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GENOTYPIC AND PHENOTYPIC CHARACTERISTICS OF p53, HER/*neu* AND Bcl-2 IN CHINESE PRIMARY BREAST CANCER

by

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ABSTRACT

GENOTYPIC AND PHENOTYPIC CHARACTERISTICS OF p53, HER/*neu* AND Bcl-2 IN CHINESE PRIMARY BREAST CANCER

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There is a need to compare the various descriptional parameters currently used to define breast cancer with the underlying genetic alterations. The work in this dissertation addresses this need by examining paraffin embedded samples of breast cancer collected previously for routine diagnosis. Two hundred and five specimens from 178 cases of primary breast cancer in patients from China, those specimens were evaluated for clinical and histopathology features of cancer. One hundred thirty-four primary invasive breast carcinomas from this group were examined for p53, HER/*neu* (*c-erbB-2*) and Bcl-2 protein expression by immunohistochemical methods; 14.18%, 23.13% and 66.42%, respectively stained positively. p53+/HER+/Bcl-2+, p53+/HER-/Bcl-2+, p53+/HER+ and p53+/Bcl-2+ were associated with poor breast cancer prognosis. HER/*neu* (*c-erbB-2*) genes were amplified and detected by differential PCR and band intensity methods; 29.10% was positive. Sixty-four of them were evaluated for p53 gene point mutations by polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP), followed by gene sequence analysis; a positivity of 6.15% was found and included 2 missense, 1 intron and 1 silent, mutations. The detected characteristics of p53 and HER/*neu* gene mutations, the proteins expressed, together with Bcl-2 protein accumulation, were used to define differences between two groups: the Western population (high breast cancer risk) and Chinese people (low breast cancer risk) these had similar genotypes and phenotypes except for the Chinese breast cancer patients tending to be younger, to have a higher frequency of infiltrating ductal cancer and have a longer disease free survival time. Aspects of these findings that might provide information as to the mechanisms involved in carcinogenesis are discussed.

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I. INTRODUCTION

Breast cancer is the most common malignant disease of women. It is a disease that in all probability has more than one cause, and indeed may be the reflection of many interacting mechanisms. Breast cancer understandably has been the subject of extensive studies. This current project is designed to compare the characteristics of p53, HER/*neu (c-erbB-2*) gene mutation, protein expression and Bcl-2 oncoprotein accumulation in a low risk breast cancer population (China) with that in a high risk population (Western countries). Furthermore, the correlative features of gene point mutations of p53, the gene amplification mutations of HER/*neu (c-erbB-2*), their protein products and Bcl-2 oncoprotein in these primary invasive breast cancers are analyzed and evaluated in the hope that this information can help to better understand breast cancer.

1.0.0 Historical Aspects of Breast Cancer

Breast cancer has had the attention of physicians for several centuries and has a place in medical history because its uncertain causes, the confusion of treatment and the often unhappy endings.

The oldest of medical records comes from ancient Egypt about 1600 BC through engravings or painted hieroglyphics on stone where were recorded the 45 case earliest known cases of breast cancer. The examiner was told that "a breast with bulging tumors, very cool to the touch, is an ailment for which there is no treatment"; Edwin Smith (1822-1906) acquired this old information about breast cancer at Thebes in 1862 (Henry, 1984).

Records existed in ancient Greece by 460-136 BCs Hippocrates (460-370), father

of medicine and the originator of the theory that the four cardinal humors of the body (blood, phlegm, yellow bile, and black bile) with the four universal elements (earth, air, water, and fire) balance maintained the health, put forward the notion that the origin of cancer was natural causes. He described a woman with carcinoma of the breast with bloody discharge from the nipple, and clearly stated that in cases of deep-seated cancer it was better to give no treatment, because treatment hastened death (Boyd, 1987).

During the Greco-Roman Period (150 BC-500 AD), Leonides, a Greek physician in the first century AD who practice at Rome, is credited with the first recorded operative treatment for breast cancer. In the same century, Aurelius Cornelius Celsus, an encyclopedist and not a physician, published the first clinical description of breast pathology: "a fixed irregular swelling with hardness or softness, dilated tortuous veins and with or without ulceration." He mentioned four clinical stages: namely malignancy (apparently simple or early), carcinoma without ulcer, ulcerated cancer, and ulcerated cancer with flower-like excrescences that bleed easily. He opposed treatment of the last three stages by any method (Wagner, 1991).

Galen (131-201 AD), who was born on the Mediterranean coast of Asia Minor, studied in Alexandria and practiced in Rome, described mammary cancer as a swelling with distended veins resembling the shape of a crab's legs. He is know for his humoral theory (i.e. " to prevent accumulation of black bile the patient should be purged and bled"), but he also claimed to have treated breast cancer by surgery in its early stage when the tumor was on the surface of the body and all the roots could be extirpated (Wagner, 1991).

There was no significant progress in this field during Medieval Period until the Renaissance. Andreas Vesalius (1514-1564), a Flemish surgeon comparative anatomist, advised wide excision and the use of ligatures instead of cautery in breast cancer operations. Ambrose Pare (1510-1590) studied medicine in Paris where he made the important observation that breast cancer often caused swelling of the axillary lymph glands. Henri Francois le Dran (1685-1770) noted that lymphatic spread worsened the prognosis of breast cancer (Robinson, 1986).

Significant changes in pathological and physiological concepts were slow to develop in the eighteenth century. Pieter Camper (1722-1789) first described and illustrated the internal mammary lymph node, and Paolo Mascagni (1752-1815) later reported the same for the pectoral lymph drainage. The textbooks described a variety of diseases: breast cancer was considered as a benign growth that under adverse circumstances could undergo malignant degeneration. Death caused by metastasis from cancer was not yet be understood at that time. Mastectomies were performed in larger numbers. Bernardine Ramazzini, an Italian physician, noted the high incidence of breast cancer in nuns and hypothesized that this was in some way related to their celibate life style (Yu 1981; Wagner, 1991).

The nineteenth century brought several important contributions to the field. In 1822, James Elliott first examined a tumor under the microscope: an axillary node removed during breast surgery and diagnosed as sarcomatous. Improvements in surgical technology occurred to assist breast cancer surgery. Bresler, William and Morton first introduced anesthesia in the United States in 1846 and Joseph Lister in Great Britain in

1867 established the principle of antisepsis. The modern radical mastectomy technique and specific investigations into the epidemiology statistics both having a great effect on the study of breast cancer were established. Methods for removing axillary nodes, cutting off the blood supply of tumor, and for taking out the entire breast no matter how small the primary tumor, were developed for use in surgical treatment (Elliott, 1822; Wagner, 1991). In 1856, Sir James Paget reported that scirrhous carcinoma patients who were not operated on lived longer than those with surgery (Paget, 1856). By the end of the century, the Halsted radical mastectomy had been established as the ideal method for surgical treatment of operable breast cancer; this method, where "the suspected tissues should be removed in one piece", was provided by William Steward Halsted (1852-1922). It is still used in today's surgical treatment. William Welch, a pathologist at Hopkins was the first to employ frozen sections in the diagnosis of breast lesions, in 1891 (Cullen, 1895). As medical education increased towards the end of the century research into breast cancer increased accordingly.

For most of the Twentieth century, the causes of breast cancer remain poorly understood, but it was known that a strong family history led to a high risk for breast cancer and it was believed that the solution was bilateral mastectomy. Cushman Dhaagensen classified breast cancer according to the size, clinical findings and nodal status and first proposed self-examination of the breast; he suggested that lobular neoplasm (*in situ*) was not actually a carcinoma (Haagensen, 1986). The modified radical technique for mastectomy in surgical treatment for carcinoma of the breast was developed in 1948 by Patey and Dyson in London. In the same year, McWhirter

introduced a simple mastectomy technique and included radiotherapy treatment, however the survival rates remained similar to tradition surgical methods (McWhirter, 1948; Patey, 1948).

In 1973 estrogen receptors and their properties were demonstrated in human breast tumors by McGuire (McGuire,1975), and in 1975, Horowitz identified progesterone receptors in hormone-dependent breast cancer (Horowitz, 1975). The presence of circulating malignant cells in the blood stream, reported in 1955 by Engell, stimulated interest for therapy through destruction of these cells by systemic administration of chemicals (Engell, 1955).

In 1895, Emile Crubbe (1875-1960), a second-year medical student in Chicago, first irradiated a breast cancer patient by X-ray, with such radiation therapy becoming the common form of enhanced standard treatment in many clinical centers until 1920 (de Moulin, 1983); the five-year survival of Stage II breast cancer patients was improved through this approach, although Stage I cases remained unchanged. Keynes (1932) in London, used radium as a source of radiation, and obtained a five-year survival of 77.1 percent in those cases with negative axillary nodes, and of 36.3 percent in those with axillary-involved breast cancer. In 1948, Robert McWhirter proposed the approach of simple mastectomy followed by radiotherapy (McWhirter, 1948). Higher X-ray voltage up to 6000 to7000 rads, including the implantation of Ir^{192}, were also used during 1960s. Even to the present day there is no certainty as to whether the more intensive surgical procedures or the addition of radiation was most benefit breast cancer patients.

During this present century, improved management of pain and infection through

the advent of anesthesia and aseptic technique have helped breast cancer control. Mammographic screening in 1978 (Nation Institutes of Health, 1978), hormonal therapy in 1939, chemotherapy in 1963, all are important additional treatments introduced for breast cancer patients; the results seen include the observation that five-year survival rates have increased particularly in premenopausal women with metastases (Bonadonna, 1981). Immunotherapy and genetherapy are not yet accepted as practical clinical treatments yet, but are under detailed research (Wagner, 1991).

2.0.0 General Aspects of Breast Cancer

2.1.0 Epidemiology

Although, globally breast cancer is the most frequent malignancy in women, international statistics reveal widely varying rates of breast cancer in different geographical areas. This may reflect different effects of the various environments or perhaps ethnic variations on gene expression and mutation in the development in this disease.

2.1.1 Geographical Influence

Each year, over 512,100 new cases of breast cancer are detected, and some 250,000 people in the world die of malignant breast cancer (WHO, 1982). However, the morbidity and mortality appear to be different between the Eastern and the Western areas of the world. For the period 1986-1988, the highest death rate in malignant diseases was from breast carcinoma and was recorded in England and Wales (29.3/100,000), followed by Denmark (28.3/100,000) and Scotland (27.5/100,000). The rate in the United States was 22.4/100,000 - 16th in a list of 50 nations. The countries at the bottom of the list were Thailand with 1.0/100,000, Korean Republic 6.0/100,000 and China with 4.7 deaths

per 100,000 population (Tavassoli, 1992). Thus there is almost a thirty fold difference between Thailand and England, the East and the West.

In relative terms as the incidence of breast cancer in the United States and Canada is six times that in Asia and Africa. In Western Europe it is four times that in Japan. South America, the Caribbean region, and Eastern Europe have rates that lie between these (Waterhouse, 1976). Of pertinence to the current study is the fact the mortality from breast cancer among Chinese women is reported to be only about one-fifth that of white females the USA (Fisher, 1978; Hoare, 1996)

2.1.2 Environmental Influences

Approximately 80 to 90% of all cancers are thought to be a result of exposure to environmental carcinogens. The importance of environmental factors can be deduced from the considerable geographical variation reported, and from the changes in rates amongst migrants. An interesting example of this occurs in Japanese who have emigrated to the continental United States and have a higher mortality rate than those living in Japan; correspondingly the rates are higher for Japanese born in the United States than for immigrants who were born in Japan (Dunn, 1977). Since changes in risk usually occur slowly, and over several generations (as illustrated by the migrants from Japan and China in the USA), it is assumed that some of the determinants of the risk must act early in life (American Cancer Society, 1988; Jigginson, 1992).

Chemical induced carcinogenesis has been noted for many areas of cancer, often related to such carcinogens present in the environment; chemicals are also purported to be causative in breast cancer. Exposure to more than one hundred chemicals have been

implicated in studies of carcinogenicity in humans (Higginson, 1992a), with 25 - 30 substances proven to cause cancer in human beings. Some pesticides are regarded as causal in breast carcinogenesis (Miller, 1977).

Environmental microorganisms are also considered as important influences in oncogenesis, and it has been suggested that viruses may act as triggers but are not sufficient alone for oncogenesis (Dmochowski, 1969, 1972; Rapp and Reed, 1977). Mammary cancer in animals can been produced by infectious viruses, with the first discovery of a B-type RNA tumor virus in mice made over 50 years ago (Higginson, 1992b). Most of the naturally occurring viral induced tumors of animals are associated with the RNA viruses, and these cause mammary cancer, leukemia, as well as sarcomas in mouse, cat and chicken (Klein and Smith, 1977). There is no convincing evidence yet of a viral agent linked to human breast cancer, despite many epidemiological and laboratory studies, and most experts favor an etiology involving nonviral carcinogens for breast cancer (Miller, 1977).

2.1.3 Racial Factors

In the United States, breast cancer is not only the leading cause of cancer mortality among Caucasian women, but also that of African American and Hispanic groups. The poorer prognoses and higher mortality rates among African-American and Hispanic women developing breast cancer are believed to be attributable to a lack of awareness about the disease and to poor-quality health care (Bair and Cayleff, 1993). The demographic features of Black and Caucasian women with breast carcinoma have been compared in several studies (Kovi, 1989; Natarajan, 1985; Polednak, 1986; Vernon,

1985). Incidence rates for female breast cancer (per 100,000) in the different racial groups in Hawaii were as follows: Caucasian, 80.3; Hawaiian, 66.2; Chinese, 54.2; Japanese, 44.2; and Filipino, 21.5 (Waterhous,1976; Mant and Vessey, 1991). In contrast to the outcome, the incidence of breast cancer is lower in black women compared to Caucasian women: for the period 1978- 1981, the age-adjusted incidence rate was 71.9/100,000 for Black women and 85.6/100,000 for Caucasian women (Tavassoli, 1992). Aside from economic social reasons, there are reports of more fundamental racial differences in that breast cancer in African-American women has a poor progression (Jacob, 1993).

2.1.4 Age

It is well known that the incidence of breast cancer increases progressively with age throughout the lifetime. The annual risk of breast cancer at age 75 is more than three times higher than the risk at age 35. It is rare for breast cancer to occur before age 15, and only 2% of breast cancers are found in women aged 30 or younger; 96% of breast cancer patients die at age 35 or older. Most breast cancer patients are aged 40 to 60, usually in the menopausal period. More than 70 percent of women over the age of 50 who present with breast cancer do not have any other identifiable risk factors. Thus women older than 50 years of age are a high risk group in Western countries (Hayes and Schnitt, 1993; Vogel and Love, 1991).

2.1.5 Gender Distribution

Breast cancer is rare in men in any country, usually averaging 100:1 female : male in population where the female to male ratio ranges from 70 to 130. In males, the

incidence rates are below 1.0/ 100,000 for most countries, and they, when compared with females, are usually not epidemiologically significant (Jigginson, 1992).

2.1.6 Trends

Death rates from breast cancer in females have generally remained stable, but the morbidities in China, in Japan, and in the USA, are all increasing. In the world as a whole, it was estimated that more than 500,000 new cases were diagnosed in 1975, but that by the year 2,000 the global total may well exceed 1,000,000 year. Clearly this form of cancer is becoming an increasingly important disease in all parts of the world (Mant and Vessey, 1991).

2.2.0 Risk Factors of Breast Cancer

The factors identified as increasing a woman's risk of breast cancer are recorded in several ways. There is the "absolute" risk and the "cumulative" risk. The absolute risk is based on the population, and is expressed as cases per 100,000. More commonly used is the cumulative, or "lifetime" risk, which assumes that all patients have the same risk and that the entire population will live to be a certain age. For breast cancer, this means that one in 10 women will develop cancer if the population lives to be 80 years of age. If one assumes that the population will live longer, for example, to age 85 or 100, the risks go up to 1 in 9 or 1 in 8, respectively, because the entire population is living longer and has more time to develop cancer.

Factors other than increasing age which significantly increase the risk of breast cancer include prior breast cancer, a strong family history, and the presence of atypical hyperplasia in a biopsy.

2.2.1 Genetic Factors (Family History)

The tendency for breast carcinoma to develop in certain families has been known for many years and it is now recognized that a positive family history of breast carcinoma constitutes a major risk factor. The risk is especially significant when the diagnosis of breast cancer is made in a first degree relative (i.e. mother, sister, daughter) (Anderson, 1977; Anderson and Badzioch, 1986).

Li-Fraumeni Syndrome, a specific gene defect, is associated with a higher frequency of breast carcinoma. Patients with this autosomal dominant syndrome are characterized by a defect in the tumor suppressor gene, p53; multiple cancers (breast, brain, sarcomas, and adrenal cortical tumors) are observed in the close relatives (Birch, 1990; Li, 1988; Malkin, 1990).

Recently a breast cancer susceptibility gene, BRCA1, was identified on chromosome 17 and appears to be involved in approximately 5% of all breast cancers. BRCA1 has been found to be associated with most inherited breast and ovarian cancer combinations. However, only 45% of the familial breast cancer appears to be linked to this gene. BRCA1 is located in same area as other genes that are known to be involved in breast cancer such as *c-erbB-2* (HER/*neu*)(Howell, 1995). *c-erbB-2* (HER/*neu*) gene correlates with a shorter time to relapse and to disease-free survival when this gene's product is overexpressed (King, 1990, 1992; Sattin, 1985). Recently a second breast cancer susceptibility gene BRCA2 has been reported (Wooster, 1995). Based on the inheritance of chromosomal markers in families with hereditary cancers that are not linked to BRCA1, BRCA2 genes have been are mapped to chromosome 13q12-13. This is a

region known to exhibit loss of heterozygosity in 20-40% of sporadic breast cancer. The new gene should provide the basis for tests to screen for cancer causing mutations in women from families with a high incidence of mammary cancers, even though no one knows yet what normal function these susceptibility genes have or how their mutation or loss many lead to cancer (Marx, 1996; Collins, 1995).

2.2.2 Hormonal Influence

A woman's own hormonal environment is likely to be a very important factor, perhaps more important than others such as diet, in considering her risk for developing breast cancer. Three major hormone-related events in the life of a women influence the risk: these are - age at onset of menstruation (menarche); secondly the age at the menopause; and lastly the age at which she has her first full-term pregnancy. Breast cancer risk is increased by an early menarche, a late menopause, or a late first full-term pregnancy (Iqbal and Taylor, 1989). Reproductive steroid hormones act mainly as promoters rather than as initiators of carcinogenesis, and therefore are most likely exert an influence on carcinogenesis by altering the kinetics of proliferation, differentiation or atrophy of the stem cell, resulting in an increase in the number of susceptible cells (Thomas, 1984).

2.2.3 Nutritional Influence

There is suggestive evidence generally relating diet to cancer (American Cancer Society, 1975), and nutrition, diet, and obesity have also been linked to an increased risk of breast cancer. This link was suggested by findings of large differences in national breast cancer incidence rates between different countries. As already mentioned, the age-

adjusted incidence rates of breast cancer are five times higher in the United States and other Western industrialized nations than in Asia; this many have a dietary component. Changes in dietary habits may also be partly responsible for the changes observed in the incidence and mortality of breast carcinoma in migrant populations (Lodon, 1992; Tammenbaum, 1942; Tavassoli, 1992).

A significant correlation between breast cancer mortality and consumption of fat and animal protein has been reported (Vorherr,1980). A number of population studies have correlated the incidence and mortality of this disease with total dietary fat (Ening, 1978). For example, in the USA most individuals derive 30 and 45 % of their calories from fat, compared with 15 per cent in China (Whittemore, 1990). Serum cholesterol levels have also been linked to breast cancer (Hiatt, 1982). Nowadays not only are single elements in food receiving attention, but also the patterns of the food consumption are being considered. In addition, investigators have found that a high total caloric intake in human beings (e.g., obesity) is associated with a modestly increased risk of developing many diseases, particularly breast cancer; alcohol and coffee intake are also linked with breast cancer risk (Willett, 1987; Harvey, 1987; Willet, 1985).

A 13 year study funded by the National Institutes of Health and the Women's Health Initiative began in 1993 to focus on whether or not a low fat diet can reduce the incidence of breast cancer; this study should present some answers to the many outstanding questions (O'Grady and Rippon, 1994).

2.2.4. Other Factors

A previous history of benign breast disease, oral hormone exposure, low

socioeconomic status, abnormal mammographic pattern, and radiation exposure, are all of concern as factors related to the development of breast cancer. Considering all the data available is not implausible to suggest that the major determinant of breast cancer risk is the total cumulative exposure of breast tissue to bioavailable estrogen, and that this exposure is influenced by nutritional factors and by reproductive history. Energy-rich diets during adolescence may enhance the occurrence of precancerous lesions, with pregnancy having a strong protective effect. Table I-1 shows the factors influencing risk of developing breast cancer.

Table I-1. Factors influencing risk of developing breast cancer

Factors	High Risk Group	Low Risk Group
SEX AND REPRODUCTION		
Sex	Female	Male
Menarche	Early age	Late age
Menopause	Late age	Early age
Ovarian Function	Artificial menopause, nulliparous	Normal, praous
Pregnancy (first)	Later age	Early age
HORMONAL EXPROSURE		
Oral Contraceptives	Use	Non-use
Postmenopausal Estrogen	Use	Non-use
HORMONE PROFILES		
Circulating Oestradiol	High	Low
RACE AND ENVIRONMENT		
Race	Westernized	Developing
Socioeconomic Status	High	Low
Body Statute	Obese	Lean
Diet	High fat calories	Low fat calories
Radiation Exposure	Childhood	None
FAMILY HISTORY		
First-degree Relative Premenopausal Bilateral Breast Cancer	Breast cancer History	No breast cancer No history
MEDICAL HISTORY		
Age	Old	Young
Previous Benign Disease, Particularly Biopsy	History	No history
Mammographic Pattern	Prominent duct dense	No prominent ducts not dense

(Forrest, 1990)

3.0.0 Clinical Diagnostic Approaches of Breast Cancer

A variety of features have been evaluated as prognostic indicators for breast

carcinoma. The use of some of these potentially can markedly influence the management of breast carcinoma.

3.1.0 Prognostic Indicators

The prognostic indicators for breast cancer are varied in their features. Some can be used to divide breast carcinoma into types with good or a poor prognosis.

Table I-2. The influence of various indicators on the prognosis of breast carcinoma.

	Good Prognosis	Poor Prognosis
Size	< 1 cm	> 5 cm
Local Extension	Absent	Present
Grade	Low	High
Receptor status	ER+, PR+	ER-, PR-
Axillary nodes	Tumor -	Tumor +

(Tavassoli, 1992). ER - eostrogen receptor; PR - progesterone receptor.

3.1.1 Tumor Staging

The staging of breast cancers is essential in the evaluation of the tumors, the determination of prognosis and in guiding the type of management used. This process is divided into pathologic staging and clinical staging. The two staging systems do not always correlate; for example, inflammatory changes in the skin of the breast do not necessarily reflect dermal lymphatic invasion in inflammatory carcinomas (Bonnier, 1995; Tavassoli, 1992).

The most widely used system is based on the TNM guidelines, where TNM stands for Tumor, Nodes and Metastases, which was proposed in 1954 by the International Union Against Cancer. The current classification was agreed upon in 1987 by both the International Union Against Cancer and the American Joint Committee on Cancer. The

TNM classification is presented in table and is derived from Sobin (1988) and Harmanek (1987).

Other classifications are usually only used within individual institutions. For example, the cTNM system, which is an evaluation of clinical presentation made before treatment, usually includes physical examination, imaging, and laboratory testing. The pTNM category is referenced as a pathological evaluation based on the macroscopic and microscopic examination of specimens, and it adds precision to clinical evaluation (Tavassoli, 1992; Hammond, 1995).

3.1.2 Tumor Size

The gross size (maximum diameter) of the invasive primary mass is one of the most important prognostic factors (Carter, 1989; Page, 1991). Tumor masses 2 cm or smaller in maximum diameter have a significantly better prognosis and survival compared to those that are larger (Palmer, 1982). Patients with tumors 1.0 cm or smaller had a 20 year survival of 86%; survival dropped to 69 % for those with tumors over 1.0 cm in size (Rosen, 1989a). The size of tumor also been a direct relationship to the probability of axillary node metastases: tumors 1.0 cm or less in diameter produced 26% axillary node metastases, whereas tumor size over 1.0 cm show 78% (Haagensen, 1986).

3.1.3 Tumor Type

Tumor type, independent from the stage, is an important indicator. Certain types of breast cancer, such as medullary, tubular, mucinous (colloid) and adenoid cystic carcinoma, are "low grade", and are associated with a low frequency or the absence of axillary node metastases and have a relatively good prognosis. In contrast, inflammatory

breast carcinoma, which a merely implies widespread dissemination and tends to develop with pregnancy (Bonnier, 1995), has a high grade, with a low five-year survival (Donegan, 1977). Moreover, recurrences are comparatively less frequent in other types such as tubular, medullary, invasive papillary, and colloidal cancer (Rosen, 1989b; Ciatto, 1990).

3.1.4 Morphologic Parameters

The morphologic parameters of breast cancer have been well defined. The morphological detection of stromal invasion is one of the most significant prognostic indicators; *in situ* or noninvasive carcinomas are almost invariably cured by mastectomy (Boring, 1992) or less radical removal of the tumor tissues. Also, intraductal proliferative lesions have been studied for an associated relative risk for subsequent development of cancer: only the development of invasive carcinoma has been useful in analyses (Page, 1985; Schintt, 1984, 1987).

3.1.5 Lymph Node Metastases

The status of the regional lymph nodes with regard to invading breast cancer cells is one of the most important prognostic indicators in breast cancer; determination that a tumor has become invasive largely establishes the survival rate (Cotran, 1994). The larger the number of cancer positive lymph nodes identified, the more serious a particular neoplasm is (Fisher, 1970), the higher the failure rate in subsequent treatment (Fisher, 1975), and the lower the survival rate (Neville, 1991).

3.2.0 Screening

Increased emphasis has been placed on breast cancer screening in the last several years, with the mortality being significantly reduced by mammography and physical

examination.

Table I-3. Randomized clinical trials of breast cancer screening (5 to 13 year follow up)

Trial	Breast Cancer Mortality Reduction in Those Screened	Type of Screening
Health Insurance Plan (New York)	29%	Annual two-view mammogram+BPE
Swedish Two County	31%	Single view mammogram every 2-3 years
Malmo	0	Initial two-view mammogram, then single- or two-view mammogram every 18-24 months
Canadian NBSS	0	Annual two-view mammogram + BPE
Combined Swedish Trials	24%	Mammogram at variable intervals

BPE = breast physical examination; NBSS = National Breast Screening Study. (Lindfors, 1995)

3.2.1 Physical Examination

Physical examination for palpable tumors is one of the most widely used methods of breast cancer screening. It is also simple and cheap as no equipment is needed and in fact, most palpable tumors are found by physical examination. Palpable breast lesions of various types can be found during physical examination of women with different kinds of breast symptoms. When a woman has detected a lump in her breast , an experienced physician must examine and evaluate the lesion. This examination includes assessment of the size, mobility, skin changes associated with the tumor, together with an assessment of the size and mobility of regional lymph nodes, and any evidence of distant metastases (Berndt, 1969; Danforth, 1988; Mackenzie, 1994; Potchen, 1993).

3.2.2 Breast Imaging

Various breast imaging procedures are widely used to detect cancer. X-ray mammography is the only proven method capable of detecting nonpalpable breast cancer. It is successful in detecting carcinomas *in situ* or those that are minimally invasive, as well as those less than 5 mm in diameter; associated with a survival rate is around a 93 percent over 20 year (Gold, 1987). Thus, X-ray mammography has the greatest emphasis in the disclosure of early, nonpalpable, and often curable cancer. Since this is the technique that has proven most successful on detecting early breast cancer, it is the standard used to screen for breast cancer and the one to which all other imaging alternatives are compared (Lindfors, 1995; Potchen, 1993, 1991).

Ultrasound is increasingly used as a possible alternative to X-rays, but has not yet proven to be superior. Diagnostic ultrasonography nevertheless stresses differentiation of benign cysts from diagnostically indeterminate solid masses, with the latter requiring biopsy (Lindfors, 1995). Ultrasound is useful when used with fine needle aspiration biopsy (FNA) technique.

Recent interest has been focused on Magnetic Resonance Imaging (MRI) as a possible alternative to mammography. Preliminary data indicates that MRI (with contrast) can reveal lesions in dense breasts that are often missed by conventional mammograms. The cost of MRI and the time required to perform it will likely prohibit its use for general screening, but it may prove to be a useful ancillary technique to mammography (Lindfors, 1995).

Transillumination light-scanning is based on the concept that breast cancer

increases blood supply and absorbs more near-infrared and red electromagnetic radiation. This procedure is rapid, noninvasive, risk-free, and relatively inexpensive, and is eliciting increasing interest. However, it is also less sensitive than x-ray mammography in the detection of nonpalpable cancer (Geslien, 1985; Sickles, 1984).

Thermography is an older procedure that seems to be only useful for advanced cancer. The cancer detection rate using thermography is only 42 percent, compared with 57 % for physical examination, and 91% for mammography; thermography gives a high number of false positive examination, and thus is clinically unacceptable (Baker, 1982; Gold, 1986).

3.2.3 Breast Biopsy

The majority of palpable breast lumps are not malignant, but it is often difficult to definitively make that determination by palpation or by mammography or both. Therefore, tissue sampling is an important adjunct for diagnosis.

Surgical excision is the classical standard because it gives a direct examination of the entire lesion through histological examination. The non-palpable lesion can be successfully examined also with mammographic localization using indicator needles. However, excisional biopsy carries the typical surgical risks of potential wound infection , hemorrhage and development of tissue scars postoperatively. Moreover, the cost, the morbidity, and the frequency of multifocal lesions requiring multiple surgical procedures, make it not always practical, nor desirable, to do such surgical biopsies.

Fine needle aspiration biopsy (FNA) is a very useful technique. It can be performed not only on palpable lesions but also on non-palpable lesions using

mammographic or ultrasound guidance. FNA is a simple, safe procedure with only little discomfort. By using X-ray guided (Hann, 1989: Lofgren, 1988; Masood, 1990) or sterotactic (Azavedo 1989; Dowlatshahi, 1987; Lofgren, 1990) procedures to ensure placement of the aspiration needle in the lesion. The sensitivity and specificity are reported to be from 70 to 100% and the specificity is 100%. Multiple lesions can be easily aspirated during the same consultation (Rosen, 1996b). The ease and accuracy of this biopsy method allows suspicious lesions to be sampled immediately, thus reducing the patient's anxiety usually associated with long drawn out clinical follow-ups. However, FNA cannot distinguish *in situ* from invasive carcinomas, and not all pathologists are equally proficient or comfortable at interpreting cytopathologic breast samples. False-negative and false-positive results may be generated by the less experienced cytopathologists.

Cutting needle biopsy or large core biopsy techniques have been regularly performed with 12 to 16 gauge cutting needles; both methods obtain a solid core of tissue for histologic diagnosis. Pathologists are generally more proficient at examining this type of specimen as it is a true tissue sample. The procedure however, unlike FNA, is very painful, produces much local hemorrhage, and may lead to tumor cell seeding along with the needle track.

Rotex needle biopsy (RNB) is performed by a needle with the inner screw cutting component covered by a 22 gauge cannula. The procedure maintains the advantage of FNA and has proved more adept at obtaining specimens for pathologic diagnoses. The false-positive and false-negative results have been reduced remarkably (Qiao, 1992).

Needle biopsy techniques can therefore be used in breast cancer diagnosis to distinguish between cysts and solid tumors, and to provide material not only for cytologic pathology evaluation, but also for immunohistochemical analysis, flow cytometric studies, morphometric analysis and molecular research (Potchen, 1993, 1991; Schnitt, 1993).

4.0.0 Breast Cancer Diagnosis of Pathology

Many delays in the diagnosis of breast cancer occur because breast masses are followed clinically without any microscopic evaluation. The best clinical laboratory support for a patient with a breast mass is based on tissue diagnostic techniques.

4.1.0 Histopathology

Histopathology is the well established method for breast cancer diagnosis. Even though there are many new theories and new methods in the today's world for understanding, detecting, and diagnosing breast cancer, histopathology remains the essential component in practical diagnosis.

4.1.1 Histochemical Pathology

Classical histopathology is a direct microscopic observation of a tissue section treated with tinctorial stains that assist in the visualization of the cellular and tissue structure. This permits observation of defining morphological characteristics of the breast lesion morphology. Histochemical stains can also be used to visualize the nucleic acids, carbohydrates, proteins, lipids and enzymes in the constituent cells. The information obtained from the histopathology forms the basics of the information to guide the breast cancer patient's prognosis and treatment.

As the Cancer Committee of the College of American Pathologists practice protocols suggests, a pathology report concerning breast carcinoma should include the anatomic extent of tissue removed, the anatomic extent of the carcinoma in the specimen, the histologic type and any other information that may be useful to the referring physician in the selection of treatment, such as the type of adjuvant, the evaluation of new types of therapy, prognosis, and analyses of outcome (Hammond, 1995).

4.1.2 Anatomical Features of Histopathology

Breast carcinomas are almost all adenocarcinomas and are usually derived from the glandular epithelium of the terminal duct lobular unit. Various subtypes derive their names from a combination of their histologic patterns and their cytologic characteristics, rather than their site of origin (Bartow, 1988).

4.1.3 Carcinoma Differentiation (Grading)

Histological grading of breast cancer is potentially of great clinical value in determining prognosis. Several different grading systems are used; however, almost all grading criteria in the different systems are based on an assessment of cytologic factors that includes tubule formation, nuclear size, nuclear pleomorphism and mitotic count (Robbins, 1995; Elston, 1987). The overall grade of a tumor is classified by a three-point scale based on from cytologic features, with the final grade depending on the specific features. Grade I is a well differentiated tumor; Grade II is moderately differentiated; and Grade III is poorly differentiated. Importantly, studies of the relationship between grade and prognosis show a strong correlation, even when data are collected from many pathologists, use different grading systems, and are without standardized guidelines

(Robbins, 1995; Henson, 1991).

Recently, thymidine labeling index (TLI), and Flow Cytometry have also been used to define tumor grades.

4.1.4 Carcinoma Classifications

The histological classification of breast tumors is descriptive, and probably the most widely used classification is that proposed by the World Health Organization (WHO).

