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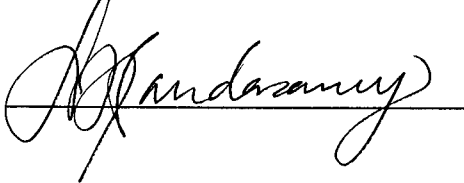DTIC QUALITY INSPECTED 4

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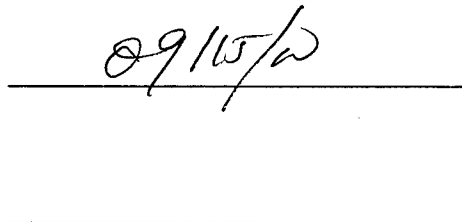
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13. ABSTRACT (Maximum 200 Words)

The purpose of the reported research was to identify novel genes whose differential pattern of expression could serve as useful diagnostic markers for human breast cancer. Using modifications of the differential display polymerase chain reaction technique, we successfully implemented a moderate-throughput method for isolating and directly sequencing novel cDNA fragments. An initial set of 150 cDNA fragments corresponded to mRNAs that were differentially expressed among five different human breast tumor cell lines. Sequence information from eight of these cDNAs was used to generate gene-specific primers. A semi-quantitative, multiplexed RT-PCR assay demonstrated that each of the eight genes had a unique pattern of expression among four, otherwise indistinguishable primary human breast tumors. This work has resulted in methodology to isolate, catalog, and characterize sets of differentially expressed genes in microdissected, primary human breast tumors. A manuscript in preparation and subsequently awarded funding will continue this work toward the goal of developing novel breast tumor markers.

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5. Introduction

Our laboratory is interested in identifying quantitative changes in gene expression that correlate with significant clinical parameters of human breast cancer. We had previously demonstrated that, using several modifications of the differential display polymerase chain reaction technique (DDPCR), we were able to create a moderate throughput system that can rapidly identify Differentially Expressed Sequence Tags (DESTs) from primary human breast tumors (1). These DESTs are cDNA fragments corresponding to mRNAs that are differentially expressed in breast tumors as compared to patient-matched normal breast tissue. A catalog of these sequences should provide a potential list of new molecular markers for phenotyping breast tumors, particularly if they are correlated with clinical information about the tumor and patient from which they were derived. In fact, we had already demonstrated that one DEST obtained in this manner is a potentially useful new molecular marker for breast cancer (2). In the report of this research project, we describe our progress in adopting our preliminary work toward the creation of a breast tumor DEST catalog, similar in scope to EST libraries that are now in existence, but with added clinical relevance to human breast cancer. We believe the results of this work will ultimately define a set of gene sequences whose unique pattern of expression ('expression fingerprint') could collectively define a breast tumor with regard to its metastatic potential, therapeutic vulnerability, and prognostic significance to the patient.

6. Body

Task 1: Isolate RNA from 12 initial breast tumor/normal pairs. Synthesize first strand cDNAs. Use these cDNAs to isolate at least 50 different DEST sequences using 40 combinations of primer pairs. Identify technical problems and modify protocol. Enter DEST sequences into DEST database.

Because of considerable technical difficulties, we elected to focus our initial efforts on identifying gene sequences that were differentially expressed among five human breast tumor cell lines. Although the cell lines were potentially less relevant to primary human breast cancer, they provided an unlimited, homogenous resource of breast cancer RNA with which to optimize our protocols.

The cell lines indicated in **Table I** were obtained from the American Type Culture Collection and grown in media formulations as recommended by the supplier. Approximately 1×10^6 cells from each cell line were isolated in duplicate. From each pair of cell samples, 5 μg of total RNA was purified, treated with RNase-free DNase, reverse transcribed, degraded with RNaseH and DNase-free RNase, and purified by spin chromatography. After considerable experimentation with different cDNA synthesis and purification schemes, we found that these steps of first strand cDNA preparation were absolutely necessary to produce reliable and reproducible DDPCR displays.

Synthesized cDNA pools from each cell line were subjected to the DDPCR technique as described previously (3, 4) except for several notable changes. First, we investigated the use of several different ^{32}P radiolabled 'Tmer' oligonucleotides to generate labeled PCR products. We compared a set of four degenerate $T_{11}VN$ oligonucleotides (where V is a mix of A, G, and C bases and N is either A, C, G, or T), a set of three specific $T_{11}N$ oligonucleotides, and a set of six (of a total possible of 12) specific $T_{11}NN$ oligonucleotides. After optimizing annealing temperatures and cycling conditions for each primer set, we concluded that the set of four degenerate $T_{11}VN$ oligonucleotides produced the most specific and reliable display pattern.

