

# Tumor suppressor genes in breast cancer

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## Introduction

The incidence of breast cancer has risen steadily over the last half a century, partially due to earlier detection. Nevertheless, the mortality rate has remained relatively constant (27 per 100 000 women). Although we are beginning to understand the risk factors for breast cancer, including environmental sources (e.g. mutagen exposure) and personal choices (e.g. late first childbirth age and high dietary fat intake), the further identification of mechanisms underlying the development and progression of breast cancer is of major public health importance.

The activation of oncogenes has been well described as one possible mechanism to transform normal cells, including breast. Now it has become clear that the inactivation of various tumor suppressor genes, which can be thought of as 'brakes' of cell growth, is at least as important in the development and progression of breast cancer. Tumor suppressor genes are considered to act mostly in a recessive fashion, i.e. some abnormality must affect both gene alleles. The classical inactivation of tumor suppressor genes, i.e. the Knudson 'Two-Hit' hypothesis (Knudson 1971), is caused by tumor suppressor gene loss due to chromosomal loss of one allele, and mutation of the other remaining allele. Chromosomal loss is mostly analyzed by karyotypic studies or loss of heterozygosity (LOH) studies, and mutations are most frequently studied by sequencing of the gene of interest or by single strand chain 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 (Baylin *et al.* 1998, Foster *et al.* 1998), increased degradation (Storey *et al.* 1998), or mislocalization (Chen *et al.* 1995, Moll *et al.* 1992, Takahashi & Suzuki 1994).

The function and role of tumor suppressor genes have been elucidated by many investigators through a combination of a number of cell biological as well as

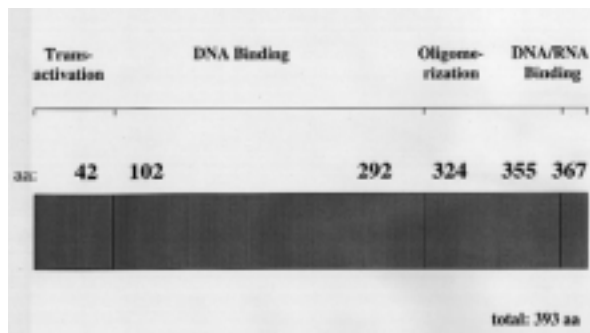
biochemical methods. Kinzler & Vogelstein (1997) have recently proposed a new system to categorize tumor suppressor genes as 'gatekeepers' and 'caretakers'. Gatekeepers are tumor suppressor genes which are directly involved in controlling proliferation by regulating cell cycle checkpoints (e.g. Rb and the INK family of cdk inhibitors). Mutations of these genes usually result in high penetrance. In contrast, caretakers are of rather low penetrance, and have an indirect effect on growth. They are responsible for genome integrity, and changes in such genes lead to genome instability. The best characterized examples are repair genes, such as MSH2 in hereditary non-polyposis colon cancer (HNPCC) (Fishel & Wilson 1997). 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  $\alpha$ -catenin) (Bullions *et al.* 1997).

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

## Tumor suppressor genes in sporadic disease

### p53

The transcription factor p53, originally discovered in the late Seventies as an oncogene binding to the large T-antigen in SV40-transformed cells (DeLeo *et al.* 1979, Lane & 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 p53 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, p53



**Figure 1** The tumor suppressor gene p53. p53 is structurally and functionally divided into four domains as indicated, and their position in the p53 protein is shown. aa, amino acids.

mutations are mostly missense mutations (reviewed in Ozbun & Butel 1995). The missense mutations occur in one allele, leading to accumulation of the p53 protein in the cell, and are followed by loss of the other allele and reduction to hemizyosity. However, there are also frameshift 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 p53 acts in a dominant negative fashion on wildtype p53, which of course is relevant for heterozygous individuals. This dominant negative role of p53 mutations ('gain of function') (Dittmer *et al.* 1993, Zambetti & Levine 1993) is consistent with the observation that transgenic mice with one mutant p53 allele accompanying two normal p53 alleles have offspring with an increased risk for cancer despite the presence of two normal p53 alleles.

The increased likelihood for tumor development in hemizygous p53 individuals with mutations in the second allele was substantiated by the results from animal studies in which the p53 allele(s) was inactivated by homologous recombination ('knockout' mice) (Donehower *et al.* 1992). Homozygous p53<sup>-/-</sup> mice are very susceptible to spontaneous tumors; 74% of the null mice developed tumors by 6 months of age. Heterozygous progeny carrying one wildtype p53 allele rarely developed tumors before 9 months of age. Somewhat surprisingly, p53<sup>-/-</sup> mice are developmentally normal, including normal mammary gland development such as duct formation and lactation.

