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HER-2/HER-3 HETERODIMERS: A QUANTITATIVE MOLECULAR

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HER-2/3 HETERODIMERS: A QUANTITATIVE MOLECULAR EPIDEMIOLOGY MARKER AND METHOD OF ANALYSIS

By

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ABSTRACT

HER-2/3 HETERODIMERS: A QUANTITATIVE MOLECULAR EPIDEMIOLOGY MARKER AND METHOD OF ANALYSIS

By

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The emerging field of quantitative molecular epidemiology promises to expand our understanding of the molecular bases of disease, and treatments that can specifically target these pathways. Closely resembling our current approach to infectious disease, molecular epidemiology has already begun to allow classification of disease on the basis of the molecular pathways leading to pathology, and thereby allowing treatment to target these pathways.

This document was derived from an application for funding to refine methods for quantifying the data contained within human tissues. Specifically, these methods will be applied to test the hypothesis that the Her-2 and Her-3 cell surface receptors must interact through non-covalent association (dimerization) to elicit pathophysiological activity in Her-2 positive human breast cancer. Through the use of fluorescent markers, the location of these proteins may be mapped as pixels within microscopy images. A molecular epidemiologic statistical parameter will be developed that will quantify the correlation of these imaged markers (colocalization–used as a proxy for the biological phenomenon of dimerization) The statistical power of this parameter to discriminate between differential prognostic groups will be measured and compared to existing methods.

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SOLI DEO GLORIA

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Chapter 1

Introduction

This work, derived from a grant application written to obtain funding for this project, describes an approach to analysis of health-related events on the molecular level, which is the aim of the field of Quantitative Molecular Epidemiology. The immediate context for this discussion is a proposed study designed to analyze specific molecular markers of breast cancer prognosis (Her-2 and Her-3 cell surface receptors). The study will test the hypothesis that measuring Her-2 alone is an imprecise prognostic measure due to the fact that Her-2 and Her-3 must interact to elicit pathophysiological activity in Her-2 positive human breast cancer. Instead, quantitative measurement of Her-2/Her-3 interaction is proposed as a more precise predictor of breast cancer prognosis. Such non-covalent interaction of two proteins to form a single functional unit (by virtue of their complementary structure and function) is referred to as dimerization. Specifically, when the two subunits are different proteins, the phenomenon is referred to as heterodimerization. The goal of the present work, then, is to quantify Her-2 and Her-3 heterodimerization and test whether this quantitative parameter represents an improved prognostic marker in breast cancer. Unfortunately, these proteins are too small to image individually using light microscopy. Instead, fluorescent markers will be used to image the density of these proteins aggregated over very small units of space (pix-

els), and the correlation of Her-2 and Her-3 density over space will be quantified using strategies detailed below. This correlation over space, or colocalization, will be used as a computational proxy for measuring heterodimerization directly.

In the balance of this chapter, Quantitative Molecular Epidemiology is further defined and placed into context, and the specific aims of the proposed study are enumerated.

1.1 Human Tissue Research: a growing imperative.

In recent years there has been an increasing emphasis on the use of human models and specimens in cancer research (1). Numerous gene and gene product markers of pathologic processes have been identified, including tumor suppressor genes such as p53 (2) and oncogenes and their products including telomerase (3), VEGF (4), and Met (5), to name a few. These markers provide substantial opportunities for new stratification and analysis of both past and future data.

This family of identified markers will certainly continue to grow over the coming years, and as it does, the potential for medical progress presented by human specimen analysis will grow as well. The increasing library of markers will allow not only investigation of the role of single components, but elucidation of complex collaborations between genes and gene products that produce normal or pathological responses via (possibly competing) multi-factorial pathways. Archived samples—when connected to treatment, outcome and survival data—will allow these markers to be analyzed in sophisticated diagnostic, prognostic, and treatment effect models.

Diagnosis and prognosis on the basis of visual examination of tissue on the microscopic level is well established (6). However, objective, quantifiable criteria

require further development (7, 8). Subjective grading by pathologists (even those trained at the same institution) exhibits a high level of inter-observer variability whether using common pathological classification systems such as WHO, Bloom-Richardson, or Nottingham, (8–10) or more complex systems such as Bayesian Belief Networks (11). This impedes the transferability of prognostic models, and may lead to either type I or type II errors in models using these subjective interpretations as predictive variables. The potential inherent in digital image analysis as a more quantitative, objective, and reproducible histologic tool has been described (12–14). The lack of objective, quantitative methodology has been cited as a significant challenge in the emerging era of molecular epidemiology (15).

As the molecular markers of disease etiology and progression become increasingly defined, our thinking about the epidemiology and treatment of disease must evolve. Consider the following hypothetical example: Figure 1.1 shows some hypothetical results from a clinical trial, which could be considered to indicate treatment failure (i.e., affirmation of the null hypothesis) since the odds ratio is essentially 1 (95% CI: 0.88-1.35), indicating no treatment effect.

However, a different picture emerges in figure 1.2. The patients have been retrospectively stratified on a "new" hypothetical marker. It emerges that the treatment may be highly efficacious for those patients who were positive for the marker. If this post-hoc finding was confirmed through one or more prospective studies, future patients could be tested for this marker, and treated accordingly. This is more than a hypothetical possibility. Genetic profiling has already allowed the identification of differential prognostic groups among B-cell lymphoma patients (16). Additionally, examination of cell lines from the National Cancer Institute's in vitro drug screen has identified genetic profiles with unique drug susceptibilities based

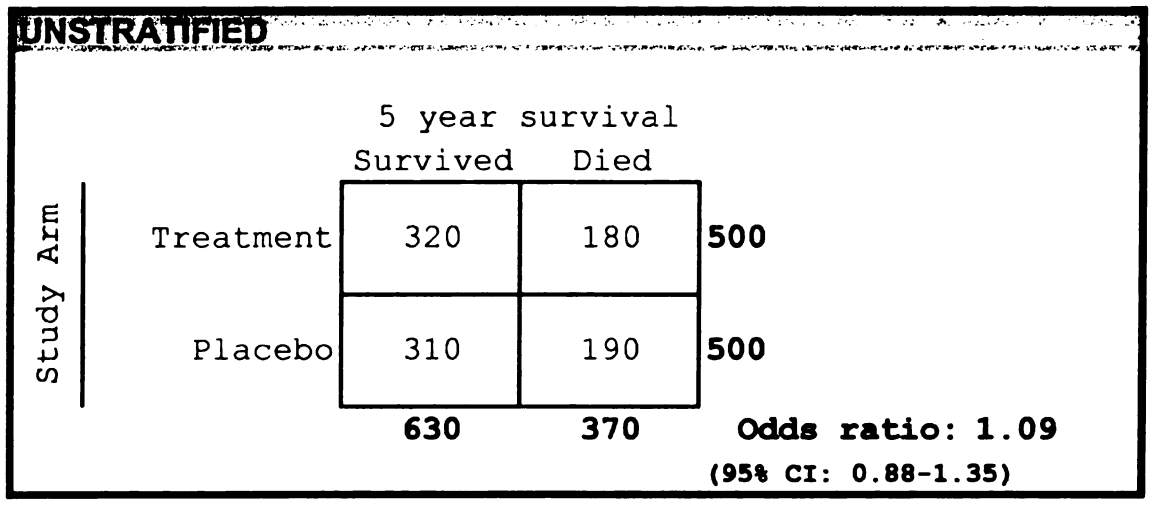


Figure 1.1: Prior to stratification, it appears that the treatment had no effect (the odds ratio is essentially 1, (95% CI: 0.88-1.35).

on activation of the ras oncogene (17). And as will be discussed fully below, Her-2 expression has been used to identify differential prognostic and treatment groups.

This sort of approach is more akin to our current methods of treating infectious diseases than traditional approaches to cancer treatment. While the approach to infectious disease treatment has long emphasized the identification of a specific etiologic agent and treatment with a compound of demonstrated efficacy against that agent, cancer treatment has typically taken the form of generalized assault against the entire patient with the hope of some differential damage to the putative cells. Molecularly targeted treatments hold great promise in elucidating optimal treatment modalities for specific groups of cancer patients while minimizing side effects. Even a retrospective look at treatment and diagnosis with new markers could be very useful and reveal new information from past studies. Recent evidence suggests that even decades old specimens contain useful biological data waiting to be tapped (18, 19).

This work describes an effort to further develop the analysis tool set that can be