Each of the $T_{11}VN$ oligonucleotides also contained a 10 nucleotide 5' 'anchor' sequence corresponding to the T7 RNA polymerase promoter for the purposes of secondary amplification of isolated PCR fragments. End-labeling oligonucleotides with T4 polynucleotide kinase (as opposed to incorporation of ^{32}P-labeled dCTP) produced superior results in terms of pattern reproducibility and theoretically ensured that all labeled (and hence visualized) PCR products were the result of priming from mRNA polyA tails. The second (10mer) primer employed in the PCR reaction was an arbitrary, ten nucleotide sequence with an additional ten nucleotide 5' 'anchor' sequence corresponding to the M13 origin of replication sequence. A number of different arbitrary sequences were incorporated in the '10mer' primer, all of which were devoid of palindromic sequences and maintained a G/C content of 60%. Although the pattern of amplified fragments differed using each '10mer' primer (see **figure 1a**), the overall performance of the reactions were not significantly affected by primer sequence composition. After considerable trial, a set of four degenerate 'Tmer' and seven '10mer' primers were established for all future work (**Table II**). Finally, conditions were optimized such that reactions could be reliably performed in a 10 µl volume and loaded directly on a 4% denaturing acrylamide sequencing gel for preparative analysis.

For one given experiment, duplicate first strand cDNA reactions were performed with each human breast tumor cell line as described above. A single display experiment involved amplification and electrophoresis of 10 duplicated pairs of cDNA samples using a single 'Tmer' primer in combination with three different '10mer' oligonucleotides (10x1x3=30 total samples). Each experiment was repeated in triplicate to verify the reproducibility of the display pattern. A representative display is shown in **figure 1a**. Experiments were then repeated for each of the seven '10mer' primers permuted with each of the four 'Tmer' sequences described above. A total of 154 bands were isolated. Potentially relevant DEST fragments were selected for reamplification, sequencing, and entry into a DEST database using a set of strict criteria. These criteria were: i) Fragment absent (loss of expression) in duplicate cDNA reactions from at least two different cell lines or ii) Fragment present (gain of expression) in duplicate cDNA reactions from at least one different cell line. In addition, fragments chosen for further analysis could not be present in the same cell line cDNA reactions that employed two different '10mer' primers. This criteria ensured that the amplified band was synthesized by the priming of both the specific '10mer' primer and specific 'Tmer', rather than the 'Tmer' alone. Bands resulting from 'Tmer'-only priming events are not candidates for direct PCR sequencing since their ends are symmetrical.

Candidate bands were excised from their acrylamide sequencing gel, eluted, and reamplified in a standard polymerase chain reaction using the original '10mer' and 'Tmer' oligonucleotide primers employed for DDPCR. We examined several other strategies that employed nested primer approaches to affect secondary amplification, but found that this was unnecessary. Overall, 86% of isolated bands could be reamplified (**Table III**). Reamplified bands were quantitated and qualitatively assessed by agarose gel electrophoresis. Approximately 20 ng of each secondarily amplified PCR product was subjected to cycle sequencing using a ^{32}P end-labeled oligonucleotide corresponding to the '10mer' used for the original DDPCR reaction. We again examined the use of nested sequencing primers to enhance sequencing read, but found that this strategy did not improve results.

Although initial results were promising, only 49 of the 154 isolated fragments were finally amenable to direct sequence analysis (**Table III**). **Figure 1b** demonstrates representative sequence results. Although many bands yielded excellent sequence information, the majority of

the bands yielded either no sequencing read or multiple, superimposed sequencing ladders. Multiple strategies to purify the amplified PCR products did not improve sequence quality. Failure to obtain sequence read was attributed to: *i)* multiple different sequences represented in the amplified PCR fragment; *ii)* generation of PCR artifacts that resulted in fragments without the corresponding forward sequencing primer site. In either case, since it was highly undesirable to clone and purify individual fragments, fragments that could not be directly sequenced were 'discarded.' After all attempted sequencing was complete, 37 unique sequences had been obtained (**Table III**).

Isolated sequence information was compared to the NCBI Genbank database (5, 6) to determine whether a DEST fragment represented a previously characterized gene, and was then entered into a computer database designed in Microsoft *Access* format (see below). A complete list of isolated and identified DEST fragments and their differential pattern of expression is presented in **Table IV**.

Task 2: Establish format for DEST database.

A Microsoft *Access* based database was created to allow the entry of DEST information. The database architecture was designed based on an existing database used for our institution's Tumor Repository and modified to accommodate DEST expression and sequence information. The object architecture is diagrammed in **figure 2a** and a sample data entry screen is shown in **figure 2b**. Because the data set is still very small, the database has not been made generally accessible. However, as our 'catalog' of sequences grows, we plan to make this information available via a web-accessible database server.

Task 3: Prepare RNA and first strand cDNA from at least 40 additional tumor/normal breast tissue pairs. Use these cDNAs to isolate at least 200 different DEST sequences using 40 combinations of primer pairs. Enter DEST sequences, clinical data, and pathology data into DEST database.

To properly conduct accurate studies of gene expression in primary solid tumors such as breast cancer, it is extremely important to isolate the malignant cells of interest from other cell types within the tissue such as fibroblasts, adipocytes, lymphocytes, and endothelial cells. To this end, a major focus of this task has been to use the *Pixcell II* laser capture microdissection instrument to isolate RNA from microdissected breast tumor specimens.

