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RELEVANCE OF CELL CYCLE REGULATORS ON CHEMOTHERAPY RESPONSE IN BREAST CANCER

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LIST OF ABBREVIATIONS

5-FU: 5-Fluorouracile

ADH: Atypical Ductal Hyperplasia

ATCC: American Type Culture Collection

ATM: Ataxia Telangiectasia Mutated

BC: Breast Cancer

BRCA1: Breast Cancer 1

BRCA2: Breast Cancer 2

BrdUrd: Bromodeoxyuridine

BSA: Bovine Serum Albumin

CDK: Cyclin-Dependent Kinase

CDKI: Cyclin-Dependent Kinases Inhibitor

cDNA: copy of DNA

CMF: Cyclophosphamide plus Methotrexate plus 5-Fluorouracil

CSPG2: Chondroitin Sulfate Proteoglycan 2

DAB: Diaminobenzidine

DAPI: 4',6-Diamidino-2-phenylindole dihydrochloride

DCIS: Ductal Carcinoma In Situ

DEPC: Diethylpyrocarbonate treated water

DFS: Disease-Free Survival

DHFR: Dihydrofolate Reductase

DMEM: Dulbecco’s Modified Eagle’s Medium

DNA: DesossiriboNucleic Acid

EDTA: EthyleneDiamine Tetra-acetic Acid

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ER: Estrogen Receptor

ErbB-2: Human Epidermal growth factor Receptor 2

ERD: Estrogen Receptor Downregulators

FBS: Fetal Bovine Serum

FITC: Fluorescein IsoThioCyanate

HCT-116: Colon cancer cell line

HELU: Hyperplastic Enlarged Lobular Units

HepG2: Human liver carcinoma cell line

HER2/neu: Human Epidermal Growth Factor Receptor 2

IBC: Invasive Breast Cancer

IL-2: Interleukin-2

LCIS: Lobular Carcinoma In Situ

LI: Labeling Index

MCF-7: estrogen receptor positive breast cancer cell line

MDA-MB-231: estrogen receptor negative breast cancer cell line

MDM2: Murine Double Minute 2

MI: Mitotic Index

mAbs: Monoclonal Antibodies

mRNA: messenger RNA

MTX: Methotrexate

PARP: Poly (ADP-ribose) Polymerase

PBS: Phosphate Buffered Saline

PDGF: Platelet-Derived Growth Factor

PR: Progesterone Receptor

pRb: Retinoblastoma protein

PTEN: Phosphatase and Tensin Homolog

RB1: Retinoblastoma gene

RNA: ribonucleic acid

RNAi: RNA interference

RPMI: Roswell Park Memorial Institute

RT: Room Temperature

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

SD: Standard Deviation

SDS-PAGE: Sodium Dodecyl Sulfate PolyAcrilammide Gel Electrophoresis

SERM: Selective Estrogen Receptors Modulators

TDLU: Terminal Duct Lobular Unit

TGF-β: Transforming Growth Factor-β

TLI: Thymidine Labeling Index

TNBC: Triple-Negative Breast Cancer

TNM: Tumor Nodes Metastasis

TP53: p53 gene

VEGF: Vascular Endothelial Growth Factor

WHO: World Health Organization

WT: Wild Type

µg/ml: micrograms per millilitre

µl: microlitre

µm: micrometer

µM: micromolar

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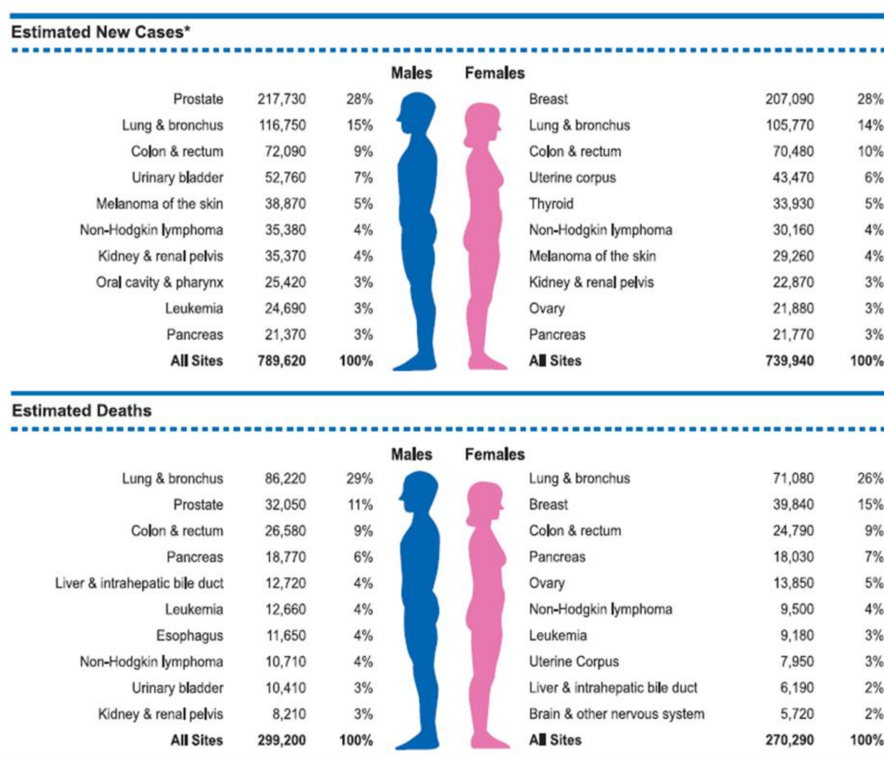
INTRODUCTION

1. BREAST CANCER

1.1. FEATURES

Breast cancer (BC) is the most frequent carcinoma in females and the second most common cause of cancer-related mortality in women, after lung cancer. According to the American Cancer Society, it is expected that the 3 most commonly diagnosed types of cancer among women in 2010 will be cancers of the breast, lung and bronchus, and colorectum, accounting for 52% of estimated cancer cases in women. Breast cancer alone is expected to account for 28% (207,090) of all new cancer cases among women, more than 1 in 4 women (Figure 1) (Jemal et al., 2010).



Estimated New Cases*

Males			Females		
Prostate	217,730	28%	Breast	207,090	28%
Lung & bronchus	116,750	15%	Lung & bronchus	105,770	14%
Colon & rectum	72,090	9%	Colon & rectum	70,480	10%
Urinary bladder	52,760	7%	Uterine corpus	43,470	6%
Melanoma of the skin	38,870	5%	Thyroid	33,930	5%
Non-Hodgkin lymphoma	35,380	4%	Non-Hodgkin lymphoma	30,160	4%
Kidney & renal pelvis	35,370	4%	Melanoma of the skin	29,260	4%
Oral cavity & pharynx	25,420	3%	Kidney & renal pelvis	22,870	3%
Leukemia	24,690	3%	Ovary	21,880	3%
Pancreas	21,370	3%	Pancreas	21,770	3%
All Sites	**789,620**	**100%**	**All Sites**	**739,940**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	86,220	29%	Lung & bronchus	71,080	26%
Prostate	32,050	11%	Breast	39,840	15%
Colon & rectum	26,580	9%	Colon & rectum	24,790	9%
Pancreas	18,770	6%	Pancreas	18,030	7%
Liver & intrahepatic bile duct	12,720	4%	Ovary	13,850	5%
Leukemia	12,660	4%	Non-Hodgkin lymphoma	9,500	4%
Esophagus	11,650	4%	Leukemia	9,180	3%
Non-Hodgkin lymphoma	10,710	4%	Uterine Corpus	7,950	3%
Urinary bladder	10,410	3%	Liver & intrahepatic bile duct	6,190	2%
Kidney & renal pelvis	8,210	3%	Brain & other nervous system	5,720	2%
All Sites	**299,200**	**100%**	**All Sites**	**270,290**	**100%**

Figure 1: Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex, 2010. *Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder. Estimates are rounded to the nearest 10.

The decrease in breast cancer incidence, and in particular mortality, has been attributed to the combination of early detection with screening programmes, breast cancer prevention interventions, a decrease in the use of post-menopausal hormone-replacement therapy and the advent of more efficacious adjuvant systemic therapy (Jemal et al., 2007). Continued advances in our understanding of the molecular biology of breast cancer progression have aided in the discovery of novel pathway-specific targeted therapeutics, and the emergence of such effective therapeutics is currently driving the 'patient-tailored' treatment planning. Knowledge gained from studying the molecular pathology of human breast cancer progression, integration and implementation of this knowledge in the clinical setting, promises to further reduce breast cancer morbidity and mortality.

1.2. EPIDEMIOLOGY AND RISK FACTORS

The cause of breast cancer is still relatively unknown, although researchers have accumulated a considerable amount of information on the factors, which may increase one's risk of developing the disease. Today, the disease, like all other forms of cancer, is considered to be the end result of many factors, both environmental and hereditary. These factors include gender, age, family history of the disease especially if there are first degree relatives affected, age at menarche and at menopause, number of full term pregnancies, the use of both oral contraceptives and hormone therapies and mutation in specific genes. Also the industrialization accompanied by environmental pollutants may contribute to breast cancer risk.

1.2.1. Gender

Breast cancer is predominantly a disease that occurs in women even if, in rare circumstances, it can develop in men. In fact, approximately one out of every 150 breast cancer cases occurs in male. It seems likely that estrogens have some role in the development of breast cancer; in fact the difference in incidence may be because estradiol is able to exert a direct biological effect on breast cells in females, whereas in males testosterone needs to be converted to estradiol before exerting any biologic effect (Endogenous Hormones and Breast Cancer Collaborative Group, 2002).

1.2.2. Age

The incidence of breast cancer, in the reproductive years, increases rapidly with age then increases at a slower rate after about the age of 50, which is average age at menopause (Figure 2).

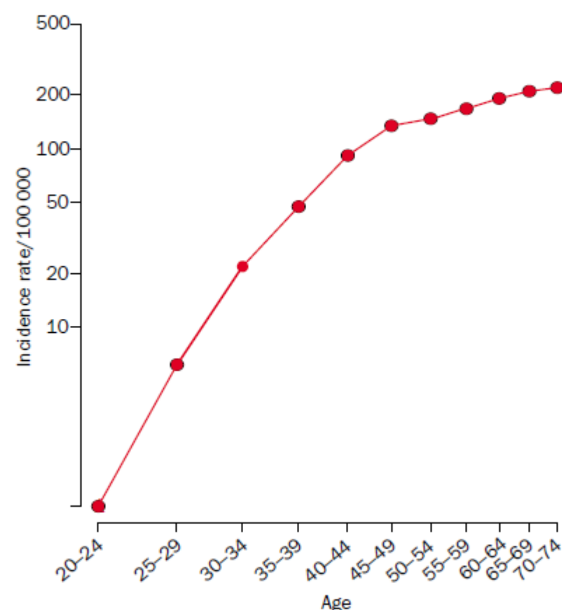


Figure 2. Age-incidence curve of breast cancer; log-log plot (from data for England and Wales 1983–87).

Younger women are not generally considered to be at risk for breast cancer: only 7% of all breast cancer cases occur in women under 40 years old, even if these women tend to have more aggressive breast cancers than older women, which may explain why often survival rates are lower among younger women. The incidence rates increased up to 10-fold by the age of 40 (Hulka and Moorman, 2001).

1.2.3. Effects of migration and geographical factors

Among populations around the world the incidence and the mortality of breast cancer vary greatly, also five-fold (Figure 3). In most of more developed countries the rates are high while in less developed countries and in Japan they are low, probably because of differences in reproductive factors. Among the migrants, the rates of those who migrate from countries with low incidence to countries with high incidence take on the higher rates of the new host country (Buell, 1973).

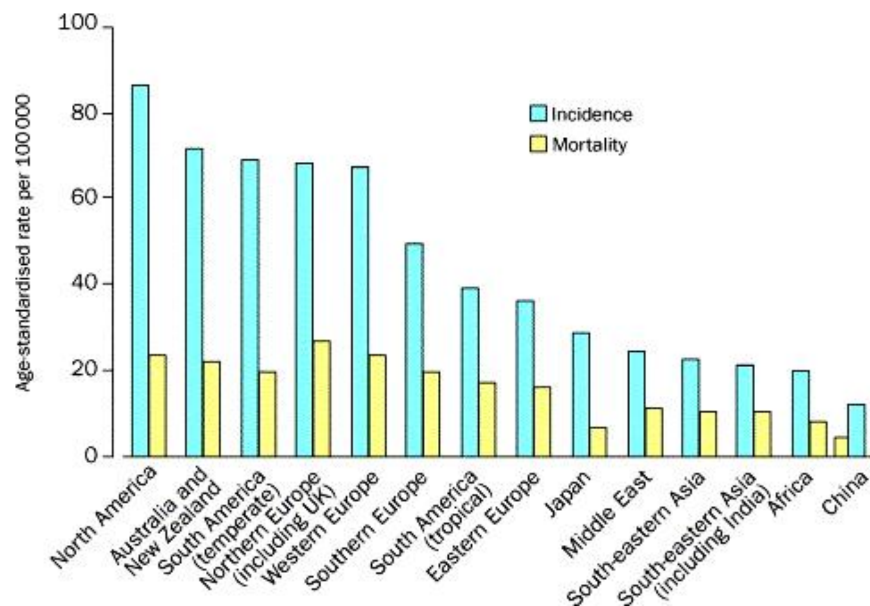


Figure 3. Worldwide variation in breast cancer rates (data from International Agency for Research on Cancer 1990).

1.2.4. Reproductive factors

Menarche and the menstrual cycle

At menarche a woman's body undergoes changes in order to accommodate the monthly cycling of sex steroid hormones and to prepare the body for childbearing. The age at menarche is inversely related to the risk for development of breast cancer (women who begin menstruating before age 13 years, have a two-fold increased risk of cancer). Some researchers have suggested that certain characteristics of the menstrual cycle, such as the time it takes for regular menstrual cycles, the length of menstrual cycles and the age at which these cycles begin, may increase the likelihood of developing breast cancer (Butler et al., 2000): for example, a short menstrual cycle of less than 28 days confers a greater risk of breast cancer than longer cycles of 28 days (Whelan et al., 1994). This is because women who have short menstrual cycles, would have more cycles throughout a year, and have more time spent in the luteal phase of the menstrual cycle and therefore an increase in time spent on cell proliferation. Moreover, if fertilization does not occur, there could also be effects on apoptosis that would occur more frequently determining a major cancer risk.

Pregnancy, breastfeeding and abort

Pregnancy and related factors, such as the age at first full term pregnancy, the number of full term births, interruptions in pregnancy (such as abortions) and breastfeeding have opposite influences on the risk of developing breast cancer.

Childbearing seems to have a dual effect on risk of breast cancer: it is increased in the period immediately after a birth, but this excess risk gradually diminishes and, in the longer term, the effect of a birth is to protect against the disease (Beral and Reeves, 1993). Compared with women who never had children (nulliparous women),

women who have had at least one full-term pregnancy have, on average, around a 25% reduction in breast-cancer risk. (Layde et al., 1989). The age at first full term pregnancy is related to breast cancer risk. The reason is that the pregnancy induces changes in the hormonal profile and these changes could result in alterations in the tissues that are under hormonal control. This renders the breast tissue less susceptible to carcinogenic stimuli and thus protects from cancer induction (Lambe et al., 1994). Furthermore, the protection rises with increasing of full-pregnancies number (Layde et al., 1989).

About the effect of breastfeeding, recent studies in less developed countries, in which the total duration of breastfeeding can be much longer, have reported substantial protective effects (women who had breastfed for a total of 25 months had a 33% lower risk of breast cancer than those who had never breastfed) (Layde et al., 1898).

Regarding the incomplete pregnancies, arising from spontaneous or induced abortions, the risk of breast cancer may be increased because the birth does not go to term, and would no longer have a protective effect. During pregnancy there is the interplay between prolactin, estrogen and progesterone which all act to promote breast growth and differentiation. If the pregnancy is interrupted, the growth and differentiation would also be incomplete and the undifferentiated structures of breast would render the breast susceptible to carcinogenesis (Russo and Russo, 1980).

Menopause

In the breast of postmenopausal women the cellular proliferation tends to be less than that of premenopausal women and this reduction of proliferation rate may be due to the decline of plasma estrogen concentrations during the menstrual cycle. The age at which menopause occurs influences breast cancer risk: women going through

menopause at a late age have a higher risk of breast cancer than those who cease menstruating earlier (Collaborative Group on Hormonal Factors in Breast Cancer, 1997).

A combination of early age at menarche and a late age at menopause would therefore prolong the time of the menstrual cycling of sex hormones, and thus would substantially increase a woman's risk of breast cancer development (Rosner et al., 1994).

1.2.5. Hormone therapies

Hormone therapies are used throughout a woman's reproductive life and decline of reproductive years, to combat a variety of ailments. They include oral contraceptives and hormones for menopausal women.

Oral contraceptives

The use of combined oral contraceptives increases the risk of breast cancer of around 25%, and the risk falls after cessation of use (10 or more years after use stops, no significant increase in risk is evident); risk does not vary significantly with duration of use, with the effect of combined oral contraceptives or with the type of estrogen or progestagen used. Women with several years of oral contraceptive use before age 25 and/or before the first full-term pregnancy, women who use oral contraceptives at age 45 or older, women with early menarche and women with a family history of breast cancer have an increased risk of breast cancer (Vessey et al., 1989).

Hormonal therapy for the menopause

Hormone replacement therapies (HRTs) are routinely prescribed for menopausal women to alleviate the symptoms of menopause and to slow the bone loss which is

associated with postmenopausal osteoporosis. Their use determines a higher risk of breast cancer than that of women who have never used these therapies and this risk increases with increasing duration of HRT use (Magnusson et al., 1999).

1.2.6. Breast tissue composition

Breast density reflects variations in breast tissue composition and can be strongly associated with breast cancer risk. Breast density is assessed by mammography and expressed as the percentage of the breast that is occupied by radiologically dense tissue. Researchers found that a major extension of mammographic density percent was associated with an increased risk of breast cancer (McCormack and dos Santos Silva, 2006). For many women, breast density will change with age or be related to factors such as relative body mass index, age at first childbirth, postmenopausal hormone replacement use and/or genetic make-up.

1.2.7. Alcohol and smoking

Observational studies have repeatedly shown that alcohol consumption is associated with only a moderate increase in the risk of breast cancer, although it depends on the amount and on the type of alcohol taken (Rohan and Bain, 1987). It has been suggested that alcohol may induce changes in the liver, which in turn may affect estrogen metabolism or may affect the level of steroid binding globulins, or for the increased secretion of pituitary stimulated hormones, such as prolactin and thyroid stimulating hormone, which would increase mitotic activity in target tissues, and hence lead to an increased susceptibility to malignancy. Another hypothesis is that the consumption of alcohol (approximately one to two alcoholic drinks per day)

increased estrogen levels in premenopausal and postmenopausal women (Ginsburg et al., 1959).

Carcinogens found in tobacco smoke pass through the alveolar membrane and into the blood stream, by means of which they may be transported to the breast via plasma lipoproteins. Due to the fact that they are lipophilic, tobacco-related carcinogens can be stored in breast adipose tissue and then metabolized and activated by human mammary epithelial cells (MacNicoll et al., 1980). As is well known, tobacco smoke contains potential human breast carcinogens (including PAHs, aromatic amines, and *N*-nitrosamines); in fact an higher prevalence of smoking-specific DNA adducts and p53 gene mutations were found in the breast tissue of smokers compared with that in nonsmokers, supporting the biological plausibility of a positive association between cigarette smoking and breast cancer risk, depending by dose and duration (Palmer and Rosenberg, 1993).

1.2.8. Diet

Foods may have several effects on the breast cancer risk. It has been demonstrated that aliments rich in omega-3 fatty acids, such as fish, suppress mammary tumour growth by blocking the tumour promoting properties of carcinogens or by inhibiting prostaglandin synthesis. Conversely, foods rich in omega-6 fatty acids, such as oil, are thought to stimulate mammary tumour growth. Both saturated and unsaturated fats are thought to act during the promotional stages of carcinogenesis and this promotion is largely dependent on the amounts and sources of fat in the diet.

A link between red meat consumption and risk for breast cancer have been reported (Toniolo et al., 1994) while an inverse associations between intakes of fruits, dietary

fibre, vegetables and breast cancer risk have been reported in several case-control studies because they are important sources of antioxidants, which may help protect against the tissue damage linked to increased cancer risk (Fund WCRL, 1997). Antioxidants include vitamin C, vitamin E, and Vitamin A such as carotenoids.

Regarding to caffeine, in a prospective studies, it has not been seen correlation between caffeine intake and breast cancer risk (Vatten et al., 1990).

1.2.9. Height, weight and exercise

Adult height shows a positive association with breast cancer risk. Average height is substantially greater in populations with high rates of breast cancer than in populations with low rates. Within populations, a 10 cm greater height is typically associated with an increase in risk of about 10%. (Hunter and Willett, 1993). Probably because height is positively correlated with energy during growth and with early menarche, and it might be a marker for the number of susceptible breast cells.

In postmenopausal women, obesity increases the risk of breast cancer; risk is about 50% higher in obese women (body-mass index >30 kg/m^2) than in lean women (body mass index 20 kg/m^2) and this association is not observed in premenopausal women (Hunter and Willett, 1993). Several studies have reported that moderate physical activity is associated with a lower risk of breast cancer. The size of the effect of high physical activity has varied widely between studies, but a typical result is a reduction in risk of around 30% in association with a few hours per week of vigorous activity versus none (Friedenreich et al., 1998) and more evident in premenopausal women.

1.2.10. Family history and genetic factors

Environmental and lifestyle factors rather than inherited genetic factors account for most cases of breast cancer, even if most women with the disease do not have a family history of it, and most women with affected relatives never develop breast cancer.

Family history

The evidence for genetic predisposition to breast cancer derives originally from observations of cancer clustering in families and cancer risk increasing in individuals with some genetically determined syndromes.

Most studies on familial risk of breast cancer have found about two-fold relative risks for first-degree relatives (mothers, sisters, daughters) of affected patients (Pharoah et al., 1997). About 13% of all patients have a first-degree relative with breast cancer. A significant increased in breast cancer risk has been observed even in second (grandmothers, aunts, grand-daughters) to fifth degree (Amundadottir et al., 2004).

High-risk mutations

About 5-10% of all breast cancers are caused by germ-line mutations in well-identified breast cancer susceptibility genes (inherited from one's mother or father). So far at least five germ line mutations that predispose to breast cancer have been identified. These include mutations in the genes *BRCA1*, *BRCA2*, *TP53*, *PTEN*, and *ATM*. Mutations in *BRCA1* and *BRCA2* can cause high risks of breast cancer because they are tumor suppressor genes and their inactivation causes genetic defects and genetic instability. Germ line mutations in *TP53* predispose to the Li-Fraumeni cancer syndrome (including childhood sarcomas and brain tumors, as well as early-

onset breast cancer) and those in *PTEN* are responsible for Cowden disease (of which breast cancer is a major feature). High-risk alleles probably account for most of the families with four or more breast cancer cases, for around 20–25% of the familial breast cancer risk overall, and for around 5% of all breast cancers (Easton, 1999). The ATM (*ataxia telangiectasia mutate)* gene control cell cycle and mutations of this gene are closely linked to a childhood disorder of the nervous system called Ataxia Telangiectasia and to breast cancer susceptibility.

1.3. DISEASE ONSET AND PROGRESSION

Breast cancer is a group of related conditions, characterized by differing microscopic appearance and biologic behavior, in which the cells of the breast escape the normal replication, growing and dividing rapidly and uncontrollably (Coe and Steadman, 1995). It is believed that this capacity of evade from the replication cycle involves the accumulation of mutations, usually in genes that regulate cell division and the accurate replication of DNA (Davis and Bradlow, 1995). Also hormones and other substances located in close proximity of the cell can stimulate abnormal cell multiplication. There are many models of human breast cancer evolution. Cytogenetic and molecular genetics analysis have revealed that the development of a primary breast carcinoma derives from a multistep process involving initiating or promoting factors characterized by the accumulation of various genetic alterations which may invoke a transformation of normal cells into malignant cells (Beckmann et al., 1997)

One of the most well-established models, published by Wellings and Jensen over 30 years ago, proposed that the cellular origin of most breast cancers occurs in the

normal **terminal duct lobular unit** (TDLU), the basic histopathologic and physiologic unit of breast, and there is an apparently continuous but non-obligatory progression from TDLUs to cancers through a series of increasingly abnormal stages over long periods of time also decades in most cases (Figure 4) (Wellings and Jensen, 1973).

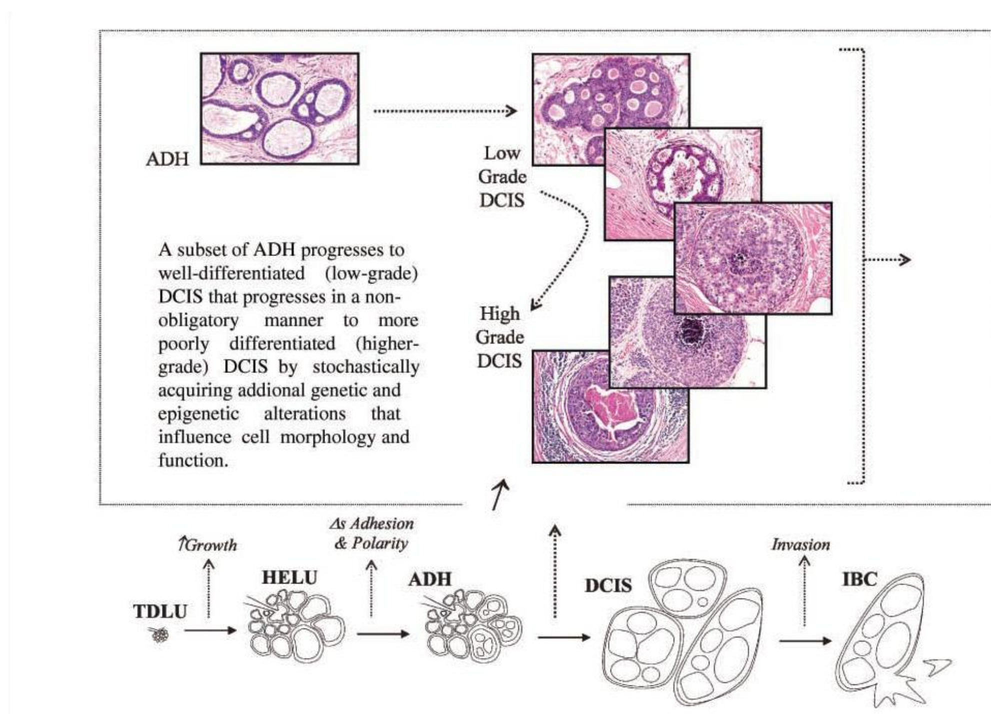


Figure 4. Revised Wellings and Jensen model of human breast cancer evolution. The original Wellings and Jensen model proposed an apparently continuous but non-obligatory linear progression from normal TDLU to IBC through a series of increasingly abnormal stages over long periods of time.

The key stages in this progression, in today's terminology, are called:

◊ **hyperplastic enlarged lobular units** (HELU);

◊ **atypical ductal hyperplasia** (ADH);

◊ **ductal carcinoma in situ** (DCIS) or **lobular carcinoma in situ** (LCIS) so called when the tumor remain confined within the basement membrane of the duct or lobule (Coe and Steadman, 1995).

If the breast cancer remains within the basement membrane and does not invade surrounding tissue or metastasize to distant organs it is said to be *in situ* (non-invasive)

◊ **invasive breast cancer** (IBC) when the tumor increases in size and the invade (or infiltrate) the normal adjacent tissue (Allred et al., 2004). When the cancer cells break away from the site of origin and penetrate the basement membrane of the epithelium, they enter the bloodstream or lymphatics located in connective tissue and may metastasize to distant organs and form secondary tumors. The major route of metastases via the lymphatic system is through the axillary nodes. Hence, the tumor extends into the central lymphatic terminus and the cancer cells enter into the venous stream. These cells can then be carried through the heart to lungs. Tumor fragments that may break loose from pulmonary vein are then carried off, back to heart and enter the bloodstream. Organs with a rich blood supply, such as the liver, spleen, adrenals and bone, are the targets for blood-bone metastases (Lu and Kang, 2007).

