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Sharjeel Mehdi Hooda   Date

Phosphorylated Mcl-1 Expression on the Prognosis of Head and Neck Cancer

By

Sharjeel Mehdi Hooda
Master of Science

Clinical Research

_____
Dong Moon Shin, M.D.
Advisor

_____
Amita K. Manatunga, Ph.D.
Committee Member

_____
Muna Qayed, M.D., M.Sc.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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By

Sharjeel Mehdi Hooda
B.S., Georgia Institute of Technology, 2006

Advisor: Dong Moon Shin, M.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in Clinical Research
2012

ABSTRACT

Phosphorylated Mcl-1 Expression on the Prognosis of Head and Neck Cancer
By Sharjeel Mehdi Hooda

Background: Head and neck cancer (HNC) accounts for an estimated 350,000 deaths
internationally each year. Mcl-1 is an anti-apoptotic Bcl-2 family protein and recent
studies have shown that phosphorylating Mcl-1 can enhance cancer cell survival
activity. However, the effect of phosphorylated Mcl-1 (pMcl-1) has not yet been studied
on clinical outcome variables for HNC.

Methods: A pilot, retrospective, cohort study was conducted with 49 patients that
presented to Emory University with HNC between 2002 and 2007. Selection criteria
included subjects who presented with Stage III, IVa, or IVb disease and underwent
surgical excision prior to radiation or chemotherapy. Protein expression levels of pMcl-1
and total Mcl-1 were evaluated via immunohistochemical staining of tumor tissue.
Expression levels, scored by a blinded pathologist, were categorized as either absent, low
(below median) or high (above median) and were correlated with disease free survival
(DFS) and overall survival (OS). Data analysis was completed with Kaplan Meier
survival analysis and Cox modeling.

Results: The median duration of follow up was 3.63 years. Tumors with undetectable
pMcl-1 levels correlated with worse DFS (p=0.0490) and OS (p=0.0162). Survival
curves were significantly different between subjects with high vs. low vs. absent
expression for OS (p=0.0368), but not for DFS (p=0.0765). Multivariate analysis for
DFS revealed a hazard ratio (HR) of 0.127 (p=0.0136) for high pMcl-1 versus absent
pMcl-1 expression and a HR of 0.232 (p=0.0276) for low pMcl-1 versus absent pMcl-1
expression.

Conclusions: Increased pMcl-1 expression was associated with improved DFS and OS.
This result was counter to the initial hypothesis and most likely related to antibody
binding selectively at the serine 159 site on Mcl-1. This suggests that upregulating Mcl-1
phosphorylation at serine 159 may improve survival for HNC patients.

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INTRODUCTION

The overall 5-year survival rates for head and neck cancer (HNC) have not significantly improved in the last 30 years and patients with HNC continue to have a poor overall prognosis. The vast majority of head and neck carcinomas are histologically of squamous cell origin; however, there are a limited number of biological factors identified that influence prognosis and treatment options for this subtype, squamous cell carcinoma of the head and neck (SCCHN). The B-cell lymphoma 2 (Bcl-2) family is a set of regulatory proteins that has been shown to have a potential effect on survival outcomes. Myeloid cell leukemia 1 (Mcl-1), an anti-apoptotic protein within the Bcl-2 family, has been noted to have a possible impact on prognosis with recent studies revealing that disease free survival (DFS) is worsened with increased expression.

While studies have shown that HNC tumors associated with a poorer prognosis have overexpression of certain Bcl-2 family biomarkers, such as Mcl-1, there has not been an investigation into whether or not these proteins are overexpressed in an activated state within tumor tissues. Protein activation primarily occurs through enzyme mediated phosphorylation, which can then affect protein activity. Moreover, the relationship between phosphorylated Mcl-1 (pMcl-1) and clinical outcomes has yet to be established.

This pilot, retrospective cohort study evaluated the role of phosphorylated Mcl-1 expression on the prognosis of head and neck cancer through measurements of disease free survival and overall survival (OS). It was hypothesized that increased pMcl-1 expression would be associated with worse DFS and OS. Protein expression levels were measured through the use of immunohistochemical staining on slides that were cut from a tissue microarray (TMA) block of SCCHN tumor samples. The effect of phosphorylated

Mcl-1 expression on these survival measurements was evaluated through both univariate and multivariate analyses, which allowed for the detection of any independent effect of phosphorylated Mcl-1 expression on HNC related disease free survival and overall survival.

BACKGROUND

Burden of Head and Neck Cancer

The impact of head and neck cancer continues to escalate annually in both the United States and worldwide. In the United States, approximately 52,610 individuals are diagnosed with and 11,500 deaths are attributed to the disease annually (1). The burden of HNC is even greater internationally, with an incidence rate above 650,000 and an annual mortality rate of 350,000 (2). Unfortunately, there has not been a significant improvement in survival rates for over 30 years. Furthermore, patients with head and neck cancer continue to have a poorer prognosis in comparison to most cancers despite aggressive treatments combining chemotherapy, radiation, and surgery.

Head and neck cancer consists of tumors that originate within the mucosa of the paranasal sinuses, nasopharynx, oral cavity, oropharynx, hypopharynx, or larynx. Over ninety percent of these tumors are of squamous cell origin, with that subtype of head and neck cancer being classified as squamous cell carcinoma of the head and neck. Over 60% of newly diagnosed SCCHN patients will present with advanced disease, stage III or IV. In addition, the 5-year survival rate for SCCHN is less than 50% (3). Similar to the general status of head and neck cancer, advanced SCCHN has seen no significant improvement in survival rates over the last three decades. Even today, 30-50% of patients will develop local or regional recurrence while 20-30% will progress to distant metastases (4). Furthermore, these recurrences occur early after initial treatment, with 80% of advanced SCCHN patients developing locoregional recurrences within the first two years (5).

Current Head and Neck Cancer Prognostic Indicators

While treatment efficacies are limited for head and neck cancer, there are certain patient/tumor characteristics and molecular factors that have been identified for prognostic value. In terms of patient characteristics, advancing age (often defined as a dichotomy variable separated at age 60), worsened performance status, and tobacco or alcohol use have been linked to poor prognosis (6). In addition, there are several tumor related factors that affect prognosis: TNM staging, tumor differentiation status, and location of primary tumor (6-7). Finally, a few molecular markers have been linked to prognosis, including EGFR copy number, the presence of a p53 mutation, HPV status, and p16 tumor expression (8-11). Nevertheless, there is significant difficulty in clearly prognosticating survival and the identified molecular markers have produced limited therapeutic targets for improving survival outcomes.

The Role of Myeloid Cell Leukemia 1 in Head and Neck Cancer

An additional set of biomarkers, the Bcl-2 family, is currently being evaluated for prognostic value in the management of head and neck cancer. This family of proteins is composed of members that play a key role in regulating both cell cycle progression and apoptosis, or programmed cell death. Certain members of the Bcl-2 family have been shown to have a pro-apoptotic effect, while others have an anti-apoptotic effect. As prevention of apoptosis is often upregulated in carcinomatous cells, the anti-apoptotic members of the Bcl-2 family have been studied in various cancer types to determine if there is an association between protein expression levels and clinical patient outcomes. Mcl-1, an anti-apoptotic member of this family, has now come under evaluation for both prognostic and therapeutic significance. First, Mcl-1 has been shown to be overexpressed

in malignant and premalignant oral tissue when compared to normal oral tissue (12). Next, Mcl-1 was identified as a prognostic variable for HNC when a study of oral squamous cell carcinoma patients revealed that low Mcl-1 expression was associated with improved disease free survival (13). Although this prognostic link has now been initially described, there have been limited studies of Mcl-1 in general as more focus has been placed on the potential for other Bcl-2 family members, such as Bcl-X_L or the Bcl-2 protein.

Although Mcl-1 expression has been connected to survival outcomes, a deeper analysis of Mcl-1 activity on prognosis for HNC has not been conducted. After protein translation, Mcl-1 can undergo four different modifications that have been shown to have an affect on cell survival activity, with the majority promoting cell survival. First, Pim and c-Myc can mediate amplification of Mcl-1 causing increased cellular stability. Second, JNK and CDKs can produce phosphorylation (activation) of Mcl-1 at serine 64 promoting cell survival. Third, ERK mediated phosphorylation of Mcl-1 at either threonine 92 or threonine 163 can also increase cellular stability. In contrast, the only modification that can promote cellular death is GSK-3B mediated phosphorylation at serine 159, which causes faster degradation of the Mcl-1 protein (14).

Several of the modifications related to Mcl-1 activity are associated with protein phosphorylation at various sites, such as threonine 163 for increased cell survival activity and serine 159 for decreased cell survival activity. In relation to oncology applications, *in vitro* studies have revealed that phosphorylation of Mcl-1 can enhance lung cancer cell survival activity (15). Furthermore, a link between smoking and phosphorylated Mcl-1 expression has been indicated as this *in vitro* study has shown that nicotine can promote

threonine 163 based phosphorylation of Mcl-1 causing the observed changes of increased

cancer cell survival activity (15). Although *in vitro* studies have not been conducted on

head and neck carcinoma cells, the prognostic significance of cigarette smoking has been

identified for both lung cancer and HNC. Thus, it is suggestive that smoking-mediated

phosphorylation of Mcl-1 may also play a role in head and neck cancer. Nevertheless,

the significance of phosphorylated Mcl-1 activity has not been evaluated for HNC

associated survival, either as an independent causal factor or an intermediate component

within a cigarette smoking causal pathway. Therefore, this study aimed to addressed the

impact of phosphorylated Mcl-1 expression, as an independent prognostic indicator

separate from both total Mcl-1 expression and previously defined prognostic variables, on

head and neck cancer associated survival.

METHODS

Study Design

This IRB approved study was a pilot, retrospective, cohort study of subjects that presented with squamous cell carcinoma of the head and neck, the major subtype of head and neck cancer, to the Winship Cancer Institute of Emory University prior to 2008. At least three years of follow-up was expected, unless subjects were lost to follow-up or were identified as having an event. The study was completed by obtaining tumor tissue for eligible subjects that met entry criteria and evaluating clinical data that corresponded to those individuals that had available tumor tissue. Clinical data corresponding to tumor tissue samples was obtained by accessing Emory University Healthcare's Cerner-based electronic medical record and the Social Security Death Index.

Characteristics of Study Population

The study inclusion criteria were the following: subjects must have been newly diagnosed with SCCHN; tumor tissue must have been surgically excised and was available at the Emory University Hospital pathology tissue bank; no systemic chemotherapy or radiation treatment to the area of the tumor was conducted prior to tumor tissue collection; subjects presented with Stage III, IVa, or IVb squamous cell carcinoma of the head and neck; and tumor tissue collection must have been conducted prior to 2008.

