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Allelic Loss Detection in Inflammatory Breast Cancer: Improvement with Laser Microdissection

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SUMMARY: Solid tumors are composed not only of tumor cells but also of stromal nonneoplastic cells. In whole tumor samples, stromal cells retaining their alleles may therefore obscure detection of loss of heterozygosity (LOH) in tumor cells. An increasing number of studies have used laser-assisted tissue microdissection to improve LOH detection, but the real gain in sensitivity has been poorly quantified. We studied a group of 16 inflammatory breast carcinomas that were submitted to both standard DNA extraction from frozen whole tumor samples and laser microdissection performed on paraffin-embedded tumor samples. Using PCR with fluorescence-labeled primers, we comparatively analyzed ten polymorphic markers with both sources of DNA. With the LOH detection threshold set at −25%, we showed that 25 LOHs could not be diagnosed with whole tumor samples out of 73 LOHs positively diagnosed in microdissected samples (34%). With the LOH detection threshold set at −50%, the respective figures were 39 LOHs not diagnosed out of 55 LOHs (71%). Measuring the intensity of the allelic decrease, we showed that the mean decrease of the lost allele is −34% with whole tumor samples and −67% with microdissected samples. The increase in sensitivity of LOH detection with microdissection is associated with the density of stromal cells. This strong improvement in LOH detection in this aggressive type of breast cancer indicates that many other molecular studies performed on heterogeneous solid tumors may benefit from a first step of laser microdissection. (*Lab Invest 2001, 81:1397–1402*).

Loss of heterozygosity (LOH) is common in human solid tumors and allows the expressivity of recessive loss-of-function mutations in tumor suppressor genes (Lasko et al, 1991). The detection of recurrent LOH in a genomic region is considered to be critical evidence for the localization of tumor suppressor genes. Because detection of LOH is based on the comparison of tumor cells and corresponding normal cells for identification of tumor cell–specific gene deletions, it is important to obtain a collection of pure tumor cells to provide the homogeneous material required for a reliable analysis. However, most solid cancers contain, not only tumor cells, but also stromal cells (eg, fibroblasts, lymphocytes, and endothelial cells) or residual nontumor cells (eg, adipocytes and normal residual ducts). These cells usually have a normal genome and therefore may obscure losses of genetic material in tumor cells when they are too numerous in whole tumor samples. To overcome this problem, several tumor cell–enrichment protocols have been developed, such as flow cytometry based on an abnormal DNA index (Abeln et al, 1994) or tissue microdissection (TM) (Bertheau et al, 1998; Sirivatanauksorn et al, 1999). Initially performed manually (Zhuang et al, 1995) or with a micromanipulator (Going and Lamb, 1996; Küppers et al, 1994), TM has evolved to laser-assisted microdissection systems that can efficiently sample various amounts of cells (Böhm et al, 1997; Emmert-Buck et al, 1996; Fend and Raffeld, 2000; Schütze et al, 1997). These systems are much easier to handle than hydraulic micromanipulators and much more precise than manual microdissection.

Several reports have stated that the use of TM is of great benefit to the detection of LOH (Fujii et al, 1996; Giercksky et al, 1997; Shen et al, 2000; Speiser et al, 1996). Only one study (Giercksky et al, 1997) has quantitatively estimated the gain in sensitivity obtained with TM. That report compared genetic changes in frozen biopsies and in manually microdissected archival material from the same colorectal liver metastases. The authors found a 54% increase in the sensitivity of detection of genetic alterations with microdissection. Thus, we decided to make a similar comparison in a group of 16 inflammatory breast carcinomas that were submitted to both standard DNA extraction from frozen whole tumor tissue and microdissection of tumor tissue embedded in paraffin. In contrast to Giercksky et al (1997), we used a laser-based tissue microdissection system, and we detected LOHs with fluorescence-labeled primers that

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allow precise quantitation of allelic decrease. Our results showed that at least one-third of LOHs present in breast tumors remain undiagnosed if the tissue has not been microdissected. Furthermore, we estimated that the sensitivity of LOH detection with TM is nearly double that without TM, especially in tumors with highly cellular stroma.

Results

Sixteen tumors were studied at 10 loci. Two methods have been compared: method 1 used whole tumor tissue, whereas method 2 used microdissected tissue (Fig. 1). For both methods, two LOH detection thresholds were tested.

