and treatment resistance by promoting cell survival during periods of nutrient deprivation, chemotherapy, or radiation-induced stress.[50] Because of this apparent paradox, there has been significant controversy regarding the role of autophagy modulation in cancer therapy.

Autophagy markers such as LC3B puncta, p62/sequestosome 1 (SQSTM1), Beclin 1, and presence of autophagic vesicles or autophagosomes (evaluated by transmission electron microscopy [TEM]) have been explored as potential predictive and

prognostic biomarkers in multiple cancer types. For example, in a series of 12 patients with melanoma treated on a phase II trial of sorafenib and temozolimide, patients whose tumors had a high "autophagic index" (defined as ≥6 autophagic vesicles per cell on EM) were less likely to respond to treatment and had shorter survival as compared to those with a low autophagic index.[51] In another series, the presence of high LC3 expression in pancreatic adenocarcinomas at time of pancreatoduodenectomy was associated inferior disease-free survival.[52] In colon cancer, overexpression of Beclin 1 has been associated with inferior overall survival amongst patients receiving adjuvant 5-fluorouracil chemotherapy.[53] While the presence of autophagy appears to be a negative prognostic marker in most cancer types, high expression of LC3 and Beclin 1 has been associated with improved response rate and superior overall survival in patients with ovarian cancer.[54] In breast cancer, studies are conflicting. In one study, the presence of LC3B (but not Beclin 1) in residual tumors following neoadjuvant chemotherapy predicted inferior relapse-free survival (RFS; multivariate HR 1.88, 95% CI 1.14-3.08) and overall survival (OS; multivariate HR 2.43, 95% CI 1.26-4.67).[55] A subsequent study found that when combined, the presence of LC3B+ puncta and nuclear HMGB1 expression in women with breast was associated with significantly improved metastasis-free survival following adjuvant chemotherapy (HR 0.49, 95% CI 0.26-0.89).[56] Conflicting studies such as these, call into question the importance of evaluating autophagy in a context-dependent manner and additionally raise concerns about the methodology for evaluating autophagy markers in patient samples. As autophagy is a dynamic process, it is important to distinguish between static measurements of autophagy from those which measure autophagic flux. This has been a major limitation of prior studies. Expert guidelines recommend that the

use of autophagy markers such as LC3B be accompanied by assays to estimate overall autophagic flux.[57] In the study by *Ladoire et al.*, the presence of cytoplasmic LC3B puncta was shown to have an inverse correlation with p62 staining, supporting the authors conclusion that in their study population high LC3B puncta reflects increased autophagic flux.[56] No studies have evaluated the predictive or prognostic significance of autophagy markers in osteosarcoma.

A wide range of current cancer drugs have been shown to modulate autophagy. For example, cytotoxic chemotherapies, hormonal therapies, and targeted therapies such as mTOR (mammalian target of rapamycin) inhibitors, HDAC (histone deacetylase) inhibitors, and multiple tyrosine kinase inhibiors (TKIs) have all been shown to induce autophagy in various cancer types.[58] The listing of approved agents by the United States Food and Drug Administration (FDA) that inhibit autophagy is much shorter, including the antimalarial drugs chloroquine and hydroxychloroquine (HCQ) which inhibit lysosomal degradation. Several clinical trials have been conducted or are currently underway utilizing HCQ to inhibit autophagy.[59] However, an important question remains: is autophagy inhibition beneficial or potentially detrimental?

The role of autophagy in cancer is context-dependent and can vary based upon tumor type, stage (premalignant vs invasive), and cancer therapy.[60] Recently studies have suggested that the role of autophagy and therefore the efficacy of autophagy inhibition may be dependent on p53 status. In a pancreas cancer genetically engineered mouse model (GEMM) of *Kras*-mutant, *Trp53* $^{-/-}$ pancreatic adenocarcinoma, loss of autophagy via tumor specific *Atg5* or *Atg7* deletion or pharmacologic inhibition with hydroxycloroquine accelerated the development of pancreatic ductal adenocarcinomas.[61]

These findings called into question the safety of autophagy inhibition in patients with *TP53*-mutant cancers.[62] Subsequent studies have refuted these findings based upon a pancreas-specific *Kras*-mutant *Trp53*$^{+/-}$ GEMM.[63] This model allows for adult p53 loss of function via loss of heterozygosity (LOH) (rather than embryonic loss of p53) and is thought to more accurately recapitulate pancreatic tumor progression in humans. In this study, *Atg5* deletion resulted in tumor suppression, with an increase premalignant lesions but preventing progression to pancreatic ductal adenocarcinoma and leading to prolonged survival. Taken together, these findings suggest that p53 status alone does not determine the role of autophagy in cancer. Rather, additional studies are needed to identify potential biomarkers that correlate with the role of autophagy in specific context (cancer type, therapy, stage) and may predict the therapeutic benefit of autophagy modulation.

Autophagy in Osteosarcoma

In osteosarcoma, most studies have focused on chemotherapy-induced autophagy as a mechanism of chemoresistance.[64] Cisplatin, doxorubicin, and methotrexate have all been shown to induce autophagy in osteosarcoma cells, which then promotes tumor cell survival by decreasing sensitivity to chemotherapy.[65-67] Autophagy inhibition, either via knockdown of key autophagy genes such as Beclin 1[68] or pharmacologic inhibition with compounds such as 3-methyladenine,[67] bafilomycin A1,[69] or chloroquine[66] have been shown to decrease cell proliferation and increase apoptosis in osteosarcoma cells treated with doxorubicin or cisplatin. Further, MAP chemotherapy results in the upregulation of high mobility group box 1 protein (HMGB1) in osteosarcoma *in vitro* and has been suggested as a potential therapeutic target in osteosarcoma.[70, 71]

Our lab has previously shown a dual role for chemotherapy-induced autophagy in osteosarcoma.[72, 73] Gemcitabine (GCB) treatment of human LM7, CCH-OS-D, and murine K7M3 cells *in vitro* and osteosarcoma lung metastases *in vivo* resulted in the formation of autophagic vesicles, conversion of LC3I to LC3II, and upregulation of Beclin 1 in both cells and tumor tissues as well as degradation of p62 and the formation of acidic vesicular organelles in tumor cells consistent with induction of autophagy. To evaluate the role of autophagy, we analyzed cell viability following treatment with chemotherapy combined with inhibition of autophagy by *BECN1* or *ATG* knockdown and pharmacologic inhibition with hydroxychloroquine (HCQ). In LM7, chemotherapy in combination with autophagy inhibition by *BECN1* knockdown or HCQ treatment resulted in decreased viability suggesting a cytoprotective role for autophagy. Conversely, autophagy inhibition prior to chemotherapy resulted in increased cell viability of CCH-OS-D and K7M3 cells consistent with cytotoxic autophagy.

Phosphorylated HSP27 as a Potential Biomarker of Chemotherapy-induced Autophagy in Osteosarcoma

We completed a human phosphokinase array to identify differentially expressed proteins and phosphoproteins at baseline and following treatment with GCB in osteosarcoma cell lines exhibiting opposing effects of autophagy and autophagy inhibition. Post-treatment expression of phosphorylated heat shock protein 27 (pHSP27) was significantly different between LM7 and CCH-OS-D cells following gemcitabine exposure and appeared to correlate with the role of chemotherapy-induced autophagy. Induction of pHSP27 following treatment with GCB correlated with a cytoprotective role

of autophagy and reduced chemosensitivity, while a failure to induce pHSP27 correlated with a cytotoxic role of autophagy. Inhibition of autophagy in cells with increased expression of pHSP27 following drug exposure resulted in increased osteosarcoma cell killing; conversely inhibition of autophagy in cells with decreased expression of pHSP27 following treatment decreased osteosarcoma cell killing.[73] These findings were validated in K7M3 cells treated with 9-nitrocamptothecin (9-NC). Previously, our lab has shown that inhibiting autophagy in K7M3 cells prior to treatment with 9-NC increases cytotoxicity whereas autophagy inhibition prior to treatment with GCB reduced cytotoxicity as compared to GCB alone.[72] In a subsequent study, we found that this effect again correlated with the post-treatment expression of pHSP27: 9-NC resulted in induction of pHSP27 (and correlated with cytoprotective autophagy) whereas pHSP27 was decreased following GCB treatment in K7M3 (correlating with cytotoxic autophagy). The correlation between pHSP27 expression and the role of chemotherapy-induced autophagy in osteosarcoma was independent of cell line, species of origin, or specific chemotherapy (summarized in Table 2). Taken together, our findings suggest that pHSP27 may be a biomarker to predict the benefit of autophagy inhibitors in the treatment of osteosarcoma.

Cell Line	Chemotherapy	pHSP27 expression	Role of Autophagy
LM7	GCB	Increase	Cell survival
CCH-OS-D	GCB	Decrease	Cell death
K7M3	GCB	Decrease	Cell death
K7M3	9-NC	Increase	Cell survival
MG63	GCB	No change	No effect

Table 2. Correlations between post-treatment expression of pHSP27 and the role of chemotherapy-induced autophagy *in vitro*. GCB – gemcitabine, 9-NC – 9-nitrocamptothecin.

In the proposed model, treatment with chemotherapy in osteosarcoma results in an induction of autophagy. This can be accompanied by an increase in pHSP27 (Figure 2, a) which is associated with chemoresistance and cytoprotective autophagy or conversely, a degradation of pHSP27 associated with cytotoxic autophagy and autophagic cell death (b). In the first scenario, autophagy inhibition with drugs such as HCQ results in increased chemosensitivity (Figure 2, c). In the second, autophagy inhibition would result in decreased drug sensitivity (d).

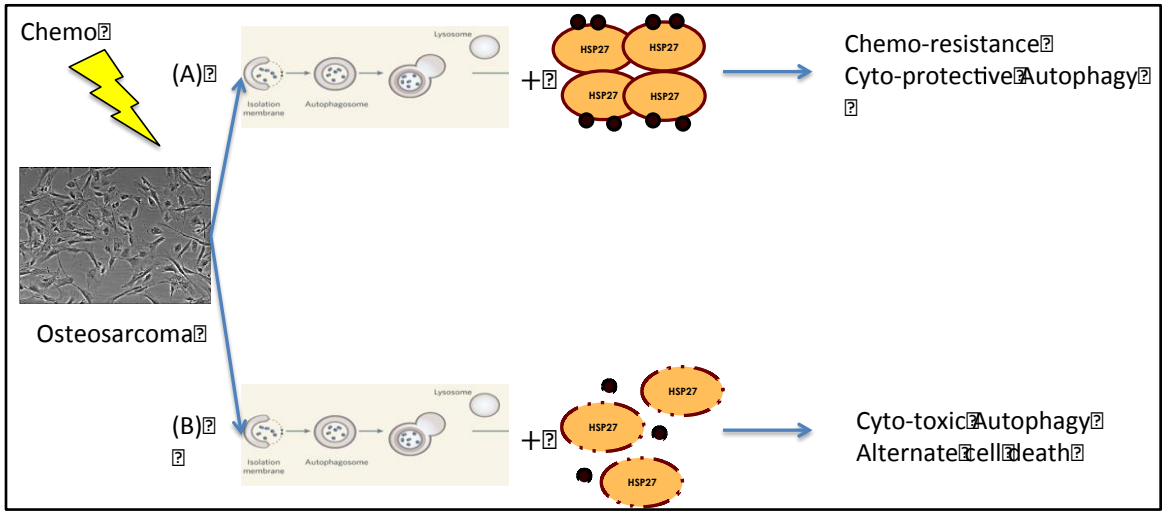

Figure 2. Proposed model for chemotherapy-induced autophagy and pHSP27 in osteosarcoma. (a) Increased pHSP27 expression following chemotherapy correlates with cytoprotective autophagy; (b) Decreased pHSP27 correlates with cytotoxic autophagy.

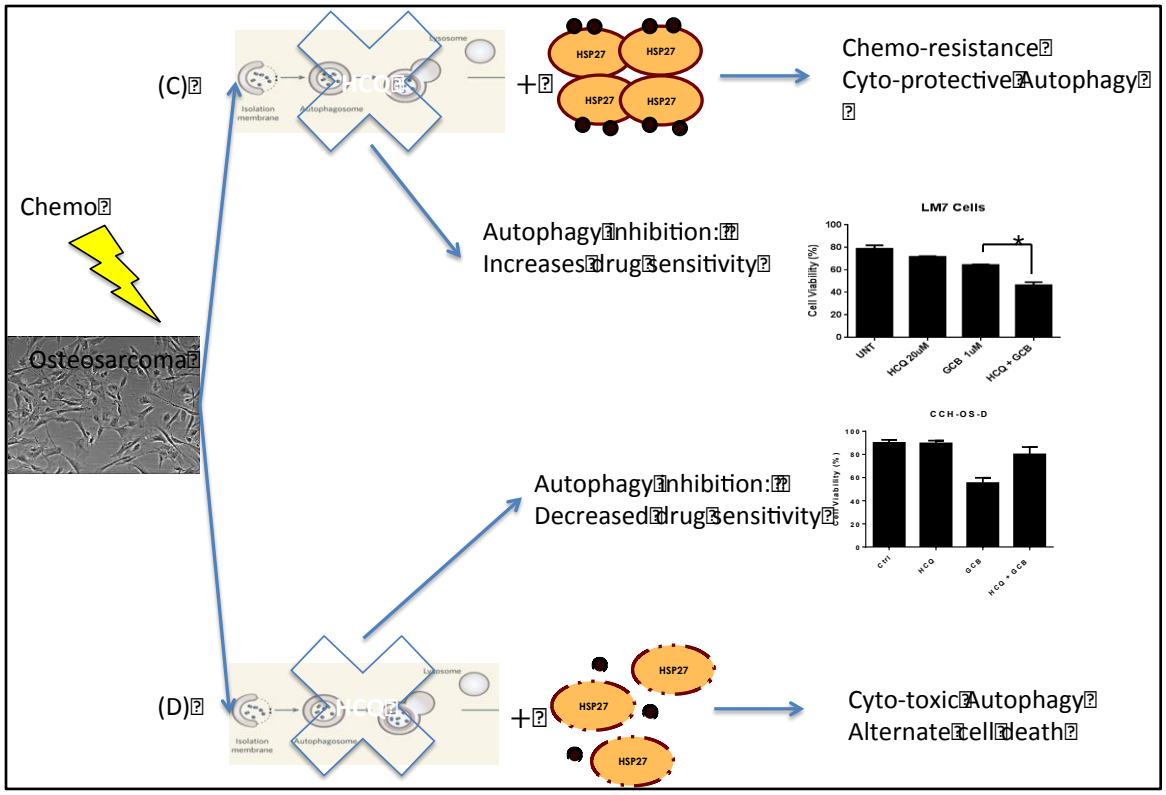

Figure 2 (continued). (c) Autophagy inhibition increases drug sensitivity (decreased cell viability). Viability studies show a decrease in LM7 viability with the combination of HCQ and GCB as compared to GCB alone; (d) Autophagy inhibition decreases drug sensitivity (increased cell viability) in osteosarcoma cells with decreased post-treatment pHSP27 expression. Viability studies show an increase in CCH-OS-D viability with the combination of HCQ and GCB as compared to GCB alone. Cell viability data reproduced with permission, courtesy Janice Santiago-O'Farrill PhD.

Heat shock Protein 27 (HSP27) in Cancer and Cancer Treatment

Heat shock proteins (HSPs) are a highly conserved class of cytoprotective proteins whose synthesis is stimulated by heat shock (as their name implies) as well as other environmental and physiologic stressors. Heat shock protein 27 (also known as HSPB1) is a small HSP (molecular weight 27 kD) that is found in both normal cells and human cancers.[74] Gene expression data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression Project (GTEx)[75] show that HSP27 is highly expressed across a range of normal tissues and tumor types including soft tissue sarcomas, with uterine and soft tissue leiomyosarcoma being most notable (Figure 3; ULMS and STLMS, respectively). Notably, osteosarcoma expression data is not included in the TCGA.