Tumor tissue collection was required to be at least prior to 2008 in order to have at least 3 years of follow-up for DFS, the primary outcome variable. Individuals that presented with Stage I or II disease were not included as event data would be limited in this subpopulation. Stage IVc was also not included as event data would be severely

skewed for a 3-year follow-up period. Tissue collection was required prior to
chemotherapy or radiation to prevent any effect of these treatment modalities on protein
expression levels in acquired tumor tissue. Eligible participants were selected based
upon meeting inclusion criteria and potential subjects were reviewed based upon year of
diagnosis starting with the most recent year, 2007, and moving backwards in time. This
process was selected in an attempt to have access to medical records and limit variability
in treatment management that could have occurred between various years.

Sample Size Calculations

As a pilot study, study population recruitment was not calculated to a particular
sample size goal. Instead, recruitment was limited based upon available tumor tissue
from the Emory University Hospital pathology tissue bank for subjects that met all
inclusion criteria. As such, a total of 49 subjects were included in the study. Power
calculations were completed in PASS 11 based upon this sample size and showed that the
study had 62% power to detect a difference of 0.40 (or hazard ratio of 0.30) when using a
two-sided log-rank with 49 subjects equally divided between groups (with 30% loss to
follow-up) and a 5% significance level. A detectable difference of 0.40 was used as it
was expected that a 40% difference between groups, assumed 20% in opposite directions
from the median, would be a significant reportable effect.

Outcome Variables

The primary outcome variable was disease free survival. An event for this
variable was defined as either relapse, or recurrence, of cancer or death from any cause.
Survival time was measured from the time of diagnosis, which was defined as time of
surgical excision and tumor sample collection, to the day of relapse or progression, day of

death in remission, or the last follow-up date for patients in remission. Event data was obtained through electronic medical record based chart reviews and a search of the Social Security Death Index.

The secondary outcome variable was overall survival. An event for this variable was defined as death from any cause. Survival time was measured from the time of diagnosis to the day of death or the last day of interaction noted by a healthcare worker on the subject's medical chart. This final interaction could include either in-person contact or verbal communication via telephone. Event data was obtained by reviewing both the electronic medical record and the Social Security Death Index.

Predictor Variables

The exposure variables were the protein expression levels for the biomarkers of interest, phosphorylated Mcl-1 and total Mcl-1. The protein expression levels were defined as categorical variables, whereby there were two categories for each biomarker: above and below the median. If there were any cases within the cohort that had no expression of a biomarker, then the expression levels would be categorized into three components: no expression, below the median level for positive expressing samples, and above the median level for positive expressing samples. The primary predictor variable for this study was the phosphorylated Mcl-1.

Additional variables evaluated in both univariate and multivariate analyses included age (categorized as either above or below 60), gender, smoking status, cancer stage, tumor differentiation, tumor classification (size), and nodal status. Interaction terms involving phosphorylated Mcl-1 and smoking status were evaluated for

multivariate analysis to determine if a relationship existed that correlated to *in vitro* findings of nicotine mediated phosphorylation of Mcl-1.

Tissue Microarrays

For each subject who met inclusion criteria, tumor tissue was evaluated by a blinded pathologist for identification of three areas that would be used as cores in tissue microarrays. The TMAs were assembled by taking core needle biopsies of 1.2 mm in diameter from individual tumor tissue paraffin blocks and constructing paraffin blocks that included several tumor samples. Two microarrays in total were constructed and sections of these blocks were mounted to slides for immunohistochemical staining. The use of tissue microarrays allowed for biomarker evaluation of several tumors simultaneously under identical conditions.

Immunohistochemistry

The constructed TMAs were evaluated for protein expression levels through immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded TMA sections were deparaffinized by heating and treatment with xylenes. They were then rehydrated through graded ethanols and microwaved in 100 mmol/L sodium citrate for 10 minutes at low power for antigen retrieval. The slides were incubated with Mcl-1 polyclonal antibody (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) or Phospho-Mcl-1 polyclonal antibody (1:50 dilution) (Cell Signaling Technology, Danvers, MA) overnight at 4°C. For total Mcl-1 detection, the staining of the antibody was observable by diaminobenzidine tetrahydrochloride peroxidase substrate solution (Vector Laboratories, Burlingame, CA). Cell nuclei were counterstained by haematoxylin QS (Vector Laboratories). Due to limitations in recognition of phosphorylated protein expression

through standard citrate based retrieval methods, detection of phosphorylated Mcl-1 was performed with the DAKO Visualization System instructions using 3,3-diaminobenzidine tetrahydrochloride substrate to visualize the protein (DAKO, Carpinteria, CA).

Immunohistochemical staining was scored by a blinded pathologist, who evaluated both percentage of positive staining in tumor cells and intensity of staining. A numerical scale (0 = no expression, 1 = weak expression, 2 = moderate expression, and 3 = strong expression) was used to measure the intensity of IHC staining. A weighted score was calculated for each core sample placed on the TMA, whereby the weighted score = % positive stain in tumor x intensity score. A subject's recorded weighted score was calculated as the average of the three tumor core biopsies placed on the TMA.

Statistical Analysis

The null hypothesis is that there is no difference in disease free survival for subjects with phosphorylated Mcl-1 tumor tissue expression levels below the cohort median in comparison to those subjects with phosphorylated Mcl-1 tumor tissue expression levels above the cohort median. Kaplan Meier survival analysis was used to estimate the survival function for both disease free survival and overall survival, and the log-rank test was used for univariate analysis to compare survival functions for categorical variables, including higher versus lower biomarker expression levels. Univariate analysis was also completed on known prognostic indicators that were included as predictor variables. A Cox proportional hazards model was used to evaluate the main exposure, phosphorylated Mcl-1 expression level, in light of the other covariates to determine if there was an independent hazard associated with the main exposure. For variables that did not meet the proportional hazards assumption, a stratified and time-

extended Cox model containing a heavyside function was developed for multivariate analysis. Interaction between the main exposure and other covariates was also evaluated in this multivariate Cox model. The main exposure, phosphorylated Mcl-1 expression level, was evaluated as both a continuous and categorical variable for all analyses. Categorical analysis was completed by evaluating quartiles and separation at the median expression level. Median expression level was the primary cutpoint used for determining conclusive findings as that is standard practice for novel oncologic biomarker evaluation; however, conclusions derived from this study were based upon evaluation of all implemented continuous and categorical based variable analysis methods. SAS 9.3 was used for all statistical analysis and statistical significance was set at an alpha of 0.05.

RESULTS

Baseline Characteristics of Study Population

49 subjects were included in the study and a total of 147 specimens were analyzed

over two tissue microarrays. Immunohistochemical staining was scored for each subject

by a blinded pathologist (Figure 1). The entire cohort, including subjects that did not

have events, had a median DFS of 1.03 years and a median OS of 1.96 years. Baseline

characteristics of study participants are reported in Table 1. In comparison of study

subjects with low and high phosphorylated Mcl-1 expression levels, in which the

dichotomy was set at the median pMcl-1 expression level, there was no significant

difference between groups based upon age, gender, clinical stage at presentation, tumor

differentiation, duration of follow-up, smoking status, site of primary disease, or total

Mcl-1 expression level. Phosphorylated Mcl-1 was noted to have low detection with a

median weighted score of 1.33 (range 0-180).

Univariate Analysis

The exposure variables (pMcl-1 and total Mcl-1) along with several covariates

known to be of prognostic significance were included in univariate analysis. Kaplan

Meier survival analysis was used to determine the association between these variables

and outcome variables, disease free survival and overall survival. Of the covariates

analyzed, tumor classification was the only statistically significant predictor (p=0.0120)

for disease free survival (Figure 2). Node status, gender, age (dichotomized at 60 years),

tumor differentiation, and smoking status were all non-significant predictors for DFS

(Figures 3-7).

The exposure variable total Mcl-1 (with groups dichotomized by the median expression level) was not a significant predictor for either disease free survival (p=0.6527) or overall survival (p=0.5945) (Figures 8-9). A significant proportion (11/49, 22%) of subjects had no phosphorylated Mcl-1 expression detectable in tumor tissue. Kaplan Meier analysis comparing those cases with no expression versus any expression revealed a statistically significant difference in survival for both DFS (p=0.0490) and OS (p=0.0162) (Figures 10-11). A three-way comparison of pMcl-1 for subjects with no expression, below median expression, and above median expression showed a significant difference in OS (p=0.0368) but not in DFS (p=0.0765) (Figures 12-13). Kaplan Meier analysis comparing pMcl-1 expression based upon a quartile categorization method revealed no significant differences between quartiles for either OS (p=0.0921) or DFS (p=0.1328); however, an observable trend was present on the survival curves in which increasing quartile was related to increased survival (Figures 14-15).

Multivariate Analysis

A Cox model was developed for multivariate analysis to determine the effect of phosphorylated Mcl-1 on DFS and OS when adjusted for known prognostic indicators. The proportional hazards assumption was met for tumor classification and phosphorylated Mcl-1 status (categorized as no expression, below median expression, and above median expression). The proportional hazards assumption was not met for the following known prognostic indicators: age (dichotomized at 60 years), tumor differentiation, clinical stage, and smoking status. A heavyside function was created for smoking status to evaluate the effect of smoking at time of surgical excision on the first 6 months of DFS and OS and the effect of smoking at time of excision on DFS and OS

beyond the first 6 months after diagnosis. A model containing interaction terms between phosphorylated Mcl-1 and smoking status showed no significant interaction. The final model was a time extended model stratified by age, tumor differentiation, and clinical stage containing the following predictors: phosphorylated Mcl-1, tumor classification, and a heavyside function for smoking status. This final model showed that both below median phosphorylated Mcl-1 expression (HR: 0.232, p=0.0276) and above median phosphorylated Mcl-1 expression (HR: 0.127, p=0.0136) were significant predictors for DFS when compared to absent phosphorylated Mcl-1 expression (Table 2). In regards to OS, the final model revealed similar results for both below median phosphorylated Mcl-1 expression (HR: 0.106, p=0.0263) and above median phosphorylated Mcl-1 expression (HR: 0.196, p=0.0285) when compared to absent phosphorylated Mcl-1 expression (Table 3).

