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# THE FUNCTIONAL ROLE OF TP53 GENE IN DEVELOPMENT OF BREAST CANCER

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**Phd Thesis**

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I dedicate this study to all women

and especially to Virgin Mary, Mother of the One trully God

under  Her, we are Her children!

## CONTENTS

Declaration and Confidentiality .............................................................. 7
Acknowledgements ............................................................................... 8
Abbreviations ..................................................................................... 9
Abstract ............................................................................................. 14
INTRODUCTION .................................................................................. 17
Breast cancer ...................................................................................... 17
Incidence ............................................................................................ 17
Etiology .............................................................................................. 18
Molecular basis of breast cancer ......................................................... 20
Diagnosis of breast cancer .................................................................. 24
Pathological techniques ...................................................................... 25
Tumor markers ................................................................................... 26
History of tumor markers .................................................................... 31
Current role of tumour markers ........................................................... 32
Breast cancer tumour markers ............................................................. 32
Measurement of breast tumour markers .............................................. 33
Serum tumour markers ........................................................................ 37
Serum antigens .................................................................................... 38
Measurement of serum antigens to diagnose cancer ............................ 40
Immune response to cancer and its use in cancer detection ................. 40
Antibodies to tumour associated antigens ............................................ 42
Microscopic types of breast cancer ...................................................... 44
Staging of breast cancer ...................................................................... 45
Grading of breast cancer ..................................................................... 47
Prognostic factors in breast cancer ...................................................... 48
Epidemiology ...................................................................................... 50
Pathogenesis of breast cancer ............................................................. 50
Methods in the genomic studies of breast cancer ................................. 52

Studies on genomic aberrations in breast cancer .................................... 52
Comparative studies using multiple techniques .................................... 56
Molecular pathology of breast cancer .................................... 57
Gene amplifications in breast cancer .................................... 59
Tumor suppressor genes .................................... 61
The genetic basis of cancer .................................... 64
P53 and Li-Fraumeni syndrome .................................... 65
Proto-oncogenes vs tumor suppressor genes .................................... 65
Cancer is a multistep process .................................... 67
Genomic alterations in cancer .................................... 68
Translocations .................................... 70
Epigenetic alterations .................................... 73
Array comparative genomic hybridization as detecting methods in cancer alterations .................................... 74
Gene expression arrays .................................... 75
Next generation sequencing .................................... 80
Cell cycle control – Tumor protein P53 .................................... 84
Vascular endothelial growth factor and TP53 .................................... 91
The role of cyclin E .................................... 93
The role of P21 (waf1/Cip1) and MDM-2 .................................... 96
Cell cycle regulators .................................... 97
The biology of TP53 .................................... 99
Stimulation and Activation effects of TP53 .................................... 100
Cell senescence .................................... 100
Apoptosis .................................... 101
Autophagy .................................... 101
Mitotic catastrophe .................................... 102
Angiogenesis .................................... 102
Regulation of P53 .................................... 103

TP53 mutations and Human cancer ........................................................ 105
P53 a sensor protein of genotoxic stress .................................................. 110
Gene therapy by TP53 ........................................................................ 112
P53 status and Anthracycline chemotherapy in breast cancer ................ 113
P53 status and Nonanthracycline chemotherapy in breast cancer......... 115
P53 and radiotherapy .......................................................................... 116
Isoforms of P53.................................................................................. 117
P53 and Its target genes...................................................................... 117
P53 as a transcription activator.......................................................... 118
P53 family .......................................................................................... 120
Regulation of P53 .............................................................................. 120
P53 at a glance ................................................................................... 121
P53 mutations..................................................................................... 122
P53 activating signals ........................................................................ 122
The P53 – mdm2 Loop........................................................................ 123
Covalent modifications of P53............................................................ 124
P53 and Oncogenic stress.................................................................... 126
P53-dependent apoptosis.................................................................... 127
Microbial or Viral inactivation of P53 protein .................................... 127
Some phychological steps for the patient and her family....................... 129
DISCUSSION....................................................................................... 130
CONCLUSIONS.................................................................................... 135
References .......................................................................................... 143
Resume................................................................................................ 182
Appendix (A) ..................................................................................... 183
Appendix (B) ...................................................................................... 184
Appendix (C)....................................................................................... 185

## DECLARATION AND CONFIDENTIALITY

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## ABBREVIATIONS

| | |
|---|---|
| AAb | Autoantibody |
| AAbs | Autoantibodies |
| ABC | Advanced Breast Cancer |
| ADH | Atypical Ductal Hyperplasia |
| ALF | Automated Laser Fluorescence |
| ALH | Atypical Lobular Hyperplasia |
| AMA | American Medical Association |
| APC | Antigen Presenting Cell |
| ASCO | American Society of Clinical Oncology |
| BCSS | Breast Cancer Specific Survival |
| Ca15.3 | Cancer Antigen 15.3 |
| Ca27-29 | Cancer Antigen 27-29 |
| CAF | Cyclophosphamide, Doxyrubicin, 5-flourouracil |
| CC | Cranio-Caudal |
| CDK-2 | Cyclin-Dependent-Kinase-2 |
| cDNA | Complementary DNA |
| CEA | Carcinoembryonic Antigen |
| CI | Confidence Interval |
| CIC | Circulating Immune Complex |
| CMF | Cyclophosphamide, Methotrexate, 5-flourouracil |
| CR | Coefficient of Reproducibility |
| CT | Computer Tomogram |
| CV | Coefficient of Variability |
| DAB | Diamino benzidin |
| DCIS | Ductal Carcinoma In-Situ |

| | |
|---|---|
| DFI | Disease Free Interval |
| DMSO | Dimethyl sulfoxide |
| DNA | DeoxyriboNucleic Acid |
| dPCR | Differential Polymerase Chain Reaction |
| ECM | Extra Cellular Matrix |
| EDTA | Ethylenediaminetetraacetic Acid |
| EIA | Enzyme Immunoassay |
| ELISA | Enzyme Linked ImmunoSorbant Assay |
| ER | Estrogen Receptor |
| ESR | Erythrocyte Sedimentation Rate |
| FASAY | Functional Analysis of Separated Alleles Yeast |
| FGF | Fibroblast Growth Factor |
| fhx | Family history |
| Fig. | Figure |
| FISH | Fluorescence In-situ Hybridisation |
| GEO | Gene Expression Omnibus |
| GIVIO | Interdisciplinary Group for Cancer Care Evaluated |
| GPs | General Practitioners |
| $H_2O_2$ | Hydrogen Peroxide |
| HER2 | Human Epidermal Growth Factor Receptor 2 |
| HGF | Hepatocyte Growth Factor |
| HLO | Medio-lateral Oblique |
| HR | Hazard Ratio |
| HRP | Horse Radish Peroxide |
| HRT | Hormone Replacement Therapy |
| IgG | Immunoglobulin G |

| | |
|---|---|
| IgM | Immunoglobulin M |
| IHC | Immunohistochemistry |
| IL | InterLeukin |
| Ki-67 | Kiel Antigen-67 |
| KS | Kolmogorov-Smirnov |
| LCIS | Lobular Carcinoma In Situ |
| LN | Lymph Node |
| MAb | Monoclonal Antibody |
| MDM-2 | Murine Double Minute-2 |
| MHC | Major Histocompatibility Complex |
| MIB-1 | Molecular Immunology Borstel-1 |
| MRI | Magnetic Resonance Imaging |
| mRNA | Messenger Ribonucleic Acid |
| N | Number |
| N/A | Not Available |
| NHSBSP | National Health Service Breast Screening Programme |
| NICE | National Institute of Clinical Health and Excellence |
| NIH | National Institute of Health |
| NPI | Nottingham Prognostic Index |
| NS | Non significant |
| NSS | Normal Swine Serum |
| NST | Non-specific Type |
| OD | Optical Density |
| ORF | Open Reading Frame |
| OS | Overall Survival |
| PAI-1 | Plasminogen Activator Inhibitor Type-1, |

| | |
|---|---|
| PBC | Primary Breast Cancer |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PEM | Polymorphic Epithelial Mucin |
| PET | Positron Emission Tomogram |
| PgR | Progesterone Receptor |
| pRB | Retinoblastoma Protein |
| PSA | Prostate Specific Antigen |
| PVP | Polyvinyl Pyrolhidone |
| RFS | Recurrence Free Survival |
| RH | Relative Hazard |
| SD | Standard Deviation |
| SDS PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SELIDI | Surface-enhanced Laser Desorption/Ionization |
| SLE | Systemic Lupus Erythematosus |
| SPF | S-Phase Fraction |
| StreptABComplex | Streptavidin Biotin Complex |
| TBS | Tris Buffered Saline |
| TGF | Transforming Growth Factor |
| TMB | 3,3',5,5' -tetramethyl-benzidine |
| TNF | Tumor Necrosis Factor |
| TNM | Tumor, Node, Metastasis |
| TPA | Tissue Polypeptide Antigen |
| TSGs | Tumor Suppressor Genes |
| Tween | Polyoxyethylenesorbitan monolaurate |

| | |
|---|---|
| UICC | International Union Against Cancer |
| uPA | Urokinase Plasminogen Activator |
| USA | United States of America |
| VEGF | Vascular Endothelial Growth Factor |
| VI | Vascular Invasion |
| VNTR | Variable Number Tandem Repeat |
| VOL | Vector Only Lysate |
| WHO | World Health Organisation |
| % | Percent |
| +ve | Positive |
| -ve | Negative |

# ABSTRACT

The incidence of breast cancer has risen steadily over the last half a century, partially due to earlier detection. Nevertheless, the mortality rate has remained relatively constant (27 per 100.000 women). Although we are beginning to understand the risk factors for breast cancer, including environmental sources (e.g. mutagen exposure) and personal choices (e.g. late first childbirth age and high dietary fat intake), the further identification of mechanisms underlying the development and progression of breast cancer is of major public health importance.

The activation of oncogenes has been well described as one possible mechanism to transform normal cells, including breast. Now it has become clear that the inactivation of various tumor suppressor genes, which can be thought of as "brakes" of cell growth, is at least as important in the development and progression of breast cancer. Tumor suppressor genes are considered to act mostly in a recessive fashion, i.e. some abnormality must affect both gene alleles. The classical inactivation of tumor suppressor genes, i.e. the Knudson "Two-Hit" hypothesis (Knudson, 1971), is caused by tumor suppressor gene loss due to chromosomal loss of one allele, and mutation of the other remaining allele. Chromosomal loss is mostly analyzed by karyotypic studies or loss of heterozygosity (LOH) studies, and mutations are most frequently studied by sequencing of the gene of interest or by single strand chain poly-morphism analysis (SSCP). In many cases, mutations can result in truncated protein products which are easy to detect. However, recently it has been shown that functional inactivation of tumor suppressor genes can be

caused by many other epigenetic mechanisms besides mutation, including hypermethylation (Baylin et al. 1998, Foster et al. 1998), increased degradation (Storey et al. 1998), or mislocalization (Chen et al. 1995, Moll et al. 1992, Takahashi and Suzuki 1994 ).

The function and role of tumor suppressor genes have been elucidated by many investigators through a combination of a number of cell biological as well as biochemical methods. Kinzler and Vogelstein (1997) have recently proposed a new system to categorize tumor suppressor genes as "gatekeepers" and "caretakers". Gatekeepers are tumor suppressor genes which are directly involved in controlling proliferation by regulating cell cycle checkpoints (e.g. Rb and the INK family of cdk inhibitors). Mutations of these genes usually result in high penetrance. In contrast, caretakers are of rather low penetrance, and have an indirect effect on growth. They are responsible for genome integrity, and changes in such genes lead to genome instability.

In the past several years we have seen an explosion of information in the field of breast cancer genetics, with regard to the identity of tumor suppressor genes that are mutated in sporadic breast cancer as well as those that are inherited in mutant forms, giving rise to a familial predisposition to cancer. In this research we will summarize informations and concentrate on tumor suppressor gene which has been proven to play a role in breast cancer *in vivo*.

Among gatekeepers genes, P53 gene is the most well known, located on chromosome band 17p13, p53 encodes a 53-Kd multifunctional transcription factor that regulates the expression of genes involved in cell cycle control, apoptosis, DNA repair and angiogenesis. In breast cancer,

most studies have shown that P53 mutation or down-regulation is associated with adverse prognosis. Also P53 wild-type protein plays an important role in cells as is shown by its fine regulation at different levels P53 is mutated in almost 30% of cases, with a higher frequency in some tumor subtypes. TP53 mutation is reported to be a factor for good prognosis in some studies, while in others it is a factor for poor prognosis. These different results can be explained based on studies in different tumor types with different therapy regimens. P53 plays a key role in integrating cellular response to damaging agents.

# INTRODUCTION

## Breast cancer

Breast cancer isn't a disease of modern society. It was recognized by the ancient Egyptians as long ago as 1600 b.c.[2], however it is still a major public health problem in both developed and underdeveloped countries. The majority of breast cancers arise from the technical duct lobular unit (TDLV) [3].

## Incidence

Breast cancer is among the most common human malignancies among women worldwide, accounting for a tenth of all new cancers and 23% of all female cancer cases[4].

In Iraq according to Iraqi cancer registry in 2002, breast carcinoma was the most frequent cancer among women. It forms 14.3% of all malignant tumors with the sharp increase in incidence of this tumor in young age group. The average age of patients with breast carcinoma in Iraqi females is 45 years [5, 6]. Breast cancer in many developing countries presents in younger patients with advanced stages at the time of diagnosis in comparison with developed countries [7]. Undoubtedly, poor screening and education programs are the main contributing factors[8] or this difference.

In the United States(U.S.), women have a 1 in 8 (12.5%) lifetime chance of developing invasive breast cancer and a 1 in 35 (3%) chance of breast cancer causing their death. In 2007 [9, 10] breast cancer caused

40,910 deaths in the U. S. (7% of cancer deaths; almost 2% of all deaths). About 182,460 women in the United States will [11] be found to have invasive breast cancer in 2008, and about 40,480 women will die from the disease this year [12].

In the United Kingdom breast cancer rates have increased by 12% in the last ten years where the age standardized incidence and mortality is the highest in the world. The incidence is increasing particularly among women aged 50-64, about 8 in 10 breast cancers are diagnosed in women aged 50 and over, probably because of breast screening in this age group. Breast cancer death rates are going down in these developed countries. This is probably the result of finding the cancer earlier and improved treatment [3, 13].

## Etiology

Several risk factors have been identified that modify a woman's likelihood of developing breast cancer [14]:

**1) Country of birth:** The incidence is high in north America and northern Europe, intermediate in southern Europe and Latin America and low in most Asian and African countries. These geographical differences appear to be environmentally rather than genetically determined [14]. Studies of migrants from Japan to Hawaii show that the rates of breast cancer immigrants assume the rate in the host country within one or two generations, indicating that environmental factors are of greater importance than genetic factors [15]

**2) Family history and genetic predisposition:** Up to 10% of breast cancer in Western countries is due to genetic predisposition. Breast cancer susceptibility is generally inherited as an autosomal dominant with limited penetrance [16]. Women who have a first degree relative with breast cancer have a risk two to three times that of general population [17]. It has been found that about half of women with hereditary breast cancer have mutations in gene BRCA1, and an additional one third has mutations in BRCA2, which are located on the long arms of chromosomes 1 7 and 13 respectively [18].

**3) Reproductive and hormonal factors:** Breast cancer may be related to total number of ovulatory menstrual cycles a women. It is increased in early menarche. Studies also showed that the risk is reduced by a young age at first live birth and increased with a late age at menopause as well as by nulliparity. It is likely that the effects of these reproductive and menstrual factors are related to hormonal pathways [19].

**4) Previous breast disease:** Benign breast diseases may be classified into non-Proliferative and Proliferative lesions. Non-proliferative lesions are generally associated with little or no increase in breast cancer risk. Proliferative lesions, on the other hand, have a two fold increase in risk provided that they show no atypical epithelium, those proliferative lesions with atypical hyperplasia may increase the risk of at least four to five folds when compared with women without breast diseases [20], women with this change and a family history of breast cancer (first degree relative) have a nine fold increase in risk [21].

**5) Hormonal replacement therapy(HRT):** Is associated with increased risk of breast cancer, particularly of the lobular type [22].Cancers diagnosed in women taking HRT tend to be less advanced clinically than those diagnosed in women who have not used HRT. Recent evidence suggests that HRT does not increase breast cancer mortality [23, 24]

**6) Ionizing radiation:** Is associated with increased risk of breast cancer. There is along latent period for radiation induced breast cancer, and the risk is related to the age at which radiation exposure occurs, only women irradiated before age 30, during breast development, appear to be affected [25], Fabio et al, (2007) [24] suggested that genomic instability triggered by irradiation before puberty might be involved in the induction of aggressive HER-2/neu over expressing breast carcinomas. These findings might rest on the ability of ionizing radiation to induce direct DNA damage, leading to gene amplification, or to affect genes involved in DNA repair, and this effect depends on the dose of radiation received.

**7) Environmental factors:** That include obesity, high fat-diet, alcohol consumption and cigarette smoking [26].

## Molecular basis of breast cancer

Breast cancer can be defined as a molecular alterations that are genetically and/or environmentally induced resulting in a cellular disorder characterized by progressive accumulation of a mass of cells, as a result of excessive production of cells not compensated by appropriate cell loss[27].

When genetic alterations develop in somatic cells it may lead to a sporadic carcinoma, but genetic damage in germ cell line causes a risk of a familial breast cancer [27]. It may have been therefore several years before it is detected and it can be successfully detected by palpation when it reaches a diameter of approximately 1 cm (one gm).By this time, it will have gone through 20-30 doublings and it is possible that metastasis has already started [27, 28]. This cellular imbalance is caused by alterations in the genetic mechanisms resulting in:

a. Activation of proto-oncogenes.
b. Alterations of genes that regulate apoptosis like Bcl2 gene.
c. Inactivation of cancer suppressor genes like p53 gene.
d. Alterations of genes regulating DNA repair, angiogenesis, etc.

**Proto-oncogenes:** Are normal cellular genes involved in the positive control of cell proliferation and differentiation [29]. Proto-oncogenes and their products include a heterogeneous family of genes and proteins with many biochemical effects including [30, 31]:

A. Growth factors: They produce the external signal to increase or maintain cell proliferation by causing cells in the resting phase (GO) to enter and proceed through the cell cycle.
B. Growth factor receptors: They recognize growth factors and receive the proliferation signal on the cell membrane.
C. Signal transducers: They transfer the signals from the cell membrane through the cytoplasm to the nucleus.
D. Nuclear factors: The induction and activation of the nuclear factors will initiate DNA transcription, which allow the entry and progression of the cell into the cell cycle, resulting in cell division.

E. Survival enhancers: They affect proliferation by prolongation of cellular life span through suppression of apoptosis; otherwise if the correct set of signals for proliferation is absent, cell cycle progression is aborted and apoptosis can result.

Alterations in the structure of proto-oncogenes can convert them into oncogenes that are characterized by the ability to promote cell growth in the absence of normal growth - promoting signals [32].

Oncogenes in general can be grouped according to the location and biochemical function of their encoded products into the following [33]:

A. Growth factors.
B. Growth factors receptors.
C. Signal transducers.
D. Nuclear proteins and transcription factors.

**Growth factor receptors (GFRs):**

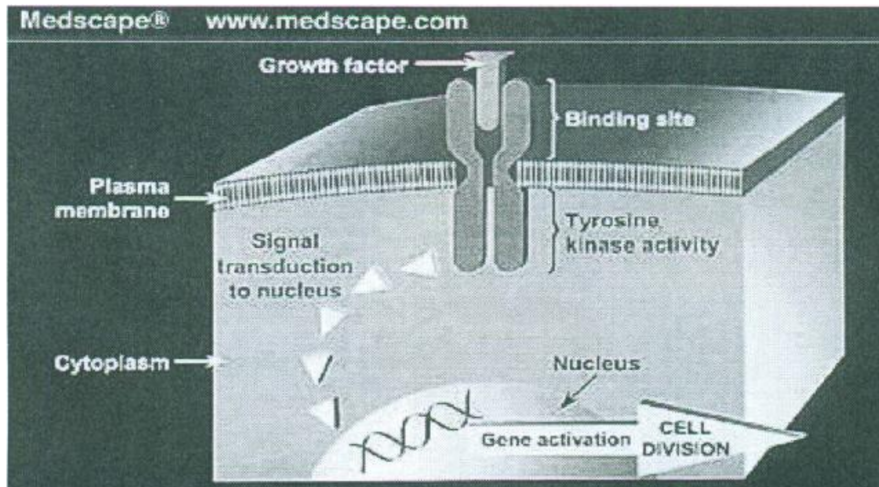
All growth factor receptors are transmembrane structures with an extra cellular ligand-binding domain and an intracellular signal-transducer domain. GFRs can be grouped as follows [34]:

1. Receptors with protein kinase activity:

- Protein tyrosine kinase as epidermal growth factor receptors (EGFRs).
- Protein serine kinase as transforming growth factor B receptors (TGF-BRs).

2. Receptors with other signaling mechanisms: e.g. receptors with single membrane domain.

The oncogenic version of GFRs is associated with persistent activation without binding to the growth factors. Oncogenic activation of these rece-




*The HER2 tyrosine kinase receptor dimer transmembrane signal transduction pathway. By www.medscape.com.*

ptors can occur as a result of mutations of extra cellular, transmembrane or intracellular domain [33].

Carcinogenesis is a multistep process where both genetic alterations and epigenetic changes can be seen in the cells. Epidemiological studies have suggested that ductal proliferation associates with carcinogenesis in breast tissue and that the majority of breast carcinomas originate from precursor lesions of various degrees of hyperplastic changes[35], which has been supported by genetic studies. Genetic alterations cause allelic imbalance and therefore the loss of heterozygosity. A trend of loss of heterozygosity accumulation has been demonstrated in a process of malignant transformation[36]. Major genetic changes seem to appear during the transition from normal breast tissue to ductal carcinoma in situ (DCIS), while the gene-expression alterations between DCIS and infiltrative cancer reveal extensive similarities. It is therefore presumed that most invasive breast carcinomas originate from in situ cancer [37]. Morphologically it is possible to distinguish a transition from normal epithelium to in situ ductal hyperplasia with various atypical characte-

ristics, further to in situ carcinoma and finally to invasive carcinoma [38]. On the other hand, it has been found that most precursor lesions will never progress to invasive carcinoma and invasive cancer may develop without evidence of concurrent precursor lesions [39]. Thus, the chronology of genetic alterations and the exact mechanism of malignant transformation in breast tissue are mainly unknown. Simpson et al., (2005), [40] suggested that breast carcinogenesis has been regarded as a complex series of stochastic genetic events that lead to divergent and distinct pathways towards infiltrative breast carcinoma.

## Diagnosis of breast cancer

**1. Physical examination:** Breast lumps are detectable in the majority of patients with breast cancer through careful palpation. The typical cancerous mass tends to be unilateral, solitary, solid, hard, irregular and not tender [41].

**2. Radiological techniques:**

**A. Mammography:** This technique detects about 85% of breast cancer cases. Although 15% of breast cancers can not be visualized with mammography, 45% of breast cancers can be seen on mammography before they are palpable [41].

Radiological characteristics of carcinoma include [42]:

a. High density of the leasion.
b. Architectural distortion and irregular margin with speculation.
c. Fine punctuate calcification.
d. Overlying skin thickening or dimpling.

**B. Ultrasonography:** The major indications of breast ultrasound are[43]:

1. The differentiation between a cyst and solid mass.
2. The exploration of a palpable abnormality not clearly visible by radio-graphy.
3. The lesion can not be radio graphed (i.e. axillary and submammary location).

**C. MRI of the breast:** Patients, who have dense breasts or a mass with normal mammography and ultrasound, may be subjected to MRI evaluation for further definition [37].

**3. Pathological techniques.**

A-Cytology: which includes:

1. Nipple secretion cytology: Nipple cytology (discharge and scraping) is a non-invasive, simple, and cost effective method. It compliments assessment of patients with nonphysiologic nipple discharge or a nipple lesion, bloody nipple discharge as well as eczematous nipple skin changes were predicted to be associated with high diagnostic yield for carcinoma [44].
2. Fine needle aspiration (FNA) cytology: This method is easy, quick, and safe. The sensitivity in diagnosing malignancy has been reported to be (90-95%) with almost no false positive results (98%-l00% specifi-city). It should be realized however, that it is impossible to dis-tinguish invasive from in situ carcinoma with these techniques [41].
3. **Needle core biopsy:** This method has the advantage of allowing histologic rather than cytologic assessment, and therefore dif-ferentiation between insitu and invasive carcinoma. However, the procedure is more time–consuming, and the equipment required for

both obtaining and processing the sample is more complex and costly than that used for aspiration cytology[41].

**B. Excisional biopsy:** This is the standard technique for the diagnosis of breast mass. When it is performed, an adequate amount of normal tissue should be removed around suspicious lesion so that the biopsy serves as a segmental mastectomy in the event that a malignancy is found. This allows for complete excision with clear margins and full histological evaluation [41].

**C. Frozen section:** The increasing use of core needle biopsy and FNA biopsy has restricted the use of frozen section technique for the evaluation of breast lumps. The main use of frozen section nowadays in breast pathology is for hormone receptors analysis and in the evaluating re-excision lumpectomy margins [45].

**Investigational tools:**

The conventional hematoxylin and eosin stained section is sufficient in most cases to establish the definitive histological diagnosis for breast tumors, however additional investigational tools may be needed to help and improve the evaluation. These tools include [46]:

Special stains, immunohistochemistry, flow cytometry cytogenetics and molecular studies.

**Tumor markers:**

Tumor markers are biochemical indicators for the presence of a tumor. They include; cell surface antigens, cytoplasmic proteins, enzymes and hormones. The technique used to detect the markers in tissue

sections is known as immunohistochemistry; it depends on the use of monoclonal antibody directed against a specific tissue antigen followed by the application of the detecting system to visualize the reaction [46].

Several tumor markers can be detected in breast carcinoma, these include:

1. **Cytokeratins [47, 48]:** The maJor diagnostic importance of cytokeratin is their ability to identify the epithelial and myoepithelial cells within the mammary glands.
   a) High molecular weight keratin [45, 48]. Tends to be distributed in myoepithelial cells and is highly expressed in lobular carcinoma (in situ and invasive) and in some cases of invasive ductal carcinoma.
   b) Low molecular weight keratin: Paget's cells can be selectively stained by the use of low molecular weight keratin [49], while the resident segment epithelium of the nipple is stained by high molecular weight.

