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Detection of Differentially Expressed Genes in Methylnitrosourea-Induced Rat Mammary Adenocarcinomas

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Abstract In this study, altered gene expression in five methylnitrosourea (MNU)-induced rat mammary adenocarcinomas was investigated using a newly developed competitive cDNA library screening assay. In order to detect the differentially expressed cDNA transcripts, three cDNA libraries (rat mammary, rat liver, and rat kidney) with over 18,000 clones were differentially screened with competing normal and neoplastic mammary cDNA probes. Ninety-eight clones indicated by competitive hybridization to be differentially expressed in tumors were verified by dot-blot hybridization analysis. Of these clones, 45 were found to be overexpressed while 53 were underexpressed in tumors. Forty-five of the confirmed clones were further analyzed by single-pass cDNA sequence determination. Four clones showed homology with cytochrome oxidase subunit I, polyoma virus PTA noncoding region, cytoplasmic beta-actin, and mouse secretory protein containing thrombospondin motifs. Further investigation into the potential roles of these identified genes should contribute significantly to our understanding of the molecular mechanism(s) of rat mammary tumorigenesis. J. Cell. Biochem. Suppls. 28/29:117–124.

Key words: rat; mammary tumor; gene expression; competitive cDNA library screening

Breast cancer affects one in eight women during their lifetime; more than 180,000 women in the United States (US) are diagnosed with this disease each year [1]. Despite advances in early detection and treatment, the mortality rate of breast cancer has remained the same for the last few decades [1]. Genetic alterations detected in human breast cancer include cancer susceptibility genes BRCA1 and BRCA2, oncogenes *int*-2, c-*myc*, EGF-R, *neu*, cyclin D1, and *Erb*-B, and tumor suppressor genes p53 and Rb. Allelic loss on chromosomes 1p & 1q, 3p, 11p, 13q, 17p, 17q, 18q has also been observed non-randomly, suggesting the involvement of tumor suppressor loci at these locations [2]. In addition to genetic changes, a new class of genes was found to be differentially expressed in breast tumors [3], and most of these genes are not mutated. Differentially expressed genes found in human breast cancer cell lines include: maspin, elafin, caveolin, Hbp17, Cx26, Cx43, FLW1, RARβ, S100L, GSTπ, α6 integrin, new protease, new cystatin, aldose reductase, parathyroid-like, protocadherin, mac 25, variant CD44, ZZ#6, ZZ#17, ZZ#38U, ZZ#38L, and ZZ#43 [3–9]. These genes are probably altered at the transcription level, and may play an important role in breast cancer through elaboration of phenotypic alterations. Identifying quantitative changes in gene expression in malignant tumors is not only significant in elucidating the mechanism(s) of mammary carcinogenesis but is also useful in generating markers for diagnosis, prognosis, treatment, and prevention of the disease.

The rat model involving the induction of mammary tumors with methylnitrosourea (MNU) is a well-established animal model for human breast cancer [10,11]. For example, the terminal end buds from which rat mammary tumors originate are histologically similar to the primary site (terminal ductal units) of human breast cancer. The majority of rat mammary

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tumors are estrogen-receptor positive and respond to treatment with ovariectomy. MNU-induced rat mammary tumors have also been shown to respond to many of the agents known as effective treatments for human breast cancer [12]. The objective of the present study was to identify and characterize genes with abnormal levels of expression in MNU-induced rat mammary tumors, to further the understanding of breast carcinogenesis and development of biomarkers for breast cancer prevention and treatment.

MATERIALS AND METHODS

Rat Mammary Tumors

Virgin female 35-day-old Sprague-Dawley rats were obtained from Harlan/Sprague-Dawley (Indianapolis, IN). The animals were given Teklad 4% rat/mouse chow as the standard diet. After a quarantine period of 1 week, animals were randomized by weight in control and treatment groups. At 50 days of age, all rats received a single i.v. injection of MNU in acidified saline (pH 5.0) at a dose of 50 mg/kg body weight. Rats that were moribund during the study were sacrificed by CO_2 asphyxiation. The remaining rats were killed at 150 days of age and the mammary tumors were weighed and frozen until RNA analysis.