Cox models were also developed for multivariate analyses to determine the effect of phosphorylated Mcl-1, as either a quartile based categorical variable or a continuous variable, on both disease free survival and overall survival. The proportional hazards assumption held same for the covariates and stratified variables included in prior cox models. The proportional hazards assumption was met for phosphorylated Mcl-1 as both a quartile-based categorical variable and a continuous variable (tested with multiple time-dependent interaction terms). A model containing interaction terms between phosphorylated Mcl-1 and smoking status showed no significant interaction. The final model for both of these methods of evaluating phosphorylated Mcl-1 was a time extended model stratified by age, tumor differentiation, and clinical stage containing the following predictors: phosphorylated Mcl-1, tumor classification, and a heavyside function for

smoking status. For the quartile based categorization of phosphorylated Mcl-1, quartile 1 (expression level below the 25th percentile) was used as the baseline quartile and quartiles 2, 3, and 4 were compared to the effect of quartile 1. This final model for a quartile based categorization of phosphorylated Mcl-1 showed that quartile 4 phosphorylated Mcl-1 expression (HR: 0.155, p=0.0424) and quartile 3 phosphorylated Mcl-1 expression (HR: 0.171, p=0.0230) were significant predictors for DFS when compared to quartile 1 phosphorylated Mcl-1 expression (Table 4). For OS, the final model revealed that quartile 3 phosphorylated expression (HR: 0.107) was a significant predictor for OS when compared to quartile 1 phosphorylated Mcl-1 expression (Table 5). For the cox model in which phosphorylated Mcl-1 expression was a continuous variable, the effect of phosphorylated Mcl-1 expression was not significant for either DFS (HR: 0.988, p=0.0552) or OS (HR: 0.990, p=0.2596) (Tables 6-7).

DISCUSSION

This study evaluated the effect of phosphorylated Mcl-1 on the prognosis of head and neck cancer, as measured by disease free survival and overall survival. Using tissue microarrays and immunohistochemistry, this study demonstrated that total Mcl-1 expression did not predict DFS and OS, but that phosphorylated Mcl-1 expression was a significant predictor for both DFS and OS. This was best seen when comparing phosphorylated Mcl-1 expression levels that were categorized by a cutpoint defined at the median expression level. This cutpoint was the primary method used for conclusive evaluation as that is standard practice for immunohistochemical-based evaluation of novel biomarkers in oncology. Nevertheless, univariate and multivariate analyses comparing pMcl-1 expression quartiles showed a trend that corroborated the effect seen based upon the standard median expression level cutpoint. This trend was less observable when phosphorylated Mcl-1 was included as a continuous variable, most likely due to the fact that significant clustering occurred for both low and absent expression.

Mcl-1, an anti-apoptotic member of the Bcl-2 family, was expected to be associated with both DFS and OS as a poor prognostic indicator for those subjects with higher total Mcl-1 expression. A statistically significant result correlating with that prediction was not seen in the data; however, a trend in that direction was observable in univariate analyses for overall survival. Phosphorylated Mcl-1 expression was similarly expected to be a poor prognostic indicator for both DFS and OS. In contrast, the effect of phosphorylated Mcl-1 was not in the anticipated direction for the outcome variables. Increased phosphorylated Mcl-1 expression in tumor tissue was a statistically significant

protective predictor for both DFS and OS in both univariate analysis and multivariate modeling adjusted for potential confounders.

Study Strengths and Limitations

There were several strengths in the conduction of this study to identify the effect of phosphorylated Mcl-1 on the prognosis of head and neck cancer. First, the utilization of TMAs allowed for evaluation of several tumor tissue specimens simultaneously under identical conditions. This limited excessive measurement variation in laboratory techniques. In addition, the tissue microarrays were placed with three cores from each tumor, which decreased the inability of TMAs to capture adequate tumor tissue for successful IHC based detection. The study was also well designed to understand the effect of phosphorylated Mcl-1 in relation to total Mcl-1 along with adjustment for known prognostic indicators, such as clinical stage and smoking status. Even though the study was limited to 49 subjects, a total of six predictors were successfully modeled when completing multivariate analyses to determine the effect of phosphorylated Mcl-1. Lastly, the use of several statistical techniques, including extended Cox models, allowed for improved understanding of the effect of phosphorylated Mcl-1 on head and neck cancer prognosis.

Although the study contained several strengths, there were clear limitations also present. The most significant limitation was the small sample size of this study, which resulted in low power to detect some statistically significant differences. Another limitation was the inability to include all known prognostic indicators for head and neck cancer survival in this study. Limited resources prevented the study from including molecular markers of interest, such as p16, and limited clinical data prevented inclusion

of clinical predictors, such as smokeless tobacco status at time of diagnosis. The study also was limited to only one blinded pathologist scoring IHC staining for biomarker detection, which could cause biased results. Finally, detection of phosphorylated Mcl-1 was significantly limited in this study. As detailed earlier, the median pMcl-1 expression level was only 1.33, whereas the normal range for scoring extends from 0 to 300. This overall low expression was due to difficulties in IHC for detecting phosphorylated biomarkers.

Hypothesis Generation

Overall, this study generated several hypotheses as to why phosphorylated Mcl-1 expression was a protective factor for both disease free survival and overall survival. The most likely explanation of these findings relates to the antibody used in immunohistochemistry to detect phosphorylated Mcl-1 expression. The Cell Signaling Technology antibody for phospho-Mcl-1 is formulated to detect phosphorylation at both the serine 159 and threonine 163 binding sites. *In vitro* studies that previously evaluated the effect of phosphorylated Mcl-1 were based upon solely the threonine 163 binding site through the creation of threonine 163 Mcl-1 mutant cell lines. It was through this pathway of phosphorylation at threonine 163 that increased cellular stability and increased cancer cell survival activity was noted with increased pMcl-1 expression (15). In contrast, phosphorylation of Mcl-1 at serine 159 has been noted to cause accelerated degradation of the Mcl-1 protein and decreased cell survival activity (14). Given that the results of this study indicated improved outcomes for those with higher levels of pMcl-1 expression, it is hypothesized that the Cell Signaling Technology phospho-Mcl-1 antibody was selectively detecting phosphorylation at the serine 159 binding site. If

binding was selective for this site, then subjects with higher expression of phosphorylated Mcl-1 would be those individuals whose tumor cells had lower cell survival activity and accordingly improved DFS and OS. Unfortunately, there has been limited overall evaluation of the serine 159 site on Mcl-1 and no evaluation of serine 159 based phosphorylation of Mcl-1 for head and neck cancer. Nevertheless, it has previously been identified that phosphorylation at serine 159 does in fact promote cellular degradation: this concept was solidified through a comparison of wild-type Mcl-1 protein with a newly developed mutant protein where alanine was substituted for serine 159, which prevented correct Mcl-1 phosphorylation and resulted in increased cell survival (16). Furthermore, there is low likelihood that the antibody in this HNC pMcl-1 study primarily detected phosphorylation of Mcl-1 at threonine 163 because there was no interaction present between pMcl-1 expression and smoking status. A relationship between these two measured variables would be expected for threonine 163 based phosphorylation of Mcl-1 given the previously described nicotine mediated enhancement of Mcl-1 phosphorylation at threonine 163 (15).

Another potential explanation for the protective effect of phosphorylated Mcl-1 is via a mutually exclusive Bcl-2 family environment. It has previously been hypothesized that the Bcl-2 protein, another anti-apoptotic member of the family, may predict improved survival outcomes with higher expression levels because tumors without Bcl-2 expression are more likely to have up-regulation of another Bcl-2 family anti-apoptotic pathway, such as Bcl-X_L (17). Therefore, another hypothesis generated from this study is that tumors with absent or lower expression of phosphorylated Mcl-1 may up-regulate

expression of another Bcl-2 family anti-apoptotic pathway that are associated with worse outcomes.

A third explanation for the protective effect seen in this study of pMcl-1 could be due to limitations in complete understanding of the molecular pathway. As noted earlier, there are molecular markers shown to have prognostic implications on survival outcomes for head and neck cancer; however, limited resources prevented those biomarkers (p16, EGFR copy number) from being evaluated in this study for confounding effects. It is possible that the observed effect of phosphorylated Mcl-1 on DFS and OS could be explained if pMcl-1 is acting as a surrogate for the effect of another significant biomarker for head and neck cancer prognosis.

Future Direction

This study revealed a statistically significant protective impact of phosphorylated Mcl-1 expression on head and neck cancer survival and generated several hypotheses to describe this observed effect. To better understand the role of phosphorylated Mcl-1 on the prognosis of head and neck cancer, it will be important to evaluate these three newly developed, data-driven hypotheses that attempt to explain the effect of pMcl-1 expression on HNC clinical outcomes.

There are at least two methods available in order to confirm the hypothesis that increased pMcl-1 expression was protective because of Cell Signaling Technologies' phosphorylated Mcl-1 antibody selectively binding at the serine 159 site. First, a site specific antibody, such as Abcam's serine 159 anti-phosphorylated Mcl-1 antibody, could be utilized for clarifying whether or not this study's results were related to antibody binding specificity. This methodology would allow for tumor tissues from this study or a

new set of tumor samples to be examined solely for serine 159 based pMcl-1 expression.

The resulting scored expression level would then be correlated to prognostic outcomes to

determine if this hypothesis explains the study results and further supports the protective

effect of increased pMcl-1 expression when evaluating phosphorylation at serine 159.

Unfortunately, there is only one commercially available serine 159 anti-phosphorylated

Mcl-1 antibody, manufactured by Abcam, and it has not yet been evaluated in any

oncology applications. Therefore, the Abcam site specific antibody would have to be

thoroughly tested and validated before reporting conclusive findings.

Another potential method of evaluating the role of site specific binding for the

Cell Signaling Technology phosphorylated Mcl-1 antibody would be to utilize an

established head and neck squamous cell carcinoma cell line so that a mutagenic cell line

could be developed in which phosphorylation at serine 159 is prevented. This process

would be completed by creating a serine 159 mutant where serine is replaced with

another amino acid, such as alanine. This would be followed by western blot analysis in

which the wild type cell line would be compared to the mutant cell line to evaluate

detection of phosphorylated Mcl-1 expression. The benefit of this methodology would be

the ability to use the previously tested and validated Cell Signaling Technology anti-

phosphorylated Mcl-1 antibody. Western blot analysis could potentially reveal inhibited

phosphorylated Mcl-1 detection for the mutant line compared to the wild-type cell line;

thus, providing increased support that the study results were identifying serine 159 based

Mcl-1 expression.

In order to evaluate the two remaining hypotheses that could potentially explain

the protective effect of phosphorylated Mcl-1 expression on head and neck cancer

survival, a larger prospective cohort study should be conducted. This study would allow for improved adjusted analysis of pMcl-1's effect on survival, in which previously identified and accepted biomarkers (such as p16 or EGFR copy number) could be included. Furthermore, sufficient tumor sample collection during surgical excision could also allow for the evaluation of both well established and less established Bcl-2 family biomarkers. This assessment would address any concerns about mutually exclusive Bcl-2 family pathways that could explain the favorable effect of increased pMcl-1 expression on HNC survival. In addition, a prospective study design would allow for improved detection of phosphorylation based biomarker expression as newly excised tumor tissue specimens could be pretreated with phosphatase inhibitors. These inhibitors would allow for improved IHC-based detection of phosphorylated biomarkers. Lastly, this prospective cohort study would also be designed to have improved power to detect differences for the prognostic effect of predictors, such as total Mcl-1.