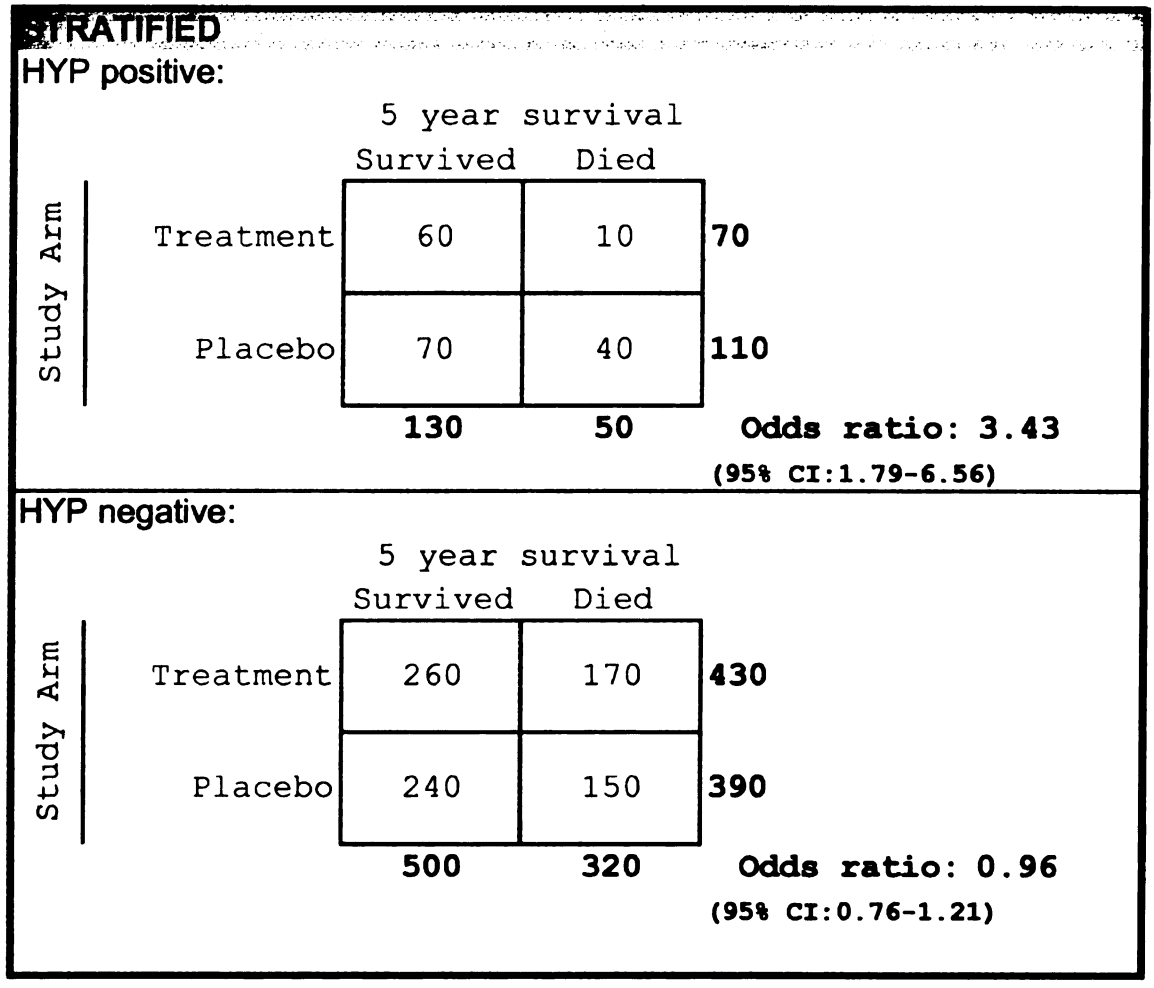


Figure 1.2: Stratification on the new genetic marker reveals a pronounced effect among the subgroup of marker positive patients (95% CI: 1.79-6.56), as opposed to the marker negative patients (95% CI: 0.76-1.21).

applied to human tissues to elucidate these molecular pathways and quantify these phenomena for use as epidemiologic markers. A quantitative, automated analysis tool to measure the interaction of two or more proteins (e.g. dimerization–the formation of single functional unit by two identical proteins–or heterodimerization–the dimerization of two different proteins) within human tissue samples is proposed. Furthermore, we propose to apply this methodology to quantifying the formation of Her-2/Her-3 heterodimers (via measurement of colocalization–the correlation or covariance of protein density over space as represented by fluores-

cent microscopy images) on the cell surface as a marker of breast cancer prognosis.

1.2 Quantitative Molecular Epidemiology Defined

Molecular epidemiology seeks to apply the tools of cellular biology to measurement of disease related states and events for the purposes of epidemiologic analysis. Quantitative molecular epidemiology takes this process one step further by quantifying these cellular phenomena as opposed to relying on subjective interpretation (an example salient to the present work is pathology, wherein molecular markers imaged microscopically may be interpreted subjectively by the pathologist or quantitatively using computer algorithms). This emerging field of quantitative molecular epidemiology promises to expand our understanding of the molecular bases of disease, and treatments that can specifically target these pathways.

Molecular epidemiology represents a paradigm shift in cancer research. As opposed to the former emphasis on anatomical site as a classification scheme (e.g. "Ductal Carcinoma In Situ"), today we are increasingly able to classify tumors based on their genetic and molecular fingerprint (e.g. "Her-2 overexpressing, estrogen receptor negative"). This allows us to direct treatments not at the imprecise target of anatomical site, but at specific causal factors elucidated through knowledge of the molecular pathway leading to carcinogenesis, invasion, growth, and metastasis.

Therefore, molecular epidemiology affords two critical opportunities:

1. The ability to classify disease based on molecular fingerprints, allowing for classification and stratification that has a high degree of granularity and physiologic relevance. This leads to an improved typology when studying cause, effect, or treatment.

2. The ability to target treatments at specific cellular events that lead directly to pathologic events

Molecular epidemiology has already elucidated predisposing genetic polymorphisms associated with increased risk (e.g., the *BRCA* family (20)) as well as proteins involved in carcinogenesis such as the growth factor HGF and its receptor Met. These molecular markers have significantly improved the resolving power of prognostication over histological interpretation alone. Camp, et al. (21) have demonstrated that high Met expression in node negative breast cancer patients is associated with increased risk of dying within five years (RR=5, p=0.03) independent of tumor size and exhibits a synerigistic relationship with nuclear grade (RR=33.4, $p<0.001$ for advanced nuclear grade combined with high Met expression). Similarly, in a land mark study, Yamashita, et al. (22) reported that in multivariate survival analysis of 258 breast cancer patients, high HGF expression was a more potent predictor of poor survival (RR=3.3, p=0.001) than any histological indicator or even nodal status (RR=2.5).

Such an approach will surely have much to offer the ongoing study of breast cancer. First, exposures such as exercise, chemicals, and hormonal events are upstream components of causal chains leading to downstream molecular pathways in the cells giving rise to tumors. By elucidating the molecular details of these pathways, exposures can be better understood and treatment responses better designed. Furthermore, existing risk factors explain only a fraction of total breast cancer risk. Much of the remaining risk may only be explained through analysis of molecular events that either lack measurable upstream causes or manifest heterogenous cellular responses to known exposures.

The story behind the HER-2 receptor illustrates wonderfully this full spectrum

of molecular epidemiologic effort, from molecular cause to molecular treatment. It further highlights new tools that must be developed in order to practice this kind of science. Some of these tools have been developed, and some will be refined through the work described in this proposal.

1.3 Specific aims

Through this study, methods for quantifying the data contained within human tissues will be refined, and details of the role of Her-2 and Her-3 in human breast cancer will be elucidated. A molecular epidemiologic parameter will be developed that will quantify the colocalization of Her-2 and Her-3 protein densities in breast cancer cells as a proxy for quantifying heterodimerization of these proteins (which are too small to image individually using light microscopy). This measure will be used to test the hypothesis that heterodimerization of these receptors is a more precise measure of pathophysiologic activity and, therefore, prognosis than measurement of Her-2 expression alone. The power of this parameter as a prognostic marker will be compared to existing methods. Specifically, this project has the following component aims:

1. Test the relative power of two approaches to quantifying Her-2 and Her-3 colocalization in cancer tissue–joint moment of standard images analysis and correlation of globally standardized images analysis–as compared to each other and traditional methods.

2. Test whether variation in a quantitative, continuous variable describing the level of Her receptor colocalization in breast cancer tissue is a significant predictor of time-to-event in univariate and multivariate models of breast cancer outcome.

3. Develop an algorithm and software that are as simple in design as possible to promote the extension of these findings to clinical practice.

In the following chapters, a synopsis of the literature pertaining to the growing role of quantitative molecular epidemiology and the role of Her-2 in breast cancer will be presented, followed by a description of the design of this study and the proposed methods to be used to execute the study.

Chapter 2

Her-2 and Breast Cancer: Epidemiology and Cell Biology

Her-2, also known as *neu* and *c-erbB-2*, is a proto-oncogene that encodes a 185kDa trans-membrane cell surface receptor (p185, or Her-2) with tyrosine kinase activity. (Note that throughout this document, gene names are placed in italics, whereas their protein products–typically bearing the same name–are rendered in plain text. I.e., *Her-2* is a gene, whereas Her-2 is a protein). It is a member of the EGFR type I family together with epidermal growth factor receptor (EGFR), Her-3 and Her-4. The story of Her-2 is highly illuminating in terms of clarifying the potential and the pitfalls inherent in the dawning era of molecular epidemiology. The science surrounding Her-2 reveals the potential these molecular markers offer to quantitatively differentiate between differing prognostic and treatment groups.

In 1974, a group of closely related oncogenes was identified in rat neuroblastoma cells(23), and was therefore termed *neu*. Over the subsequent decade, it became clear that this gene family encoded p185 (a common tumor antigen) (24–26), was homologous to but not the same as the *erbB* gene that encoded the epithelial growth factor receptor (27, 28), and encoded a growth factor receptor tyrosine kinase (18). These genes were therefore labelled erbB-2 and erbB-3. *Neu/erbB-2* was capable of transforming certain cell lines into a malignant state, and was found to

be activated in many rat neuroblastomas (29). Subsequently, similar *erbB-2* related genes were probed from human genomic libraries and isolated independently by two groups (27, 30). The human analog was termed human Human Epidermal-growth-factor-receptor-like Receptor, or *Her-2*. The matter reached its denouement with the revelation from sequencing analysis that *erbB-2*, *neu*, and *Her-2* were not only related but also, in fact, one and the same (27, 30). The gene had already been found to be elevated (i.e., amplified) in human mammary carcinoma (28).

Growth factors and their receptors are integrally linked to the cell cycle, and regulate apoptosis, locomotion, differentiation, and division. It is important to note that Her-2, like numerous other growth factor receptors and their ligands, are essential components of normal development and physiology. These proteins may become important in the development and progression of cancer, however, when they are mutated or over-expressed (either through gene amplification or break down of normal regulatory pathways). Therefore, as a growth factor receptor candidate, both the *Her-2* gene and its protein product quickly became the subject of intensive study to discover what role, if any, it may play in carcinogenesis. What has been found makes clear the fact that molecular epidemiology will continue to improve the outlook for patients even while presenting unique challenges to the field.

2.1 Descriptive Epidemiology of Breast Cancer and Her-2 expression

Metastatic breast cancer kills more than 40,000 women each year (31). The incidence of invasive breast cancer has been rising over the past several decades (32) as shown in figure 2.1. Mortality has decreased nearly 20 percent over the past decade from 15.5 deaths per 100,000 cases in 1988 to 12.6 in 1998 (figure 2.2). Both

these facts can no doubt be explained in part by improved screening and the resulting biases in detection and lead time (33–37).