Table I-4. World Health Organization (WHO) histologic classification of breast tumors

I. Epithelial Tumors
  A. Benign
    1. Intraductal papilloma
    2. Adenoma of the nipple
    3. Adenoma
      a. Tubular
      b. Lactating
  B. Malignant
    1. Noninvasive
      a. Intraductal carcinoma
      b. Lobular carcinoma *in situ*
    2. Invasive (infiltrating)
      a. Invasive ductal carcinoma
      b. Invasive ductal carcinoma with Paget's disease
      c. Invasive lobular carcinoma
      d. Mucinous carcinoma (colloid carcinoma)
      e. Medullary carcinoma
      f. Secretory (juvenile) carcinoma
      g. Tubular carcinoma
      h. Adenoid cystic carcinoma
      i. Apocrine carcinoma
      j. Invasive papillary carcinoma
      k. Carcinoma with metaplasia
        i. Squamous type
        ii. Spindle cell type
        iii. Cartilaginous and osseous type
        iv. Mixed type
      l. Others
    3. Paget's disease of the nipple
II. Mixed Connective Tissue And Epithelial Tumors
  A. Fibroadenoma
  B. Phyllode Tumor (cystosarcoma phyllodes)
  C. Carcinosarcoma
III. Miscellaneous Tumors
  A. Soft Tissue Tumors
  B. Skin Tumors

C. Tumors of Hematopoietic and Lymphoid Tissues
IV. Unclassified Tumors
V. Mammary Dysplasia/Fibrocystic Disease
VI. Tumor-like Lesions
A. Duct Ectasia
B. Inflammatory Pseudotumors
C. Hamartoma
D. Gynecomastia
E. Others

(Azzopardi, 1982; WHO, 1982).

Variations on this basic system appear in other texts, such as the following which is from Bartow and Fenoglio-Preiser (1988):

Table I-5. World Health Organization classification of carcinomas

Non-invasive
- Intraductal
- Lobular carcinoma *in situ*
- Intraductal papillary carcinoma

Invasive
- Invasive ductal carcinoma
- Paget's disease
- Invasive lobular carcinoma
- Medullary carcinoma
- Mucinous carcinoma
- Tubular (well-differentiated) carcinoma
- Invasive papillary carcinoma
- Adenoid cystic carcinoma
- Secretory carcinoma
- Apocrine carcinoma
- Carcinoma with metaplasia
- Mixed type

The classification of breast cancer used mainly in China today is that proposed in 1987 at the Third National Breast Cancer Meeting in China. This system is shown in the following Table I-6.

Table I-6. Breast carcinoma classification in China

1. Non-infiltrative carcinoma
   - Ductal carcinoma *in situ*
   - Lobular carcinoma *in situ*
2. Early infiltrative carcinoma
   - Ductal carcinoma with early invasion
   - Lobular carcinoma with early invasion
3. Infiltrative specific carcinoma
   - Papillary carcinoma
   - Medullary carcinoma with massive lymphocytic infiltration

Tubular carcinoma (well-differentiated-adenocarcinoma)
Adenoid cystic carcinoma
Mucinous adenocarcinoma
Apocrine carcinoma
Squamous cell carcinoma
Paget's disease

4. Infiltrative not specific carcinoma
   Infiltrating lobular carcinoma
   Infiltrating ductal carcinoma
   Scirrhous carcinoma
   Medullary carcinoma
   Simple Carcinoma
   Adenocarcinoma
5. Other rare carcinomas
   Signet-ring cell carcinoma
   Smell cell undifferentiated carcinoma
   Secretory carcinoma
   Carcinosarcoma
   Pseudosarcoma
   Anaplastic carcinoma
   Papillary carcinoma

(Goung, 1993; He, 1990)

Other classifications exist in other countries but are not widely used.

4.2.0 Cytopathology

There are three different types of materials for cytologic examination of the breast: nipple secretions, aspirations from cysts, and various types of needle samples from solid tumors. The more needle biopsies performed, the more important cytopathology diagnosis on breast specimens becomes to disease management. Accuracy of the cytologic diagnosis plays a major role in the breast cancer patient's life today. Clear differential diagnosis between benign and malignant lesions is possible for most breast cytologic specimens when there is careful application of the cytologic criteria for malignancy (Carter, 1990).

4.2.1 Cytochemistry on the Fine Needle Biopsy Sample

Histochemistry on cytology samples is essentially similar to that used with tissue

samples, where different chemicals stain specific cellular structures. For breast tumors, the commonly used histochemical stains are Papanicolaou, modified Wright-Giemsa, H & E, "Diff-Quik", and the modified May-Grunwald-Giemsa stains.

The "Diff-Quik" stain offers rapid results with good cellular detail on air-dried smears and usually provides a good presentation of the cytoplasm. The rapid Papanicolaou stain provides a fast staining method useful for immediate FNA diagnosis. With the Papanicolaou stain, cell nuclear and nucleolar details provide a very clean image for pathologists to used in diagnosis (Bedrossian, 1991a; Feldman, 1985).

4.2.2 Morphological Characteristics of Cytologic Samples

Features of the breast cancer cytology present in certain patterns; for example, the solid cell ball, psammoma bodies, small semi-cohesive cell clusters, and "indian files" are all seen.

The solid cell ball configuration is a typical presentation of breast carcinoma. The cell balls immediately stand out, even under low-power examination, not only because of their size, but also because of their distinct configuration. Psammoma bodies are curious structures that represent degeneration and calcification of cells commonly arranged in papillary clusters in the tumor. The calcific concretions are usually larger and more irregular than calcospherites, and commonly fragment along straight fracture lines. However, a diagnosis of malignancy should not be made unless well-preserved neoplastic cells are clearly identified. The small partially cohesive tumor cell clusters often do not have overt differentiating features in their cytoplasm. There is usually only a small amount of nonvacuolated cytoplasm exhibited by these cells and they lack a tight-ball,

acinar, or papillary configuration. The cell clusters may break up into smaller groups that have no consistent pattern of size and shape. Tandem formations of tumor cells are known as "Indian files", a phenomenon that occurs commonly in invasive breast cancer. The arrangement into rows is understandable, with the tumor cells appearing to fall into line because of the pressures from the stroma surrounding of the neoplasm. Electron microscopy can identify the intercellular connections between the various members of the rows of cells in this linear formation (Bedrossian, 1991a,b; Spriggs, 1984; Salhadin, 1976; Ashton, 1975).

The features of malignant cells in cytology include cellular and nuclear variation in size and shape, hyperchromatism, irregular chromatin clumping, abnormal chromatin distribution, nuclear molding, prominent or macro nucleoli, abnormal mitotic figures, increased nuclear:cytoplasmic (N:C) ratio, and overlapping and crowding of nuclei. Such features must be carefully observed for diagnosis (Feldman, 1985). The malignant features in breast cytology are summarized in table I-7.

Table I-7. Common cellular features of breast cancer

High cellularity
Lack of cohesion of cellular groups
Presence of isolated intact single cells
Cellular monomorphism
Variation in nuclear size from cell to cell (anisonucleosis)
Nuclear membrane infolding
Chromatin Clumping and radial dispersion of chromatin
Prominent nucleoli or macronucleoli

(Wilkinson, 1991).

4.2.3 Grading

In recent years, cytological grading of breast carcinoma specimens has been well described. Early on, a modified histological scoring grade was used in cytological

grading, but it failed to become an independent prognostic indicator after a median follow up study of 8 years (de Graaf, 1994). The grading which may be satisfactorily substituted for the histological grade, therefore, is a combination of fine needle aspiration sampleing (FNA) and mammography; it can provide information on tumor type, grade, and size before surgery (Robinson, 1994). Furthermore, the proliferating cell nuclear antigen (PCNA), a proliferation marker, detected by the PC 10 monoclonal antibody (MAb), can also be used as an indicator for cytological grading in determining prognosis (Betta, 1993).

4.2.4 Other Techniques

Recently, newer techniques have been reported with fine needle aspiration samples of breast cancer. Transmission electron microscopy as well as scanning electron microscopy, has been used. Immunocytochemical staining for keratin, estrogen receptors (ER), progesterone receptor(PR), carcinoembryonic antigen (CEA), *c-erbB2*, human milk-fat globules (HMFG) have been successfully applied in stereotictic fine needle aspiration sampling (SFNA) of breast cancer (Redard, 1989; Corkill, 1994; Bedrossian, 1991b). Immunofluorescence and immunoperoxidase techniques are also often used to detect steroid hormone receptors (Wilkinson, 1991). In addition, flow cytometric DNA analysis, and determination of thymidine labeling indices and morphometric analysis are also now common (Barbieri, 1994; Andoh, 1991). Most recently, oncogene investigations on FNA samples have been carried out, using *c-erbB2* oncogene amplification (Barbieri, 1994).

4.3.0 Immunohistochemical Pathology

Immunocytochemical pathology is the application of immunological principles and techniques to the study of the microscopic structure of diseased tissue and cells (Smith, 1993). The principle of immunohistochemical reactions is the specific recognition by an antibody of an antigen that is present, and is chemically fixed, in tissue sections or a cell suspension, followed by the visualization of the antibody (DeLellis, 1979).

Classical histopathology, using chemical stains for the visualization of the cellular and tissue structure usually cannot differentiate among specific antigenic components of breast cancer. Immunocytochemical histopathology on the other hard led to a great expansion in the range of substances identified as being relevant to diseases, and provides a higher level of specificity. For example, with immunocytochemistry it is often possible to delineate the internal architecture of the cell "ball" described above found in cytology samples.

4.3.1 Features of Immunohistochemistry of the Mammary Tissue

Immunocytochemical features of the normal breast unit have been described. The epithelial cells display a strongly positive reaction for various cytokeratins. Positivity for alpha-lactalbumin is evident in the epithelial cells during secretory activity, while this trait is much less strong and uniform in resting epithelial cells. Myoepithelial cells show a strongly positive reaction for actin and a variable degree of positivity for S-100 protein and cytokeratins. A positive reaction for actin indicates a myoepithelial cell type in the mammary duct system. The basal lamina shows positive reactions for laminin and type IV collagen (Berg, 1984; Owings, 1990).

4.3.2 The Immunomarkers Used on Breast Cancer Tissues

Monoclonal antibodies have been generated against (1) human milk-fat globule membranes, (2) human mammary tumor cell lines, and (3) extracts of human breast tumors. Recently, immunocytochemical serological and *in situ* stains for hormone receptors and tumor markers have been the most frequently used probes in either routine diagnostic or laboratory investigations of breast carcinoma (Kufe, 1988).

Estrogen (ER) and progesterone receptor (PR) immunochemical staining are used as indicators for classification, prognosis, and to provide information for the treatment of breast cancer. A breast cancer with a positive stain for ER and PR responds better for endocrine therapy, prognosis and survival rate than does a negative tumor (Roulston, 1993a). There are very many immunocytochemical markers that have been used on breast cancer for histological and cytological evaluations. Most of them are lactation-related antigens. They are listed in the table I-8.

Table I-8. Some immunohistochemical markers used to define breast cancer

Carcinoembryonic antigen (CEA)
Ferritin
Human chorionic gonadotropin (HCG)
Polyamines (putrescine, spermidine, spermine)
Hydroxyproline
Monoclonal antibodies
Tissue polypeptide antigens
Calcitonin
Casein
Alpha-Lactalbumin
Sialyl transferase, galactosyl transferase
Ceruloplasmin
Alkaline phosphatase
r-Glutamyl transpeptidase
Erythrocyte sedimentation rate
Pregnancy-associated alpha-glycoprotein (PAM)
Oncogene product
Tumor associated gene product

(Veach 1987; Klavins 1985)

4.3.3 The Immunomarkers of Specific Interest

The specific immune tumor markers of interest to this present study are p53 tumor suppressor protein, *c-erbB2* oncoprotein, and *Blc*-2 oncoprotein, and they have been selected for our immunocytochemical investigation. Comparison of these markers by immunohistochemical stain has not yet been reported in breast cancer, including for the disease in Asian countries. Neither have p53 gene point mutations and *c-erbB2* gene amplification of breast cancer patients ever been sought in any study on Chinese patients.

4.4.0 Molecular Pathology

Molecular pathology is the study at a molecular level of the causative agents and mechanisms underlying disease by molecular methods (McGee, 1992). It focuses on the DNA, RNA, and expressed proteins present in the tissues and cells, and has been used to analyze the causative agents and mechanisms in intact cells by relating phenotypes to genotypes. Most techniques used for this approach employ immunocytochemistry on both light and electron microscopic preparations. Some techniques use RNA or DNA probes for *in situ* hybridization, estimation of the DNA of index, karyotyping, and *in situ* hybridization to detect chromosomal aberrations by cytogenetics. Flow cytometry to measure DNA content is now incorporated into many experimental studies and practical diagnostic pathology. Primed *in situ* labeling, or PRINS, is used for determine the distribution of high copy number repeats within human chromosomes, and for the localization of target DNA and RNA within cells (Mackenzie, 1994).

4.4.1 *In situ* Hybridization

In situ hybridization has become a valuable tool in the world of molecular studies

on nucleic acid (DNA and RNA). The principle behind *in situ* hybridization is the specific annealing of a labeled RNA or DNA probe to complementary sequences in genes of chromosomes, in cell preparations or in fixed tissues, followed by visualization of the specific location of the probe (Lipshaw, 1995; Wilkinson, 1992). With the *in situ* hybridization technique, the nucleic acids are held within the perfectly preserved structure of the sample. This technique can be used to locate DNA sequences in chromosomes, or to detect RNA or DNA in tissues (Lux, 1990; Van den Berg, 1989). Pathologists can visualize these targets at the molecular level, and this technique has opened up a new approach for studying many diseases and their pathogenesis.

In situ hybridization can be used with light microscopy and also at the electron microscopic level (Binder, 1986). Furthermore, it and immunochemistry can be viewed as complementary techniques with the interpretation of immunochemistry findings can often be aided by *in situ* hybridization (Pringle, 1993, 1990; Ruprai, 1991).

4.4.2 *In situ* Hybridization in Malignant Diseases

The *in situ* hybridization technique has been used to study malignant tumors, permitting detection of uncontrolled cellular proliferation, terminal differentiation and excessive gene transcription. Oncogenesis is a complex process which is reflected in the fact that most malignancies probably arise from a combination of abnormalities in both proto-oncogenes and tumor-suppressor genes via a multi-step process. Extraction methods for DNA/RNA analysis and *in situ* analysis of malignancy should be viewed as being complementary to immunohistochemistry (Bishop, 1991; Fleming, 1994).

In situ hybridization has been used to investigate human proto-oncogene

expression in both non-malignant tissue and malignant tumor tissues; the products of oncogenes can be detected and localized in tissues. Examples include: c-*sis* (PDGF-2) mRNA which is overexpressed in the lung epithelial cells (Antoniades, 1990); oncogene WT1 detected in Wilms' tumor (Pritchard-Jones, 1991); N-*myc* in neuroblastoma, *c-myc* in non-Hodgkin's lymphoma, and *ras* family genes (H*a-ras*, K*i-ras*, N-*ras*) in colon, lung, pancreas and thyroid tumors (Basset-Seguin, 1991; Bishop,1991; Fleming, 1994).

C-erbB2 mRNA has been detected by ^{35}S-labelled probe in a poorly differentiated infiltrating ductal breast carcinoma. *c-myc* gene and its product have been compared with an immunolabelling and *in situ* hybridization in breast cancer (Walker, 1989) and found to have gene ampilification mutation, limited to invasive duct carcinoma type, and positivity correlated with shorter disease -free survial time; but not related to age at diagnosis, tumor grade or estrogen receptor status of the tumor (Rosen, 1995b). Similar data were also obtained for mRNA of *fms*, a growth factor and its receptor related gene (Le Roy, 1991; Fleming, 1994).

4.4.3 *in situ* Hybridization on Cytology Samples

Theoretically, any cytological specimen can be analyzed by *in situ* hybridization. In practice, the technique has not been widely applied because of difficulties in the methodology; most experience has been obtained from its use in viral detection. As yet *in situ* hybridization on cytology samples from breast cancer cases has not yet been reported, although, there are techniques for cultured cells which could be adapted for use on such cytologic specimens (Gan, 1994; Lewis, 1992; Yap, 1992).

4.4.4 Flow Cytometry on Breast Cancer Cells

Flow cytometry, especially DNA flow cytometry, is often performed for routine clinical application and provides additional information for breast cancer patient management.

The basic principle of flow cytometry is the use of particular wavelengths to excite fluorescent dyes attached to phenotype markers which are detected by a focused light beam (generally a laser), resulting in separation of the various specifically identified components of a single cell suspension. A wide variety of cellular constituents can be measured by flow cytometry, such as DNA, RNA total protein content, and the presence of specific proteins (by using monoclonal antibodies). Furthermore, intracellular pH, calcium transport, and enzyme kinetics can be measured by the flow cytometry technique (Melamed, 1990). Many diagnostic and research flow cytometric studies have been performed on breast cancer. Using DNA histograms, flow cytometry can show clearly defined G_1, S and G_2/M phases of the cell cycle: these are now routinely used on breast cancer samples. DNA aneuploid cells with a high S-phase fraction can be found in malignant breast tumors: DNA aneuploidy has been shown to be more common in high-grade tumors in virtually all published studies. Correlation of ploidy with other clinicopathological variables such as tumor size, nodal status and steroid receptor presence has yet be established (Merkel, 1990; Camplejohn, 1992; Camplejohn, 1993 a,b).

This technique can be performed on fresh cell biopsy material, fresh solid tumors, and on paraffin-embedded archival material. It is therefore a popular routine method used with breast cancer needle biopsy studies. The results of DNA flow cytometry are critical

to the care of the majority of breast cancer patients, and serve as a very useful adjunct for pathological studies and basic research (Levack, 1987; Vindelov, 1990; Camplejohn, 1992).

4.4.5 Polymerase Chain Reaction (PCR) on Cancer Studies

PCR has proven to be a valuable tool applicable to a number of research and diagnostic areas. PCR is the in vitro enzymatic replication of DNA by a repetitive cyclical process that results in approximately a millinon fold amplification of a specific DNA sequence (Spadoro, 1993).

PCR applications in cancer research include: 1) Detection of tumor viruses; 2) Detection of immunoglobulin (Ig) or TCR gene rearrangements; 3) Detection of chromosomal translocation in hematological malignancies; 4) Detection of point mutations, 5) Detection of tumor-suppressor genes, and 6) Detection of amplified oncogenes.

A variety of sample types can be used for PCR studies and mutation analysis. Venous blood, biopsy samples, cultured cells, fixed tissue specimens, dried blood spots and mouthwash samples are all suitable for PCR analysis.

PCR has been used successfully in breast carcinoma to study *c-erbB2* oncogene amplification and p53 gene point mutations. It is also a well established method in the study of the genetics of breast cancer.

4.4.6 PCR *in situ* Hybridization

PCR *in situ* hybridization combines the techniques of nucleic acid amplification and *in situ* hybridization together to increase the copy number of specific sequences for

visialization within single cell. The amplified sequence product can be either detected by detector markers as used with *in situ* hybridization or by immunohistochemistry on histological specimens presently (Komminoth, 1995; Staecker, 1994).

In situ gene amplification theoretically can be used with any DNA analysis together with appropriate sample fixation. By using reverse transcriptase *in situ*, gene amplification has successfully allowed the amplification of mRNA targets (Chen, 1993). This will be a most useful when other techniques, such as histochemistry, immunohistochemistry or *in situ* hybridization, fail to answer a scientific or diagnostic question. It allows the direct correlation of molecular analysis with cytopathology and histopathology.

PCR *in situ* has been particularly useful for detecting human papilloma virus, human immunodeficiency virus-1, and cytomegalovirus (CMV). Recently, the technique has been extended to detect nerve growth factor, T cell cytotoxic marker expression, CMV, lentivirus, EGF receptor, tumor necrosis factor, hepatitis C cDNA, and immunoglobulin gene rearrangement. Many different types of specimens, such as blood cells and formalin fixed/paraffin embedded tissue sections, can be evaluated by this procedure (Nunovo, 1994; Komminoth, 1995). However, PCR *in situ* is still essentially a research technique that is difficult to perform and has many variables. Its relatively low sensitivity when compared with other methodologies remains a challenge. Tissues fixed in solutions that contain picric acid or heavy metals such as mercury are generally not acceptable for either PCR or *in situ* hybridization, and consequently these fixatives will not support PCR *in situ* hybridization (Nunovo, 1994).

4.4.7 Other Molecular Techniques Used with Breast Cancer

Thymidine labeling index (TLI), is also utilized for breast cancer diagnosis and study. This technique determines the percent of cells within the tumor that are in S-phase; the median TLI for breast cancer is approximately 5%; the results directly relating to the histological grade. In general, low grade carcinoma such as mucinous and adenoid cystic carcinomas, have a low TLI. Carcinomas with high TLIs have the characteristics of growing rapidly, and recur relatively quickly (Tubiana, 1981; Tavassoli, 1992).

Karyotypic analysis of interphase and metaphase chromosomes from human cancer has been able to reveal interstitial deletions in several chromosomal arms. Specific information on chromosome number and structural abnormalities can be produced with all chromosomes being viewed simultaneously. However, this analysis cannot detect the large majority of the small gene deletions, and is also dependent on the culturing of the cells; it may also select for unrepresentative subpopulations (Waters, 1994; Fleming, 1994). In breast cancer, loss of heterozygosity on chromosome 17p (the location of p53 gene) has been reported (Fleming, 1994).

Primed *in situ* labeling (PRINS), and Cycling PRINS are rapid alternative methods to fluorescence *in situ* hybridization for the localization of DNA and mRNA sequences in chromosomes. They are used in the detection of unique sequences within short, synthesized oligonucleotides without the need for genomic cloning. These techniques have been reported from the human genome mapping project, and are useful in chromosome and PCR product band mapping, and for research into the location of alpha satellite DNA and RNA. Theoretically, these types of analytic techniques can be used in

many areas of breast cancer molecular evaluation (Miller, 1995).

5.0.0 Involvement of Genes in Oncogenesis

Cancer as a disease is characterized by an autonomous proliferation of neoplastic cells that are genetically dysfunctional. All cellular functions are controlled by proteins, and because these proteins are encoded by DNA organized into genes, molecular studies have shown that cancer is a paradigm of acquired genetic disease. Carcinogenic agents are known to mutate three types of genes that regulate cell growth: proto-oncogenes, tumor suppressor genes and programmed cell death regulator genes (Weinberg, 1991).

The existence of mutant proto-oncogenes which code for the growth factor related proteins has been demonstrated in human tumors (Weinberg, 1994). A change in the sequence structure of a gene has been pinpointed as being responsible for converting a proto-oncogene into an active oncogene. In addition, understanding that normal cells regulate their growth provided a possible explanation for the mechanism of non-oncogenes action in oncogenesis (Weinberg,1994). A number of tumor-suppressor genes have been discovered over the past five years (Weinberg, 1991; Weinberg, 1994), and their role in tumorgenesis appears to be as important as that of oncogenes.

Roughly 10 percent of breast cancers are due to a genetic predisposition and two breast cancer genes have been identified, BRCA1and BRCA2. They showed up in about 80% of germline breast cancer, but only in less than 5% of general breast cancer cases (Miki, 1994; Lerman, 1995; Szabo, 1995; Neuhausen, 1996).

5.1.0 p53 tumor Suppressor Gene and Product

5.1.1 Background

The p53 tumor suppressor protein was first identified in 1979 as a cellular protein that coimmunoprecipitated with the transforming protein of Siman Virus 40(SV40), the large T antigen in SV40 transformed cells. In the early experiments, p53 was thought to be a tumor antigen, because the large T antigen is needed to maintain the transformed phenotype, and p53 coprecipitated with the large T antigen during purification from SV40 (Lane,1979). Later, it was found in SV40 transformed cells and tumor derived cell lines from both human and nonhuman tumors that p53 was present in much higher levels in tumor than the normal cells (Oren, 1981).

5.1.2 Structure and Function

The p53 gene encompasses a 6 to 20 kilo base (Kb) of DNA on the short arm of human chromosome 17 at position 17p13.1. This gene is composed of 11 exons (Mcbride 1986, Miller 1986). In a cross-species comparison, the p53 protein is seen to have five highly (>90%) conserved regions within the amino acid residues 13-19, 117-142, 171-181, 234-258, and 270-286 (Soussi, 1990). These five evolutionarily conserved domains within the coding regions are regarded as essential to the function of the p53 (Pavletich, 1993).

The product of the p53 gene is a 393-amino acid, 53-Kda nuclear phosphoprotein (Soussi, 1990), containing an acidic amino terminal (amino acids 1-75) possessing the transcriptional activation region followed by a central, extended hydrophobic proline rich domain (amino acids 75-150) and a highly charged basic carboxy terminal domain (amino

acids 275-390) containing nuclear localization signals (Shaulsky, 1990; Addison, 1990), as well as the oligomerization (Milner, 1991) and DNA biding domains (Raycroft, 1991). Oligomerization refers to multiple binding sites on the protein molecules which results in formation of protein complexes (Milner, 1991). The central region is the highly conserved portion of the protein, and the majority of oncogenic mutations are located here (Soussi, 1990).

Wild type p53 is virtually undetectable in normal cells as it is known to be expressed at only low levels and has a short half-life (20-30 minutes); it is generally located in the nucleus (Oren, 1981). In serum stimulated, nontransformed Balb/c 3T3 cells, the cellular localization of the p53 protein was seen to vary during the cell cycle. This protein is found in cytoplasm during the G1 phase, then enters the nucleus during the G1/S transition where it remains until the end of the G2/M phase. After DNA synthesis, it is again found in the cytoplasm. In tumour or transformed cells, the location of p53 protein is still generally unclear but in certain cases p53 protein has been found in cytoplasm (Shaulsky, 1990).

p53 tumor suppressor protein appears to have multiple functions that include regulation of DNA synthesis (Lane, 1979, Gannon, 1987, Friedman, 1990), repair (Nelson, 1994) and programmed cell death regulation (Nelson,1994, Lane, 1993, Rand, 1996), as well as acting as a transactivator of gene expression (Vogelstein, 1992). Loss of normal p53 function predisposes cells to multiple deleterious effects. Inactivation of p53 protein can occur through mutation (Levine, 1991; Soussi, 1994; Chang, 1993), protein complex formation with viral oncoproteins (Mietz, 1992; Yew, 1992), binding to

cellular gene products (Harris, 1993; Momand, 1992; Leach 1993), and dislocation of p53 between the cell compartments (Moll, 1992). Allelic deletions (Rand, 1996; Levine, 1991; Soussi, 1994; Chang, 1993), structural rearrangements (Levine, 1991; Soussi, 1994; Chang, 1993), missionse mutations (Levine, 1991; Soussi, 1994; Chang, 1993) and germline mutations (Malkin, 1993; Santibanez-Koref, 1991; Kleihues, 1997) have all been reported for the p53 gene.

5.1.3 Oncogenic Features

There are mutational "hot spots" in p53 at amino acids 175, 248-249, 273, and 282 (Soussi, 1990); mutations at these amino acids account for approximately 30% of the total missense mutations in human tumors (Finlay, 1993). The codon 248 is also a hot spot for germline mutations (Birch, 1994). Interestingly, different "hot spots" are present in different tissues: for example, mutations at amino acid 175 have been found repeatedly in colorectal carcinomas (Baker, 1990; Rand, 1996), in lymphoma (Diller, 1990) and breast cancer (Prosser, 1991), glioblastoma (Nigro, 1989), and an esophageal squamous cell carcinoma (Hollstein, 1991a). The mutation at 175 has not yet been observed in lung carcinomas (Finlay, 1993).

Recent laboratory data suggest that specific carcinogens can cause point mutations in the p53 gene and have their specific features; for example, exposure to ultraviolet (UV) light is correlated with transition mutations at dipyrimidine sites (CC to TT double base change mutation) (Brash, 1991; Kress, 1992). Mutations resulting from G:C to T:A transversion that occur commonly in hepatocellular carcinomas, are a result of exposure to Aflatoxin (Aguilar, 1993; Bressac, 1991). Exposure to cigarette smoke is correlated with

G:C to T:A transversions in lung, esophageal, and head/neck carcinomas (Miller, 1992; Takeshima, 1993). In addition, endogenous mutagenesis can have the same effect; for example the high frequency of C to T transitions at CpG dinucleotides in colon carcinomas is consistent with an endogenous mutational mechanism due to deamination of the 5-methylcytosine residue found at CpG dinucleotides in the mammalian genome (Lehman, 1994). Thus analysis of p53 mutations may provide clues to the etiology and pathogenesis of human cancers.

The phenotypic alterations in p53 gene mutation patterns are different in different races. For example, Asians tend to have more frequent G:C to A:T transitions at the CpG site (Sasa, 1993), Blacks an A:T to G:C transition (Blaszyk, 1994), and Caucasians have G:C to A:T transversions, and rarely at CpG site (Fukushima, 1995).

5.1.4 Clinical Approaches

The p53 tumor suppressor protein was first associated with human mammary neoplasia in 1982 and it was found that 90% of patients with breast cancer, particularly those cases with visceral metastases, had circulating antibodies to human p53 protein (Crawford, 1982). An hypothesis was developed that "p53 is altered in amount, and type in breast tumors". The accumulation of p53 protein in breast carcinoma was found to be associated with point mutations within highly conserved regions of the p53 gene (Hollstein, 1991). These altered genes encode stable proteins detectable by standard immunohistochemical techniques that can detect both wild and mutant form of the p53 protein. In addition, a significant association between high levels of p53 in late stages of the disease with metastatic tumor spread was found (Humphrey, 1994; Isola, 1992).

Results of these and other studies, clearly indicate that immunohistologic expression of p53 is associated with a clinically advanced and highly aggressive forms of breast cancers (Elledge, 1993; Mazars, 1992; Iwaya, 1991).

Li-Fraumeni syndrome is a rare autosomal dominant syndrome. The disorder is characterized by diverse neoplasms at multiple sites, including breast carcinoma, adrencocortical carcinoma, soft tissue sarcoma, brain tumors, osteosarcoma, leukemia, and possibly other tumors. Tumors develop in the family members at unusually early ages, and multiple primary tumors are frequent. Members of these families carry one mutant p53 allele and one wild type allele (Malkin, 1993; Santibanez-Koref, 1991). The wild-type allele present in the normal tissues of these individuals is generally deleted in the tumor, while the mutant allele is retained. It is therefore thought that Li-Fraumeni syndrome patients are predisposed to cancer because one p53 allele is inactivated in the germline and only the remaining allele is altered by somatic mutation (Malkin, 1993; Santibanez-Koref, 1991).

p53 gene mutations and the mutant protein are frequently found on human cancers including breast cancer. It has been shown that the p53 gene is a tumor suppressor gene and that is a frequent target for inactivation in many types of tumors (Finlay, 1989; Raycroft, 1990; Ginsberg, 1991; Kern, 1992). These p53 gene mutations occur on the short arm of chromosome 17 and the point mutations frequently occur in association with neoplastic transformation in human tissues (Harris, 1990). It is believed that mutations of the p53 gene play an important role in the development of many human malignancies (Nigro, 1989; Hollstein, 1991b; Coles, 1990). Normal p53 protein has a very short half-

life and thus the protein level is too low to be identified immunohistochemically. However, in transformed cells or malignantic cells were found p53 was 100-fold higher concentration than that in normal cells, and the half-life was increased to over 6 hours instead 20-30 min, and thus can be detected by immunohistochemical methods (Finlay, 1988; Runnebaum, 1991).

Only limited data relating p53 expression to the clinical features of mammary carcinoma exists in the literature. Several investigators have failed to detect a significant correlation with age at diagnosis or menstrual status (Isola, 1992; Rosen, 1995a; Thompson, 1992; Barbareschi, 1992; Silvestrini, 1993). Clinically, mutated p53 protein appears in breast cancer tissues when it is of a poor differentiated carcinoma type. p53 has been used as a biologic gene therapy (Roth, 1996) and could be used to generate new clinically active agents (Weinstein, 1997).

5.2.0 HER/*neu* (*c-erbB-2*) Proto-oncogene and Product

5.2.1 Background

HER (c-*erbB-2* or *neu*) is an proto-oncogene. The name *neu* was defined in 1974, when female BDIX rats were injected with the chemical carcinogen N-ethyl-N-nitrosourea at 4 to10 months after birth: 93% of these animals developed central nervous system tumors such as neuroblastomas or glioblastomas (Schubert, 1974). The tumor cells were cloned and the DNA extracted from these cell lines was able to induce transformation of mouse embryo fibroblast NIH3T3 cells (Shih, 1981); they induced tumors (fibrosarcomes) when injected into nude mice (Padhy, 1982). The DNA acted as a potential transforming oncogene, and was responsible for the original induced rat

neuroblastomas during 1974 to 1982 (Schubert, 1974; Shih, 1981; Padhy, 1982). Then, another group researchers found the *neu* gene to be homologous to the avian erythroblastosis virus (AEV) transforming gene *v-erbB* (Schechter, 1984). Later, the human homologue of the rat *neu* gene was also cloned independently and named *c-erbB-2* or HER-2, or MAC117, or NGL by several laboratories (Coussens, 1985; Yamamoto, 1986; King, 1985; Semba, 1985).

5.2.2 Structure and Function

Human HER-2/*neu* gene was mapped to chromosome 17q21 (Coussens, 1985; Fukushige, 1986). It contains an open reading frame of 3765 nucleotides and has major transcript of 4.8 kb (Coussens, 1985; Fukushige, 1986; Semba, 1985), which translates into a 1255 amino acid polypeptide (Coussens, 1985; Yamamoto, 1986; King, 1985). The functional gene product of the HER-2/*neu* gene was termed p185 in accordance with its molecular weight (185,00 Daltons), while the primary translation product has a relative molecular mass (Mr) of 137,895 daltons (Padhy, 1982; Drebin, 1984). This difference in apparent molecular masses is due in part to N-linked glycosylation and probably O-linked oligosaccharides as well (Stern, 1988a). Phosphorylation has also been found to be involved in the modification of HER-2/*neu*-encoded protein.

The HER/*neu* gene sequence and its protein product are closely related to those associated with epidermal growth factor receptor (EGF-R) with show an overall homology of 50% (Schechter, 1985; Yamamoto, 1986; Bargmann, 1986). Similar to EGF-R, *neu* encoded p185 is a transmembrane glycoprotein having intrinsic tyrosine kinase activity (Coussens, 1985; Stern, 1986; Akiyama, 1986), and can be grouped as a

member of the growth factor receptor tyrosine kinase (RTK) gene family (Yarden, 1988). The p185 consists of the following domains: 1) 16 of the first 19 amino acids following the first ATG codon are hydrophobic residues, thus representing a cleaved signal peptide sequence; 2) 640 residues constitute the extracellular ligand-binding domain, containing two cysteine-rich regions that may be important for ligand binding; 3) a second hydrophobic stretch from residues 650 to 680 suggests a transmembrane domain; and 4) the remaining carboxy-terminal 580 amino acids constitute the intracellular/cytoplasmic domain (Coussens, 1985; Yamamoto, 1986; Bargmann, 1986).

The extracellular domain of p185 shows 44% homology with the ligand-binding domain of the EGF-R. Within the ligand-binding domain are two cysteine-rich regions that are completely conserved between these two *erbB* related proteins. The cytoplasmic component of the protein encompasses residues 727-986 and contains the tyrosine kinase domain with an ATP binding site. Some important structures in this region that are identical between the two proteins include threonine (Thr) 954, which is the site of phosphorylation mediated by protein kinase C (PKC), and several tyrosine residues (Y1023, Y1248, Y1139,Y1222) for autophosphorylation (Hazan, 1990). All of the autophosphorylation sites of *neu* reside within the carboxy-terminal tail (Margolis, 1989).

