Molecular Epidemiology, Racial/Ethnic Differences and Chemoprevention of Breast Cancer: Population-based Studies from Metropolitan Detroit

by

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To my Husband,
Dan,
whose support of my dreams
has been unwavering since
we were 17 years old.

To my Children,
Nathaniel Lawrence
and Grace Margaret,
may they see a
future full of joy and a time when
people are no longer afraid of cancer.

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Chapter 1

Introduction

Breast cancer is the most common potentially lethal cancer diagnosed in US women. Data from the American Cancer Society's annual report estimate that 182,460 new cases of breast cancer will occur among US women in 2008 along with 40,480 deaths due to the disease (American Cancer Society 2008). The median age at diagnosis is 61 years (American Cancer Society 2006). The incidence rate among women aged 40-49 years of age is 150/100,000 US women whereas for those age 50+ years, the incidence rate is more than double that at about 350/100,000 (American Cancer Society 2006). For the United States from 2000-2004, the annual breast cancer death rate was 26 per 100,000 females (American Cancer Society 2008). The 5-year survival for all stages is 89% for women aged 40-74 years at diagnosis (American Cancer Society 2008).

The major risk factors for female breast cancer include increased age, number of first-degree affected relatives, mammographically dense breasts, history of atypical hyperplasia, ionizing radiation exposure to the chest and factors associated with increased exposure to endogenous hormone levels, such as early menarche, late menopause and

fewer pregnancies as well as exogenous hormone exposure (i.e. hormone therapy) (Adami et al. (ed) 2002). An inherited susceptibility is substantial for women who have known BRCA1 or BRCA2 gene mutations. It is estimated carriers have a 50%-80% chance of developing breast cancer in their lifetime (Streuwing et al. 1998; Easton et al. 1995).

Surgical and adjuvant treatment of breast cancer have improved greatly since Dr. William Halsed's disfiguring radical mastectomy (Lerner 2001). Halsted's approach to early and extensive surgery was the prevailing approach to breast cancer treatment from the late 1800s until nearly the end of the 20th century. His data suggested that women who received a radical mastectomy lived longer on average than women who had less surgery. It was improved study methodologies and statistical analysis that helped Dr. Bernie Fisher eventually overturn the Halsted mastectomy (Lerner 2001).

Concurrent with Dr. Fisher's work, mammographic screening techniques were being developed (Lerner 2001). Important population-based data demonstrated that women who received regular mammograms were diagnosed with smaller and more treatable tumors (Lerner 2001). There has been great public acceptance of this screening modality.

Despite progress in our understanding of the risks associated with breast cancer and improvements in screening, detection, and treatment, there remain considerable knowledge gaps. First there are important health disparities in the incidence and mortality of the disease among ethnic groups, with little known about breast cancer outcomes in some minority groups. Second, despite mammographic screening, late

stage, aggressive, and treatment resistant tumors arise commonly. The ultimate goal of research on breast cancer would be the prevention of the disease altogether, and if the disease does arise, the prevention of increased morbidity and of mortality. The studies contained in this dissertation address these issues: health disparities in disease occurrence and outcome, markers of progression, and primary prevention of breast cancer.

1.1 Breast Cancer Disparities

The breast cancer experience is not the same across the world or across ethnic and racial groups within countries. Within the United States in the period of 1998-2002, the annual incidence rates per 100,000 women were 141.1 for whites and 119.4 for African-Americans, while the average annual death rates where 25.9 and 34.7 per 100,000 women, respectively (American Cancer Society 2006). All other major ethnic/racial groups in the US have considerably lower breast cancer incidence and mortality than whites.

The International Agency for Cancer Research (IARC) orchestrates the world-wide effort to collect standardized statistics on cancer incidence by country (Parkin et al. (eds) 2003). Between country comparisons demonstrate that more developed countries have higher breast cancer incidence rates. Countries with comparably high breast cancer incidence include the US, Sweden, The Netherlands, and the United Kingdom with rates of 80-100 cases per 100,000 women per year. The Israeli Jewish population also has a breast cancer incidence rate similar to the US (93 per 100,000) while the rate among

Israeli Arabs is considerably lower (37 per 100,000 women) (Freedman 2006). Data from developing countries are often hard to interpret because of variation in case ascertainment. Population-based tumor registries with rigorous data quality control exist only at a few locations. Most often, hospital-based reports are solely used for reporting a specific area's breast cancer experience. Seeking to understand the breast cancer experience of a large ethnic population in metropolitan Detroit, Arab women, we examined the literature regarding breast cancer incidence in Arab countries in the Middle East and Northern Africa. From these reports, it is often hard to glean if the experience women in the Arab world is exceptional or not, due to the attendant limitations of hospital-based data. Using a validated Arab name algorithm and Detroit Surveillance, Epidemiology, and End Results (SEER) tumor registry data, we examined the breast cancer experience of Arab-American women in Southeastern Michigan.

1.2 Breast Cancer Progression

The wide spread use of mammography screening has enabled early detection of breast cancer and improved survival. However, even with regular screening, aggressive and difficult to treat tumors commonly arise. Examples include small tumors (<1 cm) that have metastasized to the lymph nodes and tumors with unfavorable molecular profiles, such as "triple negative" tumors; that is, tumors that are estrogen-receptor, progesterone-receptor, and Her2 negative at diagnosis (Onitilo AA et al. 2009). Molecular profiling of aggressive vs. non-aggressive breast tumors is a strategy we employed with respect to two potential prognostic markers, RhoC and EZH2. RhoC, a

Rho family GTPase, has been clearly identified as a major phenotypic driver of inflammatory breast cancer, the most lethal form of breast cancer (van Golen et al. 1999; van Golen et al. 2000; Kleer et al. 2004). EZH2 is a histonemethyltransferase polycomb group protein, which has been implicated in the process of cellular differentiation and cancer progression. (Collett K et al. 2006) In a population-based cohort of breast cancer patients, we assessed the potential role of RhoC and EZH2, independently, in the prognosis of early stage disease.

1.3 Breast Cancer Chemoprevention

In 1998, Fisher et al. reported the findings of the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 Study that 20 mg/day of tamoxifen for 5 years reduced the risk of breast cancer by 49% (Fisher et al. 1998). The FDA approved the use of tamoxifen for breast cancer chemoprophylaxis in 1998 (FDA News 1998). Recently, results of the Study of Tamoxifen and Raloxifene (STAR) trial showed raloxifene to be equally effective to tamoxifen in preventing breast cancer (Vogel et al. 2006). The FDA approved raloxifene for this purpose, as well, in 2007 (FDA News 2007).

The investigation into these two breast cancer chemopreventive agents was initiated because of clinical observations on the recurrence of breast cancer in the contralateral breast among women treated with tamoxifen. From the earliest studies onwards, prolonged treatment with tamoxifen decreased the rate of recurrence of previously diagnosed breast cancer and it thus became the standard therapy for hormone

receptor positive breast cancer. Clinicians and researchers subsequently hypothesized tamoxifen could work to also prevent breast cancer.

A treatment investigation is currently underway at the NSABP in a trial to assess whether bisphosphonates, used regularly in the palliative treatment of breast cancer bone metastases, may prevent metastases in women with early stage breast cancer. (NSABP.org) In parallel with the tamoxifen story, this dissertation considers whether bisphosphonates may also be potentially chemopreventive for breast cancer.

This dissertation represents the use of epidemiologic methods in a multi-pronged approach to address some of the leading issues in breast cancer. Combined with molecular techniques, an understanding of tumor biology, and a sound analytic approach, we address breast cancer from several perspectives. Future research in this area will require this type of trans-disciplinary partnership in order to continue making strides in the fight against breast cancer.

Four manuscripts, Chapters 2-5, form the basis of this dissertation. At the time of this writing, Chapter 2 has been published (Hensley Alford et al. 2009), Chapter 5 has been submitted for publication, and Chapters 3 and 4 will be submitted shortly.

References

Adami HO, Hunter D, Trichopoulos D (eds) (2002) *Textbook of Cancer Epidemiology*. Oxford: Oxford University Press.

American Cancer Society. Cancer Facts & Figures 2007 (2007) Atlanta: American Cancer Society.

American Cancer Society (2006) Breast Cancer Facts & Figures 2005-2006. Atlanta: American Cancer Society; 2006.

Collett K, Eide GE, Arnes J, Stefansson IM, Eide J, Braaten A, Aas T, Otte AP, Akslen LA. (2006) Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clinical Cancer Research 12(4):1168-74.

Easton DP, Ford D, Bishop DT (1995) Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. American Journal of Human Genetics 56(1):265-71.

FDA News (1998) Tamoxifen approved for reducing breast cancer incidence. October 29, 1998. http://www.fda.gov/bbs/topics/NEWS/NEW00662.html

FDA News (2007) FDA Approves New Uses for Evista: Drug reduces risk of invasive breast cancer in postmenopausal women. September 14, 2007. www.fda.gov/bbs/topics/NEWS/2007/NEW01698.html

Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N, and other National Surgical Adjuvant Breast and Bowel Project Investigators (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. Journal of the National Cancer Institute. 90(18): 1371-1388.

Freedman LS, Edwards BK, Ries LAG, Young JL (eds) Cancer Incidence in Four Member Countries (Cyprus, Egypt, Israel, and Jordan) of the Middle East Cancer Consortium (MECC) Compared with US SEER. National Cancer Institute. NIH Pub. No. 06-5873. Bethesda, MD.

Hensley Alford S, Schwartz K, Soliman A, Johnson CC, Gruber SB, Merajver SD. (2009) Breast cancer characteristics at diagnosis and survival among Arab-American women compared to European- and African-American women. Breast Cancer Research and Treatment 114(2):339-46.

Kleer CG, Zhang Y, Pan Q, Gallagher G, Wu M, Wu ZF, Merajver SD. (2004) WISP3 and RhoC guanosine triphosphatase cooperate in the development of inflammatory breast cancer. Breast Cancer Research 6(1):R110-5.

Lerner B. (2001) The Breast Cancer Wars: Hope, Fear, and the Pursuit of a Cure in Twentieth-Century America. Oxford University Press, Inc. New York, NY.

NSABP.org http://www.nsabp.pitt.edu/NSABP_Protocol_Chart.pdf

Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. (2009) Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. Clinical Medical Research [Epub ahead of print]

Parkin DM, Wheln SL, Ferlay J, Teppo L, Thomas DB (eds) (2003) Cancer Incidence in Five Continents, Vol. VIII. IARC Scientific Publications, No. 155. Lyon, France..

Streuwing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC, Tucker MA (1997) The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. New England Journal of Medicine 336(20):1401-8.

Van Golen KL, Bao LW, Pan Q, Miller FR, Wu ZF, Merajver SD. (2002) Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer. Clinical and Experimental Metastasis 19(4):301-11.

Van Golen KL, Wu ZF, Ziao XT, Bao LW, Merajver SD. (2000) RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Research 60(20):5832-8.

Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Wade JL, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark (2006) Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial. JAMA 295:2727-2741.

Chapter 2

Breast Cancer Characteristics at Diagnosis and Survival among Arab-American Women Compared to European- and African-American Women

2.1 Background

Several papers from major treating hospitals in Arab countries have reported an early age of onset and a preponderance of aggressive breast cancer phenotypes in their patients (Akhtar et al. 1993; Al-Idrissi et al. 1992; Ibrahim et al. 1998; Chiedozi et al. 2003; Tabbane et al.1977; Tabbane et al. 1985; Mourali et al. 1980; Costa et al. 1982; El Saghir et al. 2002; El Saghir et al. 1998; Abdel-Rahman et al. 1993; Soliman et al.1999). For example, papers from Tunisia report observations of rapidly progressing breast cancer in young women, suggestive of an inflammatory breast cancer histology (Tabbane et a.1977; Tabbane et al. 1985; Mourali et al. 1980; Costa et al. 1982). In addition, others have reported that the majority (>50%) of breast cancer cases in Arab countries are diagnosed among women less than 50 years of age (Akhtar et al. 1993; Ibrahim et al. 1998; El Saghir et al. 2002); this is in comparison to US statistics which show that 22% of breast cancer cases are diagnosed in women under age 50 years of age.

While these statistics give some relative comparisons, they are difficult to interpret given the variation in the population age structure between Arab countries and the US and the lack of detailed comparative studies between the US and Arab populations. In addition, it cannot be assumed that all breast cancer cases are systematically captured in most Arab countries, given the extreme paucity of population screening efforts for early detection in those regions. The well-established tumor registry in Israel gives data for the Jewish and Arab populations separately. Age-adjusted and standardized incidence rates from this registry show that the Arab Israeli population has a lower overall incidence of breast cancer compared to the US (36.7 per 100,000 women compared to 97.2 per 100,000 women) and a later age at onset. Recently the Middle East Cancer Consortium (MECC), in conjunction with the US National Cancer Institute (NCI), published cancer statistics from four Middle Eastern countries: Israel, Cyprus, Jordan, and Egypt (Tanta) (Freedman et al. 2006). The Tanta registry in Egypt, which is also population based, reports age-adjusted and standardized incidence rates of breast cancer higher than those of the Israeli Arab population for women <60 years as well as a higher overall incidence rate of 50 per 100,000 women. It is likely that given the diversity of the populations of the Arab world, the breast cancer experience varies among groups. It is also possible that there is under-reporting in Israeli Arabs. Such variation, if it does exist, could be due to differences in genetic background, environmental exposures, or reproductive behaviors.

Metropolitan Detroit is home to the largest Arabic-speaking population outside of the Middle East. The city of Dearborn, a near west suburb of Detroit, has been a center

of Arab culture and immigration since the late 1800's. Immigrant populations include Lebanese, Yemenis, Syrians, Palestinians, Egyptians, and Iraqis, including Chaldeans (Christian Iraqis). Political unrest in other Arab countries has also contributed to the heterogeneity of the Detroit Arab population. According to the 2000 Census, which included the option to report country of origin, the metropolitan Detroit Arab population is 44% Lebanese, 32% Chaldean, 10% Iraqi, 6% Syrian, 3% Palestinian, 2% Egyptian, and 2% Jordanian (Wayne State University College of Urban, Labor, and Metropolitan Affairs 2000). These census data were collected before 9/11/2001, when fear of discrimination among American Arab and Muslim populations increased dramatically; therefore, the reported proportions are thought to be representative, despite the usual expected undercount from self-reported statistics.

Detroit has been part of the NCI's national tumor registry, the Surveillance Epidemiology and End Results (SEER) program, since the registry's inception in 1973. The large Arab-American community in Detroit gave us the opportunity to characterize in a population-based framework, the patterns of breast cancer experienced at diagnosis by Arab women versus other ethnic groups. Our work was highly facilitated by the advent of a validated name algorithm, which allowed the identification of women of Arab decent in the registry so that a comparison could be made to white, non-Hispanic (European) and African-America women (Schwartz et al. 2004). SEER racial/ethnic categories do not include "Arab" so this algorithm was required to identify this racial/ethnic group. In addition, because the US Census does not include demographics on this specific group, it was not possible to calculate incidence rates for this population.

The *a priori* hypothesis, based on the reports from the Arab world, was that Arab-American women would have poorer prognostic characteristics at the time of diagnosis than European-American women and worse survival.

2.2 Methods

In contrast to the behavior of many other migrating populations, Arabs have maintained their cultural names after immigrating and settling in the US. Using a previously published and validated algorithm to identify Arab ethnicity by name (Schwartz et al. 2004), we identified women with an Arab maiden name or surname in the Detroit SEER registry. First names were used for equivocal surnames. We used data from the start of the registry in 1973, up to and including 2003. Race is collected for the tumor registry by the registrars during medical record abstraction. Thus, the data depends on subjective clinical observations of race. We compared women identified as being Arab to non-Hispanic, non-Arab Caucasian women (henceforth termed European-American) and to African-American women. The small percentage of women with other racial identities where excluded from this analysis.

We compared Arab women to European- and African-American women on several prognostic indicators at diagnosis including age, histology, grade, estrogen (ER) and progesterone (PR) marker status, and SEER stage. For each indicator, we first tested for global association with race using a chi-square test. For indicators with a significant chi-square, we calculated the odds ratio for that factor by race to characterize the breast cancer risk.

We evaluated overall survival using Kaplan-Meier and Cox Proportional Hazard models for each racial group. Adjusted hazard ratios were calculated for each race/ethnicity adjusting for age, grade, ER/PR marker status, SEER stage, histology and year of diagnosis. We tested for interactions between race/ethnicity as well as age and each of the prognostic characteristics; significant interactions were retained in the model along with their main effects.

2.3 Results

Study Cohort

There were 80,316 women diagnosed with primary breast cancer in the Detroit SEER registry between 1973 and 2003. We excluded 9 females diagnosed < 18 years of age, 1,095 women with a race/ethnicity other than Arab-, European- or African-American, and 91 cases were excluded due to uncommon non mammary epithelial histology (e.g., melanoma of the breast). The resulting analytic sample (n=79,121) was 80% European-American, 18% African-American, and 2% Arab-American. (Table 2.1) The overall mean age at diagnosis was 60 years. In situ cases represent 12% of the cohort and 46%, 30%, and 6% had local, regional, or distant disease, respectively, with 6% of an unknown stage.

Characteristics at Diagnosis

Age. The mean ages at diagnosis were 61, 57, and 58 years, for European-American, Arab-, and African-American women, respectively (Table 2.1). We calculated a log-rank test to compare the distribution of age at diagnosis among the three ethnic

groups, which was statistically significantly different ($p<0.001$). All pairwise log-rank tests comparing each race category to each other were also significant ($p<0.001$).

Stage. The distribution (number and percent) of SEER staging categories is presented in Table 2.2a. There was a statistically significant overall chi-square for the distributions of stage at diagnosis by race ($p<0.0001$). Table 2.2b gives the unadjusted odds ratios (OR) and 95% confidence intervals (CI) for each SEER stage at diagnosis comparing African-American and Arab-American breast cancer cohorts individually to European-American women. Arab-American women were significantly less likely to be diagnosed with local disease (OR=0.82; 95% CI 0.74-0.91) and significantly more likely to be diagnosed with regional disease (OR 1.18; 95% CI 1.06-1.30). Similarly, African-American women were less likely to be diagnosed with local disease (OR=0.74; 95% CI 0.71-0.77) and more likely to be diagnosed with regional (OR=1.20; 95% CI 1.15-1.25) and distant disease (OR=1.60; 95% CI 1.50-1.72).

Histology. Table 2.3a shows the number and proportion of each histological type by race. The overall chi-square was significant at $p<0.0001$. The histological tumor type was not significantly different in any category for Arab-American women compared to European-American women (Table 2.3b). However, notable odds ratios included a protective association for invasive (i.e., not otherwise specified) (OR=0.86; 95% CI 0.73-1.02) which is not significant, and an increase OR for metaplastic (OR=2.57; 95% CI 0.61-10.76). (Table 2.3b) African-American women differed significantly from European-American women with fewer invasive (OR=0.63; 95% CI 0.59-0.67) breast cancers, but more papillary (OR=1.86; 95% CI 1.62-2.12), comedo (OR=1.26; 95% CI

1.14-1.38), medullary (OR=2.03; 95% CI 1.82-2.27), Paget's (OR=1.58; 1.16-2.15), and inflammatory (OR=1.53; 95% CI 1.30-1.81) tumors.

Marker Status. Estrogen-receptor (ER) and progesterone-receptor (PR) status at diagnosis are important prognostic factors and robust predictors of response to hormonal therapy. The distribution of ER/PR combined status is given in Table 2.4a. Overall chi-square for differences in the distribution of ER/PR between the three racial groups was $p<0.0001$. Arab-American women were more likely, though not significant, to have ER- disease compared to European-American women (OR=1.17; 95% CI 0.97-1.40); and significantly more likely to have ER-/PR- tumors (OR=1.29; 95% CI 1.08-1.54) (Table 2.4b). African-American women were significantly more likely at diagnosis to have tumors that were ER- (OR=2.29; 95% CI 2.15-2.46), PR- (OR=1.84; 95% CI 1.73-1.96) or ER and PR negativity combined ((ER-/PR-) OR=2.09; 95% CI 1.97-2.12) when compared to European-American women. Figure 2.1 in conjunction with Table 2.4b depicts the proportion of each ER/PR pair status by race and age at diagnosis dichotomized by age <50 years and ≥50 years. Of note, the proportion of ER+/PR+ tumors in African-American women ≥50 years is lower than the proportion seen in Arab- or European-American women <50 years of age.

Grade. The distribution of the grade at diagnosis for European-, Arab-, and African-American women is given in Table 2.5a. The overall chi-square for a difference in the distribution between groups was $p<0.0001$. Arab-American women were less likely to have well-differentiated (OR=0.86; 95% CI 0.71-1.05) and unknown (OR=0.86; 95% CI 0.78-0.95) tumors at diagnosis than European-American women. (Table 2.5b)

They were significantly more likely to have poorly differentiated tumors (OR=1.25; 95% CI 1.11-1.41). African-American women were significantly less likely to have either well-differentiated (OR=0.71; 95% CI 0.66-0.77) or moderately differentiated (OR=0.90; 95% CI 0.86-0.95) tumors as well as tumors with unknown differentiation (OR=0.78; 95% CI 0.75-0.81). In addition, they were significantly more likely to have poorly differentiated (OR=1.72; 95% CI 1.65-1.79) or undifferentiated (OR=1.79; 95% CI 1.52-2.11) tumors.

Small tumors with positive nodes. We hypothesized that a surrogate for biologically aggressive disease is a tumor that even though small at the primary site (< 1 cm) has evidence of nodal metastases. Table 2.6 gives the results of our analysis of small tumors with positive nodes at diagnosis. Both Arab- and African-American women were more likely to be diagnosed with this type of tumor than European-American women; however, the results for Arab-Americans were not significant for both the unadjusted or adjusted analysis. Interestingly, the odds ratios for both Arab- and African-American women increased in magnitude after adjustment.

Survival Overall and Adjusted

Figures 2.2a-2.2f are the Kaplan Meier plots for overall survival and for survival by SEER stage at diagnosis. In all graphs, Arab-American women have the best survival followed, usually closely, by European-Americans. African-American women have considerably worse survival. In Table 2.7, the unadjusted and adjusted hazard ratios (HR) from the Cox Proportional Hazards models are presented. Hazard ratios include an

interaction term for race-by-age and age-by-marker status, and were adjusted for histology, age at diagnosis, marker status, year of diagnosis, grade, and stage. Commensurate to the graphs, Arab-American women have significantly better survival (HR=0.83; 95% CI 0.74-0.92) in the unadjusted analysis. The magnitude of the estimate is similar in the adjusted analysis but is not statistically significant. Notably, African-American women have a significantly higher mortality than European-American women in the unadjusted (HR=1.21; 95% CI 1.17-1.25) and more profoundly in the adjusted (HR=2.3; 95% CI 1.75-3.03) analyses.

2.4 Discussion

Data from the Arab world vary regarding the reported breast cancer experience of women. A retrospective review of 292 patients seen at King Fahd Hospital from 1985-1995 showed that 78% of patients were younger than 50 years at diagnosis and 79% were pre-menopausal (Ibrahim et al. 1998). Similarly, in Lebanon, 49% of breast cancer cases diagnosed between 1983-1995 (n=2673) were <50 years of age (El Saghir et al. 2002). There is only one oncology clinic in Libya which maintains a tumor registry for all cases receiving consultation. Between 1981-1985, breast cancer was the most frequently diagnosed cancer in women, 72% of whom were less than 50 years of age (Akhtar et al. 1993). The data from these studies represent hospital-based observations and are thus hard to interpret without additional information. For example, it is possible that only younger women seek treatment for their breast cancer resulting in a selection bias in the data reported.

In two Egyptian studies, alternative study designs were applied with similar results. Abdel-Rahman et al. used a case-control design to assess epidemiologic features of breast cancer (1993). Results of this study were notable for age at diagnosis and several risk factors. Forty-four percent of cases were diagnosed less than or equal to 50 years of age. Cases were more likely than controls to have a family history of breast cancer, to have a history of radiation exposure, to be employed, as well as several reproductive factors including later age at first birth, lower parity, and artificial menopause. Although this study offers some potential explanations for the age distribution of cases, the cases still may have been differentially selected.

Soliman et al. conducted a review of mortality data in Egypt where death certificates are required to receive a burial permit (1999). Records reviewed for the period of January 1, 1992 to December 31, 1996 were compared to US mortality statistics (1991-1995). Results from this population-based study showed a higher age-specific mortality for breast cancer among women less than 40 years of age.

