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Identification of Differentially Expressed mRNAs in Normal and Neoplastic (Adenocarcinoma) Human Endometrium[1]

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Individual mRNA species from normal and neoplastic endometrium obtained from the same patient were comparatively studied by exploiting the "differential display" methodology. The mRNAs were extracted from tissues, reverse transcribed, and amplified by PCR using appropriate primers. The cDNA electrophoretic bands which were frankly different on the basis of their quantitative expression were excised from the gels and reamplified. Each of these sequences was subsequently screened for the capacity to hybridize to RNA preparations from normal endometrium and endometrial adenocarcinoma samples, first from the original patient and then from a group of selected patients. Of many, only two sequences, thereafter named $N_{5.5}$ and $T_{16.2}$, respectively, successfully passed the two sieving tests and were chosen for further analysis. It appears that $N_{5.5}$ recognizes an mRNA which is expressed more abundantly in normal than in neoplastic (adenocarcinoma) endometrium; in contrast, $T_{16.2}$ seems to preferentially recognize an mRNA which is expressed in tumoral tissues. These two sequences have been cloned and sequenced; they do not show any identity or significant similarity to any other known sequence.

INTRODUCTION

Endometrial adenocarcinoma is one of the most common female pelvic genital malignancy [1]. Although the primary diagnosis of this disease is very often in the first clinical and pathological stages, with regard to all other oncological diseases, it would be advisable to have more information on molecular factors involved in development and evolution of the neoplastic process. The characterization of such factors, while allowing a better understanding of the mechanisms of transformation, should also result in new diagnostic and therapeutic approaches. This will improve the clinical perspectives of this and similar diseases.

To date, some specific antigens and proteins have been found associated with endometrial cancer with variable clinical and biological correlation [2–7]. Most of these proteins or other kind of antigens, however, were described originally in other systems (ovary, breast, etc.) and their correlation with endometrial neoplasia was established as a result of tentative researches *a posteriori.* This paper reports the first attempt to directly individuate, through a methodical comparison, those mRNAs which are differentially expressed in normal and tumoral endometrium. Indeed, normal and tumoral tissues have very different morphological, biological, and biochemical traits. These features originate from selective qualitative and quantitative expression of mRNAs which clearly ensues in a concomitant variation in the expression of the encoded proteins. Therefore this different approach should give better chances for identifying more biologically meaningful and more clinically useful elements. To reach this goal we took advantage of differential display, a sensitive and powerful differential cloning technology which has been introduced quite recently by Liang and Pardee [8]. First, we have individuated several mRNAs which were differently expressed in the normal and in the neoplastic portion of an endometrium obtained from one patient. At this stage several sequences corresponding to differential mRNAs were cloned. Of many, only those which were also found to be differential when assayed against RNAs obtained from other clinical cases have been studied further.

MATERIAL AND METHODS

Collection of Clinical Specimens and RNA Isolation

Clinical specimens from patients undergoing total abdominal hysterectomy and bilateral salpingo-oophorectomy for endometrial malignancies were obtained at the time of surgery from the Obstetrics and Gynecology Department of the Medical School of the University of Reggio Calabria. From

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TABLE 1
Clinical Data for Patients with Endometrial Adenocarcinoma

Patient	Age	Histology grade	Myometrial invasion	Extrauterine metastases	Lymph node metastases	Cervical involvement
1	58	G1, well differentiated	Inner $\frac{1}{3}$	No	No	No
2	68	G3, poorly differentiated	None	No	No	No
3	80	G2, moderately differentiated	Middle $\frac{1}{3}$	No	No	No
4	63	G2, moderately differentiated	Inner $\frac{1}{3}$	No	No	No
5[a]	81	G2, moderately differentiated	Middle $\frac{1}{3}$	Ovary	No	No
6	66	G1, well differentiated	Inner $\frac{1}{3}$	No	No	No

Note. All patients were in menopausal status and did not receive specific therapy before surgery.
[a] Patient 5 was also affected by uterine leiomyoma.

each patient aliquots from normal endometrium and tumor were obtained. Each of these samples was cut in two pieces: one was immediately frozen in liquid nitrogen and stored at −80°C, the second was used for histological analysis. Only specimens from clinical cases with histological diagnosis of endometrial adenocarcinomas were selected for this study. Total cellular RNA was extracted from tissues (0.5–1.0 g, either normal or neoplastic) essentially as previously described [9]. A collection of data regarding patients selected for this study, is reported in Table 1.

