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Minireview paper

Tumor-specific DNA in plasma of breast cancer patients

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The presence of DNA fragments circulating in cancer patients was described a number of years ago. The mere presence of DNA in the circulation is not indicative of cancer. However, there are reports that apoptosis and necrosis of the cancer cells can increase the levels of circulating DNA. The study of plasma DNA with the detection of genetic abnormalities associated with specific cancers has produced some promising results. Primary cancer often harbors *ras* or p53 mutations and the detection of these mutations in free circulating DNA could indicate the presence of cancer. Other approaches have included detection of specific losses of heterozygosity (LOH), microsatellite instability (MI) and promoter hypermethylation. For breast cancer, studies published to date have focused on detecting LOH, MI and methylation of the p16INK4A promoter. Good concordance between alterations in the primary tumor and detection of the same alterations in the circulation has been observed. Also, it is encouraging to note that DNA alterations have been detected in patients with small or even *in situ* lesions, indicating that circulating tumor DNA is shed early in the disease process. If 'universal' breast-specific DNA alterations can be identified, this approach may hold significant promise for early detection of breast cancer. [© 2002 Lippincott Williams & Wilkins.]

Key words: Breast carcinoma, p53 mutation, plasma DNA, tumor marker.

Introduction

Recent advances in tumor genetics have revealed that malignant transformation follows an accumulation of multiple genetic alterations, including inactivation of tumor suppressor genes and/or activation of proto-oncogenes.[1] Several genetic changes, such as the activation of the *ras* oncogene and the inactivation of the p53 gene, are involved in the pathogenesis of breast cancer.[2] Assays based on the molecular detection of these genetic changes have been shown to be potential diagnostic and prognostic tools for breast cancer.[3,4] The identification of these genetic changes at sites away from the primary tumor may help to assess the extent of disease and overall tumor burden at initial diagnosis, as well as on follow-up care.

The presence of DNA fragments circulating in cancer patients was described a number of years ago.[5,6] There is evidence that naked DNA is released, enriched and remains stable in the blood of cancer patients.[5,6] Recently, tumor-specific DNA has been detected in the plasma of lung, head and neck, breast, and colon cancer patients.[7–10] This suggests that cell-free plasma is a source for detecting cancer-specific DNA markers. In the past, tumor-associated markers such as proteins or glycoproteins have been used for diagnosis of disease progression in patients. However, the specificity of these assays is limited because the majority of these markers are not tumor-specific and are found in normal cells. The mere presence of DNA in the circulation is not indicative of cancer. However, there are indications that apoptosis and necrosis of the cancer cells can increase the levels of circulating DNA.[13] Combining plasma DNA with the detection of genetic abnormalities associated with specific cancers has produced some promising results.

To date, tumor-specific genetic markers have been assessed primarily in tumor biopsies. However, in advanced-stage patients, surgery is not always performed, which limits the availability of tumor tissue for genetic assessment. The detection of tumor-specific genetic alterations in cancer patients at distant sites from the tumor, such as in the blood,

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provides a unique and valuable tumor marker for diagnosis and prognosis. Breast cancer is associated with different types of molecular genetic aberrations such as somatic mutations of oncogenes and tumor suppressor genes.[14–18] The determination of these abnormalities can be used as a specific tool in the histological diagnosis and possibly as a tumor marker. As observed in other tumors, the specific molecular alterations shown by the primary breast carcinoma may also be found in the plasma DNA of breast cancer patients.

DNA is present in increased amounts in plasma DNA of breast cancer patients

Leon and colleagues found that cancer patients tend to have higher levels of circulating DNA than those with non-malignant disease.[6] They demonstrated that plasma DNA concentration in the normal controls has a mean of 13 ng/ml while in the cancer patients the mean is 180 ng/ml.[12] In human breast cancer, they reported that free DNA is present at concentrations ranging between 0 and 2 ng/ml in the serum of breast cancer patients, and that it is possible to analyze the variation in the amount depending on the stage of the disease and the response to the treatment received by the patients.[12] In our recent study, we also demonstrated that the concentration of plasma DNA is much higher in breast cancer patients in comparison to healthy women (Table 1). In breast cancer patients, the level of plasma DNA also correlates with stage, lymph node metastasis and tumor size.[19]

p53 mutation of plasma DNA in breast cancer

Previous studies have identified several tumor-specific gene alterations in the plasma DNA of cancer patients. Mutant K-*ras* and p53 DNA have been detected in the plasma of patients with colorectal, pancreatic, and hematological neoplasms.[20] Several studies have shown that the presence of tumor DNA in the plasma correlates with disease stage.[17,18,20,21] Nawroz *et al.* has reported a large number of patients with advanced head and neck cancer who have detectable p53 mutations in plasma DNA.[10] Silva *et al.* have reported that there is a significant difference between breast cancer patients with or without p53 mutations in plasma DNA with regard to lymph node involvement, proliferating index and tumor cell growth.[2,3] Silva *et al.* also investigated the presence of TP53 gene mutations in plasma DNA of breast cancer patients.[22] Tumor and plasma DNA of 25 patients were studied by PCR-SSCP and direct sequencing, through exons 5, 6, 7 and 8, of TP53. They found that out of the six cases with mutations seen in tumor tissue DNA, three also have DNA mutations detected in the plasma. Mutations of the TP53 gene in plasma DNA of cancer patients may prove to be a useful new tool in the management of these patients.