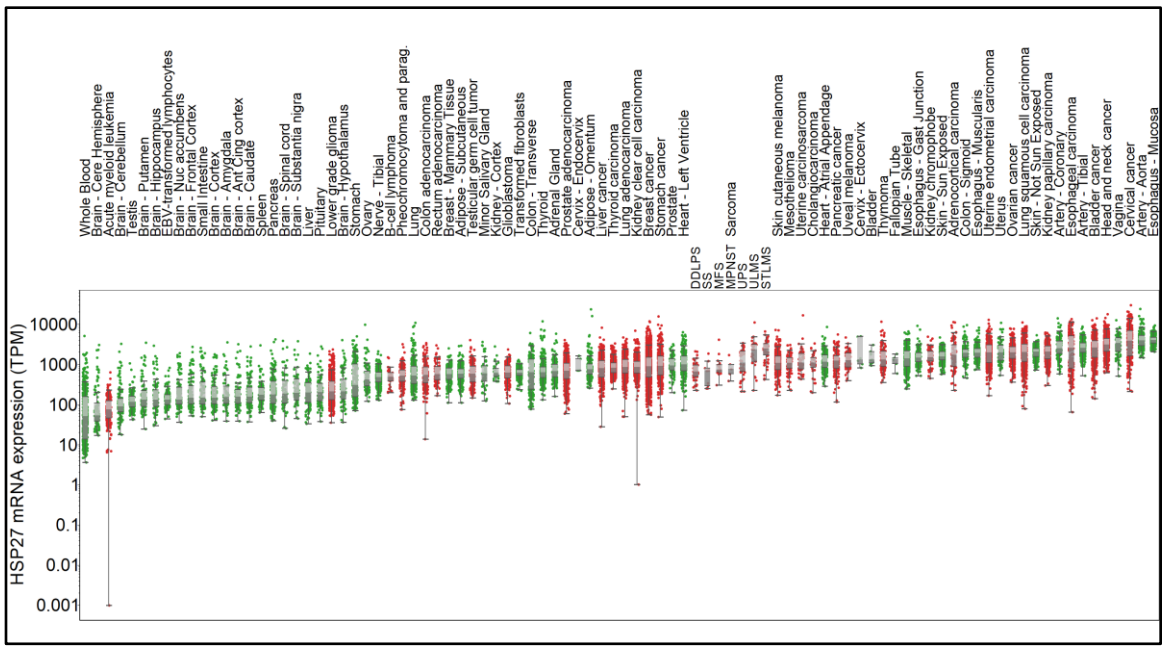

Figure 3. HSP27 expression across normal tissues and multiple cancer subtypes. The results presented here are in part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

In cancer, HSP27 expression can be induced by cytotoxic drugs and protects cells from apoptotic cell death. Overexpression of HSP27 is a poor prognostic biomarker in multiple cancers including gastric, liver, prostate, rectal and breast, and is additionally a marker of chemotherapy resistance in breast cancer and leukemia.[76, 77]

HSP27 can undergo post-translational modification via serine phosphorylation; human HSP27 is phosphorylated at *serine15, serine-78,* and *serine-82* by MAPKAP kinase 2/3 via the activation of the P38 MAPK pathway.[78] Phosphorylation is a dynamic, reversible process that can affect structure, oligimerization, and function. Heat shock as well as other stressors have been shown to induce phosphorylation. Interestingly, early studies noted that agents such as tumor necrosis factor (TNF) and leukemia inhibitory factor only induced HSP27 phosphorylation in cells that were sensitive to the agents.[74] Similarly, in human breast cancer cells, phorbol ester induces HSP27 phosphorylation and inhibits cell growth.[79] More recently, pHSP27 has been associated with gemcitabine-resistance in pancreatic cancer cells as well as 5-fluorouracil resistance in colorectal cancer.[80, 81] As a prognostic marker, pHSP27 expression is inversely correlated with tumor size and stage and was noted to decrease at progression in hepatocellular carcinoma[82] and was shown to correlate with HER-2 status and node positivity in breast cancer.[83]

While previously unactionable, several strategies are being developed to target heat shock proteins in cancer therapy.[84] Recent studies with the novel HSP27 antisense oligonucleotide OGX-427 have shown promise in multiple tumor types including reports of increased chemosensitivity to GCB in pancreatic cancer[85], erlotinib and chemotherapy

in NSCLC[86], and increased responses to docetaxel in prostate cancer[87]. However, only limited preclinical data exists for the use of OGX-427 in osteosarcoma and no sarcoma patients were included in the phase I trials.[88]

Heat Shock Protein 27 in Osteosarcoma

Two small series have evaluated the expression and prognostic significance of HSP27 in osteosarcoma. HSP27 is overexpressed in 22-24% of patients at baseline and 33-37% at resection following neo-adjuvant chemotherapy.[89, 90] Overexpression of HSP27 in pretreatment biopsy samples evaluated in 54 patients was associated with inferior overall survival in univariate analysis (HR 3.95, [1.67-9.34]) and when adjusted for tumor size and histologic subtype (HR 3.26, [1.28-8.31]).[89] In a second series from the same group examining the prognostic significance of multiple HSPs (27, 47, 60, 70, 90α, 90β), only HSP27 overexpression at biopsy (p=0.0021) and HSP27 and HSP70 expression at surgery (p=0.045, and p=0.018 respectively) were significantly associated with poor prognosis.[90] Similarly, HSP27 overexpression at biopsy was associated with inferior overall survival in a multivariate analysis (adjusted HR 2.63, [1.14-6.06]), although presumably this series included some of the same patients as the initial study. As predictive biomarkers, HSP72 expression by immunohistochemistry[91] and antibodies to HSP90[92] in patient sera prior to initiation of therapy have both been shown to correlate with better responses to neoadjuvant chemotherapy. The utility of HSP27 as a potential predictive biomarker of chemotherapy response has not been assessed in osteosarcoma.

Hypothesis and Aims

Based upon preclinical data showing a dual role for chemotherapy-induced autophagy in osteosarcoma and the correlation between autophagy inhibition and pHSP27 expression *in vitro*, my central hypothesis is that phosphorylated HSP27 determines the role of autophagy in osteosarcoma and modulates response to preoperative chemotherapy. Therefore, HSP27, pHSP27, and/or autophagy may pose novel biomarkers and potential therapeutic targets to improve the efficacy of standard MAP chemotherapy in patients with osteosarcoma.

In order to test this hypothesis, the following specific aims were studied:

Aim 1. To determine if autophagy is induced in osteosarcoma tumor specimens following preoperative chemotherapy and correlates with pathologic response, prognosis, and HSP27/pHSP27 expression.

Aim 2. To determine whether standard chemotherapy can result in either cytotoxic or cytoprotective autophagy in osteosarcoma cell lines and determine whether pHSP27 expression correlates with the cytoprotective function of chemotherapy-induced autophagy following treatment with doxorubicin or cisplatin

II. Materials and Methods:

Patients

The study population for Aim 1 included 260 pediatric and adult patients with osteosarcoma evaluated at the University of Texas MD Anderson Cancer Center between 1985 and 2012 with tissue specimens included on an institutional tissue microarray (TMA) that contains 394 specimens. In all cases, the diagnosis was established according to the World Health Organization Classification of Tumors by an expert sarcoma pathologist. [93] Patients with extraskeletal osteosarcoma were specifically excluded as this is considered a separate entity. Clinical data was collected by retrospective chart review. Institutional Review Board approval was obtained for this study and was exempt from requiring informed consent.

Construction of tissue microarrays

Decalcified FFPE tissue blocks from osteosarcoma pretreatment biopsies and surgical resection specimens (resected primary tumors and resected metastasis) were retrieved from the MD Anderson institutional tumor bank. Hematoxylin/eosin stained slides from each block were reviewed by a sarcoma pathologist to identify tumor areas. Tissue microarrays (TMAs) were constructed with 0.6mm diameter tissue cores from representative tumor areas from the FFPE blocks.

Immunohistochemical Studies

Four-micron thick unstained slides were prepared from formalin-fixed paraffin-embedded decalcified human osteosarcoma tissue microarrays. Immunohistochemical

studies were performed using an autostainer (Bond III/Rx, Leica Biosystems, Buffalo Grove, IL) with an anti-human HSP27 monoclonal antibody (1:1000, Thermo Fischer Scientific, clone MA3-15) and LC3B (1:50, NanoTools/Axorra, clone 5F10).

Immunohistochemical labeling was independently scored by 2 trained sarcoma pathologists, both of whom were blinded from the clinical data at the time of assessment. Both percentage of tumoral labeling (0-100%) and the intensity of immunostaining [0 (negative), 1 (weak), 2 (moderate), 3 (strong)]was evaluated.

LC3B was evaluated using previously validated immunohistochemical methods[94] with granular cytoplasmic or punctate staining assessed. Staining of >10% tumor cells was considered positive for HSP27 or LC3B expression. In addition, median cut-points (≥ vs < median % staining) and staining intensity (negative vs weak/moderate/strong) were examined as prognostic factors.

OS cell lines and cell culture

LM7 is a human metastatic osteosarcoma cell line that was established in the Kleinerman Lab by intravenous recycling the parental cell line (SAOS-2) through the lungs of nude mice serially through 7 iterations. CCH-OS-D is a human osteosarcoma cell line derived from an 18-year-old male with osteosarcoma lung metastasis. LM7, and CCH-OS-D metastatic OS cell lines were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum supplemented with antibiotic and nonessential amino acids. Cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Reagents and antibodies

Doxorubicin and cisplatin were obtained from the MD Anderson Cancer Center pharmacy and dissolved in PBS to desired concentrations. HCQ was purchased from Sigma Aldrich and dissolved with water to a concentration of 10mM. RIPA lysis buffer (sc-24948) was purchased from Santa Cruz Biotechnology. The following antibodies were used for Western blot analysis: Microtubule-associated protein 1 light chain LC3B (NB600-1384) was purchased from Novus Biologicals. Beclin (sc-11427) and HSP27 (sc-13132) were purchased from Santa Cruz Biotechnology. SQSTM1/p62 (5114) was obtained from Cell Signaling Technology. p-HSP27 (MAB23141) was purchased from R&D Systems.

Cell viability

Cell viability, Western blot, and human phosphokinase arrays were completed using the same methodology as prior studies from our lab.[73] Cells were seeded in 12-well plates with approximately 0.5×10^5 cells per well and allowed to attach overnight at 37°C, 5% CO_2. Cells were then treated in the following conditions: doxorubicin, cisplatin, HCQ, doxorubicin following HCQ pretreatment for 20 minutes, and cisplatin following HCQ pretreatment; cells with no treatment served as controls. Cells were trypsinized and viability was assessed by trypan blue exclusion assay using an automated cell counter (Vi-cell, Beckman Coulter). The incubation time and drug concentrations are specified in the results and respective figures.

Human phospho-kinase Antibody Array Kit

LM7 and CCH-OS-D cells were treated with doxorubicin or cisplatin for 48 hours. Untreated cells were used as a control. The cells were collected and lysed using the lysis buffer provided by the manufacturer, and protein concentration was determined using the Bradford Protein Assay from Bio-Rad. After blocking, the membranes were incubated with 400µg of protein overnight at 4°C. The membranes were washed and incubated with Detection Antibody Cocktails. HRP- conjugated Streptavidin antibodies and chemniluminescent detection reagents were used to visualize the protein. Densitometry was completed using ImageJ software (https://imagej.nih.gov/ij/).

Western blot analysis

After treatment, whole cell lysates were prepared by lysing the cells with RIPA lysis buffer for 30 minutes and centrifuging at 10,000 *g* at 4°C. Supernatants were collected and protein concentration was determined using the Bio-Rad DC protein assay kit (500-0113-0115). Equal amounts of protein were subjected to SDS-polyacrylamide gels (SDS-PAGE) and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk or 5% bovine serum albumin for 1 hour and then incubated with primary antibody against LC3 (1:1000) or p62 (1:1000). After overnight incubation with primary antibodies, membranes were washed and incubated with anti-mouse (1:2000) horseradish peroxidase linked whole antibody (from sheep, NA931V; GE Healthcare) or anti-rabbit (1:2000) horseradish peroxidase linked whole antibody (from donkey, NA934V; GE Healthcare) as a secondary antibody. Signal was detected using ECL

reagents (GE Healthcare Life Science). β-actin or GAPDH were used as loading controls. Densitometry was completed using ImageJ software (https://imagej.nih.gov/ij/).

Statistical analysis

Analyses of overall survival (OS) and relapse-free survival (RFS) were performed. OS was defined as from the time of diagnosis to the time of death or to the time of last contact. RFS was defined as from the time of surgery to the time of relapse or death, whichever occurred first or to the time of last contact. The distributions of OS, and RFS were estimated by the Kaplan-Meier method.[95] Log-rank test was performed to test the difference in survival between patient characteristics/biomarkers groups.[96] Regression analyses of survival data based on the Cox proportional hazard (PH) regression model were conducted on OS and RFS.[97] For the resection samples and good response, landmark analysis method was used and time of surgery would be the starting points of OS and RFS.[98] Stepwise method was used to build a multivariate Cox PH regression model. Functional form, PH assumption, and multicollinearity were examined. The correlation between two continuous factors was measured by Spearman correlation.[99] The Chi-square test and Fisher's exact test were used to determine whether proportions of patients with factors of interest were equal between patient characteristics/biomarkers groups.[100] Wilcoxon signed rank test was used to compare biomarker expression of paired resection with synchronous metastatic samples, and paired resection with metachronous metastatic samples. A two-sided p-value<0.05 was considered statistically significant. SAS version 9.4 was used to carry out the computations. Experimental data for cell viability studies were analyzed using Graphpad PRISM 6.0 software.

III. Results

Clinical Characteristics

There were 215 patients with localized osteosarcoma at the time of diagnosis (localized patients) and 45 patients with primary metastatic osteosarcoma (metastatic patients). The age at diagnosis of all patients ranged from 4.9 to 90.8 with mean and median of 24.2 and 18.3, respectively. Metastatic patients (mean= 24.8 years) were slightly older than the localized patients (mean= 24.0 years). Tables 3 and 4 show the clinical characteristics in all patients and by stage at presentation (localized disease and metastatic disease).

Factor	n	Min	Median	Mean	Max	SD
All patients						
Age at diagnosis (years)	260	4.9	18.3	24.2	90.8	15.8
Tumor necrosis (%) (patients with pre-op chemo)	241	0	87.0	76.0	100	27.0
Localized disease at diagnosis						
Age at diagnosis (years)	215	4.9	18.2	24.0	90.8	15.6
Tumor Necrosis (%) (patients with pre-op chemo)	205	0	86.0	76.1	100	27.2
Metastatic disease at diagnosis						
Age at diagnosis (years)	45	7.2	20	24.8	78.2	16.9
Tumor necrosis (%) (patients with pre-op chemo)	36	0	88	75.0	100	25.9

Table 3. Clinical and pathologic characteristics by stage at presentation (continuous factors).

Factor	All patients	Localized Disease	Metastatic Disease
	n (%)	n (%)	n (%)
Gender			
Male	153 (59)	126 (59)	27 (60)
Female	107 (41)	89 (41)	18 (40)
Race			
Asian	9 (3)	7 (3)	2 (4)
Black	35 (14)	28 (13)	7 (16)
Hispanic	75 (29)	63 (29)	12 (27)
White	140 (54)	116 (54)	24 (53)
Histologic Subtype			
Osteoblastic	110 (42)	91 (42)	19 (42)
Chondroblastic	49 (19)	40 (19)	9 (20)
Fibroblastic	46 (18)	39 (18)	7 (16)
Telangiectatic	23 (9)	18 (8)	5 (11)
Dedifferentiated parosteal	12 (5)	10 (5)	2 (4)
Small cell	6 (2)	4 (2)	2 (4)
High grade surface	3 (1)	3 (1)	
Other - high grade	6 (2)	5 (2)	1 (2)
Other intermediate/low grade	5 (2)	5 (2)	
Radiation Induced Osteosarcoma			
No	250 (97)	207 (96)	43 (100)
Yes	8 (3)	8 (4)	
Primary Site			
Femur	140 (54)	112 (52)	28 (62)
Tibia	43 (17)	39 (18)	4 (9)
Fibula	10 (4)	9 (4)	1 (2)
Humerus	32 (12)	24 (11)	8 (18)
Radius/ulna	3 (1)	3 (1)	
Mandible	1 (0)	1 (0)	
Rib/chest wall	7 (3)	4 (2)	3 (7)
Pelvis/acetabulum	18 (7)	17 (8)	1 (2)
Other upper extremity	2 (1)	2 (1)	
Other lower extremity	1 (0)	1 (0)	
Other axial skeleton	2 (1)	2 (1)	

Table 4. Clinical and pathologic characteristics by stage at presentation (categorical factors).

Factor		All patients	Localized Disease	Metastatic Disease
		n (%)	n (%)	n (%)
Grade				
	Low	6 (2)	6 (3)	
	Intermediate	1 (0)	1 (0)	
	High	253 (97)	208 (97)	45 (100)
Good response (patients receiving pre-op chemo)				
	No	133 (55)	113 (55)	20 (56)
	Yes	108 (45)	92 (45)	16 (44)

Table 4 (continued). Clinical and pathologic characteristics by stage at presentation (categorical factors).

Relapse-free and overall survival for osteosarcoma patients with localized disease

Amongst patients with localized disease at diagnosis, there were 122 relapses and deaths during the follow-up period. Ninety-three patients are alive without relapse. Table 5 shows the log-rank test results for clinical characteristics related to relapse-free survival. Patients with radiation induced osteosarcoma had inferior RFS than compared to patients without radiation induced osteosarcoma (Figure 4, p=0.0050).

Factor	p-value
Gender	0.435
Race	0.828
Histologic Subtype	0.737
Histologic Subtype (Osteoblastic)	0.361
Radiation Induced osteosarcoma	0.0050
Primary Site	0.192
Primary Site (femur)	0.951
Grade (High/Intermediate vs Low)	0.151
Pre-op Chemo	0.525
Good response (patients receiving pre-op chemo)*	0.301

*Landmark analysis method

Table 5. Log-rank test for relapse-free survival amongst patients with localized disease at diagnosis. Radiation-induced osteosarcoma was the only clinical or pathologic characteristic that was significantly associated with RFS.