DNA Manipulations, Vectors, and Sequence Analysis

Unless differently specified, DNA manipulations were performed according to standard procedures [10]. Cloning plasmid pT7Blue T-vector was obtained from Novagen and used according to manufacturer's instructions. Sequencing was performed with the Sequenase kit (USB, Cleveland, OH). The analysis of DNA sequences was performed using the University of Wisconsin Genetic Computer Group software package (Version 8, September 1994, Madison, WI [11]).

Primer Syntheses

Degenerate oligonucleotide primers $T_{12}XG$, $T_{12}XA$, $T_{12}XT$, and $T_{12}XC$, where X indicates A, G, and C, were synthesized by the Pharmacia Biotech facility (Milan, Italy). Commercially available random decamers OPA1 to 20 were purchased from Operon Biotechnology Inc. (Alameda, CA).

Differential Display

To remove possible contaminant DNA, purified total cellular RNA from normal and tumoral tissues of patient 1 (30 μg) was incubated for 30 min at 37°C with 15 units of DNase I and 10 units of human placental ribonuclease inhibitor (Gibco-BRL, Gaithersburg, MD) in 10 m*M* Tris–Cl, pH 8.3, 50 m*M* KCl, 1.5 *M* $MgCl_2$, and 0.01% (w/v) gelatin. RNA was then extracted with phenol–chloroform and precipitated. Aliquots of these materials were then reverse transcribed as previously described [8, 12]. Each total RNA extract (0.2 μg) was reverse transcribed using $T_{12}XG$, $T_{12}XA$, $T_{12}XT$, and $T_{12}XC$ as primers in separate reactions. cDNA products from these reactions were then PCR amplified. The reaction was performed in a total volume of 20 μl containing 2 μl of *Taq* polymerase buffer, 0.6 μl of 50 m*M* $MgCl_2$, 0.2 μl of 5 U/μl *Taq* polymerase (Polymed, Florence, Italy), 1.6 μl of dNTP 25 μM and 1 μl [α-^{35}S]dATP (1000 Ci/mmol, Amersham, Milan, Italy). Each reaction mixture also included the $T_{12}XN$ primer used to generate the cDNA and a random commercial decamer primer whose final concentration was 1 μM. The cycling parameters were as follows: 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec for 40 cycles, followed by 72°C for 5 min. The amplified cDNAs were separated on a 6% acrylamide denaturing sequencing gel. The gels were dried and autoradiographed (see next section). Visual inspection revealed some quantitative differences in the expression of cDNAs from normal and neoplastic tissue obtained from the same patient (Patient 1, Table 1). The film was then used as a guide to excise selected bands from the dried gel. The DNA recovered using this procedure was reamplified as previously described [8, 12]. This material was finally used for further labeling and cloning.

Dot and Northern Blot Hybridization

For dot-blot analysis 12.5 μg of RNA samples was denatured with 10 m*M* NaOH, 1 m*M* EDTA and applied to a Zeta-Probe GT blotting membrane (Bio-Rad) with Bio-Rad manifold apparatus. For Northern blots 30 μg of RNA was denatured in a formaldehyde/formamide-containing buffer, run on a 1.2% (w/w) agarose, 2.2 *M* formaldehyde gel, and transferred onto Zeta-Probe GT membrane [10]. The migration position of the rRNA species of the samples in the gels was determined by ethidium bromide staining. In both cases the filters were baked for 1 hr at 80°C in a vacuum oven and then prehybridized at 65°C in 0.25 *M* Na_2HPO_4, pH 7.2, 7% SDS. Hybridization was performed overnight at 65°C with labeled probe in fresh prehybridization solution. Label-

ing of cDNAs was carried out with [α-^{32}P]dATP and [α-^{32}P]dGTP by random priming with the DNA labeling kit from Boehringer Mannheim. The cDNAs used for labeling were either PCR amplification products obtained directly from differential display or inserts cut off from cloned plasmids of the amplification products. Filters were then washed four times in 20 m*M* Na_2HPO_4, pH 7.2, 1% SDS at 55°C for 30 min. All the filters were autoradiographed (Kodak X-Omat XAR-5 films) for periods ranging from overnight for dot-blots to 8–9 days for Northern blots. The hybridization of the RNA samples in dot-blots was estimated densitometrically. Films were scanned using a Discover Pharmacia Scanner equipped with a Sun Spark Classic Workstation. The amount of various RNA used in each set of hybridization experiments was approximately the same (around 12.5 μg). Indeed the relative amounts of RNA extracted from tissues were estimated by absorbance at 260 nm and validated by comparative densitometric measures of ethidium bromide-stained agarose gels. Small corrections for concentration differences were made when necessary.

