Chapter -2

Review of Literature

2.1 Prevalence of breast cancer associated genes

The incidence of breast cancer has risen steadily over the last half a century, partially due to earlier detection. Now it has become clear that the inactivation of various tumor suppressor genes, which can be thought of as 'brakes' of cell growth, is at least as important in the development and progression of breast cancer. Tumor suppressor genes are considered to act mostly in a recessive fashion, i.e. some abnormality must affect both gene alleles. According to Knudson's "two-hit" hypothesis based on studies of retinoblastoma, the inactivation of a tumor suppressor gene by mutation requires two inactivating "hits" or mutations. In the dominantly inherited form of familial cancers, one mutation is inherited by the germinal cells while the second occurs in somatic cells. In non-hereditary forms, both mutations are somatic (Knudson, 1971). In addition, a tumor suppressor gene may be inactivated by loss of heterozygosity (LOH) as reported for example in case of PTCH, *BRCA1* and *BRCA2* (Locke et al., 2006), *TP53*, *RB* and *PTEN* (Duman et al., 2013) tumor suppressor genes; or, by epigenetic silencing through hypermethylation of promoters in case of *RB, APC, MLH1, VHL, CDNK2A, CDKN2B* (Esteller, 2002), *BRCA1* and *STK11*. Thus, accordingly, Knudson's "two-hit" hypothesis may be revised to accommodate the inactivation of the first active allele of a tumor suppressor gene by localized mutation or transcriptional repression through DNA methylation, and the second hit, that is, inactivation of the second allele by LOH or transcriptional silencing, in addition to mutation. Chromosomal loss is mostly analyzed by karyotypic studies or loss of heterozygosity (LOH) studies, and mutations are most frequently studied by sequencing of the gene of interest or by single strand chain polymorphism analysis (SSCP). In many cases, mutations can result in truncated protein products which are easy to detect. However, recently it has been shown that functional inactivation of tumor suppressor genes can be caused by many other epigenetic mechanisms besides mutation, including hypermethylation (Lo et al., 2010), increased degradation (Zhang et al., 2012), or mislocalization (Chen et al., 2012).

The function and role of tumor suppressor genes have been elucidated by many investigators through a combination of a number of cells biological as well as biochemical methods. (Kinzler and Vogelstein, 1997) have recently proposed a new system to categorize tumor suppressor genes as 'gatekeepers' and 'caretakers'. Gatekeepers are tumor suppressor genes which are directly involved in controlling proliferation by regulating cell cycle checkpoints (Oliveira et al., 2005). Mutations of these genes usually result in high penetrance. In contrast, caretakers are of rather low penetrance, and have an indirect effect on growth. They are responsible for genome integrity, and changes in such genes lead to genome instability. The best characterized examples are repair genes, such as MSH2 in hereditary non-polyposis colon cancer (HNPCC). It is likely that more categories will follow, for instance 'landscaper' for genes which are involved in epithelial-stromal and epithelial-epithelial interactions (e.g. E-cadherin and a-catenin). In the past several years we have seen an explosion of information in the field of breast cancer genetics, with regard to the identity of tumor suppressor genes that are mutated in sporadic breast cancer as well as those that are inherited in mutant forms, giving rise to a familial predisposition to cancer.

2.2 Cancer associated genes in breast cancer progression

Variations of the *BRCA1, BRCA2, CDH1, STK11,* and *TTP53* genes increase the risk of developing breast cancer. The *AR, ATM, BARD1, BRIP1, CHEK2, DIRAS3, ERBB2, NBN, PALB2, RAD50* and *RAD51* genes are associated with breast cancer (Koboldt et al., 2012). In addition to specific genetic changes, researchers have identified many personal and environmental factors that may influence a person's risk of developing breast cancer. These factors include gender, age, ethnic background, a history of previous breast cancer, certain changes in breast tissue, and hormonal factors. A history of breast cancer in closely related family members is also an important risk factor, particularly if the cancer occurred at an early age. Some breast cancers that cluster in

families are associated with inherited mutations in particular genes, such as *BRCA1*. There are thousands of genes involved in cancer progression, directly or indirectly they have a role in the cancer prognosis. Here we are concentrated on *BRCA1* and *TP53* nucleotide changes.

2.2.1 *BRCA1* a strong candidate for breast cancer

Date of Receipt

The role of *BRCA1* in breast cancer progression might be important in fewer cases than originally hoped. It came as a surprise to many researchers when various studies showed that *BRCA1* is very rarely mutated in sporadic cancers. However, although *BRCA1* (and *BRCA2*) mutations account for only a few percent of total breast cancer patients, these patients constitute a large number considering that, in the USA alone, there are about 180 000 new cases per year. In an effort to quantify the various classes of mutations, DNA from 798 women with a family history of breast cancer was sequenced (Shattuck-Eidens et al., 1997). Mutations in *BRCA1* resulting in deletions were found in 12.8%, and 50% of those were unknown prior to this study. Furthermore, the mutations were scattered throughout the genome, and there was no correlation between the position of the mutation and the phenotype.

There is the obvious question of why *BRCA1* mutations seem to specifically induce breast (and ovarian) tumors, at least at a much higher rate than any other tumor types described as being increased in *BRCA1* carriers. One could speculate that *BRCA1* has some specific function in breast, perhaps related to steroid hormone receptors. Although the question has yet to be answered, there are recent findings supporting this idea. For instance, in the mouse mammary gland, *BRCA1* is up regulated during puberty and pregnancy (Welcsh and King, 2001). Another group has also described an estrogen-mediated effect on *BRCA1* expression (Kang et al., 2012). Finally, we would like to address a practical point which is important for *BRCA1* and *BRCA2* carriers. Although yearly mammography and annual or semiannual physician-administered breast examination beginning at age 25 is recommended, there is

the open question of whether mammography represents a risk factor for those mutation carriers. This arises from the finding that *BRCA1* appears to play a role in DNA repair, which would make it possible that *BRCA1* defective cells are hypersensitive to ionizing radiation. Mutations might accumulate because repair mechanisms are defective. However, this is speculative, and very hard to prove.

