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$(C\text{-}A)_n$ microsatellite repeat *D7S522* is the most commonly deleted region in human primary breast cancer

(tumor suppressor gene/chromosome 7q/loss of heterozygosity)

JEAN C. ZENKLUSEN*, IVAN BIÈCHE†, ROSETTE LIDEREAU†, AND CLAUDIO J. CONTI*‡

*The University of Texas M. D. Anderson Cancer Center Science Park–Research Division, Smithville, TX 78957; and †Laboratory of Oncogénetique, Centre René Huguenin, 35 rue Dailly, F-92211, St-Cloud, France

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ABSTRACT **Loss of heterozygosity in human chromosome 7q was studied to determine the location of a putative tumor suppressor gene. Twenty-six of 31 cases studied presented loss of heterozygosity at one or more loci on chromosome 7q. Eighty-three percent loss of heterozygosity (in 11 informative cases) was detected by using the $(C\text{-}A)_n$ microsatellite repeat marker *D7S522* at 7q31.1–7q31.2. These results suggest that a tumor suppressor gene relevant to the development of breast cancer is present in the 7q31.1–7q31.2 region, confirming our previous evidence for a tumor suppressor gene in this chromosome and frequent deletions of the long arm in human primary breast cancers.**

Although breast cancer patients accounted for 19% (1.45 million) of the total number of patients worldwide (7.6 million) affected by cancer in 1985 (1, 2), little is known about the etiology and pathogenesis of breast neoplasia. Alterations in oncogenes and tumor suppressor genes (TSG) are considered to be critical in the multistep process leading to the development of tumors (3, 4), and the succession of these events is very conserved in some types of cancer (5). Ever since the idea of recessive-acting TSG was formulated (6), cytogenetic techniques have been used to identify their possible locations. Previous cytogenetic reports indicated that several chromosomes (such as chromosomes 3, 7, 16, 17, and 20) (7–11) are frequently altered in breast cancer. However, in only a few cases has the target of these alterations been identified (12). Consistent deletions or inversions of part of a chromosome are indicative of inactivation of a nearby TSG during neoplastic progression (13). In this regard, deletions of chromosome 7 are common throughout many different types of tumors studied, such as ovarian cancer, gastric carcinomas, and malignant myeloid disorders (14–20), indicating frequent breaks near the *MET* oncogene (7q31–7q33) with a frequency of 27–41% in the case of breast cancer (21). Also, recent results using microcell-fusion transfer of human chromosome 7 to a murine-derived squamous cell carcinoma cell line indicated that the inserted chromosome can delay the onset of tumors by 2-fold to 3-fold and in some cases can even repress completely the tumorigenic potential of the squamous cell carcinoma cell line. *In situ* hybridization revealed that the clones that reverted to the malignant phenotype had expelled the inserted chromosome (22). Moreover, a recent report demonstrated that the insertion of an intact human chromosome 7 to immortalized human fibroblast cell lines having loss of heterozygosity (LOH) in the segment 7q31–7q32 will restore the senescence properties to the cells (23).

Although cytogenetic techniques are useful, they do not detect the entire spectrum of inactivating events; for example, microdeletions and homologous recombination with a defective chromatid (24, 25) are beyond the range of detection by karyotyping procedures. More sensitive molecular methods should be used to screen genetic alterations in any tumors and to determine the smallest chromosome region involved in those alterations. LOH analysis of DNA extracted from solid tumors using polymorphic genetic markers is the method of choice for small regions that harbor TSG (26).

To determine the extent and type of alterations in chromosome 7 in human breast cancer, we utilized an extensive set of highly polymorphic markers in the q21–qter segment of chromosome 7. By comparing the results obtained with tumoral and normal DNA, we have been able to determine a 2-centimorgan (cM) smallest common deleted region.

MATERIALS AND METHODS

Tumor and Blood Samples. Specimens were obtained from 31 primary breast tumors surgically removed from patients at the Centre René Huguenin; none of the patients had undergone previous radiotherapy or chemotherapy. Immediately following surgery, the tumor samples were placed in liquid nitrogen until extraction of high molecular weight DNA. A blood sample was also taken from each patient and stored in a similar way. Frozen tissue samples were ground in liquid nitrogen to a very fine powder with a mortar and pestle. High molecular weight DNA was prepared by a standard proteinase K digestion and phenol/chloroform extraction (27) from both blood and tissue samples.