5.2.3 Oncogenic Features

In the rat, *c-neu* proto-oncogene has a single point mutation of T to A at nucleotide position 2012 that results in the proto-oncogene being activated to the *neu* oncogene (Bargmann, 1988). This point mutation results in a change at amino acid residue 664 from valine (Val) to glutaminc acid (Glu) in the transmembrane domain of the

protein product and was shown to activate the intrinsic tyrosine kinase activity (Bargmann, 1986; Stern, 1988 a ; Barmann, 1988 a, b). In humans, amino acid 664 mutation is a "hot spot" for activation of increasing tyrosine kinase activity in vitro (Stern,1988b; Weiner, 1989).

The human HER/*neu* gene was originally isolated as an amplified *v-erbB* related sequence in human mammary carcinoma, salivary gland adenocarcinoma, and gastric cancer cell lines (Yamamoto, 1986; King, 1985). This gene is amplified or overexpressed in many different human primary tumors (Tal, 1988). Abnormality of the HER/*neu* proto-oncogene has been studied most frequently in human breast cancer with the HER/*neu* gene found to be amplified or rearranged in many primary breast cancers and in breast cancer cell lines (Kraus, 1987; Slamon, 1989; van de Vijver, 1987). One of the early reports described a 2 to 20 fold amplification of the HER-2/*neu* gene in about 30% of primary human breast cancer in a 189 cases study. In addition there is a significant correlation between the level of HER/*neu* gene amplification fold and its protein overexpression (Slamon,1987).

5.2.4 Clinical Approaches

HER/*neu* gene has been reported to be amplified or overexpressed many different cancers including breast cancer, ovarian cancer (Slamon, 1989; Zhang, 1989; Berchuck, 1990), gastric tumors, (Yokota, 1988; Park, 1989), colon cancer (D'Emilia, 1989), lung cancer (Schneider, 1989; Weiner, 1990), oral cancer (Hou, 1992) and cervical cancer (Mitra, 1994). In contrast to gene amplification mutation, a very low frequency of rearrangement mutation of the HER/*neu* gene has been observed in breast cancer and

gastric carcinoma (Yokota, 1988; Park, 1989).

C-erbB2 oncoprotein is already an established marker for poor prognosis in breast cancer, and may also be predictive of response to treatment (Soomro, 1993; Allan, 1993). The HER/*neu* gene was found to correlate with the number of lymph node metastases in reoccurring breast cancers, and therefore is considered to play an important role in tumor metastasis. The correlations found from clinical data have however been insufficient to prove that HER/*neu* gene expression is responsible for aggressive breast cancer (van de Vijver, 1987; Varley, 1987; Venter, 1987; Adnane, 1989; Garcia, 1987; King, 1989). Nevertheless, HER/*neu* gene amplification or overexpression in human primary breast cancers was shown to be a powerful predictor of the risk of recurrence (Nagai, 1993).

It was reported that breast cancers showing HER-2 overexpression are less responsive to chemotherapy than those with a normal amount of the gene product (Gusterson, 1992). However, the $p185^{neu}$ on the cell surface of HER-2 overexpressing tumor cells may be a good target for receptor-directed immunotherapies. Using the anti-human p185 monoclonal antibody 4D5 together with chemotherapy may increase the chemosensitivity of the p185 overexpressing breast cancer cells to Taxol and Taxotere (Cole, 1992; Moscow, 1988). Gene therapy has been studied by different groups, but more study is needed before this therapy becomes beneficial to the cancer patients (Hung, 1995; Wels, 1995; Dhingra, 1995).

5.3.0 Bcl-2 cell death regulate gene (proto-oncogene) and product

5.3.1 Background

In 1984, while studying the t (14:18) chromosome translocation that occur

frequently in B cell leukemia and non-Hodgkin's follicular lymphoma, a gene, Bcl-2, was isolated from the breakpoint of the translocation between chromosomes 14 and 18; it was found to be present in a high proportion of the most common human lymphomas, the follicular B cell lymphomas (Yunis, 1982; Tsujimoto, 1984). In 1988, it was shown that introduction of the gene into interleukin-3-dependent myeloid and lymphoid cell lines promoted survival of these cells after withdrawal of interleukin-3, but did not stimulate their proliferation (Vaux, 1988). Subsequently, the gene was shown to specifically inhibit apoptosis (Hockenbery, 1990). Thus, Bcl-2 emerged as a new type of proto-oncogene, one that suppresses cell death rather than stimulating proliferation. However, it did not inhibit the apoptosis occurring in all circumstances as it fails to block apoptosis induced by cytotoxic T-lymphocytes (Vaux, 1992).

5.3.2 Structure and Function

The Bcl-2 gene was cloned as a novel gene located at chromosome 18q21 and it has a three exon structure with an untranslated first exon, a facultative 220 bp intron I, but with an enormous 370 kb intron II (Seto, 1988).

Bcl-2 is 717 nucleotides long and codes for a Bcl-2 alpha molecule of 239 amino acids with a molecular mass of 26 Kda (Tsujimoto, 1984). It is a putative membrane-associated protein containing a hydrophobic carboxyl terminus located in the inner mitochondria membrane; its lipophilic character suggests that perhaps if is a membrane-spanning protein (Cleary, 1986; Tsujimoto, 1984). Its location suggests that it is involved in some way with the metabolic functions of the inner mitochondria membrane, i.e. oxidative phosphorylation and electron transport (Hockenbery, 1990). It has been noted

that decreasing cytosolic Ca^{2+} and increasing mitochondrial Ca^{2+} are correlated with Bcl-2 overexpression suggesting that Bcl-2 may be involved in the regulation of intracellular Ca^{2+} distribution (Bafy, 1993). However, Bcl-2 protects against apoptosis even in cells without mitochondria (Jacobson, 1993). Bcl-2 has also been localized to the nuclear membrane and to the mitotic nuclei in epithelial cell lines suggesting that it may protect DNA from damage caused by nuclease activation (Lu, 1994). It is known that Bcl-2 expression can occur at any stage of the cell cycle (Fanidi, 1992; Bissonnette, 1992), but the mechanism by which Bcl-2 inhibits apoptosis is still unclear.

The function of Bcl-2 as a cell death suppressor provided the first clue that altered gene expression could enhance cell survival without affecting cell proliferation. In the Bcl-2 gene family, a group of genes with a sequence homology to Bcl-2 produce protein that share two highly conserved domains, Bcl-2 homologue 1 and 2 (BH1,BH2). They are divided functionally into two groups: 1) cell death suppressors such as Bcl-2 and Bcl-X1, and MCL-1, and 2) cell death promoters such as bax, bcl-xs, Bak and Bad cell death promotors. BH1 and BH2 domains regulate heterodimerization and regulation of cell death by members of these gene family may be achieved through competing dimerization (Cleary, 1986; Lin, 1993; Farrow, 1995; Yang, 1995).

Normal Bcl-2 expression is associated with proliferating cells and cells which have a need for longevity. Bcl-2 is often demonstrated in glandular epithelial cells where regulation of hyperplasis and involution is controlled by hormones and growth factors (breast), in complex differentiating epithelium with long-lived stem cells (skin, intestine, memory B cells) and in fully differentiated long-lived stem cells and non-cycling cells

(neurons) (Nunez, 1990).

The protein product of the Bcl-2 gene that acts to inhibit apoptosis is maximally expressed in the normal fetal breast (Nathan, 1994a) where there is immunohistochemical localization in the basal epithelium of the developing breast bud and in the surrounding stroma; similar patterns of staining have been reported in male and female tissues. Bcl-2 reactivity is lost soon after birth and it is not present in the epithelium of the normal adult breast (Nathan, 1994b). The observations suggest that up-regulation of Bcl-2 contributes to morphogenesis of the fetal breast by its inhibitory effect on apoptosis.

Bcl-2 proto-oncogene actively blocks apoptosis and is triggered by several factors and events such as withdrawal growth-factor (Nunez, 1990), wild-type p53 protein (Chiou, 1994), and X-radiation (Sentman, 1991). Roles for Bcl-2 in epithelial differentiation, morphogenesis, its response to hormone regulation, and in tumorgenesis have been reported from different groups and it seems to be a important factor in regulating complex pathways.

5.3.3 Oncogenic Features

The most common translocation of Bcl-2 was found in human lymphomas, at t(14;18) (q32;21). This translocation point is located within exon III of the Bcl-2 gene at chromosome 18q21 and at the joining regions of the gene to immunoglobulin heavy chain (IgH) loci at chromosome 14q32; this creates a Bcl-2/IgH fusion gene resulting in high levels of Bcl-2 (Seto, 1988; Hanada, 1993). Expression of Bcl-2 was thought initially to be restricted to those B cell malignancies with the translocation, but subsequent studies have reported its expression in a wide range of lympho-proliferative diseases that lack this

chromosomal abnormality as well as in normal lymphoid cell.

The mechanism by which Bcl-2 promotes tumorgenesis is unique and the gene expression confers survival advantage to a variety of cell types by inhibiting apoptosis, an important feature in many normal biological processes.

5.3.4 Clinical Approaches

The detection of positivity for the Bcl-2 gene and its product protein in neoplastic cells has revealed a wide range of manifestations and heterogeneous staining pattern throughout the tumors. Bcl-2 oncoprotein is expressed in 79% of invasive breast carcinomas, 97% of normal breast epithelium specimens, and 91% of *in situ* cancer samples. Loss of Bcl-2 expression is generally regarded as a relatively late event in the progression of the disease. In breast cancer, its expression is associated with favorable clinicopathological features. In addition, loss of Bcl-2 gene expression tends to be linked to poor prognosis (Russel, 1994). It is an unique oncoprotein and is proving to be an independent marker for prognosis in neoplastic breast disease (Leek, 1994; Vanhaesebroeck, 1993; Joensuu, 1994; Carson, 1993; Bissonnette, 1992).

One study suggests that Bcl-2 cooperates with *c-myc* in vitro to promote proliferation and in some cases inducing tumors in nude mice (Strasser, 1990). Because Bcl-2 expression prolongs the life span of cells, it increases the risk for those cells to acquire other changes, such as chromosomal abnormality and viral infection, which may result in malignant transformation or overt tumor progression (McDonnell, 1989, 1991; Nunez, 1990).

The association of Bcl-2 expression with the sensitivity of epithelial cells to drug,

radiation and hormone therapies very much depends on the type of malignancy (Lu, 1996). Its effect on therapeutic responses and hence on prognosis is not clear cut and additional studies are needed.

5.4.0 p53, Bcl-2 and HER/*neu* (*c-erbB-2*), Apoptosis and Oncogenesis

5.4.1 Apoptosis

Apoptosis is a form of cell death where single cells are deleted in the midst of living tissue. The term drives from a Greek word to describe the dropping off of leaves from trees. It is characterized by structural changes that appear with a great fidelity in cells of widely different lineage and presumably represent a pleiotropic effector response (Kerr, 1972a; Wyllie, 1980; Arends, 1991). Most or all of the programmed death is responsible for tissue modeling in cell development, for the cell loss that accompanies atrophy of adult tissues following endocrine and other stimuli, and in some tissues for the physiological death of cells that occurs in the course of normal tissue turnover. The morphological changes in apoptosis have been extensively reviewed (Kerr, 1972a; Wyllie, 1980; Arends, 1991).

Morphological evaluation shows that apoptosis characteristically affects scattered single cells, not groups of adjoining cells, such as the case of necrosis. Early apoptosis is characterized by compaction and margination of nuclear chromatin, condensation of cytoplasm, and convolution of nuclear and cell outlines; at a later stage, the nucleus fragments, and protuberances that form on the cell surface separate to produce apoptotic bodies, which are phagocytosed by nearby cells and degraded within lysosomes. In contrast, the development of necrosis is associated with irregular clumping of chromatin,

marked swolling of organelles and focal disruption of membranes. Membranes subsequently disintegrate, but the cell usually retains its overall shape until removed by mononuclear phagocytes (Wyllie, 1980; Kerr, 1987; Arends, 1991). Apoptosis can be distinguished biochemically and morphologically from necrosis by the following summary criteria: 1) chromatin condensation; 2) membrane blebbing; 3) appearance of apoptotic bodies; and 4) fragmentation of the genomic DNA (Ellis, 1991).

Apoptotic cells do not induce an inflammatory reaction even when they present in large numbers, but they are targets of immediate phagocytosis, either by macrophages already present nearby, or by other adjacent viable cells. The compacted organelles and condensed chromatin of the apoptotic cells may be visible for a few hours but eventually reduce to large nondescript lysosomal residual bodies (Kerr, 1972b; Wyllie, 1980; Arends, 1991).

5.4.2 Apoptosis and Oncogenesis

An increase in cell number is one of the most prominent characteristics of a tumor. It may be caused by either an increase in cell proliferation or a decrease in cell death, or both. Previous studies of oncogenesis have focused predominantly on abnormalities of cell proliferation but recently it has been recognized that abnormalities in the control of cell death and survival are of equal significance (Huebner, 1969; Bishop, 1983; Oren, 1985; Lee, 1991).

The fact that apoptosis occurs in tumors is not new. It can be found in virtually all untreated malignant tumors (Searle, 1973; Wyllie, 1985; Harmon, 1987). More than 20 years ago it was suggested that apoptosis may account for much of the spontaneous

cell loss known from kinetic studies to occur in many tumors (Kerr, 1972 a, b). It has been clear for some time that abnormal apoptosis is enhanced in tumors by common treatments such as, irradiation (Kerr, 1980; Stephens, 1991; Macklis, 1992), cytotoxic chemotherapy (Searle, 1975; Dive, 1991; Gunji, 1991; Cotter, 1992; Johnston, 1992), heat (Dyson, 1986; Barry, 1990; Harmon, 1990, 1991), and hormone ablation (Szende, 1990; Kyprianou, 1991; Redding, 1992). The genetic analysis of programmed cell death during the development of the nematode Caenorhabditis elegans has proved very useful in identifying important events in the cell death program (Hale, 1996). Advances in understanding of the control of apoptosis at the molecular level have potential oncologic significance far for beyond the mere provision of a mechanistic explanation for tumor cell deletion (Marx, 1993; Wyllie, 1987).

5.4.3 p53, Bcl-2 and HER/*neu* (*c-erbB-2*) Involvement in Apoptosis and Oncogenesis

It has been suggested that exposure to environmental DNA damaging agents contributes to the development of the vast majority of human tumors (Doll, 1981). Therefore, an understanding of the molecular events involved in the cellular responses to such exposures should provide insights into mechanisms of human carcinogenesis. There are many interrelating effector events in apoptosis and the malignant phenotype, p53, HER/*neu* (*c-erbB-2*) and Bcl-2 mutations in breast cancer have attracted the great attention (Cope, 1991) to those studying this phenomenon.

The p53 tumour suppressor gene is involved on genetic regulation of apoptosis. It acts as a direct "apoptogene", a gene that causes apoptosis (Carson, 1993), and it has

been suggested that the product of the p53 gene acts as a "molecular policeman" monitoring the integrity of the genome. If a cell's DNA is damaged, p53 product accumulates through a post-translational stabilization mechanism and arrests the cell cycle at G1 to allow extra time for repair. If repair fails, p53 may trigger deletion of the cell by apoptosis (Lane, 1992). To what extent p53 is involved in regulating apoptosis under normal conditions is unknown (Donehower, 1992). A major mechanism whereby abnormalities of p53 contribute to the development and progression of tumors may be the abrogation of the normal pathway that leads to the self-destruction of mutant cells (Lane, 1993). Mutation in p53 confer resistance to apoptosis (Yonisch-Rouach, 1991; Shaw, 1992).

Bcl-2 is unique among oncogenes because it exerts its oncogenic effect via the inhibition of apoptosis and not via enhanced cell cycle progression. Bcl-2 is normally expressed in tissues characterized by extended viability or self-renewal that involves the process of apoptosis (Hockenbery, 1991). In many malignancies, Bcl-2 overexpression confers an inhibition of programmed cell death upon malignant cells, even in the absence of gene arrangements (Pezzella, 1990). Bcl-2 has been shown to inhibit apoptosis triggered by wild-type p53, and an inverse correlation between Bcl-2 expression and p53 mutation has been observed in breast cancer and other malignant diseases (Tomita, 1996).

The loss of function of p53 in cells may effect the function of Bcl-2. Tumor cell numbers would increase as a result of both unregulated proliferation and resistance to cell death. The study has suggested Bcl-2 oncoprotein expression is frequently associated with an overexpression of p53 (Siziopikou, 1996). Loss of the p53 tumour suppressor

gene or overexpression of the apoptosis inhibitor protein Bcl-2, has been reported in oncogenically transformed cells that have lost their apoptotic potential (Graeber, 1996). p53 was also found to down regulate Bcl-2 and to regulate the Bcl-2/*Bax* balance. Bax is one of member in Bcl-2 family which acts as a apoptosis promotor (Oltvai, 1993; Krajewski, 1994); wild-type p53 function may be required for the optimal expression of Bcl-2 since p53 mutation in some cell types results in a marked reduction of the Bax and an increase of Bcl-2 expression (Haldar, 1994; Lohmann, 1993).

HER/*neu* (*c-erbB-2*) is known as an EGFR proto-oncogene, playing an important role in the prognosis, and in transformation of neoplasms (Lupu, 1996); its involvement in apoptosis is thought to be limited. One report, however, demonstrated that *erbB-2* overexpression in ovarian tumor cell lines transfected with an endoplasmic reticulum form of an anti-*erB-2* single-chain antibody undergo specific cytotoxicity through the induction of apoptosis (Grim, 1996). Another study using anti-HER2 monoclonal antibodies induced tyrosine phosphorylation of HER proteins, causing cell morphology changes and apoptosis (Kita, 1996).

Most of the studies on the mechanisms of apoptosis have been in *vitro* with only limited in *vivo* studies. Overexpression of those entities in breast cancer has been demonstrated with the finding that Bcl-2 expression is negatively associated with overexpression *c-erbB-2* and p53 protein expressions in breast by immunohistochemical methods (Binder, 1996; Ceccarelli, 1995; Nathan 1994a,b); however, it is strongly associated with steroid hormone receptors. Bcl-2 negative is associated with estrogen growth factor receptor (EGFR) positivity (Gee, 1994; Leek, 1994). p53, *c-erbB-2* and

Bcl-2 are rarely co-expressed at any stage of breast cancer (Cohen, 1995; Reson, 1996a). Combined studies of Bcl-2, p53, and *c-erbB-2* are hard to find.

6.0.0 Breast Cancer Immunohistochemical and Molecular Studies in China

Breast cancer in China is not a major malignant disease. However Chinese researchers have made a number of basic and clinical investigations. The epidemiology, histopathology, immunocytochemistry and molecular biology of breast cancer in China have been discussed separately (Li, 1994). ER and PR are determined routinely in the big hospitals, and the diagnostic value of various tumor markers in breast cancer has been reported (Shi, 1988; Yiao, 1991; Song, 1993; Gong, 1993).

There are also studies from mainland China on oncogenes, tumor-suppressor genes and their products, but the most them were on the other kinds of cancer. For example, oncogene product overexpression, metastasis-suppressor gene and metastatic cell markers were all studied by immunocytochemical methods and *in situ* hybridizations with *myc*, *ras* family, *jun*, *fos*, int2, NM23, EGF, JCZ30, DCC, POR, TF, CEA, CA19-1, *c-erbB2*, and p53 (Fu, 1993; Chen, 1994; Ni, 1993; He, 1990; Liu, 1993; Lu, 1993), and the majority of the reports were published in Chinese language only. A report of using flow cytometry on breast cancer research also exists (Li, 1994; Guo, 1994). There is a single report of the detection of point mutation of p53 gene by PCR-SSCP only without DNA sequencing in paraffin-embedded breast cancer tissue: Fourteen of sixty (23.33%)cases were found SSCP abnormal shifting bands. Another group showed 13 of 60 (21.67%) cases where p53 overexpression was detected by an immunohistochemical method. However, no information on p53 gene point mutation sequence was presented, nor correlations with

other indicators, in these studies (Chen, 1994).

II. PATIENTS, MATERIAL AND METHODS

1.0.0 Patients Clinical Information

Two hundred and five formalin fixed, paraffin embedded specimens, which were originally obtained for diagnostic purposes, were examined from 178 cases of primary breast cancer patients at teaching hospitals in mainland China with the full acknowledgment of the patients as part of the clinical function of these teaching hospitals. Adjacent normal skin was also studied in 54 of these cases of primary breast cancer. All specimens were obtained from surgical operations medically directed as part of treatment with the consent of the patients. The age, sex, tumor location, size of tumor, status of lymph node metastasis and certain patient's disease free follow up for various times were recorded.

2.0.0 Histopathology

Tumors were diagnosed for type according to the World Health Organization (WHO) classification (Hartmanek, 1987) with all histological grading made by certified pathologists (in most cases more than three observers). The specimens were grouped into three groups depending on duct or tubule malformation, the nuclear morphology and the mitotic rate. The method used in the Nottingham/Tenovus breast study (shown in the table II-1) was used for our specimen evaluation (Elston, 1987; Powell, 1994).

Table II-1. Histological grading of invasive breast cancer

Feature	Score
I. Tubule formation	
Tubules with visible lumina as major component of tumor mass	1
Tubules with visible lumina as moderate component of tumor mass	2
Tubules with visible lumina as minor component of tumor mass	3
II. Nuclear morphology	
Nuclei show little variation in size and shape	1
Nuclei show moderate variation in size and shape	2
Nuclei show marker variation in size and shape	3
III. Mitotic rate	
<10 mitoses per 10 high-power fields (mitoses per 1000 cells at standardization of high-power field size).	1
10 - 19 mitoses per 10 high-power fields	2
20 or more mitoses per 10 high-power fields	3
Grade 1 (well differentiated)	3-5
Grade 2 (moderately differentiated)	6-7
Grade 3 (poorly differentiated)	8-9

(Elston, 1987; Powell, 1994)

3.0.0 Immunohistochemistry

For the immunohistochemistry studies, five 4μm sections were cut, deparaffinized in xylene, transferred to 100 %, 95%, 80%, 70%, 50% ethanol, distilled water and to Tris Buffered Saline (TBS, Commercial). These sections were digested with 0.03% Pronase E in 0.2N HCL, at 37°C for 10 min, and rinsed well for 5 min, and stained using an automated HISTOSTAINER lg immuno-stainer (Leica Instruments Gmb H, Germany).

p53 immuno-staining: The deparaffinized sections for p53 immuno-staining were treated with a 1:30 dilution of normal horse serum (Vector Co.) for 30 min; then with anti-p53, clone BP53-12 (BioGenex), 1:1000 dilution for 720 min; followed by biotinylated anti-mouse IgG (H+L) made in the horse, 1:150 (Vector Co.) dilution for 40

min; then streptavidin alkaline phosphatase (Kirkegaard & Perry Labs) for 60 min, and finally the substrate 1 (Vector Red) for 8 min. BP53-12 is an IgG_{2a} monoclonal antibody which recognizes a fixation-resistant epitope of both mutant and wild-type p53 proteins located on the amino terminus between residues 1 and 45 (out of range of most identified mutational 'hot spots'). This clone yields highly consistent results even in poorly fixed and overfixed tissue.

c-erbB-2 (HER/*neu*) immuno-staining: Deparaffinized sections were treated with a 1:30 dilution of normal horse serum for 30 min (Vector Co.); then treated with *c-erbB-2* antibody (NOVA CASTRA Co.) 1:40 dilution for 90 min; biotinylated anti-mouse 1:150 dilution for 40 min; streptavidin alkaline phosphatase for 60 min; and finally substrate 1 (Vector Red) for 8 min. The *c-erbB-2* clone CB11 is a mouse IgG_1 monoclonal antibody which recognizes a site on the internal domain of the *c-erbB-2* oncoprotein.

Bcl-2 immuno-staining : the deparaffinized tissue sections at the room temperature were immersed into a preheated Antigen Retrieval Solution (95-100°C) in the waterbath, and incubated for 40 min; the slides in the incubation container were stood at room temperature for 20 min for clone 124.3, and at 37°C for clone 100; then the sections were rinsed two to three times with distilled water. These antigen pretreated sections were then exposed to 1:30 dilution of normal horse serum for 30 min (Vector Co.), treated with Bcl-2 antibody (BIOGENEX Co.) 1:50 dilution for 720 min for clone 124.3; a 1:20 dilution of clone 100 for 120 min followed by the biotinylated horse anti-mouse IgG (H+L) (VECTOR Co.) at a 1:150 dilution for 40 min; and finally

streptavidin biotin alkaline phosphatase was applied for 60 min and substrate 1 (Vector Red) for 20 min. Both Bcl-2 clone 124.3 and 100 can positively stain the 380 cell line which carries a 14;18 chromosomal translocation mutation.

The slides were counterstained with Lerner 2 Hematoxylin for 15-30 seconds, differentiated in 1% glacial acetic acid, blued in running tap water for 3 min and dehydrated through graduated percentages of ethyl alcohol (95%, 100% several changes of each), cleared in several changes of xylene, and coverslipped immediately with synthetic mounting media.

The Cytokeratin (AE3/AE1) and Proliferating cell nuclear antigen (PCNA) antibodies were used to evaluate the quality of antigens in the breast cancer tissues as "house control" markers. AE3/AE1 monoclonal antibodies have been used in many studies to characterize an epithelial source for neoplasms and to study the distribution of keratin-containing cells in epithelia during normal development. AE3 was directed at basic keratins, and AE1 at low molecular weight cytokeratin antigens. PCNA monoclonal antibody is used to identify a proliferating cell nuclear antigen which is usually present in any normal tissue components that contain proliferating cells.

A cell was regarded as staining positively for p53 if there was red nuclear staining; for *c-erbB-2*, if there was red membrane staining; and for Bcl-2, if there was red cytoplasmic staining. Known positive and negative control specimens were stained within each run. See the figure 1 - 4.

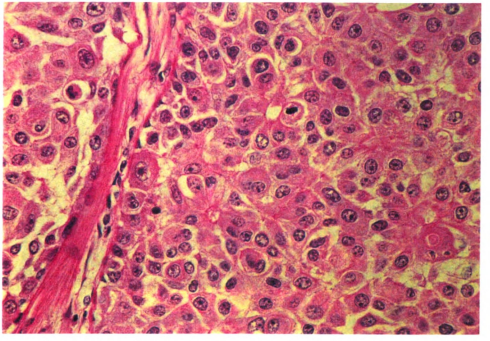

Figure 1. H & E stain section of a primary breast cancer.

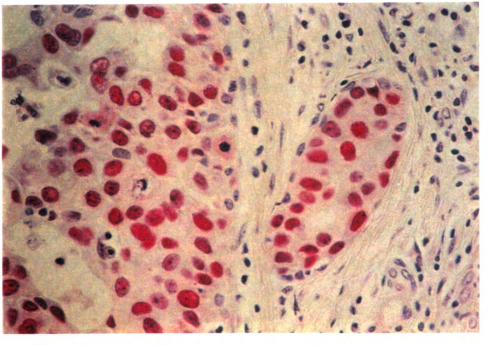

Figure 2. Immunohistochemical stain positive of p53 mutated protein overexpression in a primary breast cancer.

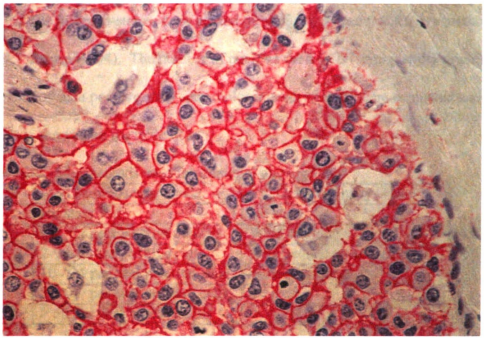

Figure 3. Immunohistochemical stain positive of HER/*neu* (*c-erbB-2)* oncoprotein overexpression in a primary breast cancer.

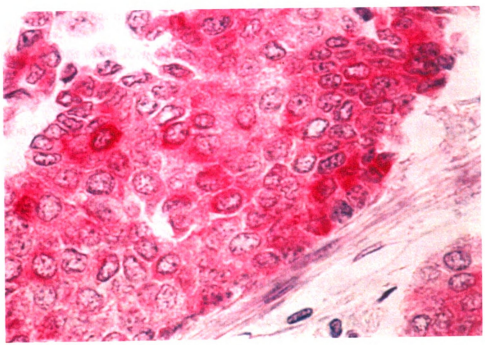

Figure 4. Immunohistochemical stain positive of Bcl-2 oncoprotein overexpression in a primary breast cancer.

The immuno-stains were graded by using two semiquantitative methods (Berg 1993; Joensu 1994). The number of positive stain cells (semiquantity grade) or color intensity of such positive stain cells (color intensity grade) were evaluated and grade methods showed on the table II-2:

Table II-2. The methods used for immuno-staining

Grade	Semiquantity Grade	Color Intensity Grade
0	No stain	No stain
1	< 1 per cent positive cells	Weak staining
2	2-9 per cent positive cells	Intermediate positive staining
3	10 - 24 per cent positive cells	Intense staining
4	25 - 49 per cent positive cells	-
5	> 50 per cent positive cells	-

4.0.0 Molecular Analysis

4.1.0 Cell Lines and Culture Conditions

The human breast cancer cell line BT474 (ATCC), known to have 4 to 8 fold amplifications of HER/*neu* gene, was used to calibrate the relationship for the differential PCR ratio. This cell line was also known to have a p53 point mutation GAG to AAG at codon 285 and was used as a positive control for SSCP at exon 8. The BT474 breast cell line was grown in RPMI 1640 medium with bovine insulin 10 μg/ml, L-glutamine medium 300 mg/ml and 90 % fetal bovine serum, 10% and maintained in a 5 % CO_2 humidified atmosphere for 10 days (Soto and Sukumar, 1992). Another breast cancer cell line T47D (provided by Dr. Cheng) known to have a p53 point mutation CTT to TTT at codon 194 and used as a positive control for SSCP at exon 6 (Soto and Sukumar, 1992).

4.2.0 DNA Extraction Method for Formalin Fixed Paraffin Embedded Breast Tissues

Five of 5μm tissue sections from the each primary breast cancer patient were put in a microcentrifuge tube, 1 ml of xylene added to each tube, mixed at room temperature for about 30 min or mixed 15 min at 56°C, pelleted the tissue by centrifugation 5 min at full speed and carefully aspirated the xylene; this was repeated with a second change of xylene and then 1 ml absolute ethanol was added; the solution mixed by inverting, centrifuging, and aspirated the ethanol; this was repeated with a second change of ethanol; finally then dried tissue pellet in a speed vacuum about 15 min.

One hundred μl (200 μl if the tissue pellet was large) digestion buffer (1M Tris-HCL, pH8.5, 0.5 M EDTA, Tween-20) containing 200 μg/ml of proteinase K was vortexed briefly with the pellet, and the solution incubated for 3 hours at 55°C or 37°C overnight. The tubes were then spun briefly, heated and inactivated proteinase K at 95°C for 8 to 10 min, centrifuged for 5 min and samples frozen at -20° C. Aliquots of the supernatant were used for amplification (typically 1 to 10 μl) (the method was provided by Dr. K Friderici).

4.3.0 DNA Extraction Method for Cell Cultures

The cell culture medium was removed and centrifuged for 10 min at 1500g; 10 ml Trypsin 0.25% with 0.03 % EDTA was added to the culture well for 10 min, or until the cells detached from the well walls and then the cells removed into a centrifuge tube; the medium was centrifuged for 10 min at 1500 g. Phosphate-buffered saline (PBS) 10 ml used to wash the cells twice with centrifugation after which the PBS was removed until about 50 μl cell solution was left. Fifty mM NaOH 100 μl was added to the pelleted

cells at 95°C for 10 min, and this placed on ice for 5 min.; 1 M Tris, pH 8, 10µl was added, and the solution was then ready to use on PCR analysis.

4.4.0 HER-2/nue Gene Amplification Analysis by Differential PCR

Differential PCR (dPCR) is a method used to detect quantitative alterations in genes within small samples of cells or paraffin embedded specimens. This procedure involves coamplification of a single copy reference gene, interferon-γ (IFN-γ) and the target gene. The level of sample gene amplification is reflected in the ratio between the results of PCR product bands of the target and reference gene (Frye, 1989; Evers, 1992).

CLONTECH's HER-2/*neu* ONCO-LYZER Kit was used for the HER/*neu* (*c-erbB2*) gene amplification analysis. The amplimer sequences were: HER/*neu* gene 5' primer: 5' CCT CTG ACG TCC ATC ATC TC3'; 3' primer: CGG ATC TTC TGC TGC CGT CG3'. IFN-γ gene 5' primer: 5'TCT TTT CTT TCC CGA TAG GT3'; 3' primer: 3' CTG GGA TGC TCT TCG ACC TC5' (Yamamoto 1986; Gray 1982).

A differential PCR amplification mixture for 50 µl final reaction volume of the following composition was used. The reagent included: distilled water 31.75 µl; 10X PCR reaction buffer 5.0 µl (PERKIN ELMER); dNTP mix (10 mM each) 1.0 µl ; 5' HER/*neu* amplimer (20 µM) 2.5 µl ; 3' HER/*neu* amplimer (20 µM) 2.5 µl; 5' IFN-γ amplimer (20 µM) 2.5 µl ; 3'IFN-γ amplimer (20 µM) 2.5 µl (CLONTECH); genomic DNA (0.25µg/µl) 2.0 µl; AmpliTaq DNA polymerase (5 U/µl) 0.25 µl (PERKIN ELMER). The temperature cycling program was as follows: segment 1: 94°C, 1 min; segment 2: 55°C,1 min; segment 3: 72°C, 1 min for 40 cycles; then 72°C, 7 min; and

finally held at 4°C (Gene Amp PCR System 9600, PERKIN ELMER).

For agarose gel electrophoresis, 8 μl PCR product was mixed with 2 μl of gel-loading solution (0.25% xylene cyanol, 0.25% bromophenl blue and 30% glycerol, in water), loaded in the wells of 2% agarose gel containing 0.5 μg/ml ethidium bromide in 0.5 X TBE (45 mM Tris-Borate-EDTA) buffer. ϕX174/Haee III (Gibco BRL) were used as the DNA size markers each time the gel was run. Electrophoresis was run in the same TBE buffer for 1-1.5 hours at 60-70 V.