2.2.1.1 Trend of *BRCA1* mutation in breast cancer

Approximately 5% of breast cancers show a familial pattern of occurrence (Rosen et al., 2003). Discovery of the genes conferring susceptibility to familial breast cancer and determination of their functional mechanisms would considerably enhance our understanding of the etiology and progression of breast tumors. In 1990, genetic studies provided initial evidence that the risk of breast cancer in some families is linked to chromosome 17q21 (Hall et al., 1990). This 17q-associated syndrome was characterized by autosomal dominant inheritance with incomplete penetrance. In fact, loss of heterozygosity (LOH) at 17q was found in most familial breast and ovarian tumors, suggesting the involvement of tumor suppressor gene(s) (Smith et al., 1992, Neuhausen and Marshall, 1994). In 1994, the breast-cancer susceptibility gene, *BRCA1*, was identified by positional cloning; subsequently, this gene has been the subject of intensive research effort (Miki et al., 1994). *BRCA1* is composed of 22 coding exons distributed over 100 kb of genomic DNA. This gene encodes 1863 amino acids, and more than 200 different germline mutations associated with cancer susceptibility have been identified. Many disease-predisposing alleles of *BRCA1* have loss-of function mutations, the majority of which result in premature truncation of the protein. Because only 45% of familial breast cancers showed evidence of linkage to *BRCA1*, the search for a second breast cancer susceptibility gene continued. In 1995, the *BRCA2* gene was identified on chromosome 13q12.3 (Wooster et al., 1994, Wooster et al., 1995). Mutations in *BRCA1* and *BRCA2* are not simply

associated with an elevated risk of breast cancer (Rahman and Stratton, 1998). Mutation carriers also have increased susceptibility to ovarian, pancreatic, prostatic, and male breast cancers. Other associations may be revealed as more epidemiological information becomes available. Surprisingly, despite the inherited predisposition to cancer associated with *BRCA1* and *BRCA2*, somatic disease-causing mutations in either of these genes are extremely rare in sporadic breast cancers (Futreal et al., 1994, Lancaster et al., 1996). Over the last 10 years, much has been learned about the structures, functions, and unique features of BRCA gene products. In particular, research into the functions of BRCA proteins has revealed that BRCA proteins interact with a number of regulatory proteins. In this article, we review recent advances in our understanding of the roles of *BRCA1* and *BRCA2* in the biological response to DNA damage.

Initial evidence suggesting a role of *BRCA1* in the repair of damaged DNA was derived from the observation that *BRCA1* is hyperphosphorylated in response to DNA damage and relocated to sites of replication forks marked by proliferating cell nuclear antigen (PCNA) (Scully et al., 1997, Thomas et al., 1997). In response to ionizing radiation, *BRCA1* is bound and phosphorylated by an ataxia-telangiectasia mutated (ATM) kinase (Cortez et al., 1999). The major target for ATM phosphorylation after ionizing radiation is Ser1387 of *BRCA1*. In response to ultraviolet irradiation, Ser1457 is primarily phosphorylated, mainly by ATM-related kinase (ATR) (Gatei et al., 2001). The G2/M control kinase, CHK2, has been shown to phosphorylate *BRCA1* at Ser988 on exposure to ionizing radiation (Chaturvedi et al., 1999, Bell et al., 1999). Other sites of *BRCA1* that are phosphorylated in response to DNA damage, such as Ser1423 and Ser1524, have been reported (Gatei et al., 2000). It is likely, therefore, that *BRCA1* is phosphorylated at multiple residues by different kinases after DNA damage. However, how each type of phosphorylation affects the functions of *BRCA1* remains obscure. Subsequent studies demonstrated the involvement of *BRCA1* and *BRCA2* in complexes

that activate the repair of double strand breaks (DSBs) and initiate homologous recombination (HR), linking the maintenance of genomic integrity to tumor suppression. *BRCA1* and *BRCA2* co-localize with Rad51 to form complexes (Scully et al., 1997, Chen et al., 1998). Eukaryotic Rad51 proteins are homologues of bacterial RecA and are required for recombination during mitosis and meiosis, as well as for HR repair of DSBs (Shinohara et al., 1992). Rad51 coats single-stranded DNA to form a nucleoprotein filament that invades and pairs with a homologous region in duplex DNA, and then activates strand exchange to generate a crossover between the juxtaposed DNA (Sung et al., 1994, Baumann et al., 1996). Co-localization of BRCAs with Rad51 at sites of recombination and DNA damage-induced foci strongly suggests that BRCAs have a role in both the detection and the repair of DSBs (Scully et al., 1997). In this regard, focus formation of Rad51 is reduced after treatment with DNA-damaging agents and is deficient during repair of DSBs by HR in *BRCA1*-deficient cells (Scully et al., 1999, Moynahan et al, 2001). However, accumulating evidence suggests that *BRCA1* might not directly regulate Rad51, since interactions between *BRCA1* and Rad51 are indirect and stoichiometrically negligible (Venkitaraman, 2001).

Other studies have shown that *BRCA1* co-localizes and coimmunoprecipitates with Rad50, together with its partners Mre11 and NBS1 (Zhong et al., 1999, Wang et al., 2000). *BRCA1* apparently functions as a regulator of the Rad50-Mre11-NBS1 complex (Wu et al., 2000). Mre11 encodes nuclease activity, which resects flush ends of DSBs to generate ssDNA tracts (Haber, 1998). *BRCA1* binds DNA directly and inhibits this Mre11 activity, regulating the length and the persistence of ss-DNA generation at sites of DNA damage (Paull, 2001). Since ss-DNA is a substrate for DNA repair by HR, *BRCA1* might play an essential role in HR-mediated repair of DSBs by inactivating Mre11. Indeed, HR is defective in *BRCA1*-deficient cells (Moynahan, 1999). Recent studies have shown that *BRCA1* co-localizes with phosphorylated H2AX (γ-H2AX) in response to DNA damage.

DSBs promote an extensive response in chromatin, demonstrated by the phosphorylation of Ser139 at the C-terminus of H2AX (Rogaku et al., 1998, Rogaku et al., 1999). This event extends for thousands of bases around a DSB and can be mediated by DNA damage signaling. γ-H2AX forms discrete foci within 10 min after DNA damage, and *BRCA1* is detectable in these foci 30 min thereafter (Paull et al., 2000). Importantly, in H2AX-deficient cells, *BRCA1* fail to form DNA damage-induced foci, suggesting that at least part of the *BRCA1* response to DSBs takes place on chromatin (Celeste et al., 2002). Forced entrapment of *BRCA1* in chromatin causes phosphorylation of H2AX by co-localization with *BRCA1* in a DNA damage-independent manner. *BRCA1* might therefore recruit kinases responsible for H2AX phosphorylation to DNA lesions and nucleate repair foci (Ye et al., 2001).

