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Carcinoma

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13. ABSTRACT (Maximum 200 Words) The long-term goal of this project is the early detection of breast cancers that are prone to develop metastasis. In this project, representational difference analysis (RDA) was used to identify the genetic alterations related to the progression of metastatic development in breast cancer. In addition to the previously reported two metastasis associated gene sequences (MAGS), we have identified a novel candidate gene sequence (MAGS IV) that was found to be associated with tumorigenicity and possibly metastasis. All these MAGS are being characterized further to use as DNA markers for breast metastasis.			
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(4) Introduction:

The development of a panel of molecular genetic markers for identifying the 13% lymph node negative mammary carcinomas that are known to develop metastases (Fisher et al. 1997) would be clearly be of considerable value in indicating those cases in need of early aggressive therapy. There would also be considerable benefit to the 87% of women with mammary carcinomas that are not likely to metastasize by sparing them the considerable physical, mental and financial costs of the treatment. In addition, further knowledge of the genetic mechanisms that play an important role in metastasis could ultimately lead to the development of improved therapeutic procedures.

(5) Body:

The technical objective of this project is to isolate genes whose loss of wild-type function represents a step in the acquisition of metastatic property by ductal mammary carcinoma cells. To accomplish this objective we have been using representational difference analysis (Lisitsyn et al. 1993; Li et al. 1997) on normal/primary and metastatic archival tissue samples from patients with breast carcinoma. Attaining this aim will result in a panel of molecular genetic markers that could be used for differentiating with high sensitivity and reliability ductal mammary carcinomas that are prone to developing metastases from those that will not.

Statement of Work (3 years)

- Task. 1: Isolation of differential sequences specific to breast metastasis using RDA.
- i. Collection of additional tissues of ductal mammary carcinoma (DMC) and continuation of ongoing RDA product characterization (Months 1-12)
 - ii. Confirmation of histopathology of tissues before LCM (Months 1-24)
 - iii. LCM of 30 cases of DMC tissues with normal, primary and metastasis cell components (Months 2-30)
 - iv. RDA of 30 cases of DMC (Months 1-14)
- Task. 2: Characterization of the differential RDA products.
- i. Cloning of RDA products isolated from the loss side (Months 1-18)
 - ii. Sequencing and Southern blot analysis of the clones (Months 1-20)
 - iii. Homology search of positive clones (Months 2-28)
 - iv. Northern blot analysis of the analysis of significant clones from the past and present RDA experiments (Months 6-25)
 - v. Isolation of complete gene sequences (Months 6-25)
- Task. 3: Further analysis of candidate genes of metastasis.
- i. Screening the metastasis-specific genes on at least 120 DMC cases and statistical analysis (Months 12-30)
 - ii. *In vitro* and *in vivo* functional assays of metastasis (Months 12-30)

- iii. Antisense oligonucleotide-mediated disruption of mRNA translational studies (months 18-34)
- iv. Compilation of data for the submission of final grant report (Months 33-36).

Second Year Progress Report:

We have undertaken all experiments that were proposed under each task for the second year. The following is the progress of the work done so far:

Task 1:

i. Breast carcinoma tissue sample collection:

This year an additional 4 cases of fresh frozen ductal breast carcinoma (DBC) consisting of normal, primary and positive lymph node samples have been obtained from the Co-operative Human Tissue Network (CHTN). We also have received matched DNA samples of normal and primary tumor of 9 DBC patients as a gift from the University of Cairo, Egypt. The matched DNA samples from the positive lymph node material from all these patients are not currently available. However we know that all these patients had developed metastasis. We also have used paired cell lines derived from normal tissue/blood and primary tumors of 3 breast carcinoma patients who are reportedly have loss of heterozygosity (LOH) for a region on the chromosome 10q encompassing PTEN gene. These cell lines were received as gift from Dr. Ramon Parson of Columbia University (Li et al. 1997).

ii. Histopathology of patient tissue samples:

Six cases of DBC from our existing archival tumor tissue collection and four cases obtained from CHTN this year were selected by Drs. Jones (Pathologist) and Klinger (Co-investigator) and histopathology of these tissue samples were reconfirmed.

iii. Additional RDA experiments:

The tissue sections of selected 10 cases of tumors were processed for LCM (Emmert-Buck et al. 1996). Six out of ten cases were found suitable for LCM. These cases were also used to screen the candidate metastasis associated gene sequences (MAGS) isolated from the previous RDA experiments. Additional cases will be processed and used next year for RDA experiments as well as for screening candidate metastasis associated gene sequences. We have undertaken 6 RDA experiments last year and another 6 this year using archival tissue samples. Except for the first RDA, all the remaining RDA experiments were performed by comparing DNA samples recovered from primary and metastatic tumor cells. We could not complete RDA of the remaining 18 cases (as proposed) so far because of two difficulties. Firstly, each RDA experiment followed by cloning and characterization of the differential products has been taking much of our time. Secondly, in the past two years we found that some of the archival samples are not suitable for LCM. We therefore are planning to try our single cell microdissection method on such cases if necessary.

iv. Total probe:

The total probe was prepared by combining differential sequences (present only in normal or primary tumor cells but missing in metastatic cells) derived from each of the 6 RDA experiments. Screening the clones isolated from the additional 6 RDA experiments using the total probe, we obtained 7 additional unique metastasis associated gene sequences (MAGS) that were not present in the total probe. Homology search, sequencing and RH mapping of these MAGS are in progress.

Task 2:

i. Further characterization of MAGS:

To determine if the MAGS obtained from our RDA experiments are parts of functional genes, RT-PCR and Dot/Northern blotting experiments are being conducted. For RT-PCR experiments primers were designed from different MAGS and used on normal human total RNA. Both MAGS IV and IX showed expression of the transcript. Dot/Northern blotting experiments also supported the RT-PCR results. Figure 1 shows RT-PCR results and figure 2 shows dot blot results of MAGS IV. Full Northern blots are being performed to confirm these results and also to determine the actual size of transcripts of these genes.

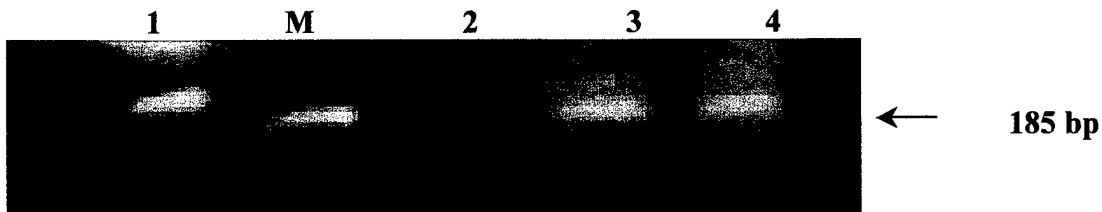


Fig. 1. RT-PCR of normal RNA using MAGS IV primers showing that the gene is expressing. Lane M: 200bp DNA marker band ; Lane 1: Positive control; Lane 2: Negative control; 3-4: Total RNA from normal human tissue digested with DNase I.

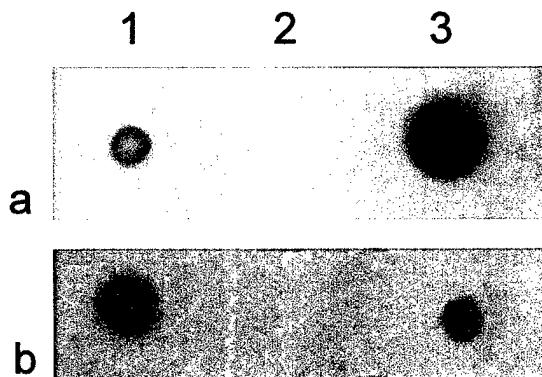


Fig. 2. Results of total RNA dot/Northern blot using $\alpha^{32}\text{P}$ -dCTP probes prepared from MAGS-IV (a) and internal control G3PDH (b) DNA. Lane 1: A positive signal is present in the normal RNA (digested with DNase I); Lane 2: Negative control; Lane 3: Positive control (DNA of MAGS IV).

ii. Isolation of complete MAGS:

In order to obtain the complete sequences of the MAGS, we used inverse-PCR and also cDNA library screening methods. The reason behind these 2 approaches is to obtain complete gene sequence or reasonably long enough sequence so that we can use these MAGS for transfection (in vitro and in vivo) studies as well as use as FISH probes (more than one kb size of DNA sequence is required to use as FISH probe) to screen normal and tumor cells simultaneously on primary and metastatic tumor tissue sections of patients.

We performed inverse-PCR using genomic DNA and reverse primers designed from the respective MAGS. The PCR products were cloned using TA cloning system (pCR 2.1) and currently are being characterized. As shown in the figure 3, four clones from MAG IV showed 4 different fragments ranging in sizes from 2-2.7 Kb. Some of them were completely sequenced and attempts are being made to use them as FISH probes.

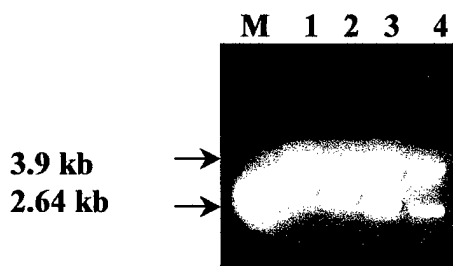


Fig. 3. Estimation of insert sizes of 4 clones obtained from inverse-PCR after digestion with EcoRI. The insert sizes (from left to right) ranged from 2.4 to 2.65Kb. Plasmid vector size is 3.9Kb. M: High molecular DNA marker.