Recently, the US NIH/National Cancer Institute partnered with the Middle East Cancer Consortium (MECC) to publish cancer incidence data from four MECC countries (Cyprus, Egypt, Israel, and Jordan) (Freedman et al. (eds) 2006). Israel, which has a diverse population, reported age-standardized breast cancer incidence rates of 93 per 100,000 women for Israeli Jews and 36.7 for Israeli Arabs. Rates per 100,000 women reported for Cyprus, Egypt, and Jordan were 57.7, 49.6, and 38.0, respectively. For comparison, the US SEER rate per 100,000 for all US women over a similar time period was 97.2. Rates for Oman and Kuwait were also available in Volume VIII of the

International Agency for Research on Cancer's Cancer Incidence in Five Continents (Parkin et al. (eds) 2003). For 1993-1997, the age-standardized breast cancer rate per 100,000 women was 12.7 in Oman. Kuwait's reported average annual age-adjusted breast cancer rate for Kuwaiti women between 1994-1997 was 32.8 per 100,000. Data quality issues for both countries were noted in the publication.

Using data from the metropolitan Detroit SEER, where an estimated 250,000 Arab-Americans reside, we present the first report of breast cancer characteristics at diagnosis among Arab-American women. Our results suggest that Arab-Americans have a different breast cancer experience from both European- and African-Americans. They were diagnosed at a younger age, had more regional disease which was poorly differentiated and tended to be more ER-/PR- than their European-American counterparts. Although not statistically significant, there was also a trend observed in our data for Arab-American women to be more likely to have small tumors with positive nodes. Importantly, these differences in disease characteristics, which would suggest poorer prognosis, did not translate into a survival disadvantage. This disparity of findings may reflect variation in environmental exposures or individual breast cancer risk factors; however, it may also represent biologically aggressive disease that is responsive to treatment and thus results in equivalent survival to the less aggressive disease more characteristic of European-Americans. With regards to genetic heterogeneity, the term "Arab" refers to an individual from any one of 23 different countries that span geographic and cultural expanses from Mauritania to Oman and from Somalia to Syria and Morocco. The rich cultural diversity of the Arab world includes differences in marriage practices,

including currently practiced consanguineous marriages, differences in social behaviors, and a wide-range of environmental exposures including those associated with oil production and agricultural practices. An interesting hypothesis that we could not investigate in this analysis is whether the length of residence within the US influences the breast cancer experience of Arab-American women. Other immigration studies have shown that recent migrants maintain the breast cancer risk profile of their native country, but subsequent generations assume the risk profile for the adopted country. SEER, which relies on medical record data, finds country of birth in less than 25% of cases in Detroit. SEER does not capture date of immigration, which is a limitation in our analysis.

It appears from our data that the Arab-American breast cancer experience for any of the characteristics at diagnosis that we examined were usually more favorable than those observed among African-American women. Our study is very robust in distinguishing the relative proportions between all combinations of estrogen and progesterone receptor status, an area of active current interest and investigation. Our results clearly reaffirm that African-Americans are much less likely than either European- or Arab-Americans to present with ER+/PR+, whereas the mixed phenotypes of hormonal receptor expression status are relatively similar amongst all the populations. This is a striking finding, especially considering that the preponderance of ER-/PR- disease was seen in this study for in African-Americans for all ages. Several recent review papers have evaluated the potential contributions to differences in breast cancer outcomes in African- and European-Americans (Smigal et al. 2006; Polite and Olopade 2005; Newman 2005; Chlebowski et al. 2005). Although it is likely that screening and

treatment differences contribute to the disparity in outcomes, it is also clear that differences in tumor biology may also be important. Polite and Olopade note in their review the evidence of significant tumor biology differences in hormone receptor and HER2 status, grade, S-phase fraction, BRCA-1/2 mutations of unknown significance, and P53 (Polite and Olopade 2005). Even when controlling for known differences in tumor biology as well as screening, treatment, and socio-demographic factors, the mortality difference between African-Americans and Caucasians can not be completely explained. Polite and Olopade conclude that this is due to as yet unidentified biological differences exist.

We found that delineating the racial distribution of the histologies from the SEER registry was a difficult task, particularly since the analysis included data from 1973 to 2003, a period during which a major revision of the SEER abstracting guidelines (1988) and changes in the clinical interpretation of the pathology took place. Due to the Tunisian reports, we wanted to examine differences in the proportion of inflammatory breast cancer among the three ethnicities. However, since there is not an ICD-O designation for inflammatory breast cancer (IBC), we used information from the extent of disease codes. Our classification of IBC using this approach is most likely imprecise. However, assuming non-differential misclassification between ethnic groups, we would surmise that the relative differences are accurate, despite the imprecision of the absolute frequency. One other study has used SEER data to evaluate racial differences in inflammatory breast cancer incidence and our results generally agree with the previous findings. (Hance et al. 2005) We found that African-American women were significantly

more likely to be diagnosed with inflammatory breast cancer (OR=1.53; 95% CI 1.30-1.81) than European-American women. Our results are consistent with those of Hance et al, who also reported a higher frequency of inflammatory breast cancer in African-Americans (Hance et al. 2005). However, ours is the first report of the proportion of IBC amongst Arab Americans, a subject of great interest, given the increased proportion of IBC in North Africa.

A limitation of our study is the small number of Arab-American women identified relative to European- and African-American women. In addition, tumor registry race/ethnicity data is captured through clinical interpretations so is not self-reported or recorded in a standardized fashion. This limitation affects subsequent generalizability of results.

It is worth noting that if denominator data were available for the Arab community, we could have calculated standardized age-adjusted incidence rates. We suspect that the underlying age distribution structure of the Arab community is younger than that of either the European- or African-American communities in Detroit. Because Arab ethnicity has been grouped with "Caucasian" in US Government population-based data collection efforts, we cannot identify the age structure or total population of Arab-Americans in Detroit or elsewhere in the US. Even if we use self-reported country of origin or language spoken at home, the population estimates are most likely an undercount. Our research within this important minority community is significantly hampered by the lack of accurate population estimates and age structure data. The current socio-political climate suggests that further Arab immigration to the US is expected. Detroit alone is

anticipating thousands of Iraqi immigrants in the next 12 months from the United Nation's efforts to resettle Iraqi refugees (UN News Service 2007; Karoub 2008). Recognition of Arabs as a separate minority group and detailed analyses of their breast cancer and other disease burdens would allow better population statistics for public health research, policy, and social support services.

Table 2.1 Study Population Characteristics

Race	n	(%)
Eur[1]	63,614	(80%)
Arb	1,652	(2%)
Afr	13,855	(18%)
SEER Stage		
In Situ	9,643	(12%)
Local	36,622	(46%)
Regional	23,754	(30%)
Distant	4,652	(6%)
Unknown	4,450	(6%)
Mean age at diagnosis (SD)	60 (14)	
Eur	61	
Afr	58	
Arb	57	

χ^2 p-value < 0.001

[1]Eur= European-American; Arb= Arab-American ; Afr= African-American

Table 2.2a Distribution of SEER Stage at Diagnosis for European-, Arab-, and African-American Women

Stage	Eur	Arb	Afr
In Situ	77,341 (12%)	209 (13%)	1,700 (12%)
Local	30,335 (48%)	708 (43%)	5,579 (40%)
Regional	18,614 (29%)	541 (33%)	4,599 (33%)
Distant	3,408 (5%)	92 (6%)	1,152 (8%)
Unknown	3,523 (6%)	102 (6%)	825 (6%)

Overall χ^2 p-value<0.0001

Table 2.2b Odds Ratios (with 95% Confidence Intervals) for SEER Stage at Diagnosis Comparing Arab-American and African-American Women Individually to European-American Women

Stage	Eur	Arb	Afr
In Situ	1.0	1.05 (0.90-1.21)	1.01 (0.95-1.07)
Local	1.0	0.82 (0.74-0.91)	0.74 (0.71-0.77)
Regional	1.0	1.18 (1.06-1.30)	1.20 (1.15-1.25)
Distant	1.0	1.04 (0.84-1.29)	1.60 (1.50-1.72)
Unknown	1.0	1.12 (0.92-1.38)	1.08 (0.99-1.17)

Table 2.3a Distribution of Tumor Histology at Diagnosis for European-, Arab-, and African-American Women

Histology	Eur	Arb	Afr
Invasive*	58,469 (92%)	1,499 (91%)	12,166 (88%)
Metaplastic	30 (0.05%)	2 (0.12%)	12 (0.09%)
Papillary	762 (1.2%)	21 (1.3%)	305 (2.2%)
Squamous	58 (0.09%)	2 (0.12%)	21 (0.15%)
Comedo	1,977 (3%)	62 (4%)	536 (4%)
Medullary	1,027 (2%)	27 (2%)	447 (3%)
Sarcomas	160 (0.2%)	5 (0.3%)	55 (0.4%)
Pagets	562 (1%)	17 (1%)	124 (1%)
Inflammatory	569 (0.9%)	17 (1.0%)	189 (1.4%)

Overall χ^2 p-value<0.0001
*Invasive, not otherwise specified

Table 2.3b Odds Ratios (with 95% Confidence Intervals) for Histology at Diagnosis Comparing Arab-American and African-American Women Individually to European-American Women

Histology	Eur	Arb	Afr
Invasive*	1.0	0.86 (0.73-1.02)	0.63 (0.59-0.67)
Metaplastic	1.0	2.57 (0.61-10.76)	1.84 (0.94-3.59)
Papillary	1.0	1.06 (0.69-1.64)	1.86 (1.62-2.12)
Squamous	1.0	1.34 (0.32-5.44)	1.66 (1.01-2.74)
Comedo	1.0	1.22 (0.94-1.57)	1.26 (1.14-1.38)
Medullary	1.0	1.01 (0.69-1.49)	2.03 (1.82-2.27)
Sarcomas	1.0	1.17 (0.72-1.89)	1.01 (0.83-1.23)
Pagets	1.0	1.20 (0.49-2.94)	1.58 (1.16-2.15)
Inflammatory	1.0	1.15 (0.71-1.87)	1.53 (1.30-1.81)

*Invasive, not otherwise specified

Table 2.4a Distribution of Marker Status at Diagnosis for European-, Arab-, and African-American Women

Marker	Eur	Arb	Afr
ER+/PR+	12,926 (64%)	382 (63%)	2,322 (47%)
ER+/PR-	2,750 (13%)	73 (12%)	607 (13%)
ER-/PR+	577 (3%)	24 (4%)	189 (4%)
ER-/PR-	4,017 (20%)	132 (21%)	1,710 (36%)

Overall χ^2 p-value<0.0001
(Note: Frequency missing is 53412 due to data capture not beginning until 1990.)

Table 2.4b Odds Ratios (with 95% Confidence Intervals) for Marker Status at Diagnosis Comparing Arab-American and African-American Women Individually to European-American Women

Marker	Eur	Arb	Afr
ER-	1.0	1.17 (0.97-1.40)	2.29 (2.15-2.46)
PR-	1.0	1.01 (0.85-1.19)	1.84 (1.73-1.96)
ER-/PR-	1.0	1.29 (1.08-1.54)	2.09 (1.97-2.12)

Table 2.5a Distribution of Grade at Diagnosis for European-, Arab-, and African-American Women

Grade	Eur	Arb	Afr
Well	4,915 (8%)	111 (7%)	780 (6%)
Moderately	11,302 (18%)	317 (19%)	2,257 (16%)
Poorly	11,023 (17%)	343 (21%)	3,668 (26%)
Undifferentiated	511 (0.8%)	13 (0.8%)	198 (1.4%)
Unknown	35,863 (56%)	868 (53%)	6,952 (50%)

Overall χ^2 p-value<0.0001

Table 2.5b Odds Ratios (with 95% Confidence Intervals) for Grade at Diagnosis Comparing Arab-American and African-American Women Individually to European-American Women

Grade	Eur	Arb	Afr
Well	1.0	0.86 (0.71-1.05)	0.71 (0.66-0.77)
Moderately	1.0	1.10 (0.97-1.24)	0.90 (0.86-0.95)
Poorly	1.0	1.25 (1.11-1.41)	1.72 (1.65-1.79)
Undifferentiated	1.0	0.98 (0.56-1.70)	1.79 (1.52-2.11)
Unknown	1.0	0.86 (0.78-0.95)	0.78 (0.75-0.81)

Table 2.6 Unadjusted and Adjusted Odds Ratio for Probability of Being Diagnosed with a Small Tumor (<1cm) with Positive Nodes

	OR	95% CI	aOR*	95% CI
Eur	1.0	---	1.0	---
Arb	1.18	(0.96-1.45)	1.27	(0.98-1.67)
Afr	1.15	(1.06-1.24)	1.37	(1.23-1.52)

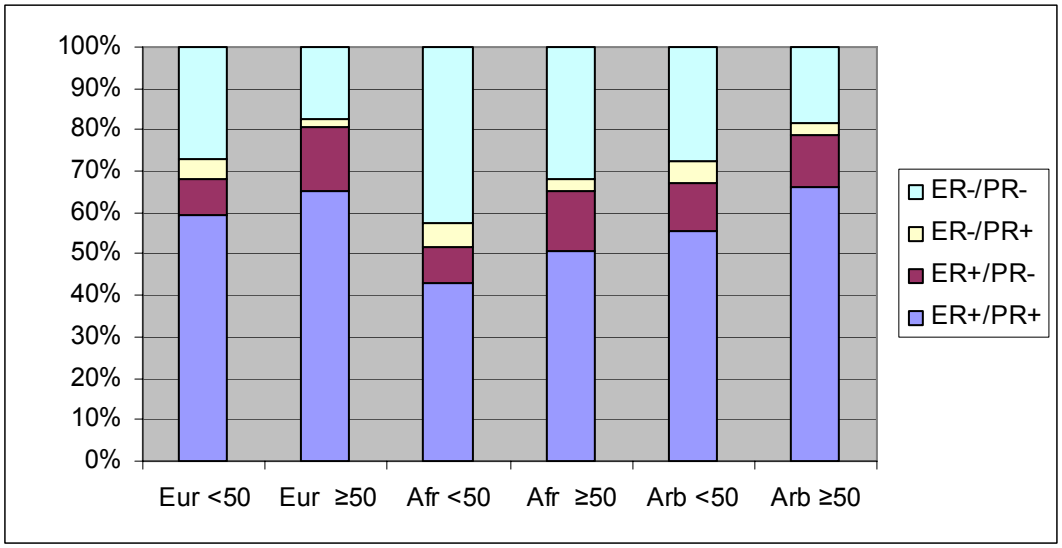
*Adjusting for age, histology, grade, marker status, year of diagnosis

Table 2.7 Cox Proportional Hazards Ratios for 5-year Survival

	HR	95% CI	aHR*	95% CI
Eur	1.0	---	1.0	---
Arb	0.83	(0.74-0.92)	0.74	(0.30-1.82)
Afr	1.21	(1.17-1.25)	2.3	(1.75-3.03)

*Adjusting for histology, age at diagnosis, marker status, year of diagnosis, grade, stage, and interaction between race and age.

Figure 2.1 Proportion of Combined Hormone Receptor Status by Race and Age at Diagnosis



Eur=European-American
Afr=African-American
Arb=Arab-American
ER=Estrogen receptor
PR=Progesterone receptor

Figure 2.2a Kaplan-Meier Curves by Race for Overall Survival

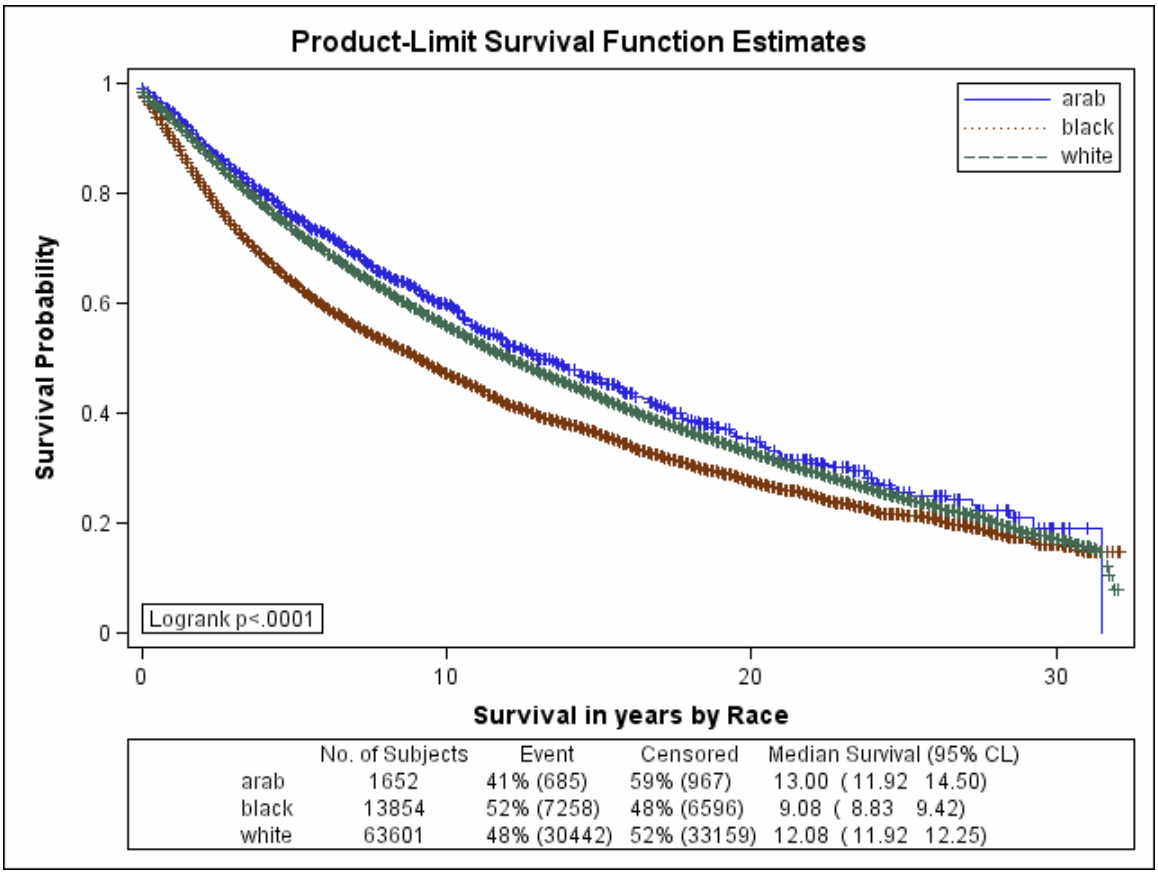


Figure 2.2b Kaplan-Meier Curves by Race for 5-year Overall Survival for In-Situ SEER Stage at Diagnosis

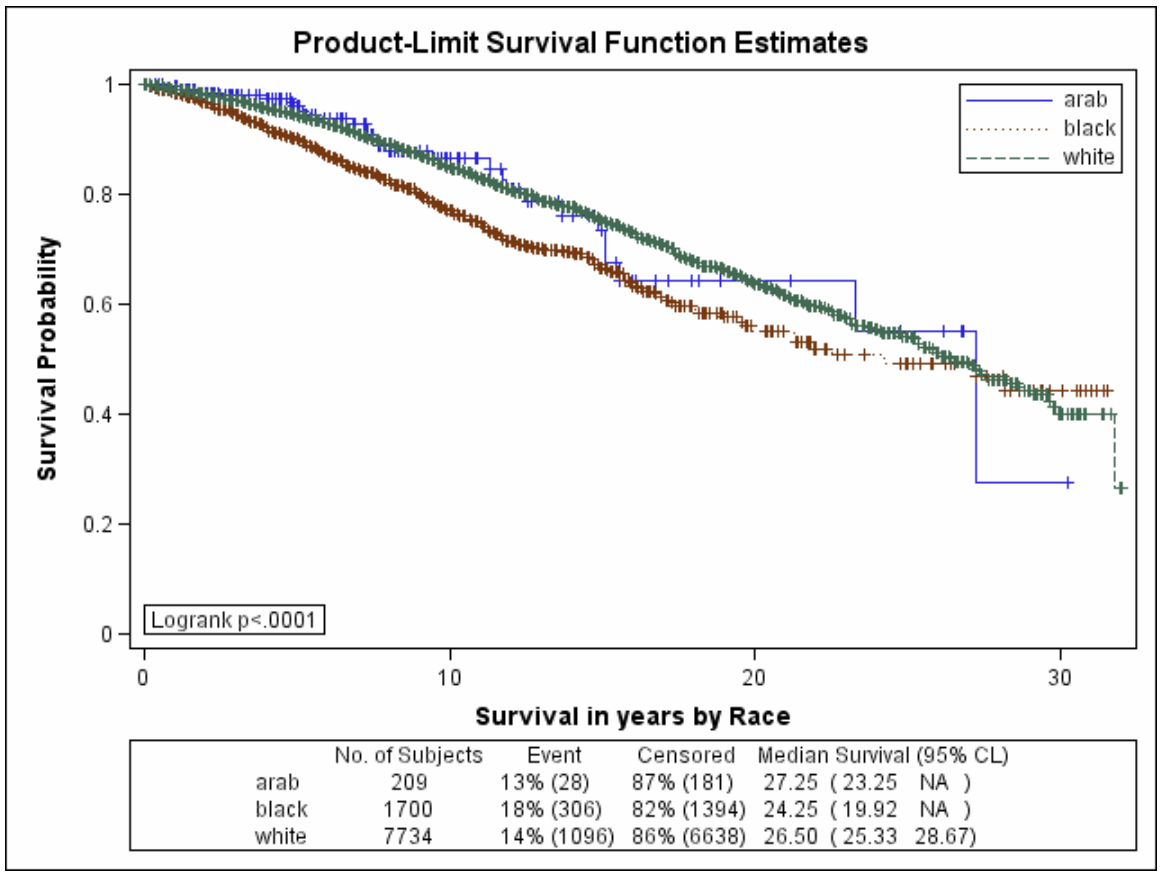


Figure 2.2c Kaplan-Meier Curves by Race for 5-year Overall Survival for Local SEER Stage at Diagnosis

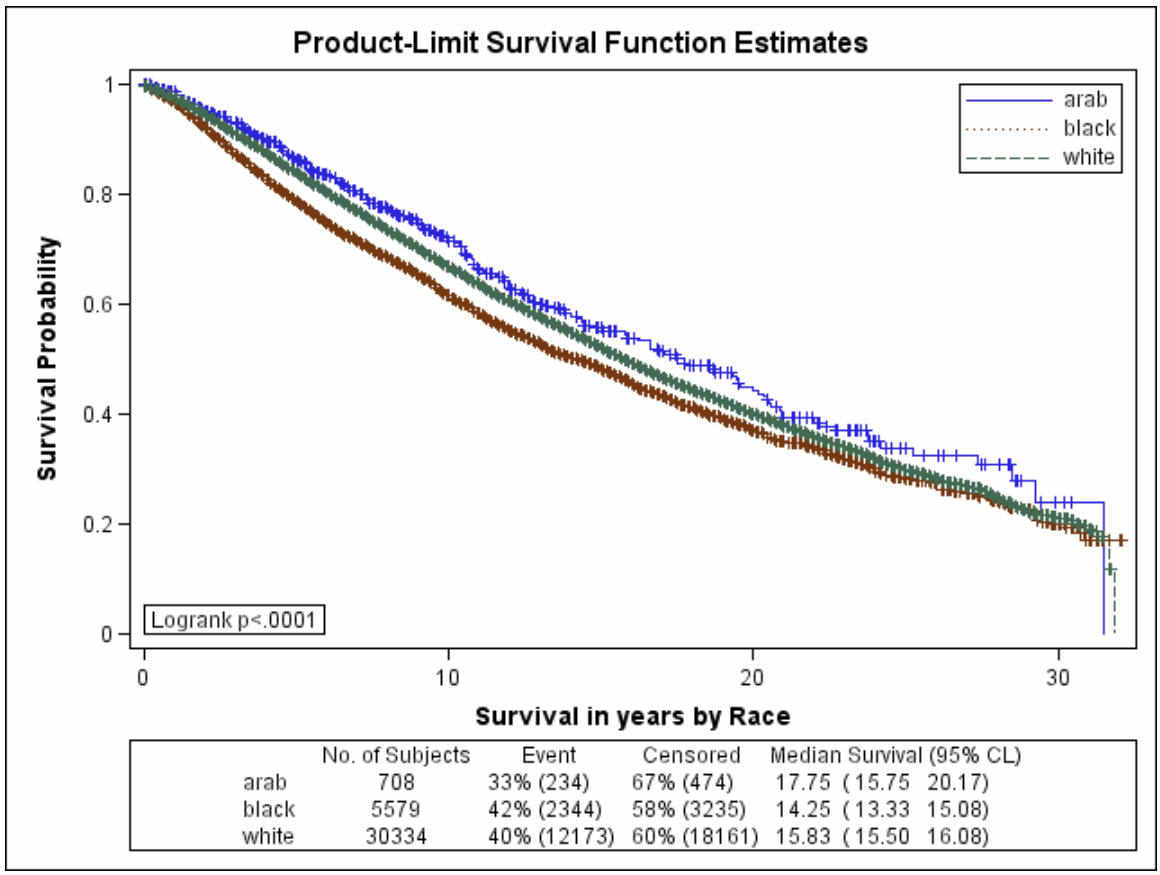


Figure 2.2d Kaplan-Meier Curves by Race for 5-year Overall Survival for Regional SEER Stage at Diagnosis