It is still an open question whether mutated p53 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 p53. However, the mouse knockout studies have to be interpreted with caution. For instance, the tumors developing in the p53<sup>-/-</sup> mice are mostly lymphomas, different from the situation in humans (breast and adrenal

carcinomas, or sarcomas). Thus, the deletion of two alleles in the p53 knockout mice may not truly reflect the hemizygous p53 patient, in which one allele is mutated and one is lost, and the development of tumors other than lymphomas could depend on p53 acting as a dominant oncogene. A role for mutant p53 as a dominant oncogene has recently been elegantly described by Gualberto *et al.* (1998), who analyzed a mutant p53 from Li-Fraumeni patients (also see below). This mutant has a defect in cell cycle checkpoint control in G2, resulting in polyploidy. Since this phenotype is not seen in p53<sup>-/-</sup> cells, this observation in a cell line with a mutated p53 supports a gain-of-function phenotype.

### Structure and functional properties of p53

The protein consists of 393 amino acids (Matlashewski *et al.* 1984, Zakut-Houri *et al.* 1985, Lamb & Crawford 1986) and has been divided structurally and functionally into four domains (Fig. 1). The domains are involved in transcriptional activation and repression, DNA and RNA binding, and oligomerization. The p53 protein is a tetramer in solution (dimer of a dimer). Posttranslational 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 p53 (Ozbun & Butel 1995). A recent paper (Woo *et al.* 1998) showed that DNA-PK is a mediator of the stress response leading to the activation of p53. However, many experiments have been performed *in vitro*, and the importance of phosphorylation for the growth-suppressing function of p53 *in vivo* is still unclear. Also, mutations at the nucleotides coding for a serine which is phosphorylated are fairly uncommon in the p53 sequence from breast tumors.

Under normal conditions, p53 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 (for review see Levine 1997). Among other factors leading to p53 activation are hypoxia, unusual genome instability, and overexpression of oncogene products. Activated p53 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 & Beach 1994), bax (Miyashita & Reed 1995), and insulin-like growth factor binding protein 3 (IGFBP-3) (Buckbinder *et al.* 1995). The activity of p53 is additionally regulated by its interaction with multiple cellular and viral proteins (reviewed in Ozbun & Butel

1995), e.g. the interaction of p53 with T-antigen, viral E6, and mdm2 leads to the abrogation of p53 function.

p53 activation results in growth suppression through either cell cycle arrest or induction of apoptosis. p53-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 (see below). More recently, p53 has also been implicated in the control of the G2/M checkpoint (Cross *et al.* 1995, Fukasawa *et al.* 1996). Wildtype p53 cells stop in G2/M when exposed to mitotic spindle inhibitors such as nocadazole. However, in the absence of wildtype p53 the cells undergo new DNA synthesis without having gone through mitosis, leading to aneuploid cells. The involvement of p53 in the G2/M checkpoint also seems to be the basis for the more recently characterized role of p53 in senescence (for review see Levine 1997). Finally, there is evidence for a transcription-independent function of p53, which is involved in G0 arrest (Del Sal *et al.* 1995).

p53's role in triggering apoptosis has been confirmed by many laboratories (Lane *et al.* 1994). For instance, thymocytes from the p53<sup>-/-</sup> mice do not undergo apoptosis as their normal counterparts do in response to DNA damage (Lowe *et al.* 1993). The details of how p53 regulates apoptosis have yet to be discovered, but it has become clear that both a p53-mediated transcriptional activity and a p53 activity not requiring transcription can play a role in apoptosis (Haupt *et al.* 1997b). The decision whether a cell undergoes p53-mediated cell cycle arrest or apoptosis seems to be based on the degree of damage and the availability of other 'survival factors' (for review see Levine 1997). It should be mentioned that other, p53-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 p53 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 p53, as p73 expression is not inducible by DNA damage.

#### *p53 mutations and breast cancer*

The p53 gene is located at 17p13.1 (McBride *et al.* 1986, Miller *et al.* 1986). Although there is a wide range of results depending on the individual study, the average LOH at this locus in breast cancer is approximately 30-40%. There are also 'hotspots' for mutations in the other

allele, mostly in the conserved region between amino acids 130 and 290 (for review see Ozbun & Butel 1995).