In the second approach we used cDNA library (λ gt 11 vector) of a human breast carcinoma (Clontech) to screen our MAGS as probes to isolate full-length cDNA clones. In the third round of screening we obtained about 9 positive plaques for MAGS IV. All of them were found to be positive by PCR using MAGS IV primers. Similar characterization with other MAGS are underway. Once the full length cDNA of these genes are isolated, we will perform transfection studies by expressing these gene products in a highly metastatic human mammary cell line, MDA-MB-435 to determine if its phenotype changes to low metastatic.

iii. Screening patient samples using MAGS by PCR:

We screened 20 patient samples so far using the primers derived from different MAGS. In the previous report 2 candidate MAGS, M-41 and M-120 (MAG IX) were reported. RH mapping indicated that MAG IX sequence is localized on human chromosome 10q arm in which PTEN gene is also located. To determine if this gene is close to PTEN gene, we screened normal tissue/blood and tumor derived cell lines from 3 patients whose tumors had LOH of a region encompassing PTEN region on 10q (Li et al. 1997). We found that MAGS IX was present in the normal and tumor samples of all the three patients indicating that it is neither a part of PTEN gene nor localized in the LOH region. The MAGS IX sequence showed 94% identity to homo sapiens chromosome 10 clone (gi-14787179; AC022541.10). While screening these three patient cell lines with other MAGS, we found that MAG IV was missing in the tumor DNA samples of one patient cell line (Fig. 4) indicating its possible involvement in tumorigenicity/metastasis. We could not determine its status in the metastatic DNA samples since they are not available in these 3 patients. With regard to homology search no matches were found for MAGS IV in the gene banks indicating that it is possibly a novel gene associated with breast cancer. In the PCR screening experiments using primers from all the MAGS on DNA of normal and primary tumor samples of 9 breast cancer patients received from the University of Cairo, none of the MAGS were found to be missing in any of these 9 tumors. We are waiting to obtain the corresponding metastatic cell DNA samples from Cairo University to determine their possible involvement in metastasis in these 9 patients.

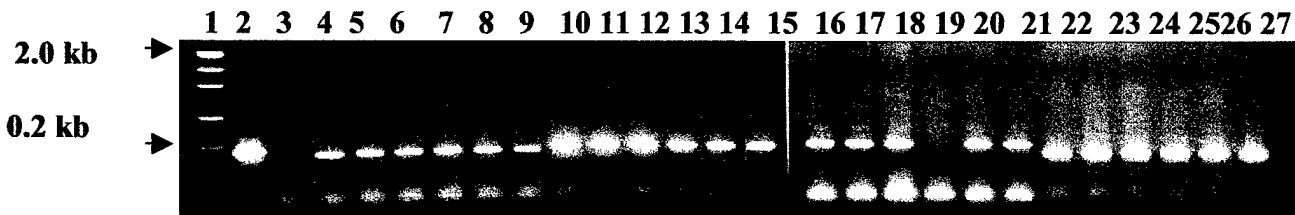


Fig. 4. PCR screening of 4 MAGS on DNA samples of paired normal cells (lanes with even numbers from 4-26) and primary tumor cells (lanes with odd numbers starting from 5-27) derived from three breast carcinoma patients with loss of heterozygosity for a region on chromosome 10q encompassing gene PTEN. Lanes 1: 2Kb DNA marker; 2: Positive control; 3: Negative control; PCR results with MAGS X with product size of 148bp (lanes 4-9); MAGS VIII with product size of 171bp (lanes 10-15); MAGS IV with product size of 185bp (lanes 16-21) and MAGS II with product size of 144bp (22-27). Lane 19 represents tumor cell line sample of a patient showing absence of a 185 bp product. G3PDH internal controls are not shown in the figure.

(6) Key Research Accomplishments:

- Collected 4 ductal breast carcinoma samples with matched normal tissue, primary and metastatic tumors (CHTN), 3 pairs of matched cell lines from normal tissue and tumors (Columbia University) and DNA from normal tissue and tumor samples from 9 breast cancer patients (Cairo University).
- “Total probe” prepared from the MAGS of first set of six RDA experiments was used as probe to screen six additional RDA experiments which yielded 7 candidate MAGS.
- A candidate sequence, MAGS IV was found to be missing in one of the three patient tumors which had LOH for a region encompassing PTEN region on 10q. Its metastatic involvement could not be confirmed due to nonavailability of matching metastatic cell DNA. Thus proposing that this MAGS IV is associated with tumorigenicity and possibly with metastasis.
- Preliminary RT-PCR and Dot/Northern blot experiments indicated that MAGS IV and IX are parts of functional genes. Full Northernblots are underway to confirm these results and also to estimate the actual sizes of the complete transcripts.
- Using inverse-PCR, sizes of sequences of MAG IV that are required for FISH were isolated. Experiments to obtain the full length of this and other MAGS are in progress.
- Isolation of full-length cDNA of MAGS IV by screening a λ gt11 human breast carcinoma cDNA library is in progress.

(7) Reportable outcomes:

Manuscripts, abstracts and presentations

Related to this grant:

Research manuscript

- A candidate metastasis associated DNA marker for ductal mammary carcinoma.
P. M. Achary, H. Zhao, Z. Fan, P. Mahadevia H. P. Klinger and B. Vikram (2001).

Note: This manuscript will be submitted next week for publication to ‘Cancer Research’ under the section “Advances in brief”. We are waiting for the figures from the graphic arts center. We will submit a copy of the manuscript to your office at the time of submission to the journal.

Presentation

- “Molecular markers of breast metastasis and cervical carcinomas”, at Calcutta University, Calcutta, India on May 15th 2001 (Invited speech); Letters attached (Annexure-I).

Directly not related to the grant (US Army IDEA grant sponsorship to the PI is acknowledged):

- i. R. Yuan, S. Fan, **Mohan P. Achary**, D. M. Stewart, I.D. Goldberg and E.M. Rosen. 2001. Altered gene expression pattern in cultured human breast cancer cells treated with hepatocyte growth factor/scatter factor in the setting of DNA damage (submitted to Cancer Research; a copy attached; Annexure-II)
- ii. **M. P. Achary**, W. Jaggernaut, E. Gross, A. Alfieri, H. P. Klinger and B. Vikram (2000). Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression. **Cytogenetics and Cell Genetics 91: 39-43.** (A reprint copy attached; Annexure-III)
- iii. Lawrence H. Herbst, Ratna Chakrabarty, Paul A. Klein, and **M. P. Achary**, 2001. Differential Gene Expression Associated with Tumorigenicity of Cultured Green Turtle Fibropapilloma-Derived Fibroblasts. **Cancer Genetics and Cytogenetics 128: 1-5** (a gally proof copy attached; Annexure-IV)

Patents and licenses applied for and/or issued

We are planning to submit the candidate metastasis associated gene sequences to database soon and also patent M-41, MAGS IV and IX sequences.

Degrees obtained that are supported by this award

None

Development of cell lines, tissue or serum repositories (core facilities)

- i. The PI has been contacting worldwide to procure all types of breast cancer tissue samples to establish a repository. The long-term goal is to make customized Tissue Arrays (Glass slides with small sections of normal, primary and metastatic tissue samples from two groups of large number of patients whose tumors did and did not develop metastasis. This will facilitate a rapid and economic way of validation of candidate metastasis associated markers by FISH and other methods. We are expecting matched DNA samples from normal tissue, primary tumor and metastatic lymph node material from about 100 breast carcinoma patients from Cairo University. So far we have normal and primary tumor DNA samples from 9 cases. These samples will be very much useful for the present study.
- ii. In our breast carcinoma tumorigenicity cum metastasis nude mouse model, we inject human mammary metastatic cell lines (MDA-MB-435) into primary mammary fat pads of the nude mice and after 6 weeks the primary tumors are surgically removed and after about 5 weeks the animals are killed to measure metastasis. In one of such experiments we have made tumor cell cultures from the metastatic lungs and after

growing them for two generations they were reinjected into mammary fat pad of nude mice. This time we found that the mice died of metastasis in lungs within 3-4 weeks indicating that these cell lines are highly metastatic compared to the original MDA-MB-435 cells. Currently we are performing cDNA microarray to determine if some genes are differentially expressed in the highly metastatic phenotype.

Informatics such as databases and animal models etc.

A tumorigenicity cum metastasis mouse model is established to evaluate breast carcinoma cell lines transfected with the candidate metastasis associated gene sequences and also to evaluate the efficacy of potential anticancer drugs.

Funding applied for based on work supported by this award

- i. Awarded a fellowship (@ \$30,000 per year) by Klinger's Fund for three years (2000-2003) to hire a post-doctoral fellow to work on breast cancer research projects in P.I's lab (copy of the letter attached; Annexure-V). Dr. Hui Zhao is currently working in my lab as a post doc sponsored by the foundation.
- ii. PI has submitted an IDEA grant to US ARMY Breast Cancer Research Program on June 13th 2001. The objective of this proposal is to evaluate the efficacy of the extract of Cynodon plant on tumorigenicity and metastasis in breast cancer using the nude mouse model developed from the current US Army grant.
- iii. PI will submit a RO-1 grant application to NIH based on the results obtained from the current US Army project to further characterize the MAGS for the October 2001 deadline.
- iv. PI submitted an Insight grant (PAR99-128) to NIH entitled "Diagnostic gene expression profiles in breast cancer" on October 13th, 1999. The long-term goal of the project is to identify the breast cancer patients who are prone to metastasis, using cDNA microarray strategy. The cDNA microarrays may identify gene expression patterns at the global level (currently 40,000 genes/ESTs) that are specific to each group of patients. The grant application was scored (priority score was 238) well but was not funded primarily due to technical problems. We have taken care of the technical problems and generated supporting data and will be submitting the revised application to NIH for the November 2001 deadline.

Employment or research opportunities applied for/or received on experiences/training supported by this award

This year 5 students (3 from Yeshiva University, 1 minority student from Bronx Community College and 1 from Bronx High School of Science competing in the Intel Prize) have satisfactorily completed their summer projects and one medical fellow (M.D.) received 4 months research training in the PI's lab. The expenses incurred by these research projects were funded by their respective Institutions.

(8) Conclusions:

We have nearly completed the work proposed for the first and second years. Several additional novel candidate metastasis associated gene sequences of ductal breast carcinoma have been isolated. So far two MAGS and one tumor associated sequence (MAG IV) were identified and are being further characterized. No significant changes are foreseen in the approach outlined in the project.

(9) References:

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J. Silver and V. Keerikatte, J. Virol. 63, 1924-1928 (1989)
B. Z. Yuan et al. Cancer Research 58: 2196-2199 (1998)



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Date.....

3- 15-2001

To
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Dear Dr. Achary :

I came to know from a reliable source that you will come to Kolkata, India, in April, 2001. If you can arrange your travel plans, you are welcome to visit our department, especially our laboratory in the Department of Zoology, University of Calcutta and you could also give a lecture to our students. I shall be highly obliged if you could accept the invitation and inform us if you could like to deliver a lecture on the topic chosen by yourself.

I am looking forward to hearing from you.