Restriction Fragment-Length Polymorphism (RFLP) Analysis. For each sample, 10 μg of genomic DNA was digested with the appropriate restriction endonuclease and fractionated by electrophoresis on 1.1% or 0.8% agarose gels.

Leukocyte and tumor DNAs from each patient were analyzed in adjacent tracks. DNA was then transferred to nylon membrane filters by standard blotting procedures (27). DNA probes were radiolabeled with [^{32}P]dCTP by nick-translation or by use of a random primer labeling system, depending upon the probe used. The membrane filters were hybridized overnight at 65°C by using standard procedures (27) with the denatured labeled probe, washed, and autoradiographed at −80°C for an appropriate period. Table 1 lists the probes and enzymes used for this analysis.

$(C\text{-}A)_n$ Microsatellite Repeats Amplification Analysis. Nineteen $(C\text{-}A)_n$ microsatellite repeats in the 7q21.3-7qqter region (38) were amplified in a Thermocycler 9600 (Perkin–Elmer) at a final reaction volume of 25 μl. The 2.5-μl reaction mixtures were composed of 10× standard PCR buffer (22) containing 100 ng of DNA, 1 unit of *Taq* polymerase, 400 pM of each primer, and 200 mM of each dNTP. The annealing temperature was 50°C for all of the primers except *D7S498*, which

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Abbreviations: TSG, tumor suppressor gene; LOH, loss of heterozygosity; RFLP, restriction fragment-length polymorphism.

‡To whom reprint requests should be addressed.

Table 1. Chromosomal locations (28, 29) of polymorphic DNA probes used to study LOH and appropriate restriction enzymes

Chromosome 7 location	Locus	Probe	Enzyme	Ref.
q22.3–q31.1	*D7S13*	pB79a	*Hin*dIII/*Msp* I	30
q22.3–q31.1	*D7S23*	pXV-2c	*Taq* I	31
q22.3–q31.1	*D7S18*	p7C22	*Eco*RI	32
q22.3–q31.1	*MET*	pmetH	*Taq* I/*Msp* I	33
q31–q32	*D7S125*	SA37	*Pst* I	34
q33–qter	*ABP1*	ABP1.1	*Hin*dIII	35
q36–qter	*D7S396*	pJCZ67	*Pvu* II/*Taq* I	36
q36–qter	*D7S22*	pLg3	*Hin*dI/*Pvu* II	37

was annealed at 45°C. We used 27 cycles of amplification comprising 20 s of denaturation at 94°C, 30 s of annealing, and 15 s of extension at 72°C. The number of cycles used was determined to be in the linear part of the amplification process (i.e., before product saturation), permitting us to assume that equal optical density of both alleles was to be expected if no LOH occurred.

The PCR products were separated in a 3.5% Metaphor agarose (FMC) gel at 5.5 V/cm^2 for 3 hr by using TBE buffer (89 mM Tris borate/89 mM boric acid/2 mM EDTA, pH 7.5) containing 0.5 mg of ethidium bromide per ml and a standard loading buffer (27). The gel was photographed with a Fotodyne (New Berlin, WI) 3-4400 UV transilluminator and Polaroid positive–negative 4×5 instant film.

Determination of Allele Loss. Allelic loss determination is only done on "informative" (I) patients. Normal DNA samples that were polymorphic at a given locus were considered to be informative, whereas the homozygotes were declared "uninformative" (N). The signal intensity of fragments was determined by densitometry or by visual examination (three reviewers) or by both techniques.

Table 2. Chromosome 7 LOH in 31 primary human breast carcinomas. The relative order of these markers has been published (28, 29, 38).

Chromosomal markers	% LOH	Informative	LOH cases
D7S527	19	21	4
D7S479	8.33	12	1
D7S518	22.20	27	6
D7S515	23.07	13	3
*D7S13**	19	21	4
*D7S23**	38.9	18	7
*D7S18**	42.9	7	3
D7S496	47	17	8
*MET**	44.4	18	8
D7S523	54.50	22	12
D7S486	33.33	15	5
D7S522	81.80	11	9
D7S480	42.85	21	9
D7S490	35.37	14	5
D7S487	27	15	4
*D7S125**	41.7	12	5
D7S514	10	10	1
D7S504	27	22	6
D7S500	16	19	3
D7S495	0	18	0
D7S498	11	18	2
D7S505	0	11	0
*ABP1**	33.33	6	2
D7S483	5	19	1
*D7S396**	27.3	22	6
*D7S22**	25.9	27	7
D7S550	0	14	0

*RFLP probes.

