Patterns of loss of heterozygosity in breast carcinoma during neoadjuvant chemotherapy

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Abstract. There is evidence indicating that resistance to some chemotherapy drugs is related to enhanced repair of DNA lesions. Microsatellite instability (MSI) and loss of heterozy-gosity (LOH) reflect genetic instability and are associated with specific DNA repair pathways. Despite the strong implication of genetic instability in breast cancer its association with chemotherapy is unknown. Thus, we analyzed microsatellite alterations with 12 markers in locally advanced breast carcinomas in relation to neoadjuvant epirubicin-cyclophosphamide-containing chemotherapy (FEC-100) and compared it to a docetaxol-based (Tax-Epi) regimen. Samples were obtained before, during and after treatments. In pre-treated samples, MSI was detected only in 2 cases (7%) whereas LOH was found in 23 of the 34 (68%) carcinomas including 10 belonging to the FEC-100 group and 13 to Tax-Epi one. LOH frequency decreased from the first course of both regimens, but differences between the patterns of LOH during treatment were found. Persistent LOH was more frequent in FEC-100 group (71% vs. 41%) that was detected only in biopsies belonging to non-responder patients. Persistent LOH were clustered at particular loci located at regions containing common fragile sites (FHIT and FRA6E). Analysis of baseline LOH with 6 markers located at 3p indicates discontinuous patterns reflecting double-strand break (DSB) lesions. These results agree with a drug-dependent link between genetic instability and chemoresistance and show that FEC-100 treatment is associated with DSB accumulation manifested as LOH in tumor cells resistant to chemotherapy in breast carcinoma.

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Introduction

Breast cancer is characterized by genomic instability, which includes amplification of proto-oncogenes, loss of heterozygosity (LOH) and microsatellite instability (MSI). Normally, genomic stability is maintained through error-free DNA replication, post-replicative proof-reading, DNA repair, and recombinational events. The study of hereditary non-polyposis colorectal carcinoma (HNPCC) revealed that loss of mismatch repair (MMR) gene products leads to MSI. *In vitro* studies have shown significant associations between MSI and drug resistance (1,2). In addition, MSI was reported to have predictive value for survival benefit from 5-fluorouracil/levamisol adjuvant chemotherapy in colon carcinomas (3,4). However, such association has not been found in other studies (5,6).

LOH is now recognized as an invaluable tool for cancer diagnosis and prognostication, regardless of whether the corresponding target genes have been identified. Several studies have analyzed the effect of LOH on the response to chemotherapy. LOH at chromosomes 9p and 17p was reported to predict low response to 5-fluorouracil/cisplatin-based neoadjuvant treatment of head and neck squamous-cell carcinomas (7). Retention of heterozygosity at 17p or 18q was found to be associated with ability to benefit from adjuvant fluorouracil in colon cancer (3,8). Conversely, a prognostic benefit of LOH at 18q was found with a single marker in colon cancer (8). The presence of LOH in the serum of patients with metastatic melanoma was associated with a poorer response to induction biochemotherapy (9). In contrast, persistence of LOH at 9p21 was detected in advanced pre-malignant head and neck lesions with complete clinical and histological response after 6 and 12 months cisplatin-IFN-tocopherol treatment (10). In addition, LOH at 17p13.1 (p53 locus), as well as a high LOH frequency (estimated on different loci throughout the genome) have been associated with a better clinical response to cisplatin-based neoadjuvant chemotherapy of gastric carcinomas (11,12).

Growing evidence indicates that LOH results from DNA double strand break (DSB) lesions (13-16). Homologous recombination and non-homologous end-joining are the two

Table I. Microsatellite markers analyzed in this study.