Figures 2 and 3 display the results. No PCR product was obtained in five tests with microdissected samples. With both methods 1 and 2, 37 tests (24%) were uninformative and 40 tests (26%) showed retention of heterozygosity. We found no cases of microsatellite instability.

Detection Threshold Set at −25%

With the detection threshold set at −25% (Fig. 2), 16 tests showed no LOH with method 1, but did show LOH with method 2 (one example is given in Fig. 4). Nine LOHs were uncertain with method 1, yet were certain using method 2. Five uncertain LOHs remained uncertain with both methods. Forty-eight tests showed LOH with both methods. Therefore 25 LOHs were not diagnosed with method 1 out of 73 LOHs diagnosed with method 2 (34% nonrecognized LOH). In the 73 tests that showed LOH with method 2, the mean decrease of the lost allele was −34% with method 1 and was −67% with method 2, indicating that method 2 is nearly twice as sensitive as method 1 to the detection of a decrease in one allele.

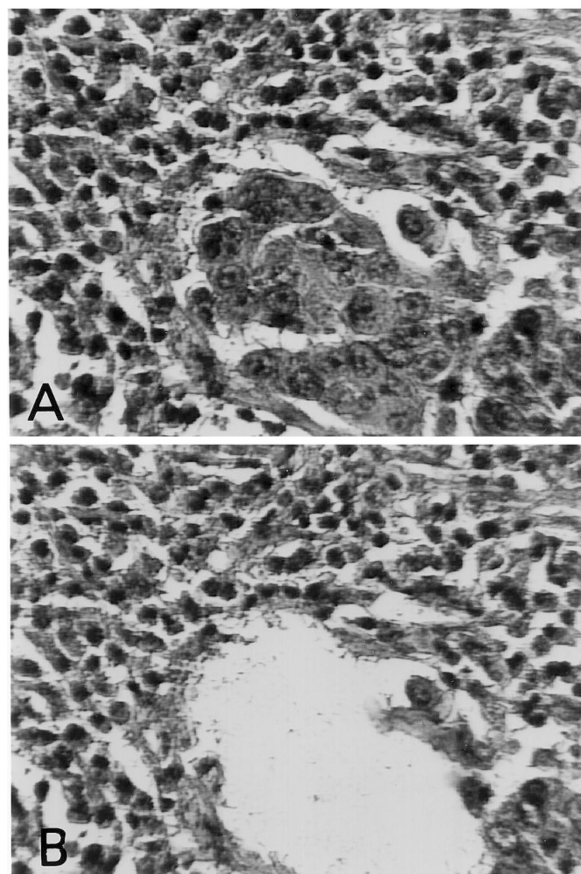

Figure 1.
Laser-assisted microdissection of breast carcinoma cells (Patient 14) on hematoxylin-eosin (H&E) paraffin section, without cover slide. A, Before microdissection, a small lobule of cancer cells is surrounded by numerous lymphocytes. B, After microdissection, only cancer cells have been removed.

Detection Threshold Set at −50%

With the detection threshold set at −50% (Fig. 3), six tests showed no LOH with method 1, but did show LOH with method 2. Thirty-three LOHs were uncertain with method 1, yet were certain with method 2. Ten uncertain LOHs remained uncertain with both methods. Sixteen tests showed LOH with both methods. Therefore, with the threshold at −50%, 39 LOHs were not diagnosed with method 1 out of 55 LOHs diagnosed with method 2 (71% nonrecognized LOH).

LOH Detection and Stromal Cellularity

The increase in sensitivity of LOH detection with method 2 is associated with the density of stromal cells. For the nine tumors with a poorly cellular stroma, the mean decrease of the allele was −40% with method 1 and −66% with method 2 (for 41 LOHs). For the five tumors with a highly cellular stroma, the figures were −23% and −67%, respectively.

Interestingly, five cases showed a greater decrease in alleles with method 1 than with method 2. For example, with Patient 11, the allele for p53CA decreased by 59% with method 1 and by 48% with method 2.

