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Breast Cancer

Oncogenes and Tumor Suppressor Genes in Breast Cancer: Potential Diagnostic and Therapeutic Applications

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LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Differentiate between the actions of oncogenes and tumor suppressor genes in the development of breast cancer.
2. Describe the results of studies with antibodies and small molecule drugs that target growth factor receptors.
3. Evaluate the current and potential roles of molecular and protein profiles of breast tumors in prognosis and in predicting response to therapy.

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ABSTRACT

Carcinogenesis is a multistep process characterized by genetic alterations that influence key cellular pathways involved in growth and development. Oncogenes refer to those genes whose alterations cause gain-of-function effects, while tumor suppressor genes cause loss-of-function effects that contribute to the malignant phenotype. The effects of these alterations are complex due to the high number of changes in a typical case of breast cancer and the interactions of the biological pathways involved. This review focuses on the more common abnormalities in oncogenes and tumor suppressor genes in human breast cancer and their known associations with clinical outcome in terms of tumor classification, prognosis, and response to specific therapies. A better understanding of these relationships has led to new therapeutic applications. Agents that target oncogenes and their associated pathways are now in clinical use, with many more undergoing preclinical and clinical testing. The availability of antibodies, small synthetic molecules, cytokines, gene therapy techniques, and even natural compounds that are screened for specific biological properties has greatly increased the number of candidate drugs. Nevertheless, clinical successes have been limited because of the redundancy of many cancer-related pathways as well as the high degree of variability in genotype and phenotype among individual tumors. Likewise, strategies to replace tumor suppressor gene functions face numerous technical hurdles. This review summarizes the current achievements and future prospects for the therapeutic targeting of oncogenes and tumor suppressor genes and new technology to better classify tumors and accurately predict responses to standard and novel agents. *The Oncologist* 2004;9:361-377

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Introduction

Breast cancer and other malignancies result from stepwise genetic alterations of normal host cells, and, possibly from other nongenetic (or epigenetic) changes in the behavior of not only malignant cells but also host cells that interact with the tumor, such as immune, vascular, and stromal cells [1-4]. A growing understanding of these changes and the associated pathways through which they operate has led to opportunities for diagnostic and therapeutic applications. Most genetic changes are acquired and present only in malignant cells, although some changes may be seen as part of a "field defect" in surrounding histologically normal cells. Less frequently, germline-inherited genetic alterations can strongly predispose some individuals to developing certain malignancies. In addition, certain inherited gene mutations or polymorphisms can increase cancer risk to a smaller extent (low penetrance) or modify the risks associated with other genes or with environmental factors (so-called modifier genes).

Genome instability appears to be one of the earliest recognizable phenotypes and may be present even in histologically normal tissue. In fact, inherited cancer syndromes often involve this phenotype—for example, the *BRCA-1* and *BRCA-2* breast/ovarian cancer risk genes are both involved in DNA repair. Genome instability, whether inherited or not, results in a greater potential to develop genetic changes such as gene loss, gene amplification, point mutations, and chromosomal translocations. While most of these subsequent changes may result in cell death, some can affect key genes involved in cell survival, proliferation, invasiveness, motility, drug resistance, and other malignant characteristics. These genomic abnormalities occur early in carcinogenesis; in the case of breast cancer, loss of heterozygosity (gene loss) and changes in gene copy number seem to sharply increase in the transition from hyperplasia to ductal carcinoma in situ (DCIS), more so in higher grades of DCIS [5, 6]. Clonal outgrowth and evolution may explain why solid tumors possess so many genetic alterations at the time of diagnosis. Moreover, cellular pathways affected by these genetic changes are highly interactive with each other. These complexities explain why diagnostic and therapeutic applications have progressed slowly.

Over the last decade, our increasing knowledge of specific genes and proteins, as well as biological pathways that are associated with the development and progression of cancer, has provided us opportunities to develop targeted therapeutics that have as their ideal aim the selective, efficient, and safe treatment of cancer. Of course, these ideals have not been fully met for several reasons:

- Cancer-related pathways are complicated, and many of these intersect and interact (crosstalk); hence, our comprehension of cancer biology is still rather limited.
- Available preclinical models are not very reflective of human disease and, therefore, do not accurately predict clinical success.
- There is considerable heterogeneity in the targets and their functions among different individuals with seemingly similar types of cancer; therefore, clinical effects of targeted therapy are variable and unpredictable.
- These targets are generally not totally confined to cancer cells; hence, effects on normal tissue and unexpected (or expected) toxicities may be seen.
- Effectiveness is limited since other factors may be overriding the target (intrinsic resistance) and because the nature of the target and its associated function may change over time (acquired resistance).

Nevertheless, the pace of clinical translation in the field of molecular oncology has accelerated in the last few years and is becoming more relevant to the practicing oncologist. This review summarizes the role of oncogenes and tumor suppressor genes in breast cancer diagnostics and therapeutics.

Oncogenes

Oncogenes refer to genes whose activation can contribute to the development of cancer. The strict definition would require that activation be proven in human cancer cases and that experimental activation of the gene in a cell culture or animal model could recapitulate the malignancy. Activation can occur through gene amplification such that more of the protein encoded by the gene is present; hence, its function is enhanced. An example of such a mode of oncogene activation is that of *HER-2*, which is seen in about 20% of primary breast cancer cases. Another mechanism of activation is a point mutation that enhances the function of the "oncoprotein." Examples include point mutations in the *ras* oncogene, seen commonly in lung, colorectal, and pancreatic (but not breast) cancer. Activating point mutations in *ras* codons 12, 13, and 61 prevent the interaction of $p21^{ras}$ with GTPase-activating protein (GAP), maintaining $p21^{ras}$ in an activated and GTP-bound form and enhancing downstream signaling events such as cell cycle entry/activation [7]. Other mechanisms of oncogene activation include chromosomal translocation, whereby a new fusion gene is transcribed into a protein, with enhanced function. Oncogenes may also act cooperatively with other genetic or epigenetic changes. In breast cancer, there has been much attention focused on oncogenic components of the cell signaling system, an example of which is the HER-2/Neu cascade [8]. While the HER-2 membrane receptor tyrosine kinase is the most studied component of this

system, many other proteins, including Ras, are involved in transducing and modulating this signal, which has many end events, including cell proliferation, alterations in drug sensitivity and DNA repair, angiogenesis, apoptosis, protease activity, and cell motility.