BIORAD's DOC1000 gel detection and image analysis system was used for the sample DNA band intensity analyses. The gel detector was set at optimal range of the sample intensity with the analysis box covering the whole density of each band; the size of the analysis area was kept constant for all the samples. Normal placenta tissue and normal skin were used as normal limitation ratio controls. The breast cancer cell line BT474 was chosen to assign positivity. The HER/*neu* gene band's intensity of sample was compared with IFN-γ band's intensity each time and the ratios between *neu* and IFN-γ were calculated for each sample. The sample ratio higher than normal placenta tissue and normal skin was considered as positive, HER/*neu* gene amplified >1 fold. A ratio higher than BT474 cell line was considered HER/*neu* gene amplified >4-8 fold. A HER/*neu* gene band with intensity brighter than the IFN-γ that sample was considered as positive gene amplification. Figure 5 and 6 show the d PCR products on 2% agarose gel contains 0.5 μg/ml ethidium bromide in 0.5 X TBE.

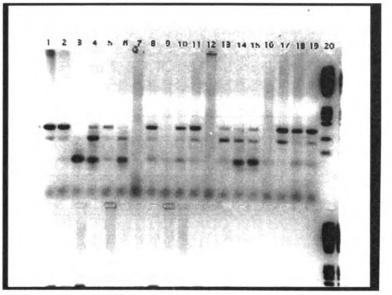

Figure 5. The HER/*neu* dPCR products are in a 2% agarose gel contains 0.5 μg/ml ethidium bromide in 0.5 X TBE. The DNA bands on the top are IFN-γ band's intensity. The DNA bands below are *neu* gene PCR products. Lane 1 to 20 is from left to right. Lane 1,2: normal placenta tissue (dPCR positive control); lane 3: dPCR negative control (water); lane 4: BT474 cell line, as a known positive control; lane 5: normal skin; lane 6-19: specimens; lane 20: ϕX174/Haee III were used as the DNA size markers. From the top of the gel picture, first band is IFN-γ and the next band below that is *neu* .

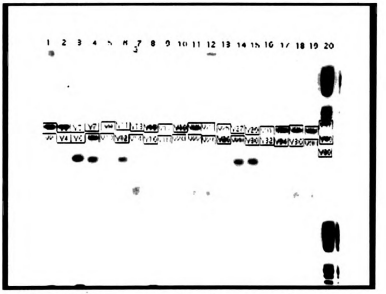

Figure 6. The HER/*neu* dPCR products are in a 2% agarose gel contains 0.5 μg/ml ethidium bromide in 0.5 X TBE. The sample intensity was defined with the analysis box covering the whole density of each band with the intensity detection box. Lane 1 to 20 is from left to right. Lane 1, 2: normal placenta tissue (dPCR positive control); lane 3: dPCR negative control (water); lane 4: BT474 cell line, as a known positive control; lane 5: normal skin; lane 6-19: specimens; lane 20: ϕX174/Haee III were used as the DNA size markers. From the top of the gel picture, first band is IFN-γ and the next band below that is *neu* .

4.5.0 p53 Gene Point Mutation Analysis

CLONTECH's Human p53 Amplimer Panels kit was used for p53 gene point mutation analysis. The exons 5, 6, 7, 8, and 9 were analyzed.

Table II-3. The amplimer sequences for p53 gene exon 5 to 9

Exon		Sequence of Amplimers
Exon 5	PU5	5'- CTC TTC CTG CAG TAC TCC CCT GC-3'
	PD5	5'- GCC CCA GCT GCT CAC CAT CGC TA-3'
Exon 6	PU6	5'- GAT TGC TCT TAG GTC TGG CCC CTC-3'
	PD6	5'- GTG TTG TCT CCT AGG TTG GCT CTG-3'
Exon 7	PU7	5'- GTG TTG TCT CCT AGG TTG GCT CTG-3'
	PD7	5'- CAA GTG GCT CCT GAC CTG GAG TC-3'
Exon 8	PU8	5'- ACC TGA TTT CCT TAC TGC CTC TGG C-3'
	PD8	5'- GTC CTG GTT GCT TAC CTC GCT TAG T-3'
Exon 9	PU9	5'- GCC TCT TTC CTA GCA CTG CCC AAC-3'
	PD9	5'- CCC AAG ACT TAG TAC CTG AAG GGT G-3'

A PCR mixture for 50 μl final reaction volume was used containing the following: distilled water 40.06 μl; 10X PCR reaction buffer (PERKIN ELMER) 5.0 μl; dNTP mix (10 mM each) 1.0 μl; 5' exon X amplimer (20 μM) 1.0 μl ; 3' exon X amplimer (20 μM) 1.0 μl; genomic DNA (0.25μg/μl) 1.0 μl; AmpliTaq® DNA polymerase (5 U/μl) 0.4μl. The temperature cycling program was: segment 1: 95°C, 30 sec; segment 2: 66°C, 45 sec; segment 3: 72°C, 1.5 min for 35 cycles; then 72°C, 7 min; soak in 4°C (Gene Amp PCR System 9600, PERKIN ELMER).

To determine whether the PCR amplification yielded product, an agarose gel containing 2.7% agarose and 0.5 μg/ml ethidium bromide in 0.5X TBE buffer was used for the agarose gel electrophoresis. Ten μl of PCR product mixed with 2 μl of gel-loading buffer was loaded into the well and ϕX174/Haee III digest used in place of the sample as size markers. The electrophoresis was run in the same 0.5X TBE buffer at 80 V for 1-1.5 hr. and the DNA bands were visualized with a UV transilluminator.

Any sample with enough PCR product was subjected to a Single-Strand Conformation Polymorphism (SSCP) study; the PCR amplification was repeated up to three times on a sample before this sample was abandoned for p53 gene point mutation analysis. Figure 7 shows p53 PCR products run on 2.7% agarose gel.

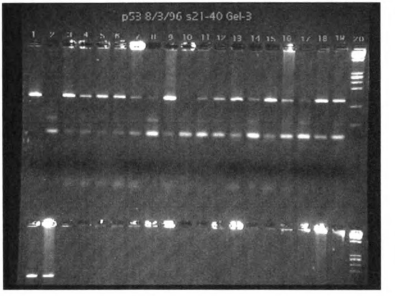

Figure 7. PCR products of p53 gene at exon 5 in 2.7% agarose gel. Lane 1: PCR positive control; lane 2: PCR negative control; lane 3-19: breast cancer specimens; lane 20: ϕX174/Hae III digest used in place of the sample as size markers. Lane 3-6, 9, 12,13, 15, 16, 18, and 19 showed optimal PCR products for SSCP and DNA sequencing. Lane 7, 8, 10, 11, 14, and 17 showed less than optimal PCR products and PCR were repeated for these samples.

For SSCP, formamide loading dye (95% formamide, 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene cyanol) of 6% polyacrylamide with and without 5%glycerol gel (Biochem Co.) was used for each sample denaturation. One μl of each PCR product was mixed with 10 μl formamide loading dye in a microcentrifuge tube, then heated at 95° C for 5 min and cooled quickly on ice for more than 5 min. A 6%-nondenaturing polyacrylamide gel containing no or 5 % (v/v) glycerol in 0.5X TBE

buffer was used for SSCP. Three μl of each denatured DNA sample was loaded on each lane, and same volume of denatured control DNA loaded in an adjacent lane. Electrophorese the gel at 0.5 W/cm for 1.5 to 2 hr at room temperature, and visualize the bands by Silver Stain (BIORAD Co.) the gel. The appearance of a band shift in a tumor sample when compared with the normal band pattern of a particular exon was characteristic of a change in the secondary structure of the mutant single strand.

The breast cancer cell line T47D (which has a known gene point mutation at codon 194 CTT to TTT) was used as a positive control for exon 6. BT474 (known to have a gene point mutation at codon 285 GAG to AAG) was used as a positive control for exon 8. (Soto and Sukumar 1992). The polymorphism bands were compared with normal controls to ascertain difference.

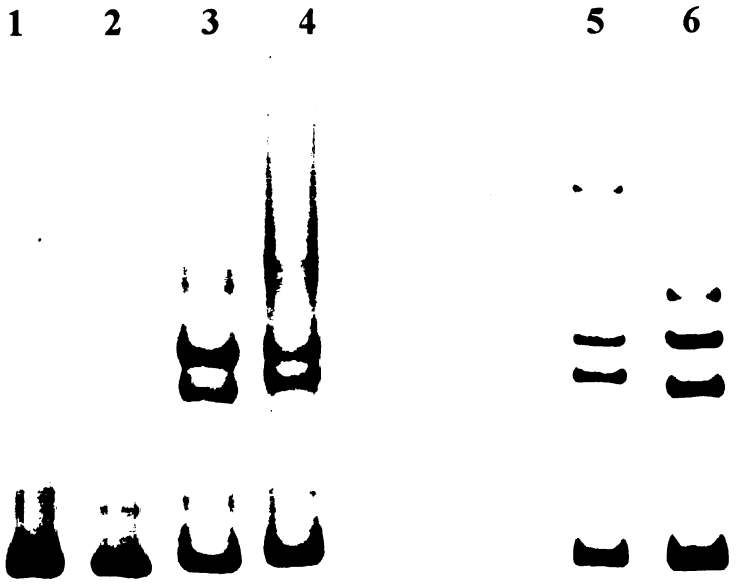

Figure 8. SSCP analysis of exon 6, T47, (lanes 1 - 4) and exon 8, BT474, (lanes 5 and 6) of the p53 gene in breast cancer cell lines in a 6% nondenaturing polyacrylamide gel in 0.5X TBE buffer. Lane 1 and 2 are nondenatured normal placenta and T47D samples. The appearances of the band mobility shift in the breast cancer cell lines compared with the normal placenta tissue bands (lanes 3 and 5) are characteristiced, because of two different conformations of the sequence.

Figure 9. SSCP analysis of exon 5 of p53 gene in breast cancer sample No. 43 in a 6% nondenaturing polyacrylamide gel in 0.5X TBE buffer. Lane 1 is denatured breast cancer tissue. The appearances of the band mobility shift are characteristiced, because of two different conformations of the sequence. Lane 2 is denatured normal skin tissue.

After detection of mutations by PCR-SSCP, DNA sequencing was used for confirmation purposes and for pinpointing the exact base change. The PCR products and primers were diluted to 1:10, then 6μl sample and 3 μl primer were sent to the Tissue Typing Laboratory for sequencing. ABI PRISM Cycle Sequencing Day Terminator with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PERKIN ELMER) are used for DNA sequencing. Each sample was sequenced from both directions.

5.0.0 Statistical Analysis

Statistical analysis for characteristics of Chinese breast cancer were performed by using Jandel Scientific program, SigmaStat, for GATEWAY2000, IBM Personal Computer. Student t test was used for age distribution between two variables. Mann-

Whitney Rank Sum test was used to compare the difference of treatments. Chi-square test, Fisher Exact test, Pearson Product Moment Correlation and Spearman Rank Order Correlation tests used for analyzing the degree of association between two or more variables Linear Regression test and Best Subsets Regression tests were applied for analyzing the significance of functional relationship between two or more variables. The Life Table test used for general five-year survival analysis.

All statistics studies were to examine the confidence interval allows a 95% confidence interval overall at a standard power (0.80) with two-tail for each test. (Armitage 1994).

III. RESULTS

The results of this study are divided to four parts: patient clinical information, histopathology, immunohistopathology and molecular pathology.

1.0.0 Patient Clinical Information

Sex: All tumors were defined as breast cancer. One hundred seventy - eight of the samples were from females and one from a male (0.57%).

Age: The age range of patients was from 22 - 79 years, with a mean of 49 years and a median of 48 years. The age distribution data summarized in the Table III-1.

Table III-1. The age and percentage distributions of breast cancer patients (n = 176)

Age Range	No. of Patients	Percentage (%)
<20	0	0
20-29	5	2.84
30-39	33	18.8
40-49	59	33.5
50-59	43	24.4
60-69	25	14.2
70-79	11	6.25
80-89	0	0

The relationship between age and lymph node metastasis was analyzed by Fisher's Exact tests: the data and the results show in the table III-2.

Table III-2. The correlation of age with lymph node metastasis (n=85)

	Age <50	Age >50
Lymph Node +	25	27
Lymph Node -	15	18
P value	0.828	

The Chi-square test was used to analyze the effect of age on survival rate. The age range groups used were 20 to 39, 40 to 59, and 60 to 79. The Chi-square test, probability value greater than 0.05 (P>0.05). Indicating that there was no significant difference

amongst the three age range groups. The effecting of young or old of age on breast cancer patient tendency were also tested by Chi-square test. The ages were grouped as mentioned before. The result of $P>0.05$ indicated that there was no significant difference between those groups.

Tumor Location: The location of tumors were recorded in fifty-eight cases and these involved the left side breast in 55.17% (32/58), and the right side in 44.83% (26/58).

Tumor Size: The sizes of the breast cancers are shown in the Figure 10. These ranged from 1.3 cm to 12 cm in diameter, with a mean of 4.5 cm. The most frequent size for palpable breast cancer detection was 3 - 4 cm.

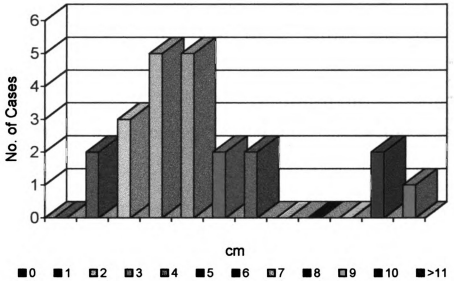

Figure 10. The size distribution of the breast cancer.

Metastasis: Lymph node metastases were assessed and found to be as follows: Positivity of 60.7%, 51/84 cases had positive lymph nodes. The number of lymph nodes evaluated in each patient was from 1 to 29 (average 17). The years of survival following the primary breast cancer were also analyzed with regard to whether or not the lymph

nodes were positive for metastases by the Chi-square test. A statistically significant difference with primary breast cancer patient's five-year survival between those with and those without lymph node metastasis was seen with a probability value of below 0.05 (P=0.006).

Table III-3. Survival information and the % results of Life Table Analysis

Survival Years	Breast Cancer		Breast Cancer with Lymph Node Metastasis		Breast Cancer without Lymph Node Metastasis	
	No. of Dead or Missed/Total	Survial %	No. of Dead or Missed/Total	Survial %	No. of Dead or Missed/Total	Survial %
1	3/41	95.12	2/26	92.31	1/15	93.33
2	8/41	83.52	6/26	71.00	2/15	80.89
3	11/41	63.15	8/26	49.16	3/15	64.71
4	6/41	55.44	5/26	39.70	1/15	60.40
5	7/41	47.33	5/26	32.07	7/15	32.21
>5	6/41	41.55	0/26	0.00	1/15	30.06

2.0.0 Histopathology

2.1.0 Types

Ten histopathological diagnostic types of breast cancer were identified in these specimens and shown in the table III-4.

Table III-4. Histopathological types and their frequency of in the Chinese breast cancer samples (n=178)

Pathologic Cancer Type	No. of Cases	%
Adenocarcinoma	2	1.12
Apocrine carcinoma	1	0.56
Carcinoma	1	0.56
Intraductal carcinoma	3	1.69
Infiltrating ductal carcinoma	153	86.0
Infiltrating lobular carcinoma	7	3.93
Infiltrating papillary carcinoma	1	0.56
in situ lobular carcinoma	2	1.12
Medullary carcinoma	3	1.69
Mucinous adenocarcinoma	5	2.80

2.2.0 Grade

The histopathological grading was recorded and the specimens scored into categories of well, moderate and poor differentiation depending on duct and tubule malformation, nuclear morphology and mitotic rate. The results are shown in the table III-5. Two *in situ* breast cancer cases were excluded in this table, because the grade system of *in situ* tumor is different with infiltrating tumor grading system.

Table III-5. Histology grading of invasive breast cancer (n=178)

Pathologic grading	Well differentiated		Moderately differentiated		Poorly differentiated	
	No.	%	No.	%	No.	%
Adenocarcinoma	1	50	0	0	1	50
Apocrine carcinoma	0	0	1	100	0	0
Carcinoma	0	0	0	0	1	100
Intraductal carcinoma	3	100	0	0	0	0
Infiltrating ductal carcinoma	51	33.33	56	36.60	46	30.07
Infiltrating lobular carcinoma	2	28.57	2	28.57	3	42.86
Infiltrating papillary carcinoma	1	100	0	0	0	0
Medullary carcinoma	0	0	2	66.67	1	33.33
Mucinous adenocarcinoma	2	40.00	3	60.00	0	0
Total number of cases	60		64		52	
%	33.71		35.96		29.21	

3.0.0 Immunohistochemistry

3.1.0 Control Used

The cytokeratin (AE3/AE1) and proliferating cell nuclear antigen (PCNA) antibodies were used as "house keeping control" markers to assess the quality of antigens in the breast cancer tissues. All the Chinese breast cancer specimens stained positively for the positive controls and all negative controls were negatively (100%).

3.2.0 Immunohistochemical Study

The overall results for the immunohistochemical study were 14.18% (19/134)

positive for p53, 23.13% (31/134) positive for *c-erbB-2* (HER/*neu*) and 66.42% (89/134) positive for Bcl-2, when assess using a semiquantification grading method. The color intensity grade was assessed and used for technique comparison only. The results are in the table III-6.

Table III-6. Immunohistochemical studies (n = 134)

Immuno-stain	Quantity Grade*						Color Intensity Grade**			
Grade	-	1+	2+	3+	4+	5+	-	1+	2+	3+
p53	99	13	3	3	5	11	99	13	14	8
c-erbB-2	95	5	3	5	7	19	95	3	10	26
Bcl-2	37	2	6	12	19	58	35	10	34	55

* data are based on number of positively stained cell, ** data are based on intensity of stain

The semiquantity grade and the color intensity grade were analyzed by Spearman Rank Order Correlation statistical technique. There were no significant relationship between the two evaluation methods ($P>0.05$). In this study, the results of the semiquantity grade were used for the rest of the studies and analyses as is reflect cell positively the best.

Mann-Whitney Rank Sum Test was used to analyze for differences between semiquantitying grade and the color intensity grade methods. For p53 and HER/*neu* immuno-stains, there were no statistically significant differences ($P = 0.704$ and $P = 0.513$). There was a statistically significant difference ($P<0.0001$) for the Bcl-2 immuno-stain.

3.3.0 Correlation Analysis of Immunohistochemical and Other Studies

3.3.1 Correlation Immunostain with Breast Cancer Types

The correlation of immuno-stain positive and with pathological cancer type were recorded and shown in the tables III-7.

A comparison of immuno-stain positive for p53, HER/*neu* and Bcl-2 with the

different type of breast cancers were analyzed by Pearson Product Moment Correlation test. Within each pattern, there was no significant relationship between the variables ($P>0.05$).

Table III-7-1. Immunostaining positivity by pathological tumor type

Pathologic diagnosis (n=134)	Frequency		p53 (+) (19/134, 14.18%)		*HER/neu* (+) (31/134, 23.13%)		Bcl-2 (+) (89/134, 66.42%)	
	No.	%	No.	%	No.	%	No.	%
Adenocarcinoma	0	0	0	0	0	0	0	0
Apocrine carcinoma	0	0	0	0	0	0	0	0
Carcinoma	1	0.74	0	0	0	0	1	100
Intraductal carcinoma	3	2.24	0	0	0	0	2	66.67
Infiltrating ductal cancer	116	86.57	18	15.52	31	26.72	74	63.79
Infiltrating lobular cancer	7	5.24	0	0	0	0	5	71.43
Infiltrating papillary cancer	1	0.74	0	0	0	0	1	100
in situ lobular carcinoma	2	1.49	0	0	0	0	2	100
Medullary carcinoma	1	0.74	1	100	0	0	1	100
Mucinous adenocarcinoma	3	2.24	0	0	0	0	3	100

Table III-7-2. Immunostaining negativity by pathological tumor type

Pathological Diagnosis (n = 134)	Frequency		p53 (-) (115/134, 85.82%)		HER/*neu* (-) (103/134, 76.87 %)		Bcl-2 (-) (45/134, 33.58%)	
	No.	%	No.	%	No.	%	No.	%
Adenocarcinoma	0	0	0	0	0	0	0	0
Apocrine carcinoma	0	0	0	0	0	0	0	0
Carcinoma	1	0.74	1	100	1	100	0	0
Intraductal carcinoma	3	2.24	3	100	3	100	1	33.33
Infiltrating ductal cancer	116	86.57	98	84.48	85	73.28	42	36.21
Infiltrating lobular cancer	7	5.24	7	100	7	100	2	28.57
Infiltrating papillary cancer	1	0.74	1	100	1	100	0	0
in situ lobular cancer	2	1.49	2	100	2	100	0	0
Medullary carcinoma	1	0.74	1	100	1	100	0	0
Mucinous adenocarcinoma	3	2.24	3	100	3	100	0	0

3.3.2 Correlation Immunostain Studies with Each Other

One Way ANOVA (Kruskal-Wallis) test was used to compare the p53, HER/*neu* (*c-erbB-2*) and Bcl-2 positive stains, and showed those immunohistochemical stains that were significantly different ($P<0.0001$). The Spearman Rank Order Correlation test was used for the correlation analysis. No significant relationships between any pair of variables

were detected (P>0.05) i.e. for immuno-stain positive results of p53, HER/*neu*, and Bcl-2.

3.3.3 Correlation Immunostain Studies with Clinical Indicators

The immunohistological results with p53, HER/*neu* and Bcl-2 were compared with clinical indicators, such as age, lymph node metastasis, cancer type, cancer cell differentiation and cell mitotic rate. (Table III-8).

Table III-8. Protein expression for p53, *c-erbB2* and Bcl-2 and their relationship to clinical indicators

Study	p53 (+) immuno-stain (19/134)		p53 (-) immuno-stain (115/134)		*c-erbB-2*(+) immuno-stain (31/134)		*c-erbB-2* (-) immuno-stain (103/134)		Bcl-2 (+) immuno-stain (89/134)		Bcl-2 (-) immuno-stain (45/134)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Grade 1	4/19	21.05	34/113	30.09	3/31	9.68	35/101	34.65	26/87	29.89	12/45	26.67
Grade 2	4/19	21.05	42/113	37.17	14/31	45.16	32/101	31.68	28/87	32.18	18/45	40.00
Grade 3	11/19	57.89	37/113	32.74	14/31	45.16	34/101	33.66	33/87	37.93	15/45	33.00
M 1	3/19	15.79	33/113	29.20	7/31	22.58	29/101	28.71	24/87	27.59	12/45	26.67
M 2	6/19	31.58	44/113	31.86	11/31	35.48	31/101	30.69	23/87	26.44	19/45	42.22
M 3	10/19	52.63	43/113	38.94	13/31	41.94	41/101	40.59	40/87	45.98	14/45	31.11
L+	9/13	69.23	43/72	59.72	18/22	81.82	33/63	52.38	20/55	36.36	20/32	62.50
L -	4/13	17.00	29/72	40.28	4/22	18.18	30/63	47.19	35/55	63.64	12/32	37.50
<50	7/19	36.84	63/115	54.78	16/31	51.61	45/103	52.43	46/89	51.69	24/45	53.53
>50	12/19	63.16	52/115	45.22	15/31	48.39	49/103	47.57	43/89	48.31	21/45	46.67
IDC	18/19	94.74	98/115	85.22	31/31	100.00	86/103	83.50	74/89	83.15	43/45	95.56
ILC	0/19	0.00	7/115	6.09	0.00	0.00	6/103	5.82	5/89	5.62	2/45	4.44
MA	0/19	0.00	3/115	2.61	0.00	0.00	3/103	2.91	3/89	3.37	0/45	0.00
in situ	0/19	0.00	2/115	1.74	0.00	0.00	0/103	0.00	2/89	2.25	0/45	0.00
MC	1/19	5.26	1/115	0.87	0.00	0.00	0/103	0.00	1/89	1.20	0/45	0.00
IC	0/19	0.00	3/115	2.61	0.00	0.00	0/103	0.00	3/89	3.37	0/45	0.00

Grade 1 = cell well differentiation, Grade 2 = cell moderate differentiation, Grade 3 = cell poor differentiation; M 1 = cell with low mitotic rate, M2 = cell with moderate mitotic rate, M3 = cell with high mitotic rate; L + = lymph node metastasis positive, L- = lymph node metastasis negative; IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer; Ca = cancer; MA = mucinous adenocarcinoma; *in situ* = *in situ* lobular carcinoma; MC = medullary cancer; IC = intraductal cancer.

The table III-9 shows these results analyzed statistically.

Table III-9. The results of Chi-square test from the table III-8 (n=134)

	p53 immuno (+) & (-) immuno-stain		HER/*neu* (+) & (-) immuno-stain		Bcl-2 (+) & (-) immuno-stain	
	X^2	P	X^2	P	X^2	P
Differentiation	4.50e+000	0.105	7.23e+000	0.671	5.43e+000	0.066
Mitotic rate	2.20e+000	0.333	5.09e-001	0.140	1.45e+000	0.484
Lymph Node +	1.14e-001	0.735	4.72e+000	0.033	3.69e+000	0.055
Age < > 50	1.45e+000	0.229	2.38e-002	0.998	6.56e-001	0.418

The clinical indicators correlated with immunostain results were also recorded and

analyzed statistically. The results show in the tables III-10.

Table III-10-1. Relationships between clinical indicators and immunostain results (n=134)

	Age				Ca in Breast				Lymph Node			
	<50		>50		Left		Right		+		-	
	No	%	No	%	No	%	No	%	No	%	No	%
Total	70	52.23	64	47.76	32	55.17	26	44.82	52	61.18	33	38.82
p53+	7	10.00	12	18.75	4	12.50	3	11.54	9	17.31	4	12.12
p53-	63	90.00	52	81.25	28	87.50	23	88.46	43	82.69	29	87.88
P value of p53	0.215				1.000				0.758			
HER+	16	22.86	15	23.44	4	12.50	3	11.54	19	36.54	3	9.09
HER-	54	77.14	49	76.56	28	87.50	23	88.46	33	63.46	30	90.91
P value of HER	1.000				1.000				0.005			
Bcl-2+	46	65.71	43	67.19	22	68.75	21	80.77	32	61.54	21	63.64
Bcl-2 -	24	34.29	21	32.81	10	31.25	5	19.23	20	38.46	12	36.36
P value of Bcl-2	1.000				0.374				1.000			

Fisher's Exact tests are used for all P value calculations. Ca = carcinoma

Table III-10-2. Relationships between clinical indicators and immunostain results (n=134)

	Cell Differentiation						Cell Mitotic Rate					
	Well		Moderate		Poor		Low		Moderate		High	
	No	%	No	%	No	%	No	%	No	%	No	%
Total	38	28.36	46	34.85	48	36.36	36	27.27	42	31.82	54	40.91
p53+	4	10.53	4	8.69	11	22.92	3	8.33	6	31.82	54	40.91
p53-	34	89.47	42	91.30	37	77.08	43	91.67	36	85.71	44	81.48
P value of p53#	1.000*		0.090**		0.161***		0.300*		0.783**		0.133***	
##	0.105****						0.209****					
HER+	3	7.89	14	30.43	14	29.17	7	19.44	11	26.19	13	24.07
HER-	35	92.11	32	69.57	34	70.83	29	80.56	31	73.81	41	75.93
P value of HER #	0.013*		1.000**		0.016***		0.593*		0.817**		0.796***	
##	*0.027****						*0.776****					
Bcl-2+	26	68.42	28	60.87	33	68.75	24	66.67	23	54.76	40	74.07
Bcl-2 -	12	31.58	18	39.13	15	31.25	12	33.33	19	45.24	14	25.93
P value of Bcl-2#	0.502*		0.518**		1.000***		0.355*		0.055**		0.483***	
##	0.247****						0.671****					

Fisher's Exact tests are used for all P value calculations. ## Chi-square tests are used for all P value calculations. * = the result of compared with Well (Low) and Moderate groups; ** = the result of compared with Well (Low) and Poor (High) groups; *** = the result of compared with Moderate and Poor (High) groups; **** = the result of compared with all groups.

3.4.0 Relationship Between Immunostain Results

Associations between immunohistochemical findings and other pathological parameters are shown in the tables III-11.

Table III-11-1. The associations between three immunostaining and clinic-pathological features

	p53+/HER+/Bcl-2+		p53+/HER+/Bcl-2-		p53+/HER-/Bcl-2-		p53+/HER-/Bcl-2+	
	No.	%	No.	%	No.	%	No.	%
Total case	5/134	3.73	2/134	1.49	7/134	5.22	5/134	3.73
Mitotic rate 1	0/5	0	1/2	50.00	2/7	28.57	0/5	0.00
Mitotic rate 2	2/5	40.00	1/2	50.00	2/7	28.57	1/5	20.00
Mitotic rate 3	3/5	60.00	0/2	0	3/7	42.86	4/5	80.00
Differentiated 1	0/5	0	1/2	50.00	3/7	42.86	1/5	20.00
Differentiated 2	1/5	20.00	1/2	50.00	2/7	28.57	0/5	0.00
Differentiated 3	4/5	80.00	0/2	0	2/7	28.57	4/5	80.00
Lymph node +	3/5	60.00	2/2	100.00	3/5	60.00	1/3	33.33
Lymph node -	2/5	40.00	0/2	0	2/5	40.00	2/3	66.67
<50 years	1/5	20.00	2/2	100.00	2/7	28.57	2/5	40.00
>50	4/5	80.00	0/2	0	5/7	71.43	3/5	60.00
IDC	5/5	100	2/2	100.0	7/7	100.00	4/5	80.00
Medullary Ca	0	0	0	0	0	0	1/5	20.00

IDC = infiltrating ductal cancer; Ca = cancer.

Table III-11-2. The associations between three immunostaining and clinic-pathological features

	p53-/HER-/Bcl-2-		p53-/HER-/Bcl-2+		p53-/HER+/Bcl-2+		p53-/HER+/Bcl-2-	
	No.	%	No.	%	No.	%	No.	%
Total case	28/134	20.90	63/134	47.01	16/134	11.94	8/134	5.97
Mitotic rate 1	8/28	28.57	19/61	31.15	5/16	31.25	1/8	12.50
Mitotic rate 2	11/28	39.24	17/61	27.87	3/16	50.00	5/8	62.50
Mitotic rate 3	9/28	32.14	25/61	40.98	8/16	12.5	2/8	25.00
Differentiated 1	9/28	32.14	23/61	37.70	2/16	43.75	0/8	0.00
Differentiated 2	10/28	35.71	20/61	32.79	7/16	43.75	5/8	62.50
Differentiated 3	9/28	32.14	18/61	29.51	7/16	25.00	3/8	37.50
Lymph node +	10/20	50.00	19/35	54.29	3/12	25.00	5/5	100.00
Lymph node -	10/20	50.00	16/63	45.71	9/12	75.00	0/5	0.00
<50 years old	17/28	60.71	38/63	60.32	8/16	50.00	5/8	62.50
>50	11/28	39.29	25/63	39.68	8/16	50.00	3/8	37.50
IDC	26/28	92.86	50/63	79.37	0/16	0.00	8/8	100.00
ILC	2/28	7.14	2/63	3.17	0/16	0.00	0/8	0.00
MA	0/28	0.00	3/63	4.76	0/16	0.00	0/8	0.00
Intraductal Ca	0/28	0.00	3/63	4.76	0/16	0.00	0/8	0.00
in situ lobular	0/28	0.00	2/63	2/63	0/16	0.00	0/8	0.00
IPC	0/28	0.00	1/63	1.59	0/16	0.00	0/8	0.00

IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer; Ca = cancer; MA = mucinous adenocarcinoma; Intraductal Ca = intraductal carcinoma; *in situ* lobular = *in situ* lobular carcinoma; IPC = infiltrating papillary carcinoma.

Table III-11-3. The associations between two immuno-stains and clinic-pathological features

	p53+/HER+		p53+/Bcl-2+		p53-/HER-	
	No.	%	No.	%	No.	%
Total case	7/134	5.22	10/134	7.46	91.134	67.91
Mitotic rate 1	1/7	14.29	0/134	0.00	27/89	30.34
Mitotic rate 2	3/7	42.86	3/10	30.00	28/89	31.46
Mitotic rate 3	3/7	42.86	7/10	70.00	34/89	38.20
Differentiated 1	1/7	14.29	1/10	10.00	32/89	35.96
Differentiated 2	2/7	28.57	1/10	10.00	30/89	37.71
Differentiated 3	4/7	57.14	8/10	80.00	27/89	30.34
Lymph node +	5/5	100.00	4/6	66.67	29/55	52.73
Lymph node -	0/5	0.00	2/6	33.33	26/55	47.27
<50 years	3/7	42.86	3/10	30.00	41/91	45.05
>50	4/7	57.14	7/10	70.00	56/91	61.54
IDC	7/7	100.00	9/10	90.00	75/91	82.42
ILC	0/7	0.00	0/10	0.00	6/91	6.59
MA	0/7	0.00	0/10	0.00	3/91	3.30
Medullary Ca	0/7	0.00	0/10	0.00	0/91	0.00
Intraductal Ca	0/7	0.00	1/10	10.00	3/91	3.30
in situ lobular	0/7	0.00	0/10	0.00	2/91	2.20
IPC	0/7	0.00	0/10	0.00	1/91	1.10

IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer; Ca = cancer; MA = mucinous adenocarcinoma; Intraductal Ca = intraductal carcinoma; *in situ* lobular = *in situ* lobular carcinoma; IPC = infiltrating papillary carcinoma.

Table III-11-4. The associations between two immunostaining and clinic-pathological features

	p53-/Bcl-2 -		HER+/Bcl-2+		HER-/Bcl-2-	
	No.	%	No.	%	No.	%
Total case	36/134	26.87	16/134	11.94	28/134	20.90
Mitotic rate 1	9/36	25.00	5/16	31.25	8/28	28.57
Mitotic rate 2	16/36	44.44	3/16	18.75	11/28	39.29
Mitotic rate 3	11/36	30.56	8/16	50.00	9/28	32.14
Differentiated 1	9/36	25.00	2/16	12.50	9/28	32.14
Differentiated 2	15/36	41.67	7/16	43.75	10/28	35.71
Differentiated 3	12/36	33.33	7/16	43.75	9/28	32.14
Lymph node +	15/25	60.00	9/12	75.00	10/20	50.00
Lymph node -	10/25	40.00	3/12	25.00	10/20	50.00
<50 years	22/36	61.11	8/16	50.00	17/28	60.71
>50	14/36	38.89	8/16	50.00	11/28	39.29
IDC	34/36	94.44	15/16	93.75	26/28	92.86
ILC	2/36	5.56	1/16	6.25	2/28	7.14

IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer.