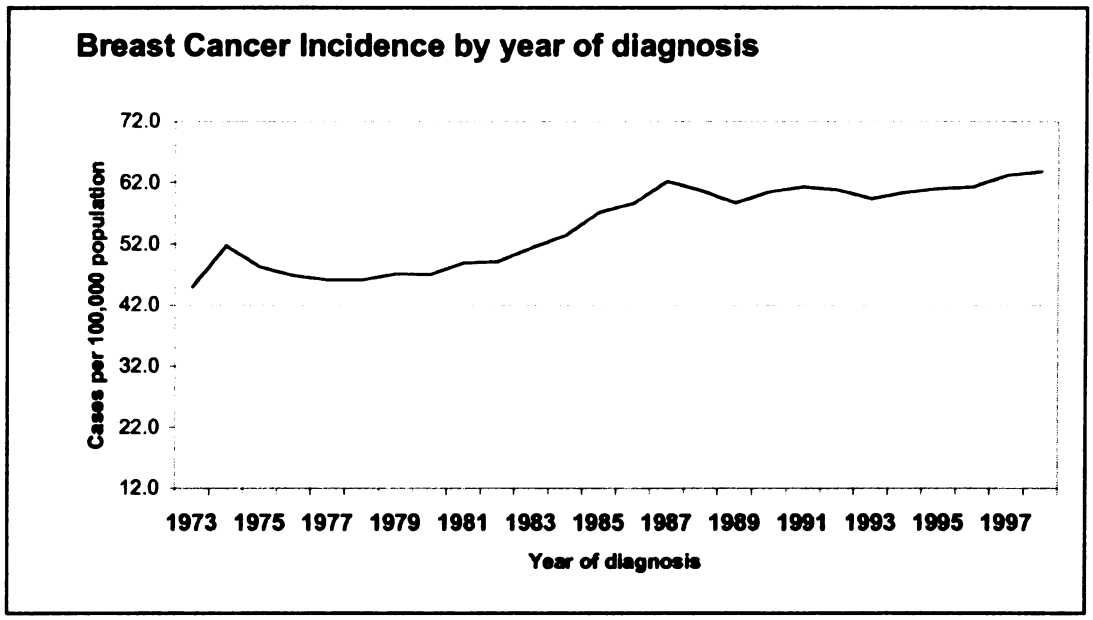


Figure 2.1: Incidence of invasive breast cancer per 100,000 females (age adjusted to 1970 standard population) by year of diagnosis. Source: SEER cancer registry data (32).

In addition to invasive breast cancer, a comparable number of women are affected by non-invasive ductal carcinoma in situ of the breast. The incidence of this diagnosis increased 14 fold between 1973 and 1998 (from 2 cases per 100,000 women in 1973 to 28 per 100,000 in 1998) (32, 38).

One area of extensive investigation is the role of the Human epidermal-growth-factor-receptor-like receptor (HER) family receptors, particularly Her-2, in breast cancers. A 1998 review of 22,616 patients indicated that about a quarter of breast cancer patients express Her-2 in tumor tissue at detectable, though not necessarily elevated, levels. Also of interest is the proportion of patients who exhibit higher than normal Her-2 levels. Elevated protein levels may occur through two pathways. One is gene amplification (an abnormally high number of copies of the

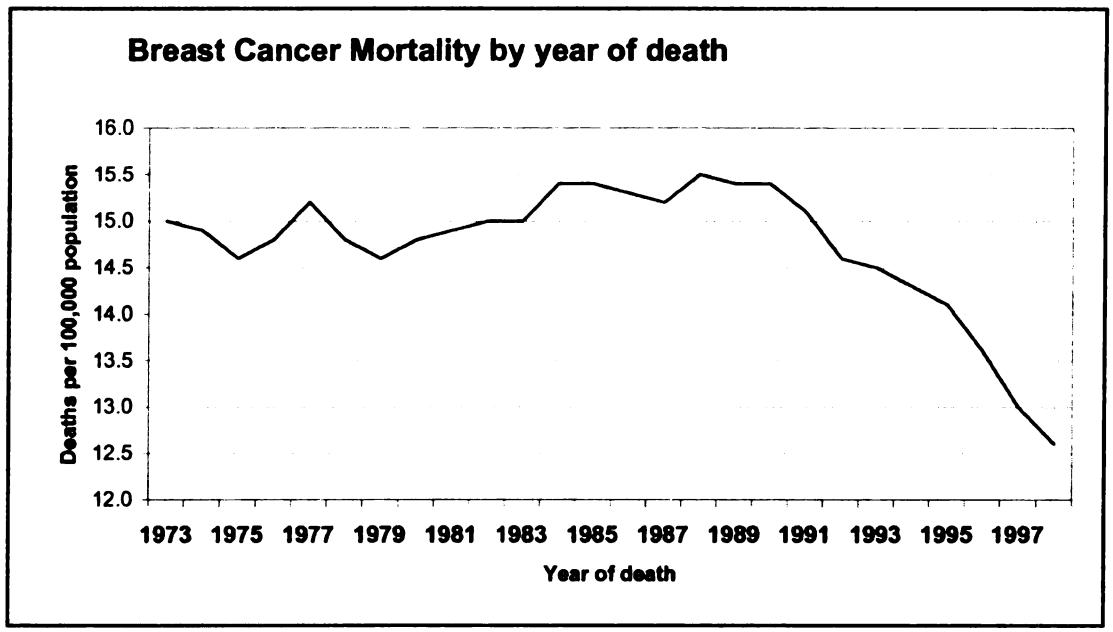


Figure 2.2: Mortality from invasive breast cancer per 100,000 female population (age adjusted to 1970 standard population) by year of death. Source: SEER cancer registry data (32).

gene in the genome as detected by microarray, fluorescent in-situ hybridization, or southern blot), and the other is over-expression (the increased production of transcription products from either a normal or abnormal amount of mRNA, as detected by western blot or immunohistochemistry). Her-2 gene amplification occurs in 20-30% of breast cancer cases (39, 40). Immunohistochemistry reveals that over-expression of the Her-2 gene product occurs in about 20% of breast cancer cases (41).

2.2 Analytic Epidemiology of Breast Cancer and Her-2 expression

2.2.1 Breast cancer risk factors

According the American Cancer Society, the following are currently accepted risk factors for breast cancer (taken from www.cancer.org):

1. Age. Less than 1% of breast cancer are diagnosed prior to age 30.

2. Genetics. About 10% of breast cancer risk can presently be accounted for by known gene mutations. (Note that in the current work we are concerned not with a gene mutation, but with excess protein production from a gene, such that Her-2 over-expression and/or dimerization would not be included in this figure).

3. Family history. Having one first degree relative with breast cancer increases a woman's risk of breast cancer 1 fold, while having two first degree relative with breast cancer increases a woman's risk 5 fold.

4. Personal history of breast cancer. In addition to recurrence, a woman with breast cancer is at a 3-4 fold increased risk of developing a second breast cancer that is not a metastasis or recurrence of the first tumor.

5. Race. White women are about 1.2 times as likely to be diagnosed with breast cancer as opposed to African American women (42).

6. Previous breast biopsy. Prior diagnosis of non-malignant hypertrophic breast disease is associated with a 1.5-5 fold increase in risk.

7. Previous breast irradiation. Prior irradiation for another cancer is associated with increased risk.

8. Menstrual periods. Early menarche or late menopause is associated with a small increase in risk.

9. Oral contraceptive use. While unclear, recent reports suggest that oral contraceptive use may be associated with a slight increase in risk (RR 1.2, $p<0.05$) (43).

10. Not having children. Having no children, or having a first child after age 30, is associated with a slight increase in risk.

11. Hormone replacement therapy (HRT). HRT has been shown to slightly increase the risk of breast cancer, however this risk is perhaps offset by other health benefits.

12. Alcohol. Consumption of 2-5 drinks per day is associated with a 1.5 fold increase in rick of breast cancer.

13. Obesity/High Fat Diet. Obesity is associated with increased risk, and some ecological studies suggest that high fat diet may increase the risk of breast cancer as well.

In addition, physical inactivity, environmental pollution, and not breast feeding have been suggested as possible risk factors by some studies.

Of course, all of these risk factors account for only a fraction of the risk of breast cancer underscoring the need for ongoing research into the causes of breast cancer. Reviews of known breast cancer risk factors demonstrate that only 30-40% of breast cancer risk has been explained (44).

2.2.2 Her-2 is elevated in a variety of cancers.

Information from published studies reporting on the relationship between Her-2 and prognosis for various cancers is presented in table 2.1. The various studies used a variety of measures to measure the effect of Her-2 expression on prognosis, including Cox regression models, Kaplan-Meier models, and simple t-tests between 5-year survival rates. For this reason, interpretation of the results of these studies must proceed with care.

Some general trends can be deduced from the literature summarized in table 2.1. It is unclear what the role of Her-2 may be in Ovarian cancers. While several of fairly large studies found over-expression to correlate with worse prognosis, including the single quantitative study, numerous other studies fail to bear this out.

Her-2 seems to play a clearer role in salivary gland tumors. Interestingly, the largest study (45) found only a "suggestive" association. However, the small number of Her-2 expressing tumors (2 of 201) calls into question the methodology or definitions used in this study, as does the fact that all the other studies (with the exception of one very small study) found a correlation between over-expression and decreased survival. Similarly, the majority of studies examining the role of Her-2 in bladder cancer report a marked decrease in survival among patients with Her-2 expressing tumors (although the failure of many of these studies to distinguish between expression and over-expression is cause for some skepticism). However, it should be noted that one study found a positive correlation between Her-2 expression and survival.

The results with regard to Esophageal cancer are more ambiguous. Nonetheless, these studies, taken together, seem to suggest that Her-2 is a prognostic factor in Barrett's adenocarcinoma, particularly those of an intestinal cell type.

Equally ambiguous are the results pertaining to renal cancer and pancreatic cancer. However, the report of correlation of her-2 expression with decreased survival in a number of large studies should motivate further investigation of the role of Her-2 in these cancers.

Expression of Her-2 appears to be an important predictor of outcome in gastrointestinal cancers, particularly those of advanced intestinal cell type.

Her-2 does not appear to be a major predictor of Lung cancer survival, with the possible exception of small cell lung carcinomas. It will be important to continue to monitor the role of known molecular markers in lung cancer given that the distribution of lung cancer pathophysiology may evolve over time as a result of changing smoking behaviors.

An overarching message that can be taken from these studies is that both methodology and definitions vary and involve considerable subjectivity. This fact underscores the importance of an approach that is not only molecular, but quantitative as well if we are to fully exploit the information inherent in the patterns of molecular pathways with respect to disease processes.

One hazard of multivariate analysis in the context of molecular epidemiology bears emphasizing. When incorporating molecular markers with environmental, demographic, or "macroscopic" clinical indicators, one is virtually guaranteed of identifying factors that share a pathway—one factor upstream, another downstream. Careful consideration must be given in selecting interaction terms in a meaningful way to ensure that covariance of upstream factors do not lead to elimination of downstream factors when those downstream factors are the predictors of interest in the study.

Table 2.1: Her-2 and survival in various cancers, by organ type. The study size (N¿ is given, followed by the number of subjects who were Her-2 positive as defined by the study measures and definitions, given in the subsequent columns. Where multiple measures were used, respective subject counts are given in the Her-2 + column. Where or not the given measures were of prognostic value is specified next, and where a positive finding was reported, a description of quantitative effect on prognosis is given as well–to the extent reported in the study. Note that "overexpression" was in almost all cases defined subjectively, with the definition varying between studies. Abbreviations: IHC, Immunohistochemistry; FISH, Flourescent In-Situ Hybridization; ELISA, Enzyme Linked Immuno-Sorbent Assay; PCR, Polymerase Chain Reaction.