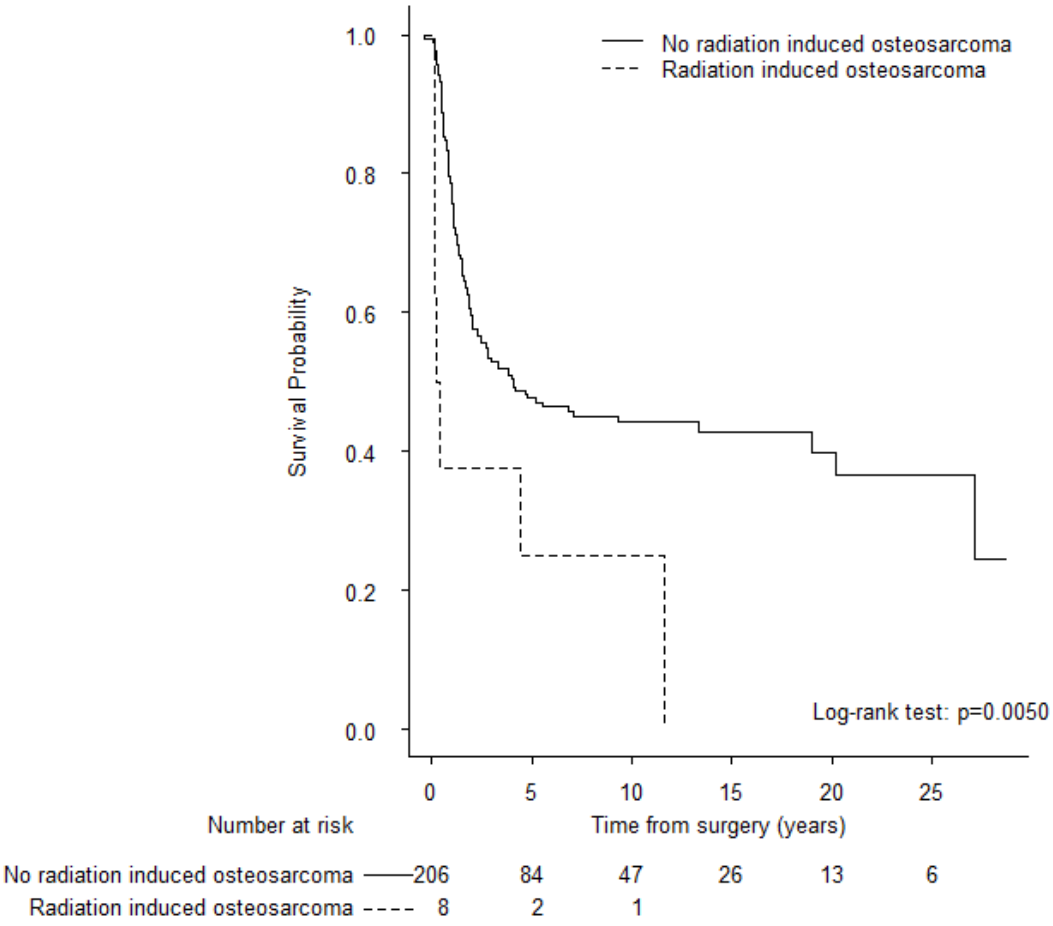

Figure 4. Relapse-free survival for patients with localized disease by radiation-induced subtype. Patients with radiation-induced osteosarcoma demonstrate inferior RFS.

Clinical and pathologic patient characteristics associated with RFS were analyzed using a univariate Cox model. Radiation induced osteosarcoma was significantly associated with poor RFS (HR: 2.85 [1.33-6.12], p=0.007) compared without radiation induced osteosarcoma (Table 6).

Factor	No. of deaths	Total n	Univariate analysis HR (95% CI)	P-value
Age at diagnosis	121	215	1.01 (1.00, 1.02)	0.133
Sex				
Female	40	89	Ref	
Male	65	126	1.16 (0.80, 1.67)	0.435
Radiation Induced Osteosarcoma				
No	98	207	Ref	
Yes	7	8	2.85 (1.33, 6.12)	0.007
Primary Site (femur vs others)				
Others	48	103	Ref	
Femur	57	112	1.01 (0.71, 1.45)	0.951
Grade (High/Intermediate vs low)				
Low	1	6	Ref	
High/ Intermediate	104	209	2.68 (0.66, 10.8)	0.168
Pre-op Chemo				
No	2	7	Ref	
Yes	103	208	1.45 (0.46, 4.56)	0.527
Good response (patients with pre-op chemo)*				
Yes	43	92	Ref	
No	59	113	1.21 (0.84, 1.75)	0.302

*Landmark analysis method

Table 6. Relapse-free survival analysis for patients with localized disease (univariate Cox regression model).

Amongst patients with localized osteosarcoma, there were 105 deaths during the follow-up period; 110 patients were alive at the time of analysis. The median follow-up time was 11.0 years. The estimated overall survival (OS) was 12.0 years (95% CI 7.03-20.3). Table 7 shows the log-rank test results for clinical characteristics related to overall survival. Patients with radiation induced osteosarcoma had inferior OS as compared to patients without radiation induced osteosarcoma (Figure 5, p=0.0003).

Factor	p-value
Gender	0.615
Race	0.597
Histologic Subtype	0.682
Histologic Subtype (Osteoblastic vs others)	0.726
Radiation Induced osteosarcoma	0.0003
Primary Site	0.162
Primary Site (femur vs others)	0.555
Grade (High/Intermediate vs Low)	0.119
Pre-op Chemo	0.574
Good response (patients with Pre-op chemo)*	0.0958

*Landmark analysis method

Table 7. Log-rank test for overall survival amongst patients with localized disease at diagnosis. Radiation-induced osteosarcoma was the only clinical or pathologic characteristic that was significantly associated with OS. Good response (pathologic tumor necrosis >90%) showed a trend toward significance.

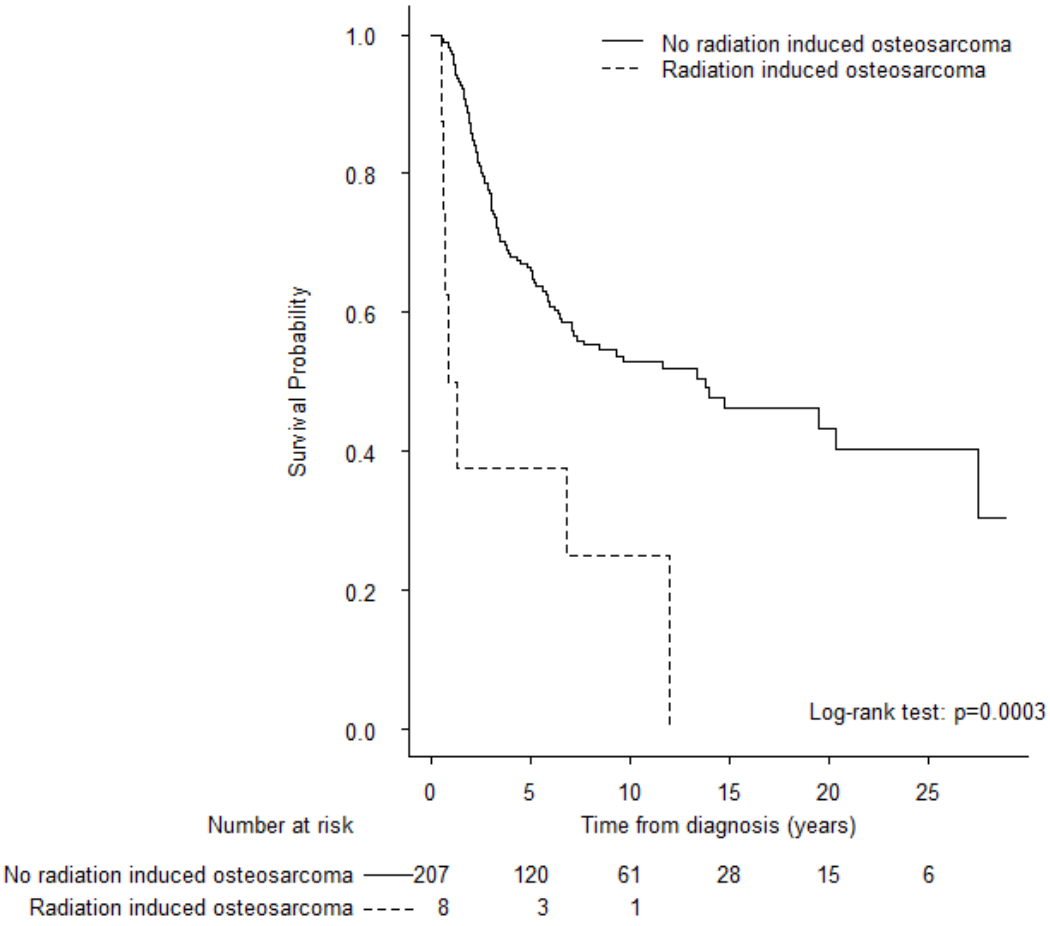

Figure 5. Overall survival for patients with localized disease by radiation-induced subtype. Patients with radiation-induced osteosarcoma demonstrate inferior OS.

Clinical and pathologic patient characteristics associated with OS amongst patients with localized osteosarcoma were analyzed using a univariate and multivariate Cox models. Higher age at diagnosis (HR 1.02 [1.00-1.03], p=0.0063) and radiation-associated osteosarcoma (HR 3.76 [1.74-8.12], p=0.001) were significantly associated with inferior overall survival in a univariate model; these factors remained significant in multivariate analysis (all $p < 0.03$, Table 8). Patients with poor response to pre-operative chemotherapy showed a trend toward inferior overall survival in a landmark analysis (HR 1.4 [0.94-2.07], p=0.097).

			Univariate analysis		Multivariate analysis	
Factor	No. of deaths	Total	HR (95% CI)	P-value	HR (95% CI)	P-value
Age at diagnosis (years)	105	215	1.02 (1.00, 1.03)	0.0063	1.01 (1.00, 1.03)	0.0294
Sex						
Female	40	89	Ref			
Male	65	126	1.11 (0.75, 1.64)	0.615		
Radiation Induced osteosarcoma						
No	98	207	Ref		Ref	
Yes	7	8	3.76 (1.74, 8.12)	0.001	3.08 (1.38, 6.86)	0.0061
Primary Site (femur vs others)						
Others	48	103	Ref			
Femur	57	112	1.10 (0.76, 1.65)	0.556		
Grade (High/Intermediate vs low)						
Low	1	6	Ref			
High/ Intermediate	104	209	4.23 (0.59, 30.4)	0.152		
Pre-op Chemo						
No	2	7	Ref			
Yes	103	208	1.49 (0.37, 6.05)	0.577		
Good response (patients with pre-op chemo)*						
Yes	43	92	Ref			
No	59	113	1.40 (0.94, 2.07)	0.097		

*Landmark analysis method

Table 8. Overall survival analysis for patients with localized disease (univariate and multivariate Cox regression models).

Progression-free and overall survival for patients with metastatic osteosarcoma

Patients with primary metastatic disease at the time of diagnosis have inferior outcomes. Because this is a well-established poor prognostic group, survival outcomes and biomarkers for patients with metastasis at diagnosis were analyzed separately from patients with localized osteosarcoma.

Among the 45 patients with primary metastatic disease, 32 (71%) died during the follow-up period, with a median follow up of 10.6 years. The median overall survival was 2.47 years (95%CI 1.70-3.51). Patients with poor response to pre-operative chemotherapy had inferior progression-free survival (HR 2.4 [1.11-5.22]; table 9). Patients who did not receive preoperative chemotherapy also had inferior PFS (2 patients, HR 6.14 [1.34- 28.1]). Failure to receive preoperative chemotherapy was associated with inferior OS (log-rank p=0.0045; Cox HR 6.82 [1.47-31.7]). This likely represents patients who were too critically ill at the time of presentation to receive chemotherapy and/or surgery. No other clinical characteristics analyzed were significantly associated with overall survival amongst patients with primary metastatic disease (Tables 10 and 11).

Factor	No. of deaths/ progression	Total n	Hazard ratio (95% CI)	P-value
Age at diagnosis			1.01 (0.99, 1.03)	0.380
sex				
Female	14	18	Ref	
Male	22	27	1.02 (0.52, 2.00)	0.945
Histologic Subtype (Osteoblastic)				
Others	20	26	Ref	
Osteoblastic	16	19	1.05 (0.54, 2.03)	0.886
Primary site (Femur)				
Others	12	17	Ref	
Femur	24	28	1.42 (0.71, 2.85)	0.323
Pre-op Chemo				
Yes	29	38	Ref	
No	2	2	6.14 (1.34, 28.1)	0.019
Good Response (with Pre-op Chemo)*				
Yes	10	16	Ref	
No	19	22	2.40 (1.11, 5.22)	0.027

*Landmark analysis method

Table 9. Progression-free survival analysis for patients with primary metastatic disease (univariate Cox regression model).

Factor	p-value
Gender	0.595
Race	0.551
Histologic Subtype	0.275
Histologic Subtype (Osteoblastic vs others)	0.669
Primary Site	0.0448
Primary Site (femur vs others)	0.444
Pre-op Chemo	0.0045
Good response (patients with pre-op Chemo)*	0.554

* *Landmark analysis method

Table 10. Log-rank test for overall survival amongst patients with metastatic disease at diagnosis. Failure to receive preoperative chemotherapy was the only factor associated with OS. Of note, only 2 patients did not receive preoperative chemotherapy.

Factor	No. of deaths	Total n	HR (95% CI)	p-value
Age at diagnosis	32	45	1.01 (0.99, 1.03)	0.496
Sex				
Male	19	27	Ref	
Female	13	18	1.21 (0.60, 2.46)	0.596
Histologic Subtype (Osteoblastic)				
Others	17	26	Ref	
Osteoblastic	15	19	1.17 (0.57, 2.37)	0.669
Primary site (Femur)				
Others	11	17	Ref	
Femur	21	28	1.33 (0.64, 2.77)	0.445
Pre-op Chemo				
Yes	25	38	Ref	
No	2	2	6.82 (1.47, 31.7)	0.014
Good Response (with Pre-op Chemo)*				
Yes	10	16	Ref	
No	14	21	1.28 (0.57, 2.89)	0.555

*Landmark analysis method

Table 11. Overall survival analysis for patients with primary metastatic disease (univariate Cox regression model).

Expression of HSP27 and LC3 in Osteosarcoma Patient Samples

The osteosarcoma tissue microarray included 394 osteosarcoma tumor specimens consisting of 116 pre-treatment biopsies, 184 primary resection specimens, and 94 metastatic tumor specimens. Metastatic specimens included a combination of synchronous metastasis (26, 28%) and metachronous metastasis (68, 72%). Due to the fragility of samples following the decalcification and staining process, not all sample were evaluable for all biomarkers analyzed.

LC3B expression in osteosarcoma patient samples

The percentage of osteosarcoma cells with clearly visible cytoplasmic LC3B+ puncta labeling were quantified, with ≥10% considered positive based upon established cutoffs in other tumor types.[56] LC3B+ expression was significantly higher in resection specimens and metastasis as compared to pre-treatment biopsies (50% and 67% respectively vs 34%, p=0.004). Examples of LC3B labeling are shown in Figure 6. The intensity of cytoplasmic LC3B staining was heterogeneous. A higher proportion of pre-treatment biopsy specimens were graded as 0 or negative (66%) as compared to resection (47%) or metastasis (30%, Table 12).

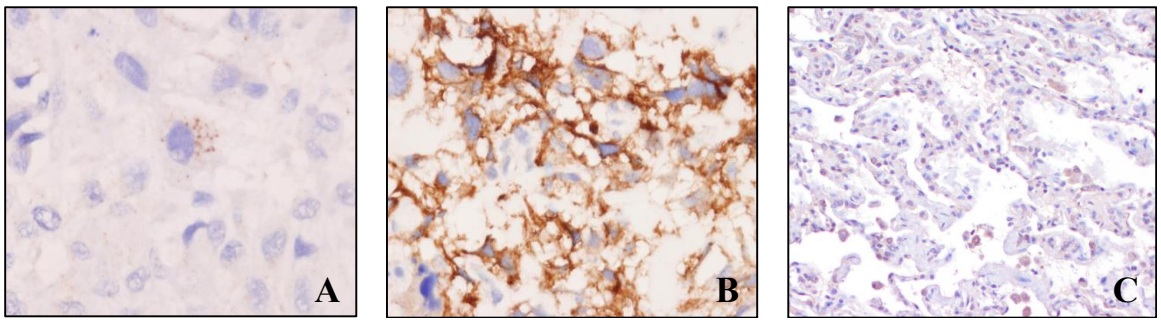

Figure 6. Examples of LC3B labeling in osteosarcoma tumor specimens and normal lung. Punctate labeling (A) as well as granular cytoplasmic labeling (B) in osteosarcoma tumor cells were considered positive. In normal human lung tissue, pneumocytes are negative for LC3B whereas macrophages stain positive (C).

LC3B Expression in Patient Samples				
Factor	All	Biopsy	Resection	Metastatic
	n (%)	n (%)	n (%)	n (%)
LC3B intensity				
None	122 (47)	38 (66)	64 (47)	20 (30)
Weak	82 (31)	7 (12)	43 (31)	32 (48)
Moderate	54 (21)	11 (19)	29 (21)	14 (21)
Strong	3 (1)	2 (3)	1 (1)	
LC3B percent (10% as cutoff)				
<10%	128 (49)	38 (66)	68 (50)	22 (33)
≥10%	133 (51)	20 (34)	69 (50)	44 (67)

Table 12. Punctate LC3B expression in osteosarcoma patient samples by IHC. Samples scored by intensity and percent of tumor cells expressing LC3B+ puncta.

Paired pre-treatment biopsy and post-treatment resection specimens were available for LC3B puncta evaluation in 18 patients, including 9 LCB+ at biopsy and 9 LC3B negative prior to treatment. Five of 9 patients (56%) who were initially negative for LC3B puncta were positive for the autophagy marker at the time of resection following chemotherapy. Conversely, 8/9 patients (89%) who were positive prior to chemotherapy remained positive at resection (Table 13). These findings suggest that the presence of LC3B puncta at resection may represent chemotherapy-induced autophagy in a subset of patients. Similarly, amongst 6 patients with negative LC3B expression in pre-treatment samples, 3 subsequently had LC3B+ puncta in metachronous metastatic specimens and 3 remained negative. Resection-metastasis pairs were available in 26 patients. Amongst 13 who were negative for LC3B expression at resection, 9 (69%) demonstrated LC3B+ puncta in metachronous metastatic specimens (Table 13). Differences in LC3B expression amongst the pairs were not statistically significant, however this may be due to the small sample size.

LC3B+ (10% cutoff) in paired biopsy-resection specimens			
	Resection		McNemar's test p-value
Biopsy	<10%	≥10%	0.103
<10%	4	5	
≥10%	1	8	
LC3B+ (10% cutoff) in paired biopsy-metastasis specimens			
	Metastasis		McNemar's test p-value
Biopsy	<10%	≥10%	0.0833
<10%	3	3	
≥10%	0	2	
LC3B+ (10% cutoff) in paired resection-metastasis specimens			
	Metastasis		McNemar's test p-value
Resection	<10%	≥10%	0.166
<10%	4	9	
≥10%	4	9	

Table 13. LC3B puncta in paired osteosarcoma tumor specimens.