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Several characteristics distinguish the breast cancer types. The transition from TDLU to HELU is characterized by increased growth due to epithelial hyperplasia. Alterations of cell adhesion and polarity distinguish ADH from HELU as the hyperplastic epithelium begins to pile up and distends acini. DCIS is characterized by further expansion of tumor volume, intraductal spread into other areas of the breast, and, most importantly, the appearance of increased histologic and biological diversity compared with earlier precursors. Invasion into surrounding stroma defines the transition of DCIS to IBC. Evidences support that most high-grade DCIS gradually evolve from lower-grade DCIS and, thus indirectly from ADH, by the

random accumulation of genetic defects, which are propagated to IBC in a manner that is largely independent of progression to invasion. Since the DCIS are the precursor of nearly all ductal IBCs (which account for 85-90% of all IBCs), then ADH is probably also a risk factor for the development of DCIS independent of its histologic and biological characteristics (Allred et al., 2008).

Since the major breast cancers evolve from precursors, identifying of biological alterations associated with early precursors, before the cancer development, may reveal strategies for the prevention of the majority of cancers or treated them early.

1.4. CLASSIFICATION AND CLINICAL PATHOLOGY

When cancer is present, a number of tests are performed to assess the behavior of the cancer, and to determine the most effective treatments.

Prognosis is defined according to several parameters: tumor size and grade, the presence/ absence of estrogen (ER) and/or progesterone (PR) receptors, HER2/neu (HER2, c-erbB2) protein, lymph node metastases and vascular or perineural tumor invasion. Other parameters, such as the proliferating index, the presence of p53, BRCA1 and 2 or EGFR alterations, may also be useful for prognostic evaluation or as predicting therapeutic response.

The TNM Classification of Malignant Tumors (**TNM**) is a cancer staging system for all solid tumors that describes the extent of cancer in a patient's body. It was devised by Pierre Denoix between 1943 and 1952 using the size and extension of the primary tumor, its lymphatic involvement, and the presence of metastases to classify the progression of cancer. The parameters are:

- **T** (range from 1 to 4) describes the size of the primary **tumour** and whether it has invaded nearby tissue:
    - T1: No evidence of primary tumour
    - T2: Tumor 2 cm or less
    - T3: Tumour more than 5cm
    - T4: Tumour of any size with extension to adjacent tissue

- **N** (range from 0 to 3) describes regional **lymph nodes** that are involved and the degree of spread:
    - N0: tumor cells absent from regional lymph nodes
    - N1: regional lymph node metastasis present; (at some sites: tumor spread to closest or small number of regional lymph nodes)
    - N2: tumor spread to an extent between N1 and N3 (N2 is not used at all sites)
    - N3: tumor spread to more distant or numerous regional lymph nodes (N3 is not used at all sites);
- **M** (0-1) represents the presence of **metastasis** (spread of cancer from one body part to another).
    - M0: no distant metastasis
    - M1: metastasis to distant organs (beyond regional lymph nodes)

1.4.1. Tumour Stage

Once a TNM classification is available for a tumour, the tumour is then classified into a clinical stage; stage I, II, III, or IV (Table 1) (Sainsbury et al., 1994).

UICC stage	TNM classification
I	T_{1-2}, N_0, M_0
II	T_{1-2}, N_1, M_0
III	T_{3-4}, N_2, M_0
IV	T_{3-4}, N_2, M_1

Table 1. The correlation of the tumour, nodes, metastases (TNM) system and the Unio Internationale Contra Cancrum (UICC) system of classification for tumours.

Survival from breast cancer is largely dependent on the stage at presentation, and the prescription of appropriate treatment is based on stage.

1.4.2. Tumour Grade

On microscopic examination, a tumour can be graded according to the degree of differentiation of the tumour from adjacent "normal" cells. The most common grading system used by pathologists is the Scarff, Bloom, and Richardson (SBR) classification and is usually used as a preference to tumour staging.

Tumour Grade Definition:

◊ Tumor grade 1: tumor well-differentiated

◊ Tumor grade 2: tumor moderately-differentiated

◊ Tumor grade 3: tumor poorly-differentiated

Grade 1 tumors are small, round, have regular nuclei and very few mitoses. Conversely, grade 3 tumors are large, have irregular nuclei and have many mitoses

Survival studies show that grade 1 tumors have a good prognosis, and thus a good response to treatment, whilst grade 3 tumors would have a poor prognosis and the response to treatment would be less successful (Elledge and McGufre, 1993).

1.4.3. Tumour size

The size of the primary tumour and the involvement of axillary nodes (which, combined, constitute the stage of the disease) in cancer development, are the most important indicators of prognosis. A good prognosis is associated with a small tumour (less than 1cm in diameter); whilst a poor prognosis accompanies a large tumour (a diameter greater than 5cm) (Stockdale, 19889. Results from the SEER program (Surveillance, Epidemiology and End Results program of the National Cancer Institute) suggest that if tumors are less than 1cm in diameter and have not progressed from the initial site of development, then there is a relatively high chance of survival, after 5 years, from the time of primary diagnosis, in comparison, tumors of greater than 5 cm in diameter, have an 82% chance of survival after 5 years from the initial time of diagnosis (Carter. Et al., 1989).

1.4.4. Estrogen and progesterone receptors

Hormone receptor assays are considered to be essential tools for the assessment, prognosis and treatment of breast cancer. Approximately 50 to 85% of breast cancers cells contain receptors that specifically bind estrogen and progesterone.

Estrogen receptors (ER) and progesterone receptors (PR) are present in higher concentrations in breast cancer tissue than in "normal" breast tissue, and are thus significantly important for planning treatment. In fact, about 75% of breast cancers

are estrogen receptor-positive (ER-positive, or ER+). About 65% of ER-positive breast cancers are also progesterone receptor-positive (PR-positive, or PR+). Cells that have receptors for one of these hormones, or both of them, are considered hormone receptor-positive. Patients with breast cancers that are shown to be ER positive, respond favorably to hormone treatments such as tamoxifen, in approximately 60-65% of cases. On the contrary, patients with negative ER assays have a less than 10% response rate to hormone therapy (Stockdale, 1988). Therefore a high concentration of these receptors is highly predictive of the response hormonal therapy.

1.4.5. Proliferation index

The proliferation index is a measure of the number of cells in a tumor that are dividing, and thus proliferating. Cell proliferation can reasonably be supposed to be related to tumor aggressiveness. Proliferative activity can be determined using various methods based on different rationales:

Ki-67 protein is an indicator strictly associated with cell proliferation. During interphase, the Ki-67 antigen can be detected only within the nucleus of cells, while in mitosis the majority of the protein is relocated to the surface of chromosomes. The Ki-67 protein is present during all active phases of cell cycle (G1, S, G2 and M) but it is absent from resting cells (G0). Ki-67 is an excellent indicator to determine the fraction of development given population of cells. The fraction of Ki-67 positive tumor cells (Ki-67 labeling index) is often correlated with the clinical course of cancer.

The **mitotic index** (MI) is the fraction of cells in mitosis at any given time. It consists in counting the number of mitotic figure on a constant sample of cells (1000 or 10000) per mm^2 of epithelium. Mitotic activity is currently used mainly as part of the tumor grading system, for women with infiltrating breast carcinoma. Several studies have indicated that mitotic activity is an important imprint of tumor evolution as it exerts a determining influence on long-term clinical outcome, regardless of type of treatment, but also they suggested that mitotic activity does not provide predictive information on response to systemic therapy (Medri et al, 2003).

The **thymidine labeling index** (TLI) is a method, which involves the incubation of fresh tissue with tritium-labeled thymidine, provides an estimate of the fraction of tumor cells that are in the S (DNA synthesis) phase of the cell cycle. Because DNA synthesis is an integral part of each cell division cycle, TLI gives an indication of the amount of proliferation taking place in a tumor and it is a strong independent predictor of survival and relapse-free survival.

Both Ki-67 and TLI are high in cancers with high nuclear and histologic grade and are higher in cancers from premenopausal women than in those from postmenopausal women (Gentili et al., 1981; McGurrin et al, 1987); tumors with high TLI or Ki-67 are frequently estrogen receptor negative (Gerdes et al, 1987).

1.4.6. HER2-neu

HER2/neu (Human Epidermal growth factor Receptor 2, also known as ErbB-2) is a member of the ErbB protein family, more commonly known as the epidermal growth factor receptor family, and it is encoded by the *ERBB2* gene. It is a cell membrane surface-bound receptor tyrosine kinase and is normally involved in the signal

transduction pathway leading to cell growth. In breast cancer approximately 30% have an amplification of *HER2/neu* gene or overexpression of its protein product, giving higher aggressiveness, increased disease recurrence and worse prognosis of breast cancer patients.

1.4.7. p53

p53 is a tumor suppressor protein that regulates the cell cycle and plays a role in genetic stability and inhibition of angiogenesis; it exerts its anti-cancer role through several mechanisms (activates DNA repair proteins, induces growth arrest and initiates apoptosis). More than 50% of human tumors contain mutations or deletions of the *TP53* gene. While the prognostic and predictive value of p53 is still matter of debate, there is an increased interest for p53-based therapies.

1.4.8. BRCA1 and BRCA2

BRCA1 and BRCA2 are two tumor suppressor genes with several functions such as repair DNA double-strand breaks, protein ubiquitylation and cell cycle checkpoint control. Germ line mutations of these two genes confer strong lifetime risks of breast cancer and the risks are influenced by the position of mutation within the gene sequence (Easton, 1997). Researchers have identified hundreds of mutations in the *BRCA1* and *BRCA2* genes, many of which are associated with an increased risk of cancer. Women with a family history of breast cancer are screened for mutations in their *BRCA1* and *BRCA2* genes.

1.4.9. EGFR

The Epidermal Growth Factor Receptor (EGFR) is a cell-surface receptor for members of epidermal growth factor family (EGF-family) of extracellular protein ligands. The binding by ligands activates EGFR dimerization and stimulates intrinsic intracellular protein-tyrosine kinase activity. The downstream signaling proteins initiate several signal transduction cascades, principally MAPK, Akt and JNK pathways leading to DNA synthesis and cell proliferation.

The expression of EGFR in models of breast cancer is associated with increased proliferation and resistance to apoptosis and with poorer prognosis. Mutations that lead to EGFR overexpression or over-activity have been associated with breast cancer: it is overespressed in 35-60% of breast cancers.

1.5. TYPES AND SUBTYPES

The normal female adult breast consists of a mixture of epithelial and stromal elements. The epithelial elements of the breast contain a series of branching ducts, which extends from the nipple, and terminates into the functional units of the breast, the lobules (DiSaia, 1993). Each breast is composed of 15-20 lobules, containing a cluster of alveoli, which are responsible for the secretion of milk during lactation. The stroma contains variable amounts of interspersed adipose tissue and fibrous connective tissue, which constitutes most of the breast volume in a non-lactational state (Carola et al*.,* 1992; DiSaia, 1993).

The two most common types of breast cancer are named after the parts of the breast in which they start (Figure 5):

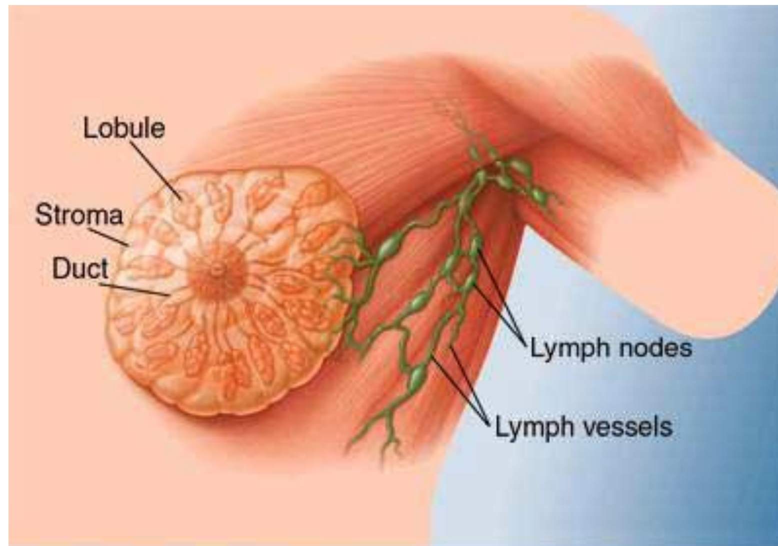


Figure 5. Anatomy of breast

● **Ductal Carcinoma in situ (DCIS):** it is the most common type of non invasive breast cancer, in fact between 85% and 90% of all breast cancers are ductal. It starts inside the milk ducts, beneath the nipple and areola and it is well contained, hasn't spread beyond the milk duct into any normal surrounding breast tissue, and it can be very successfully treated. The DCIS cancers have a higher risk for recurrence (most recurrences happen within the 5 to 10 years after initial diagnosis and the chances of a recurrence are under 30%) and for developing a new breast cancer.

● **Lobular Carcinoma:** about 8% of breast cancers are lobular. LCIS begins in the lobes, or glands which produce milk in the breast and the cancer is limited within the lobe and has not spread to surrounding tissues. Despite the fact that its name includes the term "carcinoma," LCIS is not a true breast cancer. Rather, LCIS is an indication that a person is at higher-than-average risk for getting breast cancer at some point in the future. LCIS is usually diagnosed often between the ages of 40 and 50.

These two cancer types are usually removed during a lumpectomy if the tumor margins are clear of cancer, follow-up treatment may include radiation. If ductal

cancer has broken into nearby breast tissue (invasive cancer) then a mastectomy may be needed, and also chemotherapy.

Second most common is a group of breast cancers that invade nearby tissue:

● **Invasive (Infiltrating) Breast Cancer** has the potential to spread out of the original tumor site and to invade other parts of your breast, the lymph nodes and other areas of the body. There are several types and subtypes of invasive breast cancer such as invasive ductal carcinoma and invasive lobular carcinoma. The treatments fall into two broad categories: local (surgery and radiation) or systemic (chemotherapy, hormonal and target therapy).

Other breast cancer types are:

● **Inflammatory Breast Cancer:** is the least common (1-5% of all breast cancer), but most aggressive of breast cancers, taking the form of sheets or nests, instead of lumps. It can start in the soft tissues of the breast, just under the skin, or it can appear in the skin. Unlike ductal and lobular cancers, it is treated first with chemotherapy and then with surgery. When caught early, inflammatory breast cancer can be a manageable disease, and survival rates are increasing.

● **Paget's disease of the nipple/areola** is a rare form of breast cancer, often looks like a skin rash, or rough. The itching and scabs are signs that cancer may be under the surface of the skin, and is breaking through. The cancer usually affects the ducts of the nipple first (small milk-carrying tubes), then spreads to the nipple surface and the areola. The disease usually develops after age 50 and is usually treated with a mastectomy, because the cancer has by then invaded the nipple, areola, and the milk ducts.

● **Rare types** of breast cancer include:

- Medullary breast cancer (5%)
- Mucinous (mucoid or colloid) breast cancer (2%)
- Tubular breast cancer (1%)
- Adenoid cystic carcinoma of the breast (1%)
- Metaplastic breast cancer (is a mixture of two cell types; 1%)

Human breast cancer is a heterogeneous disease, encompassing a number of distinct biological entities that are associated with specific morphological and immunohistochemical features and clinical behavior and, therefore, no golden standard therapy exists suitable for all tumors of the mammary gland (Lacroix et al., 2004). For many decades, breast carcinomas were only classified according to histological type, grade, and expression of hormone receptors as described above. However, this classification proved to be limiting for it was unable to define subgroups sharing similar prognostic and therapeutic aspects. A more recent approach to classify breast cancer subgroups is gene expression profiling, based on cDNA microarrays (Care et al., 2006; Sorlie et al., 2001), which suggests the presence of multiple molecular subtypes of breast cancer. Based on transcriptomic similarity, breast carcinomas can be distinguished into five "intrinsic" main distinct subtypes:

- **Luminal A** (ER positive, and/or PR positive, HER2 negative)
- **Luminal B** (ER positive and/or PR positive, HER2 positive)
- **Triple negative** (or also basal like) (ER negative, PR negative, HER2 negative)
- **HER2 positive** (ER negative, PR negative, HER2 positive)
- **Normal Breast-like**

Known as the ‘intrinsic subtypes of breast cancer’, these groups of tumors have revealed critical differences in incidence (Millikan et al., 2008), survival (Cheang et al., 2009; Hu et al., 2006), and response to treatment (Prat et al., 2010; Nielsen et al., 2010). For example, luminal tumors have been associated with the most favorable prognoses, while HER2-overespressing and triple-negative have been associated with the worst prognoses.

1.5.1 TRIPLE-NEGATIVE BREAST CANCER

Triple-negative breast cancers (TNBC) account for 10–17% of all breast carcinomas (Reis-Filho and Tutt, 2008) are reported to be more commonly seen in younger women, often in pre-menopausal women (<50 years), of African-American and Hispanic ethnicity (Morris et al., 2007), with BRCA1 mutations (Dent et al., 2007), an increased body weight (Trivers et al., 2009). It have been characterized by several aggressive clinicopathologic features including higher mean tumor size, higher histologic grade tumors, elevated mitotic count, ductal or mixed histology, and, in some cases, a higher rate of node positivity (Dent et al., 2007; Irvin and Carey, 2008). TNBC have a worse prognosis than the other breast cancer subtypes, high recurrence, occurring within three years of diagnosis and mortality rates are increased for five years after diagnosis, and development of recurrence and distant metastasis with a specific metastatic pattern (meninges, brain, liver and lung) (Rakha et al., 2007). Due to the absence of hormone receptors and HER2 expression, these tumors cannot take advantage from the endocrine therapy or trastuzumab treatment, chemotherapy remaining the only potential adjuvant therapeutic approach. As far as sensitivity to chemotherapy is concerned, the TNBCs exhibit higher rates of

objective response to neoadjuvant chemotherapy than other tumor types (Reis-Filho and Tutt, 2008), thus suggesting that biological features present more frequently in this group are responsible for the increased sensitivity to chemotherapy. In general, adjuvant therapeutic options for TNBC can be divided into two groups: cytotoxic agents (as anthracycline agents or platinum-containing agent) and targeted therapies (as PARP1 and EGFR or VEGF inhibitors). Although triple-negative cancers are report to have excellent response rates to neoadjuvant chemotherapy (Rouzier et al., 2005), survival of patients with such tumors is still poor and their management may therefore require a more aggressive alternative intervention and it remains an urgent need to understand the molecular and biological features of these tumors in order to develop novel therapeutic strategies to improve their clinical outcome.

1.6. THERAPY

The mainstay of breast cancer is surgery when the tumor is localized, followed by chemotherapy, radiotherapy and hormonal therapy for ER positive tumor, depending on clinical criteria. Treatments are given with increasing aggressiveness according to the prognosis and risk of recurrence.

1.6.1 Surgery

Surgery is usually the first line of attack against breast cancer. Some of the lymph nodes under the arm are usually taken out and looked at under a microscope to see if they contain cancer cells. Several types of surgery exist to remove breast cancer.

Breast-conserving surgery, an operation to remove only the cancer but not the breast itself, includes the following:

- Lumpectomy: Surgery to remove a tumour (lump) and a small amount of normal tissue around it.
- Partial mastectomy: Surgery to remove the part of the breast that has cancer and some normal tissue around it.

Other types of surgery include the following:

- Total mastectomy: Surgery to remove the whole breast that has cancer. Some of the lymph nodes under the arm may be removed for biopsy.
- Modified radical mastectomy: Surgery to remove the whole breast that has cancer, many of the lymph nodes under the arm, the lining over the chest muscles, and sometimes, part of the chest wall muscles.
- Radical mastectomy: Surgery to remove the breast that has cancer, chest wall muscles under the breast, and all of the lymph nodes under the arm

Radiation therapy

Radiation therapy is a cancer treatment that uses high-energy x-rays or other types of radiation (gamma rays). This radiation is very effective in killing cancer cells that may remain after surgery or recur where the tumor was removed.

There are two types of radiation therapy. External radiation therapy uses a machine outside the body to send radiation toward the cancer. Internal radiation therapy (or brachytherapy) uses a radioactive substance sealed in needles, seeds, wires, or catheters that are placed directly into or near the cancer. The way the radiation therapy is given depends on the type and stage of the cancer being treated. Although radiation therapy can reduce the chance of breast cancer recurrence, it is much less effective in prolonging patient survival. According to a review of six studies by the

United States' National Cancer Institute, none of them found a survival benefit for radiation therapy (Porter et al., 1993).

Chemotherapy

Chemotherapy is a cancer treatment that uses drugs to stop the growth of cancer cells. The mechanism of action of chemotherapy is to destroy fast growing or fast replicating cancer cells either by causing DNA damage upon replication or other mechanisms; these drugs also damage fast-growing normal cells where they cause serious side effects. Chemotherapy is used to treat: early-stage invasive breast cancer to get rid of any cancer cells that may be left behind after surgery and to reduce the risk of the cancer coming back; advanced-stage breast cancer to destroy or damage the cancer cells as much as possible. In some cases, chemotherapy is given before surgery to shrink the cancer.

When chemotherapy is taken by mouth or injected into a vein or muscle, the drugs enter the bloodstream and can reach cancer cells throughout the body (systemic chemotherapy). When chemotherapy is placed directly into the cerebrospinal fluid, an organ, or a body cavity such as the abdomen, the drugs mainly affect cancer cells in those areas (regional chemotherapy). The way the chemotherapy is given depends on the type and stage of the cancer being treated. Some protocols call for a cycle of treatment every three weeks; others may be more frequent.

It predominately is used for stage 2-4 disease, but may also be used to treat types of early-stage breast cancer. Many different types of chemotherapy drugs are used to treat this cancer and often they are administered in combination (regimen).

One of the most common treatments is cyclophosphamide plus doxorubicin (Adriamycin), known as AC. Sometimes a taxane drug, such as docetaxel, is added, and the regime is then known as CAT; taxane attacks the microtubules in cancer cells. Another common treatment, which produces equivalent results, is cyclophosphamide, methotrexate, and fluorouracil, known as CMF.

Hormone therapy

Hormones are substances produced by glands in the body and circulated in the bloodstream. Some hormones can cause certain cancers to grow. Hormonal therapy medicines treat hormone-receptor-positive breast cancers in two ways: by lowering the amount of the hormone estrogen in the body or by blocking the action of estrogen on breast cancer cells, stopping their growth.

If tests show that the cancer cells have places where hormones can attach (receptors), drugs, surgery, or radiation therapy are used to reduce the production of hormones or block them from working. The hormone estrogen, which makes some breast cancers grow, is made mainly by the ovaries. Treatment to stop the ovaries from making estrogen is called ovarian ablation.

Hormone therapy with tamoxifen is often given to patients with early stages of breast cancer and those with metastatic breast cancer. Hormone therapy with tamoxifen or estrogens can act on cells all over the body and may increase the chance of developing endometrial cancer. Hormone therapy with an aromatase inhibitor is given to some postmenopausal women who have hormone-dependent breast cancer. Hormone-dependent breast cancer needs the hormone estrogen to grow. Aromatase inhibitors decrease the body's estrogen by blocking an enzyme called aromatase from

turning androgen into estrogen. For the treatment of early stage breast cancer, certain aromatase inhibitors may be used as adjuvant therapy instead of tamoxifen.

Targeted therapy

Targeted therapy is a type of treatment that uses drugs or other substances to identify and attack specific cancer cells without harming normal cells. Monoclonal antibodies and tyrosine kinase inhibitors are two types of targeted therapies used in the treatment of breast cancer.

Monoclonal antibody therapy is a cancer treatment that uses antibodies made in the laboratory, from a single type of immune system cell. These antibodies can identify substances on cancer cells or normal substances that may help cancer cells grow. The antibodies attach to the substances and kill the cancer cells, block their growth, or keep them from spreading. Monoclonal antibodies are given by infusion. They may be used alone or to carry drugs, toxins, or radioactive material directly to cancer cells ant they may be used in combination with chemotherapy as adjuvant therapy.

Trastuzumab (Herceptin) is a monoclonal antibody that blocks the effects of the growth factor protein HER2, which sends growth signals to breast cancer cells. About one-fourth of patients with breast cancer have tumors that may be treated with trastuzumab combined with chemotherapy.

Another important monoclonal antibody used for the antiangiogenic therapy is Bevacizumab that blocks the VEGF receptor protein, which is involved in forming tumor blood vessels.

Tyrosine kinase inhibitors are targeted therapy drugs that block signals needed for tumors to grow. Also tyrosine kinase inhibitors may be used in combination with

other anticancer drugs as adjuvant therapy. Lapatinib is a tyrosine kinase inhibitor that blocks the effects of the HER2 protein and other proteins inside tumor cells. It may be used to treat patients with HER2-positive breast cancer that has progressed following treatment with trastuzumab.

PARP inhibitors are a type of targeted therapy that block DNA repair and may cause cancer cells to die. PARP inhibitor therapy is being studied for the treatment of triple-negative breast cancer.

Stage 1 cancers (and DCIS) have an excellent prognosis and are generally treated with lumpectomy and sometimes radiation. HER2+ cancers should be treated with the trastuzumab (Herceptin) regime (Gonzalez-Angulo et al., 2009) chemotherapy is uncommon for other types of stage 1 cancers. Stage 2 and 3 cancers with a progressively poorer prognosis and greater risk of recurrence are generally treated with surgery (lumpectomy or mastectomy with or without lymph node removal), chemotherapy (plus trastuzumab for HER2+ cancers) and sometimes radiation (particularly following large cancers, multiple positive nodes or lumpectomy). Stage 4, metastatic cancer, (i.e. spread to distant sites) has poor prognosis and is managed by various combination of all treatments from surgery, radiation, chemotherapy and targeted therapies.

2. CHEMOTHERAPY

2.1. FEATURES

Chemotherapy for the treatment of cancer was introduced into the clinic more than fifty years ago. Chemotherapy refers to antineoplastic drugs or chemical used to treat cancer. Chemotherapeutic drugs acts by killing cells that divide rapidly, one of the main properties of most cancer cells. Since malignant cells divide without control or order, these drugs effectively target cancerous growths. Ideally, chemotherapeutic drugs should specifically target only neoplastic cells and should decrease tumor burden by inducing cyto-endotoxic and/or cytostatic effects with minimal "collateral damage" to normal cells. Indeed, chemotherapy inadvertently also harms healthy cells that divide rapidly under normal circumstances: cells in the bone marrow, digestive tract and hair follicles; this results in the most common side effects of chemotherapy: myelosuppression (decreased production of blood cells, hence also immunosuppression), mucositis (inflammation of the lining of the digestive tract), and alopecia (hair loss).

There are various types of cancer those need different type of drugs that kill cancer cell in different ways at various phases in the cell cycle. Depending on the type, size, and location of the cancer, as well as your overall health, there are different strategies in the administration of chemotherapeutic drugs:

● **Neoadjuvant Chemotherapy:** refers to the administration of therapeutic agents prior to the main treatment, that usually it is the surgery. The aim is to reduce the size or extent of the cancer before employing radical treatment intervention, thus making procedures easier and more likely to be successful, and reducing the consequences of a more extensive treatments technique.

● **Adjuvant chemotherapy:** refers to additional treatment, usually given after primary therapy (surgery or radiotherapy) where all detectable disease has been removed, but where there remains a statistical risk of relapse due to occult disease. This treatment strategy permit to kill any remaining cancer cells in the body.

● **Palliative chemotherapy**: is given to patients who develop metastatic disease (cancer that spreads throughout the body) which are generally not curable. New advances in drug therapies, however, can help shrink tumors, prolong survival, and improve quality of life**.** Palliative treatments are also used to help relieve cancer-related symptoms, improving the patient's quality of life.

First line chemotherapy is treatment with chemotherapeutic drugs that has, through research studies and clinical trials, been determined to have the best probability of treating a given cancer. This may also be called "standard therapy".

Second line chemotherapy: is chemotherapy that is given if a disease has not responded or reoccurred after first line chemotherapy. In some cases, this may also be referred to as "salvage therapy".

Multiple chemotherapeutic agents may be used in combination to treat patients with breast cancer. Determining the appropriate regimen to use depends on many factors; such as, the character of the tumor, lymph node status, and the age and health of the patient. In general, chemotherapy has increasing side effects as the patient's age passes.

