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Presence of an allelic *Eco*RI restriction fragment of the c-*mos* locus in leukocyte and tumor cell DNAs of breast cancer patients

(protooncogenes/human malignancies/gene polymorphism)

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ABSTRACT **Structure of the human c-*mos* protooncogene in DNAs from breast tumors, leukemic cells, and lymphocytes from normal individuals was analyzed by restriction enzyme digestion and Southern blot. In 6 of 75 breast tumor DNAs, we found an *Eco*RI 5-kilobase extra band hybridizing with a human c-*mos* probe containing all of the sequences homologous to v-*mos* oncogene. This band was also found in lymphocyte DNA from 3 of these patients, indicating a restriction fragment length polymorphism. This polymorphism was not found in a series of 69 lymphocyte DNAs from the unaffected population. Moreover, 1 of 73 leukemic cell DNAs exhibited the 5-kilobase band. These results indicate that this rare polymorphism is significantly more frequently found in patients with breast cancer than in the rest of the population ($P < 0.05$, by a χ^2 test with Yates correction).**

The normal human genome, as in other eukaryotes, contains a limited number of specific genes named protooncogenes or proto-*onc* that, after activation processes such as punctual mutation, chromosome translocation, amplification, etc., are more or less directly involved in genesis and evolution of human malignant tumors (1). On the other hand, the possibility of a genetic susceptibility to cancer has been evoked as one of the multiple factors participating in the malignant transformation process. In that respect, restriction fragment length polymorphism (RFLP) analysis constitutes an efficient tool for studying genetic heterogeneity. This approach has been documented in a recent work (2): a polymorphism of the proto-*onc* Ha-*ras* significantly has been found more frequently in lymphocyte DNA from patients bearing different varieties of malignancies than in lymphocytes from a nonaffected population.

Using such a methodology, we found that DNA from human breast tumors exhibits in 8% of the cases a supernumerary *Eco*RI band hybridizing with c-*mos* proto-*onc* sequences. This band corresponds to a RFLP because it is also present in lymphocyte DNA from these patients. In contrast, it was not found in DNA from unaffected individuals, indicating that this sort of polymorphism is rare. Moreover, this polymorphism could be specific to patients with breast tumors, as it was observed in only 1 of 73 cases of patients with leukemia.

MATERIALS AND METHODS

Tumor Cells. Breast biopsies were immediately frozen in liquid nitrogen.

Lymphocytes from Tumor Breast Patients. Twenty milliliters of peripheral blood was taken in the presence of heparin. Lymphocytes were separated by sedimentation through Ficoll/Hypaque. Pellets were stored at −80°C before DNA extraction.

Leukemic Cells. Peripheral blood samples were mixed with Plasmagel (Laboratoire Roger Bellon, France; 1:4, vol/vol). After a 1-hr incubation, the supernatant phase was decanted and sedimented. Pellets were washed and immediately treated for DNA isolation.

Cell DNA Isolation. Frozen tumor fragments were reduced to powder in a cold metallic mortar. Homogenization was in Tris/EDTA buffer (0.02 M Tris/0.01 M NaCl/0.01 M EDTA, pH 7.4). Sodium dodecyl sulfate (0.5%) and proteinase K (250 μg/ml) were added, and lysates were incubated for 24 hr at 37°C. Deproteinization was completed by phenol/chloroform treatments, and DNA was recovered by ethanol-precipitation.

For blood-cell DNA, initial tissue disruption was omitted.

Lymphocyte DNA from Normal Population. DNA samples were provided by the "Centre d'Etudes du Polymorphisme Humain" (CEPH). DNA was extracted from buffy coat by proteinase K and phenol and chloroform treatment.

Restriction Enzyme Digestions. They were made according to the specifications of the enzyme suppliers (Boehringer).

c-*mos* Probes. Two probes representative of the human c-*mos* locus were prepared (3). Probe A consisted of a 2.5-kilobase (kb) *Eco*RI fragment subcloned in pBR322 and containing all v-*mos*-specific sequences (Fig. 1). Probe B was a 2.0-kb *Hin*dIII fragment recognizing the 5′ end of the c-*mos* locus (Fig. 1). This fragment was prepared from clone pHM1 (3) kindly supplied by G. Vande Woude.