Numerous oncogenes have been characterized in human cancers, but relatively few have been found to be crucial in the progression of breast cancer. Certain oncogenes are known to cause mammary cancer when overexpressed in transgenic mouse models, and specific oncogenes lead to distinct phenotypes in mice [9]. Amplification and overexpression of these oncogenes and oncogene products are the major mechanisms through which these genes participate in carcinogenesis. Amplification may involve short chromosomal regions to chromosomal arms, involving hundreds of genes, to entire chromosomes. The following are descriptions of those oncogenes and proto-oncogenes (preactivation) for which there is rather uniform agreement as to their role in breast cancer carcinogenesis.

The *HER-2* Oncogene

The *HER-2* (human epithelial receptor 2, also known as *HER-2*/*neu* or *erb*B-2) gene is located on chromosome 17q and encodes a 185-kDa transmembrane tyrosine kinase growth factor receptor [10]. Growth factor receptor activation is initiated by binding to specific ligands, or autonomously, if present in sufficient receptor density on the cell membrane, followed by dimerization and receptor autophosphorylation, which leads to multiple transduction cascades acting through a variety of pathways. These include the mitogen-activated protein (MAP) kinase and 3-kinase (PI3K)/Akt pathways, which eventuate in proliferation, angiogenesis, altered cell-cell interactions, increased cell motility, metastases, and resistance to apoptosis [11]. The discovery of *HER-2* gene amplification and overexpression in primary human breast cancer and its association with more aggressive clinical behavior led to early interest in diagnostic and therapeutic applications [12]. The *HER-2* gene is rarely amplified in benign breast disease, and its expression varies by histologic subtype, as it is almost exclusively found in the primary breast cancers of ductal origin in contrast to those of lobular origin. The *HER-2* gene is amplified and overexpressed in 20%-30% of invasive breast cancer and, interestingly, in the majority of high-grade DCIS cases [12, 13]. Numerous studies have strongly suggested its association with higher recurrence risk in early-stage breast cancer and, to a lesser extent, increased resistance to hormonal therapy (perhaps more so with tamoxifen than with aromatase inhibitors), resistance to nonanthracycline therapy, enhanced sensitivity to doxorubicin, and, in some series, to taxane-based therapy [14]. At this time, however, *HER-2* status is not generally recommended for decision making other than for identifying patients for trastuzumab therapy. In early-stage breast cancer, it increases the risk of breast cancer recurrence risk and may, therefore, influence adjuvant therapy choice.

Antibodies to growth factor receptors were shown to inhibit growth in several preclinical models [15]. Trastuzumab (Herceptin®; Genentech, Inc.; South San Francisco, CA) is a humanized recombinant monoclonal antibody directed against the extracellular portion of the HER-2 protein [16]. The mechanism of action of trastuzumab from animal models is presumed to be modulatory effects on cell signaling, but there is also evidence of an immunological effect [17]. Table 1 and Table 2 summarize the results of the early trastuzumab trials [18-21]. Response rates of 11%-26% were seen when trastuzumab was used as a single agent, and this activity was higher (35%) in patients who, in retrospect, had truly HER-2$^+$ tumors by updated immunohistochemical (IHC) or gene amplification criteria. Greater activity was observed when trastuzumab was combined with chemotherapy, with the pivotal randomized trial showing improvements in response rate, time to disease progression, duration of response, and survival. Cardiomyopathy that is usually transient and improves over time has been noted with the use of trastuzumab, especially in combination with anthracycline therapy. This is an illustration of the difficulty of predicting effects of targeted therapy. While HER-2 is expressed at low

Table 1. Results of single-agent trastuzumab trials

Prior chemotherapy for advanced disease	*n*	RR	MDR (months)	MTTP (months)	Median survival (months)	Study
Any	43	12%	6.6	5.1	14	*Baselga et al*. [18]
1 or 2 prior regimens[a]	222	15%	9.1	3.0	13	*Cobleigh et al*. [19]
None[b]	114	26%	> 12	3.5	24	*Vogel et al*. [20]

Abbreviations: RR = response rate; MDR = median duration of response; MTTP = median time to disease progression.

[a]Trastuzumab was given as a loading dose of 4 mg/kg followed by 2 mg/kg i.v. every week.

[b]Patients were randomized to receive either 4 mg/kg followed by 2 mg/kg i.v. every week or 8 mg/kg followed by 4 mg/kg every week.

Table 2. Summary results of the pivotal randomized trial comparing chemotherapy alone with chemotherapy plus trastuzumab [21]

Therapy	*n*	RR	MDR (months)	MTTP (months)	Median survival (months)
Chemotherapy	234	32%	6.1	4.6	20.3
Chemotherapy plus trastuzumab	235	50%	9.1	7.4	25.1
	p value	<0.001	<0.001	<0.001	0.046
Subsets					
AC	138	42%	6.7	6.1	21.4
AC plus trastuzumab	143	56%	9.1	7.8	26.8
	p value	0.02	0.005	<0.001	0.16
Paclitaxel	96	17	4.5	3.0	18.4
Paclitaxel plus trastuzumab	92	41	10.5	6.9	22.1
	p value	<0.001	<0.01	<0.001	0.17

Abbreviations: RR = response rate; MDR = median duration of response; MTTP = median time to disease progression; AC = anthracycline (doxorubicin or epirubicin) plus cyclophosphamide.

levels in adult myocytes, the HER signaling system is known to be important in embryonic neural and cardiac development and also may be involved in stress responses and remodeling in the adult heart [22].

Growth factor receptor pathways interact with other pathways, such as those involved in hormone-receptor signaling and DNA repair, suggesting that some combinations of trastuzumab and conventional agents used in breast cancer may be synergistic or additive. In preclinical models, platinum agents, docetaxel, and vinorelbine have been found to exhibit the greatest degrees of synergy, but different results were obtained by other investigators using different cell lines [23]. Phase II clinical trials have shown the greatest degree of activity of trastuzumab when used in combination with vinorelbine as well as with docetaxel and, to a lesser extent, with gemcitabine, yet larger comparative trials are needed to optimize these regimens [24-26]. One such study has preliminarily shown a superior response and time to progression with the addition of carboplatin [27]. Likewise, trastuzumab and other HER-family-targeted drugs are being used to reverse resistance, or improve the effectiveness of hormonal agents [28].

A new anti-HER-2 antibody, pertuzumab (2C4), also binds the extracellular portion of HER-2, but also causes steric hindrance and impairs receptor dimerization. In preclinical models, this antibody was shown to inhibit the growth of cells expressing lower levels of HER-2, presumably by interfering with HER family heterodimer formation [29]. Phase I testing has shown activity (3/21 patients, 15%) in solid tumors [30], and testing in breast cancer, both HER-2$^-$ and trastuzumab-refractory HER-2$^+$ breast cancer, is under way.