2.2. CHEMOTHERAPEUTIC DRUGS ANS MECHANISM OF ACTION

Currently there are many drugs, about a hundred, which can be used in cancer treatment. The majority of chemotherapeutic drugs can be divided into:

Alkylating agents: are drugs that act directly on DNA, causing cross-linking of DNA strands, abnormal base pairing, or DNA strand breaks, thus preventing the cell from dividing. Alkylating agents are generally considered to be cell cycle phase non-specific, meaning that the kill the cell in various and multiple phases of the cell cycle. Although alkylating agents may be used for most types of cancer, they are generally of greatest value in treating slow-growing cancers. Examples of these drugs are:

- classical alkylating agents, that are drugs with true alkyl groups, which including three subgroups: nitrogen mustards such as cyclophosphamide and melphalan, nitrosoureas such as carmustine, and alkyl sulfonates such as busulfan;
- alkylating-like agents that are platinum-based drugs, don't have an alkyl group but nevertheless damage DNA (Cruet-Hennequart et al., 2008) and including cisplatin, oxaliplatin and carboplatin.

Antimetabolites: are chemical that interfere with the formation or use of a normal cellular metabolites, interfering with DNA or RNA production and therefore cell division and the tumor growth. Antimetabolites are cell cycle specific, in fact they are most effective during S-phase of cell division because they primarily act upon cells undergoing synthesis of new DNA for formation of new cells. Indeed

antimetabolites masquerade as a purine or a pyrimidine chemicals which become the building blocks of DNA and they prevent these substance becoming incorporated in to DNA during S phase stopping normal development and division. The toxicities associated with these drugs are seen in cells that are growing and dividing quickly.

Examples of antimetabolites include:

- purine antagonists (act by mimicking the structure of metabolic purines) such as 6-mercaptopurine;
- pyrimidine antagonists (act by mimicking the pyrimidine structures) such as 5-fluorouracil, Gemcitabine and Cytarabine;
- folate antagonists (impair the acid folic function) such as Methotrexate.

Methotrexate is one of the most commonly used chemotherapy agents and works on the S-phase of the cell cycle. It is an analogous of folic acid and acts by inhibiting dihydrofolate reductase (DHFR) and, therefore, the metabolism of folic acid required for DNA synthesis and also for RNA and proteins.

5-Fluorouracil (or 5-FU) is a pyrimidine analogous which works through non competitive inhibition of thymidylate synthase, blocking the synthesis of the thymidine required for DNA replication, inducing cell cycle arrest.

Anti-tumor antibiotics: have several mechanisms of action to block cell growth, by interfering with DNA and RNA synthesis, and they work in all phase of the cell cycle. Example of anti-tumor antibiotics including:

- anthracyclines (act by inhibiting DNA and RNA synthesis by intercalating between base pairs of DNA/RNA strand preventing the replication of rapidly-growing cancer cells or by creating iron-mediated free oxygen radicals that damage the DNA

and cell membranes) that include doxorubicin;

- actinomycins (act by binding DNA at the transcription initiation complex preventing the elongation by RNA polymerase) including actinomycin-D;
- bleomycins (act by inducing DNA strand breaks).

Doxorubicin (or also Adriamycin) is used to treat wide range of cancer (carcinomas, sarcomas and hematological malignancies) and acts with DNA by intercalation (Fornari et al., 1994) and by inhibition of macromolecular biosynthesis (Momparler et al., 1976). Doxorubicin stabilizes the topoisomerase II complex preventing the DNA double helix from being released and thereby stopping the process of replication.

Mitotic inhibitors are drugs derived from plants and other natural products that block cell division by preventing microtubule functions during mitosis. Microtubules are polymers made of tubulin protein. They are created during normal cell functions; they move and separate the chromosomes and other components of the cell for mitosis. Therefore they are vital for cell division and, without them, cell division cannot occur, triggering the apoptosis. These drugs interfere with the assembly and disassembly of tubulin into microtubules and act primarily during M-phase of cell cycle, but they can also do so in all phases. The main examples are:

- vinca alkaloids derived from periwinkle plant, *vinca rosea* (act by binding to specific sites on tubulin inhibiting the assembly of tubulin into microtubules) such as Vincristine;
- taxanes derived from Pacific yew tree (act by destroying the microtubule function preventing the separation of chromosomes during anaphase) including paclitaxel;

- podophyllotoxins extracted from American May Apple tree (prevent the cell from entering the G1 phase and the replication of DNA and is the pharmacological precursor for etoposide agent).

Topoisomerase inhibitors: are agents designed to interfere with the action of topoisomerase enzymes (I and II), which are enzymes that control the changes in DNA structure, maintaining the topology of DNA and control the integrity of the genetic material during transcription, replication and recombination processes during the normal cell cycle. Topoisomerase inhibitors interfere with both transcription and replication of DNA, by upsetting proper DNA supercoiling, and can be divided according to which type of enzyme they inhibit:

- topoisomerase I inhibitors such as irinotecan, topotecan and camptothecin;
- topoisomerase II inhibitors including etoposide and mitoxantrone.

Hormone therapy

Drugs in this category are sex hormones, or hormone-like drugs, that alter the action or production of female or male hormones. The concept of this therapy is that the cancer cells of an organ sensitive to hormones may be subjected to hormonal control and an altered hormonal environment, blocking use of hormones or preventing the body from making them, inhibition produces a remission of tumor.

There are several types of hormonal therapy including:

- aromatase inhibitors (work blocking the enzyme aromatase which turns the hormone androgen into small amounts of estrogen in the body) such as letrozole;
- selective estrogen receptors modulators (SERMs) (work by sitting in the estrogen

receptors of cancer cells and so estrogen can't attach to the cell and this can't grow) such as tamoxifen;

- estrogen receptor downregulators (ERDs) (enter in the estrogen receptors of cell and so estrogen cannot attach to the cell and the cell can't grow but also it reduce the number of estrogen receptors) such as fulvestrant.

Some of the abbreviations used for chemotherapy drug combinations (regimens) refer to drug classes rather than drug names. For example, regimens that contain an anthracycline drug (such as doxorubicin) use the letter "A," and regimens that contain a taxane drug (such as docetaxel) use the letter "T." Cyclophosphamide (Cytoxan), fluorouracil (5-FU), and methotrexate (MTX) are standard cancer drugs used in many breast cancer chemotherapy regimens.

Chemotherapy regimens usually consist of 4-6 cycles of treatment given over 3-6 months. Common chemotherapy regimens for early-stage breast cancer include:

- AC (Doxorubicin and cyclophosphamide)
- AC followed by T (Doxorubicin and cylophosphamide followed by paclitaxel)
- CAF (Cyclophosphamide, doxorubicin, and 5-FU)
- CMF (Cyclophosphamide, methotrexate, and 5-FU)
- TAC (Docetaxel, doxorubicin, and cyclophosphamide

3. CELL CYCLE

3.1. CELL CYCLE AND CANCER

More than 50 years have passed since Howard and Pele in 1951 first described the cell cycle and its phases. Nevertheless, there are only more recent studies that have revealed that the cell cycle is a highly conserved and ordered set of events, culminating in cell growth and division. Cell cycle is tightly controlled by many regulatory mechanisms that either permit or restrain its progression (Gali-Muhtasib and Bakkar, 2002). Therefore, cell cycle is a process in which it grows and divides to create two genetically identical cells. In mammalian cells, the whole cell cycle takes around 24 hours from start to finish. Some cells, such as skin cells, are constantly going through the cell cycle while other cells may divide rarely as the neurons that don't grow and divide. The basic cell cycle consists of four distinct phases (Figure 6):

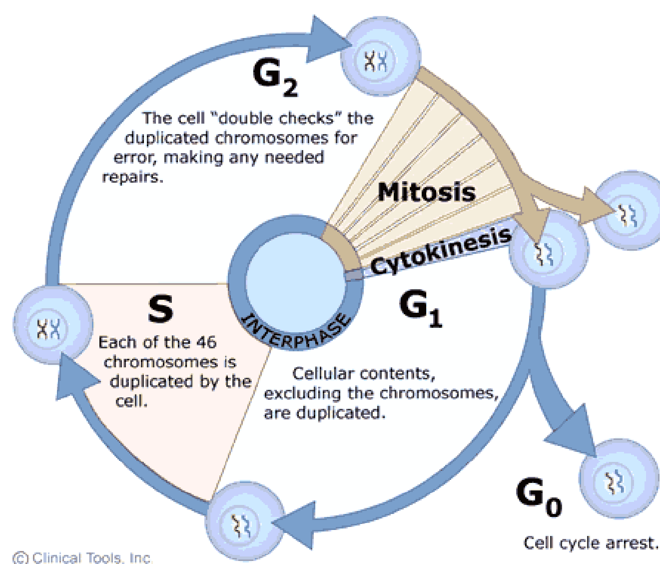


Figure 6. Phases of cell cycle

- **G1 phase** (the interval between the M phase and the beginning of S phase) in

which cells respond to extracellular cues that ultimately determine whether cells will make the decision to replicate DNA and divide or, alternatively, to exit the cell cycle into a quiescent state (G0). G1 phase is characterized by metabolic changes that prepare the cell for division; in fact this phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication. Duration of G_1 is highly variable, even among different cells of the same species (Smith and Martin, 1973)

- **S phase** (S for synthesis) in which the genetic material is duplicated (each chromosome now consist of two sister chromatids);
- **G2 phase** (the interval between the end of S phase and the beginning of M phase) in which metabolic changes assemble the cytoplasmic materials necessary for mitosis and cytokinesis;

the period between mitotic division, which consists of G1, S, G2 phases, is known as interphase;

- **M phase** (M for mitosis) in which phase a nuclear division (**mitosis**) is followed by a cell division (**cytokinesis**) (Gorbsky, 1997).

Mitosis is conventionally divided into five stages (Figure 7):

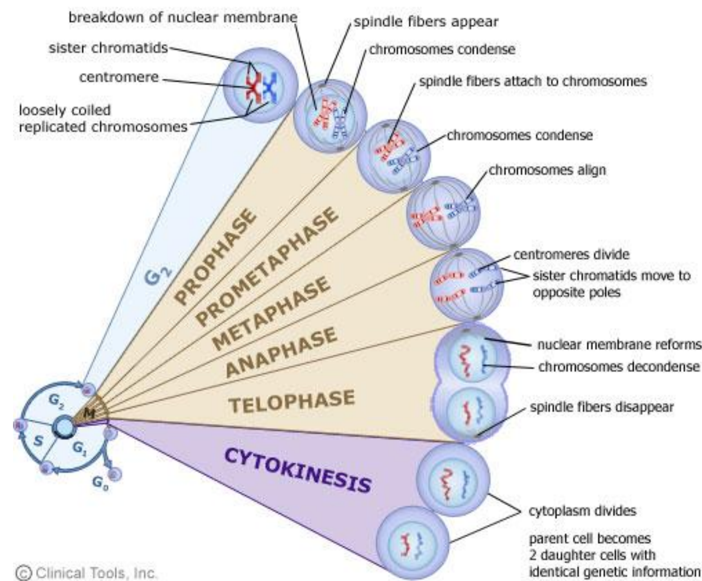


Figure 7. Steps in mitosis

- prophase: in which the nuclear membrane breaks and the centrosome duplicate itself to form two daughter centrosome that migrate to opposite ends of the cell; the centrosomes organized the production of microtubules that form the spindle fibers that constitute the mitotic spindle; each replicated chromosome can now be seen to consist of two identical chromatids, or sister chromatids, held together by the centromere;
- prometaphase: in which the chromosome migrate to the equatorial plane in the midline of cell, in the metaphase plate;
- metaphase: in which the chromosome align themselves along the metaphase plate of the spindle apparatus;
- anaphase: in which the centromeres divide and the sister chromatids are pulled apart and pulled in opposite sides of the cell;
- telophase: in which the nuclear envelope reassembles around the two new set of separate chromosome to form two nuclei;

Cytokinesis is the time in which the other components of the cell (membranes, cytoskeleton, organelles) are distributed to the two daughter cells.

When cells cease proliferation, either due to specific antimitogenic signals or to the absence of proper mitogenic signalling, they exit the cycle and enter a non-dividing, quiescent state known as **G0.**

Activation of each phase is dependent on the proper progression and completion of the previous one. In the typical dividing eukaryotic cell, G1 phase lasts approximately 15 hours, S phase 6 to 8 hours, G2 phase 3 to 6 hours, and mitosis about 30 minutes, although the exact length of each phase varies with the cell type and growth conditions (Pardee et al., 19878; Murray and Hunt T, 1993).

In the cell, there are control systems, independent by cell cycle events, that operate even if those events fail or in response to genetic damage. Both intracellular (oncogenes and anti-oncogenes) and extracellular (environmental signals, growth factors) inputs trigger molecular events that regulate normal progress through the stages of the cell cycle.

The main families of intrinsic regulatory proteins that play key roles in controlling cell cycle progression are the cyclin-dependent kinases (CDKs), cyclins, CDK inhibitors (CDKIs) and are actively involved two tumor suppressor protein, p53 and pRb (Gali-Muhtasib and Bakkar, 2002).

The **cyclin-dependent kinase** (CDK) is a family of serine/threonine protein kinases (Morgan, 1997) that regulates cell cycle and mRNA transcription and processing. All CDKs share the feature that their enzymatic activation requires the binding of a specific regulatory cyclin subunit (Table 2).

Phase	Cyclin	CDK
G0	C	CDK3
G1	D,E	CDK4, 2, 6
S	A,E	CDK2
G2	A	CDK2,1
M	B	CDK1

Table 2. Cyclins and CDKs by cell cycle phase

CDK regulators can also control cell-cycle commitment: they include activators, mainly the cyclins, and inhibitors, generically known as CDKI.

The **cyclins** are a family of proteins centrally involved in cell cycle regulation and structurally identified by conserved 'cyclin box' regions.

Cyclins are regulatory subunits of holoenzyme CDK complexes controlling progression through cell cycle checkpoints by phosphorylation and inactivating target substrates and they are so named because their concentration varies in a cyclical fashion during the cell cycle (Figure 8).

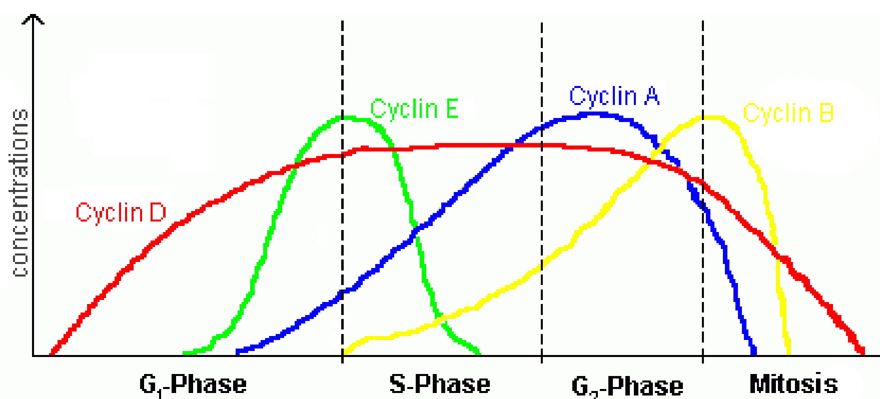


Figure 8. Expression of human cyclins through the cell cycle

There are several different cyclins that are active in different parts of the cell cycle and that cause the CDK to phosphorylate different substrates. There are also several "orphan" cyclins for which no Cdk partner has been identified.

There are two main groups of cyclins:

◊ **G1-S cyclins**: these ccyclins rise in late G1 and fall in early S phase. The Cdk-G1/S cyclin complex begins to induce the initial processes of DNA replication, primarily by arresting systems that prevent S phase Cdk activity in G1; they are Cyclins D and E involved in the transition from G1 to S phase (bind to CDK4 an CDK 6) and Cyclins A, active in S-phase (bind to CDK2);

◊ **G2/M cyclins:** M cyclin concentrations rise as the cell begins to enter mitosis and the concentrations peak at metaphase. Cell changes in the cell cycle like the assembly of mitotic spindles and alignment of sister-chromatids along the spindles are induced by M cyclin-CDK complexes. The destruction of M cyclins during anaphase causes the exit of mitosis and cytokinesis. They are Cyclins B (bind to CDK1).

The **CDK inhibitors** (CDKI) are protein that that serve as negative regulators of the cell cycle and stop the cell from proceeding to the next phase of the cell cycle, interacting with the cyclin-CDK complex blocking the kinase activity. There are two major CDKI families: the INK4 family (named for their ability to inhibit CDK4), comprising four members ($p16^{Ink4a}$, $p18^{Ink4c}$, $p15^{Ink4b}$, $p19^{Ink4d}$) which inhibit the activity of cyclin D-dependent kinases to prevent the phosphorylation of pRb family proteins and Cip/Kip family comprising three members ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$).

In addition to intrinsic controls, many external controls affect cell division. For example, the hormone estrogen affects the development of a wide variety of cell types in women and it exerts its effects on a receptive cell by binding to a specific receptor protein on the cell's nuclear membrane, initiating a cascade of biochemical reactions that lead to changes in the cell-cycle program. Also growth factors, such as TGF-β, PDGF, EGF and IL-2, stimulate cell proliferation and cell cycle progression.

The independence from specific growth factors is a common occurrence in transformed cells, which leads to a growth advantage on normal cells (Baserga et al., 1993).

To ensure proper progression through the cell cycle, cells have developed a series of **checkpoints** that prevent them from entering into a new phase until they have successfully completed the previous one (Hartwell and Weinert, 1989). It is likely that newly divided or quiescent cells must also pass certain checkpoints before they can enter the cycle. For instance, cells must make sure that they have reached their homeostatic size, otherwise cells will become smaller with each round of division.

The checkpoints are three in the normal cells:

- the **G1-S checkpoint** (or Start or restriction point) is located between mid and late G1 phase, just before entry into S phase. This is the point at which the cell ascertains whether it has received the necessary growth signals so that it can pass out of G1 into S phase, replicates its DNA and completes one round of cell division (Planas-Silva and Weinberg, 1997). If the cell has not received the appropriate cues, it will not pass the restriction point and will instead enter G0.

The cell may also arrest later in S phase due to incomplete DNA replication or DNA damage. The main controllers of this restriction point, which are pRb (retinoblastoma protein), p53, p16INK4a, be discussed in detail in the next section;

- the **G2 checkpoint** is located at the end of G2 phase, controlling the triggering of M phase. This point monitors the fidelity of DNA replication and is also an important sensor of DNA damage;
- the **metaphase checkpoint** (or spindle checkpoint) is activated during mitosis and control appropriate formation of spindle microtubule structure, chromosome alignments, sister-chromatids segregation, and completion of mitosis and cytokinesis.

If these cell cycle checkpoints are not in place then inappropriate proliferation can occur, which is one of the hallmarks of cancer. Several genes encoding regulatory activities that govern the cell cycle are targets for genetic and epigenetic alterations that underlie the development of many human cancers (Sherr, 1996). Molecular analysis of human tumours has shown that cell-cycle regulators are frequently mutated in human neoplasias (Figure 9), underscoring the importance of cell-cycle regulation in the prevention of cancer. These alterations include overexpression of cyclins (i.e. D1 and E1) and CDKs (i.e. CDK4 and CDK6), as well as loss of CDKI (i.e. p16, p15 and p27) and pRb expression. Tumour-associated changes in the expression of these regulators frequently result from chromosome alterations (amplification of cyclin D1 or CDK4, translocation of CDK6 and deletions of INK4 proteins or pRb) or epigenetic inactivation (methylation of *INK4* or *RB1* promoters) (Sherr, 2000; Wölfel et al., 1995).

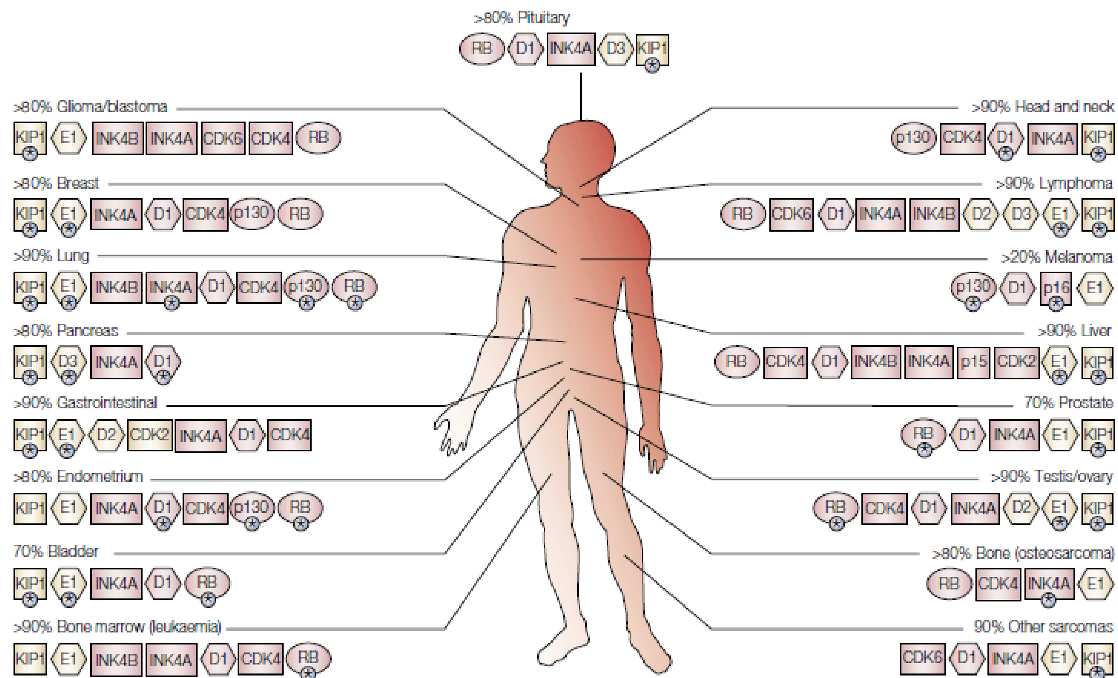


Figure 9. Mutations of G1-S regulators in human cancer

3.2. THE p53, pRb AND p16INK4a PATHWAYS IN CANCER

Most, if not all, human cancers contain genetic alterations in the p53, pRb and p16INK4a tumor suppressor pathway (Hanahan and Weinberg, 2000).

The **p53** tumour suppressor (known as "the guardian of genome") is a transcription factor responsible for the blockage of the cell cycle at the G1/S and G2/M checkpoints and/or inducing apoptosis in proliferating cells that are subjected to a variety of stressful events.

p53 belongs to a small family of related proteins that includes two other members: p63 and p73. Although structurally and functionally related, p63 and p73 have clear roles in normal development (Irwin, M. S. & Kaelin, W. G. p53 family update: p73 and p63 develop their own identities. *Cell Growth Differ.* **12**, 337–349 (2001).),

whereas p53 seems to have evolved in higher organisms to prevent tumour development. The steady state level of p53 is low in the absence of cellular stress and its turnover rate is rapid (less than 30 minutes). However, in response to a variety of stress signals, both intrinsic and extrinsic, the p53 protein is activated and, in turn, it can induce its downstream pathway. Gamma or UV radiation, alkylation or depurination of DNA, reaction with oxidative free radicals, ribosomal stress, oncogene activation, chemotherapeutic agents, altering DNA in different ways, but also hypoxia, microtubule disruption and loss of normal cell contacts cause damages and different repair mechanisms are employed by the cell. In each case, the damage activates and stabilizes p53, which migrates to the nucleus. These effects are determined by post-translational modifications of p53, such as phosphorylation, acetylation, methylation, ubiquitination or sumolation (Figure 10) (Appella and Anderson, 2001).

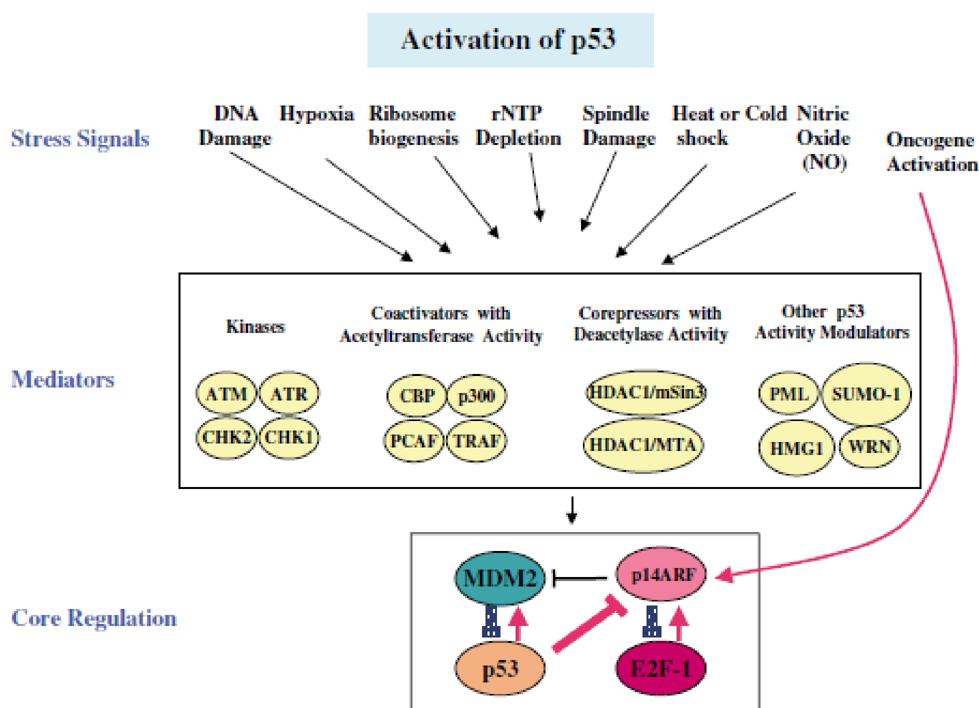


Figure 10. Diversity of cancer-related signals that activate p53 contributes to the central role the p53 protein as a tumor suppressor.

Different types of DNA damage activate different enzyme activities that modify the p53 protein on different amino-acidic residues. These modifications alter the p53 protein in two ways: first, by increasing the half-life of p53 in the cell (from 6-20 minutes to 1 hours), and this results in a 3-10 fold increase in p53 protein quantity in the cell; second, by enhancing the ability of p53 to bind to DNA sequences.

In fact, once that p53 is activated, it binds to specific DNA sequences and activates genes that are part of one of three stress response programs: cell cycle arrest (such as p21, GADD45 genes) to buy time to repair the DNA damage, cellular apoptosis (such as Bax, Puma genes), the programmed cell death when DNA damage proves to be irreparable, senescence (such as CSPG2 gene) promoting irreversible growth arrest (Figure 11) (Balint and Vousden, 2002; Giaccia and Kastan, 1998).

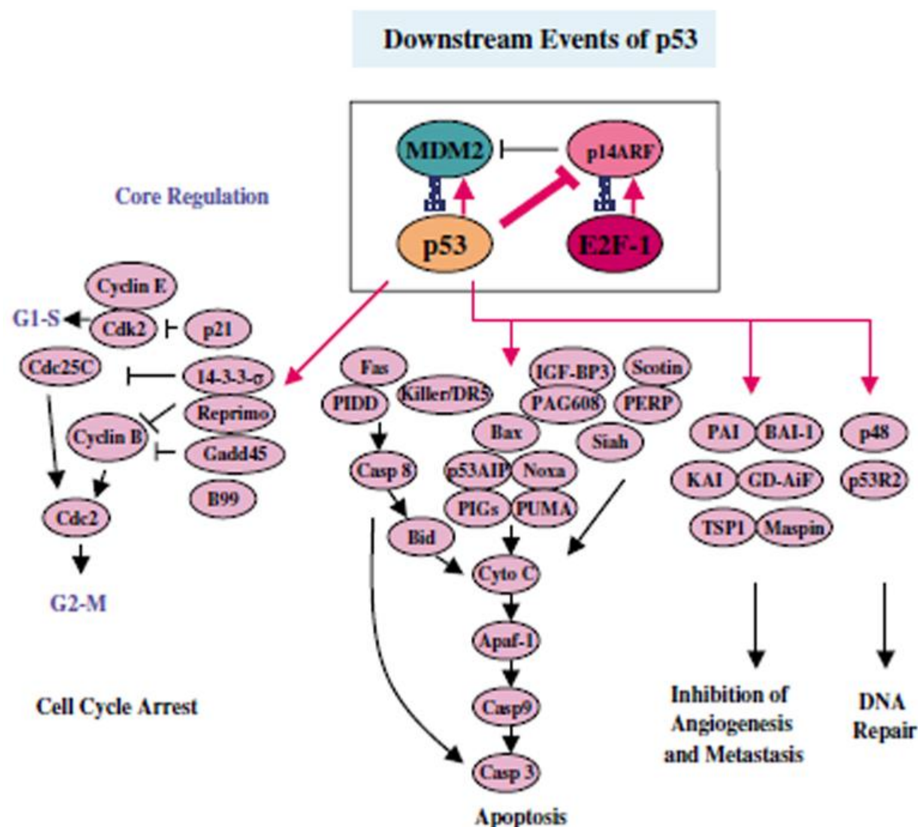


Figure 11. Downstream targets of the p53 transcription factor mediate its different biological outcomes.