Snap frozen tumor specimens were cryosectioned at 6 μm, placed on uncharged glass slides, post-fixed in 70% ethanol, and lightly stained with Mayer's hemotoxylin and Eosin as previously described (7). After air drying without cover slips, sections were visualized on the *Pixcell* II Laser Capture Microdissection Instrument (Arcturus Engineering). Clusters of breast tumor cells were transferred to *Capsure* transfer film using a 15 μm laser spot size and 30 mW laser power. For each sample, 1000 laser 'shots' (corresponding to approximately 5000 malignant epithelial cells) was dissected and captured onto the transfer film. **Figure 3a** demonstrates three examples of this process. Microdissected tissue was eluted from the transfer film using RNA lysis buffer and total RNA was isolated using the Micro RNA Isolation kit (Stratagene) and by following the manufacturer's protocol. The entire RNA sample from each microdissected tissue was converted to cDNA using the SensiScript System (Qiagen) and following the manufacturer's protocol. Five percent of each cDNA was then used in a standard

PCR reaction with a primer pair specific for the glyceraldehyde phosphate dehydrogenase (GAPDH) gene. The success of tissue microdissection, RNA recovery, and cDNA synthesis was judged by successful amplification using the GAPDH primers (8).

A major technical difficulty in this task has been the ability to routinely generate viable cDNA from microdissected tissue. As shown in **figure 3b,** successful amplification from microdissected tissue was highly variable. We therefore examined, different staining and tissue processing procedures, different RNA isolation protocols, and different cDNA synthesis kits to develop an optimal protocol for synthesis of cDNA from microdissected breast tumor tissue. At this point, we have now established a fairly robust protocol and we are proceeding with **task 3** (with funding from a new grant award) as originally described. All cDNA samples from microdissected tumors that are successfully amplifiable with GAPDH will be used for the differential display technique as described in **task 1**.

Task 4: Synthesize RNA standards. Create preliminary array of 44 DEST sequences. Test hybridization conditions and quantification of gene expression using radiolabled cDNAs from 2-5 sets of breast tumor/normal pairs. Identify technical problems and modify protocol.

Again, to properly conduct these expression studies in primary breast tumors we immediately realized that it would be necessary to microdissect tumor cells from surrounding non-malignant, non-epithelial cells present in breast tumor tissue. Unfortunately, the resulting quantities of cellular RNA obtained from this approach (50-100 ng) would be far too little to effectively hybridize to a cDNA array. Therefore, without changing the aim or scope of **task 4-6**, we modified the experimental approach to increase the likelihood of obtaining meaningful results. Rather than using a hybridization-based approach to quantitate DEST gene expression in breast tumor / normal pairs, we used a semi-quantitative (soon to be quantitative) RT-PCR assay to quantitate gene expression among cohorts of breast tumors.

From the 37 DEST sequences isolated in **task 1**, seven sequences were used to develop gene specific primers for performing multiplexed, semi-quantitative RT-PCR. Each gene-specific primer pair was designed to generate a uniquely sized PCR product (**Table V**). Initially, RNA from six human breast tumor cell lines (five of which were used in **task 1**) were used in an RT-PCR assay with all seven pairs of radiolabled, gene-specific primer pairs in addition to a radiolabled pair of GAPDH-specific primers. The resulting PCR products were size fractionated on a 6% polyacrylamide sequencing gel (**figure 4b,** *left panel*), subjected to autoradiography, and quantitated by subjecting the autoradiographic signal to scanning densitometry (**figure 4c**). As shown in **figure 4**, each cell line demonstrated a unique pattern of DEST expression and the pattern recapitulated the pattern seen from the initial differential display experiment (*data not shown*). This demonstrated that the isolated DESTs were truly differentially expressed in the cell lines from which they were isolated and that the semi-quantitative RT-PCR approach was suitable for assessing expression levels of these sequences.

Next, this same experiment was repeated using four primary human breast tumor specimens (**figure 4a**). A single 6 μm section of the indicated breast tumor was microdissected. Tissue was used for RNA isolation, cDNA synthesis, and the multiplexed, semi-quantitative RT-PCR DEST assay as described above. As shown in **figure 4b** (*right panel*) and **figure 4c**, each primary tumor specimen also displayed a relatively unique DEST expression profile. This disparity in expression pattern was surprising since the four tumors had been chosen for their nearly identical histological type, tumor grade, and clinical stage.

This preliminary data suggests that selection of DEST sequences and characterization of their expression patterns in microdissected breast tumor specimens is a feasible and useful means of potentially sub-classifying breast tumors based on their patterns of gene expression.

Task 5: Prepare RNA and complex radiolabled cDNA from at least 40 additional tumor/normal breast tissue pairs. Hybridize to preliminary DEST array and quantitate expression. Enter patterns of expression, clinical data, and pathology data into DEST database.

Although we have modified the experimental approach, we have developed the methodology to perform the analogous experiment described in the proposal using RT-PCR technology. Currently, we are preparing cDNA from at least 50 different microdissected breast tumor specimens. We are also developing new gene-specific primer sets for a subset of the remaining DEST sequences isolated in **task 1**. We plan on testing the set of 50 tumor specimens against this extended DEST panel. This work will be funded by a newly awarded grant. All data will be entered into the database described in **task 2**, and when it reaches sufficient size to be useful to breast cancer researchers, this data will be made publicly available on a web-based database server.