RNA Isolation

Total RNA was isolated as previously described [13]. The quantity and purity of the RNA was determined spectrophotometrically at wavelengths of 260 nm/280 nm, and the quality of the RNA was assessed by electrophoresis on a formaldehyde agarose gel.

Labeling of cDNA by Reverse Transcription (RT)

For hybridization probe set no. 1, 2 µg of total RNA from a rat mammary tumor was labeled with [α-^{32}P]dCTP during the first strand cDNA synthesis from oligo-dT primer, and mixed with a competing unlabeled cDNA made from 2 µg of RNA isolated from normal mammary glands. For hybridization probe set no. 2, 2 µg of total RNA from a normal mammary gland was labeled with [α-^{32}P]dCTP, and used to compete with a cold cDNA probe made with 2 µg of total RNA isolated from a normal mammary gland. The cDNA labeling conditions were as follows: 2 µg of total RNA was mixed with a oligo-dT primer (Promega, Madison, WI) in 10 µl of 2 x reverse transcription buffer (40 mM Tris-HCl, pH 8.4, 10 mM KCl, 5 mM MgCl2, 10 mM DTT, 0.2 mg BSA, 0.5 mM of dATP, dGTP, dTTP, and 0.45 mM of either 10µCi [α-^{32}P]dCTP or 0.5 mM cold dCTP) containing 200 U of superscript II reverse transcriptase (BRL-Life Technologies Co., Gaithersburg, MD). The reaction mixtures were incubated at 37°C for 1 hour. Prior to hybridization, the probes were column (G50) purified.

cDNA Libraries as Targets for Competitive Hybridization

Three rat cDNA libraries (mammary, liver, and kidney) were used as targets. Each library contains approximately 1.5 million clones. A total of 60 plates with 300 clones on each plate was used with 20 plates from each library. These libraries were plated and amplified on LB plates, and transferred to nitrocellulose filters (2 replicas were made for each plate) which were denatured in 1.5 M NaCl/0.5 N NaOH, and then neutralized in 2.5 M NaCl/1 M Tris (pH 7.4). Replicas were hybridized either in a solution containing the labeling mixture for hybridization set no. 1 or 2. Hybridization conditions were as follows: 5 x SSC, 1 x Denhardt's solution, 100 µg herring sperm DNA, 50% formamide, and cDNA probes (labeled and competing unlabeled). The hybridization was allowed to proceed at 42°C overnight with shaking. Washing stringency was 1 x SSC/1% SDS 3 times for 20 minutes at room temperature, followed by 0.1 x SSC/0.1% SDS twice for 30 minutes at 55°C. Filters were then exposed to X-ray film for 1–3 days at −80°C.

Dot-Blot Analysis

Clones found to be differentially expressed in mammary tumors were selected for confirmation using dot-blot analysis. Small-scale isolation of the clones from rat mammary plasmid cDNA libraries was performed as described [13]. Clones from phage cDNA libraries (liver and kidney) were isolated by using the wizard lambda preps DNA purification system (Promega, Madison, WI). Approximately 2 µg DNA was denatured by adding 0.1 volume of 2 M NaOH, 2 mM EDTA, and neutralized by adding 0.1 volume of 3 M sodium acetate (pH 5.0). The denatured DNA from these clones was spotted onto nitrocellulose membranes using the HYBRI.DOT Manifold Apparatus (Life Technologies Co.). Two identical membranes were made for each set of clones. These membranes

were hybridized with probes prepared as described above for differential screening of cDNA libraries.