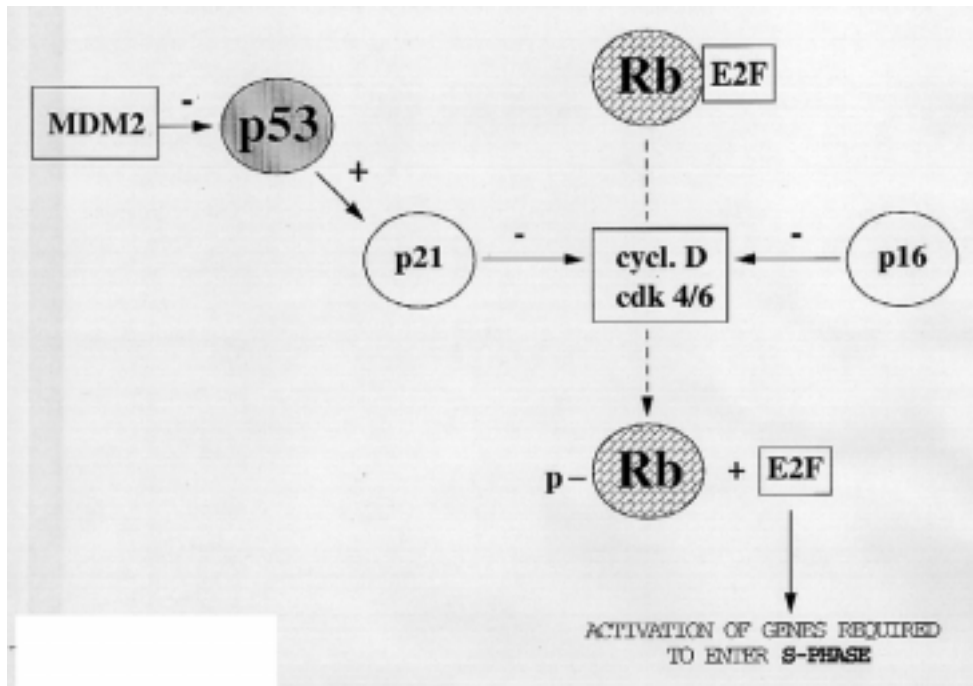
Many other mechanisms besides chromosomal deletion and gene mutation can lead to the inactivation of p53, such as nuclear exclusion (Takahashi & Suzuki 1994), increased degradation (Storey *et al.* 1998), and inactivation through binding to amplified mdm2 (Haupt *et al.* 1997a, Shieh *et al.* 1997). It is therefore difficult to design a single test to determine accurately and reliably the p53 status in tissue. Many laboratories use immunohistochemistry measuring increased p53 levels as a criterion for mutated p53 (Hall *et al.* 1990), but we now know that not all mutations necessarily lead to an increase in half life. On the other hand, sequencing of p53 misses defects in the p53 pathway caused by other epigenetic changes. It would therefore be desirable to design a functional test(s) for various p53 activities, such as the activation of the p21 promoter or ability to participate in protein-protein interactions in a reporter assay.

Some laboratories have recently shown that p53 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 p53, since chemotherapy kills cells by causing DNA damage and thus stimulating apoptosis. Thus, therapy aimed at restoring wild type p53 would not only inhibit tumor growth by restoring cell cycle checkpoint control, but would also make the tumor cells more chemosensitive.

#### **The retinoblastoma gene (Rb)**

##### *Structure and functional properties of Rb*

Retinoblastoma is a childhood ocular tumor, which is inherited in an autosomal dominant fashion (reviewed in Goodrich & Lee 1993). Positional cloning based on the physical localization of the gene to chromosomal band 13q14 (Yunis & Ramsay 1978) led to the isolation of the tumor suppressor gene Rb (Dryja *et al.* 1986, Friend *et al.* 1986, Fung *et al.* 1987, Lee *et al.* 1987a). The Rb protein is a 105 kDa nuclear phosphoprotein (Lee *et al.* 1987b) that is differentially phosphorylated on Ser and Thr residues during the cell cycle. Hypophosphorylated Rb predominates in G1, and phosphorylation by cyclin-dependent kinases leads to entry into S-phase (Buchkovich *et al.* 1989, Mihara *et al.* 1989). The generation of homozygous Rb-deficient mice allowed further insights into the role of Rb in normal development and differentiation (Clarke *et al.* 1992, Jacks *et al.* 1992, Lee *et al.* 1992). Rb<sup>-/-</sup>embryos die by gestational day 16 and suffer defects in development of hematopoietic and central nervous system cell lineages. Interestingly, heterozygous mice do not develop retinoblastoma, but rather, pituitary gland tumors. There are several possible



**Figure 2** The p53 pathway. A complex interaction between oncogenes (squares) and tumor suppressor genes (circles) regulates the G1-S cell cycle checkpoint. MDM2 negatively regulates p53 activity, which in turn activates the cdk inhibitor p21. p21 and p16 inhibit the cyclin D (cycl.D)-cdk4/6 complex, thereby preventing phosphorylation of Rb. Upon phosphorylation of Rb(p), E2F is released from Rb and activates genes which are involved in cell cycle progression.

explanations for this unexpected finding. Currently, the theory of 'functional compensation' is favored by many investigators, where Rb's other family members (p107 and p130) could replace Rb's function when Rb is lost. This in fact seems to be the case, as Rb and p107 deficient chimera develop retinoblastoma (Robanus-Maandag *et al.* 1998).