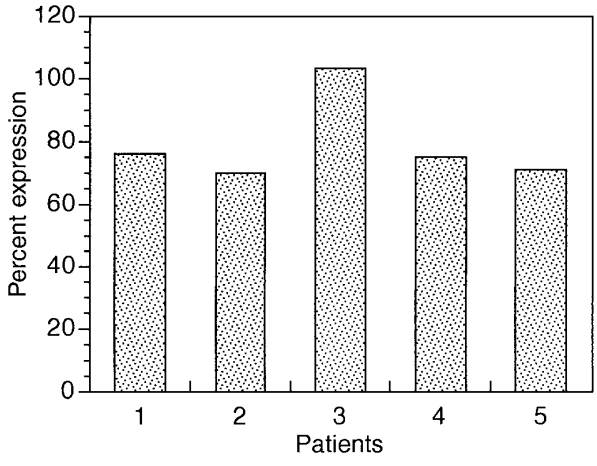

FIG. 1. Differential hybridization of $N_{5.5}$ probe, obtained from normal tissue of patient 1, to RNA preparations from tissues of patients. Values represent the relative expression of the $N_{5.5}$ in tumoral tissue with respect to the normal tissue, both obtained from the same patient. A 25–30% inhibition in the expression of $N_{5.5}$ species is observed in tumoral tissues in all but one case (patient 3). Bars are the average of three separate experiments. Standard deviations are ±0.07, ±0.03, ±0.015, ±0.025, and ±0.03, respectively.

RESULTS

Selection and Cloning of Differentially Expressed mRNAs

Differential display. The screening by differential display was performed to compare the RNA pattern of the normal endometrium from patient 1 to the RNA pattern of the tumoral tissue from the same individual. Forty parallel amplifications were performed using each of the $T_{12}XN$ primers with 1 of 10 different arbitrary decamers. The analysis by several denaturing gel electrophoresis of the amplification products visualized a total of ~3500 bands which are likely to be corresponding to a similar number of different mRNA species. Therefore, assuming ~15,000 as the approximate number of mRNA molecular species in a cell [8], we estimate that our analysis covered between one-fifth and one-sixth of the tissue mRNA species. The comparison of the amplification products obtained for each couple of $T_{12}XN$-arbitrary decamers allowed the detection of 61 differential bands. Bands were selected when they appeared exclusively present or differentially abundant in one of the electrophoretical lanes either from the normal tissue or from the tumor. The further evaluation of the amplification products extracted from gel bands regarded first the possibility of their reamplification, then their grade of hybridization to the original RNA from patient 1, and finally their grade of hybridization to other RNAs also obtained from the other patients. This successive screening allowed the progressive reduction of the number of products. More than two-thirds of the selected original amplification product was discarded either because it did not reamplify or because it did not show differential hybridization when reassayed against the RNAs (from normal and/or tumoral tissue of patient 1) from which they were originated. Indeed, these products were considered possible artifacts derived from the technology [8, 12]. For this reason, these products were not investigated further.

Cloning. Twenty sequences were chosen first on the basis of the criteria stated above; the subsequent cloning was attempted by the pT7Blue plasmid vector. Thirteen species were successfully obtained as individual clones. To pick sequences potentially related with the pathological or normal state, an additional selection criterion was used. In this respect, the ability of the labeled inserts from the cloned plasmids to hybridize to RNA extracts from tissues obtained from other patients with endometrial adenocarcinoma of similar state was evaluated (Table 1). Indeed, the largest number of inserts obtained did not significantly hybridize differentially to most of the samples from the clinical cases under evaluation. Therefore, only clones $N_{5.5}$ and $T_{16.2}$ were finally recognized as the most significant species and were subsequently investigated.

Analysis of $N_{5.5}$ and $T_{16.2}$ mRNAs in Clinical Samples

RNA preparations from normal endometrium and tumor of the clinical cases analyzed were hybridized to the labeled inserts from clone $N_{5.5}$ and $T_{16.2}$ (Figs. 1 and 2). In detail, it appears that the $N_{5.5}$ mRNA (Fig. 1), which was originally identified as a messenger preferentially expressed by the normal tissue from patient 1, is again preferentially expressed in normal compared to neoplastic tissues in all but one patients. Indeed, a small overexpression is observed in tumor 3. The other species, i.e., the mRNA $T_{16.2}$, originally identified as a component of the tumoral tissue from the patient 1, is more abundant in 3 of 4 patients (Fig. 2) studied.