Task 6: Create an algorithm to identify significant and insignificant patterns of DEST expression. Identify DEST sequences with significant patterns of expression. Remove insignificant DEST [oligonucleotides from the RT-PCR panel] and replace with additional DEST sequences isolated from Task 3. [Perform RT-PCR analysis] with additional tumor samples.

Even by casual inspection of the preliminary quantitative data presented in **figure 4c**, analysis of DEST gene expression profiles will be complicated. This has certainly been true for experiments using nucleic acid arrays (9) where a number of hierarchical clustering and self-organizing mapping algorithms have been developed (10, 11). Fortunately, because our research strategy uses a directed 'array' of a much smaller number of gene sequences, data analysis should be less complicated. As the DEST database described in **task 2** becomes larger, we will be developing algorithms to sort the data collected into meaningful expression patterns that could potential correlate with clinical or pathological features. To this end, we are collaborating with a clinical biostatistician, Dr. William Shannon, in the Division of General Medical Sciences at Washington University School of Medicine to create novel computational algorithms to sort expression data and correlate it with other clinical and pathology data sets. This work will be supported by funding received by our laboratory and possibly funding applied for by Dr. Shannon.

7. Key Research Accomplishments

- Development of a method for moderate throughput isolation and direct identification of differentially expressed gene sequences. The protocol is robust, reproducible, and moderately efficient.

- Development of a data infrastructure to catalog isolated and identified gene sequences and relate gene expression information with clinical and pathology data.

- Isolation of 37 known and anonymous gene sequences that are differentially expressed among a set of phenotypically distinct human breast tumor cell lines.

- Development of a method to isolate RNA and synthesize cDNA from enriched populations of microdissected primary human breast tumor cells. This technique will be used in future studies to perform differential message display from primary human breast tumors.

- Characterization of the composite expression pattern of seven identified differentially expressed sequence tags in microdissected primary human breast tumors. This approach will be applied to a larger number of primary breast tumors in future studies.

8. Reportable Outcomes

- "Directed Expression Profiling Of Microdissected Human Breast Tumors," Voss, L., Watson, M. *manuscript in preparation.*

- Breast DEST expression database. This database will be made accessible on a web-based server when a sufficient amount of useful information has been gathered.

- <u>Molecular Typing of Human Breast Cancer Using Gene Expression Fingerprints</u>. Project proposal was awarded funding by the Mary Kay Ash charitable foundation for the period 7/1/99 - 6/30/01. This award will be used to continue the research described in this report.

9. Conclusions

Although progress has been slower than anticipated due to a number of technical difficulties, we have developed the methodology and demonstrated our ability to isolate and identify differentially expressed gene sequences from enriched populations of microdissected human breast tumors. We are now beginning to apply this technology to larger populations of clinical samples and this should allow us to generate a catalog of gene sequences as originally conceptualized in the study proposal.

This approach for 'gene discovery' is an excellent complement to the emerging nucleic acid array technology (9) because it utilizes small amounts of RNA such is available from microdissected histological sections of tumors and because it allows for *de novo* gene discovery of both abundant and rare transcripts that may be present in primary breast tumor samples. Finally, the technology is relatively inexpensive and allows the rapid, simultaneous comparison of many clinical samples.

In the future, we will expand our catalog of DEST sequences to include tumor specimens with accompanying patient data describing treatment response, disease-free survival, and overall survival. Ultimately, we believe that this approach can be used to create a nearly complete set of genetic markers whose pattern of expression would define the clinical course and therapeutic vulnerability of individual patient tumors. Obviously, such a diagnostic tool would have tremendous impact on the diagnosis and treatment of breast cancer patients.

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Appendix A - Figures and Tables

T47D
MCF-7
MDA-MB361
MDA-MB415
MDA-MB468

Table I. Human Breast Carcinoma Cell Lines Used for Generating a DEST catalog for Human Breast Cancer.

Primer Name	Sequence
T7T$_{11}$VA	5'-CACTATAGGGTTTTTTTTTTTVA-3'
T7T$_{11}$VC	5'-CACTATAGGGTTTTTTTTTTTVC-3'
T7T$_{11}$VG	5'-CACTATAGGGTTTTTTTTTTTVG-3'
T7T$_{11}$VT	5'-CACTATAGGGTTTTTTTTTTTVT-3'
MF1	5'-CAGTCACGACGACCGCTTGT-3'
MF2	5'-CAGTCACGACAGGTGACCGT-3'
MF3	5'-CAGTCACGACTCCGGCTGAA-3'
MF4	5'-CAGTCACGACGCTGCGTGAT-3'
MF5	5'-CAGTCACGACGGTGCCTGAA-3'
MF6	5'-CAGTCACGACCTGCGGTGAT-3'
MF7	5'-CAGTCACGACCGGCTGTGAA-3'

Table II. Primer Sequences Used for Differential Display PCR.