HSP27 and pHSP27 expression in osteosarcoma patient samples

The percent and intensity of HSP27 expression was variable, with some tumor having strong diffuse expression in tumor cells and others having no appreciable HSP27 expression (Figure 7).

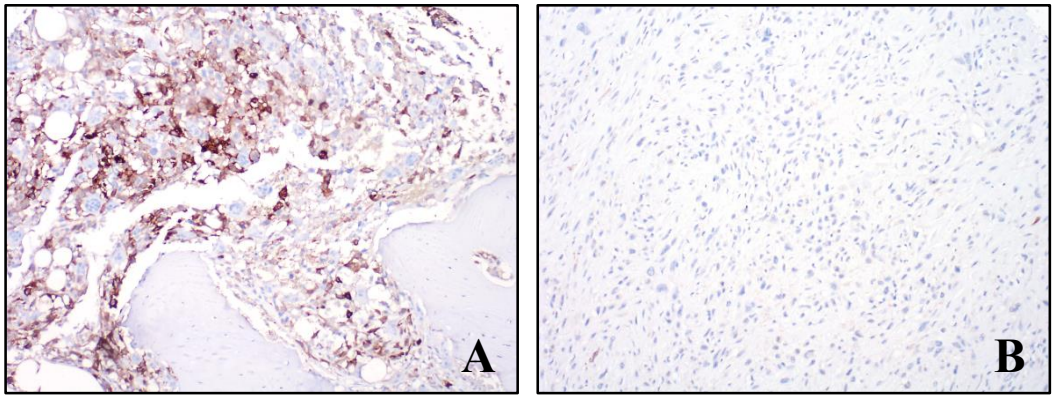

Figure 7. Representative expression of HSP27 in osteosarcoma tumor specimens. Expression was variable, with some tumor having strong, diffuse staining (A) and others showing no expression (B).

The percentage of osteosarcoma cells with HSP27 staining was quantified, with ≥10% considered positive. Two-thirds of all tumor specimens analyzed were scored as positive for HSP27 expression. The proportion of HSP27+ tumors was higher in pre-treatment biopsy (85%) and metastasis specimens (79%) as compared to resection (52%), although this was not statistically significant. The intensity of HSP27 staining was variable across all groups. There was no significant correlation between HSP27 expression and LC3B expression (Spearman correlation 0.006, p=0.945). HSP27 expression is summarized in table 14.

HSP27 Expression in Patient Samples				
Factor	All	Biopsy	Resection	Metastatic
	n (%)	n (%)	n (%)	n (%)
HSP27 intensity				
None	41 (16)	9 (16)	28 (21)	4 (6)
Weak	144 (57)	28 (48)	74 (56)	42 (67)
Moderate	38 (15)	16 (28)	13 (10)	9 (14)
Strong	29 (12)	5 (9)	16 (12)	8 (13)
HSP27 percent (10% as cutoff)				
<10%	85 (34)	9 (15)	63 (48)	13 (21)
≥10%	168 (66)	50 (85)	68 (52)	50 (79)

Table 14. Heat shock protein 27 (HSP27) expression in osteosarcoma patient samples by IHC. Samples scored by intensity and percent of tumor cells expressing HSP27.

The percentage and intensity of osteosarcoma cells with phosphorylated HSP27 (pHSP27 S78/S82) staining was quantified. The expression of pHSP27 was very limited (representative staining shown in figure 8) at all time points, with only 16% of all specimens tested showing any staining and 8% considered positive (pHSP27 >10%; table 15). This likely reflects a degradation of phosphoproteins in the acquisition and processing (decalcification) of bone tumor specimens, rather than a true lack of pHSP27. Due to the limited labeling, no significant inferences could be made regarding the prognostic significance of pHSP27 or its correlation with other biomarkers of interest.

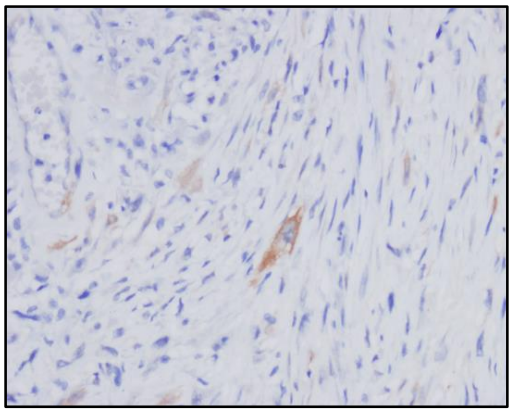

Figure 8. Representative expression of pHSP27 in osteosarcoma tumor specimens.

pHSP27 Expression in Patient Samples				
Factor	All	Biopsy	Resection	Metastatic
	n (%)	n (%)	n (%)	n (%)
pHSP27 intensity				
None	241 (84)	44 (77)	133 (88)	64 (82)
Weak	39 (14)	11 (19)	16 (11)	12 (15)
Moderate	4 (1)	2 (4)	2 (1)	
Strong	3 (1)		1 (1)	2 (3)
pHSP27 percent (10% as cutoff)				
<10%	264 (92)	44 (77)	143 (94)	77 (99)
≥10%	23 (8)	13 (23)	9 (6)	1 (1)

Table 15. Phosphorylated heat shock protein 27 (pHSP27 S78/S82) expression in osteosarcoma patient samples by IHC. Samples scored by intensity and percent of tumor cells expressing HSP27.

Prognostic Significance of HSP27 Expression and LC3B Puncta in Osteosarcoma Patients

LC3B as a prognostic biomarker in localized osteosarcoma

The presence of LC3B puncta labeling in pre-treatment specimens was not associated with relapse-free or overall survival. Localized osteosarcoma patients with LC3B puncta staining present at resection (which was found in 50% of cases) had superior overall survival (Figure 9, p=0.031).

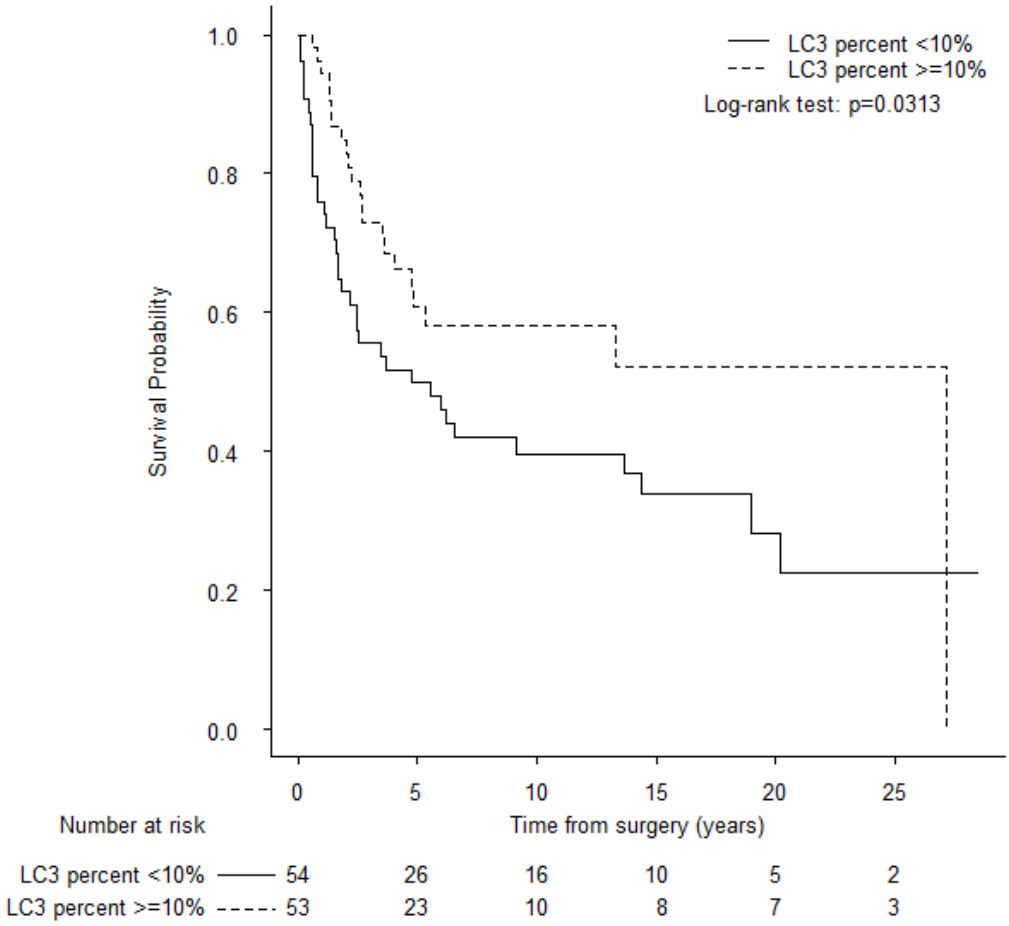

Figure 9. Overall survival for localized osteosarcoma patients stratified according to cytoplasmic LC3B puncta expression at resection (>10% positive, <10% negative)

The lack of LCB puncta labeling at resection was associated with inferior relapse-free survival in univariate analysis (HR 1.81 [1.10-2.98], p=0.019) but only maintained borderline significance when adjusted for radiation-induced osteosarcoma in the multivariate model (HR 1.65 [0.99-2.75], p=0.053; table 16). This lack of LC3B at resection following chemotherapy was significantly associated with inferior overall survival in both the univariate (HR 1.78 [1.05-3.03], p=0.034) and multivariate analysis (HR 1.75 [1.01-3.04], p=0.045) when adjusted for age and radiation-induced osteosarcoma (Table 17). A median cutpoint was also explored and found to yield prognostic significance: patients with LC3B ≤ median at resection had inferior relapse free (lor rank p=0.004) and overall survival (log rank p=0.0068). Taken together, our findings suggest that presence of LC3B puncta can be considered an independent prognostic biomarker of improved survival following preoperative chemotherapy.

Factor	No. of deaths	Total n	Univariate HR (95% CI)	Univariate P-value	Multivariate* HR (95% CI)	Multivariate* P-value
HSP27 intensity						
None	10	22	Ref		Ref	
Weak, moderate, strong	52	81	1.76 (0.89, 3.49)	0.107	1.68 (0.84, 3.33)	0.141
HSP27 intensity						
None, weak	43	78	Ref		Ref	
Moderate, strong	19	25	1.70 (0.98, 2.96)	0.0598	1.86 (1.06, 3.26)	0.0307
HSP27 percent (median)						
≤ median	28	55	Ref		Ref	
> median	34	48	1.74 (1.04, 2.91)	0.0336	1.57 (0.93, 2.66)	0.0912
HSP27 percent (10%)						
<10%	22	47	Ref		Ref	
≥10%	40	56	1.92 (1.13, 3.28)	0.016	1.76 (1.03, 3.03)	0.0398
pHSP27 intensity						
None	63	103	Ref		Ref	
Weak, moderate, strong	11	17	1.21 (0.63, 2.29)	0.568	1.28 (0.67, 2.44)	0.456
pHSP27 percent (median)						
≤ median	63	103	Ref		Ref	
> median	11	17	1.21 (0.63, 2.29)	0.568	1.28 (0.67, 2.44)	0.456
pHSP27 percent (10%)						
<10%	68	111	Ref		Ref	
≥10%	6	9	1.25 (0.54, 2.89)	0.6	1.30 (0.56, 3.01)	0.538
LC3 intensity						
Weak, moderate, strong	28	56	Ref		Ref	
None	38	51	1.82 (1.11, 2.97)	0.0174	1.65 (1.00, 2.73)	0.0525
LC3 intensity						
Moderate, strong	13	27	Ref		Ref	
None, weak	53	80	1.32 (0.72, 2.43)	0.374	1.24 (0.67, 2.29)	0.488
LC3 percent (median)						
> median	22	48	Ref		Ref	
≤ median	44	59	2.09 (1.25, 3.50)	0.0051	1.93 (1.14, 3.25)	0.0142
LC3 percent (10%)						
≥10%	26	53	Ref		Ref	
<10%	40	54	1.81 (1.10, 2.98)	0.0192	1.65 (0.99, 2.75)	0.0527

*Adjusting for radiation induced osteosarcoma

Table 16. Univariate and multivariate analyses (Cox regression) for biomarkers at resection associated with RFS.

Factor	No. of deaths	Total n	Univariate HR (95% CI)	Univariate P-value	Multivariate* HR (95% CI)	Multivariate* P-value
HSP27 intensity						
None	7	22	Ref		Ref	
Weak, moderate, strong	46	81	2.11 (0.95, 4.73)	0.0683	2.13 (0.95, 4.78)	0.0661
HSP27 intensity						
None, weak	35	78	Ref		Ref	
Moderate, strong	18	25	2.02 (1.14, 3.59)	0.0166	2.27 (1.26, 4.11)	0.0065
HSP27 percent (median)						
≤ median	23	55	Ref		Ref	
> median	30	48	1.72 (0.99, 2.98)	0.0541	1.53 (0.87, 2.68)	0.137
HSP27 percent (10%)						
<10%	18	47	Ref		Ref	
≥10%	35	56	2.00 (1.12, 3.56)	0.0197	1.85 (1.03, 3.33)	0.0395
pHSP27 intensity						
None	54	103	Ref		Ref	
Weak, moderate, strong	10	17	1.33 (0.67, 2.61)	0.413	1.32 (0.67, 2.63)	0.423
pHSP27 percent (median)						
≤ median	54	103	Ref		Ref	
> median	10	17	1.33 (0.67, 2.61)	0.413	1.32 (0.67, 2.63)	0.423
pHSP27 percent (10%)						
<10%	59	111	Ref		Ref	
≥10%	5	9	1.09 (0.44, 2.71)	0.860	1.14 (0.46, 2.85)	0.778
LC3 intensity						
Weak, moderate, strong	24	56	Ref		Ref	
None	34	51	1.78 (1.05, 3.00)	0.0315	1.69 (0.99, 2.90)	0.0556
LC3 intensity						
Moderate, strong	11	27	Ref		Ref	
None, weak	47	80	1.34 (0.69, 2.60)	0.382	1.34 (0.69, 2.61)	0.395
LC3 percent (median)						
> median	18	48	Ref		Ref	
≤ median	40	59	2.12 (1.21, 3.70)	0.0082	2.15 (1.21, 3.81)	0.0089
LC3 percent (10%)						
≥10%	22	53	Ref		Ref	
<10%	36	54	1.78 (1.05, 3.03)	0.0337	1.75 (1.01, 3.04)	0.0448

*Adjusting for age and radiation induced osteosarcoma

Table 17. Univariate and multivariate analyses (Cox regression) for biomarkers at resection associated with OS.

HSP27 as a prognostic biomarker in localized osteosarcoma

The majority of osteosarcoma specimens were scored as HSP27+ in pre-treatment samples. While HSP27+ in pre-treatment samples was not significant in univariate analysis for relapse free survival or overall survival, it was associated with inferior relapse-free survival (HR: 12.5 [1.34-116], p=0.027) and overall survival (HR: 26.7 [1.47-484], p=0.0263) in multivariate analysis (tables 18 and 19, respectively). At resection, patients with HSP27+ osteosarcoma had inferior overall survival (Figure 10, p=0.017). HSP27+ in resection specimens was associated with inferior relapse-free survival in both the univariate (HR 1.92 [1.13-3.28], p=0.016) and multivariate models (HR 1.76 [1.03-3.03], p=0.039; table 16) as well as worse overall survival (univariate HR 2.00 [1.12-3.56], p=0.0197, multivariate HR: 1.85 [1.03-3.33], p=0.039; table 17). A median cutpoint and stratification by intensity (none/weak vs. moderate/strong) was also explored and showed a similar trend with high expression of HSP27 associated with inferior outcomes. However neither measure was statistically significant in both the univariate and multivariate models for both RFS and OS (at resection summarized in tables 16 and 17; pre-treatment specimens in tables 18 and 19).

Phosphorylated HSP27 was evaluated as a potential biomarker based upon the initial hypothesis. There were no significant associations between pHSP27 expression based upon percent staining, median cutoff, or intensity prior to treatment or at resection in relation to either RFS or OS. Further, there was no correlation between pHSP27 positivity (any criteria) and total HSP27 nor LC3B.

Factor	No. of deaths	Total n	Univariate HR (95% CI)	Univariate P-value	Multivariate* HR (95% CI)	Multivariate* P-value
HSP27 intensity						
None	2	8	Ref		Ref	
Weak, moderate, strong	18	41	2.41 (0.55, 10.6)	0.243	11.5 (1.27, 105)	0.0299
HSP27 intensity						
None, weak	10	30	Ref		Ref	
Moderate, strong	10	19	2.01 (0.84, 4.85)	0.119	2.60 (1.01, 6.73)	0.0487
HSP27 percent (median)						
≤ median	11	27	Ref		Ref	
> median	10	23	1.12 (0.47, 2.64)	0.798	1.35 (0.55, 3.32)	0.512
HSP27 percent (10%)						
<10%	2	8	Ref		Ref	
≥10%	19	42	2.48 (0.57, 10.8)	0.226	12.5 (1.34, 116)	0.0265
pHSP27 intensity						
Weak, moderate, strong	4	12	Ref		Ref	
None	15	36	1.29 (0.43, 3.88)	0.655	1.16 (0.38, 3.53)	0.801
pHSP27 percent (median)						
> median	4	12	Ref		Ref	
≤ median	15	36	1.29 (0.43, 3.88)	0.655	1.16 (0.38, 3.53)	0.801
pHSP27 percent (10%)						
≥10%	4	12	Ref		Ref	
<10%	15	36	1.29 (0.43, 3.88)	0.655	1.16 (0.38, 3.53)	0.801
LC3 intensity						
Weak, moderate, strong	4	16	Ref		Ref	
None	13	34	1.62 (0.53, 4.99)	0.399	1.50 (0.48, 4.67)	0.483
LC3 intensity						
Moderate, strong	2	10	Ref		Ref	
None, weak	15	40	2.03 (0.46, 8.89)	0.35	1.90 (0.43, 8.39)	0.398
LC3 percent (median)						
> median	4	16	Ref		Ref	
≤ median	13	34	1.62 (0.53, 4.99)	0.399	1.50 (0.48, 4.67)	0.483
LC3 percent (10%)						
≥10%	4	16	Ref		Ref	
<10%	13	34	1.62 (0.53, 4.99)	0.399	1.50 (0.48, 4.67)	0.483

*Adjusting for radiation induced osteosarcoma

Table 18. Univariate and multivariate analyses (Cox regression) for biomarkers assessed in pre-treatment biopsy specimens associated with RFS.