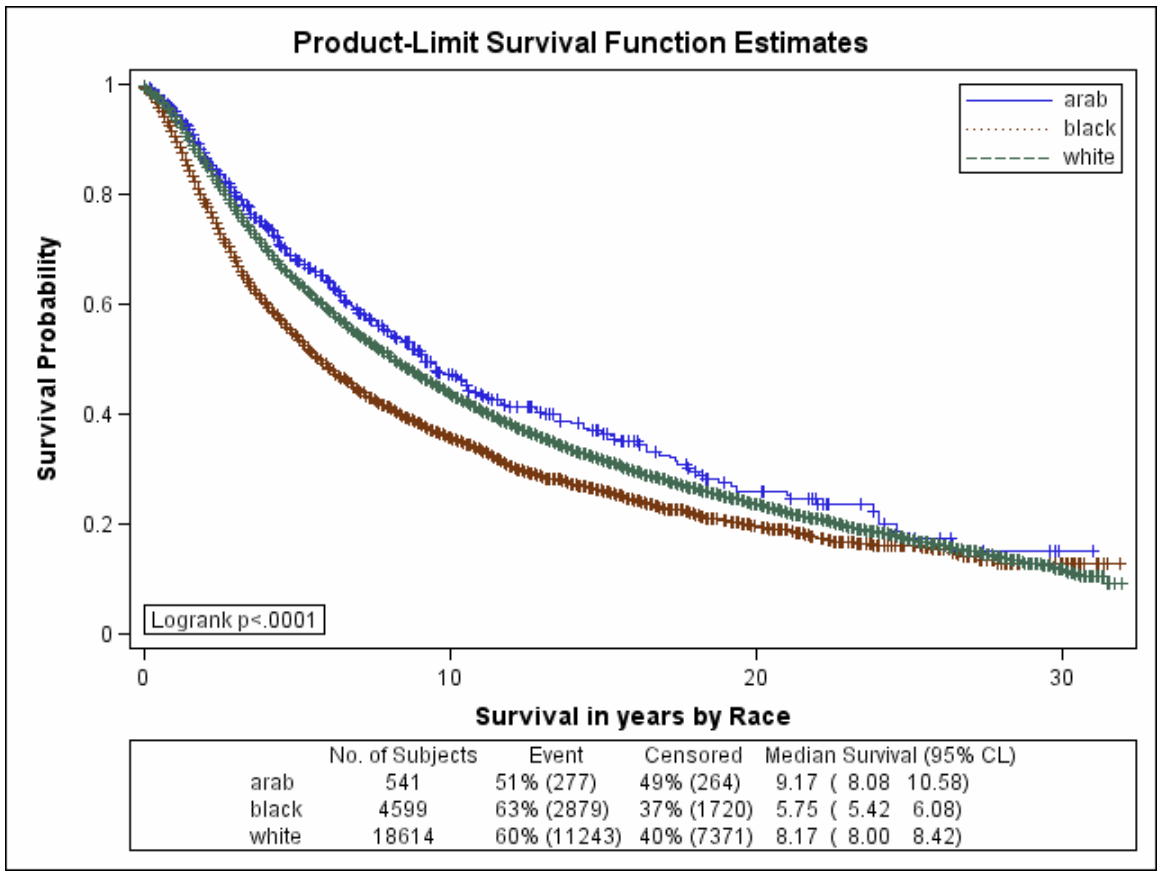


Figure 2.2e Kaplan-Meier Curves by Race for 5-year Overall Survival for Distant SEER Stage at Diagnosis

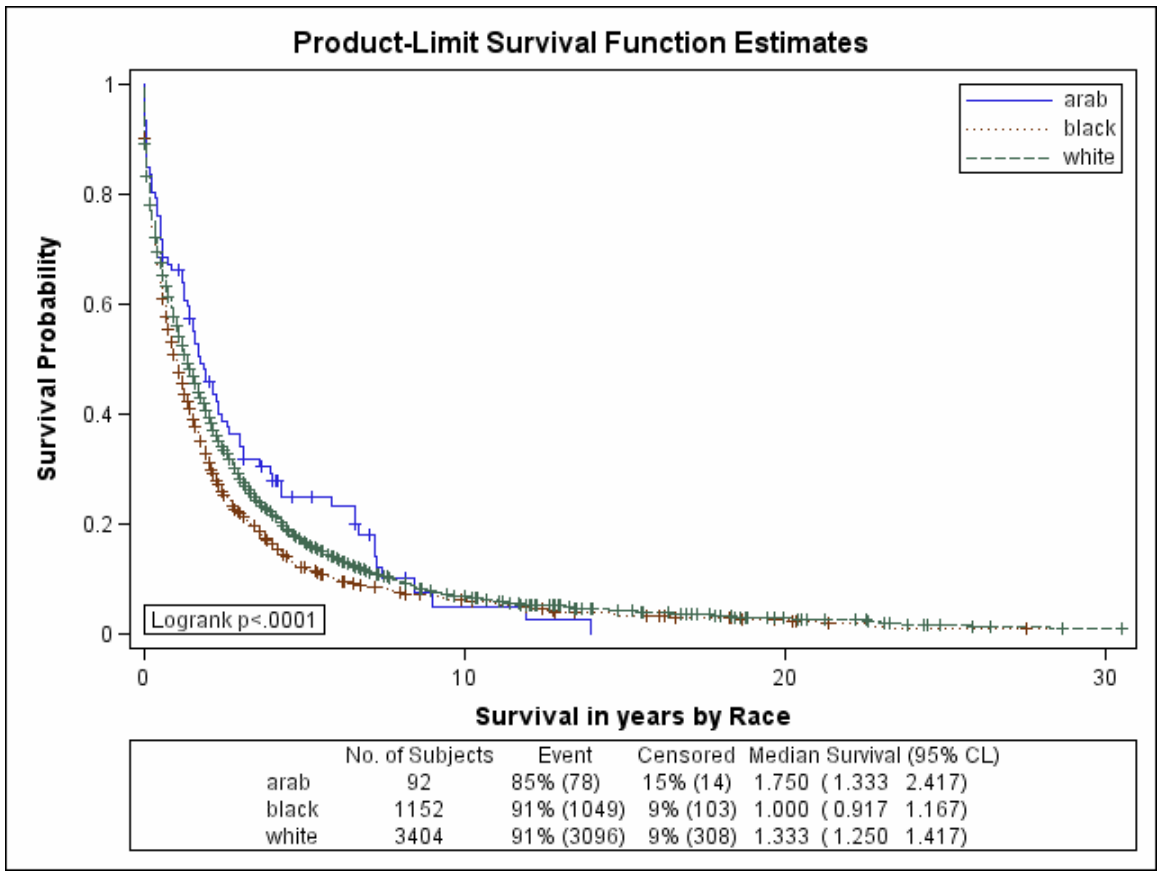
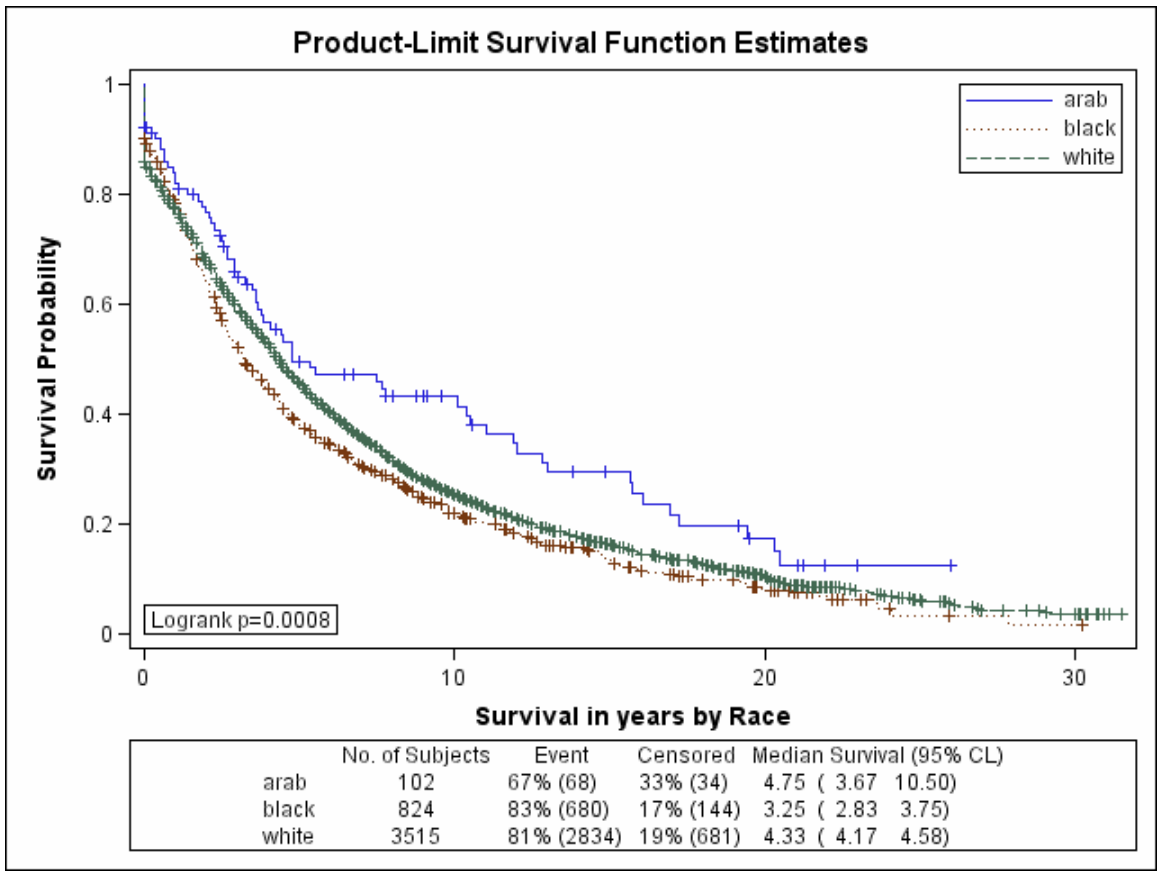


Figure 2.2f Kaplan-Meier Curves by Race for 5-year Overall Survival for Unknown SEER Stage at Diagnosis



References

Abdel-Rahman HA, Moustafa R, Shoulah ARS, Wassif OM, Salih MA, El-Gendy SD, Abdo AS (1993) An epidemiological study of breast cancer in greater Cairo. Health Assoc 68(1-2):119-142

Akhtar SS, Abu Bakr MA, Dawi SA, Ikram-ul-Huq (1993) Cancer in Libya- A retrospective study (1981-1985). Afr J Med Med Sci 22:17-24

Al-Idrissi HY, Ibrahim EM, Kurashi NY, Sowayan SA (1992) Breast cancer in a low-risk population. The influence of age and menstrual status on disease pattern and in survival in Saudi Arabia. Int J Cancer 52:48-51

Chiedozi LC, El-Hag IA, Kollur SM (2003) Breast diseases in the norther region of Saudi Arabia. Saudi Med J 6:623-627

Chlebowski RT, Chen Z, Anderson GL, Rohan T, Aragaki A, Lane D, Dolan NC, Paskett ED, McTiernan A, Hubbell A, Adams-Campvell LL, Prentice R (2005) Ethnicity and breast cancer: Factors influencing differences in incidence and outcome. Journal of the National Cancer Institute 97(6):439-448

Costa J, Webber BL, Levine PH, Muenz L, O'Connor GT, Tabbane F, Belhassen S, Kamaraju LS, Mourali N (1982) Histopathological features of rapidly progressing breast carcinoma in Tunisia: A study of 94 cases. Int Cancer 30:35-37

El Saghir NS, Adib S, Mufarru A, Kahwaji S, Taher A, Issa P, Shamseddine AI (1998) Cancer in Lebanon: Ananlysis of 10220 cases from the American University of Beirut medical center. Leb Med J 46:4-11

El Saghir NS, Shamseddine AI, Geara F, Bikhazi K, Rahal B, Salem ZMK, Taher A, Tawil A, El Khatib Z, Abbas J, Hourani M, Seoud M (2002) Age distribution of breast cancer in Lebanon: Increased percentages and age adjusted incidence rates of younger-aged groups at presentation. J Med Lib 50:3-9

Freedman LS, Edwards BK, Ries LAG, Young JL (eds) Cancer incidence in four member countries (Cyprus, Egypt, Israel, and Jordan) of the Middle East cancer consortium (MECC) compared with US SEER. National Cancer Institute. NIH Pub. No. 06-5873. Bethesday, MD

Hance KW, Anderson WF, Devesa SS, Young HA, Levine PH (2005) Trends in inflammatory breast carcinoma incidence and survival: The Surveillance, Epidemiology, and End Results program at the National Cancer Institute. Journal of the National Cancer Institute 97(13):966-975

Ibrahim EM, Al-Mulhim FA, Al-Amri A, Al-Muhanna FA, Ezzat AA, Stuart RK, Ajarim D (1998) Breast Cancer in the eastern province of Saudi Arabia. Med Onco 15:241-247

Karoub, J (2008) ABC News. Detroit expects half of Iraqi refugees. http://abcnews.go.com/print?id=3233636

Mourali N, Muenz LR, Tabbane F, Belhassen S, Bahi J, Levine PH (1980) Epidemiologic features of a rapidly progressing breast cancer in Tunisia. Cancer 46:2741-2746

Newman L (2005) Breast cancer in African-American women. Oncologist 10:1-14

Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DB (eds) (2003) Cancer incidence in five continents Vol. VIII. International Agency for Research in Cancer. IARC Scientific Publication No. 155. Lyon, France

Polite B, Olopade O (2005) Breast cancer and race: A rising tide does not lift all boats equally. Perspectives in Biology and Medicine 48(1):S166

Schwartz KL, Kulwicki A, Weiss LK, Fakhouri H, Sakr W, Kau G, Severson RK (2004) Cancer among Arab Americans in the metropolitan Detroit area. Ethnicity & Disease 14:141-146

Smigal C, Jemal A, Ward E, Cokkinides V, Smith R, Howe HL, Thun M (2006) Trends in breast cancer by race and ethnicity: Update 2006. A Cancer Journal for Clinicians 56:168-183

Soliman A, Bondy ML, Raouf AA, Makram MA, Johnston DA, Levin B (1999) Cancer mortality rates in Menofeia, Egypt: comparison with US mortality rates. Cancer Cases and Control 10:345-347

Tabbane F, El May A, Hachiche M, Bahi J, Jaziri J, Cammoun M, Mourali N (1985) Breast cancer in women under 30 years of age. Breast Cancer Res Treat 6:137-144

Tabbane F, Muenz L, Jazira M, Cammoun M, Belhassen S, Mourali N (1977) Clinical and prognostic features of a rapidly progressing breast cancer in Tunisia. Cancer 40:376-382

UN News Service (2007) UN officials lauds US decision to shelter 7,000 of the most vulnerable Iraqi refugees. 15 Feb 2007. http://www.un.org/apps/news/printnewsAr.asp?nid=21585

Wayne State University College of Urban, Labor, and Metropolitan Affairs. 2000 Census: Ethnic profile of Arab and Chaldean populations in metropolitan Detroit.

Chapter 3

Role of RhoC in Early Stage Breast Cancer

3.1 Background

Several studies, in vivo and in vitro, have demonstrated the important role of Rho family GTPases in the metastatic potential of certain breast cancers (Negrini et al. 2008; Hakem et al. 2005; Wu et al. 2004). This family of proteins, and RhoC in particular, have been associated with high grade, positive lymph nodes, Her2 overexpression, and negative hormonal receptor status (Kleer et al. 2005). Specifically, overexpression of RhoC has been associated with metastatic cellular characteristics including adhesion, invasion, and migration (Kusama et al. 2006; Lang et al. 2005; Simpson et al. 2004). As a group, the Rho proteins are involved in controlling cytoskeletal reorganization, cell motility, membrance ruffling, cell trafficking, and certain aspects of cellular proliferation and apoptosis (Pille et al. 2005; van Golen et al. 2002; van Golen et al. 2000).

RhoC overexpression has been strongly associated with inflammatory breast cancer (IBC), a particularly aggressive and lethal form of breast cancer (van Golen et al. 1999; van Golen et al. 2000; Kleer et al. 2004). A study of Egyptian IBC cases demonstrated a higher level of RhoC expression with the most phenotypically aggressive

IBC cases (Lo et al. 2008). It has been suggested that overexpression of RhoC be included in the definition of inflammatory breast cancer (van den Eynden et al. 2006). However, it has also been shown that RhoC expression in non-IBC small tumors is also associated with metastatic potential (Kleer et al. 2002) for patients treated uniformly.

In light of these data, we investigated how RhoC expression is integrated with other prognostic factors in early stage breast cancers and interrogated the sample for any association of RhoC expression with outcomes. We used a population-based cohort of patients identified from Henry Ford Hospital (HFH) in Detroit, MI. We hypothesized that increased RhoC expression in early stage disease might be predictive of aggressive disease with worse outcomes. The clinical resources available, including electronic medical records, archived surgical slides and blocks, and a SEER reporting tumor registry, as well as the availability of long-term patient follow-up made HFH a suitable setting for this type of research.

3.2 Methods

Using a managed health system tumor registry, we identified early stage breast cancer cases diagnosed and treated at Henry Ford Hospital (HFH) between 1996 and 2002. Stage I cases were identified between 1996 and 2002. We also collected node positive Stage II cases between 1999-2002. Medical record data were reviewed for all cases and available archived tumor tissue was retrieved. Using archived paraffin blocks we constructed tumor microarrays (TMAs) to assess the protein expression of RhoC in patient samples and compared this to patient and tumor characteristics abstracted from

the medical record. All aspects of this study were approved by the Institutional Review Boards at Henry Ford Hospital and University of Michigan.

Medical Record Abstraction

The Henry Ford Health System maintains an electronic medical record for each patient. The medical record captures all patient encounters and test results. For each patient identified through the HFH tumor registry, we reviewed the medical record to confirm eligibility. Patients were eligible for the study if they had been diagnosed and treated for a primary, initial invasive breast cancer. Patients were excluded if primary treatment was not received at HFH, the cancer was bilateral, patient was pregnant at the time of diagnosis, or if there was a prior breast cancer. We also excluded patients with any other clinically active malignancy. Medical records were reviewed for eligible cases to collect clinical-pathologic and demographic data. Variables abstracted included age at diagnosis, race, family history, gravidity, parity, age of first live birth, age at menarche, age at menopause, tumor characteristics, treatment received, and tumor recurrence. If Her2 status was not available in the medical record then we used immunohistochemistry (as discussed below) and then included this data in the analysis.

Microarray Construction and Immunohistochemistry

We used previously validated methods for tumor microarray (TMA) construction and RhoC staining (van den Eynden GG et al. 2004; Kleer et al. 2002). Briefly, archived paraffin-embedded tissue blocks and their corresponding H&E slides were retrieved from

storage in the Department of Pathology. Each H&E slide was reviewed by a breast pathologist to identify the most appropriate tissue sample available for tissue coring. Optimally, three 0.4 mm cores were taken from each patient's sample. Tissue cores where used to build high-density tumor microarrays (TMAs) which were cut to make 4μm slides for immunohistochemistry.

Immunohistochemistry was performed on the TMAs by using a standard biotin-avidin complex technique and a polyclonal antibody against RhoC that was previously validated by immunoblot and immunohistochemistry (Kleer et al. 2002). Since 3 core samples were obtained for each patient, the highest value of the 3 scores was used for subsequent analysis. At least two authors scored each tumor core blinded to the pathological or clinical characteristics of the case. As previously observed (Kleer et al. 2002), RhoC protein is strongly expressed in the cytoplasm of myoepithelial cells and vascular smooth muscle cells, which served as consistent internal positive controls. Cytoplasmic RhoC expression was scored from 0 to 3+ by comparison to the positive internal controls. Strong, diffuse staining was considered score =3+, whereas moderate and low diffuse staining was scored as 2 and 1, respectively. Negative staining was scored as 0. Based on previous work dealing with the biological characterization of RhoC as an oncogene, we defined high RhoC expression when there is strong or moderate staining (score=3 or 2) and low RhoC expression, when staining is weak or negative (scores=0-1).

Statistical Analysis

The *a priori* planned analysis was to explore the relationship between RhoC+ expression with each clinicopathologic variable available for the cohort. For each variable, we assessed the association with RhoC expression using chi-square and logistic regression. Survival analysis was conducted using Kaplan-Meier curves for any recurrence and development of distant metastases. Cox Proportional Hazards Models supplement the Kaplan-Meier curves for analysis of the outcomes. Hazard ratios (HR) were calculated for univariate and multivariate analyses. Finally, we compared the clinico-pathologic variables of interest between eligible cases included on the TMAs vs. not included on a TMA.

3.3 Results

We identified a total of 906 cases through the HFH tumor registry that met the stage and year of diagnosis criteria for inclusion in the study. Of these, 637 were Stage I cases and 269 were node positive Stage II cases (Figure 3.1). After medical record review, 137 (15%) were excluded from the study because they did not received all treatment at HFH or, for example, they had another active malignancy. Of the 769 eligible cases, tumor specimens were not available for 233 (30%). Of the 536 cases with blocks available we have successfully stained and scored 379 (71%). Table 3.1 gives the p-vlaues of the $\chi 2$ analysis comparing eligible cases included on the TMAs and those eligible cases not available for TMA analysis. There were significantly more ER negative, high grade, and

larger tumors included on the TMA. More of the TMA cases had distant metastasis. There were also fewer cases with family history.

To explore the association of RhoC with patient characteristics, tumor characteristics, and outcomes of interest, we calculated $\chi 2$ statistics and univariate logistic regression odds ratios (OR) with 95% confidence interval (CI). The $\chi 2$ results are present in Tables 3.2 and 3.3. The univariate results from the logistic regression are presented in Table 3.4. There was not a significant association with any characteristic considered except for estrogen-receptor (ER) and progesterone-receptor (PR) status. Those with ER positive tumors were 2.47 times more likely to have RhoC positive tumors (95% CI 1.51-4.04). Likewise, those with PR positive tumors were 1.84 times more likely to have RhoC positive tumors (95% CI 1.15-2.93).

Results of the Kaplan-Meier analysis are presented for any recurrence in Figure 3.2 and for distant metastases in Figure 3.3. The log-rank p-value for the association of RhoC expression with any recurrence and distant metastasis was 0.12 and 0.08, respectively. Because RhoC expression was associated with ER, we stratified the Kaplan-Meier analysis by ER status. The log-rank p-value for any recurrence for ER positive and negative tumors was 0.12 and 0.76, respectively (Figures 3.4 and 3.5). The log-rank p-value for distant metastasis was 0.21 for ER positive tumors and 0.81 for ER negative tumors (Figures 3.6 and 3.7). Given the results of the stratified analysis, there was not evidence of an interaction between ER or PR status and RhoC expression.

We calculated univariate Cox Proportional Hazards and 95% CI for both outcomes. The hazard ratio (HR) was 0.62 (95% CI 0.35-1.12) for any recurrence and 0.50 (95% CI

0.23-1.11) for distant metastasis. These results are presented in Table 3.5 with the multivariate analysis.

3.4 Discussion

RhoC has been shown in a number of previous studies to be an important marker of aggressive disease. Based on this body of previous work, we hypothesized that RhoC expression in early stage disease (Stage I and Stage IIA) would be associated with recurrence, particularly distant metastases. Having included approximately 49% of the analyzable samples, our results are so far inconclusive. Several factors may contribute to an explanation of why our results are not conclusive. First, our study population was limited to very early stage disease. In particular, the T1 N1 samples were rare in our cohort. All prior work with human tissue has been among either exclusively later stage and inflammatory breast cancer cases (Lo et al. 2008; van den Eynden et al. 2006; van den Eynden et al. 2004; Turpin et al. 2002; van Golen et al. 2002) or with the full spectrum of disease (Kleer et al. 2005; Pan et al. 2005; Kleer et al. 2002). Kleer et al. (2002) did find a significant association between RhoC expression and metastasis among cases where the primary tumor was < 1 cm. In that study, less than 20% of the Stage I cases positively expressed RhoC. In contrast, 69% of our study's Stage I and Stage IIA cases positively expressed RhoC. Furthermore, only 49% of our eligible samples were analyzed. Moreover, the samples that were not analyzed were statistically significantly different from the analyzed samples in terms of ER status, distant metastasis, family history information, tumor grade and tumor size. Therefore, it is possible that we had a

selection bias that affected the possible dynamic range of outcomes. This impacts the potential significance we can achieve.