(Prof. R. N. Chatterjee)

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University of Calcutta**

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5- 15-2001

To whom it may Concern

This is to certify that Dr. Mohan P. Achary, Ph.D. Assistant Professor and Cancer Biologist, Department of Radiation oncology, has delivered a lecture entitled "Molecular markers of breast metastasis and cervical carcinomas in our department. Our students and a large number of research fellows have benefited from his deliver. I wish him every success in life.

Bernice
15.5.01
(Prof. B. Manna)

Head

Department of Zoology

Prof. & Head
Department of Zoology
University of Calcutta

Submitted to: Cancer Research, May 16, 2001

Subject Category: Tumor Biology

Altered Gene Expression Pattern in Cultured Human Breast Cancer Cells Treated with Hepatocyte Growth Factor/Scatter Factor (HGF/SF) in the Setting of DNA Damage

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Keywords: scatter factor (SF), hepatocyte growth factor (HGF), HGF/SF, c-Met, breast cancer, MDA-MB-453, adriamycin (doxorubicin), microarray

Running Title: Altered Gene Expression in SF Treated Cells

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ABSTRACT

The cytokine hepatocyte growth factor/scatter factor (HGF/SF) protects epithelial and cancer cells against DNA damaging agents via a pathway involving signaling from c-Met → phosphatidylinositol-3-kinase → c-Akt. However, the down-stream alterations in gene expression resulting from this pathway have not been established. Based on cDNA microarray and semi-quantitative RT-PCR assays, we found that MDA-MB-453 human breast cancer cells pre-incubated with HGF/SF and then exposed to adriamycin (ADR), a DNA topoisomerase II inhibitor, exhibit an altered pattern of gene expression, as compared with cells treated with ADR only. [HGF/SF+ADR]-treated cells showed altered expression of genes involved in the DNA damage response, cell cycle regulation, signal transduction, metabolism, and development. Some of these alterations suggest mechanisms by which HGF/SF may exert its protective activity: *eg.*, up-regulation of polycystic kidney disease-1 (a survival-promoting component of cadherin-catenin complexes), down-regulation of 51C (an inositol polyphosphate-5-phosphatase), and down-regulation of TOPBP1 (a topoisomerase IIB binding protein). We showed that enforced expression of the cdc42-interacting protein CIP4 - a cytoskeleton-associated protein for which expression was decreased in [HGF/SF+ADR]-treated cells - inhibited HGF/SF-mediated protection against ADR. The cDNA microarray approach may open up new avenues for investigation of the DNA damage response and its regulation by HGF/SF.

INTRODUCTION

The cytokine hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic mediator of multiple biologic functions, that plays significant roles in embryonic development, tissue and organ repair, tumorigenesis, and angiogenesis. HGF/SF has been found to protect various cell types against apoptosis induced by a variety of stimuli, including loss of contact with the substratum (1), exposure to staurosporine (a protein kinase inhibitor) (2,3), and DNA damage (4-7). We have previously reported that various epithelial and carcinoma cell lines are protected by HGF/SF against apoptotic cell deaths induced by DNA-damaging agents, including ionizing radiation, ultraviolet (UV-C) radiation, and adriamycin (ADR, also known as doxorubicin) (5). ADR is a DNA intercalator and a DNA topoisomerase II α inhibitor that induces single- and double-strand DNA breaks similar to those induced by ionizing radiation.

Interestingly, pre-incubation with HGF/SF also reduced the number of residual DNA strand breaks at 24 hr after exposure to ADR or ionizing radiation, suggesting that HGF/SF may also enhance the rate of DNA repair (*ie.*, strand rejoining) (6). The increased DNA repair and the cell protection against DNA damage appeared to be due, at least in part, to: 1) activation of a cell survival pathway involving phosphatidylinositol-3-kinase (PI3K) and c-Akt (protein kinase B); and 2) subsequent stabilization of the protein levels of the anti-apoptotic mitochondrial pore-forming protein Bcl-X_L (5,6).

These studies have not revealed the down-stream effector genes that mediate cytoprotection by HGF/SF. Cytoprotection by HGF/SF might involve non-nuclear events, such as inactivation of pro-apoptotic effectors (eg., Bad and caspase-9) by c-Akt mediated protein phosphorylation events (8,9). However, it might also involve prolonged patterns of altered gene expression induced by HGF/SF in the DNA damaged cells. The latter possibility was suggested

by the observation that maximal protection required a pre-incubation of cells with HGF/SF for at least 48 hr prior to exposure to ADR (5). Shorter pre-incubation periods yielded less protection, and application of HGF/SF only at the time of ADR treatment and during the 72 hr post-incubation period gave no protection.

To investigate the potential alterations of gene expression that might contribute to HGF/SF-mediated cell protection, we have utilized a cDNA microassay approach, using a previously studied model for HGF/SF protection (5). MDA-MB-453 human breast cancer cells were pre-incubated with HGF/SF, exposed to ADR, and then post-incubated in ADR-free culture medium for 72 hr to allow the repair processes to proceed. Alterations of mRNA expression were examined in cells treated with [HGF/SF+ADR], in comparison with cells treated with ADR alone.

MATERIALS AND METHODS

Sources of Reagents and Vectors. Sources of reagents and antibodies. Recombinant human two-chain HGF/SF was generously provided by Dr. Ralph Schwall, Department of Endocrine Research, Genentech, Inc. (South San Francisco, CA). Adriamycin (doxorubicin hydrochloride) and MTT dye (thioazyl blue) were purchased from Sigma Chemical Co. (St. Louis, MO). Expression vectors encoding full-length and truncated or deleted forms of human CIP4 have been described earlier (10). These CIP4 cDNAs were cloned into the pRK5-myc mammalian expression vector, which provides an amino-terminal myc epitope tag.

Cell Lines and Culture. MDA-MB-453 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal calf serum (5% v/v), non-essential amino acids (100 mM), L-glutamine (5 mM), streptomycin (100 μ g/ml), and penicillin (100 units/ml) (all from BioWhittaker, Walkersville, MD). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Adriamycin [ADR] treatment. Subconfluent proliferating cells in 100 mm plastic dishes or 96-well plates were pre-incubated in the absence or presence of HGF/SF (100 ng/ml x 48 hr) in serum-free DMEM and then sham-treated (control) or treated with ADR (10 μ M x 2 hr, at 37°C) in complete culture medium (DMEM plus 5% fetal calf serum). Cultures were then washed three times to remove the ADR and post-incubated in fresh drug-free complete culture medium at 37°C for 72 hr (again in the absence or presence of HGF/SF, respectively). Cultures were then harvested for isolation of total cell RNA and cDNA microarray or semi-quantitative RT-PCR analyses.

Transient Transfections. Subconfluent proliferating cells were transfected overnight using Lipofectamine (GibcoBRL Life Technologies, Rockville, MD) (10 μ g plasmid DNA per 100 mm dish) and then washed to remove the excess vector and Lipofectamine. As a control for transfection efficiency, cultures were co-transfected with 10 μ g of a β -galactosidase expression vector (pSV- β -gal, Promega, Madison, WI) under parallel conditions; and β -galactosidase was detected using an X-gal staining kit (Gene Therapy Systems, Inc., San Diego, CA).

MTT Cell Viability Assay. This assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] into an insoluble formazan precipitate, which is dissolved in dimethyl sulfoxide and quantitated by spectrophotometry (11). To test the effect of CIP4 on HGF/SF-mediated cell protection, cells transiently transfected with CIP4 expression vectors (see above) were harvested using trypsin and seeded into 96-well dishes (2000 cells per well) in standard growth medium, incubated for 24-48 hr to allow attachment and entry into the cell cycle, pre-incubated \pm HGF/SF (100 ng/ml x 48 hr), treated with ADR (10 or 20 μ M x 2 hr), post-incubated for 72 hr, and tested for MTT dye conversion. Cell viability was calculated as the amount of MTT dye conversion relative to sham-treated control cells. Ten replicate wells were tested for each experimental condition. Statistical comparisons were made using the two-tailed Student's t-test.

Isolation of RNA. After cell treatments \pm ADR \pm HGF/SF, the total cellular RNA was extracted using TRIzol[®] Reagent (GibcoBRL), according to the manufacturer's instructions. The RNA was treated with DNase and precipitated using 95% ethanol prior to cDNA synthesis. Isolated RNA was electrophoresed through 1.0% agarose-formaldehyde gels to verify the quality of the RNA, and RNA concentrations were determined from optical density measurements at 260 and 280 nm.

cDNA Synthesis and Microarray Hybridization. One hundred micrograms of total cellular RNA was annealed to oligo(dT) and reverse-transcribed in the presence of Cy3-labeled or of Cy5-labeled dUTP (Amersham Pharmacia Biotech, Piscataway, NJ), using 10,000 units/ml of Superscript II reverse transcriptase (GibcoBRL). The resulting Cy3- and Cy5-labeled cDNAs were treated with RNase One (Promega, Madison, WI) for 10 min at 37°C, combined, purified by using a Centricon-50 filtration spin column (Millipore, Bedford, MA), and concentrated to final volume of 6.5 µl. The cDNA was then combined with 12.5 µl of hybridization solution and 1.0 µl of blocking solution to a final volume of 20 µl. The mixture was heated at 94°C for 2 min and centrifuged at 13,000 rpm for 10 min; and the supernatant was transferred to a clean tube and incubated at 50°C for one hour.

Hybridizations were performed on cDNA microarray glass slides prepared at the Albert Einstein College of Medicine microarray facility. Each slide contained 9,216 unique human cDNA clones. The hybridization solution was placed on a pre-treated microarray slide, covered with Hybri-slip, and then incubated in a hybridization chamber overnight at 50°C. After hybridization, the slide was washed at room temperature, first with 0.2 x SSC, 0.1% SDS for 20 min with gently shaking, and then with 0.2 x SSC two times (20 min each time). Slide was dried by spinning at low speed in a centrifuge for 5 min.