Table III-11-5. The associations between two immunostaining and clinic-pathological features

	p53-/HER+		p53+/Bcl-2-		p53+/HER-	
	No.	%	No.	%	No.	%
Total case	24/14	17.91	9/134	6.72	12/134	8.96
Mitotic rate 1	6/24	25.00	3/9	33.33	2/12	16.67
Mitotic rate 2	8/24	33.00	3/9	33.33	3/12	25.00
Mitotic rate 3	10/24	41.67	3/9	33.34	7/12	58.33
Differentiated 1	2/24	8.33	3/9	33.33	3/12	25.00
Differentiated 2	12/24	50.00	3/9	33.33	2/12	16.67
Differentiated 3	10/24	41.67	3/9	33.34	7/12	58.33
Lymph node +	3/17	17.65	5/7	71.43	4/8	50.00
Lymph node -	14/17	82.35	2/7	28.57	4/8	50.00
<50 years	13/24	54.17	4/9	44.44	4/12	33.33
>50	11/24	45.83	5/9	55.56	8/12	66.67
IDC	24/24	100.00	9/9	100.00	11/12	91.67
ILC	0/24	0.00	0/9	0.00	0/12	0.00
MA	0/24	0.00	0/9	0.00	0/12	0.00
Medullary Ca	0/24	0.00	0/9	0.00	1/12	8.33
Intraductal Ca	0/24	0.00	0/9	0.00	0/12	0.00
in situ lobular	0/24	0.00	0/9	0.00	0/12	0.00
IPC	0/24	0.00	0/9	0.00	0/12	0.00

IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer; Ca = cancer; MA = mucinous adenocarcinoma; Intraductal Ca = intraductal carcinoma; *in situ* lobular = *in situ* lobular carcinoma; IPC = infiltrating papillary carcinoma.

Table III-11-6. The associations between two immunostaining and clinic-pathological features

	p53-/Bcl-2+		HER+/Bcl-2-		HER-/Bcl-2+	
	No.	%	No.	%	No.	%
Total case	78/134	58.21	10/134	7.46	68/134	50.75
Mitotic rate 1	23/76	30.26	2/10	20.00	19/66	28.79
Mitotic rate 2	20/76	26.32	6/10	60.00	18/66	27.79
Mitotic rate 3	33/76	43.42	2/10	20.00	29/66	43.94
Differentiated 1	24/76	31.58	1/10	10.00	24/66	36.36
Differentiated 2	26/76	34.21	6/10	60.00	20/66	30.30
Differentiated 3	25/76	32.89	3/10	30.00	22/66	33.33
Lymph node +	24/34	70.59	7/7	100.00	20/38	52.63
Lymph node -	10/34	29.41	0/7	0.00	18/38	47.37
<50 years	40/74	54.05	6/9	66.67	37/68	54.41
>50	34/74	45.83	3/9	33.33	31/68	45.59
IDC	61/78	78.21	10/10	100.00	54/68	79.41
ILC	5/78	6.41	0/10	0.00	4/68	5.88
MA	3/78	3.85	0/10	0.00	3/68	4.41
Medullary Ca	0/78	0.00	0/10	0.00	1/68	1.47
Intraductal Ca	3/78	3.85	0/10	0.00	3/68	4.41
in situ lobular	2/78	2.56	0/10	0.00	2/68	2.94
IPC	1/78	1.28	0/10	0.00	1/68	1.47

IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer; Ca = cancer; MA = mucinous adenocarcinoma; Intraductal Ca = intraductal carcinoma; *in situ* lobular = *in situ* lobular carcinoma; IPC = infiltrating papillary carcinoma.

4.0.0 Molecular Analysis

4.1.0 HER/*neu* Gene Amplification Mutation

When tissues were examined for HER/*neu* (*c-erbB-2*) gene amplification, thirty-nine specimens (29.10%) had HER/*neu* gene amplification equal to and/or greater than two fold as detected by a differential PCR technique (Frye, 1989; Evers, 1992). Two folds of HER/*neu* gene amplification was the most frequent gene amplification mutation 21/39 (53.85%) compared with 3 fold 7/39 (17.95%), 4 fold 5/39 (12.82%), and greater than 4 fold 6/39 (15.38%). Relationships between HER/*neu* gene amplification rate and clinical information is shown in the tables III-12.

Table III-12-1. Amplification of HER/*neu* gene mutation related to clinical information

	Normal rate ≤1		HER/*neu* rate 2		HER/*neu* rate 3		HER/*neu* rate 4		INF/*neu* rate >4	
	No.	%	No.	%	No.	%	No.	%	No.	%
Total case	95/134	70.90	21/134	15.67	7/134	5.22	5/134	3.73	6/134	4.48
Grade 1	30/94	32.26	6/20	30.00	1/7	14.29	1/5	20.00	0/6	0.00
Grade 2	27/94	29.03	8/20	40.00	6/7	85.71	3/5	60.00	2/6	33.33
Grade 3	37/94	39.78	6/20	30.00	0/7	0.00	1/5	20.00	4/6	66.67
Mitosis 1	27/94	29.03	5/20	25.00	3/7	42.86	1/5	20.00	0/6	0.00
Mitosis 2	27/94	29.03	7/20	35.00	3/7	42.86	3/5	60.00	2/6	33.33
Mitosis 3	40/94	43.01	8/20	40.00	1/7	14.29	1/5	20.00	4/6	66.67
Lymph Node +	33/60	55.00	7/12	58.33	6/6	100	2/2	100.0	4/5	80.00
Lymph Node -	27/60	45.00	5/12	41.67	0/6	0.00	0/2	0.00	1/5	20.00
<50	47/95	49.47	13/21	61.90	4/7	57.14	2/3	66.67	1/6	27.78
>50	48/95	50.53	8/21	38.10	3/7	42.86	1/3	33.33	5/6	83.33
IDC	82/95	86.32	17/21	80.95	7/7	100.0	5/5	100.0	6/6	100
ILC	5/95	5.26	2/21	9.52	0/7	0.00	0/5	0.00	0/6	0.00
MA	2/95	2.11	1/21	4.76	0/7	0.00	0/5	0.00	0/6	0.00
Medullary Ca	1/95	1.05	0/21	0.00	0/7	0.00	0/5	0.00	0/6	0.00
Intraductal Ca	2/95	2.11	0/21	0.00	0/7	0.00	0/5	0.00	0/6	0.00
in situ lobular	2/95	2.11	1/21	4.76	0/7	0.00	0/5	0.00	0/6	0.00
IPC	1/95	1.05	0/21	0.00	0/7	0.00	0/5	0.00	0/6	0.00

IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer; MA = mucinous adenocarcinoma; Intraductal Ca = intraductal carcinoma; *in situ* lobular = *in situ* lobular carcinoma; IPC = infiltrating papillary carcinoma.

Table III-12-2. Amplification of HER/*neu* gene mutation related to clinical information

	Age				Cancer in Side of Breast				Lymph Node Metastasis			
	<50		>50		Left		Right		+		-	
	No	%	No	%	No	%	No	%	No	%	No	%
Total case	70	52.24	64	47.76	32	55.17	26	44.83	52	61.18	33	38.82
HER gene rate<1	26	37.14	46	71.88	13	40.63	11	42.31	30	57.69	28	84.85
HER gene rate 1	21	30.00	2	3.13	12	37.50	7	26.92	2	3.85	1	3.03
HER gene rate 2	13	18.57	1	1.56	7	21.88	6	23.08	1	1.92	1	3.03
HER gene rate 3	4	5.71	1	1.56	0	0.00	1	3.85	2	3.85	1	3.03
HER gene rate 4	4	5.71	4	6.25	0	0.00	1	3.85	4	7.69	0	0.00
HER gene rate>4	2	2.86	10	15.63	0	0.00	0	0.00	13	25.00	2	6.06
P value	0.263				0.550				0.013			

Fisher's Exact tests are used for all P value calculations. P value is comparison of HER gene rate ≤1 and ≥2.

Table III-12-3. Amplification of HER/*neu* gene mutation related to clinical information

	Cell Differentiation						Cell Mitotic Rate					
	Well		Moderate		Poor		Low		Moderate		High	
	No	%	No	%	No	%	No	%	No	%	No	%
Total case	38	28.79	46	34.86	48	36.36	36	27.27	42	30.95	54	40.91
HER gene rate<1	9	23.68	17	36.96	17	35.42	9	25.00	13	31.82	21	38.89
HER gene rate 1	21	55.26	10	21.74	20	41.67	18	50.00	14	33.33	19	35.19
HER gene rate 2	6	15.79	8	17.39	6	12.50	5	13.89	7	16.67	8	14.81
HER gene rate 3	1	2.63	6	13.04	0	0.00	3	8.33	3	7.14	1	1.85
HER gene rate 4	1	2.63	3	6.52	1	2.08	1	2.78	3	7.14	1	1.85
HER gene rate>4	0	0.00	2	4.35	4	8.33	0	0.00	2	4.76	4	7.41
P value #	*0.062		**1.000		***0.767		* 0.336		**1.000		***0.372	
P value ##	0.066						0.484					

P value is comparison of HER gene rate ≤1 and ≥2. # = Fisher's Exact tests are used. ## = Chi-square tests are used. * = the result of compared with Well (Low) and Moderate groups; ** = the result of compared with Well (Low) and Poor (High) groups; *** = the result of compared with Moderate and Poor (High) groups; **** = the result of compared with all groups.

How the amplification results for HER/*neu* gene mutations relate to clinical indictors were analyzed by Chi-square test. The characteristics that define in the table were not significantly related (P>0.05) except lymph node metastasis.

4.2.0 p53 Gene Mutations

A total of 4 cases (6.15%) from 65 samples were found to have p53 tumor suppressor gene mutations in our study. The features of p53 gene mutation and the mutations related to clinical and histopathological information are in the table III-13

Table III-13. p53 gene point mutation related to other information

Case No.	43	57	73	80
Age	62	52	64	39
Sex	female	female	female	female
No. of lymph node metastasis	2	18	5	19
Type of breast cancer	IDC	IDC	IDC	IDC
Cell differentiation	poor	poor	poor	well
Cell mitotic rate	high	high	high	low
Survive year	>5 years	>2 years	unknown	3 years
p53 immunostain	-	+	-	-
c-erbB-2 immunostain	+	+	-	+
Bcl-2 immunostain	+	+	-	+
HER/*neu* gene amplification mutation	+	-	-	-
p53 gene mutation	c→t	t→c	t→a	g→a
p53 gene mutation location (codon)	151 (exon5)	14436 (intron7)	309 (exon8)	248 (exon8)
Characteristic of Mutation	transition, CpG site	transition	transversion	transition non-CpG site
Amino acid sequence change	Pro→Ser (ccc→ tcc)	-	Cys→Ser (tgc→agc)	Arg (agg→aga)
Types of mutations	missense	intron	missense	silent

IDC = infiltrating ductal carcinoma

4.3.0 Correlation of Gene Mutation with Clinical Indicators

The relationship of p53 gene and HER/neu gene mutations with breast cancer clinical indicators were recorded and showed on the tables III-14.

Table III-14-1. p53 gene and HER/*neu* gene mutations related to clinical information

	Age				Ca in Breast				Lymph Node			
	<50		>50		Left		Right		+		-	
	No	%	No	%	No	%	No	%	No	%	No	%
p53 gene +	1	25.0	3	75.0	-	-	-	-	4	100	0	0.00
p53 gene -	37	60.7	24	39.3	23	57.5	17	42.5	18	72.0	7	28.0
P value	0.30				-				0.55			
HER gene +	23	32.86	16	25.00	7	21.88	8	30.77	19	36.54	6	18.18
HER gene -	47	67.14	48	75.00	25	78.13	18	69.23	33	63.46	27	81.82
P value	0.346				0.550				0.089			

Fisher's Exact tests are used for all P value calculations. Ca = carcinoma.

Table III-14-2. p53 gene and HER/*neu* gene mutations related to clinical information

	Cell Differentiation						Cell Mitotic Rate					
	Well		Moderate		Poor		Low		Moderate		High	
	No	%	No	%	No	%	No	%	No	%	No	%
p53 gene +	1		0		3		1		0		3	
p53 gene -	20		26		15		20		23		18	
P value #	0.446*		0.318**		0.062***		0.477*		0.606**		0.100***	
P value ##	0.074						0.137					
HER gene +	8	21.05	19	41.30	11	22.92	9	85.00	15	35.71	14	25.93
HER gene -	30	78.95	27	58.70	37	77.08	27	75.00	27	64.29	40	74.07
P value #	0.062*		0.077**		1.000***		0.336*		0.372**		1.000***	
P value ##	0.066****						0.484****					

Fisher's Exact tests are used for all P value calculations. ## Chi-square tests are used for all P value calculations. * = the result of compared with Well (Low) and Moderate groups; ** = the result of compared with Well (Low) and Poor (High) groups; *** = the result of compared with Moderate and Poor (High) groups; **** = the result of compared with all groups.

Table III-14-3. Sum of the results of the gene mutations and clinical information

	HER/*neu* gene with amplification (39/134)		Normal HER/*neu* gene (95/134)		p53 gene with mutation (4/65)		p53 gene with no mutaion (61/65)	
	No.	%	No.	%	No.	%	No.	%
Grade 1	8/38	20.5	30/94	31.9	1/4	25.0	20/61	32.8
Grade 2	19/38	50.0	27/94	28.7	0/4	0.00	26/61	42.6
Grade 3	11/38	29.0	37/94	39.4	3/4	75.0	15/61	24.6
Mitosis 1	9/38	23.7	27/94	28.7	1/4	25.0	20/61	32.8
Mitosis 2	15/38	39.5	27/94	28.7	0/4	0.00	23/61	37.7
Mitosis 3	14/38	36.8	40/94	42.6	3/4	75.0	18/61	29.5
Lymph Node +	21/27	77.8	33/60	55.0	4/4	100	18/46	39.1
Lymph Node -	6/27	22.2	27/60	45.0	0/4	0.00	2846	60.9
<50	23/39	59.0	47/95	49.5	1/4	25.0	37/58	63.8
>50	16/39	41.0	48/95	50.5	3/4	75.0	24/58	41.4
IDC	35/39	89.7	81/95	85.3	4/4	100	53/61	86.9
ILC	2/39	5.1	5/95	5.3	0.00	0.00	5/61	8.20
MA	1/39	2.6	2/95	2.1	0.00	0.00	1/61	1.64
in situ	1/39	2.6	1/95	1.1	0.00	0.00	0/61	0.00
Medullary Ca	0/39	0.00	0/95	0.00	0.00	0.00	0	0.00
Intraductal Ca	0/39	0.00	0/95	0.00	0.00	0.00	2/61	3.28

IDC = infiltrating ductal cancer; ILC = infiltrating lobular cancer; Ca = cancer; MA = mucinous adenocarcinoma; *in situ* = *in situ* lobular carcinoma.

VI. DISCUSSION

Earlier studies reported that Asian countries had a lower morbidity rate of breast cancer than Western countries. In particular, that patients with breast cancer in Asia have better prognosis than in the USA (Sakamoto, 1979). However, considerable caution is required when comparing Chinese and Western data through comparison of literature as the diagnostic criteria used often are different. Therefore, in this present study, the most commonly used criteria and methods are employed.

1.0.0 Comparison of Characteristics of High Risk (Western Countries) with Low Risk (Chinese) Breast Cancer Populations

Although breast cancer is globally the most frequent malignancy in women, international statistics reveal that the rates of breast cancer vary widely in different geographical areas.

1.1.0 General Aspects of Breast Cancer

1.1.1 Age Difference

The most frequent age of patients with breast cancer in the USA is 40-50 years (Potchen, 1993). The American Cancer Society's Cancer Prevention Study reported: 21% of breast cancer cases occur in the 30-54 age group and 79% of cases in the 55-84 age group (Seidman, 1982). Mettlin's (1994) study showed that only 4.8% of breast cancer in the United States occurred in women with the age of 30 –39, 17.8% with in ages 40-49, 23.8% of ages 50-59, 26.3% in ages 60 -70; and 2.66% in women older than 70; this report thus indicates that 95% of all breast cancer was found in women in the US who were older than 40. In the Leis's report, only 1.8% of breast cancers occur below the age of 30 and 4% below the age of 35 with 75% of breast cancers appearing after the age of

40 (Leis, 1970).

From the records of our 178 primary breast cancer patients, the mean and median ages of Chinese breast cancer patients is younger, than the findings from the Michigan State University (MSU) data bank (data not shown) and from that reviewed in the literature. In addition, the Student t test confirmed that there is a statistically significant difference in age distribution between Chinese primary breast cancer group and MSU group (personal observation). However, the obvious differences between these groups make such comparisons difficult, for example the patients from MSU group have non-palpable lesions and are in early stage of breast cancer; whereas the patients from Chinese group all have palpable lesions with tumor sizes from 1.3 to 12 cm (average size 4.5 cm), some with lymph node metastases; therefore they are at a later stage than those in MSU group.

It is clear from our results and observations that primary breast cancer occurs earlier in Chinese women than in the Western women. Reasons for this are undoubtedly complicated, and probably include the environment, life style, as well as genetic reasons. Further study and investigation of a larger sample size are needed to confirm these current findings.

Some factors may be similar between Western and Chinese women. The peak age distribution in both Chinese and the USA groups is in the over 40 years olds, which suggests that breast cancer may occur by 40 years or older that not only in the Western population, but also in the Chinese. By the age of 40 menopausal influences is a major involvement, supporting the contention that female hormones may play important roles in

both populations with regards oncogenesis.

1.1.2 Size of Tumor

Tumor size is one of the most important indicators for evaluating breast cancer development. The breast cancer specimens from China were all from breast surgical operations where the tumors were palpable masses. A 3 cm sized breast tumor is the most often detected size of tumor in physical examinations and this has been reported by Robbins (1994), Tavassoli (1990) and Lagios (1982) which similar to our study. Mammography screening has been widely used in the West for patients over 40 years old, and this has decreased the detectable size of breast cancer dramatically with many more *in situ* breast carcinomas detected than occurs in China.

1.1.3 Survival Rate

Only some of the Chinese breast cancer patients in this current study had followed up investigations involving more than five years. The Life Table analysis, a method for reporting clinical trials and presenting data from comprehensive registries, and Chi-square tests are both used for our data analyses (Mueller, 1994). The general five-year survival rate in our Chinese group is 47.33%. Others report breast cancer five-year survival rate in Chinese populations as 58.1% (826 cases) (Liu, 1992), 59% and 47.6% (626 cases) (Fisher, 1978). In the USA, the survival rates on primary breast cancer are 40.1% (1,335 cases) and 44% (1,700 cases). Rates in Southeast England were 42% (16,516 cases) and 43% (1,045 cases) (Mueller, 1994). The Mann-Whitney Rank Sum test analysis showed there is a statistically significant difference in survival rate between Chinese and Western breast cancer patients ($P = 0.0286$).

As previous stated, patients with breast cancer in Asia are known to have a better prognosis than that of the patients in the USA. In our present study, this figure seems to indicate that the Chinese have a higher five-year survival rate. However, the method of analysis, the size of the samples, the method of treatment and the other factors may effect the final result. The approach taken in our study will hopefully enrich knowledge of the breast cancer, and will serve as a reference for the breast cancer research and investigation of the future.

Analysis of our results showed that the patient's age does not effect her survival chance from breast cancer. Whether a patient has breast cancer at 20, or 40, or at 60 years of age does not appear to influence the patient's subsequent survival time. Support for this contention exists in the literature as to the age of breast cancer patients not being a good clinical prognostic indicator, as has be reported in the West for breast cancer in women (Potchen, 1993; Leis, 1970; Seidman, 1982; Mettlin, 1994). In summary, the age of breast cancer patient can not be used as a prognostic indicator in the Western or in the Chinese populations.

We also analyzed the effect of lymph node metastases from a primary breast cancer on patient survival rate. As expected, there was a significant difference between the primary breast cancer patients with and without lymph node metastases. The primary breast cancer patient with positive lymph node metastasis had a poor prognosis when compared with lymph node negative breast cancer patients. These results support the concept that lymph node metastases in primary breast cancer patients can be used as a general prognostic indicator for different racial groups, and a fact well accepted in the

West (Tavassoli, 1992).

In our study, the sample size of the patients with primary breast carcinoma and a five-year survival rate are too small to be fully analyzed. Only 3 cases of well differentiated, 2 of moderately differentiated, and 3 cases of poorly differentiated group were recorded for their five-year survival rate. For same reason the influence of the type of breast carcinoma on five-year survival rate, with only 17 cases of infiltrating ductal carcinoma, can not be adequately assessed.

1.2.0 Histopathology of Breast Cancer

In our study, one hundred seventy-eight primary breast cancer cases were evaluated for the type of breast cancer, the cell mitotic rate and the degree of cell differentiation and compared with Western breast cancer patients.

1.2.1 Types of Breast Cancer

The type of breast carcinoma is regarded as another important prognostic indicator. It can divide breast cancers into favorable or unfavorable types in relation to progression. The classifications of breast cancer in various countries used are not exactly same, even though WHO has a standard classification for breast tumors. The variety of breast cancer classifications and sampling methods used by different groups makes comparing diagnostic data between Chinese and Western breast cancer groups somewhat difficult. Thus we only used direct comparison for this part of study and those which followed WHO guide lines.

The frequencies of histological types of invasive breast cancer in three large series in the Western woman are showed in the table IV-1.

Table IV-1. The frequency of breast cancer types in the USA

Types of Breast Cancer	Fisher (1,000 cases)	Bartow (unknown No. of cases)	Rosen (1024 cases)
Infiltrating ductal cancer	81.0 %	52.6%	66.0 %
Infiltrating lobular cancer	5.00 %	4.90%	6.48%
Medullary carcinoma	6.00 %	6.20%	5.66%
Mucinous carcinoma	2.00%	2.40%	-
Tubular carcinoma	1.00 %	-	-
Others	5.00 %	5.90%	21.86%

(Fisher, 1975; Bartow, 1988; Rosen, 1982).

Invasive, or infiltrating, ductal carcinoma is by far the most common form of breast cancer in the world when considering all sources of data: for Chinese it is 85.96%, and for Western women reports range between 81% and 52.6%, with an average of 70%. There is apparently no racial influence on the type of tumor which is the most common. Infiltrating lobular carcinoma is the second most common type of breast cancer found by most observers, with all other types of breast cancer considerably less common. These studies demonstrated that racial grouping probably dose not influence the frequency of primary breast cancer types. In addition, when comparing the differences between these two racial groups, the average percentage of the infiltrating ductal cancers in Chinese and Western women by Chi-square test, had probability values with a wide range (0.098 to 0.0013). Comparison of the averages from these groups shows a statistically significant difference between the Chinese and the Western women ($P = 0.0454$). Furthermore, from our results, it is suggested that Chinese have a higher frequency of infiltrating ductal cancer than do the Western group.

1.2.2 Breast Cancer Histological Grading

The grade of a breast cancer is another important prognostic indicator though it is not usually used as a parameter in staging a tumor. The results from other Chinese investigators and from USA studies are summarized in table IV-2.

Table IV-2. The frequency of breast cancer grades

Cell Differentiation	MSU (134) (Chinese)	Liu (826) (Chinese)	Keshgegian (135) (Western)	Fisher (1000) (Western)
Well	33.5%	35.2%	31.0%	2.3%
Moderately	35.8%	49.9%	48.0%	28.1%
Poorly	30.7%	14.9%	21.0%	69.0%

(Keshgegian, 1995; Fisher, 1975; Liu, 1992).

The results from different reports are quite variant for both Chinese women as well as Western women, and statistical analysis shows that those groups are not a significantly different (all $P>0.05$). In a recent report, interobserver agreement in histological grading of breast cancer study has been investigated. Complete agreement in grade in different studies range from 54 - 80 %. An adequate training as a pathologist, the use of optimally fixed tissue, and adaptation of precise guidelines are, all useful quality guidelines for the diagnostic histopathologist to pursue. Fortunately, statistical studies of the relationship between grade and prognosis show a strong correlation, even with data collected from many pathologists in the USA, and even when different grading systems are used without standardized guidelines. However, it is inevitable that some disagreement in breast cancer histological grading will occur among the individual pathologists. Special training following proper guidelines for pathologists will be helpful for limiting the vary in the breast cancer grading (Robbins, 1995; Davis, 1986; Elston, 1991; Henson, 1991).

1.3.0 Immunohistochemical Studies

Immunohistochemical methods were used to detect p53 mutated protein, HER/*neu* (*c-erbB-2*) oncoprotein, and Bcl-2 oncoprotein in our investigation. The results were evaluated and a comparison made between Chinese breast cancer and reports of Western breast cancer.

1.3.1 General Aspect

The positivity of the immunohistochemical studies on protein overexpression from our Chinese breast cancer tissues are in the similar range to that of the Western (table IV-3).

Table IV-3. Comparison of different groups

Study	MSU (134) (Chinese)	Alsabeh* (208)	Luna-More* (33)	Marks* (230)	Ceccarelli* (291)
p53	14.18%	52.7%	12.1%	24%	30.6%
c-erbB-2	23.13%	18.9%	36.3%	17%	31.6%
Bcl-2	66.42%	79.3%	69.6%	-	85.9%

*The Western group. (Alsabeh, 1996; Marks, 1995; Luna-More, 1996; Ceccarelli, 1995)

Overexpression of p53 mutated tumor suppressor protein was very common in patients age of >50 regardless of the type of cancer. Similarly overexpression of p53 was common in these with the types of infiltrating ductal and medullary breast cancers, with the highest cell mitotic rate, with poor differentiation, and in the cancers with lymph node metastases regardless of age. However the data was not clearly statistically significant different and thus we report trend rather than a clear statistical differences.

HER/*neu* (*c-erbB-2*) oncoprotein overexpression was present in around half of both <50 and >50 year ie. apparent different with age. Overexpression was usual in those tumors of the well differentiated cell group, with poorly and moderated differentiated cell

groups being similar (45%). Overexpression appear and increase with mitotic rate. The most significant correlations were with stage of differentiation (P=0.027) and lymph node metastases (P=0.005). Lymph node positivity was strongly related to the presence of erbB2 positivity

Bcl-2 oncoprotein expression was present in similar frequency in the breast cancer patients age <50 and >50, with regard to breast cancer type the most commonly present in infiltrating ductal cancer, and with other forms presenting positive much less common in infiltrating lobular cancer, mucinous adenocarcinoma, intraductal carcinoma, *in situ* lobular carcinoma, infiltrating papillary cancer, and medullary carcinoma. Bcl-2 positivity was with common in those with the moderate cell mitotic rate, poorly differentiation, and the those cancers without lymph node metastasis (63.64%) compare with Bcl-2 negativity common with lymph node metastasis (62.50%). These results are not statistically significant different between Bcl-2 oncoprotein expression positive and negative, however, it gives us the useful information in breast cancer prognosis.

Results from different groups indicate that p53 protein overexpression occurs in 13% to 53% cases (Rosen, 1996a; Elledge, 1993). Several investigators failed to detect any significant correlation with age (Isola, 1992; Rosen, 1995a,b; Silvestrini, 1993), tumor size or stage (Isola, 1992; Rosen, 1995a,b). p53 expression was more frequent in ER-negative tumors (Isola, 1992; Rosen, 1995a,b; Iwaya, 1991).

HER/*neu* protein overexpression is an established marker for aggressive breast cancer in Western women (Van de Vijver, 1987; King, 1989; Adnane 1989). This is also true for our Chinese breast cancer group as it was associated with poorly differentiated

cancer and lymph node metastasis.

Bcl-2 protein overexpression was detected by an immunohistochemical method in our study; the positive results seem to be lower than cases studied in the West (Leek, 1994; Vanhaesebroeck, 1993; Joensu, 1994; Carson, 1993). An explanation may be the immuno-staining technique, itself, or perhaps racial differences could be in play. In addition, our study shows that Bcl-2 immuno-stain positivity appears to correlate with the poor prognostic indicator, lymph node metastasis, that has been reported in a study in Spain (Sierra, 1995). In contrast many studies have indicated that Bcl-2 presence relates to a good prognosis (Luna-More, 1996; Ceccarelli, 1995).

Protein overexpression by p53, *c-erbB-2* and Bcl-2 may not be of significant benefit to the pathologist in clinical diagnosis if used individually. However, if used in combinations they may have more value. They could be useful as an alternative method to confirm those which are not "clean cut" breast cancer cases, especially where the breast cancer cytological diagnosis (especially the needle biopsy) is becoming more and more popular.

From our experience, it is clear that standardized criteria for classifying and evaluating immunohistochemical results is urgently needed to allow comparison of various studies of the immunohistochemical studies today.

1.3.2 The Combination of Immunostaining Results

In our study, we compared the significance of associations between either all three or just two of p53, HER/*neu* and Bcl-2 protein overexpressions in each breast cancer case. Each combination group appears to have its own specific associations.

1.3.2.1 The Effects of Combining Three Immunostaining Results

When combining three protein overexpressions. p53-/HER-/Bcl-2+ was found to the most frequent combination pattern; and p53+/HER+/Bcl-2- was the least frequent combination pattern in breast cancer tissues (refer to the tables III-10). The combination of p53+/HER+/Bcl-2- pattern is the most common in breast cancer patient age of <50; and p53+/HER+/Bcl-2+ pattern relate to shows with the highest percentage at breast cancer patient age >50. p53+/HER+/Bcl-2- and p53-/HER+/Bcl-2- patterns are with same the highest percentage in the breast cancer cases with lymph node metastases, when p53-/HER+/Bcl-2+ pattern is with the highest percentage in the breast cancer cases without lymph node metastasis.

When all the three combinations are considered, the characteristics in the different combination pattern are found in the breast cancer patient age, the lymph node metastasis, and the feature of histopathology. All combination patterns were shown with the highest percentage in infiltrating ductal carcinoma; the combinations of p53+/HER+/Bcl-2+, p53+/HER+/Bcl-2-, p53+/HER-/Bcl-2-, and p53-/HER+/Bcl-2- patterns are detected in infiltrating ductal carcinoma type only. p53+/HER-/Bcl-2+ is with the highest percentage in medullary cancer; p53-/HER-/Bcl-2- is with the highest percentage in infiltrating lobular cancer; p53-/HER-/Bcl-2+ is found with the highest percentage in mucinous adenocarcinoma, intraductal carcinoma, in situ lobular carcinoma and infiltrating papillary carcinoma.

The characteristics of the histopathological evaluations, p53+/HER-/Bcl-2+ is with the highest percentage in the cell high mitotic rate, while p53+/HER+/Bcl-2- shown

with the highest percentage in the low mitotic rate; p53+/HER+/Bcl-2+ and p53+/HER-/Bcl-2+ showing with same the highest percentage in cell poorly differentiated group, and p53+/HER+/Bcl-2- is with the highest percentage in cell well differentiated group.

The information of the combination of p53, HER/*neu*, and Bcl-2 protein overexpression correlated with clinical, histopathological features, and lymph node metastases are not available in literature for the different population comparison.

1.3.2.2 The Effects of Combining Two Immunostaining Results

With the combining two of p53, HER/neu and Bcl-2 protein overexpression in the breast cancer are records. p53-/HER- pattern is the most frequent combination, and p53+/HER- pattern is least frequent combination in our present study.

Every combination of staining was seen, usually as the highest percentage, in infiltrating ductal carcinomas; indeed the combination patterns of p53+/HER+, p53-/HER+, p53+/Bcl-2-, and HER+/Bcl-2- were detected in infiltrating ductal carcinoma type only. The highest percentage of p53+/HER- showed in medullary cancer and in situ lobular carcinoma; HER-/Bcl-2+ was in infiltrating lobular cancer; and p53+/HER+ was found within intraductal carcinoma, *in situ* lobular carcinoma and infiltrating papillary carcinoma; and p53-/HER- was the most common in infiltrating papillary carcinomas.

With regards in the hisotopathological characteristics, p53+/Bcl-2+ was highest percentage in the group with the high cell mitotic rate, while p53+/Bcl-2- was seen with a low mitotic rate; p53+/HER- was associated with poor cell differentiation; and HER-/Bcl-2+ occurred with a high percentage of well differentiated cells.

The interaction of p53 and HER/*neu* has been extensively investigated, but

combination studies are not very common. Iwaya, et al reported that the majority of tumors were p53-/HER-, and that the least frequent combination was p53 +/HER+. Combinations of p53-/HER+ and p53+/HER- occurred with intermediate frequency (Iwaya, 1991); our data essentially showed the same results as the Iwaya group. Combined expression of p53 and HER was significantly associated with the histological characteristics of the carcinomas in the USA (Rosen, 1995a,b) and that was true also in the most cases in our studies. In addition, medullary carcinomas are almost always p53 +/HER- and very rarely p53- /HER+ in the Western group as well as in Chinese; infiltrating lobular carcinomas tend to be p53-/HER-.

The joint expression of p53 and HER was not found by others to be significantly related to prognosis in the USA; patients with the favorable stage, T1N0M0 and T2N0M0, were found to have a immunostain profile of p53+/HER+ (Rosen, 1995a,b, 1996a). Our study does not have enough information for such comparison.

1.4.0 Gene Mutation Studies

Genetic change is essential to the role of genotype and phenotype in process of oncogenesis. p53 tumor suppressor gene point mutations and in HER/*neu* oncogene amplification mutations are both investigated in this study.

1.4.1 HER/*neu* Gene Amplification

Several investigators have suggested that HER/*neu* (*c-erbB-2)* amplification is an independent variable predictive for shorter disease-free survival, high metastatic spread and time to give remove all tissue in breast (Dickson, 1987). The results of the molecular analysis of gene amplification procedures from different researches vary. The frequency of

gene amplification of HER/*neu* (*c -erbB-2*) averages 20-30% (Zhou, 1987; Clark, 1991; Gasparini, 1992; Guerin, 1988; Rosen, 1995b; Slamon, 1987; van de Vijver, 1987; Varley, 1987; Marks, 1995) with a range of 9% (Digiovanna, 1996) to 52% (Tandon, 1989).

Clinical and pathological studies of the expression of the HER/*neu* oncogene in human breast carcinomas have employed molecular and immunohistochemical procedures. Molecular analysis of gene amplification offers a more quantitative approach, and there appears to be a close correlation between amplification (reflected in increased copy number) and HER/*neu* immunohistochempositivity (Borg, 1990; Tsuda, 1990). Furthermore, the HER/*neu* gene amplification occurred in a higher frequency in primary breast cancers (22.5%), and in those breast cancers with lymph node metastases (50%). HER/*neu* gene amplification can thus be used as a molecular prognosis indicator for cancer (Borresen 1990).

From the results of our study, the Chinese population is seen to be no exception from other areas having a positive detection percentage in the same range of the USA and another Asian group, the Japanese (Tsuda, 1990). HER/*neu* gene amplification mutation tends to be more frequent in those whose age <50, with moderate differentiation, with moderate mitotic rate, with positive lymph node metastasis that frequency is higher than the Western group. However, the results are not strongly statistically significant different.

In addition, our study showed that HER/*neu* oncogene amplification mutation cases correlate with immunohistochemical stain positivity. Our semiquantifying evaluation of the immunohistochemical positivity (using HER/*neu* oncogene amplification >2 fold) suggests that the higher fold the gene is amplified, the higher level of the protein is

expressed. Immunohistochemical studies of HER/*neu* protein expression can thus be used to screen for HER/*neu* gene amplification. By the same token, the HER/*neu* gene amplification can provide information on HER/*neu* oncoprotein expression.