Locus	Repeat	Location	Associated gene	Primers
BAT26	A	2p22	*hMSH2*	Kit linkage mapping set (applied)
D3S1300	CA	3p14.2	*FHIT (FRA3B)*	Kit linkage mapping set (applied)
D3S1514	GAAA	3p21-14.3	-	GGC AAC AGA GCA AGA TGC CCA GCC AGC AGA ATT ATG A
D3S1478	CA	3p21.3-21.2	-	GAT GAA ACT GTG ATA GCA CC CTG CCA GTA ATG TAA ATC TCC
D3S1029	CA	3p21.31-21.2	-	ATA CTC TGG ACC CAG ATT GAT TAC TAA TTC CCA AAT GGT TTA GGG GAG
D3S1612 -	CA	3p24-22	*hMLH1*	TCT TTT AGT CAG CAG TTA TGT C CCCATT AAG AAA TGT TAC TCT AC
D3S1244	CATT	3p24.2	*FA-D*	GTG CCC TTC CAG GAG TT AGT GAG GCA TCC ACT ACC
D6S264	CA	6q27-25.2	*FRA6E*	Kit linkage mapping set (applied)
D8S256	CA	8q24.13	-	GTT CAA GGG CTC AGG GTT CT CTT CCA CCT TTA GCC AAG GA
D10S197	CA	10p11.2	-	Kit linkage mapping set (applied)
TH01	TCAT	11p15.5	*Tyrosine Hydroxylase*	GTG GGC TGA AAA GCT CCC GAT TAT ATT CAA AGG GTA TCT GGG CTC TGG
D11S2179	CA	11q23	*ATM*	TAG GCA ATA CAG CAA GAC CCT G GCA CTG GAA TAC GAT TCT AGC AC
TP53	CA	17p13.1	*P53*	ACT GCC ACT CCT TGC CCC ATT C AGG GAT ACT ATT CAG CCC GAG GTG
TP53 Penta AGC	AAAAT	17p13.1	*P53*	ACT CCA GCC TGG GCA ATA AGA GCT ACA AAA CAT CCC CTA CCA AAC
D17S855	CA	17q21	*BRCA1*	GGA TGG CCT TTT AGA AAG TGG ACA CAG ACT TGT CCT ACT GCC
AR	CAG	Xq13	*Androg*en *Receptor*	TGG GGA GAA CCA TCC TCA CC TCC AGA ATC TGT TCC AGA GC

major pathways for repairing DNA DSB in mammalian cells. DSB accumulation and overactivated recombinational repair mechanisms in tumor cells lead to resistance to many DNA damaging agents (17-19). Among the mechanisms of chemotherapy cytotoxicity (for example anthracyclines), figure the induction of DSB via the generation of reactive oxygen species (20-22). Repair of DNA crosslinks caused by crosslinking agents such as alkylating chemotherapeutic drugs (for example cyclophosphamide) is achieved by these recombinational repair mechanisms (19). The use of drugs that specifically inhibit DNA repair pathways in combination with chemotherapy has also been proposed (17-19).

In breast cancer, both anthracyclines and cyclophosphamide were used for treatment, and despite the high implication of LOH in this lesion its association with chemotherapy is unknown. In this study, LOH profile patterns were analyzed in breast carcinomas in relation to neoadjuvant epirubicin-cyclophosphamide-containing (FEC-100) chemotherapy and compared to a docetaxol-based (Tax-Epi) regimen.

Materials and methods

Patients and tumors. We studied a population of 34 patients (age 18-65 years) with non-metastatic large tumor (T2, T3N0 or T3N1), unilateral, non-inflammatory, operable breast cancer requiring mastectomy (wishing to conserve the breast) and who were treated with neoadjuvant chemotherapy at the Centre Georges François Leclerc (Dijon, France) between 1999 and 2000. Among the 34 carcinomas analyzed, 18 belong to patients who received a FEC-100 regimen (6 courses every 21 days): 5-fluorouracil (500 mg/m^2), epirubicin (100 mg/m^2) and cyclophosphamide (500 mg/m^2). The other 16 carcinomas