Although the precise mechanism by which Rb suppresses growth is still unclear, its interaction with various proteins, including E2F and ATF-2, seems to play a major role. E2F is a growth stimulator and can promote oncogenic transformation, probably via transcriptional activation of various genes involved in cell cycle progression (see Fig. 2). Rb's binding to E2F blocks this transcriptional activity (Helin *et al.* 1992). The recent identification of other E2F family members might help to elucidate further the tissue-specific effect of Rb (for reviews see Adams & Kaelin 1996, Slansky & Farnham 1996).

#### *Rb in breast cancer*

The Rb gene is located on chromosome 13q14, and loss of this region has been implicated in breast cancer progression (T'Ang *et al.* 1988, Andersen *et al.* 1992, Lee *et al.*

1988). Structural abnormalities including chromosomal loss and mutation of the Rb gene have been reported in approximately 20-30% of breast cancers. Besides chromosomal loss and mutation, there are various other mechanisms for Rb inactivation. For instance, recent studies show that hypermethylation of the Rb promoter (Stirzaker *et al.* 1997) results in significant down-regulation of the Rb product. Indeed, it has been shown that Rb can be inactivated in tumors by the loss of one allele and hypermethylation of the other allele. In addition, recent studies suggest that estrogen regulation of Rb might play an important role in breast carcinogenesis. Estrogen treatment of breast cancer cells leads to phosphorylation of Rb, which conversely can be blocked with antiestrogens (Watts *et al.* 1995, Foster *et al.* 1998). Furthermore, not only phosphorylation but also Rb mRNA and protein levels are directly increased as a result of estrogen exposure of estrogen-dependent breast cancer cell lines (Hurd *et al.* 1997).

Also, the inactivation of other genes in the Rb pathway, but not necessarily Rb itself, might be sufficient to give cells a growth advantage. This includes amplification of cyclin D and inactivation of cdk inhibitors. cdk inhibitors are divided into two families: the INK family

(p15, p16, p18, p19) and the CIP family (p21, p27, p57) (Biggs & Kraft 1995, Hengst & Reed 1998). Many of these proteins are being intensely studied as potential tumor suppressor genes. In breast cancer, most studies have been performed on p16, a CDK4 and CDK6 inhibitor (for review see Serrano 1997). p16 maps to 9p21, a chromosomal region that is frequently lost in breast cancer. However, many studies show that mutation of p16 occurs only infrequently. This paradox has recently been solved by showing inactivation of p16 by hypermethylation (Baylin *et al.* 1998, Foster *et al.* 1998), i.e. methylation of CpG islands in the p16 promoter results in the reduction of p16 expression. Summarizing, it is likely that the vast majority of tumors have a defect in at least one of the members of the Rb pathway. However, the 'defect' does not necessarily have to be a mutation.

### Other tumor suppressor genes with potential importance in breast cancer development and progression

#### The Wilms tumor genes (WT-1)

Wilms tumor is a pediatric kidney cancer in which the tumor suppressor action of WT-1 is disturbed (for reviews see Grundy 1997, Pritchard-Jones 1997). WT-1 is a transcription factor (Wang *et al.* 1993) containing a proline-rich transactivation domain and four zinc fingers (Rauscher 1993). Furthermore, it has an RNA binding domain and copurifies with spliceosomal proteins (Kennedy *et al.* 1996). Alternative splicing (Larsson *et al.* 1995), most frequently a 3-amino acid insertion, leads to changes in WT-1's transcriptional activity.

Unlike Rb-affected individuals, WT patients are not especially susceptible to other tumors. However, the finding that WT-1 negatively regulates insulin-like growth factor-I receptor (IGF-IR), IGF-II, and transforming growth factor  $\beta$  (TGF- $\beta$ ) led Siberstein *et al.* (1997) to investigate WT-1 in normal and cancerous breast. They found (1) that a high percentage of tumor cells lacked WT-1, (2) that WT-1 was mislocated from the nucleus to the cytoplasm in breast cancer cells, and (3) that mRNA splice usage is commonly perturbed in tumors. Furthermore, lack of WT-1 seemed to correlate with the expression of the estrogen receptor (ER), i.e. ER-positive cells were more likely to lack WT-1. Unfortunately, there are very few other reports on WT-1 in breast cancer. However, its chromosomal locus (11p13) has been implicated in the progression of breast cancer (Garcia *et al.* 1991), and further studies are awaited to define its role.