2. **Lactoalbumin, casein:** There is a great discrepancy in the reported incidence of lactoalbumine and casein positivity in breast cancer ranging from nil-100% [50].

3. **Milk -Fat Globule Membrane Antigen (MFMA):** The difference in the breast cancer as compared to normal breast cells is of diagnostic value. In normal breast cells, this antigen is restricted to the apical membrane, while in malignant breast cells, the positivity may be observed at any Point around the cell surface [51].

4. **Gross Cystic Disease Fluid Protein-(GCDFP-15):** This displays high specificity but limited sensitivity thus can not be used as a general screen for breast cancer in tissue substance. It reacts only with apocrine epithelium and a subset of breast carcinoma known as apocrine carcinoma [52].

5. **Actin and Myosin:** These stain selectively the myoepithelial cells. They are used to distinguish benign proliferative lesions (such as sclerosing adenosis, and tubular adenosis) from tubular carcinoma. The latter lacks myoepithelial cells [49].

6. **Polymorphic Epithelial Mucins (PEM):** This is used as a marker for the detection of occult metastatic carcinoma cells in bone marrow preparation [53].

7. **Carcinoembryonic Antigen (CEA):** Is positive in about 70% of breast carcinoma. In situ carcinoma and invasive lobular carcinoma are frequently CEA negative [54].

8. **S-100 protein:** Ten to forty five percent invasive breast carcinomas are immunoreactive for S-100 protein, especially medullary carcinoma [55].

9. **Prostatic Specific Antigen (PSA):** PSA immunoreactivity has been recently demonstrated in about one third of breast tumors [56].

10. **Cathepsin D:** Since cathepsin D lyses protein in the stroma around the tumor, a cell containing large amounts, could more easily invade and metastize [57].

11. **Collagenase:** In both in situ and invasive breast carcinomas there is amplification of type IV collagenase with strong cytoplasmic staining of the tumor cells [58].

12. **P-glycoprotein:** It is responsible for multidrug resistance, so tumors that are resistant to chemotherapy express a high level of P-glyco-protein [46].

13. **Estrogen and progesterone receptors** (ER and PR) [59, 60]: The degree of positivity of ER and PR is proportional to the differentiation of the lesion, the less differentiated invasive ductal and lobular carcinoma tumors show significantly lower levels of ER and PR. There is a positive correlation between the amount of ER and PR in mammary carcinoma and its probability of response to endocrine therapy. ER and PR also have prognostic value.

14. **Beta subunit of Human Chorionic Gonadotropin (BHCG)** [61, 62]. Mammary carcinomas have been shown to stain positivity for B-HCG. There is a relationship between bHCG and PR by the suggestion that progestins regulate the expression of B-HCG in breast epithelial cells, however, no strong correlation is found between B-HCG and ER.

15. **Genetic markers,** these include:

   a) P53: The mutant form of the tumor suppressor gene (P53) can be identified by Immunohistochemistry in breast carcinoma. About 25-40% of sporadic human breast cancer contains P53 mutation. It is usually associated with aggressive tumor phenotype, poor prognosis, short overall survival and poor response to endocrine therapy [63].

   b) BRCA 1 and BRCA2: Both are associated with early onset of familial breast cancer, and breast/ovarian carcinoma syndrome [64].

   c) Bcl2: The gene of this marker is located on chromosome 18, which is one of the genes regulating apoptosis. It is over expressed in about 70% of human breast cancer and its expression associated with better prognosis [65] ·

   d) C-erbB2(HER-2/neu):HER-2/neu overexpression, usually attributable to HER-2/neu gene amplification occurs in 25-30% of breast cancer patients and is associated with a poor prognosis [1].

16. **DNA ploidy and proliferative markers (Ki67):** They are cell proliferation associated nuclear antigens, and are regarded as direct indicators of cell growth fraction [66].

## History of Tumour Markers

As a normal cell transforms to a neoplastic cell, changes occur both within and on the surface of the cell that could potentially be detected and used as a tumour marker. This could provide valuable information on the status of the cell at the given point thus enabling early detection, which is key to cancer cure and prevention (Srinivas et al, 2001). Although there are many modalities available to detect early tumour e.g. computer tomogram (CT) and MRI, laboratory based detection has an added advantage that it is relatively inexpensive. The costbenefit analysis is favourable for this type of investigation as the unit cost of the test is low and will reduce further with more high-throughput assay innovations (Srivastava and GopalSrivastava, 2002).

Current tumour markers are generally tumour associated-antigens. The first tumour marker noted was in 1960s with the description of carcinoembryonic antigen (CEA) (Gold and Freedman, 1965). It was noted that this was present in the serum of patients with gastrointestinal malignancies but not in normal mature tissues. It was hoped that this and other markers would be highly sensitive and specific to the tumour in question. It was thought they could be used not only for diagnosis but also in screening. However it was later realised that the same tumour markers were not only detected in other malignant conditions but were also found in various quantities in normal cells (Thomson, 1972). Their role in distinguishing malignant from benign thus became unclear.

## Current role of tumour markers

Current clinical application of tumour markers is limited to diagnosis of recurrent or metastatic disease such as CEA in colorectal cancer and CA15.3 in breast cancer. Tumour markers are also used to monitor response to systemic therapy in certain cancer patients.

If tumour markers are to be clinically applicable for screening or diagnosis then the marker must be present in the serum of the at-risk individual in sufficient quantity and not be present in the normal population. The assays used to measure these markers need to have a high sensitivity and specificity for the detection of markers. The assay also has to be relatively inexpensive and the disease tested is common and causes significant morbidity and/or mortality if left unchecked. A positive assay should result in definitive treatment with survival advantage in those treated compared to the untreated group (Daar and Aluwihare, 2000).

## Breast cancer tumour markers

Unfortunately breast cancer has yielded no such simple screening blood test to-date. The overwhelming prevalence of breast cancer in the north European and American populations and its morbidity and mortality and some limitations of current screening methods demand a simple and reliable test similar to the prostate specific antigen (PSA).

Tumour markers in breast cancer are extremely various in number and type. Mucins e.g. CA15.3 (Clinton et al, 2003; Safi et al, 1991) and CA 27-29 (Frenette et al, 1994), oncofoetal proteins (e.g. CEA) (Esteban et al, 1994; Sundblad et al, 1996), oncoproteins e.g. HER2 (lmoto et al, 2007;

Muller et al, 2006; Kong et al, 2006; Hudelist et al, 2006) c-myc (Breuer et al, 1994). and p53 (Balogh et al, 2006; Hassapoglidou et al, 1993), cyto-keratins e.g. TPA (Nicolini et al, 2006; Sliwowska et al 2006) and ESR (Robertson et al, 1991 and 1999; Rubach et al, 1997) are among the many proposed as a tumour marker for breast cancer. More recent tumour markers described in the literature include Mammaglobin (Watson et al, 1996), survivin ( Goksel et al, 2007; Yagihashi et al, 2005), livin (Yagihashi et al, 2005), NYES0-1 (Bandic et al, 2006), Endostatin (Balasubramanian et al, 2007), Hsp90 (Pick et al, 2007), p62 (Rolland et al, 2007) and koc (Zhang et al, 2003).

These and other various antigen markers have been used with only limited success. These tumour antigen markers are either over-expressed and therefore produced in excessive amounts or are the mutated form of a corresponding "wild type". Normal form and amounts of antigen can also be found but in abnormal compartments of the cancer cell or in extra cellular spaces. Normal functions of these wild type markers vary dependent on the marker and the cell that produces it. However aberrant markers can actually be involved in the pathogenesis of the tumour itself.

## Measurement of breast tumour markers

There is a wide range of methods used to test breast tumour markers dependent on the marker itself. These assays include solid matrix-blotting, immunohistochemistry (IHC), fluorescence in-situ hybridisation (FISH), enzyme immunoassay (EIA) and enzyme linked immunosorbant assay (ELISA). The different assays can be used to measure various targets related to the tumour marker, such as DNA or gene copy number

(FISH, Southern blot), mRNA (Northern blot), cell surface protein (Western blot, cell surface ELISA and IHC) and circulating protein (serum ELISA and EIA). Furthermore different tissues can be used depending on the assay used: fresh frozen tissue for Southern, Northern and Western blots and IHC; formalin-fixed, paraffin-embedded tissue for IHC and FISH; and serum or tissue extracts for ELISA and EIA.

There are advantages and disadvantages to using the various methods. For example, IHC is performed using specific antibodies against the tumour marker and depending on the specificity of the antibody IHC may be able to discriminate between normal and abnormal copies of tumour marker and can precisely localize the marker in cells and tissues. However even where an antibody shows promise in distinguishing cancer from normal cells there are many technical issues with IHC such as antigen loss that can occur in stored formalin-fixed, paraffin-embedded tissue samples. This loss is variable and depends on time and nature of fixation; method of tissue processing; temperature of paraffin embedding; duration of storage; the particular antibody used for detection; and the staining procedure used. Therefore variability in results using IHC is partly related to antigen loss as well as use of different antibodies to the same marker.

ELISA can be used to measure breast tumour markers in either fresh tumour cytosolic fractions or in circulating serum as shed antigens or detection of the immune response, as antibodies, to such antigens. The convenience of serum ELISA is that a serum sample can be taken at any time and on repeated occasions whereas tissue samples of primary tumour are usually obtained following biopsy or surgery. Unfortunately

one disadvantage of ELISA is that histological information cannot be obtained using ELISA and furthermore an ELISA blood test may measure a different marker endpoint to IHC.

Dependent on the marker, these can either be present in cancer cell nucleus, cytosol or the cell membrane. Tissue concentrations of markers may also correspond to tumour load thereby quantifying tumour burden. Depending on the actual marker it may indicate prognosis or predict tumour behaviour.

The expression of some markers such as HER2 within the tumour has been reported to correlate with a rise in serum levels of the marker at an advanced stage of breast cancer (Narita et al, 1994; Molina et al, 1996). Molina and colleagues reported on 200 women treated for primary breast cancers that were followed up with sequential blood samples for measurement of three tumour markers. In 18% of patients the first sign of recurrence in terms of blood antigen measurements was a rise in HER2 in the blood. Further tests showed that serum HER2 was elevated in 80% of patients who were found to be HER2 positive in their primary tumour and in only 3.3% of patients who had a HER2 negative tumour. However this apparent link between tissue and serum antigen expression is not the case for all tumour antigen markers (Cannon et al, 1993).

Tumour marker levels in the tissue cannot therefore accurately predict its presence or level in the serum for the majority of markers and so direct measurement in the serum is necessary. A further disadvantage of measuring tissue markers is that these markers only confer a static view of the tumour. This is in contrast to using serum markers whose detection reflect a dynamic situation and can be repeated as often as required.

Tumour markers may be measured singularly or in combination. However, recently whole cell proteins have been measured as biomarkers of cell events (Belhajjame et al, 2005). Since cancer cells have altered oncogene expression, the protein products are also altered. This can be measured as microarrays of multiple proteins.

Individual proteins can also be identified using various techniques such as 2-D gel electrophoresis (Arora et al, 2005) thereby allowing the detection of new single protein tumour marker. This promising field for early tumour detection is aided by various new techniques that detect these proteoms e.g. surface enhanced laser desorption/ionisation (SELDI) (Mazzatti et al, 2007), biochips (Hervas 2004) and mass spectrometer (de Souza et al, 2006).

The measurement of the protein products of oncogenes rather than the genes themselves has several advantages. Proteins are regarded as the dynamic consequence of cellular events and therefore will indicate the cell condition at a given time. Furthermore a single protein translated from a gene may undergo multiple further procedures that can be at fault at any stage and therefore measurement of proteins should be a more accurate reflection of what has gone wrong. This is not possible simply by measuring oncogenes.

The potential uses for proteomics in breast cancer diagnosis, prognosis and monitoring, although undoubtedly huge, are currently undetermined. Although there are many modalities available to detect early tumours e.g. CT and MRI, laboratory-based detection has an added advantage that it is relatively inexpensive (Srivastava and Gopal-Srivastava 2002).

Despite such potential benefits getting results from proteomics takes time. Protein analysis can be laborious. It often requires separating multitude of proteins and determining their individual molecular weight and electric charge. This downside of proteomics may limit its general use. Proteomics may also yield an array of proteins, which are highly specific to the individual patient. It therefore limits general clinical application of the procedure.

## Serum Tumour Markers

Some tumour markers are originally contained within tumour cells. They can be found within the nucleus, cytosol or are membrane bound with extracellular domains.

Nuclear proteins, which can be measured as markers of the cancer, may be sequestered in the cytosol as part of carcinogenesis. Sequestration in an abnormal compartment may prevent the normal function of the cell, thus resulting in eventual carcinogenesis. These intra-cellular markers are released into the serum via non-apoptotic and apoptotic cancer cell death, where they can be measured as serum markers. Other markers may be cleaved from its original membrane bound configuration and shed into the extra cellular domain.

Detection of these serum markers may therefore reflect the overall antigen load of the organism i.e. its cancer burden or the degree of proteolytic activity owing to growth rate, necrosis and cell degeneration.

Serum marker measurement can be by techniques such as ELISA (Cordiano et al, 1995) and SELDI (Mazzatti et al, 2007), which also allow quantification of the marker present.

SELDI is an affinity-based mass spectrometric method in which proteins of interest are selectively adsorbed to a chemically modified surface on a biochip. Impurities are removed by washing with buffer. These proteins can then be measured on a reader (Li et al, 2002). This therefore allows accurate measurement and profiling of the proteins available in the serum.

Marker levels in the serum are dynamic in that they reflect overall tumour burden as some tumour markers are seen to decrease in level in the serum after excision of the primary tumour. This decrease is not demonstrated in pre and postoperative levels of markers found in non-neoplastic conditions (Reis et al, 2002). This correlation between the markers noted in the serum and actual tumour load is also seen in measuring the parameters of an active extrinsic coagulating pathway. Breast cancer activates this pathway thus resulting in elevated plasma D-dimers that can be directly measured. Elevated plasma levels of this and other markers of the coagulation pathway also correlate with the number of metastatic sites as well as progression kinetics of the tumour (Dirix et al, 2002).

## Serum Antigens

Serum antigen measurement allows a dynamic overview of disease burden in an individual with respect to progression of the disease as well as response to therapy. Its current clinical role therefore is in the diagnosis of symptomatic metastatic breast cancer (Robertson et al, 1999 Jan) and also in the monitoring of response during therapy (Murray et al, 1995; Safi et al, 1991; Tondini et al, 1988).

The role of serum antigens in the follow up of patients with primary breast cancer is disputed. GIVIO investigators concluded that intensive follow up of breast cancer patients, clinically and with serum markers did not improve overall survival (GIVIO investigators, 1998). This has also been confirmed by Sato (Sato et al, 2003). Tumour antigen levels in the serum reflect tumour load, therefore in early disease where tumour burden is low, detection of serum antigens as markers can be negligible.

However Molina et al (1995) detected metastases in 40% of those progressing from primary breast cancer to metastatic disease with a lead-time of 4.9 months using serial measurements of serum CA15.3 and CEA. The specificity of these markers for metastatic disease was 99% (Molina et al, 1995).

Data from Nicolini showed that early treatment based on rising tumour markers can result in delaying the onset of symptoms of metastasis (up to 13.5 months) and longer survival compared to those who are treated dependent on onset of symptoms (42.9% vs 13.6% at 30 months) (Nicolini et al, 1997). They concluded that the clinical use of serum antigen markers has advantages over the generally accepted UICC assessment of response (Hayward et al, 1977), which is the preferred method of response assessment in many centres.

It is seen that biochemical progression often occurs ahead of clinical and or radiological progression as the tumour burden required to elicit a positive response in the serum is lower than that required to be noticed radiologically or clinically (Gion.1992). Furthermore disease stabilization and survival as well as improved quality of life has been noted where markerdirected chemotherapy has been utilised over current method of

UICC response assessment. There is also significant cost-savings achieved since earlier discontinuation of expensive chemotherapeutic agents can be directed by tumour marker results.

## Measurement of serum antigens to diagnose cancer

Despite the importance of the measurement of tumour markers in the serum for disease monitoring, the potential use in the diagnosis of cancer is limited. They appear to be neither sufficiently sensitive nor specific for the detection of early breast cancer as they are more a measurement of tumour load. Molina and colleagues reported that only 13% of patients with primary breast cancer had an elevated serum CEA whilst 18.8% had a rise in CA15.3 (Molina et al, 2003).

Using more than one tumour antigen markers in combination may increase the sensitivity but may also result in decreased specificity and their measurement, as a cancer screening tool had yet to be established.

## Immune response to cancer and its use in cancer detection

Malignant transformation of cells is the end result of altered expression of genes that are essential in regulating normal cell growth and differentiation. Oncogenic antigens are the expressed proteins of these altered genes. These gene alterations include both somatic DNA mutation and gene translocation, both resulting in the expression of "foreign" proteins. An immune reaction to oncogenic proteins has been recognised for sometime with the detection of AAbs to p53 by Crawford in 1982 (Crawford et al, 1982).

Immune response to cancer cells require the host immune system recognising foreign antigens, which are captured, processed and presented by antigen presenting cells (APCs) to the humoral system via the major histocompatability complex (MHC) class I and II systems.

It is seen that although oncogenic antigens are contained within numerous cellular compartments they can be shed into extracellular space by enzymatic cleavage or expelled out after tumour-induced necrosis or apoptosis of cells. For this reason AAbs to both intracellular as well as extracellular components of transmembrane receptors have been noted.

Some gene alterations are amplifications rather than mutations thus resulting in over expression of normal proteins. Despite no obvious protein abnormality, the increased availability of protein results in peptides from the protein being presented in higher concentrations by MHC molecules. It therefore renders a non-immunogenic protein immunogenic (Cheever et al, 1995). Thus oncogenic proteins, whether abnormal in structure or quantity can elicit the production of AAbs.

Evidence of humoral immune response can be seen by the isolation of a B-cell producing AAbs against an oncogenic antigen (Polymorphic epithelial mucin (PEM) in this case) in a patient with known ovarian carcinoma (Rughetti et al, 1993). Cellular immune response is also involved as cytotoxic T cells have been shown to recognise oncogenic antigens and mediate lysis of tumour targets in-vitro (Jerome et al, 1991). The immune response in cancer can therefore be used to aid diagnosis and perhaps also screening.

Measurement of AAbs produced by the humoral immune response may provide an in vivo amplification of tumour antigen markers at an

early stage of the disease and therefore provide high sensitivity in terms of early detection. This potential use of AAbs in screening, diagnosis and prognosis of breast cancer is one of basic principles in breast cancer issues.

## Antibodies to tumour associated antigens

Based on the measurement of the presence of MUC1, p53 and c-myc AAbs in the normal, primary breast cancer (PBC) and at-risk populations who were followed up. We determined the role of the AAb either as individual or within a panel in screening in this group for cancer detection. Calculation of lead-time, test sensitivity and specificity of the assay in screening and diagnosis was performed. We also endeavoured to assess the potential to use the novel assay in establishing diagnosis and prognosis of primary breast cancer patients who had longer follow-up data than previously.

The HER2 AAb assay was not included in this study. The p53 and c-myc proteins were produced in a bacterial expression vector that allowed the antigens to be expressed in large quantities and produced in a biotinylated form. Biotinylation allowed the oncoproteins to be immobilised onto a neutravidin coated plastic well during ELISA (Cordiano et al, 1995). This form of immobilisation enabled the antigens to be more accessible for AAb binding in the ELlSA.

Since breast cancer is a heterogeneous disease, these tumours express many aberrant proteins and there are an increasing number Measuring individual markers either as antigens or antibodies gives low sensitivity irrespective of the cancer type or the marker measured in most reported studies due to the heterogeneity of the disease. No single antigen is likely

to demonstrate an AAb response in all patients. Two reviews from Zhang demonstrated that combining greater numbers of tumour-associated antigens within a panel will enhance the detection of the specific cancer using autoantibody assays (Zhang, 2004 and 2007) and different cancers may require different panel of markers (Zhang, 2007). However combination of markers in a panel may decrease the specificity of the panel. Worldwide various combinations have been established by different groups.

Within a panel, combining various mucins has a limited value, as different mucins appear to give equivalent sensitivities (Steger et al, 1989). Since cancer evolution is a multi-step process, it seems reasonable to speculate that choosing markers within a panel that are all formed from different stages will give increased sensitivity than markers from the same stage of carcinogenesis.

It has been identified a panel of markers that were involved in various steps of carcinogenesis and could therefore be utilised in screening and early diagnosis of breast cancer. The initial study (Cheung 2001) highlighted four antigens (MUC1, p53, c-myc and HER2) that were present in small amounts in most patients with early disease (Robertson 1990 and 1991a). We have speculated that such small amounts of antigen can induce the production of a larger number of AAbs in the early phase of cancer evolution, which can be detected readily with an ELl SA assay employing a novel means of antigen presentation.

We continued to use MUC1, p53 and c-myc as the antigens within a novel ELISA assay. All three antigens are involved in different cell cycle function and therefore at various steps during carcinogenesis.

## Microscopic types of breast cancer:

These include the followings according to World Health Organization (WHO) classification system(2003) [67]:

I. Precursor leasions:
   1. Lobular neoplasia(Lobular carcinoma in situ).
   2. Intraductal proliferative lesions.
      Atypical ductal hyperplasia
      Ductal carcinoma insitu.
   3. Microinvasive carcinoma.
   4. Intraductal papillary neoplasms.

II. Invasive carcinoma:
   1. Invasive ductal carcinoma Most are not otherwise specified (NOS).
   2. Invasive lobular carcinoma.
   3. Tubular carcinoma.
   4. Invasive cribriform carcinoma.
   5. Medullary carcinoma.
   6. Mucinous carcinoma.
   7. Invasive papillary carcinoma.
   8. Apocrine carcinoma.
   9. Inflammatory carcinoma.
   10. Bilateral breast carcinoma.
   11. Mesenchymal tumors(including sarcoma).

## Staging of breast cancer:

The American Joint Committee on Cancer (AJCC) staging system provides a strategy for grouping patients with respect to prognosis and therapeutic decisions [68]. The AJCC has designated staging by TNM classification as shown in the following table:

### The American Joint Committee on Cancer (AJCC) staging system

| TNM definitions of breast cancer | |
|---|---|
| Primary Tumor (T): | |
| T0 | No evidence of primary tumor |
| Tis | Carcinoma in situ ;intraductal carcinoma, lobular carcinoma in situ, or paget's disease of the nipple with no associated tumor. |
| T1 | Tumor 2.0 cm. or less in greatest dimension. |
| T2 | Tumor more than 2.0 cm. but not more than 5.0 cm. In greatest dimension. |
| T3 | Tumor more than 5. 0 cm. in greatest dimension. |
| T4 | Tumor of any size with direct extension to (a) chest wall or (b) skin. |
| Regional Lymph nodes (N): | |
| N0 | No regional lymph node metastasis. |
| N1 | Metastasis to movable ipsilateral axillary lymph node(s) |
| N2 | Metastasis to ipsilateral axillary lymph node(s) fixed to each other or to other structures. |
| N3 | Metastasis to ipsilateral internal mammary lymph node(s). |
| Distant Metastasis (M): | |
| M0 | No distant metastasis. |
| M1 | Distant metastasis present. |

| Pathological classification (PN) | | | |
|---|---|---|---|
| Clinical stage grouping | | | |
| PNX | Regional lymph nodes cannot be assessed (not removed for pathological study or previously removed). | Stage 0 | Tis, N0, M0 |
| PN0 | No regional lymph node metastasis. | Stage I | T1, N0, M0 |
| PN1 | Metastasis to movable ipsilateral axillary lymph nodes (s) | Stage IIA | T0, N1, M0<br>T1, N1, M0/<br>T2, N2, M0 |
| PN1a | Only micro metastasis (none larger than 0.2 cm.) | Stage IIB | T2, N2, M0<br>T3, N0, M0 |
| PN1b | Metastasis to lymph node (s), any larger then 0.2 cm. | Stage IIIA | T0, N2, M0<br>T1, N2, M0/<br>T2, N2, M0<br>T3, N1, M0/<br>T3, N2, M0 |
| PN1bi | Metastasis in 1 to 3 lymph nodes, any more than 0.2 cm and all less than 2.0 cm in greater dimension. | Stage IIIB | T4, Any N, M0<br>Any T, N3, M0 |
| PN1bii | Metastasis to 4 or more lymph nodes, any more than 0.2 cm and all less than 2.0 cm in greatest dimension. | Stage IV | Any T, N3, M1 |
| PN1biii | Extension of tumor beyond the capsule of a lymph node metastasis less than 2.0 cm. in greatest dimension. | | |
| PN1biv | Metastasis to lymph node 2.0 cm. or more in greatest dimension. | | |
| PN2 | Metastasis to ipsilateral axillary lymph node( s) fixed to each other or to other structures. | | |
| PN3 | Metastasis to ipsilateral internal mammary lymph node (s). | | |

## Grading of breast cancer

The currently employed grading scheme is the Nottingham Modification of bloom-Richardson Grading Scheme, which is elicited by Elston.

**The Nottingham Modification of Bloom-Richardson Grading**

| Histological Feature | Score 1 | Score 2 | Score 3 |
|---|---|---|---|
| Tumor tubule formation | if >75 of tumor cells arranged in tubules | if >10% and <75% | if 10% |
| Number of mitosis* | if <10 mitoses in 10 HPF | if >10 and <20 mitosis / 10 PF | if >20 mitosis /10 HPF |
| Nuclear pleomorphism | If cell nuclei are uniform in size, shape relatively small, dispersed Chromatin Pattern, and are without prominent nucleoli | Cell nuclei are somewhat Pleomorphic have nucleoli and are of intermediate size | Cell nuclei are relatively large, have prominent nucleoli or multiple nucleoli coarse chromatin pattern and vary in size and shape |

* Number of mitosis {via low power scanning (x 100) locates the most mitotically active area of tumor and proceeds to high power (x 400)}.