DNA Sequence Analysis

Candidate clones of differentially expressed genes were sequenced directly using phage DNA as template and the fmol sequencing kit (Promega) as suggested by the manufacturer. Plasmids were sequenced with a Sequenase version 2.0 DNA sequencing kit purchased from USB (Cleveland, OH). In both cases, the sequencing primers were synthesized to match the cloning vector arms. DNA sequences from these clones were submitted to the NIH/GenBank/blast program for comparison with known sequences.

Northern Blot Analysis

Approximately 15 µg of total RNA was separated on a formaldehyde/formamide denaturing 1.2% agarose gel and subsequently transferred to a nitrocellulose membrane. The membrane was hybridized with probes specific to individual differentially expressed clones. Clones were labeled with [α-^{32}P]dCTP by random prime labeling with a Multiprime DNA labeling system (Amersham Inc., Arlington Heights, IL). Membranes were then washed twice in buffer containing 0.1% SDS and 0.1 x SSC, performing each wash step at 55°C for 30 minutes. The membranes were exposed to X-ray films and the autoradiograms were quantitated by densitometry (Shimatzu Dual-Wavelength TLC scanner CS-930).

Quantitative RT-PCR

Two µg of total RNA were used to synthesize cDNA in a total volume of 30 µl. After incubating the RNA in DEPC-treated water at 65°C for 10 minutes, the following components were added: 45 nM oligo-dT, 1 µl of 5 x reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl), 0.6 µl of 50 U/ul RNase inhibitor, and 2 µl of 200 U/µl M-MLV reverse transcriptase (BRL-Life Technologies Co., Gaithersburg, MD). The reaction mixture was incubated at 37°C for 1 hour, after which it was terminated by heating at 95°C for 10 minutes. A 1 µl aliquot was used to perform a multiplex PCR (18 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute) in 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1 µM downstream clone-specific primer, 1 µM upstream clone-specific primer, a pair of primers specific for the GAPDH gene, and 0.025 U Taq DNA Polymerase (Promega). The gels were quantitated following the same procedure as for Northern blot analysis.

RESULTS

All of the MNU-induced rat mammary tumors were diagnosed as adenocarcinomas. Five of the tumors were used to screen for differentially expressed genes against normal mammary glands by using the Competitive cDNA Library Screening (CCLS) assay. CCLS is a procedure in which the cDNA from tumor tissue is labeled radioactively and used in cDNA library screening in competition with the cDNA made from normal tissue and vice versa. Equal amounts of both cDNAs are used in a competitive hybridization reaction. After extensive washing, the hybridized filter will then be analyzed for a quantitive difference. Figure 1 shows a typical CCLS assay of a rat mammary tumor. After CCLS screening, 309 clones were found to have altered expression in rat mammary tumors. Further confirmations were found to be necessary to avoid false-positive clones identified during the first round of screening by CCLS. Of the 309 clones, 136 more distinctive clones were selected for further analysis. Ninety-eight of these clones were confirmed by dot-blot hybridization analysis. Figure 2 shows a competitive dot-blot analysis of the differentially expressed clones. Following dot-blot analysis, two clones, T311 and T1811, were also subjected to Northern blot analysis. As shown in Figure 3, clone T311 was overexpressed in all five tumors (Lanes T1 to T5; 2.6- to 13.2-fold) but absent (Lane N1) or underexpressed (Lane N2) in normal tissues. Clone T1811 was overexpressed in three of five tumors (Lanes T1, T3, and T4; 3.5- to 6.0-fold). Forty-five clones were subjected to sequence analysis. Additional clones were analyzed using quantitative RT-PCR. As shown in Figure 4, both clone T611 (3/5 tumors; Lanes T1 to T3; 2.1- to 5.3-fold) and IIIT155 (5/5 tumors; Lanes T1 to T5; 2.5- to 4.5-fold) were overexpressed in rat mammary tumors when GAPDH was used as a control. As summarized in Table I and Figure 5, all four overexpressed clones have sequence homologies with known genes in the GenBank. They are rat cytochrome oxidase subunit I (T311), polyoma virus PTA noncoding region (T611), rat cytoplasmic beta-actin (T1811), and mouse secretory protein containing thrombospondin motifs (IIIT155).