Scanning, Griding, and Analysis. The slides were scanned using a Microarray Scanner 4000A (Axon Instruments) at the Albert Einstein College of Medicine Cancer Center microarray facility. The scanner output images were localized by overlaying a grid on the fluorescent images, using the ScanAlyze software by Michael Eisen, Stanford University (eisen@genome.stanford.edu, 1998-1999). The fluorescent intensities were then calculated, using the program Copy of FUBAR! (the easy way out). The final reported intensity was the difference between average probe intensity and average local background intensity. Both final

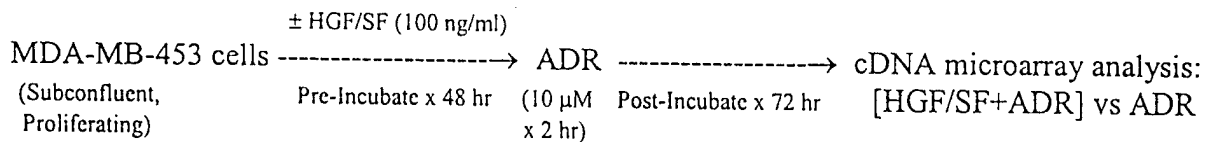
reported intensities (green and red) were filtered and the spots with intensity less than 1.5 were eliminated. The ratios of the red intensity to the green intensity and green intensity to red intensity for all targets were determined.

Semi-quantitative RT-PCR Analysis. Aliquots of total cellular RNA (1.0 μg) were subjected to first strand cDNA synthesis using Superscript II reverse transcriptase (GibcoBRL); and the cDNA was diluted 5 times with water. One μl of the diluted cDNA was used for each PCR reaction. PCR amplifications were performed using a Perkin-Elmer DNA thermal cycler. The PCR primer sets utilized in this study are shown in Table 1. The PCR reaction conditions were individually optimized for each gene product studied. For each gene product, the cycle number was adjusted so that the reactions fell within the linear range of product amplification. PCR reaction conditions and cycle numbers are shown in Table 2. The β -actin and β_2 -microglobulin genes were utilized as controls for loading. PCR products were analyzed by electrophoresis through 1.2% agarose gels containing 0.1 mg/ml of ethidium bromide; and the gels were photographed under ultraviolet illumination. The amplified cDNA product bands were quantitated by densitometry.

Western Blotting. Whole cell lysates were prepared and Western blotting was performed as described earlier (5). Equal aliquots of total cell protein (50 μg per lane) were electrophoresed, transferred, and blotted using an anti-myc mouse monoclonal antibody (Invitrogen, Carlsbad, CA) at a 1:1500 dilution, to detect the myc epitope tagged wild-type and mutant CIP4 proteins.

RESULTS

cDNA microarray analyses. The purpose of this study was to identify candidate genes whose expression is altered by HGF/SF in the setting of DNA damage, that might contribute to the HGF/SF-mediated protection against adriamycin (ADR). ADR is a DNA topoisomerase II α inhibitor that induces single- and double-stranded DNA breakage. The basic experimental protocol is described in the Materials and Methods section and is summarized in the diagram shown below:



This design was chosen for several reasons. The main comparison was between [HGF/SF+ADR] vs ADR alone, to identify genes for which expression was altered by HGF/SF during the response to DNA damage, since it is likely that some of these alterations may contribute to HGF/SF-mediated cell protection. However, a comparison of cells treated with HGF/SF vs CONTROL (sham-treatment only) was also made. A post-incubation period of T=72 hr after removal of ADR was utilized in order to examine well-established alterations in gene expression rather than transient changes occurring immediately after DNA damage. Furthermore, alterations in mRNA levels observed at T=72 hr are more likely to reflect changes in protein levels, since the mRNA alterations are of a prolonged duration.

An illustration of cDNA microarrays comparing gene expression in cells treated with [HGF/SF+ADR] vs ADR alone and in cells treated with HGF/SF vs 0 (control) is provided in Fig. 1. Gene products whose expression was consistently increased in [HGF/SF+ADR]-treated cells, relative to cells treated with ADR alone, by an average ratio of greater than 1.7 in at least two out of three independent experiments are listed in Table 3. Those gene products for which expression was consistently decreased in cells treated with [HGF/SF+ADR] relative to ADR

alone (ratio less than 0.7 in at least two out of three independent experiments) are listed in Table 4. The ratio values shown in these tables represent the means \pm ranges (N=2) or means \pm SDs (N=3). Some of the cDNA sequences contained on the microarray slides corresponded to expressed sequence tags (ESTs) for which the full-length sequence is not available in public domain databases. Alterations in the expression of cDNAs corresponding to these cDNAs, for which there is little or no information available on the structure-function of the putative gene product, are not included in Tables 3 and 4.

Although the HGF/SF-induced alterations in gene expression in the setting of DNA damage were not usually very large [(1.7-4.0)-fold increases and (0.41-0.67)-fold decreases], these changes were very reproducible. Elevated mRNA levels in the [HGF/SF+ADR] group (relative to ADR alone) were observed for various different functional classes of genes, including genes involved in the DNA damage response (*eg.*, ATM, FEN1) and cell cycle regulation (*eg.*, Hs-cul-3, HsGAK), signal transduction (*eg.*, RHO B, CSBP1), protein/RNA synthesis and metabolism (*eg.*, eIF3, U1 snRNP70), development and cellular differentiation (*eg.*, PKD1, IRX-2a), general cellular metabolism (*eg.*, LDH-A, PGK1), and other functional categories (see Table 3). The abbreviations for these gene products are defined and their functions (or putative functions) are shown in Table 4.

Genes for which the mRNA levels were reproducibly decreased in [HGF/SF+ADR]-treated cells (relative to ADR alone) included those in similar functional classes: including DNA damage response (*eg.*, TOPBP1), cell cycle regulation (*eg.*, c-Myc, CIP-4), signal transduction (*eg.*, 51C, STK2), protein and RNA metabolism (*eg.*, human Gu protein). Few or no gene products for which expression was reduced were observed in several functional classes, including development and differentiation, transcriptional regulation, and general cellular metabolism. However, in interpreting the significance of the lack of genes whose expression was decreased in certain functional classes, it should be noted that: 1) the number of genes included in each functional class is influenced by the ratio cutoffs, which is arbitrary; 2) fewer

genes showed decreased than increased expression, based on the ratio criteria chosen; and 3) the inclusion of genes in the different functional categories was somewhat arbitrary, since some genes could be included in more than one category.

Table 5 shows a cDNA microarray comparison of gene expression in MDA-MB-453 cells treated with HGF/SF relative to untreated control cells. These data indicate that the number of genes whose expression is reproducibly altered and the magnitude of the alterations are relatively small when the experiment is performed in the absence of treatment with ADR. However, it was noted that 51C (INPPL1), which was decreased in [HGF/SF+ADR]-treated cells relative to ADR alone, was also decreased in HGF/SF-treated cells relative to control.

RT-PCR assays. Since false positive results are commonly observed in cDNA microarray analyses, we sought to confirm some of the gene expression alterations shown in Tables 3 and 4, via semi-quantitative RT-PCR assays, utilizing techniques described before by us (12,13). The PCR primers and reaction conditions are provided in Tables 1 and 2, respectively. For each PCR assay, the reaction conditions and cycle numbers were individually optimized and adjusted so that the reaction fell within the linear range of product amplification. β -actin and β 2-microglobulin, two genes whose expression was not altered, were used as controls for loading. The levels of amplified PCR products were quantitated by densitometry and expressed relative to β -actin. Figures 2 and 3 show semi-quantitative RT-PCR results for genes whose expression was either increased (Fig. 2) or decreased (Fig. 3) in cells treated with [HGF/SF+ADR] relative to ADR alone.

In general, qualitative agreement between the cDNA microarray and RT-PCR results was quite good, although there were differences in the quantitative extent of the gene expression alterations between the two assay methodologies. Figs. 2 and 3 show 16 different genes for which expression was either increased (N=7) or decreased (N=9) in [HGF/SF+ADR]-treated cells by both cDNA microarray and semi-quantitative RT-PCR analyses. Genes confirmed to be

increased in the [HGF/SF+ADR] group included: ATM (ataxia-telangiectasia mutated), PKD1 (polycystic kidney disease-1), lysyl hydroxylase, LDH-A (lactate dehydrogenase-A), U1 snRNP70 (U1 small nuclear riboprotein, 70 kDa), VEGF (vascular endothelial growth factor), and PGK1 (phosphoglycerate kinase). Genes confirmed to be decreased in the [HGF/SF+ADR] group included: c-Myc, CIP4 (cdc42-interacting protein-4), S100A9 (calgranulin), B94 (a TNF-inducible gene product), 51C (an inositol polyphosphate-5-phosphatase, *aka.*, INPPL1 and SHIP-2), TOPBP1 (a DNA topoisomerase IIB binding protein), STK2 (a serine/threonine protein kinase), PTPN2 (a protein tyrosine phosphatase), and Gu protein (an RNA helicase).

Some of these alterations, although novel and not otherwise predictable, make sense within the context of explaining how HGF/SF may protect DNA-damage cells, as will be considered in depth in the Discussion. The down-regulation of 51C in [HGF/SF+ADR]-treated cells was of particular interest because: 1) a decrease in 51C mRNA levels was also noted in cells treated with HGF/SF alone (related to sham-treated control cells); and 2) 51C is a lipid phosphatase, analogous to PTEN, except that 51C removes the 5-phosphate while PTEN removes the 3-phosphate (14). Thus 51C, like PTEN (15), might be expected to inhibit c-Akt activation (see Discussion). Thus, we also examined 51C expression levels by semi-quantitative RT-PCR using a completely different set of primers. Very similar results were obtained for 51C using both sets of PCR primers (see Fig. 3).

Finally, it is noted that the RT-PCR assays provide additional information not obtained in the microarray comparisons. Thus, the RT-PCR assays allow comparisons of gene expression in cells treated with ADR, relative to control, a comparison not made by cDNA micorarray analysis. Thus, in Fig. 2, it was observed that in most cases, the main effect of HGF/SF was not to alter gene expression by itself, but to block the ADR-induced reduction of mRNA levels that were observed in the absence of HGF/SF. In Fig. 3, with the exception of 51C and PTPN2, HGF/SF by itself did not significantly alter gene expression; but its main effect was to block the

ADR-induced up-regulation of mRNA levels. However in some cases, the mRNA levels in [HGF/SF+ADR]-treated cells were reduced to below control levels (eg., CIP4, TOPBP1).