This result in a total score of 3 to 9 points by adding the score from tubules formation plus number of mitosis plus nuclear pleomorphism;

If the score is 3-5 points = Grade I (well differentiated).

If the score is 6-7 points = Grade II (moderately differentiated).

If the score is 8-9 points = Grade III (poorly differentiated).

## Prognostic factors in breast cancer

A number of factors have been identified that are related to prognosis. It should be noted that when determining actual prognosis. It is the combination of factors rather than an individual one on its own that is important.

1. **Axillary lymph nodes status:** It has repeatedly been shown to be the single most important predictor of disease-free survival in breast cancer. Only 20%-30% of node-negative patients will develop recurrence within 10 years, compared with about 70% of patients with axillary nodal involvement. The absolute number of involved nodes is also of prognostic importance; patients with 4 or more involved nodes have a worse prognosis than those with fewer than 4 involved nodes [70].
2. **Tumor size:** The diameter of the primary tumor shows a good correlation with the incidence of nodal metastases and with survival rate. More than 98% of women with tumors less than 1 cm survive for 5 years [71].
3. **Histologic subtypes:** The 30-years survival of women with special types of invasive carcinomas (tubular, colloid, medullary and papillary) is more than 60%, compared with less than 20% for women with cancer of non otherwise specified ductal carcinoma (NOS) [72].
4. **Tumor grade:** The most commonly used grading system (Modified Bloom and Richardson system). More than 80% of women with grade I tumor survive 16 years, whereas less than 60% of women with Grade III survive for the same period [70].

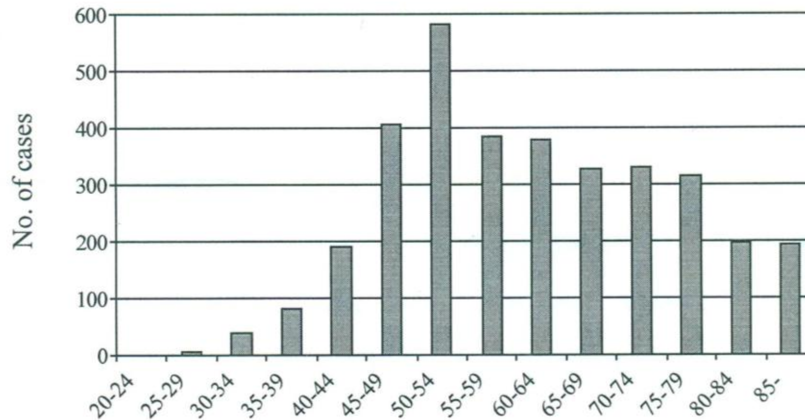
5. **Angiogenesis:** Most studies have shown a correlation between vessel density and the subsequent development of metastasis [73].
6. **Estrogen and Progesterone receptors:** Cancers with high levels of hormone receptors have a slightly better prognosis than those without receptors [74].
7. **Molecular genetic alterations:** It is well accepted that malignant tumors develop as a consequence of multiple critical gene abnormalities.
   - **a)** HER-2/neu overexpression of this oncogenic protein is associated with poor prognosis [75]. It is highly correlated with tumor grade [76]
   - **b)** Changes in expression of c-myc, ras and P53 are associated with a poorer prognosis and are commonly found in cancers with other poor prognostic factors [70, 77, 78]
8. **DNA ploidy and proliferative markers:** Proliferative markers such as mitotic count, Ki-67. Determination of S-DNA synthesis phase fraction by flow cytometry, many published literatures supports an association between high S-phase fraction and increased risk of recurrence and mortality for patients with both node-negative and node-positive invasive breast cancer.

# Epidemiology

The number of female breast cancer patients in Western World has been increasing over the past decade. It has been estimated that every tenth woman will be diagnosed with breast cancer at some point in her life (Pukkala et al. 1997). During the years 1994-98, the mean annual number of new breast cancer cases was 3236, rising by one hundred per year, reaching a peak of 3426 in 1998 (according to Finnish Cancer Registry 2000). The latest preliminary results on new cancer cases show a steady increase, with 3554 new breast cancer cases diagnosed in 1999. In 1998 about half of the breast cancers were diagnosed in women between the ages of 45 to 64 years (1744 cases out of the 3426 cases, 50.9%), but there still remains marked variation of the age of onset. The highest peak incidence was in the age group of 50-54 years old women (579, 16.9% of all the cases).

## Pathogenesis of breast cancer

The studies on colorectal cancer have constructed a pioneering model for the multistep nature of human carcinogenesis (Fearon and Vogelstein 1990). This has occurred because it is possible to conduct histopathological definition of the different stages of the disease. There is some evidence also for the sequential progression in breast cancer; the transformation of normal cells via the steps of adenoma, premalignant changes and *in situ* carcinoma to invasive carcinoma (Micale et al. 1994, Beckman et al. 1997). In spite of extensive efforts, the evolutionary steps of breast carcinoma are still not well understood. On next table you can see the changes based on Finnish Cancer Registry.




*The number of new cases of female breast cancer in 1998 by 5-year age groups (statistics from the Finnish Cancer Registry, 2000).*

Various risk factors for breast cancer have been proposed and tested over the years, factors like diet, oral contraception, postmenopausal substituent treatment with estrogen, breast irradiation, geography, occupation, etc. Epidemiologic studies have found statistical significance of estrogen stimuli, and chemical and physical carcinogenic stimuli (carcinogenic agents in food or the environment, mechanical stimuli such as irradiation, etc.). However, the underlying cytobiological processes of how these candidate factors cause carcinogenicity have remained unknown. A family history of breast cancer remains the main risk factor in breast cancer (reviewed in Biéche and Lidereau 1995).

Heterogeneity and complexity are in many respects the most characteristic phenomena in breast cancer (e.g. Escot et al. 1986). The age of onset, clinical course, hormone sensitivity and genomic instability vary greatly from one breast cancer patient to the next. Histopathologic variance is also seen, although almost 80% of all cases are histologically classified as ductal carcinoma. Hereditary breast cancers (about 5-10% of all breast carcinomas) manifest with earlier onset: the mean age at diagnosis is less than 45 years (reviewed in van de Vijver 2000).

## Methods in the genomic studies of breast cancer

Oncogenes manifest themselves at the cellular level either by increasing the protein production through amplification of the gene or by altering the gene transcription e.g. by translocating the gene to an active transcribing position, resulting in overactive or otherwise abnormal protein function. **Immunohistochemistry** (IHC) has been used to detect overexpressed proteins in tumor tissues, giving indirectly a gross evaluation of the gene amplification through the transcription activity. The widespread use of IHC is partly due to the fact that the IHC technique itself is widely available in pathology laboratories, and that the analysis using a brightfield microscope is easily performed. However, IHC is a relatively insensitive method, due to background signaling. In the recent years there has been an intense search for ways to improve the pretreatment methods for recovering the antigenicity of tissues and gaining optimal results (Shi et al. 2001).

The genomic alterations may be studied directly using methods such as Southern blotting and karyotyping. The more recent studies have utilized techniques like loss of heterozygosity (LOH), fluorescence in situ hybridization (FISH) and its new multicolor variants, including spectral karyotyping (SKY), polymerase chain reaction (PCR) comparative genomic hybridization (CGH), mutation detection methods, etc.

### Studies on genomic aberrations in breast cancer

The conventional cytogenetics has been applied to the identification of chromosomal abnormalities. **Karyotyping** is based on short-term cultured cells arrested in metaphase, and Giemsa stain to make the

chromosomal banding visible. For many years, this was the only method to characterize chromosomal abnormalities. The complexity of the abnormal karyotypes in breast tumors makes it difficult to characterize the whole genetic "profile" of any given tumor, and to reliably define the frequency of the genomic changes found in the samples. Most of the studies revealing the karyotypes of primary breast tumors have been case reports with only one to three patients, but a few larger studies involving up to a hundred cases or more have been conducted, focusing on slightly different aspects.

In one of the earliest modern studies on chromosomal abnormalities in breast cancer (Dutrillaux et al. 1990), gain of chromosome 1 q and loss of 16q were found most frequently in the less rearranged tumors. In cases with more anomalies, gains of 1q and 8q were the most frequent, whereas deletions involving many chromosomes were detected. It was concluded that the trisomy 1 q and monosomy 16q were early changes, whereas other deletions and gain of 8q represented secondary changes. Later on, the group of Dutrillaux (1991) analyzed cytogenetically 113 breast carcinomas. They found cytogenetic evidence on the steps of genetic evolution from diploid to hyperploid, and in general, towards LOH at multiple sites in the genome. Rohen et al. (1995) found clonal or non-clonal chromosomal abnormalities in 118 out of 185 (64%) cases of breast cancers. Pandis et al. (1995) found also polyclonality, 2-8 clones in 79 out of 97 cases of breast cancers (81 %).

Mertens et al. (1997) surveyed the karyotypic data on 3185 neoplasms, including 508 breast carcinomas. In that material, overall losses were more frequent than gains, and the only chromosomal areas more often

gained than deleted were 1q, 7, 12q and 20. Adeyinka et al. (1999) studied the associations of chromosomal aberrations with metastatic phenotype, identifying differences among the genetic lesions present in node positive (66 cases studied) and node negative (63 cases) breast cancers.

The majority of the oncogene studies on breast cancer have applied **Southern blotting,** which was one of the first direct methods to detect gene amplification (Southern 1975). However, its relatively low sensitivity has resulted in widely variable results in oncogene amplification studies. There are several weaknesses in this technique: first, normal cells "contaminate" the tumor sample, i.e. DNA from non-malignant cells dilutes the DNA of malignant cells, thereby decreasing its sensitivity to detect amplified gene copies (Nesbit et al. 1999). Second, the specificity and sensitivity of Southern blotting on formalin-fixed tissue samples has been questioned. After the initiation of use of the PCR techniques, there are now very few investigators performing Southern blotting on formalin-fixed tissue (Reinartz et al. 2000).

**Northern blot hybridization** is a variant of Southern blotting, detecting mRNA from the sample instead of DNA. The target RNA is extracted from freshly frozen tissue, digested and size-fractionated before hybridization. The result gives a gross evaluation of the transcription level of a specific gene.

The analysis of chromosomal aberrations was boosted with the CGH method, which was developed in 1992 (Kallioniemi et al. 1992). CGH is applicable for analyzing the numerical changes in the whole genome at the same time, and it may be used as a screening method for novel chromosomal areas either showing copy number gains or losses. CGH

can also work with genomic DNA from either formalin fixed and paraffin embedded tissue, or freshly frozen tissue, and it can efficiently reveal chromosomal copy number changes (gains and losses) with a minimum size of 10 Mb in one hybridization (Bentz et al. 1998), when the chromosomal aberration is present in at least 60 % of the cells studied (Kallioniemi OP. et al. 1994). After screening by CGH, the newly identified chromosomal areas may be studied by other methods, such as locus-specific FISH and sequence analysis, for more specifically restricted amplifications or deletions, i.e. for new oncogenes and tumor suppressor genes.

To detect balanced changes, such as reciprocal translocations - which are also important pathways of oncogene activation - newer methods, such as **SKY** and different variations of **FISH** have been developed. SKY is a method that detects genetic material from each chromosome by individual chromosome labeling, thus being able to detect both translocation partners at the same hybridization. For SKY, metaphase spreads from short term cultured cells are needed. The specific probes used in FISH give even more detailed information about which subregions of chromosomes are involved. FISH is accessible for formalin fixed tissue samples or cells from short-term cultures, arrested either in interphase or in metaphase, depending on the aim of the study. The multitissue arrays have brought new possibilities to utilize FISH, while large tumor selections, either on the same type of tumor or completely different types and tissues, may be gathered for screening for specific probes for oncogenes.

## Comparative studies using multiple techniques

Before a novel technique is accepted for research, confirmation of its reproducibility is required, preferably along with comparative studies with the known methods as reference on the same samples. The comparison of the different methods is not always straightforward, because of the fact that they are focusing on the chromosomes from different perspects. For example in the same tumor, LOH can indicate loss of one allele, while CGH and FISH may show normal results. The reason for this discrepancy is that loss of one allele may be combined with duplication of the other and this will result in a diploid gene copy number. CGH is not capable of detecting small allelic losses, while FISH can be used for the detection, with a locus-specific probe.

Persson et al. (1999) evaluated the frequencies of gains and losses found by G-banding cytogenetics and CGH by studying 29 invasive breast cancers with three different methods. The comparisons between karyotyping (after converting the karyotypes to net gains and losses) and CGH, and DNA flow cytometry and CGH were only partly concordant. Only 56% of the cases (15/29) were in agreement by both methods. Ten out of 12 discordant cases showed a higher DNA index in flow cytometry compared to CGH, and 9 had a "simple" abnormal karyotype. Ploidy discordance was also detected in 12 cases; all the cases were cytogenetically diploid, but nondiploid by flow cytometry, showing disagreement in DNA and chromosome indices. Possible explanation for the CGH discrepancies given by the authors was that different cell populations were used in the various studies. CGH detected the predominant, often aneuploid cell clone, whereas the near-diploid minor cell clones had a

growth advantage in vitro, and were used for G-banding cytogenetics. This is in line with earlier findings on the detection of chromosomal abnormalities: e.g. in the study of Steinarsdottir et al. (1995), complex chromosomal changes were yielded in 87 % of the cases by direct harvesting, compared to 44% after culture of digested tissue ($P < 0.01$). Also polyploidy was more common in direct-harvested samples.

## Molecular pathology of breast cancer

The multistep nature of carcinogenesis in general, as well as in breast carcinoma, has been widely accepted. Similarly to other solid tumors, the development of breast cancer is thought to be initiated after multiple successive changes in the genome of the cells in the "target" tissue (reviewed in Biéche and Lidereau 1995, Courjal and Theillet 1997). Early studies of the age-dependence of cancer suggested that on average 6-7 successive somatic mutations are needed to convert a normal cell into an invasive carcinoma cell (Renan et al. 1993, Strachan and Read 1999).

Clinically, the genomic changes manifest as pathologically aggressive growth, invasion and metastatic behavior of the cells. The main genomic changes in breast carcinogenesis are activation of proto-oncogenes mainly through amplification, and coordinated inactivation of tumor suppressor genes (reviewed in Biéche and Lidereau 1995, Beckman et al. 1997, reviewed in Mertens et al. 1997). When CGH is used, the most common chromosomal imbalances detected in sporadic breast cancers are gains of 1q, 8q, 16p, 17q, and losses involving 8p, 13q, 18q, and 16q (Isola et al. 1995, Ried et al. 1995, Rohen et al. 1995, Tirkkonen et al. 1998, reviewed in Knuutila et al. 1998 and 1999). In sporadic cancer, when near-normal

breast cells evolve to malignancy, the genetic abnormalities developed are so diverse that it has been suggested that no two tumors or tumor cells in any one tumor are likely to be genetically identical (Lengauer et al. 1998).

Many of the somatic genetic changes seen in sporadic cancers are present more frequently in BRCA1 and BRCA2 epigenetically mutated cells, confirming the nature of these breast cancer susceptibility genes as DNA stability conserving genes (reviewed in Ingvarsson 1999). Epigenetic lesions have been shown to drive genetic lesions in cancer (Esteller 2000). This may also be seen in the more malignant nature of the hereditary breast cancers as a whole.

The development of breast cancer is known to involve many types of activated or inactivated genes in order to promote malignancy. The sequential steps, however, are far less understood than what is known of the best example of tumor progression, colorectal carcinoma (reviewed in Ingvarsson 1999, Fearon and Vogelstein 1990). Studies on LOH in synchronous benign and malignant lesions, intraductal (DCIS) and invasive breast cancer (IDC) mostly favor the theory of progression from DCIS to IDC (O' Connell et al. 1994, Munn et al. 1995, Radford et al. 1995, Fujii et al. 1996a,b), as well as there being a common clonal origin of DCIS and IDC. However, some studies have not found any common mutations in DCIS and IDC cells (Munn et al. 1996). There are also recent studies suggesting more complex pathways to breast carcinoma than the straightforward linear progression model (Kuukasjärvi et al. 1997). Nevertheless, it has been shown that both the structural and numerical

aberrations tend to be more complex in more malignant and more aggressive tumors than in intraductal or less aggressive types of invasive carcinomas (Kuukasjärvi et al. 1997, Adeyinka et al. 1998, Aubele et al. 2000, Cuny et al. 2000).

## Gene amplifications in breast cancer

To date, there are at least ten known oncogenes found frequently amplified in breast cancer (see the following table): EGFR (at 7p13) in 3% of breast cancers, FGFR1 (at 8p12) in 10%, c-myc (at 8q24) in 10%, FGFR2 (at 10q26) in 12%, CCND1 and EMS (at 11q13) in 15%, HER-2/neu (also known as ERBB2, at 17q12) in 20 %, PS6K (at 17q22-24) in 10%, and AIB1 and CAS (at 20q11-13) in 15% of breast cancers (reviewed in van de Vijver 2000).

In addition, there are various chromosomal regions, which are frequently amplified, but no specific oncogene driving these amplifications has yet been identified. The arm amplification of entire 1q (in up to 40-60 %of breast cancers) has long been detected for, but no oncogene involved in breast carcinogenesis in this region has been identified. In addition to c-myc at 8q24, 8q12-22 is amplified in 10 % of breast tumors. Region 12q13 is found amplified in 6 % of breast cancers, including proto-oncogene MDM2, a downstream regulator of p53. However, MDM2 expression (using immunohistochemistry) has shown clinical relevance in breast cancer (Bankfalvi et al. 2000), which suggests that there may be another oncogene in the same region. Region 16p11-12 is found amplified by CGH in 20 % of breast cancers (Courjal and Theillet 1997). The possible oncogenes responsible for these amplifications are still not known.

**Breast cancer linked oncogenes and amplified chromosomal regions (according to van de Vijver 2000).**

| Amplified region | Breast cancer linked oncogenes | Incidence in breast cancer (%) |
|---|---|---|
| 7p13 | EGFR | 3 |
| 8q12 | FGFRI | 10 |
| 8q24 | c-myc | 10 |
| 10q26 | FGFR2 | 12 |
| 11q13 | CCNDI<br>EMS | 15 |
| 17q12 | HER-2/neu<br>also known as ERBB2 | 20 |
| 17q22-24 | PS6K | 10 |
| 20q13 | AIB1 and CAS<br>(others?) | 15 |
| 1q | ? | 40 |
| 8q12-22 | ? | 10 |
| 16p11-12 | ? | 20 |
| 20q11 | ? | 6 |

Like HER-2/neu and c-myc, also many other oncogenes are more active in the early stages of the development of human embryo and fetus (Downs et al. 1989). Their proteins act in the early development and in adults in many crucial steps in the differentiation and growth of the cells, including apoptosis (e.g. c-myc), cell cycle regulation CCND1, c-myc,

MDM2), promoting cells to move from G1 to S-phase, etc. In tumorigenesis, amplification and activation of a quiet proto-oncogene results in overexpression of its protein(s), which leads to malignant features like cell cycle excitation, pathological growth and invasion of the tumor cells, lower stage of apoptosis and reduced cell differentiation.

After the introduction of a novel anti-cancer drug trastuzumab (Herceptin®), analysis of the HER-2/neu oncogene amplification by FISH has become an integral part of breast cancer diagnostics (Tubbs et al. 2001). Although no other oncogene has yet been employed in the therapy of breast cancer, many research groups are focusing on the prognostic significance and clinical relevance of many of the oncogenes and also in finding new candidate oncogenes in the amplified regions.

## Tumor suppressor genes

The other main line of genetic changes in tumorigenesis is the inactivation of the cell "protector" genes, tumor suppressor genes. These genes function mainly to repair damaged DNA. One group is called the mismatch repair genes (e.g. MSH2, MLH1 in colorectal carcinoma, Caluseriu et al. 2001). Replication errors involving the defects in DNA mismatch repair genes (RERs) are key features in hereditary non-polyposis colorectal cancer (HNPCC), whereas in breast cancer they have been reported only at a low frequency (reviewed in Ingvarsson et al. 1999). The following table explains the frequency of breast cancer appearance:

**Tumor suppressor genes known to be linked to sporadic breast cancer (according to van de Vijver 2000).**

| Rearranged region | Tumor suppressor genes | Incidence in breast cancer (%) |
|---|---|---|
| 6q26-27 | IGF-II | <10 |
| 9q21 | p16INK4a (p16) | (rare) |
| 10q23 | PTEN | 1 |
| 17p12 | p53 | 20 |
| 16q22.1 | E-cadherin gene | ? |

There are to date five known tumor suppressor genes affected in sporadic breast cancer, two of which are more frequent: TP53 (at 17p12) and E-cadherin gene, CDH1 (at 16q22.1) especially in lobular carcinomas (reviewed in van de Vijver 2000). In hereditary breast cancer, there are germline mutations in the susceptibility genes, such as BRCA1 (at 17q21) and BRCA2 (at 13q12) (Miki et al. 1994, Wooster et al. 1994, Zheng et al. 2000). These genes function as tumor suppressor genes in the maintenance of genetic stability through participating in the cellular response to DNA damage (Zheng et al. 2000). No mutations in BRCA1 and BRCA2 have been reported in sporadic late onset breast cancers.

In tumor progression, the first mutation is critical: it should provide an otherwise normal cell with a growth advantage over the adjacent cells. The gatekeeper hypothesis of Kinzler and Vogelstein (1996, 1997) suggests that some certain gene is responsible in any given tissue for maintaining a constant cell number. A mutation of a gatekeeper leads to a

permanent imbalance of cell division over cell death, offering a growth advantage, while mutations in other genes have no long-term effects. No gatekeeper gene has been proposed so far for breast cancer (reviewed in Ingvarsson et al. 1999).

In many cases, one tumor suppressor gene allele is mutated by a relatively subtle mutation (point mutation, small insertion or deletion), while the other allele is completely lost. LOH studies have been the main source of information on tumor suppressor gene changes; in addition to the known genes, a relatively large number of other regions have been detected by LOH, where efforts are being dedicated to identify the tumor suppressor genes (reviewed in van de Vijver 2000). Other means to identify tumor suppressor genes are further elucidation of the lost regions seen by CGH, or studying the rare familial cancers - as in the case of aggressive retinoblastoma (tumor suppressor gene RB1 at 13q14)- following the Knudson's two-hit mechanism (Knudson 1971). According to this theory, two successive mutations are needed to turn a normal cell into a tumor cell. This has been confirmed to be a valid mechanism in tumor progression in the studies of tumor suppressor genes (Strachan and Ried 1999), but also contradictory results have been reported, e.g. in a study on E-cadherin (CDH1) (Cheng et al. 2001).

Inactivation of tumor suppressor gene is possible through a few mechanisms, e.g. point mutations and deletions. Recently, also gene inactivation by promoter region hypermethylation leading to transcriptional silencing has been demonstrated (Jones and Laird 1999, Krop et al. 2001). Examples of abnormal methylation in the promoter region of genes include the first tumor suppressor gene described, the retinoblastoma

(RB1) gene (Jones and Laird 1999). Abnormal methylation has been detected also in some genes involved in breast cancer: the p16 (INK4a), the estrogen and progesterone receptors, and E-cadherin (Esteller 2000), as well as a putative tumor suppressor gene HIN-1, which is frequently inactivated in the early stages of tumorigenesis (Krop et al. 2001). Methylation of DNA may play an important role in cancer pathogenesis, because this change is epigenetically transferred to the next generation. Furthermore, the frequency of changes in methylation has probably been underestimated, because methylated bases can not be detected by the standard techniques for mutation screening (Strachan and Ried 1999).

## The genetic basis of cancer

Cancer is the general name for over 100 medical conditions involving uncontrolled and dangerous cell growth. A cancer generally derives from a single cell that is changed dramatically by a series of genetic alterations.

A healthy cell has a well-defined shape and fits neatly within the ordered array of cells surrounding it. lt responds to the environment, giving rise to daughter cells solely when the balance of stimulatory and inhibitory signals from the outside favors cell division. But the process of replication, carries the constant hazard of random genetic mutations which can impair the regulatory circuits of a cell. [81]

Genetic abnormalities found in cancer typically affect two general classes of genes. Cancerpromoting "oncogenes" are typically activated in cancer cells, giving those cells new properties (gain of function). "Tumor suppressor genes" are then inactivated in cancer cells, resulting in the loss of normal functions in those cells.

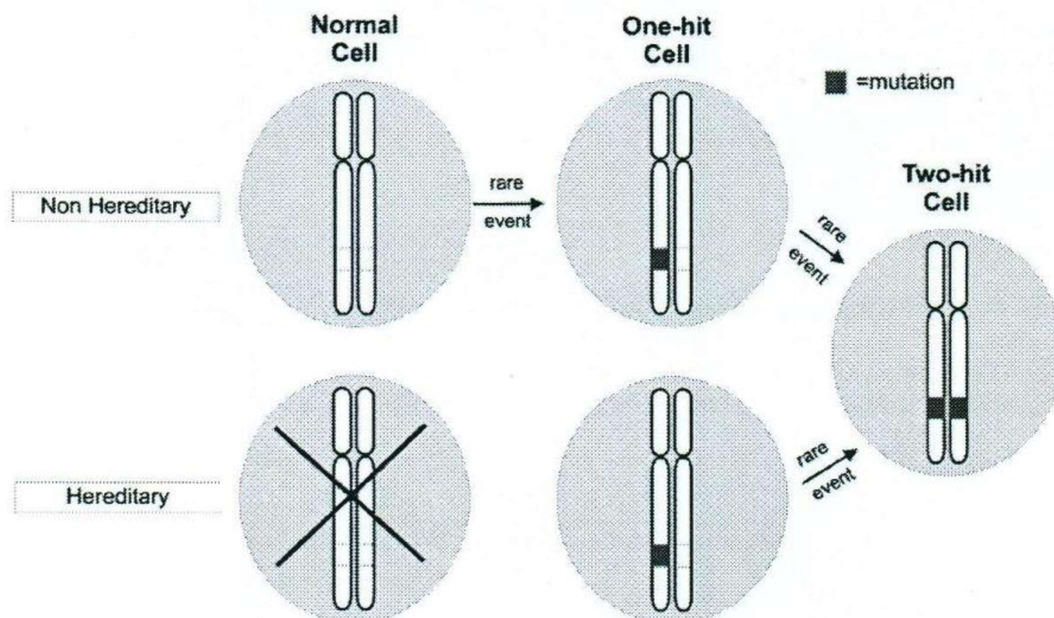
## P53 and Li-Fraumeni Syndome

On British Journal of Cancer (2010), 102: 719-726 among others referred inherited mutations cases in p53 with Li Fraumeni families. In 1969, Li and Fraumeni originally described families with soft tissue sarcoma and increased risk of other malignacies at an early age, including breast cancer. Studies followed showing that, in at least half of the families fulfilling "Li-Fraumeni" definitions, p53 was mutated (Malkin et al., 1990), therefore being a major contributor to the occurence of inherited breast cancer in the specific setting of Li-Fraumeni syndrome.