Other HER Family Members

Other HER family genes, such as the one encoding the epidermal growth factor receptor (EGFR, also known as HER-1), are relevant in breast cancer. *EGFR* is expressed very commonly in lung and head and neck cancer and, to a lesser extent, in breast cancer. Expression of *EGFR* has been reported in some studies to be associated with a worse clinical outcome as well as estrogen-receptor (ER) negativity [31]. Antibodies to EGFR are being assessed in clinical trials, with some reports of activity in combination with radiation therapy for head and neck cancer [32] and with chemotherapy in colorectal cancer [33], but no activity has yet been reported in breast cancer. Since HER-2 can heterodimerize with EGFR and initiate signal transduction, there has been ongoing interest in targeting this receptor in breast cancer. EMD 72000 is a humanized anti-EGFR antibody undergoing trials in head and neck, colorectal, and ovarian cancers.

Newer small molecules with high and specific potency against the cytoplasmic tyrosine kinase domain of the HER family of receptors (Table 3) have been tested in several malignancies, and gefitinib (Iressa®; AstraZeneca Pharmaceuticals; Wilmington, DE) is currently approved for chemotherapy-refractory lung cancer [34]. Small phase II trials of gefitinib and the related erlotinib (Tarceva™; Genentech, Inc. and OSI Pharmaceuticals; Melville, NY) in breast cancer have yielded rather low activity [35-38], and it is not clear that surrogate cell signaling markers might help identify appropriate candidates (Table 4). Agents with a spectrum HER-1/HER-2 or panHER inhibition might be more suitable for breast cancer trials, and several of these agents are now in phase II and phase III testing. Also, these drugs are being explored in combination with other drugs, such as hormonal agents, trastuzumab, and antiangiogenic drugs to take advantage of crosstalk between these pathway modulators.

Table 3. HER family small molecule tyrosine kinase inhibitors in clinical trials

Agent	Class	Target	Mode of action	Manufacturer
Gefitinib	I	HER-1 (EGFR)	Reversible	AstraZeneca Pharmaceuticals; Willmington, DE
Erlotinib	I	HER-1	Reversible	Genentech, Inc.; South San Francisco, CA
PKI-166	I	HER-1	Reversible	Novartis Pharmaceuticals; East Hanover, NJ
EKB-569	II	HER-2 > HER-1	Irreversible	Wyeth Laboratories; Philadelphia, PA
Lapatinib	III	HER-1, HER-2	Reversible	GlaxoSmithKline; Research Triangle Park, NC
CI-1033	IV	HER-1, HER-2, HER-4	Irreversible	Pfizer Inc.; Groton, CT

Table 4. Responses to HER-1 tyrosine kinase inhibitors in breast cancer

Agent	*n*	Prior therapy	Response rate	Clinical benefit	Study
Gefitinib	63	Any	1.6%	4.8%	*Albain et al*. [35]
Gefitinib	27	Tamoxifen	7.4%	29.6%	*Robertson et al*. [36]
Gefitinib	31	Any	0%	9.7%	*Baselga et al*. [37]
Erlotinib	76	Doxorubicin, taxane	1.3%	NR	*Winer et al*. [38]
Erlotinib	22	Doxorubicin, taxane, capecitabine	4.5%	NR	*Winer et al*. [38]

NR = not reported.

Downstream Signal Transduction Modulators

Growth-factor-mediated signal transduction activates several key kinases that serve as master switches and can control numerous pathways. Other receptors can also initiate signals, so the targeting of downstream mediators may result in clinically beneficial results that cannot be achieved by growth factor receptor blockade but, by the same token, may cause additional toxicities. The mammalian target of rapamycin (mTOR) is a pivotal downstream kinase that couples growth stimuli from receptors or cytoplasmic kinases to regulation of the cell cycle. Rapamycin and its analogues inhibit phosphorylation of mTOR, thus blocking the downstream activation of S6 kinase and 4E binding protein-1, which in turn reduces translation of key protein synthesis machinery components and cell cycle regulatory proteins (such as c-Myc and cyclin D1), respectively [39]. CCI-779 is a rapamycin analogue that has undergone phase I testing and has demonstrated toxicities that include myelosuppression, dermatitis, and liver enzyme elevation. On a weekly schedule, which appeared to be the best tolerated, responses were seen in several tumor types, including breast [40]. A phase II trial comparing 75 mg CCI-779 with 250 mg CCI-779 i.v. weekly for doxorubicin- and/or taxane-refractory breast cancer showed preliminary aggregate results of nine responses out of 106 patients (8.5%), with a <10% incidence of grade III/IV hepatic, skin, and hematologic toxicities [41]. Further study is needed to assess its potential when used earlier in the course of disease or as combination therapy and to determine which biological subsets of patients are more likely to respond.

Another downstream central acting protein, Ras, activates the PI3K/Akt and MAP kinase pathways. Farnesyl transferase inhibitors (FTIs) prevent the translocation of Ras to the inner membrane, where it is activated. Although some oncogenic forms of Ras are not inhibited by FTIs and ras mutations are generally not seen in breast cancer, there may still be a rationale for FTIs in breast cancer since growth factor receptor and other pathways signal through Ras. The FTI tipifarnib (Zarnestra™; Ortho Biotech; Bridgewater, NJ) yielded a 12% response rate and a 24% clinical benefit rate in a phase II trial of 76 patients, with neurotoxicity, thrombocytopenia, and granulocytopenia seen [42]. Small molecule and antisense inhibitors of Ras downstream components (e.g., Raf, MAP/extracellular signal-regulated kinase [MEK] kinase) are also being investigated, although results in breast cancer are not yet available.

Cyclins and Cell Cycle Modulators

Entrance into cell cycling and active proliferation is a tightly regulated process. Cyclin-dependent kinases (CDKs) are a group of proteins placed strategically throughout the phases of the cell cycle. When activated, CDKs promote phosphorylation of other proteins, especially retinoblastoma protein (pRb), a primary gatekeeper that allows the cell to pass from a resting state, G_0, into active cycling and mitosis. CDKs are regulated positively by cyclins and negatively by cyclin-dependent kinase inhibitors (CKIs). Cyclin D1 and cyclin E expression levels oscillate according to the cell cycle, and both play a key role in the progression of the cell from the G_1 to S phase [43].