Role of CIP4 in HGF/SF-mediated protection against ADR. The cdc42-interacting protein-4 (CIP4) was originally identified as a protein that binds to the activated form of cdc42, a Rho-like small GTPase, and was subsequently found to bind to the Wiskott-Aldrich syndrome protein (WASP) through its carboxyl terminus and to microtubules through its amino terminus (10,16) (illustrated in Fig. 4A). Although CIP4 is not known to be involved in cell survival or apoptosis pathways, the finding that CIP4 mRNA expression is up-regulated by ADR and that HGF/SF blocks the ADR-induced up-regulation CIP4 raises this possibility. To determine if CIP4 could modulate the survival of MDA-MB-453 cells in response to ADR or HGF/SF, MDA-MB-453 cells were transfected with expression vectors encoding wild-type (wt) or mutant (truncated or deleted) forms of CIP4 containing an amino-terminal myc epitope tag and then assayed for their survival response. The MTT assay, which measures cytotoxicity as the loss of mitochondrial function (ie., the ability to reduce a tetrazolium dye to formazan) was used to quantitate cell viability (11). Expression of these proteins was confirmed by Western blotting of transfected cells using an anti-myc antibody (see Fig. 4B).

Cells transfected with wild-type CIP4 (wtCIP4) showed an increased sensitivity to ADR, as well as a significantly decreased degree of cytoprotection by HGF/SF (Fig. 4C), consistent with a role as a modulator of DNA damage or apoptosis response pathways. The decrease in cell survival in wtCIP4 transfected cells (relative to the empty vector transfected control) treated with ADR alone was greater at 10 μ M ADR than at 20 μ M ADR. This finding might reflect a greater degree of up-regulation of endogenous CIP4 expression at the higher dose of ADR, so that the transfected wtCIP4 has a smaller effect. The quantitative degrees of cell protection by HGF/SF at doses of 10 μ M and 20 μ M ADR were averaged and plotted in the bottom panel of Fig. 4C. Based on these calculations, transfection of wtCIP4 reduced the HGF/SF-mediated cell

protection from about 85% to 40%. On the other hand, there was no effect of wtCIP4 on cell viability in the absence of ADR (100% of control).

Expression vectors encoding mutant forms of CIP4 included a deletion of the microtubule binding domain (CIP4 118-545), a deletion missing the cdc42 binding region (CIP4 Δ 383-481) and a deletion of the carboxyl-terminal WASP binding domain (Fig. 4A). In general, these deletion mutants had little or no effect on the degree of HGF/SF-mediated cell protection, nor did they affect cell viability in the absence of ADR (Fig. 4C). However, cells transfected with the mutant CIP4 cDNAs did show an increase in cell viability (by \cong 15-20%) at 20 μ M ADR in the absence of HGF/SF. This finding may be due to their function as dominant inhibitors of the endogenous wild-type CIP4, although that conclusion cannot be made from this experiment alone.

Similar findings were obtained utilizing another cell type that is also protected against ADR-induced DNA damage by pre-incubation with HGF/SF, DU-145 human prostate cancer cells (6). Thus, wtCIP4, but not the mutant or truncated forms of CIP4, blocked the HGF/SF-mediated protection against ADR (data not shown). These findings are consistent with a role for CIP4 as a regulator or modulator of cell survival in the setting of DNA damage.

DISCUSSION

These studies revealed an interesting pattern of up-regulation and down-regulation of genes in MDA-MB-453 cells treated with [HGF/SF + ADR], as compared with ADR alone. Admittedly, some of these gene products may be altered simply because of the higher proportion of surviving cells in the [HGF/SF+ADR]-treated group relative to the ADR-treated group. Gene products of this type might include lactate dehydrogenase [LDH-A] and phosphoglycerate kinase [PGK1], which were increased in [HGF/SF+ADR]-treated cells. However, the complexity of the findings, including many genes that were either increased or decreased in ADR-treated cells, suggest a more selective pattern of altered gene regulation.

We have previously reported that in addition to protecting cells against cytotoxicity and apoptosis induced by DNA damage, HGF/SF enhanced the ability of carcinoma cells, including MDA-MB-453 cells, to repair DNA strand breaks induced by adriamycin or X-rays (6). The observation that cells treated with [HGF/SF+ADR] show altered expression of certain gene products involved in DNA damage response pathways is consistent with that prior finding. For example, ATM (ataxia-telangectasia mutated), a nuclear protein kinase involved in DNA damage signaling (17), and FEN1 (flap endonuclease-1), an enzyme implicated in the base excision repair pathway (18), were up-regulated in [HGF/SF+ADR]-treated cells. A mutation or deletion of the ATM gene leads to a defect in the repair of double-strand DNA breaks and increased sensitivity to ionizing radiation.

We also found that ADR caused the down-regulation of the PKD1 (polycystic kidney disease-1) gene product, and HGF/SF blocked the ADR-induced down-regulation of PKD1 expression. PKD1 has been identified as a developmentally regulated gene, the absence of which is linked to type I autosomal dominant polycystic kidney disease (19). The function of

this gene is not well understood, but PKD1 was found to encode a large cell membrane protein associated with the cadherin-catenin cell:cell adhesion complex (20). Interestingly, the PKD1 gene product was recently shown to play roles in maintaining the structural integrity of blood vessels (21) and in protecting MDCK epithelial cells against apoptosis (22). We had previously reported that HGF/SF protects both vascular endothelial and MDCK epithelial cells against DNA damage-induced apoptosis (4,5). Thus, inhibition of the down-regulation of PKD1 by HGF/SF may be a cytoprotective function, one which merits further investigation.

On the other hand, the expression of the topoisomerase binding protein TOPBP1, which binds DNA topoisomerase IIB and also shows DNA strand break binding activity (23-25), was decreased in cells treated with [HGF/SF+ADR]. ADR causes DNA strand breakage in part by converting the DNA topology enzyme topoisomerase II into a DNA cleaving enzyme (26). It is thought that topoisomerase binding proteins such as TOPBP1 may contribute to or potentiate ADR-mediated DNA damage, but the role of TOPBP1 in this process remains to be established. The finding that ADR up-regulates TOPBP1 expression and that the up-regulation is blocked by HGF/SF is provocative, since it suggests a potential mechanism by which HGF/SF might modulate the DNA damage and repair process, upstream of DNA-damage induced apoptosis. HGF/SF blocked the ADR-induced up-regulation of the human Gu protein. Gu is a DEXD box nucleolar RNA helicase, which presumably participates in aspects of RNA synthesis and processing (27). This finding is interesting because recent evidence suggests that, like topoisomerase II, Gu may be a target of ADR (28). However, the significance of this finding relative to HGF/SF-mediated cell protection remains to be determined.

A number of gene products implicated in signal transduction pathways were found to be up-regulated (eg. RhoB, RAB5A) or down-regulated [eg., STK2 (a serine/threonine kinase), PTPN2 (*aka.* T cell protein tyrosine phosphatase, TCPTP) and 51C (*aka.* INPPL1 or SHIP-2)].

Expression of the 51C gene, which encodes an the inositol polyphosphate-5-phosphatase (29), was decreased in both HGF/SF-treated cells (relative to control) and [HGF/SF+ADR]-treated cells (relative to ADR alone). This finding is of particular interest because of previous studies demonstrating a requirement for PI3K \rightarrow c-Akt signaling in the HGF/SF-mediated protection of breast cancer (MDA-MB-453) and glioma cell lines against apoptosis (6,7,30).

It had previously been reported that the tumor suppressor PTEN/MMAC1, an inositol polyphosphate-3-phosphatase, inhibited the PI3K/Akt pathway through its lipid phosphatase activity (15). Recently, 51C was similarly found to act as an inhibitor of the PI3K/Akt pathway, presumably also by reducing the levels of phosphatidylinositol-3,4,5-phosphate [PI(3,4,5)P₃], which is generated through the lipid kinase activity of PI3K (31). Thus, the reduced expression of 51C in HGF/SF-treated cells should have the effect of maintaining the levels of PI(3,4,5)P₃, which is essential for the activation and proper localization of c-Akt.

Interestingly, it has been demonstrated that one of the splice variants of the protein tyrosine phosphatase PTPN2/TCPTP, TC45, can inhibit epidermal growth factor receptor-mediated activation of PI3K/c-Akt signaling (32). Although the role of PTPN2 in c-Met receptor signaling and the important *in vivo* substrates for PTPN2 are unclear, the finding that HGF/SF down-regulates PTPN2 gene expression again raises the possibility that PTPN2 is a target for the HGF/SF-mediated protection against DNA damaging agents.

A cytoskeleton-associated cdc42-interacting protein, CIP4, was found to be up-regulated in ADR-treated cells, while HGF/SF blocked the up-regulation of CIP4. The function of CIP4 has not been definitively established, but CIP4 may function, in part, to carry the Wiskott-Aldrich syndrome protein (WASP) - a multi-domain protein involved in cytoskeletal organization - from actin filaments to microtubules (10). We showed that forced expression of

wild-type human CIP4 reduced the degree of HGF/SF-mediated protection of MDA-MB-453 cells to 50% or less of that observed in untransfected or empty vector-transfected control cells. On the other hand, expression of internally deleted or truncated CIP4 proteins did not inhibit cell protection. These findings suggest a role for CIP4 in cell survival/apoptosis pathways, a finding that is not obvious based on its known activities and protein interactions.

Although we have focussed on some of the more novel findings of this study, not all of the cDNA microarray and RT-PCR results were unexpected. For example, the finding that ADR up-regulates c-Myc mRNA expression and that the up-regulation was blocked by HGF/SF was not unexpected. We reported similar results based on Western blotting of MDA-MB-453 cells (5). The transcription factor c-Myc has been implicated in a variety of cellular processes, including proliferation, differentiation, transformation, and apoptosis. Over-expression of c-Myc renders cells more susceptible to apoptosis through both p53 dependent and p53-independent mechanisms (33,34). Thus, theoretically, down-regulation of c-Myc by HGF/SF in the setting of DNA damage, might be expected to confer protection against apoptosis.

We had also reported that ADR down-regulates the protein levels of the anti-apoptotic protein Bcl-X_L, while HGF/SF blocks the ADR-induced down-regulation of Bcl-X_L protein in MDA-MB-453 cells (1998). Bcl-X_L was not present among the cDNAs spotted onto the microarrays slides utilized in this study. However, we examined the Bcl-X_L mRNA expression by semi-quantitative RT-PCR analysis and found no ADR or HGF/SF alterations in Bcl-X_L mRNA levels in multiple repeat assays (unpublished results). Thus, the alterations in Bcl-X_L protein levels probably occur through translational or post-translational mechanisms. This finding suggests that some of the protection conferred by HGF/SF may be due to alterations in protein processing and metabolism. We had also noted that cell protection required a relatively long pre-incubation with HGF/SF of ≥ 24 hr for some protection and ≥ 48 hr for maximal

protection (5). This consideration suggests that the ability of HGF/SF to block the reduction of Bcl-X_L protein levels induced by ADR might be due to alterations in the expression of genes involved in the processing or metabolism of Bcl-X_L.