In our recent study, we demonstrated that the status of p53 mutations in plasma DNA strongly correlates with clinical stage, tumor size, lymph node metastasis and estrogen receptor status.[19] Our study also shows that patients with plasma p53 mutations have a shorter probability of survival than those with the wild-type p53 gene. Patients with p53 mutations both in the primary tumor and the plasma DNA have the worst outcome. The results of our study indicate that the presence of p53 mutations both in the plasma DNA and primary tumor in breast cancer patients is significantly associated with highly malignant lesions and shorter survival. The status of p53 mutations in plasma DNA could be used as a marker of cancer recurrence or metastasis. Our study shows that over 97% of patients have undetectable p53 mutations in plasma DNA after complete excision of their primary tumors. However, in 22 patients with recurrence or metastasis, 13 patients who previously had p53 mutations in plasma DNA before surgery again develop detectable p53 mutations in plasma DNA. This would strongly suggest that the detection of p53 mutation in plasma DNA after primary treatment could be used as a marker of recurrence or distant metastasis.[19]

Table 1. Clinical characteristics and the concentration of plasma DNA in healthy women and breast cancer patients

	Breast cancer	Healthy
Number	126	92
Age range (median)	27–71 (47)	29–69 (51)
Plasma DNA concentration		
range (ng/ml)	21–329	0–52
mean (ng/ml)	211	21

Specific losses of heterozygosity (LOH) in plasma DNA of breast cancer

Chromosomal abnormalities are associated with the development of breast cancer, and widespread allelic loss or imbalance is frequently found in tumor tissues taken from patients with this disease.[23]

Several studies have demonstrated specific LOH in plasma DNA of breast cancer.[24–26] Using different markers, Chen *et al.* studied a total of 61 patients (divided into three groups) for the presence of LOH in plasma DNA.[25] Of their initial 27 patients, 35% of the tumor samples displayed LOH, whereas 15% had identical alterations in the corresponding plasma samples. In addition, the adjacent normal breast tissue of two patients also displayed LOH. In their second group of 11 patients, 45% of the tumors displayed LOH and 27% displayed identical plasma DNA alterations; one case displayed an identical LOH in adjacent non-tumor tissue. In their third series of 23 patients also studied with tetranucleotide repeats, 81% of the tumor samples displayed LOH, whereas 48% had LOH in the corresponding serum samples. The fact that small tumors (T1) of histoprognostic grade 1 or *in situ* carcinomas could present DNA alterations in the plasma/serum at an early stage, allied to the widely increased range of available microsatellite markers, suggests that plasma or serum DNA may become a useful diagnostic tool for early and potentially curable breast cancer.[25] Shaw *et al.* reported that breast cancer plasma DNA displayed frequent LOH (31.3%) and microsatellite instability (MI) (11.6%) supported by the same alteration in microdissected tumor DNA.[26] Most notably in their study, 10 of the 39 patients with primary breast cancer showed LOH (n=6).[26] The authors compared plasma tumor DNA, plasma and bone marrow QPCR, and blood and bone marrow immunocytochemistry in 32 of the patients with primary cancer. Of these, only one patient had immunocytochemically detectable carcinoma cells in the blood and three showed abnormally high levels of plasma CK19 mRNA. All four of these patients had plasma DNA alterations.[26]

Microsatellite alterations in plasma DNA of breast cancer

Microsatellites are short repetitive nucleotide sequences that, through mutation, can undergo either expansion or contraction. This novel mutational mechanism known as MI may play a role in carcinogenesis. A number of studies have shown MI in plasma DNA of breast cancer patients.[27–34] Rush *et al.* investigated the incidence of MI in a series of primary breast carcinoma surgical specimens.[27] They analyzed 46 pairs of normal and primary breast tumor samples at seven different microsatellite loci, five of which are located on chromosome 17. They found that 13 out of 46 tumors (28.2%) demonstrate MI. Five tumors (10.8%) were unstable at two or more loci; and of those, four (8.7%) were unstable at different loci on different chromosomes. Their findings indicate that MI is present in primary breast cancer populations and, although the mechanism of action has yet to be elucidated, may play a role in breast carcinogenesis.[27] Several polymorphic markers including D17S855, D17S654, D16S421, TH2, D10S197 and D9S161 have been found to have a high rate of alterations in breast cancer.[3] Silva *et al.* identified 56 cases (90%) with at least one molecular event in tumor DNA and 41 cases (66%) with a similar alteration in plasma DNA. Comparison of the clinicopathological parameters between patients with and without plasma DNA revealed significant differences in the axillary involvement, rate of invasive ductal carcinoma, high proliferative index, and the parameter comprised of lymph node metastases, histological grade II and peritumoral vessel involvement. A high proportion of breast cancer patients exhibited plasma DNA changes similar to tumor DNA and its presence correlated significantly with pathological parameters associated with a poor prognosis.