#### Von Hippel-Lindau disease (VHL)

VHL is a multisystem disorder with marked phenotypic variability, but retinal and central nervous system hemangio-

blastoma are very common (Maher *et al.* 1990b). Using genetic linkage studies, the VHL gene was mapped to 3p25 (Maher *et al.* 1990a, Seizinger *et al.* 1988), and soon after the gene was cloned (Latif *et al.* 1993). The sequence is not highly similar to that of any known genes, but the identification of VHL-interacting proteins suggests that it is involved in transcriptional elongation. Breast tumors show frequent loss of 3p, and therefore it is possible that VHL plays a role in breast cancer progression.

#### Adenomatous polyposis coli (APC)

Familial adenomatous polyposis (FAP) is a dominantly inherited disorder characterized by early onset of multiple adenomatous polyps of the colon and increased predisposition to colon cancer. Aberrations of the APC gene are responsible for FAP, and loss at the APC locus (5q21) (Grodin *et al.* 1991, Kinzler *et al.* 1991) has been described in various other cancers, including lung and gastric cancers.

There are a few studies describing LOH at the APC locus in breast cancer patients; the rates vary from 11% up to 38% (Thompson *et al.* 1993, Kashiwaba *et al.* 1994, Medeiros *et al.* 1994). Furthermore, one study actually reports mutations in the APC gene in primary breast cancer specimens (Kashiwaba *et al.* 1994). However, a definite answer as to whether APC inactivation contributes to breast cancer development cannot yet be made.

#### Insulin-like growth factor receptor 2 (IGFR2)

Members of the IGF and IGFR family have been known to play an important role in breast cancer development and progression for a long time (Lee *et al.* 1995). Recently it has been shown that the IGFR2 locus (6q26-27) displays LOH in breast cancer and early breast disease (Hankins *et al.* 1996). Furthermore, in two out of five early breast lesions with LOH at 6q26-27 the remaining allele was mutated, suggesting that IGFR2 functions as a tumor suppressor gene in breast cancer.

#### Tumor suppressor genes in familial disease

Epidemiological literature shows that there is a two- to threefold increase in breast cancer among sisters and mothers of breast cancer patients (Colditz *et al.* 1993). The magnitude of risk depends on age at diagnosis, closeness of relationship, and laterality, e.g. presence of bilateral breast cancer is a sign for the presence of familial disease. Data from many groups demonstrates that there is an autosomal dominant pattern of inheritance for a small percentage of breast cancers (5%-10%), and the cloning of two tumor suppressor genes important in familial breast cancer supports this theory.

## BRCA1

### *Gene structure and possible function of BRCA1*

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) (for review see Tavtigian *et al.* 1998).

BRCA1<sup>-/-</sup> 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<sup>-/-</sup> phenotype can be partially rescued by crossing the mice with p53<sup>-/-</sup> 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 p53-mediated growth arrest and thereby allowing for continuous proliferation. Furthermore and surprisingly, the heterozygous BRCA1<sup>+/-</sup> 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<sup>-/-</sup> mice fail to proliferate in culture. Secondly, the overexpression of such a large gene makes confirmation of wildtype 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 p53 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 & 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 p53 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 a ubiquitin-hydrolase function. The further characterization of this and other BRCA1-interacting proteins will certainly help to gain clearer insights into BRCA1's function.

### *BRCA1 in breast cancer*

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 upregulated during puberty and pregnancy, and estrogen treatment in oophorectomized mice also results in upregulation of BRCA1 (Marquis *et al.* 1995). Another group has also described an estrogen-mediated effect on BRCA1 expression (Romagnolo *et al.* 1998).

Finally, we would like to address a practical point which is important for BRCA1 and BRCA2 carriers.

Although yearly mammography and annual or semi-annual 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. Further studies, e.g. examining various radiation schedules and doses, are needed to clarify this important point.

