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MINIREVIEW

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Chromosome 3p and breast cancer

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Abstract Solid tumors in humans are now believed to develop through a multistep process that activates oncogenes and inactivates tumor suppressor genes. Loss of heterozygosity at chromosomes 3p25, 3p22–24, 3p21.3, 3p21.2–21.3, 3p14.2, 3p14.3, and 3p12 has been reported in breast cancers. Retinoid acid receptor β2 (3p24), thyroid hormone receptor β1 (3p24.3), Ras association domain family 1A (3p21.3), and the fragile histidine triad gene (3p14.2) have been considered as tumor suppressor genes (TSGs) for breast cancers. Epigenetic change may play an important role for the inactivation of these TSGs. Screens for promoter hypermethylation may be able to identify other TSGs in chromosome 3p. Alternatively, use of an "epigenetic modifier" may enhance the response to another type of agent for breast cancer.

Key words Breast cancer · Chromosome 3p · Loss of heterozygosity · Hypermethylation · RARβ2 · TRβ1 · RASSF1 · FHIT

Introduction

Breast cancer is one of the most common malignancies among women, and its cumulative risk by age 85 years is 1 in 8 women in the United States and 1 in 40 women in Japan (American Cancer Society 1994). Solid tumors in humans are now believed to develop through a multistep process that activates oncogenes and inactivates tumor suppressor genes (Lopez-Otin and Diamandis 1998). Inactivation of a tumor suppressor gene (TSG) often involves mutation of one allele and loss or replacement of a chromosomal segment containing another allele. Loss of heterozygosity (LOH) has been found on chromosomes 1p, 1q, 3p, 6q, 7p, 11q, 13q, 16q, 17p, 17q, 18p, 18q, and 22q in breast cancer (Smith et al. 1993; Callahan et al. 1992; Sato et al. 1990; Hirano et al. 2001a); the commonly deleted regions include 3p, 6q, 7p, 11q, 16q, and 17p (Smith et al. 1993; Hirano et al. 2001a). In addition, other gross alterations such as the aneuploidy state and gene amplification, and small changes including point mutations, small insertions, or deletions in multiple genes are scattered through the genome of cancer cells. In fact, most cancer research on the neoplastic cell in the last 20 years has focused on genetic aspects. However, the malignant cell has also acquired a different epigenotype. The inheritance of information on the basis of gene expression levels is known as epigenetics, as opposed to genetics, which refers to information transmitted on the basis of gene sequence. The main epigenetic modification in mammals, and in particular in humans, is the methylation of the cytosine nucleotide residue.

DNA methylation is an enzymatic modification performed by DNA methyltransferases. The added methyl group does not affect the base pairing itself, but the protruding of methyl groups into the major groove can affect DNA–protein interactions (Razin and Riggs 1980). In eukaryotes, two different types of DNA methyltransferases have been characterized: de novo methyltransferases, such as Dnmt3a and Dmnt3b (Okano et al. 1998), which use nonmethylated DNA as a subtrate; and maintenance methyltransferases, such as Dnmt1 (Bestor et al. 1988), which methylate hemimethylated DNA that is generated by replication of methylated sites (Bestor 2000). Maintenance methylation implies copying of the existing methylation pattern of the old DNA strand onto the new one. Therefore, DNA methylation can be heritable and can serve as an epigenetic mark that is transmitted by mitotic or cell division onto the progeny. DNA methylation of TSGs is a

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Table 1. LOH, sensitive microsatellite markers, and candidate TSGs in chromosome 3p

Regions of LOH	Microsatellite markers	Candidate TSGs
3p25		*VHL*
	D3S1351	
	D3S1597	
	D3S1111	
3p24		*RARβ2, TRβ1*
	D3S 1286	
	D3S 1293	
	D3S 1283	
	D3S 2432	
	D3S 1537	
3p21.3		*RASSF1*
	D3S 4597	
	D3S 4604	
	D3S 4614	
	D3S 4622	
	D3S 4623	
	D3S 4624	
3p14.2		*FHIT*
	D3S 1295	
	D3S 1234	
	D3S 4103	
	D3S 1300	
	D3S 1600	
3p12		*DUTT1*
	D3S 1284	
	D3S 1274	
	D3S 1511	