A recent study has revealed that *BRCA1* contributes to the regulation of c-Abl activity (Foray et al., 2002). c-Abl tyrosine kinase is ubiquitously expressed and localized in the cytoplasm and nucleus. Nuclear c-Abl is activated by diverse genotoxic agents and induces apoptosis mediated by p73 or Rad9 (Yuan et al., 1999, Yoshida et al., 2002). c-Abl is also implicated as a regulator of transcription and DNA repair. *BRCA1* and c-Abl form a complex constitutively, and exposure to ionizing radiation triggers an ATM-dependent disruption of this *BRCA1*-c-Abl complex, coinciding with the activation of c-Abl kinase activity (Foray et al., 2002). Loss of *BRCA1* results in constitutively elevated c-Abl kinase activity, suggesting that *BRCA1* is involved in the control of such activity. These findings suggest a route by which *BRCA1* affects cellular responses to DNA damage, distinct from a direct role in DNA repair or a role in cell cycle checkpoint control. The locus for *BRCA1* was identified by linkage analysis from breast cancer families to be 17q21 (Hall et al., 1990), and the gene was cloned in 1994 (Miki et al., 1994).

The gene encompasses 24 exons in approximately 81 kb of genomic DNA. The protein is coded by 1863 amino acids, and contains several different domains: a RING finger domain, two nuclear localization signals,

and a tandem repeat of sequence elements near the C-terminus (BCRT) (Tavtigian et al., 1998). *BRCA1* -/- embryos die on day 5-6 during gestation. The cells show reduced proliferation concomitant with an increase in the cell cycle inhibitor p21 (Hakem et al., 1996). This is somewhat surprising, as one would expect unrestrained cell proliferation to be associated with cancer. The *BRCA1* -/- phenotype can be partially rescued by crossing the mice with *TP53* -/- mice (Hakem et al., 1997, Ludwig et al., 1997). The crossing results in delayed embryonic lethality (embryo days 8.5-9.5), possibly caused by abolishing a *TP53*-mediated growth arrest and thereby allowing for continuous proliferation. Furthermore and surprisingly, the heterozygous *BRCA1* +/-mice did not exhibit any clear tumor predisposition, either in the mammary gland or anywhere else. Various complications have been encountered when trying to overexpress *BRCA1* in cell lines. First, no *BRCA1*-null cell line has been identified, and cells from the *BRCA1* -/- mice fail to proliferate in culture. Secondly, the overexpression of such a large gene makes confirmation of wild type sequence difficult, and thirdly, *BRCA1* overexpression seems to be toxic to many cell types. However, various groups have succeeded in overexpressing *BRCA1* in cells, resulting in reduced proliferation. Furthermore, overexpression in breast and ovarian cell lines reduced their ability to form tumors in mice (Holt et al., 1996). Although it has been shown that *TP53* and p21 levels can be regulated by *BRCA1*, many questions remain on how *BRCA1* regulates proliferation.

Defining *BRCA1*'s biochemical function within the cell might give clues to these questions. At the moment there are two roles described for *BRCA1* which are not mutually exclusive. First, there are findings which suggest a transcriptional function for *BRCA1* (Chapman and Verma, 1996). These include the identification of a transcriptional activator function in the *BRCA1* C-terminus in yeast and mammalian cells, the structure of *BRCA1* with its RING finger and an acidic domain, its proposed association with RNA polymerase II, and its ability to regulate the p21 promoter and a *TP53* binding

site in reporter assays. The second role is an involvement in DNA repair processes, which would allow *BRCA1* defective cells to accumulate mutations, thereby fostering cancer development. *BRCA1* was found to interact indirectly and to colocalize with Rad51 (Scully et al., 1997b), a protein involved in DNA repair and recombination. Also, *BRCA1* is found in discrete nuclear foci (dots), which are disturbed by treatment of the cells with DNA damaging agents such as hydroxyurea, UV or gamma irradiation (Scully et al., 1997a). Further exploration of these dots is undoubtedly in progress. Finally, consistent with this proposed role in repair, tumors from patients with *BRCA1* mutations have a higher frequency of chromosomal amplifications and deletions than do sporadic tumors. Many groups are trying to identify other *BRCA1*- interacting proteins. One of them is *BRCA1*-associated protein-1 (BAP-1) (Jensen et al., 1998), which was identified by yeast two-hybrid screening. BAP-1 is a nuclear protein which has an ubiquitin-hydrolase function. The further characterization of this and other *BRCA1*- interacting proteins will certainly help to gain clearer insights into *BRCA1*'s function.

2.2.2 *TP53* as gatekeeper for breast cancer

The *TP53* gene was discovered in 1979 as a gene coding for a 53 kDa nuclear phosphoprotein bound to the large T antigen of the simian virus 40 (SV40) DNA virus (Lane and Crawford, 1979; Linzer and Levine, 1979). Since it was known that the T antigen is essential for both the initiation and maintenance of the transformed state in SV40-infected cells, *TP53* were thought to be a transforming oncogene. This view was further corroborated by the finding that *TP53* is overexpressed in methylcholanthrene-induced sarcoma, leukemias, virus-transformed cell lines and spontaneously transformed fibroblasts (DeLeo et al., 1979). Studies also demonstrated that cloned murine *TP53* cDNA could immortalize cells thereby leading to their susceptibility to transformation by the *ras* oncogene (Jenkins et al., 1984), and that *TP53* co-operated with the activated *Ha-ras* oncogene to transform normal embryonic cells (Eliyahu et

al., 1984). However, in 1984 itself Maltzman and Czyzyk demonstrated that *TP53* production is stimulated and *TP53* protein undergoes post-translational stabilization in non-transformed cells upon ultraviolet irradiation. It was observed that only mutant *TP53* could co-operate with *ras* during cellular transformation, and wild-type *TP53* could in fact inhibit transformation induced by the combined effect mutant *TP53* with E1A antigen or *ras*. It was also demonstrated that colorectal carcinomas in which one allele of the *TP53* gene is lost, also harbored mutations in the remaining allele, a characteristic hallmark of loss of tumor suppressor function according to Knudson's hypothesis. This pattern was found to be true for other cancers, and it was observed that introduction of wild-type *TP53* resulted in suppression of growth of human colorectal cell lines (Velculescu and El-Deiry, 1996). Thus, by 1989 there was substantial evidence to support the role of wild-type *TP53* as a tumor suppressor gene rather than an oncogene.