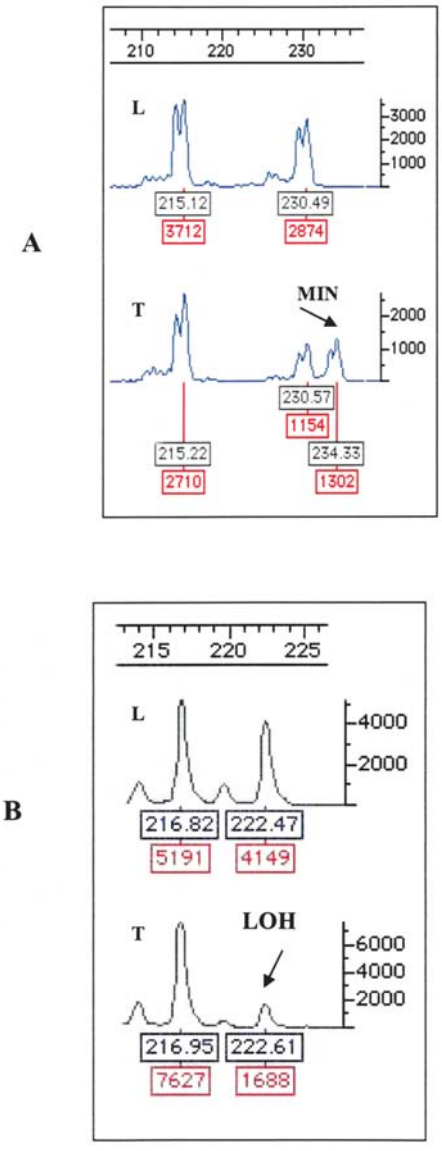

Figure 1. Representative microsatellite analysis in breast carcinoma (T) compared with their corresponding peripheral blood lymphocytes (L) MIN, microsatellite instability detected with D3S1514 marker. LOH, loss of heterozygosity obtained with AR marker.

belong to patients who received a Tax-Epi regimen (6 courses every 21 days): docetaxel (75 mg/m^2) and epirubicin (100 mg/m^2). Three biopsy specimens were analyzed for each patient corresponding respectively to 17, 15 and 12 before, during and after FEC-100 treatment, whereas 13, 16 and 12 samples were analyzed before, during and after Tax-Epi treatment. Thus, a total of 85 samples were analyzed for the two treatments. Histological control for tumor cell frequencies was evaluated for each sample. All tissue samples were frozen and stored in liquid nitrogen. Blood samples were obtained from each patient, and peripheral leukocytes were used as normal controls. This work was done with the approval of the local boards governing research on human subjects.

Response evaluation. Assessment of histological response (HR) in the surgical specimens was based on a classification proposed by Sataloff *et al* (23). This classification allows evaluating the extent of therapeutic effect in the primary tumor site and axillary lymph nodes. HR is graded as complete if total or near total therapeutic effect on the tumor and negative nodes is present. Carcinomas are classified as partially resistant to the treatment if >50% therapeutic effect in tumor and negative or positive nodes with therapeutic effect is present. Carcinomas are classified as resistant to the treatment if <50% therapeutic effect in tumor, whatever the node status.

DNA isolation and microsatellite analysis. DNA extraction was performed as described previously (24). Microsatellite markers were representative of mono-, di-, tri-, tetra- and pentanucleotide repeats localized on 10 chromosome arms including: 2p, 3p, 6q, 8q, 10p, 11p, 11q, 17p, 17 and Xq. Characterization of the microsatellite markers used is reported in Table I.

The PCR products were analyzed on an ABI prism 310 automated genetic analyzer (Applied Biosystems). The data collected were further analyzed with Genescan and Genotyper softwares. Peak heights were compared between cancer and normal cells as described previously (24).

Scoring of MSI or LOH. MSI was determined by the presence of novel alleles in the tumor comparing to the corresponding normal tissue. LOH was determined only in cases with no evidence of MIN. Allele ratio was calculated in informative cases using peak height for each normal and tumor sample and then the normal ratio was divided by the tumor ratio as follows N2:N1/T2:T1. N2 and T2 are the height values of the longer allele product peak for the normal and tumor samples, respectively, and N1 and T1 are the height values of the shorter allele product peak for the normal and tumor samples, respectively. LOH was scored when allele ratios were ≤0.5 or ≥2. Frequency of LOH for each locus was calculated by dividing the number of cases presenting LOH by the total number of informative cases. BAT26 is a non-informative marker and was therefore not evaluated for LOH. All suspect samples were analyzed twice by different PCR reactions.