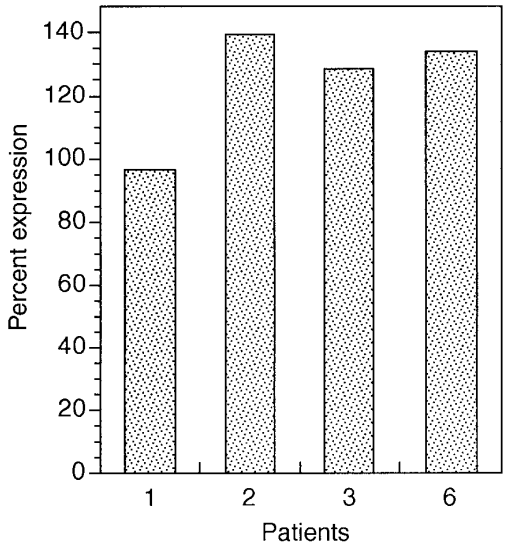

FIG. 2. Differential hybridization of $T_{16.2}$ probe, obtained from neoplastic tissue of patient 1, to RNA preparations from tissues of patients. Values represent the relative expression of the $T_{16.2}$ in tumoral tissue with respect to the normal tissue, both obtained from the same patient. A 30–40% enhancement in the expression of $T_{16.2}$ species is observed in tumoral tissues in all but one case (patient 1). Bars are the average of three separate experiments. Standard deviations are ±0.11, ±0.08, ±0.03, and ±0.07, respectively.

To determine the molecular size of the RNA hybridizing to $N_{5.5}$ probe, a Northern blot analysis of the normal and tumoral samples from patient 2 was also conducted (not shown). This analysis was performed with the RNA preparations from a single patient because only in this case was the amount available enough to carry out the assay. As extrapolated from the migration of the rRNA bands, the $N_{5.5}$ probe recognized an ~25,000-base band and a faint ~1000-base species; the $T_{16.2}$ probe recognized a single band of ~20,000 bases.

Sequencing of $N_{5.5}$ and $T_{16.2}$ Clones

The sequences of the inserts of the plasmids $N_{5.5}$ and $T_{16.2}$ were determined and are reported in Fig. 3. The analysis of the sequences did not reveal significant open reading frames. This is not an unexpected result since the features of this cloning method are inclined to select noncoding 3′-end regions from mRNAs. In fact, the primers used for the reverse transcription reaction to generate cDNA make use of poly(T)-containing oligonucleotides, i.e., transcription is initiated at the level of the mRNA poly (A).

A search through EMBL (European Molecular Biology Laboratory) and GenBank sequence databases (February 1996) indicated that there is no significant sequence homology with any sequence of known function.

DISCUSSION

The diagnosis of endometrial adenocarcinoma is generally consequent to the onset of postmenopausal bleeding, while currently available reference markers for this disease do not allow early diagnoses. In particular, there is no pathologic marker (cytologic atypia and grading) able to preoperatively discriminate which early lesions will develop into endometrial adenocarcinoma and the stage I cancers with lower malignancy, virtually treatable by a more conservative surgery. Nevertheless, it is evident that the biological and morphological differences between neoplastic tissue and the original normal tissue must correspond to a different qualitative and quantitative molecular constitution, particularly at the level of functional and structural proteins and, then, of their relative mRNAs.

This paper describes the cloning and sequencing of two mRNA bands which are apparently overexpressed in normal or neoplastic (adenocarcinoma) endometrium, respectively. So far, we have studied six clinical cases. In detail, the $N_{5.5}$ mRNA is preferentially expressed in the normal endometrium and therefore it is represented to a lesser extent upon transformation of the tissue. This change (decrease), which is remarkably constant, irrespective of the tumor histological grade, has been observed in four of five clinical cases (Fig. 1). In addition, the only observed increase (patient 3 in the figure) is, indeed, very small. In contrast, the $T_{16.2}$ mRNA appears to be preferentially expressed in tumoral tissue, in three of four cases studied (Fig. 2).

The quantitative changes observed for $N_{5.5}$ mRNA may possibly correspond to quantitative variations of its encoded protein. Because this difference has been found by means of a selection among clinical samples having the endometrial neoplasia as a common feature, it seems that the decrease of $N_{5.5}$ mRNA is involved either in the causative or in the consequential molecular mechanisms of the disease. Several antioncogene-encoded proteins have been described in tumors as reduced and their role has been characterized [13]. However, in those cases the identification of the genes has been accomplished on the basis of their function in inhibiting transformation, while in our case a quantitative criterion was used. At the moment, it is not possible to make hypotheses, or even speculate, on the function of the related protein.