Another reason that our results may turn out to be discordant with previously published results may be due to the short observation period for this stage of disease, where prognosis is very good; thus, we captured very few events. Indeed, there were only 87 recurrences within our cohort (11%) and even fewer distant metastases (n=25; 5%). Our cohort had 260 cases (69% of n=379) with RhoC overexpression which means we had 83% power to detect a rate ratio of 1.8 or greater assuming 10% of early stage cases will develop a recurrence within this period. If RhoC expression can potentially discern aggressive from non-aggressive early stage disease, more power may be needed in order to decipher the true relationship. In addition, we point out that our study did not show the well known difference in outcomes between ER positive and ER negative early stage breast cancers, a strong indication that our study was underpowered to discern more subtle differences.

Studies by Kleer et al. (2005) and Cestac et al. (2005) have demonstrated an association between hormonally negative tumors and RhoC overexpression. However, our study showed a positive association with ER and PR expression and RhoC. Kleer et al. (2005) used 280 tissue samples from a wide spectrum of breast disease ranging from normal noncancerous breast tissue to tissue from metastasic sites. She and her colleagues reported that high RhoC expression was associated with several features typical of aggressive breast cancer, including high grade, positive lymph nodes, and negative hormonal receptor status. Cestac et al. used transfected cell lines to demonstrate

prenylated proteins RhoA, RhoB and/or RhoC antagonize the ability of the cancer cell to stimulate ER transcriptional activity. We found that ER positive tumors were 0.69 times less likely to develop a recurrence (95% CI 0.43-1.12) in our cohort. The fact that this is not significant may reflect the variation in ER histological assessments from 1996 to 2002; meaning, that perhaps the quality, accuracy, or reporting methods for clinical ER status may not have been consistent over time. In recent years, more and more studies of prognostic markers are restaining samples at standardized labs to avoid this variability.

The median observation time for our cohort was 6.9 years with a range of 2 weeks to 12 years. In total, our cohort represents 2,514 person-years of observation, which is a longer observation time than other RhoC cohorts have reported so far. All prior human studies have been from major referral hospital populations, whereas this study is from a health system that provides primary and specialty care to a stable patient population; therefore, it is less likely that bias toward more aggressiveness cancer is inherent in our data. It is a possibility that this study is a closer representation of the true RhoC distribution in a population-based cohort, with nearly one-third of cases being African-American, of early stage breast cancer.

With polyclonal antibodies, certain variability in antibody affinity is possible depending on processing equipment. Our study used a non-commercial antibody which may have introduced greater variability in the results as different lots were employed in this study than had been used previously. In addition, it is also possible that there was some variability in the staining techniques that may also have contributed to our lack of statistically significant or predicted findings. Our results represent work with 4 different

TMAs that were stained on three separate occasions which could have potentially affected the consistency. To test this, we did run the results with the two TMAs that were stained simultaneously. The results did not materially change (data not shown). In addition, we ran the results counting only 3+ scores as positive (as opposed to 2-3+) in an effort to account for possible variation in antibody affinity. Again, the results did not change substantially (data not shown).

The potential for RhoC to be a significant predictor of subsequent recurrence and/or distant metastasis for T1 breast cancers remains unresolved based on our study. Future studies should consider a larger sample size with a broader representation in stage, especially important would be inclusion of more T1 N1 cases and a longer observation period. The exclusion of later stage cases from our study limited our comparisons with other studies. Inclusion of later stages would have allowed us to confirm our findings in this more studied group.

Table 3.1 Comparison of Patient and Tumor Characteristics as well as Recurrence Outcomes for Cases Included on the TMA vs. Not

Characteristic	$\chi 2$ p-value	on TMA
ER status	0.03	more ER neg
PR status	0.53	
Her2 status	0.87	
Recurrence	0.09	more recurrence
Distant Mets	0.008	more mets
Stage	0.09	more Stage I
Family HX	0.008	less family hx
Family HX 1st degree	0.18	
Grade	0.004	more high grade
Race	0.89	
Node status	0.07	less node positive
Age of 1st birth	0.25	
Age of menarche	0.98	
Tumor size	0.0002	more $\geq$1 cm
Age at diagnosis	0.62	
Parity	0.84	

Table 3.2 Study Population Characteristics

Characteristic	N (%)	RhoC+	RhoC-	$\chi 2$ p-value
Menarche				
≤ 12	179 (47%)	121 (47%)	58 (49%)	
> 12	200 (53%)	139 (53%)	61 (51%)	0.69
Age of First Birth				
≤ 30	247 (65%)	170 (65%)	77 (65%)	
> 30	132 (35%)	90 (35%)	42 (35%)	0.90
Parity[1]				
Childless	57 (28%)	39 (27%)	18 (30%)	
More than 1 child	148 (72%)	106 (73%)	42 (70%)	0.65
Race				
White	254 (68%)	176 (69%)	78 (67%)	
Black	117 (32%)	78 (31%)	39 (33%)	0.61
Age at Diagnosis				
≤ 50	83 (22%)	60 (23%)	23 (19%)	
> 50	296 (78%)	200 (77%)	96 (81%)	0.41
Family History[2]				
Any	147 (39%)	96 (37%)	51 (43%)	
None	232 (61%)	164 (63%)	68 (57%)	0.27
Family History[2]				
Any 1st Degree Relative	82 (22%)	56 (22%)	26 (22%)	
None or No 1st Degree	297 (78%)	204 (78%)	93 (78%)	0.95

[1] Parity was missing for several cases.
[2] Family History of breast and/or ovarian cancer.

Table 3.3 Breast Cancer Characteristics and Outcomes for Study Population

Characteristic	N(%)	RhoC+	RhoC-	$\chi 2$ p-value
Stage				
I	330 (87%)	229 (88%)	101 (85%)	
II (T1, N1)	49 (13%)	31 (12%)	18 (15%)	0.39
Grade				
High (=3)	116 (31%)	74 (28%)	42 (35%)	
Low (<3)	203 (69%)	186 (72%)	77 (65%)	0.18
Tumor Size				
≤ 1 cm	122 (32%)	89 (34%)	33 (28%)	
> 1 cm	257 (68%)	171 (66%)	86 (72%)	0.21
Nodal Status				
Positive	51 (13%)	33 (13%)	15 (15%)	
Negative	327 (87%)	226 (87%)	101 (85%)	0.53
Estrogen Receptor				
Positive	290 (77%)	213 (82%)	77 (65%)	
Negative	89 (23%)	47 (18%)	42 (35%)	0.0002
Progesterone Receptor				
Positive	272 (72%)	197 (76%)	75 (63%)	
Negative	107 (28%)	63 (24%)	44 (37%)	0.01
Her2neu Status				
Positive	74 (20%)	52 (21%)	22 (19%)	
Negative	289 (80%)	196 (79%)	93 (81%)	0.69
Recurrence				
Any	51 (13%)	32 (12%)	19 (16%)	
None	328 (87%)	228 (88%)	100 (84%)	0.33
Distant Metastasis				
Yes	25 (7%)	14 (5%)	11 (9%)	
No	354 (93%)	246 (95%)	108 (91%)	0.16

Table 3.4 Unadjusted Odds Ratios and 95% Confidence Intervals (CI) for Patient Characteristics, Tumor Characteristics and Outcomes with RhoC Overexpression

Patient characteristics	N	OR	95% CI
Age of menarche	379	1.09	0.71-1.69
Parity (0 vs. any)	205	1.17	0.60-2.26
Fam HX (any)	379	0.78	0.50-1.21
Fam HX (first degree)	379	0.98	0.58-1.66
Age first birth	379	0.97	0.62-1.53
Age at diagnosis (<50 vs. ge 50)	379	0.80	0.47-1.37
Race (black vs. white)	371	0.89	0.56-1.42
Tumor Characteristics			
ER status (pos vs. neg)	379	2.47	1.51-4.04
PR status (pos vs. neg)	379	1.84	1.15-2.93
Her2 status (pos vs. neg)	363	1.12	0.64-1.96
Size (≤ 1cm vs. >1 cm)	379	0.65	0.39-1.06
Nodal status (pos vs. neg)	378	0.82	0.44-1.52
Grade (high vs. not)	379	0.73	0.46-1.16
Stage (I vs. IIA)	379	0.76	0.41-1.42
Outcomes			
Any Recurrence	379	0.74	0.40-1.37
Distant Mets	379	0.56	0.25-1.27

Table 3.5 Unadjusted and Adjusted Hazard Ratios (HR and aHR, respectively) for Any Recurrence and Distant Metastasis

	HR (95% CI)	aHR[1] (95% CI)
Any Recurrence	0.62 (0.35-1.12)	0.66 (0.36-1.23)
Distant Metastasis	0.50 (0.23-1.11)	0.59 (0.25-1.39)