Results

Microsatellite alterations. MSI was found in only 2 of the 34 (7%) carcinomas with the dinucleotide repeats D3S1514 and D10S197. MSI profiles consisted in one extra allele (Fig. 1A). No MSI was detected with the mononucleotide marker BAT26. Since a very low frequency of MSI was observed, no further analysis was made for this alteration.

LOH was found in 23 of the 34 (68%) carcinomas. The most frequently affected microsatellite markers in the pretherapeutic carcinomas was D3S1300 (32%), TH01 (27%) and TP53CA (25%). The most affected loci for FEC-100 group were D3S1300 (45%), TH01 (31%) and D3S1612 (23%), and for Tax-Epi, TP53 penta (38%), D11S2179 (36%) and D8S256 (33%). The LOH was generally accumulated within the same carcinoma. For example, in the FEC-100 group, 10 of the 18 (56%) patients exhibited LOH in at least one of their biopsy specimen, while no LOH was observed in the remaining 8 samples. In the Tax-Epi group, 13 of the 16 (80%) patients had LOH, and no LOH was observed in the remaining 3 samples. A representative analysis of LOH detection is illustrated in Fig. 1B.

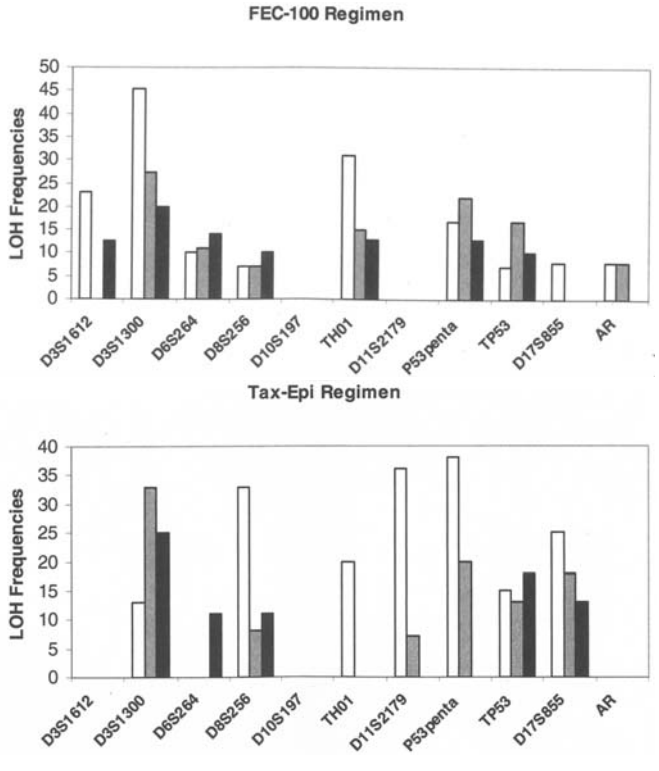

Figure 2. LOH frequencies detected before (white), during (grey) and after treatment (black) for each chemotherapy regimen.

LOH patterns during chemotherapy. The number of affected loci decreased during and after treatment for both regimens. Thus, overall LOH frequencies were 15, 9 and 7%, respectively before, during and after FEC-100 treatment, and a decrease from 12, to 6 to 5% was observed for Tax-Epi. However, there are differences between the patterns of LOH in the two groups (Fig. 2). For example, LOH at TH01, which persisted after FEC-100 treatment, disappeared from the first course of Tax-Epi, whereas LOH at D17S855 showed the opposite pattern.