Conclusion

Phosphorylated Mcl-1 expression has been identified in this study as a potentially protective prognostic indicator for both DFS and OS in patients diagnosed with advanced head and neck cancer. An analysis of the unanticipated study results has provided an opportunity to better understand the Bcl-2 family of biomarkers by generating several hypotheses explaining the observed effect of pMcl-1 expression. If it is subsequently confirmed that there is a protective effect of Mcl-1 phosphorylation on prognostic variables, then further evaluation of phosphorylated Mcl-1 could lead to potential chemotherapeutic targets for patients diagnosed with head and neck cancer. Furthermore, if an independent, protective effect of phosphorylated Mcl-1 is confirmed for survival,

then the binding site serine 159 on Mcl-1 could be targeted for up-regulation (through either direct or indirect pathways). This targeted therapy could potentially lead to an improvement in head and neck cancer survival, which has yet to significantly improve in over 30 years.

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TABLES/FIGURES

Table 1. Baseline patient characteristics comparing subjects with low phosphorylated Mcl-1 (pMcl-1) expression to those with high pMcl-1 expression in which the two groups were based upon a cutpoint at the median expression level.

	Low pMcl-1 (n=25)	High pMcl-1 (n=24)	P-value*
Age in years: Mean (standard deviation)	57.92 (14.14)	60.08 (11.91)	0.5648[1]
Gender (count):			0.2121[2]
Male	17	20	
Female	8	4	
Stage (count):			0.4962[3]
III	6	4	
IV	18	21	
Tumor Differentiation (count):			0.3418[3]
Well	3	1	
Moderate	17	14	
Poor	3	6	
Non-specific	1	4	
Duration of follow-up in years (median, range):	3.72 (0.09 – 8.93)	3.55 (0.09 – 9.43)	0.7644[4]
Smoking Status (count):			0.6800[2]
Smoker	11	10	
Non-smoker	13	15	
Primary Site (count):			0.3940[3]
Oral Cavity	18	18	
Oropharynx	3	6	
Laryngeal	3	1	
Biomarker Expression Level (median, range):			
Total Mcl-1	112.5 (15 – 289)	100 (10 – 297)	0.8774[1]
Phosphorylated Mcl-1	1.33 (0 – 180)		N/A

*α = 0.05
1 Pooled two-sample t-test
2 Chi-square test
3 Fisher's exact test
4 Wilcoxon rank sum test

Table 2. Extended Cox model for disease free survival with phosphorylated Mcl-1 expression, tumor classification, and a heavyside function for smoking status. The model was stratified by age (dichotomized at age 60), tumor differentiation (classified as either poor, moderately, or well differentiated), and clinical stage (classified as stage III or IV).

	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq*	Hazard Ratio (HR)	95% HR Confidence Limits
pMcl1 High vs. None	-2.0658	0.8375	6.0842	0.0136	0.127	0.025 – 0.654
pMcl1 Low vs. None	-1.4591	0.6625	4.8505	0.0276	0.232	0.063 – 0.852
T_Smoking1	-1.5182	1.1174	1.8460	0.1742	0.219	0.025 – 1.958
T_Smoking2	2.2865	0.9613	5.6580	0.0174	9.840	1.496 – 64.750
T3/T4 vs. T1/T2	1.7698	0.5977	8.7693	0.0031	5.870	1.819 – 18.939

*$\alpha = 0.05$, H_0: hazard ratio = 1

pMcl-1 High = Above median phosphorylated Mcl-1 expression (median defined as only those with positive expression levels)
pMcl-1 Low = Below median phosphorylated Mcl-1 expression (median defined as only those with positive expression levels)

T_smoking1 = The effect of smoking at time of surgical excision (or within the previous year) on disease free survival within the first six months of follow-up after excision. Effect determined with a heavyside function.
T_smoking2 = The effect of smoking at time of surgical excision (or within the previous year) on disease free survival beyond the initial six months of follow-up. Effect determined with a heavyside function.

T3/T4 = Tumor classification status as T3 or T4
T1/T2 = Tumor classification status as T1 or T2

Table 3. Extended Cox model for overall survival with phosphorylated Mcl-1 expression, tumor classification, and a heavyside function for smoking status. The model was stratified by age (dichotomized at age 60), tumor differentiation (classified as either poor, moderately, or well differentiated), and clinical stage (classified as stage III or IV).

	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq*	Hazard Ratio (HR)	95% HR Confidence Limits
pMcl1 High vs. None	-2.2411	1.0234	4.7952	0.0263	0.106	0.014 – 0.790
pMcl1 Low vs. None	-1.6306	0.7341	4.9346	0.0285	0.196	0.046 – 0.825
T_Smoking1	2.2259	1.0008	4.9463	0.0261	9.262	1.302 – 65.857
T_Smoking2	0.5488	0.7041	0.6074	0.4358	1.731	0.435 – 6.882
T3/T4 vs. T1/T2	1.7258	0.6970	6.1317	0.0133	5.617	1.433 – 22.017

*α = 0.05, H_0: hazard ratio = 1

pMcl-1 High = Above median phosphorylated Mcl-1 expression (median defined as only those with positive expression levels)
pMcl-1 Low = Below median phosphorylated Mcl-1 expression (median defined as only those with positive expression levels)

T_smoking1 = The effect of smoking at time of surgical excision (or within the previous year) on overall survival within the first six months of follow-up after excision. Effect determined with a heavyside function.
T_smoking2 = The effect of smoking at time of surgical excision (or within the previous year) on overall survival beyond the initial six months of follow-up. Effect determined with a heavyside function.

T3/T4 = Tumor classification status as T3 or T4
T1/T2 = Tumor classification status as T1 or T2

Table 4. Extended Cox model for disease free survival with phosphorylated Mcl-1 expression (categorized into quartiles), tumor classification, and a heavyside function for smoking status. The model was stratified by age (dichotomized at age 60), tumor differentiation (classified as either poor, moderately, or well differentiated), and clinical stage (classified as stage III or IV).

	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq*	Hazard Ratio (HR)	95% HR Confidence Limits
pMcl1 Quartile4 vs. Quartile1	-1.86247	0.91786	4.1174	0.0424	0.155	0.026 – 0.938
pMcl1 Quartile3 vs. Quartile1	-1.76335	0.77553	5.1699	0.0230	0.171	0.038 – 0.784
pMcl1 Quartile2 vs. Quartile1	-1.26813	0.75427	2.8267	0.0927	0.281	0.064 – 1.234
T_Smoking1	-1.42734	1.15679	1.5225	0.2172	0.240	0.025 – 2.316
T_Smoking2	2.23692	1.04147	4.6132	0.0317	9.364	1.216 – 72.108
T3/T4 vs. T1/T2	1.82823	0.60455	9.1454	0.0025	6.223	1.433 – 22.017

*$\alpha = 0.05$, H_0: hazard ratio = 1

pMcl-1 Quartile4 = Expression level >= 75th percentile
pMcl-1 Quartile3 = 50th percentile <= Expression level < 75th percentile
pMcl-1 Quartile2 = 25th percentile <= Expression level < 50th percentile
pMcl-1 Quartile1 = Expression level < 25th percentile

T_smoking1 = The effect of smoking at time of surgical excision (or within the previous year) on disease free survival within the first six months of follow-up after excision. Effect determined with a heavyside function.
T_smoking2 = The effect of smoking at time of surgical excision (or within the previous year) on disease free survival beyond the initial six months of follow-up. Effect determined with a heavyside function.

T3/T4 = Tumor classification status as T3 or T4
T1/T2 = Tumor classification status as T1 or T2

Table 5. Extended Cox model for overall survival with phosphorylated Mcl-1 expression (categorized into quartiles), tumor classification, and a heavyside function for smoking status. The model was stratified by age (dichotomized at age 60), tumor differentiation (classified as either poor, moderately, or well differentiated), and clinical stage (classified as stage III or IV).

	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq*	Hazard Ratio (HR)	95% HR Confidence Limits
pMcl1 Quartile4 vs. Quartile1	-2.19813	1.23865	3.1493	0.0760	0.111	0.010 – 1.258
pMcl1 Quartile3 vs. Quartile1	-2.23705	0.90066	6.1693	0.0130	0.107	0.018 – 0.624
pMcl1 Quartile2 vs. Quartile1	-1.15594	0.81459	2.0137	0.1559	0.315	0.064 – 1.554
T_Smoking1	2.17446	1.06512	4.1678	0.0412	8.797	1.091 – 70.956
T_Smoking2	0.48586	0.80590	0.3635	0.5466	1.626	0.335 – 7.888
T3/T4 vs. T1/T2	1.90732	0.72896	6.8460	0.0089	6.735	1.614 – 28.108

*$\alpha = 0.05$, H_0: hazard ratio = 1

pMcl-1 Quartile4 = Expression level >= 75th percentile
pMcl-1 Quartile3 = 50th percentile <= Expression level < 75th percentile
pMcl-1 Quartile2 = 25th percentile <= Expression level < 50th percentile
pMcl-1 Quartile1 = Expression level < 25th percentile

T_smoking1 = The effect of smoking at time of surgical excision (or within the previous year) on overall survival within the first six months of follow-up after excision. Effect determined with a heavyside function.
T_smoking2 = The effect of smoking at time of surgical excision (or within the previous year) on overall survival beyond the initial six months of follow-up. Effect determined with a heavyside function.

T3/T4 = Tumor classification status as T3 or T4
T1/T2 = Tumor classification status as T1 or T2

Table 6. Extended Cox model for disease free survival with phosphorylated Mcl-1 expression (as a continuous variable), tumor classification, and a heavyside function for smoking status. The model was stratified by age (dichotomized at age 60), tumor differentiation (classified as either poor, moderately, or well differentiated), and clinical stage (classified as stage III or IV).

	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq*	Hazard Ratio (HR)	95% HR Confidence Limits
pMcl1	-0.01229	0.00641	3.6771	0.0552	0.988	0.975 – 1.000
T_Smoking1	-1.46432	0.96363	2.3091	0.1286	0.231	0.035 – 1.529
T_Smoking2	1.89171	0.94353	4.0197	0.0450	6.631	1.043 – 42.140
T3/T4 vs. T1/T2	1.69044	0.58978	8.2153	0.0042	5.422	1.707 – 17.225

*$\alpha = 0.05$, H_0: hazard ratio = 1

pMcl1 = pMcl1 expression level (scored from 0 – 300)

T_smoking1 = The effect of smoking at time of surgical excision (or within the previous year) on disease free survival within the first six months of follow-up after excision. Effect determined with a heavyside function.
T_smoking2 = The effect of smoking at time of surgical excision (or within the previous year) on disease free survival beyond the initial six months of follow-up. Effect determined with a heavyside function.