### BRCA2

BRCA2 has been mapped to 13q12-13 by linkage analysis in families in which BRCA1 has been excluded as a cause of breast cancer, and like BRCA1, it is a very large gene, consisting of 27 exons spanning 70 kb of genomic DNA, and encoding a protein of 3418 amino acids (Wooster *et al.* 1995, Tavtigian *et al.* 1996). There is no strong homology between BRCA1 and BRCA2, although both genes have a large exon 11 which seems to be crucial for function. However, the function of the two genes seems to be similar. Like BRCA1, BRCA2 binds to Rad 51, possibly putting both genes in the same pathway (Mizuta *et al.* 1997).

A BRCA2 knockout results in embryonic lethality at gestational day 6.5 (Sharan *et al.* 1997). Proliferation stops in the BRCA2<sup>-/-</sup> cells. The checkpoints may be dysfunctional, since repair processes are disturbed. If BRCA2<sup>-/-</sup> cells are taken into culture, they display an increased radiation sensitivity compared with BRCA2<sup>+/-</sup> cells. Other BRCA2 knockout studies with less severe phenotypes are being performed to further study the protein *in vivo*.

Finally, we would like to state that there is some evidence for a role of BRCA1 and BRCA2 in sporadic disease. Mutations in BRCA1 and BRCA2 are very rare (Lancaster *et al.* 1996, Garcia-Patino *et al.* 1998, Miki *et al.* 1996, Papa *et al.* 1998); however there is recent evidence that aberrant cytosine methylation of the CpG island promoter may be one mechanism of BRCA1 repression in sporadic breast cancer (Rice *et al.* 1998).

### BRCA3

BRCA1 and BRCA2 genes only contribute to a fraction of familial breast cancer cases; women with neither of these genes often report a family history. Therefore, other more common but less penetrant factors are probably involved. There is evidence for an additional breast cancer susceptibility gene (BRCA3) in families at high risk for breast cancer which do not have linkage to either BRCA1 or BRCA2. The published studies strengthen the possibility

of a BRCA3 locus on the short arm of chromosome 8 (8p12-22) (Kerangueven *et al.* 1995).

### Ataxia telangiectasia gene (ATM)

Another candidate for a breast cancer tumor suppressor gene has been thought to be the ATM. Ataxia-telangiectasia (AT) is an autosomal recessive disease characterized by cerebellar ataxia, oculocutaneous telangiectasia, immune defects, and a predisposition to cancer (for review see Shiloh 1995). To date, it is controversial whether AT heterozygotes have an increased risk of breast cancer. ATM is located on 11q23 (Pecker *et al.* 1996), and high rates of LOH have been reported in breast cancer patients. However, Laake *et al.* (1997) showed recently that two other genes distal and close to the ATM locus might be involved in breast cancer progression. There are as many studies showing an increased risk of breast cancer in AT families, as there are studies where no evidence for AT in breast cancer was found (Bebb *et al.* 1997). Why are there such discrepant results? AT heterozygotes represent about 1% of the total population, and the individuals are clinically normal. Therefore, large studies have to be performed to have the statistical power to detect small risk increases. Furthermore, the size of ATM ( $\approx 350$  kDa) (Savitsky *et al.* 1995a,b), its complexity, and its lack of mutational hotspots inhibit the fast development of a screening test. Finally, truncated protein products seem to be unstable.

There is certainly no question that individuals with AT mutations have a very high risk for cancer (Swift *et al.* 1991), making ATM a putative tumor suppressor gene. Recent laboratory findings also support this hypothesis. ATM knockout mice exhibit severe defects in T-cell maturation and display a high level of double strand breaks (Elson *et al.* 1996). AT cells fail to induce appropriate p53 expression in response to ionizing radiation, so that the cell cycle arrest that normally allows for DNA repair is lost. Therefore, AT heterozygosity might represent a mild mutator phenotype leading to increased accumulation of genomic damage.

It is clearly an important issue, and the involvement of ATM in breast cancer has to be clarified. This research is even more relevant, since AT heterozygotes might display increased radiosensitivity. Such an association would most likely still justify therapeutic or even diagnostic radiation for targeted individuals or older patients, but screening programs, such as mammography, for younger women might have to be reconsidered for AT heterozygotes.

### Li-Fraumeni

In 1969, Li and Fraumeni originally described families with soft tissue sarcoma and increased risk of other

malignancies at an early age, including breast cancer. Studies followed showing that, in at least half of the families fulfilling 'Li-Fraumeni' definitions, p53 was mutated (Malkin *et al.* 1990), therefore being a major contributor to the occurrence of inherited breast cancer in the specific setting of Li-Fraumeni syndrome.

### Cowden

Cowden disease was originally described in 1963 as an autosomal dominant disease characterized by various facial and central nervous system abnormalities as well as breast and thyroid cancer (Lloyd & Dennis 1963). The disease is very rare - approximately 200 cases have been identified, and 30% of those patients have breast cancer. A genomic search mapped the Cowden locus to 10q22-23 (Nelen *et al.* 1996).