Persistent LOH after chemotherapy. Although LOH frequency decreased during chemotherapy, persistent LOH after treatment was observed regardless of the chemotherapy regimen. For FEC-100 group, 5 of 7 cases harbored at least one persistent LOH (Table II: cases 1, 2, 3, 5 and 6). Similarly, for Tax-Epi group, 4 of 9 cases harbored at least one persistent LOH (Table III: cases 2, 5, 8 and 9). Thus, frequency of persistent LOH differed between the two groups (71% and 44%, respectively). In addition, persistent LOH in FEC group appeared closely related to the presence of viable tumor cells in post-treatment biopsy samples (Table II). In contrast, in Tax-Epi group, some LOH disappeared even in biopsies showing high fraction of viable tumor cells (cases 4, 6 and 7; Table III). Moreover, all 5 cases with persistent LOH after FEC-100 chemotherapy were non-responders to the treatment. In

Table II. LOH patterns in lesion biopsy specimens taken before, during and after FEC-100 chemotherapy.

Patient	Time of biopsy	D3S1612	D3S1300	D6S264	D8S256	D10S197	TH01	D11S2179	TP53 Penta	TP53	D17S855	AR	HC	HR
1	Baseline	❍	●S	●S	/	/	●L	❍	❍	❍	❍	/	ND	NR
	6 months	❍	●S	●S	/	/	❍	❍	❍	❍	❍	/	ND	
2	1 month	/	●S	●L	●S	/	/	❍	/	/	❍	●L	3	NR
	6 months	/	❍	●L	●S	/	/	❍	/	/	❍	❍	3	
3	Baseline	❍	●L	/	❍	❍	●S	/	●S	❍	❍	/	3	NR
	1 month	❍	●L	/	❍	❍	●S	/	●S	●S	❍	/	2	
	6 months	●S	❍	/	❍	❍	❍	/	●S	●S	❍	/	3	
4	Baseline	●L	/	❍	❍	❍	❍	❍	/	❍	❍	/	3	PR
	1 month	❍	/	❍	❍	❍	❍	❍	/	❍	❍	/	2	
	6 months	❍	/	❍	❍	❍	❍	❍	/	❍	❍	/	0	
5	Baseline	/	❍	/	/	/	❍	❍	/	❍	/	❍	3	NR
	1 month	/	❍	/	/	/	❍	❍	/	❍	/	❍	3	
	6 months	/	❍	/	/	/	●S	❍	/	❍	/	❍	3	
6	Baseline	❍	●L	/	❍	❍	❍	/	/	❍	/	❍	3	NR
	1 month	❍	●L	/	❍	❍	❍	/	/	●S	/	❍	3	
	6 months	❍	●L	/	❍	❍	❍	/	/	❍	/	❍	1	
7	Baseline	●S	/	●S	❍	/	❍	❍	●L	❍	❍	●S	2	CR
	1 month	❍	/	❍	❍	/	●L	❍	●L	❍	❍	❍	3	
	6 months	❍	/	❍	❍	/	❍	❍	❍	❍	❍	❍	0	

●, LOH at the shorter (S) or the longer (L) allele. ❍, retention of two alleles; /, not informative. ND, not determined. HC, histological control of the presence of tumor cells graded as ≥80% (3), 50-80% (2) or ≤50% (1). HR, histological response corresponding to complete (CR), partial (PR) or no response (NR).

Table III. LOH patterns in lesion biopsy specimens taken before, during and after Tax-Epi chemotherapy.

