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Therapeutic Potential of Tumor Suppressors in Treating Breast Cancer

Research Article

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Abstract

Purpose: Tumor suppressor genes participate in a variety of critical and highly conserved cell functions, including regulation of the cell cycle and apoptosis, differentiation, surveillance of genomic integrity and repair of DNA errors, signal transduction, and cell adhesion. Genome instability appears to be one of the earliest recognizable phenotypes which appear in carcinogenesis. Studies have shown that the inactivation or deletion of certain tumor suppressor genes contribute to tumor development. Targeted strategies aim to recognize altered proteins and their associated pathways which revealed tremendous therapeutic potential for the cancer treatment. Restoring normal expression of the missing tumor suppressor gene in cancer cells is one of the emerging targeted therapeutic strategies to inhibit tumor development. The genetic determinants for most breast cancer cases remain elusive.

Methods: Targeted strategies aim to recognize altered proteins and their associated pathways which revealed tremendous therapeutic potential for the cancer treatment. Restoring normal expression of the missing tumor suppressor gene in cancer cells is one of the emerging targeted therapeutic strategies to inhibit tumor development.

Results: The genetic determinants for most breast cancer cases remain elusive. However, a mutation in tumor suppressor genes has been determined to be one mechanism of breast carcinogenesis.

Conclusions: This review focuses the therapeutic potential of tumor suppressor genes in breast cancer inhibition.

Keywords: p53; p27; BRCA1; BRCA2.

Abbreviations: MDM2: Mouse Double Minute 2; LFS: Li-Fraumeni Syndrome; HIC: Hypermethylated in Cancer; KAI-1; Kangai-1; LOH: Loss of Heterozygosity; GOF: Gain-of-Function; ASPP: Apoptosis-Stimulating Protein of P53; ER: Estrogen Receptor; CKI: Cyclin-dependent Kinase Inhibitor; BASC: BRCA1-Associated Genome Surveillance Complex; RNF: RING Fingers; ORF: Open Reading Frame; HR: Homologous Recombination; ICLs: Intrastrand Crosslinks; ATFs: Artificial Transcription Factors; PDGFRβ: Platelet-Derived Growth Factor Receptor-β.

Introduction

Despite significant advances in early detection and treatment, breast cancer remains a major cause of morbidity and mortality in women. The etiology of breast cancer involves a complex interplay of genetic, hormonal and dietary factors [1]. Recent studies have provided greater insights into the molecular mechanisms of breast carcinogenesis, which has enabled novel therapeutic strategies that target the molecular and genetic processes triggering neoplastic transformation. Genes affecting the cellular processes involved in neoplasia are classified as proto-oncogenes and tumor suppressor genes, and they regulate proteins involved in cell growth and proliferation. Therefore, mutations in these genes can contribute to the development of cancer [2]. Some oncoproteins and tumor suppressors directly regulate cell proliferation (either promoting or inhibiting), programmed cell death or apoptosis, and DNA repair [6]. Increasing knowledge of these genes and their involvement with the neoplastic pathways has provided greater opportunities to develop targeted therapeutics, which offer higher specificity,

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efficiency, and safety in cancer treatment. Mutations in tumor suppressor genes are one of the drivers of breast carcinogenesis and inherited mutations in p53, BRCA1 and BRCA2 significantly contribute to breast cancer risk [3, 4].

Tumor suppressor genes normally inhibit cell proliferation, and their loss or inactivation during neoplastic transformation results in abnormal proliferation of the tumor cells [5]. Mutations in tumor suppressor genes also enhance the invasiveness and metastatic potential of tumor cells. In some cancers, tumor suppressor function is disrupted not by mutations in the encoding gene, but other regulatory mechanisms that inhibit its expression in the tumor cells, such as promoter methylation, increased proteasomal degradation, and even abnormalities in other proteins that interact with the tumor suppressors [7]. The tumor suppressor genes identified from hereditary/familial tumors are also involved in the formation of sporadic tumors. For example, the RB gene is commonly mutated in familial breast cancers and predisposes the carriers to some rare forms such as retinoblastoma in breast cancers [5]. The 'two hit' hypothesis was proposed to explain hereditary tumor development. Two copies of normal tumor suppressor genes are present in normal diploid cells, and therefore, two mutations are required to inactivate the gene completely. In heterozygous carriers, the first hit is inherited, and only one additional hit is required to completely inactivate the gene [8]. Since one mutation pre-exists, the hereditary cancer syndrome is characterized by early onset, multiple tumor foci and incomplete penetrance.