The gene encoding cyclin D1 is located on chromosome 11q13 and has been found to be overexpressed in 40%-50% of invasive breast cancers and amplified in 10%-20% of cases [44]. When cyclin D1 is complexed with its CDK partner, the pRb tumor suppressor protein is phosphorylated, releasing the transcriptional factor E2F and inducing proteins required for DNA synthesis. High cyclin D1 expression level appears to be positively associated with ER positivity and an increased proliferative index [45].

The gene encoding cyclin E is located on chromosome 19q12 and is rarely amplified in breast cancer (~2%); however, overexpression and alterations of the degradation pathway resulting in the accumulation of low-molecular-weight isoforms have been demonstrated in 20%-30% of breast cancers [46]. Rarely, both cyclin D1 and cyclin E are concomitantly overexpressed. Like cyclin D1, overexpressed cyclin E results in hyperphosphorylation of pRb and increased proliferative activity. However, in contrast to high cyclin D1 tumors, high cyclin E tumors are, in addition, able to induce S phase independently of pRb phosphorylation and E2F activation. The overall result of this is a marked reduction in cell cycle control and a significant dysregulation of proliferation. High cyclin E tumors are more likely to be of a higher grade than high cyclin D1 tumors, are typically hormone-receptor negative, have a more marked proliferative index, and are associated with a worse outcome [45, 46]. Several features associated with high cyclin E levels may help to explain this more aggressive phenotype. As mentioned above, cyclin E overexpressed tumors are able to bypass the pRb node, allowing for pronounced active cell cycling. Additionally, high cyclin E levels, in contrast to high cyclin D1 levels, have been associated with increased genomic instability. Furthermore, elastase, the enzyme that cleaves cyclin E into its low-molecular-weight isoforms, has also been associated with an increased propensity for invasion and metastases and may, in part, explain the more aggressive phenotype [46]. However, the routine use of cyclins or their isoforms for prognostic or therapeutic decisions remains unproven.

The targeting of cell cycle regulation is appealing, since this represents a relevant end point of numerous signaling pathways [47]. Flavopiridol is a semisynthetic flavone analog of rohitukine, an anticancer compound from an Indian tree, and is a nonspecific CDK inhibitor [48]. This compound appears to work through competing with CDKs for ATP binding and disruption of P-TEFb (the CDK9-cyclin T complex), resulting in apoptosis, possibly a consequence of downregulation of various antiapoptotic proteins. Phase I studies have shown secretory diarrhea and hypotension to be dose limiting. A phase II trial in mantle cell lymphoma, which is associated with cyclin D1 overexpression, resulted in three responses in 28 evaluable patients (11%), with diarrhea, fatigue, and nausea commonly seen along with mild hematologic toxicity [49]. Trials of flavopiridol in combination with several chemotherapeutic agents are ongoing in breast cancer, and early results from a phase I trial of flavopiridol with docetaxel show this combination to be well tolerated [50]. Since growth factor receptor signaling eventually triggers entry into the cell cycle, targeting the proximal and distal aspects of this axis with trastuzumab in combination with flavopiridol was investigated in HER-2$^+$ cell lines, and indeed, synergistic cytotoxicity was seen [51]. Thus, trials that capitalize on both early and midpoint signaling coupled with direct cell cycle modulators may be able to provide better cell kills with less host toxicity.

Ro 31-7453 is another nonspecific, oral cell-cycle inhibitor with in vitro efficacy against a wide range of tumor cell lines. It weakly inhibits CDK1, CDK2, and CDK4 and tubulin polymerization, and causes failure of mitotic spindle formation in proliferating cells, leading to M-phase arrest. In a phase II study of taxane- and anthracycline-resistant breast cancer, 2 of 32 (6%) patients responded, with diarrhea and nausea reported as the primary side effects [52]. UCN-01 (7-hydroxy-staurosporine) is also a broad inhibitor of CDKs and 3-phosphoinositide-dependent protein kinase-1 (PDK1); phase I trials have shown hypotension to be a side effect, with no responses seen when used as a single agent in renal cell cancer [53]. Combinations with chemotherapy are being tested. More specific CDK4 and CDK6 inhibitors have been developed and will be entering clinical trials.

Since many CKIs and other negative regulatory proteins are normally regulated by ubiquitination and proteasomal degradation, proteasomal inhibitors have been of interest [54] and are in clinical testing for breast cancer. These drugs affect not only CKIs but many other short-lived proteins, such as the inhibitor of NF-κB, a key mediator of stress and immune response pathways, and thus may downregulate other signaling pathways as well. A phase II study of the proteasome inhibitor bortezomib (Velcade®; Millenium Pharmaceuticals, Inc.; Cambridge, MA) with docetaxel yielded responses in 6 of 14 patients (43%) in anthracyline-pretreated breast cancer [55].

c-*myc* Oncogene

The c-*myc* oncogene has been localized to chromosome 8q24 and encodes a nuclear phosphoprotein that acts as a transcriptional regulator involved in cellular proliferation, differentiation, and apoptosis. It is amplified and overexpressed in 15%-25% of breast tumors [56] and, in some series, has been associated with a worse prognosis or more aggressive clinical features [57]. While many agree that overexpression of this gene is clearly associated with breast cancer, there continues to be controversy as to whether or

not aberrant Myc expression alone is sufficient for breast carcinogenesis. There also appears to be a role of Myc in hormone responsiveness and chemotherapy resistance [58]. Antisense to c-*myc* can affect cell growth rate in some breast cancer line models, and antisense trials targeting c-*myc* are in progress.

Tumor Suppressor Genes

Tumor suppressor genes refer to those genes whose loss of function results in the promotion of malignancy. Tumor suppressor genes are usually negative regulators of growth or other functions that may affect invasive and metastatic potential, such as cell adhesion and regulation of protease activity. Although inherited abnormalities account for a minority of breast cancer cases, these germline mutations occur in tumor suppressor genes. These same genes can harbor sporadic acquired somatic mutations. In both cases, the tumor typically contains a mutation in one allele and a deletion of the remaining allele in keeping with the long-standing "two-hit" hypothesis formulated by *Alfred Knudson* in reference to retinoblastoma, which states that both gene alleles must be lost to unmask the malignant phenotype [59]. In some cases, there may not be a mutation of the tumor suppressor gene, but rather some other mechanism that interferes with its expression or function. This may include methylation of the gene promoter that suppresses its transcription, an increased rate of proteasomal degradation, or abnormalities in other proteins that interact with the gene product. Tumor suppressor genes have not been extremely useful in diagnostic applications, with the exception of inherited susceptibility genes. Likewise, therapeutic approaches that would entail replacing the function of the lost gene have been stymied by the technical hurdles of efficient gene delivery.