Patient	Time of biopsy	D3S1612	D3S1300	D6S264	D8S256	D10S197	TH01	D11S2179	TP53 Penta	TP53	D17S855	AR	HC	HR
1	1 month	/	●L	❍	❍	❍	❍	❍	/	❍	❍	❍	0	NR
	6 months	/	❍	❍	❍	❍	❍	❍	/	❍	❍	❍	1	
2	Baseline	/	❍	ND	ND	ND	/	ND	ND	❍	ND	❍	3	PR
	1 month	/	❍	❍	●S	❍	/	❍	❍	❍	❍	❍	3	
	6 months	/	❍	●L	●S	❍	/	❍	❍	❍	❍	❍	3	
3	Baseline	ND	❍	ND	/	❍	/	❍	❍	❍	❍	ND	3	CR
	1 month	❍	●S	❍	/	❍	/	❍	❍	❍	❍	❍	2	
	6 months	❍	❍	❍	/	❍	/	❍	❍	❍	❍	❍	1	
4	Baseline	❍	/	❍	❍	❍	/	❍	❍	❍	❍	❍	3	NR
	1 month	❍	/	❍	❍	❍	/	●L	●S	❍	❍	❍	3	
	6 months	❍	/	❍	❍	❍	/	❍	❍	❍	❍	❍	3	
5	Baseline	❍	●L	❍	/	❍	❍	●S	●L	●S	/	❍	2	NR
	1 month	❍	❍	❍	/	❍	❍	❍	●L	❍	/	❍	3	
	6 months	❍	●L	❍	/	❍	❍	❍	❍	●S	/	❍	ND	
6	Baseline	/	/	ND	ND	/	❍	❍	●S	❍	/	❍	0	NR
	1 month	/	/	❍	❍	/	❍	❍	❍	❍	/	❍	3	
	6 months	/	/	❍	❍	/	❍	❍	❍	❍	/	❍	3	
7	Baseline	❍	❍	❍	❍	/	❍	●L	❍	❍	●S	❍	3	NR
	1 month	❍	●L	❍	❍	/	❍	❍	❍	❍	❍	❍	3	
	6 months	❍	❍	❍	❍	/	❍	❍	❍	❍	❍	❍	3	
8	Baseline	❍	❍	❍	❍	/	❍	❍	/	●L	●L	❍	3	NR
	1 month	❍	❍	❍	❍	/	❍	❍	/	●L	●L	❍	3	
	6 months	❍	❍	❍	❍	/	❍	❍	/	●L	●L	❍	3	
9	Baseline	/	❍	/	❍	❍	❍	❍	/	❍	❍	❍	3	PR
	1 month	/	❍	/	❍	❍	❍	❍	/	●L	●S	❍	2	
	6 months	/	●L	/	❍	❍	❍	❍	/	❍	❍	❍	2	

●, LOH at the shorter (S) or the longer (L) allele. ❍, retention of two alleles; /, not informative. ND, not determined. HC, histological control of the tumor cells presence graded as ≥80% (3), 50-80% (2) or ≤50% (1). HR, histological response corresponding to complete (CR), partial (PR) or no response (NR).

Tax-Epi group, LOH pattern seemed independent from histological response status. Interestingly, persistent LOH was clustered at particular loci located at chromosomes 3p14.2 and 6q27.

3p region analysis. Discontinuous pattern of LOH was frequently observed in breast carcinomas and was attributed to interstitial deletion resulting from DSB formation (16,25). To determine whether LOH at 3p observed in our study could also have a discontinuous pattern, we analyzed 4 additional microsatellite markers located in this region (Fig. 3). The results showed that the majority of cases had discontinuous LOH with regions of 3p loss separated by intervening regions of retention of heretozygosity (example, cases 7, 8, 16, 22) or LOH affected alternatively the shorter or the longer allele (example, nos. 2, 4, 9, 10, 11, 17). Only one case (no. 5) exhibited an eventual loss of the entire region showing LOH for the longer allele with the 5 markers. In other cases (nos. 3, 18, 19), LOH affected the more telomeric loci analyzed in this study and thus the patterns could not be determined.

Discussion

Recombinational repair is an essential mechanism for the cell in order to protect its DNA from common sources of DSB, such as DNA replication and endogenous reactive oxygen species (14,26). LOH constitutes a hallmark of DSB (13,25), and represent the most frequent alteration detected in breast carcinoma. Our results show, for the first time, the existence of a link between LOH and chemotherapy-drugs used in breast carcinoma.