aHR[1]: adjusting for ER, PR, Her2, tumor size, nodal status, grade, and race

Figure 3.1 Data flow

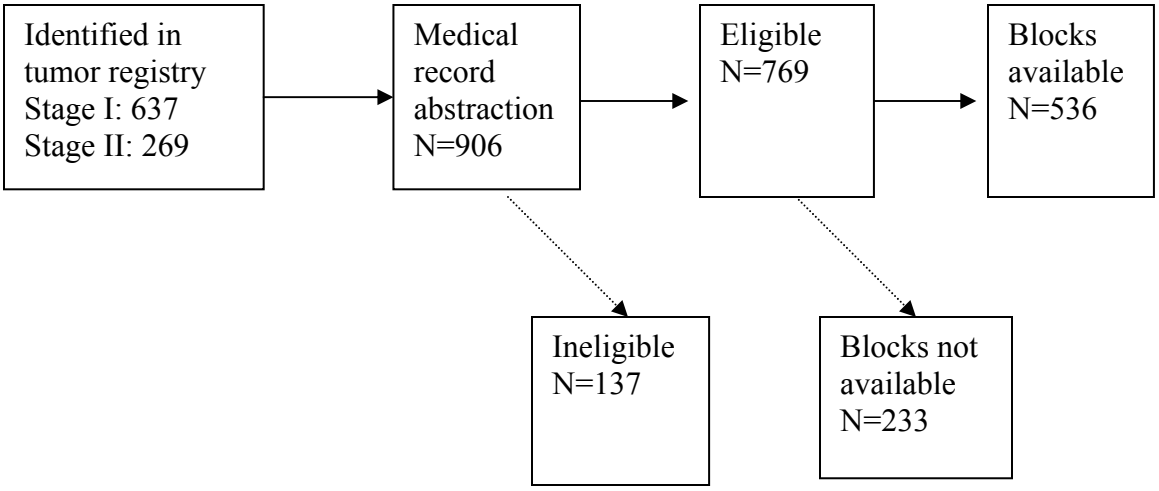


Figure 3.2 Kaplan-Meier Curve for Recurrence by RhoC Status

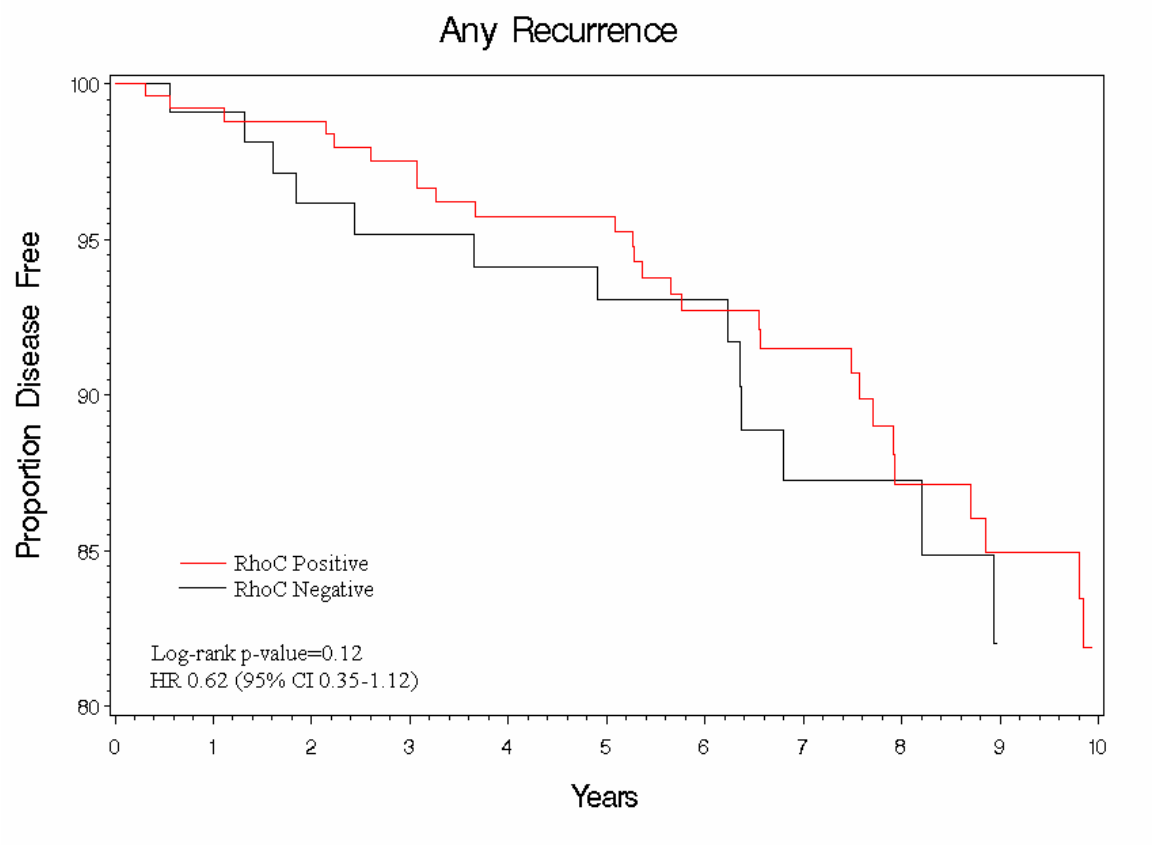


Figure 3.3 Kaplan-Meier Curve for Distant Metastasis by RhoC Status

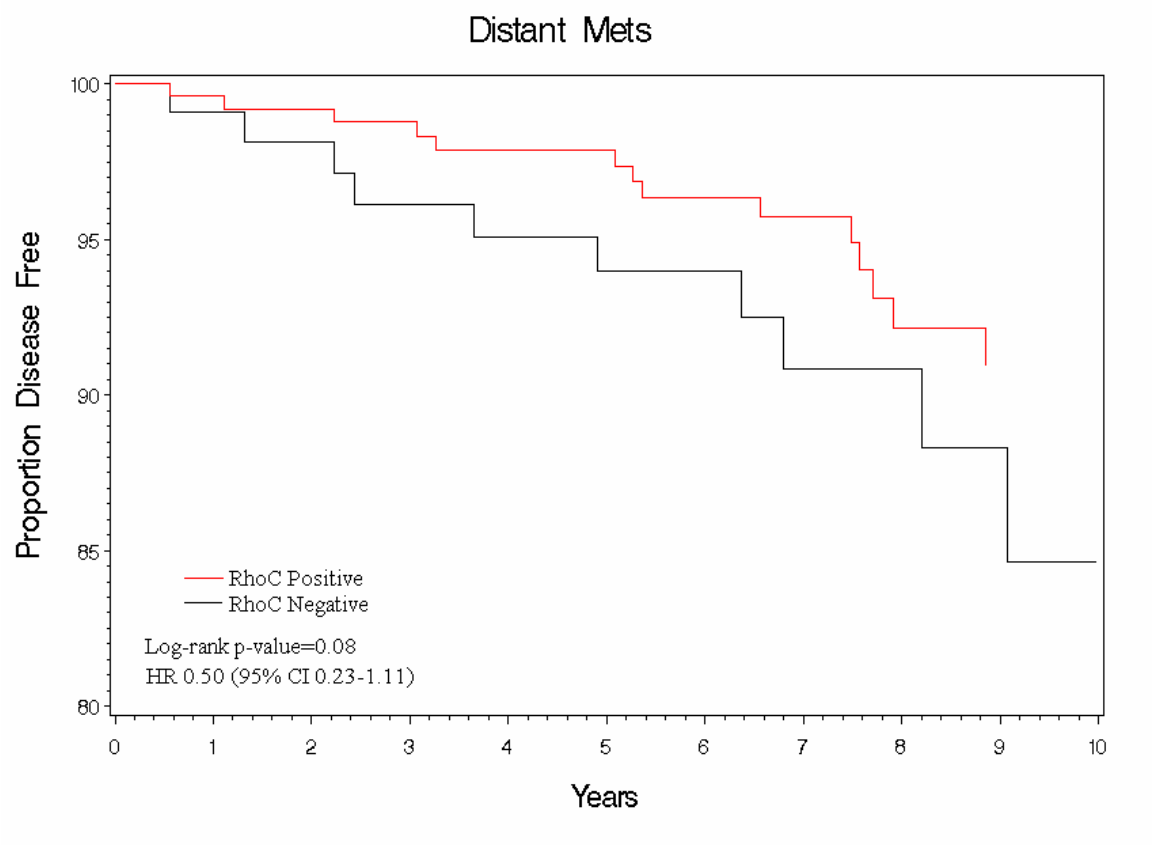


Figure 3.4 Kaplan-Meier Curve for Any Recurrence Among ER Positive Cases

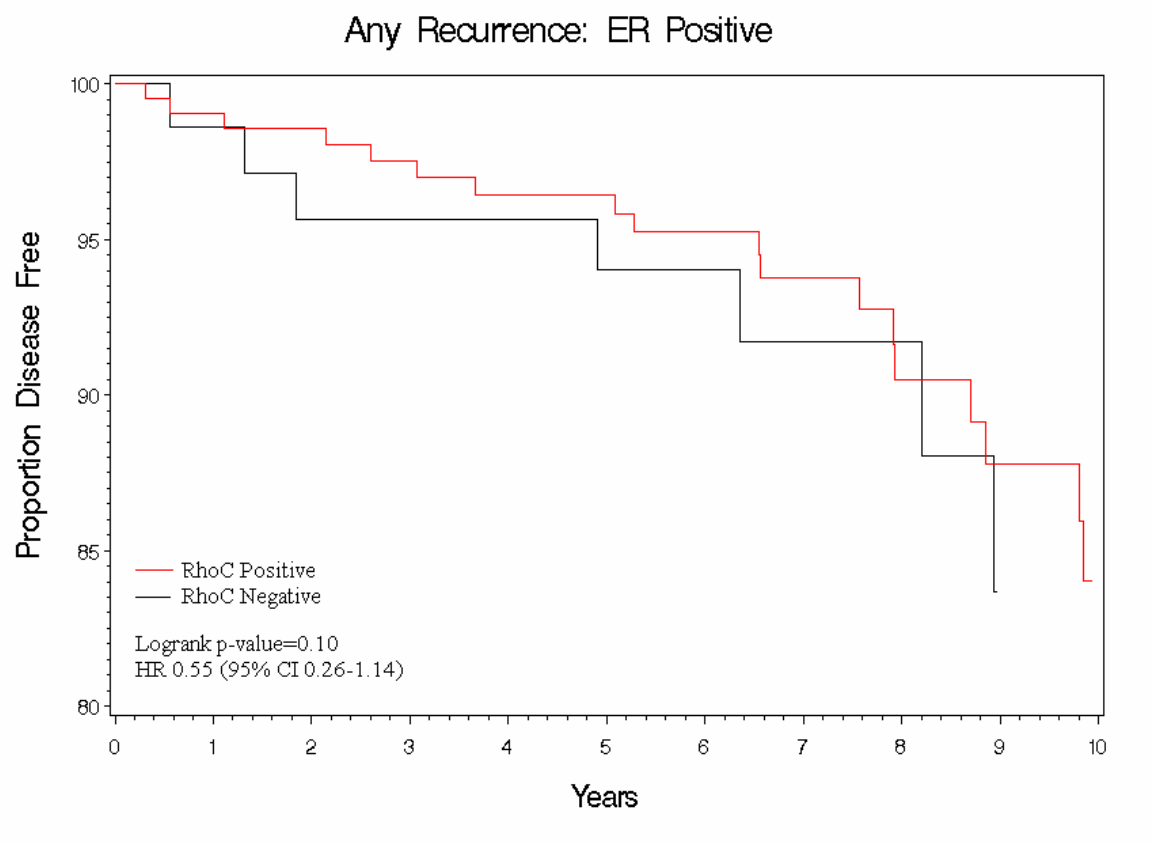


Figure 3.5 Kaplan-Meier Curve for Any Recurrence Among ER Negative Cases

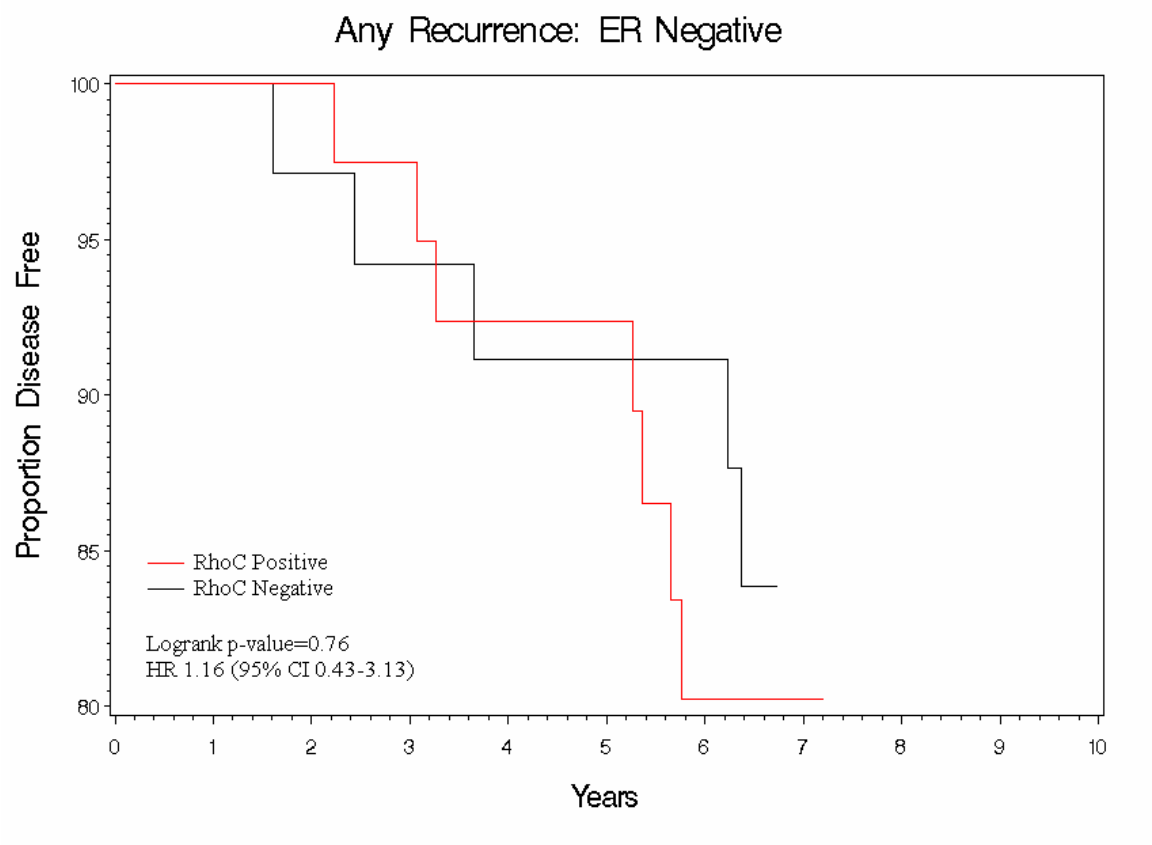


Figure 3.6 Kaplan-Meier Curve for Distant Metastasis Among ER Positive Cases

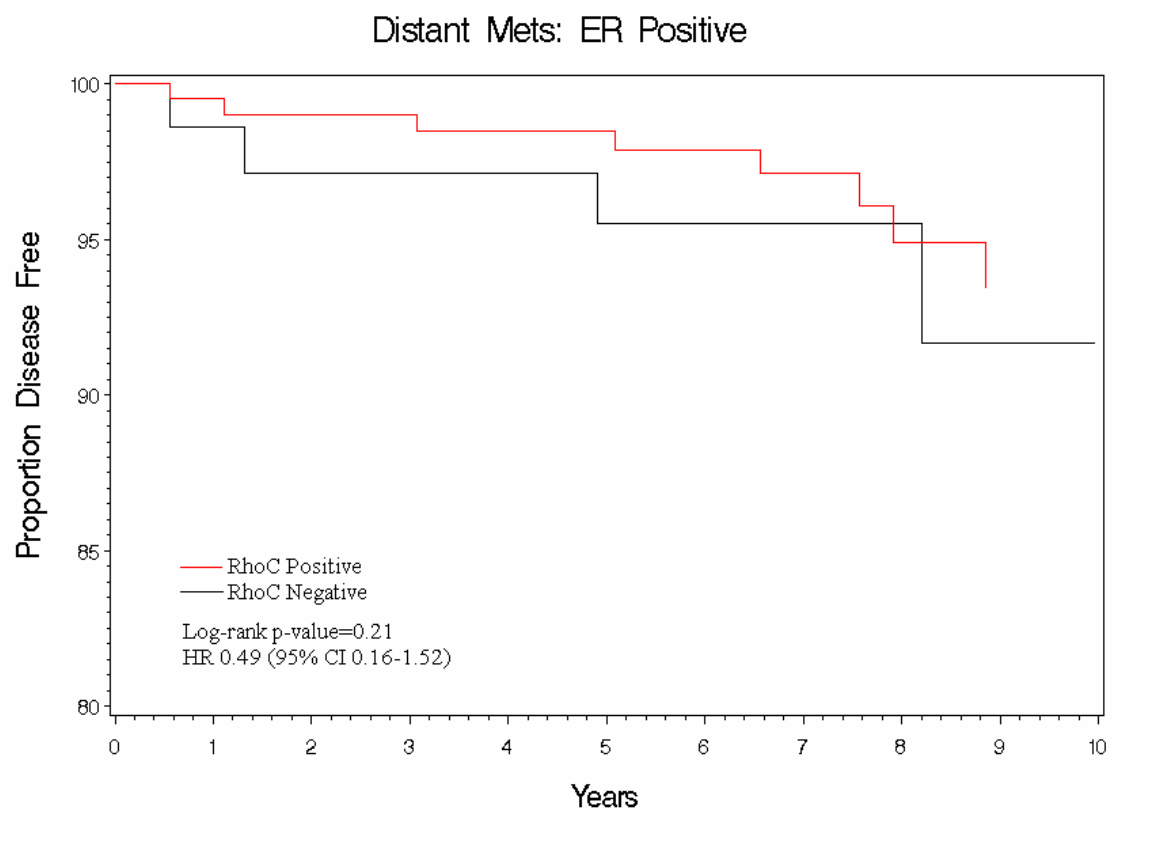
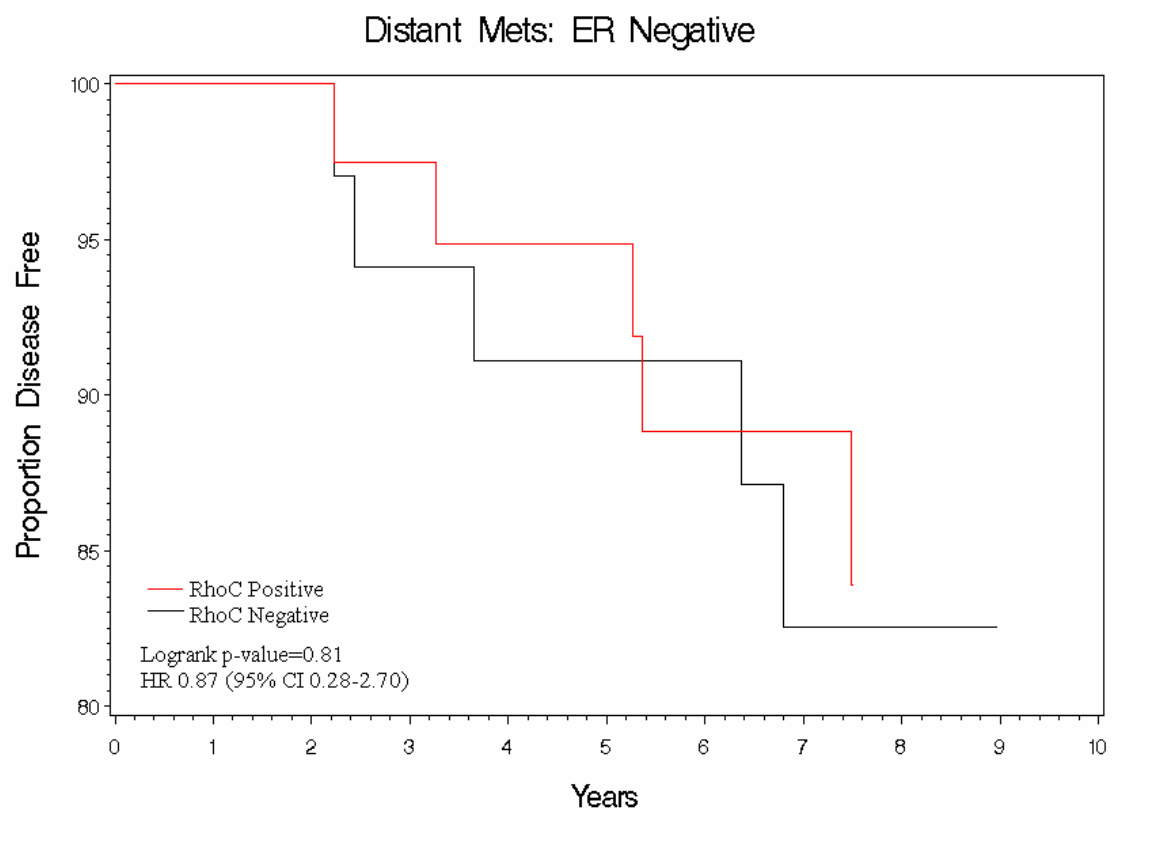


Figure 3.7 Kaplan-Meier Curve for Distant Metastasis Among ER Negative Cases



References

Cestac P, Sarrabayrouse G, Medale-Giamarchi C, Rochaix P, Balaguer P, Favre G, Faye JC, Doisneau-Sixou S. (2005) Prenylation inhibitors stimulate both estrogen receptor alpha transcriptional activity through AF-1 and AF-2 and estrogen receptor beta transcriptional activity. Breast Cancer Research 7(1):R60-70.

Hakem A, Sanchez-Sweatman O, You-Ten A, Duncan G, Wakeham A, Khokha R, Mak TW. (2005) RhoC is dispensable for embryogenesis and tumor initiation but essential for metastasis.

Kleer CG, Griffith KA, Sabel MS, Gallagher G, van Golen KL, Wu ZF, Merajver SD. (2005) RhoC-GTPase is a novel tissue biomarker associated with biologically aggressive carcinomas of the breast. Breast Cancer Research and Treatment 93(2):101-10.

Kleer CG, Zhang Y, Pan Q, Gallagher G, Wu M, Wu ZF, Merajver SD. (2004) WISP3 and RhoC guanosine triphosphatase cooperate in the development of inflammatory breast cancer. Breast Cancer Research 6(1):R110-5.

Kleer CG, van Golen KL, Zhang Y, Wu ZF, Rubin MA, Merajver SD. (2002) Characterization of RhoC expression in benign and malignant breast disease: a potential new marker for small breast carcinomas with metastatic ability. American Journal of Pathology 160(2):579-84.

Kusama T, Mukai M, Tatsuta M, Nakamura H, Inoue M. (2006) Inhibition of transendothelial migration and invasion of human cells by preventing geranylgeranylation of Rho. International Journal of Oncology 29(1):217-23.

Lang JY, Chen H, Zhou J, Zhang YX, Zhang XW, Li MH, Lin LP, Zhang JS, Waalkes MP, Ding J. (2005) Antimetastatic effect of salvicine on human breast cancer MDA-MB-435 orthotopic xenograft is closelyh related to Rho-dependent pathway. Clinical Cancer Research 11(9):3455-64.

Lo AC, Lkeer CG, Banerjee M, Omar S, Khaled H, Eissa S, Hablas A, Douglas JA, Alford SH, Merajver SD, Soliman AS. (2008) Molecular epidemiologic features of inflammatory breast cancer: a comparison between Egyptian and US patients. Breast Cancer Research and Treatment 112(1):141-7.

Negrini M and Calin GA. (2008) Breast cancer metastasis: a microRNA story. Breast Cancer Research 10(2):203.

Pan Q, Bao LW, Kleer CG, Sabel MS, Griffith KA, Teknos TN, Merajver SD. (2005) Protein kinase C epsilon is a predictive biomarker of aggressive breast cancer and a

validated target for RNA interference anticancer therapy. Cancer Research 65(18):8366-71.

Pille JY, Denoyelle C, Varet J, Bertrand JR, Soria J, Opolon P, Lu H, Pritchard LL, Vannier JP, Malvy C, Soria C, Li H. (2005) Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. Molecular Therapy 11(2):267-74.

Simpson KJ, Dugan AS, Mercurio AM. (2004) Functional analysis of the contribution of RhoA and RhoC GTPase to invasive breast carcinoma. Cancer Research 64(23):8694-701.

Turpin E, Bieche I, Bertheau P, Plassa LF, Lerebours F, de Roquancourt A, Olivi M, Espie M, Marty M, Lidereau R, Vidaud M, de The H. (2002) Increased incidence of ERBB2 overexpression and TP53 mutation in inflammatory breast cancer. Oncogene 21(49):7593-7.

Van den Eynden GG, Van Laere SJ, Van der Auwera I, Merajver SD, Van Marck EA, van Dam P, Vermeulen PB, Dirix LY, van Golen KL. (2006) Overexpression of caveolin-1 and -2 in cell lines and human samples of inflammatory breast cancer. Breast Cancer Research and Treatment 95(3):219-28.

Van den Eynden GG, Van der Auwer I, Van Laere S, Colpaert CG, van Dam P, Merajver S, Kleer CG, Harris AL, Van Marck EA, Dirix LY, Vermeulen PB. (2004) Validation of a tissue microarray to study differential protein expression in inflammatory and non-inflammatory breast cancer. Breast Cancer Research and Treatment 85(1):13-22.

Van Golen KL, Bao LW, Pan Q, Miller FR, Wu ZF, Merajver SD. (2002) Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer. Clinical and Experimental Metastasis 19(4):301-11.

Van Golen KL, Wu ZF, Qiao XT, Bao L, Merajver SD. (2000) RhoC GTPase overexpression modulates induction of angiogenic factors in breast cells. Neoplasia 2(5):418-25.

Van Golen KL, Wu ZF, Ziao XT, Bao LW, Merajver SD. (2000) RhoC GTPase, a novel transforming oncogene for human mammary epithelial cells that partially recapitulates the inflammatory breast cancer phenotype. Cancer Research 60(20):5832-8.

Van Golen KL, Davies S, Wu ZF, Wang Y, Bucana CD, Root H, Chandrasekharappa S, Strawderman M, Ethier SP, Merajver SD. (1999) A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer),

and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clinical Cancer Research 5(9):2511-9.

Wu M, Wu ZF, Kumar-Sinha C, Chinnaiyan A, Merajver SD. (2004) RhoC induces differential expression of genes involved in invasion and metastasis in MCF10A breast cells. Breast Cancer Research and Treatment 84(1):3-12.

Chapter 4

EZH2 as a Potential Breast Cancer Prognostic Marker

4.1 Background

The Polycomb Group Protein EZH2 (Enhancer of zeste-2) is a histone methyltransferase involved in controlling cellular memory. Overexpression and/or gene amplification has been associated with a number of cancer types. (Rajasekhar VK and Begemann M 2007; Bachmann et al. 2006; Wei Y et al. 2008; Yu J et al. 2007) EZH2 appears to be involved with cellular proliferation and DNA repair. (Reynolds PA et al. 2006; Collett K et al. 2006; Zeidler M et al. 2005; Bachmann IM et al. 2006) Several studies have considered EZH2's effects on the development and progression of breast cancer. In breast cancer, EZH2 overexpression has been associated with poorly differentiated tumors (Raaphorst FM et al. 2003) and estrogen receptor (ER) negativity (Gonzalez ME et al. 2009; Hwang C et al. 2007; Hwang C et al. 2008). In addition, EZH2 has been linked to locally advanced disease, distant metastasis, and decreased survival. (Collett K et al. 2006; Arnes JB, et al. 2008; Wei Y et al. 2008)

Given these prior studies, we hypothesized that over-expression of EZH2 would result in worse outcomes in early stage breast cancer (Stage I and IIA). We evaluated

EZH2 status in 373 breast cancer cases and assessed the relationship of expression with clinicopathologic variables at diagnosis and disease outcomes over a 10 year period.

4.2 Methods

Using a managed health system tumor registry, we identified early stage breast cancer cases diagnosed and treated at Henry Ford Hospital (HFH) between 1996 and 2002. Stage I cases were identified between 1996 and 2002. We also collected node positive Stage II cases between 1999-2002. Medical record data were reviewed for all cases and available archived tumor tissue was retrieved. Using archived paraffin blocks we constructed tumor microarrays (TMAs) to assess the protein expression of EZH2 in patient samples and compared this to patient and tumor characteristics abstracted from the medical record. All aspects of this study were approved by the Institutional Review Boards at Henry Ford Hospital and University of Michigan.

Medical Record Abstraction

The Henry Ford Health System maintains an electronic medical record for each patient. The medical record captures all patient encounters and test results. For each patient identified through the HFH tumor registry, we reviewed the medical record to confirm eligibility. Patients were eligible for the study if they had been diagnosed and treated for a primary, initial invasive breast cancer. Patients were excluded if primary treatment was not received at HFH, the cancer was bilateral, patient was pregnant at the time of diagnosis, or if there was a prior breast cancer. We also excluded patients with

any other clinically active malignancy. Medical records were reviewed for eligible cases to collect clinical-pathologic and demographic data. Variables abstracted include age at diagnosis, race, family history, gravidity, parity, age of first live birth, age at menarche, age at menopause, tumor characteristics, treatment received, and tumor recurrence.

Microarray Construction and Immunohistochemistry

We used previously validated methods for tumor microarray (TMA) construction and EZH2 staining. (van den eynden GG et al. 2004; Dhanasekara et al. 2001; Perrone EE et al. 2000; Varambally S et al. 2002) Briefly, archived paraffin-embedded tissue blocks and their corresponding H&E slides were retrieved from storage in the Department of Pathology. Each H&E slide was reviewed by a breast pathologist to identify the most appropriate tissue sample available for tissue coring. Optimally, three 0.4 mm cores were taken from each patient's sample. Tissue cores where used to build high-density tumor microarrays (TMAs) which were cut to make 4μm slides for immunohistochemistry.

Immunohistochemistry was performed on the TMAs by using a standard biotin-avidin complex technique and by using a standard polyclonal antibody against EZH2 that was previously validated by immunoblot analysis. (Varambally S et al. 2002) Since 3 core samples were obtained for each patient, the value of the 3 scores was used for subsequent analysis. At least two authors scored each tumor core blinded to the pathological or clinical characteristics of the case. Nuclear EZH2 expression was scored by using a validated system (Varambally et al. 2002; Kleer et al. 2001; Rhodes et al.

2003) as negative (score=1, no staining); weak (score 2, <25% of nuclei staining, any intensity); moderate (score=3, 25-75% of nuclei staining, any intensity); and strong (score=4, >75% of nuclei staining, any intensity). Positive EZH2 expression was defined as scores 3 and 4; negative EZH2 was defined as scores 1 and 2.

Stastistical Analysis

The *a priori* planned analysis was to explore the relationship between EZH2+ expression with each clinicopathologic variable available for the cohort. For each variable, we assessed the association with EZH2 expression using chi-square and logistic regression. Survival analysis was conducted using Kaplan-Meier curves for any recurrence and development of distant metastases. Cox Proportional Hazards Models supplement the Kaplan-Meier curves for analysis of the outcomes.

4.3 Results

We identified a total of 906 cases through the HFH tumor registry that met the stage and year of diagnosis criteria for inclusion in the study. Of these, 637 were Stage I cases and 269 were node positive Stage II cases. (Figure 3.1) After medical record review, 137 (15%) were excluded from the study. Of the 769 eligible cases, tumor specimens were not available for 233 (30%). Of the 536 cases with blocks available we successfully stained and scored 373 (71%). Table 4.1 gives the p-vlaues of the $\chi 2$ analysis comparing eligible cases included on the TMAs and those eligible cases not available for TMA analysis. There were significantly more ER negative, high grade, and larger tumors

included on the TMA. More of the TMA cases had distant metastasis. There were also fewer cases with family history.

To explore the association of EZH2 with patient characteristics, tumor characteristics, and outcomes of interest, we calculated $\chi 2$ statistics and univariate logistic regression odds ratios with 95% confidence interval (CI). The $\chi 2$ results are present in Tables 4.2 and 4.3. The univariate results from the logistic regression are presented in Table 4.4. For the patient characteristics, there is the suggestion of a potential relationship between EZH2 positivity with race ($\chi 2$ p-value=0.06) and 1st degree family history ($\chi 2$ p-value=0.07). Among the tumor characteristics examined, EZH2 positivity was associated with high grade ($p<0.0001$), tumor size > 1cm ($p=0.0008$), negative estrogen receptor (ER) status ($p<0.0001$), negative progesterone receptor (PR) status ($p<0.0001$) and positive Her2 status ($p=0.004$). The odds ratios (OR) and 95% confidence intervals (CI) for these significant relationships were 8.34 (95% CI 4.49-15.50) for ER, 3.56 (95% CI 2.19-5.78) for PR, 2.17 (95% CI 1.27-3.70) for Her2, 1.82 (95% CI 1.16-2.86) for tumor size, and 5.88 (95% CI 3.57-10.0) for grade. These results are presented in Table 4.4. The analysis of EZH2 and the outcomes any recurrence (OR=1.51; 95% CI 0.83-2.77) and distant metastasis (OR=1.79; 0.76-1.16) were not significant.

Table 4.5 gives the Hazard Ratios (HR) and 95% CIs for the univariate and multivariate Cox Proportional Hazards Regression models. Results for four different models are presented. The first model is the univariate analysis demonstrating a HR of 1.61 (95% CI 0.91-2.84) for any recurrence and 1.93 (95% CI 080-4.65) for distant

metastasis. The following models gradually add additional covariates with the final model considered the full model. The model adjusting for only Her2 status were nearly significant with a HR of 1.64 (95% CI 0.90-2.99) for any recurrence and a HR of 2.53 (95% CI 0.95-6.78) for distant metastasis. While none of the results where significant, the hazard ratios for distant metastasis were consistently higher in magnitude than those for any recurrence.

Kaplan-Meier curves for the time to either any recurrence or distant metastasis are given in Figures 4.1-4.10. The first two curves are for any recurrence (Figure 4.1) and distant metastasis (Figure 4.2) by EZH2 status. These are followed by curves stratified by ER status. Figures 4.3 and 4.4 are for ER positive cases and Figures 4.5 and 4.6 are for ER negative cases. In each pair, time to any recurrence is presented first and time to distant metastasis is presented second. The final four curves are for each outcome stratified by Her2 status.

The Log-Rank p-value (p=0.03) is significant for time to any recurrence by EZH2 status (Figure 4.1); however, there was not a significant difference for time to distant metastasis (p-value=0.24). ER positive cases were no more likely to recur (Log-Rank p-value=0.34) or develop distant metastasis (Log-Rank p-value=0.56) if they were EZH2 positive or negative. The curves of ER negative cases are notable for the consistently poorer outcomes among the EZH2 positive cases; however, neither time to recurrence (Log-Rank p-value=0.43) or time to distant metastasis (Log-Rank p-value=0.79) were significant. Likewise, for Her2 positive cases neither outcome was significant. But for Her2 negative cases, there was a significant Log-Rank p-value for time to recurrence

(p=0.02) and a nearly significant Log-Rank p-value for time to distant metastasis (p=0.06).

4.4 Discussion

Our results support some of the previous findings regarding EZH2 and its relationship with breast cancer. We found that, even in very early stage disease, EZH2 overexpression is strongly associated with ER negativity. Those cases positive for EZH2 in our study were 8.34 (4.49-15.5) times more likely to be ER negative. Gonzalez et al. demonstrated in ER-negative breast cancer cell lines MDA-MB-231 and CAL51 the relationship between EZH2 overexpression and ER (Gonzalez ME et al. 2009). Results of the Gonzalez study showed that invasive ER-negative breast cancers have overexpression of EZH2 and downregulation of BRCA1.

Interestingly, we found that women in our cohort with an affected 1st degree relative were more likely, although not significant, to have EZH2 positive breast cancers. Given the results from the Gonzalez study on the relationship of EZH2 with BRCA1—a gene implicated in inherited breast cancer, our results seem to confirm this prior finding as well. However, inheritance of BRCA1 deleterious mutations is a rare event so this potential association needs to be considered cautiously. We could find no other study that has considered the relationship between self-reported family history and EZH2 expression.

Similarly, to our knowledge, no other study has investigated the relationship between EZH2 and Her2 status. We found that Her2 positive tumors were 2.17 times

more likely to be EZH2 positive. However, it was Her2 negative tumors that were EZH2 positive that had worse outcomes. The Kaplan-Meier curves for any recurrence and distant metastasis among Her2 negatives cases had Log-Rank p-values of 0.02 and 0.06, respectively. Additionally, the hazard ratios for recurrence and distant metastasis adjusting for EZH2 and Her2 status were nearly significant, 1.64 (95% CI 0.90-2.99) and 2.53 (95% CI 0.95-6.78), respectively, with a noticeably stronger relationship with distant metastasis.

Nearly a third of the patients in our cohort were African-American. African-American women are known to have a lower incidence of breast cancer but a higher mortality from the disease. Our study, which was also the first to consider racial/ethnic variation in EZH2, found a nearly significant relationship between race and EZH2 status. African-American women were 1.54 (95% CI 0.98-2.38) times more likely to be EZH2 positive than white women. African-American women are also more likely to have hormone receptor negative disease which we have already discussed being associated with EZH2 positivity. Additional studies are needed to see if EZH2 varies between white and African-American women with hormonally negative breast cancer.

Prior work has demonstrated that EZH2 expression is associated with cellular proliferation. In our study EZH2 overexpression was associated with higher grade tumors (OR=5.88; 95% CI 1.27-10.0) and larger tumor size (OR=1.82; 95% CI 1.16-2.86), both indications of the level of proliferation of the tumor.

It is worth pointing out that there were statistically significant differences between those cases for which pathologic samples were available and successfully stained versus

those that could not be analyzed. The significant differences were that more ER negative cases were included, more cases with distant metastases were included, and more tumors 1 cm or larger were included. In contrast, we had fewer cases with family history information. These differences represent a potential selection bias that could affect the generalizability and future replication of our results.

Our results suggest a possible association between EZH2 and recurrence and late development of distant metastasis. In total 183 of our cases (49%) are EZH2 positive. With 11% of our cases having a recurrence, we have 80% power to detect a rate ratio of 1.8 or greater. When analysis is complete, we plan to submit our results for publication in a peer-reviewed journal.

Table 4.1 Comparison of Patient and Tumor Characteristics as well as Recurrence Outcomes for Cases Included on the TMA vs. Not

Characteristic	$\chi 2$ p-value	on TMA
ER status	0.03	more ER neg
PR status	0.53	
Her2 status	0.87	
Recurrence	0.09	more recurrence
Distant Mets	0.008	more mets
Stage	0.09	more Stage I
Family HX	0.008	less family hx
Family HX 1st degree	0.18	
Grade	0.004	more high grade
Race	0.89	
Node status	0.07	less node positive
Age of 1st birth	0.25	
Age of menarche	0.98	
Tumor size	0.0002	more $\geq$1 cm
Age at diagnosis	0.62	
Parity	0.84	

Table 4.2. Study Population Characteristics

Characteristic	N (%)	EZH2+	EZH2-	$\chi 2$ p-value
Menarche				
≤ 12	182 (49%)	90 (49%)	92 (48%)	
> 12	191 (51%)	93 (51%)	98 (52%)	0.88
Age of First Birth				
≤ 30	245 (66%)	127 (69%)	118 (62%)	
> 30	128 (34%)	56 (31%)	72 (38%)	0.14
Parity[1]				
Childless	55 (28%)	29 (30%)	26 (25%)	
More than 1 child	144 (72%)	67 (70%)	77 (75%)	0.43
Race				
White	249 (68%)	113 (63%)	136 (73%)	
Black	116 (32%)	65 (37%)	51 (27%)	0.06
Age at Diagnosis				
≤ 50	86 (23%)	42 (23%)	44 (23%)	
> 50	287 (77%)	141 (77%)	146 (77%)	0.96
Family History[2]				
Any	149 (40%)	77 (42%)	72 (38%)	
None	224 (60%)	106 (58%)	118 (62%)	0.41
Family History[2]				
Any 1st Degree Relative	83 (22%)	48 (26%)	35 (18%)	
None or No 1st Degree	290 (78%)	135 (74%)	155 (82%)	0.07

[1] Parity was missing for several cases.
[2] Family History of breast and/or ovarian cancer.