					Correlation to Survival			
Ref	N	Her-2 +	Measure	Exposure definition	Overexpression	Amplification	Outcome	Diagnosis
OVARY								
(46)	73	23	IHC	High expression compared to normal controls	15 vs. 32 months mean survival, (p=0.001)		Survival	Advanced epithelial ovarian cancer
(47)	40	28/4	IHC	Moderate/Weak staining	No		Survival	Early epithelial ovarian cancer (overexpression was of borderline significance on multivariate analysis, p=0.09).
(48)	105	25	IHC	Strong Staining	No		Survival	Grade III or IV epithelial ovarian cancer.
(49)	196	78	PCR	Any amplification		No	Survival	Ovarian Cancer
(50)	32	11	PCR	Any amplification		No	Survival	Ovarian Carcinomas
(51)	42	9	IHC	Overexpression	No		Survival	Ovarian Cancer (overexpression correlated with Grade, but not survival)
(52)	57	6	Serum level	Elevated level	7 vs. 29 weeks months median survival ((p=0.02)		Survival	Ovarian Cancer
(53)	44	16	IHC	Overexpression	No		Survival	Primary ovarian cancer
(54)	43	10	FISH	Moderate to High amplification		No	Survival	Ovarian Cancer
(55)	42	10	FISH	Amplification		No	Survival	Ovarian Cancer patients receiving platin chemotherapy

Table 2.1: Her-2 and Survival in various cancers, *continued*.

Ref	N	Her-2 +	Measure	Exposure definition	Correlation to Survival: Overexpression	Correlation to Survival: Amplification	Outcome	Diagnosis
(56)	208	46	IHC	Any detection	17 vs., 26 months median survival, (p=0.0003)		Survival	Ovarian cancer. (Note: Her-2 and cym-c expression together predictive of survival)
(57)	108	35	Southern Blot	Amplification		3 year survival 28% vs 58% (p=0.016)	Survival	Primary epithelial ovarian cancer
(58)	65/52	9/23	PCR/IHC	Amplification/Overexpression	(p=0.04, Kaplan-Meier)	No	Survival	Ovarian eptithelial cancer (univariate effect of amplification on survival disappeared on multivariate analysis, rates not reported)
(59)	100	31/8	IHC/Southern Blot	Expression/Amplification	No	No	Survival	Ovarian carcinoma (a significant interaction between Her-2 and Ras was noted)
(60)	74	43	FISH	Any amplification		No	Survival	Ovarian epithelial tumors of low malignant potential and ovarian carcinoma
(61)	29	19	IHC	Any detection	No		Survival	Advanced stage transitional cell carcinoma
(62)	82	79	IHC	Any detection	No		Recurrence	female genital tract carcinosarcomas
(63)	34	N/A	ELISA	Cytosolic concentration > .43 U/microgram	1 year survival 20% vs. 56%, p<0.05)		Survival	Primary advanced HCC
SALIVARY GLAND								
(64)	58	22/12	IHC/FISH	Membrane staining/Amplification	28 months median survival vs. 162, p<0.0001)	18 months median survival vs. 125 (G6p<0.0001)	Survival	Mucoepidermoid carcinoma of salivary gland
(45)	201	2	IHC	Any detection	Suggestive			Primary salivary gland tumors
(65)	42	16	IHC	Overexpression	11.6 months median survival vs. 22.7, (p=0.009)		Survival	Minor salivary tumors of the palate. Note: Her-2 expression also was predictive of malignant disease compared to non-malignant cases.
(66)	59	13	IHC	Any detection	20% 5 year survival vs. 58%, p<0.05)		EFS	Adenocarcinoma/ adenocarcinoma in pleomorphic adenoma of major salivary glands
(67)	25	9	IHC	Any detection	"trend with grade"		Grade	Mucoepidermoid carcinoma of the salivary glands
(68)	9	9	IHC	Overexpression	No		Survival	Salivary duct carcinoma
BLADDER								

Table 2.1: Her-2 and Survival in various cancers, *continued*.

Ref	N	Her-2 +	Measure	Exposure definition	Correlation to Survival: Overexpression	Correlation to Survival: Amplification	Outcome	Diagnosis
(69)	91	2	IHC	"Moderate or Heavy" staining	0.9 years mean survival, vs. 9, (p=0.038)		Survival	Primary transitional cell cancers. Note: not correlated to recurrence or progression
(70)	43	21	IHC	Any detection	Shorter survival by Log rank test ($p<0.05$)		Survival	Invasive bladder cancers treated by cystectomy. Note: Survival rates not reported.
(71)	57	18	PCR	Amplification		Shorter survival by Kaplan-Meier (p=0.0001)	Survival	Bladder cancer. Note: Survival rates not reported. Her-2 amplification also correlated with grade and stage.
(72)	82	29	IHC	Any detection	decreased survival in Cox model, ($p<0.05$)		Survival	Transitional cell carcinoma. Note: Survival rates were not reported.
(73)	249	99/31	IHC	"Heavy" staining	No		Survival	Transitional cell bladder cancers.
(74)	92	24	PCR	Amplification	No		Survival	Transitional cell carcinoma. Note: Her-2 amplification was associated with tumor grade.
(75)	106	42	IHC	Overexpression	RR=0.47, (p=0.0202)		Survival	Transitional cell carcinoma. Note: positive Her-2 staining was predictive of improved survival.
(76)	256	21	IHC	Any detection	No		Recurrence	Primary Ta/T1 superficial bladder tumors
(77)	83	22	IHC	Any detection	9.7% 5 year survival vs. 48.5%, $p<0.01$)		Survival	Bladder carcinoma (operative patients)
(78)	163	37	PCR	Amplification		RR=3.67 (G22p <0.002)	Survival	Transitional cell carcinoma. Note: Her-2 expression was also correlated to tumor stage and grade.
(79)	95	20/4	IHC / Dot Blot	"Strong" staining / Amplification	No	No	Recurrence	Transitional cell cancer. Note: Her-2 expression/amplification not correlated to stage or grade.
ESOPHAGUS								
(80)	66	7	IHC	Membrane staining	0% 5 year survival vs. 33%, $p<0.05$)		Survival	Barrett's adenocarcinomas. Note: Her-2 expression was not correlated to tumor grade.

Table 2.1: Her-2 and Survival in various cancers, *continued*.

Ref	N	Her-2 +	Measure	Exposure definition	Correlation to Survival: Overexpression	Amplification	Outcome	Diagnosis
(81)	80	15	IHC	Overexpression	relative risk of death = 4.06 ($p<0.05$–see note)		Survival	Barrett's adenocarcinomas. Note: Her-2 expression was correlated to grade and recurrence, however the correlation to survival disappeared upon multivariate analysis.
(82)	42	18	IHC	Any detection	No		Survival	Adenocarcinoma of the esophagus and esophagogastric junction.
(83)	41	10	IHC	Overexpression	No		Survival	Adenocarcinoma of the esophagus and/or gastroesophageal junction. Note: Her-2 expression was correlated to stage, but not independantly correlated to survival
KIDNEY								
(84)	30	30	IHC	Expression Level	No		Survival / Recurrence	Renal Cell Carcinoma
(85)	123	12	IHC	Any detection	No		Survival	Renal adenocarcinoma
(86)	184	NA	Immunoassay	Soluble domain serum level above median	Kaplan-Meier method, ($p=0.003$)		Survival	Renal Cell Carcinoma. Note: Rates not reported
LUNG								
(87)	107	14	IHC	Positive staining	23 days median survival vs. 274 days, ($p=0.0031$)		Survival	Small Cell Lung Cancer
(88)	408		IHC	Overexpression	hazard ratio =1.43, ($p=0.044$)		Survival / Recurrence	Small Cell Lung Cancer
(89)	42	21	IHC	Overexpression	Yes		Survival/ Recurrence	Surgical Adenocarcinoma of the Lung
(90)	238	100	IHC	Overexpression	No		Survival	Non-small cell lung cancer (significant interaction with BCL-2)
(91)	271	58	IHC	Overexpression	45% 5 year survival vs. 67%, $p<0.001$)		Survival	Non-small cell lung cancer
(92)	150	17/1	IHC / Dot Blot	Positive staining/amplifications	No		Survival	Localized adenocarcinoma
(93)	81	28	IHC	Expression	No		Survival	Squamous cell carcinoma

Table 2.1: Her-2 and Survival in various cancers, *continued*.

Ref	N	Her-2 +	Measure	Exposure definition	Correlation to Survival: Overexpression	Amplification	Outcome	Diagnosis
(94)	55	26	IHC	Overexpression	84 weeks mean survival vs. 189, (p=0.01)			Adenocarcinomas
(95)	40	NA	IHC	Overexpression	No		Survival	Stage I non-small cell lung cancer
(96)	107	50/20	IHC	"Strong" staining	No		Survival	Surgical stage I non-small cell lung cancer. Note: Membrane staining not assessed.
GASTRO-INTESTINAL								
(97)	87	8	IHC	Membrane staining	No (+)		Survival	Gastric carcinoma. Membrane positve cases showed a trend towards improved survival, but this finding was not significant at the 0.05 level.
(98)	56	35/14	IHC	Cytoplasmic / Membrane staining	387 days mean survival vs. 763 (p<0.05)			Gastric carcinoma. Survival correlated with any Her-2 staining (cytoplasmic or membrane).
(99)	58	22	IHC	Membrane staining	see note)		Survival	Gastric carcinoma. Correlation was limited to advanced stage intestinal-type carcinoma.
(100)	120	55	IHC	Any detection	No		Survival	Colon cancer
(101)	93	11	IHC	Membrane staining	60 month mean survival vs. 12 F94(p=0.05)		Survival	Gastric carcinoma. Note that Her-2 positive patients had improved prognosis.
PANCREAS								
(102)	109	37	Immunoassay	Serum positivity	154 days mean survival vs. 220 (p<0.05)		Survival	Pancreatic cancer
(103)	79	63 / 46	IHC	Any / Moderate to Strong	No		Survival	Pancreatic adenocarcinoma
(104)	157	52	IHC	Any detection	No		Survival	Pancreatic ductal adenocarcinoma
(105)	21	10	IHC	Moderate to Strong staining	7 months mean survival vs. 19 (F41p<0.001)		Survival	Pancreatic cancer of ductal origin

2.2.3 Her-2 may distinguish between prognostic groups.

A year 2000 review of 47 studies presenting data on Her-2 abnormalities (amplification or over-expression) measured in over 15,000 breast cancer patients indicates that Her-2 overexpression or amplification is correlated with worse prognosis (106). Several smaller studies in the late 1980s had failed to show any Her-2 effect in multivariate models, or even in univariate models in about half of the studies. However, since then, the overwhelming evidence of numerous large studies suggests that level of Her-2 expression is an independent predictor of poor outcome, though the magnitude of the effect is difficult to quantify due the variability in the study designs (and for this reason the review does not attempt to quantify the magnitude of effect).