For preparation of the probe, plasmid DNA was purified, digested with *Eco*RI, and fractionated by electrophoresis in 1% agarose gel. The 2.5-kb *Eco*RI fragment was electroeluted, precipitated, and stored before use. It was ^{32}P-nick-translated as described by Maniatis *et al.* (4).

Other Probes. c-*myc*-specific, c-*erbA*-specific, and c-*erbB*-specific probes used for testing the quality of enzyme digestions were generously provided by D. Stehelin and S. Saule.

Southern Blot and Hybridization. Conditions were those of Maniatis *et al.* (4); 10–20 μg of DNA were used in each test.

RESULTS

Seventy-five DNA samples from breast tumors histologically defined as invasive ductal carcinomas (this type represents approximately 85% of breast cancers) were digested with *Eco*RI, blotted, and analyzed with a c-*mos* *Eco*RI probe containing all of the sequences homologous to v-*mos* (Fig. 1).

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Abbreviations: kb, kilobase(s); RFLP, restriction fragment length polymorphism; ANLL, acute nonlymphoblastic leukemias.

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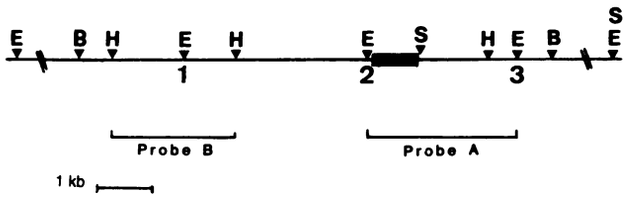

FIG. 1. Restriction map of human c-*mos* (3). Only restriction sites useful for understanding have been indicated (see text). The bracketed regions correspond to coding sequences homologous to a v-*mos* probe. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sma* I. *Eco*RI sites are numbered 1, 2, and 3 (see *Discussion*). EcoRI and/or *Sma* I sites at each end are defined by size of the DNA fragments (see text).

In 69 cases (67 women and 2 men), a unique 2.5-kb band was detected, representing the *Eco*RI fragment itself (Fig. 2*A*, lanes 1, 3, and 4). The 6 other DNAs contained an additional 5-kb band whose intensity was approximately equal to that of the 2.5-kb band in each DNA (Fig. 2*A*, lane 2). Moreover, in these 6 samples, intensities of the 2.5-kb and 5-kb bands were approximately half of that of the 2.5-kb unique band in other samples (same DNA amounts were used in tests).

In order to show that the 5-kb fragment did not result from a DNA partial digestion in these 6 particular cases, the same blots were hybridized successively with other probes corresponding to c-*myc*, c-*erbA* and c-*erbB* (not shown). With c-*myc* a unique 12.8-kb band corresponding to the normal alleles (5) was found. As for c-*erbA*, a unique 9-kb fragment was detected. Presence of this fragment has been reported in normal human DNA (6, 7). Finally, in 25 DNA samples, the c-*erbB* probe recognized a 8.7-kb fragment as published elsewhere (8). These results and the fact that the 5-kb c-*mos* fragment was repeatedly found in separate experiments excluded an artifact due to incomplete hydrolysis.

Complete digestions of the 6 DNAs containing the 5-kb fragment were also done with *Pst* I, *Hin*dIII, *Pvu* II, and *Bam*HI. These enzymes were selected for their function in the c-*mos* restriction map. In no case did the fragments hybridizing with the c-*mos* probe differ from those resulting from digestion by the same enzymes of DNA that did not possess a 5-kb fragment (not shown). This made it unlikely that the 5-kb fragment corrresponded to a rearranged c-*mos* locus.

Since the relative intensities of the 2.5-kb and 5-kb bands were in a ratio of 1:1, we suspected the existence of a RFLP in these 6 tumor DNAs. For this purpose, we were able to analyze lymphocyte DNAs from 3 of the patients (Fig. 2*B*).