Factor	No. of deaths	Total n	Univariate HR (95% CI)	Univariate P-value	Multivariate* HR (95% CI)	Multivariate* P-value
HSP27 intensity						
None	1	8	Ref		Ref	
Weak, moderate, strong	15	41	3.68 (0.49, 28.0)	0.207	23.1 (1.34, 397)	0.0306
HSP27 intensity						
None, weak	8	30	Ref		Ref	
Moderate, strong	8	19	1.90 (0.71, 5.09)	0.201	2.39 (0.84, 7.10)	0.117
HSP27 percent (median as cutoff)						
≤ median	8	27	Ref		Ref	
> median	9	23	1.52 (0.59, 3.97)	0.388	1.85 (0.68, 5.01)	0.227
HSP27 percent (10%)						
<10%	1	8	Ref		Ref	
≥10%	16	42	3.78 (0.50, 28.6)	0.198	26.7 (1.47, 484)	0.0263
pHSP27 intensity						
Weak, moderate, strong	3	12	Ref		Ref	
None	11	36	1.18 (0.33, 4.27)	0.796	1.20 (0.32, 4.49)	0.787
pHSP27 percent (median as cutoff)						
> median	3	12	Ref		Ref	
≤ median	11	36	1.18 (0.33, 4.27)	0.796	1.20 (0.32, 4.49)	0.787
pHSP27 percent (10%)						
≥10%	3	12	Ref		Ref	
<10%	11	36	1.18 (0.33, 4.27)	0.796	1.20 (0.32, 4.49)	0.787
LC3 intensity						
Weak, moderate, strong	3	16	Ref		Ref	
None	9	34	1.38 (0.37, 5.12)	0.631	1.40 (0.34, 5.82)	0.646
LC3 intensity						
Moderate, strong	2	10	Ref		Ref	
None, weak	10	40	1.10 (0.24, 5.06)	0.905	1.02 (0.21, 4.84)	0.983
LC3 percent (median)						
> median	3	16	Ref		Ref	
≤ median	9	34	1.38 (0.37, 5.12)	0.631	1.40 (0.34, 5.82)	0.646
LC3 percent (10%)						
≥10%	3	16	Ref		Ref	
<10%	9	34	1.38 (0.37, 5.12)	0.631	1.40 (0.34, 5.82)	0.646

*Adjusting for age and radiation induced osteosarcoma

Table 19. Univariate and multivariate analyses (Cox regression) for biomarkers assessed in pre-treatment biopsy specimens associated with OS.

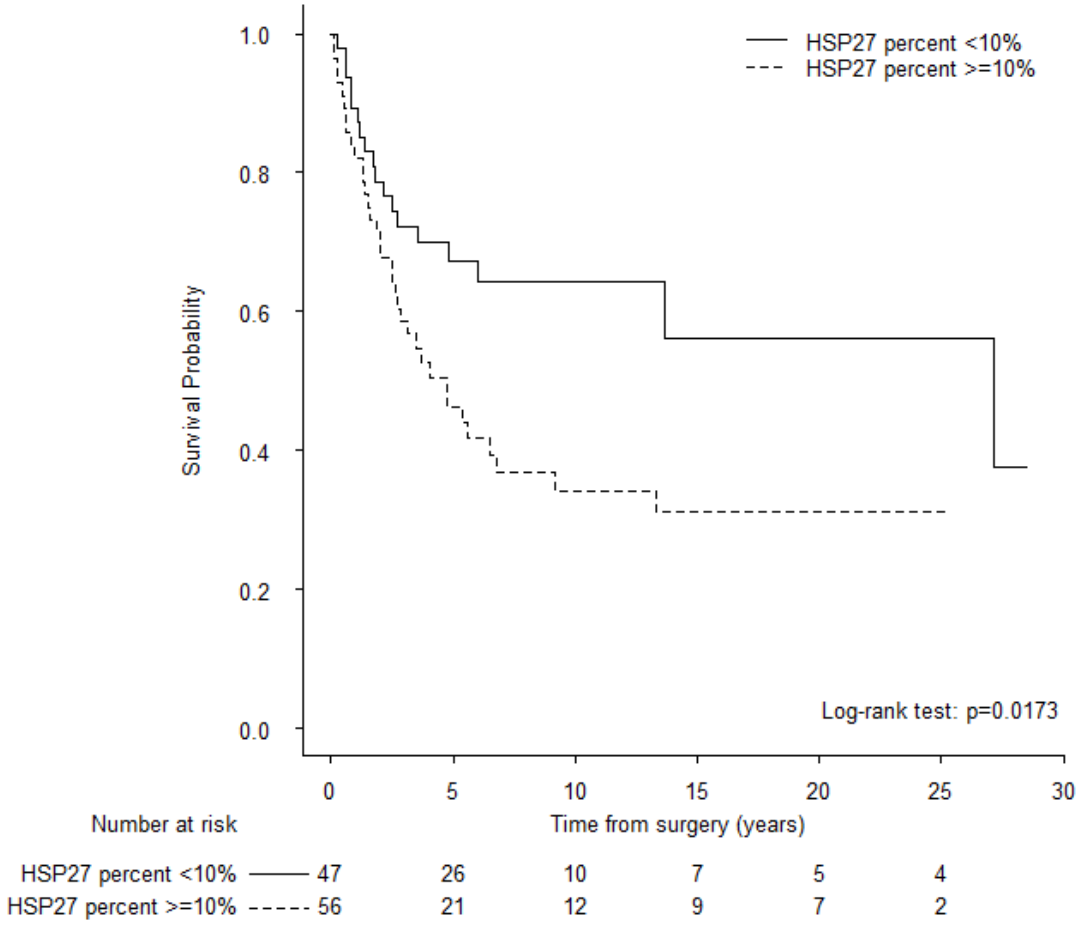

Figure 10. Overall survival for localized osteosarcoma patients stratified according to HSP27 expression at resection (>10% positive, <10% negative)

Combined analysis of LC3B puncta and HSP27 was evaluated for further risk stratification amongst patients with localized disease. In pre-treatment biopsy samples from 32 evaluable patients, the combination of HSP27+/LC3B- was associated with a trend towards inferior survival (log rank p=0.087). Ninety-two patients with localized disease had resection specimens with HSP27 and LC3B biomarker data available for analysis. Patients with HSP27-/LC3B+ tumors had significantly improved overall survival as compared to all others (p=0.012, Figure 11). This was stratified into 4 groups with HSP27-/LC3B+ having favorable outcomes, HSP27+/LC3B+ or HSP27-/LC3B- with intermediate risk, and HSP27+/LC3B- having the worst overall survival (p=0.018, Figure 12).

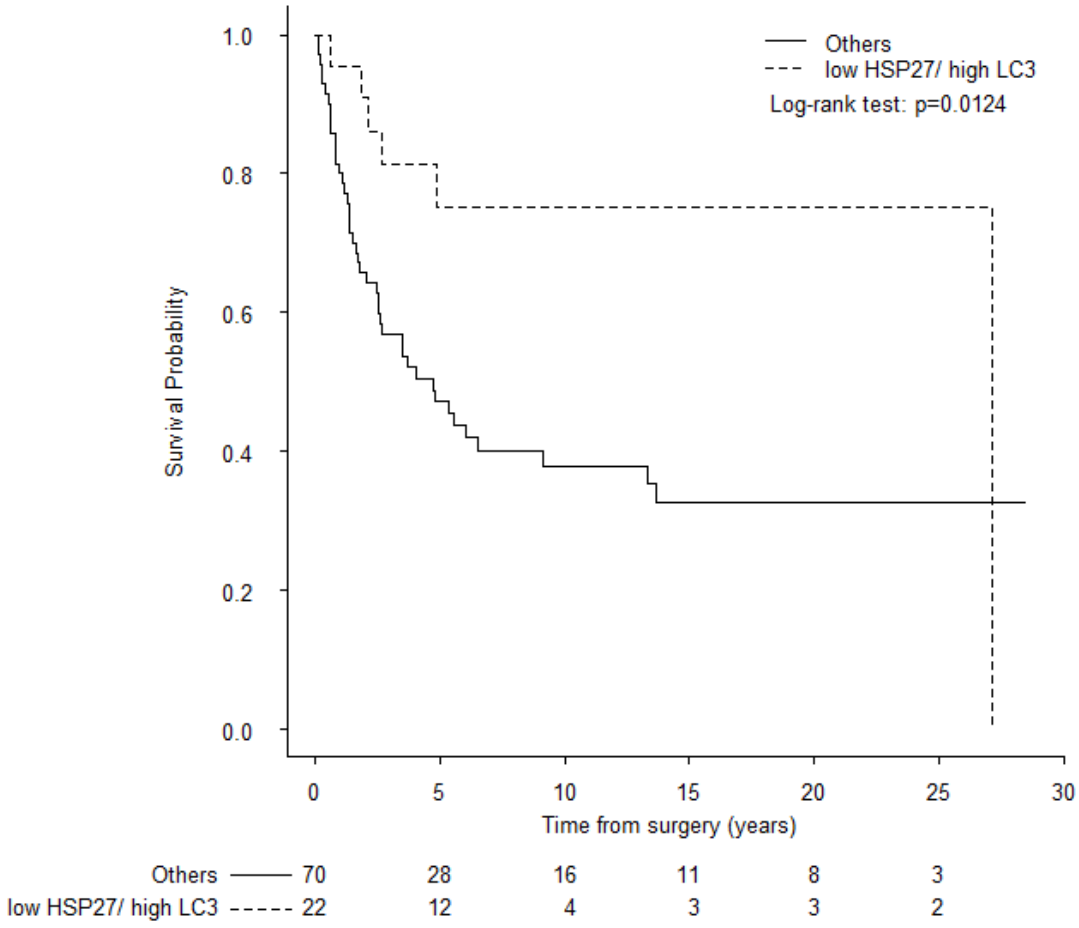

Figure 11. Overall survival for localized osteosarcoma patients combined biomarker analysis at resection, HSP27-/LC3B+ vs all others. HSP27-/LC3B+ patients have significantly improved OS as compared to all others.

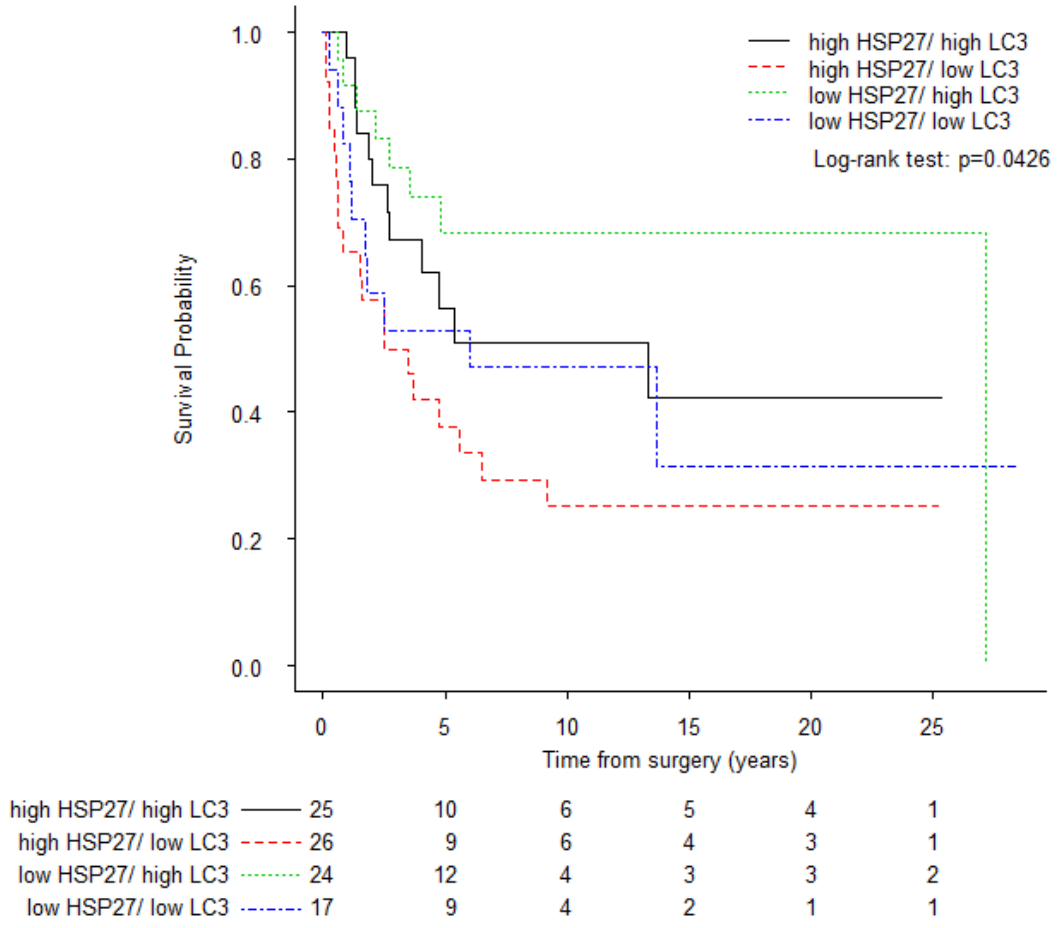

Figure 12. Overall survival for localized osteosarcoma patients stratified by combined biomarker analysis at resection yields 3 risk groups: good risk (HSP27-/LC3B+), intermediate risk (HSP27+/LC3B+ or HSP27-/LC3B-), or poor risk (HSP27+/LC3B-).

Pathologic treatment response assessed by percent tumor necrosis following neoadjuvant chemotherapy is the most well established prognostic marker in patients with localized osteosarcoma. Neither LC3B percentage nor HSP27 percentage expression in pre-treatment specimens correlated with percent tumor necrosis following neoadjuvant chemotherapy (Spearman correlation -0.12, p=0.384 and -0.067, p=0.655 respectively). Further, there was no association between LC3B+ puncta in pre-treatment samples and a good pathologic response to chemotherapy (p=0.391) nor between HSP27+ and good pathologic response (p=1.00, Table 20). Additionally, no significant association was seen between either marker at resection and a good response to therapy (data not shown). In a subset of localized OS patients with poor response to therapy (tumor necrosis <90%), the lack of LC3B puncta was associated with a trend toward inferior overall survival (HR: 1.87 [0.96-3.64], p=0.0665; Figure 13).

Factor	Poor response n (Column %)	Good response n (Column %)	Poor response n (row %)	Good response n (row %)	P-value
HSP27 intensity					1[a]
None	3 (14)	4 (13)	3 (43)	4 (57)	
Weak, moderate, strong	19 (86)	26 (87)	19 (42)	26 (58)	
HSP27 intensity					0.756[b]
None	13 (59)	19 (63)	13 (41)	19 (59)	
Weak, moderate, strong	9 (41)	11 (37)	9 (45)	11 (55)	
HSP27 percent (median)					0.758[b]
≤ median	13 (59)	17 (55)	13 (43)	17 (57)	
> median	9 (41)	14 (45)	9 (39)	14 (61)	
HSP27 percent (10%)					1[a]
<10%	3 (14)	4 (13)	3 (43)	4 (57)	
≥10%	19 (86)	27 (87)	19 (41)	27 (59)	
LC3 intensity					0.391[b]
None	16 (62)	21 (72)	16 (43)	21 (57)	
Weak, moderate, strong	10 (38)	8 (28)	10 (56)	8 (44)	
LC3 intensity					0.831[b]
None, weak	20 (77)	23 (79)	20 (47)	23 (53)	
Moderate, strong	6 (23)	6 (21)	6 (50)	6 (50)	
LC3 percent (median)					0.391[b]
≤ median	16 (62)	21 (72)	16 (43)	21 (57)	
> median	10 (38)	8 (28)	10 (56)	8 (44)	
LC3 percent (10%)					0.391[b]
<10%	16 (62)	21 (72)	16 (43)	21 (57)	
≥10%	10 (38)	8 (28)	10 (56)	8 (44)	

a: Fisher's exact test, b: Chi-square test

Table 20. Association between pre-treatment biomarker expression and pathologic treatment response to preoperative chemotherapy. Good pathologic response is defined as ≥ 90% tumor necrosis at the time of resection.