Although tumor suppressor genes that predispose to breast cancer are yet to be identified, mutations in several tumor suppressor genes are common in breast carcinoma. This is consistent with the hypothesis that multiple genetic alterations are involved in tumor development. This review summarizes the major tumor suppressor genes associated with breast cancer and the therapeutic potential of these genes in breast cancer.

p53

Tumor protein 53 or p53 acts as a sensor of various cellular stresses, including DNA damage, hypoxia, oncogene expression, nutrient deprivation and ribosome dysfunction, and limits (tumor) cell proliferation under these adverse conditions [9]. Mutations in p53 are the most common genetic aberrations seen in human neoplasia, occurring in almost 50% of all tumors and in approximately 20%-30% of breast cancers [7]. It operates within a complex signaling pathway and senses a plethora of stress signals originating from dysregulated oncogenes, DNA damage, metabolic deprivation or telomere erosion [10]. Depending on the type of cell and the stress, p53 activation can trigger apoptosis, DNA repair, transient or permanent cell cycle arrest, and metabolic homeostasis.

The *TP53* gene is located on the short arm of chromosome 17 and encodes a 375 amino acid-long protein that is regulated via phosphorylation at different sites [7]. The primary negative regulator of the p53 protein is the mouse double minute 2 (MDM2) ligase, which binds p53 in an inactive complex [13]. The primary transcript of *TP53* consists of 11 exons, of which the exons 2-11 encode the protein. There are 5 conserved domains in exons 1, 4, 5, 7 and 8 which are considered essential for normal p53 function. Approximately 90% of disease-associated mutations occur in these domains, and those in five specific codons (175, 245, 248, 249, and 273) account for approximately 20% of all mutations reported to date [14]. Somatic mutations in *TP53* lead to inactivation of the gene, loss of tumor suppressor function, and in some cases generation of a dominant negative form of p53 [8]. Furthermore, germline mutations in *TP53* are associated with dominantly inherited Li-Fraumeni syndrome (LFS), a rare autosomal dominant syndrome which increases the risk of early-onset sarcomas of bone and soft tissues, carcinomas of the breast and adrenal cortex, brain tumors, and acute leukemias [11, 12]. In addition, carriers of germ-line p53 mutations may also be at an increased risk of other cancers.

In physiological conditions, p53 regulates cell division and proliferation by directly binding to the promoter sites of checkpoint genes such as CKI *p21* and by inducing a temporary cell-cycle arrest at the G1 or G2/M phase to allow DNA repair before mitosis [15]. It also interacts with other signaling pathways to trigger apoptosis or differentiation. In addition, p53 also regulates the expression of other tumor suppressors or regulators of angiogenesis and metastasis, such asma spin, hypermethylated in cancer (HIC)-1 and Kangai-1 (KAI-1) [7]. Therefore, p53 mutations during neoplastic transformation endow the cells with growth and survival advantages. Some of these mutations are frequently followed by loss of heterozygosity (LOH) during cancer progression [8]. Furthermore, several mutant p53 isoforms can exert additional oncogenic activity by a gain-of-function (GOF) mechanism [16]. Mutant p53 proteins almost always have defective DNA binding ability, which transactivates the genes downregulated by the wild-type protein. Interestingly, the proportion of missense mutations in p53 is higher than that seen in other tumor suppressor genes, suggesting that expression of p53 mutants may confer some additional selective advantage to the tumor cells beyond the loss of wild-type function [8].