One of the genes induced by p53 is p21, which play a pivotal role in G1 arrest by inhibiting cyclin D-CDK4/6 activity, reducing the pRb phosphorylation and blocking cell cycle.

Furthermore, p53 is regulated by different regulatory mechanisms. The p53 is a short-lived protein, its level kept low in most normally proliferating cells by rapid protein degradation. One of the key components regulating p53 stability is MDM2 (murine double minutes 2), a protein that functions as an ubiquitin ligase for p53, promoting the rapid degradation of p53 via the ubiquitin-proteasome pathway (Kubbutat et al., 1997). MDM2 is also a transcriptional target of p53 and therefore it functions in a negative regulatory feedback loop in which p53 activates the expression of MDM2, which in turn inactivates p53 by targeting p53 for degradation (Momand et al., 1992). Therefore, the function of p53 is to prevent the propagation of abnormal cells at risk of becoming cancer cells, blocking their cell cycle progression. The loss of p53 function occurs in > 50% of human cancer, thus representing the most frequent gene alteration in cancers (Harris and Levine, 2005; Vousden and Lu, 2002), by various mechanisms, including lesions that prevent activation of p53, missense, deletions and insertions mutations within the *TP53* gene (which encodes p53) itself or mutations of downstream mediators of p53 function.

In human breast tumors p53 gene mutation is the most common genetic alterations identified: mutations or over-expression of p53 protein in up to 52% of primary breast cancer specimens were observed indicating p53 as potential marker for studying the relationship between mutant p53 expression and tumor development, progression, and response to treatment and disease outcome.

Of the remainder breast carcinomas in which p53 gene mutation is not observed, half or more express wild-type p53 protein at high levels. In these cases, events independent of direct mutation of p53 may interfere with the normal function of the tumor suppressor during mammary tumorigenesis. Several studies have suggested that p53 status is an important determinant of tumour responsiveness to anti-neoplastic agents (Lowe et al., 1994; Clahsen et al., 1998). Specific mutations in p53 have been associated with poor response to primary systemic therapy (Aas et al., 1996) or overall survival (Borresen et al., 1995). Since many anti-cancer agents function is to activate cell death/apoptosis (Carson and Ribeiro, 1993), loss of normal p53 function can potentially result in the relative resistance of breast cancers to chemotherapeutic agents, due to the loss of the apoptotic properties of p53 (Bates and Vousden, 1999). This is possibly the reason why alterations of the p53 gene in breast cancer are associated with an unfavorable prognosis. Therefore, designing alternative treatment strategies aimed specifically at either restoring p53 function, or inducing optimal cellular response to damage, is a promising, rapidly-developing field in cancer research. Among these strategies are the gene therapy transfer of a 'minigene' encoding wild-type p53 with a viral vectors, or designing p53-reactivating drugs in tumors with an inactived p53, or restoring the p53 function by alternative approaches which aim to promote p53 transcriptional and tumour suppressor activities.

The pRb pathway consists of five families of proteins: CDKI, cyclin-D, CDK (CDK4 and CDK6), pRb-family of pocket protein (pRb, p107, p130), E2F-family of transcription factors (Figure 12).

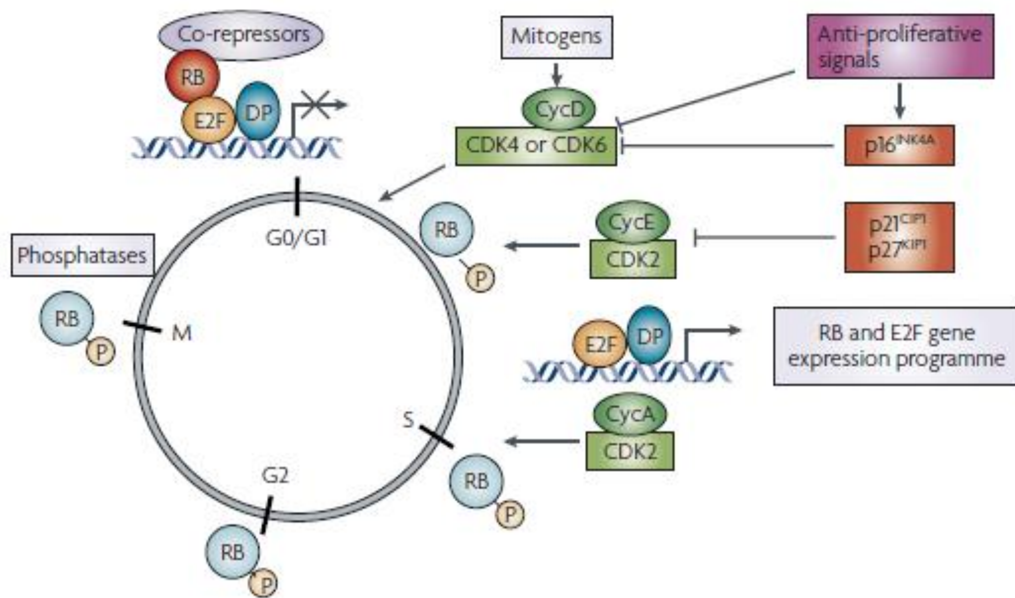


Figure 12. Schematic of pRb pathway in cell cycle control.

The pRb pathway is the major controller of cell cycle progression and of cell proliferation, and its constituents are activated by growth-promoting and inhibited by growth-suppressing signals. In quiescent cells, pRb is in its actively growth-suppressing hypophosphorylated state, and inhibits the cell cycle progression through the interaction with E2F factors, a family of transcriptional regulators that control the expression of genes whose products are important for entry and progression through S phase (Sherr and Roberts, 1999; David-Pfeuty, 2006). In response to mitogenic factors, pRb is inactivated through its phosphorylation on multiple sites. In its hyper-phosphorylated form, pRb leaves the E2Fs free to activate the target genes involved in cell cycle progression (e.g. Cyclins E) and DNA synthesis (i.e. thymidylate synthase, dihydrofolate reductase, thymidine kinase, ribonucleotide reductase, myc and DNA polymerase α), thus suggesting that E2F family member may be responsible for transversing the G/S checkpoint (Harbour et al., 1999; Zhang et al.,

2000). pRb phosphorylation is triggered in the early G1 phase by the cyclin D–CDK-4 and CDK-6 complexes and is completed, at the end of the G1 phase, by cyclin E–CDK-2 complexes. The activities of the CDKs are in turn constrained by the CDK inhibitors: CDK-4 and CDK-6 are inhibited mainly by p16(INK4a), whereas CDK-2 is negatively regulated by p21 and p27 (Sherr and McCormick, 2002), p53 negatively affects the cell cycle progression by inducing the p21 expression. The components of the regulatory machinery that controls G1-S phase transition behave as tumor suppressors or proto-oncogenes and are frequently altered in cancer cells. *RB1* (the gene encoding pRb) mutation or deletion, *p16INK4a* mutation and/or epigenetic silencing, and cyclin D1 or CDK4 overexpression and/or amplification characterize many human cancers (Figure 13) (Sherr and McCormick, 2002).

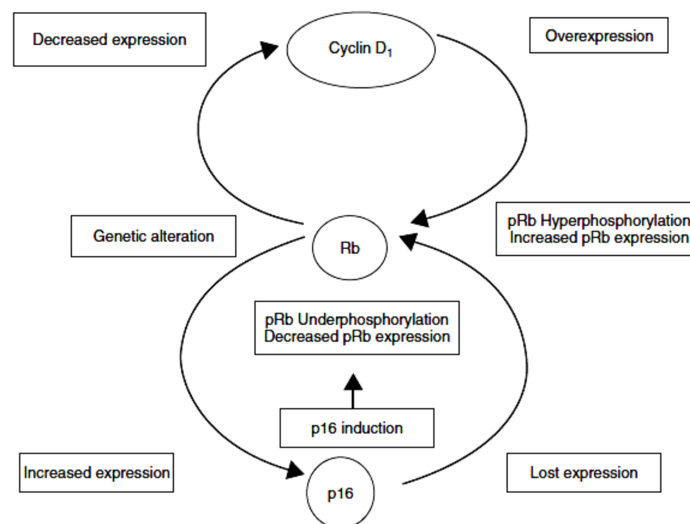


Figure 13. Example of alterations in the pRb pathway

These changes, causing either pRb loss or hyperphosphorylation, render the major control mechanism of the G1-S phase checkpoint out of order. Indeed, inactivation of the pRb tumor-suppressor pathways is associated with tumorigenesis and

characterizes a large fraction of many types of cancers (Sherr, 2000; Vogelstein and Kinzler, 2004).

Loss of normal pRb function is associated with 20% of human breast cancers. In the 80% of breast carcinomas in which pRb gene mutation is not observed, alterations in components of the signaling pathways that regulate pRb are frequently noted (Varley et al., 1989). For example, cyclin D1 and cyclin E overexpression, CDK4 gene amplification, or deletion of p16 have all been associated with primary breast carcinomas. Nearly 50% of invasive breast cancers examined have elevated cyclin D1 expression (Buckley et al., 1993).

p16INK4a (also known as CDKN2A) belongs to the INK4 family, which includes p16 ^{Ink4a}, p18 ^{Ink4c}, p15 ^{Ink4b}, and p19^{Ink4d}. It is an inhibitor of cyclin-CDK4 or cyclin-CDK6 complexes, blocking their kinase activity, and so interfering with the pRb phosphorylation, and inhibiting the progression to the S-phase of cell cycle (Ruas and Peters, 1998). p16INK4a is a potent tumor suppressor and alterations leading to its inactivation result in the deregulation of cell proliferation through loss of G1 arrest control, contribute to the formation of cancer and may influence tumour response to chemotherapy. In fact p16INK4a is commonly mutated, deleted or hypermethylated, resulting in the reduction or absence of its expression, in human cancers (Medema et al., 1995).The absence of p16INK4a expression is seen predominantly in cells that retain wild-type pRb (Otterson et al., 1994). However, *p16INK4a* can be up-regulated or overexpressed in cancer cell lines and tumors in which pRb is dysfunctional (Dublin et al., 1998) providing evidence for a negative

feedback loop in which the functionally inactive pRb fails to sequester transcription factors, which, in turn, induce *p16INK4a* gene expression.

The ability of p16INK4a to arrest the cell cycle in G1 phase depends upon the presence of a functional pRb, implying that by inhibiting cyclin D-dependent kinases, pRb remains hypophosphorylated and able to repress transcription of S-phase genes (Medema et al., 1995). The loss of p16 expression is necessary to bypass the G1 checkpoint in cancer cells during tumor progression (Shapiro et al., 1998). In mammary carcinomas, the etiological role of p16 is far from understood: although p16 inactivation is observed in several breast cancer cell lines, mutation or deletion of the *p16INK4a* gene are rare events in breast cancer (Quesnel et al., 1995). The only study available in the literature specifically examining the prognostic significance of p16INK4a in breast cancer reported that poor outcome was associated with high expression of p16 protein assessed by immunohistochemical staining (Dublin et al, 1998).

The hallmark of cancer is deranged growth control (Pardee et al., 1978), because checkpoints are defective in cancer cells (Hartwell and Kastan, 1994). As previously stated, control mechanisms are often lost due to mutations in tumor suppressor genes, e.g. mutated p53 gene, or alterations in one of the pRb pathway components. A relationship between pRb and p53 exists in cell cycle regulation based on the action of the two genes regulated by p53: MDM2 and p21.

MDM2 contains a p53-binding site, but also a pRb-binding site: by interacting with pRb, MDM2 restrains its functions by altering the conformation of the pocket region (Xiao et al., 1995). It is postulated that overespression of MDM2 inactivates both

p53 and pRb. p21 is an effector of cell cycle arrest in response to activation of the p53 G1 phase checkpoint pathway that acts through inactivation of the cyclin-CDK complexes that are responsible for pRb phosphorylation.

These findings imply a potential link between pRb (p16-pRb-cyclin D1) and p53 (p53-MDM2-p21) pathways in cell cycle regulation and apoptosis and it play a critical role in tumorigenesis (Figure 14).

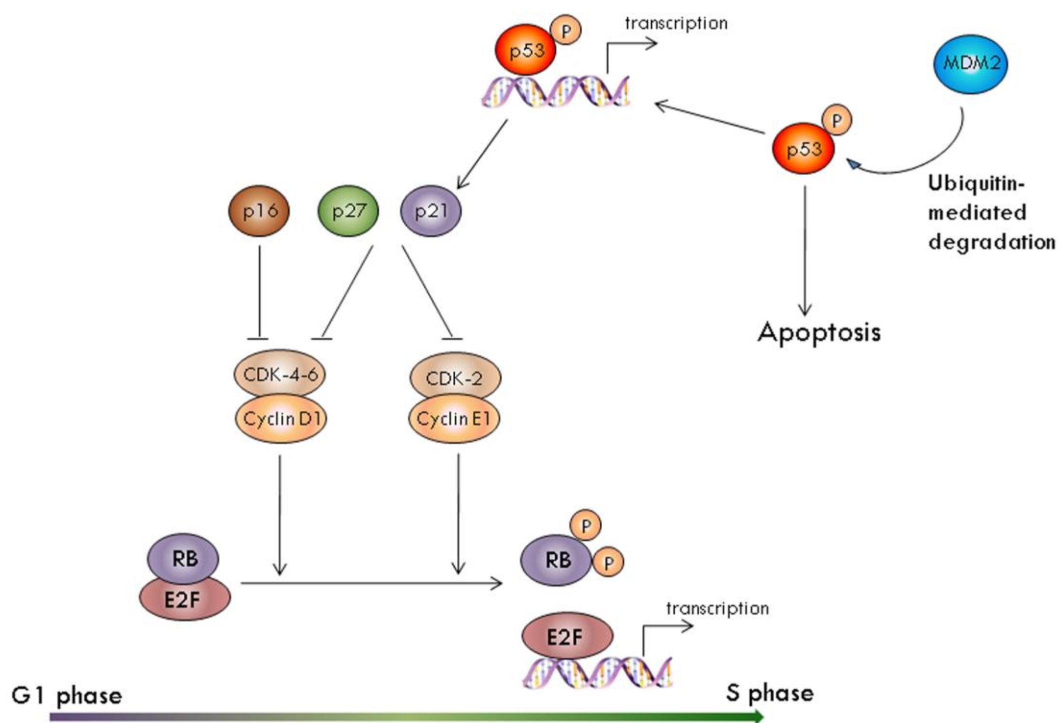


Figure 14. Schematic representation of the molecular networking model for p53 and pRb

4. AIMS OF THE THESIS

Chemotherapy is used to treat various tumor types, including breast cancers; chemotherapeutic agents kill cancer cells in different ways, inducing cell cycle arrest and/or apoptosis. Cells respond to drug-induced damages mainly by activation and stabilization of p53 protein and its downstream pathway (Johnstone et al., 2002).

Because chemotherapy commonly induces p53 activation, as a matter of principle, the presence of a normally functioning p53 in cancer cells could be important for both the response to treatment and the prognosis of patients. However, the assessment of p53 status has produced contradictory results regarding its prognostic/predictive value in human breast cancer (Hall and McCluggage, 2006). We hypothesized that these conflicting results could be a consequence of the fact that in cancer cells the p53-downstream pathway may be altered, nullifying or changing the effect of p53 stabilization after chemotherapy treatment. The most important downstream pathway of p53 is represented by pRb, which is often altered in cancer cells, influencing the p53-mediated effect of chemotherapy (Knudsen and Knudsen, 2008). Cancers characterized by pRb alteration, from the clinical point of view, are generally more aggressive than those with a normally functioning pRb pathway (Cordon-Cardo, 1995) probably because the pRb inactivation causes chromosome instability, genetic changes facilitating tumor progression and an up-regulation of proliferation cell rate. Moreover, it is also known that the pRb status influences the response to DNA-damaging agents in human breast cancer cell lines and in xenografts models (Bosco et al., 2007). In order to gain information on the influence

of the pRb status in p53-mediated response to chemotherapy, we conducted a prospective study on series of patients with primary breast cancer treated with chemotherapy, in which we investigated their clinical outcome according to the p53 and pRb status. We also evaluated the role of pRb status on the p53-mediated response to chemotherapeutic drugs in human cancer cells lines treated either with 5-FU plus MTX or doxorubicin where pRb was down-regulated. Since, in this study we demonstrated that tumors characterized by pRb loss were more sensitive to chemotherapy independently by p53 status, we then investigated the effects on cell cycle progression of pRb deficiency in cancer cell lines after chemotherapy treatments.

There is evidence that a particular subtype of breast carcinomas, the triple-negative breast cancer (TNBC), is very sensitive to chemotherapy than other tumor subtypes (Reis-Filho and Tutt, 2008). Since we have shown that breast cancers lacking pRb expression were more sensitive to adjuvant chemotherapy, we sought to ascertain whether in TNBCs, the high sensitivity to chemotherapeutic drugs could be due to the loss of pRb.

Therefore we evaluated the prevalence of pRb loss and the chemosensitivity in a large series of triple-negative breast cancer patients treated with chemotherapy, in according to the pRb status. We also studied the relevance of pRb loss on chemosensitivity in a triple-negative derived cell line.

5. MATERIALS AND METHODS

5.1. Patients

We studied a total of 518 consecutive patients who underwent surgical resection for primary invasive breast carcinoma at the Department of Surgery, University of Bologna, between 1991 and 1995. Patients' age ranged from 25 to 89 years, with an average (±SD) of 60 (±12.9) years (median value, 61 years). Tumors were histologically classified and staged according to the WHO and the Unio Internationale Contra Cancrum tumor-node-metastasis systems, respectively. Histologic grading (G) was done in ductal carcinomas according to Elston and Ellis (Elston and Ellis, 1991). Due to patient age, axillary dissection was not done in 7 patients (1.3%): in the remaining 511 cases, axillary lymph node metastases were reported as absent (N0) or present (N+). Estrogen receptor (ER) and progesterone receptor (PR) status; Ki67 antigen expression; and p53, HER2, and pRb status were assessed on histologic sections by standard immunohistochemistry, as reported below. All immunohistochemical analyses were done at the time of diagnosis. Patients were then regularly followed up every 6 mo for a median observation time of 109 mo (range 4-142 mo).

The present study was approved by the Senior Staff Committee, the board, which, at the time of patient enrollment, regulated non interventional studies and was comparable with an institutional Review Board.

5.2. Adjuvant treatments

Three hundred and forty-two patients underwent mastectomy and 176 patients underwent conservative breast surgery. One hundred and forty-five received six cycles of the cyclophosphamide, methotrexate, and 5-FU (CMF) chemotherapy regimen that was given on days 1 and 8 of each treatment cycle. The dose of cyclophosphamide and fluorouracil was 600 mg/m^2 of body surface area and the dose of methotrexate was 40 mg/m^2. Each of the three drugs was repeated every 28 d. 231 patients who did not receive systemic chemotherapy received adjuvant endocrine therapy alone (tamoxifen, 20 mg daily, for at least 2 y). A total of 49 patients received radiotherapy only and 93 patients did not receive any kind of adjuvant therapy.

5.3. Immunohistochemical assessment

From each case, one block of formalin-fixed, paraffin-embedded tissue was selected, including a representative tumor area. Four-micrometer-thin serial sections were cut, collected on 3-ethoxy-aminoethyl-silane-treated slides, and allowed to dry overnight at 37°C. Tissue sections were then processed for immunohistochemistry and the immunostaining reaction was then developed according to the SABC (Streptavidin-Biotin-Peroxidase Complex) method, combined with antigen retrieval pretreatment in citrate buffer solution (pH 6), and highlighted using a peroxidase/ 3,3'-diaminobenzidine (DAB) enzymatic reaction.

The following monoclonal antibodies (mAbs) were used: anti-p53 (clone BP53-12.1), anti-Ki67 (clone MIB-1), anti-HER2 internal domain (clone CB11), anti-ER (clone 1D5) and anti-progesterone receptor (anti-PR; clone 1A6), all from BioGenex

Laboratories. pRb immunostaining was assessed using two different mAbs: clone G3-245 (BioGenex Laboratories), which specifically recognizes the phosphorylated pRb form, and clone 1F8/Rb1 (Neomarkers, Lab Vision), which identifies all forms of pRb (phosphorylated as well as unphosphorylated or underphosphorylated).

The pRb status was assessed by evaluating both the percentage of cells with phosphorylated pRb and of cells exhibiting total pRb.

The pRb phosphorylation level was evaluated using an anti-pRb monoclonal antibody (mAb) (clone G3-245) that specifically recognizes ppRb form.

The phosphorylated pRb-LI variable was dichotomized using the cutoff point of 25%, according to Derenzini *et al* (Derenzini et al., 2004) in which chose the 25% cutoff because pRb hyperphosphorylation is found mainly in the late G1, S, and G2 phases, whose duration in human cancers is not longer than one quarter of the cell cycle length. Therefore, the presence of a pRb LI > 25% is strongly indicative of an alteration of pRb phosphorylation control.

Because in our series 40 cases (7.7%) showed a very low positivity for phosphorylated pRb (ppRb LI <1%), these cases were assumed to include two kinds of tumors: (a) tumors in which pRb was present but phosphorylated only in a few cells and (b) tumors in which both the pRb forms were absent, very likely due to *RB1* deletion. To differentiate between these two groups, the 40 cases were investigated for the presence of total pRb, using a specific mAb (clone 1F8/Rb1) that recognizes both the phosphorylated and the unphosphorylated or underphosphorylated pRb forms. Nine cases showed positive immunostaining in some cancer cells, whereas the remaining 31 cases showed no immunostaining. The latter cases were definitively

regarded as *RB1* deleted and were included in the RB negative (RB-) group. The remaining 487 cases were included in the RB positive (RB+) group.

The p53 status was evaluated by measuring the percentage of immunostained nuclei (p53-LI). We considered samples with at least 10% of nuclear staining to be characterized by an altered p53 status, according to Esrig *et al* (Esrig et al., 1993).

p21 expression was evaluated using anti-p21 mAb (Dako Cytomation, Glostrup, Denmark) measuring the percentage of immunostained nuclei (p21-LI). All mAbs were applied overnight at room temperature at the predetermined optimal concentrations.

The nuclear immunostaining of ER, PR, Ki67, p53 and pRb was assessed by image cytometry, using the Cytometrica program (C&V, Bologna, Italy) as detailed by Faccioli et al. (Faccioli et al., 1996). Staining was expressed as the percentage of labeled nuclear area over the total neoplastic nuclear area in the section [labelling index (LI)]. HER2 membrane immunostaining pattern and intensity were assessed by direct microscope evaluation, following the four class scoring system (0, +1, +2, +3) according to published protocols (Ellis et al., 2004). For each case, at least 2,000 cells were evaluated. All the immunohistochemical analyses were performed at the time of diagnosis.

5.4. Cell lines and growth conditions

The human breast cancer cell line MCF-7 was maintained in RPMI 1640 (Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Euroclone); the human colon cancer cell line HCT-116 and the human breast cancer cell line MDA-MD-231 were maintained in DMEM supplemented with 10% FBS; the human

hepatocellular carcinoma cell line HepG2 was maintained in RPMI 1640 supplemented with 10% FBS and sodium pyruvate (Euroclone). All cell lines were from the American Type Culture Collection (ATCC). FBS was inactivated by heat (56°C for 30 minutes).

All cell lines were cultured in monolayer at 37°C in humidified atmosphere containing 5% CO_2 in medium with L-glutamine (Euroclone) 2mM, penicillin 100 U/ mL and streptomycin 100 mg/ mL (Euroclone).

5.5. Production of HCT-116-derived cells with stably inactivated p53

HCT-116 cells stably expressing p53DD, a truncated, dominant-negative form of murine p53 (Shaulian et al., 1995) and the related empty vector-transduced control cells (pBABE), were obtained as described by Morgenstern JP and Land H (Morgenstern and Land, 1990). These cell lines were maintained in DMEM supplemented with 10% FBS and selected with puromycin antibiotic (Sigma-Aldrich, Milan, Italy).

5.6. Drugs and cell treatment protocols

A drug cocktail of 5-Fluorouracil (5-FU; Fluorouracil, Teva Pharma B.V. Milan, Italy) and methotrexate (MTX; Metotrexato, Mayne-Mayne Pharma, Naples, Italy) at doses of 20 µg/ml and 0.1 µg/ml, respectively, was used to treat MCF-7, MDA-MB-231, HCT-116 wild-type (wt) and HCT-116-derived cell lines. Doxorubicin (Doxorubicin Hydrochloride Injection, USP, Pfizer Italia, Rome, Italy) was used at a concentration of 1 µM on MCF-7 and MDA-MB-231, 3 µM on HepG2 and 0.3 µM on HCT-116-derived cell lines. Both drugs were diluted directly from stock solutions

and mixed in RPMI or in DMEM with 10% FBS. Cells were exposed to either doxorubicin or 5-FU plus MTX for 1 or 2 h at 37°C. After the drug treatments, in an initial set of experiments, the cells were washed extensively with PBS, fed with fresh medium for 6 h and then harvested. In a second set of experiments the cells were exposed to the drugs for 2 h daily for 4 consecutive days and fixed in formalin 24 h after the last treatment.

5.7. Genes silencing by RNAi transfection

The day before transfection, cells were seeded in antibiotic-free growth medium. Transfections were performed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, UK) in Opti-MEM medium (Invitrogen) following the manufacturer's protocol. After 4 hours, the Opti-MEM is been replaced with the appropriate growth medium.

Silencing of *RB1, TP53* and *p16INK4a* was obtained by transient transfection of cells with specific interferent RNA oligos (RNAi). Transfections were performed with Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM medium (Invitrogen) accordingly to manufacturer's procedures. *RB1* and *TP53* genes were silenced using Stealth RNAi Select kits (Invitrogen), while sequences of RNAi for *p16INK4a* silencing (Invitrogen) were from Lau *et al* (Lau et al., 2007). Controls for *RB1*- and *TP53*-silenced cells were transfected with equivalent amounts of Stealth RNAi Negative Control (Invitrogen), while controls of *p16INK4a* silenced cells were transfected with an RNA oligo sequence (obtained by scrambling the sequence of p16-specific RNAi) that is not complementary to any known human transcript (screened on NCBI BLAST). The concentrations of siRNAs used resulted to be lowest one capable to reduce the mRNA levels to at least the 80% of control for

duration of 120 h. The RNAi specific for *RB1* was used at a final concentration 80 nM, while those for *TP53* and *p16INK4a* genes in 40 nM concentration. The Lipofectamine is being used with a ratio of 1 μL every 15 picomoles of RNAi.

5.8. RNA extraction, cDNA synthesis and real-time RT–PCR analysis

Cells were harvested and total RNA was extracted from cells 48 and 120 h after siRNA transfection with TRI reagent (Ambion, Austin, TX, USA) according to manufacturer instructions. The cells were homogenized in TRI Reagent solution, collected in eppendorf tubes and incubate for 5 minutes at room temperature (RT). After that, the homogenate were centrifuged, incubated with 160 µl chloroform for 10 minutes at RT to generate the phase separation, re-centrifuged again and the aqueous phase containing the RNA was transferred in a fresh tube. The RNA was precipitated by adding isopropyl alcohol, incubated at RT for 10 minutes, spinned to allowed RNA to precipitate. After discard the supernatant, the gel-like bottom RNA was washed in 75% alcohol for washing, centrifuged, air-dried for 10 minutes e dissolve in DEPC water. Extracted RNAs were quantified with a Nanodrop spectrophotometer (ND1000). The quality of RNA extracted was evaluated measuring the A260/A280 ratio. Reverse transcription reactions were performed in a 25 µl volume using 2 µg of total RNA with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. cDNAs obtained were diluted in DEPC water and were subjected to real-time PCR analysis in a Gene Amp 7000 Sequence Detection System (Applied Biosystems) using the TaqMan Universal PCR mastermix (Applied Biosystems) diluted in 20 µl of total volume for well. For each sample, three replicates were analyzed. Cycling

conditions were as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 15 s, and 60°C for 1 min.

Primers and probes for *RB1, TP53* and *p16INK4a* were purchased from Applied Biosystems (Assay on Demand); human-*β*-glucuronidase was used as an endogenous control gene (Applied Biosystems). All primers were used at a final concentration of 5 μM.