PTEN is a protein phosphatase with homology to tensin, which has been mapped to 10q23. Various groups have described germline mutations of PTEN in families with Cowden disease (Tsou *et al.* 1998). Consequently, investigators set out to analyze whether PTEN was mutated in breast cancers, independently of Cowden disease. Indeed, some recent studies indicate that PTEN could play a role in breast cancer, since mutations were found in breast cancer cell lines as well as in tumors (Chen & Lindblom 1998, Li *et al.* 1997, Lynch *et al.* 1997, Rhei *et al.* 1997, Singh *et al.* 1998, Ueda *et al.* 1998). The incidence of mutations is rather low, and more studies are necessary to further delineate the role of PTEN in breast cancer.

### Tumor suppressor genes in early breast disease

During the past few years there has been an increase in the number of patients diagnosed with benign breast disease. Benign breast disease is both common and heterogeneous. We know that some types, e.g. usual and typical hyperplasias, are putative precursors for breast cancer and carry a low, but well-established increased risk (1.5- to 2-fold and 4- to 5-fold respectively) for the subsequent development of invasive cancer (Dupont & Page 1985, Page *et al.* 1985, Dupont *et al.* 1993). Little is known, however, about the genetic changes that distinguish those lesions which will progress to cancer. We were the first to report LOH in hyperplastic breast disease (O'Connell *et al.* 1994), suggesting that these lesions are really benign neoplasms whose development involves the inactivation of tumor suppressor genes. We have since performed a more comprehensive follow-up analysis of 399 putative precursor lesions for LOH at 15 polymorphic genetic loci known to exhibit high rates of LOH in invasive breast cancer (O'Connell *et al.* 1998). LOH was detected in 37% of usual hyperplasia lesions and in 42% of atypical

hyperplasias from noncancerous breasts, which supports our original premise (O'Connell *et al.* 1994) and results from other studies (Lakhani *et al.* 1995, 1996, Rosenberg *et al.* 1996, Kasami *et al.* 1997) that tumor suppressor genes play an important role in the early development of these lesions. Furthermore, in comparing precursor lesions from noncancerous breasts to lesions in cancerous breasts, certain chromosomal loci (2p, 11p, and 17q) showed very high rates of loss, suggesting that candidate suppressor genes at these sites may be particularly important in the early development of breast cancer. Allred & Hilsenbeck (1998) recently demonstrated that altered p53 tumor suppressor gene expression in benign breast disease was associated with a significant increased risk of developing breast cancer, in a case control study. This finding is of special interest since, in other cancers such as colon and rectum, the loss of p53 is believed to be a relatively late event. Thus, the validation of this result of p53 as a risk factor in early disease, and the eventual cloning of other candidate genes at implicated loci may help to identify high-risk genotypes for possible treatment intervention.

### Recent progress in tumor suppressor gene discovery methods

The analysis of genetic changes using techniques such as LOH is problematic in the breast because of the presence of normal epithelial cells, stroma, myoepithelium, etc., and manual microdissection of the relevant cell is laborious. Laser capture microdissection (LCM) is an alternative approach which has recently been developed (Simone *et al.* 1998) that provides a reliable method of obtaining pure populations of cells. The cells of interest are easily transferred to a polymer film that is activated by laser pulses, both DNA and RNA can be subsequently isolated, and the technique requires much less time to perform. There are several new technologies which can then be coupled with LCM to evaluate for the presence of tumor suppressor genes. These new technologies include fluorescence *in situ* hybridization (FISH) and DNA chips (Southern 1996).

Recent advances in FISH such as multi-color chromosome painting (Raap 1998) and comparative genomic hybridization (CGH) (Kallioniemi *et al.* 1992) are particularly suited for the identification of tumor suppressor loci. We found that both the total number of aberrations detected by CGH, and especially regions with decreased DNA copy number (indicative of a site of LOH) were significantly higher in node-negative breast cancer with a poor prognosis and early recurrence (Isola *et al.* 1995). Others have similarly used CGH to localize specific regions of loss in primary and metastatic breast cancer (Nishizaki *et al.* 1997, Tirkkonen *et al.* 1998), but the regions have yet to be defined in more detail.