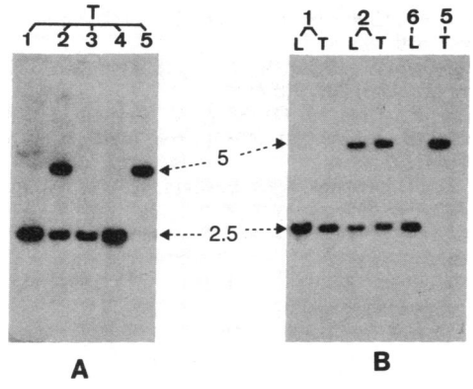

FIG. 2. (*A*) Southern blot analysis of c-*mos* in *Eco*RI-digested human DNA from four different breast tumors (lanes 1–4) and in blast cell DNA from a patient with an ANLL. (*B*) DNA blots from lymphocytes (lanes L) and breast tumor cells (lanes T) of patients 1 and 2 in *A*, from blast cells of patient with an ANLL (same as in *A*) (lane 5), and from lymphocytes of a normal donor (lane 6).

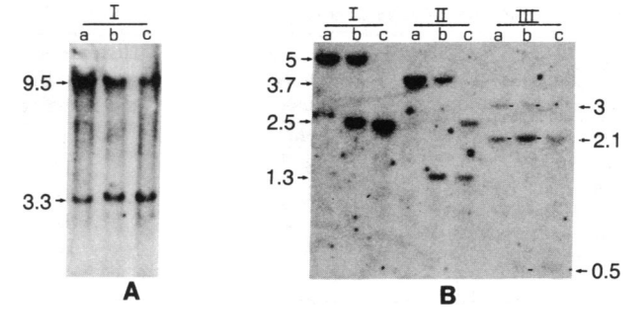

FIG. 3. (*A*) Southern blot hybridization of *Eco*RI-digested DNA with probe B. Lanes: a, polymorphic DNA from the patient with an ANLL; b, polymorphic DNA from the patient with breast tumor; c, control DNA from a normal donor. (*B*) Southern blot hybridization of the same DNAs with probe A. The DNAs were digested with *Eco*RI (lanes I), with *Eco*RI/*Sma* I (lanes II), with *Eco*RI/*Hin*dIII (lanes III). Because of their close sizes, the two *Eco*RI/*Hin*dIII fragments running at 1.3 kb are not resolved in this gel.

Again, these 3 DNAs exhibited the same 5-kb band (lane 2). These results, while they excluded a rearrangement acquired by tumor cells, were compatible with a constitutive c-*mos* polymorphism. If that were so, the same RFLP should be observed in a normal population. DNA samples from 69 normal individuals were analyzed under the same conditions. No RFLP was found (not shown), suggesting that it was more frequent in patients with breast cancers.

Three *Eco*RI sites exist in the vicinity of the v-*mos* homologous sequences (designated 1, 2, and 3 in Fig. 1). To decide which site was implicated in the RFLP, *Eco*RI-digested DNA was hybridized with probe B, covering 2.0 kb upstream of the v-*mos* sequences. Two bands running at 3.3 and 9.5 kb were detected in control DNA and in polymorphic DNA, suggesting that sites 1 and 2 were not modified (Fig. 3*A*). The same DNAs were submitted to a double digestion with *Eco*RI and *Sma* I enzymes and hybridized with probe A (Fig. 3*B*). As expected, control DNA generated a doublet of 1.1 and of 1.3 kb. In contrast, an additional 3.7-kb band was observed in the pattern of polymorphic DNA, indicating that *Eco*RI site 3 was impaired or missing. A similar result was obtained by DNA double digestion with *Eco*RI and *Hin*dIII. In addition to normal bands of 2.1 and 0.5 kb, a third band of 3 kb occurred on the blot of polymorphic DNA. This band also resulted from the disappearance of *Eco*RI site 3.