LOH, Loss of heterozygosity; TSGs, tumor suppressor genes

frequent mechanism of transcriptional silencing in cancer (Jones and Laird 1999; Baylin and Herman 2000). Recently, Rhee et al. (2002) demonstrated that Dnmt1 and Dnmt3b cooperate to silence genes in human cancer cells. The molecular mechanisms underlying the specificity of methylation are largely unknown. Croce et al. (2002) reported that leukemia-promoting promyelocytic leukemia-retinoic acid receptor fusion protein induces gene hypermethylation and silencing by recruiting DNA methyltransferases to target promoters, and that hypermethylation contributes to its leukemogenic potential. Retinoic acid treatment induces promoter demethylation, gene reexpression, and reversion of the transformed phenotype. The mechanistic link between genetic and epigenetic changes during transformation suggests that hypermethylation contributes to the early steps of carcinogenesis (Croce et al. 2002).

Chromosome 3p allele loss is a frequent event in breast cancer, and some candidate TSGs have been located in this region (Table 1). Notably, epigenetic changes can mediate inactivation of these TSGs (Bovenzi et al. 1999; Sirchia et al. 2000; Widschwendter et al. 2000; Li et al. 2002; Dammann et al. 2001; Zochbauer-Muller et al. 2001). We would like to outline the genetic and epigenetic analysis of chromosome 3p in breast cancer.

LOH analysis of chromosome 3p

LOH at chromosome 3p occurs in many human malignancies including breast cancers (Ali et al. 1989; Devilee et al. 1989). It is important to elucidate the region within 3p that is involved in breast cancer, which could provide information on genetic alterations in tumors and help to localize sites of potential TSGs. Ali et al. (1989) reported a 30% LOH of markers at 3p21–25. Sato et al. (1990) reported LOH at 3p in 47% of 219 tumors analyzed by restriction fragment length polymorphism. Matsumoto et al. (1997) reported a frequency of 52% 3p LOH in 196 tumors analyzed by conventional allelotyping. Maitra et al. (2001) performed high-resolution allelotyping for LOH analysis on microdissected samples from 45 primary breast cancers; allele loss in some regions of chromosome 3p was detected in 39 cases (87%). Chen et al. (1994) demonstrated that two separate deletion regions (3p13–14 and 3p24–26) existed on chromosome 3p. Mastumoto et al. (1997) were able to define two noncontiguous regions of frequent LOH in their analysis (3p24.3–25.1 and 3p14.2–21.1). Maitra et al. (2001) revealed frequent LOH in 3p21.3, 3p22–24, 3p21.2–21.3, 3p25, 3p14.2, 3p14.3, and 3p12.

The chromosomal regions where allelic losses have been detected are thought to include specific target genes whose inactivation either is essential for transformation or provides a selective advantage associated with progression. However, it is also possible for an LOH to be randomly acquired and irrelevant but coselected with other specific mutations important for tumor development. It is still tempting to speculate that these discrete areas in chromosome 3p may harbor one or more TSGs critical to breast carcinogenesis.

Candidate tumor suppressor genes in chromosome 3p

Retinoid acid receptor β2

Retinoid acid receptor β2 (RARβ2) maps to chromosome 3p24 (Chambon 1996), a region that exhibits a high frequency of LOH (Deng et al. 1996). Deng et al. (1996) reported that LOH in normal terminal ductal–lobular units adjacent to the tumor was seen in six of the ten cases with confirmed LOH at 3p24 in the cancer. Maitra et al. (2001) detected 3p24 LOH in precursor lesions. Retroviral transduction of breast tumor cell lines with RARβ2 results in inhibition of tumor cell proliferation (Seewaldt et al. 1995). RARβ2 levels were found to be decreased or suppressed in a number of malignant tumors, including lung cancer, squamous cell cancer of the head and neck, and breast cancer (Picard et al. 1999; Xu et al. 1997, 1999). These findings suggest that RARβ2 plays an important role in limiting the growth of many cell types, and that the loss of this regulatory activity is associated with tumorigenesis. RARβ2 inhibits the metastatic cascade in a mouse mammary gland xenograft tumor model and is a potential candidate for therapeutic intervention in human breast cancer (Treutin et al. 2002). RARβ2 can mediate retinoid action in breast cancer cells by promoting apoptosis (Liu et al. 1996). In vitro (Liu et al. 1996) and in vivo (Toma et al. 2000)