## Proto-oncogenes vs tumor suppressor genes

Oncogenes are the altered forms of proto-oncogenes. Proto-oncogenes are found in normal cells and encode proteins involved in the control of replication, apoptosis (cell death) or both. They are involved in the normal function of the cell, but can turn a cell into a cancer cell when activated. Activation of proto-oncogenes by chromosomal rearrangements, mutations, or gene amplification confers a growth advantage or increased survival of cells carrying such alterations. All three mechanisms cause either an alteration in the oncogene structure or an increase in or deregulation of its expression [82].

Tumor suppressor genes (TSGs) are targeted by genetic alterations in the opposite way as proto-oncogenes. The affected cell loses one of his functions like accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system. According to Knudson's two hit hypothesis [83] inactivation of both TSG alleles is necessary for tumor development. This scheme shows Knudson's in details.




Knudson's two-hit hypothesis

*In hereditary tumor syndromes, the initial inactivation of one allele is present in the germ cells. To start tumorigenesis an additional "hit" or somatic inactivation of the second allele is required. Somatic inactivation events include subchromosomal deletions, mitotic recombination, nondisjunctional chromosome loss with or without reduplication of the chromosome carrying the affected TSG, intragenic mutation or an epigenetic event. In sporadic tumors, the initial and second inactivating event occurs in the same somatic cell of an individual.*

Knudson suggested that multiple "hits" are necessary to cause cancer. The first inactivation is inherited and any second mutation would rapidly lead to cancer. In non-inherited cancer, two "hits" need to take place before tumor development, explaining the higher age of onset compared to inherited cancer.

TSGs can be subdivided into several classes according to their normal gene function, i.e. gatekeepers, caretakers and landscapers [84, 85] Gate-

keepers act directly by inhibiting cell growth. Caretakers are involved in maintaining DNA integrity and repairing DNA damage. Mutations in these caretakers have no direct effect on the proliferation, but they result in an accelerated accumulation of other mutations and will eventually lead to genomic instability. The landscapers, the third subgroup of TSGs are genes, which act by modulating the micro-environment rather than the tumor itself.

## Cancer is a multistep process

Later it became clear that carcinogenesis depends on more than the activation of protooncogenes or deactivation of tumor suppressor genes. A first "hit" in an oncogene will not necessarily lead to cancer, as normally functioning TSGs would still counterbalance this impetus; only additional damage to TSGs would lead to unchecked proliferation. Conversely, a damaged TSG would not lead to cancer unless there is a growth impetus from an activated oncogene. Generally, the normal cell has multiple independent mechanisms that regulate its growth and differentiation potential and several separate events are therefore needed to override all the control mechanisms, as well as induce other aspects of the transformed phenotype, like metastasis.

## Genomic Alterations in Cancer

Practically all cancer genomes are altered by different combinations of point mutations, copy number changes and chromosomal rearrangements [86]. Aneuploidy, defined as the gain or loss of whole chromosomes, is almost ubiquitous in cancer. It is frequently caused by chromosomal instability (CIN), the inability of a cell to correctly divide the chromosomes into daughter cells. In that case, aneuploidy is an evolving process, with every cell division potentially altering the chromosome composition of the daughter cells. However, a cancer may also have a stable though aneuploid karyotype, with no continuing CIN [87]. In solid tumors, CIN in general is associated with poor prognosis [88]. The relationship may not be directly linear, however, as in breast cancer, tumors with the highest level of CIN have a better prognosis than those with a more moderate level of instability [89]. The manner in which aneuploid contributes to cancer development is still unclear [90, 91]. Aneuploidy carries a replicative penalty in both normal and cancer cells [92, 93], yet it is seen in nearly all cancers. In mice with widespread aneuploidy due to haploinsufficiency in mitotic checkpoint genes, such as Rae1 and Bub3, aneuploidy has been shown to increase carcinogen induced tumorigenesis [94], even when aneuploidy itself does not increase cancer incidence [95]. Aneuploidy may also be a mechanism by which the cell can get rid of a remaining wt TSG allele or duplicate, and thereby increase the dosage of, a mutated oncogene. In addition, gain or loss of whole chromosomes or chromosome arms may contribute to cancer through dosage effects on a large number of genes, e.g. if they

create additional genomic instability or provide a buffer of extra copies of essential genes, such that functional copies of them are more likely to be available even if the mutation rate is high [91]. The importance of the last hypothesis could be tested in cancer types, such as lung cancer, that are known to carry a large number of mutations due to mutagen exposure [96] or individual tumors that have a high mutation rate. If the buffering hypothesis would be true, these cancers should, on average, be more aneuploid.

**Genomic aberrations commonly occurring in cancer**

| Genomic alteration | Description |
|---|---|
| point mutation | A change of a single nucleotide to another. |
| Insertion/deletion | Addition or loss of one or more consecutive nucleotides. |
| Aneuploidy | An abnornal chromosome number. In the context of cancer, usually somatically acquired. |
| Amplification | An increase in copy number of a genomic region. |
| Deletion | Loss, either of one or both copies of a genomic region. |
| Chromosomal rearrangement | A general term for various chromosomal aberration, including inversions and translocations. |
| Transclocation | Fusion of part of one chromosome to another, non-homologous, chromosome. |

Even under normal circumstances, the genome of every cell diary receives and repairs thousands of DNA lesions of various kinds [97, 98]. In most cases, the lesions are either repaired or, if their extent is too large,

the cell goes into apoptosis or senescence [99]. Either way, mutations are not transmitted to daughter cells. In cancer, single nucleotide mutations, small insertions and deletions (indels) and e.g. microsatellite instability and larger genomic rearrangements, such as amplifications and translocations occur due to different types of mistakes during DNA repair [100]. Single nucleotide mutations and small indels can arise during DNA replication if the DNA polymerase makes a mistake [101] or at any other point during the cell cycle, mostly as a consequence of normal cellular metabolism. External factors, such as mutagenic chemicals and radiation can also cause DNA damage [97]. The central role of DNA damage repair in cancer formation is exemplified by the large number of tumor suppressor genes that code for proteins involved in DNA damage response.

## Translocations

Many solid tumors, including breast cancers contain amplifications or deletions of genomic regions of various sizes [102]. Amplifications are thought to arise primarily through breakage-fusion-bridge cycles, caused e.g. by telomere attrition, and via the formation of double minute chromosomes [103]. High-level amplifications affect cancer development by upregulating the expression of one or more genes in the amplicon [102, 104, 105]. Examples of amplification targets include ERBB2 and CCND1 in breast cancer [203, 107] and the Myc family genes MYCN, MYCL1 and MYC in a variety of cancers [108, 109]. Amplicons containing multiple interacting oncogenes are also known [110, 111], and fusion genes can also be formed within or at the borders of high level,

amplifications [112]. Additionally, presumably independently of the specific genes amplified, the pattern of complex genomic rearrangements in a breast tumor is prognostic of outcome [113].

Homozygous deletions occur frequently in cancers and are thought to primarily affect cancer development through inactivation of TSGs. As an alternative to TSG inactivation, deletions may also lead to fusion gene formation, as has been shown for TMPRSS2-ERG [114] However, as recurrent homozygous deletions also occur at fragile sites, i.e. parts of the genome that are prone to genomic breaks due to some inherent feature, homozygous loss of a gene as such is not conclusive evidence of cancer relevance. Recent studies indeed suggest that a majority of homozygous deletions occur at fragile sites [115, 116]

Recurrent chromosomal translocations and the resulting gene fusions are well known mechanisms for oncogene activation and occur frequently in leukemias, lymphomas and sarcomas [117, 118]. Translocations form through double stranded breaks, which can be generated by e.g. immunoglobulin gene processing in B cells [119, 120], DNA damage e.g. caused by agents or occuring during DNA replication, double stranded breaks caused by chromosome segregation errors [121], chromotripsis [122, 123] and during amplification formation, [112, 124, 125]. Essentially any process that gives rise to two or more double stranded breaks that are then not repaired correctly is capable of generating translocations. In addition, the two sequences that are fused may need to be in close proximity in the nucleus [126]. The best studied example is BCR-ABL in chronic myelogenous leukemia [127, 128], which is formed by a translocation between chromosomes 9 and 22. The discovery of translocations

involving Ets-family members in prostate cancer [129], EML4-ALK in lung cancer [130] CD44-SLC1A2 in gastric cancer [131] now suggests that fusion genes may play a more prominent role in the development of epithelial cancers than previously anticipated. In breast cancer, both primary tumors and cell lines have been found to contain fusion genes, but recurrent fusions have only been known in rare subtypes, such as ETV6-NTRK3 in secretory breast carcinoma [134] and MYB-NFIB in adenoid cystic carcinoma of the breast [135]. Recently, rare but recurrent rearrangements of NOTCH and MAST family genes as well as the recurrent RPS6KB1-VMP1 fusion have been reported in breast cancer [133, 136].Individual examples of both fusion categories have also been reported previously, e.g. NOTCH1-NUP214 and RPS6KB1-VMP1 (previously known as RPS6KB1-TMEM49) [112] and ARID1A-MAST2 [132]. However, in regard to RPS6KB1-VMP1, Inaki et al. [133] suggest that it may rather be a marker of genomic instability or amplification of the 17q23 locus in which both genes are located, than an oncogenic fusion transcript.

Although there is significant intertumoral variation in the number of mutations they carry, most tumors contain a large number of mutations, especially point mutations [137]. Mutations that attain a significant frequency in a tumor are unlikely to have a negative impact on cancer cell growth, as cells carrying these would have been removed by negative selection. Mutations can therefore be divided into two categories, drivers and passengers, based on whether they increase the net growth rate of a developing cancer cell, or whether they are selectively neutral [137]. The average selective growth advantage of an individual driver mutation has

been estimated to be only ~0,4% [138], suggesting a developing tumor must accumulate a surprisingly large number of driver mutations, before becoming life threathening. The percentage of all mutations in a tumor that are drivers is poorly known, but results from glioblastoma multiforme suggest 8% of missense mutations may be drivers [139].

## Epigenetic alterations

In addition to the DNA changing alterations described above, epigenetic changes are also common in cancers [140] and cancer genomes as a whole are frequently hypomethylated [140]. However, hypermethylation of CpG islands close to the promoters of genes leads to their silencing, and this is a common mechanism for TSG inactivation in cancer. Genome wide, several cancer types show alterations in CpG island methylation boundaries and significantly increased between tumor heterogeneity in the methylation status of a large number of specific genomic regions, compared to their tissues of origin [141]. This indicates a general loss of epigenetic stability in cancer and results in both inter- and intratumoral heterogeneity through its effects on gene expression levels [141]. Methylated cytosines in CpG dinucleotides are also more prone to mutation, either spontaneously or when exposed to ultraviolet light or tobacco carcinogenes [140]. The importance of altered methylation in cancer development is also supported by the recent discovery of frequent mutations in e.g. the DNMT3A DNA methyltransferase in acute myeloid leukemia, myelodysplastic syndrome and T -cell lymphoma [142, 143].

## Array comparative genomic hybridization as detecting methods in cancer alterations

Array comparative genomic hybridization is based on the concept of competitive hybridization of DNA from two samples to the probes on a microarray [144, 145]. Both cDNAs, bacterial artificial chromosomes (BACs) and synthetic oligonucleotides have been used as probes, typically printed or synthesize onto glass microscope slides [146, 147]. If some part of the genome is not present in equal number of copies in both samples, this will be visible as either a gain or loss of fluorescent signal from probes measuring that region, indicating the presence of an amplification or deletion. aCGH is always comparative, in other words, gains and losses are defined in relation to a reference sample. This applies also to Affymetrix SNP microarray derived copy number data, even if the hybridizations themselves are done with one sample per microarray and therefore are not competitive. Current aCGH microarrays can contain up to 1 million probes (Agilent SurePrint G3 Human High-Resolution Discovery 1M arrays), providing an average resolution of 3kb across the genome. aCGH does not detect balanced genomic rearrangements, such as translocations, in which no genetic material is gained or lost. In practice, however, it seems that many, if not most, translocations are accompanied by small copy number changes (either deletions or gains), which may be visible using aCGH [112, 148]. aCGH has been used most widely m cancer research [145]. Compared to G-band karyotyping, aCGH is able to identify much smaller copy number variants, and is therefore increasingly used in the diagnosis of e.g. idiopathic mental retardation and developmental malformations [149] as well as in prenatal diagnosis [150]. Beyond medical applications, aCGH has been used to study

population wide copy number variation in several species, including humans [151, 152] various great apes [153] and dogs [154].

As aCGH data is comparative, results are nearly universally reported as ratios of sample divided by reference, frequently log-transformed to make them symmetric around zero. Visualization of the ratios in the context of their genomic positions then allows the determination of copy number profiles for all examined chromosomes. Simultaneous analysis of copy number profiles from multiple samples can be used to identify minimal common regions of amplification and deletion, the locations of potential oncogenes and TSGs [155, 156], minimal common region identification rests rests on dividing the genome into non-overlapping regions of differing copy number by segmentation [157, 158]. Segmentation provides smoothed DNA copy number estimates for genomic regions by using the ratios from multiple adjacently located probes to derive an average copy number value for the region. Gene level copy number values, for integration with e.g. gene expression data, can be derived directly from the values of the segment in which the gene is located. Alternatively, gene level copy number data can be calculated, on a gene by gene level, from probes located in a specified window surrounding the gene's location [159]. Recently, next generation whole genome - or exome sequencing data has also been used for estimation of copy number [204, 160, 161], and this may come to replace aCGH in areas of research in which sequencing becomes common.

## Gene expression arrays

Gene expression microarrays are miniaturized assays that enable measuring the expression of nearly all protein coding genes in the human genome in a single experiment. Expression arrays can be divided into two

main types. One is based on the competitive hybridization of two samples on the same array, as done with aCGH [162]. In the other type, only one sample is hybridized onto the microarray, and the quantified signal is therefore the absolute fluorescent intensity measured, not a ratio of signal from two samples, [163, 164]. As with aCGH, the probes may be either cDNAs or synthesized oligos, the latter being used almost exclusively these days. Oligobased expression arrays range from relatively simple designs using long 60 base pair (bp) oligos (e.g. Agilent) to the more complex short oligo-based design of Affymetrix. Expression arrays have been used extensively in cancer research, contributing to identifying, in breast cancer alone, new subtypes [165, 166, 167], expression profiles predictive of disease outcome [168] and the impact of DNA copy number changes on expression levels [104, 105]. Outside of cancer research, they have been used in anything from researching the effects of parabolic flight on plant gene expression [169] to stydying gene expression changes in the brain caused by the domestication of dogs from wolves [170].

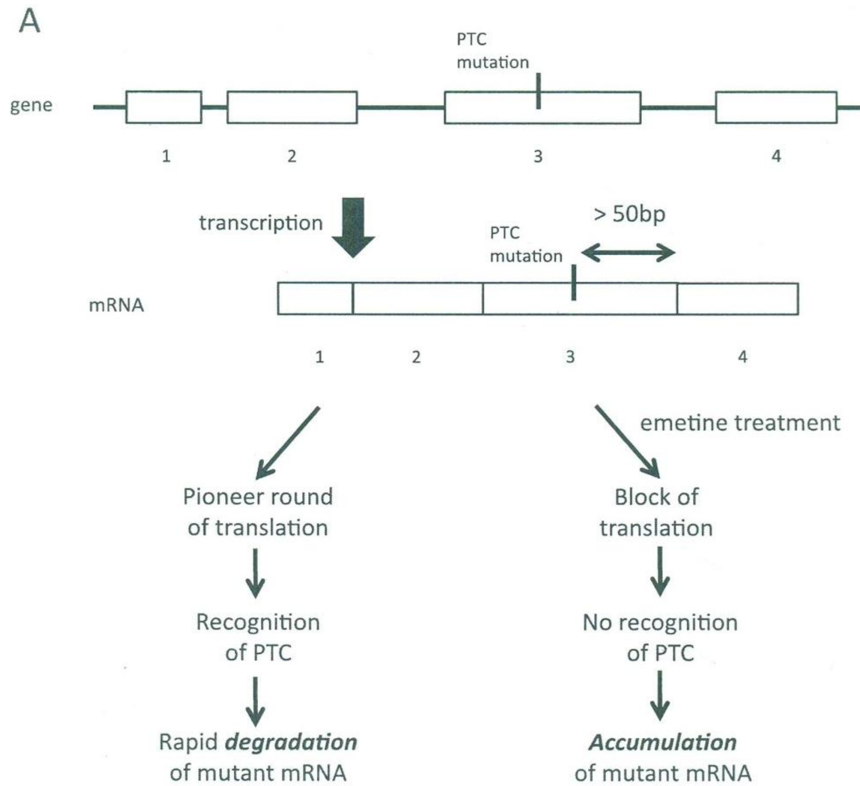
Although the bioinformatic methods used to analyze microarray data are almost as varied as the hypotheses being studied, all analyses start with preprocessing the microarray data [171]. The first step in data aquisition is the segmentation of scanned microarray images to obtain signal intensities for all probes [172]. In the literature, the post signal aquisition steps in microarray data preprocessing are frequently simply called "data normalization", although formally normalization is only one of the steps in preprocessing. Microarray preprocessing methods vary depending on the type of microarray, but all aim at correcting for technical noise and variation in the data [173]. For single-color microarrays, such as Affy-

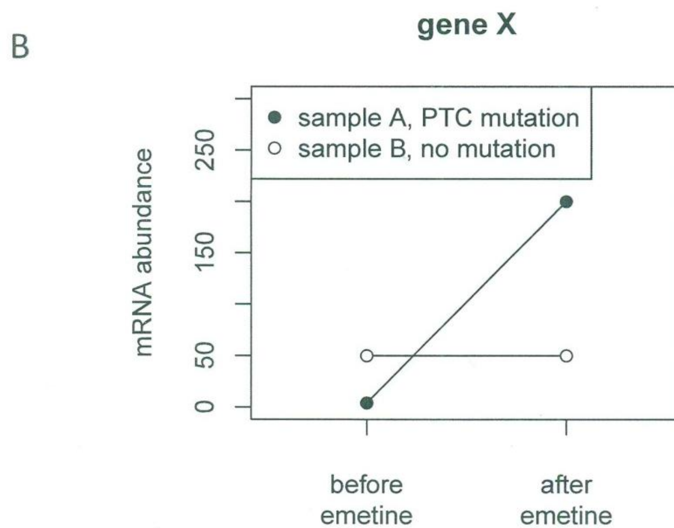
metrix, one of the most commonly used preprocessing methods is the Robust Multiarray Average [174]. RMA consists of three processing steps. The first step is background adjustment, in which an estimate of background signal intensity is subtracted from probe signals, under the assumption that background signal represents nonspecific hybridization. After background adjustment, probe intensities are normalized using quantile normalization. Finally, data is summarized at the level of probe sets or other probe groupings, such as Ensemble gene definitions [175, 176].

Clustering and classification methods, also termed unsupervised and supervised classification, have frequently been used in microarray data analysis, the former especially in exploratory data analysis. Examples include the previously mentioned identification of breast cancer subtypes using hierarchical clustering [166] and definition of new subtypes of diffuse large B-cell lymphoma [177]. One major aim of microarray data analysis is the identification genes that are differentially expressed between two or more groups of samples, e.g. samples subjected to a treatment compared to an untreated control group. Methods range from simple log fold change calculation [171] to more complex methods, such as Gene Set Enrichment Analysis [178, 179], that do not rely on defining a ratio cutoff for differential gene expression, but rather identify simultaneous changes in groups of genes that share a biological function. During the last decade, the data from tens of of thousands of microarray hybridizations has been made public through repositories such as Gene Expression Omnibus (GEO) [180] and Array Express [181]. This has prompted the development of meta-analysis methods to integrate data

across multiple studies to be able to answer questions that no single study is powered to answer. Examples include GeneSapiens [182] and Oncomine [183], both of which concentrate on integrating data from cancer microarray studies. GeneSapiens normalizes Affymetrix gene expression data for altogether 9783 healthy, cancer and other disease samples onto the same scale, enabling e.g. studying the expression profile of all kinases across ~56oo different healthy and malignant tissue samples [184] as well as determining the origin of cancers of unknown primary origin [185].

Nonsense-mediated messenger RNA (mRNA) decay (NMD) is an eukaryotic quality control mechanism that triggers the decay of m RNAs that contain premature termination codons (PTCs) [186]. In addition, NMD also regulates the expression of a set of target transcripts under normal physiological conditions [187]. A PTC mutation is an effective way for a cancer cell to inactivate one copy of a TSG. Methods to identify such mutations based on the stabilization of PTC carrying mRNAs after either chemical (emetine with or without actinomycin D) [159, 188, 189] or siRNA-based [190] inhibition of NMD have therefore been developed. One of the strengths of the gene identification by nonsense inhibition (GINI) method is that no a priori information about candidate genes or location in the genome is necessary, although if available, this information can be integrated with the GINI data [188]. Mutations have been found using an NMD-based approach in e.g. EPHB2 in prostate cancer [189], RIC8A and ARID1A in breast cancer cell lines [159, 191], as well as several genes in colon [192] and prostate cancer [193], mantle cell lymphoma [194] and melanoma [195].




**Principle of emetine mediated NMD block.**

**A)** *If a PTC mutation occurs at least 50-54 bp before the last exon-exon junction, the mutation will be recognized during the pioneer round of translation and the m RNA molecule is degraded. Emetine is a general inhibitor of the translation process. Emetine treatment therefore also blocks the pioneer round of translation and prevents the NMD machinery from recognizing and degrading the mutated transcript, leading to accumulation of mutation carrying transcripts.*

**B)** *An idealized example of the effects of emetine treatment on the abundance of mRNA transcripts of gene X. In sample A that carries a PTC mutation in X, emetine treatment leads to an increase in mRNA from gene X. Conversely, in sample B that has no mutation, transcript levels of X are not affected by emetine treatment. Note also that, compared to sample B, continued degradation of mutated transcripts from X in sample A leads to lower expression of the gene in the untreated state.*

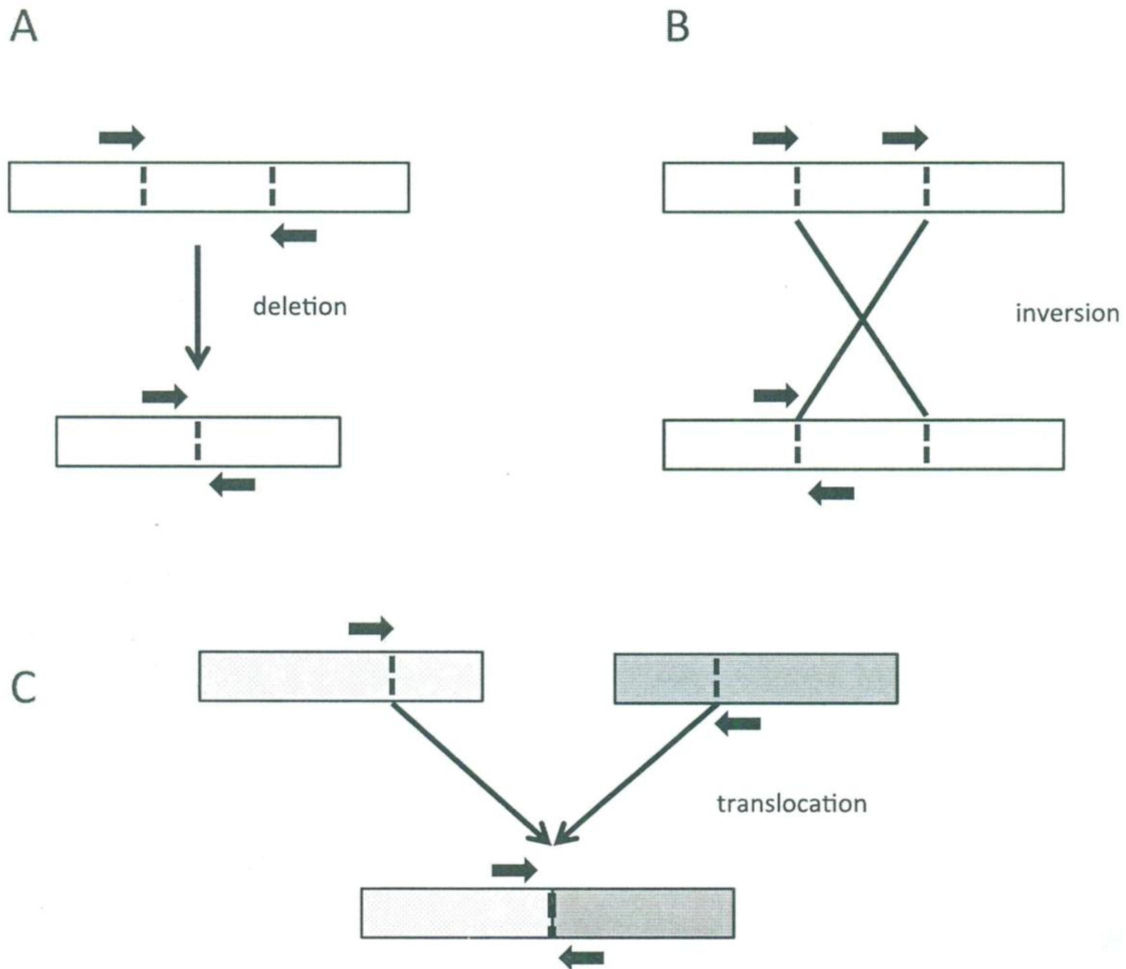
NMD microarray data analysis is in principle simple: a matter of identifying the mRNA transcripts that increase in amount following inhibition of NMD. In practice, however, a large number of transcripts are induced by NMD inhibition, whether chemical or siRNA-based [159, 188]. The main task of data analysis is therefore to prioritize a short list of the most likely mutation carrying genes. Several of the above mentioned studies have arrived at similar filtering algorithms. One of the two main filtering criteria follows; increased transcript level in only one out of several cell lines studied, this rests on the assumption that only one of the cell lines is likely to have inactivated a gene through a PTC, and transcripts upregulated in multiple cell lines are therefore likely to be physiological NMD targets. The second main criteria is that in untreated cells, expression of the transcript should be low compared to other samples, as would be expected based on a PTC containing transcript being degraded when NMD is intact [159, 194] A futher criterion used in several publications is that the candidate gene should be located in a region of heterozygous deletion or loss of herozygosity [159, 189, 191].