For the RFLP analysis, we used the accepted definition of LOH: one band having an intensity <50% of its normal counterpart. For the PCR experiments, we considered a sample to have LOH when an entire band was absent or the band had <20% of the normal intensity (21). Although PCR amplification cannot be considered quantitative, we optimized the PCR conditions so that equal amounts of template produced equal amounts of amplified product. We used 27 amplification cycles, which we demonstrated to be in the linear part of the amplification process—i.e., before product saturation (data not shown). We also conducted a series of titrations using different proportions of homozygous and heterozygous templates to assess the influence of stromal-tissue contamination of our amplification reactions. We determined that we could detect as little as 40% contamination by heterozygous template in the homozygous DNA (data not shown). Thus, our limit of 20% intensity for LOH is very conservative.

Statistical Analysis. The normality of the percentage LOH distributions was tested by using the Kolmogorov–Smirnov continuous cumulative distribution test (39). The distributions obtained with $(C\text{-}A)_n$ microsatellite repeats and RFLP probes were compared by using a paired *t*-test analysis.

RESULTS AND DISCUSSION

RFLP analysis as well as amplification of $(C\text{-}A)_n$ microsatellite repeats (38) by PCR were used to screen 31 breast samples for LOH in the 7q21–7qter segment. In all, we used eight RFLP probes and 19 $(C\text{-}A)_n$ microsatellite repeats mapping to the long arm of chromosome 7 (Table 2). Figs. 1 and 2 show representative photographs of RFLP analysis and $(C\text{-}A)_n$ microsatellite amplifications, respectively.

Our results indicated that the loss of part or all of the chromosome 7q arm is a common event in human primary breast cancer. LOH occurred in at least one locus on the long arm of chromosome 7 in 26 of 31 tumors (83.9%). This incidence is higher than the incidence of other frequently deleted regions in breast tumors [3p13–3p14.3, 16p22–16p23,

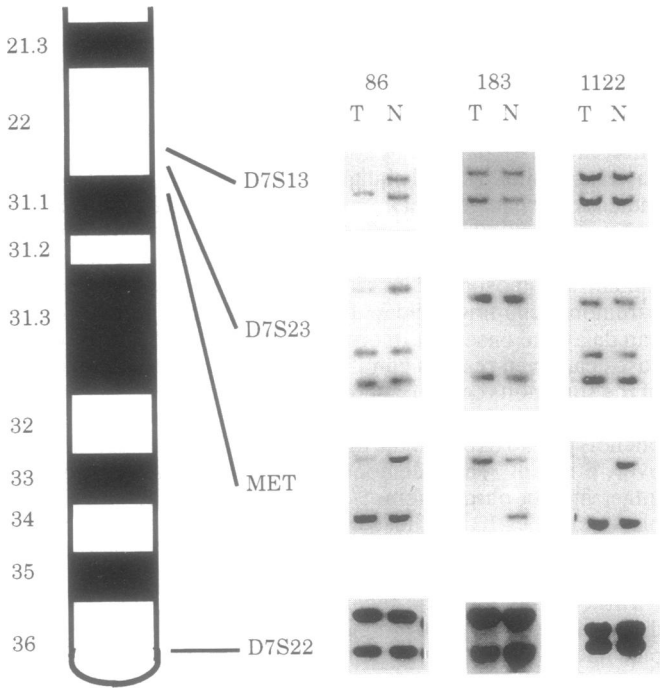

FIG. 1. Representative autoradiographies of the RFLP analysis. Lanes T and N indicate matched DNA samples isolated respectively from tumor tissue and peripheral leukocytes.