The relative amount of the target gene in the cells transfected with the specific siRNAs compared with that of scrambled sequences of transfected cells was evaluated by the ΔΔCt method (Schmittgen et al., 2000).

5.9. Proteins extraction and Western blot analysis

For Western blot analysis, cells were lysed in a lysis buffer consisted of 0.1 M KH2PO4 (pH 7.5), 1% Igepal (NP-40), 0.1 mM β-glycerophosphate and complete protease inhibitor cocktail (Roche Diagnostics) 1X. Cells were incubated 25 min on ice and centrifuged at 14,000xg for 25 minutes at 4°C. After the supernatants were collected for analysis. Protein concentrations in supernatants were evaluated using Bradford assay (using Bio-Rad Protein Assay). All steps were done at 4°C.

For each sample, 30 μg of lysate proteins (or 50 μg to assess protein expressed little) were resuspended in Laemmli buffer. Denatured protein samples were separated in 10% or 14% SDS polyacrylamide gels and transferred to cellulose nitrate membranes (Hybond C Extra, Amersham). Filters were then saturated with 5% non fat dry milk powered dissolved in TBS-T solution for 1 h at room temperature. TBS is constituted by 20 mM Tris-HCl, 137 mM NaCl (pH 7.6) and is added 0.1% Tween 20 (Sigma) for the final TBT-T solution. After the saturation of the membranes are washed with

TBS-T (2 washes of 5 minutes) at RT and incubated overnight at 4°C with primary antibodies in 3.5% bovine serum albumin TBS-T. The following antibodies were used: anti-total pRb (1:200, clone 1F8; Lab Vision Corporation), anti-phospho pRb (1:250, Ser780, Cell Signalling Technology, Beverly, MA, USA), anti-p16 (1:200, Santa Cruz Biotechnology), anti-p53 (1:1000, clone BP53-12, Novocastra), anti-p21 (1:100, clone SX118, Dako Cytomation), anti–phospho-H2AX histone (1:1000, Ser139, clone 20E3, Cell Signaling Technology) and anti-β-actin (1:4000, Sigma Chemical Co.). The next day, the membranes were washed 1x10 min and 2x5 min in TBS-T to remove unbound antibody, and were incubated for 1 h in the presence of horseradish peroxidase–labeled secondary antibody (dilution 1:10.000 in 5% milk TBS-T) at RT. After 3 washes of 10 minutes, the horseradish peroxidase activity was detected using an enhanced chemiluminescence kit ECL (GE) and was revealed on Hyperfilm enhanced chemiluminescence films (Amersham). The intensity of the bands was evaluated with the densitometric software GelPro analyzer 3.0 (Media Cybernetics). Normalization was made against β-actin expression.

5.10. Immunocytochemical analysis

HCT-116-derived and MCF-7 cells seeded on glass coverslips were silencing and 48 h after the end of the silencing procedure were fixed and permeabilized in PBS containing 2% paraformaldehyde and 1% Triton X-100 for 10 minutes at room temperature; after this process the cells were washed 3 times in PBS. For immunocytochemical staining, cells were treated with 1.5% H_2O_2 for 5 min in the dark in order to suppress endogenous peroxidase activity. After this the slips were washed in PBS. The slips were incubated for 30 minutes at room temperature in PBS

containing 1% bovine serum albumin (BSA) to block aspecific staining, then washed and incubated overnight with primary anti-p53 monoclonal antibody (1:150, clone BP53-12, Novocastra) and anti-pRb monoclonal antibody (1:150, clone 1F8; Lab Vision Corporation) diluted in PBS containing 1% BSA at 4°C in a humidified chamber. After overnight incubation, slips were washed in PBS and incubated first with a biotinylated secondary antibody (Vector Laboratoires) in PBS 1% BSA for 30 min, washed and then incubated with the streptavidin-peroxidase conjugate (Biospa) in PBS 1% BSA for 30 min. The streptavidin-peroxidase complex was visualized by dark incubation with diaminobenzydine DAB (Sigma-Aldrich) for 6 minutes. The reaction is blocked by immersing slides in H_2Od before to proceed with the dehydration and the assembly through sequential steps in 70% ethanol, 96%, 100% and xylene. The slides are mounted on the glass with Canada balsam (Sigma). The number of positive cells (and hence the proportion of cells in active progression in the S phase) is assessed at the microscope in 10 fields per sample using Image-Pro Plus software (Media Cybernetics).

5.11. Evaluation of cell population growth

The crystal violet is a substance of violet color able to bind to DNA and allows to assessment of cellular population growth *in vitro*.

For the evaluation of cell population growth inhibition after treatment with doxorubicin or 5FU-MTX cocktail, 40.000-100.000 cells, depending on cell type, were seeded in 12-well plates and drugs were given for 4 consecutive days, 2 h/day, starting 48 h after transfection for silenced cells. 24 h after the last drug treatment, treated and untreated cells were formalin-fixed for their quantitative growth

evaluation, which was carried out using the crystal violet assay as described in Carnero *et al.* (Carnero et al., 2000). Briefly, cells were washed twice with PBS, formalin-fixed overnight at 4°C, washed with distilled water and stained for 30 minutes with 0.1% Crystal Violet in a 20% methanol solution in agitation. Then they were washed 4 times in double-distilled water before, photographed, then resolubilized in 10% acetic acid solution, for 15 min at room temperature and quantified spectrophotometrically at 595 nm, in triplicate. The absorbance is proportional to the number of cells because it depends on the quantities of crystal violet bound to DNA.

5.12. Evaluation of cell death rate

Trypan blue is a vital stain obtained from toluidine that is absorbed by the macrophages of the reticuloendothelial system and is therefore used for staining cells to selectively color dead tissues or cells blue.

MCF-7 and HCT-116 cells either silenced for *RB1* or transfected with scrambled sequences were treated with 5-FU and MTX for 1 h. Twenty-four hours after the end of drug treatment, the floating cells in the medium of each flask were transferred to centrifuge tubes. After detachment of the adherent cells with trypsin, the cells were mixed with the corresponding floating cells before centrifugation. The cells were then stained with 0.4% trypan blue, and the numbers of trypan blue-positive and trypan blue-negative cells were counted on a hemocytometer by light microscopy. The experiments were carried out in triplicate.

5.13. Cell cycle progression analysis by dual-parameter flow cytometry

To define the effect of 5-FU and MTX treatment on cell cycle progression, the MCF-7 cell line was used. Dual-parameter flow cytometry for the simultaneous evaluation of DNA content and Bromodeoxyuridine (BrdUrd) incorporation was done. Asynchronously growing MCF-7 cells were either silenced for *RB1* expression or transfected with scrambled sequences. Seventy-two hours after the end of silencing procedure, BrdUrd was added at a final concentration of 20 µmol/L for 1 h, and then removed and fresh medium was added. Twelve hours later, cells were treated with 5-FU and methotrexate at doses of 10 and 0.05 µg/mL for 1 h. Cells were harvested 12 and 24 h later. Untreated cells were used as control. Cells were collected by centrifugation and fixed in 70% alcohol. Dual-parameter flow cytometry was done by a direct labeling of incorporated BrdUrd by FITC monoclonal antibody followed by propidium iodide-DNA counterstaining (Mazzini et al., 1996). Cytofluorimetric analyses were carried out in triplicate. Measurements were done by means of a Partec PAS II flow cytometer equipped with dual excitation system (argon ion laser and HBO100Warc lamp). The 488-nm blue line of the laser has been used to excite propidium iodide intercalated into the DNA and the FITC bound to BrdUrd. A preliminary instrument alignment and control has always been set up (with rat thymocytes stained with propidium iodide) to assure best instrumental analytic performances. Immediately before measurement, each sample has been filtered by ‘‘Filcons’’ 100 (ConsulTS) to remove cell clusters. For a sample measurement, a minimum of 20,000 events was acquired. The green (BrdUrd-FITC) and red (DNA-propidium iodide) fluorescence emission bands were collected, converted, and stored as DNA distribution values (histogram) or dual-parameter correlated dot plots by

means of a dedicated computer integrated into the instrument. Data were elaborated and plotted thanks to the ‘‘Flow Max’’ software installed in the computer. Cell cycle analyses and the relative statistical data (coefficient of variation of the DNA distributions) were done by means of a dedicated software.

5.14. Effect of drug treatment on p53 activation and DNA double-strand breaks accumulation

MCF-7 cells silenced for *RB1* and transfected with scrambled sequences were used 48 h after the end of the transfection procedure. Cells were treated for 1 h with the 5-FU and MTX and harvested 6, 12, and 24 h after the end of treatment, along with an untreated control sample for every condition. The experiments were conducted in triplicate. Proteins were extracted for Western blot analysis as described above.

5.15. Statistical analysis

Differences between groups were evaluated by Student’s t -test. Comparison of proportions between groups was assessed using the two-sample Z-test of proportions. Disease-free survival (DFS) curves were generated using the Kaplan–Meier method and compared using the log-rank test. Multivariate analyses for DFS were performed by applying the Cox proportional hazards regression model. All statistics were obtained using the SPSS statistical software package (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA). $p<0.05$ was regarded as statistically significant.

5.16. RNAi sequences

RB1: RB1-HSS109090 Fw UCAAGAUUCUGAGAUGUACUUCUGC
RB1-HSS109090 Rev GCAGAAGUACAUCUCAGAAUCUUGA
RB1-HSS109091 Fw AUAAAGGUGAAUCUGAGAGCCAUGC
RB1-HSS109091 Rev GCAUGGCUCUCAGAUUCACCUUUAU
RB1-HSS109092 Fw UUCAGUCUCUGCAUGAAGACCGAGU
RB1-HSS109092 Rev ACUCGGUCUUCAUGCAGAGACUGAA

TP53: TP53 RNAi-1 Fw CCAUCCACUACAACUACAUGUGUAA
TP53 RNAi-1 Rev UUACACAUGUAGUUGUAGUGGAUGG
TP53 RNAi-2 Fw CCAGUGGUAAUCUACUGGGACGGAA
TP53 RNAi-2 Rev UUCCGUCCCAGUAGAUUACCACUGG

p16INK4a: p16INK4a RNAi Fw 5'-CGCACCGAAUAGUUACGGUTT-3'
P16INK4a RNAi Rev 5'-ACCGUAACUAUUCGGUGCGTT-3'.

6. RESULTS

6.1. The p53-mediated sensitivity of cancer cells to chemotherapeutic agents is conditioned by status of the pRb protein

6.1.1. Assessment of pRb and p53 status

We analyzed 518 consecutive patients who underwent surgical resection for primary invasive breast carcinomas.

The pRb status was assessed by immunohistochemistry by evaluating the percentage either of cells with phosphorylated pRb (using a mAb antibody which specifically recognizes the phosphorylated pRb form) or of cells exhibiting total pRb (using a mAb antibody which recognizes all form of pRb) as described in detail in Material and Methods. We could distinguish three pRb forms: pRb underphosphorylated, pRb hyperphosphorylated, pRb deleted (Figure 15).

The p53 status was evaluated by measuring the percentage of immunostained nuclei (p53-LI) as described in Material and Methods and we distinguished two p53 forms: p53 normal or wild-type (wt) and p53 alterated (Figure 16). Since there is evidence that a series of local cell injuries may occur in tumour tissues causing wild-type p53 stabilization, in order to identify the cases really characterized by the presence of mutated p53, we also evaluated the expression of p21, target of activated p53, in the p53-accumulating tumours. In fact, p53-positive tumours, which also express p21, might be not characterized by mutated p53 (Nenutil et al, 2005).

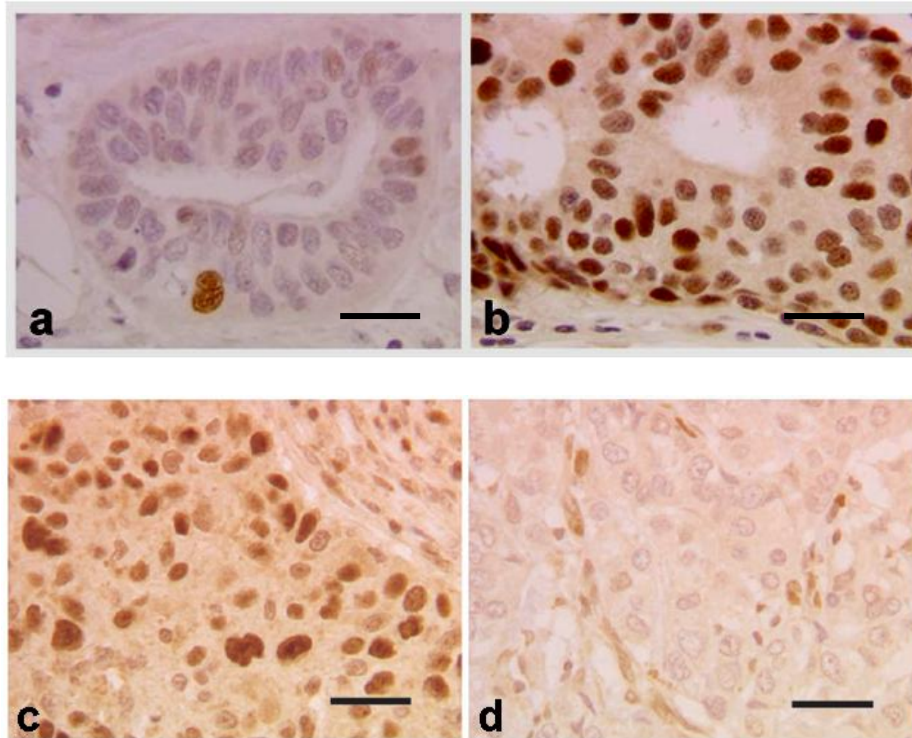


Figure 15: pRb immunostaining of breast cancers. a-b): pRb immunostaining using pRb monoclonal antibody which specifically recognizes the phosphorylated pRb form and (a) is indicative of low positivity for ppRb, these cancers are analyzed also for pRb total form (b) is positive for ppRb form, these cancers are considered with ppRb. c-d): pRb immunostaining using pRb antibody which recognized the total pRb protein. (c) is indicative of normal expression of pRb, (d) is indicative of a presumable *RB1* gene deletion. In both cases, stromal cells -considered as positive internal controls- are clearly immunostained. Bar, 25 µm

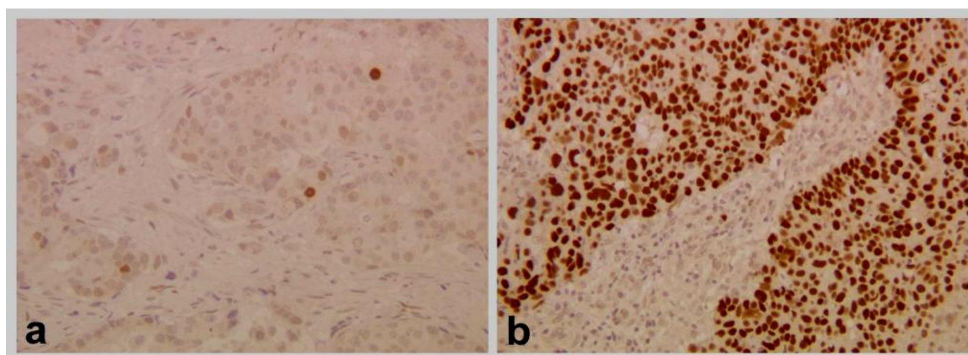


Figure 16. p53 expression of breast cancers. a-b): Two carcinomas were shown after p53 immunostaining. Note the absence of expression reported in (a), representing a wild-type expression of p53, and the p53 accumulation in (b), representing a p53 alteration

The p21 expression was evaluated in the p53-positive tumours by measuring the percentage of immunostained nuclei (p21-LI). Fourteen p53-positive tumours were found to be characterized by a p21-LI>10% and were therefore excluded from the group of p53 putatively-mutated tumours and not considered for the statistical analyses. Among the 518 patients, in this study we considered only the 145 patients treated with CMF chemotherapy. All the features of population enrolled are reported in Table 3.

Variable	n (%)
Age	
< 50%	63 (43.4)
≥ 50%	82 (56.6)
Histological diagnosis	
ductal carcinomas	132 (91)
lobular carcinomas	7 (4.8)
medullary carcinomas	1 (0.7)
mucoid carcinomas	3 (2.1)
sarcomatoid carcinomas	2 (1.4)
Tumour size	
pT1	78 (53.8)
pT2	48 (33.1)
pT3	7 (4.8)
pT4	12 (8.3)
Histological grade	
G1	15 (10.3)
G2	37 (25.5)
G3	93 (64.1)
N-status (*)	
N0	38 (26.8)
N+	104 (73.2)
ER-LI	
< 10%	58 (40)
≥ 10%	87 (60)
PGR-LI	
< 10%	93 (64.1)
≥ 10%	52 (35.9)
HER2-status	
negative	69 (47.6)
positive	76 (52.4)
Ki67-LI	
< 20 %	44 (30.3)
≥ 20 %	101 (69.7)
pRb status	
loss	16 (11)
under- phosphorylated	85 (58.6)
hyper-phosphorylated	44 (30.3)
p53-LI	
< 10 %	96 (66.2)
≥ 10 %	49 (33.8)

Table 3. Clinical and histopathological characteristics of the enrolled population, treated with chemotherapy

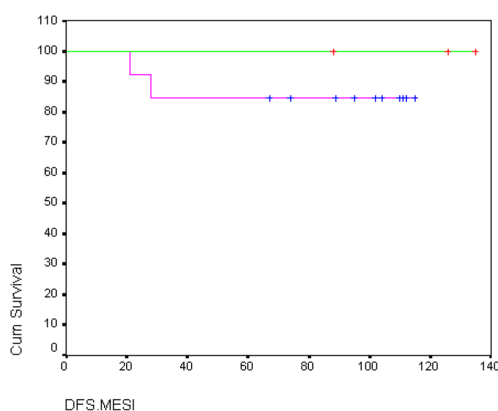
6.1.2. Relationship between p53 and pRb in tumor prognosis

We assessed the prognostic relevance of p53 in the whole series and in patients according to the pRb status, treated with chemotherapy (Table 4; Figure 17, 18 and 19).

Treatment	Cases (n)	DFS (%)	$\chi 2$	p
Whole series				
p53-LI <10%	96	57.29	0.97	= 0.3259
p53-LI ≥10% *	35	48.57		
Patients with pRb loss				
p53-LI <10%	3	100	0.57	= 0.4492
p53-LI ≥10% *	11	81.82		
Patients with underphosphorylated pRb				
p53-LI <10%	65	61.54	6.63	**= 0.0100**
p53-LI ≥10% *	16	31.25		
Patients with hyperphosphorylated pRb				
p53-LI <10%	28	42.86	0.75	= 0.6246
p53-LI ≥10% *	8	37.50		

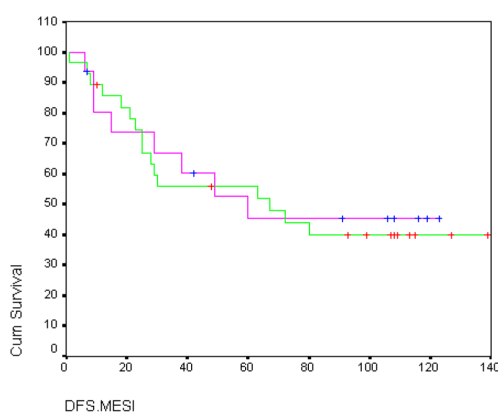
Table 4. Prognostic relevance of p53 in the whole series and in patients considered according to the pRb status, investigated by the log-rank test. * Cases with a p21-LI >10% were excluded from this group

Univariate analysis of DFS indicated that in the whole patient's series, in patients with pRb loss (Figure 17) and with hyperphosphorylated pRb (Figure 18) the p53 variable was not associated with the clinical outcome; the only association of p53 with prognosis was in patients with normally functioning pRb pathway (Figure 19).



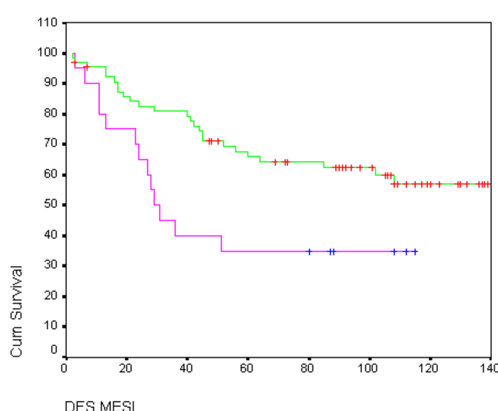
	Total	Number Events	Number Censored	Percent Censored	Significance
p53 wt	**3**	**0**	**3**	**100,00**	
p53 mutated	**13**	**2**	**11**	**84,62**	
Overall	**16**	**2**	**14**	**87,50**	**0,4881**

Figure 17. Disease-free survival curves (Kaplan–Meier estimates) in patients with pRb loss, according to p53 status, treated with adjuvant chemotherapy



	Total	Number Events	Number Censored	Percent Censored	Significance
p53 wt	**28**	**16**	**12**	**42,86**	
p53 mutated	**16**	**8**	**8**	**50,00**	
Overall	**44**	**24**	**20**	**45,45**	**0,8314**

Figure 18. Disease-free survival curves (Kaplan–Meier estimates) in patients with hyperphosphorylated pRb, according to p53 status, treated with adjuvant chemotherapy



	Total	Number Events	Number Censored	Percent Censored	Significance
p53 wt	65	25	40	61,54	
p53 mutated	20	13	7	35,00	
Overall	85	38	47	55,29	0,0142

Figure 19. Disease-free survival curves (Kaplan–Meier estimates) in patients with normally function of pRb, according to p53 status, treated with adjuvant chemotherapy

Then, in this group, a multivariate analysis of DFS, including the histopathological variables associated with the clinical outcome such as tumor size, histopathological grade, node status, ER-, PR- and Ki67-LI, and HER2, demonstrated that p53 status was the only factor significantly associated with the DFS (Table 5). Also, without correcting the definition of the p53 status by the evaluation of p21 expression, p53-LI >10% was associated with a worse clinical outcome in the univariate analysis of DFS (p=0.0142) and the p53 status was found to be the only factor significantly associated with patient clinical outcome in the multivariate analysis of DFS (p=0.0190; data not shown).

variable	Patients treated with chemotherapy	
	hazard ratio (95% CI)	**p-value**
p53-LI		
< 10%	1.00	
≥ 10%	3.02 (1.30–7.01)	= **0.0099**
Tumor size		
pT1	1.00	
pT2	1.72 (0.74–3.99)	= 0.2047
pT3 + pT4	1.93 (0.67–5.55)	= 0.2194
Histological grade		
G1	1.00	
G2	1.37 (0.27–6.85)	= 0.6975
G3	1.79 (0.32–9.96)	= 0.5061
N-status		
N0	1.00	
N+	1.92 (0.66–5.51)	= 0.2250
ER status (LI)		
≥ 10%	1.00	
< 10%	0.87 (0.33–2.29)	=0.7792
PR status (LI)		
≥ 10%	1.00	
< 10%	0. 95 (0.45–2.02)	= 0.9109
HER2-status		
negative	1.00	
positive	1.70 (0.81–3.55)	=0.1562
Ki67-LI		
< 20%	1.00	
≥ 20%	1.51 (0.62–3.66)	=0.3618

Table 5. Prognostic relevance of p53 in patients with cancer with normally functioning pRb pathway: multivariate DFS analysis

6.1.3. Evaluation of p53-mediate chemosensitivity and pRb pathway status in cancer cells

In order to demonstrate the relevance of the pRb pathway status in the p53-mediated sensitivity to chemotherapeutic agents, we studied the response to the drugs used in breast cancer chemotherapy in human cancer cell lines with either wild-type or abrogated p53 function, where the function of pRb was down-regulated either inducing a pRb loss either a pRb hyperphosphorylation. Two methods were used to inhibit p53 activity:

- interference with siRNAs specific for *TP53* mRNA expression in MCF-7 and HepG2 cells;

- inducement of the expression of an inactive truncated, dominant negative form of murine p53 (p53DD) in HCT-116 cells.

The level of *TP53* mRNA was evaluated by Real-Time RT-PCR and it was strongly reduced in MCF-7 and HepG2 cells at 48 h after the interference procedure and remained very low up to 120 h (Figure 20 a, b, upper). In order to evaluate the effect of *TP53* mRNA interference on both p53 expression in MCF-7 and HepG2 cells, we exposed these cells to doxorubicin and p53 expression was measured by Western blot analysis. *TP53*-silenced cells did not show any accumulation of p53, unlike control cells (Figure 20 a, b, lower).

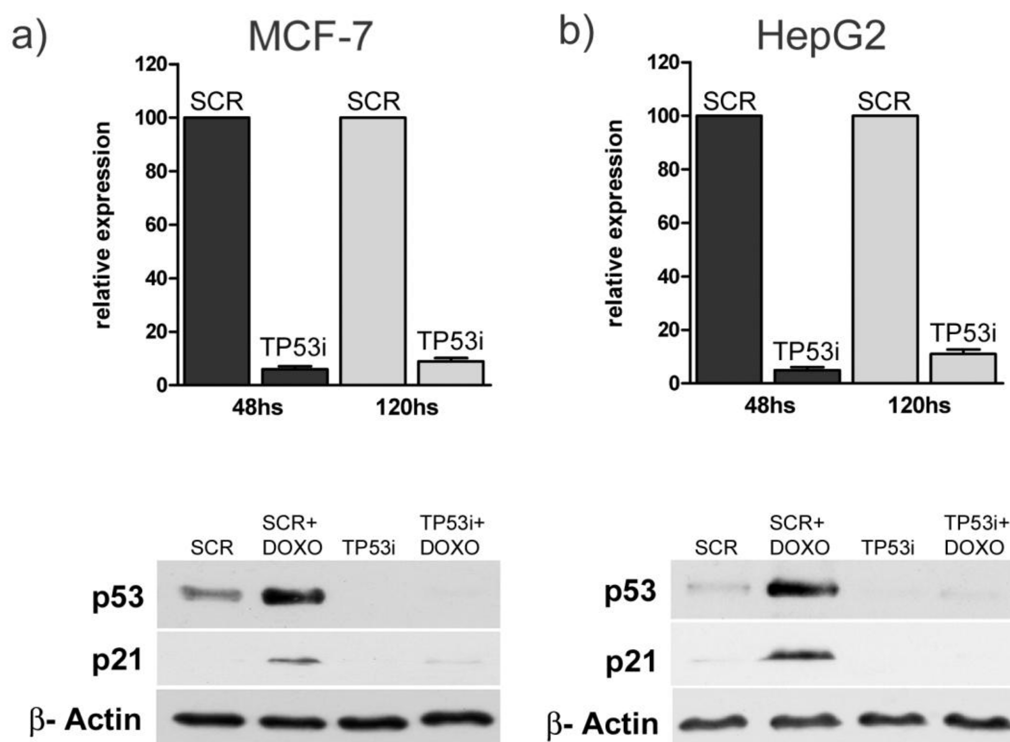


Figure 20. p53 inactivation in MCF-7 and HepG2 cells. MCF-7 (a) and HepG2 (b) cells were silenced for p53 expression by RNA interference. *TP53* mRNA level was evaluated in cells transfected with control scrambled sequences (SCR) and in cells silenced for p53 (TP53i), at 48 and 120 h after the end of the silencing procedure. Note the high reduction of *TP53* mRNA in *TP53*-silenced cells at both evaluation times. Histograms show the values (mean ± SD) of three independent experiments. Representative Western blots of p53 and p21 expression in controls and *TP53*-silenced MCF-7 (Figure 20a) and HepG2 (Figure 20 b) cells, 48 h after the end of the silencing procedure, show the absence of p53 and p21 accumulation after doxorubicin treatment in *TP53*-silenced cells (TP53i), as compared to cells transfected with control scrambled sequences (SCR). The expression of *β*-actin was used as a control

To check the activity of p53 in the p53DD HCT-116 cells, in which the truncated form of p53 induced an accumulation of inactive protein, we evaluated the expression of p53 by p53 immunocytochemical staining (Figure 21, upper) and the expression of p21, the target of p53, by Western blot analysis after either 5-FU plus MTX or doxorubicin treatment. p21 was expressed only in pBABE, not in p53DD

HCT-116 cells, demonstrating that both methods were effective to abolishing p53 activity (Figure 21, lower).