This same review points out that immunohistochemical methods were more likely to result in negative or univariate only results compared to gene-based methods (9 studies vs. 3). However, gene-based studies can not identify overexpression, only amplification, and they can not quantify the interaction of protein products. Nonetheless, this finding underscores the need for objective, quantitative methods to be applied to immunohistochemical techniques.

2.2.4 Her-2 may distinguish between treatment groups.

Several studies have indicated that Her-2 status is a powerful predictor of tamoxifin resistance (107–109). This is likely due to the fact that the Her-2 pathway initiates kinase-dependent cell proliferation pathways independent of estrogen receptors blocked by tamoxifen.

2.3 Her-2 exhibits complex ligand-receptor relationships.

Despite the homology of Her-2 to EGFR, EGFR ligands such as TNF-alpha and EGF do not activate erbB-2. However, the following Her-2 agonist ligands have been suggested:

1. Gp30, a 30kDa TNF-a like protein, homologous to heregulin (110)
2. 50kDa factor (111)
3. Neu differentiation factor, also known as heregulin and p44 (110)

The role of Gp30 and 50kDa factor are unclear. Heregulin acts as an agonist ligand in breast and colon tissues, but not in ovarian tissue, despite demonstrated comparability of the amino acid sequences of the receptors (112). This indicates that the presence of Her-2/heregulin interaction alone is not sufficient for stimulating the tyrosine kinase activity of the receptor (112). Further study of the Her-2 receptor has elucidated additional intriguing characteristics of its ligand-receptor relationships. First, it was found that divalent antibodies directed against Her-2 can induce its kinase activity, but monovalent fragments do not (113). Second, molecular studies of Her-2 indicate that activating mutations observed in vitro involve trans-membrane domains likely involved in receptor-receptor interactions (114). Third, disruption of Her-2 dimers by herceptin (see below) inhibits tyrosine kinase activity and growth of transformed cells (115).

These findings suggested that Her-2 is activated via dimerization. Subsequent work has demonstrated that Her-2 preferentially forms heterodimers with Her-3 (116, 117), that these heterodimers exhibit enzymatic activity not present when only one or the other receptor is expressed alone (118), and that these heterodimers can lead to neoplasia when activated by heregulin (119). It has been shown that

Her-3, and not Her-2, binds heregulin (120, 121), explaining the lack of heregulin induced kinase activity in ovarian cells lacking Her-3. Interestingly, Her-3 has limited kinase activity (122) but numerous ligand docking sites, while Her-2 has substantial kinase activity but no ligand of known pathophysiologic importance. The formation of a heterodimer results in a catalytic unit with high ligand affinity and high kinase activity. Unlike Her-2, Her-3 and Her-4 amplification has not been observed, indicating that the availability of Her-2 kinase activity is rate limiting. It is presumably for this reason that the limited evidence available suggests that Her-3 expression level is not associated with cancer risk (reviewed in (123)). Nonetheless, Her-3 targeted treatment has been suggested, with an aim towards interrupting Her-2/Her-3 heterodimers (118). Interestingly, heterodimerization with EGFR inactivates Her-2 in vitro (122).

Dimerization dependent kinase activity is not unique to Her-2 and Her-3. EGFR, another member of the Her family, also exhibits dimerization dependent phosphorylation. However, homodimerization of EGFR is not sufficient for phosphorylation, as induced homodimerization in the absence of agonist ligands does not result in kinase activity (122).

2.4 Her-2 has no confirmed human, cancer-specific mutations

Her-2 gene amplification and protein overexpresion have been identified in a subset of breast cancers as described above. Until very recently, no cancer related Her-2 *mutations* (i.e., a change in the genotype, or DNA sequence, leading to a change in the protein sequence of Her-2) had been discovered in human cancer tissues (124, 125), despite the fact that oncogenic Her-2 point mutations and deletions have been identified in animal and cell line models (126–130). This is in contrast to

other human oncogenes such as ras for which human oncogenic polymorphisms have been identified (17). A population based study has suggested that a valine to isoleucine point mutation at codon 655 of the Her-2 receptor may be associated with increased breast cancer risk, but the relative risk was of borderline significance (131). Nonetheless, this continues to be an important area of investigation. In the mean time, the lack of clearly oncogenic polymorphisms means that genetic testing is presently of limited use with respect to Her-2, because there are no clearly prognostic mutations to look for as there are with other genes such as the BRCA group. Instead, novel quantitative techniques paired with statistical methods that fully account for the interaction of continuous variables must be applied to detect those patients who may be at increased risk of disease progression or treatment failure, based not on genotype, but on gene copy, expression level, and interaction (i.e., based on phenotype). The present study seeks to expand current abilities to measure protein expression patterns in order to better quantify phenotype in this fashion.

2.5 Her-2 is the target of the first receptor-specific cancer treatment.

Antibodies directed against Her-2 result in homodimerization and sequestration of the receptor, and subsequent inhibition of cancer cell growth (132–135) via cell cycle arrest at the G1 phase (136). This observation led to the development of herceptin, an antibody based treatment with demonstrated efficacy in the treatment of breast cancer both alone (137) and in combination with other therapies (138). In these studies, 12-20% of patients with severe metastatic breast cancer unresponsive to traditional therapies responded to herceptin treatment ("response" was determined by a blinded review board, but was not clearly defined), and the combina-

tion therapy was shown to improve response rate (17% vs. 42%, p = 0.001), prolong the response duration (from 4.5 to 10.4 months, p=0.01) prolong time to disease progression (from 3.0 to 6.9 months, p=0.0001). These findings demonstrate the full potential of the molecular approach to diagnosis and treatment: patients receive treatments not based on anatomy, but based on the specifics of the metabolic pathways causing the disease.

2.6 Issues for study

As can be seen, a great deal is known about Her-2. Its role in the molecular pathway of breast cancer has been identified, and it has been targeted in treatment intervention. However, there is still more information to be gleaned about this interesting receptor. As described above, Her-2 exhibits complex receptor-ligand interactions. Her-2/Her-3 heterodimers exhibit biological function not exhibited by either receptor alone, and, therefore, these heterodimers should be the unit of analysis if the most accurate measure of receptor activity is desired. Therefore, methods to quantitate these interactions are required.

In a static environment such as a fixed tissue section, interaction of proteins at the moment of fixation can be quantified by staining the proteins if interest with different fluorophore-conjugated antibodies and quantifying the resulting fluorescent intensities within tiny spatial regions (pixels) and the interaction of these intensities. Careful consideration must be given, of course, to how the surgical handling and subsequent fixation and staining of the tissue may have impacted the spatial relationships of the proteins of interest. In this project, we propose to modify and further develop these methods for application to Her-2 and Her-3 interaction (dimerization), and use the resulting data in multi-variate models of

prognosis.

Because of the nature of its possible role in cancer, Her-2 and Her-3 are particularly amenable to quantitative molecular epidemiologic techniques. In particular, the fact that Her-2/3 heterodimers are believed to play an interactive role in heregulin activated kinase dependent cell processes makes these proteins an extremely interesting candidate for colocalization analysis.

If successful, such analytic methodology could clearly be applied to other interesting protein interactions as well.

Chapter 3

Design

As discussed above, Her-2 has no known pathophysiologically relevant ligand, and Her-3 has little enzymatic ability. We hypothesize that interaction of these two proteins is necessary for signal transduction. Therefore, measuring the interaction of these proteins, rather than the level of Her-2 alone, will more accurately portray the level of activation of this potentially neoplastic pathway. In this way, we may improve the resolving power of Her-2 expression as a prognostic factor in breast cancer.

In the proposed study, we will use flourescently tagged antibodies against Her-2 and Her-3 to allow microscopic imaging of the location of these proteins within cancer and control tissues. Quantitative algorithms will be applied to determine the level to which these two protein are interacting. These concepts are further discussed below.

3.1 Images as Data Sets

Digitized images are stored as an array of pixel intensities, with the format of these arrays and associated file information arranged in a file format dependant manner. Each pixel represents a small square area of the image and the pixel is displayed as a uniformly colored area (i.e. the pixel displays a single color, which in the case of

a grey scale image will be some shade of the color white). The digital image is then a mosaic of pixels. However, unless highly magnified, the digital image appears as a coherent image as opposed to a jagged array of square pixels thanks to the very small size of the pixel and the facilities of the human visual system. An example of this can be seen in figure 3.1.

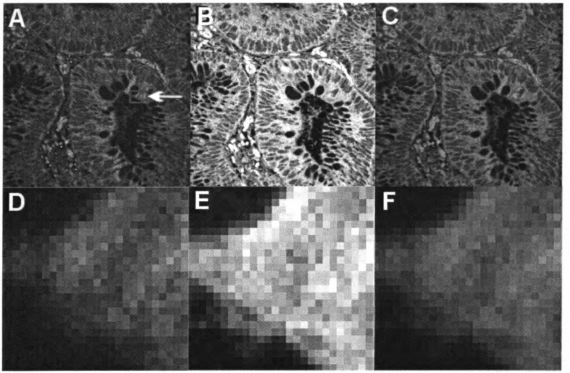


Figure 3.1: A representative confocal microscopy image of intestinal epithelium. A. Green fluorescent antibodies against the Met receptor are excited and digitally captured. B. Red fluorescent antibodies against HGF are excited and digitally captured. C. The previous two images are inserted into the Red and Green channels, respectively, of an RGB image to allow for simultaneous viewing of the location (and possible colocation) of Met and HGF. D-E. The area indicated by the arrow in A for each image A-C is magnified to allow visualization of individual pixels within each field.

Such a representation of data is similar to that employed in ecological studies. For example, smoking or population density may be measured and aggregated for some given geographic subdivision (say for example, census block groups). The resulting data may be visualized by color coding each geographic unit according to the level of the variable of interest. An illustration is given in figure 3.2, where

population density is represented by the gray level of each block group. This data could then be correlated to an outcome of interest–say, cancer rate for each census block group. In this example, the map is simply a way of visualizing the numerical data. In the same way, fluorescent markers may be conjugated to antibodies against specific proteins, and the resulting fluorescent intensity measured and aggregated on a pixel by pixel basis. The result is a digital map of labelled tissues, with each pixel color coded according to protein density. The numerical data underlying this visual representation may be analyzed statistically–just as with the geographic map, the graphical representation is simply a tool for visualizing the data. However, minimal attention has traditionally been given to the numerical data underlying the visual representation of the image. Instead, subjective scoring of the visual representation is typically performed by pathologists. It is as if we did not calculate a correlation coefficient between population density and cancer rate, but rather simply looked at the color coded maps and subjectively determined the degree to which cancer and population density were covariant over space.