## Next generation sequencing

Next generation sequencing is a collective term used to describe several different new sequencing technologies that utilize massive parallelization to achieve large increases in sequencing throughput in comparison to traditional capillary sequencing (Sanger sequencing) using e.g. ABI Prism 3730 DNA Sequencer instruments (Applied Biosystems). Currently, the main technologies in use are provided by Illumina (HiSeq, MiSeq, GA-family of instruments), Applied Biosystems (SOLiD), Roche

(454), Life Technologies (Ion Torrent) and the technology of Complete Genomics [196, 197]. Massively parallel sequencing of RNA allows the comprehensive characterization of the features of a transcriptome, including gene expression levels, alternative splicing, identification of new transcripts as well as chimeric RNA molecules [198, 199]. Chimeric RNAs, such as fusion transcripts, can be detected using paired-end sequencing of mRNA or ribosomal RNA (rRNA) depleted total RNA, in which 35-150 bp are sequenced from both ends of DNA molecules in the sequencing library (typically 200-500bp long). Whole-genome sequencing is also able to identify chromosomal rearrangements that potentially can create fusion genes. However, RNA-seq can directly identify the expressed fusion genes, out of a potentially large set of rearrangements, and is therefore a more cost effective and straightforward method for detecting potentially oncogenic gene fusions.

Several pipelines have been published for fusion gene identification [112, 132, 200, 201], but most methods that achieve a high specificity converge on very similar solutions. In all approaches, paired-end reads are first aligned and filtered to identify those pairs, in which the reads align to two different genes. This, however, does not distinguish between true fusion genes and readthrough transcription between genes that lie next to each other in the genome. Various solutions for filtering out transcriptional readthrough have been proposed, such as excluding all gene-gene pairs that lie closer to each other than some specified bp distance [132] or only considering gene-gene pairs that are separated by at least one other gene that lies between them [112]. The exon-exon junction at which the fusion occurs is then identified by searching non-aligned single-end reads for




**Identification of deletions, inversions and translocations using paired-end DNA sequencing data.**

*Vertical arrows indicate sequence read pairs, and arrow directions show the strand they align on (arrow pointing to the right: forward strand). Vertical dashed lines indicate chromosomal breakpoints. In each subgraph A-C, the lower part shows the chromosome after the rearrangement, i.e. the state assayed by sequencing. The upper parts of each subgraph show how the reads in the readpair align to a normal reference genome. Rearrangements are identified as follows.* ***A)*** *When sequencing across a deletion point, the reads align further away from each other on the reference genome than would be expected. If the insert size is e.g. on average 300bp, reads that align 10 kb from each other on the reference genome are likely to flank a roughly 9-10 kb deletion.* ***B)*** *When sequencing across an inversion point, both reads will align on the forward strand when aligning them to a normal reference genome. In addition, depending on the size of the inversion, the reads may align further from each other than expected.* ***C)*** *When sequencing across a translocation point, both reads will align to different chromosomes in a normal reference genome.*

ones that align partially to exons from both genes. This search is typically done by bioinformatically constructing a library of all possible exon-exon junctions, i.e. potential fusion junctions, between a candidate gene-gene pair, against which alignments are performed. Fusion gene validation is then typically performed by polymerase chain reaction (PCR) and Sanger sequencing across the predicted fusion junction(s). Additional filtering criteria employed by some pipelines include filtering out gene-gene pairs with high sequence similarity, on the assumption that they are false positives derived from misaligned sequence reads [112, 132]. Additionally, the locations of alignment start positions for fusion junction spanning reads have proven to be a good criterion for excluding false positive fusion candidates [112]. One of the main points at which pipelines differ is whether they can identify fusions that do not occur at known exon-exon junctions. Here, the TopHat-Fusion [202] algorithm seems to provide the most robust detection of fusion junctions, in which one or both fusion breakpoints reside within exons.

## Cell cycle control – Tumor protein P53

The TP53 gene provides instructions for making a protein called tumor protein p53. This protein acts as a tumor suppressor, which means that it regulates cell division by keeping cells from growing and dividing too fast or in an uncontrolled way.

Tumor protein p53 is located in the nucleus of cells throughout the body, where it binds directly to DNA. When the DNA in a cell becomes damaged by agents such as toxic chemicals, radiation, or ultraviolet (UV) rays from sunlight, this protein plays a critical role in determining whether the DNA will be repaired or the damaged cell will self-destruct (undergo apoptosis). If the DNA can be repaired, tumor protein p53 activates other genes to fix the damage. If the DNA cannot be repaired, this protein prevents the cell from dividing and signals it to undergo apoptosis. This process prevents cells with mutated or damaged DNA from dividing, which helps prevent the development of tumors.

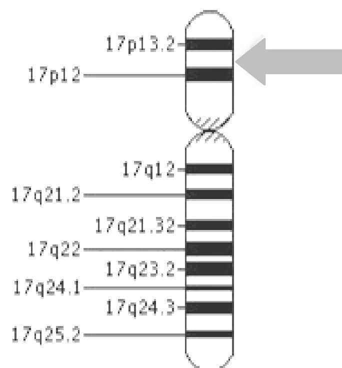
Because tumor protein p53 is essential for regulating cell division and preventing tumor formation, it has been nicknamed the "guardian of the genome."

So any changes in the TP53 gene greatly increase the risk of developing breast cancer as part of a rare inherited cancer syndrome called Li-Fraumeni syndrome. These inherited mutations are thought to account for less than 1 percent of all breast cancer cases.

Somatic mutations in the TP53 gene are much more common, occurring in approximately 20 percent to 40 percent of all breast cancer cases. These cancers are typically not inherited and do not occur as part of

a cancer syndrome. Many of these mutations change a single protein building block (amino acid) in tumor protein p53. These mutations lead to the production of a nonfunctional version of this protein. The defective protein builds up in cells and cannot regulate cell growth and division.

In some cases of breast cancer, one copy of the TP53 gene is lost and the remaining copy has a mutation that prevents the cell from producing any tumor protein p53. Without this protein DNA damage accumulates and cells divide in an uncontrolled way, leading to a cancerous tumor. Mutations in the TP53 gene are assosiated with larger tumors and more advanced disease than breast cancers without TP53 mutations. Recurring tumors are more likely to have mutations in the TP53 gene. The TP53 gene is located on the short (p) arm of chromosome 17 at position 13.1: base pairs 7, 571, 719 to 7, 590, 867.




*The molecular position of TP53 gene on chromosome 17.*

The majority of genetic alterations in the TP53 gene found in breast cancer tumors are point mutations leading to translation of a stable, mal-functional protein with extended half-life which accumulates in the cell, and is therefore detectable by imunohistochemistry (IHC) reviewed in (Borresen-Dale, 2003).

During the initial studies on the prognostic value of TP53 for breast cancer mainly IHC was used for TP53 determination, as an accessible substitute for direct mutation detection. Some studies using IHC reported that TP53 overexpression was associated with worse outcome, especially in node negative patients. (Allred et al., 1993; Silvestrini et al., 1993). However, overall only one-third of such studies observed a positive association (reviewed in a meta-analysis including over 9000 patients by Barbareschi in 1996). A very likely explanation for these results is that mutation types such as insertion, deletions or stop codon point mutations may result in truncated proteins, undetectable by IHC, as shown by Klaar (formerly Sjögren) et al (Sjögren et al., 1996) comparing TP53 IHC with cDNA based sequencing of all coding exons in the TP53 gene. Elevated expression of wild-type TP53 due to DNA damage, or unspecific antibody-binding may also influence TP53 determination by IHC. (Borresen-Dale, 2003) Klaar et al observed that IHC produced a rate of 33% false negative and 30% false positive cases when compared with TP53 gene sequencing data. (Sjögren et al., 1996) Studies directly correlating TP53 gene mutations with patient prognosis, such as Bergh et al (Bergh et al., 1995) or Blaszyk et al (Blaszyk H, 2000), have in general observed strong associations between TP53 and outcome.

A meta-analysis with inclusion of data from 2319 patients from eleven studies, investigating the association between somatic TP53 mutations and outcome demonstrated the combined relative hazard (RH) of 2,0 (CI95% 1,7-2,5) for overall fatal outcome for patients with TP53 mutated breast cancers. (Pharoah et al., 1999). TP53 is also a potential predictive marker for breast cancer. Adjuvant radiotherapy along with systemic

adjuvant therapy, especially tamoxifen, has been reported to be of less effect in lymph-node positive patients with mutated TP53, (Bergh et al., 1995). Tamoxifen resistance has also been associated with TP53 mutations that affect the DNA-binding region or mutations in the zinc-binding domain L3 (Berns et al., 1998) and with concomitant VEGF overexpression and mutated TP53 in advanced breast cancer. (Berns et al., 2003; Linderholm et al., 1998; Linderholm et al., 2001; Linderholm et al., 2003).

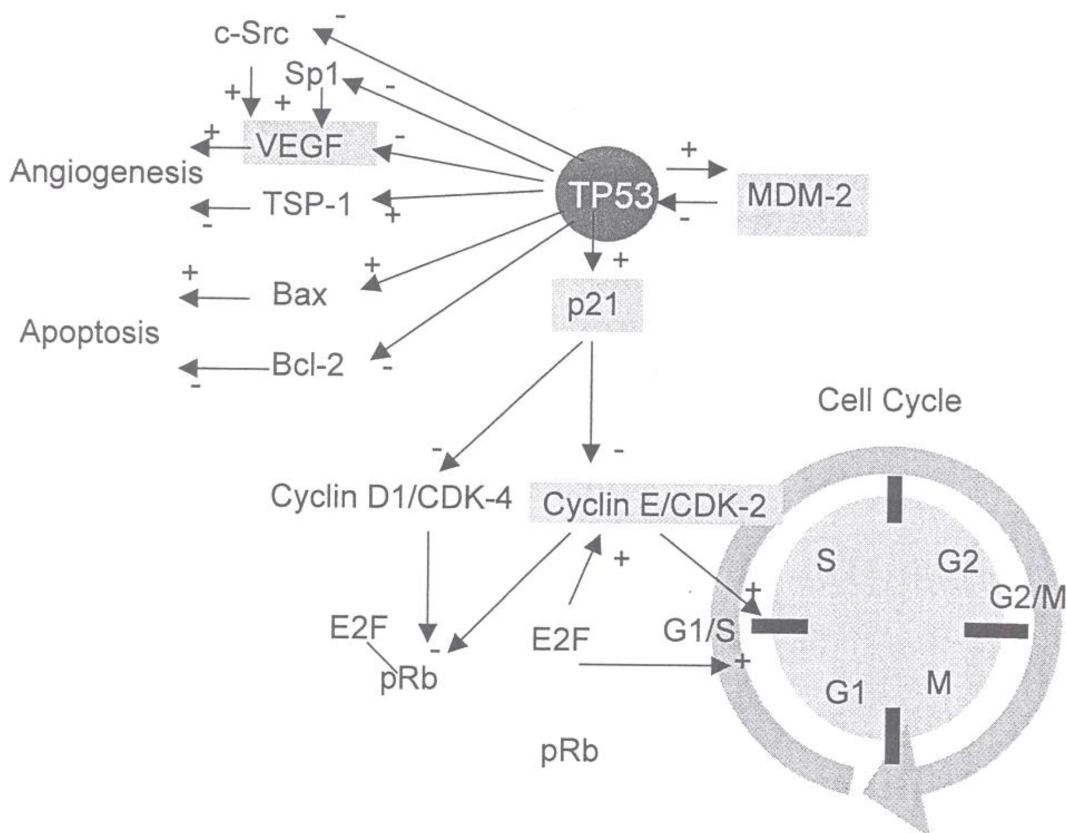
Opposing results on the predictive value of TP53 for tamoxifen therapy have also been published (Archer et al., 1995; Elledge et al., 1997) thus suggesting further validation before clinical recommendation. Smaller studies have observed associations between mutated TP53 and effect of chemotherapeutic agents, such as low response to doxorubicin (Geisler et al., 2001), 5-fluorouracil and mitomycin (Geisler et al., 2003) or paclitaxel (Schmidt et al., 2003) while others demonstrated opposite results. (Bertheau et al., 2002).

Although the biological properties of TP53 suggest potential clinical usefulness and many study results are promising, but the documentation of the predictive and prognostic value for TP53 is not yet solid enough, for the recommendation to include TP53 determination in routine clinical management of breast cancer patients. (Bast et al., 2001).

The proliferation of eukaryotic cells is tightly controlled by several checkpoints during the process of DNA duplication and mitosis, the cell cycle. Loss of cell cycle control result in unrestricted cellular proliferation, genetic instability and inappropriate cell survival, allowing proliferation and evolution of cells with genetic damage. (Malumbres and Barbacid,

2001) Escape from cell cycle control is essential for tumor or development. (Hartwell and Kastan, 1994).

The tumor suppressor gene TP53 has a key role in the cell cycle control system. The human TP53 gene is located at the short arm of chromosome 17 (17pl3.1). (McBride OW, 1986). The TP53 gene has 11 exons (the first is not translated) and encodes a 53kDa nuclear transcription factor. (Lamb and Crawford, 1986). The open reading frame of TP53 is 393 amino acids long, and the central region contains the DNA-binding domain. (Cho et al., 1994). A mutation in the TP53 gene is the most common mutation found in malignant cells, and somatic mutations are present in 20-30% of all breast cancers. (Soussi and Beroud, 2001; Soussi et al., 2000). Expression of the TP53 gene is induced by diverse forms of cellular stress such as hypoxia or DNA damage caused by carcinogens, ionizing radiation and UV light. (Graeber TG, 1994; Hall et al., 1993; Harris, 1996; MacCallum et al., 1996). Induction of TP53 triggers either cell cycle arrest to allow for DNA repair, or execution of programmed cell death if the DNA damage is beyond repair (reviewed in (Schwartz and Rotter, 1998). The TP53 dependent cell cycle arrest is mediated through TP53 dependent induction ofp21(wafl/cipl/CDKN1). The p21 gene is located at chromosome 6p21.2 and contains a transcription responsive TP53 binding site in its promoter, ( el-Deiry et al., 1993) p21 inhibits G 1-cyclins/cyclin dependent kinase-complexes to facilitate cell cycle arrest at the G l/S-phase checkpoint. (Gartel et al., 1996). TP53 dependent induction ofp21 is also necessary for maintained arrest at the G2 checkpoint after DNA damage. (Bunz et al., 1998).




*A schematic overview of the TP53 network and the links between TP53 and VEGF, MDM-2 and Cyclin E*

Programmed cell death proceeds through at least two main pathways, which both can be regulated at multiple levels. The extrinsic apoptotic pathway consists of cell surface receptors (death receptors), their inhibitory counterparts (decoy death receptors) and downstream cytoplasmic proteins such as caspase activators (reviewed in Peter and Krammer, 2003). The intrinsic apoptotic pathway is focused on the mitochondria, which contains several apoptogenic factors. (Kroemer, 1999). TP53 can induce apoptosis through both pathways by activating transcription of pro-apoptotic genes, although the intrinsic pathways contribution to TP53-mediated cell death is not clearly defined (reviewed in Fridman and Lowe, 2003). The best described link between TP53 and apoptosis is the

TP53 mediated regulation of transcription of proapoptotic members of the Bcl-2 family, Bax, Bid, Noxa and Puma. (Miyashita et al., 1994; Nakano and Vousden, 2001; Oda et al., 2000; Sax et al., 2002). The exact action of these proteins downstream of TP53 is not clearly defined, but the net effect is to increase the ratio of pro-versus anti-apoptotic Bcl-2 proteins. (Fridman and Lowe, 2003).

MDM-2 (Mouse Double Minute 2) is a TP53 induced phosphoprotein that acts as a major regulator of the TP53 by targeting its destruction, thus forming an autoregulatory loop with TP53. (Piette et al., 1997). The MDM-2 gene is mapped to the 12q13-q14 region and encodes a 90kDa protein. (Oliner et al., 1992). Binding of MDM-2 to TP53 results in ubiquitination and rapid degradation ofTP53. (Piette et al., 1997). During DNA damage is TP53 phosphorylated at amino acid ser15, which induces a conformational change that makes MDM2 unable to bind TP53 and results in the relief of the inhibitory effect of MDM2 on TP53. (Shieh et al., 1997).

The cyclin E protein is involved in cell cycle control downstream of TP53. Cyclin E is induced by the transcription factor E2F1 at the transition from G1 into S-phase, and rapidly degraded in early S-phase by an ubiquitin-mediated degradation. (Pestell et al., 1999). The cyclin E gene is positioned at 19q13 (Demetrick et al., 1995) and encode a protein that binds and activate a catalytic subunit, the cyclin-dependentkinase-2 (CDK-2). (Sherr, 1994).

During the G1 phase, phosphorylation of the retinoblastoma protein (pRB) by mainly cyclin D-cyclin-dependent kinase-4 complexes, releases pRb from E2F. (Morris et al., 2000) Cyclin E/CDK-2 may induce E2F by phosphorylation of pRb which abolishes pRb binding to E2F response

elements, (Keenan et al., 2003). Cell cycle progression into S-phase is suggested to be facilitated through E2F mediated recruitment of the p300/CBP family of co-activators, which binding to E2F is stabilized by phosphorylation of E2F by cyclin E/Cdk2, (Morris et al., 2000). Both cyclin-E/CDK-2 and E2F can also initiate S-phase independent of one another. (Leone et al., 1999) Cyclin E/CDK-2 is also playing a role in the initiation of DNA replication (Krude et al., 1997) and centrosome duplication. (Hinchcliffe et al., 1999; Mussman et al., 2000).

## Vascular endothelial growth factor and TP53

The formation of new blood vessels, angiogenesis, is a necessity for growth of both primary and metastatic tumors, since tumor growth beyond 1-2mm$^3$ requires development of an adequate blood supply due to the oxygen diffusion limit between a capillary and cells. (Folkman, 1990). Angiogenesis is a highly regulated process involving sequential activation of series of receptors by various ligands, in order to initiate degradation of the basement membrane, endothelial cell proliferation, cell migration and tube formation. (Ferrara et al., 2003; Scott et al., 1998).

The existence of angiogenic factors was first proposed in 1939 by Gordon Ide Ode, 1939) and colleagues after observing tumor growth accompanied by rapid and extensive neovascularisation in transplanted tumors (reviewed in Ferrara, 2002). Today a large number of molecules have been associated with pro-angiogenic capacity, including the vascular endothelial growth factor (VEGF) family, acidic fibroblast growth factor (FGF), basic FGF, transforming growth factor alpha (TGF-α), TGF-β, hepatocyte growth factor (HGF), tumor necrosis factor alpha

(TNF-α), angiogenin and interleukin-8 (IL-8). (Folkman and Shing, 1992).VEGF signalling has often been shown to be a critical rate limiting step in physiological angiogenesis and also associated with pathological angiogenesis, such as tumor neo-vascularisation, (Ferrara et al., 2003; Yancopoulos et al., 2000). VEGF is a polypeptide cytokine induced by hypoxia, with exclusive and potent mitogenic effect on endothelial cells, (Ferrara et al., 2003) There is a family of growing number of VEGF homologues (VEGF A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF), with specific affinity for three tyrosine kinase receptors, VEGFR1, R2 and R3, (reviewed in Clauss, 2000). VEGF A was the first discovered family member and the gene is located at 6p21.3. (Senger et al., 1986; Vincenti et al., 1996) Alternative splicing produce four different isoforms of which VEGF is believed to be the predominate one, (Ferrara, 1996).

Wild-type TP53 inhibit angiogenesis in vitro through induction of the endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1), (Dameron et al., 1994). It has also been reported that TP53 down-regulate VEGF in vitro by inhibitory binding to the VEGF transcription factor Sp 1 or by inhibiting c-Src dependent VEGF expression, (Pal et al., 2001).

Elevated VEGF has been demonstrated to be associated with a worse outcome for primary breast cancer in several retrospective studies, despite methodological differences, (Eppenberger et al., 1998; Gasparini et al., 1997; Gasparini et al., 1999; Linderholm et al., 2000; Linderholm et al., 1998; Linderholm et al., 2001; Linderholm et al., 2003).

## The role of cyclin E

The cell cycle regulatory protein cyclin E has recently been described as a strong prognostic factor E for breast cancer, (Keyomarsi et al., 2002) reported that patients with high cyclin E values had more than thirteen times higher risk for breast cancer caused death (Relative hazard 13,3 95%CI: 5,8-30,2) compared with patients with low cyclin E. In addition, among the 112 stage I patients in the study none of the 102 patients with low cyclin E had died of breast cancer at five years follow up, whereas all 12 patients with high cyclin E had died from breast cancer within that period. In multivariate analysis, including lymph node, high cyclin E was the strongest prognostic marker.

When reviewing the literature on cyclin E and breast cancer outcome, conflicting results have been reported: Nine (Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kuhling et al., 2003; Lindahl et al., 2003; Loden et al., 2002; Nielsen et al., 1996; Porter et al., 1997; Rudolph et al., 2003) out of twelve (Bukholm et al., 2001; Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kim et al., 2001; Kuhling et al., 2003; Lindahl et al., 2003; Loden et al., 2002; Nielsen et al., 1996; Porter et al., 1997; Rudolph et al.1 2003; Span et al., 2003) studies have observed statistically significant association between cyclin E and overall outcome. In four (Han et al., 2003; Keyomarsi et al., 2002; Lindahl et al., 2003; Porter et al., 1997) of nine (Bukholm et al., 2001; Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kuhling et al., 2003; Lindahl et al., 2003; Porter et al., 1997; Rudolph et al., 2003) multivariate survival analyses did cyclin E remain statistically significant. See the table on next page.

### Overview of the reported prognostic value of cyclin E

| Study | No. cases | | Cyclin E method | Univariate OS | Multivariate OS |
|---|---|---|---|---|---|
| Nielsen et al, 1996 | 100 | Stage I-III | IHC | 0,0002 | N/A |
| Porter et al, 1997 | 287 | Node negative | IHC* | 0,001 | RH 2,4 (1,1-5,2) |
| Bukholm et al, 2001 | 137 | Unselected | IHC*** | 0,40 | p=0,57, RH N/A |
| Kim et al, 2001 | 128 | I-III | IHC* | 0,32 | N/A |
| Donnellan et al, 2001 | 157 | Unselected | IHC | <0,0001 | NS |
| Loden et al, 2002 | 113 | Stage I-IV | WB* | 0,011 | N/A |
| Keyomarsi et al, 2002 | 395 | Stage I-IV | WB* | 0,0001 | RH 4,3** (95% CI 2,2-8,4) |
| Lindahl et al, 2003 | 270 | Stage I-IV | IHC* | 0,0002 | RH 2,4 (CI 1,3 – 4,5) |
| Kühling et al, 2003 | 332 | Node negative | IHC | <0,0001[1] | NS |
| Rudolph et al, 2003 | 273 | Node negative | IHC | 0,0006/N.S[2] | NS |
| Span et al, 2003 | 277 | Unselected | RT-PCR* | 0,54 | NS/RH 3,0*** |
| Han et al, 2003 | 175 | Node negative | TA-IHC* | 0,028 | RH 2,7 (95% CI 1,4-3,5) |

**Abbreviations:**

Overall survival (OS), Immunohistochemistry (IHC),
Western blot (WB), Real time polymerase chain reaction (RT-PCR),
Relative Hazards (RH), Confidence interval (Cl), Not significant (NS),
Tissue microarray immunohistochemistry (TA-IHC)

* Used antibody/method estimates content of both full length Cyclin E and shorter isoforms.

** Breast cancer specific HR 13,3 (95% Cl 5,8-30,2)

*** Type of anti-cyclin E antibody not disclosed.

**** Tamoxifen treated patients with high Cyclin E (HR 3,0, 95%CI 1,3-7,1). No statistically significant association for OS total study population.

1. Breast cancer specific survival
2. Breast cancer specific survival. p=0,0006 in postmenopausal patients. p=0,82 in premenopausal patients.

Keyomarsi et al (Keyomarsi et al., 2002) proposed that studies that does not determine cyclin E by methods that detect both full length cyclin E and the shorter isoforms, may underestimate the association between

cyclin E and outcome. The five short cyclin E isoforms (size 34 to 49 kDa) are generated by proteolytic cleavage. They lack the amino terminus and are biologically hyperactive in inducing progression from G1 to S-phase. (Porter et al., 2001) Keyomarsi et al. (Keyomarsi et al., 2002) used Western Blot with the HE12 mouse monoclonal anti-cyclin E antibody, which is directed at the C-terminal of the cyclin E protein. The use of cyclin E in clinical breast cancer management is not recommended by the ASCO guidelines. (Bast et al., 2001).

The content of Cyclin E protein was determined by IHC on paraffin embedded primary breast cancer samples. The paraffin sections were deparaffinized and microwave treated. Immunostaining was performed using the monoclonal mouse anti-Cyclin E antibody HE 12 (Santa Cruz Inc., U.S.A.) and an automated Immunohistochemistry-staining machine (Ventana 320-202, Ventana Inc., AZ, U.S.A.). The IHC reactivity were divided into three levels, before statistical analysis, according to the percentage of tumor cells stained; low (0-4%), medium (5-49%) and high (50-100%). The cut-off levels were chosen in order to achieve distinct separation between patients with high and low Cyclin E expression. This selection was done before any statistical analyses were performed. All glasses were read without knowledge of previously determined tumor characteristics or patient outcome.