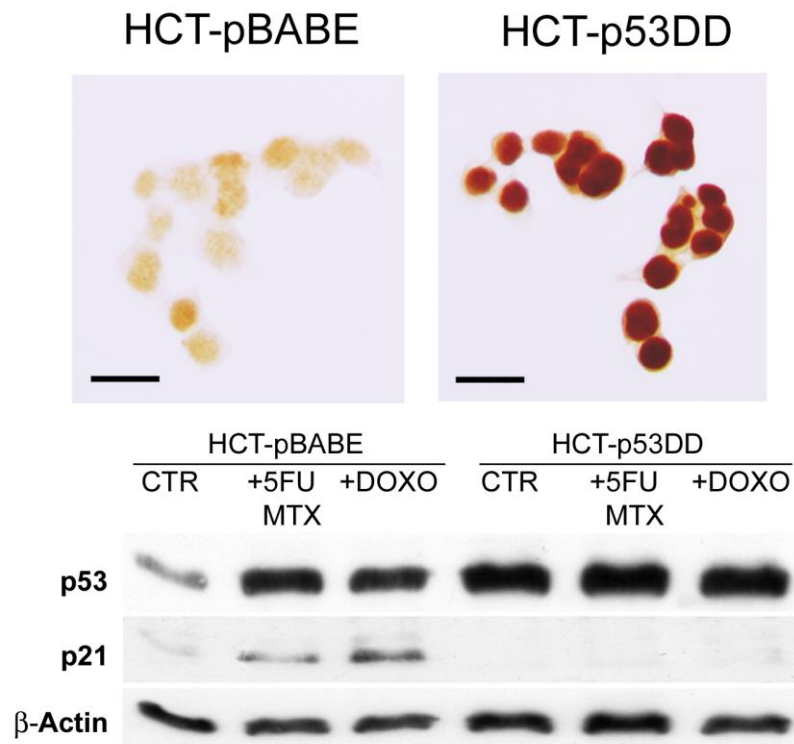


Figure 21. p53 inactivation in HCT-116 cells. p53 inactivation in HCT-116 cells was induced by expressing a truncated, dominant-negative form of p53 (p53DD). p53 immunocytochemical staining exhibits a more intense nuclear positivity in cells expressing p53DD as compared to cells transduced with appropriate control sequences (pBABE). Bar = 25 μm. Representative Western blots of p53 and p21 expression in pBABE and p53DD HCT-116 cells treated with either 5-FU plus MTX or doxorubicin show the absence of p21expression in p53DD HCT-116 cells, as compared to pBABE HCT-116 cells

To down-regulate the pRb function, we silenced *RB1* in MCF-7 and HCT-116 cells, while we induced pRb hyperphosphorylation by *p16INK4a* silencing in HepG2 cells, because in MCF-7 and HCT-116 cells the *p16INK4a* gene was not expressed (Musgrove et al., 1995; Myöhänen et al., 1998). The effect of *RB1* silencing in MCF-7 and HCT-116 pBABE and p53DD cells was evaluated by both Real Time-RT PCR

and by Western blot analysis; 48 and 120 h after the RNA interference procedure, a strong reduction of *RB1* mRNA expression occurred in both cell lines (Figure 22 a). Western blot analysis for pRb expression confirmed that 48 h after the *RB1* silencing procedure, the level of pRb was markedly reduced compared to control samples in both cell lines (Figure 22 b).

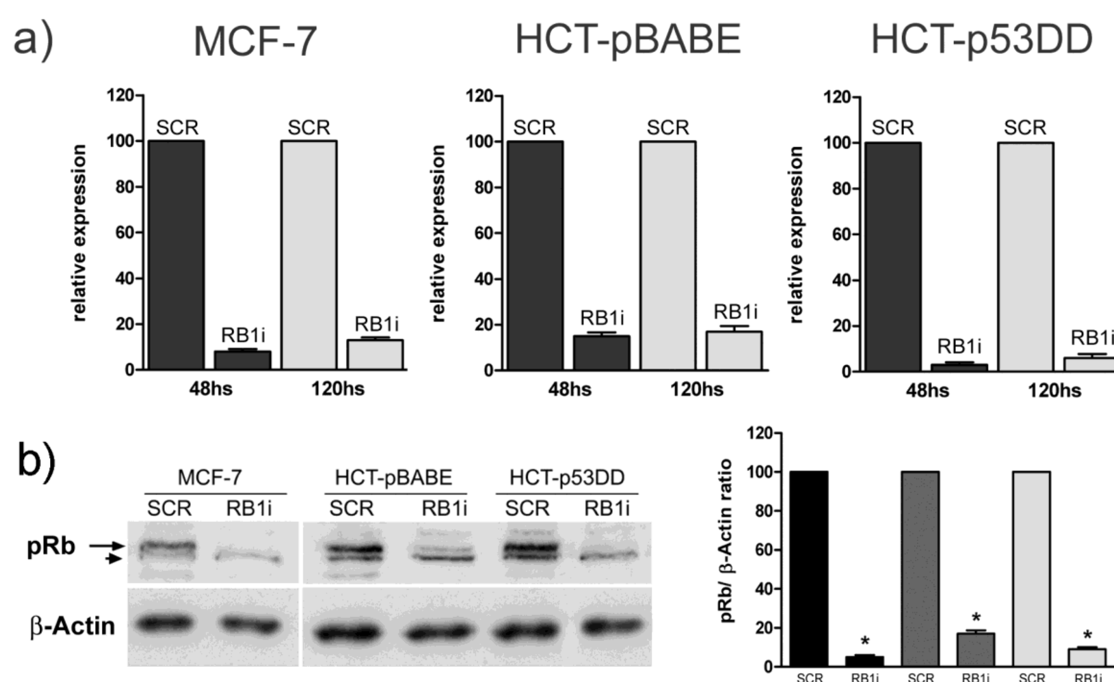


Figure 22. pRb inactivation in MCF-7and HCT-116 cells. a,b) MCF-7 and HCT-116 (pBABE and p53DD) cells were silenced for *RB1* expression by RNA interference. a) *RB1* mRNA level was evaluated in cells transfected with control scrambled sequences (SCR) and in cells silenced for *RB1* (RB1i) at 48 and 120 h after the end of the silencing procedure. Note the high reduction of *RB1* mRNA in *RB1*-silenced cells at both evaluation times. Histograms show the values (mean ± SD) of three independent experiments. b) Representative western blots of pRb expression in MCF-7, pBABE HCT-116 and p53DD HCT-116 cells, silenced for *RB1* expression, 48 h after the end of the silencing procedure, show the strong reduction of pRb expression in *RB1*-silenced cells (RB1i) as compared to cells transfected with control scrambled sequences (SCR). pRb is indicated by the pointer, and the background staining is indicated by an arrowhead. The expression of *β*-actin was used as a control. The histogram shows the densitometric values (mean ± SD) of three independent experiments. Each value concerns the pRb/*β*-actin ratio, which was set to 100, in untreated cells transfected with scrambled sequences. *$p < 0.05$

The silencing of *p16INK4a* was also confirmed by these two techniques. 48 h after the interference procedure, the *p16INK4a* mRNA level was very low and the expression of p16INK4a protein was strongly reduced, while the amount of phosphorylated pRb was increased as compared to control, in HepG2 cells (Figure 23 a, b).

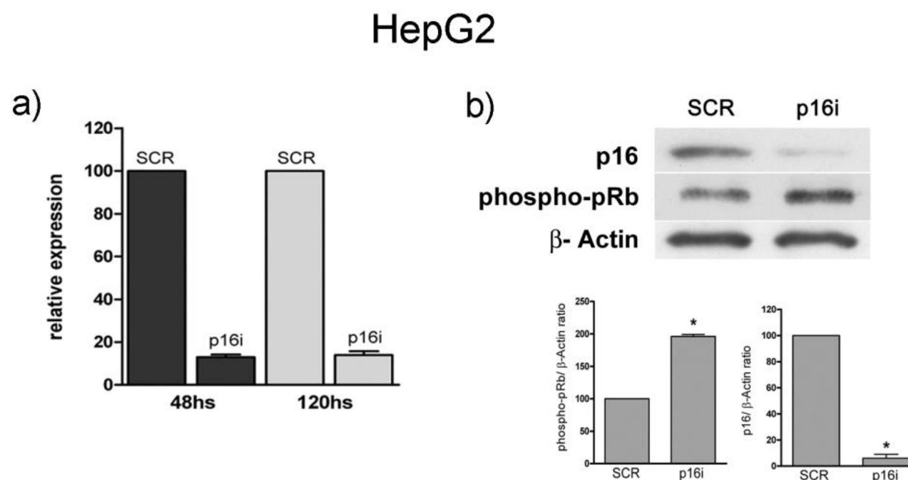


Figure 23. pRb inactivation in HepG2 cells. a, b) HepG2 cells were silenced for p16INK4a expression by RNA interference. a) *p16INK4a* mRNA level was evaluated in cells transfected with control scrambled sequences (SCR) and in *p16INK4a*-silenced cells at 48 and 120 h after the end of the silencing procedure (p16i). Note the high reduction of *p16INK4a* mRNA in *p16INK4a*-silenced cells at both evaluation times. Histograms show the values (mean ± SD) of three independent experiments. b) Representative western blots of p16 and phosphorylated pRb expression in controls (SCR) and *p16INK4a*-silenced (p16i) HepG2 cells, 48 h after the end of the silencing procedure, show a high reduction of p16 expression together with the increased level of phosphorylated RB in *p16INK4a*-silenced cells as compared to control HepG2 cells. The expression of *β*-actin was used as a control. The histogram shows the densitometric values (mean ± SD) of three independent experiments. In each analysis, values concern the protein:*β*-actin ratio, which was set to 100, in untreated cells transfected with scrambled sequences (SCR). *$p < 0.05$

To assess whether the drug treatment could affect the pRb phosphorylation, after *p16INK4a*-silencing, we evaluated, by Western blot analysis, the expression of p53,

p21 and phospho-pRb in control (SCR) or *p16INK4a*-silenced HepG2 cells treated with doxorubicin for 8 hours (Figure 24). We confirmed that the drug treatment induced a reduction of phospho-pRb protein level in similarly to not interfered cells.

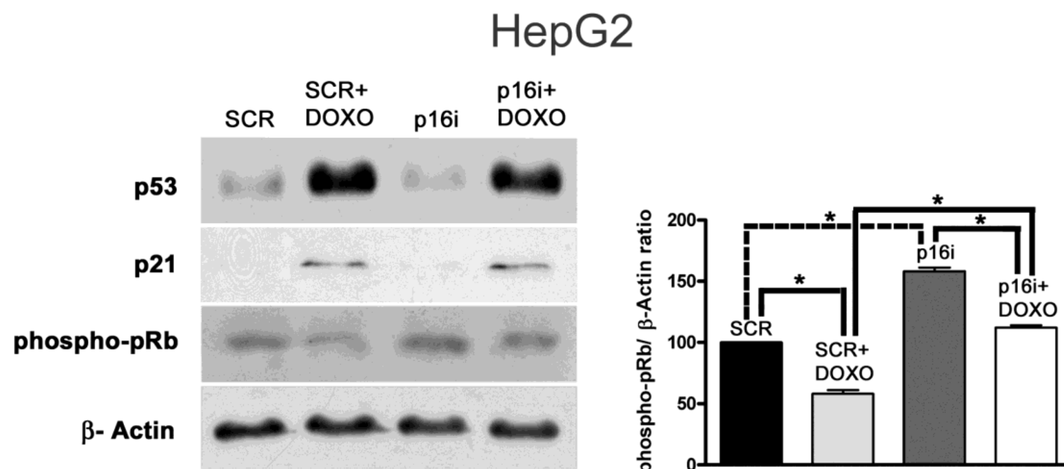


Figure 24. Effect of p53 stabilization on p21 and phosphorylated pRb expression in *p16INK4a*-silenced HepG2 cells. Cells were treated with Doxorubicin for 8 hours to induce p53 stabilization. Representative Western blots of p53, p21 and phosphorylated pRb expression in controls and *p16INK4a*-silenced HepG2 cells, 48 hours after the end of the silencing procedure, show that the drug treatment induced an increased level of p53 and p21 expression in controls (SCR) and *p16INK4a*-silenced (p16i) cells, as compared to untreated cells. Both in control and in *p16INK4a*-silenced cells, Doxorubicin reduced the level of phosphorylated pRb expression as compared to the respective untreated cells. Nevertheless, the expression of phosphorylated pRb after drug treatment appeared to be at the same level in *p16INK4a*-silenced cells as in control, untreated cells. The expression of β actin was used as a control. The histogram shows the densitometric values (mean ± S.D.) of three independent experiments. In each analysis, values concern the ppRb/β-actin ratio, which was set to 100, in untreated cells transfected with scrambled sequences. Statistical significance ($p<0.05$) is indicated (*)

We then investigated the long-term effect of 5-FU plus MTX and of doxorubicin treatment on the cell population growth in controls and p53-deficient MCF-7 and HCT-116, either silenced or not for *RB1* expression, and in controls and p53-deficient HepG2 cells, either silenced or not for *p16INK4a* expression.

In MCF-7 cells, the 5-FU plus MTX, but also the doxorubicin treatment, significantly reduced the cell population growth in control *TP53*-silenced and *RB1*-silenced cells ($p<0.01$). In cells silenced for both *TP53* and *RB1* expression, the drug treatments induce a growth rate reduction that was significantly greater than caused in *TP53*-silenced cells alone ($z=3.300$; $p<0.001$) and not significantly differ from that of control cells (Figure 25).

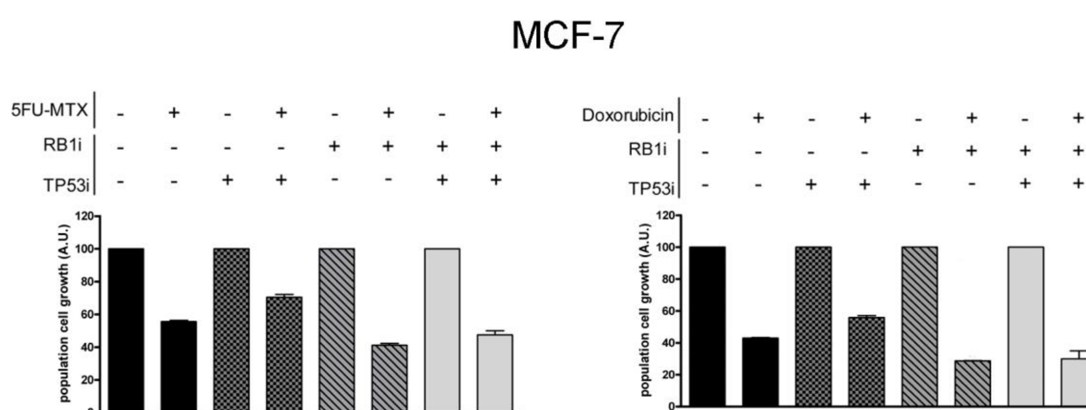


Figure 25. Effect of *TP53*- and *RB1*-silencing on the growth rate of MCF-7 cells exposed to chemotherapeutic agents. The cells were exposed to either 5-FU plus MTX or doxorubicin for 2 h daily for 4 consecutive days, and 24 h after the last treatment were formalin-fixed for the crystal violet assay for growth rate evaluation. Values relative to samples not treated with drugs were normalized to 100. Cells were silenced for either *RB1* (RB1i+) or *TP53* (TP53i+) expression or for both tumour suppressors. Cells transfected with scrambled sequences were used as controls (RB1i−, TP53i−). (Left panel) 5-FU plus MTX treatment strongly hindered the proliferation of controls and *RB1*-silenced cells, and to a lesser extent that of *TP53*-silenced cells. After drug treatment, cells with both tumour suppressors silenced had a growth rate significantly lower than that of cells silenced for p53 expression alone. Also, doxorubicin treatment (right panel) strongly reduced the proliferation rate of control and *RB1*-silenced cells. The drug significantly hindered the proliferation rate of *TP53*-silenced cells, although to a lesser extent than in control and *RB1*-silenced cells. After drug exposure, the proliferation rate of cells silenced for both tumour suppressors was significantly lower than that of cells silenced for p53 expression alone

The result obtained in HCT-116 cells were similar: 5-FU plus MTX treatments significantly reduced the growth rate in control and to greater extent of *RB1*-silenced cells, but in p53DD cells, the drug treatments did not significantly reduce the growth rate. In *RB1*-silenced p53DD cells, after the drug treatment, the cell growth reduction was significantly greater than that in p53DD cells (z=4.591; p<0.001), being similar to that induced in control cells. The effect of doxorubicin treatment on the cell population growth rate of HCT-116 cells was similar to those obtained using 5-FU plus MTX (Figure 26).

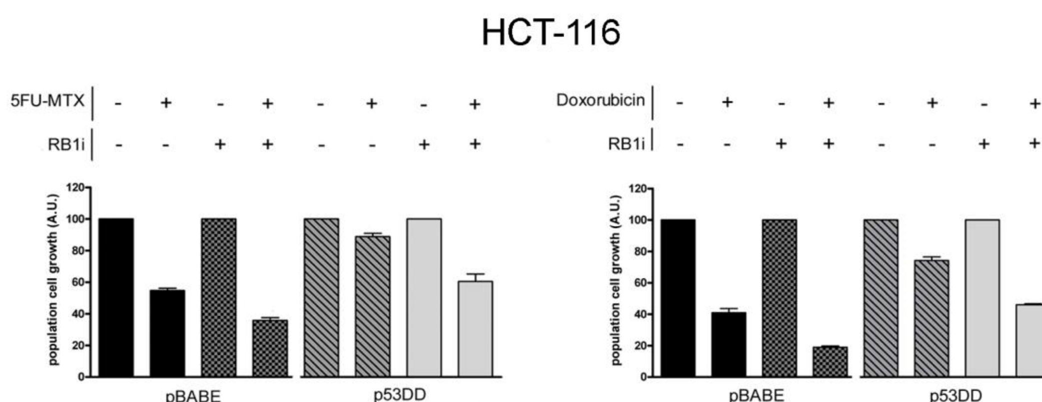


Figure 26. Effect of p53 and pRb inactivation on the growth rate of pBABE and p53DD HCT-116 cells exposed to chemotherapeutic agents. The cells were exposed to either 5-FU plus MTX or doxorubicin for 2 h daily for 4 consecutive days, and 24 h after the last treatment were formalin-fixed for the crystal violet assay for growth rate evaluation. Values relative to samples not treated with drugs were normalized to 100. Effect of 5-FU plus MTX (left panel), and doxorubicin (right panel) treatment on cell population growth of pBABE and p53DD HCT-116 cells, whether or not silenced for pRb expression. Cells harboring the truncated form of p53 (p53DD) were significantly less sensitive to the drugs than pBABE cells. *RB1* interference (RB1i+) increased the sensitivity to the drugs in pBABE and p53DD cells. After *RB1* interference, both drug treatments reduced the growth rate of p53DD cells to the same level as that of control pBABE cells

The HepG2 cells were treated only with doxorubicin, as a consequence of their low sensitivity to 5-FU plus MTX. The drug significantly reduced the growth rate of

HepG2 cells independently of TP53 and p16INK4a expression ($p<0.001$). After doxorubicin treatment, the difference between the growth rate of cells silenced for both *TP53* and *p16INK4a* expression and cells silenced for *p16INK4a* alone was significantly lower than that observed between control and *TP53*-silenced HepG2 cells ($z=7.720; p<0.001$) (Figure 27).

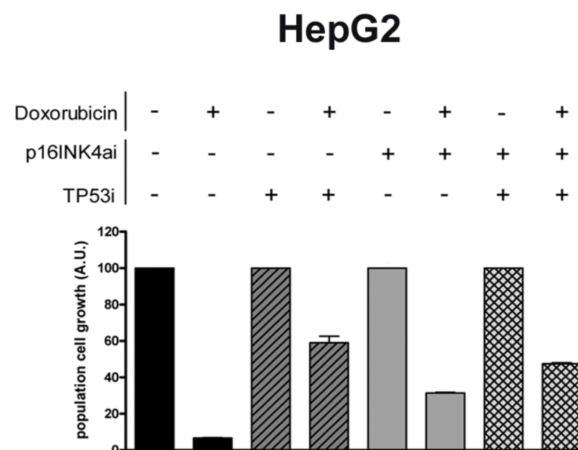


Figure 27. Effect of p53 and pRb inactivation on the growth rate of HepG2 exposed to doxorubicin. The cells were exposed to doxorubicin for 2 h daily for 4 consecutive days, and 24 h after the last treatment were formalin-fixed for the crystal violet assay for growth rate evaluation. Values relative to samples not treated with drugs were normalized to 100. Doxorubicin greatly reduced the growth rate of control and, to a much lesser extent, of *TP53*-silenced HepG2 cells (TP53i+). The drug exposure also reduced the growth rate of *p16INK4a*-silenced cells alone (p16INK4ai+) and of cells silenced for both *p16INK4a* and *TP53* expression. On the other hand, the difference in drug sensitivity between the cells silenced for *p16INK4a* alone and cells silenced for both *TP53* and *p16INK4a* expression was significantly lower than that observed between control and *TP53*-silenced HepG2 cells. The histograms show the values (mean ± SD) of three independent experiments

6.2. Loss of pRb protein makes human breast cancer cells more sensitive to antimetabolites exposure

6.2.1. Immunohistochemical definition of pRb status and determination of its prognostic value in a large series of primary breast cancer patients

We studied 518 consecutive patients who underwent surgical resection for primary invasive breast carcinomas. The pRb status was assessed by immunohistochemistry as described in Material and Methods. The cases regarded as *RB1* deleted (31 cases) were included in the RB negative (RB-) group; the remaining 487 cases were included in the RB positive (RB+) group (Table 6).

variable	n (%)
Age	
< 50%	117 (22.6)
≥ 50%	401 (77.4)
Histological diagnosis	
ductal carcinomas	451 (87.1)
lobular carcinomas	44 (8.5)
medullary carcinomas	16 (3.1)
mucoid carcinomas	4 (0.8)
sarcomatoid carcinomas	3 (0.6)
Tumor size	
pT1	323 (62.4)
pT2	142 (27.4)
pT3	13 (2.5)
pT4	40 (7.7)
Histological grade	
G1	59 (11.4)
G2	339 (65.4)
G3	120 (23.2)
N-status (*)	
N0	275 (53.8)
N+	237 (46.2)
ER-LI	
< 10%	123 (23.7)
≥ 10%	395 (76.3)
PGR-LI	
< 10%	280 (54.1)
≥ 10%	238 (45.9)
HER2-status	
negative	331 (65.0)
positive	178 (35.0)
Ki67-LI	
< 20 %	277 (53.5)
≥ 20 %	241 (46.5)
RB status	
deleted	31 (6.0)
under- phosphorylated	406 (78.4)
hyper-phosphorylated	81 (15.6)
p53-LI	
< 10 %	407 (78.6)
≥ 10 %	111 (21.4)
Adjuvant therapy	
none	93 (18.0)
radiotherapy	49 (9.5)
endocrine therapy alone	231 (44.6)
chemotherapy	145 (28.0)

Table 6. Clinical and histopathological characteristics of the enrolled population. (*) N-status was available for 511 cases since, due to patient age, axillary dissection was not performed in 7 patients

6.2.2. Prognostic value of pRb expression and phosphorylation

We evaluated the prognostic effect (univariate DFS analysis) of the pRb protein expression and phosphorylation in the whole series of patients and in patients who

received chemotherapy (145 cases). In the whole series, the pRb protein expression (RB+ or RB-) did not show an significant correlation with prognosis, whereas it became a significant predictor of DFS in patients treated with chemotherapy (table 7). In fact the absence of pRb expression was associated with a better clinical outcome in patients treated with chemotherapy.

factor	whole series of patients (n = 518)			patients treated with chemotherapy (n = 145)		
	No patients	hazard ratio (95% CI)	p-value	No patients	hazard ratio (95% CI)	p-value
pRb expression						
RB-	31	1.00		16	1.00	
RB+	487	0.79 (0.43 – 1.47)	= 0.469	129	5.10 (1.24 –20.86)	= **0.023**
ppRb LI						
< 25%	406	1.00		94	1.00	
≥ 25%	81	1.95 (1.34 – 2.85)	**< 0.001**	35	1.44 (0.84 – 2.45)	= 0.178

Table 7. Univariate analysis of the pRb and ppRb variables for DFS applied to the whole series of cases and to patients treated with chemotherapy Abbreviation: 95% CI, 95% confidence interval

To evaluate the relationship between the pRb phosphorylation and the patient clinical outcome, the ppRb LI variable was analyzed. The ppRb variable was significantly associated with DFS in the whole series, whereas it did not significantly in patients receiving chemotherapy (Table 7). These results indicated that the lack of pRb and not its inactivation by phosphorylation represented a predictive variable of DFS in patients who received chemotherapy.

6.2.3. The absence of pRb expression is the only predictive factor of good clinical outcome in patients treated with adjuvant chemotherapy

We have further investigated the relationship between pRb expression and the clinical outcome in these two groups of patients (RB- and RB+), considering the possibility that the significant predictive effect of pRb found for chemotherapy-

treated patients might be related to other clinical and histopathologic variables associated with the clinical outcome that can confound the results of the statistical analysis. We compared the relative predictive value of these variables with that of pRb status in a multivariate analysis. The multivariate DFS analysis indicated that the absence of pRb expression resulted to be the only significant variable predicting the clinical outcome in patients treated with chemotherapy (Table 8).

variable	Patients treated with chemotherapy	
	hazard ratio (95% CI)	p-value
pRb expression RB- RB+	 1.00 5.56 (1.17-23.71)	 = **0.030**
p53-LI < 10% ≥ 10%	 1.00 1.49 (0.84 – 1.52)	 = 0.169
Tumor size pT1 pT2 pT3 + pT4	 1.00 0.84 (0.47 – 1.51) 1.11 (0.50 – 2.42)	 = 0.574 = 0.792
Histological grade G1 G2 G3	 1.00 1.01 (0.30 – 3.34) 1.47 (0.41 – 5.28)	 = 0.980 = 0.549
N-status N0 N+	 1.00 2.10 (0.95 – 4.60)	 = 0.063
ER status (LI) ≥ 10% < 10%	 1.00 1.00 (0.51 – 1.95)	 = 0.986
PR status (LI) ≥ 10% < 10%	 1.00 0.84 (0.46 – 1.52)	 = 0.569
HER2-status negative positive	 1.00 1.75 (0.97 – 3.14)	 = 0.061
Ki67-LI < 20% ≥ 20%	 1.00 1.24 (0.97 – 3.14)	 = 0.570

Table 8. Multivariate DFS analysis applied to patients treated with chemotherapy

Furthermore because the number of RB- patients treated with chemotherapy was low (n=16), we did a DFS analysis comparing the population of patients with RB- cancer with a population of patients with RB+ cancer exhibiting the same characteristics

(high histologic grade (G3), high ki67-LI (>30%), absence of ER) (Table 9). The RB- cancers were then matched with RB+ cancers according to these three variables. DFS analysis indicated that the pRb expression remained a highly predictive factor of a better clinical outcome (Figure 28).

variable	**whole series of cases treated with chemotherapy (n=145)**	**RB- cases treated with chemotherapy (n=16)**
	n (%)	**n (%)**
Age		
< 50%	63 (43.4)	11 (68.8)
≥ 50%	82 (56.6)	5 (31.2)
Histological diagnosis		
ductal carcinomas	132 (91)	16 (100)
lobular carcinomas	7 (4.8)	-
medullary carcinomas	3 (2.1)	-
mucoid carcinomas	2 (1.4)	-
sarcomatoid carcinomas	1 (0.7)	-
p53-LI		
< 10%	96 (66.2)	3 (18.8)
≥ 10%	49 (33.8)	13 (81.2)
Tumor size		
pT1	78 (53.8)	7 (43.8)
pT2	48 (33.1)	6 (37.5)
pT3 + pT4	19 (13.1)	3 (18.8)
Histological grade		
G1	15 (10.3)	-
G2	37 (25.5)	-
G3	93 (64.1)	16 (100)
N status		
N0	38 (26.2)	8 (50)
N+	104 (73.8)	8 (50)
ER status (LI)		
≥ 10%	58 (40.0)	16 (100)
< 10%	87 (60.0)	-
PR status (LI)		
≥ 10%	93 (64.1)	15 (96.8)
< 10%	49 (33.8)	1 (3.2)
HER2 status		
negative	70 (48.3)	11 (68.8)
positive	75 (51.7)	5 (31.2)
Ki67-LI		
< 20%	44 (30.3)	-
≥ 20%	101 (69.7)	16 (100)

Table 9. Multivariate DFS analysis applied to patients treated with chemotherapy

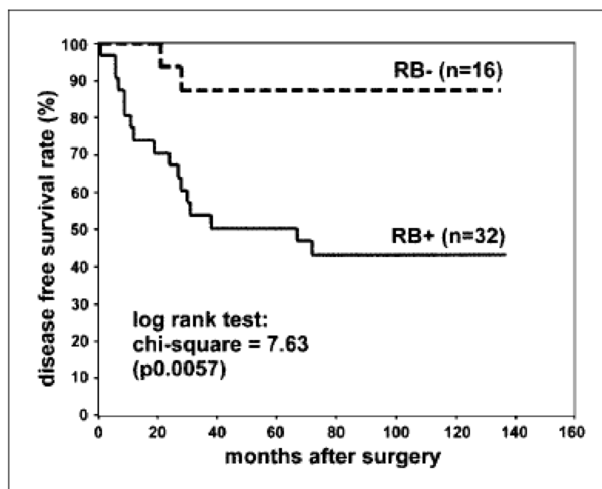


Figure 28. Effect of pRb expression on the clinical outcome of patients treated with chemotherapy according with the histologic grade, ki67 LI, ER status. DFS curves (Kaplan-Meier estimates) for patients treated with chemotherapy with reference to the pRb expression. The16 patients with cancer lacking pRb (RB-) showed a better clinical outcome when treated with chemotherapy in comparison with 32 patients with RB+ cancer, matched according to histologic grade, Ki67 LI, and ER status

6.2.4. 5-FU and MTX treatment hindered cell population growth of *RB1*-silenced MCF-7 and HCT-116 cells

To ascertain whether the better prognosis of pRb-deficient tumors treated with adjuvant chemotherapy might be the consequence of a higher sensitivity of pRb-deficient cells to the drugs used, we studied the response to the 5-FU plus MTX drugs in MCF-7 and HCT-116 cells, where the function of pRb was down-regulated by *RB1*-silencing. We evaluated the effect of *RB1* silencing on MCF7 (similar data were obtained using HCT-116 cells, data not shown) after 48 and 120 hours after the RNA interference procedure, both by Real Time RT-PCR, by immunocytochemistry and by Western blot analysis (Figure 29).