HER/*neu* gene amplification is thought to be a poor prognosis for breast cancer (Prost, 1994; Ravdin, 1995). It is our impression that HER/*neu* gene amplification can be used as a molecular marker and an independent predictor for breast cancer management and study. The number of amplifications of HER/*neu* gene seems to be not as important as the gene amplification mutation itself. In the present study, there are no statistically significant differences between different amplification rates (2 to >4 fold) for HER/*neu* gene amplification it related to age 50, cell differentiation, cell mitotic rate and lymph node metastasis.

In our study, there were more cases of HER/*neu* gene amplification mutation recorded than HER/*neu* protein overexpression. There are at least two possible explanations for this phenomenon. (1) the dPCR method is more sensitive in detection the gene DNA amplification mutation than the immuno-stain method used to detect the mutated protein; (2) there are other factors acting in the controlling mechanism pathway of gene expression, as has suggested from in vitro study (Slamon, 1989).

1.4.2 p53 Gene Point Mutation

Mutations in the human tumor suppressor gene p53 have been found in most human cancers. In breast cancer, the mutation in p53 have been proposed to occur in 16-50% and appeared to be a strong indicator of poor prognosis, independent of other risk factors (Callahan, 1992; Eyfjord, 1992; Friedrichs, 1993). Missense mutations are the

most common finding and more than 90% of missense mutations are clustered in four mutation "hot spot" regions (Pavletich, 1993).

In this study, p53 gene mutations have been detected in 4 cases (6%) of 65 Chinese primary breast cancer patients, a percentage which is lower than that in the USA. Our group detected two missense, 1 intron and 1 silent mutations. Three mutations in exons were associated with p53 immuno-stain negativity, however, the mutation in intron was associated with p53 immuno-stain positivity. Most breast cancer cases with p53 gene mutations were found in age >50 (75%), with infiltrating ductal carcinoma (100%), lymph node metastasis (100%), 75% with poorly differentiated cell and with a high cell mitotic rate. p53 gene point mutation was not statistically associated with its protein overexpression, lymph node metastasis, cell differentiation, and cell mitotic rate. A possible explanation is that only a few cases with positivity of p53 gene mutation were studied, and the sample size is too small to analyses.

In our study, the most common mutation type is the transition mutation (50%). One case mutation being found in a "hot spot" (25%). One case mutation was found in CpG site (25%), reportedly a less common mutation for Western women. One mutation was found at base 14436 in intron 7, with the expected T but a novel C. The primers used in our study were to detect exon mutation only, but with exon 8 primer also included an end part of intron 7, from 14404 to 14452; SSCP and both direction DNA sequencing confirmed this mutation. This intron mutation site has not been previously reported. Intronic point mutation was noted in 1990, one study identifying p53 intronic mutation in intron 3 where mutation inactivation of p53 tumor suppressor gene during cell

transformation in a cancer cell line occurred (Takahashi, 1990). Later, there were reports of intron 3, 4, and 10 mutations in breast cancer (Sjalander, 1996; Wazer, 1994, Buller, 1995). Our finding indicates that for detection of p53 gene mutation, intronic sequencing is also as important as exon sequencing.

2.0.0 Characteristics of p53, HER/*neu* (*c-erbB-2*)and Bcl-2 and Their Relationship to Oncogenesis

2.1.0 General Concept of Oncogensis

Foulds (1954) codified and expanded the concept of multistage oncogenesis. Evidence obtained twenty years after Rous's work indicating that the first stage, initiation, is characterized by damage to DNA, and showed that the second stage, promotion, usually does not involve damage to the DNA, but rather a stimulation of cellular proliferation. Prior to the promotional stage, the process is reversible and exhibits a distinct dose response and a measurable threshold. The third stage, progression, leads to morphological changes and increased grades of malignant behavior, such as invasion, metastasis, and drug resistance (Robbins, 1994). Carcinogenic agents can mutate two type of genes that regulate cell growth: proto-oncogenes that code for growth factors (Hirsh-Ginsberg, 1995), and tumor suppressor genes which code for growth suppression (Weinberg, 1991). Human carcinogenesis can involve several steps, including activation of an oncogene, inactivation of a tumor suppressor gene, and abnormal activation of a cell death suppressor gene.

The illustration below shows the contribution of genotype and phenotype to risk determination for cancer. It reveals the primary importance of genotype, which influences

not only the characteristics of the tumor but also those of the host. It also indicates the effect of the host on the tumor's characteristics, the oncogene, tumor suppressor gene and cell death suppressor gene mutations. The immune and endocrine systems are the most likely to be important modulating mechanisms. The magnitude of risk could be assessed in a more refined way either by a sophisticated integration of all known phenotypic characteristics or, alternatively, by pinpointing the crucial locus in the DNA - either a single gene of an unidentifiable factor which responsible for the phenotype. Integrated knowledge and quantification of the phenotypic and genotypic characteristics may provide the ultimate risk assessment (Ragaz and Ariel, 1989).

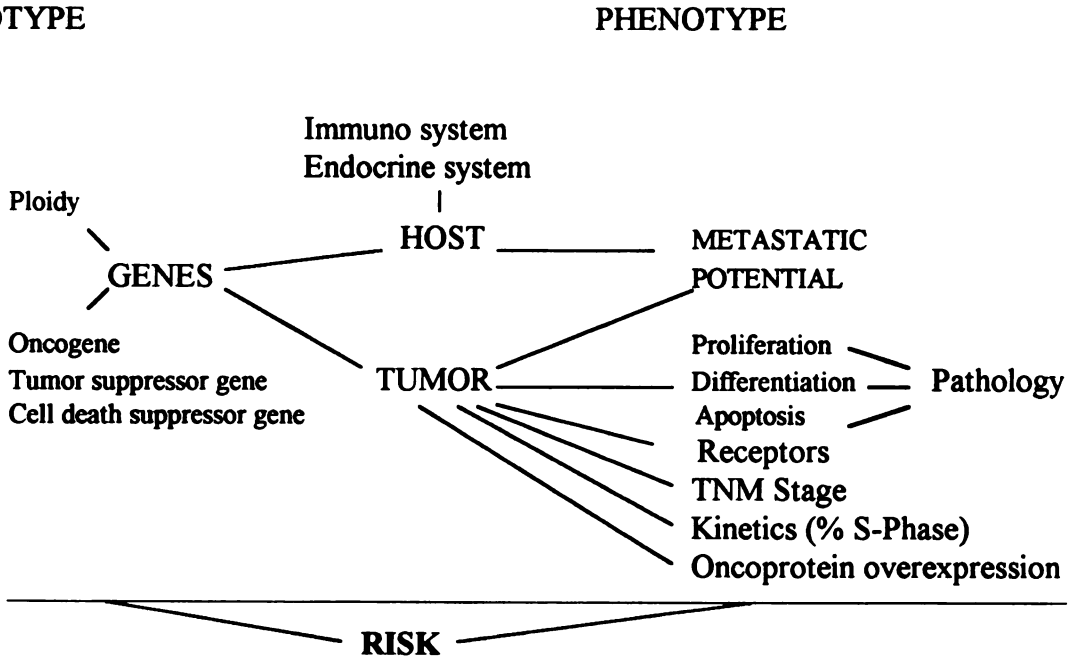

Figure 11. Contribution of geneotype and phenotype to risk determination.

The characteristics of the genotype and the phenotype are major players. In the understanding of the oncogenesis of breast cancer, behaviors of the p53 tumor suppressor

gene and HER/*neu* oncogenes, both genotype and phenotype, are investigated in this study. The gene product overexpression of the proto-oncogene, HER/*neu*, the tumor suppressor gene, p53; and the cell death suppressor gene, Bcl-2, which is also called proto-oncogene, are all investigated with our primary breast cancers. These elements have been reported to involve the alteration of breast genotype and phenotype in many complex ways. HER/*neu* acts as a transmembrane growth factor receptor pathway, whilst p53 and Bcl-2 regulate though the apoptosis pathway and they are affected and regulated in many ways.

2.2.0 Our Findings in Oncogensis

Our studies produced some interesting phenomena which may help to understand oncogenesis of breast cancer.

2.2.1 p53

The p53 mutated protein, the HER/*neu* (*c-erbB-2*) oncoprotein overexpression without p53 tumor suppressor gene point mutation and HER/*neu* oncogene amplification revealed in our study have been previously reported in human breast cancer as well as in established mammary carcinoma cell lines (Guerin, 1988; Kraus, 1987; Elledge, 1993). Many different mechanisms possibly explain the overexpression of a gene without gene mutation, and which involve the regulation of gene expression at the transcriptional level. p53 gene and HER/*neu* gene overexpression in the absence of gene point mutation or amplification mutation were found in a few of our cases, suggesting that mechanisms other than gene amplification or gene point mutation can contribute to gene overexpression. In vitro studies, involving the use of a novel transcription factor OB2-1, was found to be

required for HER/*neu* overexpression in several breast cancer cell lines (Hollywood, 1993). Also, the gene promoter, the hormone regulator, and transforming growth factor (TGF) are all involved in protein expression. The expression of human HER/*neu* gene has been shown to be inhibited by estrogens (Read, 1990; Dati, 1990; Russell, 1992) and by ligand-activated thyroid hormone and retinoic acid receptors (Hudson, 1990).

The intronic base mutation of p53 gene attracts attention recently. Our finding indicates that the p53 gene mutation in the intron may be related to p53 protein overexpression. It may serve as a marker of risk for malignancy or as a clinical prognosis indicator is uncertain and needs more complete researches.

2.2.2 HER/*neu*

Two interesting phenomena were found in our study. (1) The immuno-stain profile for HER/*neu* oncoprotein overexpression when compared with the cases that have, or do not have, HER/*neu* gene amplification mutation showed that HER/*neu* gene amplification was common in well and moderated cell differentiated groups, and when its oncoprotein overexpression frequent was more in poorly differentiated group. (2) The behaving of HER/*neu* gene amplification, by our record, suggests differently with protein expression during the carcinogenesis. This characteristic, such as, HER/*neu* gene amplification, is not correlated with other oncogenesis factors, such as p53 gene mutation, p53 or Bcl-2 protein overexpression. The question is whether the HER/*neu* gene amplification, which has a different mediate potential in HER/*neu* protein expression and this specific potential could effect the cancer cell differentiation, or this just shows the gene mutation present in early oncogenesis stage and the protein change in the later

cancergenesis stage. This information has not be reported before. (3) There is significant correlation between the number of HER/*neu* gene amplifications and HER/*neu* oncoprotein overexpression, indicating that with more copies of amplificated DNA sequence, there is more oncoprotein expression in the cancer cells. In addition, the number of HER/*neu* gene amplifications is not correlated with specific characteristics of genotype and phenotype. The impression seems to be that overexpression protein level is a more impotent change that occurs during the processing of breast oncogenesis. However, the phenotypic HER/*neu* gene is associated with lymph node metastases and as strong as the oncoprotein dose. This suggests that HER/*neu* gene mutation has the metastatic potential in the breast cancer.

2.2.3 Correlation of p53, HER/*neu* and Bcl-2

The third interesting finding relates to the basic fact that breast cancer oncogenesis has multiple elements involved and is a complicated process. Our consideration of joint protein expressions of p53, HER/*neu* and Bcl-2 in breast cancer provides novel information to help understand the mediating mechanisms. We found that p53, HER/*neu* and Bcl-2 are acting individually, and there are no apparent correlations among them. Each combination pattern had its specific feature related to clinical, pathological, and immunohistological evaluations. In the study of the combination of three immuno-stains, the combinations p53+/HER+/Bcl-2+ and p53+/HER-/Bcl-2+ indicated a bad breast cancer prognosis; with the cancer cells poorly differentiated, highest in mitotic rate, and likely to metastasize to lymph nodes, as well as tending to be of the infiltrating ductal cancer type. In the two immuno-stain combination, p53+/HER+ and p53+/Bcl-2+ were

associated with the worse characteristics of poor differentiation, high cell mitotic rate, a likelihood of lymph node metastasis and a tendency to be infiltrating ductal cancer. These results with multiple immuno-stain studies indicate that abnormal or lack of action in the regulation of the normal cell apoptosis pathway (p53 and Bcl-2) and a transmembrane growth factor receptor pathway (HER/*neu*) have a strong potential for oncogenesis. Features of multiple difference in breast phenotype from genotype are the worse patterns. However, many breast cancer cases in our study show that there is no evidence of an association with p53, HER/*neu*, or Bcl-2, either gene mutation or protein expression. This phenomenon may suggest the specific factors which we detected and their pathways are only involved in a particular period during oncogenesis procession. In addition, we observed that p53-/HER-/Bcl-2+ combination is the most frequent in our combinational analysis. That seems to demonstrate that Bcl-2 may play an alternative rule during oncogenesis; Bcl-2 is down regulated by p53 during cell apoptosis (Diebold, 1996; Haldar, 1994). Therefore, the t(14)(18) traslocation mutation of the Bcl-2 gene, with its mutated protein expression, seems to change more frequently during oncogenesis. That event may indicate that beside cell differentiation, the cell apoptosis regulation is very important for breast cancer oncogenesis.

2.2.4 The Feature of p53 Gene Point Mutation in Chinese Primary Breast Cancer

Finally, in our study 2 missense, 1 intron, 1 silent mutation were detected. The missense mutation is the most common mutation as reported in the USA. The intronic point mutation was detected in intron 7 this phenotypic characteristic has not been previously reported, even p53 gene mutation in intron 3, 4, 6 and 10 in cancer that have

been reported in a few studies (Merlo, 1994; Takahasi, 1990; Sjalander, 1996). The sequence features of the p53 gene point mutation found in case 80 which is an non-CpG site mutation in the "hot spot". By the contract, case 43, which is a CpG site, is not a common "hot spot" of p53 gene mutation location. However, both of the mutation are in the conserved domain area (Harris, 1996). An endogenous mutational mechanism due to deamination of the methylcytosine could be the cause of this mutation (see figure 12) (Antonarakis, 1996; Lehamn, 1994).

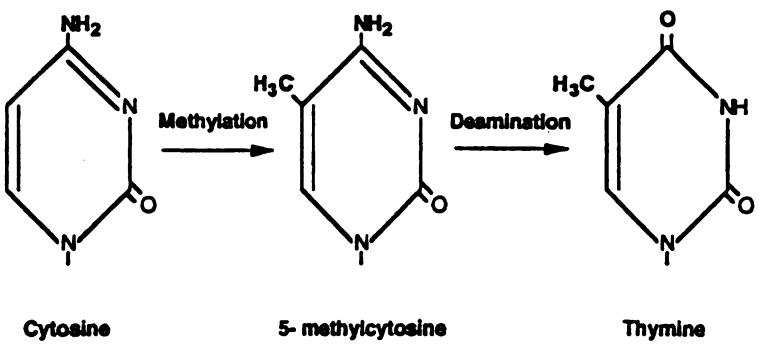

Figure 12. Schematic representation of structure of cytosine, 5'-methylcytosine and thymine, and the chemical events for the transformation of mutational mechanism for cytosine to thymine due to methylation and deamination reactions.

Both cases were not associated with mutated p53 protein overexpression. There could be three explanations: (1) gene mutation present earlier than protein does; (2) protein overexpression not associated with gene mutation; (3) the pathway was dysfunctional between gene mutation and mutated protein overexpression,.

Indeed, our data showing the differences and similarities between Chinese and Western women in terms of their breast cancer are still early evaluation. This underscores

the need for more studies such as those with large sample sizes, more complete clinical information, with long-term follow-up, in the near future for a better understanding of breast oncogenesis and the benefit for the breast cancer detection, diagnosis and treatment of molecular techniques.

3.0.0 Clinical Approach

Since p53 and HER/*neu* (*c-erbB-2)* protein overexpression occurs in less than 24% of our primary breast cancer specimens, and Bcl-2 appears in a high percentage of normal tissues, the immuno-staining can only be used to help pathologists in unclear diagnostic cases. Positive staining of p53, HER/*neu* and Bcl-2 can provide cancer prognostic information for the clinical management. Since there is no correlation among the overexpression of each protein that can be of benefit to pathologist on clinical diagnosis for use of them individually as a immunohistochemical tumor markers; or to use them in combinations to increase positive stain case numbers from p53's 14.18%, HER/*neu*'s 23.13% up to 32.09% (which is 1.27 fold higher than us p53 immuno-stain alone and 0.72 fold higher than by using HER/*neu* immuno-stain alone).

HER/*neu* gene amplification mutations detected by dPCR method provide more positive finding of primary breast cancer cases than does immunohisto-staining methods. It can thus be used as a molecular breast cancer marker for detecting and studying this gene in the breast cancer and the other malignant diseases.

Today, clinical screenings are becoming very important part in breast cancer management. It follows from a increasing number mammograms in breast cancer

screening, and the stereo-mammography guiding breast needle biopsy, that more cytology samples are expected for the pathologists. With the breast cytology samples, the pathological diagnosis is more difficult when compared with the surgical biopsy samples, p53 and HER/*neu* can be used as the immunohistological marker, and HER/*neu* gene amplification mutation could be used as the molecular marker to help the pathological diagnosis.

Immuno therapy and the gene therapy are novel treatments for the fight with the malignant diseases. The immunochemical and the molecular tumor markers are very important information for those therapies. p53 and HER/*neu* are the most common factors studied and are already used on immuno therapy and gene therapy (Hung, 1995; Roth, 1996). In a clinical or pathologic review, when a immunohistochemically positive case shows, this case most likely also has the HER/*neu* gene amplification mutation, and that can be a useful reference information for the gene therapy application in the future. Following the continuing maturity of those treatments, p53 and HER/*neu* genes and their proteins will be very useful clinically.

4.0.0. Technique Approach in The Study

The various types of tissue preparation, the different antibodies, and the lack of standardized criteria for classifying carcinomas by immunohistochemistry evaluation, makes comparisons very difficult.

For a large sample size breast cancer study, most specimens will probably be paraffin embedded tissues. Preparations needs on these specimens can vary and some

specimens may for technical reasons not be able to be worked with. Therefore, for immunohistochemical methods or molecular analysis, it is important to compare different methods and select the most suitable one.

By using differential PCR to analyze HER/*neu* gene amplification mutations, each PCR run and gel electrophoresis is compared with the normal controls and to known positive control sample to provide a confident interpretation.

Interpretation of SSCP is often difficult without experience. From our experiences, the pattern of denatured DNA bands are a major concerned for positive results. Whereas, the band intensity is not important in gene mutation finding.

5.0.0 Conclusion

The genotypic and phenotypic characteristics of breast cancer in Western (high risk) and Chinese (low risk) population are similar, such as, sex , tumor location of side breast, size of palpable tumor, survival rate of lymph node metastasis, type of tumor, grade of tumor, protein expression of p53 and HER/*neu* and HER/*neu* gene mutation. There are however variations between Western and Chinese populations in terms of phenotypic characteristics of breast cancer on age, survival rate, frequency distribution of tumor types, Bcl-2 protein expression, p53 gene point mutation.

Two missense, 1 intron and 1 silent, mutations of p53 gene were detected. p53 gene point mutation does not correlate with overexpression of its protein or clinical indicators. p53 protein overexpression was detected in those samples with the highest percentage of cell poor differentiation and cell mitotic rate, and had showed in infiltrating ductal and medullary carcinomas. HER/*neu* gene amplification mutation is correlated with

HER/*neu* oncoprotein overexpression. HER/*neu* gene mutation tends to occur in moderately differentiated tumors, and its oncoprotein tends to be over-expressed in the group with poor cell differentiation. HER/*neu* gene amplification mutations occurred in infiltrating ductal, infiltrating lobular carcinoma, mucinous adenocarcinoma as well as in situ lobular carcinomas. HER/*neu* protein overexpression was detected in infiltrating ductal carcinoma only. HER/*neu* gene amplification mutation and its product overexpression is strongly correlated with lymph node metastasis. Bcl-2 oncoprotein overexpression is correlated with lymph node metastasis and was seen in infiltrating ductal, infiltrating lobular, in situ lobular, medullary, intraductal carcinoma and mucinous adenocarcinomas. p53, HER/*neu* and Bcl-2 protein overexpressions are not correlated to each other in breast cancer tissues.

p53-/HER-/Bcl2+ and p53-/HER- are the most frequent combination patterns; p53+/HER+/Bcl-2- and p53+/HER- are the least frequent combination patterns. p53+/HER+/Bcl-2+ , p53+/HER-/Bcl-2-, p53+/HER+ and p53+/Bcl-2+ were associated with the poor in breast cancer prognosis and considered as a "bad indicator" for a breast cancer patient

V. REFERENCE

Addison C, Jenkins JR, Sturzbecher HW: 1990. The p53 nuclear localization signal is structurally linked to a p34cdc2 kinase mortification. *Oncogene* 5:423-426.

Adnane J, Gaudray P, Simon M, et al: 1989. Proto-oncogene amplification and human breast tumor phenotype. *Oncogene* 4:1389-1395.

Aguilar F, Hussain SP, Cerutti P: 1993. Aflatoxin B1 induces the transversion of G→T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proceedings of National Academy of Science of the United States of America* 90:8586-8590.

Akiyama T, Sudo C, Ogawara H, et al: 1986. The product of the human *c-erbB-2* gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* 232:1644-1646.

Ali IU, Campbell G, Libereau R: 1988. Lack of evidence for the prognostic significance of *c-erbB-2* amplification in human breast carcinoma. *Oncogene Research* 3:139-146.

Allan S, Dean D, Fernando I, et al: 1993. Radioimmunolocalisation in breast cancer using the gene product of *c-erbB2* as the target antigen. *British Journal of Cancer* 67:706-712.

Almoguera C, Shibata D, Forrester K, et al: 1988. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell* 53:549-554.

Alsabeh R, Wilson CS, Ahn CW, et al: 1996. Expression of bcl-2 by breast cancer: a possible diagnostic application. *Modern Pathology* 9:439-444.

American Cancer Society: 1975. A symposium sponsored by the American Cancer Society and the National Cancer Institute on Nutrition in the Causation of Cancer. *Cancer Research* 35:3231-3543.

American Cancer Society, Silverberg E, Lubera A: 1988. Cancer statistics. *CA: A Cancer Journal for Clinicians* 38:5-22.

Anderson DE, Badzioch MD: 1986. Survival in familial breast cancer patients. *Cancer* 58:360-365.

Anderson DE: 1977. Breast cancer in families. *Cancer* 40:1855-1860.

Andoh Y, Kimijima I, Takita K, et al: 1991. Flow cytometric DNA analysis using fine needle aspiration sample in breast cancer. *Journal of Japan Surgical society* 92:228-232.

Antonarakis SE: 1996. Mutations in human disease: nature and consequences. In *Principles and Practice of Medical Genetics* Third Edition. Edited by Rimoin DL, Connor JM and Pyeritz RE. Churchill Livingstone. pp53-66.

Antoniades HN, Bravo MA, Galanopoulos T, et al: 1990. Platelet-derived growth factor in idiopathic pulmonaryfibrosis. *Journal of Clinic Investigation* 86:1055-1064.

Armitage P, Gerry G: 1994. Probability. In *Statistical Methods in Medical Research* Edited by Armitage P and Berry G. Third Deition. Blackwell Scientific Publications. pp41-76.

Arends MJ, Wyllie AH: 1991. Apoptosis: mechanisms an roles in pathology. *International Review of Experimental Pathology* 32:223-254.

Ashton PR, Hollingsworth AS Jr, Johnston WW: 1975. The cytopathology of metastatic breast cancer. *Acta Cytologica* 19:1-6.

Azavedo E, Svane G, Auer G: 1989. Stereotactic fine-needle biopsy in 2294 mammographically detected nonpalpable lesions. *Lancet* 1:1033-1036.

Azzopardi JG, Chepick OF, Hartmann WH et al: 1982. The World Health Organization histological typing of breast tumors-second edition. *American Journal of Clinical Pathology* 78:806-816.

Bafy G, Miyashita T, Williamson JR, et al: 1993. Apoptosis induced by with drawl of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced *Bcl-2* oncoprotein production. *Journal of Biologic Chemistry* 286:6511-6519.

Bair B and Cayleff S: 1993. Part 4 Breast cancer: images of self. In *Wings of Gauze Women of Color an the Experience of Health and Illness*. Edited by Bair, Barbara and Cayleff, Susan E. Wayne State University Press. Detroit. pp241-243.

Baker SJ, Preisinger AC, Jessup JM, et al: 1990. P53 gene mutations occur in combination with 17 p allelic deletions as late events in colorectal tumorgenesis. *Cancer Research* 50:7717-7722.

Baker LH: 1982. Breast cancer detection demonstration project: five-year summary report. *Cancer* 32:196-225.

Barbareschi M, Leonardi E, Mauri FA, et al: 1992. P53 and erbB-2 protein expression in breast carcinomas. *American Journal of Clinical Pathology* 98:408-418.

Barbieri F, Parodi G, Massa T, et al: 1994. Feasibility of thymidine labeling index on fine needle aspirates from breast cancer: comparison with surgical samples. *Anticancer Researches* 14(5A):1973-1977.

Barry MA, Behnke CA, Eastman A: 1990. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochemical Pharmacology* 40:2353-2362.

Bargmann CI, Hung M-C, Weinberg RA: 1986. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* 319:226-230.

Bargmann CI, Weinberg RA: 1988a. Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene. *Proceedings of National Academy of Science of the United States of America* 85:5394-5398.

Bargmann CI, Weinberg RA: 1988b. Oncogenic activation of the neu-encoded receptor protein by point mutation and deletion *Engineering in Medicine and Biology Journal* 7:2043-2052.

Bargmann CI, Hung M-C, Weinberg RA: 1986. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* 319:226-230.

Bartow SA and Fenoglio-Preiser C: 1988. The breast. In *Pathology* Edited by Rubin E and Farber JL. J.B Lippincott Company Philadelphia pp990-1013.

Basset-Seguin W, Escot C, Moles JP, et al: 1991. *c-fos* and *c-jun* proto-oncogene expression is decreased in psoriasis: a *in situ quantitative analysis. Journal of Investigation of Dermatology* 97:672-678.

Bedrossian CWM: 1991a. Epithelial Malignancies. In *Malignant Effusions A Multimodal Approach to Cytologic Diagnosis* Edited by Bedrossian, C.W.M. IGAKU-SHOIN Medical Publishers, Inc. New York. pp120-147.

Bedrossian CWM: 1991b. In search of the primary site. In *Malignant Effusions A Multimodal Approach to Cytologic Diagnosis* Edited by Bedrossian, C.W.M. IGAKU-SHOIN Medical Publishers, Inc. New York. pp190-210.

Berchuck A. Kamel A, Whitaker R, et al: 1990. Overexpression of HER/*neu* is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Research* 50:4187-4091.

Berg FM, Baas IO, Polak OO, et al: 1993. Detection of p53 overexpression in routinely paraffin-embedded tissue of human car5cinomas using a novel target unmasking fluid. *American Journal of Pathology* 142:381-385.

Berg JW: 1984. Clinical implications of risk factors for breast cancer. *Cancer* 53:589-591.

Berndt H, Titze U: 1969. TNM clinical stage classification of breast cancer. *International Journal of Cancer* 4:837-844.

Betta PG, Bottero G, Pavesi M, et al: 1993. Cell proliferation in breast carcinoma assessed by a PCNA grading system and its relation to other prognostic variables. *Surgical Oncology* 2:59-63.

Binder C, Marx D, Binder L, et al: 1996. Expression of Bzx in relation to *Bcl-2* and other predictive parameters in breast cancer. *Annals of Oncology* 7:129-133.

Binder M., Tourmente S, et al: 1986. *in situ* hybridization at the electron microscope level: localization of transcripts on ultrathin sections of Lowicry K4M-embedded tissue using biotinylated probes and protein A-Gold complexes. *Journal of Cell Biology* 102:1646-1653.

Birch JM, Horrley AL, Tricker KJ, et al: 1994. Prevalence and diversity of constitutional mutations in the p53 gene among 21 Li-Fraumeni families. *Cancer Research* 54:1298-1304.

Birch JM: 1990. The Li-Fraumeni cancer family syndrome. *Journal of Pathology* 161:1-2.

Bishop JM: 1983. Cellular oncogenes and retroviruses. *Annals Review of Biochemistry* 52:301-354.

Bishop JM.: 1991. Molecular themes in oncogenesis. *Cell* 64:235-248.

Bissonnette RP, Echeverri F, Mahboubi A: 1992. Apoptotic cell death induced by *c-myc* is inhibited by *Bcl-2*. *Nature* 359:552-556.

Blaszyk H, Vaughn CB, Hartmann A, et al: 1994. Novel pattern of p53 gene mutations in an American black cohort with high mortality from breast cancer. *Lancet* 343:1195-1197.

Bonadonna G, Valagussa P: 1981. Dose-response effect of adjunct chemotherapy in breast cancer. *New England Journal of Medicine* 304:1-15.

Bonnier P; Charpin C; Lejeune C, et al: 1995. Inflammatory carcinomas of the breast: a clinical, pathological, or a clinical and pathological definition? *International Journal of Cancer* 62:382-385.

Borg A, Tandon AK, Sigurdsson H, et al: 1990. HER/*neu* amplification predicts poor survival in node-negative breast cancer. *Cancer Research* 50:4332-4337.

Boring CC, Squires TS, Tong T: 1992. Cancer statistics. *CA*: *A Cancer Journal for Clinicians* 42:19-38.

Boyd NF: 1987. The epidemiology of cancer: principles and methods. In *The Basic Science of Oncology*. Edited by Hill RP, Tannock LF, Pergamon Books, Inc. pp1-4.

Borresen AL, Ottestad L, Gaustad A, et al: 1990. Amplification and protein over-expression of the neu/HER-2/c-erbB-2 protooncogene in human breast crcinomas: relationship to loss of gene sequences on chromosome 17, family history ad prognosis. *British Journal of Cancer* 62:585-90.

Bragmann CI Hung M, Weinberg RA: 1986. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* 45:649-657.

Brash DE, Rudolph JA, Simon JA, et al: 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proceedings of National Academy of Science of the United States of America* 88:10120-10128.

Bressac B, Kew M, Wands J, et al: 1991. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 350:429-431.

Callahan R, Cropp CS, Merlo GR, et al: 1992. Somatic mutations an dhuman breast cancer. *Cancer* 69:1582-1588.

Camplejohn RS: 1993a. A role for proliferative measurements in clinical oncology? *Annals of Oncology* 4:184-186.

Camplejohn RS, Brock A, Barnes DM, et al: 1993b. A novel proliferative marker: flow cytometric assessment of staining in human breast carcinoma cells. *British Journal Cancer* 67:657-662.

Camplejohn RS: 1992. Flow cytometry in clinical pathology. In *Diagnostic Molecular Pathology A Practical Approach* volume I. Edited by Herrington CS and McGee JO'D IRL PRESS pp239-258.

Carson DA, Ribeiro JM: 1993. Apoptosis and disease. *Lancet* 34:1251-1254.

Carter CL, Allen C, Henson DE: 1989. Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. *Cancer* 63:181-187.

Carter D: 1990. Biopsy. In *Interpretation of Breast Biopsy 2nd Edition Biopsy Interpretation Series* Edited by Carte D. Raven Press New York pp9-28.

Ceccarelli C, Santini D, Chieco P, et al: 1995. Multiple expression patterns of biopathological markers in primary invasive breast cancer: a useful tool for elucidating its biological behavior. *Annals of Oncology* 6:275-282.

Chang F, Syrjanen, Kurvinen K, et al: 1993. The p53 tumor suppressor gene as a common cellular target in human carcinogenesis. *American Journal of Gastroenterology* 88:174-1186.

Chen YH, Dong JP, Zhang SJ: 1994. Detection of point mutation of p53 gene by non-isotopic PCR-SSCP in paraffin-embedded breast cancer tissue. Chung Hua Ping Li Hsueh Tsa Chih 23:327-329.

Chen RH, Fuggle SV: 1993. *in situ* cDNA polymerase chain reaction a novel technique for detecting mRNA expression. *American Journal of Pathology* 143:1527-1534.

Chiou SK, Rao L, White E: 1994. *Bcl-2* blocks p53-dependent apoptosis. *Molecular and Cellular Biology*. 14:2556-2563.

Ciatto S, Biggeri A, Del Turco CR, et al: 1990. Risk of breast subsequent to proven gross cystic disease. *The European Journal of Cancer* 26:555-557.

Clark GM, McGuire WL: 1991. Follow-up study of HER/*neu* amplification in primary breast cancer. *Cancer Research* 51:1944-1948.

Cleary MI, Smith SD, Sklar J: 1986. Cloning and structural analysis of cDNAs for *Bcl-2* and a hybrid *Bcl-2*/immunoglobulin transcript resulting from the t(14:18) translocation. *Cell.* 47:19-28.

Cohen RJ, Cooper K, Haffejee Z, et al: 1995. Immunohistochemical detection of oncogene proteins and neuroendocrine differentiation in different stages of prostate cancer. *Pathology* 27:229-232.

Cole SPC, Bhardwaj G, Gerlach JH, et al: 1992. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 258:1650-1654.

Coles C, Thompson A, Elder P, et al: 1990. Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. *Lancet* 336:761-763.

Collins N, McManus R, Wooster R, et al: 1995. Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12-13. *Oncogene* 10:1673-1675.

Cope FO, Wille JJ: 1991. Carcinogenesis and apoptosis: paradigms and paradoxes in cell cycle and differentiation. In *Apoptosis: The Molecular Basis of Cell Death*. Edited by Tome LD, Cope FO. Cold Spring Harbor Laboratory Press. pp61-86.

Corkill EM, Katz R: 1994. Immunocytochemical staining of *c-erbB2* oncogene in fine-needle aspirates of breast carcinoma: a comparison with tissue sections and other breast cancer prognostic factors. *Diagnosis of Cytopathology* 11:250-254.

Cotran RS, Kumar V, Ribbins SL: 1994. Chapter twenty-four. The breast. In *Robbins, Pathologic Basis of Disease*, 5th Edition. Edited by Schoen FJ. W.B. Saunders Company pp1089-1111.

Cotter TG, Glynn JM, Echeverri F, et al: 1992. The induction of apoptosis by chemotherapeutic agents occurs in all phases of the cell cycle. *Anticancer Research* 12:773-780.

Coussens L, Yang-Feng TL, Liao YC, et al: 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 230:1132-1139.

Crawford DC, Pim DC, Bulbrook RD: 1982. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. *International Journal of Cancer*. 30:403-408.

Cullen IS: 1895. A rapid method of making permanent specimens from frozen sections by the use of formalin. *Bull J Hopkins Hospital* 6:67-73.

Danforth D, Lichter AS, Lippmann ME: 1988. The diagnosis of breast cancer In *Diagnosis and Management of Breast Cancer* Edited by Lippmann ME, Lichter AS, and Danforth D. WB Saunders, Philadelphia. pp50-94.

Dati C, Antoniotti S, Taverna D, et al: 1990. Inhibition of *c-erbB-2* oncogene expression by estrogens in human breast cancer cells. *Oncogene* 5:1001-1006.