Our findings suggest the viability of the cDNA microarray approach - coupled with additional studies to confirm gene expression alterations and functional studies to evaluate the significance of the findings - as a means of identifying novel and interesting genes that may be involved in HGF/SF cell protection pathways. It is likely that some of the genes for which expression was altered by HGF/SF in the setting of DNA damage are not involved in cell survival or apoptosis pathways. Alterations in these gene products may reflect other activities of HGF/SF than promotion of cell survival, or may be a passive consequence of cell survival rather than a cause of survival. On the other hand, it is also likely that genes not previously implicated in cell survival or apoptosis mechanisms will be found to play roles in these processes (eg., CIP4).

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Table 1. Primers used for semi-quantitative RT-PCR analyses.

Gene Name	Primer Sequences (5'→3')	Genebank Accession No.	Position in cDNA Sequence	Expected Size of Product (bp)
ATM (ataxia telangiectasia mutated)	Sense: ctcagatggcagaagtgtgaggc Antisense: tacactgcgcgtataagccaatgc	NM_000051	8030-8757	728
polycystic kidney disease-1 (PKD-1)	Sense: ctctatcttgtgacagtcaaccg Antisense: gtccagctgtaggagacgttggtgc	NM_000296	4528-5211	684
lysyl hydroxylase (LH)	Sense: cgctgatccctaattggccaggcc Antisense: aagatcgagctgtgcacagatgcc	L06419	2372-2986	615
lactate dehydrogenase type A (LDH-A)	Sense: tagttctgccacctctgacgcacc Antisense: tataaaccttgatagttggttc	X02152.1	1330-1628	299
U1 snRNP70	Sense: cgagatggcaagaagattgatggc Antisense: aciccggctgcttcgccgttccgg	NM_003089	1700-2096	397
Vascular endothelial growth factor (VEGF)	Sense: atgtctatcagcgcagctactgcc Antisense: caagctgcctcgccttgaacgcg	XM_004512	150-548	399
phosphoglycerate kinase I (PGK-1)	Sense: ggtagtctctatgagccacatagcc Antisense: cagccagcaggtatgccagaagcc	XM_010102	250-1011	762
c-Myc	Sense: cacatcagcacaactacgcagegc Antisense: gactcagccaaggtgtgaggttgc	K02276	1331-1847	517
Cdc42-interacting protein (CIP-4)	Sense: caagacatggatgaacgcagg Antisense: gagatagtcctcctgctgg	AJ000414	688-1550	863
S100A9 (calgranulin B)	Sense: aggagttcatcatgctgatggcg Antisense: tggcctggcctcctgattatgg	NM_002965	275-479	205
TNF-inducible gene product B94	Sense: gagtgcagtgccctggtcatggc Antisense: tctgactcagcactgcagagcc	M92357	3306-3944	639
51C (inositol-5'-polyphosphate phosphatase like-1, INPPL1) <i>Primer Set #1</i>	Sense: cttccttcgattcagtgaggagg Antisense: ccttatcaatgctgacactcg	L36818	2062-2804	743
51C (inositol-5'-polyphosphate phosphatase like-1, INPPL1) <i>Primer Set #2</i>	Sense: tcagggcagtatctctctgcc Antisense: accccaataatattaaggtgc	Y14385	4077-4522	446
topoisomerase binding protein-1 (TOPBP1)	Sense: cgacctagagtacactaatgc Antisense: gcttcctcattaaacctgtgc	NM_007027	4630-5123	494
protein ser/thr kinase STK2	Sense: caacttacagtgtgaaagctgcc Antisense: cttaaggttattaacaatagcagg	NM_003157	2646-3144	499
protein tyrosine phosphatase (PTPN2)	Sense: ctaaggaagacttatctcctgcc Antisense: tgtagcactgtcagttactagtg	NM_002828	938-1359	422
human Gu protein	Sense: acaggcagagctggaaggac Antisense: actgatgcgtaggtacatc	U41387	1636-2123	488
β -actin	Sense: tagcggggttcaccacactgtgcccatcta Antisense: ctagaagcatttgggtggaccgatggaggg	XM_004814	541-1201	661
β_2 -microglobulin	Sense: ctccgctactctctcttcc Antisense: tgtcggatggatgaaaccag	XM_007650	41-176	136

Table 2. PCR reaction conditions for semi-quantitative RT-PCR assays.

Gene Name	PCR Cycle Parameters	Number of Cycles
ATM	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
PKD-1	94°C (1 min); 72°C (2 min)	28
lysyl hydroxylase	94°C (1 min); 60°C (1 min); 72°C (1 min)	28
LDH-A	94°C (30 sec); 57°C (30 sec); 72°C (1 min)	25
U1 snRNP70	94°C (1 min); 60°C (1 min); 72°C (1 min)	30
VEGF	94°C (1 min); 57°C (1 min); 72°C (1 min)	28
phosphoglycerate kinase-1 (PGK-1)	94°C (1 min); 60°C (1 min); 72°C (1 min)	25
c-Myc	94°C (1 min); 57°C (1 min); 72°C (1 min)	25
CIP-4	94°C (1 min); 59°C (1 min); 72°C (1 min)	35
S100A9	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
B94	94°C (1 min); 65°C (1 min); 72°C (1 min)	31
51C [INPPL1] <i>Primer Set #1</i> (743-bp)	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
51C [INPPL1] <i>Primer Set #2</i> (446-bp)	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	30
TOPBP1	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	30
STK2	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
PTPN2	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	33
human Gu protein	94°C (30 sec); 55°C (30 sec); 72°C (1 min)	30
β -actin	94°C (30 sec); 56°C (30 sec); 72°C (1 min)	23
β_2 -microglobulin	94°C (1 min); 54°C (1 min); 72°C (1 min)	28

Table 3. Genes whose expression is increased in [HGF/SF+ADR]-treated cells relative to ADR alone.

Gene Name	Function	Ratio
<i>DNA damage response</i>		
ATM (ataxia-telangiectasia mutated)	DNA damage signaling, nuclear PI-3-kinase domain protein	2.9 ± 0.0
FEN1	flap endonuclease-1, implicated in base excision repair pathway	2.1 ± 0.04
<i>cell cycle regulation</i>		
CENP-F kinetochore	microtubule motor protein, component of centromere	2.7 ± 0.3
Hs-cul-3	homology to cullin/cdc53 family, ? role in cell proliferation control	2.4 ± 0.15
HsGAK	ubiquitously expressed perinuclear cyclin G-associated kinase	2.3 ± 0.4
NuMa gene (clone T33)	nuclear mitotic protein, mitotic centromere function	2.1 ± 0.04
cell growth regulator CGR19	Ring finger gene induced by p53	2.0 ± 0.2
cyclin G2	may mediate proteolysis of G1 family cyclins	1.9 ± 0.2
<i>signal transduction-related</i>		
RHO B transforming protein	endosomal Rho protein, roles in receptor trafficking and apoptosis	4.0 ± 1.7
dual specificity tyr phosphorylat. regulated kinase	homolog of Drosophila kinase midbrain, ? role in brain development	2.1 ± 0.2
CSAids binding protein-1 [CSBP1]	aka. p38, homolog of yeast Hog1 MAPK, stress response signaling	2.1 ± 0.15
protein phosphatase PPP2R2A [PR53]	53 kDa regulatory subunit of ser/thr protein phosphatase 2A	1.9 ± 0.2
RAB5A	ras-related small GTPase, regulator of vesicle trafficking	1.6 ± 0.1
<i>protein and RNA metabolism</i>		
lysyl hydroxylase [LH1, aka. PLOD]	collagen modification, defective in Ehlers-Danlos syndrome VI	2.4 ± 0.7
eIF3	eukaryotic translation initiation factor	2.2 ± 0.3
U1 snRNP70 (small nuclear ribonucleoprotein)	associated with RNA processing and ubiquitination	2.1 ± 0.3
SAP49	spliceosomal associated protein, RNA processing	2.1 ± 0.5
cellular and nucleic acid binding protein		2.0 ± 0.3
SNC19	putative novel human serine protease mapping to chr. 11q24-25	1.9 ± 0.2
beta COP	Golgi transport protein, component of COT1 complex	1.8 ± 0.02
<i>cytokine and cytokine-induced</i>		
vascular endothelial growth factor (VEGF)	stimulates endothelial cell proliferation and angiogenesis	2.8 ± 0.9
VEGF-related protein [VRP]	FLT4 ligand, VEGF family protein	2.1 ± 0.4
interferon-induced 17 kDa protein	precursor of 15 kDa protein homologous to ubiquitin	1.8 ± 0.2
<i>development and differentiation</i>		
keratin 17	soft epithelial keratin 9 (eg., hair follicle)	3.8 ± 1.5
B4-2 protein	proline-rich natural killer cell protein	2.6 ± 0.8
keratin 19	intermediate filament protein	2.5 ± 0.7
Iroquois class homeodomain protein IRX-2a	transcription factor involved in embryonic patterning, regionalization	2.3 ± 0.4
polycystic kidney disease-1 [PKD1]	component of cadherin-catenin complex, endothelial survival	2.0 ± 0.4
cancellous bone osteoblast	mRNA expressed in osteoblasts, function unknown	2.0 ± 0.1
SM22α homolog [TAGLN2]	marker of differentiated smooth muscle (SM)-like cells	1.7 ± 0.2
<i>transcriptional regulation</i>		
RIP140	nuclear receptor-interacting protein, transcriptional coactivator	2.1 ± 0.1
hkf-1	novel zinc finger protein isolated from a brain cDNA library	2.1 ± 0.0
DGS-1	DiGeorge (velocardiofacial) syndrome candidate gene	2.0 ± 0.45
<i>general cellular metabolism</i>		
lactate dehydrogenase-A [LDH-A]	enzyme involved in anaerobic glycolysis	4.1 ± 1.0
phosphoglycerate kinase [PGK1]	glycolytic enzyme, induced by hypoxia-inducible factor HIF-1	4.0 ± 1.4
hexokinase-1	early glucose metabolic enzyme	2.2 ± 0.3
glucosylceramidase precursor	degradation of GlcCer, mutated in Gaucher's disease	2.0 ± 0.4
phosphoglycerate mutase 1 [PGAM1]	late glycolytic pathway enzyme	1.7 ± 0.25
<i>cytoskeletal and structural proteins</i>		
ezrin-radixin-moesin phosphoprotein 50 [EBP50]	PDZ phosphoprotein, linkage of cell membrane to cytoskeleton	3.8 ± 1.0
p16-Arc [ARC16]	Arp 2/3 complex subunit, control of actin polymerization	1.8 ± 0.1
<i>miscellaneous and unknown function</i>		
XAP-5	unknown function	3.1 ± 0.7
OriP binding protein [OBP1]	binds to Epstein Barr virus replication origin	2.1 ± 0.2
JTV-1	gene overlapping PMS2, function unknown	1.9 ± 0.2
MAC30 (3' end)	meningioma expressed protein	1.9 ± 0.2
Sm-like (CaSm)	cancer-associated Sm motif-like domain protein	1.7 ± 0.2

Table 4. Genes whose expression is decreased in [HGF/SF+ADR]-treated cells, relative to ADR alone.