The transcription factor *TP53*, originally discovered in the late Seventies as an oncogene binding to the large Tantigen in SV40-transformed cells (DeLeo et al., 1979; Lane and Crawford, 1979) is the most studied tumor suppressor gene in cancer today. Clearly, the fact that more than 50% of all cancers contain mutations in the *TP53* gene (Hollstein et al., 1991) justifies this intense research. In contrast to most other tumor suppressor genes in which mutations result in truncated protein products, *TP53*mutations are mostly missense mutations (Ozbun & Butel, 1995). The missense mutations occur in one allele, leading to accumulation of the *TP53* protein in the cell, and are followed by loss of the other allele and reduction to hemizygosity. However, there are also frame shift mutations leading to premature stop codons. Finally, there are also some rare cases of a different missense mutation on each allele. Another difference from most tumor suppressor genes is the finding that mutant *TP53* acts in a dominant negative fashion on wildtype *TP53*, which of course is relevant for heterozygous individuals. This dominant negative role of *TP53* mutations ('gain of function') (Dittmer et al.,

1993; Zambetti and Levine 1993) is consistent with the observation that transgenic mice with one mutant *TP53* allele accompanying two normal *TP53* alleles have offspring with an increased risk for cancer despite the presence of two normal *TP53* alleles.

The increased likelihood for tumor development in hemizygous *TP53* individuals with mutations in the second allele was substantiated by the results from animal studies in which the *TP53* allele(s) was inactivated by homologous recombination ('knockout' mice) (Donehower et al., 1992). Homozygous *TP53* -/- mice are very susceptible to spontaneous tumors; 74% of the null mice developed tumors by 6 months of age. Heterozygous progeny carrying one wild type *TP53* allele rarely developed tumors before 9 months of age. Somewhat surprisingly, *TP53*-/- mice are developmentally normal, including normal mammary gland development such as duct formation and lactation. It is still an open question whether mutated *TP53* acts as a dominant oncogene ('gain of function'). The results from the knockout studies argue against this theory, since tumors develop without the presence of a mutated *TP53*. However, the mouse knockout studies have to be interpreted with caution. For instance, the tumors developing in the *TP53* -/- mice are mostly lymphomas, different from the situation in humans (breast and adrenal carcinomas, or sarcomas). Thus, the deletion of two alleles in the *TP53* knockout mice may not truly reflect the hemizygous *TP53* patient, in which one allele is mutated and one is lost, and the development of tumors other than lymphomas could depend on *TP53* acting as a dominant oncogene. A role for mutant *TP53* as a dominant oncogene has recently been elegantly described by Gualberto (Gualberto et al., 1998), who analyzed a mutant *TP53* from Li-Fraumeni patients. This mutant has a defect in cell cycle checkpoint control in G2, resulting in polyploidy. Since this phenotype is not seen in *TP53* -/- cells, this observation in a cell line with a mutated *TP53* supports a gain-of-function phenotype.

2.2.2.1 Trend of *TP53* mutation in breast cancer

The protein consists of 393 amino acids (Matlashewski et al., 1984; Zakut-Houri et al., 1985; Lamb and Crawford 1986) and has been divided structurally and functionally into four domains (Figure 1.3). The domains are involved in transcriptional activation and repression, DNA and RNA binding, and oligomerization. The *TP53* protein is a tetramer in solution (dimer of a dimer). Post-translational modifications have been studied, especially cell cycle dependent phosphorylation. Various kinases such as casein kinase II, cell division cycle 2 (cdc2), and double stranded DNA-activated protein kinase (DNA-PK) have been implicated in phosphorylating *TP53* (Ozbun and Butel 1995). A paper (Woo et al., 1998) showed that DNA-PK is a mediator of the stress response leading to the activation of *TP53*. However, many experiments have been performed in vitro, and the importance of phosphorylation for the growth-suppressing function of *TP53 in-vivo* is still unclear. Also, mutations at the nucleotides coding for a serine which is phosphorylated are fairly uncommon in the *TP53* sequence from breast tumors.

Under normal conditions, *TP53* is kept at very low concentrations in the cell, regulated by ubiquitin dependent degradation ($t_{1/2}$=20-40 min). Also, it seems to exist in an inactive form, and has to be activated to regulate transcription. Activators are DNA damage such as a double-strand break after gamma-irradiation or DNA repair intermediates after UV irradiation or chemical damage (Levine, 1997). Among other factors leading to *TP53* activation are hypoxia, unusual genome instability, and overexpression of oncogene products. Activated *TP53* then influences the transcriptional activity of a number of genes, including p21 (el-Deiry et al., 1993; Harper et al., 1993), mdm2 (Momand et al., 1992; Barak et al., 1993), GADD45 (Smith et al., 1994), cyclin G (Okamoto and Beach, 1994), bax (Miyashita and Reed, 1995), and insulin-like growth factor binding protein 3 (IGFBP- 3)

(Buckbinder et al., 1995). The activity of *TP53* is additionally regulated by its interaction with multiple cellular and viral proteins (White et al., 2012), e.g. the interaction of *TP53* with T-antigen, viral E6, and mdm2 leads to the abrogation of *TP53* function.

TP53 activation results in growth suppression through either cell cycle arrest or induction of apoptosis. *TP53*-mediated growth suppression has mainly been attributed to a cell cycle block in G1 through the transcriptional activation or repression of the above mentioned genes and yet to be identified genes. G1 arrest in the mammalian cell involves a rather complicated interrelationship among many genes, and we will go into more detail in the section on another tumor suppressor gene, *RB*. More recently, *TP53* has also been implicated in the control of the G2/M checkpoint (Cross et al., 1995; Fukasawa et al. 1996). Wildtype *TP53* cells stop in G2/M when exposed to mitotic spindle inhibitors such as nocadazole. However, in the absence of wildtype *TP53* the cells undergo new DNA synthesis without having gone through mitosis, leading to aneuploid cells. The involvement of *TP53* in the G2/M checkpoint also seems to be the basis for the more recently characterized role of *TP53* in senescence (Levine, 1997). Finally, there is evidence for a transcription independent function of *TP53*, which is involved in G0 arrest (Del et al., 1995).