Isolated Fragments	154
Reamplified Fragments	136 (86 %)
Sequenced Fragments	49 (32 %)
Unique Fragments	39 (25 %)
"Interesting" Candidates	22 (14 %)
Confirmed Differential Expression	10 (6 %)

Table III. Number and Percent Yield of Sequences Isolated from Seven Human Breast Tumor Cell Lines as Described in Task 1.

| DEST	IDENTIFICATION	T47-D	MCF-7	MB415	MB468	MB361
27	Lacrimal Gland mRNA	○	○	●	○	○
31	Human Mucin-Like Gene	○	○	●	○	○
45	Taxol Resistant Associated Protein	○	○	○	●	○
3	EST (F. Hrt / Prt)	○	○	●	○	○
5	EST (Tst / Ovy / F. Hrt / F. Liv / F. Spl / Col)	○	○	○	●	○
12	EST (Tst / Pth / Ovy / Fib / F. Liv / F. Spl)	●	○	○	○	○
13	EST (Squamous Cell CA / F. Hrt / Tst)	○	○	○	●	○
19	EST (F. Lng)	○	○	●	○	○
32	EST (Sarc. / Ovy / Uts / Prt / Hrt / Pth / F. Liv / F. Spl)	○	○	○	●	○
33	EST (Plc)	○	○	○	○	○
34	EST (Sarc. / Ovy / Uts / Prt / Hrt / Pth / F. Liv / F. Spl)	●	○	○	●	○
35	EST (Sarc. / Ovy / Uts / Prt / Hrt / Pth / F. Liv / F. Spl)	●	○	○	○	○
36	EST (Sarc. / Ovy / Uts / Prt / Hrt / Pth / F. Liv / F. Spl)	○	○	○	●	○
37	EST (Infant Brain / Total Fetus / Lng / Tst / Col)	○	●	○	○	●
40	EST (Tst / Prt / Brt / Ovy / Parathyroid / Col / F. Lng)	●	○	●	○	○
42	EST (Tst / Brt / Uts / Thy / Pth / Lng / Kid)	○	●	○	○	○
22	mRNA from Chromosome 5q21-22	●	●	○	○	○
6, 15, 16	Chromosome 6p24 PAC Clone	○	○	○	○	●
21	Chromosome 17 clone	●	●	●	○	○
4	Ferratin	●	○	○	○	○
9, 10	Amplaxin	○	○	○	○	○
23, 24	Human β-Adaptin mRNA	○	●	○	○	●
39	Flow Induced Endothelial G Protein Coupled Receptor	●	○	○	○	○
20	NEFA DNA Binding Protein	○	●	○	○	○
43	Histone H3.3b	●	○	○	○	○
7	Ribosome Protein L22	○	●	○	○	○
28	Ribosomal Protein S14	●	○	●	○	●
38	Ribosomal Protein L41	○	●	○	○	○
26	16s rRNA Gene	●	○	○	○	○
25	Lysyl Oxidase Related Protein	○	○	○	○	○
11	GAPDH	○	●	●	○	●
18	NADH Dehydrogenase	●	○	○	●	○
1, 2, 8, 14, 17	ATP Synthetase β-Subunit	●	○	●	○	●
30	Unknown	○	○	○	○	○
41	Unknown	○	○	○	○	●
29	Vector Sequence	○	○	○	●	●
44	Luciferace Expression Vector	○	○	○	●	○

Table IV. List, identity, and expression pattern of DEST sequences isolated from five different human breast tumor cell lines as described in Task 1.

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DEST	Forward Primer	Reverse Primer
GAPDH	5'-TGGCCTCCAAGGAGTAAG-3'	5'-GATGGTACATGACAAGGTGC-3'
2	5'-CCAAACGGATGAAACTCTG-3'	5'-AGAAAGAGAAGGTGTGGTTTG-3'
9/10	5'-TTCTCATCATCCTTGCTTTAC-3'	5'-CCTATGGAGGGTGTGCCTAC-3'
19	5'-CGATAAACCTGGTCACCTG-3'	5'-TGCTGTGTGCTATTTCAATTAC-3'
21	5'-CCAGTTACGACTGAAGTCAGTG-3'	5'-CCTGTCTCTTTAGGAAGAGGTG-3'
27	5'-AGCATCATCATTCTTCCAGAGG-3'	5'-ATTACCAGAGTGGTTGCTCCTG-3'
31	5'-TTAATGCTGACATCACCTTC-3'	5'-CCTCACAATGAGAACTCAT-3'
48	5'-CCGGGAATTACTGGCTGTC-3'	5'-CCGGGAATTACTGGCTGTC-3'