Size changes in microsatellite sequences have been detected in many types of cancer, but the influence of this form of genetic instability on disease progression remains unclear. Paulson *et al.* determined the incidence of MI in breast cancer by comparing PCR-amplified sequences from paraffin-embedded samples of normal and tumor tissue from affected individuals.[28] Their analysis showed that at least 30% of breast cancers exhibit MI. Of importance, MI correlated with indicators commonly associated with poor disease prognosis, including lymph node status, tumor size and advanced tumor stage. Individuals with MI^+ tumors also showed significantly reduced disease-free and overall survival. These data contrast with studies showing that MI correlates with improved prognosis in colon and gastric cancers. MI may promote disease progression and result in a poor prognosis in breast cancer. Also, a significant correlation was observed between MI and negative expression of both estrogen and progesterone receptors ($p<0.02$), indicating a possible relationship between specific genetic changes at these microsatellite regions and hormonal deregulation in the progression of breast cancer.[29]

Promoter hypermethylation and others

Promoter hypermethylation may be one of the mechanisms of the inactivation of some important

genes.[34–37] The $p16^{INK4a}$ is a D-type cyclin-dependent kinase (cdk) inhibitor that blocks the ability of cdk4 to interact with cyclin D_1 and stimulate the progression of eukaryotic cells through G_1 phase of the cell cycle.[31] This gene has been found to be inactivated in a large percentage of tumor cells and primary tumors. In Silva's report, hypermethylation of exon 1 of $p16^{INK4a}$ was examined in tumor and plasma DNA of a series of breast cancer patients. *De novo* methylation was observed in the tumors of eight patients (23%) and in plasma DNA in five (14%) of these eight patients. Their data show that *de novo* methylation of exon 1 of p16INK4a can be demonstrated in plasma DNA of breast cancer patients, which may provide additional evidence of the tumor-related origin of free plasma DNA in cancer patients.[35]

Hypermethylation of the BRCA1 promoter region also has been examined in breast cancer.[36] Esteller *et al.* examined methylation patterns of the BRCA1 promoter in breast cancer cell lines, xenografts and 215 primary breast and ovarian carcinomas by methylation-specific PCR. They found that the BRCA1 promoter was unmethylated in all normal tissues and cancer cell lines tested. However, BRCA1 promoter hypermethylation was present in two breast cancer xenografts, both of which had loss of the BRCA1 transcript. BRCA1 promoter hypermethylation was present in 11 (13%) of 84 unselected primary breast carcinomas. BRCA1 methylation was strikingly associated with the medullary and mucinous subtypes, which are over-represented in BRCA1 families. In a second series of 66 ductal breast tumors, 20% of tumors with LOH had BRCA1 hypermethylation, while 5% without LOH were methylated. Silencing of the BRCA1 gene by promoter hypermethylation occurs in primary breast and ovarian carcinomas, especially in the presence of LOH and in specific histopathologic subgroups. These findings support a role for this tumor suppressor gene in sporadic breast and ovarian tumorigenesis.

In addition to tumor-derived DNA, RNA has also been found circulating in the plasma of normal subjects and cancer patients.[38] In Chen's study, the expression pattern of human telomerase RNA subunits in plasma of human breast cancer patients was examined using RT-PCR.[38] They found that these RNA subunits were present in the serum of some patients with breast cancer. The presence of amplifiable RNA was confirmed in all tissue and serum samples using RT-PCR of glyceraldehyde-3-phosphate dehydrogenase RNA. Telomerase RNA template (hTR) was found in 17 of 18 tumors (94%) and five of 18 serum samples (28%). Telomerase reverse transcriptase protein (hTERT) was also detected in 17 of 18 tumors (94%) and in four of 16 available serum samples (25%). hTR and hTERT were undetectable in tissues and sera taken from two patients with benign disease and in the sera of 21 normal subjects. They concluded that RNA is detectable in the serum of breast cancer patients and that tumor-derived mRNA can be extracted and amplified using RT-PCR, even in patients with localized disease, which may have implications for cancer diagnosis and follow-up in the future.[38]

Conclusion

Plasma DNA with the detection of genetic abnormalities associated with specific cancers has produced some promising results. Detection of p53 mutations, specific LOH, MI and promoter hypermethylation in free circulating DNA could indicate the presence of breast cancer. Good concordance between alterations in the primary tumor and detection of the same alteration in circulation has been observed. Also, it is encouraging to note that DNA alterations have been detected in patients with small or even *in situ* lesions, indicating that circulating tumor DNA is shed early in the disease process. If 'universal' breast-specific DNA alterations can be identified, this approach may hold significant promise for early detection of breast cancer.

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