Presence of p53 mutations in breast cancer is associated with more aggressive disease and worse overall survival. Mutant p53 proteins have been demonstrated in breast cancer cell lines, and LOH in the *TP53* gene is a common event in primary breast carcinomas and is also accompanied by mutation in the residual allele in some cases. Germline mutations in the gene encoding BRCA1, a transcriptional co-activator for p53, confer a high risk of breast cancer [17]. BRCA1 is phosphorylated after DNA damage by the ATM, ATR and Chk2 kinases, and binds to the C-terminus of wild-type p53 and stimulates transcription via the p53-responsive promoters. BRCA1 mutants lack this ability which leads to the proliferation of the cells. In addition to the DNA-damage cascade kinases (ATM, ATR and Chk2) that regulate the stability and function of p53 through phosphorylation, another functionally distinct group of proteins has recently been implicated as co-stimulatory factors of the wild-type p53. One such family of proteins, with possible involvement in breast cancer, is the apoptosis-stimulating protein of p53 (ASPP) [19]. The pro-apoptotic activity of p53 is tightly regulated by the ASPP members like ASPP1, ASPP2 and iASPP. Overexpression of either ASPP1 or ASPP2 stimulates the pro-apoptotic function of wild-type p53 by increasing p53-dependent induction of apoptotic effectors such as Bax and PIG3. Downregulation of the ASPP proteins attenuates p53-dependent apoptosis, thus conferring a selective advantage to breast carcinoma cells with intact p53 [18]. ASPP Overexpression has been linked to estrogen

receptor (ER) negativity, a strong predictor of negative outcome in breast cancer. Independent of ER status, mutationsin *TP53* increase the relative risk of relapse in breast cancer by ~33% [7]. There are conflicting data regarding p53 as a predictor of therapeutic response, although it has been ranked as a category II prognostic marker in breast carcinoma [20-22]. Since p53 is a multifunctional protein and mutations in different domains may have distinct consequences, the analysis of the mutation status of *TP53* may be more informative than analysis of p53 protein levels.

Since p53 is the master regulator of various tumor suppressive pathways, it is imperative to study the means of reactivating or restoring p53 function in breast cancer cells in order to reverse their chemo-resistance. Many anticancer drugs induce apoptosis through multiple pathways that are at least partially dependent on functional p53 activation [23, 24]. Studies show that the introduction of wild-type *TP53* gene into various human cancer cells inhibits proliferation and induces apoptosis. In line with this, multiple p53-based therapeutic strategies are currently being studied [25].

p27

Cyclin-dependent kinase inhibitor (CKI)1B or p27 belongs to a family of CKIs known as Cip/Kip, which also includes p21 and p57 [26], and is regulated at the post-transcriptional level through protein translation and degradation. p27 binds to a number of unique cyclin/CDK complexes to attenuate their activity, and induce cell-cycle arrest at the G1 phase. It has separate binding sites for cyclin and CDK2, and binding results in conformational changes in the catalytic cleft of CDK2 [27, 28]. Decreased expression of p27 has been observed in a number of human cancer cell lines, which interferes with the cell cycle check-points, and leads to the accumulation of additional genetic alterations and increased malignancy [29, 30]. However, mutations in the *p27* gene are rare and have been observed in only 1% of tumors [7]. The expression level of p27 has a prognostic value in the tumors of the lungs and colon. Proteins are often regulated by phosphorylation and poly-ubiquitination [31]. Pin1, a peptidyl-prolyl isomerase, recognizes and stabilizes p27 when phosphorylated on Thr187 by inducing a conformational change. The inhibitory actions of p27 on cyclin/CDK complexes are weakened by phosphorylation at other sites by kinases of signal transduction pathways [32]. Any disruption in these regulatory axis leads to degradation of p27 and can trigger cancer development. If these oncogenic signaling pathways are inhibited, the tumor suppressive functions of p27 can be restored [31]. Two studies reported that p27 heterozygous (+/-) mice were more susceptible to mammary and prostate tumors than p27 null (-/-) mice, indicating a certain pro-oncogenic role as well [31, 33, 34]. In breast cancer, a diminished expression of p27 is associated with shorter overall survival and shorter time to progression, and it is a stronger independent predictor of outcome than either p53 mutations or tumor grade [35]. The function of p27 is impaired in breast and other human cancers due to accelerated p27 proteolysis, sequestration by cyclin D-cdks, and mislocalization in the cytoplasm. Stepwise loss of p27 expression may trigger the transition of a normal cell to the premalignant and then malignant phenotypes [36]. The poor prognosis conferred by loss of p27 expression may be partially related to its modulatory effect on cell-cell adhesion and, therefore, a pro-metastatic role.

The S-phase kinase-associated protein Skp2 is required for the ubiquitin-mediated degradation of p27 and has been shown to increase oncogenicity and resistance to anti-estrogens *in vitro* [37]. Skp2 may also be preferentially overexpressed in ER^- and $HER\text{-}2^-$ breast cancer, a subset recently defined as the "basal phenotype" by gene profiling [7].