Table 4.3 Breast Cancer Characteristics and Outcomes for Study Population

Characteristic	N(%)	EZH2+	EZH2-	$\chi 2$ p-value
Stage				
I	326 (87%)	156 (85%)	170 (89%)	
II (T1, N1)	47 (13%)	27 (15%)	20 (11%)	0.22
Grade				
High (=3)	115 (31%)	89 (49%)	26 (14%)	
Low (<3)	258 (69%)	94 (51%)	164 (86%)	<0.0001
Tumor Size				
≤ 1 cm	118 (32%)	46 (25%)	72 (38%)	
> 1 cm	255 (68%)	137 (75%)	118 (62%)	0.008
Nodal Status				
Positive	49 (13%)	28 (15%)	21 (11%)	
Negative	324 (87%)	155 (85%)	169 (89%)	0.22
Estrogen Receptor				
Positive	286 (77%)	110 (60%)	176 (93%)	
Negative	87 (23%)	73 (40%)	14 (7%)	<0.0001
Progesterone Receptor				
Positive	267 (72%)	108 (59%)	159 (84%)	
Negative	106 (28%)	75 (41%)	31 (14%)	<0.0001
Her2neu Status				
Positive	72 (20%)	46 (27%)	26 (14%)	
Negative	283 (80%)	127 (73%)	156 (86%)	0.004
Recurrence				
Any	50 (13%)	29 (16%)	21 (11%)	
None	323 (87%)	154 (84%)	169 (89%)	0.17
Distant Metastasis				
Yes	24 (6%)	15 (8%)	9 (5%)	
No	349 (94%)	168 (92%)	181 (95%)	0.17

Table 4.4 Unadjusted Odds Ratios and 95% Confidence Intervals (CI) for Patient Characteristics, Tumor Characteristics and Outcomes with EZH2 Overexpression

Patient Characteristics	N	OR	95% CI
Age of menarche (≤12 vs. >12)	373	1.03	0.69-1.55
Parity (none vs. 1+)	199	1.29	0.69-2.39
Fam HX (any vs. none)	373	1.19	0.78-1.79
Fam HX (first degree vs. other)	373	1.57	0.96-2.56
Age first birth (≤30 vs. >30)	373	1.38	0.90-2.13
Age at diagnosis (≤50 vs. 50)	373	0.99	0.61-1.60
Race (White vs. Black)	365	0.65	0.42-1.02
Tumor Characteristics			
ER status (neg vs. pos)	373	8.34	4.49-15.50
PR status (neg vs. pos)	373	3.56	2.19-5.78
Her2 status (pos vs. neg)	355	2.17	1.27-3.70
Size (>1 cm vs. ≤ 1 cm)	373	1.82	1.16-2.86
Nodal status (pos vs. neg)	373	1.45	0.79-2.63
Grade (high vs. low)	373	5.88	1.27-10.0
Stage (I vs. IIA)	373	0.68	0.37-1.26
Outcome			
Any Recurrence	373	1.51	0.83-2.77
Distant Metastases	373	1.79	0.76-1.16

Table 4.5 Unadjusted and Adjusted Hazard Ratios (HR and aHR, respectively) with 95% Confidence Intervals (CI) for EZH2 Status

Outcome	HR (95% CI)	aHR[1] (95% CI)	aHR[2] (95% CI)	aHR[3] (95% CI)
Any Recurrence	1.61 (0.91-2.84)	1.64 (0.90-2.99)	1.35 (0.69-2.64)	1.40 (0.71-2.75)
Distant Metastasis	1.93 (0.80-4.65)	2.53 (0.95-6.78)	1.75 (0.59-5.24)	2.29 (0.71-7.38)

[1]Adjusting for Her2 status.
[2]Adjusting for Her2 status, ER status, tumor size, grade, and race.
[3]Adjusting for Her2 status, ER status, tumor size, grade, race, node status, and age at diagnosis.

Figure 4.1 Kaplan-Meier Curve for Time to Any Recurrence by EZH2 Status

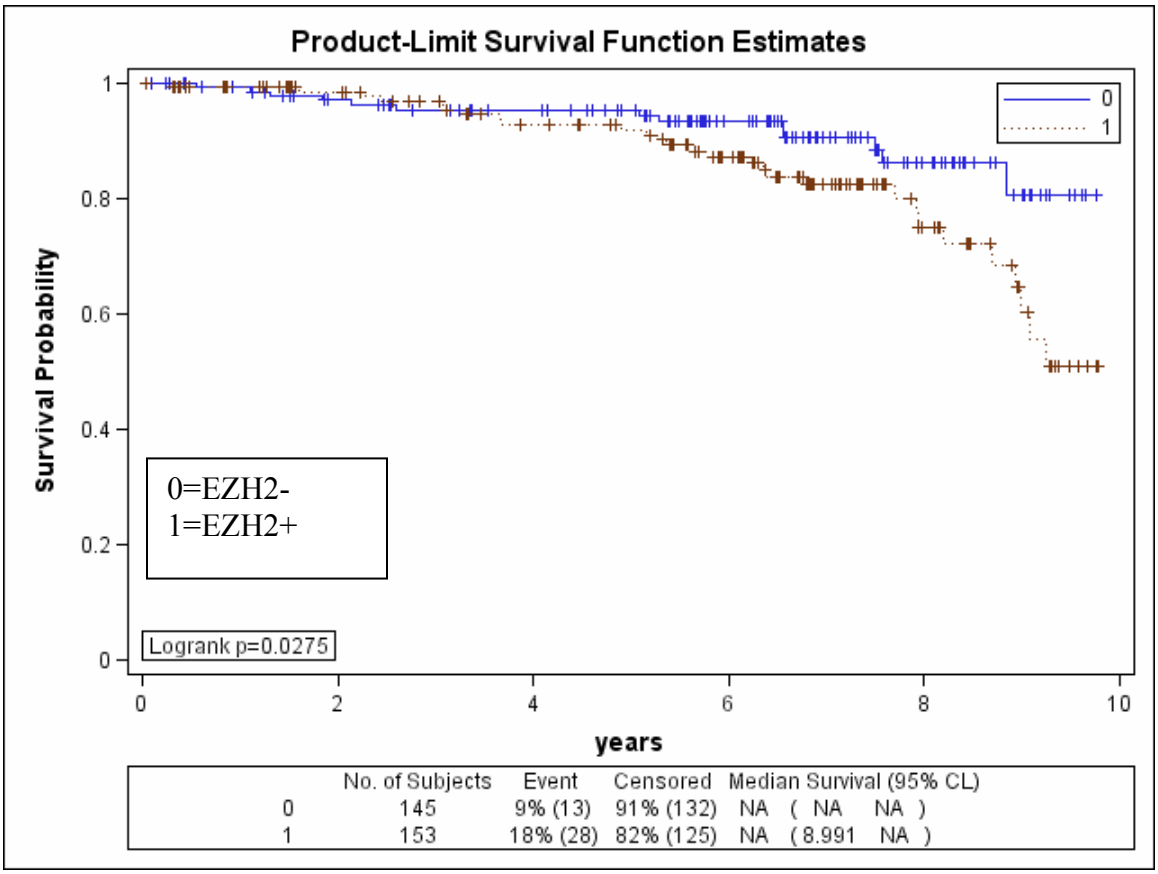


Figure 4.2 Kaplan-Meier Curve for Time to Distant Metastasis by EZH2 Status

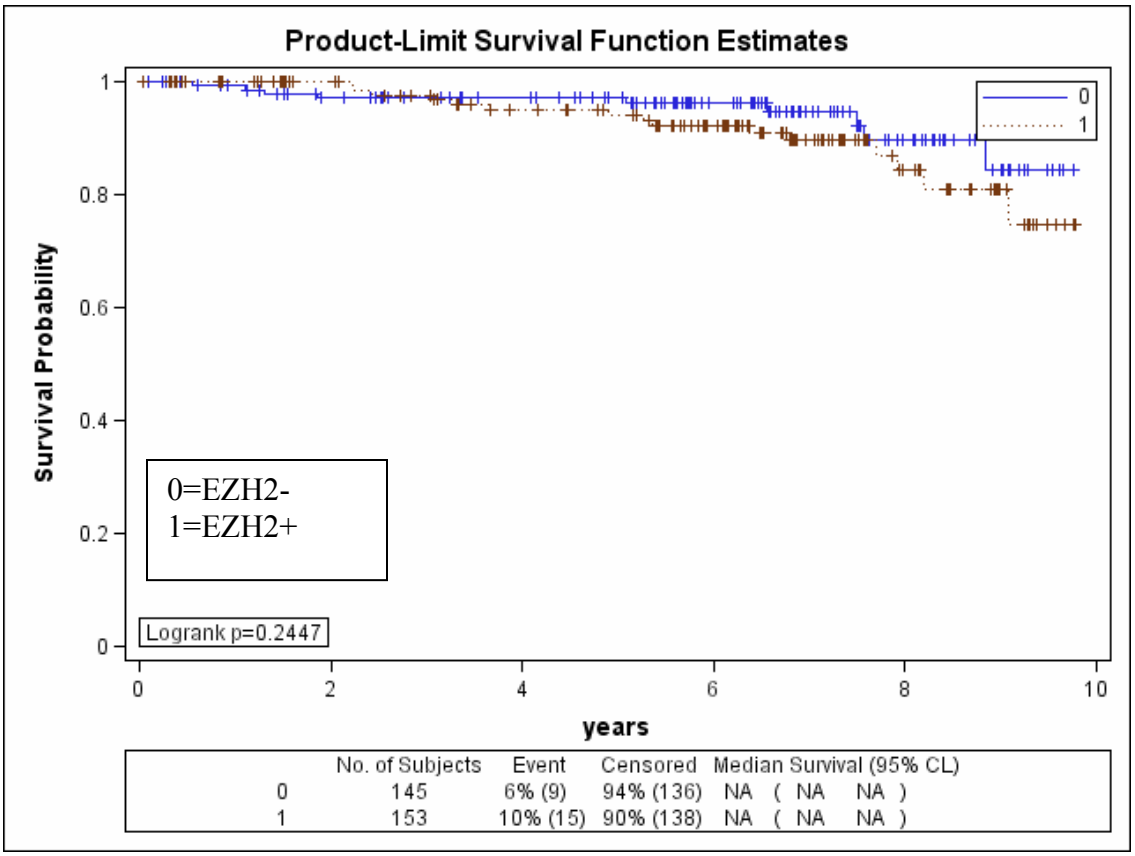


Figure 4.3 Kaplan-Meier Curve for Time to Any Recurrence for ER Positive Cases by EZH2 Status

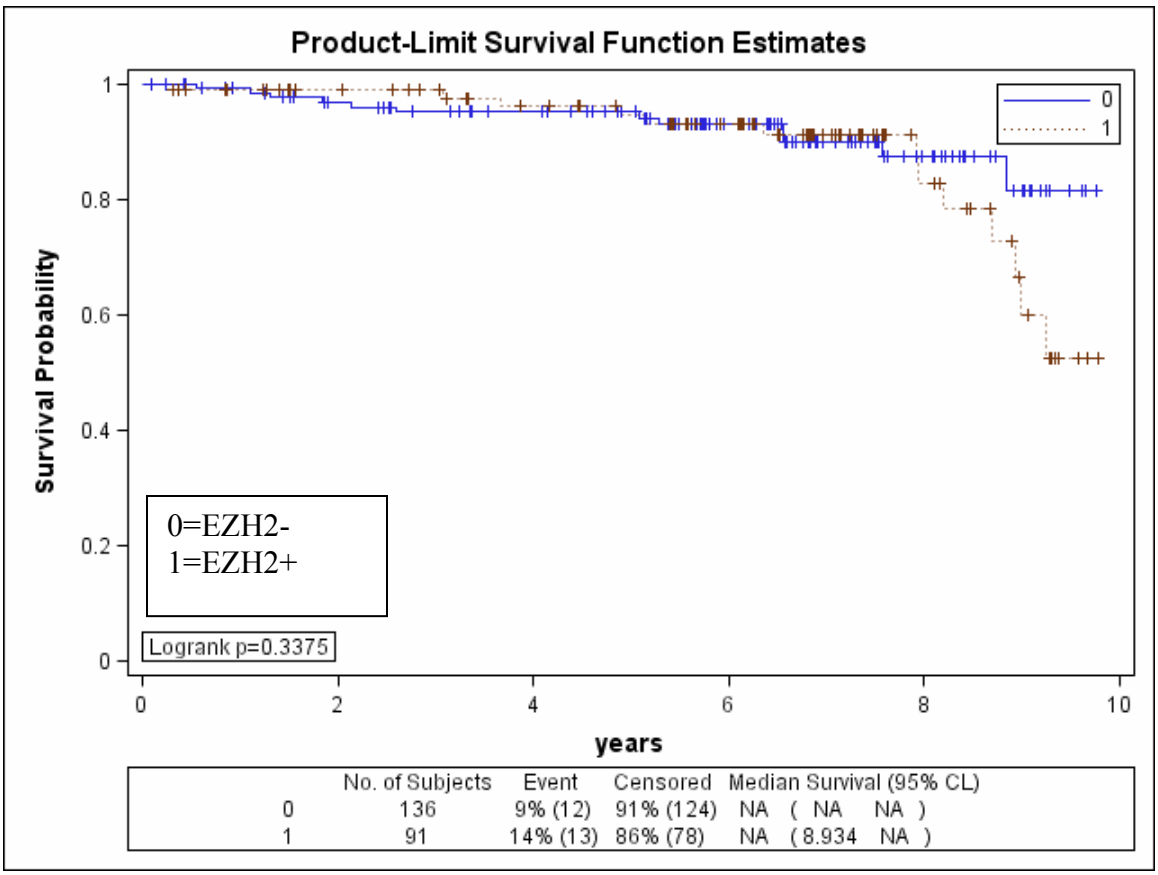


Figure 4.4 Kaplan-Meier Curve for Time to Distant Metastasis for ER Positive Cases by EZH2 Status

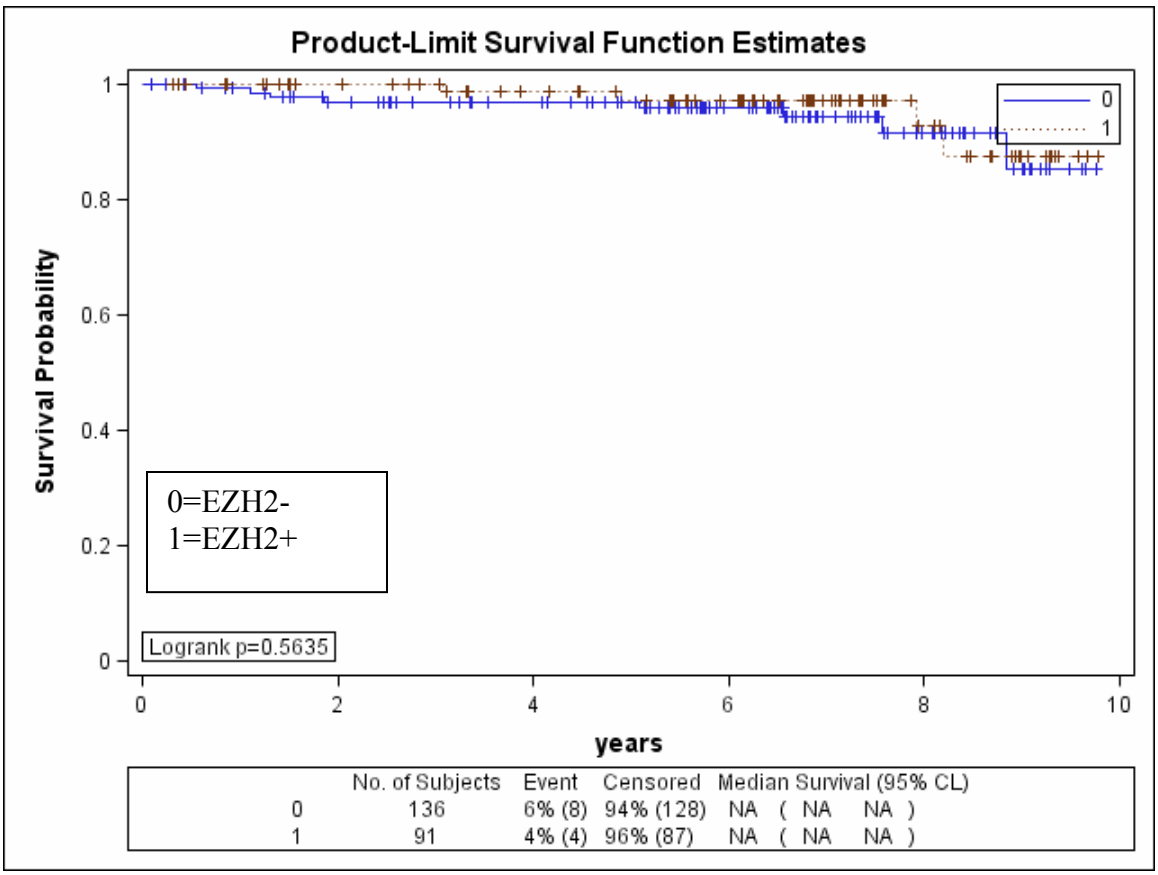


Figure 4.5 Kaplan-Meier Curve for Time to Any Recurrence for ER Negative Cases by EZH2 Status

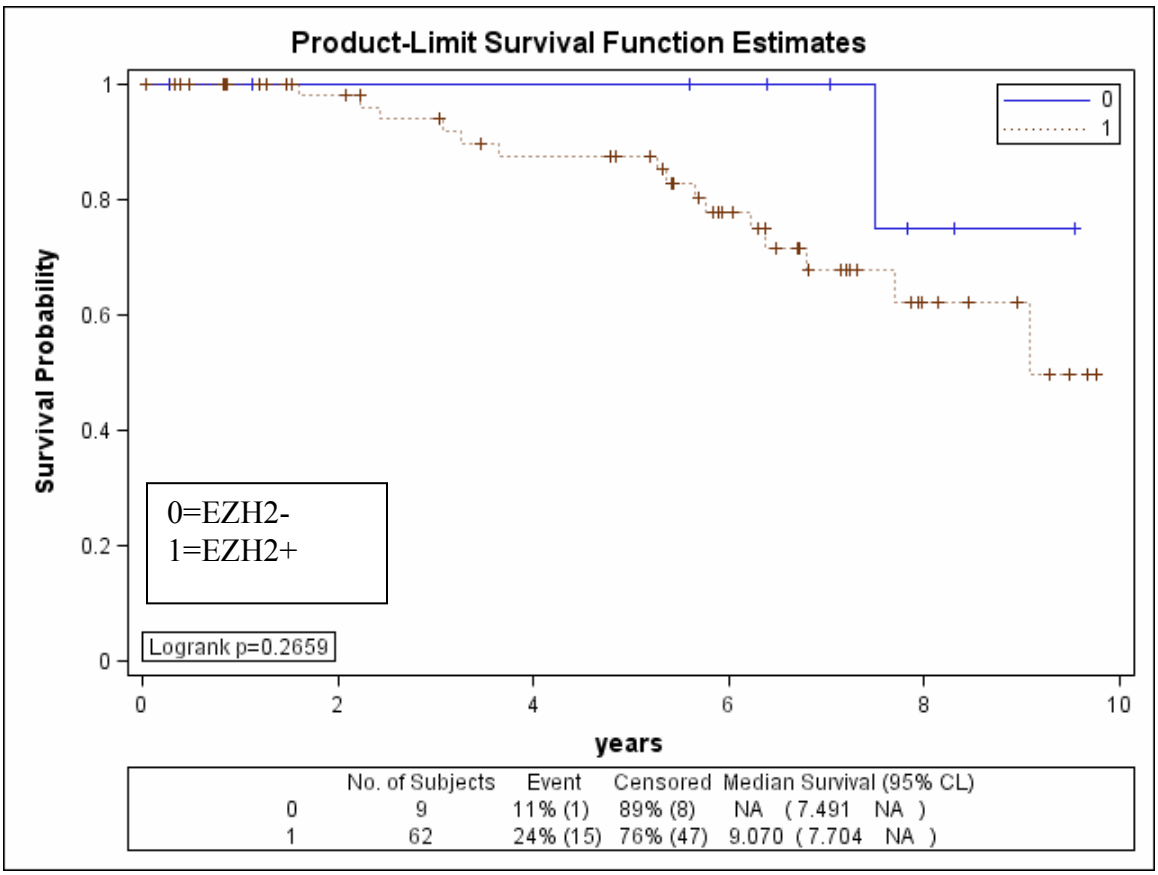


Figure 4.6 Kaplan-Meier Curve for Time to Distant Metastasis for ER Negative Cases by EZH2 Status

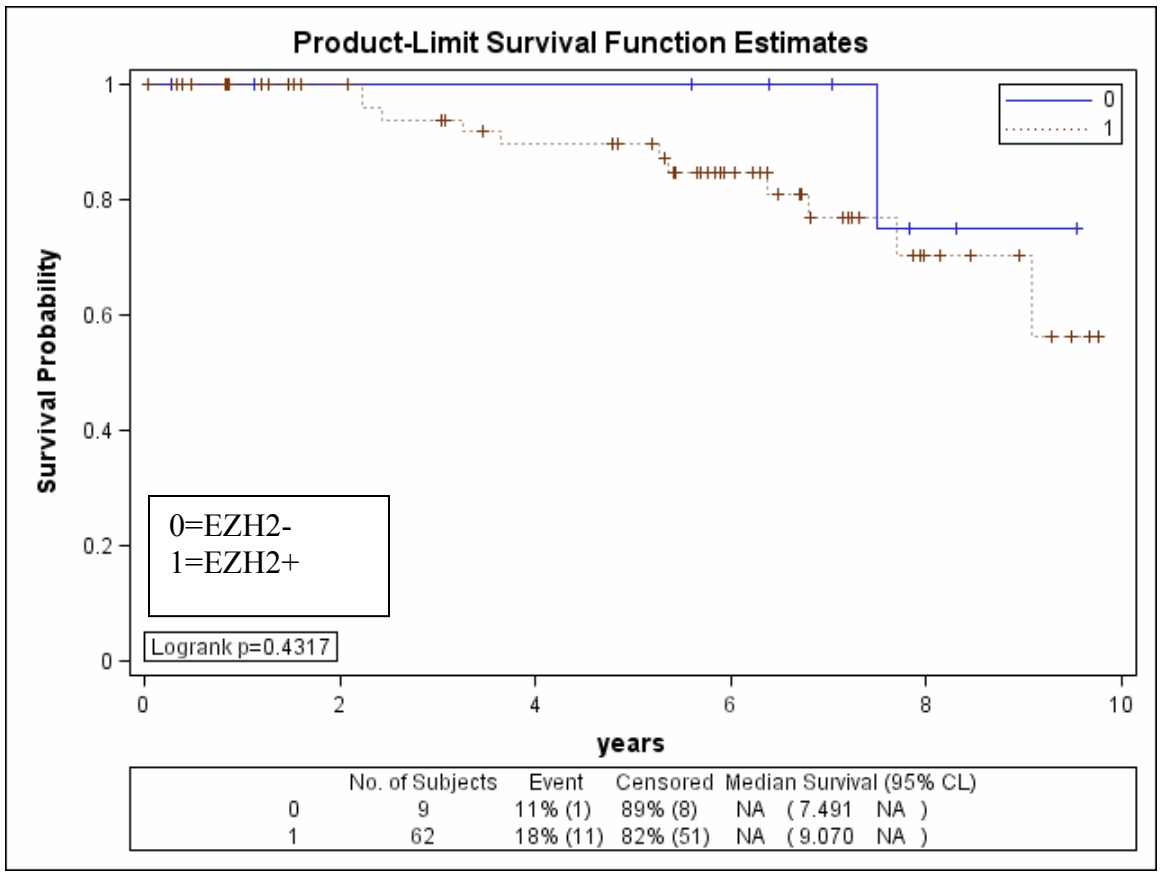


Figure 4.7 Kaplan-Meier Curve for Time to Any Recurrence for HER2 Positive Cases by EZH2 Status