studies suggest that RARβ2 could be induced by all-trans-retinoic acid in breast cancer. All-*trans*-*N*-(4-hydroxyphenyl)retinamide (4HPR), a synthetic retinoid acid, is an activator of RARβ2. 4HPR is a promising drug for the chemoprevention of breast cancer (Fisher et al. 1998). This synthetic retinoid has been shown to be more effective and less toxic than other retinoids for chemoprevention of mammary cancer in animals (Moon et al. 1979). 4HPR was the subject of a recent 5-year clinical trial conducted to assess its usefulness in preventing contralateral breast cancer in a population of patients previously operated on for breast cancer (Veronesi et al. 1999). The combined administration of tamoxifen and 4HPR has proven to be additive or synergistic in both the growth inhibition of the breast cancer cell line MCF-7 and the prevention of *N*-methyl-*N*-nitrosourea-induced mammary carcinoma in the rat (Ratko et al. 1989). Because retinoids do not require estrogen receptors (ERs) for their action, they may affect neoplastic transformation in ER-negative cells (Fraker et al. 1984), in contrast to tamoxifen, whose primary mechanism of action is through the ER.

To understand why RARβ2 activity is down-regulated or lost in malignant tumors, researchers have made intense efforts to identify possible alterations that affect either the RARβ2 promoter or its regulatory factors (Seewaldt et al. 1995; Xu et al. 1997; Wu et al. 1997). We assessed LOH on chromosome band 3p24 to correlate it with RARβ2 expression and other established prognostic parameters in primary breast cancers (Yang et al. 2001a). Based on three microsatellites, D3S 1283, D3S 1293, and D3S 1286, all of the tumors were informative; of these, 12 (23%) exhibited LOH. RARβ2 expression was lost in 42% (19/45) of detected samples. We found that LOH on chromosome band 3p24 was not correlated with loss of RARβ2. Moreover, no mutations were found in the promoter of breast cancer (Widschwendter et al. 1997; Yang et al. 2001b). These results suggest that it is unlikely that changes in gene structure could completely explain the altered RARβ2 expression. A possible mechanism to be considered is suppression of the RARβ2 gene by methylation in the promoter (Bovenzi et al. 1999; Sirchia et al. 2000; Widschwendter et al. 2000). We demonstrated that biallelic inactivation of the RARβ2 gene could result either from epigenetic inactivation of both parental alleles, or from epigenetic modification of one allele and deletion of the remaining allele (Yang et al. 2001b). Unexpectedly, methylation status could not account completely for suppression of RARβ2 expression in breast cancer tumors (Widschwendter et al. 2000; Yang et al. 2001c). Changes altering RARβ2 transcription in breast cancer might be prevented in the presence of supraphysiological levels of retinoic acid. This hypothesis was supported by the findings that RARβ2 expression is selectively reduced in several organs when vitamin A is deficient, and is enhanced by retinoic acid (Kato et al. 1992; Verma et al. 1992). In addition, transcription of RARβ2 is also regulated by corepressors and coactivators; thus, its suppression may be due to aberrant expression of these cofactors. Previous studies have indicated that orphan receptor chicken ovalbumin upstream promoter transcription factor is required for induction of RARβ2 in several types of cancer cells including the breast (Lin et al. 2000).

Thyroid hormone receptor β1

Our findings that LOH of the 3p24 region harboring the RARβ2 gene does not correlate with lack of RARβ2 expression may indicate that other genes of these regions, probably not only *RARβ2*, play a role in determining the pathological characteristics of the tumors (Yang et al. 2001a). The frequent region of allelic loss at 3p24.3 in morphologically normal terminal ductal–lobular units also encompasses the thyroid hormone receptor β1 (*TRβ1*) gene (Deng et al. 1996; Li et al. 1997). *TRβ1* regulates gene expression when bound to thyroid response elements in the proximity of target genes (Mangelsdorf et al. 2000). On the basis of the presence or absence of the ligand, thyroid hormone triiodothyronine (T_3), *TRβ1* can act as a transcriptional activator or silencer (Damm et al. 1989). Increasing evidence has suggested that aberrant expression and/or mutations in TR genes could be associated with carcinogenesis. A reduction in the expression of mRNA for *TRβ1* and *TRβ2* was implicated in inappropriate expression of the glycoprotein hormone α-subunit gene in nonfunctioning tumors of the anterior pituitary and was proposed to contribute to uncontrolled tumor growth (Gittoes et al. 1997; McCabe et al. 1999). Reduced expression of *TRβ1* was also found in poorly differentiated fibroblast-like osteosarcoma (Williams et al. 1994). However, in poorly differentiated hepatocarcinomas, overexpression of *TRβ1* was correlated with enhanced T_3-induced proliferation (Lin et al. 1990, 1994). In neuroblastoma cells, Lebel et al. (1994) have also demonstrated that T_3 treatment of *TRβ1*-overexpressing cultures arrests proliferaton in the G0/G1 phase of the cell cycle, and induces morphological and functional differentiation. These results suggest that aberrant expression of *TRβ1* may be associated with different types of tumors and/or different states of differentiation. Functionally impaired *β1* mutants have been detected in thyroid papillary cancer (Puzianowska- Kuznicka). Li et al. (2002) could not detect *TRβ*1 mutations but observed a variable degree of *TRβ*1 promoter hypermethylation in two of five breast cancer cell lines and in all 11 cases of primary breast cancer examined. Moreover, biallelic inactivation of *TRβ1* by LOH and/or methylation was also suggested.