BRCA1

BRCA1 gene encodes a nuclear phosphoprotein that maintains genomic stability and interacts with other tumor suppressors, DNA damage sensors and signal transducers to form a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC). It also associates with RNA polymerase II, and through its C-terminal domain, with histone deacetylase complexes. Therefore, *BRCA1* plays a role in transcription, DNA repair of double-stranded breaks, and recombination. Mutations in the BRCA1 gene are seen in ~40% of inherited breast cancers and in more than 80% of the inherited breast and ovarian cancers. Based on linkage analysis of families with a history of breast cancer, the locus of *BRCA1* was first identified in 1990 on the long (q) arm of chromosome 17 at position 21 (17q21) and was subsequently designated as such in 1994 [38]. It has been estimated that approximately 0.12% of the general population carries a mutation of *BRCA-1*; however, this rate varies depending on different ethnic groups. In a meta-analysis of such case-based studies, by age 70 years, in BRCA1 carriers breast cancer risk was 65% (95% CI 51–75%) and ovarian cancer risk was 39% [39].

The BRCA1 protein is 1,863 amino acid long and belongs to the RNF (RING-type zinc fingers) family of proteins where in the cysteine and histidine residues fold around and hold azinc ion. This configuration makes the protein highly stable and enables its binding to downstream targets like BARD1 and E2F1 at the N-terminus, which is necessary for DNA repair. In addition, BRCA1 may function independently as a tumor suppressor. Two repeats in the C-terminus of BRCA1 are similar to those seen in many DNA repair enzymes including Rad9. Following genotoxic insult, BRCA1, along with BARD1 and Rad51, localizes to areas of damaged DNA, which regulates transcription as well as repair of double-stranded DNA [40, 41].

Over 200 individual *BRCA-1* mutations have been identified, including deletions, substitutions, and insertions. They are found throughout the length of the gene, although some areas do appear to be mutational hotspots. Approximately 80% of these events result in abnormal truncation of the BRCA1 protein [42, 43]. The severity of disease can be linked to the location of the mutation, with those involving the N-or C-terminus associated with more aggressive tumors.

BRCA2

The *BRCA2* gene is longer than *BRCA1* and has a 10.3 kb open reading frame (ORF) encoding a 384 kDa nuclear protein. It does not share a high degree of sequence homology with other known genes, and the BRCA2 protein consists of domains that are as yet undefined. However, since the BRCA1 and BRCA2 proteins share functional similarities, mutations in the encoding genes result in similar and specific hereditary predisposition to breast

and ovarian cancer.

BRCA2 plays an essential role in several DNA repair pathways, including DSB repair by homologous recombination (HR) and DNA crosslink repair by the FA pathway, and maintains genome stability after binding to BRCA1 and PALB2. BRCA2 is a key player in the repair of DNA lesions including DSBs and intra-strand crosslinks (ICLs), and independent of its DNA repair function, prevents nucleolytic degradation at stalled replication forks. Both functions are directly or indirectly involved in telomere maintenance. In addition, BRCA2 is required for the processing of R-loops along with the TREX-2 complex [44].

BRCA2 has been linked to six different germline mutations in familial breast cancer and is typically disruption at the transcriptional unit 17 of the ORF. These mutations, especially deletions and/or frameshifts, result in premature stop codons and thus interrupt protein translation. Currently, more than 1800 mutations have been identified in *BRCA2*, including frameshift deletions, insertions, or nonsense mutations that lead to premature truncation of proteins. These events are consistent with the loss of function that is expected in mutations of tumor suppressor genes. Carriers of *BRCA2* mutations also have a higher risk of gallbladder, bile duct and stomach cancer, and melanoma [40].