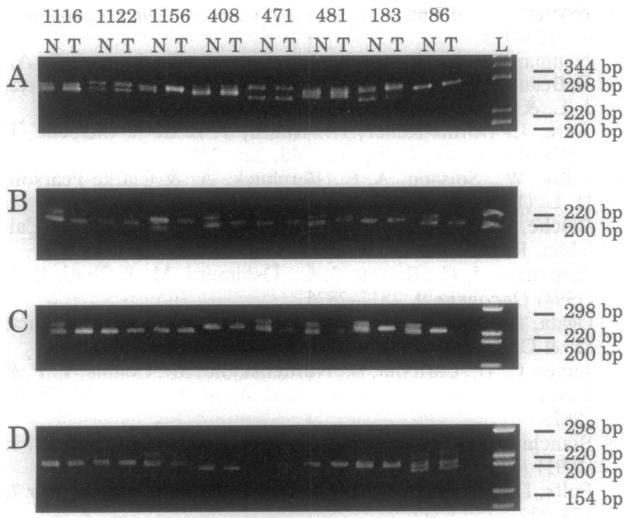

FIG. 2. Representative PCR amplifications of the $(C\text{-}A)_n$ microsatellite repeats. Case numbers are shown atop the respective lanes. Lanes T and N indicate matched DNA samples isolated respectively from tumor tissue and peripheral leukocytes. *(A) D7S527. (B) D7S523. (C) D7S522. (D) D7S550.*

17p13, and 17q21 (7–11)]. The most frequent LOH (81.8%) among 27 markers was observed with microsatellite *D7S522*

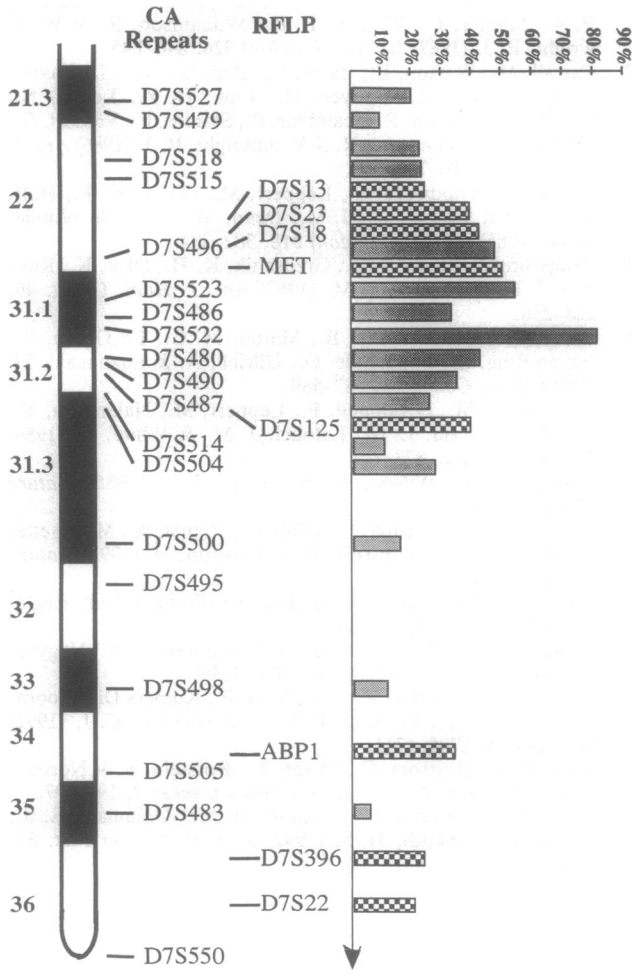

FIG. 3. *(Left)* Representation of 7q21.3–7qter and approximate position of the microsatellite repeats and RFLP probes (28, 29, 38). *(Right)* Histogram showing the percentage LOH for each of these microsatellites (solid bars) and RFLP probes (checkered bars).