Discussion

Inflammatory breast cancer (IBC) is a very aggressive subtype of breast carcinoma that so far has been poorly characterized biologically. Inflammatory breast cancers are clinically defined by the presence of signs suggesting inflammation, such as breast redness, edema, and pain. Histologically however, IBC are not significantly different from noninflammatory breast cancers, except that they often contain dermal lymphatic emboli. It is crucial to find prognostic or predictive criteria for these tumors, which are treated by induction chemotherapy followed by surgery. We randomly selected 16 cases in a large population of IBC currently under investigation for a genome-wide search for specific LOHs.

Ten microsatellite markers were selected using two criteria. First, the frequency of allelic losses at each locus had to be previously described in sporadic breast cancers, and second, the PCR product length had to be less than 250 bp. PCR products longer than 250 bp are too difficult to obtain with microdissected, paraffin-embedded, formalin-fixed tissue. Despite

patient number	D3S1573 method 1	D3S1573 method 2	D7S490 method 1	D7S490 method 2	D8S261 method 1	D8S261 method 2	D8S1820 method 1	D8S1820 method 2	D11S860 method 1	D11S860 method 2	D11S1356 method 1	D11S1356 method 2	D13S171 method 1	D13S171 method 2	D16S496 method 1	D16S496 method 2	D17S855 method 1	D17S855 method 2	p53CA method 1	p53CA method 2	stromal cells density
1	?												?								1
2									?	?											1
3																	?				1
4			?										?	?							1
5																npp					1
6																					1
7																					1
8											?	?									1
9								npp											?	?	1
10																	?		?		2
11										npp											2
12	?												?	npp							3
13													?								3
14																			?		3
15											?	npp									3
16			?	?																	3

Figure 2.

Results in all 16 patients tested for 10 loci with loss of heterozygosity (LOH) threshold at −25%. Method 1: with whole tumor tissue; method 2: with microdissected tissue. Stromal cell density is scored from 1 to 3 (see "Materials and Methods"). ■, LOH (peak decrease more than 25%); [?], uncertain LOH (decrease between 10% and 25%); □, retained (peak decrease less than 10%); ▩, uninformative cases; npp, no PCR product.

patient number	D3S1573 method 1	D3S1573 method 2	D7S490 method 1	D7S490 method 2	D8S261 method 1	D8S261 method 2	D8S1820 method 1	D8S1820 method 2	D11S860 method 1	D11S860 method 2	D11S1356 method 1	D11S1356 method 2	D13S171 method 1	D13S171 method 2	D16S496 method 1	D16S496 method 2	D17S855 method 1	D17S855 method 2	p53CA method 1	p53CA method 2	stromal cells density
1	?						?		?		?		?	?		?					1
2	?								?	?	?						?		?		1
3																	?				1
4			?	?			?	?			?	?	?	?							1
5					?		?							?	?	npp			?		1
6	?													?					?		1
7	?											?							?		1
8		?									?	?						?			1
9							?	npp											?	?	1
10		?															?		?		2
11								?	?	npp			?							?	2
12	?									?			?	npp			?		?		3
13	?								?		?		?								3
14				?			?				?		?				?	?	?		3
15	?		?								?	npp					?				3
16			?	?												?				?	3

Figure 3.

Results in all 16 patients tested for 10 loci with LOH threshold at −50%. Method 1: with whole tumor tissue; method 2: with microdissected tissue. Stromal cell density is scored from 1 to 3 (see "Materials and Methods"). ■, LOH (peak decrease more than 50%); [?], uncertain LOH (decrease between 10% and 50%); □, retained (peak decrease less than 10%); ▩, uninformative cases; npp, no PCR product.

these precautions, five tests with microdissected samples did not give any PCR product (Figs. 2 and 3) and were excluded from the analysis. Frozen tissues are not always available in routine practice, and therefore, we preferred using paraffin sections.

A stronger difference between the two methods might have been noted if the whole tumor tissue method (method 1) had been performed with frozen tissue not histologically controlled. This would have allowed us to study cases with only a few tumor cells or cases with much necrosis.