One final important concept is that of the image "channel". As described above, multiple markers may be applied to a tissue, and individually imaged (by using the appropriate excitation wavelength and filters to elicit and capture the fluorescence of only one marker at a time). Therefore, at a given pixel, data on the intensity of each marker can be stored. This can be conceptualized as a stack of images, each depicting the same slice of material but representing the density of different proteins. Each slice is referred to as a "channel" of the composite image, and stores data unique to an individual marker. To return to our ecological example, one could have one map representing population density, and a second map which, while depicting the identical geographical area, is color coded to represent

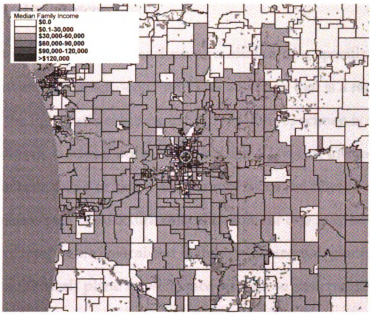


Figure 3.2: A familiar example of a color coded image representing data (population density) aggregated for each subdivision. This is precisely analogous to microscopy images which allow us to visualize data (protein density) at each subdivision (pixel) of the image. Both types of images allow visualization of underlying numeric data.

cancer incidence. The two maps could be printed on transparencies and superimposed to visualize the correspondence of the two variables, or the underlying numerical data could be computationally manipulated. In the same way, multiple image channels could be superimposed to allow visualization of the covariance of multiple proteins (by means of, for example, a color image where the detected fluorescence for one labelled protein is loaded into the red channel, and another into the green channel–areas where both protein densities are high would then be bright yellow due to the interaction of red and green). However, more important than the visualization is the fact that these multiple channels can be used to computationally measure the influence of one protein upon another by analyzing the interaction of the multiple data points corresponding to multiple proteins for any given pixel or group of pixels.

3.1.1 Confocal microscopy

Confocal microscopy allows for exclusive resolution of a specific slice within a specimen. Conventional microscopy provides an image that is a representation of all material in the light path, resulting in blurring if the specimen is relatively thick or dense. However, confocal microscopy isolates a layer of interest and fluorescence from markers above or below the plain of focus are filtered out (139). This allows for measurement of structure colocalization, for one may be assured that if the structures of interest are colocalized in the x-y (horizontal) plane, they are colocalized in the z (vertical) plane as well since only those structures in the plane of focus are being visualized. As an illustration, consider the four pennies depicted in figure 3.3. When viewed from above (3.3-A), the upper two pennies and the lower two pennies appear to be closer together than the middle two pennies. However, when viewed from the side (3.3-B), it become apparent that the middle two pennies are in fact closer together. By filtering out those pennies above or below the plane of focus (3.3-C), only truly colocalized pennies remain visible.

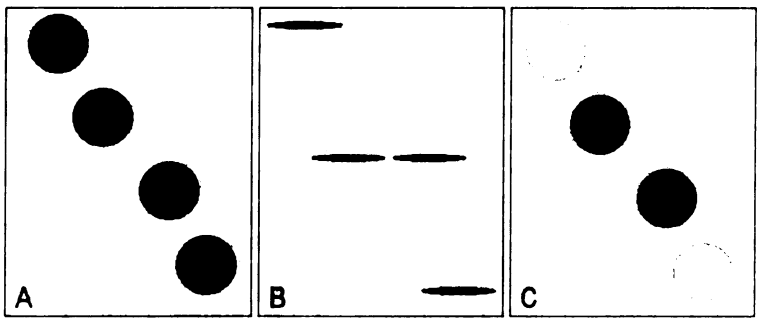


Figure 3.3: A conceptualized illustration of confocal microscopy. A. Viewed from above, the top two pennies and the bottom two pennies appear to be closer together (more colocalized) than the middle two pennies. B. However, when viewed from the side, it becomes apparent that this is not the case due to their differing heights (or position on the z-axis). C. Confocal microscopy optically eliminates items outside the plane of focus, such that objects near each other in the horizontal (or x-y) plane, can be assumed to be near each other in the vertical (or z) plane as well.

By using multiple fluorescent markers, the interaction of multiple compounds

may be visualized using confocal methods(139). One fluorescent marker may be excited and imaged, and then the second marker excited and imaged. This produces two slices or channels of the same field, with each channel documenting the density of a different protein. At any given pixel, then, the density of one protein can be compared to the density of the other protein, and these interactions may be quantified in various ways. This technique has been applied to elucidate protein and gene interactions in a variety of cellular processes. The use of multiple laser frequencies to image up to three markers has been employed for a decade (140–142). Most of these efforts have described such colocalization qualitatively. However, some techniques for quantifying confocal microscopy images have been described (143, 144). Typically, these methods have been applied to examine the interaction of cellular structures (membranes and chromosomes, etc), as opposed to molecular interactions.

3.1.2 Colocalization as an image phenomenon

Colocalization as an image phenomenon can be conceptualized as correlated variations in intensity of two or more different markers over a region of interest, indicating that the concentration or density of the marked compounds are spatially related over that region. Of particular interest, then, is quantifying the degree to which the intensity of the respective markers are varying over space in a related fashion. In the proposed study, we will stain her-2 and Her-3 in sections of breast tumor tissue with different fluorescent antibodies, allowing the density of each protein to be imaged. The colocalization of Her-2 and Her-3 proteins will be measured by applying traditional measures of covariance to the fluctuation of Her-2 and Her-3 protein density (as measured by fluorescent marker intensity at each pixel) over space. An idealized representation of this is given in figure 3.4. In 3.4-

A we see an idealized nucleated cell. In 3.4-B, fluorescent labelling of one protein is depicted, as would be visualized in a microscopic image of the cell–a grid of pixel intensities which may be represented either visually (as shown), or simply as an array or matrix of numerical data. In 3.4-C, the same methodology is used to measure the density of a second protein throughout the cell. As can be seen, both proteins are somewhat localized to the membrane region of the cell (more so for the protein in 3.4-C), and no protein is seen in the nuclear region. (This may be verified computationally by measuring the intensity of the proteins' fluorescence in the region indicated by the nuclear fluorescent dye represented in 3.4-D. In this case, where the nuclear marker fluorescence is greater than 0, there is little or no fluorescence in this region in images 3.4-B and 3.4-C). There seems to be some colocalization of the protein (both proteins exhibit bright fluorescence at some of the same pixels), but certainly not perfect colocalization.

To quantify this colocalization, we will calculate the rate of change in protein density (also known as the "moment"–further described below) at each pixel for each protein, and then calculate the degree to which the Her-2 and Her-3 moments are correlated for the entire image. By calculating a correlation coefficient between the Her-2 moments and the Her-3 moments, we can quantify the degree to which the presence of one protein is correlated with the presence of the second protein (as would be the case if they are forming dimers). This overall strategy is summarized in figure 3.5. The resulting correlation coefficient provides a quantitative score for each image which may then be included in survival analysis to test if the parameter–and therefore the colocalization and, by proxy, the heterodimerization of these two proteins–correlates to survival.

To accomplish this, the joint moment of the pixels from two or more markers

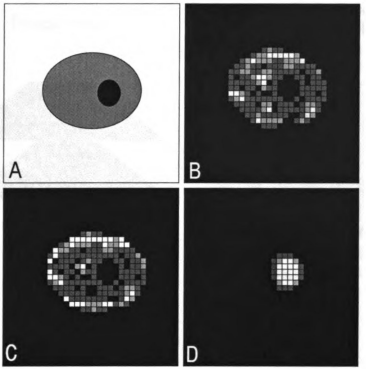
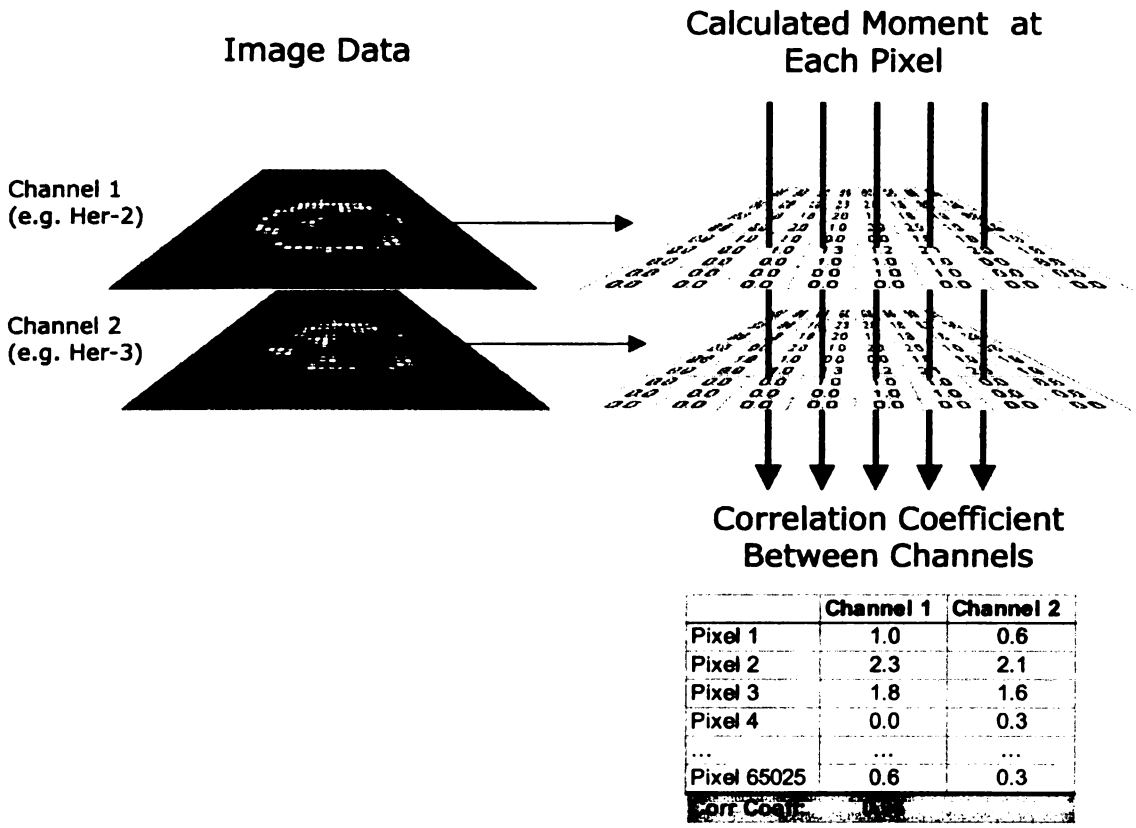