***p53* Oncogene**

Beginning with the detection of a mutated form in lung and colon cancers some 14 years ago, *p53* has become, perhaps, the most-studied tumor suppressor gene [60, 61]. Mutations of *p53* are estimated to occur in up to half of all human cancers and in approximately 20%-30% of breast cancers [62]. Li-Fraumeni syndrome, a rare familial predisposition to a variety of malignancies including breast cancer, sarcomas, leukemia, and brain tumors as early as the second and third decades of life, has been shown to result from germline alterations of *p53*, and multiple mutation sites have been reported. The risk of cancer in these patients has been estimated to be 50% by the fourth decade of life and as high as 90% by the eighth [63, 64].

The *p53* gene is located on chromosome 17p; it codes a 393-kDa protein that has multiple functions that are regulated via phosphorylation at different sites. Downregulation of the protein is largely through the mouse double minute 2 (MDM2) ligase, which usually exists in a complex with p53. The Myc pathway is capable of enhancing p53 levels through the *ARF/INK4* gene product, which binds the p53-MDM2 complex.

Under normal conditions, p53 acts as a regulating mechanism for cell division. Insults to DNA, such as chemotherapy or gamma irradiation, are associated with rapid increases in cellular content of the protein [65]. When activated, p53 can directly interact with DNA to yield transcription of a number of genes, including the CKI *p21*, and a temporary arrest of the cell cycle in the G_1 or G_2/M phase, prior to mitosis to allow for DNA repair. p53 is also capable of interacting with other cellular pathways to trigger apoptosis or differentiation [66]. p53 has also been shown to factor in the expression of other proposed tumor suppressors or regulators of angiogenesis and metastasis, including the proteins maspin, hypermethylated in cancer (HIC)-1, and Kangai-1 (KAI-1) [67-69].

Since most *p53* mutations result in increased protein stability, overexpression of p53 has been used as a surrogate of *p53* dysfunction. However, some mutations result in the absence of the protein and may be missed by IHC assays. Abnormalities of *p53* expression have been associated with worse prognoses in cases of breast cancer [70]. Overexpression has been linked to ER negativity, a strong predictor of outcome [71]. Independent of ER status, mutation of *p53* increases the relative risk of relapse by roughly 33%. There are conflicting data regarding p53 as a predictor of response to therapy. Although less commonly analyzed, gene mutation analysis of *p53* may be more informative than IHC assays of protein levels for this purpose, since p53 is a multifunctional protein and mutations in different domains may have specific consequences. The gene has been ranked as a category II prognostic marker in breast carcinoma [72].

p27* and *Skp2

Negative regulators of the cell cycle are considered tumor suppressor genes in that a loss of their function can contribute to malignant behavior. First isolated in 1993, p27 belongs to a family of cyclin-dependent protein kinase inhibitors (CKIs) known as Cip/Kip, whose other members are p21 and p57. In general, CKIs slow the progression of the cell cycle; p27 is capable of binding to a number of unique cyclin/CDK complexes to attenuate their activity, typically directing the cell toward arrest in the G_1 phase. It has been demonstrated that p27 has separate binding sites for cyclin and CDK2 and that binding to this complex results in conformational changes of the catalytic cleft of CDK2 [73]. Many roles of p27 have been proposed, including functions in modulation of drug resistance, cell differentiation, and

protection from inflammation [74-76]. Supporting the role of *p27* as a tumor suppressor, decreased expression has been documented in a wide range of human cancer cell lines. However, mutations of *p27* appear to be uncommon events in malignancy, occurring in only 1% of tumors in one study [77].

p27 expression has been shown to have prognostic value in a variety of tumors, including lung and colon [78, 79]. In breast cancer, diminished expression of p27 is associated with shorter overall survival and shorter time to progression, and this seems to be a stronger independent predictor of outcome than either p53 alterations or tumor grade [80, 81]. Stepwise loss of p27 expression may be an event that parallels the transition of a cell from the normal to premalignant to malignant phenotypes [82]. Some degree of the poor prognosis conferred by loss of p27 expression may be related in part to a role in modulating cell-cell adhesion, and thus, tendency for metastatic spread. In one series, analyzing tumors smaller than 1 cm in diameter, p27 underexpression was found to be a strong predictor of lymphatic spread [83]. Affording further support for the importance of p27 in tumor behavior is the finding that antiestrogen compounds result in increased inhibition of CDK activity [84].

The S-phase kinase-associated protein Skp2 is required for the ubiquitin-mediated degradation of p27 and has been shown to experimentally increase oncogenicity and resistance to antiestrogens in vitro [85]. Skp2 may also be preferentially overexpressed in ER^- and $HER\text{-}2^-$ breast cancer, a subset of breast cancer recently defined as the "basal phenotype" by gene profile analysis (see **Molecular Diagnostics**).

BRCA-1

Based upon linkage analysis of families with multiple breast cancers, the locus of an associated gene at 17q21 was reported in 1990, and the gene that came to be known as *BRCA-1* was subsequently identified in 1994 [86, 87]. Is has been estimated that approximately 0.12% of the general population carries a mutation of *BRCA-1*, but this rate is much higher in certain groups. Among Ashkenazi Jews, there is a 1% rate of heterozygosity for the mutation *185delAG* and a smaller (0.1%) rate for a separate mutation (*5382insC*) [88, 89]. *BRCA-1* mutations have been estimated to account for slightly more than 5% of all breast cancer cases occurring in women under the age of 40, but this figure rises to over 90% for cases that arise in a family that has a history of four or more cases of breast cancer and more than one case of ovarian cancer [88, 90]. The lifetime risk of breast cancer in patients with a mutation of *BRCA-1* has been estimated to be 49%-73% by age 50 and 71%-87% by age 70, along with a 20%-30% lifetime risk of ovarian cancer [91, 92]. Colorectal and prostate cancer incidences may also be increased, but *BRCA-1* alterations are not associated with an increased risk of male breast cancer.

BRCA-1 codes a protein of 1,863 amino acids with several structural domains that hint at its function [87]. A RING finger domain encodes a protein-binding domain at the amino terminus [93]. BRCA-associated ring domain (BARD1) is a protein found to interact with BRCA-1 in the RING domain, and it may prove to have a tumor suppressor function of its own. Two repeats in the carboxy terminus are similar to those seen in many DNA repair enzymes including Rad9. Following genotoxic insult, BRCA-1 protein, along with BARD1 and Rad51, has been shown to localize to areas of damaged DNA, supporting a role in regulation of transcription as well as in repair of double-stranded DNA [94].