Table V. Primer Sequences Used for DEST RT-PCR Assay.
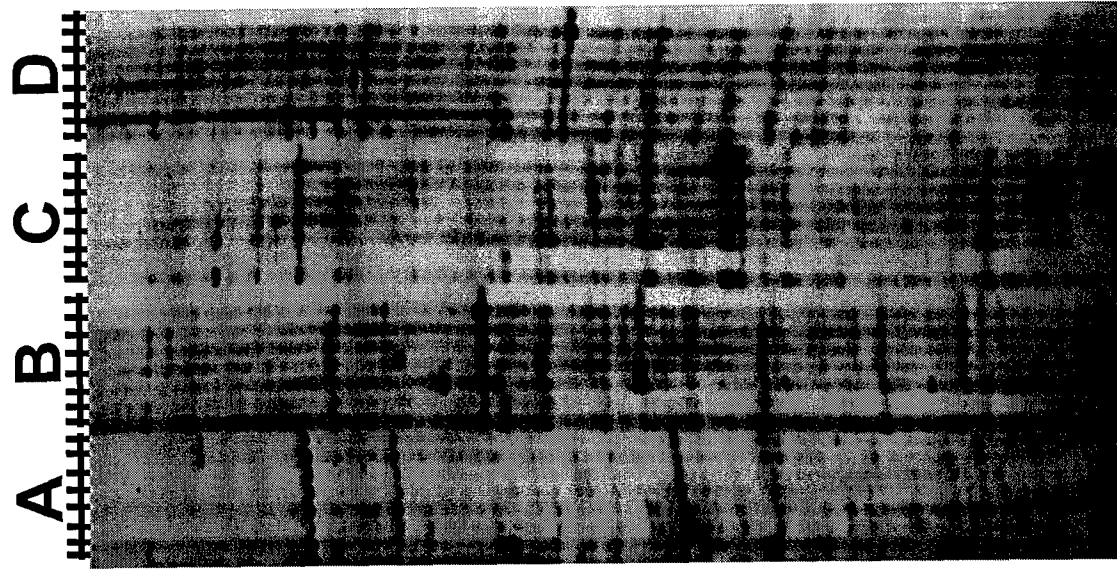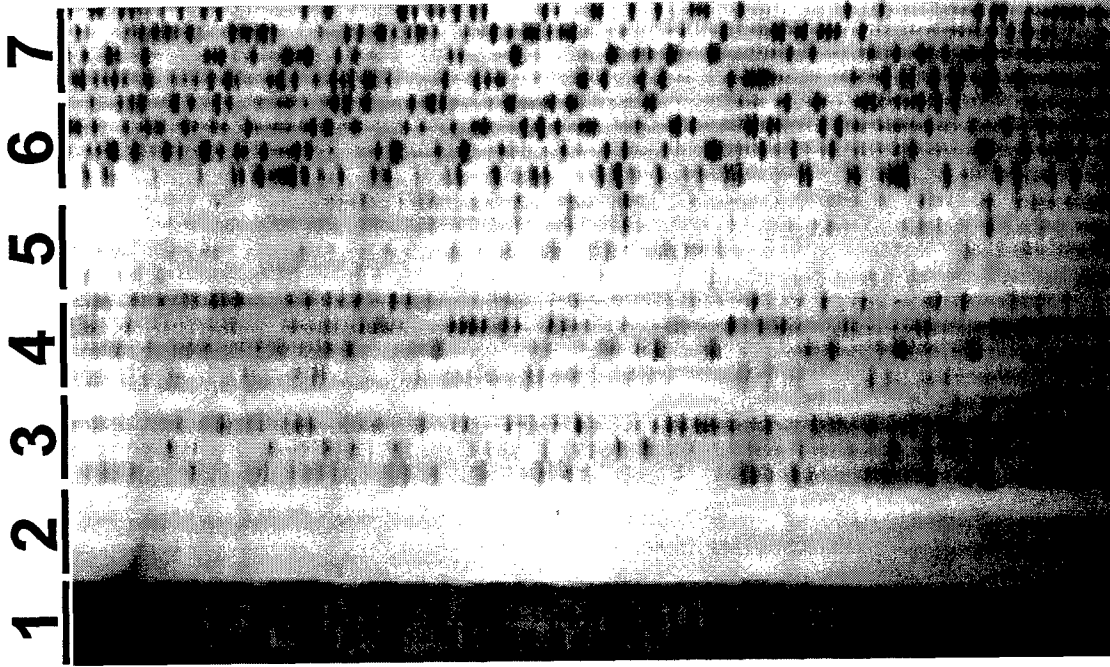
FIGURE 1: Representative Differential Display (A) and Direct Sequencing (B) Results from Human Breast Carcinoma Cell Lines. cDNAs were prepared from four or five different cell lines (Table I) in duplicate and subjected to differential display analysis using a single $T_{11}MN$ labeled primer and different 10mer primers (A-B) as described in the text. Candidate bands were identified, excised from the gel, reamplified, and subjected to direct cycle sequencing (1-7).