DNA chips are microarrays of immobilized DNA or oligonucleotides which are fabricated by high-speed robotics on glass or nylon substrates. Labeled DNA or cDNA probes are then used to determine complementary binding, allowing comparative gene expression studies (Skena *et al.* 1995), accurate sequencing (Drmanac *et al.* 1998), and large scale mapping or genotyping of the human genome (Wang *et al.* 1993, Chee *et al.* 1996). The use of LCM should further advance the utilization of DNA chips in cancer research. Gene chips have already been fabricated to detect mutations in exon 11 of the BRCA1 gene (Hacia *et al.* 1996), and the p53 tumor suppressor gene. DNA chip technology is rapidly advancing and should prove extremely useful in the near future in the identification of new tumor suppressor loci and genes in clinical material, along with the confirmation of their role in breast cancer once they become readily available to the individual investigator.

### Role of tumor suppressor genes for improved diagnosis and therapy

Researchers have learned a great deal about how to inactivate oncogenes. A recent example is the farnesyl-transferase inhibitor targeting the ras oncogene which is now in clinical trials. Antitumor strategies aimed at restoring tumor suppressor gene function seem more complicated, at least at this point in time, since gene therapy approaches still display major limitations. However, there is very active research on ways to improve tumor-specific delivery of genes, and to increase efficiency of transduction and gene expression.

One of the most hotly pursued gene therapy approaches involves replacement of wildtype p53, and clinical trials are ongoing in lung and in head and neck cancer. Clinical trials in breast cancer will undoubtedly follow in the near future. Another gene therapy strategy uses the loss of wildtype p53 as an advantage. The engineered 'Onyx' adenovirus lacks the protein disabling p53, which is a necessary step before the virus can replicate in the cell. Therefore, only cells lacking p53 function allow the virus to replicate, resulting in selective killing of tumor cells lacking p53. Preliminary clinical trials in head and neck cancer patients are promising, and further trials will follow (Heise *et al.* 1997).

Another approach could use the fact that some tumor cells carry both a wildtype and a mutated tumor suppressor allele. If the tumor suppressor gene acts in a dominant-negative fashion, the inactivation of the single mutated allele would be beneficial for the patient. Therefore, targeting the mutated allele with strategies such as antisense RNA or triple DNA helix molecules could result in a therapeutic benefit.

Although traditional therapies are likely to remain a part of antitumor treatment, these new approaches targeting tumor suppressor genes will certainly modify and revolutionize treatment strategies. Furthermore, even before there is a gene therapy or pharmaceutical approach to restore tumor suppressor gene functions, we have the ability to use information about the status of specific tumor suppressor genes in patients to predict outcome and therefore manage therapy better. Currently there is a large debate in the scientific/medical community as well as in the general population, since the knowledge of a genetic test result, i.e. BRCA1 and BRCA2 mutations, certainly has both advantages and disadvantages. The advantages include relief from anxiety and ability to make choices about childbearing without genetic constraints for those who do not have a mutation. For those who have a positive result, one might opt for prophylactic mastectomy or aggressive surgical treatment of a first tumor as a prophylactic measure against subsequent tumors. Furthermore, patients might choose intensive cancer surveillance and changes in lifestyle. However, there are disadvantages which are rather complex and extensive. First, there are limitations to the tests being performed, including sensitivity and quality control. A negative result could be a false-negative result, and a positive result does not necessarily equal higher chance of breast cancer risk, since a missense mutation could reflect a polymorphism (it is estimated that at least 60% of people carry at least one polymorphism within the BRCA1 exons). There are only few data correlating specific BRCA1 and BRCA2 mutations with cancer risk. Finally, there are certainly more breast cancer genes besides BRCA1 and BRCA2, test costs are high, and positive results might cause potential problems with insurability and employability. Therefore, genetic testing should only be done in conjunction with informed consent, patient education, and support and counseling.

Finally, the introduction of microarrays ('chips', also see above) will change the way a tumor will be analyzed by physicians in the future. The tumor DNA will be analyzed for specific patterns, e.g. inactivation of specific tumor suppressor genes and their downstream targets and pathways, and thereby therapy will be based on the individual biology of the breast tumor. Therefore, in the future, treatment decisions might be directed less by tissue origin of the malignancy, and more by the set of mutations displayed by the individual tumor.

### Conclusions

This review displays the complexity of tumor suppressor gene function and expression in breast cancer cells. Tumor suppressor genes are involved in a diversity of cellular processes such as cell cycle control, replication, recom-

ination, signal transduction, repair, differentiation and aging. The further identification and characterization of abnormalities of gene products in breast cancer might eventually lead to the design of treatment strategies that specifically target cell cycle checkpoints and apoptosis in cancer cells, without affecting normal cells, thereby leading to decreased death rate from cancer. Recent improvements in technology such as the development of chips/microarrays will certainly advance research efforts and will ultimately result in improved treatment.

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