TP53's role in triggering apoptosis has been confirmed by many laboratories (Lane et al., 1994). For instance, thymocytes from the *TP53*-/- mice do not undergo apoptosis as their normal counterparts do in response to DNA damage (Lowe et al., 1993). The details of how *TP53* regulates apoptosis have yet to be discovered, but it has become clear that both a *TP53*-mediated transcriptional activity and a *TP53* activity not requiring transcription can play a role in apoptosis (Haupt et al., 1997b). The decision whether a cell undergoes *TP53*-mediated cell cycle arrest or apoptosis seems to be based on the degree of damage and the availability of other 'survival factors' (Levine, 1997). It should be mentioned that other, *TP53*- independent

apoptosis-inducing pathways exist, one of them involving the transcription factor interferon regulatory factor-1. The existence of such pathways is encouraging for the discovery of additional options to restore apoptotic potential to tumor cells.

Finally, other *TP53* family members, designated p73a and b (Jost et al., 1997; Kaelin, 1998) have recently been described. At this point it is not clear which role they play in tumorigenesis; however, there seem to be differences compared with *TP53*, as p73 expression is not inducible by DNA damage.

Some laboratories have recently shown that *TP53* mutations can lead to drug resistance, including doxorubicin resistance in breast cancer cells (Aas et al., 1996). This is a result of inhibition of drug-induced apoptosis in cells with defects in *TP53*, since chemotherapy kills cells by causing DNA damage and thus stimulating apoptosis. Thus, therapy aimed at restoring wild type *TP53* would not only inhibit tumor growth by restoring cell cycle checkpoint control, but would also make the tumor cells more chemosensitive.

2.3 Screening of breast cancer

2.3.1 Cytopathological screening

A specific and extremely useful subfield of anatomical pathology is that of cytopathology. Cytopathology by definition is the study of disease through microscopic examination of tissues and their component parts. It is a vital component in the overall clinical pathway of many diseases and is an important tool in the research of many medical topics.

2.3.1.1 Haematoxylin and eosin staining in breast cancer

Haematoxylin is the most important and most used dye in the histopathology and histochemistry laboratories (Avwioro, 2010). It is especially used in Erhlich's haematoxylin and eosin technique for the demonstration of general

tissue structures where it stains tissue in shades of blue, pink and red enabling recognition of malignant and non-malignant cells as well as several intracellular and extracellular substances necessary for diagnosis of disease conditions in tissues and cells (Avwioro, 2010). The color of the dye can be altered by mixing it with other agents such as iron alum (black), potash alum (blue) and salts of tin (red) (Lillie and Fullmer, 1976). Haematoxylin which has been used extensively as a nuclear counter stain and for the staining of specific intracellular and extracellular substances has also been used in the textile industries, although to a lesser extent. Haematoxylin is obtained from the logwood, haematoxylon campechianum (Willis, 1951). The crude product is obtained from the milled heartwood by hot water or steam, purified by ether extraction, dried and crystallized from water (Lalor and Martin, 1959). Alternatively, the aqueous extract is precipitated from solution with urea (Stevens, 1990). Haematoxylin has also been prepared synthetically (Morsingh and Robinson, 1970), but it is not widely available. Haematoxylin has no staining properties, unless it is oxidized in a process called ripening to yield haematein, which is then combined with certain chemicals called mordants (Avwioro, 2010). The process of oxidizing haematoxylin to hematin (Culling, 1974), which is the active staining ingredient can be achieved naturally and artificially. In the natural process, prepared haematoxylin solution is exposed to air and sunlight for 6 to 8 weeks before it is used (Drury and Wallington, 1980). The advantage of the natural process of oxidation is that the solution lasts longer because oxidation is slow and gradual. In the artificial process of oxidation, oxidising agents such as sodium iodate, hydrogen peroxide, mercuric oxide and potassium permanganate are added to the solution of haematoxylin. These effect instant oxidation of haematoxylin to haematein and the solution may be used immediately, although with a shorter life span because dye solution is quickly over oxidized. Haematoxylin cannot stain a tissue unless a mordant is incorporated into the dye (Clark, 1974). Mordants are metallic salts, which act as a bridge between the stain and tissue enabling staining to take place (Avwioro, 2010). The colour of the staining reaction

depends on the constituents of the staining solution and the type of mordant used. Some staining solutions, which contain aluminium alum and potassium alum as the mordant, give a blue nuclear staining while those which contain iron give a black staining reaction. Other metallic salts which have been combined with haematoxylin in special staining techniques are chrome alum for the staining of lipoproteins (Gurr, 1962), myelin, phospholipids and cytoplasmic granules in B cells of the anterior pituitary and pancreatic islet (Hrapchak, 1976), molybdenum for the staining of collagen (Bulmer, 1962) and neural tissue (Mallory, 1891). Copper haematoxylin has been used to stain fatty acids (Gurr, 1962), myelin sheaths and mitochondria. Leadhaematoxylin solution has been used for the staining of axis cylinders, although staining may be up to 6 weeks.

Eosin is a fluorescent red dye resulting from the action of bromine on fluorescein. It can be used to stain cytoplasm, collagen and muscle fibers for examination under the microscope. Structures that stain readily with eosin are termed eosinophilic.Eosin is most often used as a counterstain to haematoxylin in H&E (haematoxylin and eosin) staining. Eosin stains red blood cells intensely red. Eosin is an acidic dye and shows up in the basic parts of the cell, ie the cytoplasm. For staining, eosin Y is typically used in concentrations of 1 to 5 percent weight by volume, dissolved in water or ethanol. For prevention of mold growth in aqueous solutions, thymol is sometimes added. A small concentration (0.5 percent) of acetic acid usually gives a deeper red stain to the tissue. Other colors, e.g. yellow and brown, can be present in the sample; they are caused by intrinsic pigments, e.g. melanin. Some structures do not stain well. Basal laminae need to be stained by PAS stain or some silver stains in order to exhibit appropriate contrast. Reticular fibers also require silver stain. Hydrophobic structures also tend to remain clear; these are usually rich in fats, eg. adipocytes, myelin around neuron axons, and Golgi apparatus membranes.