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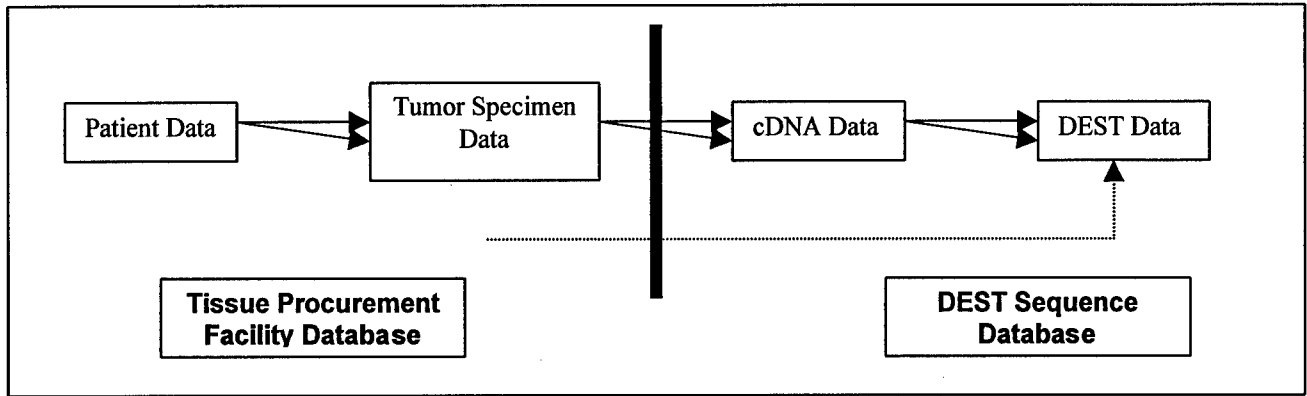
FIGURE 2a: Data Table Relationships. This figure shows the relationships between data in our institution's Tissue Procurement Facility database which stores clinical and pathology information and the DEST database described in this report which stores sequence and expression information for each DEST. The vertical bar represents a 'firewall' which is designed to prevent experimental data from being connected directly to patient data and patient identifiers from being connected directly to DEST experimental data.

FIGURE 2b: Sample Data Entry Form from DEST database. This figure shows the data entry form for each DEST sequence identified. Other forms are accessible for entering information about each cDNA pool used for display experiments.
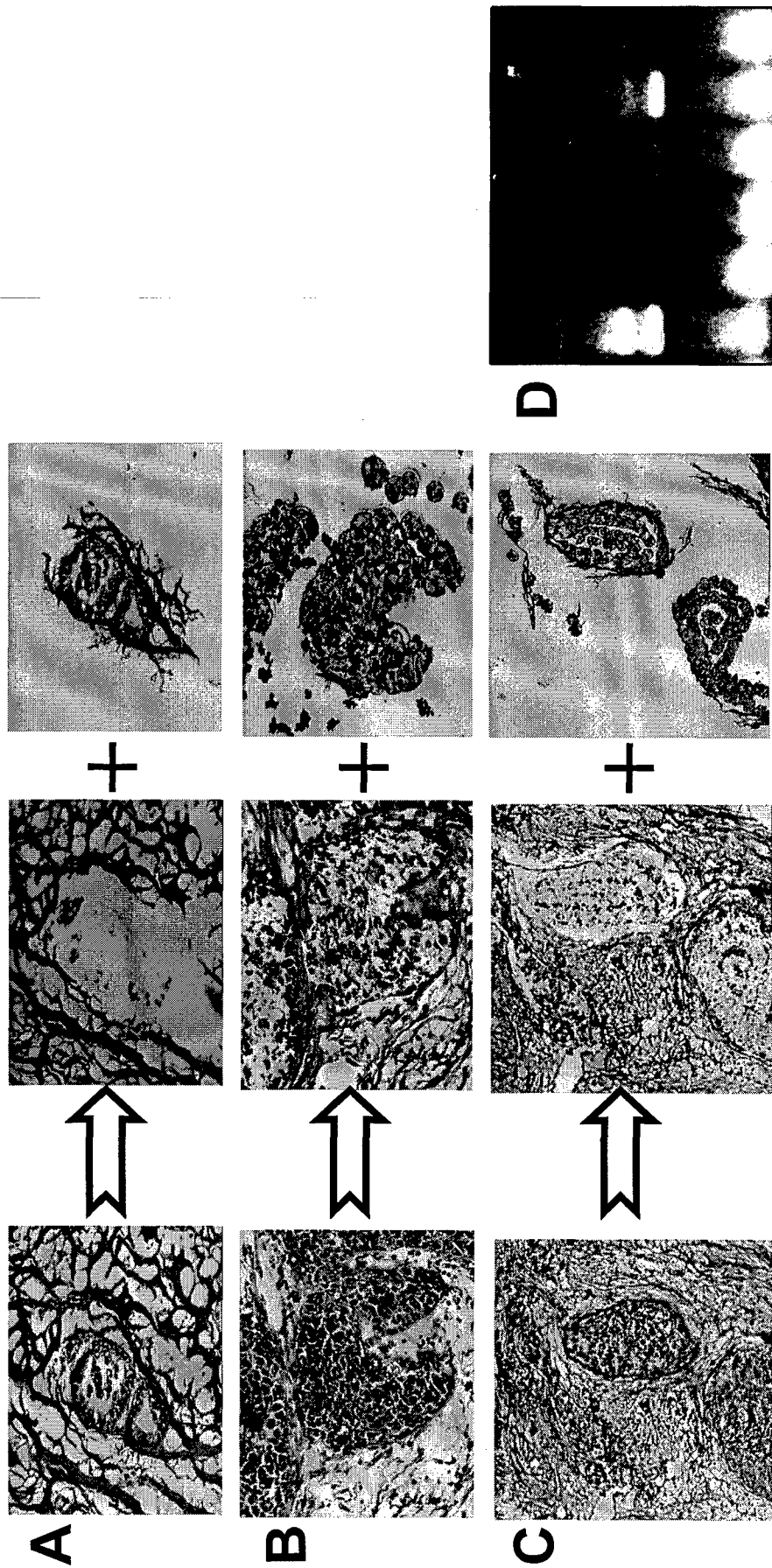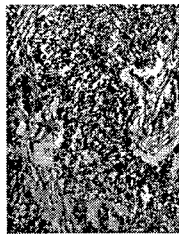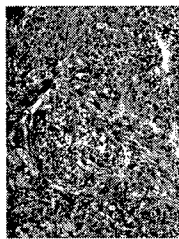
FIGURE 3: RT-PCR from Microdissected Breast Tumor Specimens. (*A, B,* C) Three examples of laser capture microdissection performed on histological sections of breast cancer. Each panel (*L-R*) demonstrates the tissue before dissection, the tissue after dissection, and the isolated tissue attached to the transfer film. (*D*) RT-PCR results of GAPDH amplification from positive control RNA (+), microdissected tissue specimens (1-3), a single total tissue section (T), and a negative control (-).