TABLE I. Detection of Genes Differentially Expressed in MNU-induced Rat Mammary Adenocarcinomas

No.	Clone	Sequenced Length(bp)	Putative identification	Identity
1	T311	172	Rat cytochrome oxidase subunit I	99%
2	T611	89	Polyoma virus PTA noncoding region	100%
3	T1811	341	Rat cytoplasmic beta-actin	99%
4	IIIT155	167	Mouse secretory protein containing thrombospondin motifs	91%

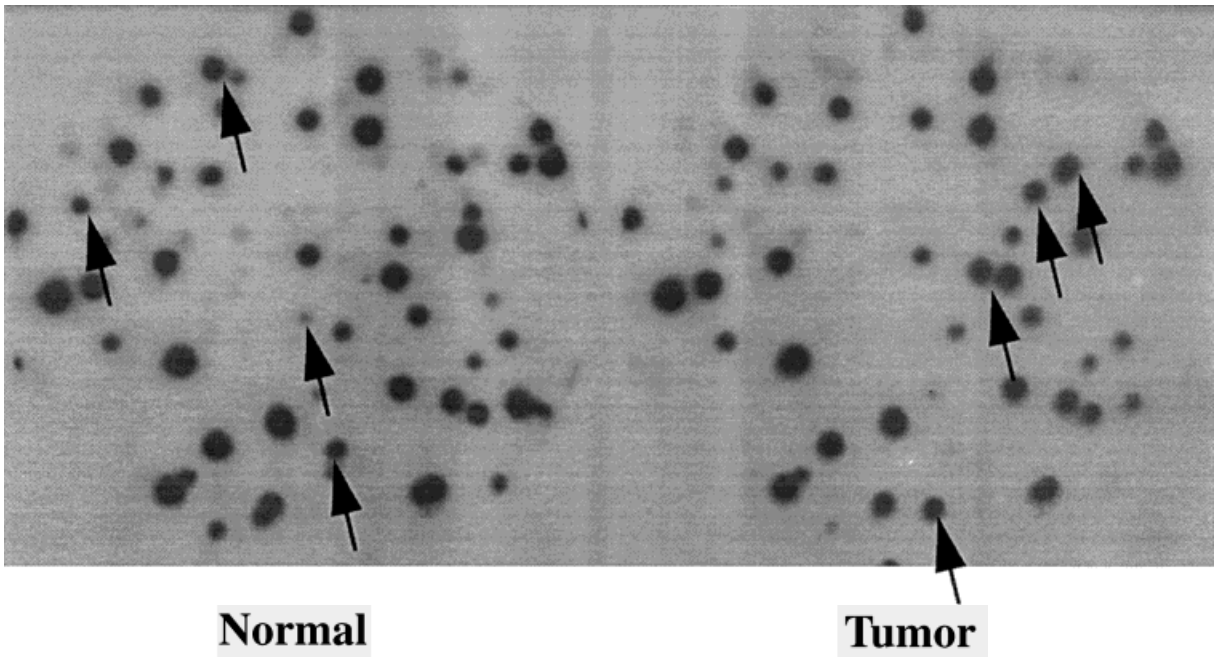

Fig. 1. Competitive cDNA library screening of rat mammary adenocarcinomas. Arrows indicate clones that are differentially expressed in a tumor as compared to its normal surrounding tissue.