Davis BW, Gelber RD, Goldhirsch A, et al: 1986. Prognostic significance of tumor grade in clinical trials of adjutant therapy for breast cancer with axillary lymph node metastasis. *Cancer* 58:2662-2670.

de Graaf H, Willemse P, Ladde BE, et al: 1994. Evaluation of a cytological scoring system for predicting histological grade and disease-free survival in primary breast cancer. *Cytopathology* 5:294-300.

de Moulin D: 1983. A short history of breast cancer. The Hague, Martinus Nijhoff, pp99.

DeLellis RA, Sternberger LA, Mann RB, et al: 1979. Immunoperoxidase techniques in diagnostic pathology. *American Journal of Clinical Pathology* 71:83.

D'Emilia J, Bulovas K, D'Ercole K, et al: 1989. Expression of the c-erbB-2 gene product (p185) at different stages of neoplastic progression in the colon. *Oncogene* 4:1233-1239.

Dhingra K, Hittelman WN, Nortobagyi GH: 1995. Genetic changes in breast cancer - consequences for therapy? *Gene* 159:59-63.

Dickson RB, Aitken S, Lippmen ME, et al: 1987. Part II: Peptide Growth Factors. Assay of mitogen-induced effects on cellular thymidine incorporation. In *Methods in Enzymology Hormone Action*. Edited by Barnes D, Sirbasku DA . New York, Academic Press, pp327-430.

Diebold J, Baretton G, Felchner M, et al: 1996. Bcl-2 expression, p53 accumulation, and apoptosis in ovarian carcinomas. *American Journal of Clinical Pathology* 105:341-349.

Digiovanna MP, Carter D, Flynn SD, et al: 1996. Functional assay for HER-2/neu demonstrates active signalling in a minority of HER-2/neu-over expressing invasive human breast tumours. *British Journal of Cancer* 74:802-806.

Diller L, Kassel J, Nelson CE, et al: 1990. P53 function as a cell cycle control protein in osteosarcomase. *Molecular and Cellular Biology* 10:5772-5781.

Dive C, Hickman JA: 1991. Drug-target interactions: only the first step in the commitment to programmed cell death? *British Journal of Cancer* 64: 192-196.

Dmochowski L, Seman G, Gallager H.S: 1969. Viruses as possible etiologic factors in human breast cancer. *Cancer* 24:1241-1249.

Dmochowski L: 1972. Viruses and breast cancer. *Hospital Practice* 7:73-81.

Doll R, Peto P: 1981. The causes of cancer in the United States today. *Journal of the National Cancer Institute* 66:1192-1196.

Donegan EL: 1977. The influence of untreated internal mammary metastases upon the course of mammary cancer. *Cancer* 39:533-538.

Donehower LA, Harvey M, Slagle BL, et al: 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature* 356.215-221.

Douc-Rasy S, et al: 1994. Association of c-erbB2-gene amplification with poor prognosis in non-inflammatory breast carcinomas but not in carcinomas of the inflammatory type. *International Journal of Cancer* 58:763-768.

Dowlatshahi K, Jokich PM, Schmidt R, et al: 1987. Cytologic diagnosis of occult breast lesions using stereotaxic needle aspiration. A preliminary report. *Archives of Surgery* 122:1343-1346.

Drebin AJ, Stern DF, Link VC, et al : 1984. Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene. *Nature* 321:545-548.

Dunn JE: 1977. Breast cancer among American Japanese in the San Francisco Bay area. *National Cancer Institute Manager* 7:157-166.

Dyson JED, Simmons DM, Daniel J, et al: 1986. Kinetic and physical studies of cell death induced by chemotherapeutic agents or hyperthermia. *Cell and Tissue Research* 19:311-324.

Elledge, RM, Fuqua SA, Clark GM, et al: 1993. The role and prognostic significance of p53 gene alterations in breast cancer. *Breast Cancer Research and Treatment* 27:95-102.

Elliott J: 1822. Sarcomatous tumour of the breast successfully removed. *London Medicine Physical Journal* 48:305-308.

Ellis RE, Yuan J, Horvitz HR: 1991. Mechanisms and functions of cell death. *Annals Review of Cell Biology*, 7:663-698.

Elston CW, Ellis IO: 1991. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. *Histopathology* 19:403-410.

Elston CW: 1987. Grading of invasive carcinoma of the breast. In *Diagnostic Histopathology of the Breast.* Edited by Page DL and Anderson TJ. Churchill Livingstone. Edinburgh London Melbourne and New York. pp300-311.

Engell HC: 1955. Cancer cells in the circulating blood. *Acta Chirurgiae Scandinavica* 207:1-70.

Ening MG, Munn RJ, Keeney M: 1978. Dietary fats and cancer trends-a critique. *Federation of Proceeds* 37:2215-2220.

Evers BM, Rady PL, Tyring SK, et al: 1992. Amplification of the HER/neu protooncogene in human endocrine tumors. *Surgery* 112:211-218

Eyfjord JE and Thorlacius S: 1992. Genetic changes in breast carcinomas in an Icelandic population. *Pharmacogenetics* 2:309-316.

Fanidi A, Harrington EA, Evan GI: 1992. Cooperative interaction between c-my and *Bcl-2* proto-oncogenes. *Nature* 359:554-556.

Farrow SN, White JHM, Martinou I, et al: 1995. Cloning of *Bcl-2* homologue by interaction with adenovirus E1B 19 K. *Nature* 374:731-733.

Fearon ER, Vogelstein B: 1990. A genetic model for colorectal tumorigenesis. *Cell* 61:759-767.

Feldman PS, Covell JL: 1985. Technique and Interpretation. In *Fine Needle Aspiration Cytology and its Clinical Applications: Breast & Lung*. American Society of Clinical Pathologists Press. Chicago. pp25-44.

Feto M, Jaeger U, Hockett RD, et al : 1988. Alternative promoters and exons, somatic mutation and deregulation of the *Bcl-2*-Ig fusion gene in lymphoma. *Engineering in Medicine and Biology Journal* 7:123-131.

Finlay CA: 1993. Chapter 17. Normal and malignant growth control of p53. In oncogene and Tumor Suppressor genes in human malignancies. Edtied by Benz CC and Liu ET. By Kluwer Academic Publisher: Boston. pp327-344.

Finlay C, Hinds P, Levine A: 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell* 57:1083-1093.

Finlay C, Hinds P, Tan T, et al: 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Molecular and Cellular Biology* 8:531-539.

Fisher TC, Milner AE, Gregory ED et al: 1993. *Bcl-2* modulation of apoptosis induced by anticancer drugs: resistance pathways. *Cancer Research* 53:3321-3326.

Fisher B: 1978. Cancer of the breast. In *Cancer in China.* Edited by Kaplan HS and Tsuchitani PJ. ALAN R.LISS, INC. New York. pp143-147.

Fisher B, Slack N, Katrych D, et al: 1975. Ten year follow-up results of patients with carcinoma of the breast in a cooperative clinical trial evaluating surgical adjutant chemotherapy. *Surgery, Gynecology and Obstetrics* 140:528-534.

Fisher B, Slack NH: 1970. Number of lymph modes examined and the prognosis of breast carcinoma. *Surgery, Gynecology and Obstetrics* 131:79-88.

Fleming KA, Morey AL, Yap EPH: 1994. Genetic analysis of malignancy in histopathological material using filter and *in situ* hybridization. In *Molecular Biology in Histopathology* Edited by Crocker J. JOHN WILEY & SONS. pp15-38.

Fobbins CK: 1993. Neoplasia. In *Pathology Basis of Disease*. W.B.Saunders Company. pp239-435.

Forrest SP: 1990. Questions of morbidity and cost. In *Breast Cancer: the Decision to Screen*. Edited by Forrest SP. Nuffield Provincial Hospitals Trust London. pp147-178.

Foulds L: 1954. A Review. *Cancer Research* 14:327-339.

Friedman PN, Kern SE, Vogelstein B, et al: 1990. Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. *Proceedings of National Academy of Science of the United States of America* 87:9275-9279.

Friedrichs K, Gluba S, Eidtmann H, et al: 1993. Overexpression of p53 and prognosis in breast cancer. *Cancer* 72:3641-3647.

Frye RA, Benz CC, Liu E: 1989. Detection of amplified oncogenes by differential polymerase chain reaction. *Oncogene* 4:1153-1157.

Fukushige SI, Matsubar KI, Yeshiva M, et al: 1986. Localization of novel v-erbB-2 related gene, *c-erbB-2*, on human chromosome 17 and its amplification in a gastric cancer cell line. *Molecular and Cellular Biology* 6:955-058.

Fukushima T, Onda M, Abe R, et al: 1995. P53 mutations and overexpressions in Japanese breast cancer. *European Journal of Surgical Oncology* 21:595-600.

Gan FY, Luk GD, Gesell MS: 1994. Nonradioactive in situ hybridization techniques for routinely prepared pathology specimens and cultured cells. *The Journal of Histotechnology* 17:313-319.

Gannon JV, Lane DP: 1987. P53 and DNA polymerase alpha compete for binding to SV40 T antigen. *Nature*. 329:456-458.

Garcia G, Dietrich M, Aapro M, et al: 1987. Genetic alterations of c-myc, cerbB-2, and cHa-ras protooncogenes and clinical associations in human breast carcinomas. *Cancer Research* 49:6675-6679.

Gasparini G, Gullick WJ, Bevilacqua P, et al: 1992. Human breast cancer. Prognostic significance of the *c-erbB-2* oncoprotein compared with epidermal growth factor receptor, DNA ploidy, and conventional pathologic features. *Journal of Clinical Oncology* 10:686-695.

Gee JM, Robertson JF, Ellis IO, et al: 1994. Immunocytochemical localization of *Bcl-2* protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. *International Journal of Cancer* 59:619-628.

Geneva, Wildenhain Y, Pawson T, Blackstein M, et al: 1990. $p185^{neu}$ is phosphorylated on tyrosine in human primary breast tumors which overexpression neu/erbB-2. *Oncogene* 5:879-883.

Geslien GE, Fisher JR, DeLaney C: 1985. Transillumination in breast cancer detection: Screening failures and potential. *American Journal of Radiology* 144:691-622.

Ginsberg D, Mechta F, Yaniv M, et al: 1991. Wild-type p53 can down-modulate the activity of various promoters. *Proceedings of National Academy of Science of the United States of America* 88:9979-9983.

Goelz SE, Hamilton SR, VogelsteinB: 1985. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochemistry and Biophysics Research Communication* 130:118-126.

Gold RH, Bassett LW, Kimme-Smith C: 1986. Breast imagining: state of the art. *Investigation of Radiology* 21:298-304.

Gold RH, Bassett LW, Kimme-Smith C: 1987. Introduction to breast imaging: state of the art and future directions. In *Breast Cancer Detection: Mammography and Other Methods in Breast Imaging*. 2nd Edition. Edited by Bassett LW and Gold RH. Grunt & Stratton, Inc. Harcourt Brace Jovanovich, Publishers Orlando, New York, San Diego, London, San Francisco, Tokyo, Sydney, Toronto. pp1-5.

Goung XY, Wang BT, et al: 1993. Breast tumor. In *Atlas of Practical Tumor Histopathology* (Chinese) AnHuei Science and Technology Press. pp288-321.

Graeber TG, Osmanian C, Jacks T, et al: 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 379:88-91.

Gray PW, Goeddel DV: 1982. Structure of the human immune interferon gene. *Nature* 298(5877):859-63.

Grim J, Deshane J, Feng M, et al: 1996. erbB-2 knockout employing an intracellular single-chain antibody (sFv) accomplishes specific toxicity in erbB-2-expressing lung cancer cells. *American Journal of Respiration Cell Molecular Biology* 15;348-354.

Guerin M, Barrois M, Terrier MJ, et al: 1988. Overexpression of either c-myc or *c-erbB-2/neu* photo-oncogenes in human breast carcinomas; correlation with poor prognosis. *Oncogene Research* 3:21-31.

Gunji H, Kharbanda S, Kufe D: 1991. Induction of inernucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β- D-arabinofuranosylcytosine. *Cancer Research* 51:741-743.

Guo WD, Chow WH, Zheng W: 1994. Diet, serum markers and breast cancer mortality in China. *Japan Journal of Cancer Research* 85:572-577.

Gusterson BA, Gelber RD, Goldhirsch A, et al: 1992. Prognostic importance of *c-erbB-2* expression in breast cancer. *Journal of Clinical Oncology* 10:1049-1056.

Haagensen CD: 1986. *Diseases of the Breast*, edition 3, Philadelphia, WB Saunders, pp656.

Haldar S, Negrini M, Monne M, et al: 1994. Down-regulation of *Bcl-2* by p53 in breast cancer cells. *Cancer Research* 54:2059-2097.

Hale AJ, Smith CA, Sutherland LC, et al: 1996. Apoptosis: molecular regulation of cell death. *Europeanp Journal of Biochemistry* 236:1-26.

Hammond EH, Henson DE: 1995. The role of pathologists in cancer patient staging. *American Journal of Clinical Pathology* June:679-680.

Hanada M, Delia D, Aiello A, et al : 1993. *Bcl-2* gene hypomethylation and high-level expression in B- cell chronic lymphocytic leukemia. *Blood* 82(6):1820-1828.

Hann L, Ducatman BS, Wang HH, et al: 1989. Non-palpable breast lesions: Evaluation by means of fine-needle aspiration cytology. *Radiology* 171:373-376.

Harmanek P, Sobin LH: 1987. *TNM Classification of Malignant Tumors*, 4th edition. International Union against Cancer, Berlin.

Harmon BV, Takano YS, Winterford CM, et al: 1991 The role of apoptosis in human and murine tumour cell lines: a study using electron microscopy and DNA gel electrophoresis. *Journal of Pathology* 163:329-336.

Harmon BV, Corder AM, Collins RJ, et al: 1990. Cell death induced in a mourine mastocytoma by 42-47°C heating in vitro: evidence that the form of death changes from apoptosis to necrosis above a critical heat load. *International Journal of Radiation Biology* 58:845-858.

Harmon BV: 1987. An ultrastuctureal study of spontaneous cell death in a mouse mastocytoma with particular reference to dark cells. *Journal of Pathology* 153:345-355.

Harris CC, Hollstein M: 1993. Clinical implications of the p53 tumor-suppressor gene. *New England Medicine* 329:1318-1327.

Harris CC: 1996. p53 tumor suppressor gene: from the basic research laboratory to the clinic - an abriged historical perspective. *Carcinogenesis* 17:1187-1198.

Harris A: 1990. Mutant p53: The commonest genetic abnormality in human cancer? *Journal of Pathology* 162:5-6.

Harvey EB, Schairer C, Brinton LA, et al: 1987. Alcohol consumption and breast cancer. *The Journal of National Cancer Institute* 78:657-661.

Hayes DF, Schnitt SJ: 1993. Risk factors, epidemiology, and development of breast cancer. In *Atlas of Breast Cancer*. Edited by Hayes DF. Published by Mosby Europe Limited London, St. Louis, Baltimore, Boston, Chicago, Philadelphia, Sudney, and Toronto. pp2.2-2.9.

Hazan R, Margolis B, Domblagian M, et al: 1990. Identification of autophosphorylation sites of HER/neu. *Cell Growth and Differentiation* 1:3-7.

He GH, et al: 1990. Breast cancer. In *Diagnosis and Treatment of Malignant Tumor* (Chinese) JingXi Science & Technology Press pp124-133.

Henson DE, Ries L, Freedman LS, et al: 1991. Relationship among outcome, stage of disease and histologic grade for 22,616 cases of breast cancer. The basis for a prognostic index. *Cancer* 68:2142-2149.

Henry J: 1984. In The Edwin Smith Papyrus. The Classics of Medicine Library. p405.

Hiatt RA, Friedman GD, Bawol RD, et al: 1982. Breast cancer and serum cholesterol. *The Journal of the National Cancer Institute* 60:955-960.

Hiatt RA, Friedman GD, Bawol RD, et al: 1982. Breast cancer and serum cholesterol. Journal of National Cancer Institute 68: 885-9.

Higginson J, Muir CS, Munoz N: 1992a. Chemical factors. In *Human Cancer: Epidemiology and Environmental Causes*. CAMBRIDGE UNIVERSITY PRESS New York pp75-96.

Higginson J, Muir CS, Munoz N: 1992b. Breast and genitourinary system. In *Human Cancer: Epidemiology and Environmental Causes*. CAMBRIDGE UNIVERSITY PRESS New Yor pp375-397.

Hilkers J, Buijs F, Hilger J: 1984. Monoclonal antibodies against human milk-fat globule membranes detecting differentiation antigens of the mammary gland and its tumour. *International Journal of Cancer* 34:197-206.

Hirsh-Ginsberg CF, Stass SA, and Freireich EJ: 1995. Principles of Molecular Oncology. In *Molecular Basis of Oncology*. Edited by Freireich EF, Stass SA. Blackwell Science. pp1-21.

Hoare T: 1996. Breast screening and ethnic minorities. *British Journal of Cancer* 74:38-41.

Hockenbery DM, Oltval ZN, Yin XM, et al: 1993. *Bcl-2* functions in an antioxidant pathway to prevent apoptosis. *Cell* 75:241-251.

Hockenbery DM, ZutlerM, Hickey, et al: 1991. *Bcl-2* protein is topographically restricted in tissues characterized by apoptotic cell death. *Proceedings of National Academy of Science of the United States of America* 88:6961-6965.

Hockenbery D, Nunez G, Milliman C: 1990. *Bcl-2* is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, 348: 334-336.

Hollstein MC, Peri L, Mandard AM, et al : 1991a. Genetic analysis of human esophageal tumors from two high incidence geographic areas: frequent p53 base substitutions and absence of ras mutations. *Cancer Research* 51:4102-4106.

Hollstein M., Sidransky D, Vogelstein B, et al: 1991b. p53 mutations in human cancers. *Science* 253:49-53.

Hollywood DP, Hurst HC: 1993. A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene ce-erbB-2 in mammary tumor lines. *Engineering in Medicine and Biology Journal* 12:2369-2375.

Horowitz KB: 1975. Progesterone receptors and hormone dependent breast cancer. *Doctoral Dissertation*. University of Texas Southwestern Medical School, Dallas.

Hou L, Shi D, Tu SM, et al: 1992. Oral cancer progression and c-erbB-2/neu proto-oncogene expression. *Cancer Letter* 65:215-220.

Howell LP: 1995. The pathway to cancer. In *A Practical Approach to Breast Disease* Edited by O'Grady LF, Lindfors KK, Howell LP, and Rippon MB. Little, Brown and Company. Boston, New York, Toronto, London. pp23-39.

Hudson LG, Santon JB, Glass CK: 1990. Ligand-activated thyroid hormone and retinoic acid receptors inhibit growth factor receptor promoter expression. *Cell* 62:1165-1175.

Huebner RJ, Todaro GJ: 1969. Oncogenes of RNA tumor viruses as determinants of cancer. *Proceedings of National Academy of Science of the United States of America* 64:1087-1094.

Humphrey PA, Franquemont DW, et al: 1994. *Applied Immunohistochemistry*. 2:22-28.

Hung MC, Matin A, Zhang YJ, et al: 1995. HER/neu-targeting gene therapy- a review. *Gene* 159:65-71.

Iqbal MJ, Taylor W: 1989. Hormonal and reproductive factors - new evidence. In *Women at High Risk to Breast Cancer*. Eedited by Stoll BA. KLWER ACADEMIC PUBLISHERS. Dordrecht, Boston, London. pp41-45.

Isola J, Visakorpi T, Holi K, et al: 1992. Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node-negative breast cancer patients. *Journal of National Cancer Institute* 84:1109-1117.

Isola J, Visakorpi T, Holli K, et al: 1992. Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node-negative breast cancer patients. *Journal of National Cancer Institute* 84:1109-1114.

Iwaya K, Tsuda H, Hiraide G, et al : 1991. Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. *Japanese Journal of Cancer Research* 82:835-840.

Jacob TC, Spieth LE, Penn NE: 1993. Breast cancer, breast self-examination, and African-American women. In *Wings of Gauze Women of Color an the Experience of Health and Illness*. Edited by Bair B and Cayleff S. Wayne State University Press. Detroit. pp244-265.

Jacobson MD, Burne JF, King MP et al: 1993. *Bcl-2* blocks apoptosis in cells lacking mitochondrial DNA. *Nature*, 361:365-369.

Jigginson J, Muir CS, Munoz,N 1: 1992. Part X Breast and genitourinary system. 40 Breast In *Human Cancer: Epidemiology and Environmental Causes*. CAMBRIDGE UNIVERSITY PRESS. Great Britain. pp377-387.

Joensu H, Pylkkanen L, Toikkanen S: 1994. *Bcl-2* protein expression and long-term survival in breast cancer. *American Journal of Pathology* 145:1191-1198.

Johnston JB, Lee K, Verburg L, et al: 1992. Induction of apoptosis in CD4+ prolymphocytic leukemia by deoxyadenosine and 2'-deoxycoformycin. *Leukemia Research* 16:781-788.

Kern S, Pietenpol J, Thiagalingam S, et al: 1992. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* 256:827-830.

Kerr JFR, Searle J: 1980. Apoptosis: its nature and kinetic role. In *Radiation Biology in Cancer Research.* Edited by Meyn RE, Withers HR. Raven Press, New York 1980:367-384.

Kerr JFK, Wyllie AH, Currie AH: 1972a. Apoptosis, a basic biological phenomenon with wider implications in tissue kinetics. *British Journal of Cancer* 26:239-245.

Kerr JFR, Searle J, Harmon BV, et al: 1987. Apoptosis. In *Perspectives on Mammalian Cell Death.* Edited by Potten CS. Oxford: Oxford University Press 1987:93-128.

Kerr JFR, Searle J: 1972b. A suggested explanation for the paradoxically slow growth rate of basal-cell carcinomas that contain muerous mitotic figures. *Journal of Pathology* 107:41-44.

Keshgegian AA, Cnaan A: 1995. Proliferation markers in breast carcinoma. *American Journal of Clinical Pathology* 104:42-49.

Keynes GL: 1932. The radium treatment of carcinoma of the breast. *British Journal of Surgery* 19:415-480.

King CR, Kraus MH, Aaronson SA: 1985. Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science* 229:974-976.

King CR, Rwain SM, Porter L, et al: 1989. Heterogeneous expression of erbB-2 messenger in human breast cancer. *Cancer Research* 49:4185-4191.

King MC: 1990. Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 250:1684.

King MC: 1992. Breast cancer genes: How many, where, and who are they? *Nature Genetics* 2:125.

Kinzler KW, Vogelstein B: 1996. What's mice got to do with it. *Nature* 382;670-671.

Kita Y, Tseng J, Horan T, et al: 1996. ErbB receptor activation , cell morphology changes, and apoptosis induced by anti-Her2 monoclonal antibodies. *Biochemical and Biophysical Research Communications* 226:59-69.

Klavins JV: 1985. Carcinomas of various organs and organ systems. In *Tumor Markers Clinical and Laboratory Studies* Edited by Klavins, J.V. ALAN R. LISS INC. New York. pp29-39.

Kleihues P, Schauble B, zur Hausen A, et al: 1997. Tumors associated with p53 germline mutations: a synopsis of 91 families. *American Journal of Pathology* 150:1-13.

Klein PA, Smith RT: 1977. The role of oncogenic viruses in neoplasia. *Annual Review of Medicine* 28:311-327.

Komminoth P: 1995. *in situ* gene amplification - theory and practice. In *situ Applications Guide*. SHANDON LIPASHAW pp53-75.

Kovi J, Mohla S, Norris HJ, et al: 1989. Breast lesions in black women. *Annual of Pathology* 24(pt 1):199-218.

Krajewski S, Krajewska M, Shabaik A, et al: 1994. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of *Bcl-2*. *American Journal of Pathology* 145:1323-1336.

Kraus MH, Popescu NC, Amsbaugh SC, et al: 1987. Overexpression of the EGF receptor-relate proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *Engerning in Medicine and Biology Journal* 6:605-610.

Kress S, Sutter C, Strickland PT, et al: 1992. Carcinogen-specific mutational pattern in the p53 gene in ultraviolet B radiation-induced squamous cell carcinomas of mouse skin. *Cancer Research* 52:6400-6403.

Kufe D, Inghirami G, Abe M, et al: 1984. Differential reactivity of a monoclonal antibody (DF3) wit human malignant versus benign breast tumors. *Hybridoma* 3:223-232.

Kufe DW: 1988. Monoclonal antibody assays for breast cancer. In *Cancer Diagnosis In Vitro Using Monoclonal Antibodies*. Edited by Kupchik HZ. MARCEL DEKKER, Inc. New York and Basel. pp68-70.

Kute T, Shao Z, Keith Sugg N, et al: 1992. Cathepsin D as prognostic indicator for node-negative breast cancer patients using both immunoassays and enzymatic assays. *Cancer Research* 52:5198-5203.

Kyprianou N, Englis HF, Davidson NE, et al: 1991. Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation.. *Cancer Research* 51:162-166.

Lagios MD, Westdahl PR, Margolin FR, et al: 1982. Duct carcinoma *in situ*. Relationship of extent of noninvasive disease to the frequency of occult invasion, multicentricity. lymph node metastases and short-term treatment failures. *Cancer* 50:1309-1314.

Lane DP, Crawford LV: 1979. T antigen is bound to a host protein in SV 40-transformed cells. *Nature* 278:261-263.

Lane DP: 1992. p53, guardian of the genome. *Nature* 358:15-16.

Lane DP: 1993. A death in the life of p53. *Nature* 362:786-787.

Le Roy X, Escot C, Brouillet JP, et al: 1991. Decrease of *c-erbB-2* and *c-myc* RNA levels in tamoxifen-treated breast cancer. *Oncogene* 6:431-437.

Leach FX, Tokino T, Meltzer P, et al: 1993. P53 mutation and MDM2 amplification inhuman soft tissue sarcomas. *Cancer Research*. 53:2231-2234.

Lee ET-HP: 1991. Tumor suppressor genes: Anew era for molecular genetic studies of cancer. *Breast Cancer Research and Treatment* 19:3-13.

Leek RD, Kaklamanis L, Pezzella F, et al: 1994. *Bcl-2* in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and in situ cancer. *British Journal of Cancer* 69:135-139.

Lehman TA, Greenblatt M, Bennett WP, et al: 1994. Mutational spectrum of the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Drug Metabolism Review* 26:221-235.

Leis HP Jr.: 1970. The pill and the breast. *NY State Journal of Medicine* 70:2911-2918.

Lerman C, Seay J, Balshem A, et al: 1995. Interest in genetic testing among first-degree relatives of breast cancer patients. *Amercan Joural of Medicine Genetics* 57:385-92

Levack PA, Mullen P, Anderson TJ, et al: 1987. *British Journal of Cancer* 56:643.

Levine AJ, Momand J, Finlay CA: 1991. p53 tumour suppressor gene. *Nature*. 351:453-456.

Lewis FA, Wells M: 1992. Detection of virus in infected human tissue by *in situ* hybridization. In *in situ Hybridization A Practical Approach*. Edited by Wilkinson DG IRL PRESS Oxford University Press Oxford New York Tokyo pp121-136.

Li FP, Fraumeni JF Jr, Mulvihill JJ, et al: 1988. A cancer family syndrome in twenty-four kindred. *Cancer Research* 48:5358-5362.

Li SL: 1994. Review of protection and treatment on breast cancer. In *Studies of the Research and Clinic Progresses of Malignant Tumors*. Edited by Suan JK (Chinese) China Medical University and Beijing Medical University Press. pp53-62.

Lin EY, Orlofsky A, Bergar M, et al: 1993. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to *Bcl-2*. *Journal of Immunology* 151:1979-1988.

Lindfors KK: 1995. Screening for breast cancer. In *A Practical Approach to Breast Disease* Edited by O'Grady LF, Lindfors KK, Howell LP, and Rippon MB. Little, Brown and Company. Boston, New York, Toronto, London. pp41-58.

Lipshaw S: 1995. Introduction In *in situ Applications Guide*. Edited by Lipshaw S. handon, Inc. pp1-3.

Liu FS, Li X, Change X, et al: 1992. Pathological investigation of the breast cancer. *The monograph of International Meeting* pp32-33.

Lodon SJ, Willet WC: 1992. Relationship to diet and body size. In *Women at High Risk to Breast Cancer* edited by Stoll, B.A. KLUWER ACADEMIC PUBLISHERS. Dordrecht, Boston, London. pp47-56.

Lofgren M, Andersson I, Bondeson L, et al: 1988. X-ray guided fine-needle aspiration for the cytologic diagnosis of nonpalpable breast lesions. *Cancer* 61:1032-1037.

Lofgren M, Andersson I, Lindholm K, et al: 1990. Sterotactic fine-needle aspiration for cytologic diagnosis of nonpalpable breast lesions. *American Journal of Roentgenology* 154:1191-1195.

Lohmann DR, Fesseler B, Putz B, et al: 1993. Infrequent mutation of the p53 gene in pulmonary carcinoid tumors. *Cancer Research* 53:5797-5801.

Lu QL, Abel P, Foster CS, et al: 1996. *Bcl-2*: role in epithelial differentiation and oncogenesis. *Human of Pathology* 27:102-110.

Lu QL, Hanby AM, Hajibagheri MA, et al : 1994. *Bcl-2* protein localizes to the chromosomes of mitotic nuclei and is correlated with the cell cycle in cultured epithelial cell lines. *Journal of Cell Sciences* 107: 363-371.

Luna-More S, de los Santos F, Breton JJ, et al: 1996. Estrogen and progesterone receptors, *c-erbB-2*, p53, and *Bcl-2* in thirty-three invasive micrpapillary breast carcinomas. *Pathology, Research and Practice* 192:27-32.

Lupu R, Cardillo M, Cho C, et al: 1996. The significance of heregulin in breast cancer tumor progression and drug resistance. *Breast Cancer Research and Treatment* 38:57-66.

Lux SE, Tse WT, et al: 1990. Hereditary spherocytosis associated with deletion of human erythrocyte ankyrin gene on chromosome 8. *Nature* 345:736-739.

Mackenzie CD: 1994. Molecular diagnosis of breast cancer. In *Molecular Pathology Course*.

Macklis RM, Lin JY, Beresford B, et al: 1992. Cellular kinetics, dosimetry, and radiobiology of œ-particle radioimmunotherapy: induction of apoptosis. *Radiation Research* 130:220-226.

MacMahon CE, Cahill JL: 1840. Practical Surgery. 3rd ed. London Churchill, pp336.

Malkin D: 1993. P53 and the Li-Fraumeni syndrome. *Cancer Genet Cytogenet.* 66:83-92.

Malkin D, Li FP, Strong LC, et al: 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 250:1233-1238.

Mant D, Vessey MP: 1991. Section V Epidemiology and primary prevention of breast cancer. In *The Breast: Comprehensive Management of Benign and Malignant Diseases.* Edited by Bland KI, Copeland EMIII. W.B. Saunders Company. Philadelphia. pp235-246.

Margolis BL, Lax I, Kris R, et al: 1989. All autophosphorylation sites of epidermal growth factor (EGF) receptor and her2/neu are located in their carboxyl-terminal tails. Identification of a novel site in EGF receptor. *Journal of Biology and Chemistry* 264:10667-1071.

Marks JR, Humphrey PA, Wu K, et al: 1995. Overexpression of p53 and HER-2/neu proteins as prognostic markers in early stage breast cancer. *Annals Surgery* 221:433-435.

Marshall E: 1996. Rifkin's latest target: genetic testing. *Science* 272 1904.

Marx J: 1993. Cell death studies yield cancer clues. *Science* 259:760-761.

Marx J: 1996. A second breast cancer susceptibility gene is found. *Science* 271:30-31.

Mazars R, Spinardi L, BenCheikh M, et al: 1992. p53 mutations occur in aggressive breast cancer. *Cancer Research* 52(14):3918-3923.

Masood S, Frykerg ER, McLellan GL, et al: 1990. Prospective evaluation of radiologically directed fine-needle aspiration biopsy of nonpalpable breast lesions. *Cancer* 66:1480-1487.

Mcbride OW, Merry D, Givol D: 1986. The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). *Proceedings of National Academy of Science of the United States of America* 83:130-134.

McDonnell TJ, Deane N, Platt FM, et al: 1989. *Bcl-2*-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 57:79-88.

McDonnell TJ, Korsmeyer SJ: 1991. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). *Nature* 349:254-256.

McGee JO'D: 1992. Molecular techniques in laboratory medicine. In *Diagnostic Molecular Pathology A Practical Approach* Edited by Herrington CS, McGee JO'D. Oxford University Press. Oxford, New York, Tokyo pp1-6.

McGuire WL, De La Garza M: 1975. Similarity of estrogen receptors in human and rat mammary carcinoma. *Journal of Clinical Endocrinology and Metabolism* 36:548-552.

McWhirter R: 1948. The value of simple mastectomy and radiotherapy in the treatment of cancer of the breast. *British Journal of Radiology* 21:599-610.

Melamed MR, Lindmo T, Mendelsohn ML, et al (ed): 1990. *Flow Cytometry and Sorting* 2nd edition. Wiley-Liss Publish. New York.

Merkel DE, McGuire WL: 1990. Ploidy, proliferative activity and prognosis: DNA flow cytometry of solid tumors. *Cancer* 65:1149-1205.

Merlo GR, Venesio T, Taverna D, et al: 1994. Growth suppression of normal mammary epithelial cells by wild-type p53. *Oncogene* 9:443-453.

Mettlin C: 1994. The relationship of breast cancer epidemiology to screening recommendations. *Cancer* 74:228-30.

Mietz JA, Unger T, Huibregtse JM, et al: 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *Engerning in Medicine and Biology Journal* 11:5013-5020.

MikiY, Swensen J, Shattuck-Eldens D,Futreal PA et al: 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266:66-71.

Miller RW: 1977. Relationship between human teratogens and carcinogens. *Journal of National Cancer Institute* 58:471-474.

Miller C, Mohands T, Wolf D, et al: 1986. Human p53 gene localized to short arm of chromosome 17. *Nature* 319:783-784.

Miller CW, Simon K, Aslo A, et al: 1992. P53 mutations in human lung tumors. *Cancer Research* 52:1695-1698.

Miller B: 1995. PRINS & Cycling PRINS Theory & Practice In *IN SITU Applications Guide* SHANDON LIPSHAW Pittsburgh pp37-50.

Milner J, Medcalf EA, Cook AC: 1991. Tumor suppressor p53: analysis of wild-type and mutant p53 complexes. *Molecular and Cellular Biology* 11:12-19.

Milner J, Medcalf EA, Cook AC: 1991. Tumor suppressor p53: analysis of wild-type and mutant p53 complexes. *Molecular and Cellular Biology* 11:12-9.

Mitra AB, Murty VV, Pratap M, et al: 1994. ERBB2 (HER2/neu) oncogene is frequently amplified in squamous cell carcinoma fo the uterine cervix. *Cancer Research* 54:637-639.

Moll UM, Riou G, Levine AJ, et al: 1992. Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proceedings of National Academy of Science of the United States of America* 89:7262-7266.