Over 200 individual *BRCA-1* mutations have been described, including deletions, substitutions, and insertions. They are found throughout the length of the gene, although some areas do appear to be hot spots for mutation. Roughly 80% of these events yield abnormal truncation of the BRCA-1 protein [95]. It has been suggested that the severity of disease can be linked to the location of the mutation, with mutations involving the amino or carboxy terminus of the gene associated with tumors that display higher proliferation rates [96].

Whether there is an impact on risk of distant recurrence related to *BRCA-1* compared with sporadic cases remains unclear. On average, tumors due to *BRCA-1* are higher grade, but there is also an increased incidence of medullary histology, which carries a more favorable prognosis [97]. *BRCA-1*-related cancers are less likely to show ER positivity and may also tend to exhibit amplification of the *myc* gene along with a "basal phenotype" expression pattern [98-100].

Although it is one of the most frequently identified causes for familial breast cancer, *BRCA-1* is rarely found to be mutated in sporadic cases. However, methylation of the gene has been found in sporadic cases and in familial cases with a normal *BRCA-1* sequence [101].

BRCA-2

After it became apparent that *BRCA-1* accounted for only a minority of inherited cases of breast cancer, a search for other culprits led to the discovery of a gene localized to 13q12-q13, dubbed *BRCA-2* [102]. The gene was cloned the following year by the same group [103].

The *BRCA-2* gene shares many features with *BRCA-1*, although its structure is dissimilar. BRCA-2 protein binds Rad51 and its paralogues needed for the high-fidelity phase of DNA repair, which requires sister chromatid template proofreading. Both genes confer a greater risk for female breast and ovarian cancers when mutated. Interestingly, each demonstrates only 60% conservation compared with the corresponding murine gene, a level lower than that found when most human oncogenes are compared with their mouse counterparts.

Over 100 unique mutations of *BRCA-2* have been described to date, most of them causing premature truncation of the protein, as is the case for *BRCA-1*. The incidence of *BRCA-2* heterozygotes in the general population is felt to be similar to that for *BRCA-1*, but the specific mutation *6174delT* occurs at a rate of 1.5% in the Ashkenazi Jewish population. The Icelandic population carries a separate mutation, *999del5*, at a rate of 0.5%, and it is present in 40% of cases of male breast cancer there [104]. In the U. S., however, the gene was found to be mutated in only 4% of men with breast cancer [105]. As with *BRCA-1*, sporadic mutations of *BRCA-2* appear to be quite rare.

The lifetime risk for developing breast or ovarian cancer is roughly similar for women with mutations in *BRCA-2* and those with mutations in *BRCA-1*. *BRCA-2* mutation has not been as strongly associated with higher tumor grade as has *BRCA-1*, but the malignant cells do show less tubule formation. Unlike *BRCA-1*-related tumors, no lymphocytic infiltrate is typically seen [97]. Mutation of *BRCA-2* also confers an increased risk for a number of other cancers, including melanoma, prostate cancer, gastric cancer, and cancer of the biliary tree [97]. Gene-expression profiling also reveals significant differences between *BRCA-1*- and *BRCA-2*-related tumors [106].

Risk-assessment tools have been described for prescreening patients with family histories suggestive of *BRCA-1* or *BRCA-2* mutations [95]. Genetic assays to test patients for specific mutations are commercially available, but their use remains somewhat controversial and they should not be applied to the population at large [107]. Testing should involve genetic counseling and must reflect individual patient preferences and risk trade-offs, since there are no data to suggest that genetically screening patients impacts mortality, even though occurrence is lowered. When carriers are identified, it is appropriate to offer radiological studies at an increased frequency and beginning at an earlier age, as well as to discuss the option of prophylactic surgery and counseling/testing other family members [108].

Other Tumor Suppressor Genes

A number of other tumor suppressors of importance in breast cancer are known, and others are continuing to be identified. The existence of a *BRCA-3* gene has been postulated, based upon the incidence of families with a strong breast cancer history but no detectable mutation of *BRCA-1* or *BRCA-2*; however, no firm localization of this gene has been made [109].

PTEN encodes a phosphatase that serves as a negative regulator to Akt. Loss of *PTEN* function augments the Akt cell survival signal [108]. Inherited *PTEN* mutations, seen in Cowden syndrome, have been shown to increase the risk of breast and ovarian cancers (among others), although mutation of this gene in sporadic cases is uncommon [110, 111].

Cell cycle checkpoint kinase (CHK2) is a serine threonine kinase that is mutated in some families that have a high breast cancer risk with a Li-Fraumeni syndrome phenotype and normal *p53*, *BRCA-1*, and *BRCA-2* sequences [112]. This kinase is activated by the ataxia-telangiectasia mutated (ATM) protein in response to DNA damage and then phosphorylates p53 and BRCA-1. One truncating mutation was found in 1% of a Finnish cohort of patients, and based on the breast cancer frequency in this cohort, it is considered to be a low-penetrance breast cancer susceptibility gene [113].

The *ATM* gene senses DNA damage and activates checkpoints and DNA repair pathways through rapid phosphorylation of several substrates including p53, BRCA-1, and CHK2 [114]. Loss of both alleles of the *ATM* gene causes ataxia-telangiectasia, a syndrome of progressive cerebellar degeneration, blood vessel fragility, immunological defects, and predisposition to lymphoid malignancies. There has been some debate as to whether the carrier state (about 1%-2% of the population) is at a greater risk for breast cancer and DNA damage from radiation. Estimates of cancer risk in heterozygotes are variable, with certain variants exhibiting up to a twelvefold higher risk, suggesting that the risk may be dependent on the type of mutation [115].

The retinoblastoma (*Rb*) gene was the first tumor suppressor to be discovered [116]. It is eponymous with the tumor in which it was first identified, but changes in the gene are estimated to occur in over half of all malignancies. In breast cancer, mutation or loss of *Rb* is present in up to 30% of cases, and it has been associated with a greater likelihood of progression [117].

Gene Therapy for Breast Cancer

The straightforward concept of replacing missing critical gene functions has long been used in the laboratory to study tumor suppressor gene functions. Gene therapy can also be used to "turn off" oncogenes through antisense (complementary) DNA sequences or genes that otherwise interfere with oncogene expression [118]. In the clinic, however, efficient gene delivery that specifically targets cancer cells and leads to sustained expression of the gene product has been difficult to achieve. As of early 2003, *The Journal of Gene Medicine* has tracked more than 400 completed, ongoing, and pending clinical trials that involve gene therapy for the treatment of cancer (http://www.wiley.co.uk/genetherapy/clinical/). Engineered viral vectors, initially retroviral vectors and, more recently, adenoviruses or adeno-associated viruses, have typically been used.

