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Loss of heterozygosity in tumor tissue in hormonal receptor genes is associated with poor prognostic criteria in breast cancer

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The estrogen receptors (ESRα and β) and the androgen receptor (AR) mediate genomic and non-genomic effects on breast tumor growth and proliferation. We analyzed 101 breast cancer patients for allelic loss in microsatellites located in regulatory regions of the *ESRs* and *AR* genes in breast cancer tumors. The loss of heterozygosity (LOH) at these loci was found in 36.2% of tumor tissues (ductal carcinoma cases), for 19% of cases at the *ESRα* locus, for 16% at the *ESRβ* locus, and for 10% at the *AR* locus. The LOH in at least one of the two *ESR* loci was correlated to poor prognosis criteria: ESR-negative status ($P = 0.007$), PR-negative status ($P = 0.003$), high Scarff-Bloom-Richardson (SBR) grade ($P = 0.0007$), high MIB-1 proliferation index ($P = 0.02$), and diminished apoptosis potential (TP53-positive status, $P = 0.018$). When *AR* was also considered, the LOH in at least one of the three loci was associated with ESR-negative status ($P = 0.036$), PR-negative status ($P = 0.027$), high SBR grade ($P = 0.005$), high mitotic index ($P = 0.0002$), TP53-positive status ($P = 0.029$), and proliferating index (high MIB-1, $P = 0.03$). Allelic loss was observed in 26% of normal tissue adjacent to tumor with LOH at the *ESRα* locus and in 7.1% of tumors with LOH at the *ESRβ* locus. The LOH in tumor tissue in the regulatory regions of *ESRα*, *ESRβ*, and *AR* genes has potentially synergistic effects on tumor proliferation, histological aggressiveness, down-regulation of *ESRα* and progesterone receptor (*PR*) genes, and is an early genetic alteration in cancer that is possibly involved in passage to estrogen independence.

Keywords Tandem repeats, allele length polymorphism, estrogen-receptor down-regulation, genetic marker

Among breast cancer risk factors, the hormonal exposure remains one of the highest. In fact, estrogens and androgens are known to have significant effects on breast tumorigenesis (1,2), which is mediated by their cognate receptors—the estrogen receptors α and β (ESRα, ESRβ) and the androgen receptor (AR), respectively—acting as transcription factors with downstream effects on the cell cycle, proliferation, or apoptosis of breast cells. Moreover, it has been clearly demonstrated that ESRα expression is one of the very few biomarkers of prognosis in breast cancer and is a predictor for a good response to endocrine therapy (3), whereas diminished ESRβ expression is predictive of tamoxifen resistance (4,5). The AR expression, independently of ESR expression, is correlated to prognostic factors, such as tumor grade, and has good prognostic value in univariate analysis of ESR-negative tumors (6). However, only two thirds of tumors express ESRα (3), and the heterogeneity in hormonal receptor expression has not been totally explained.

Several authors have demonstrated that in the complex gene regulation network, gene expression is influenced by genetic alterations, such as allelic loss. In the case of the *EGFR* gene, microdeletions of part or the total sequence of microsatellite repeats (CA) in intron 1 affect the *EGFR* expression in breast cancer tissue: EGFR expression was

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higher when the longer CAn allele was lost, compared with loss of the short allele (7). Recently, Wang et al. genotyped a CAn sequence located in the *Cyr61* promoter using hepatocellular carcinoma tissues. They found instability (loss of heterozygosity (LOH) or somatic mosaicism) in this specific locus in 32% of carcinomas, whereas other microsatellites were not affected (8). In addition, down-regulation of Cyr61 expression in tumor samples was observed, even if it was not to a great extent.

More evidence on the functional significance of CAn repeats in regulating gene expression comes from in vitro experiments. Promoter reporter assays demonstrated that longer CAn sequences in the gene promoter down-regulated the expression of *Cyr6* (8), or modified the expression of *EGFR* (9), *MMP 9* (10), *IFNγ* (11), and housekeeping genes (12). Finally, evidence also exists that intronic CAn repeats, particularly in the first intron, may play a role in stimulation of recombination (13) and splicing (14).

Genetic loss occurs in the context of genomic instability, which is a hallmark of cancer. It leads to various alterations such as LOH, allelic imbalance, and allelic instability, as well as to rearrangements at the chromosomal level (changes in copy number or in DNA content). Microsatellites are the first to be affected because their repetitive structure is particularly prone to strand slippage during replication. Thus, microsatellites are considered as a barometer of DNA stability (15).