2.3.1.2 Immunohistochemistry in breast cancer diagnosis

Immunohistochemistry is an umbrella term that encompasses many methods used to determine tissue constituents (the antigens) with the employment of specific antibodies that can be visualized through staining (Brandtzaeg, 1998). The history of immunostaining methods began when Marrack produced reagents against typhus and cholera microorganisms, using a red stain conjugated to benzidin tetraedro (Marrack, 1934). However, Professor Albert H. Coons from Harvard School of Medicine-Boston, U.S.A. believed that the antigen detection provided by red color in tissue slices had very low sensitivity under optical microscopy and, in the early nineteen forties demonstrated that localizing antigens, especially microorganisms, was possible in tissue slices using antibodies against Streptococcus pneumoniae stained with fluorescein, visualized by ultra-violet light (fluorescence microscopy) (Coons et al., 1941).

Subsequently, the introduction of enzymes as marked antibodies, developed by Nakane, heralded a new and important era for immunohistochemistry, since it was possible to see these reactions through optical microscopy. These results had great impact and were much awaited in the nineteen sixties (Nakane, 1968). This innovation took immunohistochemistry beyond the exclusive sphere of laboratories equipped with fluorescence microscopes, and the technique spread to a broad group of researchers and pathologists (Haines, 2005). The following discoveries of the unlabelled antibody peroxidase-antiperoxidase (PAP) method by Sternberger (Sternberger et al., 1970) and the alkaline phosphatase-antialkaline phosphatase (APAAP) method by Mason et al (Mason DY and Sammons R., 1978) significantly expanded the application of immunohistochemistry technique (Cordell et al., 1984). The diaminobenzidine molecule (DAB) was also conjugated to antibodies during the same period,(Singer, 1959) currently representing the most used chromogen for peroxidase, and as it produces an

electrodense precipitate which is also used in electronic microscopy, substituting ferritin. Subsequently, gold colloidal particles were introduced as immunohistochemical colorations (Faulk and Taylor, 1971) and this finding rapidly led to an important method of subcellular immunostaining (Roth, 1982). The discovery of antigen retrieval methods (exposure of antigen epitopes present in study tissue, favoring the antigen-antibody reactions for the next stages of the technique) by Huang et al, (Huang et al., 1976) and also the systems of secondary antibody detection (for example the avidin-biotin-peroxidase complex-ABC and the labeled streptavidin-biotin complex-LSAB) by Hsu et al (Hsu and Raine, 1981) allowed immunohistochemistry to be used in fresh specimens as well as in fixed tissues, which further increased the applicability of the technique in pathology diagnostic routines. However, only after the presence of tissue antigens could be demonstrated by the immunoperoxidase technique in tissues fixed in formalin and embedded in paraffin, did immunohistochemistry really became incorporated into the diagnostic routine of pathological anatomy (Werner B., 2005).

This fact reflects the position that immunohistochemistry currently holds in a pathological anatomy laboratory. It is an important tool for scientific research and also a complementary technique in the elucidation of differential diagnosis which are not determinable by conventional analysis with hematoxylin and eosin. The great improvement in the contribution and application of immunohistochemistry in pathological anatomy became known as the "brown revolution" of the histopathology laboratory.

Importance of the immunohistochemical reactions

The immunohistochemical reactions can be used in different situations within research or pathological anatomy laboratories. The most important are: 1) histogenetic diagnosis of morphologically non-differentiated neoplasias; 2) subtyping of neoplasias (such as lymphomas, for example); 3) characterization of primary site of malignant neoplasias; 4) research for prognostic factors and

therapeutic indications ofsome diseases; 5) discrimination of benign versus the malign nature of certain cell proliferations; identification of structures, organisms and materials secreted by cells (Jaffer and Bleiweiss, 2004). Werner and colleagues (Werner et al., 2005) evaluated the reason for employment and number of cases in which immunohistochemistry aided the diagnosis of neoplasias and pseudo-neoplastic lesions.

Limitations, difficulties and problems

Although a relatively simple technique, immunohistochemistry has some particularities and its outcome depends on many factors. The usefulness and contribution of immunohistochemistry in solving problems in pathological anatomy is directly proportionate to the experience of the hands that perform the reactions and also the eyes that interpret the results (Jaffer and Bleiweiss, 2004). Therefore, even though very simple in concept, immunostaining methods requires rigor of execution and may present significant bias. Hence, its outcomes must be interpreted with caution. A review (Yaziji and Barry, 2006) discusses the main bias that may follow the analysis of immunohistochemistry reactions. These are didactically divided into reaction bias (examples: specimen fixation, tissue processing, antigen retrieval and detection system) and interpretation bias (examples: selection of antibody panels, sensitivity of the chosen panel, choice of antibody types and clones, results and literature interpretation). A wide variety of protocols for standardizing the immunohistochemistry technique are being proposed to minimize undesirable effects. The Committee of Quality Control in Immunohistochemistry of the French Pathology Society published a report in 1997 demonstrating that two of the main causes of diagnosis mistakes in immunohistochemistry are the non-employment of antigen retrieval techniques and the use of amplifying methods with low power. The acquisition, handling, fixation, specimen delivery to the laboratory and antigen retrieval are all critical factors. Fresh specimens that are inadvertently submitted to long periods of fixation may significant lose antigenicity (Shi,