All microsatellite loci analyzed have been reported to be frequently altered in breast carcinomas except for BAT26. This is a good marker of high microsatellite instability in colon cancer and was included as an indicator of eventual

µsat/Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
D3S1244	O	O	/	/	L	/	O	S	/	/	S	/	O	O	/	S
D3S1612	nd	O	O	O	L	L	O	S	O	S	L	L	O	L	L	O
D3S1029	nd	S	O	/	L	/	O	O	L	L	L	S	/	O	O	L
D3S1478	nd	L	O	L	L	O	O	L	O	O	S	S	S	/	O	L
D3S1514	L	S	O	L	L	O	S	/	S	S	L	L	/	L	S	O
D3S1300	nd	L	L	S	/	O	O	O	O	L	S	L	L	/	S	O

µsat/Patient	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
D3S1244	L	L	L	L	O	O	/	/	/	/	O	O	L	S	L	L	/
D3S1612	S	O	O	/	O	O	nd	O	O	/	O	/	/	/	/	/	S
D3S1029	L	O	/	O	O	O	L	O	/	O	S	O	L	O	nd	S	O
D3S1478	O	O	O	O	O	S	O	O	/	S	O	O	O	O	nd	S	/
D3S1514	L	O	O	/	O	O	O	S	S	O	O	O	O	S	L	L	S
D3S1300	/	O	/	/	S	nd	nd	nd	nd	nd	O	L	O	L	nd	/	O

Figure 3. Chromosome 3p analysis of pre-treated breast carcinomas using 6 microsatellite markers. S, LOH at the shorter allele; L, LOH at the longer allele. ❍, retention of two alleles; /, not informative; nd, not determined.

unstable cases. In our series, MSI is a less frequent event than LOH, and corresponds to the low frequency generally detected in breast cancer which did not fulfill the established criteria to be classified as mismatch repair deficient (25,27-29). Conversely, LOH was found in 68% of cases, and this prevalence is in agreement with other studies (25,29,30). In spite of the role that MMR genes may have in breast cancer development, MSI corresponds to a background noise of instability, of as yet unknown origin, also present in other human cancers such as gastric and head and neck squamous-cell carcinomas (31). In contrast, high frequencies of LOH in individual breast carcinomas have been shown to occur non-randomly at certain chromosome loci, suggesting the involvement of specific region and regulatory genes (32).

During each treatment analyzed, LOH frequency decreased since the first course of chemotherapy. This decrease could be the result of decreasing tumor clones harboring these alterations and not a reflection of the absence of viable tumor as confirmed by the histological controls. The identical alleles affected suggested that the same tumor clones were maintained during treatments.

However, there are differences between the patterns of LOH in the two groups treated with different agents. In particular, persistent LOH appeared more frequently in the FEC-100 group. Additionally, it was detected only in post-treatment biopsies with viable tumor and non response to treatment, suggesting that persistent LOH after treatment could be a factor of poor prognosis following FEC-100 chemotherapy. The results indicate that clones harboring these genomic abnormalities were resistant to FEC-100, and that epirubicin-cyclophosphamide either played a role in selecting these particular clones or in inducing chromosomal instability.

Persistent LOH was clustered at particular loci located at chromosomes 3p14.2 (D3S1300) and 6q27-25 (D6S264), the region contain the *FHIT* and *FRA6E* genes, respectively. LOH at 3p is one of the frequent alterations observed in breast lesions. Located at 3p14.2, the *FHIT* gene encodes a protein involved in the cellular damage response, cell cycle and genetic stability regulation (33) and encom-passing the FRA3B common fragile site (34). LOH at 6q25.2-27 was also frequently observed in breast carcinoma. Interestingly, this locus contains the FRA6E common fragile site. It has been shown that common fragile sites exhibit increased fragility in cancer cells subjected to chemotherapy (35). Another study has also shown that DSBs are formed at these sites and that their repair was regulated by recombinational mechanisms (36).

Consistent with the above, persistent LOH in FEC-100 tumors reflects DSB accumulation and over-activated homologous recombination repair. The accumulation of DSB by Tax-Epi could be attenuated by the presence of docetaxol. Taken together, our results show that FEC-100 treatment leads to the accumulation of DSB manifested as LOH in tumor cells resistant to chemotherapy. Thus, these findings would be of great utility in the management of locally advanced breast carcinomas in order to estimate the efficacy of currently used chemotherapeutic treatments.

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