Tissue sectioning results in nuclear truncation, thus affecting any calculated DNA yield. For the microdissection method (method 2), we therefore took special

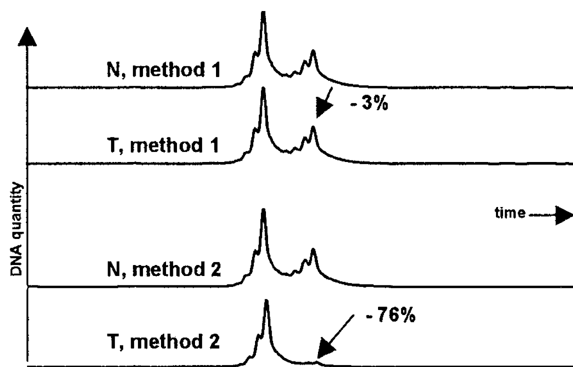

Figure 4.
Patient 10, locus D8S1820. The decrease of the right allele in the tumor compared with normal is −3% with DNA extracted from whole tissue (method 1) and −76% with microdissected tissue (method 2). N, method 1, normal sample with method 1 (see "Materials and Methods"); T, method 1, tumor sample with method 1; N, method 2, normal sample with method 2 (see "Materials and Methods"); T, method 2, tumor sample with method 2.

care to sample enough cells (nearly 500 cell profiles for each PCR) to avoid artificial allelic losses ("allelic dropout ").

The detection threshold for LOH assessment varies greatly among studies (Amari et al, 1999; Fujii et al, 1996; Kerangueven et al, 1997; Marsh and Varley, 1998). We decided to use two different threshold values and were able to show that the sensitivity gain with microdissection was higher with the −50% threshold. This is because most LOHs diagnosed with microdissection show peak decreases beyond −50%. However, only 55 LOHs could be diagnosed with the −50% threshold, compared with 73 LOHs with the −25% threshold.

It is likely that TM increases the sensitivity of LOH detection, not only in inflammatory breast cancer, but also in most other types of solid tumors. However, the gain in sensitivity is probably higher in breast cancer than in other cancers because breast cancer is more histologically heterogeneous, consisting of infiltrating tumor cells, noninfiltrating tumor cells (intraductal carcinoma, lobular carcinoma in situ), stromal cells, adipocytes, and residual epithelial cells.

It is interesting to ask why microdissection, especially with a laser system, does not systematically give −100% allelic decrease in cases of LOH. One possible explanation is that, even with a very careful use of a laser system, it is likely that a few stromal cells, perhaps very closely associated with tumor cells, will be sampled along with tumor cells. A second reason is intratumoral heterogeneity. It is now well known that cancer is a juxtaposition of subclones (Fey and Tobler, 1996) and that LOH distribution among tumor cells is heterogeneous (Chen et al, 1992; Hugel and Wernert, 1999; Yatabe et al, 2000). Our study supports the notion that some LOHs are not distributed homogeneously in tumor cells. This is because, in several tests, method 2 (with microdissection) showed smaller allelic decrease than method 1 (without microdissection). Also, cells microdissected in a single area of the tumor may contain proportionally fewer deletions than the whole tumor cell population. In our study, the small size of the biopsy samples (usually less than 1 cm) did not allow us to microdissect several tumor areas.

Our results clearly show that the gain in sensitivity is much greater in tumors with dense stroma because of the low sensitivity of method 1 in these tumors. However, the mean allelic decrease obtained with TM is not dependent on the cellularity of the stroma (−66% for tumors with poorly cellular stroma versus −67% for tumors with highly cellular stroma). Our results demonstrate that TM is precise enough to allow enrichment in tumor cells whatever the number of inflammatory cells.

Gains in sensitivity with TM are important for losses of genetic material, but TM also can improve the detection of mutations and amplifications (Lehmann et al, 2000; Pappalardo et al, 1998). TM also allows better assessment of gene expression (Specht et al, 2001) and better protein analysis (Emmert-Buck et al, 2000) in heterogeneous tumor tissues. Laser-assisted microdissection is likely to have a profound impact on molecular pathology and may soon be a prerequisite for many molecular studies that benefit from the pure cell populations.

Materials and Methods

Patients and Tissues

From 1993 to 1998, 72 patients were referred to Hospital Saint-Louis for inflammatory breast cancer. All these patients underwent frozen section examination at the time of initial diagnostic biopsy. Ten subsequent frozen sections (10 μm thick) were pooled in DNA extraction buffer, and the remaining tissue was fixed in AFA (Carlo Erba, Rodano, Italy), a mix of 2% formalin 40% (v/v), 5% acetic acid (v/v), 75% ethanol (v/v), and 18% water (v/v) and then embedded in paraffin for histopathological diagnosis. For our study, we randomly selected 16 patients in that population. All 16 tumors were infiltrating ductal carcinomas; 5 were Grade 2 and 11 were Grade 3 (Elston, 1987).