## The role of P21 (waf1) / (Cip1) and MDM-2

Immunohistochemistry and paraffin embedded tumor samples was also used for determination of p21(waf1/Cip1) and MDM-2. Out of the 311 original patients 276 and 257 had tumor material available for IHC determination of p21 and MDM-2, respectively. The tumor sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water.and then microwave treated in citrate buffer. Immunostaining was performed using a commercial Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) directed against mouse IgG. Blocking serum was applied for 15 minutes followed by overnight incubation with the diluted monoclonal primary antibody MDM-2 1:100 (clone IF2, Oncogene Research products, Cambridge, MA), p21 1 :200 (WAF 1 protein, clone 4D10, Novocastra Laboratories Ltd, Newcastle uponTyne, UK). The sections were then incubated with the biotinylated second antibody and the peroxidase-labelled ABC for 30 minutes each. Sections stained with p21 and mdm2 were further stained with biotinyl tyramide and streptavidin conjugated to horseradish peroxidase, using these two reagents from a commercial amplification kit (Catalysed Signal Amplification, CSA, Dako, Glostrup, Denmark). Bound peroxidase was visualized in all slides with a 3-amino-9-ethyl-carbazole (AEC) solution. Finally, the sections were lightly counterstained in Mayer' s hematoxylin and mounted in Aquamount Mountant (BDH Ltd, Poole, United Kingdom).

Known positive sections for MDM-2 and p21 were included in every staining batch as positive controls. Slides stained with PBS substituting the primary antibody were used as negative controls. An experienced

pathologist, without knowledge of previously determined tumor characteristics or patient outcome read all glasses. All breast cancers with nuclear p21 or MDM-2 immunostaining were regarded as positive. The percentage of tumor or cells with positive nuclear staining was also noted for each slide.

## Cell cycle regulators

Some of major regulators of cell cycle progression are Cyclin E, p21 and p27. These expression and activity of these regulators have been demonstrated to have the potential to significantly impact tamoxifen sensitivity and resistance. For instance, Cyclin D1 inhibits Rb early in G1 phase, and the transcription factor E2F strongly induces the expression of cyclin E, which associates with CDK2 to form an active complex that promotes entry into S phase [205]. Cyclin E and CDK2 activity is antagonized by the CDK inhibitors p21 and p27. The contribution of cyclin E to tamoxifen resistance is unclear, but over-expression of cyclin E in MCF-7 cells partially counteracts the growth arrest mediated by tamoxifen [206] More studies are necessary to determine the mechanisms by which cyclin E is induced in breast cancer and its role in the induction of resistance.

P21 and p27 are CDK inhibitors and are negative regulators of cell cycle progression. These proteins counteract the activities of cyclin D1 and cyclin E. In MCF-7 cells, tamoxifen increase the expression of p21 and p27 during cell cycle arrest, but down-regulation of these by antisense inhibition prevents the growth inhibitory effects [207]. P27 induction in breast cancer cells by tamoxifen induces quiescence and insensitivity to growth stimulation by growth factors such as IGF-I and EGF [208]. Somatic dele-

tion of the p21 gene in human breast cancer cells resulted in hyperphosphorylation of ERa causing an increased gene expression of ER regulated genes [209]. These studies demonstrate that p27 and p21 are critical for the inhibitory effects of tamoxifen in inhibiting breast cancer cell growth.

c-Myc, a transcription factor whose expression is frequently altered in human breast cancer, also plays a role in regulating the activity of p21. Increased c-Myc expression can rescue the growth arrest mediated by anti-estrogen treatment by activating CDK2/Cyclin E complex [210]. In vitro over-expression of c-Myc reduces the expression of p21 in response to tamoxifen potentially mediating tamoxifen resistance [211] They also demonstrate that p21 expression in anti-estrogen resistant cells is increased when treated with c-Myc siRNAs.

Clinical data also supports a role of these CDK inhibitors in response to tamoxifen treatment. In premenopausal women with early breast cancer, an increase in p27/KIP1 expression was able to predict better relapse free survival upon tamoxifen combination treatment [212]. A multivariate analysis of their data revealed decreased p27 expression to be correlated with poor outcome upon combination endocrine therapy. Localization of these CDK inhibitors has also been implicated in the development of resistance. Perex-Tenorio et al. showed that increased activity of the PI3K and MAPK pathway promotes p21 localization into cytoplasm through phosphorylation of residues within their nuclear localization sequences [213]. The significance of their in vitro data is supported by immunohistochemistry in frozen human tumors. They demonstrated that increased cytoplasmic localization of p21 is correlated with a poorer response to tamoxifen treatment in a cohort of 280 women.

# The biology of TP53

In 1979, three teams led by A. Levine, P. May and L. Old discovered the p53 protein, a protein that is, highly conserved across animal species, which is encoded by the TP53 gene located on the short arm of chromosome 17 (17p13.1). Its sequence, about 20 Kb, contains 11 exons, but the first exon does not encode and is located about 10 Kb from other exons [214]. In 1989, Vogelstein's team discovered that the TP53 gene is inactivated in human cancers [215].

The p53 protein contains 393 amino acids (AA), is divided into regions highly conserved during evolution [216], and its role in numerous regulatory mechanisms has been well established. The protein is composed of: (i) an N-terminal region (AA 1–42), (ii) a region rich in proline residues (AA 63–97) involved in the induction of apoptosis [217], (iii) a core domain necessary for binding to DNA (AA 102–292), containing most of the inactivating mutations found in different types of human cancers [218], (iv) a tetramerization domain (AA 323–356), and (v) a C-terminal region (AA 363–393). This C-terminal region of p53 binds to the N-terminal domain of Mdm2 (murine double minute 2). In addition, there are also sequences for exporting to the cytoplasm at the N- and C-terminal ends (NES, nuclear export signal), as well as nuclear localization sequences at the C-terminal end (NLS, nuclear localization signal), enabling the regulation of subcellular localization of p53 [219, 220].

## Stimulation and activation effects of TP53

Multiple stimuli such as ionizing radiations, DNA lesions, nitric oxide, hypoxia, chemotherapeutic agents, or oncogenic stimuli can activate 53 [221, 222]. In response to various stimuli, p53 undergoes different changes and this activation could induce different effects. P53 is a transcription factor involved in the control of G1/S and G2/M phase transition, in DNA repair, and in induction of senescence, apoptosis, autophagy, mitotic catastrophe, and angiogenesis.

## Cell senescence

TP53 regulates the control of the G1 checkpoint and can induce an arrest of the cell cycle, repair or apoptosis if DNA lesions are extensive [223]. Wild-type p53 protein can transcriptionally transactivate, a potent inhibitor of most cyclin-dependent kinases, involved in the cell cycle arrest [224]. P53 also stimulates the expression of the 14-3-3σ protein that sequesters the cyclin B1/CDK1 complex to block the transition G2/M. But p53 also induces the expression of many others genes such as GADD45, which interacts with PCNA to inhibit the passage to S phase, or Reprimo to block the cell cycle in G2 phase [225].

Cellular senescence is thought to play an important role in tumor suppression and to contribute to cellular aging [226]. The p53 tumor suppressor is also a critical mediator of senescence, and it seems to play a critical role in the induction and maintenance of cellular senescence. The first information about the importance of p53 on cell senescence was provided by the studies using T antigens of SV40 virus which inactivate p53. P53-null fibroblasts remain immortal when propagated in vitro. P53

activation is an essential step in the induction of senescence following DNA damage or other forms of stress. In the context of senescence, p53 is controlled by ATM/ATR and Chk1/Chk2 proteins which cause the posttranslational stabilization of p53 through its phosphorylation [227].

## Apoptosis

Apoptosis is one of the principal functions of p53. It has been shown that p53 can transactivate the cell death receptors CD95 or TNF which induce the formation of the DISC complex and finally activate caspase 8. P53 also activates proapoptotic members of the Bcl2 family: Bax, Noxa, and Puma-involved in the permeabilization of the outer mitochondrial membrane [228]. Moreover, p53 has also been reported to have a direct role in cell death initiation by localizing to mitochondria and regulating mitochondrial outer membrane permeabilisation directly. Thus, p53 protein can directly induce permeabilisation of the outer mitochondrial membrane by forming complexes with the protective BclXL and Bcl2 proteins, resulting in cytochrome C release [229, 230].

### Autophagy

Autophagy is a process suppressing tumor initiation and reducing genomic instability. Autophagy consists in the lysosomal degradation of intracellular components leading to the generation of new metabolic substrates, thus favouring adaptation to stress and cell survival [231]. P53 can activate but also inhibit autophagy. Under stress, p53 can activate its target gene in the nucleus, such as AMPK β1 and β2 (AMP-activated protein kinase) [232], DAPK-1 (death-associated protein kinase1), and

DRAM (damage-regulated autophagy modulator) [233]. Cytoplasmic, but not nuclear, p53 is able to repress autophagy [234, 235].

## Mitotic catastrophe

Mitotic catastrophe is a biological state that precedes cell death. In response to DNA damage, checkpoints are activated to delay cell cycle progression and to coordinate repair. Reports have suggested that the absence of p53 might increase mitotic catastrophe [236]. P53-deficient cells in an unchecked tetraploid G1 state reduplicate their DNA, leading to polyploidy and subsequent chromosomal instability. In the presence of wild-type p53, the polyploidy causes an irreversible arrest in the cell cycle, or in cell death, thus preventing the propagation of aneuploidy [237].

## Angiogenesis

The formation of new blood capillaries (angiogenesis) is closely regulated by proangiogenic and antiangiogenic factors [238]. The p53 protein has been shown to limit angiogenesis by few mechanisms: (1) interfering with central regulators of hypoxia that mediate angiogenesis, (2) inhibiting the production of proangiogenic factors, and (3) directly increasing the production of endogenous angiogenesis inhibitors. The combination of these effects allows p53 to efficiently shut down the angiogenic potential of cancer cells [239]. Wild-type p53 plays a role in limiting tumor vascularization as demonstrated by some clinical studies [240]. Mutant p53 plays a central role in promoting angiogenesis in colon cancer progression [241], and tumors carrying p53 mutations are more highly

vascularised than tumors harboring wild-type p53. The loss of TP53 appears to amplify the HIF (Hypoxia Inducible Factor) pathway. HIF-1α has been shown to be physically associated with p53 in immuno-precipitation experiments. P53 promotes MDM2-mediated ubiquitination and degradation of HIF-1α, while loss of p53 leads to amplification of the HIF response [242].

## Regulation of p53

The protein p53 can be regulated at different levels:

(i) by post translational modifications, such as phosphorylation, sumoylation, or acetylation of the protein [243, 244],

(ii) by increasing the protein concentration: One of the key regulators of p53 is Mdm2 which targets p53 for breakdown by the proteasome [245],

(iii) by cellular localization: Import and nuclear export is closely regulated because the functions of p53 depend on its nuclear localization. Efficient transfer to the cytoplasm depends on Mdm2 forming a complex with p53, which is why ubiquitin ligase activity of Mdm2 is essential for nuclear export of p53 [246]. The ubiquitinilation of p53 by Mdm2 occurs in the C-terminus domain, and it has been shown that mutations in lysine residues inhibit the nuclear export of p53 by Mdm2 [219].

In a large majority of studies, detection of p53 is highlighted by the protein in the nucleus using immunohistochemistry techniques. This method of detection could give false positive results from stabilization of wild-type p53 proteins due to cellular stress or could give false negatives

due to codon stop, frameshifts, or other destabilizing mutations. Lack of immunostaining for p53 despite mutation of the TP53 gene was particularly seen in tumors harboring nonsense mutations or deletions/splices [247] while other studies have shown that the identification of positivity for p53 solely detected by immunohisto-chemistry did not always reflect a p53 mutation [248].

Another way to determine TP53 status is the FASAY test (Functional Analysis of Separated Alleles in Yeast) [249]. After the extraction of mRNA from whole blood or from tissue (normal or tumoral) reverse transcription by RT-PCR is carried out. The DNA binding domain is amplified and the PCR product is cloned by homologous recombination into yeast with a linearized expression plasmid vector carrying the 5′ and 3′ ends of the TP53 open reading frame. The plasmid, thus, has a constitutive expression of human TP53. The yeast contains an open reading frame (ORF) for adenine regulated by a promoter under the control of TP53. The yeasts are selected on a selective medium lacking leucine, but containing adenine. When TP53 is wild-type, a complete metabolism of adenine occurs and the colonies are white. The cells containing mutant TP53 fail to express adenine, and, consequently, the colonies are red because of the accumulation of an intermediate adenine metabolite. These colonies are also smaller than normal because adenine limits growth. Thus, the TP53 status can be easily determined by the color of transfected yeast cells [249].

Some studies analyzed TP53 status in breast tumors using a robust and sensitive approach combining three different methods: P53 immuno-histochemistry, FASAY test, and sequencing of the coding sequence.

Tumors were considered TP53 mutant when (i) more than 15% of the yeast colonies were red (ii) analysis using the split versions of the test could identify the defect in the 5′ or 3′ parts of the gene, and (iii) sequence analysis from mutant yeast colonies could identify an unambiguous genetic defect (mutation, deletion, splicing defects) [250]. FASAY provided a major contribution to the analysis by revealing several TP53 mutations not detected by direct sequencing, principally in samples highly contaminated with stromal cells [251, 252].

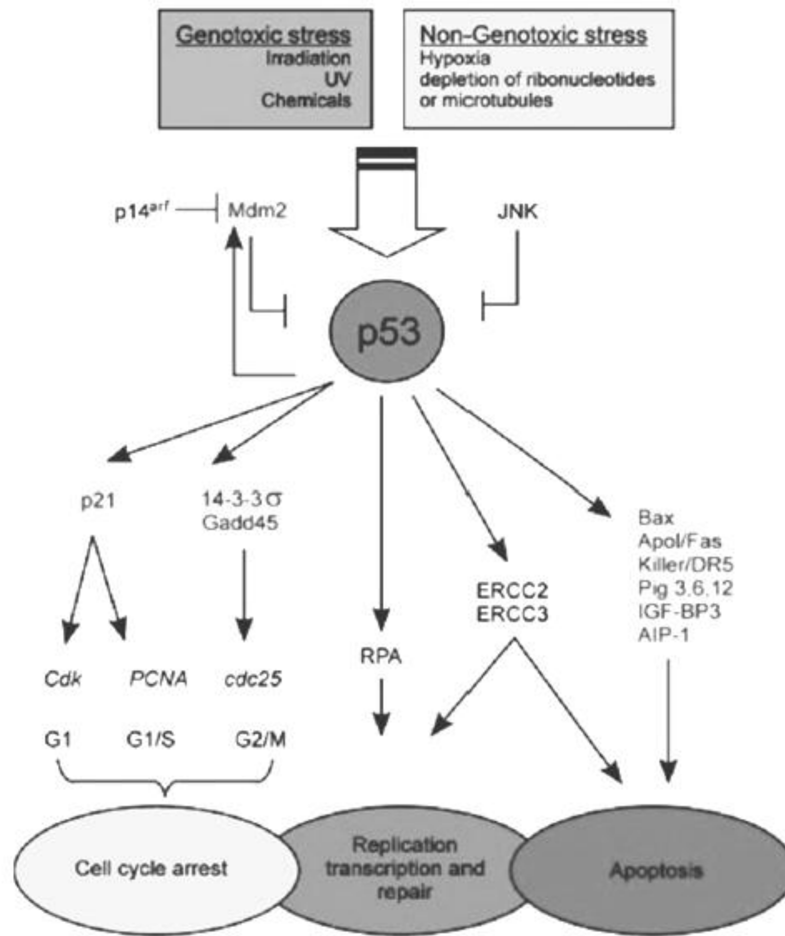
## TP53 mutations and Human cancer

It has been told that the human TP53 gene is located in 20 kb of chromosome band 17p13.1. The gene is composed of 11 exons, the first of which is non-coding. The product of the gene is a 53kD nuclear phosphoprotein, composed of 393 amino acids. The functional molecule is a tetramer and acts as a transcriptional factor. It's is involved in cell cycle checkpoints, apotosis, genomic instability, and DNA repair.

The p53 protein is activated in response to genotoxic (DNA-damaging) and nongenotoxic stresses. The stability of the protein is controlled by Mdm2 and by JNK. After activation, p53 regulates genes and proteins involved in cell cycle arrest (in G1, G1/S and G2/M) in replication, transcription, repair, and apoptosis. The p53 protein is constitutively expressed in almost all cell types but has a very rapid turnover and appears to be latent under normal conditions. However, p53 is rapidly converted to an active form in response to a number of physical or chemical DNA-damaging agents such as gamma-irradiation, UV rays, oxidizing agents, cytotoxic drugs, and cancer causing chemicals. Induction of p53 implies

nuclear retention, accumulation of the protein as a result of post-translational stabilization, and allosteric conversion to a form with high sequence-specific DNA-binding capacity. This has led to the concept that p53 is specifically activated in response to DNA-damage thus acting as a "guardian" against genotoxic stress.

The p53 protein is a sequence specific transcription factor which binds DNA sequences corresponding to repeats of the consensus motif RRRC (A/T)(T/A)GYYY (where R is a purine and Y pyrimidine). The protein has five structural and functional domains: aN-terminal, transcriptional activation domain, a proline-rich regulatory domain, a sequence-specific DNA-binding domain, an oligomerization domain, and a C-terminal domain involved in the regulation of DNA binding. In terms of three dimensional structure, the protein is made of a scaffold of beta-sheets that support flexible loops and helixes which are in direct contact with DNA. The position of these loops and helixes is stabilized by the binding of an atom of zinc. The protein p53 contains several functional domains, as indicated. The number of mutations detected in the human cancer that falls within each of these domains is given. The most frequently mutated portion is the sequence-specific DNA-binding domain. Within this domain, several residues are "hotspots" for mutation. The three most frequently mutated residues in human cancers are represented using a space-fill model in which each atom is pictured as a small sphere. The target DNA that p53 binds to is outlined.




*The p53 signaling pathway.*

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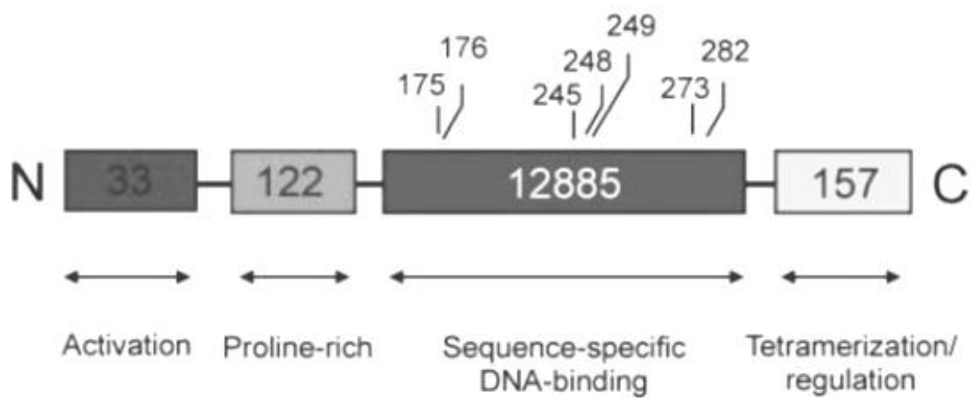
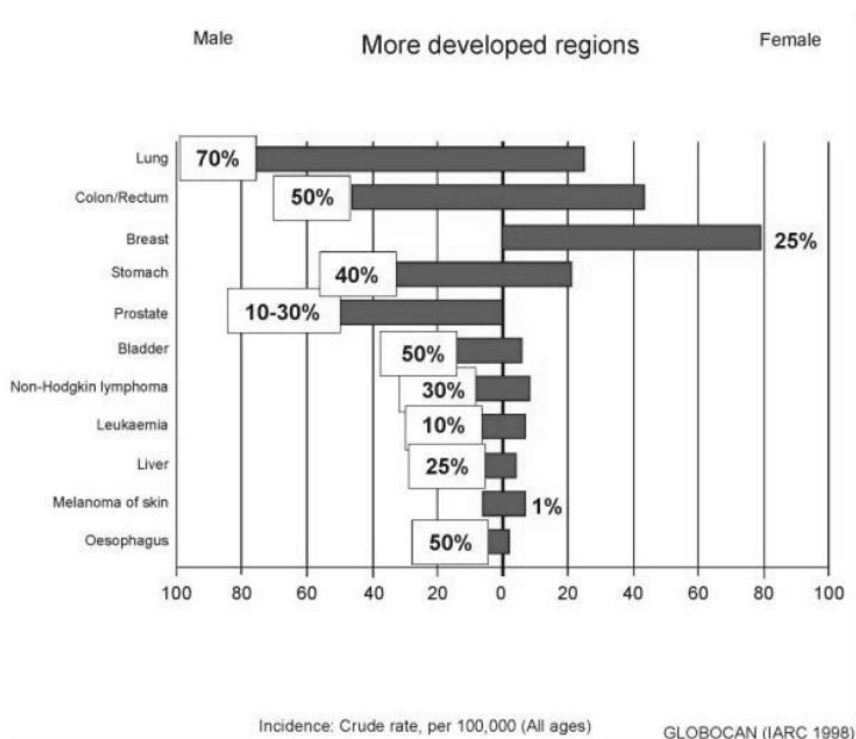



*Structure of the p53 protein.*

In cancer, inactivation of p53 occurs through various mechanisms, including genetic alteration (mutation, deletion), inactivation of the protein by binding to viral or cellular oncoprotein, and sequestration in the cytoplasm. The DNA binding domain contains 93% of all mutations identified to date. This high frequency may be overestimated, since after initial reports that mutations tended to cluster in the central portion of the coding sequence (DNA binding domain), most investigators have limited their analysis to exon 5 to 8. A database of all published mutations is maintained at the International Agency for Research on Cancer. The most frequently mutated residues are conserved among species and play an important, direct or indirect, role in the contacts between the protein and target DNA. All these mutations result in impaired DNA -binding and loss of transcriptional activity.




*Incidence of cancers in developed countries*

Mutations in the TP53 are found in almost every kind of human tumor. Malignancies in which the mutation prevalence is higher than 50% include skin cancer (except melanoma), late stage cancer of bladder cancers, and carcinomas of the aero-digestive tract. Lymphomas and tumors of the brain, breast, prostate, and liver show an intermediate mutation frequency (15 to 35%). Malignancies with low mutation frequency include leukemia (10%), testicular cancer, and malignant melanoma (both less than 5%). In cancers such as breast and colon, TP53 mutations seem to occur late in tumorigenesis. In several other cancers (head and neck, lung, skin), mutations occur very early and may even precede tumor development. The nature and type of mutations is often informative of the mutagenic mechanisms that have caused them, making TP53 an interesting gene to study in molecular epidemiology.

Mutations in the p53 protein can have at least three phenotypic effects: Loss of function, in which a missense mutation abrogates p53's ability to block cell division or reverse a transfonned phenotype; gain of function (or dominant-positive effect), where mutant p53 acquires novel functions as demonstrated with the introduction of a mutant p53 gene into cells lacking wild-type p53 allele, which induces a tumorigenic phenotype: trans-dominant mutation (dominant-negative effect), seen when a mutant p53 allele is introduced into cells bearing a wtp53 allele, resulting in the ability of mutant p53 to drive wtp53 to a mutant conformation overriding of the normal inhibitory function of p53.

## P53 a sensor protein of genotoxic stress

In most cells, p53 is almost undetectable because it is rapidly degraded by the proteasome. Upon activation, the protein escapes degradation and accumulates in the nucleus. At the same time, it is turned from a latent to an active form by conformational changes which activate its capacity to transactivate target genes. The main factor controlling p53 accumulation is Mdm2, a protein encoded by a gene which is itself a transcriptional target of p53. Mdm2 acts as a ubiquitin ligase to direct p53 out of the nucleus to the proteasome, where it is degraded.

Various types of genotoxic and non-genotoxic stresses can lead to p53 activation, including agents that create single or double-strand breaks in DNA (irradiation, oxidative stress), mutagens-aflatoxins, benzo(a)pyrene, alkylating agents-and inhibitors of topoisomerases. Moreover, damage to the mitotic spindle, ribonucleotide depletion, hypoxia heat shock, and exposure to nitric oxide can also induce p53. Induction follows a different time-course, depending upon the nature and intensity of the stress.

Induction in response to stress is a multi-step process. It involves phosphorylation of p53 in the N-terminus (e.g. by kinases activated after DNA-damage such as Atm or Chk-2). and dissociation of p53-Mdm2 interactions. Other changes in the protein include acetylation of the C-terminus (by acetyl -transferases of CBP/p300 family), conformational changes in the C-terminus leading to the unmasking of the DNAbinding domain, and changes in oxidation-reduction in the DNA-binding domain. All these changes turn the protein into an active form which binds DNA with high affinity.

Once activated, p53 can trigger several cellular events via two distinct and parallel pathways, transcription-dependent or transcription-independent. Examples of transcription-independent pathways include binding of p53 to components of the DNA replication/repair machinery such as the helicases ERCC2 and ERCC3, or the replication protein RPA. Genes transcriptionally regulated by p53 include cell cycle regulators in Gl and in G2 phases (p21/waf-l, 14-3-3s, GADD45), regulators of apoptosis (BAX, CD95/FAS, KILLER/DR5, p53AIP1, PIG3, IGF-BP3), and genes involved in cellular responses to stress such as inducible forms of nitric oxide synthase ($NOS_2$) and cyclooxygenase (COX2), which are both repressed by p53. How p53 selects from the set of alternative responses (e.g. choosing between cell cycle arrest or apoptosis) depends upon the nature and the amplitude of the inducting signal, as well as of the cell and tissue type.

An important aspect of the role of p53 in cancer treatment is the fact that the function of p53 is crucial for the cytotoxic response of cancer cells to radio- or chemotherapy. There is evidence that many anti-cancer drugs induce apoptosis through a p53-dependent pathway. However, in clinical terms the presence of a wild-type TP53 gene is not always correlated with good response to treatment, as many other factors can also influence this response. On the other hand, in certain cell types activation of p53 by therapeutic agents may induce cell cycle arrest (and DNA repair) rather than apoptosis, thus resulting in a form of protection of cancer cells against the effects of therapy. Thus activation of p53 may be seen as both a chemo-sensitizer or a chemo-protective mechanism, depending on the cellular context. This is why current, experimental approaches that target p53 for cancer treatment include attempts to activate p53 (and thus

induce apoptosis) as well as to inactivate p53 (and thus prevent destruction of normal cells by cytotoxic therapies).