Figure 3.4: Idealized representation of digital imaging of flourescently labelled proteins. A. An idealized nucleated cell. B. The same cell, with a protein flourescently labelled, and the resulting fluorescence digitally captured (pixels enlarged for clarity). C. Same as B., but now with a different protein labelled. D. Here, a fluorescent nuclear dye has been applied to allow imaging of the nuclear region. Such an exercise is useful to enable the computer to count nuclei, and thereby count cells, or two compare nuclear vs. cytoplasmic expression of protein (the dye tells the computer where the nucleus is, so it can separate nuclear expression from cytoplasmic expression in the other channels)

can be compared. The moment at a given pixel simply refers to the rate of change of pixel intensity near that pixel. In other words, is the specified pixel much different from its neighbors? This can be quantified by calculating the deviation of a given pixel's intensity from the mean of its neighbors (the "local" mean). This concept is illustrated in figure 3.6 and expressed in mathematical terms in the methods section below. Where the intensities are "flat"—as in large regions of constant intensity—the moment can be defined as zero because the denominator (the variance) is zero. Where the intensities are changing over small intervals of space (edges of structures, etc), the moment is non-zero because the variance is



	Channel 1	Channel 2
Pixel 1	1.0	0.6
Pixel 2	2.3	2.1
Pixel 3	1.8	1.6
Pixel 4	0.0	0.3
...	...	...
Pixel 65025	0.6	0.3

Figure 3.5: Summary of quantification strategy. First, flourescently labeled proteins are imaged (with her-2 fluorescence captured in one channel, and Her-3 fluorescence captured in a second channel). The moments are calculated (as described in the next chapter) for each pixel. Then, a correlation coefficient is calculated describing the correlation of the her-2 and Her-3 moments to eachother.

non-zero. The joint moment then refers the relatedness of the moments of two channels at each pixel. The moments for each marker at each pixel can be tested for correlation using standard (or novel) statistical techniques (143, 145, 146). If the intensities of the markers are rising and falling, as measured by their moments at each pixel, in an unrelated fashion, the compounds they mark can be assumed to be non-colocalized. The degree to which this is the case can be assigned a numerical score (such as a Spearman's correlation coefficient), and this score can be included in multivariate analysis. This method is called the "Joint Moment of Standardized Images" or JMSI analysis and has been described previously (147).

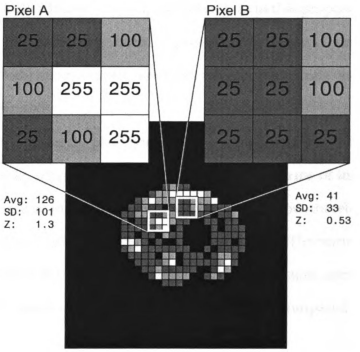


Figure 3.6: channels)Illustration of the moment at two pixels. Here, the moments of two pixels of one of the channels from figure 3.4 is shown. A 3x3 window is used to calculate the local mean in the neighborhood of each pixel. These neighborhoods are blown up for clarity, and the numerical intensity of each pixel is specified in each pixel. By calculating the standard deviation of the 9 pixels in each neighborhood, the deviation of the intensity of the central pixel (the pixel of interest) from the local mean may be expressed in standard deviations (the z-score). This value represents the moment, or the degree of flux near the pixel of interest. These values for the two pixels shown are given in the tables. As can be seen, "flat" areas (such as surrounding the pixel on the right) have smaller moments than pixels in regions of fluctuating intensity (as on the left). By correlating these values for each pixel in two or more channels, the joint moment for the entire image may be calculated.

However, by comparing the value of a pixel to the mean of its neighbors effectively blurs the image (the larger that neighborhood used to calculate the local mean, the more blurred the image becomes). In fact, a similar algorithm is used to digitally retouch images by softening them using a gaussian filter. This is fine where the coincidence of relatively large structures are of interest (for example, the localization of mRNA to a nuclear membrane), and is a particularly effective method for detecting edges of cellular structures, which are relatively large. Where

interactions of individual proteins are of interest, as in this proposal, such methodology may not be appropriate since the proteins are somewhat smaller than a single pixel–therefore, incorporating data from neighboring pixels via local averaging may not be indicated.

Instead, simple pixel by pixel correlation within globally standardized images may be preferable, wherein each pixel is expressed in terms of its deviation from the mean of the entire image field–not a local neighborhood–thereby standardizing the pixel intensities without reference to local windows. The normalized intensity of each pixel can then be correlated between the two images using the Spearman rank correlation coefficient. These two methods will be compared in the proposed analysis.

3.2 Application to Her-2/Her-3 colocalization

In the proposed study, we will use digital image analysis to determine the degree of colocalization of Her-2 and Her-3 within tissues. As described above, we will store the fluorescent intensity resulting from fluorophore conjugated anti-Her-2 antibody in one color channel, and that resulting from anti-Her-3 antibody in another color channel. At each pixel within the field, then, we can determine the relative level of Her-2 protein and the relative level of Her-3 protein based on the intensity of fluorescence stored in their respective color channels at each pixel. If the intensities or moments (depending on which of the two methods described is employed) in each pixel within a given image are correlated, this suggests that the presence of one of these proteins is some how driving the presence of the other (or perhaps they are synergistically causing eachother to be located in the same place), as would be the case if molecular interactions were occurring leading to formation

of heterodimers between the receptors. If, on the other hand, there were no attractive forces between these receptors, their location (and, therefore, there relative intensities at all pixels within the image) would not be expected to be correlated.

As described above, there is more than one way to quantify the correlation of pixel intensities. This proposal describes two methods, and will compare their efficacy in conducting this type of molecular interaction analysis. In future work, these findings could be compared to other methodologies. Most notable among alternatives is Fluorescent Resonance Energy Transfer which allows much finer resolution of fluorescent particles. This technology has been rejected for the current study because of its complexity, but it will provide an important point of reference for future study. Furthermore, other epidemiological fields have approaches to offer. For example, Kulldorff, et al. (148) describe an approach to detection of geographic clustering of epidemiologic phenomena. One of the strengths of their approach is its independence of ad hoc assumptions about the nature of location of the clustering, an objective shared by the approach described in the present work. Extension of their approach to microscopic events may prove fruitful in the future.

Chapter 4

Methods

4.1 Phase one: algorithm development and data protocol development

In phase one, we will further develop and test our computer algorithm for quantifying colocalization of the proteins of interest. Sections of test cases will be obtained and stained to optimize the parameters and algorithm, as described below, to ensure that the algorithm captures molecular events of interests. The methods for these analyses are further described below.

The second major task for phase one is to develop, in cooperation with the Grand Rapids Clinical Oncology Program, a mechanism whereby supplemental outcome data can be added to our dataset in a manner that protects the anonymity and confidentiality of patients. This mechanism will need to be fully reviewed and approved by the IRBs of both our institutions.

4.1.1 Tissues

Breast carcinoma tissue specimens will be obtained from the VARI tissue repository using our web-based query system. These samples will be prepared according to existing protocols in our lab and as described previously (143). Briefly, the paraffin block will be sectioned in our laboratory at 5μm. These sections will be

flourescently stained for Her-2 and Her-3. We proposed using Fitc and Rhodamine markers (each having a unique excitation profile allowing them to be individually isolated optically). The exact array of antibodies and markers used may require adaptation and optimization through initial testing of the system.

4.2 Imaging

After the tissues are prepared as described above, each section will be imaged by our Zeiss/LSM-410 upright confocal microscope in 4 areas at 40x: two tumor tissue fields and two fields of surrounding normal tissue. Each field will be imaged two times. The first image of each field will be imaged using a 665nm low pass filter and 633nm excitation to elicit fluorescence of the Cy5 marker. The second image of each field will be imaged using a 590nm low pass filter and 568nm excitation to elicit fluorescence of the rhodamine marker. (Again, this protocol may require optimization).

The images will be stored as 512 x 512 pixel tagged inline file format (TIFF) files. Each marker will be imaged as a separate 8 bit (256 shades of gray) tiff files (multilayer tiffs could be used to store both images in a single file, but we have found separate files to be simpler to deal with in the image processing phase).

4.3 Image analysis

4.3.1 Data Modeling

Two key questions need to be addressed in conducting the analyses described below. First, to what extent does the arbitrariness arising from the orientation of the tissue within the tissue block (and, therefore, within the section being imaged with the microscope) as well as the selection of fields within the section to be imaged

introduce additional variance into the analysis? Second, how should variance of pixel intensities be modelled? Are these intensities normally distributed? If not, how are they distributed and how can they be modelled?

To answer the first question, a test series of cases will be analyzed. Stacks of images (say, at 0.1 micron intervals) representing the entire thickness of each tissue section will be isolated by means of confocal microscopy and imaged. These image stacks can then be used to reconstruct a three dimensional model of the tissue section which can be computationally rotated in space. Then, new sections taken at different orientations through this virtual tissue block can be computationally created. The analysis can be conducted on these various sections to see to what extent the orientation of the tissue affects the analysis. If it is found that there is high variability between orientations, this will need to be factored into our analysis, and may point out the need to conduct the analysis in three dimensions rather than two. Since proteins clearly interact in three dimensions, such an approach may be preferably any way. However, the computational burden of such an approach is an order of magnitude greater than the approach here described. Therefore, if the two dimensional approach proves to provide an effective predictor of prognosis, it would be preferable by virtue of its relative simplicity.

To answer the second question, the distributions of the pixel intensities will be analyzed, both in terms of entire images and small windows used in the joint moment of standardized images method described below. As shown in figure 4.1, these images tend not to be normally distributed on the whole. It may be possible to restore a normal distribution through various techniques (background correction, or analysis of the square root of the intensity of each pixel, or ranking the intensities of the pixels and analyzing the rank rather than the raw intensity). The

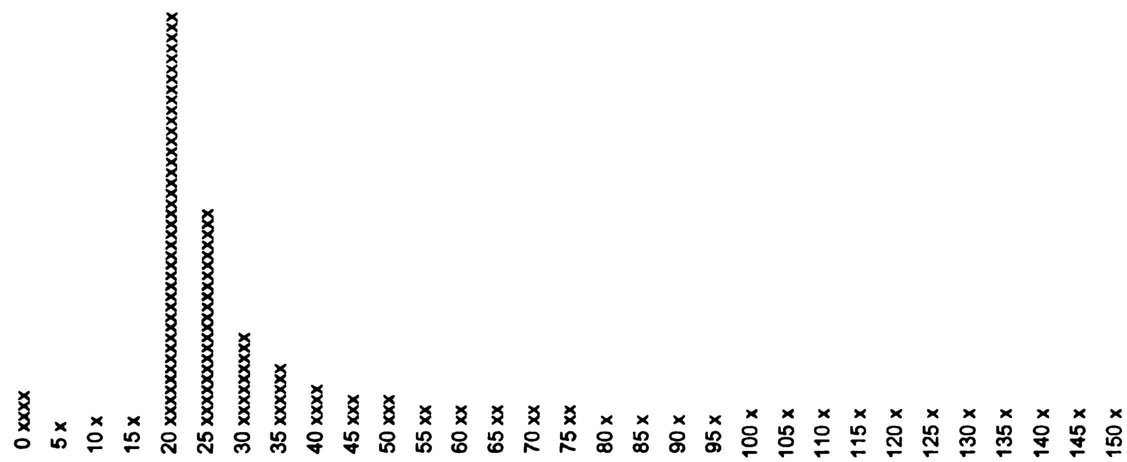


Figure 4.1: Histogram of a representative image. Note the chi-square distribution of intensities. Each x represents 1% of all pixels.

discussion that follows assumes that normally distributed data can be extracted from the images for the purposes of measuring the variance of the intensities of the pixels over space. If this is not possible, non-parametric measures of central tendency and variance will need to be employed instead of the mean and Z-score as described below.