Adenovirus-mediated *p53* gene therapy was initially tested in lung cancer patients, with early trials showing modest

transfection levels and some clinical responses [119], although, to this day, it is not clear whether the responses were due to p53 function or a subsequent immune reaction to viral-mediated *p53* expression. A randomized phase II/III trial of p53 viral vector given intraperitoneally for p53-overexpressing ovarian cancer was stopped due to no difference in outcome [120]. It has been postulated that the mutant *p53* in some cases may have either oncogenic functions or could interfere with wildtype *p53* activity. Adenoviral *p53* gene therapy (INGN 201; Advexin®; Introgen Therapeutics, Inc.; Houston, TX) is currently being tested in combination with docetaxel in advanced breast cancer. A trial assessing intralesion p53 is also in progress.

BRCA-1 gene replacement strategies have also been pursued. Intraperitoneal administration of the retroviral LXSN-BRCA1 vector in *BRCA-1*-deficient ovarian cancer yielded no clinical responses, and this may have been due to the instability of the vector in vivo [121]. However, applications of *BRCA-1* or *BRCA-2* gene replacement in breast cancer are not currently in progress.

Antioncogenic therapy using antisense sequences to c-*fos* and c-*myc* is currently under investigation in breast cancer. Numerous other genes relevant in breast cancer are being targeted using antisense techniques in preclinical models [122]. Gene therapy with the adenoviral *E1A* gene, which binds the *HER-2* promoter and downregulates its expression, has been investigated as a form of antioncogenic therapy. A phase I trial of intracavitary liposome-complexed *E1A* gene in breast and ovarian cancer demonstrated a reduction in HER-2 protein and increased *E1A* expression in both cancer and normal cells as well as a reduction in pleural or peritoneal cancer cells and apoptosis [123]. A phase II trial is currently in progress.

Other ongoing nononcogene/tumor suppressor-based gene therapy trials include transfection of immune modulators, such as antigens and cytokines, as well as the protection of stem cells with the multidrug resistance (*MDR-1*) gene or purging stem cells with the proapoptotic *bcl-xs* gene or with herpes virus thymidine kinase (*HSV-TK*) gene and gancyclovir.

Multiparametric Molecular Diagnostics in Breast Cancer

New advances in technology now allow for the unprecedented ability to perform large-scale analysis at the genomic, gene expression, and protein levels. At the genome and gene expression levels, one can label genomic DNA or RNA, respectively, with different colored fluorochromes for each tumor sample and reference sample (normal cells or pooled cell lines). These are then hybridized to known genes or gene fragments arrayed on a slide, followed by scanning and measuring the intensity of both colors on each spotted gene. The relative levels of gene copy or gene expression can then be calculated. A very dense representation of the genome (for gene copy number, gene gain, or gene loss) or relative expression levels of all known genes can be accomplished on one or two "chips." Proteins in serum and tumor can also be characterized using special surfaces or matrices and laser desorption/ionization time of flight mass spectroscopy (SELDI or MALDI-TOF MS) to generate a profile of protein peaks. Expression array analyses of human breast tumors suggest that a subset of genes, especially those that vary substantially among different patient cases, can effectively define profiles that can classify tumors according to recognized biological phenotypes, such as ER positivity or degree of differentiation [124]. Moreover, small case series suggest that clinical behavior, such as prognosis and risk of recurrence following therapy for early-stage disease, may be predicted more efficiently than with conventional factors such as tumor stage or histopathologic characteristics [125, 126]. In the case of proteomic analysis, there is early evidence that serum profiles can efficiently distinguish between patients with and without prostate or ovarian cancers [127]. This technology is also being explored in breast cancer, examining both serum and ductal lavage fluid [128-130].

One of the potentially most applicable aspects of these techniques is to develop accurate tests that would be predictive of response to specific therapies. Using the statistical method of cluster analysis, one can develop patterns based on differences and similarities in the gene-expression profiles when the response to therapy is known (test set) and then apply the patterns associated with response or resistance to predict outcome in a different group of patients (validative set). In a preliminary small study, gene-expression profiles were obtained using fine-needle biopsies on patients who underwent preoperative combination chemotherapy, and data on subsequent pathologic response were used to generate the test set and predictive patterns. The accuracy when applied to the validative set was 71%, with a sensitivity of 43% and specificity of 100% [131]. Likewise, profiles that discriminate between responsiveness and resistance to docetaxel have been described, with an accuracy of 88% [132]. In that study, sensitive tumors tended to express genes involved in stress, DNA repair, apoptosis, adhesion, cytoskeletal function, protein transport, signal transduction, and RNA splicing or transport. In both those studies, the number of genes that drove the discrimination was under 100, out of several thousand assessed. This is encouraging, although many more refinements in analytical and statistical techniques need to be undertaken. Since many of the newer targeted therapies may only work in defined subsets of patients whose cancer cells heavily rely on the pathway of interest, it will become

increasingly important to use new technology to define those patients most likely to benefit. Furthermore, surrogate markers, such as gene or protein induction after therapy, may allow for much more rapid screening and testing of new drugs.

While these techniques are already showing potential to develop pattern recognition strategies to better classify tumors and choose appropriate agents, another major objective would be to use this technology to identify new targets that are critical to cancer cell growth and survival. The well-studied oncogenes and tumor suppressor genes may not necessarily represent the most effective targets. Gene discovery represents a larger challenge to high throughput, large-scale approaches, mostly due to the statistical and bioinformatics constraints in properly analyzing mega-data. Some examples are already surfacing. Using comparative genomic hybridization (CGH), high-density data on gene copy number at chromosome 22q13 revealed what was thought to be a large amplicon (amplified segment of chromosome spanning many genes) to actually contain discreet areas of gene amplification and gene loss [133]. Functional testing of amplified genes in this region demonstrated that the amplified *ZNF217* gene encodes a transcriptional factor that can immortalize human mammary cells [134], and *CYP24* encodes a protein that binds vitamin D and interferes with its prodifferentiating effects [135], both of these being hallmarks of oncogenes. These candidate oncogenes can then be validated by gene knockout studies and, ultimately, pharmacologically targeted using small molecule, antibody, or gene therapy approaches.