**Gene therapy by TP53**

The capacity of wild-type TP53 to arrest the proliferation of cultured cells and induce apoptosis has raised an enormous interest in the possibility that restoring TP53 function in tumor cells may block tumor development. In addition, the finding that the p53 protein is a key factor in determining the response of cancer cells to therapy, has led to the concept that re-introduction of a normal protein may sensitize cells to cytotoxic killing and thus improve therapeutic response. Over the past ten year, several efforts have been made to translate these laboratory findings into clinical applications. One of the most popular approaches to achieve this goal is gene therapy. Below, we summarize the various modalities of TP53-based gene therapy have been described in the recent literature.

The function of TP53 is lost in many cancers through mutation or loss of alleles. Therefore it seems reasonable to try to restore TP53 function by replacing the mutant gene with a functional, wild-type copy. The primary requirement to treat cancer with such replacement gene therapies is the necessity for highly efficient delivery of the wild-type TP53 into tumor cells in vivo. There must also be sufficient expression of functional p53 protein to mediate tumor suppression either through a direct mechanism involving cell death or growth arrest, or by increasing sensitivity to conventional antitumor agents. Other critical success factors include a low level of toxicity towards normal cells and the absence of a host immune response against the gene delivery system. The mechanisms of gene delivery can be subdivided in two broad categories: viral and non-viral.

## p53 status and Anthracycline chemotherapy in breast cancer

In 50 noninflammatory locally advanced breast cancers that were treated with dose-intense epirubicin-cyclophosphamide combination, eight complete pathological response (pCR) were shown in the 14 patients with tumors containing mutated TP53, whereas none of the 36 patients with a wild-type TP53 status had a pCR after chemotherapy [282]. In 80 patients with noninflammatory breast cancers treated with front-line chemotherapy comprising epirubicin and cyclophosphamide, 28 had TP53-mutant tumors. Fifteen out of these 28 patients exhibited pCR while none of the 52 patients with TP53 wild-type tumors had a pCR. Moreover, nine out of ten of the highly aggressive basal subtypes showed pCRs. This demonstrates that, in noninflammatory breast cancers, TP53 status could be a key predictive factor for response to this chemotherapy treatment and further suggests that the basal subtype is exquisitely sensitive to this association [283]. Research on stage II-III breast cancer patients treated front line with epirubicin-based regimens of various cyclophosphamide dose intensities suggest that cyclophosphamide dose intensification in ER negative and TP53 mutated patients could significantly improve their response [284]. All these studies show an increased response in tumors with a mutation in TP53. Recently, it was shown on in vivo models that epirubicin-cyclophosphamide treatment induces senescence-like features in TP53 wild-type tumor, probably accounting for cell cycle arrest and subsequent resistance to treatment. Conversely in TP53 mutated tumors, chemotherapy induces mitotic catastrophe and tumor death, accounting for complete response to this association exclusively in patients with TP53 mutated tumors [285].

In contrast, in a study on 63 patients with locally advanced breast cancer and treated with doxorubicin, a correlation was observed between the presence of mutations in the zinc finger domain of the p53 protein and resistance to treatment [286]. These results were confirmed in another study involving 90 patients [247]. Some clinical studies showed that mutant p53 confers chemoresistance in patients with breast cancer. Patients with missense mutations located in zinc-binding regions had significantly decreased disease-free and overall survival relative to patients whose tumors had mutations in other domains [287]. It has been suggested that codon polymorphism 72 (Arg/Pro) could affect the response to chemotherapy in tumor cells through the interaction between p53 and p73 [288]. Protein p73 belongs to the same family as p53 and p63 and shows a striking homology within both the DNA binding domain and oligomerization domain. P73 presents a wide array of splicing variants α, β, γ, δ, ε, ζ [289]. p73 has proapoptotic and antiapoptotic properties. P73alpha mRNAs encode two types of isoform (TAp73alpha and DeltaNp73alpha) resulting from the use of two different promoters, and eliciting or lacking NH(2)-terminal transactivation domain, respectively. DeltaNp73alpha inhibits p53 proapoptotic function [290]. Patients with breast cancer with a variant Pro/Pro TP53 are less sensitive to anthracycline-based therapy than those with a variant Pro/Arg or Arg/Arg [291]. These studies show that mutations in TP53 could induce a resistance to treatment based on anthracyclines.

These results are not contradictory, they rather result from studies exploring different tumor types and different regimens. TP53 status may have a different predictive value for efficacy of anthracycline/alkylating

agents-based regimen in each molecular subclass [292]. In 630 patients with breast cancer, the clinical outcome was significantly different for different TP53 mutation types but also for different tumors [293].

A quick look at EORTC 10994 "p53 trial indicates that mutant P53 primary breast cancer responds better to taxanes than anthracyclines (Kandiolier-Eckersberger 2000). If anyone see further influences relation in bibliography between drugs dosages, stages, tissue types, histological types can easily conclude that mutant P53 has poor response to tamoxifen (Bergh, 1995), to doxorubicin (Aas 1996), to 5FU, MMC (Geisler, 2003), to CMF (Andersson 2005). For EORTC 10994 P53 trial profile has been used functional yeast assay with frozen tissues and biological assays examining the retrospective analysis of p53 mutation on pretreatment samples v prediction and prognostication. 1856 patients cooperate on this, p53 mutations assessed on 1486. 825 (56,2%) with wtp 53 and 644 (43,8%) with mp53.

## P53 status and Nonanthracycline chemotherapy in breast cancer

In 67 tumors treated with 5-FU, epirubicin, cyclophosphamide, or paclitaxel, combined sequencing and immunohistochemistry showed a significant association between the presence of TP53 mutation and response to paclitaxel. The efficacy of paclitaxel during mitosis is induced by the fact that there is no stop in G1 phase, because of absence of p53 [250]. Trastuzumab, an HER2-targeted monoclonal antibody, induces growth arrest and apoptosis in a p53-independent manner. A retrospective study on 104 patients receiving trastuzumab shows that p53 status is not a predictor of the clinical efficacy [294].

Some studies suggested that p53 may influence response to antihormonal treatments. TP53 mutations are less frequent in patients with ER-positive breast cancers, but they are associated with a poorer prognostic in these patients. In vitro studies on human breast cancer cell lines, MN1 (p53WT) and MDD2 (p53MUT) derived from MCF-7, it was shown that p53 mutated cells were more resistant to cytotoxic effects of 4-hydroxy-tamoxifen compared to p53 wild-type cells [295]. Clinical studies on patients with locally advanced breast cancer treated with tamoxifen or primary chemotherapy showed that mutations in the TP53 gene are associated with a poor survival [296]. In a meta-analysis of 4,683 patients with breast cancer, the overexpression of p53 was correlated with poor outcome in premenopausal women treated with tamoxifen after chemotherapy [297].

## P53 and Radiotherapy

Tumor cell death following exposure to radiotherapy occurs by apoptosis and is a p53-dependent event [298]. Preclinical studies were realized on immunocompromised mice engrafted with fibrosarcoma tumors expressing a functional or TP53-deficient gene. Tumors with functional TP53 contained a large proportion of apoptotic cells and regressed after treatment with gamma radiation or adriamycin. p53-deficient tumors treated with the same regimens continued to enlarge and contained few apoptotic cells. Reduced levels of functional p53 would prevent radiotherapy-induced cell death, while mutant p53 is a marker for resistance. The defects in apoptosis due to inactivation of p53

can produce treatment-resistant tumors, suggesting that p53 status could be important in determining tumor response [299].

In conclusion, TP53 status shows a strong prognosis impact and this could be useful in the choosing the best treatment for breast cancer. Generally, TP53 mutated is associated with a poor response to chemo-therapy, hormonotherapy or radiotherapy. Discordant studies concerning its predictive value exist, and this is linked to method of detection of TP53 status. We show that FASAY test and sequencing of TP53 are better than immunohistochemistry to determine if TP53 is mutated or not. Prospective studies using these two methods could better determine its predictive value according to response to treatments.

## Isoforms of p53

The human p53 gene can encode at least nine different p53 protein isoforms:

1. p53, p53β, p53γ due to alternative splicing of intron. 9.
2. Δ133 p53, Δ133 p53β and Δ133 p53γ due to alternative splicing of intron 9 and usage of alternative promoter in intron 4.
3. Δ04 p53 due to alternative splicing of intron 2 and alternative initiation of translation [300].

### P53 and Its target genes

P53 controls cellular functions like cell cycle control, DNA repair, apoptosis, angiogenesis and cellular stress response through iits target genes, shown in the following table [301].

**Target genes of p53**

| No | Cellular response | Target genes |
|---|---|---|
| 1 | p53 control | Mdm2 |
| 2 | Cell cycle control | P21, WAF1/CIP1, GADD45, WIP1, Mdm2, EGFR, PCNA, TFG. Cyclin D1, Cyclin G. |
| 3 | DNA repair | GADD4, PCNA, P21, WAF1/C1P1 |
| 4 | Apoptosis | BAX, Bc1-L, FAS1, FASL, IGF-BP3, PAG608, DR5/KILLER, GML, P2XM |
| 5 | Angiogenesis | TSP-1, BA11 |
| 6 | Cellular stress response | TP53TG1, CSR, PIG3 |

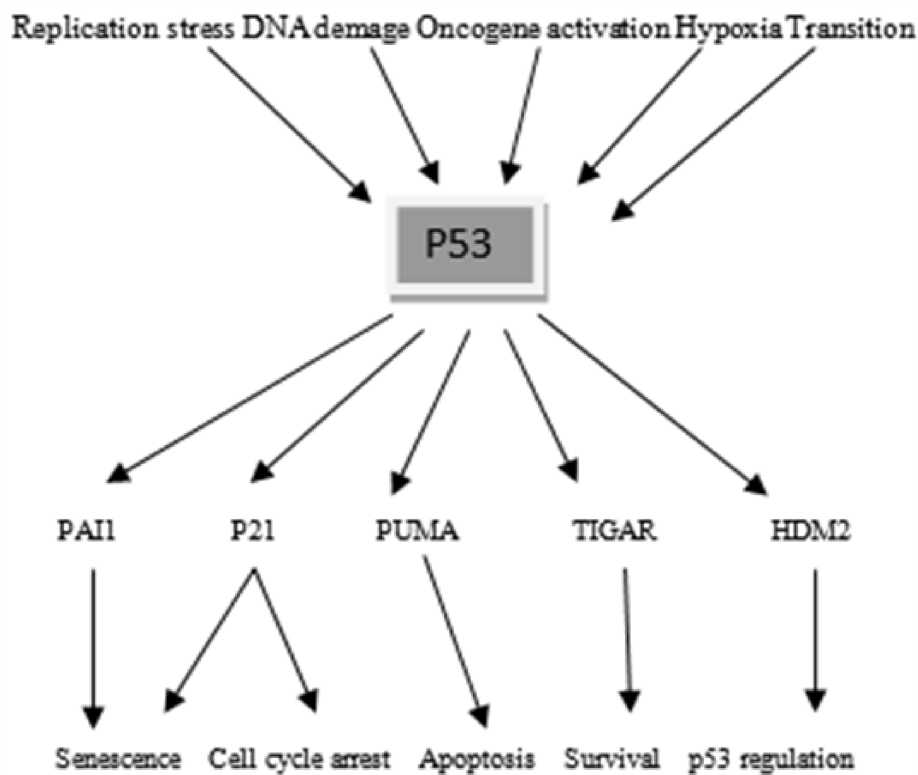
Mdm2 has been well characterized as a negative regulator of tumor suppressor p53. It is clear that E3 ubiquit in ligase, Mdm2, is a primary regulator and suppressor of p53 activity. In the absence of cellular stress and damage, Mdm2 bind to p53 and targets it for proteosomal degradation through ubiquitination[302], whereas Mdmx inhibits p53 by binding to and masking the transcriptional activation domain of p53, without causing its degradation. However, Mdm2 and Mdmx have been shown to function collaboratively[303].

## P53 as a transcription activator

The protein functions mainly as a transcription factor. P53 interacts with DNA via its DNA binding domain. This domain has been defined by numerous biochemical studies and its interaction with DNA has been visualized by X-ray crystallography, which supported that DNA binding is critical for the biological activity of p53. More than 90% of p53 mutations found in human tumors reside in the DNA binding domain. This implies the importance of the transcription factor function of p53 in growth control. Not only the DNA binding domain, but also the C-terminal part

has been implicated in DNA binding. Besides the DNA binding domain, p53 consists of several other functional domains: transactivation domain, a proline rich domain, a nuclear localization signalling domain and a tetramerization domain. The two tandem transactivation domains are located at the N-terminal part of p53, which are responsible for activation or repression of target genes. Thus p53 acts as a transcription factor mostly by sequence specific binding to the DNA and and thereby activating or repressing target genes to control cellular outcome [106].

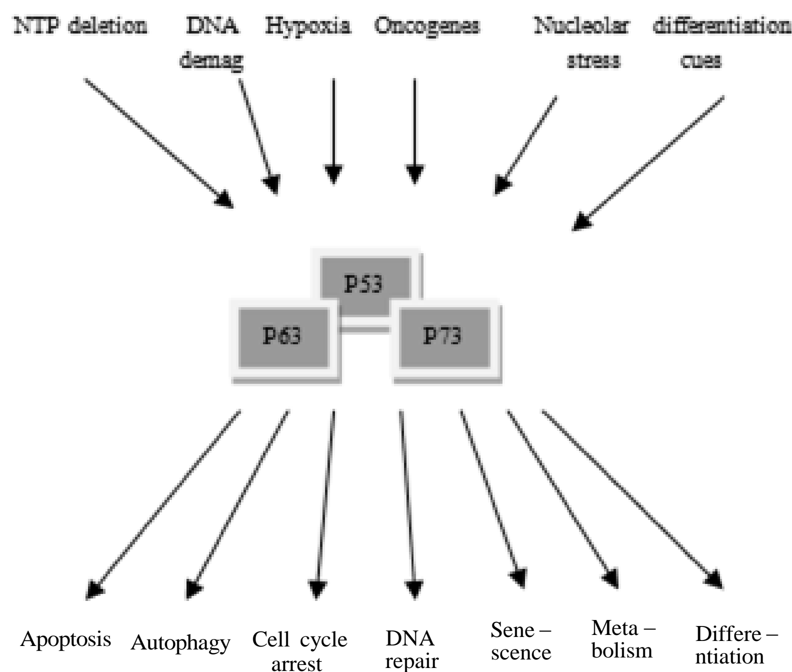
Of the target gene promoter, among others. The transcriptional output of p53 is responsible for determining which cellular process in response to different genotoxic insults. P53 lies at the centre of the complex signalling network as shown in the following figure [304].




*Transcriptional regulation by p53*

## P53 family

More than 15 years after discovery of p53, two p53 related genes were identified: p63 and p73. Interestingly, p63 and p73 are structurally similar and functionally related to p53, and hence the entire p53 family may be regarded as a unique signalling network controlling cell proliferation, differentiation and death as shown in the following figure [305].




*p53 family as a network*

## Regulation of p53

The p53 protein can be regulated at different levels:

1. Posttranslational modifications such as phosphorylation, sumoylation or acetylation of protein.
2. Increasing the protein concentration: one of the key regulators of p53 is Mdm2 which targets p53 for breakdown by the proteosome.
3. Cellular localization: important and nuclear export is closely regulated because the functions of p53 depend on its nuclear localization.

Efficient transfer to the cytoplasm depends on Mdm2 forming a comlex with p53, which is why ubiquitin ligase activity of Mdm2 occurs in the C-terminus domain, and it has been shown that mutations in lysine residues inhibit the nuclear export of p53 by Mdm2 [306].

## P53 at a glance

**p53 in Physiology** [307]

| | |
|---|---|
| Aging | P53 has been implicated in both inhibiting and promoting longevity |
| Development | Fraction of p53 null embryos display defects in neural tube closure |
| Differentiation | P53 can inhibit or promote differentiation depending on cell type |
| Fertility | P53 promotes LIF expression and embryo implantation |
| Skin pigmentation and tanning | P53 upregulates POMC and KITL in kerotinocytes and induce pigmentation |
| Stem cell function | P53 represses self renewal of various stem cell types and inhibit IPS cell generation |
| Tissue homeostasis | P53 preserves tissue integrity in response to DNA demage and limits liver fibrosis |
| chemotherapy and radiation therapy | P53 dependent apoptosis promotes deleterious side effects of these treatments |
| Diabetes | P53 promotes senescence response in adipose tissue, which lead to insuline resistance |
| Ischemic stroke | Acute injury induces p53 mediated apoptosis in neurons |
| Neurodegenerative disorder | Alzhiemer’s disease, parkinson’s disease, huntington’s disease. P53 induces neuronal apoptosis, causing pathologies associated in these diseases |
| Myocardial infarction | P53 promotes apoptosis after acute injury |
| Ribosome biogenesis disorder | Treacher Collins syndrome, diamond blackfan anemia, 5q syndrome, ribosome dysfunction induces p53 dependent programmes of apoptosis and cell cycle arrest |

## p53 mutations

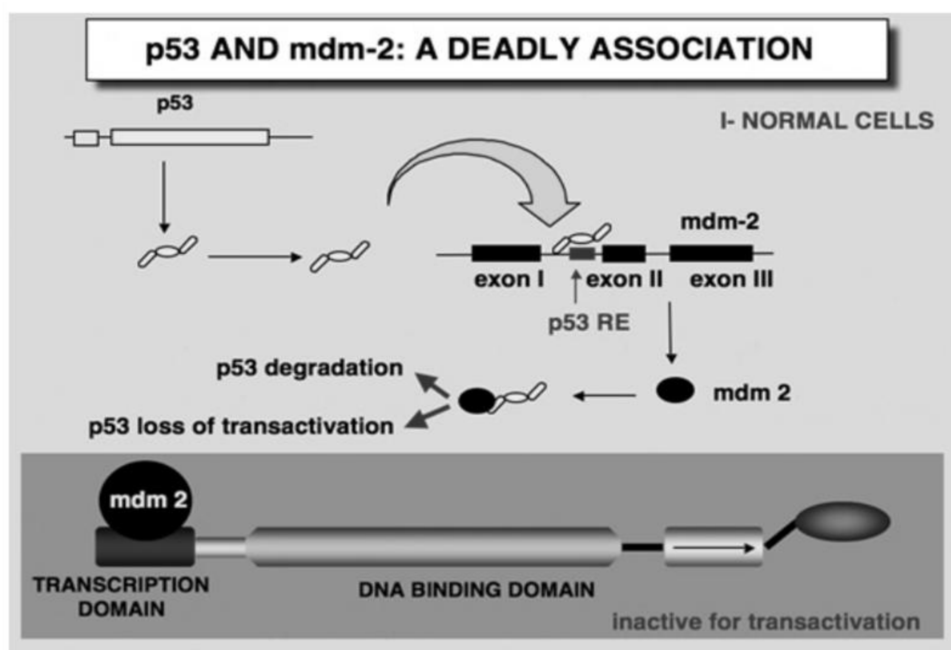
The p53 pathway is ubiquitously abnormal in human cancers, either through mutation of the p53 gene or via modification of the p53 function by interaction with oncogenic cellular or viral patients. Somatic p53 gene mutations, found in about 25% of breast cancers, are associated with poor prognosis [308].

## p53-activating signals

Under normal conditions, p53 is latent. It does not interfere with cell cycle progression and cell survival. p53 is not essential for the normal performance of cells within the body. A variety of conditions can lead to rapid induction of p53 activity, which represents 5 types of stress, that are likely to favor the emergence of cancer-bound cells. Such conditions include direct DNA damage as well as damage to components involved in the proper handling and segregation of the cellular genetic material (e.g. The mitotic spindle, ribonucleotide depletion, hypoxia, heat shock, and exposure to nitric oxide (NO). Accumulation of genomic aberrations is a key carcinogenic mechanism; the rapid induction of p53 activity in response to genomic damage thus serves to ensure that cells carrying such damage are effectively taken care of. Furthermore, p53 may also contribute directly or indirectly, to particular DNA repair processes. In addition, p53 activity is triggered by a variety of oncogenic proteins, including Myc, Ras, adenovirus E1A, and β-catenin. P53 activation may also involve a change in subcellular localization; whereas latent p53 may often be cytoplasmic, at least during part of the cell cycle, exposure to stress results in its accumulation in the nucleus, where it is expected to exert its biochemical activities.

## The p53 - mdm2 Loop

A key player in the regulation of p53 is the Mdm2 protein. Mdm2 is the product of an oncogene, whose excess activity facilitates several types of human cancer. Mdm2 exhibits a unique relationship with p53. On the other hand, the Mdm2 protein binds to p53 and inactivates it. The binding occurs right within the p53 transactivation domain, interfering with recruitment of basal transcription machinery components.




*p53 and mdm-2 association*

Mdm2 can actively repress transcription when tethered to p53. Importantly, Mdm2 binding can also lead to complete elimination of p53 through proteolytic degradation. On the other hand, p53 binds specifically to the mdm2 gene and stimulates its transcription. This duality defines a negative feed back loop, which probably serves to keep p53 in tight check and to terminate the p53 signal once the triggering stress has been effectively dealt with. In the absence of functional Mdm2 protein,

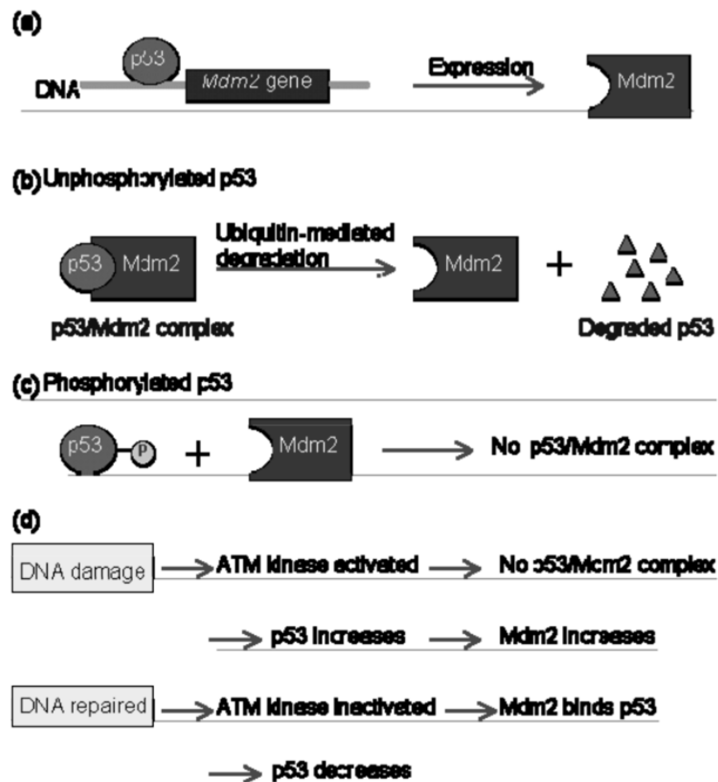
p53 becomes strongly deregulated. In certain human cancers; excessive Mdm2 expression, achieved through mdm2 gene amplification or other mechanisms, can lead to constitutive inhibition of p53 and thereby promote cancer without a need to alter the p53 gene itself. Excess Mdm2 can also promote cancer independently of p53.

**Covalent modifications of p53**

The rapid stabilization and activation of the p53 protein upon stress also involves stress-induced covalent modifications of p53. P53 becomes phosphorylated on multiple sites in vivo in response to various types of stress, and many stree activated kinases can phosphorylate p53 in vitro.

A potential outcome of such phosphorylation might be the stabilization of p53 through inhibition of p53 ubiquitination and degradation. In the case of p53, several candidate sites within its Mdm2-binding domain have been identified which are modified in response to DNA damage and whose phosphorylation reduces the affinity of p53 for Mdm2.

(a) Expression of Mdm2 is activated by p53.
(b) Binding of p53 by Mdm2 can trigger the degradation of p53 via the ubiquitin system.
(c) Phosphorylation of p53 at Ser15, Thr18 or Ser20 will disrupt its binding with Mdm2. In normal cells, these three residues are not phosphorylated, and p53 is maintained at low level by Mdm2.




*Covalent Modifications of p53*

(d) DNA damage may activate protein kinase (such as ATM, DNA-PK, or CHK2) to phosphorylate p53 at one of these three residues, thereby increasing p53 level. Since Mdm2 expression is activated by p53, the increase of p53 also increases Mdm2, but they have no effect while p53 is phosphorylated. After the DNA damage is repaired, the ATM kinase is no longer active. p53 will be quickly dephosphorylated and destroyed by the accumulated Mdm2.

For example, DNA- damaging agents activate phosphorylation at serine ( Ser) 15, likely by a family of protein kinases including ATM and ATR, and Ser20 by the Chk2 kinase. (Chk2 is a protein kinase that phosphorylates serine 20. Defects in the Chk2 gene cause a predisposition to cancer.) These phosphorylation events are believed to contribute

to p53 stabilization by preventing the binding of Mdm2 and rendering p53 more resistant to Mdm2.

In addition to potentially regulating Mdm2 binding, phosphorylation was also shown to modulate the transcriptional activity of p53. For example,phosphorylation at Ser15 stimulates p53 interaction with its transcriptional co-activators p300 and CBP, and a mutation that eliminates this phosphorylation leads to p53 transcriptional defects.

Another potential mechanism that may play a critical role in p53 activation is acetylation. Multiple lysine (K) residues in p53 are acetylated by p300 and its family member CBP or by P/CAF.

## p53 and Oncogenic stress

Oncogenic stress, such as the deregulated expression of oncoproteins like Myc, Ras, adenovirus E1A, and β-catenin activates p53 response. Excess activity of these oncoproteins leads to massive induction of ARF protein that arises through translation of an alternative reading frame derived from the INK4A tumor suppressor gene. This induction is primarily because of enhanced transcription, some of which is mediated through the E2F transcription factor.

The induced ARF protein then binds to Mdm2, and inhibits the p53 biquitin ligase activity of Mdm2. Because the ubiquitin ligase activity of Mdm2 appears to be essential for the degradation of p53, it is possible that by directly binding and inactivating Mdm2, p14ARF bypasses the need for phosphorylation in p53 activation. Loss of the p14ARF gene causes Mdm2 to increase in concentration leading to a decrease in the levels of p53.