T3/T4 = Tumor classification status as T3 or T4
T1/T2 = Tumor classification status as T1 or T2

Table 7. Extended Cox model for overall survival with phosphorylated Mcl-1 expression (as a continuous variable), tumor classification, and a heavyside function for smoking status. The model was stratified by age (dichotomized at age 60), tumor differentiation (classified as either poor, moderately, or well differentiated), and clinical stage (classified as stage III or IV).

	Parameter Estimate	Standard Error	Chi-Square	Pr > ChiSq*	Hazard Ratio (HR)	95% HR Confidence Limits
pMcl1	-0.00963	0.00855	1.2707	0.2596	0.990	0.974 – 1.007
T_Smoking1	1.70015	0.94693	3.2236	0.0726	5.475	0.856 – 35.026
T_Smoking2	0.26094	0.64699	0.1627	0.6867	1.298	0.365 – 4.614
T3/T4 vs. T1/T2	1.13605	0.58022	3.8336	0.0502	3.114	0.999 – 9.711

*$\alpha = 0.05$, H_0: hazard ratio = 1

pMcl1 = pMcl1 expression level (scored from 0 – 300)

T_smoking1 = The effect of smoking at time of surgical excision (or within the previous year) on overall survival within the first six months of follow-up after excision. Effect determined with a heavyside function.
T_smoking2 = The effect of smoking at time of surgical excision (or within the previous year) on overall survival beyond the initial six months of follow-up. Effect determined with a heavyside function.

T3/T4 = Tumor classification status as T3 or T4
T1/T2 = Tumor classification status as T1 or T2

Figure 1. Representative sample of immunohistochemical staining for phosphorylated Mcl-1. Stain intensity is a numerical scale from 0 to 3+, where 0 represents absent biomarker expression and 3+ represents strong expression.
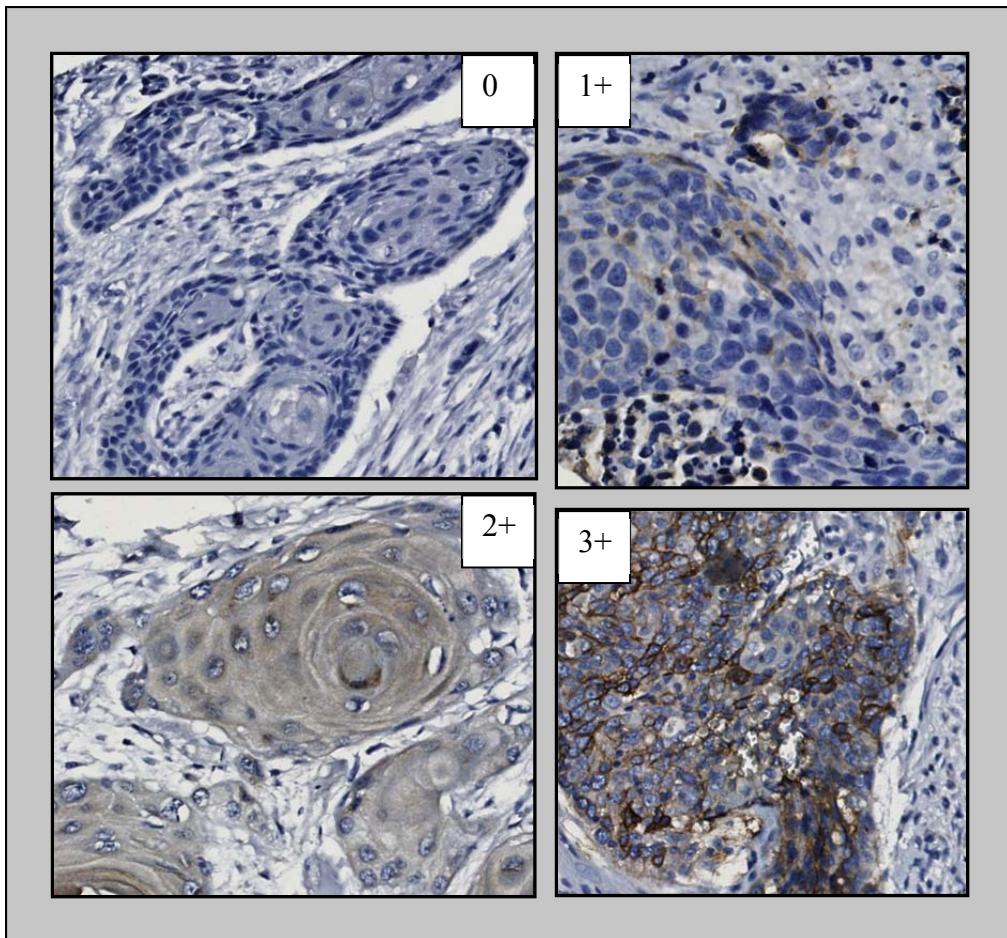
Figure 2. Kaplan Meier survival curves for disease free survival by tumor classification, defined as either T1-T2 or T3-T4.
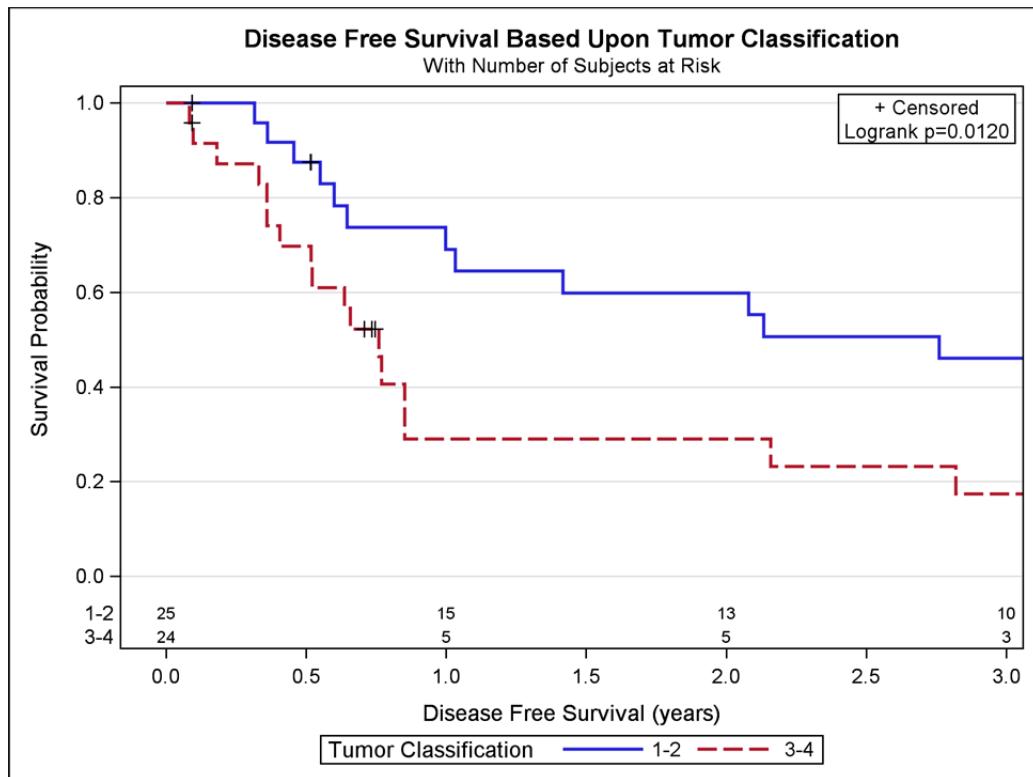
1-2 = T1 and T2
3-4 = T3 and T4
$\alpha = 0.05$

Figure 3. Kaplan Meier survival curves for disease free survival by node classification, defined as either N0 or N1-N3.
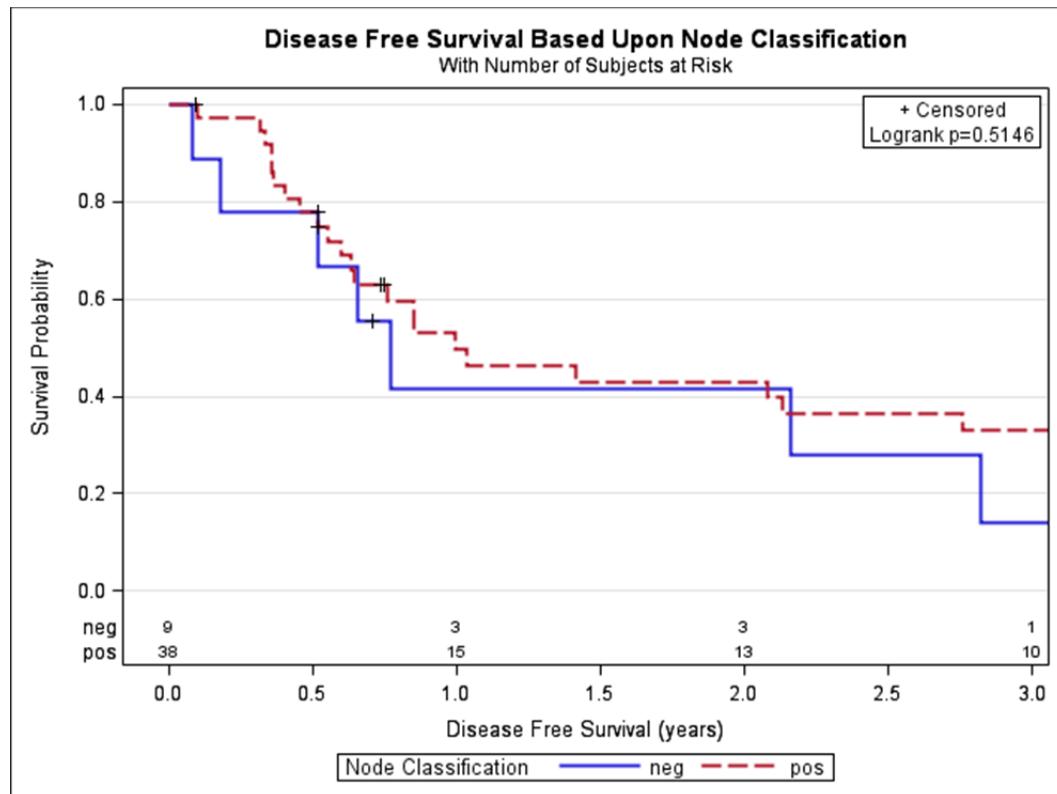
neg = N0
pos = N1, N2, or N3
$\alpha = 0.05$