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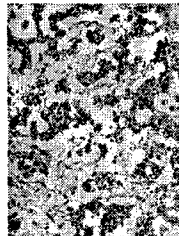
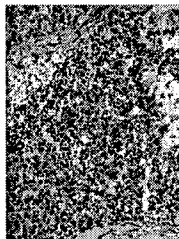17

A
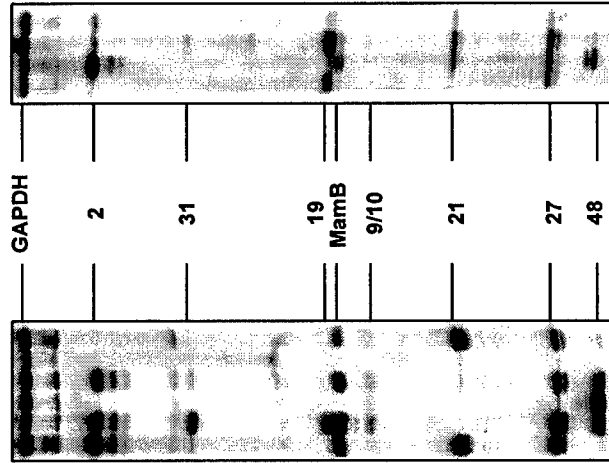

2083

1059

934

1121

B

GAPDH
2
31
19
MamB
9/10
21
27
48

C

Sample	Diagnosis	Stage	Grade	ER	PR	2	31	19	MamB	9/10	21	27	48
MCF7	Cell Line	--	--	+		0	2	10	63	22	112	101	17
T47D	Cell Line	--	--	+		15	0	7	13	7	45	50	13
ZR75	Cell Line	--	--	+		116	22	14	83	36	28	98	100
MB468	Cell Line	--	--	-		14	0	7	4	14	0	55	121
MB415	Cell Line	--	--	-		119	79	100	123	51	10	117	97
MB361	Cell Line	--	--	-		119	21	21	91	18	109	132	16
2083	Inv. Ductal	T1/N0	III / III	+	+/-	50	0	48	27	0	37	35	0
1059	Inv. Ductal	T2/N0	III / III	-	-	41	6	122	64	0	92	95	20
934	Inv. Ductal	T2/N0	III / III	+	+	125	0	92	98	0	103	132	97
1121	Inv. Ductal	T2/N0	III / III	-	-	44	0	88	32	0	19	79	6

Figure 4. (previous page) DEST Expression Profile in Primary Human Breast Cancer. Gene-specific primers derived from seven different DEST sequences and one known gene previously isolated in a separate study (MamB) were used in a radiolabled, semi-quantitative RT-PCR assay as described in the text. The amplification reactions were pooled and analyzed by gel electrophoresis. Each amplified product migrates at a unique size as indicated (*B*). Six different cell line RNAs were analyzed, five of which were the same cell lines used to initially isolate the DEST sequences. The expression pattern of each DEST in this assay recapitulates that seen in the original message display (*B, Left Panel*). Histological sections of four different tumors were analyzed in a similar fashion (*A, B, Right Panel*). The table at the bottom of the figure (*C*) summarizes expression data (quantitated and normalized to GAPDH signal) and basic pathology data from each tumor specimen. Note that despite similar pathological diagnosis, each tumor displays a unique DEST expression fingerprint. The necessity for complex, multi-dimensional statistical data analysis is also apparent, even using this small set of pilot data.

Appendix B - Bibliography and Personnel

Bibliography

Voss, L.E., and Watson, M.A. Directed Expression Profiling Of Microdissected Human Breast Tumors. *manuscript in preparation.*

Personnel

Mark A. Watson	Principal Investigator	20% Effort
Lora E. Voss	Research Technician I	100% Effort

MCMR-RMI-S (70-1y)   1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

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