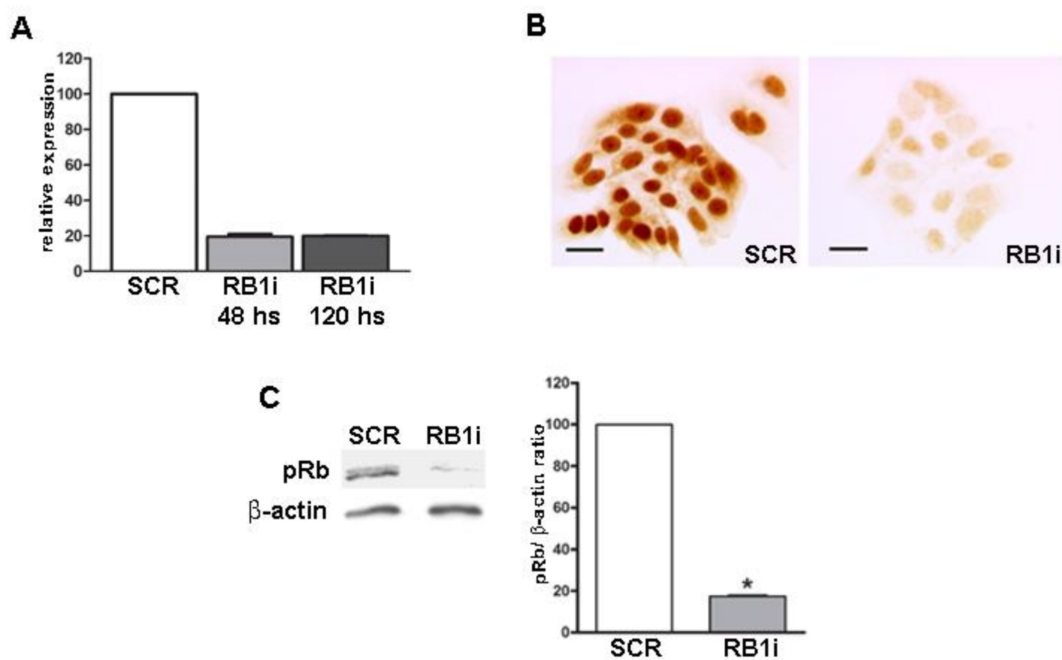


Figure 29. Effect of *RB1* interference on RB1 mRNA and pRb protein expression in MCF-7 cells. A) MCF 7 cells were silenced for *RB1* by RNA interference. *RB1* mRNA level in cells transfected with scrambled sequences (SCR) and in cells silenced for *RB1*at 48 and120 h after the end of the silencing procedure. Note the high reduction of *RB1* mRNA in *RB1*-silenced cells at both the times evaluated. B) immunocytochemical pRb staining: Cells transfected with scrambled sequences (SCR) showed an intense staining reaction, which was absent in cells silenced for *RB1* (RB1i) by 48h after the end of the silencing procedure. Bar, 25 μm. C) Western blot analysis of pRb expression in control (SCR) and *RB1*-silenced cells, 48 h after the end of the silencing procedure. Note the strong reduction of pRb expression in *RB1*-silenced cells (RB1i), in comparison with cells transfected with scrambled sequences (SCR).The expression of β-actin was used as a control. Histogram shows the densitometric values of three independent experiments. Columns, mean; bars, SD. Each value is relative to the pRb to β-actin ratio in untreated cells transfected with scrambled sequences (SCR), which was set to 100. *, $P < 0.05$, statistical significance

48h and 120 h after the RNA interference procedure, a strong reduction in *RB1* mRNA expression occurred (Fig. 29 A). Immunocytochemical analysis for pRb expression revealed that, as early as 48 hours after the *RB1* interference procedure, the intensity of the immunostaining was markedly reduced in comparison with

control samples (Fig. 29 B) and Western blot analysis confirmed the reduction of pRb expression (Fig. 29 C).

We investigate the long-term effect of 5-FU and MTX treatment on the cell population growth in control (SCR) and *RB1*-silenced MCF-7 and HCT-116 cells (Figure 30 A, B). The cell population growth of both MCF-7 and HCT-116 cells silenced for *RB1* was significantly hindered. On the contrary, regarding the control cells, the 5-FU and MTX treatment induced a not significant reduction in the MCF-7 cell population growth and no reduction at all in the HCT-116 cells. To investigate the reason for the reduced growth rate of *RB1*-silenced MCF-7 and HCT-116 cells after drug treatment, we also evaluated the cell death rate in these cells and in control cells 24 hours after the end of 5-FU and methotrexate exposure. We found that the drug treatment was responsible for a significantly greater mortality in *RB1*-silenced MCF-7 and HCT-116 cells than in control cells (Figure 30 B, D).

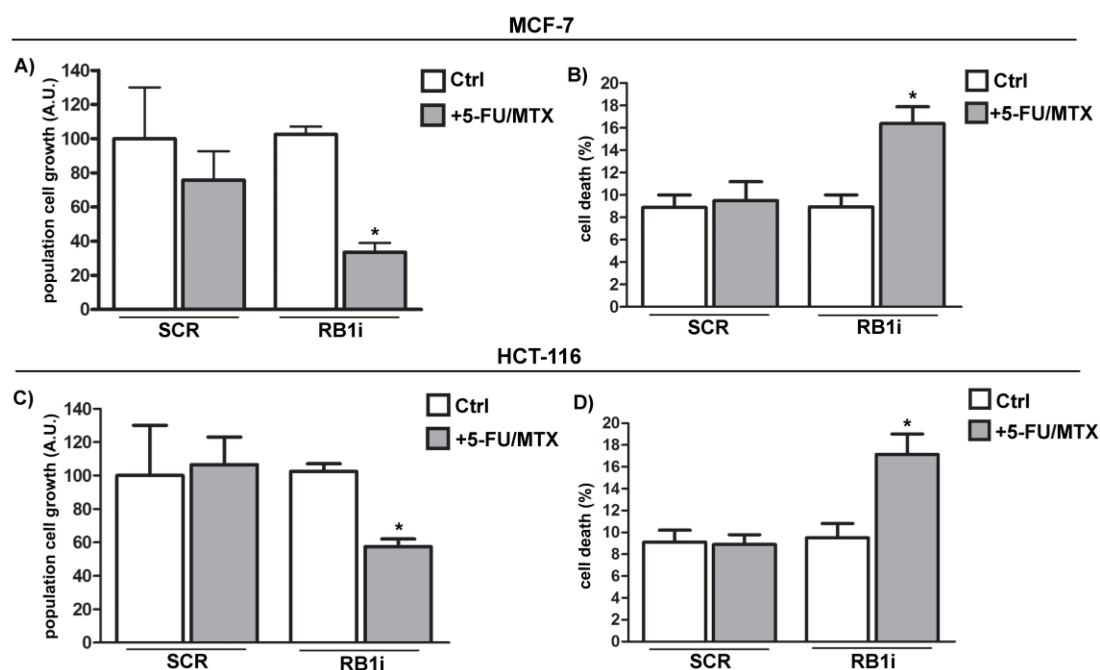


Fig. 30. Effect of *RB1* interference on the growth and mortality rate of MCF-7 and HCT-116 cells treated with 5-FU and methotrexate. A) and C) the effect of 1-h treatment with 5-FU (20 µg/mL) and MTX (0.10 µg/mL) on cell population growth of MCF-7 (A) and HCT-116 (C) cells either silenced for *RB1*expression (RB1-) or transfected with scrambled sequences (RB1+). Cell number was evaluated 72 h after the end of drug treatment. Drug treatment strongly hindered the proliferation of *RB1*-silenced cells. On the contrary, the drugs only slightly reduced the proliferation of the RB1+MCF-7 cells (P = 0.313) and had no effect on RB1+ HCT-116 cells. B) and D) the effect of 1-h treatment with 5-FU (20 µg/mL) and methotrexate (0.1 µg/mL) on cell mortality rate. Cell number was evaluated 24 h after the end of drug treatment. The percentage of dead cells was greater in the drug-treated RB1i MCF-7 and HCT-116 cells than in untreated cells. Drug-treated and untreated RB+ cells exhibited the same percentage of dead cells. *, P < 0.05, statistical significance

6.2.5. 5-FU and MTX treatment caused a cell cycle arrest in control but not in *RB1*-silenced cells

To obtain information on the cause of the higher sensitivity of *RB1*-silenced cells to 5-FU and methotrexate treatment, we evaluated the effect of the drug exposure on the cell cycle progression of control and *RB1*-silenced asynchronously MCF-7 cells, by a dual-parameter flow cytometry analysis for DNA content and incorporated BrdUrd evaluation (Figure 31).

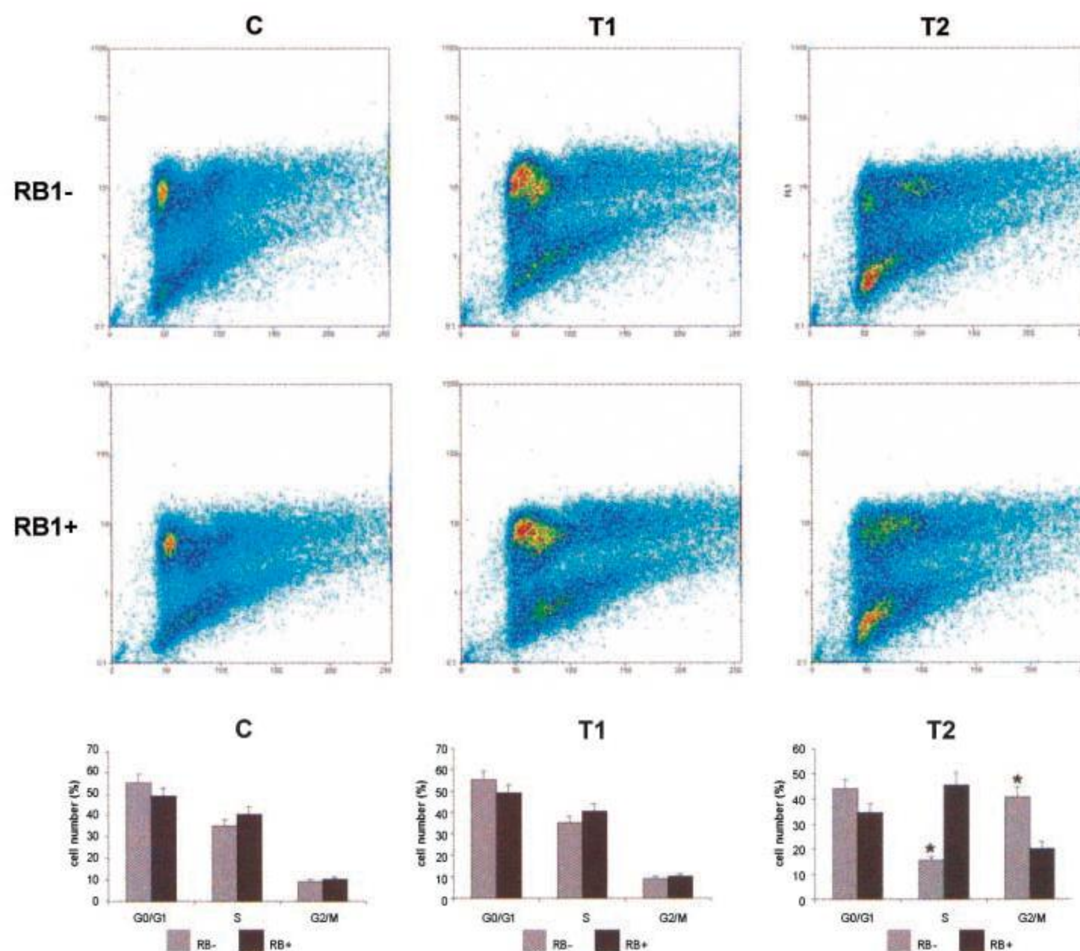


Fig. 31. Effect of 5-FU and methotrexate treatment on cell cycle progression of RB-silenced and control MCF-7 cells. Representative dual-parameter flow cytometry analysis of DNA content (horizontal) and incorporated BrdUrd (vertical) of asynchronously growing MCF-7 cells either silenced for *RB1* (RB1-) or transfected with scrambled sequences (RB1+).The cells were labeled with BrdUrd for1h, and12h later were either harvested (C) or treated with 5-FU and MTX for1h; these cells were processed 12 and 24 h later (T1 and T2, respectively). Each dot plot represents the distribution of correlated red (propidium iodide) and green (FITC) fluorescence of 20,000 analyzed cells. Top row, MCF-7 cells silenced for *RB1*. Twelve hours after the end of BrdUrd labeling, both RB1- and RB1+ BrdUrd-labeled cells (C) are located in the G0-G1region of the cell cycle. After drug treatment, the BrdUrd-labeled cells silenced for *RB1*seem to move through the S phase (T1) and finally accumulate in G2-M (T2), whereas BrdUrd-labeled cells transfected with scrambled sequences seem to be arrested in the early S-phase region, without entering the G2-M compartment. Columns, mean percentage of cells in theG1, S, and G2-M compartments relative to three independent experiments; bars, SD. *, $P < 0.05$, statistical significance

For this purpose, both control and *RB1*-silenced cells, 72 hours after the end of the silencing procedure, were labeled with BrdUrd for 1 hour. Twelve hours later, when most of the labeled cells were passed to the G1 phase, the cells were either immediately harvested (control cells) or treated with 5-FU and MTX for 1 hour and harvested 12 and 24 hours later for dual-parameter flow cytometry analysis. The control cells were mainly located in the G0-G1 region. Twelve hours after the exposure to 5-FU and MTX, the BrdUrd-labeled *RB1*-silenced cells seemed to move to the S phase and, 24 hours after the end of drug treatment, were accumulated in the G2-M region (Fig. 31 A, T1 and T2). On the other hand, at the same time, the BrdUrd-labeled, drug treated control cells were prevalently confined to the early S-phase region and only a limited aliquot was able to reach the G2-M compartment, without any accumulation in the G2-M phase compartment (Fig. 31 B, T1 and T2). These results indicated that 5-FU and MTX treatment caused an arrest of cell cycle progression in control cells but not in *RB1*-silenced cells. The arrest of cell cycle progression in control cells was removed 36 hours after the end of drug treatment.

6.2.6. The p53/p21 pathway was normally activated in *RB1*-silenced cells treated with 5-FU and MTX

After we investigated whether in *RB1*-silenced cells the p53-p21 pathway, which is involved in genotoxic-induced arrest of cell cycle, was hindered. We evaluated the expression of p53 and p21, by Western blot analysis, after 1-hour treatment with 5-FU plus MTX in control and *RB1*-silenced MCF-7 cells. We found that in both control and *RB1*-silenced cells, the amount of p53 was greatly increased 6 hours after

the drug treatment and progressively decreased thereafter. The expression of p21 reflected the p53 time course (Figure 32).

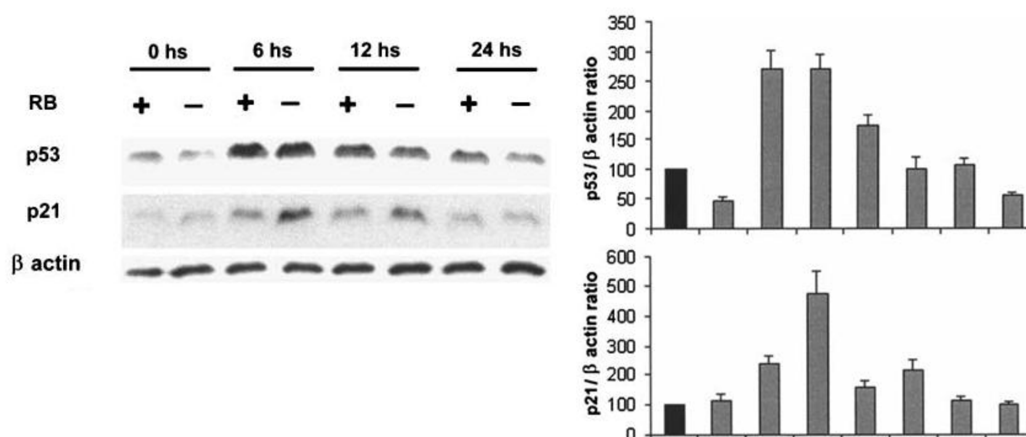


Figure. 32. Effect of 5-FU and MTX treatment on p53 expression in *RB1*-silenced MCF-7 cells. Representative time course Western blot of p53 and p21 in cells transfected with scrambled sequences (RB+) and in cells silenced for *RB1* expression (RB-). Cells were either untreated or treated with 5-FU and MTX for 1h. An increased amount of p53 was visible 6 h after the treatment and progressively decreased thereafter. The expression of p21 reflected the p53 time course. No differences were observed in p53 and p21expression between control and silenced cells. Histograms show the densitometric values of three independent experiments. Columns, mean; bars, SD. Each value is relative to the p53 or p21/h-actin ratio in untreated cells transfected with scrambled sequences, which was set to 100

6.2.7. *RB1*-silenced cells accumulated DNA double-strand breaks

We also investigated whether the higher sensitivity of *RB1*-silenced cells to drug exposure might be the consequence of their reduced capacity for repairing the drug-induced DNA changes in comparison with control cells. For this purpose, we carried out a Western blot analysis with anti-phospho-H2AX antibody to reveal the accumulation of DNA double-strand breaks in drug-treated and untreated control and *RB1*-silenced MCF-7 cells (Figure 33). We observed that *RB1* silencing caused untreated cells markedly to accumulate phosphorylated (γ) H2AX, thus suggesting a

failure to repair the endogenously arising double strand breaks promptly enough. The level of γ-H2AX seemed not to be increased after drug treatment. Control cells showed a very low level of γ-H2AX, which was not modified by drug exposure.

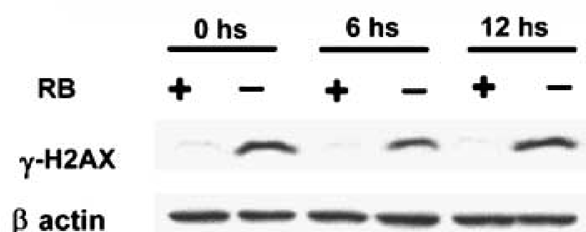


Figure 33. Effect of 5-FU and MTX treatment on γ-H2AX accumulation in *RB1*-silenced MCF-7 cells. Time course Western blot analysis of γ-H2AX expression in control (RB+) and *RB1*-silenced (RB-) cells. Cells were either untreated or treated with 5-FU and MTX for 1h. Note the high expression of γ-H2AX in drug-untreated *RB1*-silenced cells. Drug treatment did not modify the expression of γ-H2AX either in control or in *RB1*-silenced cells

6.3. High prevalence of retinoblastoma protein loss in triple-negative breast cancers and its association with a good prognosis in patients treated with adjuvant chemotherapy

6.3.1. Valuation of pRb status and its association of the clinical outcome of chemotherapy-treated patients with triple-negative tumors

In our breast cancer series (518 patients), we identified four immunohistochemical profiles according to the expression of hormone receptors and HER2:

53 tumors as triple-negative cancers, 61 cases pertaining to the ER-, PR- and HER2+ subtypes, 284 cases to luminal A (ER+ and/or PR+ and HER2-) and 120 cases to luminal B (ER+ and/ or PR+ and HER2+) subtypes (Table 10). The features of population enrolled are resumed in table 11.

Immunohistochemical subtypes	Patients *n*
Triple negative	53
ER-/PR-/HER2+	61
Luminal A	284
Luminal B	120

Table 10. Immunohistochemical subtypes identified in the population enrolled

variables	whole series 518 pz	triple-negative 53 (10,2%)	non triple-negative 465 (89,8%)
Age			
< 50	117 (22.6)	19 (35.8)	98 (21.1)
≥ 50	401 (77.4)	34 (64.2)	367 (78.9)
Histological grade			
G1	59 (11.4)	3 (5.7)	101 (21.7)
G2	339 (65.4)	9 (17.0)	183 (39.4)
G3	120 (23.2)	41 (77.4)	181 (38.9)
N-status (*)			
N0	275 (53.8)	30 (57.7)	240 (53.2)
N+	237 (46.2)	22 (42.3)	211 (46.8)
ER-status (LI)			
< 10%	123 (23.7)	53 (100.0)	70 (15.1)
≥ 10%	395 (76.3)	-	395 (84.9)
PGR-status (LI)			
< 10%	280 (54.1)	53 (100.0)	227 (48.8)
≥ 10%	238 (45.9)	-	238 (51.2)
HER2-status			
negative	331 (65.0)	53 (100.0)	284 (61.1)
positive	178 (35.0)	-	181 (38.9)
p53-LI			
< 10 %	407 (78.6)	22 (41.5)	385 (82.8)
≥ 10 %	111 (21.4)	31 (58.5)	80 (17.2)
Ki67-LI			
< 20 %	277 (53.5)	9 (17.0)	268 (57.6)
≥ 20 %	241 (46.5)	44 (83.0)	197 (42.4)
RB status			
RB deleted	31 (6.0)	20 (37.7)	11 (2.4)
RB under- phosphorylated	406 (78.4)	19 (35.8)	387 (83.2)
RB hyper-phosphorylated	81 (15.6)	14 (26.4)	67 (14.4)
Adjuvant therapy			
none	93 (18.0)	7 (13.2)	86 (18.5)
radiotherapy	49 (9.5)	7 (13.2)	42 (9.0)
endocrine therapy alone	231 (44.6)	15 (28.3)	216 (46.5)
chemotherapy	145 (28.0)	24 (45.3)	121 (26.0)

Table 11. Clinical and histopathological characteristics of triple-negative compared with those with other cancer subtypes gathered together in one group (the non triple-negative group). * N status was available for 511 cases since, due to patient age, axillary dissection was not carried out in seven patients

After we evaluated the clinical outcome (univariate DFS analysis) of the four subtypes of tumors, independently of the adjuvant treatment. After a mean follow-up time of 109 months, the best prognosis was associated with the luminal A type, followed by the triple-negative tumors, whereas a poor clinical outcome was associated with both the luminal B and ER2/PR2/HER2+ subtypes.

Moreover, adverse events were concentrated, in the triple-negative tumors, in the first 40 months after surgery, whereas in other tumor subtypes they were distributed throughout the entire follow-up period (Figure 34).

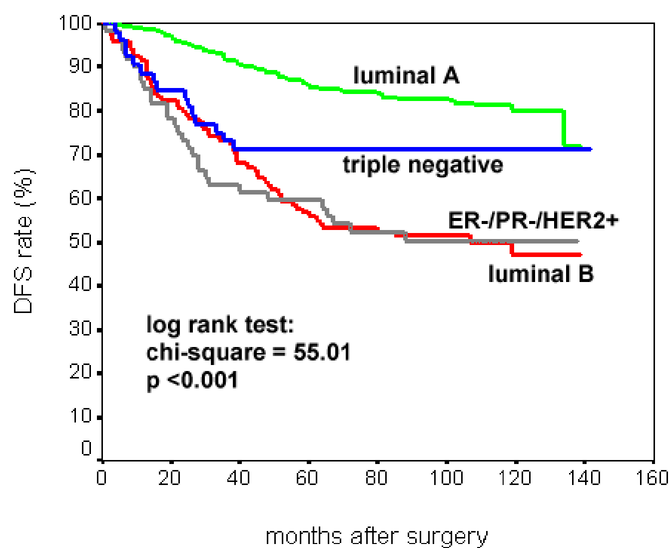


Figure 34. Disease-free survival curves (Kaplan-Meier estimates) according to breast cancer subtypes

After we analyzed the clinical outcome of patients according to chemotherapeutic treatment. Chemotherapy-treated patients with triple-negative tumors (n=24) maintained an optimal prognosis in comparison to those affected by other tumor subtypes (Table 12).

Immunohistochemical subtypes	Patients treated with chemotherapy		
	n	**DFS rates (%)**	**Long-rank test: χ^2 (P)**
Triple negative	24	**75.0**	13.19 (=0,004)
ER-/PR-/HER2+	30	50.0	
Luminal A	45	68.89	
Luminal B	46	39.96	

Table 12. Univariate DFS analysis of different tumor subtypes in patients treated with chemotherapy

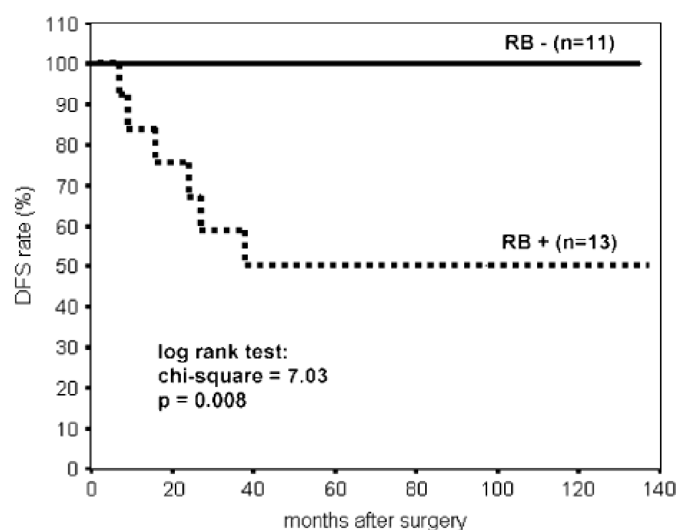
6.3.2. pRb status and the clinical outcome of triple negative tumors treated with chemotherapy

After we evaluated, by immunohistochemical analysis, the pRb status in the different tumor subtypes and we distinguished three pRb forms: pRb underphosphorylated, pRb hyperphosphorylated, pRb deleted (Table 13).

	Triple negative	**ER-/PR-HER2+**	**Luminal A**	**Luminal B**
pRb loss (*n*=31)	20 (64,5%)	7 (22,6%)	2 (6,5%)	2 (6,5%)
pRb underphosphorylated (*n*=406)	19 (4,7%)	26 (6,4%)	265(65,3%)	96 (23,6%)
pRb hyperphosphorylated (*n*= 81)	14 (17,3%)	28 (34,6%)	17 (21,0%)	22 (27,2%)

Table 13. pRb status in different tumor subtypes

The percentage of tumors without pRb expression was significantly higher in the triple-negative subtype (64.5%) than in other tumor subtypes. After we evaluated the clinical outcome of patients with triple-negative tumors treated with chemotherapy dividing the cancers in two groups: one characterized by presence of pRb expression including the under- and hyper- phosphorylated pRb form, and one with absence of pRb expression. All patients with pRb loss were found to be disease free, whereas those with normal or hyperphosphorylated-pRb had a significantly poorer prognosis (Figure 35).