Figure 4. Kaplan Meier survival curves for disease free survival by gender.
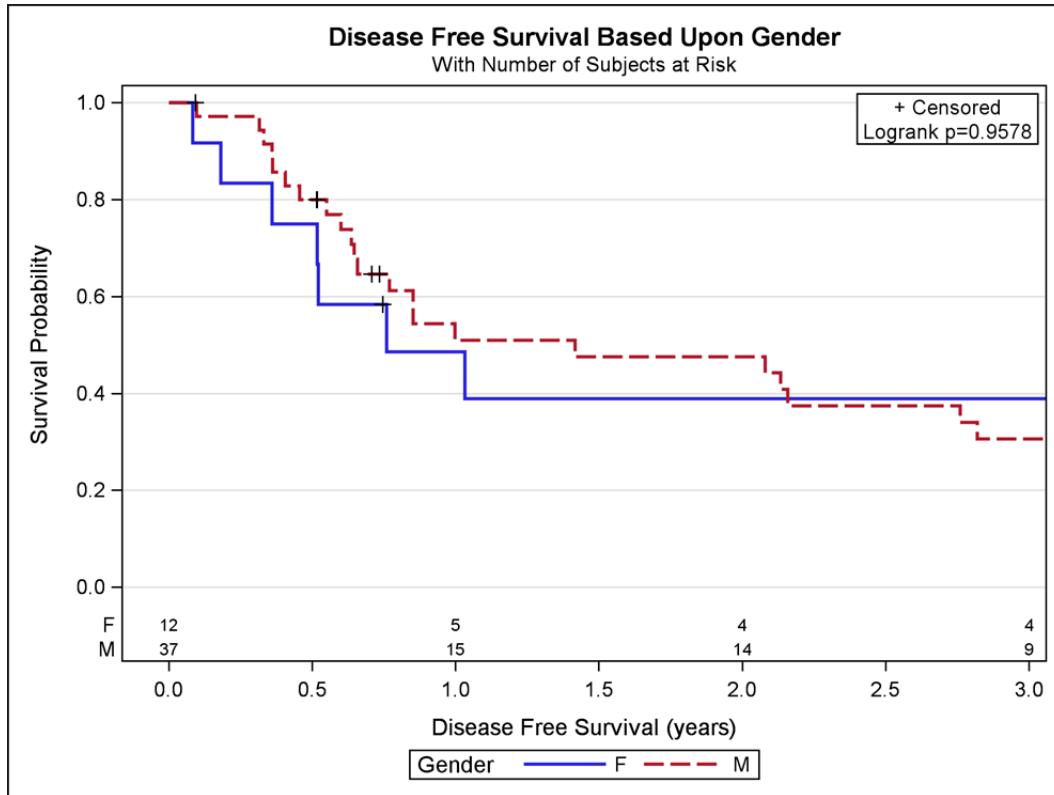
M = Male
F = Female
$\alpha = 0.05$

Figure 5. Kaplan Meier survival curves for disease free survival by age, classified as a dichotomous variable with cutpoint at age 60.
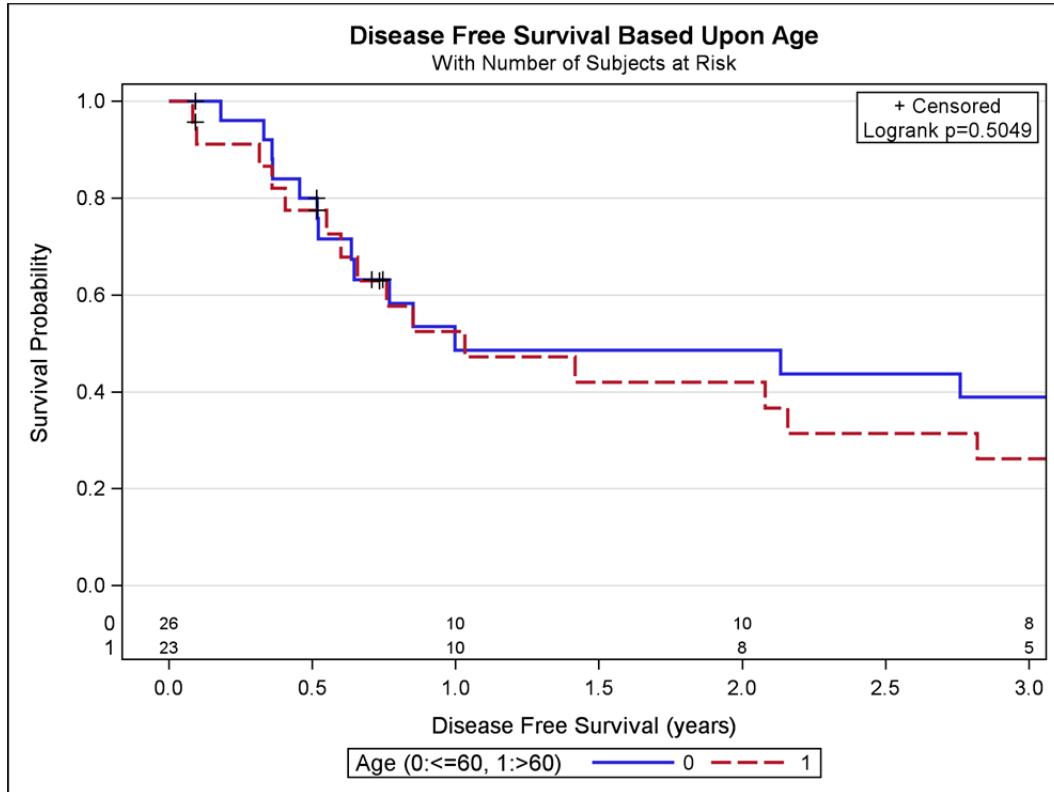
$\alpha = 0.05$

Figure 6. Kaplan Meier survival curves for disease free survival by tumor differentiation, classified as either poor, moderately, or well differentiated.
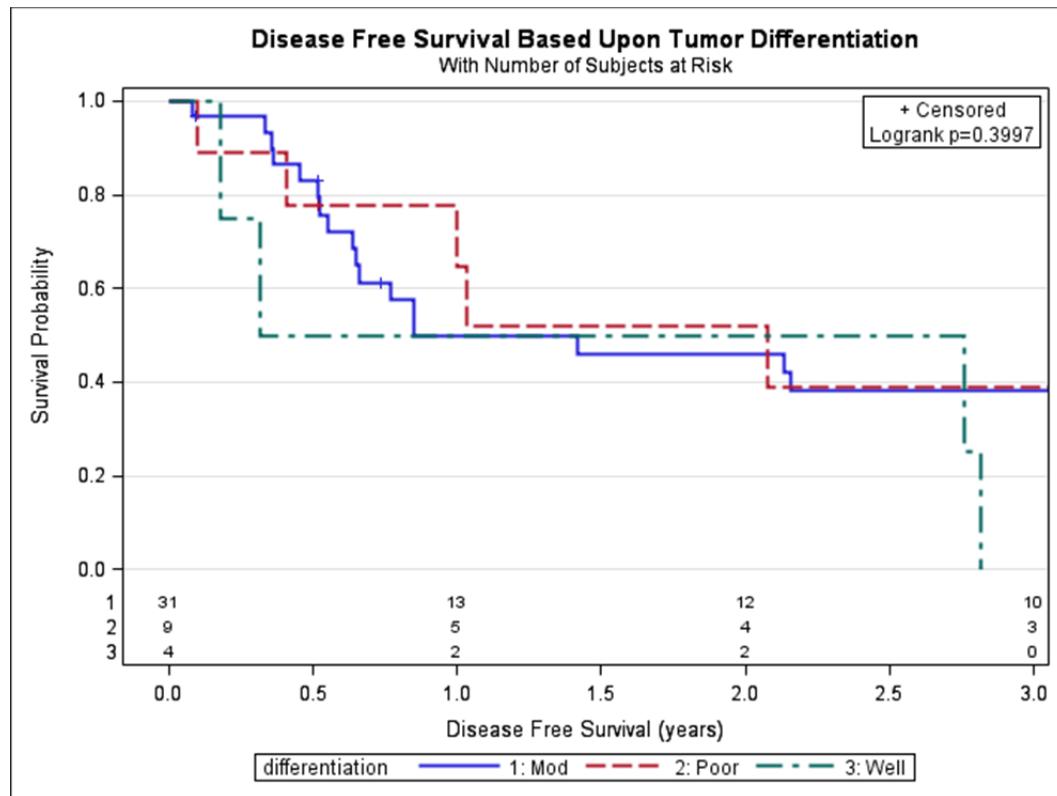
Mod = Moderately
α = 0.05

Figure 7. Kaplan Meier survival curves for disease free survival by smoking status. Smoking status was classified by whether or not the subject was a smoker at time of surgical excision. Subjects were classified as a non-current smoker if they never smoked or completed smoking cessation at least one year prior.
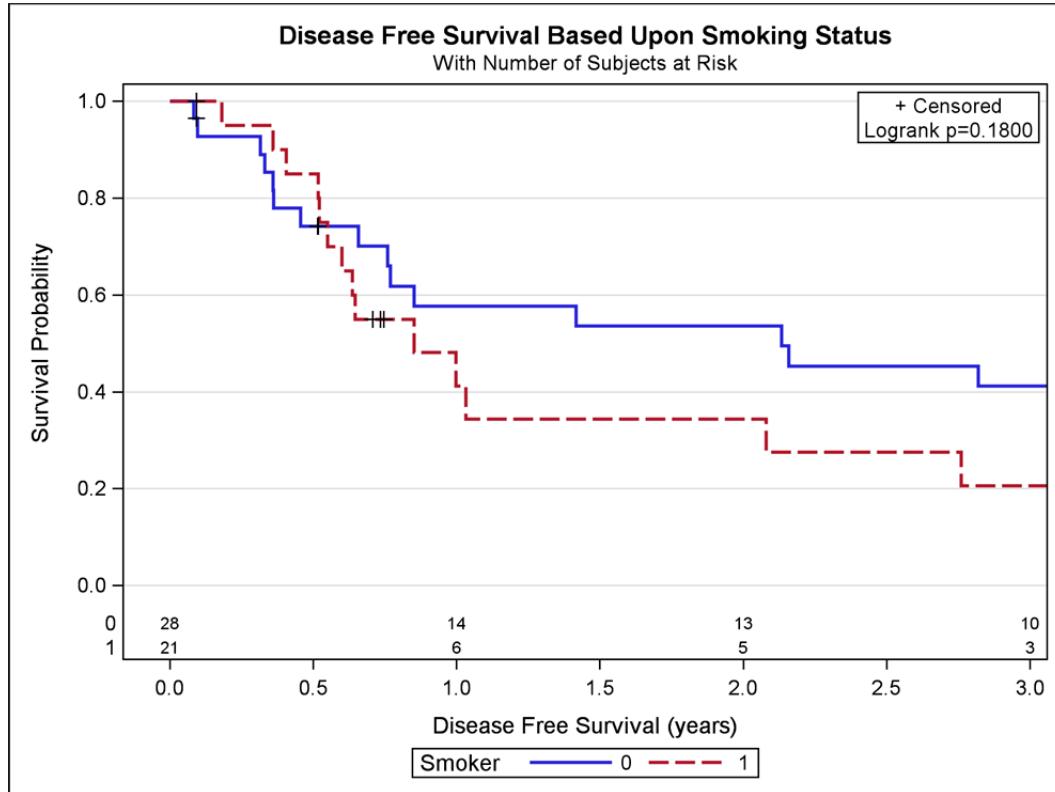
0 = non-smoker or former smoker (quit at least 1 year before date of surgical excision)
1 = current smoker at time of surgical excision
α = 0.05