Polymorphisms in certain genes directly or indirectly involved in cancer pathways, also known as modifier genes, are being investigated, as they may alter risk attributable to inherited predisposition or to environmental factors. For example, specific single nucleotide polymorphisms (SNPs) in estrogen biosynthetic enzymes and signal transduction pathways, particularly those that affect protein function, are being investigated in relationship to the development and clinical course of breast cancer [136]. Similarly, SNPs in genes that regulate metabolism, distribution, and membrane transport of cancer drugs, now known as the field of pharmacogenomics, will increasingly help define target populations that may ideally benefit or that may be at added risk of toxicity to specific therapies [137]. Recently, certain polymorphisms in cytochrome p450 genes and glutathione-S-transferase genes were shown to be associated with differential outcomes in patients treated with high-dose cyclophosphamide and thiotepa for node-positive breast cancer [138].

Oncogenes and tumor suppressor genes represent alterations within our own genome that propel cells toward malignancy. Key functions necessary for growth and development can undergo changes on the basis of genetic and epigenetic alterations and can accumulate if there is a selective advantage. A better appreciation of the individual gene functions and their interrelationships with other genes and the environment is now being translated into better diagnostics and therapeutics. Tables 5 and 6 list examples of treatment strategies geared towards oncogenes and tumor suppressor genes. A schematic of therapies targeting specific pathways that are either in clinical use or in clinical trials is shown in Figure 1. The next few years promise to be an era of applications in human cancer control and treatment that will affect, and require participation from, laboratory scientists, clinical investigators, practicing oncologists, and the public alike.

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Table 5. Oncogenes, their functions, and targeted therapies in breast cancer

Oncogene	Function	Targeted therapy
HER-2	Tyrosine kinase receptor	Anti HER-2 antibodies (trastuzumab, pertuzumab) Kinase inhibitors (CI-1003, EKB-569, lapatinib) *E1A* adenoviral gene therapy
Ras	G-protein	Farnesyl transferase inhibitors (tipifarnib)
PI3K	Kinase	Rapamycin/rapamycin analogues (CCI-779, RAD 001, AP23573)
Akt	Kinase	
ElF-4E	Initiator of protein translation	
Cyclin D1	Cell-cycle mediator	Flavopiridol, UCN-01 (7-OH staurosporine),
Cyclin E	Cell-cycle mediator	Ro 31-7453, specific CDK inhibitors
c-*myc*	Transcription factor	Antisense
c-*fos*		

Table 6. Tumor suppressor genes, their functions, and targeted therapies in breast cancer

Tumor suppressor gene	Normal function	Current clinical trials
p53	Induces cell-cycle arrest, cell-cycle checkpoint activation	Phase II p53 peptide vaccine with or without interleukin-2 (NCI-99-C-0138)
	Triggers/facilitates apoptosis	Phase II—Ad5CMV-p53 gene therapy (INGN 201) + docetaxel
p27	Inhibit cyclin-dependent protein kinases; arrest cell cycle in G_1 phase	—
BRCA-1	Regulates DNA transcription; acts to repair damaged DNA	—
BRCA-2	Repairs damaged DNA	—
CHK2	Cell cycle checkpoint kinase, activates p53 after DNA damage	—
ATM	Checkpoint kinase, activates CHK2	—
PTEN	Phosphatase, negative regulator of Akt kinase	—
Rb	Retinoblastoma gene, repressor of cell cycle and protein translation	—

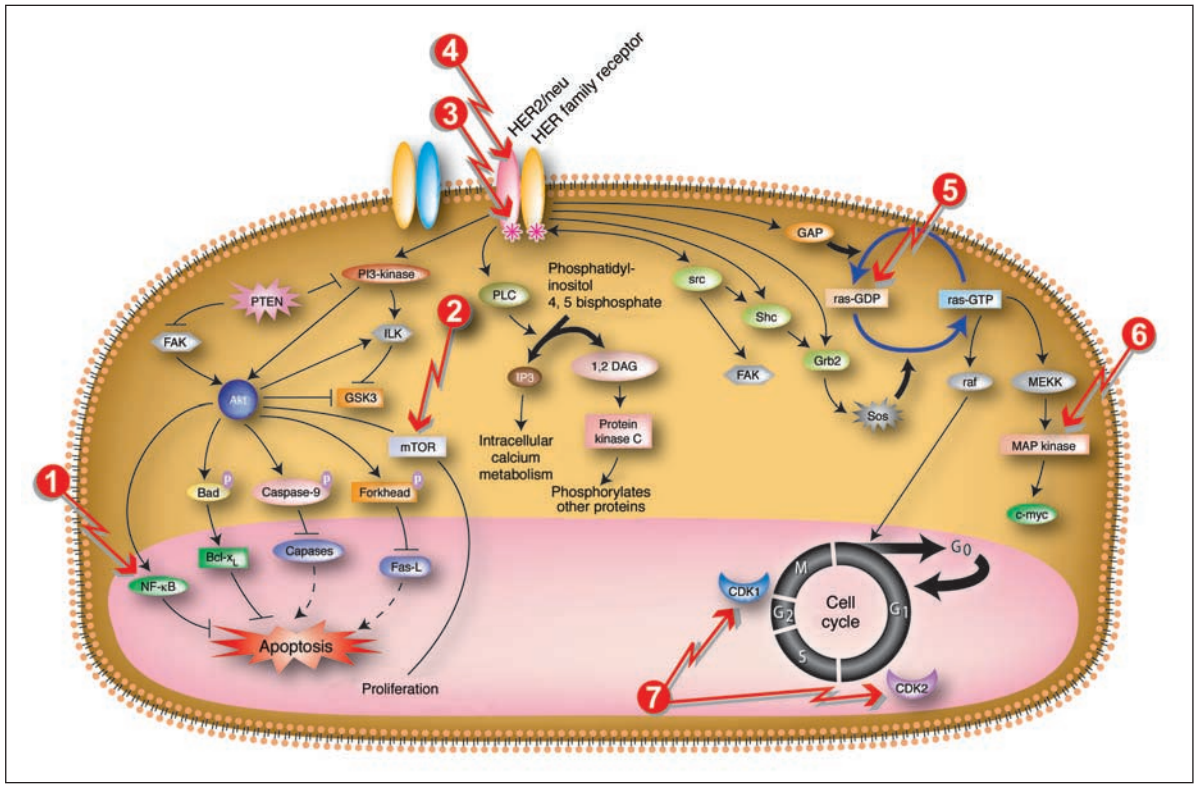

Figure 1. Multiple signaling pathways involved in cancer and action of targeted therapeutics. *1) Proteasome inhibitors (among other targets, NF-κB inhibitor IKB); 2) mTOR inhibitors; 3) Receptor tyrosine kinase inhibitors; 4) Growth factor receptor antibodies; 5) Farnesyl transferase inhibitors; 6) MEK inhibitors; 7) CDK inhibitors.*

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