4.3.2 Algorithm Implementation

Software already developed by the author for performing the computational analysis here described will be used to quantify the colocalization between the two markers. Two methods will be employed and compared:

1. Joint moment of standardized images (JMSI) (147)

- The moments at each pixel for each of the two markers (her-2 and Her-3) will be quantified in terms of the variability of the pixel intensities within a local "window" centered on the pixel of interest. The local window will be a small region (for example, 5 x 5 pixels) around the region of interest. If (as discussed above), the intensities of the pixels in these windows can be rep-

resented in a normally distributed fashion, the moment will be quantified as the Z-score of each pixel (it's deviance from the mean intensity of the local window expressed in terms of the variance of the local window). From this point forward, it will be assumed that this is the case (if not, adjustments will need to be made as mentioned above).

- A correlation score will then be assigned to the image by calculating the Spearman Rank-Correlation Coefficient between the moments of the her-2 and Her-3 markers flourescent markers. The Spearman Rank-Correlation Coefficient will be used because the intensities of such images are not normally distributed, and normality is not required by this non-parametric statistical test.

- All pixels with an intensity less than an empirically determined threshold will be discarded as background (non-tissue areas).

2. Correlation of globally standardized images (CGSI)

- The images will be standardized by assigning a Z-score to each pixel as calculated as the deviation of the pixel from the mean of the entire image (not a local window) mean, measured in standard deviations.

- A correlation score will then be assigned to the image by calculating the Spearman Rank-Correlation Coefficient between the z-scores of each of the two markers (her-2 and Her-3). This will be the measure of colocalization for each image.

In either case, each pixel will be assigned a score based on its variation from either the local or global mean. Spearman Rank-Correlation Coefficients will be used because even if the pixel intensities are represented in a normally distributed

fashion, the manipulation of this data (which involves squaring deviations) will almost certainly result in a chi-square distribution of Z-scores. The Spearman Rank-Correlation Coefficient will be calculated as follows:

1. Pixel Z-scores will be sorted in ascending order.

2. Each pixel will be assigned a rank.

3. Where multiple pixels have identical scores, they will each be assigned the mean rank for the entire group.

4. The correlation coefficient is then calculated as follows:

$$r_s = \frac{L_{xy}}{\sqrt{L_{xx} \times L_{yy}}} \tag{4.1}$$

Where

$$L_{xx} = \sum_{i=1}^{n} (p_{rx_i} - \bar{p}_{rx})^2$$

$$L_{yy} = \sum_{i=1}^{n} (p_{ry_i} - \bar{p}_{ry})^2$$

$$L_{xy} = \sum_{i=1}^{n} (p_{rx_i} - \bar{p}_{rx}) \times (p_{ry_i} - \bar{p}_{ry})$$

And p_{rx_i} is the rank of the score of the i^{th} pixel in image x (and respectively for y), and $\bar{p}_{rx}$ is the mean rank for all pixel scores in image x (and respectively for y).

4.4 Phase Two: Retrospective survival analysis of a cohort of breast cancer cases

In phase two, the technologies and data transfer protocols will be applied to full analysis of a cohort of breast cancer tissues from the repository at VARI.

4.4.1 Followup data

The exact protocol for data handling in this study will be developed in cooperation with the Grand Rapids Clinical Oncology Program, as described in phase one. The protocol could follow the following strategy, but the final strategy will be agreed upon in the course of Phase 1, and approved by the IRBs at both institutions.

The Van Andel Research Institute maintains a repository of paraffin fixed human tissue specimens from surrounding pathology departments. Each specimen retains the original accession number imprinted on its cassette, and is linked to a data file that contains age, gender, and diagnosis field from the pathology report. Sections from the blocks to be used in this study will be cut, and a new number placed on the resulting slides. The accession number for specimens identified through our online query tool together with the new number assigned to the slides will be transmitted securely to the Grand Rapids Clinical Oncology Program, who could add treatment and survival data (what treatment, if any, was attempted, date of last contact, status at last contact) and strip the file of the accession number, leaving only the new identifier. This file would then be transmitted back to the Van Andel Research Institute. The file linking the slide identifier to the accession number would be destroyed, removing any means of linking the slides and associated data to the original accession number or medical record.

4.4.2 Statistical analysis

1. Subject selection and censoring

Survival analysis will be conducted upon patients corresponding to the breast cancer blocks in our repository. This analysis will be done retrospectively. Survival time will be calculated from the date of diagnosis. For each patient, the time to

event (see dependant variables below) will either be known through the medical record or vital record, or the patient will be censored as of last contact with the data system (i.e. the last medical record date that confirms the disposition of the patient as of that date).

2. Dependent variables

Two dependent variables will be individually and separately analyzed:

- Survival
- Time to metastasis

3. Models The JMSI and CGSI coefficients will be separately analyzed as independent variables in Cox proportional hazard models. This model has been selected because of its robustness—i.e., it performs well under a variety of distributions citepkleinsurv—and the distribution of our correlation coefficients is not likely to be normal.

Initial covariates included in the model will be:

1. Age
2. Nodal status
3. Tumor size
4. Tumor grade
5. Treatment
6. Mean Her-2 intensity
7. Mean Her-3 intensity

Thus, our initial hazard model will be as follows:

$$h(t, \mathbf{X}) = h_0(t)e^{\beta_1 r_s + \sum_{c=2}^{8} \beta_c X_c + \sum_{i=2}^{8} \beta_i (X_i \times r_s)} \tag{4.2}$$

Where β_1 is the coefficient for the correlation coefficient r_s calculated as described above, β_c is the coefficient for each covariate, X_c, above, and β_i is the coefficient for each interaction term between r_s and each covariate listed above.

Beginning with the least significant interaction term, non-contributory parameters will be removed from the model in a step-wise fashion. At each step, the integrity of the model will be assessed using the likelihood ratio test (the alternative—the Wald test—has been rejected at the behest of statistical texts such as (149)). For each parameter removed, the following calculation will be performed:

$$LR = (-2logL_{\mathbf{X}*}) - (-2logL_{\mathbf{X}}) \tag{4.3}$$

This number has a chi-square distribution, and should be non-significant if the subtraction of the parameter (in vector X*) did not impact the predictive power of the hazard function. Alternatively, entire sets of parameters (such as all interaction terms) may be removed so long as the resulting difference in LR is non-significant. The point estimate of interest is the adjusted hazard ratio due to the colocalization of Her-2 and Her-3 receptors. The hazard ratio is calculated as follows:

$$\widehat{HR} = e^{\beta_{r_s}} \tag{4.4}$$

Where the confidence interval is given by exponentiating $\beta_{r_s} \pm 1.96 \times SE$ (SE being the standard error of the correlation coefficient as calculated from the data set). If this interval is substantially narrower with the inclusion of a parameter that is non-significant by the LR test above, it may be retained.

Note that the model must at all times be well formed. That is, no term may be removed, even if non-significant by the LR test, if an interaction term involving that term has been retained. For example, the Nodal Status term must be retained if Nodal Status $\times r_s$ has been retained for any reason.

This analysis will be performed using the Proc Logisitic procedure of the SAS statistical analysis program.

If the level of interaction between Her-2 and Her-3 is truly prognostic, we would expect the hazard ratio as given above to be significantly greater than 1. Furthermore, the the CGSI method is indeed superior to the JMSI method for this sort of application, the hazard ratio from the correlation coefficient as calculated by the CGSI method should be significantly larger than that from the JMSI method.

We have hypothesized that this analysis will provide additional information beyond simple measurement of Her-2 level. By including the Her-2 and Her-3 terms in our model, we will be able to identify any independent predictive power of the colocalization term above and beyond that of measuring the intensities of either or both proteins without attempting to account for their interaction in space.

Chapter 5

Denouement

It was the intent of this work to describe the approach of quantitative molecular epidemiology, motivate its implementation, and demonstrate its application through a proposed study of the role of the interaction of two receptors, Her-2 and Her-3, as a quantitative prognostic parameter in breast cancer. This work arose from a grant application to the National Institutes of Health, which as of this date remains pending. Regardless of the outcome of this application, the author and his colleagues will continue to pursue the vital agenda of quantitative molecular epidemiology, and they invite the inquiry and/or collaboration of interested parties.

Glossary

Because of the cross-disciplinary nature of this work, some key terminology is here defined:

Amplification - The increase in gene copy of a specified gene in the genomic DNA. This may be detected by Flourescent In-Situ Hybridization (FISH), a technique that hybridizes flourescent probes targeted to specific gene sequences to detect how many copies of the gene are in the chromosomes.

Dimer - The molecular interaction (via non-covalent forces such as hydrogen bonding) of two molecules to form a complex. These molecules may cell surface receptors or other molecules. **Dimerization** is the process by which this occurs.

Colocalization - An imaging phenomena in which imaged markers are found in the same point in space as documented by the multi-channel image of those markers. Flourescent tags attached to dimerized proteins would be expected to be colocalized in an image of those tags.

Granularity - The ability of a measure to distinguish fine gradations in state. For example a True/False question has less granularity than a multiple choice question with 5 possible answers, which in turn has less granularity than an essay question with respect to measuring exactly what a student knows about a given topic.

Heterodimer - A dimer composed of two different proteins.

Homodimer - A dimer composed of a pair of identical proteins.

Overexpression - The production of protein above normal physiologic levels re-

sulting from gene amplification, or an increase in transcription products (mRNA), or an increase in translation products (these latter two manifesting a breakdown in the normal regulation of these processes).

Proto-oncogene - A gene encoding a protein necessary for normal physiologic function but which may become carcinogenic as a result of mutation or disruption of normal regulatory processes.

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