Figure 8. Kaplan Meier survival curves for disease free survival by total Mcl-1 expression, dichotomized at the median expression level for the cohort.
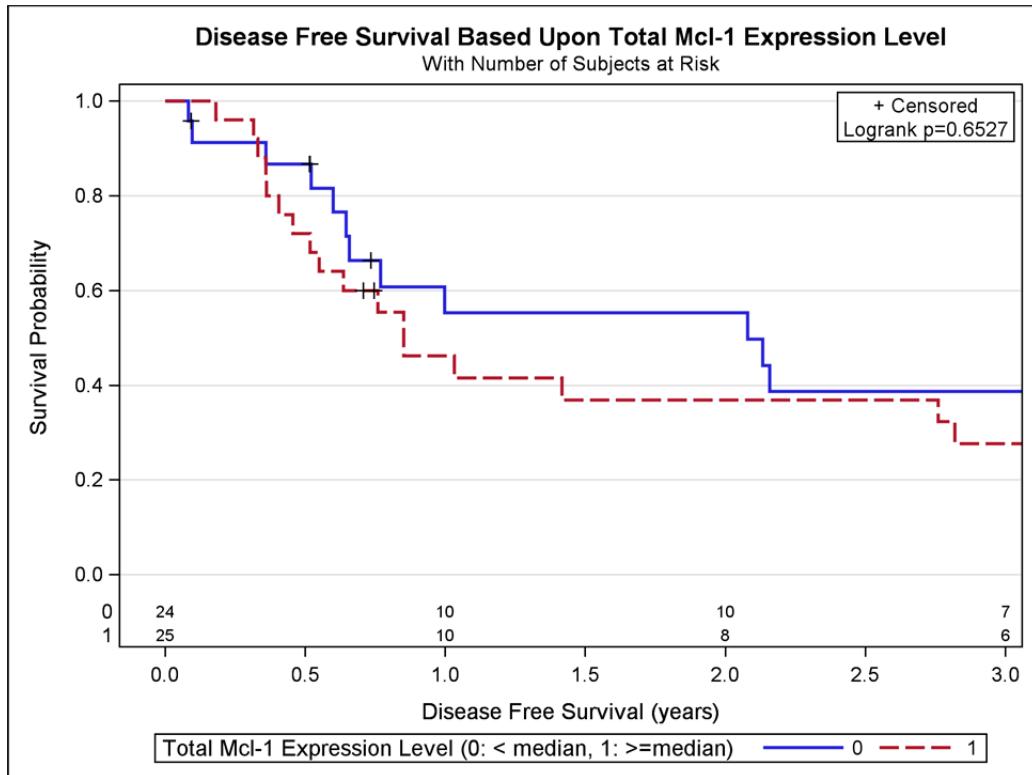
$\alpha = 0.05$

Figure 9. Kaplan Meier survival curves for overall survival by total Mcl-1 expression, dichotomized at the median expression level for the cohort.
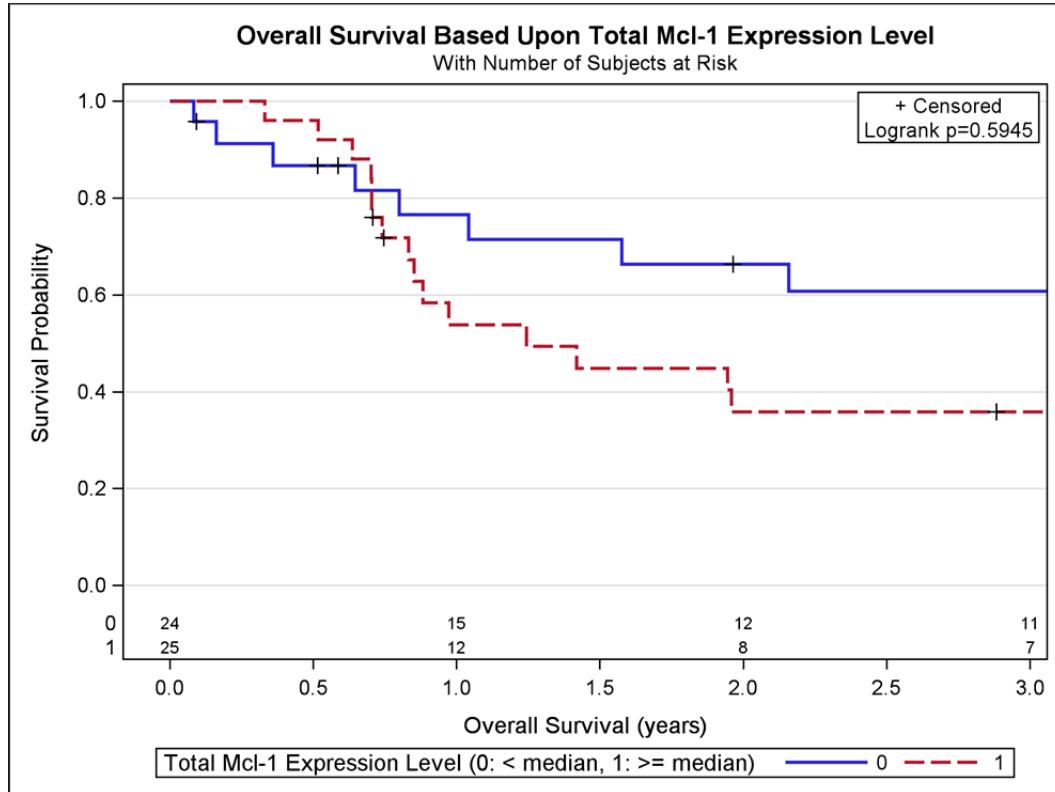

$\alpha = 0.05$

Figure 10. Kaplan Meier survival curves for disease free survival by phosphorylated Mcl-1 (pMcl-1) expression, categorized by whether or not any pMcl-1 expression was detectable.
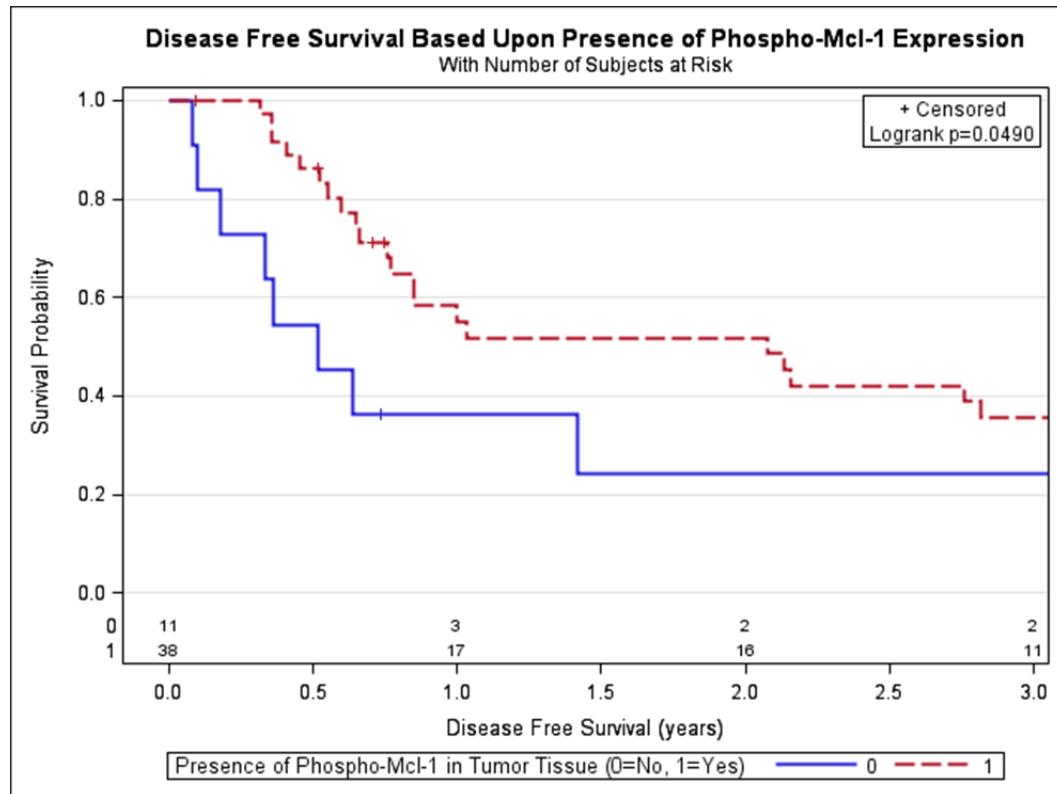
$\alpha = 0.05$

Figure 11. Kaplan Meier survival curves for overall survival by phosphorylated Mcl-1 (pMcl-1) expression, categorized by whether or not any pMcl-1 expression was detectable.
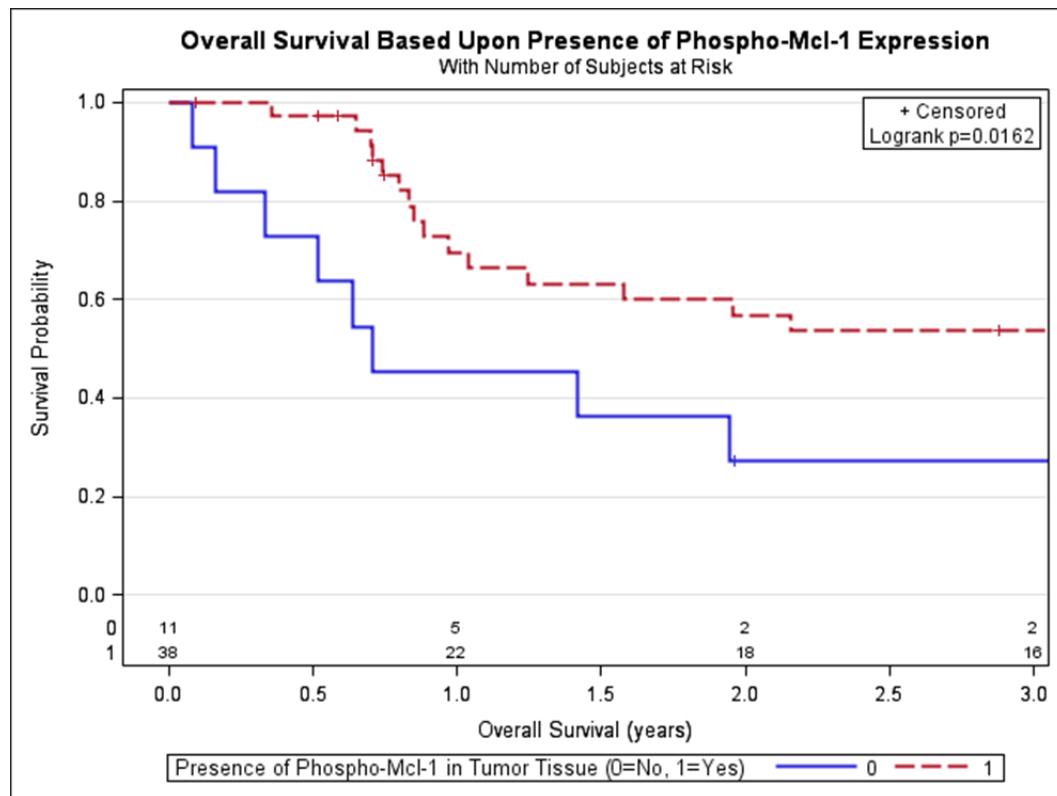