The inhibitory effects of p53 are not triggered when Myc or Ras proteins are recruited as part of a properly orchestrated growth response, initiated by the binding of a growth factor to its receptor, or else such cells would not be able to execute a mitogenic response. When a cell is exposed to a growth factor, one arm of the response drives the neutralization of p53 concurrently with the activation of Myc, Ras, and E2F by the other arm.

## p53-dependent Apoptosis

p53 trancriptionally activates genes leading to cell cycle arrest or cell death(apoptosis). P21WAF1/CIP1 is a G1 cyclin/cyclin-dependant protein kinase inhibitor, which blocks the activity of a G1cyclin- dependent protein kinase. This results in cell cycle arrest. P53-binding sites in the regulatory region of the gene directly activate transcription of the Bax gene, which is located in mitochondria. When over induced, they induce apoptosis.

There are several potential mediators of p53-induced apoptosis. The bax protein is an apoptosis inducing member of the Bcl-2 protein family. p53-binding sites in the regulatory region of the gene directly activate transcription of the bax gene. Bax is located in mitochondria. When over expressed, Bax induces apoptosis.

### Microbial or Viral inactivation of P53 protein

Infection with viruses or even microbes introduces foreign DNA into the cells. P53 along with other proteins is responsible for the cell's response to the presence of foreign DNA, which include shutting down

cell division and cell death. An example is the Simian Virus 40 (SV40). Upon infection with SV40, viral proteins are produced with in the cell cytoplasm. One of the proteins produced is termed the large T antigen. This, protein function is the binding and inactivation of the P53 protein. Similar functions provided from Hepatitis Virus and Human Papillomavirus by production of similar proteins. Clinical trials have suggested that cancer growth can be arrested or reversed by gene treatment with the propriate vectors which carry a single growth inhibitory or pro-apoptotic gene or a gene that can recruit immune responses against tumor. Many of these gene transfer vectors are modified viruses that retain the capability of the viruses for efficient gene delivery but are safer than the native viruses due to modifications that eliminate or alter one or more essential viral functions. The field of viral-based gene transfer vectors for the treatment of cancer has now entered the final stage of clinical testing prior to possible product approvals. Three viral vectors are currently undergoing Phases, clinical testing for cancer treatment. All three of these vectors are based on adenovirus, a common human virus that in its native state can cause cold or flue-like symptoms. In two of these vectors, genes essential for viral replication have been replaced with the wild-type p53 tumor suppressor gene, a gene that is deleted or mutated in over 50% of human cancers and which, when transferred into tumor cells, can induce cell death. The three vectors represent two of the approaches now being taken to develop viral-based gene transfer vectors for cancer treatment.Additional approaches include the tranfer of the genes capable of converting non-toxic prodrugs into toxic forms, using anti-angiogenic gene transfer to block the transfer to block the formation of tumor blood

vessels, inhibiting the activity of oncogenes through blocks to transcription or translation, stimulating the body's own immune system with immunomodulatory genes, and "cancer vaccination" with genes for tumor antigens.

Future research thoughts or plans could expand to similar agents such as Microbes (Gram + and -). May the results help patients and health community. Depends on researchers to open this experimental page for humanity.

**Some psychological steps for the patient and her family**

After diagnosis of breast cancer, no matter how begins the treatment from the expertise doctor. It is important to be informed the rest of the family. Things are difficult for the new members. So it is a first step to be surrounded by specialists and understanding people. In nowadays, hospital care units have great programs for kids, where they can meet the doctors, see and ask lots of questions. Most importantly, they can meet other kids in the same boat as them. So every new member has the feeling of not being alone in this. On the other hand, seeing the patients their kids be entirely self-centered by these programs, feel that all is normal. Any help offering by experienced scientists could help a lot female patients.

# DISCUSSION

The TP53 gene is commonly altered by mutation in many types of human cancers. The p53 protein is an inducible transcription factor that plays multiple, anti-proliferative roles in response to exposure to many forms of stress, including in particular various classes of DNA-damaging agents. Thus, p53 function is essential for the genetic homeostasis of cells exposed to mutagens. In a physiological context, the status of p53 controls the sensitivity of cells to environmental mutagens. In a pathological context, the status of p53 is considered as a key factor in the response to cancer cells to cytotoxic therapies. Thus, control of p53 functions is a very promising target for cancer management.

Given the central importance of TP53 in carcinogenesis, there have already been many attempts at restoring or modulating p53 protein functions through genetic or pharmacological approaches. First, gene therapy experiments using either retroviral or adenoviral vectos have given a proof of principle for the restoration of wild-type TP53 function in cancer cells containing mutant alleles. However, the therapeutic efficiency of such approaches is currently limited by difficulties in targeting cancer cells, in obtaining high expression levels of the transgene, and in maximizing so-called by-stander effects. Alternative approaches to "replacement" gene therapies are also being developed. For example, the ONYX-015 viral vector is a defective adenovirus that can selectively kill cancer cells with deficient TP53 functions. This vector is currently under clinical evaluation.

The rapid accumulation of knowledge on p53 protein functions has led to the development of a number of pharmacological approaches. One

of the most promising makes use of small peptides that target specific regulatory domains of the protein to either activate wild-type p53 or restore function of mutant p53. Other methods address specific bio-chemical properties of p53, such as sensitivity to redox signals.

It should be kept in mind that the TP53 gene and its product can be used as biomarker for cancer detection, diagnosis and prognosis. This can be achieved by the analysis of gene or protein status in cancer or pre-cancer lesions, as well as by detection of gene fragments or antibodies in plasma or serum. All these aspects are currently the topic of intense research efforts. However, their outcome on the clinical management of cancer will not be available for several years.

The TP53 gene was discovered twenty years ago, but it took about ten years before a clear idea of its function eventually emerged in the early 1990s. Experimental studies then demonstrated that this gene encodes a tu-mor suppressor, and molecular pathological approaches showed that this suppressor was inactive in a majority of human cancers. Since then, the TP53 gene and its product, the p53 protein, have occupied the center stage of the molecular biology of cancer, and have raised great expectations for applications leading to better cancer management or therapy.

TP53 is special among cancer genes in at least three respects. First, most of its alterations in cancers are missense mutations. This is uncommon for suppressor genes, which are classically inactivated by deletions or non-sence mutations. Second, it is altered at a significant frequency (between 20 and 80%) in almost every human cancer, irrespect-ive of the organ site or the historical type. This observation stresses the central role of p53 as one of the basic elements of the cellular growth control machinery. Third, the protein itself is apparently essential for

many aspects of normal life. This also contrasts with many tumor suppressors, which encode “vital” proteins. Strikingly, mice deficient in TP53 by homologous recombination show essentially normal development and behavior. However, when they reach 20 or 30 weeks of age, most of them die from multiple, early cancers. Thus, TP53 may be considered as, in the words of M. Oren, the “ultimate tumor suppressor gene”, the function of which is essentially to protect cells against the occurrence and development of cancer.

This very special position of p53 in the control of cell proliferation is due to two biological characteristics. First, p53 is an inducible protein at the post-transcriptional level. It is almost absent, or “latent”, in most normal cells and tissues, but becomes stabilized and activated in response to many forms of cellular stress, in particular stress inducing the formation of DNA-damage. Moreover, p53 is capable of regulating many overlapping pathways. P53 is a transcription factor with more than 30 known target genes in pathways such as cell cycle control, apoptosis, DNA repair, differentiation and senescence. The protein also acts through direct, complex formation with other cellular components, further increasing the range of responses elicited by p53 activation. Overall, p53 appears to sit at the center of a network of signals that connect stress response (in particular to DNA damage) with growth regulation. This special function has earned p53 the nickname of “guardian of the genome”. Lose of p53 function thus eliminates a protection system by which cells normally regulate their capacity to proliferate in stressful conditions, and increases the likelihood that such cells may acquire other genetic changes during cancer progression.

Therefore, p53 represents an interesting target for genetic or pharmacological intervention in cancer treatment. The reality is that numerous approaches have been applied to generate p53 – based anti-cancer therapies. Such approaches include retrovirus – or adenovirus – mediated gene therapy to restore p53 function, killing of p53-dificient cells with modified adenoviruses and pharmacological modulation of p53-protein functions (Bouchet et al., 2006). Studies have also taken place to identify drugs and mechanisms that activate p63 and p73, since these proteins are not mutated in cancers and as such are potential candidates for replacing p53 in p53-deficient cells (Alsafadi et al. , 2009).

Recent genetic studies in mouse models have shown that reactivation of the p53 pathway in tumours with reduced or no p53 activity promotes tumour clearance, renewing interest in and providing further strong evidence for designing anti-cancer drugs that restore p53 function (Ventura et al., 2007; Xue et al ., 2007; Vazquez et al., 2008; Shangary and Wang, 2009). Among the different strategies for restoring p53 function, targeting the MDM2-p53 interaction by small molecules has proven to be popular. MDM2 has been shown to inhibit p53 by regulating its subcellular location, its stability, and its transactivation function (Vaz-quez et al, 2008; Shangary and Wang, 2009). Historically, disruption of protein-protein interactions has been a daunting task due to the typically large binding region of the protein partners. However, the MDM2-p53 interaction has been mapped to a small, well-defined interface, opening the door to the possibility of interference by small molecule inhibitors. Different approaches have been used to identify and design small-molecule inhibitors of the MDM2-p53 interaction. These include: 3D database screening of large chemical libraries, experimental screening of

chemical libraries, and structure-based *de novo* design (Shangary and Wang, 2009). These approaches have generated a number of potential therapeutic agents (Nutlins, benzodiazepines, reactivation of p53 and induction of tumour cell apoptosis [RITA], spiro-oxindoles, and quinolinols) for interference with the MDM2-p53 interaction, however, the efficacies of such treatments in humans remain to be determined (Vazquez et al., 2008).

Interestingly, accumulating observations of p53 activity *in vivo* in experimental animals indicate that the same p53 tumour suppressive functions can be harmful under conditions of systemic genotoxic stress such as total body irradiation or injection of genotoxic anti-cancer drugs (Gudkov and Komarova, 2007). By comparing tumour models differing in stromal p53 status, Burdelya et al. (2006) showed that tumours with p53-deficient stroma were significantly more sensitive to experimental chemo and radiotherapy than tumours with wildtype p53 stroma. Thus, temporary and reversible suppression of p53 may be beneficial for prevention and treatment of acute conditions associated with severe genotoxic stress (Gudkov and Komarova, 2007).

Despite the intensive p53-based therapeutic research and numerous discoveries presented above, reality dictates that significant challenges and unresolved issues need to be addressed before p53-targeted therapies find clinical application. Examples of such obstacles include: premature aging, unwanted side effects in normal tissues, appearance of p53-resistant tumours, establishment of optimal dose and time of treatment, and standardization of administration in the clinical setting (Bouchet et al., 2006; Fuster et al., 2007).

# CONCLUSIONS

Since its discovery in 1979, p53 has become the focus of intensive cancer-based research in laboratories around the world. The p53 protein mediates critical cellular functions including the response to genotoxic stress, differentiation, senescence, and apoptosis, and has been shown to be mutated in a large proportion of human cancers. These observations led many to speculate that targeting the p53 pathway would result in the development of successful anticancer treatments. ln spite of this, 30 years later, p53 has yet to fulfill this promise. However, new insights into small molecule combination therapies, microRNA regulation, structuring of clinical trials, and potential involvement in stem cell regulation may help p53 reach its potential.

This protein was identified by coimmunoprecipitation of p53 with T-antigen in SV40-transformed cells (Chang et al., 1979; Kress et al ., 1979; Lane and Crawford, 1979; Linzer and Levine , 1979; Melero et al., 1979). lt was further noted that the p53 protein was overexpressed not only in SV40-transformed cells, but also in carcinoma cell lines (Linzer and Levine, 1979; for a detailed historical review of p53, see Hainaut and Wiman, 2009).

Early work implicated p53 as an oncogene (Eliyahu et al., 1984; Parada et al., 1984); however, it was subsequently determined that a mutant version of p53 was utilized in these studies (Hinds et al., 1989). Successive studies using wildtype p53 supported the conclusion that p53 acts instead as a tumour suppressor (Baker et al., 1989; Finlay et al., 1989; Hinds et al., 1989). Following these discoveries, p53 was dubbed

"guardian of the genome" (Lane, 1992) and Science's "Molecule of the Year 1993".

Since then, p53 has been shown to play a role in response to genotoxic stress, differentiation, senescence, and apoptosis, and is one of the most commonly altered proteins in human cancer (Chari et al., 2009). Consequently, many laboratories have dedicated considerable time and resources with the intention of developing therapies aimed at restoring wildtype p53 activity in cells with mutated p53 or by inhibiting a key negative regulator of p53, such as murine double minute 2 (MDM2; Vazquez et al., 2008). Though a wealth of information has been accumulated in this area, p53-based research has not yet had a wide impact on cancer management and therapy (Hainaut and Wiman, 2009) and the question remains as to whether the promise of p53-based anti-cancer treatments will turn out to be an empty one.

Recent discoveries complement the last 30 years of p53-based research. lnsights into small molecule combination treatments, microRNA regulation of p53, potential involvement of p53 in stem cell regulation, and coordinated restructuring of clinical trials with compatible comprehensive databases will likely accelerate the development of p53- based anti-cancer therapies. Due to the recognized heterogeneous nature of cancer, combination therapies are increasingly being proposed as more effective strategies. ln the case of p53-based treatment approaches, promising results have been seen by combining small molecule inhibitors with various other anti-cancer agents. For example, Graat et al. (2007) demonstrated enhanced tumour cell kill with a combination of the MDM2 antagonist Nutlin and adenovirus-mediated p53 gene therapy.

The authors also tested Nutlin in combination with oncolytic adenovirus-infected cancer cells, revealing accelerated viral progeny burst and a 10-1000-fold augmented eradication of p53 wildtype cancer cells. Cheok et al. (2007) also highlighted the potential success of Nutlins as therapeutic team members, but in a different manner. ln this case, statistical measurement of the combination of cyclin-dependent inhibitors and Nutlin-3a demonstrated an additive effect on the reduction of cell viability and apoptotic induction in breast adenocarcinoma. Clinical trials will be necessary to evaluate combinatorial effects for future therapeutic usage.

MicroRNAs (miRNAs) are small (18-25 nt), noncoding RNAs that function by controlling protein expression of other genes (Metias et al., 2009). miRNAs have recently stolen some thunder from small interfering RNAs (siRNAs; Campbell and Choy, 2005) as potential diagnostic and therapeutic tools. Specific miRNAs have been identified as inappropriately expressed in a variety of different tumours, leading to the speculation of linkage to cancer (Chari et. al. , 2009). Several members of the miRNA-34 family have been shown to be downstream mediators of p53-induced apoptosis, cell cycle arrest, and senescence (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al ., 2007; Raver-Shapira et al., 2007; Chari et al., 2009). Recently, the presence of a positive feedback loop was demonstrated in which p53 upregulated miRNA-34a, which then repressed the NAD-dependent deacetylase silent information regulator 1 (SIRT 1), resulting in increased levels of p53 and amplification of the apoptotic signal (Yamakuchi et al., 2008). Such observations provide the impetus to move forward with manipulation of microRNAs for the development of p53-based anti-cancer treatments.

Published in late 2009, results from five independent laboratories identified p53 as a critical checkpoint during the multifactor reprogramming process whereby induced pluripotent stem cells are derived from differentiated adult cells (Hong et al. , 2009; Kawamura et al., 2009; Li et al ., 2009; Marion et al. , 2009; Utikal et al ., 2009). Absence of functional p53 enhanced the yield of induced pluripotent stem cells in each case, implicating p53 as a major gatekeeper of self-renewal (Aparicio and Eaves, 2009). This both complicates and enhances the role p53 may play in anti-cancer therapies. For example, if cancer is shown to arise directly through reprogramming-like processes, then further studies into reprogramming and the subsequent role of p53 may eventually point towards new, effective treatment for cancers (Krizhanovsky and Lowe, 2009).

Hainaut and Wiman (2009) stress the need for large, structured clinical trials in which patients with defined p53 status are specifically recruited, randomly assigned to predetermined treatment regimens, and followed up for long-term therapeutic and clinical endpoints. They advocate that detailed understanding of the clinical significance of p53 status will come from pooled analyses and metaanalyses assessing the strength of evidence across large data sets and different study contexts. This restructuring and amalgamation of clinical trials will expedite the process for determining the prognostic and predictive value of p53 mutations, as well as contribute to the eventual pharmacological control of P53 in cancer therapy, improving both survival and quality of life for cancer patients.

The truth is p53 gene is mutated in about 50% of human cancers and the non-mutaed allele is generally lost, the frequency and the type of mutation may vary from one tumor type to another and the mutations tend to cluster in central DNA-binding domain. Important environmental role is the value of mutational events which are related to carcinogens that affect p53 such as ultraviolet radiations and cigarette smoke. So mutations are often dominant negatives, since p53 acts as a tetramer. Different effects from p53's alterations depending on the location of alteration. For example, a mutation in the promoter region can result in the decrease or absence of p53 in the cell. Mutations occur in the protein-coding region of the gene can have impact in the expression of the gene, It's important to say in some sarcomas amplify another gene, called mdm-2 which produces a protein that binds to p53 and inactivates it, much the way the DNA tumor viruses do. Use of tumor suppresses genes as anticancer therapeutics has been investigated rigorously in both experimental and clinical researches. Transfer of various tumor suppressor genes directly to cancer cells has been demonstrated to suppressor tumor growth via induction of apoptosis and cell cycle arrest and, in some cases, with evidence for by stander effect. Various studies have shown that combination of tumor suppressor gene therapy with conventional anticancer therapy can yield synergistic therapeutic benefits. Clinical trials with p53 gene, have demonstrated that the treatment is well tolerated, and; favorable clinical responses, have been observed in a subset of patients with advanced diseases or with cancers resistant to conventional therapy. Yet, current gene replacement approaches in cancer gene therapy must be improved if they are to have a boarders

clinical impact. Efficient systemic gene delivery systems will be required ultimately for the treatment of metastatic disease.

Inhibiting the p53-MDM2 interaction is a promising approach for activating p53, because this association is well characterized at the structural and biological levels. MDM2 inhibits p53 transcriptional.

*In vitro,* the interaction of TP53 with cancer associated/HPV viral proteins lead to ubiquitination and degradation of TP53 giving a possible model for cell growth regulation. This complex formation requires an additional factor, E6-AP, which stably associates with TP53 in the presence of E6. C-terminus interacts with TAF1, when TAF1 is part of TFIID complex.

Several gene therapeutic strategies have been employed in the attempt to restore p53 function to cancerous cells. These approaches include introduction of wild type p53 into the cells with mutant p53; the use of small molecules to stabilize mutant p53 in wild type, active conformation; and the introduction of agents to prevent degradation of p53 by proteins that normally targets it. In addition, because mutant p53 has oncogenic gain of function activity, several approaches have been investigated to selectively target and kill cells harboring mutant p53 and the introduction of gene that, in the absence of functional p53, produces toxic product. Many obstacles remain to optimize these strategies for use in humans, but, despite these, restoration of p53 function is a promising anti-cancer therapeutic approach. activity, favors its nuclear export and stimulates its degradation, so inhibiting the p53-MDM2 interaction with synthetic molecules should lead to p53-mediated cell-cycle arrest or apoptosis in p53-positive stressed cells.

Two recent strategies have been proposed to exploit p53's unique death-regulating activity in opposite directions and improve cancer treatment. One approach seeks to inhibit p53 in normal cells there by diminishing therapy-related, p53-dependent toxicity. The other utilizes a peptide derived from the C-terminus of p53 to activate wild type or mutant p53 proteins, triggering apoptosis with selectivity for transformed cells. These novel approaches hold promise for targeting p53 in cancer therapy and may shed light on mechanisms underlying the role of p53 in cancer cell survival.

Change in expression and mutations of gene p53 cause variations of cellular p53 protein concentration. Higher cellular protein p53 levels are associated with increased protein transfer to the extracellular liquid and to blood. It has been observed that increased blood serum protein p53 concentrations may have a prognostic value in early diagnosis of lung cancer. The results of a number of studies confirm that accumulation of a mutated form of protein p53, and presumably also large quantities of wild forms of that protein in the cells, may be a factor that triggers the production of anti-p53 antibodies. Statistical analysis showed that anti-p53 antibodies could be regarded as a specific biomarker of cancer process. The prevalence of anti-p53 antibodies correlated with the degree of cancer malignancy. The increased incidence of anti-p53 antibodies was also associated with the higher frequency of mutations in gene p53.

It's true that p53 holds tumor suppressor activities. P53 contains 393 aminoacids and a single amino acid substitution lead to loss of function of the gene. Mutations at amino acids 175, 248, and 273 can lead to loss of function and changes at 273 (13%) are the most common. All these act

as recessive mutations. Dominant gain-of-function mutations have also found that lead to uncontrolled cell division. Because these mutations can be expressed in heterozygous conditions, they are often associated with cancers. This genetic function of this gene is to prevent cell division of cells with damaged DNA. Damaged DNA could contain genetic changes that promote uncontrolled cell growth. Therefore, preventing cell division until damaged DNA is repaired is one mechanism of preventing the onset of cancer. About 50% of human cancers can be associated with a p53 mutation including cancers of the bladder, breast, cervix, colon, lung, liver, prostate, and skin. P53 related cancers are also more aggressive and have a higher degree of fatalities.

Several gene therapeutic strategies have been employed in the attempt to restore p53 function to cancerous cells. These approaches include introduction of wild-type p53 into p53 mutant cells; the use of small molecules to stabilize mutant p53 in a wild-type, active conformation; and the introduction of agents to prevent degradation of p53 by proteins that normally target it. In addition, because mutant p53 has gain of function activity, several approaches have been investigated to selectively target and kill cells harboring mutant p53. These include the introduction of mutant viruses that cause cell death only in cells with mutant p53 and the introduction of a gene that, in the absence of functional p53, produces a toxic product. Many obstacles remain to optimize these strategies for use in humans, but, despite these, restoration of p53 function is a promising anti-cancer therapeutic approach.

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## RESUME

Dr. Georgios J. Foutsitzis was born in Thessaloniki. He graduated from the 20th General High School of Thessaloniki (1992). He completed his studies at a recognized Private Technical Vocational School with honors in the specialty: "Dental Technician" (1996-1997). After tickets Panhellenic Examinations, introduced in ATEI (Higher Technological Institution) - Department of Medical Laboratory, completed his studies (2001) with honors. After his graduation of technological studies in Medical Laboratory Science continued his postgraduate studies at the Higher School Robert De Sorbon-France, on the subject of microbiology and became a postgraduate (DEA) in 2005. In 2012 he became BSc graduate in Natural Medicine from the Center for Natural Studies, Institute for Natural Medicine. He was a member of national and international organizations abroad. He attended seminars, wrote articles in general health magazines Also he worked as an instructor hourly paid professor at accredited private educational institute training, named: P.I.V.E-PASTER ( Thessaloniki-Greece). He is a certified instructor in E.KE.PI.S. He also worked as a medical laboratory technologist (seasonal) at Center Veterinary Institutions of Thessaloniki Laboratory of Transmissible Spongiform Encephalopathy Institute of Infectious and Parasitic Diseases, Thessaloniki. He is currently a Professor of Titulaire in Microbiology at Higher School Robert De Sorbon-France since 2010.

## Appendix (A)

## Equipment and materials

The following equipment was used throughout the research:

1. Water bath, drying oven capable of maintaining 65c or less, humidity chamber and hot plate.
2. Automatic micropipettes of different capacities with tips, pastures, pipettes and eppendrof tubes.
3. Timer with alarm, gloves, cotton swabs, and tissue papers.
4. Binocular light microscopy.
5. Positively charged slides, cover slides, and slide holders.
6. Staining jars of different sizes and callipered cylinders.
7. Pap pen, accessories; calibrated test tube, plastic Pasteur pipette.
8. Buffer solution: phosphate buffer saline.
9. Xylene, hematoxyline stain, distilled water, ethanol of different concentrations, and DPX.

## Appendix (B)

**Herceptest manufacturing staining kit**

Immunotech. Marseille, France. Cat. No. 2765.

The contents of Herceptest manufacturing staining kit are:

1. Peroxidase Block, lx 8ml blocking agent, 3% hydrogen peroxide in water.
2. Biotinylated Link, , biotin labeled affinity isolated goat anti-rabbit and goat anti-mouse immunoglobulins in phosphate buffered saline (PBS).
3. Streptavidin-Peroxidase, lx5ml, Streptavidin conjugated to horse-radish peroxidase in PBS containing stabilizing protein and anti-microbial agents.
4. DAB substrate buffer, lx8 ml, 3,3'-diaminobenzidine.
5. DAB chromogen, lx8 ml, 3,3'-diaminobenzidine in chromogen solution, the chromogen solution should be prepared immediately prior to use and discard after use.

## Appendix (C)

### Hematoxyline and Eosin stain[161]

This routine staining method used in all types of histology laboratories. A good H & E stains will demonstrate a number of a structure including nuclei, cytoplasm, RBC and other connective tissues. Hematoxylin must be used to stain the nuclei and then accurately drained so as to leave no excess stain in the background.

A section properly stained with eosin will demonstrate structures in various shades of red and pink. Sections need to be over stained with eosin, well drained in running tap water, slowly dehydrated through gradual cleave in xylol and mounted in a resinous medium.

**Staining methods:**

Solutions:

1. Hematoxyline solution
2. Eosin 10 gm
3. Distilled water 1000 ml
4. 0.5 ml acetic acid may be added to the above solutions to sharpen the staining moulds, but these are harmless and can be filtered off, or thymol may be added to the solution.

**Method:**

1. Sections were de waxed in xylol then treated with graded alcohol 100%, 95%, 70% and placed in water.

2. Remove fixation pigment if necessary.
3. Haematoxylin for 15 minutes.
4. Wash well in running tap water till section go darker blue (5 minutes).
5. Back ground staining and excess stain were removed by differentiating in 1% acid alcohol (1% HCL in 70% alcohol) just dipping.
6. Wash well in tap water until section regains blue color (5 minutes).
7. 1% eosin for 1 0-15 second then used.
8. Wash in running tap water for 5 minutes.
9. Dehydrate slowly through graded alcohols 70%, 95%, and 100% to xylol for at least 10 minutes.
10. Mounted in DPX.