Next, we wanted to see whether this RFLP did exist in another tumor variety. DNAs from leukemic cells [63 acute nonlymphoblastic leukemias (ANLL) belonging to each category of the FAB classification, 7 chronic myelogenous leukemias (CML), and 3 acute lymphoblastic leukemias (ALL)] were blotted and analyzed with the c-*mos* probe. Only the 2.5-kb fragment was detected with the exception of 1 case (Fig. 2*A* and *B*, lanes 5, and Fig. 3*B*, lanes a): a single 5-kb band occurred on the blot, whereas the 2.5-kb band was absent. Because of the death of this patient, it was impossible to examine DNA from normal lymphocytes. So, we could not decide whether this abnormal apparent homozygosity was constitutive or acquired in the leukemic cells. A cytogenetic study of leukemic cells indicated that two chromosomes 8 were present and did not exhibit any apparent abnormality.

DISCUSSION

Results presented in this report indicate that a significant proportion of patients with breast cancer contains an *Eco*RI RFLP at the c-*mos* locus. That it is indeed a polymorphism is assessed by the presence of the same 5-kb band in normal lymphocyte DNA from 3 of the patients who exhibit it in their tumor cell DNA, and by absence of extra bands in DNA

Table 1. Some parameters characterizing the six breast tumor patients with a polymorphic c-*mos* allele

Patient no.	Age at tumor detection, yr	Hormonal status*	Children	Histoprognostic grading (Bloom and Richardson)	Oestrogen receptors†	Progesteron receptors†	Invasive lymph nodes	Cancer family story
92	47	nM	3	I	0	177	2	Mother with breast tumor
121	79	48	1	II	115	50	7	No cancer
213‡	62	51	3	II	311	0	ND	Other familial carcinoma
111	63	50	2	II	636	225	5	Unknown
167	54	49 (nonnatural)	2	III	0	0	0	3 other familial carcinomas
93	41	nM	1	II	18	0	3	No cancer

ND, not determined.
*nM, nonmenopausal; numbers give the age of the patient at the time of menopause (natural or provoked).
†Expressed as femtomol/mg of cytosol proteins.
‡Deceased.

digests with other restriction enzymes cutting c-*mos* close to or within v-*mos* homologous sequences.

Two simple hypotheses can explain this RFLP: either an *Eco*RI site has disappeared in one allele, or it is, for an unknown reason, resistant to digestion. Three *Eco*RI sites exist in the vicinity of the v-*mos* homologous sequences (referred to as sites 1, 2, and 3 in Fig. 1). Our results show that it is the site 3, located 3′ to v-*mos* sequences, that is missing. At the moment, it cannot be decided if the absence of this site is due to a point mutation affecting one of the nucleotides that constitute the site or reflects a larger modification of the primary structure of the region spanning the site. A definite answer will be provided by sequencing the 5.0-kb fragment.

The fact that 8% of the DNA from patients with breast tumors contains this RFLP and that 69 normal lymphocyte DNAs and 72 of 73 leukemic cell DNAs do not possess it deserves several comments. First, this is clearly a rare RFLP in the normal population. Second, its presence in breast tumor patients suggests a particular susceptibility of individuals having it to this variety of cancer. Table 1 summarizes some data of the record of each patient. The individual status of these 6 patients was representative of the whole population tested in this study: 49 of 75 patients had invasive lymph nodes; 46 of 75 possessed hormonal receptors; 23 of 73 women were nonmenopausal, 46 were postmenopausal, and the 4 others were perimenopausal. For 3 patients, a family cancer story revealed the presence of breast cancers in relatives. This can constitute an indication to look for the presence of the 5-kb *Eco*RI fragment in such families.

To date, only a few reports have been published concerning a c-*mos* implication in animal tumors (with the exception of sarcoma virus-induced sarcoma). The only documented cases concern murine plasmacytoma in which transposition of particle A long terminal repeat (LTR) close to v-*mos* sequences has been reported, resulting in the expression of these sequences (9–12). It will be useful to screen DNA containing the *mos* RFLP with a LTR-homologous probe. A polymorphic c-*mos* gene has also been described in DNA from a monocyte–macrophage cell line established from the bone marrow of a young patient with congenital hypoplastic anemia (R. P. Revoltella, personal communication). Size of the *Eco*RI fragment is compatible with our results.

Finally, it would be interesting to look for *Eco*RI c-*mos* RFLP in other histological types of breast cancers and in benign lesions.

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