Therapeutic Approaches Involving Tumor Suppressors

Gene Therapy

Both experimental and clinical studies have focused on tumor-suppressor genes as potential anti-cancer therapeutic targets. Exogenous expression of various tumor-suppressor genes in cancer cells suppresses tumor growth via apoptosis induction and cell-cycle arrest. Clinical trials so far, especially with the p53 gene, have demonstrated pathologically complete responses and minimal adverse effects in patients with advanced or refractory cancer [46]. Gene replacement therapy strategies use a viral vector, such as a replication-deficient adenovirus, to introduce wild-type tumor suppressor genes into cancer cells. These vectors can be administered intratumorally, intraperitoneally or intravesically, and are minimally toxic to normal cells since the introduction of a TSG at physiological levels would not be expected to have any significant effect. Although early-phase clinical trials show good tolerance by the patients, the major limitation of gene therapy is low efficacy. The viral vectors used for gene therapy have not been able to achieve the necessary efficiency of transduction into tumor cells to be therapeutically significant. Furthermore, repeated administration of attenuated viruses activates the host immune response to the viral vectors [47]. A novel p53-related gene therapy currently underway uses an E1B-deleted adenovirus called ONYX-015, which selectively replicates in p53-deficient cancer cells and subsequently lyses the cells. Preclinical studies showed anti-tumor activity of ONYX-015 both *in vitro* and *in vivo*, especially in combination with chemotherapy or radiation therapy [48].

Specific reactivation of endogenous tumor suppressors is another important therapeutic strategy that has been tested to block tumor growth and progression. It can be achieved by constructing artificial transcription factors (ATFs) targeted against the promoter sequences of the respective tumor suppressor genes. Blancafort et al., found that ATF induced apoptosis and inhibited *in vitro* invasiveness of breast cancer cells [49]. Other strategies that are still in developmental phases include tumor suppressor gene silencing to alter mutation frequency, inhibition of signaling pathways that are abnormally activated by mutations in these genes, and restoration of the normal tumor suppressor gene which turns on an apoptosis or senescence pathway.

Targeting the Downstream Sequences

Several downstream mediators of tumor suppressor genes have been identified, which opens up the possibility of new therapeutic targets. For example, mutant p53 facilitates a pro-metastatic phenotype in a pancreatic adenocarcinoma model [50], by inducing the expression of platelet-derived growth factor receptor-β (*PDGFRβ*) which in turn mediates invasion and metastasis. Pharmacological inhibition of *PDGFRβ* with crenolanib or imatinib significantly reduced the invasive potential of pancreatic adenocarcinoma cells [47].

Anti-Tumor Cell Vaccines

Anti-cancer vaccines are also currently in the experimental stage. This approach is based on the observation that cancer patients often produce antibodies and reactive T-cells against p53. Vaccines containing multiple p53 peptides can generate a T-helper type I response in patients, although they are as yet not potent enough to be clinically beneficial. More recently, vaccines derived from dendritic cells transfected with the *TP53* gene have been shown to generate stronger immune responses. Related approaches use dendritic cells loaded with human leukocyte antigen class I p53 peptides, which target the immune regulatory mechanisms. Nevertheless, a continuing challenge is to overcome the strong immune suppressive mechanisms in cancer patients [51].

Conclusion

Tumor suppressor genes negatively regulate oncogenes, cell cycle checkpoint factors, or metabolic enzymes that are needed to complete a cell cycle in the absence of stress. Tumor suppressor gene mutations include deletions, nonsense mutations, frame-shift mutations, insertions, as well as missense mutations that functionally inactivate a protein. Furthermore, they are recessive, loss-of-function mutations that occur in both alleles. However, mutations in both alleles of the gene in the same cell are a very rare event (the square of the independent probabilities). Instead, a tumor suppressor gene undergoes "reduction to homozygosity," which is mediated by either gene conversion (via replication or recombination) or loss of the chromosome carrying the wild-type allele and duplication of the chromosome with a mutant allele. Inherited mutations in tumor suppressor genes like p53, P27, BRCA1 and BRCA2 significantly contribute to breast cancer risk. In addition, sporadic mutations in p53 are also common in breast cancer cells. Although these genes have different functions, they are all involved in maintaining genomic stability after DNA damage, and it is highly likely that mutations in the above genes trigger breast cancer development via this mechanism. Bioinformatics offers the possibility of analyzing exon sequencing data of different tumor suppressor genes indifferent breast cancer variants. This approach has helped identify tumor-specific

peptides and neo antigens which arise as a consequence of tumor-specific mutations. Further research on tumor suppressor gene biology, DNA damage repair mechanisms, signaling pathways and the immune system is needed to improve the therapeutic prospects of breast cancer.

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