	Total	Number Events	Number Censored	Percent Censored
RB -	**11**	**0**	**11**	**100,00**
RB +	**13**	**6**	**7**	**53,85**
Overall	**24**	**6**	**18**	**75,00**

Figure 35. Disease-free survival curves (Kaplan–Meier estimates) according to pRb status of triple-negative patients treated with chemotherapy. RB-: tumors with pRb loss; RB+: pRb expressing tumors

We have considered the possibility that the highly favorable clinical outcome of chemotherapy-treated patients might be related to other anatomo-clinical parameters associated with an aggressive phenotype that can confound the results of the statistical analysis. For this reason, we analyzed the prognostic relevance of the node status, tumor size, histological grade, Ki67- and p53-LI which are well established tumor-related factors which might also influence the clinical outcome of the patients treated with adjuvant therapy. None of these variables, with the exception or pRb status, resulted to be significantly associated with the clinical outcome (Table 14).

Variables	*n*	DFS rate (%)	Long-rank test: χ^2	*P*
Tumor size				
pT1	9	77,78	1,74	= 0,4197
pT2	11	63,64		
pT3 + pT4	4	100		
Histological grade				
G1 + G2	2	50	0,83	= 0,3625
G3	22	77,27		
N-status (*)				
N0	12	83,33	0,90	= 0,3440
N+	11	64,64		
p53-LI				
< 10 %	10	80	0,05	= 0,8210
≥ 10 %	14	71,43		
Ki67-LI				
< 20 %	2	50	0,83	= 0,3625
≥ 20 %	22	77,27		
RB status				
RB-	11	100	7,03	= **0,0080**
RB+	13	53,85		

Table 14. Prognostic relevance of tumor size, histological grade, N status, p53 status, proliferation rate and pRb status in triple-negative tumors treated with adjuvant chemotherapy (n = 24): univariate DFS analysis

6.3.3. Relevance of pRb status on sensitivity to doxorubicin in MDA-MB-231 triple-negative derived cells

In order to demonstrate the relevance of the pRb status in the sensitivity to chemotherapeutic agents in TNBCs, we also studied the sensitivity to 5-FU and MTX as well as to doxorubicin exposure, in a human triple-negative derived cancer cell lines, MDA-MB-231, in which the function of pRb was down-regulated by silencing *RB1*. The effect of *RB1*-silencing in MDA-MB-231 cells was checked by both Real Time-RT PCR and by Western blot analysis (Figure 36).

48 and 120 h after the RNA interference procedure, a strong reduction of pRb mRNA expression occurred in MDA-MB-231 cells (Figure 36 a). Western blot analysis for pRb expression confirmed that 48 h after the *RB1* silencing procedure, the level of pRb was markedly reduced compared to control samples (Figure 36 b).

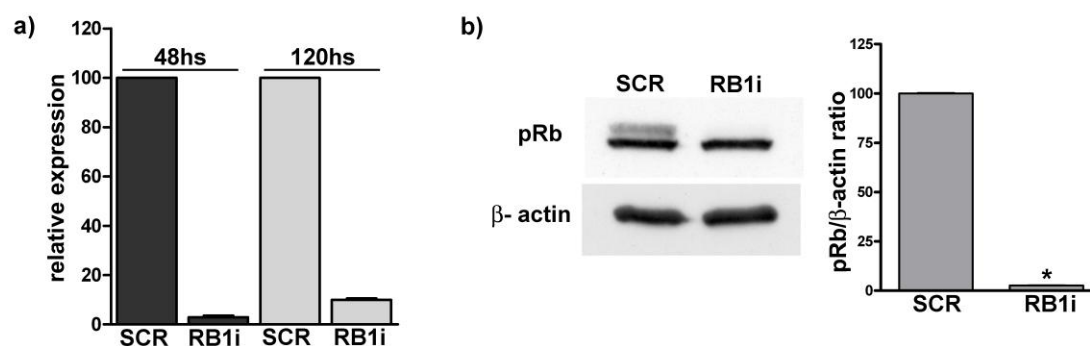


Figure 36. Effect of *RB1* interference on *RB1*mRNA and pRb protein expression on MDA-MB-231 cells. a) Asynchronously MDA-MB-231 cells were either silenced for *RB1*expression or transfected with control scrambled sequences by RNA interference. The *RB1* mRNA level was evaluated in cells transfected with control scrambled sequences (SCR) and in cells silenced for *RB1* (RB1i) at 48 and 120 h after the end of the silencing procedure. Note the high reduction of *RB1* mRNA in *RB1*-silenced cells at both evaluation times. Histograms show the values (mean ± SD) of three independent experiments. b) Representative Western blots of pRb expression in MDA-MB-231 cells, silenced for *RB1* expression, 48 h after the end of the silencing procedure, show the strong reduction of pRb expression in *RB1*-silenced cells (RB1i) as compared to cells transfected with control scrambled sequences (SCR). The expression of *β*-actin was used as a control. The histogram shows the densitometric values (mean ± SD) of three independent experiments. Each value concerns the pRb:*β*-actin ratio, which was set to 100, in untreated cells transfected with scrambled sequences. *$p < 0.05$

We then investigated the long-term effect of 5-FU plus MTX or doxorubicin treatments on the cell population growth in controls and *RB1*-silenced MDA-MB-231 cells (Figure 37).

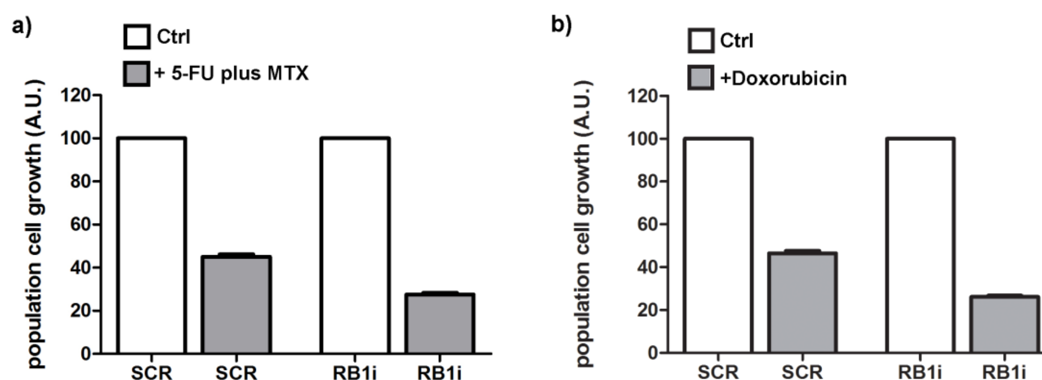


Figure 37. Effect of *RB1* interference on the growth rate of MDA-MB-231 cells exposed to chemotherapeutic agents. The cells were exposed to either 5-FU plus MTX (a) or doxorubicin (b) for 2 h daily for 4 consecutive days. The drug treatments started 48 hours after *RB1* silencing procedure was completed and 24 h after the last treatment the cells were formalin-fixed for the crystal violet assay for growth rate evaluation. Values relative to samples not treated with drugs were normalized to 100. The histograms show the values (mean ± SD) of three independent experiments. statistical significance was: *A= 0,0003 *B< 0.0001

The drug treatments significantly reduced cell population growth both in control and *RB1*-silenced cells, but when pRb was down-regulated the sensitivity to drugs was greater.

7. DISCUSSION

These data show that in breast cancer the response to chemotherapy is conditioned both by p53 and pRb status. In fact, when pRb pathway is normally functioning, p53 is the only independent factor capable to predict the patient clinical outcome after adjuvant chemotherapy treatment. pRb alteration characterized by pRb hyperphosphorylation reduces the chemosensitivity of cancer cells, independently by p53 status, while pRb loss increases the chemosensitivity, always independently by p53 status. These data suggest that the assessment of these two genes is necessary to have a prognostic indication of response to chemotherapy in breast cancer patients.

Going into detail, clear-cut evidence that the p53 status may influence the response to chemotherapeutic agents, and therefore the clinical outcome of breast cancer patients, was still lacking. As far as breast cancer is concerned, no consensus was established on the predictive role of p53. Several studies, using either immunohistochemistry or *TP53* gene sequencing for p53 status analysis, supported the role of p53 as prognostic marker (Silvestrini et al., 1993; MacGrogan et al., 1995; Silvestrini et al., 1996; Thor et al., 1998; Chappuis et al., 1999) and many others failed to demonstrated this role (Elledge et al., 1995; Sjögren et al., 1998; Clahsen et al., 1998; Bro¨et et al., 1999; Penault-Llorca et al., 2003). We demonstrated that these conflicting data were the results of an alteration of the p53-downstream pathway which is frequently disrupted in human cancer cells for *RB1* mutation or deletion, overexpression of cyclin D1, CDK4, *p16INK4a* mutation (Knudsen and Knudsen, 2008). These changes, by causing either pRb loss or pRb

hyperphosphorylation, could either nullify the effect of p53 stabilization after chemotherapy treatment or change the sensitivity to chemotherapeutic agents.

7.1. In breast cancer with a normally function of pRb pathway, the p53 status was the only independent factor capable to predicting the patient clinical outcome after adjuvant chemotherapy treatment

First, we evaluated the prognostic relevance of p53 in a series of patients according to the pRb status, after chemotherapy treatment (5-FU plus MTX). In this series the p53 status, considered independently of the pRb status, proved to have a null prognostic value.

Also, in patients with pRb loss and with hyperphosphorylated pRb, the p53 variable was not associated with the clinical outcome. As far as the patients with cancer with normally functioning pRb pathway (underphosphorylated pRb) was concerned, univariate analysis of DFS indicated a significant association of p53 status with prognosis, the putatively mutated p53 being associated with a worse clinical outcome. In this group, a multivariate analysis of DFS, including the other clinical and histopathological variables associated with the clinical outcome such as tumor size, histopathological grade, node status, ER-, PR- and Ki67-LI, and HER2, that could have confounded the results of the statistical analysis, confirmed that the p53 status was the only factor significantly associated with the DFS, when pRb was normally functioning.

7.2. The absence but not functional inactivation of pRb predicted the clinical outcome of patients treated with 5-FU and MTX adjuvant therapy

We evaluated, in a univariate analysis for the DFS, the predictive value of both the expression of pRb and the degree of its phosphorylation in the whole series of patients and in the patients who received standard chemotherapy regimen (5-FU plus MTX). In fact, there is evidence that from the functional point of view, hyperphosphorylation abolishes the tumor suppressor activity of pRb (Sherr and McCormick, 2002). Thus, regarding the biological behavior of cancer cells, both the lack of pRb expression and pRb hyperphosphorylation might have similar effects.

We subdivided the tumors into two groups: one characterized by the presence of pRb expression, which included the underphosphorylated and hyperphosphorylated pRb form *(RB+),* one with a deleted pRb status *(RB-).*

Regarding the relationship between pRb expression and patient clinical outcome, we found that this pRb variable (RB- or RB+) was not a significant prognostic parameter in the whole series of patients. However, among the patients who received chemotherapy, those whose cancers lacked pRb (RB-) had a better prognosis than those expressing the tumor suppressor protein.

About the relationship between the level of pRb phosphorylation and prognosis, we found that the level of pRb phosphorylation not correlated with the clinical outcome in patients who received chemotherapy. Therefore, only the loss of pRb, but not its inactivation for hyperphosphorylation, was a predictive factor of the clinical outcome of breast cancer patients, when treated with chemotherapy.

7.3. Lack of pRb expression was the only independent factor predicting a good clinical outcome in patients treated with adjuvant chemotherapy

We further investigated this relationship between pRb expression and the clinical outcome in patients treated with chemotherapy, considering the possibility that the significant predictive effect of pRb found might have been related to other clinical and histopathological variables associated with the clinical outcome, such as node status, tumor size, histologic grade, ER-, PR-, Ki67-, and p53- LI and HER2 status, confounding these results. So we carried out a multivariate analysis for DFS and we found that the pRb expression resulted to be the only significant predictive factor associated with the prognosis in patients treated with chemotherapy: the group of patients with RB- cancers having a better clinical outcome than those with RB+ cancer. Furthermore, because the number of breast cancers lacking pRb was small (n = 16), to validate the significant association between pRb expression and prognosis the 16 RB- tumors were matched with 32 RB+ tumors according those variables that characterized all the RB- tumors (high histologic grade, high proliferation rate, and absence of ER). Also, in this data set, patients with tumors lacking pRb expression had a significantly better clinical outcome than patients with RB+ tumors. Therefore, even if the number of breast cancers lacking pRb expression is only a small fraction of total breast cancers, altogether these data indicated the only independent factor predicting a good clinical outcome in patients treated with adjuvant chemotherapy was the loss of pRb.

In order to demonstrate that a normally functioning pRb pathway was necessary to allow wild-type p53 to induce a cytostatic activity after the exposure to

chemotherapeutic agents, we analyzed the response to chemotherapeutic drugs, used in breast cancer therapy, in human cancer cell lines with either wild-type or abrogated p53 function (to inhibit p53 activity we have both interfered *TP53* gene and used an inactive truncated-dominant negative form of murine p53 (p53DD)) where the function of pRb was down-regulated either by abolishing the expression of pRb by *RB1*-silencing, or by inducing pRb hyperphosphorylation by *p16INK4a*-silencing. Therefore, we first evaluated the effects of the loss of pRb on the cell proliferation rate of a p53-deficient and p53-proficient cell lines treated either with 5-FU plus MTX or doxorubicin. As for the effect of the loss of pRb on the sensitivity of p53-deficient and p53-proficient cells to drug exposure, we found that in cell lines where p53 was inactivated, the inhibitory effect of drugs on the cell population growth rate was greatly reduced as compared to cells harboring wild-type p53. *RB1* silencing restored the high sensitivity to drugs in cells with inactivated p53 and the cell population growth rate being the same as that of cells with wild-type p53.

There is evidence that the loss of pRb actually increases cell sensitivity to both DNA-damaging agents and drugs targeting the thymidylate biosynthesis pathway (Knudsen and Knudsen, 2008) The present results demonstrated that the high sensitivity of pRb-deficient breast cancer cells, both to drugs targeting the thymidylate biosynthesis pathway and to doxorubicin, were not influenced by p53 status and explain why tumours with mutated p53 could strongly benefit from chemotherapy if they were also characterized by the loss of pRb.

As for the effect of pRb pathway inactivation, our results showed that pRb hyperphosphorylation, caused by *p16INK4a* silencing, reduced the sensitivity to doxorubicin in p53-proficient HepG2 cells. Since drug treatment of p53-proficient

HepG2 cells caused the disappearance of the phosphorylated form of pRb in controls but not in *p16INK4a*-silenced cells, our results demonstrate that p53 stabilization had a lower cytostatic effect in *p16INK4a*-silenced cells, which was very likely due to the persistence of phosphorylated pRb within cancer cells. These results were consistent with the established mechanism of cell cycle progression blockage induced by the activation of the p53-p21 pathway leading to the inhibition of pRb phosphorylation: pRb hyperphosphorylation hinders p53-mediated cell cycle arrest (Knudsen and Knudsen, 2008). They also explain well the observations that breast cancer patients with hyperphosphorylated pRb and treated with adjuvant chemotherapy were characterized by a poor prognosis that was independent of the p53 status. Our results indicated that in breast cancers, as it has also been previously reported to occur in non-small cell lung cancer (Burke et al., 2005), the complexity of the cell cycle protein interaction warrants caution in interpreting survival results when specific protein abnormalities are taken in isolation.

7.4. The greater sensitivity of pRb deficient cells to 5-FU plus MTX exposure was due to the absence of a DNA damage checkpoint and DNA repair mechanisms

To ascertain the mechanism at the basis of the enhanced sensitivity of pRb negative tumors to antimetabolites action, we analyzed the effect of 5-FU and MTX treatment on cell cycle progression in MCF-7 cells.

Analysis of the cytofluorimetric results indicated that 1-hour drug treatment caused an arrest of cell cycle progression in control cells but not in *RB1*-silenced MCF-7 cell. These data were consistent with the available evidence indicating that several

DNA damage inducers used in human tumor chemotherapy inhibit G1- and S-phase progression in pRb-proficient but not in pRb-deficient cells (Knudsen KE et al., 2000; Angus SP et al., 2002). Specifically, it has been shown that pRb-proficient cells exposed to 5-FU failed to accumulate in any phase of the cell cycle, indicating that the drug was responsible for the arrest in all phases of the cell cycle (Mayhew et al., 2004). We also investigated whether in *RB1*-silenced cells the p53/p21 pathway, which is usually involved in the genotoxic-induced arrest of cell cycle progression (Sherr CJ and McCormick F, 2002) was hindered. We evaluated the expression of p53 after 1-hour treatment with 5-FU and MTX in control and *RB1*-silenced MCF-7 cells and we found that in both control and *RB1*-silenced cells, the amount of p53 was greatly increased after the drug treatment, indicating a functional p53 pathway.

We also investigated whether the higher sensitivity of pRb-deficient cells to drug exposure could have been the consequence of their reduced capacity for repairing the drug-induced DNA changes. We demonstrated that *RB1*-silenced cells exhibited elevated levels of γ-H2AX, indicative of defects in the DNA repair machinery, whereas the control cells did not shown accumulation of double strand breaks, thus indicating a normal DNA repairing activity. In other words, pRb-proficient cells may be more resistant to anti-metabolite exposure than pRb-deficient cells because they have the time for repairing the 5-FU-induced damage by possessing functioning cell cycle checkpoint and DNA repair mechanisms. This repair would be impossible for cells lacking pRb in which the DNA damaging agents do not induce arrest of cell cycle progression and DNA repair mechanisms are hindered.

7.5. High prevalence of retinoblastoma protein loss in triple-negative breast cancers was responsible for a good prognosis in patients treated with adjuvant chemotherapy

The triple negative breast cancers (TNBCs) are a particular subtype of breast carcinomas. They are very aggressive and due to absence of hormone receptors and HER2, they are treated only with adjuvant chemotherapy.

It is worth noting that they exhibit higher rates of objective response to chemotherapy then other tumor types. This suggests that the biological features present more frequently in this subtype are responsible for their increased sensitivity to chemotherapy.

Since we demonstrated that breast cancers lacking pRb expression were more sensitive to adjuvant chemotherapy, we investigated whether the high sensitivity to chemotherapy of TNBCs could be due to the loss of pRb.

We carried out an immunohistochemical analysis on a large consecutive series of primary breast cancer to identify the breast cancer subtypes. In our breast cancer series, we identified 53 tumors as triple negative cancers, corresponding to 10.2% of the 518 cases taken into account. This value was within the range (10%–17%) reported for the frequency of triple-negative cancers among all breast cancers (Reis-Filho and Tutt, 2008). Then we evaluated the clinical outcome of the four subtypes of tumors independently of the adjuvant treatment received. After a mean follow-up time of 109 months, the best prognosis was associated with the luminal A type, followed by the triple-negative tumors. Therefore, in our series, triple negative tumors did not appear to be characterized by a more aggressive clinical behavior compared with other types of breast cancer. According to the previous results (Dent

et al., 2007), we found that in patients with triple-negative tumors, adverse events were concentrated in the first 40 months after surgery, whereas in other tumor subtypes they were distributed throughout the entire follow-up period. We also analyzed the clinical outcome of patients according to their adjuvant therapy treatment. Chemotherapy-treated patients with triple-negative tumors were characterized by a very good prognosis in comparison to those affected by other tumor subtypes.

We evaluated the pRb status on the four breast cancer subtypes and we found that 64.5% of pRb-deficient tumors were triple-negative cases and that 37.7% of triple-negative tumors were pRb deficient compared with 2.3% of other cancer types. Regarding pRb inactivation by hyperphosphorylation, the percentage of TNBCs with hyperphosphorylated pRb was not significantly different to that of other cancer subtypes, thus indicating that pRb loss, but not pRb functional inactivation by hyperphosphorylation, represented a frequent biological characteristic of triple-negative tumors. We evaluated the clinical outcome of patients with triple-negative tumors treated with chemotherapy according to the presence or absence of pRb expression. We subdivided the tumors into two groups: one characterized by the presence of pRb expression, which included the underphosphorylated and hyperphosphorylated pRb form *(RB+),* one with a deleted pRb status *(RB-).*

We demonstrated that all patients with pRb loss were found to be disease free, whereas those with normal or hyperphosphorylated-pRb had a significantly poorer prognosis, indicating that the lack of pRb expression represented a strong predictive parameter of DFS in TNBC patients who received chemotherapy.

We considered the possibility that the highly favorable clinical outcome of chemotherapy-treated patients could be related to other anatomo-clinical parameters associated with an aggressive phenotype, confusing the results of the statistical analysis. For this reason, we analyzed the prognostic relevance of the node status, tumor size, histological grade, Ki67- and p53-LI which were well established tumor-related factors which could influence the clinical outcome of the patients treated with adjuvant therapy. We confirmed that none of these variables resulted to be associated with the clinical outcome, with the exception of pRb status which was the only predictive factor significantly associated with the clinical outcome.

We also confirmed the effect of loss of pRb on the sensitivity to drug exposure in a triple-negative derived cell lines, the MDA-MB-231.

In conclusion, triple negative cancers seemed to harbor a biological feature that, when present, made them highly sensitive to chemotherapy. In the absence of this specific feature, the highly aggressive phenotype of these cancers would determine the poor clinical outcome for patients. In the present study we found that the lack of pRb expression was more frequent in TNBCs than in other cancer subtypes, and patients with triple-negative tumors lacking pRb had a very favorable clinical outcome if treated with adjuvant chemotherapy. Therefore, we suggested that the loss of pRb expression was this biological feature.

In conclusion, taken together, these data indicate that p53 and pRb are key elements for the determination and prediction of response to chemotherapy, in particular in breast cancer, just because their function is to control the cell cycle and to respond to

any damages, including those induced by chemotherapy drugs (Figure 38) Alterations of p53-pRb pathway may influence the chemosensitivity.

We observed that in breast cancer with a normally functioning pRb pathway, p53 was the only independent factor capable of predicting the patient clinical outcome after adjuvant chemotherapy treatment. Regarding the pRb alterations, we found that the pRb functional inactivation (pRb hyperphosphorylated) reduced the chemosensitivity, independently by p53 status; whereas the cancers with pRb loss increased the sensitivity to chemotherapy, always independently by p53 status, and the patients had a better prognosis (Figure 39). Therefore, the pRb loss was the only predictive factors of a good clinical outcome for patients treated with adjuvant chemotherapy, especially in a particular subtype of breast cancers, the triple-negative tumors, characterized by a large amount of pRb-deleted tissues.

Therefore, the systemic chemotherapy should be considered to represent the first choice adjuvant treatment for patients with pRb negative cancers.

These studies allow us to suggest the introduction into clinical practice, beyond the already known assessment of p53, also the concomitant evaluation of the pRb expression because together they represent two important, related and strong prognostic and predictive parameters of clinical outcome of patients with breast cancers and treated with chemotherapy.

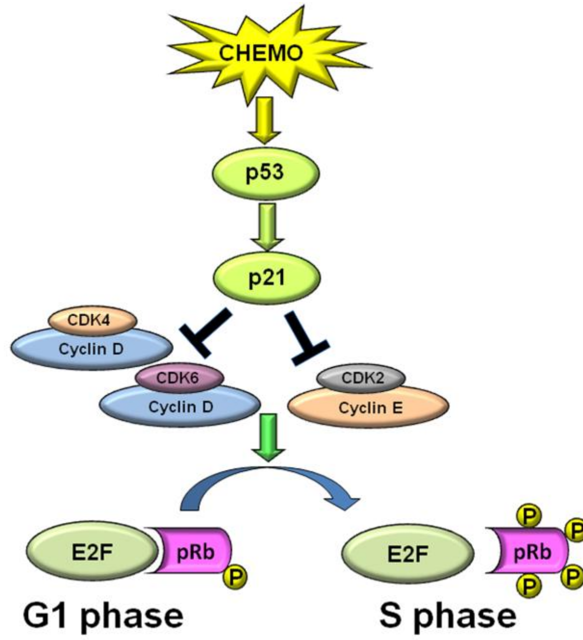


Figure 38. Schematic representation of the p53-pRb pathway activated by chemotherapeutic treatments

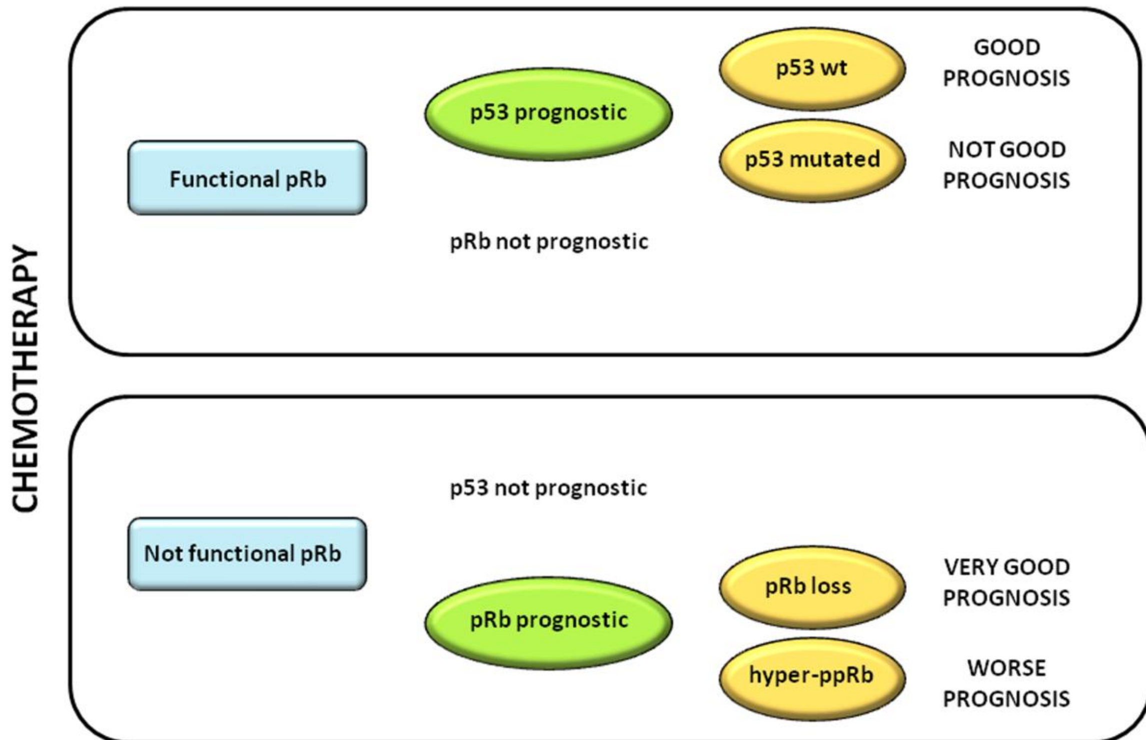


Figure 39. Schematic representation of prognostic relevance of pRb and p53 status to predict the clinical outcome of breast cancer patients treated with chemotherapy

8. NOTES

During the three years of PhD studies, I coauthored these work:

- M Derenzini, **E Brighenti**, G Donati, M Vici, C Ceccarelli, D Santini, M Taffurelli, L Montanaro, D Treré. *The p53-mediated sensitivity of cancer cells to chemotherapeutic agents is conditioned by the status of the retinoblastoma protein.* J Pathol. 2009 Nov; 219(3):373-82.

- D Trerè, **E Brighenti**, G Donati, C Ceccarelli, D Santini, M Taffurelli, L Montanaro and M Derenzini. *High prevalence of retinoblastoma protein loss in triple-negative breast cancers and its association with a good prognosis in patients treated with adjuvant chemotherapy.* Ann Oncol. 2009 Nov; 20(11):1818-23. Epub 2009 Jun 25.

I have also been involved in another research project. This work was published at the beginning of 2011 in Oncogene:

- G Donati*, S Bertoni*, **E Brighenti**, M Vici, D Treré, S Volarevic, L Montanaro, M Derenzini. *The balance between rRNA and ribosomal protein synthesis up- and down-regulates the tumour suppressor p53 in mammalian cells.* Oncogene in press.

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