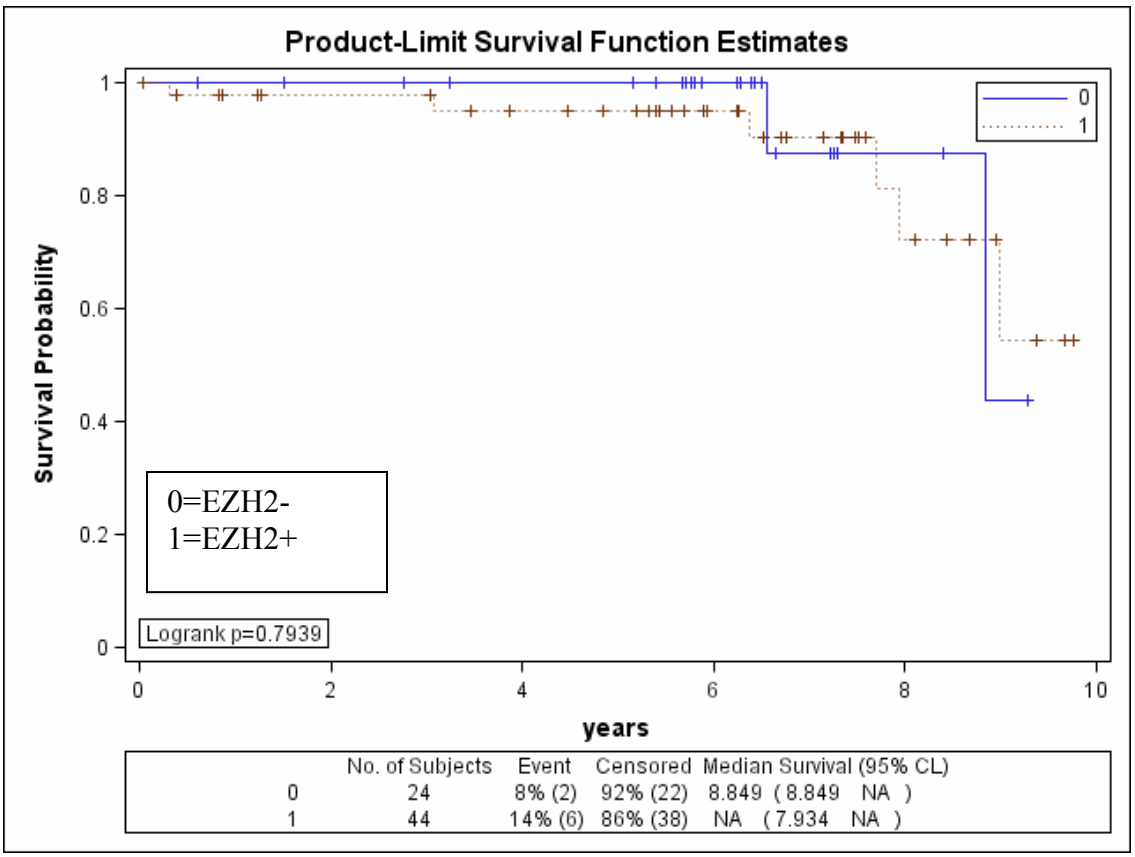


Figure 4.8 Kaplan-Meier Curve for Time to Distant Metastasis for HER2 Positive Cases by EZH2 Status

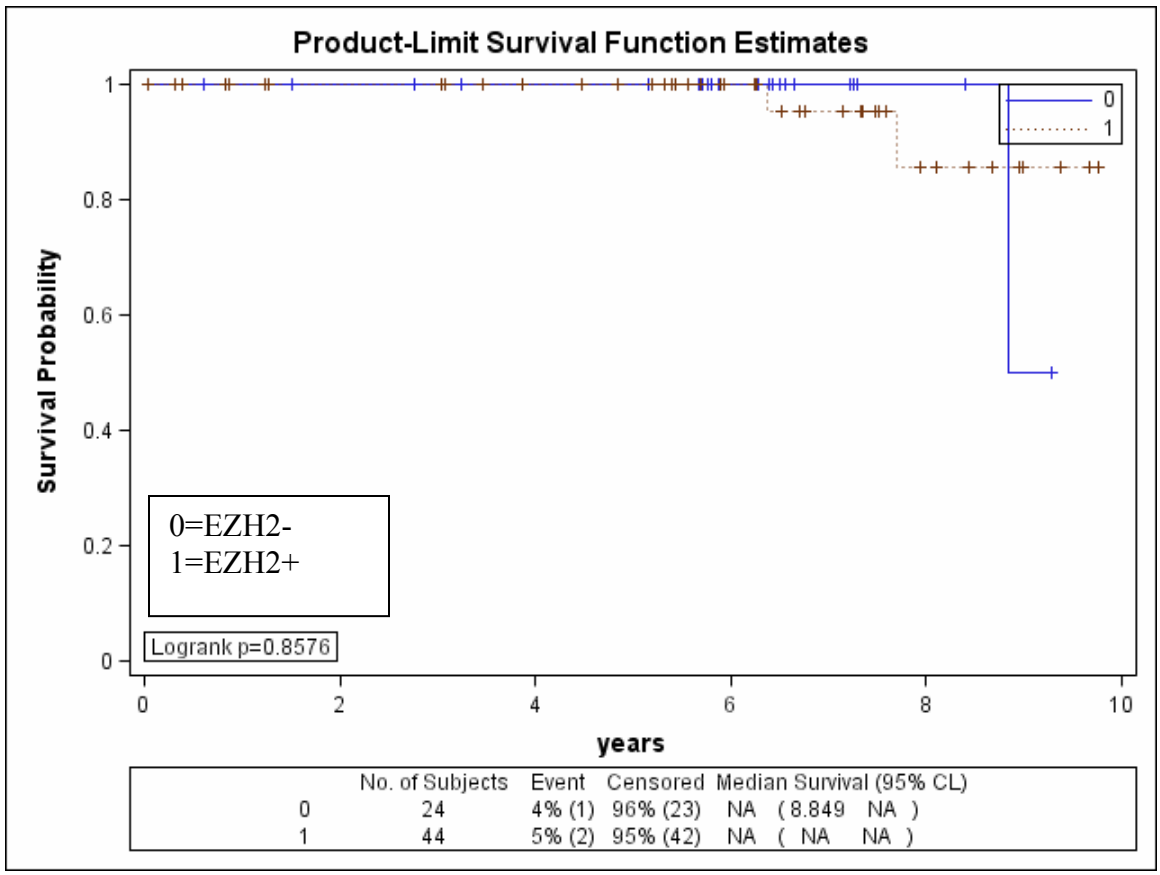


Figure 4.9 Kaplan-Meier Curve for Time to Any Recurrence for HER2 Negative Cases by EZH2 Status

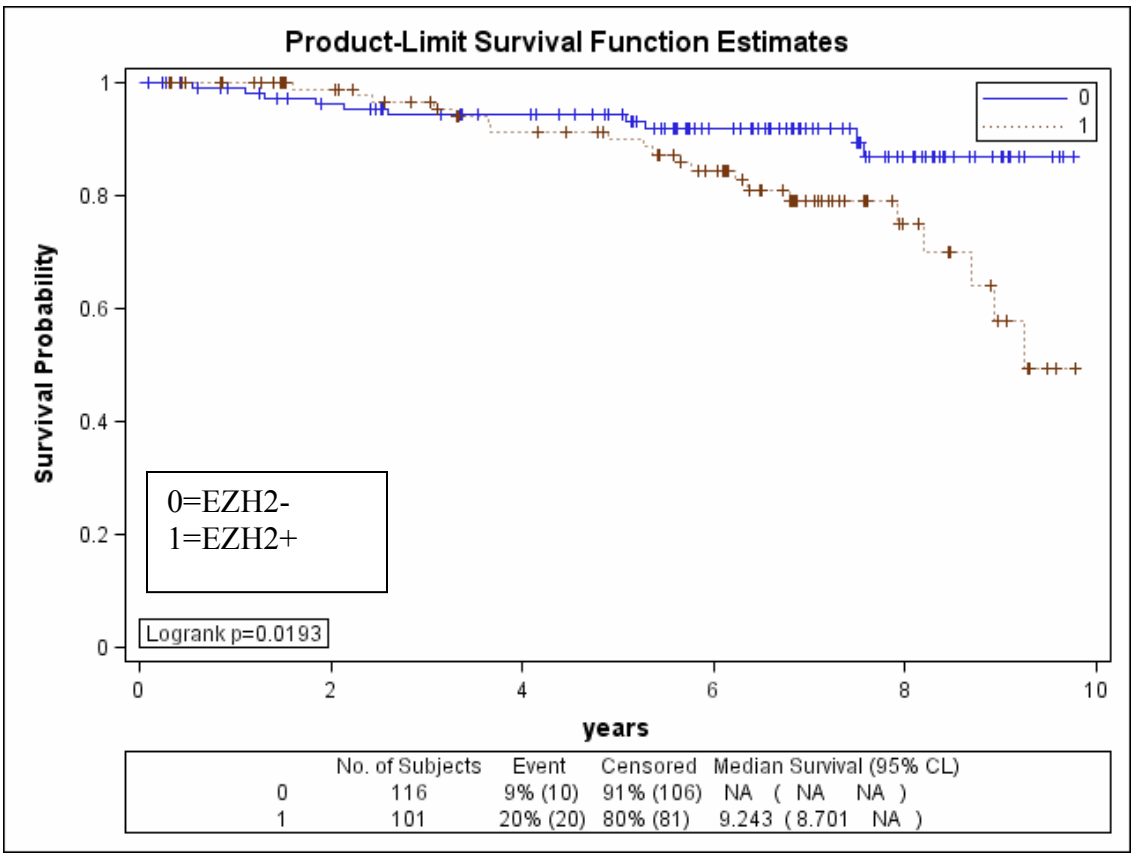
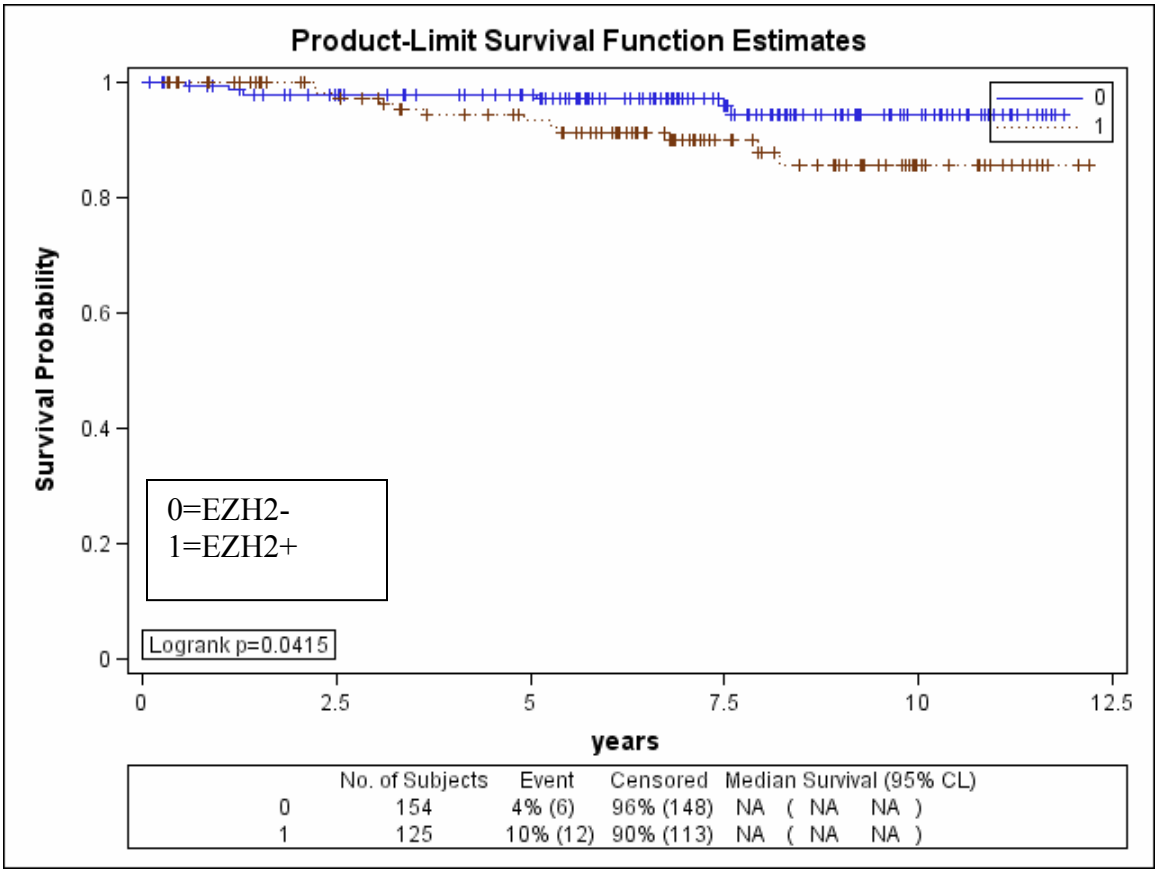


Figure 4.10 Kaplan-Meier Curve for Time to Distant Metastasis for HER2 Negative Case by EZH2 Status



References

Arnes JB, Collett K, Akslen LA. (2008) Independent prognostic value of the basal-like phenotype of breast cancer and associations with EGFR and candidate stem cell marker BMI-1. Histopathology 52(3): 370-80.

Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, Salvesen HB, Otte AP, Akslen LA. (2006) EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. Journal of Clinical Oncology 24(2):268-73.

Collett K, Eide GE, Arnes J, Stefansson IM, Eide J, Braaten A, Aas T, Otte AP, Akslen LA. (2006) Expression of enhancer of zeste homologue 2 is significantly associatied with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clinical Cancer Research 12(4):1168-74

Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM. (2001) Delineation of prognostic biomarkers in prostate cancer. Nature 412;822-826.

Ding L, Erdmann C, Chinnaiyan AM, Merajver SD, Kleer CG. (2006) Identification of EZH2 as a molecular marker for the precancerous state in morphologically normal breast tissues. Cancer Research 66(8): 4095-9.

Ding L, Kleer CG. (2006) Enhancer of Zeste 2 as a marker of preneoplastic progression in the breast. Cancer Research 66(19): 9352-5.

Gonzalez ME, Li Z, Toy K, DuPrie M, Ventura AC, Banerjee M, Ljungman M, MerajverSD, Kleer CG. (2009) Downregulation of EZH2 decreases growth of estrogen receptor-negative invasive breast carcinoma and requires BRCA1. Oncogene 28(6): 843-53.

Hwang C, Giri VN, Wilkinson JC, Wright CW, Wilkinson AS, Cooney KA, Duckett CS.(2008) EZH2 regulates the transcription of estrogen-responsive genes through association with REA, an estrogen receptor corepressor. Breast Cancer Research Treatment 107(2): 235-42.

Kleer, C. G., Giordano, T. J., Braun, T. & Oberman, H. A. (2001) Pathologic, immunohisstochemical, and molecular features of benign and malignant phyllodes tumors of the breasr. Mod. Pathol. 14(3):185-190.

Perrone EE, Theoharis C, Mucci NR, Hayasaka S, Taylon JM, Cooney KA, Rubin MA. (2000) Tissue microarray assessment of prostate cancer tumor proliferation in

African-Ameerican and white men. Journal of the National Cancer Institute 92(11): 937-939.

Pieterson AM, Horlings HM, Hauptmann M, Langerod A, Ajouaou A, Cornelissen-Steijger P, Wessels LF, Jonkers J, Vijver MJ, van Lohuizen M. (2008) EZH2 and BMI1 inversely correlate with prognosis and TP53 mutation in breast cancer. Breast Cancer Research 10(6): R109.

Raaphorst FM, Meijer CJ, Fieret E, Blokzijl T, Mommers E, Buerger H, Packeisen J,Sewalt RA, Otte AP, van Diest PJ. (2003) Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia 5(6):481-8.

Reynolds PA, Sigaroudinia M, Zardo G, Wilson MB, Benton GM, Miller CJ, Hong C,Fridlyand J, Costello JF, Tlsty TD. (2006) Tumor suppressor p16ink4A regulates polycomb-mediated DNA hypermethylation in human mammary epithelial cells. Journal of Biological Chemistry 281(34): 24790-802.

Rhodes, D. R., Sanda, M. G., Otte, A. P., Chinnaiyan, A. M. & Rubin, M. A. (2003) Multiplex biomarker approach for determining approach for prostate-specific antigen-defined recurrence of prostate cancer. Journal of the National Cancer Institute 95(9): 661-668.

Shi B, Liang J, Yang X, Wang Y, Zhao Y, Wu H, Sun L, Zhang Y, Chen Y, Li R, ZhangY, Hong M, Shang Y. (2007) Integration of estrogen and Wnt signaling circuits by the polycomb group protein EZH2 in breast cancer cells. Molecular Cell Biology 27(14): 5105-19.

Tonini T, D'Andrilli G, Fucito A, Gaspa L, Bagella L. (2008) Importance of EZH2 polycomb protein in tumorigenesis process interfering with the pathway of growth suppressive key elements. Journal of Cell Physiology 214(2)295-300.

Van den Eynden GG, Van der Auwer I, Van Laere S, Colpaert CG, van Dam P, MerajverS, Kleer CG, Harris AL, Van Marck EA, Dirix LY, Vermeulen PB. (2004) Validation of a tissue microarray to study differential protein expression in inflammatory and non-inflammatory breast cancer. Breast Cancer Research and Treatment 85(1):13-22

Varambally S, Dhanasekaran SM, Zhou M, Baarrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, et al. (2002) The polycomb group protein EZH2 is involved in the progression of prostate cancer. Nature 419;624-629.

Wei Y, Xia W, Zhang Z, Liu J, Wang H, Adsay NV, Albarracin C, Yu D, Abbruzzese JL,Mills GB, Bast RC Jr, Hortobagyi GN, Hung MC. (2008) Loss of trimethylation

at lysine 27 and histone H3 is a predictor of poor outcome in breast, ovarian, and pancreatic cancers. Molecular Carcinogens 47(9): 701-6.

Yu J, Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G, Mehra R, Wang X, Ghosh D, Shah RB, Varambally S, Pienta KJ, Chinnaiyan AM. (2007) A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer Research 67(22): 10657-63.

Zeidler M, Kleer CG. (2006) The Polycomb group protein Enhancer of Zeste 2: its links to DNA repair and breast cancer. Journal of Molecular Histology 37(5-7):219-23.

Zeidler M, Varambally S, Cao Q, Channaiyan AM, Ferguson DO, Merajver SD, KleerCG. (2005) The Polycomb group protein EZH2 impairs DNA repain in breast epithelial cells. Neoplasia 7(11):1011-9.

Chapter 5

Breast Cancer Chemoprevention with Bisphosphonates

5.1 Introduction

Primary prevention of breast cancer is still a much desired goal both from the clinical and public health perspective. The FDA has approved two drugs, tamoxifen and raloxifene, as chemopreventive agents for use in high risk women. Tamoxifen is an antiestrogen that was first approved for use in the palliative setting but has since been realized to have beneficial effects in the adjuvant setting. In 1991, Nayfield et al. suggested that tamoxifen might be an effective chemopreventive agent based on the pharmacology, laboratory research, and clinical experience from use in early-stage disease (Nayfield et al. 1991). Preclinical studies have demonstrated a number of anti-tumor effects of bisphosphonates on breast cancer cells. These studies have suggested that bisphosphonates inhibit tumor cell proliferation, decrease cancer cell adhesion, block angiogenesis, inhibit invasion, induce cancer cell apoptosis and block degradation of the tumor microenvironment (Green & Clezardin 2002; Caraglia et al. 2007; Oades et al. 2003).

In this study, we suggest that women exposed to bisphosphonates may have a reduced risk of breast cancer. Typically used in the palliative care of women with breast cancer bone metastasis, here we present the first reported analysis of bisphosponates as potential chemopreventive breast cancer agents.

5.2 Methods

We conducted a retrospective cohort study using computerized data from Henry Ford Health System (HFHS). HFHS is a large, integrated health system serving the health care needs of a large group of residents in metropolitan Detroit. Most of the care given under the HFHS umbrella is provided by the Henry Ford Medical Group (HFMG), a system-affiliated, multi-specialty, physician group practice. In addition, HFHS is affiliated with the Health Alliance Plan (HAP), a large nonprofit, mixed-model HMO in southeastern Michigan. At the time of study, among 490,000 HAP members, approximately 350,000 members were HFMG-assigned and made up the source population from which our retrospective cohort was identified.

HFHS maintains a centralized system of computerized databases including outpatient pharmacy, electronic medical records, encounter and claims records. There is a single electronic medical record for each patient with a unique medical record number that can easily be linked to the individual's HAP number to develop both a care-based and claim-based review of an individual patient's utilization.

<u>Study Population</u>

Patients included in the study were women selected from HFMG-HAP members enrolled on or before 1/1/2002. An index date was assigned the day after a woman had accumulated one year of continuous enrollment or at the time of reaching 50 years of age given a year of previous enrollment. Women who had no prior documented use of bisphosphonates, tamoxifen, or raloxifene were included in the cohort. Women were followed until disenrollment from HAP or cancer diagnosis.

Assessment of Potential Confounders

We considered age, race, year of index date, number of screening mammograms, and any exposure to hormone replacement therapy as potential confounders. Screening mammograms were identified using claims data and procedure codes specific for screening mammograms. Only one mammogram was counted per twelve months to account for coding of additional views as screening mammograms despite being taken during separate visits. Using pharmacy data, we classified women as being exposed to hormone replacement therapy if a single prescription was filled prior to or during the study period. Our definition of exposure included oral or topical hormone related drugs.

Identification of Breast Cancer Cases

The cohort was merged with the system's tumor registry to identify newly diagnosed, incident breast cancer. Henry Ford Health System has been a participating member of the Detroit SEER since the registry's inception in 1973. Any cancer diagnosed prior to the index date was considered an exclusion criterion.

Classification of Medication Exposure

Since enrollment in HAP was required for study inclusion and individuals were censored at disenrollment, we were able to capture all covered prescriptions filled during the study period. Exposure to bisphosphonates was determined using pharmacy claims data, which includes data elements for National Drug Code (NDC), brand name, generic name, class, quantity, dose, route, date of dispensing, and days supply. Exposure was limited to oral drugs. We calculated exposure from the date of the first bisphosphonate fill date plus the days supply for each prescription. Exposure for less than 35 days, a single prescription period, was not counted as exposure. Continuous exposure was defined by sequential (at least 2 or more) prescription fills. To account for off-label dispensing of the prescription, we allowed up to a 45-day lapse in supply between prescriptions. Because hypercalcemia maybe the first sign of breast cancer noticed by practitioners, we did not count as exposure any initiation of bisphosphonates that occurred 35 days or less before a cancer diagnosis.

Data Analysis

Women were followed until either disenrollment, cancer diagnosis, or the end of the study period (12/31/2007). Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using logistic regression or Cox proportional hazards regression models. The p-values in the univariate analysis were based on the log-rank or chi-square test. A t-test was used to judge the difference in mean age between those exposed and unexposed.

Multivariable Cox proportional hazards models for breast cancer were modeled with age at index date, race, year of index date, any use of hormone replacement therapy and number of screening mammograms received as confounders. We also modeled bisphosphonate exposure as a time-dependent covariate in the Cox proportional hazards analysis to assess confounding by length of observation. We tested whether marker status, stage or grade at diagnosis were different between those ever exposed to bisphosphonates and never exposed. In addition we assessed length of cumulative exposure in an effort to assess potential length-of-exposure effects.

5.3 Results

A total of 43,267 women were included in the cohort of which 3,423 (8%) had at least 2 months exposure to bisphosphonates. The majority of patients were 50-59 years of age (n=24,367; 56%) with 21% (n=8,881) between 60-69 years old, 16% (n=7,066) between 70-79 years old, and 7% (n=2,953) 80 years and older. The racial distribution of the cohort was 59% (n=25,742) white, 34% (n=14,737) black and 6% (n=2,755) were another race (Table 5.1). The age and race distribution was significantly different between those exposed and those unexposed. However, it is worth noting that there was a strong representation of African-Americans in our cohort, with 34% of the total cohort and 28% of those exposed being black. Eight percent (n=3,423) of the cohort was exposed to bisphosphonates, with the majority (55%) exposed for less than 1 year (n=1,866). There were 713 (21% of exposed) women exposed from 1 year to less than 2 years and 844 (25% of exposed) women exposed for 2 or more years. The mean follow-

up time for the cohort was 3.8 years; however this did vary by exposure status with a mean of 3.7 among those unexposed and 4.5 among the exposed (p-value <0.0001; data not shown). There were a total 1,542 cancers diagnosed in the cohort of which 494 (32%) were breast cancer. Of the breast cancers diagnosed, 25 were among those exposed ($\chi 2$ p-value=0.02).

Results of our analysis on stage, grade and marker status of tumors diagnosed within the cohort can be found in Table 5.2. Those ever exposed to bisphosphonates were less likely to be diagnosed with a Stage III or IV (OR=0.39; 95% 0.05-2.98) or high grade (OR=0.72; 95% CI 0.30-1.71) breast cancer, although neither of these results reached significance. Exposed women had a trend towards being less likely to have estrogen or progesterone negative tumors (OR=0.63 and OR=0.55, respectively) but these findings were not significant. The significance of the marker findings did not change when we included mixed tumors and compared double negative (OR=0.66; 95% CI 0.22-1.97) or double positive (OR=1.83; 95% CI 0.77-4.32) to the mixed hormonal receptor status phenotypes.

Among those defined as ever exposed to bisphosphonates, the hazard ratio was 0.51 for breast cancer with a 95% CI of 0.34-0.76. (Table 5.3) After adjusting for age, race, index year, number of screening mammograms, and any exposure to hormone replacement therapy, the hazard ratio was 0.35 (95% CI 0.23-0.53). The duration of exposure analysis showed a decrease in risk from 0.58 (95% CI 0.35-0.98) to 0.38 (95% CI 0.16-0.93) when exposure increased to 2 or more years (Cochrane-Armitage Test for trend p-value=0.02; data not shown). Stratifying by length of exposure and adjusting for

covariates, the protective association was 0.48 (95% CI 0.29-0.78) for < 1 year of exposure, 0.36 (95% CI 0.15-0.87) for exposure from 1 year to less than 2 years, and 0.28 (95% CI 0.12-0.67) for more than 2 years of exposure (Table 5.3).