2006). As an example, Jacobs and colleagues (Jacobs et al., 1996) showed that there is progressive loss of antigenicity upon only 12 week storage of breast cancer histological slices on slides stored in ambient temperature for the detection of *TP53*, Bcl-2, estrogen receptor and factor VIII proteins. However, the same was not observed in recent histological slices of specimens in paraffin blocks for periods of over 10 years (Manne et al., 1997). The specimen fixation in formaldehyde and its consequent inclusion in paraffin are the internationally most used histological processing procedures. Some specialists propose that this procedure should be the standard for comparing diagnostic outcomes among immunohistochemistry reactions (Wick, 1995). However, formaldehyde fixation results in a variably reversible loss of immunoreactivity by its masking or damaging some antibody binding sites (Rickert et al., 1989). Although such epitopes may be demasked by several epitope retrieval methods, the immunohistochemical detection system must still be sensitive enough to produce a strong signal. For some epitopes, the duration of the formaldehyde fixation is critical. With some antibodies, depending on the resistance of its target epitope to autolytic change, delay in fixation may cause loss of immunoreactivity (Wasielewski et al., 1998). Other fixatives often used in pathology include alcohol and alcohol-based fixatives such as acetone. Alves et al (Alves et al., 1992) studied the fixation in ethanol and formalin for trypsin digestion in immunohistochemical detection of cytokeratins and vimentin in a case of ovarian cystadenofibrocarcinoma. They found superior reactivity for both markers in achieved ethanol-fixed sections, even in samples stocked up to 60 days. Cytokeratin reactions in formalin-fixed sections were better when trypsin was used. However, this digestion was deleterious to vimentin detection. This was an import work to alert surgeons and oncologists on the relevance of fixation of specimens suspicious for neoplasia, since different epitopes may require different fixatives and the inadequate choice in the operative room may impart difficulties when immunohistochemistry is necessary. It is important to emphasize that in tissue processing, inclusion in paraffin at high temperatures (in general, over 60^0C)

may compromise the specimen antigenicity. Another important point addresses the preparation of slides. The block slices must preferentially present a thickness ranging between 3 and 7 μm and must be deposited on slides previously prepared with some kind of adhesive (the most used are silane and polylysine). Slices less than 3 μm thick could result in very weak immunostaining while those thicker than 7 μm may lead to loss of tissue on the glass slide or may hamper analysis of the resultant immunostaining (Yaziji and Barry, 2006). The amount of material to be analyzed is being discussed, especially now that pathologists are expected to reach a precise diagnosis with small samples. In the majority of situations a block is sufficient, preferentially when it contains a fragment of the tumor-surrounding parenchyma interface (prepared in the macroscopic examination), distally to hemorrhagic or extensively necrotic areas, as well as a fragment representative of the tissue distal to the neoplasm (Balaton et al., 1996). Whenever possible, tissue that was previously submitted for frozen examination must be avoided. Regarding antigen retrieval, the simplification of procedures, costs and technical error risk reduction are important factors. Irradiation techniques with microwaves or by humid heat in pressure or vapor pan, with exposition times adapted to offer the same pattern of staining in a group of case-controls has been suggested (Cattoretti et al., 1992). The use of detection systems (secondary antibodies) is also considered valuable in error reduction. Among high discharge amplification systems, the avidinbiotin-peroxidase complex (ABC) and the labeled streptavidin-biotin complex (LSAB) are the most important (Elias et al., 1989). Specific situations require adaptations and even the use of alternative detection methods. The selection of an adequate method is one of the great technical responsibilities faced in an immunohistochemistry laboratory. The advance in the technique, with systems of epitope retrieval through heat (HIER) and amplification methods, as well as the reactions performed in a single stage (EPOS) (Pastore et al.,1995) and the method of catalyzed product deposition (CARD), (Bobrow et al., 1992) have introduced a paradox in immunohistochemistry.

On the one hand numerous cases hitherto unsolved because of negativity in many panels, became positive and began to permit precise diagnosis. On the other hand, antibodies that were expressed characteristically in certain neoplasias began to react non-specifically in other situations. Concerned about the so called "anarchy" then introduced, Swanson proposed that no method should be universally applicable (Swanson, 1997), the choice should be based on the technique that, in the experience of the laboratory or of the school followed by researchers, best solves the diagnostic question (Alves et al., 1999). Due to their flexibility and relatively low cost, the most used protocols currently (such as the ABC method, for example) are indirect and therefore require many stages of incubation. High sensitivity could be obtained with the application of immunological principles, enzymatic amplification reactions and/or the employment of avidin-biotin complex; however the various steps required must be rigorously followed in order to avoid non-desirable interactions. It is fundamental that, on technical planning, all reagents follow the sequence rigorously established, where the employment of work flow charts for such stages are very useful in avoiding false results. Making notes of all reaction stages and pattern of each antibody are equally important and are suggested in patterning technique programs question (Alves et al., 1999). The ability of the specialized technician who performs the reactions is a guarantee against the introduction of crossed immunological reactions with endogenous immunoglobulins during the test preparations, or with different sequence experiments of immunostaining with many colors. The selection of antibody panels is one of the most important aspects for optimal applicability of immunohistochemistry question (Alves et al., 1999). Studies from Jensen and colleagues (Jensen et al., 1997) concluded that the selection of the antibody panel and the interpretation of the reaction patterns of each case were the most important factors for the final diagnostic outcome (Jensen et al., 1997). This observation was fundamental because the detection sensitivity of the chosen panel evidently increases with increased practice and experience of the pathologist who indicates the method, combined with the

clinical data analysis by the researchers (Yaziji and Barry, 2006). Prescott and colleagues (Prescott et al., 1995) attributed 42.1% of the diagnostic discrepancies in immunohistochemistry to poor antibody selection.

The knowledge of each reagent's characteristics, especially those of antibodies, requires new titration in each new batch or clone, selecting the dilution that offers the greatest "true/background positivity" contrast (Yaziji and Barry, 2006). The primary antibodies can be divided into two categories: poly or monoclonal. The polyclonal group is those obtained from animal immunization (example: rabbit, goat, monkey, rat, mouse, ewe etc) and results in antibodies that are capable of recognizing many epitopes of the same antigen, generating higher detection sensitivity. The monoclonal type, however, is developed from hybrids and provide antibodies against only one antigen epitope, yielding more specific results (Lipman et al., 2005). Regarding the validation of findings and their interpretation, it is necessary to observe the reactivity patterns of the negative and positive, internal and external controls. The external controls (histological slices of specific tissues for each antibody) must be included in each panel, prepared from the samples fixed under the same conditions as the test cases and submitted to the same stages of the reaction. Attention must also be paid to the reactivity of structures present on the slide of the case being studied that may be used as internal positive controls, such as the reactivity of vessels for vimentin, muscle and endothelial markers, or breast ducts adjacent to the neoplasm for estrogen and progesterone receptors. Similarly, structures knowingly negative for a marker offer an excellent internal negative control, since they were submitted to the same treatment as the test-tissue, for example the erythrocytes within blood vessels a great endogenous source of peroxidase (Taylor, 1994).