Ras association domain family 1A

Ras association domain family 1 (*RASSF1*) maps to chromosome 3p21.3. It spans 7.6 kb of genomic DNA, has a predicted Ras association domain and homology to the Ras effector Nore 1 (Dammann et al. 2000). The *RASSF1* gene encodes two major transcripts, RASSF1A and RASSF1C, which are produced by alternative promoter selection and alternative messenger RNA splicing. *RASSF1A* is encoded by *RASSF1* exons 1A, 1C, and 2–5. *RASSF1C* is encoded by RASSF1 exons 1–5. The start sites for *RASSF1A* and *RASSF1C* are approximately 2 kb apart and have two inde-

pendent CpG island-containing putative promoter regions. *RASSF1A* is predicted to encode a 39-kDa peptide that contains an N-terminal diacylglycerol-binding domain and a Ras association domain. *RARSSF1A* promoter hypermethylation was detected in many breast cancer cell lines and 49%–62% of primary breast cancers (Burbee et al. 2001; Dammann et al. 2001). However, Agathanggelou et al. (2001) reported *RASSF1A* promoter hypermethylation in 9% of primary breast cancers; the lower hypermethylation frequency may result from sample selection bias. Burbee et al. (2001) demonstrated that the frequency of *RASSF1A* gene silencing virtually parallels that of the incidence of the LOH for chromosome 3p21, the region that harbors the gene. Methylation and LOH may be the major loss of function pathways for the *RASSF1A* gene because somatic mutations appear to be rare in this gene (Dammann et al. 2000). Dammann et al. (2001) detected a constant methylation frequency of *RASSF1A* in all of the different grades of the mammary cancers. *RASSF1A* inactivation was already very high in grade I tumors. Furthermore, some methylation is also detected in 7.5% of the samples, which were classified as normal tissue removed with tumor surgery. Thus, methylation of *RASSF1A* may be an early event during breast cancer pathogenesis.

The fragile histidine triad gene

The fragile histidine triad (*FHIT*) gene has been mapped to 3p14.2 of human chromosome 3 (Ohta et al. 1996). The most common fragile site of the human genome, FRA3B, maps within the *FHIT* gene (Ohta et al. 1996). The presence of the FRA3B fragile site within *FHIT* suggests that the fragility of this gene may make *FHIT* susceptible to rearrangements induced by a variety of environmental carcinogens. It also suggests that the degree of chromosomal fragility at this site may contribute to the degree of cancer susceptibility. In sporadic breast cancer, LOH within the *FHIT* gene has been observed at different frequencies (Ahmadian et al. 1997; Man et al. 1996; Ingvarsson et al. 1999; Huiping et al. 2000; Yang et al. 2002). Similar deletions of the *FHIT* gene have been observed in preneoplastic lesions (Ahmadian et al. 1997), suggesting that *FHIT* deletions could be an early event in a significant fraction of breast cancer. LOH at *FHIT* is associated with tumor progression and patient survival (Ingvarsson et al. 2001). Gatalica et al. (2000) have demonstrated that the expression of Fhit protein is related inversely to disease progression in patients with breast cancer. Campiglio et al. (1999) analyzed Fhit expression in 185 breast cancers and indicated that a decrease or an absence of Fhit protein expression is associated with high proliferation and large tumor size. We assessed Fhit expression using immunohistochemistry in 166 invasive breast cancers (Yang et al. 2001d) and found that loss of Fhit expression is associated with higher malignant phenotypes and appears to be a prognostic factor in breast cancer.