We modeled bisphosphonate exposure as a time-dependent covariate and repeated the Cox Proportional hazards analysis reported in Table 5.3. Results from this revised analysis are given in Table 5.4. The findings are generally the same as from the prior models with some results not reaching significance, likely due to the extra power needed when considering time-dependent covariates.

Figure 5.1 is the Kaplan-Meier curve for breast cancer for ever exposed compared to unexposed women. Over a five-year period of observation, the log-rank p-value for disease free survival was <0.0001. Figure 5.2 compares disease free status over the five years by duration of exposure. Those exposed more than two-years were the least likely to be diagnosed with breast cancer (adjusted HR=0.28; 95% CI 0.12-0.67).

5.4 Discussion

Bisphosphonates are known to interfere with osteoclast bone resorption, a fact that has been exploited clinically for treating and preventing osteoporosis for several decades. In oncology, bisphosphonates have traditionally been used in the palliative care of bone metastasis, primarily for breast and prostate cancer and multiple myeloma, as well as other cancers. Early clinical studies of the adjuvant use of bisphosphonates in breast cancer were contradictory resulting in the initiation of the National Surgical

Adjuvant Breast and Bowel Project's (NSABP) B-34 trial of adjuvant clodronate in early stage breast cancer (Paterson AGG 2006).

Preclinical studies have demonstrated the anti-tumor effects of bisphosphonates, including inhibition of tumor cell proliferation, adhesion, angiogenesis, and invasion (Clezardin 2002; Green & Clezardin 2002; Clezardin et al. 2003; Boissier et al. 2000; Caraglia et al. 2007). In addition, bisphosphonates affect tumor microenvironment degradation proteins (i.e. matrix metalloproteinases) and induce apoptosis (Heikkila et al. 2002; Teronen et al. 1999; Ueda et al. 2003; Ueno et al. 1997; Sato et al. 1997; Oades et al. 2003). Some of these effects are modulated through the mevalonate pathway but a better understanding of the molecular mechanisms remains unclear.

Given the clinical observations and preclinical evidence, we hypothesized that bisphosphonates might also work in the chemoprevention of breast cancer. Using a retrospective cohort of insured women for whom all medical, claims, and pharmacy data were electronically available, we were able to assess breast cancer incidence among women ever or never exposed to bisphosphonates. Our cohort of over 40,000 women was followed for up to 5 years. Results reported here strongly support that bisphosphonates are associated with reduced risk of breast cancer. Indeed, our data suggest that exposure to bisphosphonates may lower breast cancer risk by half or more. The magnitude of the HR is comparable to the results of the NSABP-P1 trial of tamoxifen (Fisher BJ, et al. 1998). In addition, our data suggest that, unlike tamoxifen, bisphosphonates appear to affect both hormone receptor positive and negative tumors.

Tamoxifen and raloxifene are the only two FDA-approved chemopreventive agents for breast cancer. We also know that women with a prior exposure to hormone replacement therapy have a residual increased risk of breast cancer (Rossouw et al. 2002). Bisphosphonates may prove to be another option for patients and clinicians in the management of breast cancer risk, possibly with more acceptable side effects, especially in pre-menopausal women. Our results suggest that even with adjustment for hormone replacement therapy exposure, bisphosphonates reduce the risk of incident breast cancer significantly, by half or more. The direction of the association of bisphosphonates with stage and grade of newly diagnosed breast cancers suggests that women exposed to bisphosphonates may have lower stage disease and well differentiated tumors; however, this finding was not significant.

It has been reported that women with a higher bone density are less likely to develop osteoporosis but more likely to develop breast cancer (Chen Z et al. 2008). This is ascribed, without direct proof, to circulating estrogen levels that increase bone density but increase the risk of breast cancer. To assess whether our results were therefore confounded by this relationship we conducted a sensitivity analysis among only women who had at least one ICD-9 coded encounter for osteoporosis or had at least one prescription filled for a bisphosphonate. The results were, in essence, unchanged with an adjusted hazard ratio of 0.26 (95% CI 0.17-0.40) among the osteoporosis cohort (data not shown). In other words, even considering women hypothesized to have lower circulation estrogen (by virtue of having osteoporosis), we were able to discern a robust inverse association between bisphosphonates and breast cancer.

The Women's Health Initiative recently reported a minimal residual risk of breast cancer among women who had taken hormone replacement therapy within the last three years (Heiss G. et al. 2008). Given the breast cancer risks associated with hormone replacement therapy use, we stratified the original analysis by HRT exposure to assess confounding of our findings by HRT use. Since the results were unchanged (HR 0.43 95% CI 0.28-0.64 among the HRT free cohort) we decided to include all women regardless of HRT exposure and adjusted for exposure in the multivariable analysis.

It is especially crucial to consider side effects when ascertaining a potentially chemopreventive drug. The most common side effect with bisphosphonates is esophageal reflux, which can be minimized with the newer once-monthly formulations. A rare but serious side effect of bisphosphonate exposure is osteonecrosis of the jaw (ONJ) Recently a study has also suggested that women exposed to bisphosphonates may be at increased risk of atrial fibrillation (Heckbert SR, et al. 2008; Black DM, et al. 2007). Further clinical trials of bisphosphonates will be needed to accurately determine if the benefits outweigh the risks.

Because our study is performed on an observational cohort of insured women, we wanted to be sure that the breast cancer incidence within our cohort reflected the general population's experience. Breast cancer risk varies by race and age; therefore, we calculated standardized incidence ratios (SIR) by race and age. The overall SIR for our cohort was 1.09 suggesting that our cohort's breast cancer experience was very similar to that of the general population.

Another limitation of observational cohort studies is that patients are not randomized to exposure groups. We found that there were statistically significant differences in our study cohort by age and race. While we did adjust for these covariates in our multivariate analysis, it is likely that unmeasured, but important, breast cancer risk factors also varied by exposure status. These unmeasured and uncontrolled for factors may confound our findings; therefore, our results should be interpreted with this limitation in mind.

A strength of our study is the use of a retrospective cohort design to assess the potential role of bisphosphonate exposure on breast cancer incidence. Using automated data that already existed allowed for an inexpensive and time efficient study of our novel hypothesis. With a cohort of over 40,000 women for whom 5 years of follow-up was available, our study was robust enough to assess the chemopreventive effects of bisphosphonates. Our data, however, are not sufficiently conclusive to change clinical management of breast cancer risk. It is strong enough, however, to support a randomized prevention trial of bisphosphonates in order to explore the risks and benefits of bisphosphonates in the prevention of breast cancer.

Table 5.1. Population Characteristics

Characteristic	Exposed N(%)[1]	Unexposed N (%)	p-value[2]
Age			<0.0001
50-59	1,266 (37%)	23,101 (58%)	
60-69	853 (25%)	8,028 (20%)	
70-79	968 (28%)	6,098 (15%)	
80+	337 (9%)	2,616 (7%)	
Mean	66 (SD 10.4)	61 (SD 10.5)	<0.0001
Race			<0.0001
Black	957 (28%)	13,780 (35%)	
White	2,265 (66%)	23,477 (59%)	
Other	202 (6%)	2,586 (6%)	
Breast Cancer	25 (5%)[3]	469 (95%)	0.02

[1] Row percent
[2] Chi-square or t-test p-value
[3] Column percent

Table 5.2. Odds Ratios (OR) with 95% Confidence Intervals (CI) for Bisphosphonate Exposure and Stage, Grade, and Marker Status of Breast Cancer Cases.

	N	Exposed	Unexposed	OR (95% CI)
Stage				
0, 1, or 2	402	22 (96%)	380 (90%)	Referent
3 or 4	45	1 (4%)	44 (10%)	0.39 (0.05-2.98)
Grade				
1 or 2	280	16 (67%)	264 (59%)	Referent
3	192	8 (33%)	184 (41%)	0.72 (0.30-1.71)
Estrogen Receptor				
Positive	303	17 (81%)	286 (73%)	Referent
Negative	111	4 (19%)	107 (27%)	0.63 (0.21-1.91)
Progesterone Receptor				
Positive	269	17 (77%)	252 (65%)	Referent
Negative	140	5 (23%)	135 (35%)	0.55 (0.20-1.52)
Double Negative	109	4 (16%)	105 (22%)	0.66 (0.22-1.97)
v. Not	388	21 (84%)	364 (78%)	Referent
Double Positive	269	17 (68%)	252 (54%)	1.83 (0.77-4.32)
v. Not	225	8 (32%)	217 (46%)	Referent

Table 5.3. Adjusted and Unadjusted Breast Cancer Hazard Ratio (aHR and HR, respectively) and 95% Confidence Interval (CI) Overall and by Duration of Exposure.

	N (%)	HR (95% CI)	aHR* (95% CI)
Unexposed	39,844 (92%)	Referent	Referent
Exposed	3,423 (8%)	0.51 (0.34-0.76)	0.35 (0.23-0.53)
Duration of Exposure			
None to < 2 months	39,844 (92%)	Referent	Referent
2 months to 1 year	1,866 (4%)	0.58 (0.35-0.98)	0.48 (0.29-0.78)
1 to < 2 years	713 (2%)	0.48 (0.20-1.16)	0.36 (0.15-0.87)
2 years or more	844 (2%)	0.38 (0.16-0.93)	0.28 (0.12-0.67)

* Adjusted for age, race, index year, number of screening mammograms and any exposure to hormone replacement therapy.

Table 5.4. Adjusted and Unadjusted Breast Cancer Hazard Ratio (HR and aHR, respectively) with 95% Confidence Interval (CI) with Bisphosphonate Exposure Modeled as Time-Dependent Covariate.

	N (%)	HR (95% CI)	aHR (95% CI)
Unexposed	39,844 (92%)	Referent	Referent
Exposed	3,423 (8%)	0.48 (0.35-0.67)	0.66 (0.47-0.92)
Duration of Exposure			
None to < 2 months	39,844 (92%)	Referent	Referent
2 months to 1 year	1,866 (4%)	0.48 (0.35-0.67)	0.66 (0.47-0.92)
1 to < 2 years	713 (2%)	0.39 (0.16-1.00)	0.41 (0.16-0.51)
2 years or more	844 (2%)	0.53 (0.21-1.36)	0.55 (0.22-1.42)

* Adjusted for age, race, index year, number of screening mammograms and any exposure to hormone replacement therapy.

Figure 5.1 Kaplan-Meier Curve for Breast Cancer for Ever or Never Exposed to Bisphosphonates.

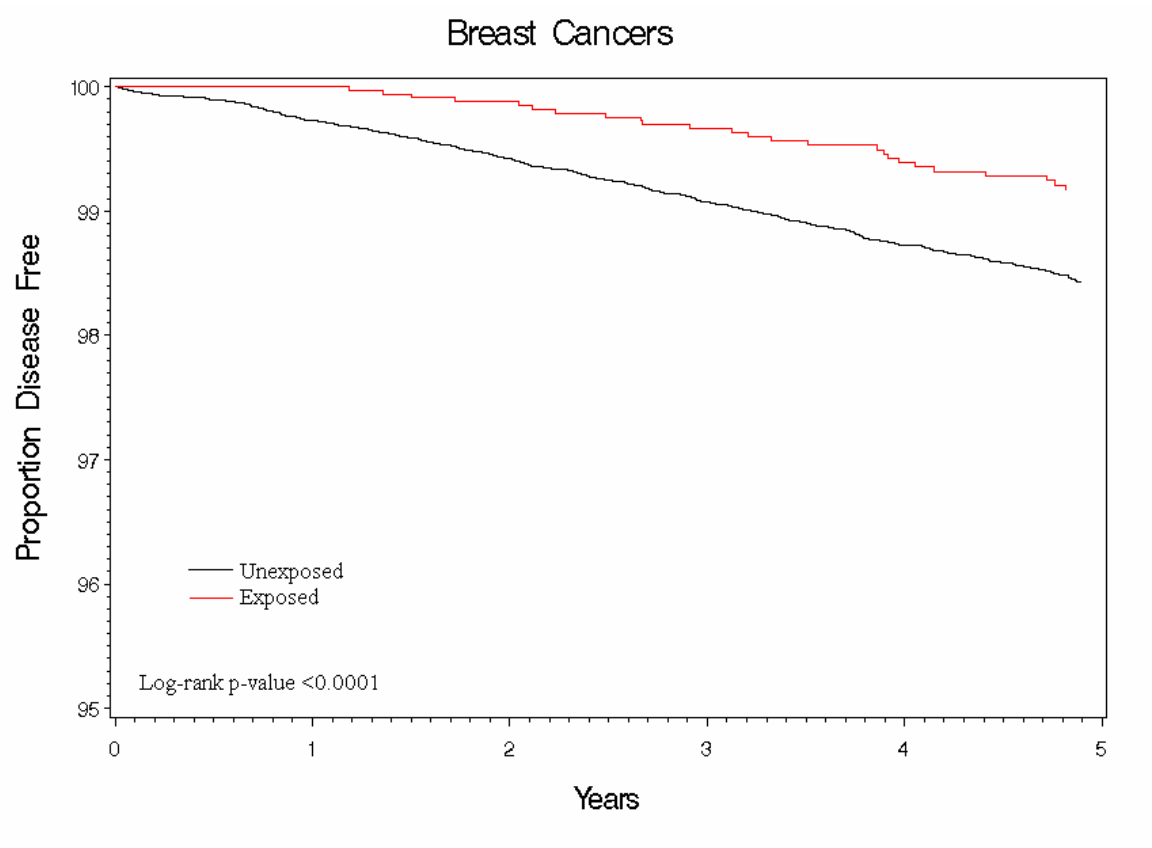
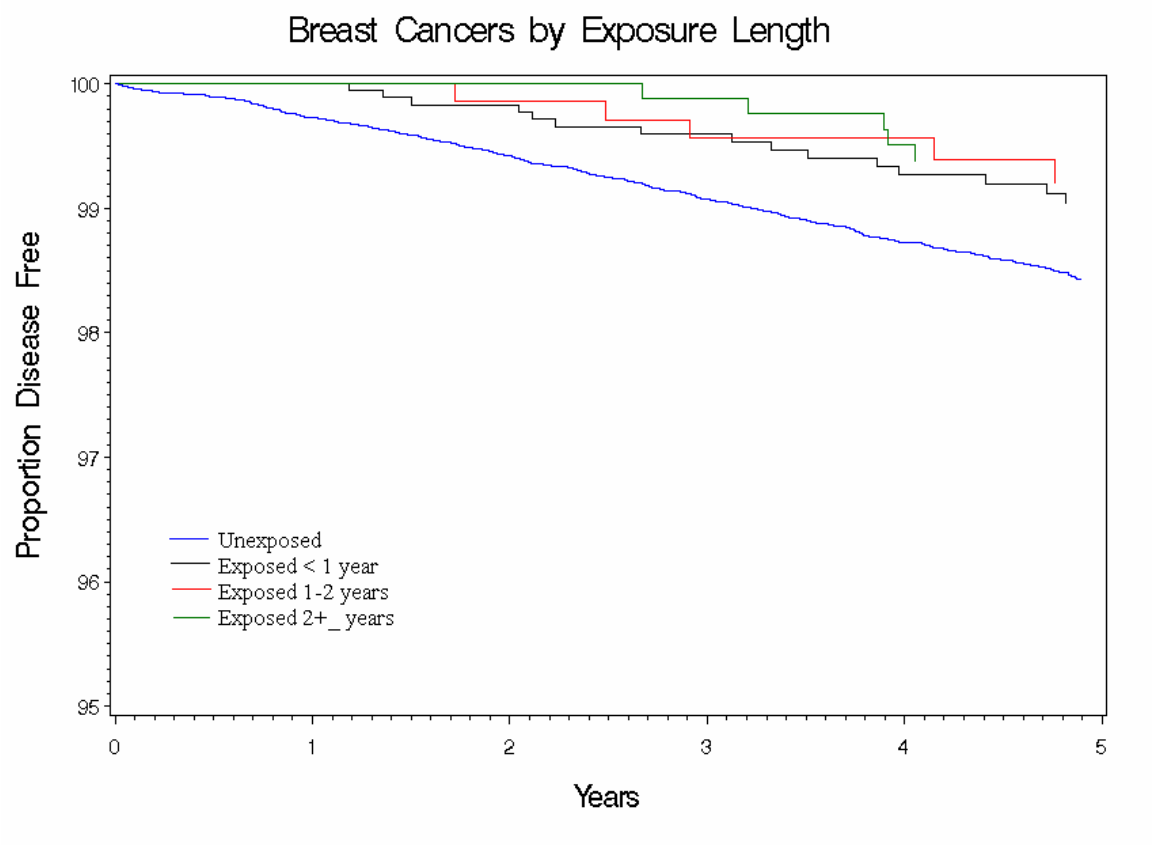


Figure 5.2 Kaplan-Meier Curve for Breast Cancer by Cumulative Exposure Status.



References

Black DM, Delmas PD, Eastell R, Reid IR, Boonen S, Cauley JA, Cosman F, Lakatos P, Leung PC, Man Z, Mautalen C, Mesenbrink P, Hu H, Caminis J, Tong K, Rosario-Jansen T, Krasnow J, Hue TF, Sellmeyer D, Eriksen EF, Cummings SR, HORIZON Pivotal Fracture Trial. (2007) Once-yearly Zoledronic Acid for Treatment of Postmenopausal Osteoporosis. New England Journal of Medicine 356(18):1809-22.

Boissier S, Ferreras M, Peyruchaud O, Magnetto S, Ebetino FH, Colombel M, Delmas P, Delaisse JM, Clezardin P. (2000) Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Research 60(11):2949-54.

Caraglia M, Marra M, Leonetti C, Meo G, D'Alessandro Am, BValdi A, Santini D, Tonini G, Bertieri R, Zupi G, Budillon A, Abbruzzese A. (2007) R115777 (Zarnestra)/Zoledronic acid (Zometa) cooperation on inhibition of prostate cancer proliferation is paralleled by Erk/Akt inactivation and reduced Bcl-2 and bad phosphorylation. Journal of Cell Physiology 211(2):533-43.

Chen Z, Arendell L, Aickin M, Cauley J, Lewis CE, Chlebowski R. (2008) Hip bone density predicts breast cancer risk independently of Gail score: results from the Women's Health Initiative. Cancer 113(5):907-15.

Clezardin P. (2002) The antitumor potential of bisphosphonates. Seminars in Oncology 29(6 Suppl 21):33-42.

Clezardin P, Fournier P, Boissier S, Peyruchaud O. (2003) In vitro and in vivo antitumor effects of bisphosphonates. Current Medical Chemistry 10(2):173-80.

Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, E Tan-Chiu, Ford L, Wolmark N. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. Journal of the National Cancer Institute 90(18):1371-88.

Green JR, Clezardin P. (2002) Mechanisms of bisphosphonate effects on osteoclasts, tumor cell growth, and metastasis. American Journal of Clinical Oncology 25 (6 Suppl 1):S3-9.

Heckbert SR, Li G, Cummings SR, Smith NL, Psaty BM. (2008) Use of alendronate and risk of incident atrial fibrillation in women. Archives of Internal Medicine 168(8):826-831.

Heikkila P, Teronen O, Moilanen M, Konttinen YT, Hanemaaijer R, Laitinen M, Maisi P, van der Pluijm, G, Barlett JD, Salo T, Sorsa T. (2002) Bisphosphonates inhibit stromelysin-1 (MMP-3), matrix metalloelastase (MMP-12), collagenase-2 (MMP-13) and enamelysin (MMP-20), but not urokinase-type plasminogen activator, and dimish invasion and migration of malignant and endothelial cell lines. Anticancer Drugs 13(3):245-54.

Heiss G, Wallace R, Anderson GL, Aragaki A, Beresford SA, Brzyski R, Chlebowski RT, Gass M, LaCroix A, Manson JE, Prentice RL, Rossouw J, Stefanick ML, WHI Investigators. (2008) Health risks and benefits 3 years after stopping randomized treatment with estrogen and progestin. JAMA 299(9):1036-45.

Oades GM, Senaratne SG, Clarke IA, Kirby RS, Colston KW. (2003) Nitrogen containing bisphosphonates induce apoptosis and inhibit the mevalonate pathway, impairing Ras membrane localization in prostate cancer cells. Journal of Urology 170(1):246-52.

Paterson AHG. (2006) The role of bisphosphonates in early breast cancer. The Oncologist 11(suppl 1):13-19.

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J; Writing Group for the Women's Health Initiative Investigators. (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA 288(3):321-33.

Sato H, Okada Y, Seiki M. (1997) Membrane-type matrix metalloproteinases (MT-MMPs) in cell invasion. Thromb Haemost 78(1):497-500

Teronen O, Heikkila P, Konttinen YT, Laitinen M, Salo T, Hanemaaijer R, Teronen A, Maisi P, Sorsa T. (1999) MMP inhibition and downregulation by bisphosphonates. Annual of New York Academy of Science 878:453-65.

Ueda J, Kajita M, Suenaga N, Fujii K, Seiki M. (2003) Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. Oncogene 22(54):8716-22.

Ueno H, Nakamura H, Inoue M, Imai K, Noguchi M, Sato H, Seiki M, Okada Y. (1997) Expression and tissue localization of membrane-types 1, 2, and 2 matrix metalloproteinases in human invasive breast carcinomas. Cancer Research 57(10):2055-60.

Chapter 6

Conclusion

In the US, cancer accounts for 1 of every 4 deaths. In US women, breast cancer is the cancer most often diagnosed and the second most common cause of cancer death. Irrespective of technological and pharmaceutical advances, breast cancer treatment still results in significant short-term toxicities and long-term comorbidities. And despite enduring these effects, some women are still claimed by the disease. Breast cancer is a frightening diagnosis for a woman and even when a "cure" is achieved, she remains concerned about recurrence, resulting in a life-long psychological burden. This dissertation, though it represents several years of work, is a humble contribution to the larger scientific effort to lessen suffering from breast cancer.

In Chapter 2, I addressed breast cancer diagnosis in an under-studied racial/ethnic group, Arab-American women. This racial/ethnic group is not well enumerated since US standard population data techniques include "Arab" in the "Caucasian" category. As a result, disease incidence rates specific to this population are impossible to calculate. We used data from the metropolitan Detroit SEER registry to study breast cancer

characteristics at diagnosis among Arab-American women. Our results suggest that Arab-American women have a different breast cancer experience from both European- and African-Americans. Arab-American women were diagnosed at a younger age and had more regional disease with a more aggressive phenotype than their European-American counterparts. Although not statistically significant, there was also a trend observed in our data for Arab-American women to be more likely to have small tumors with positive nodes. Importantly, these differences in disease characteristics which would suggest poorer prognosis did not translate into a survival disadvantage. This could mean that breast cancer in Arab-American women is more responsive to treatment.

Recognition of Arabs as a separate minority group and detailed analyses of their breast cancer burden would allow better population statistics for public health research and planning for policy and social support services. At the time of completing this dissertation, this study has been published in the journal *Breast Cancer, Research, and Treatment*. (2009)

Chapters 3 and 4 are molecular studies which were undertaken to identify potential prognostic markers that might discriminate indolent tumors from aggressive breast cancer. Using a population-based cohort of early stage breast cancer, we investigated expression two genes, RhoC and EZH2, with clinicopathologic features at breast cancer diagnosis. We also investigated whether expression of either of these genes was related to the development of any recurrence or distant metastasis. Rho proteins are involved in controlling cytoskeletal reorganization, cell motility, membrane ruffling, cell trafficking, and certain aspects of cellular proliferation and apoptosis. RhoC has been

associated with metastatic cellular features like adhesion, invasion, and migration. EZH2 is a polycomb group protein that functions in cell memory and differation. Overexpression has been associated with cancer progression and disease outcomes.

Using a tumor microarray of breast tissue samples from our cohort, we examined the expression of both RhoC and EZH2, independently, with characteristics at diagnosis and disease outcomes. The RhoC analysis is currently inconclusive but we will continue to pursue this study. There were some novel findings from the EZH2 analysis. To our knowledge, we are the first to find an association with EZH2 positivity and family history, race, and Her2 status. Our results support prior research demonstrating a relationship between EZH2 and indicators of poor prognosis and outcomes.

Finally, Chapter 5 explores the potential use of a commonly used drug for osteoporosis for breast cancer chemoprevention. Bisphosphonates are known to interfere with osteoclast bone resorption, a fact that has been exploited clinically for preventing and treating osteoporosis for several decades. Preclinical studies have demonstrated the anti-tumor effects of bisphosphonates, including inhibition of tumor cell proliferation, adhesion, angiogenesis, and invasion. Given the anti-tumor effects, we investigated whether women exposed to bisphosphonates might be at a decreased risk of breast cancer. Our results suggest that breast cancer risk is reduced over half for those women exposed. These are exciting findings. After a successful pre-submission inquiry to the New England Journal of Medicine, we are currently in the process of formally submitting this work.

The collective work presented here is what I hope will represent the beginning of a long and productive career dedicated to using cancer epidemiology as a tool to reduce the burden of breast cancer in the population.