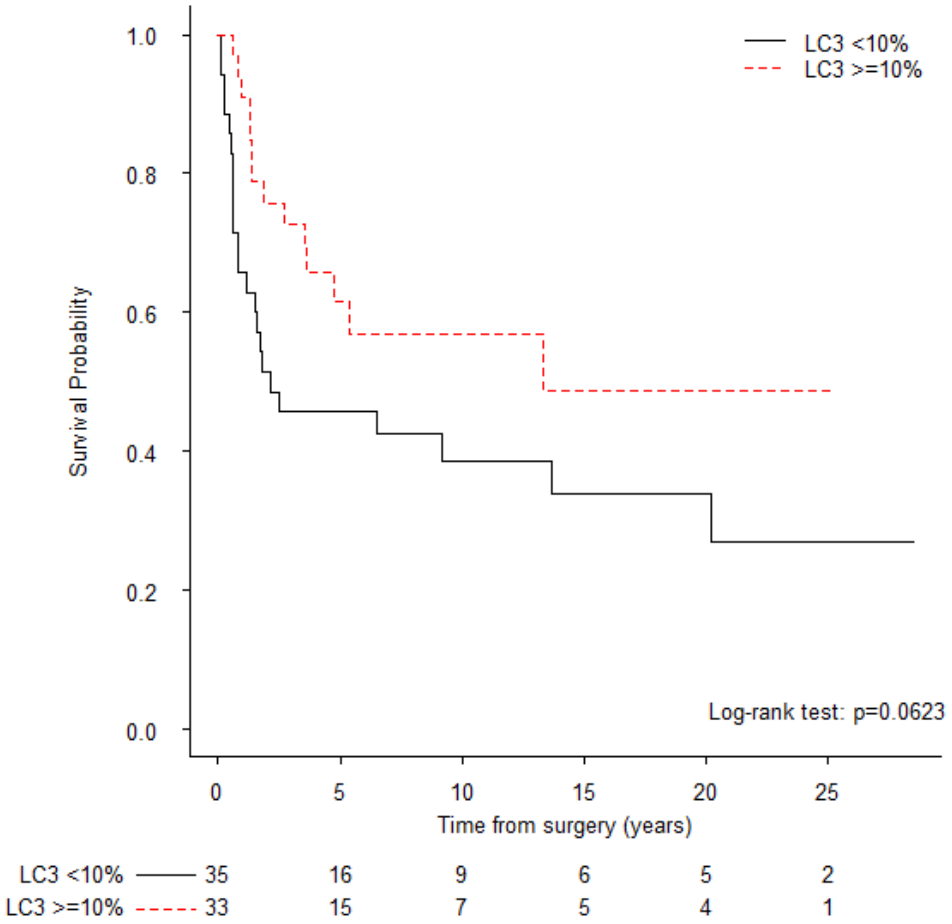

Figure 13. Overall survival for localized osteosarcoma patients with poor response pathologic response to preoperative chemotherapy stratified according LC3B+ at resection. Amongst poor responders, presence of LC3B+ puncta is associated with a trend towards improved OS.

HSP27 and LC3B as prognostic biomarkers in primary metastatic osteosarcoma

The prognostic significance of HSP27 and LC3B in patients with primary metastatic disease was evaluated at the time of diagnosis (pre-treatment) and at resection for patients who underwent resection of the primary tumor. Due to the limited number of samples, no meaningful inferences could be made based upon pretreatment expression of either marker. For example, amongst 9 patients with metastatic osteosarcoma who had evaluable pretreatment samples, 8/9 (89%) were positive for HSP27 (>10% expression). Similarly, at resection, neither LC3B+ (any criteria) nor HSP27+ (any criteria) was associated with either PFS or OS.

The Dual Role of Chemotherapy-induced Autophagy in Osteosarcoma Cells

Several studies have evaluated the presence and significance of chemotherapy-induced autophagy in osteosarcoma cell lines and to a lesser extent, mouse xenograft models.[64] The majority of these works have focused on autophagy as a mechanism of chemoresistance and therefore explore autophagy inhibition as a means to enhance chemotherapeutic efficacy. In prior studies from our lab, we have shown that chemotherapy-induced autophagy in osteosarcoma can have a dual role, either cytoprotective or cytotoxic, following treatment with 9-nitrocamptothecin (9-NC)[72] or gemcitabine (GCB).[73] Further, in a recent paper from our group, we showed that the post-treatment expression of pHSP27 correlated with the role of autophagy (and treatment effect of autophagy inhibition with hydroxychloroquine [HCQ]) *in vitro* for both 9-NC and GCB.

Both cisplatin and doxorubicin have previously been shown to induce autophagy in multiple osteosarcoma cell lines including MG-63, SaOS-2, and U-2 OS amongst others. In these studies, standard MAP chemotherapy resulted in chemoresistance, and the inhibition of autophagy (either pharmacologic or by shRNA knockdown of key autophagy genes) resulted in enhanced chemosensitivity.[65-67, 70, 101] To our knowledge, however, no preclinical models or *in vitro* experiments have shown a cytotoxic role for autophagy following doxorubin or cisplatin. Based upon our preliminary data with 9-NC and GCB in osteosarcoma cell lines in conjunction with the clinical biomarker data suggesting a favorable role for autophagy following standard chemotherapy, we sought to determine whether standard chemotherapy can result in either cytototxic or cytoprotective autophagy in osteosarcoma cell lines and determine whether pHSP27 expression

correlates with the cytoprotective function of chemotherapy-induced autophagy following treatment with doxorubicin or cisplatin.

Doxorubicin and cisplatin induce autophagy in LM7 and CCH-OS-D

Changes in key autophagy proteins were evaluated by Western blot to confirm the induction of autophagy following treatment with either doxorubicin or cisplatin treatment. LC3 and p62 are common markers of autophagy and autophagic flux. Upon induction of autophagy, LC3I is conjugated to phosphatidylenthanolamine to form LC3II which is then recruited to the autophagosomal membrane and subsequently degraded in the autolysosome. This process results in an increase in the LC3II/LC3I ratio that is indicative of activation or induction of autophagy. p62, also known as SQSTM1, is often used as a protein marker of autophagic flux. When autophagy is induced, p62 is incorporated into the autophagosome and is degraded. Thus the combination of an increased LC3II/LC3I ratio and decrement in p62 are suggestive of an increase in autophagy, and more specifically autophagic flux.[57] Finally, the lysosomal inhibitor hydroxychloroquine blocks the late-stages of autophagy resulting in an increase in LC3II/LC3I.

Initially, LM7 cells were treated with either doxorubicin (DOX, 0.2 ug/mL), cisplatin (CDDP, 20uM), hydroxychloroquine alone (HCQ, 20 uM), or the combination of DOX+HCQ, or CDDP+HCQ and collected at 48 hours post-treatment. For the combination, cells were pretreated with HCQ for 20 minutes prior to the addition of DOX. Evaluation of LC3 showed a modest increase in the LC3II/LC3I ratio for DOX and CDDP alone, and as expected, a significant increase LC3II accumulation in all groups containing HCQ (Figure 14).

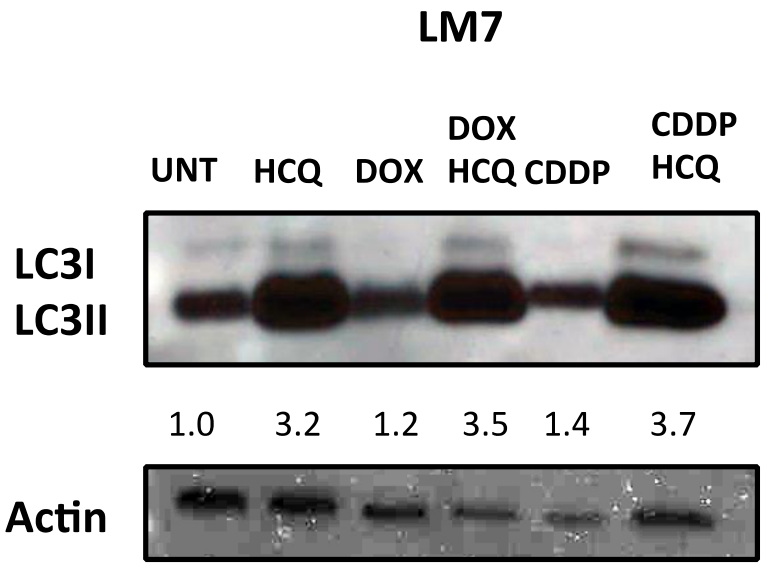

Figure 14. DOXO and CDDP increase LC3II expression in LM7 cells. Osteosarcoma cells were grown until 70-80% confluent and treated with HCQ (20 uM), DOXO (0.2 ug/mL), HCQ+DOXO (pretreatment with HCQ for 20 minutes, followed by DOXO), CDDP (20 uM), or HCQ+CDDP (pretreatment with HCQ for 20 minutes, followed by CDDP). Cells were collected and lysed using RIPA buffer. Equal amounts of total protein were resolved in SDS-PAGE, transferred to a nitrocellulose membrane and blotted using specific antibodies for LC3. Beta-actin is used as a loading control. Western blot analysis and densitometry show increase in LC3II/LC3I ratio following treatment with DOXO or CDDP compared to untreated control consistent with induction of autophagy. LC3II accumulation is seen in all HCQ containing conditions, consistent with late-autophagy inhibition. p62 is needed to confirm the presence of autophagic flux, but was not able to be performed.

Next, this experiment was repeated for LM7 cells with DOX alone (0.2ug/mL) for 48 and 72 hours. Under these conditions, induction of autophagy was not readily apparent (qualitative expression of p62 and LC3 shown in figure 15). While the HCQ either alone or in combination with DOX resulted in an increase accumulation of LC3II, DOX alone did not significantly alter either p62 or LC3 expression in comparison to untreated controls.

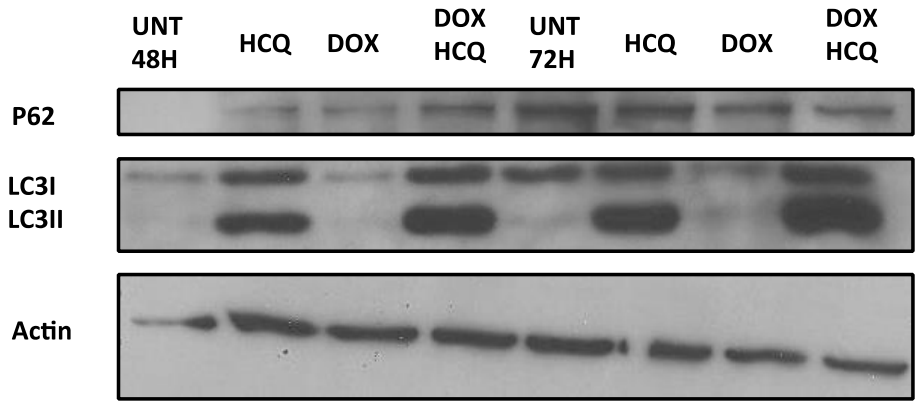

Figure 15. Autophagy induction is not apparent with standard doses of DOXO at 48 and 72 hours in LM7 cells. Osteosarcoma cells were grown until 70-80% confluent and treated with HCQ (20 uM), DOXO (0.2 ug/mL), HCQ+DOXO (pretreatment with HCQ for 20 minutes, followed by DOXO). Cells were collected and lysed using RIPA buffer. Equal amounts of total protein were resolved in SDS-PAGE, transferred to a nitrocellulose membrane and blotted using specific antibodies for LC3 and p62. Beta-actin is used as a loading control. Qualitative changes in p62 and LC3II/LC3I were not consistent with autophagy induction, where a decrement in p62 and increase in LC3II would be expected. HCQ containing conditions result in increased LC3II accumulation.

Subsequently, LM7 cells were treated with escalating doses of DOX (0.2-0.8 ug/mL) and collected at 24, 48, and 72 hours post-treatment. LC3I to LC3II conversion was assessed; DOX+HCQ was used as a positive control. This demonstrated a relative increase in the LC3II/LC3I ratio with increasing doses and longer drug exposure (Figure 16).

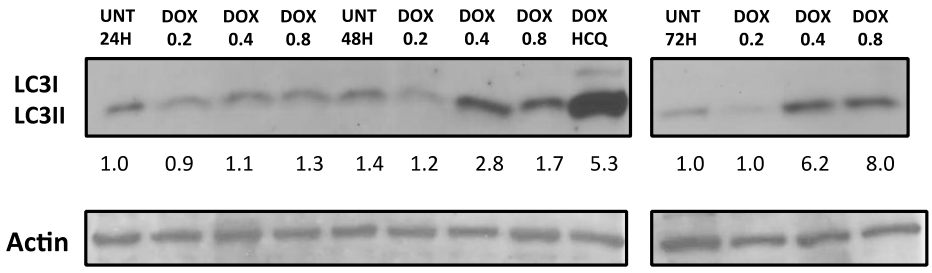

Figure 16. Autophagy induction with DOXO increase with dose and time in LM7 cells. Osteosarcoma cells were grown until 70-80% confluent and treated with increasing doses of DOXO (0.2 – 0.8 ug/mL) for 24, 48, and 72 hours. DOXO+HCQ was used as a positive control. Cells were collected and lysed using RIPA buffer. Equal amounts of total protein were resolved in SDS-PAGE, transferred to a nitrocellulose membrane and blotted using specific antibodies for LC3. Beta-actin is used as a loading control. Western blot analysis and densitometry show increase in LC3II/LC3I ratio at later time points and higher doses consistent with induction of autophagy. LC3II accumulation is seen in DOX+HCQ consistent with late-autophagy inhibition.

Our prior experience with LM7 has shown this cell line to be relatively chemoresistant to multiple chemotherapies, having higher IC50 concentrations as compared to other human and murine osteosarcoma cell lines. We therefore repeated the Western blots for LM7 cells treated at higher doses (DOX 1ug/mL and CDDP 40uM) for 48 and 72 hours. DOX resulted in an increase in the LC3II/I ratio at both 48 and 72 hours but without a concomitant decrease in p62 (Figure 17). CDDP treatment resulted in an increase in LC3II/I and a decrement in p62 at 48 but not 72 hours (Figure 18). Overall, these finding suggest that both DOX and CDDP can induce autophagy in LM7 cells, however the optimal dose and time point for maximal autophaguc flux was not clearly identified.

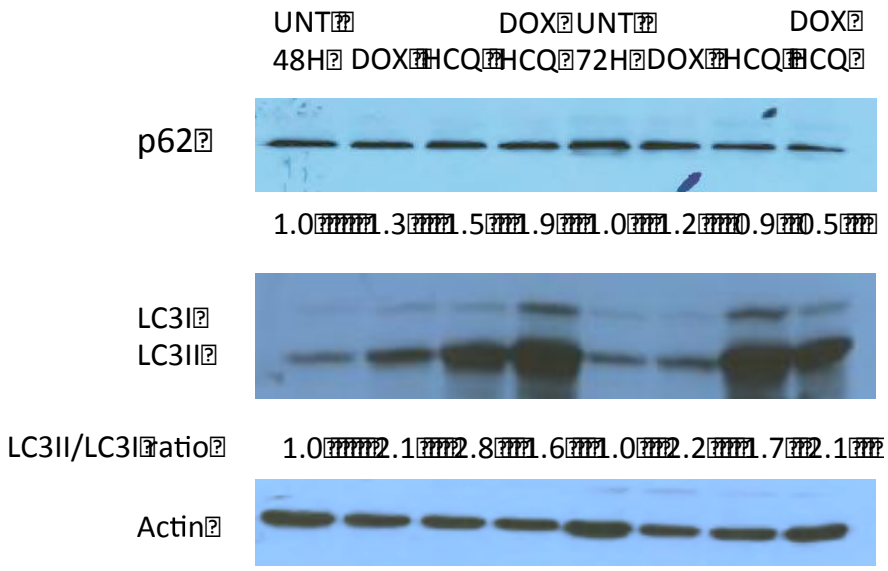

Figure 17. DOXO induces autophagy in LM7 cells. Osteosarcoma cells were grown until 70-80% confluent and treated DOXO (1 ug/mL), HCQ (20 uM), HCQ+DOXO (pretreatment with HCQ for 20 minutes, followed by DOXO) and collected at 48 and 72 hours. Cells were collected and lysed using RIPA buffer. Equal amounts of total protein were resolved in SDS-PAGE, transferred to a nitrocellulose membrane and blotted using specific antibodies for LC3 and p62. Actin is used as a loading control. Western blot analysis and densitometry show increase in LC3II/LC3I ratio with DOX treatment at 48 and 72 hours compared to untreated controls; DOX did not result in a decrease in p62. Note, densitometry values were normalized to untreated cells at each respective time point.

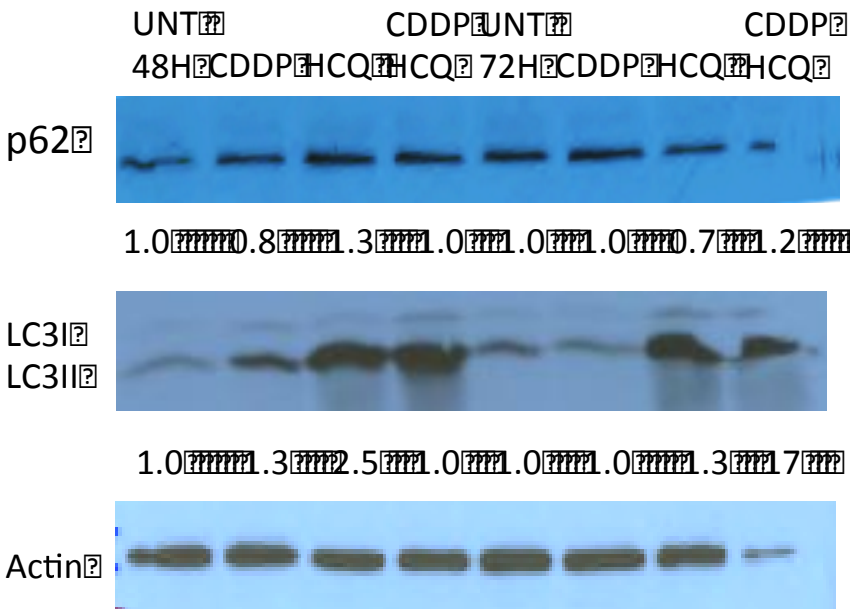

Figure 18. CDDP induces autophagy in LM7 cells. Osteosarcoma cells were grown until 70-80% confluent and treated CDDP (40 uM), HCQ (20 uM), HCQ+CDDP (pretreatment with HCQ for 20 minutes, followed by CDDP) and collected at 48 and 72 hours. Cells were collected and lysed using RIPA buffer. Equal amounts of total protein were resolved in SDS-PAGE, transferred to a nitrocellulose membrane and blotted using specific antibodies for LC3 and p62. Actin is used as a loading control. Western blot analysis and densitometry show increase in LC3II/LC3I ratio and decrease in p62 with CDDP treatment at 48 as compared to untreated controls consistent with induction of autophagy. Note, densitometry values were normalized to untreated cells at each respective time point.