Interestingly, the *ESRα* gene (6q25) contains a TA repetitive sequence in one of its two promoters (promoter A), which is 1,174 base pairs upstream of exon 1, whereas the *ESRβ* gene (14q22) contains a highly polymorphic CA repeat sequence at its 3′end, which is correlated with testosterone levels in sera (16). Similarly, *AR* (Xq11) contains a CAG microsatellite in the first exon whose length was related to its transactivation power (17): reduced number of CAG repeats was associated with enhanced coactivator mediated signaling (18,19). In addition, somatic alterations of CAG repeats in *AR* were found to be related to positive growth selection of colon cancer cells (20).

In breast cancer, apart from the existence of several hot points of genomic instability (LOH of microsatellites in regions 1p, 1q, 3p, 6q, 9p, 11p, 13q, 16q, 17p, 17q, 18q, Xq25 (21–30)), little data is available regarding the stability of intragenic microsatellites located in potential regulatory regions. Allelic imbalances in these intragenic regulatory regions may play a significant role in breast carcinogenesis.

We questioned whether local tumoral changes could influence tumor cell features with clinical significance: Are the microsatellites located within the *ESRα*, *ESRβ*, and *AR* genes subject to instability in the tumor tissue? Is the length variation in microsatellites or the repetitive sequence loss related to tumor cell features such as ESRα and PR expression, mitotic index, and MIB-1 and p53 expression? In routine pathological examination, MIB-1 is used as an indicator of proliferative activity. The accumulation of p53 is a poor prognostic factor, since it is involved in regulating cell proliferation, inducing apoptosis, and in promoting chromosomal stability.

For this purpose, we undertook the genotyping of microsatellite sequences in tumor tissue, paired normal surrounding tissue and peripheral blood cells, and compared it to bring out the local changes in the tumor tissue. Furthermore, we analyzed ESRα and AR expression by immunohistochemistry in tumor cells and adjacent normal tissue.

Patients and methods

A total of 101 patients who were diagnosed with breast cancer (90 ductal carcinomas and 11 lobular carcinomas) at the University Hospital of Saint-Étienne were selected. The investigations were conducted after Human Experimentation Review by the local in charge committee. Eligible cases were considered from the patients who gave their informed consent to participate. This study was performed on human samples drawn for diagnosis and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

The patients' ages ranged from 36 to 94 years old (mean 68, median 68).

Microsatellite genotyping

DNA samples were extracted from breast tumor and normal breast tissue (30 mg each), which was selected by an experienced pathologist after histological confirmation on a slide from the core of the sample. Thus, the samples destined for microsatellite genotyping were systematically controlled by optical microscopy to assess histological normality in the case of normal breast tissue and the high percentage of tumor cell content in the case of tumor samples. The microscopy examination also allowed us to ensure the histological characteristics of non-tumor tissue (i.e., normal breast parenchyma, epithelial hyperplasia, mastosis, and atypical hyperplasia) and to avoid inappropriate sampling.

Control DNA was obtained from white cells of blood samples collected on EDTA in each patient. DNA was extracted using the QIAamp Mini Kit (Qiagen, Hilden, Germany). Microsatellites were amplified with the use of one end-labeled primer (D2 dye for the CAn and CAGn microsatellites, and D3 dye for the TAn microsatellite; dyes from Beckman Coulter, Fullerton, CA, USA) and one unlabeled primer. The primer sequences were those used by the Westberg genotyping protocol (16). Approximately 150 ng of genomic DNA was amplified in a 25-μL reaction volume that contained 1.25U Taq polymerase (Eppendorf, Hamburg, Germany), 5 pmols of each primer, and 1.5 mM $MgCl_2$ (Eppendorf) on a thermocycler (Eppendorf). The thermocycling conditions comprised 2 minutes of initial denaturation at 94°C, followed by 35 cycles with 45 seconds at 94°C, 45 seconds at 53°C for CA, 50°C for TA, and 61°C for CAG and 45 seconds at 72°C; a final extension was performed at 72°C for 2 minutes. PCR products were analyzed by capillary electrophoresis on a sequencer (Beckman Coulter). Allele sizes were scored by comparison with standard DNA of known sizes (22 DNA fragments) using Fragment Analysis Software (Beckman Coulter). A control sample (Fragment Analysis Test from Beckman Coulter) was included in each series of analysis. For each patient, we analysed the peak height of alleles in ESRα, ESRβ and AR locus in the 3 samples: blood, normal tissue and tumour tissue, in order to detect the peak height imbalance between 2 alleles of a locus. Each experiment was repeated three times independently using the same DNA preparation, and the results were found reproducible (i.e., allelic peak height