2.3.2 Molecular screening of breast cancer

The completion of the human genome project, along with the ancillary technologies derived from this effort, provides the ability to comprehensively

analyze patient tumors as well as the individual patient's own genetic make-up at the DNA, RNA, and protein level. As a result, novel molecular screening techniques have the potential to push the boundaries of detection to even smaller tumors and also to allow accurate risk assessment, cancer prevention, and treatment planning in individual women (Stemke-Hale et al., 2006). Genetic testing for breast cancer susceptibility has become an integral part of medical management. At-risk individuals can be provided with regular surveillance to identify breast cancer at an early stage. Prophylactic surgery aims to prevent the development of cancer, and in the near future, moving in the direction of individualized medicine, targeted therapies for affected mutation carriers will be available. The essential step towards a rational approach is how to identify individuals who will benefit from testing, without straining the financial budget of the national health system, although recently established techniques like next generation sequencing may significantly reduce the costs. It is well known that genetic factors play an important role in the development of breast cancer (Foulkes, 2008; Ripperger et al., 2009). Five to ten percent of breast cancers are believed to be caused by a genetic predisposition due to a mutation in a highly penetrant breast cancer gene (Chen et al., 2006). According to the autosomal-dominant manner of inheritance, there is a 50% probability for each offspring to inherit the mutation and therefore to carry the elevated risk for breast and ovarian cancer. Once a pathogenic mutation has been identified in a family, predictive testing for healthy relatives becomes possible. Because of the substantially lower age of onset of hereditary tumours compared to sporadic breast cancer, women at risk need access to surveillance at a younger age.

All cancers carry somatic mutations in their genomes. A subset, known as driver mutations, confer clonal selective advantage on cancer cells and are causally implicated in oncogenesis, and the remainder are passenger mutations. The driver mutations and mutational processes operative in breast cancer have not yet been comprehensively explored. The number of somatic

mutations varied markedly between individual tumours. Stephens et al., found strong correlations between mutation number, age at which cancer was diagnosed and cancer histological grade, and observed multiple mutational signatures, including one present in about ten per cent of tumours characterized by numerous mutations of cytosine at TpC dinucleotides (Stephens et al., 2012). Ripperger suggested (Ripperger et al., 2009) that genetic counselling of *BRCA1/BRCA2/TP53* mutation carriers regarding modifier polymorphisms such as FGFR2, TOX3, MAP3K1 and RAD51, may be the easiest task because (i) the additional analysis of certain polymorphisms may specify the breast cancer risk and allow further individualization of the clinical management regarding the surveillance programme, decision-making concerning risk-reducing surgery and therapy, (ii) this is relevant for a small group of about 3% of all breast cancer patients, and most importantly, (iii) clinical programmes to meet this high risk have been set up in most countries (Silva et al. 2008). At present, complete sequencing of intermediate penetrance genes such as *ATM, BRIP1, CHEK2, PALB2* or *RAD50* in *BRCA1/BRCA2-TP53* negative high-risk breast cancer families does not appear to be justified. Mutations in these genes are extremely rare in most populations and, even more importantly, the clinical consequences are not yet clear, especially regarding the incomplete segregation of the known mutation with breast cancer in affected families (Renwick et al., 2006). It has to be considered that mutations, at least in some of these genes, are not specific for *BRCA1/BRCA2/TP53* mutation-negative familial cases, but can also occur in sporadic breast cancer cases (Blay et al., 2013). Therefore, it is likely that predisposing mutations with moderate penetrance in other genes will be identified in families with multiple cases. Subsequently, this knowledge as well as the potential risk-modifying interaction of these mutations with predisposing polymorphisms may be an attractive tool for risk prediction.

Nevertheless, in the near future, as soon as costs and time of sequencing are reduced to a more reasonable level, it may become cost-

effective to screen all genes involved in DNA repair or other breast cancer-associated pathways in high-risk families. In addition to the identification of new polymorphisms and the validation of their relative risk in different large cohorts, it will be essential to understand the combined effect of different polymorphisms, and to establish reliable risk prediction models before stepping forward to routine investigations. Given seven established common breast cancer susceptibility SNPs, there are 2187 possible combinations of genotypes. Although risks conferred by individual loci are rather small, some risk alleles seem to act multiplicatively. Thus, the breast cancer risk among women carrying 14 risk alleles is estimated to be six times as high as among women carrying none of these risk alleles (Pharoah et al., 2008). In the future, screening programmes may be adapted to the individual genetic risk, for example, modifying age of initiation and investigation intervals of mammography, integration of magnetic resonance imaging and/or risk-reducing surgery. It is to be hoped that this will lead to an intensified screening in high-risk individuals, but spare unnecessary and even potentially harmful screening in women with very low-risk profiles. However, the clinical utility of such an individualized genome-based approach has to be validated, and principles for the implementation of genetic testing of new susceptibility mutations and/or polymorphisms have to be established from an individual as well as from a population and health-care system point of view (Ginsburg et al., 2008). Further studies will probably identify polymorphisms associated with different response rates to certain therapies or histological subtypes and particular SNPs may even serve as therapeutic targets (Gracia-Closas et al., 2008). Taking full advantage of these polymorphisms will require functional characterization of the polymorphisms, as for instance for an SNP in intron 2 of FGFR2 leading to an altered binding affinity for transcriptional factors and thereby, to an increased FGFR2 expression (Meyer et al., 2008).

Meanwhile, guidelines have been established on how to identify individuals at risk for familial breast cancer. Regulation of the field was

initiated by the respective national leading stakeholders to ensure and control the quality of the process. Although a similar frequency of *BRCA1, BRCA2* and *TP53* mutation is assumed within developed countries, each country has established its own approaches on how to proceed in the case of familial breast cancer. The individual guidelines and recommendations are roughly similar. Nevertheless, they are a result of a growing superordinate process of how to deal with genetic information in accordance with the public attitude and the organization of the respective national health system: in this case, regarding the gatekeepers for the genetic service, differences are obvious.