The results for CCH-OS-D cells were more apparent. CCH-OS-D cells were treated with either doxorubicin (DOX, 0.2 ug/mL) or cisplatin (CDDP, 20 uM) for 48 hours. GCB treatment was used as a positive control. All treatment groups resulted in an increase in the LC3II/LC3I ratio and decrement in p62 relative to untreated cells consistent with induction of autophagy (Figure 19).

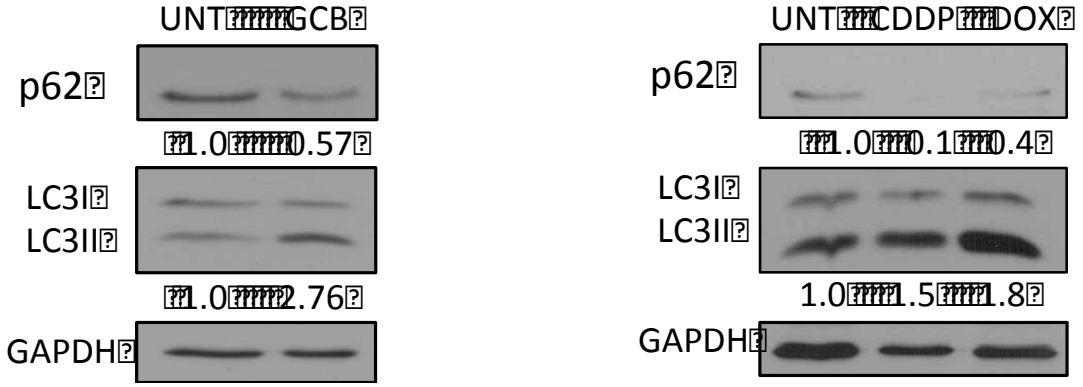

Figure 19. DOXO and CDDP induce autophagy in CCH-OS-D cells. Osteosarcoma cells were grown until confluent and treated with GCB (1 uM), DOXO (0.2 ug/mL), or CDDP (20 uM) for 48 hours. Cells were collected and lysed using RIPA buffer. Equal amounts of total protein were resolved in SDS-PAGE, transferred to a nitrocellulose membrane and blotted using specific antibodies for LC3 and p62. GAPDH is used as a loading control. Western blot analysis and densitometry show increase in LC3II/LC3I ratio and decrease in p62 following treatment with GCB, DOXO, or CDDP compared to untreated controls consistent with induction of autophagy.

While autophagy induction was evidenced by changes in autophagy proteins for CCH-OS-D cells treated with either DOX or CDDP, autophagy induction in LM7 cells as evaluated by Western blot was only modest at best. Autophagy induction appeared to be greater at later time points and higher doses of DOX in LM7, however additional studies are needed to validate these findings and optimize conditions for chemotherapy-induced autophagy with DOX in LM7 cells. We have previously correlated the findings of Western blot analysis for autophagy induction in LM7 and CCH-OS-D cell lines treated with GCB and 9-NC by both acridine orange staining for AVOs and electron microscopy. These additional assays would allow further evaluation of chemotherapy-induced autophagy in both cell lines following DOX or CDDP and may validate the findings on Western blot analysis.

Chemotherapy-induced authophagy serves a dual role in osteosarcoma cells treated with doxorubicin or cisplatin

The effect of autophagy inhibition can give insights into the role of chemotherapy-induced autophagy. When chemotherapy induces cytoprotective autophagy and chemoresistance, the addition of autophagy inhibition to chemotherapy results in increased chemosensitivity and decreased cell viability. Conversely, if autophagy serves a cytotoxic role and is a mechanism of cell death, blocking autophagy in addition to chemotherapy is expected to result in increased cell viability. To determine the role of chemotherapy-induced autophagy in LM7 and CCH-OS-D treated with DOX and CDDP as either cytoprotective or cytotoxic, viability studies were completed using chemotherapy alone or in combination with the autophagy inhibitor HCQ.

LM7 and CCH-OS-D cells were treated with HCQ, DOX alone, CDDP alone, or combination DOX+HCQ pretreatment or CDDP+HCQ pretreatment and collected at 48 and 72 hours. Viability was assessed using trypan blue exclusion via ViCell and compared to untreated controls at each time point. All conditions were conducted with 3 replicates and experiments were repeated 3 times. Combining HCQ with either DOX (0.2 ug/mL) or CDDP (20 uM) resulted in a trend towards decreased cell viability for LM7 (Figures 20 and 21, respectively). While this effect was not statistically significant, this may be in part due to inadequate autophagy induction at the specified dose and time point of analysis.

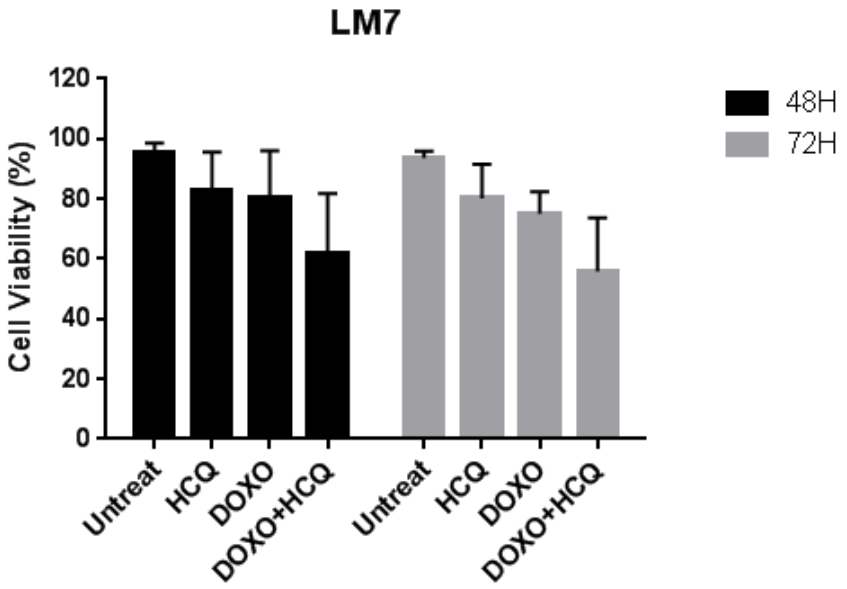

Figure 20. Pharmacologic inhibition of autophagy prior to DOX results in a trend towards increased chemosensitivity in LM7 cells. Cells were treated with HCQ (20 uM), DOX (0.2 ug/mL), or pretreated with HCQ for 20 minutes prior to DOX treatment for 48 and 72 hours. Cell viability was measured by trypan blue exclusion assay.

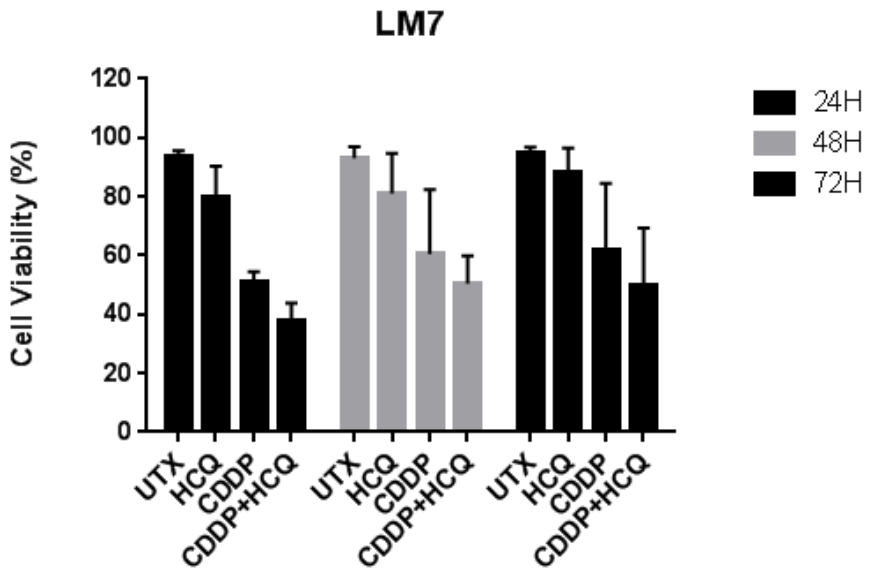

Figure 21. Pharmacologic inhibition of autophagy prior to CDDP results in a trend towards increased chemosensitivity in LM7 cells. Cells were treated with HCQ (20 uM), CDDP (20 uM), or pretreated with HCQ for 20 minutes prior to CDDP treatment for 24, 48, and 72 hours. Cell viability was measured by trypan blue exclusion assay.

Viability studies in LM7 were repeated with higher doses of DOX (1 ug/mL) and CDDP (40 uM) alone or in combination with HCQ for 48 and 72 hours. There were no significant differences in viability at 48 hours amongst the treatment groups (data not shown). However, at 72 hours combining HCQ with DOX resulted in a trend toward decreased viability (Figure 22, DOX vs DOX+HCQ p=0.094); combining HCQ with CDDP also decreased viability (Figure 23, CDDP vs CDDP+HCQ p=0.022). These findings support a cytoprotective role for autophagy in LM7 cells treated with DOX or CDDP.

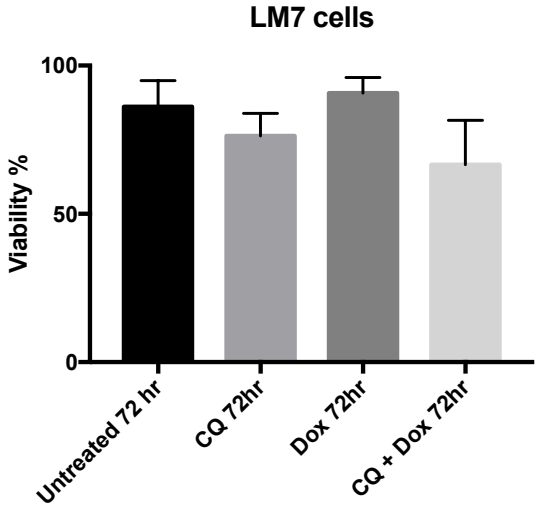

Figure 22. Pharmacologic inhibition of autophagy prior to higher doses of DOX results in a trend towards increased chemosensitivity in LM7 cells at 72 hours. Cells were treated with hydroxychloroquine (CQ), DOX (1ug/mL), or pretreated with CQ for 20 minutes prior to DOX treatment for 72 hours. Cell viability was measured by trypan blue exclusion assay. There was no difference in viability between untreated controls and HCQ alone. Pretreatment with HCQ prior to DOX resulted in a trend towards decreased viability as compared to DOX alone (p=0.094).

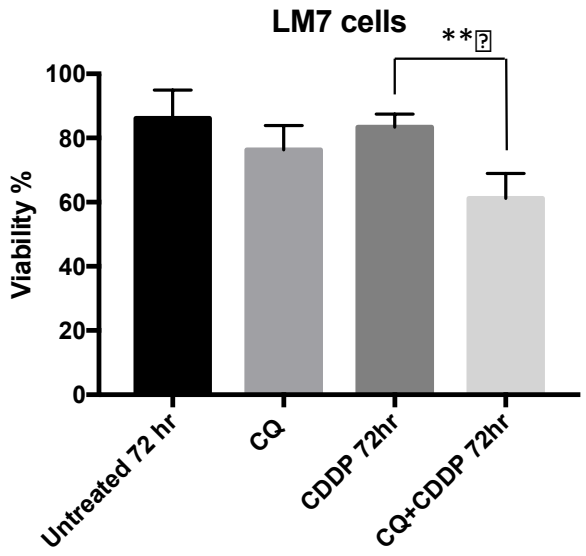

Figure 23. Pharmacologic inhibition of autophagy prior to higher doses of CDDP results in increased chemosensitivity in LM7 cells. Cells were treated with hydroxychloroquine (CQ), CDDP (40 uM), or pretreated with HCQ for 20 minutes prior to CDDP treatment for 72 hours. Cell viability was measured by trypan blue exclusion assay. There was no difference in viability between untreated controls and HCQ alone. Pretreatment with HCQ prior to CDDP resulted in decreased cell viability as compared to CDDP alone (p=0.022).

Conversely, the pretreatment with HCQ prior to DOX in CCH-OS-D resulted in increased cell viability at 48 and 72 hours (Figure 24). Combining in HCQ with CDDP in CCHOSD cells resulted in no change in viability (Figure 25). Notably, treatment with CDDP alone resulted in significant cytotoxicity at both 48 and 72 hours in CCH-OS-D cells which may have limited the apparent effect adding the autophagy inhibitor HCQ.

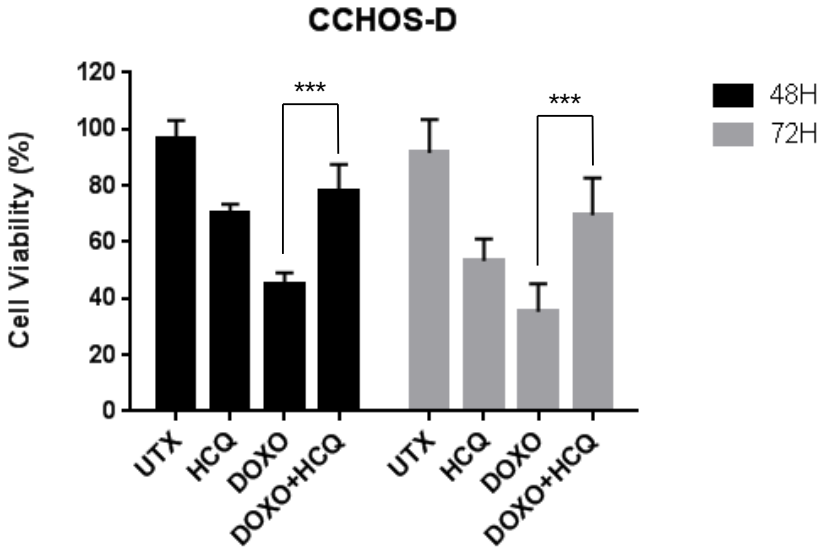

Figure 24. Pharmacologic inhibition of autophagy prior to DOX results in decreased chemosensitivity in CCH-OS-D cells. Cells were treated with HCQ (20 uM), DOX (0.2 ug/mL), or pretreated with HCQ for 20 minutes prior to DOX treatment for 48 and 72 hours. Cell viability was measured by trypan blue exclusion assay. Pretreatment with HCQ prior to DOX resulted in increased cell viability as compared to DOX alone ($p<0.001$).

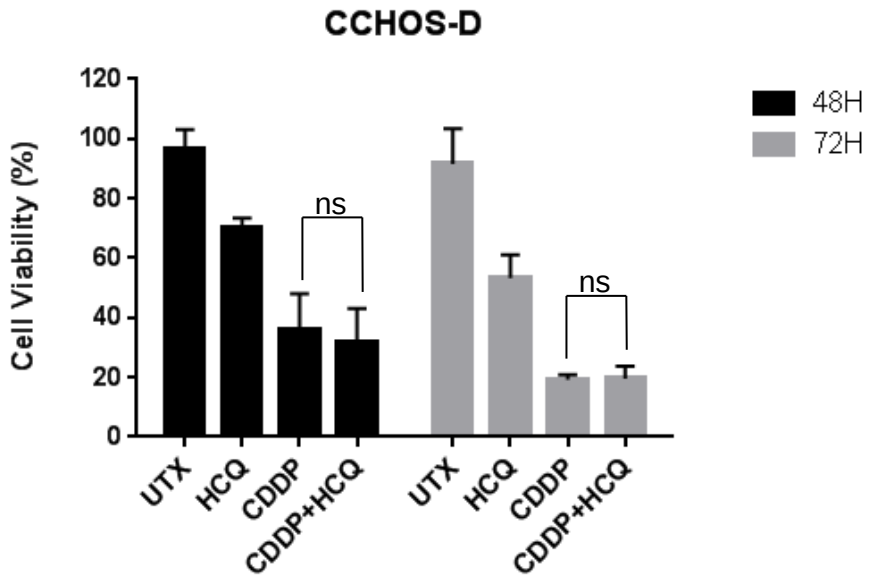

Figure 25. Pharmacologic inhibition of autophagy prior to CDDP does not alter chemosensitivity in CCH-OS-D cells. Cells were treated with HCQ (20 uM), CDDP (20 uM), or pretreated with HCQ for 20 minutes prior to CDDP treatment for 48 and 72 hours. Cell viability was measured by trypan blue exclusion assay. CDDP alone significantly decreased viability at both 48 and 72 hours; pretreatment with HCQ did not change viability at either time point.