Previous studies have demonstrated that point mutations are very infrequent in *FHIT* (Ahmadian et al. 1997; Gonzalez et al. 1998; Kannan et al. 2000). Hypermethylation seems to play an important role in *FHIT* inactivation (Zochbauer-Muller et al. 2001).

Discussion and future directions

In addition to the candidate TSGs described earlier, *DUTT1* (3p12) may be a new candidate TSG for breast cancer (Sundaresan et al. 1998). Its tumor-suppressing activity and protein patterns in tumors are unknown. Chromosome 3p25 has been reported to have a significantly adverse effect on postoperative survival (Matsumoto et al. 1999; Hirano et al. 2001a, 2001b). Lininger et al. (1999) also suggest that TSG in this region contributes to carcinogenesis of apocrine cancer of the breast. The Von Hippel Lindau (*VHL*) gene may be a candidate. Kim et al. (1998) have demonstrated that *VHL* controls cell cycle progression by regulation of p27Kip1 at both the mRNA and protein levels. However, no mutations were identified in the *VHL* gene regions studied in breast cancer cell lines (Bailly et al. 1995). It is possible that the *VHL* gene or another putative TSG also undergoes inactivation by the increasingly apparent epigenetic pathway of promoter methylation rather than by mutation, but this remains to be determined. Identification of nested 3p21.3 homozygous deletions in small cell lung cancers and a breast cancer line directed positional cloning efforts to a 630-kb region, which was narrowed subsequently to a 120-kb subregion by a breast cancer homozygous deletion (Lerman and Minna 2000; Sekido et al. 1998). A group of candidate tumor suppressor genes (designated *CACNA2D2*, *PL6*, *101F6*, *NPRL2*, *BLU*, *RASSF1*, *FUS1*, *HYAL2*, and *HYAL1*) has been identified in a 120-kb critical tumor homozygous deletion region (found in lung and breast cancers) of human chromosome 3p21.3. The *RASSF1A* isoform of the *RASSF1* gene, which has been studied extensively for promoter methylation in a variety of tumors, including lung and breast cancer, was found to be frequently epigenetically inactivated in these tumors, and shows the ability to suppress lung cancer malignant growth (Burbee et al. 2001; Dammann et al. 2000). Ji et al. (2002) studied the effects of six of these 3p21.3 genes (*101F6*, *NPRL2*, *BLU*, *FUS1*, *HYAL2*, and *HYAL1*) on tumor cell proliferation and apoptosis in human lung cancer cells by recombinant adenovirus-mediated gene transfer in vitro and in vivo. They found that forced expression of wild-type *FUS1*, *101F6*, and *NPRL2* genes significantly inhibited tumor cell growth by induction of apoptosis and alteration of cell cycle processes. Further research should be addressed if these genes are TSGs for breast cancer.

Frequent hypermethylation has been identified in the TSGs on chromosome 3p (Yang et al. 2001b; Bovenzi et al. 1999; Sirchia et al. 2000; Widschwendter et al. 2000; Li et al. 2002; Dammann et al. 2000; Burbee et al. 2001; Dammann et al. 2001; Zochbauer-Muller et al. 2001). It will be interesting to investigate whether this methylation occurs as part of the aging process, a phenomenon that has been described

for other genes (Ahuja et al. 1998). Hypermethylation and LOH may be the major loss of function pathways for these TSGs because somatic mutations appear to be rare, and the mechanisms fit the revised Knudson two-hit theory (Jones and Laird 1999). Similar to LOH (Maitra et al. 2001), hypermethylation also occurs in precursor lesions (Lehmann et al. 2002). This raises the question regarding which event, LOH or epigenetic change, occurs first in breast cancer development. The development of sporadic breast cancers is driven by heritable phenotypic changes, which are due to both genetic and epigenetic events. Perhaps either one is effective in initiating the disease process. However initiated, the molecular and mechanistic heterogeneity of sporadic cancers suggests that the cause of a tumor may be as specific as the individual in which it has arisen. Therefore, both diagnostic and treatment options need to be tailored to address this aspect.

Epigenetically mediated gene silencing in breast cancer heavily impacts future research in this area. Screens for promoter hypermethylation should be considered as one of the important methods for searching for TSGs in breast cancer. Although heritable, epigenetic changes are potentially reversible. A better understanding of epigenetic regulation of TSGs in gene-specific fashion will help efforts to modulate gene expression selectively, with the ultimate goal of improved breast cancer prevention and therapy.

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