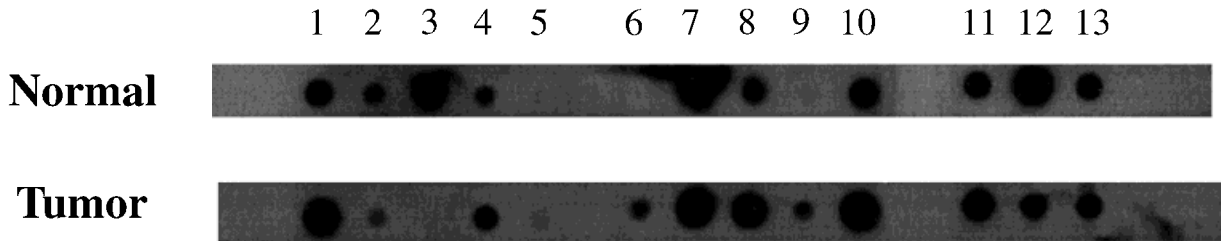

Fig. 2. Dot-blot analysis of differentially expressed clones. **Lanes 1**, T1811; **2**, N 641; **3**, WH511; **4**, T932; **5**, T611; **6**, T1511; **7**, T332; **8**, T311; **9**, T513; **10**, T621; **11**, T211; **12**, N321; and **13**, T111.

DISCUSSION

In this study, we have shown that CCLS, a modified form of the microarray technique, is a powerful tool for screening differentially expressed genes in tumors. Using CCLS, 309 differentially expressed clones were detected in MNU-induced rat mammary tumors. Forty-five clones were partially sequenced. Twenty-five of the 45 clones were overexpressed and 20 were underexpressed in rat mammary tumors. Four of the overexpressed clones were identified by homology with cytochrome oxidase subunit I, polyoma virus PTA noncoding region, cytoplasmic beta-actin, and mouse secretory protein containing thrombospondin motifs (Table I, Fig. 5). Further evaluation of these clones for their potential role in rat mammary tumorigenesis is underway.

Several techniques have been developed for screening differentially expressed genes in tu-

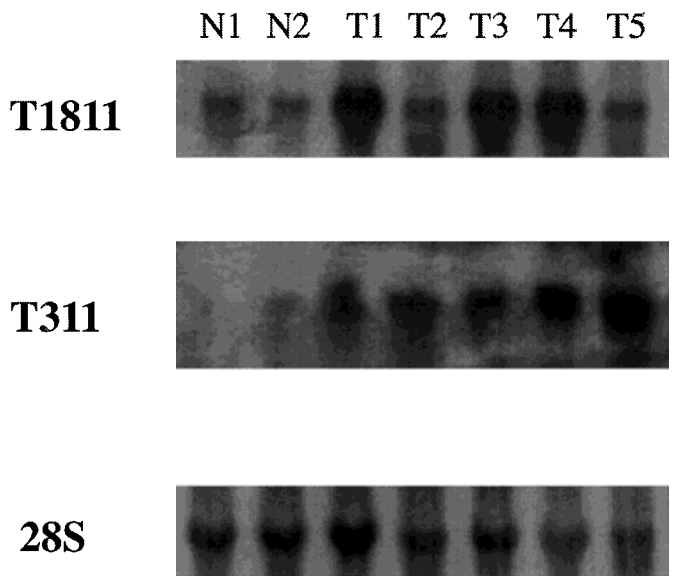

Fig. 3. Northern blot analysis of clones T311 and T1811. **Lanes N1, N2**, normal rat mammary tissues; **T1–T5**, MNU-induced rat mammary adenocarcinomas. Clone T1811 was used as the probe (**top**) and clone T311 was used as the probe (**middle**). **Bottom:** Loading control (28S rRNA).