$\alpha = 0.05$

Figure 12. Kaplan Meier survival curves for overall survival by phosphorylated Mcl-1 expression, categorized as either no detectable expression, expression below the median (of expressive tumors), and expression above the median (of expressive tumors).
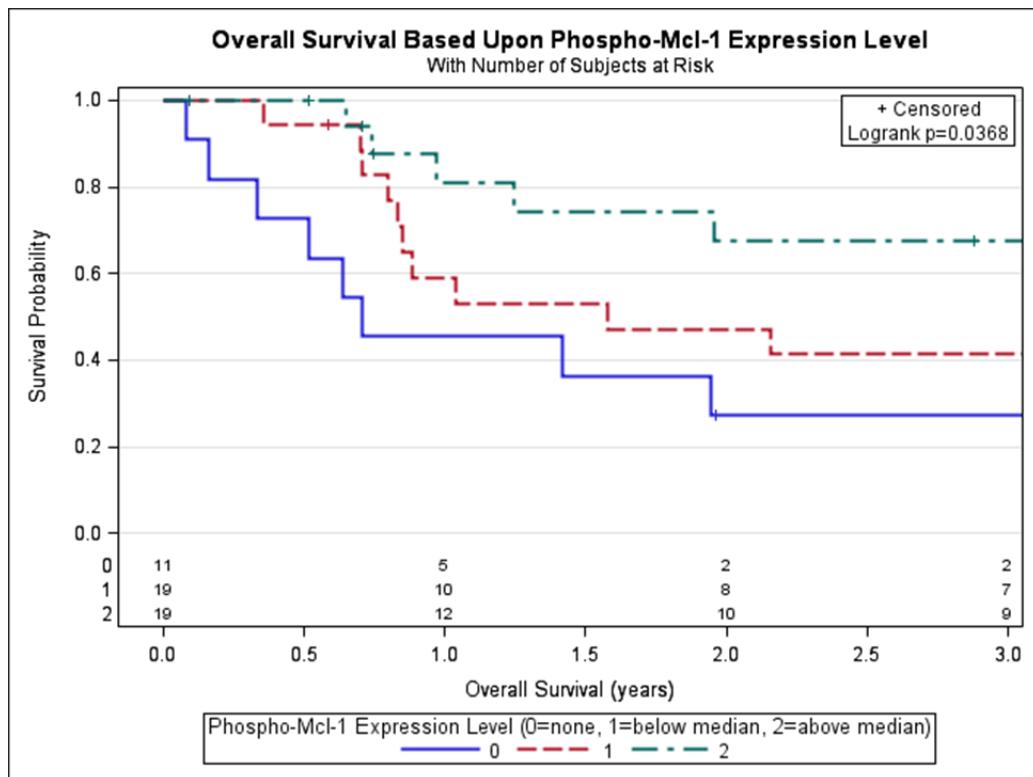

$\alpha = 0.05$

Figure 13. Kaplan Meier survival curves for disease free survival by phosphorylated Mcl-1 expression, categorized as either no detectable expression, expression below the median (of expressive tumors), and expression above the median (of expressive tumors).
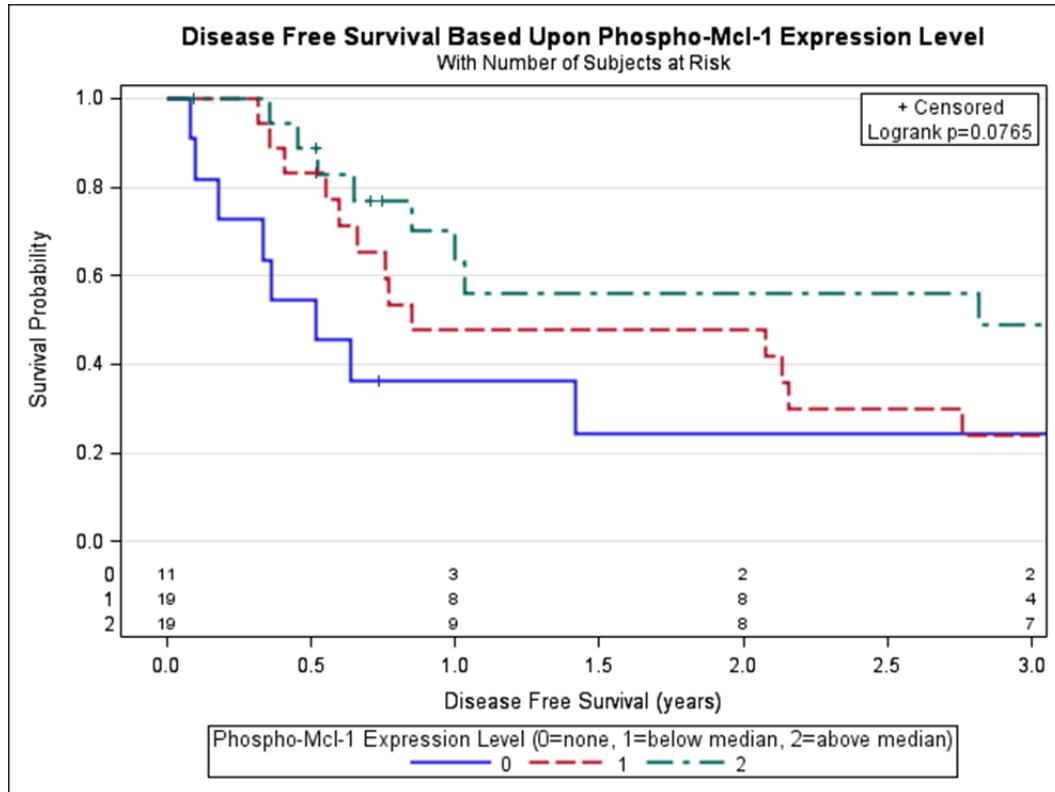
$\alpha = 0.05$

Figure 14. Kaplan Meier survival curves for overall survival by phosphorylated Mcl-1 expression, where expression level is categorized by quartiles.


α = 0.05
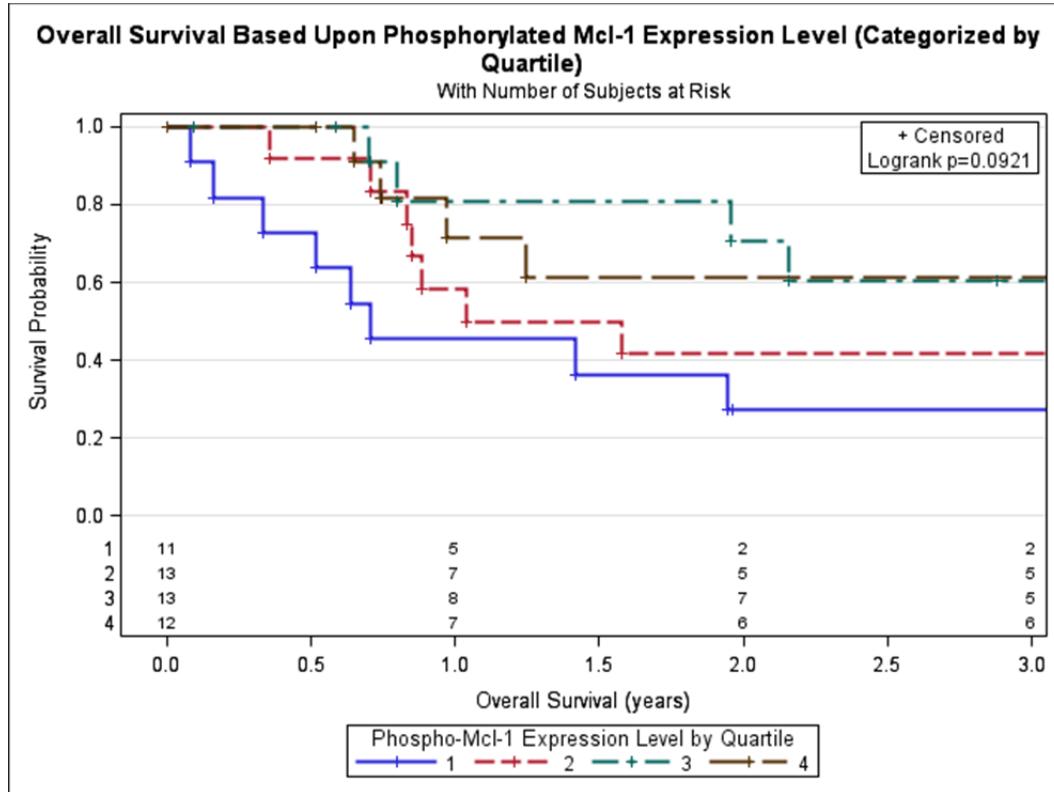
Phospho-Mcl-1 Quartile4 = Expression level >= 75th percentile
Phospho-Mcl-1 Quartile3 = 50th percentile <= Expression level < 75th percentile
Phospho-Mcl-1 Quartile2 = 25th percentile <= Expression level < 50th percentile
Phospho-Mcl-1 Quartile1 = Expression level < 25th percentile

Figure 15. Kaplan Meier survival curves for disease free survival by phosphorylated Mcl-1 expression, where expression level is categorized by quartiles.

$\alpha = 0.05$

Phospho-Mcl-1 Quartile4 = Expression level $>= 75^{th}$ percentile
Phospho-Mcl-1 Quartile3 = 50th percentile $<=$ Expression level $< 75^{th}$ percentile
Phospho-Mcl-1 Quartile2 = 25th percentile $<=$ Expression level $< 50^{th}$ percentile
Phospho-Mcl-1 Quartile1 = Expression level $< 25th$ percentile