$N_{5.5}$

GAAACGGGTG GGTAGTGGTA GTGTACACAC ACTGGAATAA GGGACGATTC
ACAATTTTAG ATCTTCTGAA GTATTTATTT CTGGAATTTT CCATTTAATA
AGATATACAG AGGGGACTAC TGTATGGAAT TAAAATATGT AATTGGAAAA
AACAGGCCTG TCGATCAACT TTTCTATGAA ATCAGAGACT CTAAAGTAGT
GAAAATGTAT TGTTTAATTA GAAATGTAAT AAATAAATGT ATTAAAATAT
TTTGATATCT AACTTAAAAA ATGGATCCAC AGGCACCCGT TTC

$T_{16.2}$

GTGACGTAGG CGGACAGCTA AAGGGAGTGG AGCGGAGAAA AAACTACAAA
CAAGGGTAGT ATAAAATACT CCTACGTCAC

FIG. 3. Nucleotide sequence of $N_{5.5}$ and $T_{16.2}$ clones.

A similar point may be noted about $T_{16.2}$ mRNA, but with the opposite view. The expression of this mRNA is increased in tumors but this does not necessarily mean that it encodes a transforming protein. Upon differential display, this sequence resulted more abundant in tumor in respect to normal tissue from patient 1. However, when the sequence was cloned, its hybridization to RNAs from the same patient (Fig. 2) was slightly higher in normal tissue. It is possible that the originally obtained amplification product was actually a mixture of sequences from which $T_{16.2}$ was cloned, and that the isolated sequence then acts differently as a probe. Such findings are often observed when using the differential display technique as confirmed by both our personal experience and the current literature [12, 13]. In any case, once isolated, the sequence results increased in the majority of the clinical cases analyzed and, also in this instance, this suggests its possible involvement in tumor development mechanisms.

An apparent limitation of this work regards the limited number of cases studied: indeed, although the number of apparently available samples was quite high, we have restricted the analysis to only the few samples whose state (handling and/or storage of the material from the time of excision to our lab bench, histological analysis) was sufficiently definite and clear. Also under these conditions, the use of tissues derived from surgical specimens to be analyzed by means of differential display is biased by the contamination from different cellular types (stromal cells, macrophages, etc.). This means that the selection of a suitable material is particularly difficult; notwithstanding, we expect a variety of interesting outcomes from the study of a larger clinical sample, as long as this will be available. A few relevant considerations sustain this point. The clinical cases collected for this study are fairly homogeneous in terms of progression of the disease and, at the same time, the amounts of assayed $N_{5.5}$ and/or $T_{16.2}$ mRNA do not correlate with the histological grade of the neoplasia (Table 1). In addition, the strategy adopted in this work, consisting of combining two separate criteria for selecting differential RNAs, has been designed to reduce the chance of possible artifacts. The first directly looks for differential messenger RNAs within tissues obtained from the same patient (namely patient 1, see Table 1). The second proceeds to the selection of those messengers which result differentially with respect to other clinical cases. The RNAs which were found to be altered within the transformation of a normal tissue of a single individual do not necessarily reflect a general phenomenon of that type of neoplasia. Each tumor is in fact a single complex event affecting directly or indirectly many genes and their products, although similar clinical cases can be classified as matching for staging, grading, etc. Therefore, it might be expected that the simultaneous comparison of the RNA patterns of normal and tumoral tissues from the same individual may also select species which are different because of the individual variability of the tumor and not necessarily because of their relationship with the more general events of endometrial adenocarcinoma development. However, the comparison of the RNA patterns of normal and tumoral tissues from several individuals should identify, in addition to tumor-related sequences, also species which are differential only as a consequence of general individual variability. For those reasons we considered that it was more convenient to use and combine both selection criteria. This strategy should also increase the possibility of getting rid of all the sequences produced from the technical artifacts often observed making use of differential display.

The comparison of the two sequences with the EMBL and GenBank databases did not allow identification of their mRNA and making any hypothesis on their function because they did not show any similarity to any known sequence. Nevertheless, the finding that the expression of $N_{5.5}$ is reduced in neoplastic tissue may suggest that the encoded protein is an endometrial cellular marker of normal state.

As soon as a large number of suitable specimens become available to us, the significance of the differences observed, studying $N_{5.5}$ and $T_{16.2}$ mRNAs as clinical markers, will be further evaluated.

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