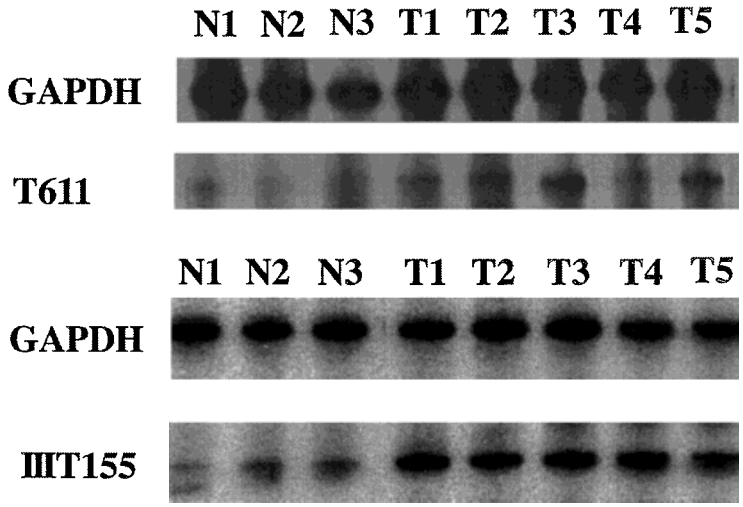

Fig. 4. RT-PCR analysis of clones T611 and IIIT155. **Lanes N1–N3**, normal rat mammary tissues; **T1–T5,** MNU-induced rat mammary adenocarcinomas. PCR primers specific for GAPDH were used as internal control.

mors, such as differential display [14–19], subtractive hybridization [20–21], serial analysis of gene expression (SAGE) [22–24], and microarray assay [25–28]. Differential display and subtractive hybridization have been used successfully to detect differentially expressed genes in rat mammary tumors [21,29,30]. Using the differential display technique, Lu et al. identified five overexpressed genes in MNU-induced rat mammary carcinomas which include: galectin-7, human melanoma inhibitory activity protein/bovine chondrocyte-derived retinoic acid sensitive protein, mouse endo B cytokeratin/human cytokeratin 18, mouse/human DNA primase small unit, and mouse stearyl-CoA desaturase-2 [29]. Similarly, Yoo et al. detected the RMT-1 gene which was overly expressed in 33 of 38 rat mammary tumors [30]. Using the substractive hybridization, Young et al. reported that calcyclin and a rat endogenous retrovirus were overexpressed, while a cDNA isolated from HL-60 cells and transferrin were underexpressed in rat mammary tumors [21].

As shown in Table I and Figure 5, four clones were homologous to known genes. Clone T311 is nearly identical (99%) to rat cytochrome oxi-

A

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T311      aagtactctcaatttcctactcttcaactaacctagaatgactgcatggatgccccccac    60
COXI      ....G.. ..................................................  2918

T311      cttaccacacattcgaagaaccttcctatgtaaaagttaaataagaaaggaaggaatcga   120
COXI      ............................C...............................  2978

T311      ccccctacaactggtttcaagccaatttcataaccattatgtctttctcaat           172
COXI      ..................................................            3030
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B

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T611      ttttgcaagaggaagcaaaaagcctctccacccaggcctagaatgtttccacccaatcat    60
P.V.      ..........................................................    265

T611      tactatgacaacagctgtttttttagta                                   89
P.V.      ............................                                  314
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C

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T1811     catagatggttacaggaagtccctcaccctcccaaaagccacccccaactcctaagggga    60
β-actin   ..........................................................  3515

T1811     ggatggctgcatccatgccctgagtccacaccggggaaggtgacagcattgcttctgtgt   120
β-actin   ................................-.............................  3574

T1811     aaattatgtac-tgcaaacatttttttaaatcttccgccttaatacttcatttttgtttt   179
β-actin   ...........T..................................................  3634

T1811     taatttctgaatggtcagccattcgtggcctgccccttttttttgtccccccaacttgat   239
β-actin   ..........................................................  3694

T1811     gtatgaaggctttggtctccctgggagtggtttgaggtgttgaggcagccagggctggcc   299
β-actin   ..............................................-.............  3753

T1811     tgtacactgacgtgagaccgttttaataaaagtgcacacctt                     341
β-actin   ....................................                        3795
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D