Gene Name	Function	Ratio
DNA damage response		
P glycoprotein 3/MDR3 [PGY3]	homolog of multidrug resistance protein MDR-1, drug transport	0.51 ± 0.07
topoisomerase binding protein-1 [TOPBP1]	BRCT domain protein, binds DNA topoisomerase IIB	0.61 ± 0.05
cell cycle regulation		
c-Myc	proto-oncogene, functions in growth, differentiation, apoptosis	0.41 ± 0.04
CIP4 (cdc42-interacting protein)	interacts with Wiskott-Aldrich protein, localized in cytoskeleton	0.42 ± 0.08
ras inhibitor (3' end)	effector or regulator of H-Ras activity	0.60 ± 0.07
signal transduction-related		
180 kDa transmembrane PLA2 receptor	receptor for secretory phospholipases A2, internalizes PLA2	0.51 ± 0.05
protein tyrosine phosphatase PTPN2	aka. PT PTP (T cell protein tyrosine phosphatase)	0.57 ± 0.17
proto-oncogene c-mer [MERTK]	member of Axl subfamily of receptor tyrosine kinases	0.57 ± 0.10
protein serine/threonine kinase STK2	homolog of cell cycle regulatory kinase NIMA	0.60 ± 0.05
51C [INPPL1]	inositol polyphosphate-5'-phosphatase-like (aka. SHIP-2)	0.61 ± 0.10
apoptosis-related		
CD40L receptor	receptor for CD154, member of TNF death receptor family	0.57 ± 0.08
protein and RNA metabolism		
human Gu protein	RNA helicase, member of DEXD box family, target of adriamycin	0.46 ± 0.01
cathepsin K precursor	lysosomal acid cysteine protease, mediates proteolysis of bone	0.49 ± 0.10
cytokine and cytokine-induced		
B94	TNF-induced gene product, unknown function	0.56 ± 0.07
tazarotene-induced gene 2 [TIG2]	novel retinoid-responsive gene, deficient in psoriatic skin	0.57 ± 0.02
IGF-1 (somatomedin-C)	insulin-like growth factor-1	0.58 ± 0.02
FGF-7 (fibroblast growth factor-7)	aka. keratinocyte growth factor, epithelial-specific growth factor	0.59 ± 0.07
development and differentiation		
none		
transcriptional regulation		
none		
general cellular metabolism		
none		
cytoskeletal and structural proteins		
S100A9 (calgranulin B)	secretory protein, ? roles in inflammation, eicosanoid metabolism	0.42 ± 0.25
human triadin	integral membrane protein, binds calsequestrin	0.58 ± 0.16
vascular cell adhesion molecule VCAM1	Ig superfamily, interacts with α -4 integrins, cell trafficking	0.58 ± 0.07
ankyrin G	axon nodal protein involved in assembly of specialized structures	0.59 ± 0.13
Miscellaneous and unknown function		
hORC2L (origin recognition complex)	putative replication initiation protein	0.58 ± 0.05
CHD2	chromodomain helicase DNA-binding protein 2	0.60 ± 0.13
Rip-1 (Rev-interacting protein)	interacts with HIV Rev protein, ? function	0.67 ± 0.01

Table 5. Genes whose expression is altered in HGF/SF-treated cells, relative to untreated control cells.

Gene Name	Function	Ratio
<i>Gene products increased in HGF/SF treated cells</i>		
interleukin-8 (IL-8)	pro-inflammatory & angiogenic cytokine, neutrophil chemotaxis	1.6 ± 0.01
(clone ch13 lambda 7) α-tubulin	microtubule protein	1.6 ± 0.02
cytochrome c oxidase VIIc subunit [COX7C]	subunit of COX holoenzyme, mitochondrial energy production	1.5 ± 0.01
tubulin β-1 chain	microtubule protein	1.5 ± 0.01
<i>Gene products decreased in HGF/SF-treated cells</i>		
51C [INPPL1]	inositol polyphosphate-5'-phosphatase-like (<i>aka.</i> SHIP-2)	0.43 ± 0.0
il-TMP (intestine/liver tetraspan protein)	integral membrane protein, density-dependent growth regulation	0.57 ± 0.13
integrin α-8 subunit, 3' end	integrin expressed in developing brain and mesangial cells	0.58 ± 0.02
topoisomerase IIB [TOP2B]	nuclear enzyme involved in DNA replication and transcription	0.65 ± 0.01
corticotrophin releasing factor receptor precursor	mediates release of corticotrophin (ACTH)	0.69 ± 0.01
osteoblast mRNA for osteonidogen	basement membrane component, entactin/nidogen family	0.69 ± 0.05
Janus kinase 1 [JAK1]	mediates tyrosine phosphorylation of STAT1	0.70 ± 0.01
MutS homolog 3 [MSH3]	DNA mismatch repair enzyme	0.72 ± 0.01

FIGURE LEGENDS

Fig. 1. Illustration of cDNA microarray grids comparing gene expression in MDA-MB-453 cells treated with [(HGF/SF+ADR) vs ADR alone] (top panels) or with [HGF/SF vs 0 (control)] (bottom panels). The panels on the right show magnified views corresponding to the boxed regions of the array on the left. cDNAs isolated from cells treated with [HGF/SF+ADR] or HGF/SF were labeled with Cy5 (red dye), while cDNAs from cells treated with ADR alone or 0 were labeled with Cy3 (green dye). Spots showing red (or green) fluorescence correspond to genes over-expressed (under-expressed) in cells treated with [HGF/SF+ADR] relative to ADR alone and with HGF/SF relative to 0. Yellow spots correspond to genes equally expressed under the conditions being compared; while the absence of fluorescence indicates genes under either experimental condition. Note that alterations in gene expression, indicated by red or green spots, are more prominent in the comparison of [(HGF/SF+ADR) vs ADR] than [HGF/SF vs 0].

Fig. 2. Semi-quantitative RT-PCR analyses of genes for which expression was increased in MDA-MB-453 cells treated with [HGF/SF+ADR] relative to ADR alone. Subconfluent proliferating cells were pre-incubated \pm HGF/SF (100 ng/ml x 48 hr), treated \pm ADR (10 μ M x 2 hr), washed x 3 to remove the ADR, and post-incubated for 72 hr in fresh drug-free medium, as described in the text. RNA was collected, and RT-PCR assays were performed (see Materials and Methods section and Tables 1 and 2 for methodological details). β -actin and β_2 -microglobulin were utilized as controls for loading. The amplified PCR products were quantitated by densitometry and expressed relative to β -actin, as a percent of the control (0 HGF/SF, 0 ADR).

Fig. 3. Semi-quantitative RT-PCR analyses of genes for which expression was decreased in MDA-MB-453 cells treated with [HGF/SF+ADR] relative to ADR alone. Assays were performed as described in Fig. 2 legend. Note that %1C was analyzed using two completely different sets of PCR primers.

Fig. 4. Effect of genetic manipulation of cdc42-interacting protein (CIP4) expression on HGF/SF-mediated protection of MDA-MB-453 cells.

- A. Schematic diagrams of CIP4 expression vectors.** The human CIP4 cDNAs were cloned into the pRK5-myc mammalian expression vector, which provides an amino-terminal myc epitope tag.
- B. Expression of wild-type and mutant CIP4 proteins.** Cells were transfected with the different CIP4 expression vectors as described below (panel C), and the dishes were incubated for 24 hr to allow expression of the encoded proteins. Proteins of the expected sizes were detected by Western blotting, using an antibody against the myc epitope tag. Cells transfected with the empty pRK5-myc vector showed no myc-tagged proteins.
- C. Effect of transient expression of wild-type (wt) and mutant CIP4 proteins on HGF/SF-mediated cell protection.** Subconfluent proliferating cells in 100 mm dishes were transiently transfected overnight with 10 μ g of each vector, in the presence of Lipofectamine. Cells were washed, subcultured into 96-well dishes, pre-incubated \pm HGF/SF (100 ng/ml x 48 hr), exposed to ADR (10 or 20 μ M x 2 hr), washed, post-incubated for 72 hr in fresh drug-free medium, and assayed for MTT dye conversion. Cell viability values (means \pm SEMs) are based on ten replicate wells. For each experimental condition, cells treated with [HGF/SF+ADR] showed higher viability than those treated with ADR alone ($P < 0.001$, two-tailed t-test). The viability of cells transfected with wtCIP4 and treated with [HGF/SF+ADR] was significantly reduced, compared to similarly treated untransfected or empty vector-transfected cells ($P < 0.001$).