Taken together, these findings support a dual role for chemotherapy-induced autophagy in osteosarcoma. The data with LM7 cells are in line with other studies that have firmly established the induction of cytoprotective autophagy with either DOX or CDDP in osteosarcoma cell lines. We found that in CCH-OS-D cells autophagy inhibition with HCQ prior to DOX treatment resulted in increased cell viability suggesting a cytotoxic role for autophagy in this context. This presents a valuable model system for evaluating the mechanisms of cytoprotective vs cytotoxic autophagy and for exploring the effects of autophagy modulation. Additional studies are needed to confirm these findings. While we have shown this duality in other osteosarcoma cell lines and with various chemotherapeutic agents, these findings would benefit greatly from validation *in vivo.*

Post-treatment phosphorylated HSP27 expression correlates with the role of autophagy and effect of autophagy inhibition in osteosarcoma cells

Previously, we identified that treatment of LM7 and CCH-OS-D cells with GCB resulted in opposing effects of autophagy. A human phosphokinase array was used to identify differentially expressed proteins and phosphoproteins that could serve as potential biomarkers and to gain mechanistic insights into the opposing roles of chemotherapy-induced autophagy. Post-treatment expression of pHSP27 correlated with the role of autophagy and effect of autophagy inhibition independent of cell line, species of origin (murine or human osteosarcoma), or chemotherapeutic agent. An increase in pHSP27 (but not total HSP27) following chemotherapy correlated with a cytoprotective role for autophagy and therefore was associated with a benefit to adding HCQ to chemotherapy. Decreased expression of pHSP27 following treatment correlated with

cytotoxic autophagy and alternate cell death. In this setting, the addition of HCQ was associated with increased cell viability. These findings may have important biomarker implications for the treatment of patients with osteosarcoma with autophagy inhibitors. We therefore sought to validate these findings in osteosarcoma cells following treatment with standard chemotherapy, DOX and CDDP.

LM7 and CCHOSD cells were treated with DOX or CDDP for 48 hours; untreated cells were used as a control. Phosphoprotein expression was determined using the human phosphokinase antibody array kit (R&D Systems) and analyzed by densitometry. Multiple phosphoproteins demonstrated changes in expression following treatment with chemotherapy (data not shown). Specifically, pHSP27 (s78/82) was decreased in CCHOSD cells following DOX or CDDP and increased in LM7 with both agents (Figure 26). In comparison, heat shock protein 60 (HSP60) was increased with DOXO in both cells lines and relatively unchanged following CDDP treatment.

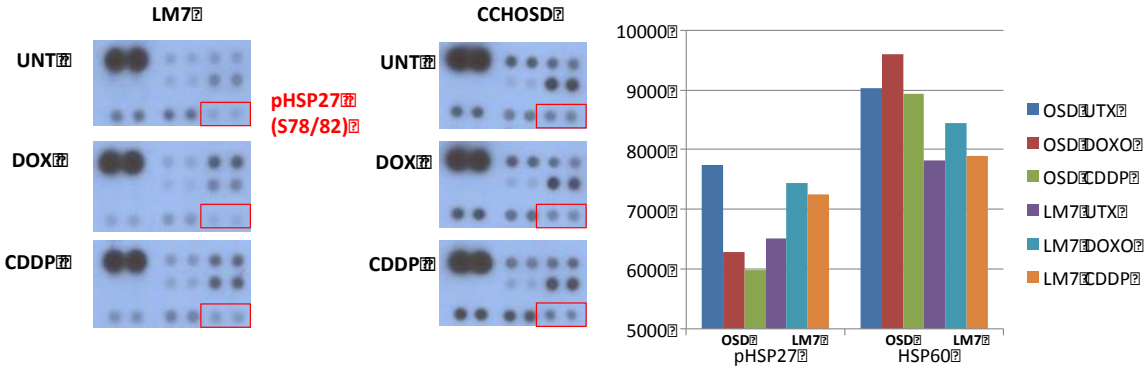

Figure 26. Post-treatment expression of phosphorylated HSP27 varies by osteosarcoma cell line. LM7 and CCH-OS-D cells were treated with DOX (0.2ug/mL) or CDDP (20 uM) for 48 hours. Proteins were detected using a human phosphokinase antibody array kit (R&D Systems). DOX and CDDP resulted in increased expression of pHSP27 (red boxes, left; relative densitometry, right) in LM7 cells as compared to untreated controls. Conversely, pHSP27 expression was decreased in CCH-OS-D treated with DOX or CDDP. HSP60 expression is shown for comparison. Additional proteins/phosphoproteins not shown.

These findings are consistent with prior work from our lab, demonstrating induction of pHSP27 following treatment correlated with a cytprotective role of autophagy in osteosarcoma. However, the limited number of replicates and semiquantitative approach did not allow for evaluating the statistical significance of these findings. Functional proteomic profiling by reverse phase protein lysate array is currently underway for both cell lines treated with DOX, CDDP, and GCB to validate these findings. This platform includes a panel of >290 proteins and phosphoproteins including HSP72, pHSP27, HMGB1, as well as other key autophagy-related and apoptotic pathway proteins. These studies will allow for further exploration of specific pathways associated with cytotoxic vs cytoprotective autophagy in osteosarcoma.

Summary of *in vitro* Studies

Both doxorubicin and cisplatin are able to induce autophagy in LM7 and CCH-OS-D cell lines, however the dose and time point for maximal chemotherapy-induced autophagy or increased autophagic flux may vary depending on the cell line or chemotherapeutic agent used. Further, autophagy inhibition can result in either increased chemosensitivity and decreased viability (in the case of doxorubicin or cisplatin in LM7) or alternatively, can reduce chemosensitivity and increase viability (for doxorubicin in CCH-OS-D). These findings imply a dual role for chemotherapy-induced autophagy in osteosarcoma, either as a cytoprotective mechanism promoting cell survival or cytotoxic leading to cell death. Finally, we demonstrated that the post-treatment expression of pHSP27 correlated with the role of autophagy and effect of autophagy inhibition. In LM7 cells, doxorubicin and cisplatin result in increased expression of pHSP27 and induction

of cytoprotective autophagy. In CCH-OS-D where autophagy appears to be cytotoxic, pHSP27 expression decreases following doxorubicin exposure. These findings are summarized in Table 21.

	LM7		CCHOSD	
	Doxorubicin	**Cisplatin**	**Doxorubicin**	**Cisplatin**
Autophagy	Varying degrees of autophagy induction in both cell lines with both agents			
Effect of autophagy inhibition	Decreased viability	Decreased viability	Increased viability	No change in viability
Post-treatment pHSP27 expression	Increased	Increased	Decrease	Decrease
Proposed Role of Autophagy	Cell survival	Cell survival	Cell death	No effect

Table 21. Summary of *in vitro* studies.

IV. Discussion

The paradoxical role of autophagy in cancer has been well described, however the key determinants of autophagy as either a mechanism of chemoresistance or promoting cancer cell death have not been defined. Defining the role of autophagy in a specific context and identifying potential biomarkers that could therefore predict benefit (or potentially harm) for combining autophagy inhibitors with cytotoxic chemotherapy is needed. In this project, we sought to understand the role and prognostic significance of chemotherapy-induced autophagy in osteosarcoma patients treated with standard MAP chemotherapy as well as in osteosarcoma cell lines and to correlate these findings with expression of HSP27/pHSP27.

First, we show that both cytoplasmic LC3B puncta and HSP27 expression are relevant prognostic biomarkers in patients with localized osteosarcoma. Patients that lack LC3B+ puncta (<10% of cells) at resection following preoperative chemotherapy have a poor prognosis, as did patients who expressed HSP27 (>10% of cells). By combining these 2 markers at resection, we were able to identify a favorable risk group (HSP27-/LC3B+) and those with particularly poor risk (HSP27+/LC3B-) with standard therapy. These markers may be valuable in risk stratification and support the development of clinical trials targeting either HSP27 or modulating autophagy in osteosarcoma.

Although several studies have examined the role of chemotherapy-induced autophagy in osteosarcoma cell lines, to our knowledge, the presence and prognostic significance of autophagy markers such as LC3B has not been previously reported in osteosarcoma patients. While the majority of preclinical data suggest that chemotherapy-

induced autophagy is a mechanism of chemoresistance to standard MAP chemotherapy in osteosarcoma,[65, 70, 101] and the expression LC3B has been shown to be a poor prognostic marker in multiple other cancer types,[102] we found the opposite. The presence of LC3B puncta following chemotherapy was a positive prognostic marker in osteosarcoma. One large study of >1600 breast cancer patients similarly showed the presence of cytoplasmic LC3B to be a favorable prognostic marker following adjuvant chemotherapy.[56] Using a similar methodology to evaluate LC3B puncta in tumor cells and the same >10% cutoff, Laoire *et al* demonstrated that the combined positivity to LC3B+ puncta and presence of nuclear $HMGB_1$ was associated with prolonged metastasis-free and breast cancer specific survival. Additionally, the authors showed that LC3B+ puncta correlated with a reduction in SQSTM1/p62, suggesting that a high percentage of LC3B+ puncta reflects increased autophagic flux.

In our study, a limited number of patients demonstrated LC3B puncta on pre-treatment specimens suggestive of basal autophagy in a subset of primary osteosarcoma. However, this finding was not associated with relapse-free survival, overall survival, or pathologic treatment response. Overall, the proportion of osteosarcoma with LC3B+ puncta was significantly higher in post-treatment specimens, though the majority of these specimens were not paired. In the nine patients with paired pre-/post-chemotherapy specimens who were initially negative for cytoplasmic LC3Bpuncta, >50% were positive following chemotherapy. Taken together, these findings suggest that standard chemotherapy can induce autophagy in a subset of patients with osteosarcoma and that chemotherapy-induced autophagy may be favorable in the primary treatment of osteosarcoma. The presence of LC3B+ either prior to treatment or at resection did not

correlate with percent tumor necrosis nor was it significantly associated with a "good" pathologic response, suggesting that LC3B+ is an independent prognostic biomarker rather than a surrogate marker for pathologic treatment response. Even amongst the subset of poor responders, patients lacking LC3B following chemotherapy had a trend towards inferior overall survival.

The association of post-treatment LC3B puncta and favorable outcomes suggests that chemotherapy-induced autophagy following MAP may serve as mechanism of alternate cell death as opposed to chemoresistance in primary osteosarcoma. Rather than combining chemotherapy with autophagy inhibitors,[2, 66, 67, 69, 71] strategies that induce autophagy such as mTOR inhibition[103, 104] or other agents[58] that are currently being explored in osteosarcoma may prove beneficial when added to primary therapy. The implications for targeting autophagy (either promoting or inhibiting it) in recurrent/metastatic osteosarcoma remain unclear. LC3B puncta was observed in the majority of metastasis specimens (67%). Further, in a limited number of metachronous biopsy-metastasis and resection-metastasis pairs, LC3B became positive in the majority of patients who were initially scored as negative. Given the conflicting data regarding the association of LC3B puncta with improved relapse free and overall survival but its increased occurrence in osteosarcoma metastasis, it would be premature to conclude whether autophagy, as evidenced by LC3B, is a driver of osteosarcoma metastasis and therefore should be inhibited. Additional mechanistic studies are needed.

In regard to HSP27, our study is in agreement with prior studies that have shown overexpression of HSP27+ at biopsy as an independent poor prognostic factor.[89, 90] However, in these 2 small series the proportion of HSP27+ tumors (>10% of tumor cells)

was much lower both at biopsy (22-24%) and resection (33-37%) as compared to our finding (85% and 52%, respectively). While Uozaki *et al* found an association between HSP27+ at resection and poor response among 19 patients evaluated, HSP27+ did not correlate with pathologic treatment response in our analysis. Although not a predictive biomarker of pathologic response, the negative prognostic implications of HSP27 overexpression at diagnosis support strategies combining HSP27 inhibition with primary MAP chemotherapy. Several preclinical studies support the rationale for targeting HSP27 in osteosarcoma.[105-107]

We examined the combination of HSP27 and LC3B in osteosarcoma patient samples based upon preclinical data suggesting an association between HSP27, pHSP27, and autophagy. The ER stress response/ubiquitin-proteosome system, mitochondrial autophagy (mitophagy), and the Akt/mTOR pathway have been proposed as mechanisms linking HSP27 and autophagy.[108, 109] In addition, HMGB1 which has been shown to promote drug resistance in osteosarcoma cell lines by inducing autophagy, regulates the expression HSP27. [108]

In osteosarcoma cell lines, both doxorubicin and cisplatin can induce autophagy. However, this may lead to either cell survival or cell death and therefore opposing effects of autophagy inhibition. We previously demonstrated that increased expression of pHSP27 following chemotherapy exposure was associated with cytoprotecitve autophagy and chemoresistance.[73] Here we found consistent findings when LM7 and CCH-OS-D cells were treated with either doxorubicin or cisplatin. Notably, however, the association between the role of autophagy, effect of autophagy inhibition, and total HSP27 in osteosarcoma cell lines has been inconsistent. We sought to evaluate pHSP27 in

osteosarcoma tumor specimens based upon the initial hypothesis but labeling was severely limited likely due to the decalcification protocols used in processing bone tumor specimens in FFPE tissue. We found no correlation between the LC3B puncta and HSP27+ in patient specimens.

There are several limitations to the current study. In regard to the biomarker analysis, all samples were analyzed retrospectively from a single institution and there were a limited number of pretreatment biopsy specimens. This significantly limited the number of pre-treatment/post-treatment paired samples available for analysis and therefore limited our ability to specifically assess chemotherapy-induced autophagy. Further, based upon the initial hypothesis and cell line data, we sought to explore pre-/post-treatment changes in HSP27 and pHSP27 expression (rather than absolute expression) in relation to autophagy markers. This analysis could not be conducted given the lack of paired samples and our inability to accurately measure pHSP27 in decalcified bone tumor specimens. Finally, autophagy was assessed by a single marker, LC3B puncta. The addition of other autophagy markers such as p62 to confirm the presence of autophagic flux or the autophagy-related DNA binding protein HMB1 to this analysis would serve to validate these findings and is currently underway.

In regard to analysis of chemotherapy-induced autophagy in osteosarcoma cell lines, optimal conditions for autophagy induction with doxorubicin and cisplatin in LM7 cells were not defined. While both agents were shown to induce some degree of autophagy, insufficient autophagy induction may limit the implications of the viability studies and post-treatment pHSP27 expression data. Finally, while we have demonstrated a correlation between the role of chemotherapy-induced autophagy and pHSP27 in

osteosarcoma cell lines, the functional significance and potential mechanistic basis of these findings have not been explored.

This study adds to our prior work identifying a context-dependent dual role for chemotherapy-induced autophagy in osteosarcoma that is independent of cell line, species of origin, or chemotherapeutic agent. These findings establish a valuable model system to further evaluate the mechanisms underlying of cytotoxic vs. cytoprotective autophagy. While pHSP27 was initially identified as a differentially expressed protein that consistently correlates with the role of autophagy, its functional significance remains unclear. Future studies stemming from this project will undertake functional proteomic profiling by RPPA to identify additional proteins and key pathways associated with the opposing effects of autophagy as well as functional studies using shHSP27 knockdowns in LM7 and overexpression of HSP27 in CCH-OS-D cells to determine if HSP27 is indeed necessary for cytoprotective autophagy in osteosarcoma. Given that pHSP27 (but not total HSP27) correlates with the role of autophagy, identifying the specific kinases involved in phosphorylating HSP27 in each context may give additional mechanistic insights. Finally, *in vivo* studies combining autophagy inhibitors with chemotherapy in the treatment of osteosarcoma will be a key step in translating these findings to the clinic.

Independent of their potential mechanistic link, HSP27 and LC3B can be considered as independent biomarkers in osteosarcoma; if analyzed together they allow for improved risk stratification for localized osteosarcoma patients following preoperative chemotherapy. One of the limitations of this approach, however, is awaiting evaluation of these markers at resection ~12 weeks into therapy. Predictive biomarkers of response to conventional MAP chemotherapy are needed. Such markers would allow for

modification or intensification of preoperative therapy for poor risk patients in a window trial paradigm that would allow for paired biomarker assessment. Prospective analysis of paired-samples may be beneficial in validating HSP27 and LC3B puncta as prognostic and potentially as predictive biomarkers in osteosarcoma. This would justify the addition of HSP27 targeted therapies or agents that promote autophagy to standard chemotherapy in poor risk patients.

V. Conclusions

While previously thought to be primarily a mechanism of chemoresistance, chemotherapy-induced autophagy can serve a dual role in osteosarcoma. Autophagy following standard chemotherapy as evidenced by the presence of LCB puncta in osteosarcoma tumor cells is associated with favorable outcomes. Conversely, overexpression of HSP27 is a negative prognostic marker prior to treatment or following preoperative chemotherapy. When evaluated together, the combination of LC3B-/HSP27+ identifies particularly poor risk patients following preoperative chemotherapy. These findings not only establish HSP27 and LC3B as prognostic biomarkers in osteosarcoma but serve as a rationale for future studies targeting either HSP27 or modulating autophagy in osteosarcoma treatment.

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Vita

John Andrew “Andy” Livingston was born in Arlington, Texas on December, 31, 1983, the son of James Loyd Livingston Jr. and Zo Lynn Livingston. After graduating from Smoky Hill High School in Aurora, Colorado, he enrolled at the University of Colorado in Boulder, Colorado. He graduated with a Bachelor of Arts with distinction in biochemistry with a minor in religious studies from CU in 2005. Andy enrolled at the University of Texas Medical Branch in Galveston, Texas in 2005 to study medicine. He completed his studies and earned his Doctorate of Medicine with High Honors in 2009. Following medical school, he went on to post-graduate training at Duke University where he completed a combined internship and residency in internal medicine and pediatrics. In 2013, he was accepted into the Hematology and Medical Oncology Fellowship Program at the University of Texas MD Anderson Cancer Center. He served as Chief Fellow for the program and completed his fellowship training in 2016. He began his work in the University of Texas Graduate School of Biomedical Sciences in 2014 under the mentorship of Eugenie Kleinerman, MD. He is currently an assistant professor in the Department of Sarcoma Medical Oncology and Department of Pediatrics at MD Anderson Cancer Center.