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IIIT155   tgtctgtcccatgatggcggtgtgttatcaaatgagagctgtgatcctttgaagaaa-cc    59
ADAMTS-1  ...G.......C.....G..C.................................G..  3253

IIIT155   aaagcattacattgacttttgcatactgacacagtgcagttaagaggtttt-gag-acaa   117
ADAMTS-1  ......................C......................CG..A...G....  3313

IIIT155   tgtagcgtggg-aggg-ctgatactactgaaagcaagagtgctggagggatc           167
ADAMTS-1  G..........G....G.......-.....GT........A...........          3364
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Fig. 5. Comparison of nucleotide sequence between genes identified in this study and known sequences in Genbank. **A:** Clone T311 and rat cytochrome oxidase subunit I. **B:** T611 and polyoma virus PTA noncoding region. **C:** T1811 and β-actin. **D:** IIIT155 and mouse ADAMTS-1.

dase subunit I(COX I). COX I is involved in cellular mitochondrial energy metabolism. The expression of the gene encoding COX I has been found to be elevated in several tumor types, presumably due to increased aerobic glycolysis rate in tumor cells [31,32]. T611 was found to have 100% homology with the Polyoma virus PTA noncoding region. Polyoma virus, a DNA virus, has been shown to transform normal cells to malignant cells and induce cancer in experimental animals [33]. Three early proteins expressed by polyoma virus contribute to

the malignant transformation. Cahill and Carmichael and Freund et al. [34,35] reported that a PTA noncoding region acts to increase tumor frequencies and diversity. Overexpression of T611 in mammary adenocarcinoma suggests that it is a potential oncogene.

The clone IIIT155 is approximately 91% homologous to mouse secretary protein containing thrombospondin motif, also named as adisintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-1) [36]. Mouse ADAMTS-1 was selectively expressed in cachexigenic tumors and its expression can be enhanced by IL-1 stimulation [36]. ADAMTS-1 is a putative secretary protein and the thrombospondin type I motif in ADAMTS-1 can bind to heparin [36]. Thus, ADAMTS-1 may function through its incorporation into extracellular matrix via interaction with sulfated glycoconjugates (such as heparin and heperin sulfate) [36]. Our observation that expression of a gene that is homologous to mouse ADAMTS-1 is elevated in rat mammary adenocarcinomas provides further evidence that ADAMTS-1 and its homologues may play an important role in carcinogenesis and inflammation [36]. T1811 is nearly identical to β-actin, which is potentially associated with tumorigenesis [37]. In our present study, most of the differentially expressed genes detected in rat mammary adenocarcinomas have not been previously identified. These novel changes in gene expression may provide significant insights into the mechanism(s) of mammary tumorigenesis.

One of the direct applications of the CCLS technique is the development of surrogate endpoint biomarkers for clinical trials in cancer chemoprevention [38,39]. Chemopreventive agent-specific biomarkers can be identified in tumor tissues treated with the agent. For example, Ariazi and Gould identified several differentially expressed genes in MNU-induced mammary tumors treated with monoterpene using a subtractive display assay [38]. Genes induced by monoterpene included: cytochrome c oxidase subunit II (COX II), lipocortin 1, calmodulin, sperm membrane protein, TGF-β type II receptor, calcium transporting ATPase, IGF II receptor, and fast myosin alkali light chains; neuroligin 1 was repressed by treatment with monoterpene [38]. Recently, we used the CCLS technique to screen differentially expressed genes in MNU/dehydroepiandrosterone-induced rat mammary tumors [40]. Five genes were up-regulated and three genes were down-regulated by treatment with dehydroepiandrosterone. One of the repressed genes was homologous to *N*-acetylglucosaminyl-transferase, which has been found to be down-regulated in several other cancer types, including melanoma, colon cancer, and lymphoma [40]. Thus, genes differentially expressed by treatment with chemopreventive agents may serve as chemopreventive agent-specific markers in the rat mammary tumor model.

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