Momand J, Zambetti GP, Olson DC, et al 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 69:1237-1245.

Moscow JA, Cowan KH: 1988. Multidrug resistance. *Journal of National Cancer Institute* 80:14.

Mueller CB: 1994. Breast cancer: reporting results with inflationary arithmetic. *American Journal of Clinic Pathology* 17:86-92.

Nagai MA, Marques LA, Torloni H, et al: 1993. Genetic alterations in *c-erbB-2* protooncogene as prognostic markers in human primary breast tumors. *Oncology* 50:412-417.

Natarajan N, Nemoto T, Metllin C, et al: 1985. Race-related differences in breast cancer patients. Results of the 1982 national survey of breast cancer by the American College of Surgeons. *Cancer* 56:1704-1709.

Nathan B, Anbazhagan R, Clarkson P, et al: 1994a. Expression of *BCL-2* in the developing human fetal and infant breast. *Histopathology* 24:73-76.

Nathan B, Gusterson B, Jadayel D, et al: 1994b. Expression of *Bcl-2* in primary breast cancer and its correlation with tumour phenotype. For the International breast cancer study group. *Annals of Oncology* 5:409-414.

National Institutes of Health: 1978. National Institutes of Health /National Cancer Institute Consensus Development Meeting on Breast Cancer: Issues and Recommendations. *Journal of National Cancer Institute* 60:6.

Nelson WG, Kastan MB: 1994. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Molecular and Cellular Biology* 14:1815-1823.

Neubauer A, He M, et al: 1992. Analysis of gene amplification in archival tissue by differential polymerase chain reaction. *Oncogene* 7:1019-1025.

Neuhausen S, Gilewski T, Norton L, et al: 1996. Recurrent BRCA2 6174delT mutations in Ashkenazi Jewish women affected by breast cancer. *National Genetics* 13:126-128

Neville AM, Price KN, Gleber RD, et al: 1991. Axillary node micrometastases and breast cancer. *Lancet* 337:1110-117..

Nigro JM, Baker SJ, Preisinger AC, et al: 1989. Mutation in the p53 gene occur in diverse human tumour types. *Nature* 342:705-708.

Nunez G, London L, Hockenbery D et al: 1990. Deregulated *Bcl-2* gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *Journal of Immunology* 144:3602-3610.

Nunovo GJ: 1994. PCR *in situ* hybridization. In *PCR in situ Hybridization Protocols and Applications* Second Edition RAVEN PRESS New York pp169-213.

O'Grady LF, Rippon MB: 1994. The high-risk patient. In *A Practical Approach to Breast Disease*. Edited by O'Grady, L.F., Lindfors, K.K., Howell, L.P., and Rippon, M.B. Little, Brown and Company. Boston, New York, Toronto, London. pp173-182.

Oltvai ZN, Milliman CL, Korsmeyer SJ: 1993. *Bcl-2* heterodimerizes in vivo with a conserved homology, Bax, that accelerates programmed cell death. *Cell* 74:609-619.

Oren M, Maltzman W, Levine AJ: 1981. Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. *Molecular and Cellular Biology* 1:101-110.

Oren M: 1985. The p53 cellular tumor antigen: Gene structure, expression and protein properties. *Biochemistry and Biophysics Acta* 823:67-78.

Orita M., Iwahana H, Kanazawa H, et al: 1989. Detection of polymorphisms of human CNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of National Academy of Science of the United States of America* 86:2766-2770.

Owings D, Hann L, Schnitt SJ: 1990. How thoroughly should needle localization breast biopsies be sampled for microscopic examination? A prospective mammographic-pathologic correlative study. *American Journal of Surgical Pathology* 14:578-583.

Padhy LC, Shih C, Cowing D, et al: 1982. Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas. *Cell* 28:865-871.

Page DL, Dupont WD, Rogers, L.W., and et al: 1985. Atypical hyperplastic lesions of the female breast. *Cancer* 55:1563-1571.

Page DL: 1991. Prognosis and beast cancer. Recognition of lethal and favorable prognostic types. *The American Surgical Pathology* 15:334-349.

Paget J: 1856. On the average duration of life in patients with scirrhous cancer of the breast. *Lancet* 1:62-63.

Palmer MK, Lythgoe JP, Smith A: 1982. Prognostic factors in breast cancer. *British Journal of Surgery* 69:697-698.

Park JB, Rhim JS, Park SG, et al 1989. Expression of the *c-erbB-2* gene product (p185) at different stages of neoplastic progression in the colon. *Oncogene* 4:1233-1239.

Patey DH, Dyson WH: 1948. The prognosis of carcinoma of the breast relation to the type of operation performed. *British Journal Cancer* 2:7-13.

Pavletich NP, Chambers KA, Pabo CO: 1993. The DBA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. *Genes and Development* 7:2556-2564.

Pezzella F, Tse AG, Cordell JL, et al: 1990. Expression of the *Bcl-2* oncogene protein is not specific for the 14;18 chromosomal translocation. *American Journal of Pathology* 137:225-232.

Polednak AP: 1986. Breast cancer in black and white women in New York state. Case distribution and incidence rates by clinical stage at diagnosis. *Cancer* 57:807-815.

Posser J, Thompson AM, Cranston G, et al: 1990. Evidence that p53 behaves as a tumor suppressor gene in sporadic breast tumors. *Oncogene* 5:1573-1579.

Potchen, EJ: 1993. Investigation of breast abnormalities. In *Screening Mammography Breast Cancer Diagnosis in Asymptomatic Women* Edited by Svane G, Potchen EJ, Sierra A, and Azavedo E. MOSBY St. Louis, Baltimore, Boston, Chicago, London, Philadelphia, Sydney, Toronto. pp237-254.

Potchen EJ, Sierra A, Mackenzie CD, et al: 1991. Svane localization of non-palpable breast lesions. *Lancet* 338(8770):816.

Powell DE, Stelling DB: 1994. Chapter 12 Carcinoma of breast In *The Diagnosis and Detection of Breast Cancer* Published by Dedicated to Publishing Excellence. St. Louis, Baltimore, Boston, Chicago, London, Madrid, Philadelphia, Sydney, Toronto. pp289.

Pringle, J.H., Baker, J., and et al: 1993. The detection of cell proliferation in normal and malignant formalin-fixed paraffin-embedded tissue sections using *in situ* hybridization for histone mRNA. *Journal of pathologic Supplementum* 169:155A.

Pringle, J.H., Ruprai, A.K., and et al: 1990. *in situ* hybridization of immunoglobulin light chain mRNA in paraffin section using biotinylated or hapten-labelled oligonucleotide probes. *Journal of Pathology* 162:197-207.

Pritchard-Jones, K. and Fleming, S.: 1991. Cell types expressing the Wilms' tumor gene (WT1) in Wilms' tumors: implications for tumor histogenesis. *Oncogene* 6:2211-2220.

Prosser J, Thompson AM, Cranston G, et al: 1991. Evans-HJ Evidence that p53 behaves as a tumour suppressor gene in sporadic breast tumours. *Oncogene* 5:1573-9.

Qiao XT: 1992. Chapter one. Tisse sampling: a comparison of the weight and the cellular nature of the material obtained by fine needle aspiration and Rotex needle biopys techniques. In *Techniques in the Detection and Treatment of Breast Cancer*. Thesis for the Degree of M.S. MSU. pp7-48.

Ragaz J, Ariel IM: 1989. High-risk breast cancer: definition of the risk. In *High-Risk Breast Cancer* Edited by Ragaz J and Ariel IM. Springer-Verlag Beidelberg New York. Pp3-23.

Rand A, Glenn KS, Alvares CP, et al: 1996. P53 function loss in a colon cancer cell line with two missions mutations (218eu and 248trp) on separate alleles. *Cancer Letters*. 98:183-191.

Rapp F, Reed CL: 1977. The viral etiology of cancer. A realistic approach. *Cancer* 40:419-429.

Ravdin P and Chamness G: 1995. The c-erbB-2 proto-oncogene as a prognostic and predictive marker in breast cancer: a paradigm for the development of other macromolecular markers - a review. *Gene* 159:19-27.

Raycroft L, Schmidt JR, Yoas K, et al: 1991. Analysis of p53 mutants for transcriptional activity. *Molecular and Cellular Biology* 11:6067-6074.

Raycroft L, Wu H, Lozano G: 1990. Transcriptional Activation by wild-type but not transforming mutants of the p53 anti-oncogene. *Science* 249:1049-1051.

Read LD, Keith DJr, Slamon DJ, et al: 1990. Hormonal modulation of HER-2/*neu* proto-oncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. *Cancer Research* 50:3947-3951.

Redard M, Vassikos P, Weintraub JA: 1989. A simple method for estrogen receptor antigen preservation in cytologic specimens containing breast carcinoma cells. *Diagnosis of Cytologic Pathology* 5:18-193.

Redding TW, Schally AV, Radulovic S, et al: 1992. Sustained release formulations of luteinizing hormone-releasing hormone antagonist SB-75 inhibit proliferation and enhance apoptotic cell death of human prostate carcinoma (PC-82) in male nude mice. *Cancer Research* 52:2538-2844.

Resnick RM, Cornelissen MT, Wright DK, et al: 1990. Detection and typing of human papilloma virus in archival cervical cancer specimens by DNA amplification with consensus primers. *Journal of National Cancer Institute* 82:1477-1484.

Robbins P, Pinder BS, Klerk N, et al: 1995. Histological grading of breast carcinomas: a study of interobserver agreement. *Human Pathology* 26:873-879.

Robinson IA, McKee G, Nicholson A: 1994. Prognostic value of cytological grading of fine-needle aspirates from breast carcinomas. *Lancet* 343:947-949.

Robinson JO: 1986. Treatment of breast cancer through the ages. *American Journal of Surgery* 151:321-327..

Robbins SL : 1994. The breast. In *Pathologic Basis of Disease* 5th Edition Edited by Cotran RS, Kumar V and Robbins SL. W. B. SAUNDERS COMPANY pp1089-1112.

Rogel A, Popliker M, Webb CG, et al: 1985. P53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. *Molecular and Cellular Biology*. 5:2851-2855.

Rosen PP: 1996a. Biological markers of prognosis. In *Breast Pathology*. Edited by Rosen PP. Lippincott-Raven Publishers. Philadelphia. pp295-320.

Rosen PP: 1996b. Role of cytology and needle biopsy in diagnosis. In *Breast Pathology*. Edited by Rosen PP. Lippincott-Raven Publishers. Philadelphia. pp817-835.

Rosen PP, Lesser ML, Arroyo CD, et al: 1995a. P53 in node-negative breast carcinoma: an immunohistochemical study of epidemiologic risk factors, histologic features and prognosis. *Journal of Clinical Oncology* 13:821-830.

Rosen PP, Lesser ML, Arroyo CD, et al: 1995b. Immunohistochemical detection of HER-2/ neu in node-negative breast carcinoma: a study of epidemiologic risk factors, histologic features and prognosis. *Cancer* 75:1320-1326.

Rosen PP, Goshen S, Saigo PE, et al: 1989a. A long-term follow-up of survival in Stage I (T1N0M0) and Stage II (T1N1M0) breast carcinoma. *Journal of Clinical Oncology* 7:355-366.

Rosen PP, Goshen S, Saigo PE, et al: 1989b. Pathologic prognostic factors in stage I (T1N0M0) and stage II (T1N1M0) breast carcinoma: a study of 655 patients with median follow-up of 18 years. *Journal of Clinical Oncology* 7:1239-1251.

Rosen PP, Lesser ML, Senie R, et al: 1982. Epidemiology of breast carcinoma IV: age and histologic tumor type. *Journal of Surgical Oncology* 19:44-47.

Roth JA, Nguyen D, Lawrence DD, et al: 1996. Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nature Medicine* 2(9):985-991.

Roulston JE, Leonard RCF: 1993a. Breast cancer. In *Serological Tumor Markers An Introduction* Edited by Roulston JE and Leonard RCF. Churchill Livingstone Edinburgh, London , Madrid, Melbourne, New York, and Tokyo. pp39-46.

Roulston JE, Leonard RCF: 1993b. Breast cancer. In *Serological Tumor Markers An Introduction*. Edited by Roulston JE and Leonard RCF. Churchill Livingstone Edinburgh, London, Madrid, Melbourne, New York, and Tokyo. pp75-85.

Runnebaum I, Nagarajan M, Bowman M, et al: 1991. Mutations in p53 as potential molecular markers for human breast cancer. *Proceeding of the National Academy of Sciences of the United Stated of America* 88(23):10657-10661.

Ruprai AK, Pringle JH, et al: 1991. Localization of immunoglobulin light chain mRNA expression in Hodgkin's disease by *in situ* hybridization. *Journal of Pathology* 164:37-40.

Russel DL, Kaklamanisl L, Pezzella F et al: 1994. *Bcl-2* in normal human breast cancer carcinoma, association with oestrgen receptor-positive , epidermal growth factor receptor-negative tumours and in situ cancer. *British Journal of Cancer* 69:135-139.

Russell KS, Hung MC: 1992. Transcriptional repression of the neu protooncogene by estrogen. *Cancer Research* 52:6624-6629.

Sakamoto G, Sugano H, Kasumi F: 1987. Bilateral breast cancer and familial aggregations. *Preventive Medicine* 7(2): 225-229.

Salhadin A, Nasiell M, Nasiell K, et al: 1976. The unique cytologic picture of oat cell carcinoma in effusions. *Acta Cytology* 20:298.

Santibanez-Koref MF, Birch JM, Hartley AL, et al: 1991. P53 gremlin mutations in Li-Fraumeni syndrome. *Lancet* 338:1490-1491.

Sasa M, Kondo K, Komaki K, et al: 1993. Frequency of spontaneous p53 mutations (CpG site) in breast cancer in Japan. *Breast Cancer Research and Treatment* 27:247-251.

Sattin RW, et al: 1985. Family history and the risk of breast cancer. *Journal of the American Medical Association* 253:1908-1913.

Schechter AL, Hung M-C, Vaidyanathan L, et al: 1985. The neu gene: an erbB-2 homologous gene distinct from and unlinked to the encoding the EGF receptor. *Science* 229:976-978.

Schechter AL, Stern DF, Vaidyanathan L, et al: 1984. The neu oncogene: an erb-B-2 related gene encoding a 185,000-Mr tumour antigen. *Nature* 312:513-516.

Schneider PM, Hung MC, Chiocca SM, et al: 1989. Differential expression of the *c-erbB-2* gene in human small cell and non-small cell lung cancer. *Cancer Research* 49:4968-4971.

Schnitt SJ: 1993. Processing of breast biopsies. In *Atlas of Breast Cancer*. Edited by Hayes DF. Mosby Europe Limited London, St. Louis, Baltimore, Boston, Chicago, Philadelphia, Sudney, and Toronto. pp6.2-6.6.

Schnitt SJ, Connoly JL, Khettry U: 1987. Pathologic findings on re-excision of the primary site in breast cancer patients considered for treatment by primary radiation therapy. *Cancer* 59:675-681.

Schnitt SJ, Connoly JL, Harris JR, et al: 1984. Pathologic predictors of early local recurrence in stage I and II breast cancer treated by primary radiation therapy. *Cancer* 53:1094-1057.

Schubert D, Heinemann S, Carlisle W, et al. 1974. Clonal cell lines the rat central nervous system. *Nature* 249:224-227.

Schubert, E.L., and et al: 1993. A method to isolate DNA from small archival tissue samples for p53 gene analysis. *Human Mutation* 2:123-126.

Searle I, Lawson TA, Abbott PJ, et al: 1975. An electron-microscope study of the mode of cell death induced a cancer-chemotherapeutic agents in populations of proliferating normal and neoplasic cells. *Journal of Pathology* 116: 129-138.

Searle J, Collins DJ, Harmon B, et al: 1973. The spontaneous occurrence of apoptosis in squamous carcinomas of the uterine cervix. *Pathology* 5:163-169.

Seidman H, Stellman SD, Mushinski MH: 1982. A different perspective on breast cancer risk factors: some implications of the non-attributable risk. *CA: A Cancer Journal of Clinic* 32:301-313.

Semba K, Kamata N, Toyoshima K, et al: 1985. A v-erbB-related protooncogene, *c-erbB-2*, is distinct from the c-erbB-1/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proceedings of National Academy of Science of the United States of America* 82:6497-6501.

Sentman CL, Shutter JR, Hockenbery D et al: 1991. *Bcl-2* inhibits multiple forms of apoptosis by not negative selection in thymocytes. *Cell* 68:879-888.

Seto M, Jaeger U, Hockett RD, et al: 1988. Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. EMBO Journal 7(1):1123-131.

Shaulsky G, Golfinger N, Ben Ze'ev A, et al: 1990. Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorgenesis. *Molecular and Cellular Biology* 10:6565-6577.

Shaw P, Bovey R, Tardy S, et al: 1992. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proceedings of National Academy of Science of the United States of America* 89:4495-4499.

Shibata D, Brynes RK, Nathwani B, et al: 1989. Human immunodeficiency viral DNA is readily found in lymph node biopsies from seropositive individuals. Analysis of fixed tissue using the polymerase chain reaction. *American Journal of Pathology* 135:697-702.

Shih C, Padhy LC, Murray M, et al: 1981. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* 290:261-264.

Sierra A, Lloveras B, Castellsague X, et al: 1995. Bcl-2 expression is associated with lymph node metastasis in human ductal breast carcinoma. *International Journal of Cancer* 60:54-60.

Sickles EA.: 1984. Breast cancer detection with transillumination and mammography. *American Journal of Radiology* 142:841-844.

Silvestrini R, Benini E, Daidone MG, et al: 1993. P53 as an independent prognostic marker in lymph node-negative breast cancer patients. *Journal of National Cancer Institute* 85:965-970.

Siziopikou KP, Prioleau JE, Harris JR, et al: 1996. Bcl-2 expression in the spectrum of preinvasive breast lesions. *Cancer* 77:499-506

Sjalander A, Birgander R, Hallmans G, et al: 1996. p53 polymorphisms and haplotypes in breast cancer. *Carcinogenesis* 17:1313-1316.

Slamon DJ, Godolphin W, Jones LA, et al: 1989a. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244:707-712.

Slamon DJ, Press MF, Godolphin W, et al: 1989b. Studies of the HER-2/neu proto-oncogene in human breast cancer. In *Cancer Cell*. Edited by Fourth M and Greaves M. COLD SPRING HARBOR LABORATORY PRESS. pp371-340

Slamon DJ, Clark GM, Wong SG, et al: 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235:177-182.

Smith CA: 1993. Monoclonal antibodies in pathological investigations. In *Molecular and Antibody Probes in Diagnosis*. Edited by Walker MR and Rapley R. JOHN WUKEY & SONS Chichester, New York, Brisbane, Toronto, Singapore. pp215-228.

Sobin LH, Hermanek P, Hutter RVP: 1988. TNM classification of malignant tumors. A comparison between the new (1987) and old editions. *Cancer* 61:2310-14.

Soomro S, Taylor P, Shepard H, et al: 1993. *c-erbB2* oncoprotein in screen-detected breast carcinoma: an immunohistological study. *International Journal of Cancer* 55:63-65.

Soto D, Sukumar S: 1992 Improved detection of mutations in the p53 gene in human tumors as single-stranded conformation polymorphs and double-stranded heteroduplex DNA. *PCR Methods and Application* 2:96-98.

Soussi T, Caron de Fromentelc, May P: 1990. Structural aspects of the p53 protein in relation to gene evolution. *Oncogene* 5:945-953.

Soussi T, Legros Y, Lubin R, et al: 1994. Multifactorial analysis of p53 alteration in human cancer: a review. *International Journal of Cancer* 57:1-9.

Spadoro JP, Dragon EA: 1993. Quality control of the polymerase chain reaction. In *Molecular Biology and Pathology A Guidebook for Quality Control* Edited by Farkas, D.H. Academic Press, Inc. pp149-158.

Spinardi L, Mazars R, Theillet C: 1991. Protocols for an improved detection of point mutations by SSCP. *Nucleic Acids Research* 19:4009.

Spriggs AI: 1984. The architecture of tumor cell clusters in serious effusions. In *Advances in Clinical Cytology* vol. 2. Edited by Koss LG and Coleman DV. New York, Masson. pp267-290.

Staecker H, Cammer M, Rubinstein R, et al: 1994. A procedure for RT-PCR amplification of mRNAs on histological specimens. *BioTechniques* 16:76-80.

Stephens LC, Ang KK, Schultheiss TE, et al: 1991. Apoptosis in irradiated murine tumors. *Radiation Research* 127:308-316.

Stern DF, Heffernan PA, Weinberg RA: 1986. p185, a product of the neu proto-oncogene, is a receptor like protein associated with tyrosine kinase activity. *Molecular and Cellular Biology* 6:1729-1740.

Stern DF, Kamps Mp, Cao H: 1988a. Oncogenic activation of p185 neu stimulates tyrosine phosphorylation in vivo. *Molecular and Cellular Biology* 8:3969-3973.

Stern DF, Kamps MP: 1988b. EGF-stimulated tyrosine phosphorylation of p185: a potential model for receptor interactions. *Engineering in Medicine and Biology Journal* 7:995-1001.

Strasser A, Harris AW, Bath ML, et al: 1990. Novel primitive lymphoid tumors induced in transgenic mice by cooperation between *myc* and *Bcl-2*. *Nature* 348:331-333.

Szabo CI, King MC: 1995. Inherited breast and ovarian cancer. *Human Molecular Genetics* 4: 1811-1817.

Szende B, Srkalovic G, Groot K, et al: 1990. Growth inhibition of mouse MXT mammary tumor by the luteinizing hormone-releasing hormone antagonist SB-75. *Journal of National Cancer Institute* 82:513-517.

Takahashi T, D'Amico D, Chiba I, et al: 1990. Identification of intronic point mutations as a altermative mechanism for p53 inactivation in lung cancer. *Journal of Clinical Investigation* 86:363-369.

Takeshima Y, Seyama T, Bennett WP, et al: 1993. P53 mutations in lung cancers from non-smoking atomicbomb survivors. *Lancet* 342:1520-1521.

Tal M, Wetzler M, Josefberg Z, et al: 1988. Sporadic amplification of the HER2/neu protooncogene in adenocarcinomas of various tissues. *Cancer Research* 48:1517-1520.

Tammenbaum, A.: 1942. The genesis and growth of tumors, III effects of high fat diet. *Cancer Research* 2:468-475.

Tandon AK, Clark GM, Chamness GC, et al: 1989. HER/neu oncogene protein and prognosis in breast cancer. *Journal of Clinical Oncololgy* 7:1120-1128.

Tavassoli FA, Norris HJ: 1990. A comparsion of the results of long-term follow-up for atypical intraductal carcinma of the breast. *Cancer* 65:518-529.

Tavassoli FA: 1992. Part 2. General Considerations In *Pathology of the Breast.* Appleton & LANGE. Norwalk, Connecticut. pp25-55.

Tetu B, Fradet Y, Allard P, et al: 1996. Prevalence and clinical significance of HER/2neu, p53 and Rb expression in primary superficial bladder cancer. *Journal of Urology* 155:1784-1788.

Thomas DB: 1984. Do hormones cause breast cancer? *Cancer* 53:595-604.

Thompson AM, Anderson TJ, Condie A, et al: 1992. P53 allele losses, mutation and expression in breast cancer and their relationship to clinicopathologic parameters. *International Journal of Cancer* 50:528-532.

Tomita Y, Bilim V, Kawasaki T, et al: 1996. Frequent expression of Bcl-2 in renal-cell carcinomas carrying wil-type p53. *International Journal of Cancer* 66:322-325.

Tsuda H, Hirohashi S, Shimosato Y, et al: 1990. Immunohistochemical study on overexpression of *c-erbB-2* protein in human breast cancer: its correlation with gene amplification and long-term survival of patients. *Japanese Journal of Cancer Research.* 81:327-332.

Tsujimoto Y, Finger LR, Yunis J, et al: 1984. Cloning of the chromosome break point of neoplastic B cell with the t(14,18) chromosome translocation. *Science* 226:1097-1099.

Tubiana M, Pejovic MH, Renaud A, et al: 1981. Kinetic parameters and the course of the disease in breast cancer. *Cancer* 47:937-943.

van de Vijver M, van de Bersselaar R, Devillee P, et al: 1987. Amplification of the neu (*c-erbB-2*) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-erbA oncogene. *Molecular and Cellular Biology* 7:2019-2023.

Van den Berg F, Schipper M, et al: 1989. Implausibility of an etiological association between cytomegalovirus and Kaposi's sarcoma shown by four techniques. *Journal of Clinical Pathology* 42:128-131.

Vanhaesebroeck B, Reed JC, Valck DD, et al: 1993. Effect of *Bcl-2* proto-oncogene expression on cellular sensitivity to tumor necrosis factor-mediated cytotoxicity. *Oncogene* 8:1075-1081.

Varley JM, Swallow JE, Brammar WJ, et al: 1987. Alterations to either *c-erbB-2*(neu) or c-myc proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. *Oncogene* 1:423-430.

Vaux DL, Aguila HL, Weissman IL: 1992. *Bcl-2* prevents death of factor-deprived cells but fails to prevent apoptosis in stargets of cell mediated killing. *International Immunology* 4:821-824.

Vaux DL, Cory S, Adams JM: 1988. *Bcl-2* gene promoters haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335:440-442.

Veach SR, Ghosh BC: 1987. Biologic markers in breast cancer. In *Tumor Markers and Tumor-Associated Antigens*. Edited by Ghosh BC and Ghosh L. McGRAW-HILL BOOK COMPANY. New York, et al. pp87-101.

Venter DJ, Tuzi NL, Kumar S, et al: 1987. Overexpression of the cerbB-2 oncoprotein in human breast carcinomas: immunohistological assessment correlates with gene amplification. *Lancer*. July 69-71.

Vernon SW, Tilley BC, Neale V, et al: 1985. Ethnicity, survival, and delay in seeking treatment for symptoms of breast cancer. *Cancer* 55:1563-15711.

Vindelov LL, Christensen IJ: 1990. A review of techniques and results obtained in one laboratory by an integrated system of methods designed for routine clinical flow cytometric DNA analysis. *Cytometry* 11: 753-70.

Vogel VG, Love RR: 1991. High risk groups and cost strategies. In *Approaches to Breast Cancer Prevention*. Edited by Stoll BA. Kluwer Academic Publishers. Dordrecht, Boston, London. pp207-220.

Vogelsteinie B, Kinzler KW: 1992. p53 function and dysfunction. *Cell* 70:523-526.

Vorherr H: 1980. Breast cancer in relation to diet, overnutrition, and obesity. In *Breast Cancer: Epidemiology Endocrinology Biochemistry and Pathobiology*. Urban & Schwarzenberg, Inc. Baltimore-Munich pp37-53.

Wagner FB Jr.: 1991. History of breast disease and its treatment. In *The Breast*. Edited by Bland KI and Copeland EM III. W.B. Saunders Company. pp1-16.

Walker RA, Senior PV, Jones JL, et al: 1989. An immunohistochemical and *in situ* hybridization study of *c-myc* and *c-erbB2* expression in primary human breast carcinomas. *Journal of Pathology* 158:97-105.

Waterhouse J, Muir C, Correa P, et al: 1976. Cancer incidence in five continents. In *Scientific Publication* No.15, Lyon, IARC.

Waters JJ, Long SG: 1994. Interphase cytogenetics. In *Molecular Biology in Histopathology* Edited by Crocker, J. JOHN WILEY & SONS. pp57-70.

Wazer DE, Chu Q, Liu SL, et al: 1994. Loss of p53 protein during radiation transformation of primary human mammary epithelial cells. *Molecular and Cellular Biology* 14:2468-2478.

Weinberg RA: 1991. Tumor suppressor genes. *Science* 245:1138-1145.

Weinberg RA: 1994. You can't get there from here: the tortuous road to basic research. *academic Medicine* 69(6):441-444.

Weiner DB, Kokai Y, Wada T, et al : 1989. Linkage of tyrosine kinase activity with transforming ability of the p185 neu oncoprotein. *Oncogene* 4:1175-1183.

Weiner DB, Nordberg J, Robinson R, et al: 1990. Expression of the neu gene-encoded protein p185neuP in human non-small cell carcinomas of the lung. *Cancer Research* 50:421-425.

Weinstein JN, Myers TG, O'Connor PM, et al: 1997. An information -intensive approach to the molecular pharmacology of cancer. *Science* 275:343-349.

Wels W, Moritz D, Schmidt M, et al: 1995. Biotechnological and gene therapeutic strategies in cancer treatment. *Gene* 159:73-80.

Whittemore AS, Wu-Williams AH, Lee M, et al: 1990. Diet, physical activity, and colorectal cancer among Chinese in North America and China. *Journal of national Cancer Institute* 82:915-926.

WHO: 1982. The World Health Organization Histological Typing of Breast Tumors--Second Edition. *American Journal of Clinical Pathology* 78: 806-816

Wilkinson DG: 1992. The theory and practice of *in situ* hybridization. In *in situ Hybridization A Practical Approach* Edited by Wilkinson DG. IRL PRESS at Oxford University Press Oxford, New York, Tokyo. pp1-14.

Wilkinson EJ, Franzini DA, Masood S: 1991. Cytological needle sampling of the breast: techniques and end results. In *The Breast Comprehensive management of benign and malignant diseases.* Edited by Bland KI and Copeland III EM. W.B. SAUNDERS COMPANY Philadelphia, London, Toronto, Montreal, Sydney, Tokyo. pp475-498.

Willet WC, Browne ML, Bain C, et al: 1985. Relative weight and risk of breast cancer among premenopausal women. *American Journal of Epidemiology* 122:731-740.

Willett WC, Stampfer MJ, Colditz GA, et al: 1987. Moderate alcohol consumption and

the risk of breast cancer. *New England Journal of Medicine* 316:1174-1179.

Winer DB, Liu J, Cohen JA, et al: 1989. A point mutation in the neu oncogene minces lignd induction of receptor aggregation. *Nature* 339:230-231.

Wooster R, Bignell G, Lancaster J, et al: 1995. Indentification of the breast cancer susceptibility gene BRCA2. *Nature* 378:789-792.

Wyllie AH, Rose KA, Morris RG, et al: 1987. Rodent fibroblast tumours expressing human myc and ras genes: growth, metastasis and endogenous oncogene expression. *British Journal of Cancer* 56:251-259.

Wyllie AH: 1985. The biology of cell death in tumours. *Anticancer Research* 5:131-136.

Wyllie AH, Ker JFR, Currie AR: 1980. Cell death: the significance of apoptosis. *International Review of Cytology* 68:251-306.

Yamamoto T, Ikawa S, Akiyama T, et al: 1986. Similarity of protein encoded by the human *c-erbB-2* gene to epidermal growth factor. *Nature* 319:230-234.

Yang E, Zha JP, Jockel J, et al: 1995. Bad, a heterodimeric partner for Bcl-x1 and *Bcl-2*, displaces Bax and promotes cell-death. *Cell* 80:285-291.

Yap EPH., Martinez-Montero JC, McGee JO'D: 1992. mRNA detection in clinical samples by non-isotopic *in situ* hybridization. In *Diagnostic Molecular Pathology A Practical Approach* Volume I Edited by Herrington CS and McGee JO'D. IRL PRESS at Oxford University Press. Oxford, New York, Tokyo. pp187-204.

Yap EP, McGee JO: 1992. Nonisotopic SSCP detection in PCR products by ethidium bromide staining. *Trends in Genetics* 8:49.

Yarden Y, Ullrich A: 1988. Growth factor receptor tyrosine kinases. *Annual Review of Biochemistry* 57:443-478.

Yeatman TJ, Bland KI: 1991. Clinical and pathological staging of breast cancer and prognostic factors. In *The Breast Comprehensive Management of Benign and Malignant Diseases*. Edited by Bland KI and Copeland III EM. W.B. SAUNDERS COMPANY Harcourt Brace Jovanovich, Inc. Philadelphia, London, Toronto, Montreal, Sydney, and Tokyo. pp313-350.

Yew PR, Berk AJ: 1992. Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* 357:82-85.

Yokota J, Yamamoto T, Miyalima N, et al: 1988. Genetic alterations of the *c-erbB-2* oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of the v-erbA homologue. *Oncogene* 2:283-287.

Yonisch-Rouach E, Resnitzky D, Lotem J, et al: 1991. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* 352;345-347.

Yunis JJ, Oken MM, Kaplan ME, et al: 1982. Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphoma. *New England Journal of Medicine* 307:1231-1236.

Yu MC, Gerkins VR, Henderson BE, et al: 1981 Elevated levels of prolactin in nulliparous women. *British Journal of Cancer* 43:826-831.

Zeillinger R, Kury F, Czerwenka K, et al: 1989. HER-2 amplification, steroid receptors and epidermal growth factor receptor in primary breast cancer. *Oncogene* 4:109-114.

Zhang X, Siva E, Gershenson D, et al: 1989. Amplification and rearrangement of c-erbB proto-oncogene in cancer of human female genital tract. *Oncogene* 4:985-989.

Zhou D, Battifora H, Yokota J, et al: 1987. Association of multiple copies of the *c-erbB-2* oncogene with spread of breast cancer. *Cancer Research* 47:6123-6125.

Song, 1993. 宋继竭，张道荣，王恩华: 1993. 第十四章 乳腺疾病 *临床组织病理学彩色图谱*. 医疗科学技术出版社，p392-420。

Yiao, 1991. 姚先莹: 1991. 第七章 性激素受体的临床应用 *激素受体及临床应用*. 曹泽毅主编. 北京医科大学中国协和 医科大学联合出版, p177-189。

Ni, 1993. 倪灿荣 王江: 1993. 癌基因蛋白产物在肿瘤的表达及意义 *免疫组织 化学 实验 新技术及应用*. 倪灿荣主编. p313-324。

Fu, 1993. 付明德: 1993. 酶受体分析法 *激素受体及临床应用*. 曹泽毅主编. 北京医科大学中国协和 医科大学联合出版社, p169-176。

He, 1990. 贺国辉: 1990. 恶性肿瘤的病理学 *恶性肿瘤的诊断与治疗*. 江西 科学 技术出版社, p11-28。

Gong, 1993. 龚西瑜: 1993. 乳腺肿瘤 *实用肿瘤病理组织学彩色图谱*. 龚西瑜, 王保泰, 倪慧蓉 主编. 安徽科学技术出版社, p288-317。

Liu, 1993. 刘德培: 1993. 肿瘤基 因治疗 *现代分子生物学实验技术* 卢圣栋 主编. 高等教育出版社。

Lu, 1993. 卢圣栋: 1993. 基因工程与分子生物学的进展及展望 *现代分子生物学 实验技术* 卢圣栋 主编. 高等教育出版社, p1-30。

Hu, 1993. 胡晓年: 1993. 癌 基 因 产物与基 因 表达控制 *现代分子生物学 实验技术* 卢圣栋 主编. 高等教育出版社, p460-462。

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