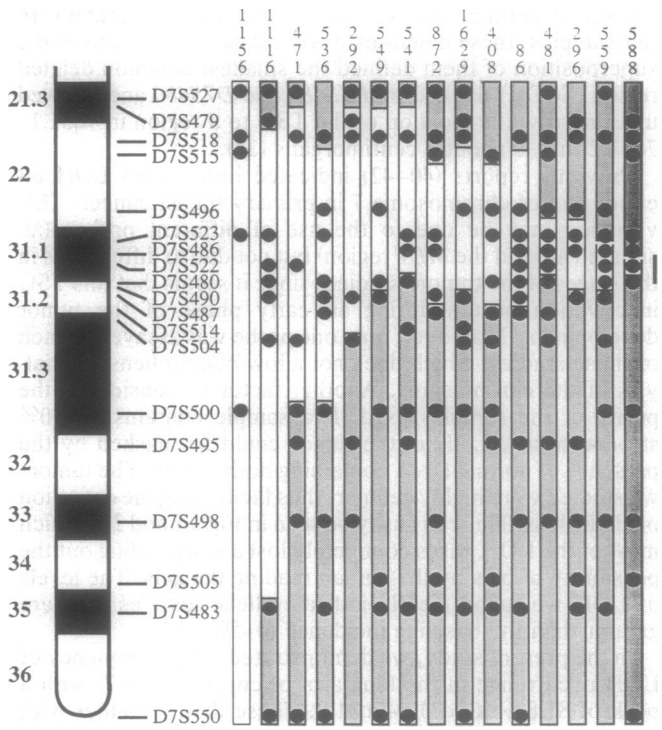

FIG. 4. Deletions suggested by a series of successive allele losses. *(Left)* $(C\text{-}A)_n$ microsatellite repeats as in Fig. 3. *(Right)* Shaded bars indicate that the fragment is retained; empty bars indicate fragment losses. Black circles in shaded bars indicate informative markers with retention of both alleles; black circles in empty bars indicate LOH. Noninformative cases for each marker are symbolized by the absence of circles at that position. The thick vertical line on the right side indicates the smallest common deleted region obtained by this analysis.

(7q31.1–7q31.2) (Fig. 3 and Table 2). None of the tumors studied showed LOH at any other microsatellite while being heterozygous for the *D7S522* marker. Furthermore, the segment limited by the $(C\text{-}A)_n$ repeats *D7S496* to *D7S480* (7q22–7q31.2), which includes the aforementioned marker, has an average percentage LOH of 52.4%. The RFLP markers in this area also gave the highest LOH of the RFLP analysis; the maximum of 44.4% was reached at the marker *MET* (Table 2), which is the nearest RFLP marker to *D7S522*.

Seventy percent of the samples studied were stage II tumors, the remaining tumors being stage III (20%) or stage I (10%); no correlation was found between stage and LOH in the *D7S522* marker. The high incidence of LOH in low-grade tumors indicates that this TSG is affected as an early step during the breast carcinoma development.

Analysis of the histograms of the data from each technique indicated that they both had a normal distribution [Kolmogorov–Smirnov (39), $P > 0.612$ for $(C\text{-}A)_n$ repeats and $P > 0.763$ for RFLP probes] as would be expected of a stochastic process such as the inactivation of a TSG. Comparison of the two distributions in a paired *t* test (39) revealed that they were indistinguishable ($P < 0.01$), demonstrating that the results obtained by both techniques are quantitatively identical. Thus, both PCR and RFLP techniques revealed a high frequency of LOH in 7q31.1–7q31.2, confirming previous reports of deletions on chromosome 7 in breast cancer (21) and in other types of cancer (14–20).

Fig. 4 shows the LOH data in 15 cases studied. In these cases, large deletions can be predicted by these molecular techniques. The probability of three or more allelic losses in the same fragment caused by independent events is very low; thus the occurrence of such a series of LOH in contiguous

markers is certainly due to deletion of the entire segment. In our samples, these deletions were frequently interstitial; the superposition of them defined the smallest common deleted region (SCDR) flanked by *D7S486* and *D7S480* and allowed us to narrow the location of the TSG to a region in 7q31.1–7q31.2 that is about 2 centimorgans (38).

Previous reports (40–42) indicated little or no LOH on either arm of chromosome 7 in primary breast cancer. This variation may be due to the use of different probes (all situated around the *MET* region) but not due to differences in the stages of the tumors studied, since it seems that this TSG inactivation occurs during an early phase of the tumor development. Moreover, only one probe was assayed in each of those studies, which does not allow comprehensive analysis of the chromosome. Another factor to consider is the purity of the samples used. If a sample contains 30–40% stromal tissue, LOH in the tumor could be masked by the presence of normal DNA containing both alleles. The tumors we studied were highly cellular, thus facilitating the detection of allelic loss. This can easily be seen in Figs. 1 and 2 in which most of the LOH cases completely lost a band, ruling out the possibility of bias by the person reading the gels. The levels of LOH we found clearly indicate allelic loss, as they are greater than the baseline incidence (0–5%) (43).

In the present study, we demonstrated a high frequency of LOH occurrence in the long arm of chromosome 7, with a peak of 81.8% at 7q31.1–7q31.2. These data, together with the results of our studies of microcell-mediated transfer of chromosome 7 (21) and reports of similar chromosome 7 deletions in several other neoplasias (14–21), strongly suggest that there is a TSG distal to *MET* near 7q31.1–7q31.2, which is relevant to several human neoplasias.

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