Stromal Cellular Density

On the tissue block from which frozen sections had been made for DNA extraction, the density of stromal cells (mostly small lymphocytes) was evaluated semiquantitatively on one H&E section according to the following criteria: 1 = less than 10% stromal cells, 2 = 10% to 50% stromal cells, and 3 = over 50% stromal cells. The stroma was scored "1" in 9 cases, "2" in 2 cases, and " 3" in 5 cases (Figs. 2 and 3).

DNA Extraction

Frozen tumor sections were immersed in a buffer containing 8 M urea, 0.3 M NaCl, 10 mM EDTA, 2% SDS, and 10 mM Tris-HCl (pH 7.5) and then submitted to phenol chloroform DNA extraction. Control normal DNA was prepared from peripheral blood as previously described (Muniz et al, 1994). Tumor DNA and

normal DNA were stored in 1 mM EDTA and 10 mM Tris-HCl (pH 7.5) at 4° C until further use.

Tissue Microdissection

Six-micrometer-thick paraffin sections were spread on nonpretreated glass slides and stained with H&E (Fig. 1). Tissue microdissection was performed with the laser microbeam microdissection system (PALM, Bernried, Germany) (Schütze et al, 1997). Briefly, a 337 nm UV-laser is used to "catapult" small tissue fragments directly into the cap of a sample tube without any mechanical contact. We used no membrane on the slide. For each tumor, at least 5000 infiltrating tumor cells microdissected in a single area of the section and 5000 nontumor cells (lymphocytes, adipocytes, and normal breast epithelial cells) were catapulted in separate vials containing 30 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5% Tween20, 0.2 mg/ml proteinase K). Cells were then incubated overnight at 37° C and proteinase K was inactivated by heating at 95° C for ten minutes. No further DNA extraction was performed.

PCR

PCRs were performed in 25 μl final volume with either 5 ng of extracted DNA (method 1) or 3 μl of lysed microdissected cells (method 2) corresponding to nearly 500 cell profiles. Ten polymorphic microsatellite markers were used in this study. Primers for PCR amplification of the following markers were designed based on the nucleotide sequences obtained from Internet databases (http://www.gdb.org, http://www3.ncbi.nlm.nih.gov): D3S1573, D7S490, D8S261, D8S1820, D11S860, D11S1356, D13S171, D16S496, and D17S855. Another marker, p53CA, is a dinucleotide repeat at the *p53* locus (Jones and Nakamura, 1992).

The PCR mix contained 1U *Taq* Gold (Applied Biosystems, Foster City, California), 2.5 to 4 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM Cy5-labeled primers, and 0.2 μM nonlabeled primers. Thirty-five cycles were performed.

LOH Analysis

We used an automated DNA analysis system, ALFexpress II (Amersham Pharmacia Biotech, Uppsala, Sweden), that separates fluorescently labeled DNA fragments by electrophoresis. The detection range is 50 attomol to 45 femtomol DNA. PCR products were run on a 0.3-mm-thick UV-polymerized polyacrylamide gel (Reprogel, Amersham Pharmacia Biotech). For automated allele quantification, we used the software AlleleLocator 1.03 (Amersham Pharmacia Biotech). The intensity of fluorescence for each peak, and therefore for each allele, is directly computed by the system. We compared the intensity of fluorescence peaks between blood DNA and whole tumor DNA (method 1) and between microdissected nontumor cells and microdissected tumor cells (method 2). We used two different thresholds for the detection of LOH. With the −25% threshold, LOH was considered certain when one allele was decreased by at least 25% as compared to the normal profile. Decreases ranging from −10% to −25% were classified "uncertain LOH." Decreases of less than 10% indicated "retention of heterozygosity." With the −50% threshold, LOH was considered certain when one allele was decreased by at least 50% compared with the normal profile, and decreases ranging from −10% to −50% were classified "uncertain LOH."

All PCRs with "certain LOH" or "uncertain LOH" were done twice. PCRs with "retention of heterozygosity" or with homozygosity were done only once.

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