Fig. 1

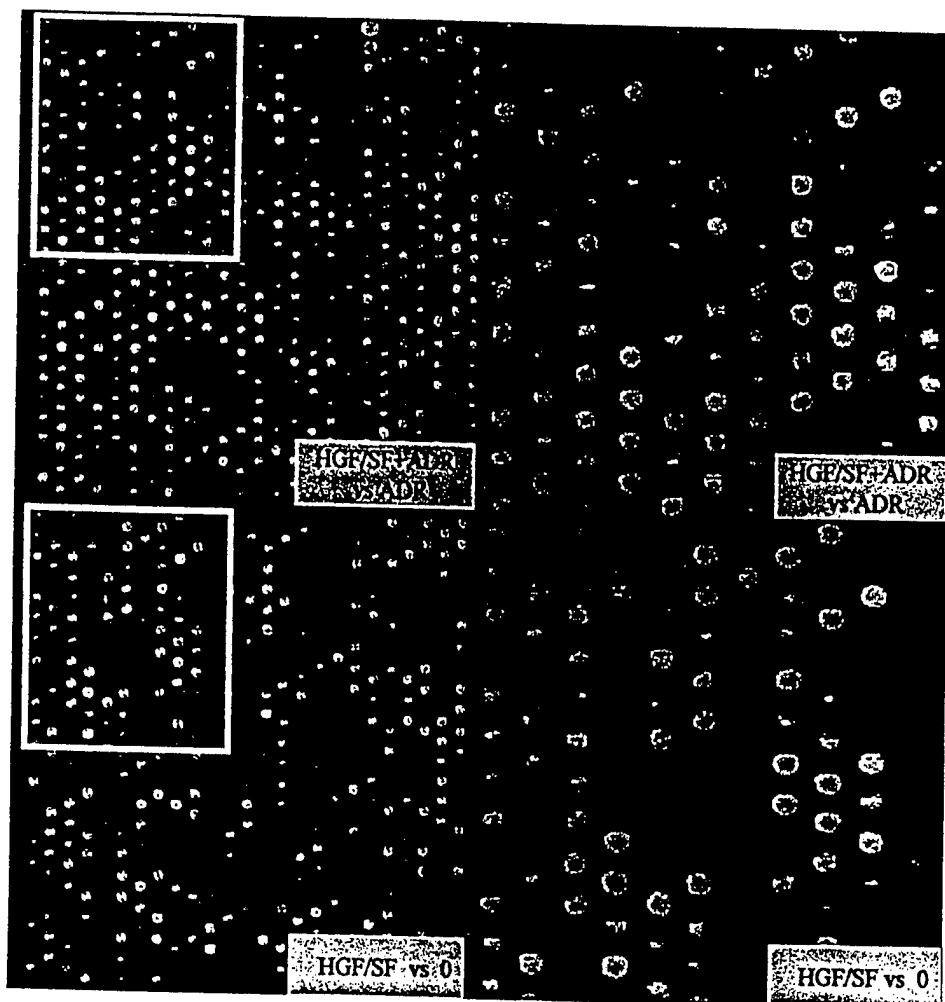


Fig. 2

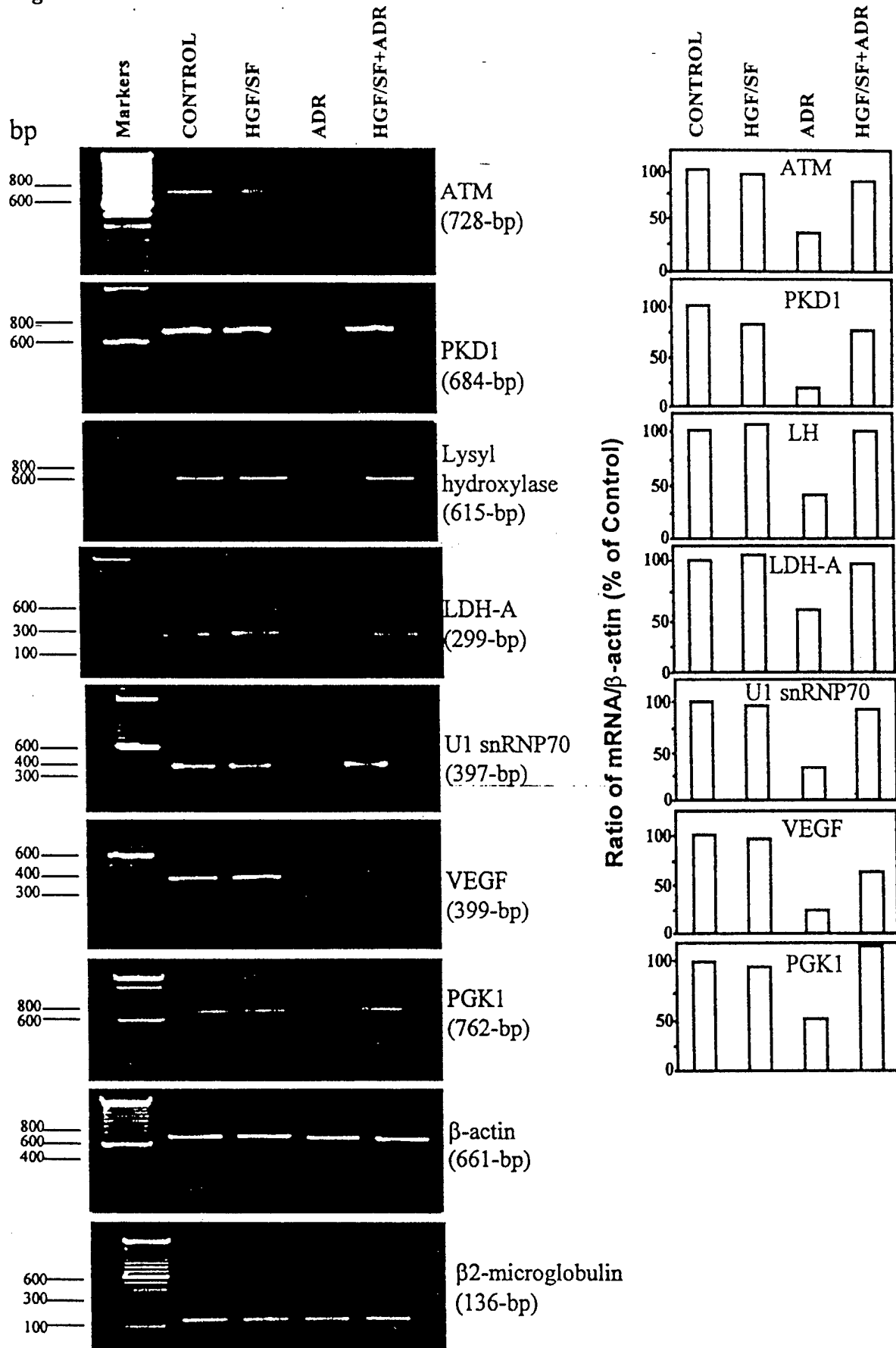


Fig. 3

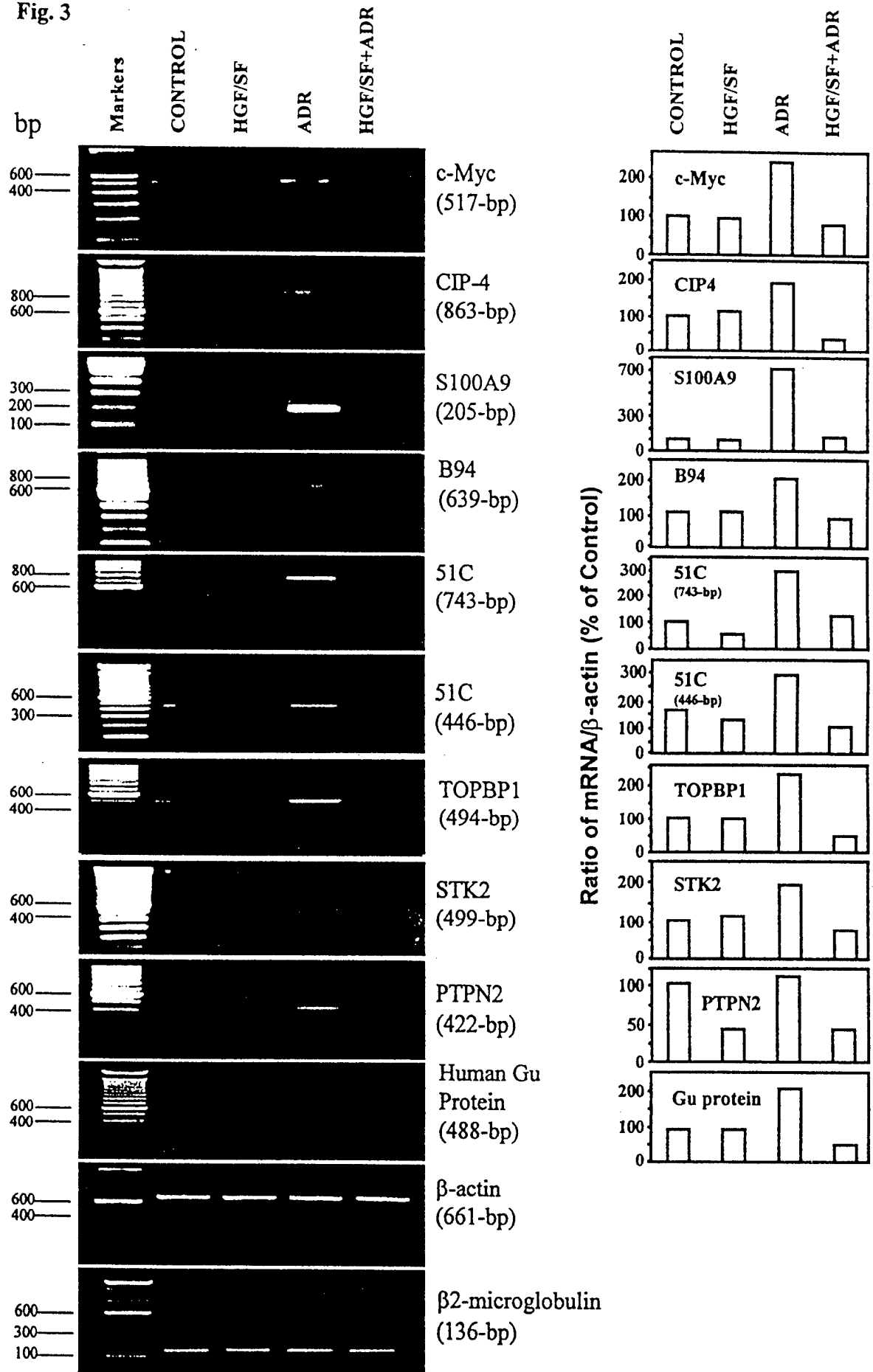


Fig. 4a

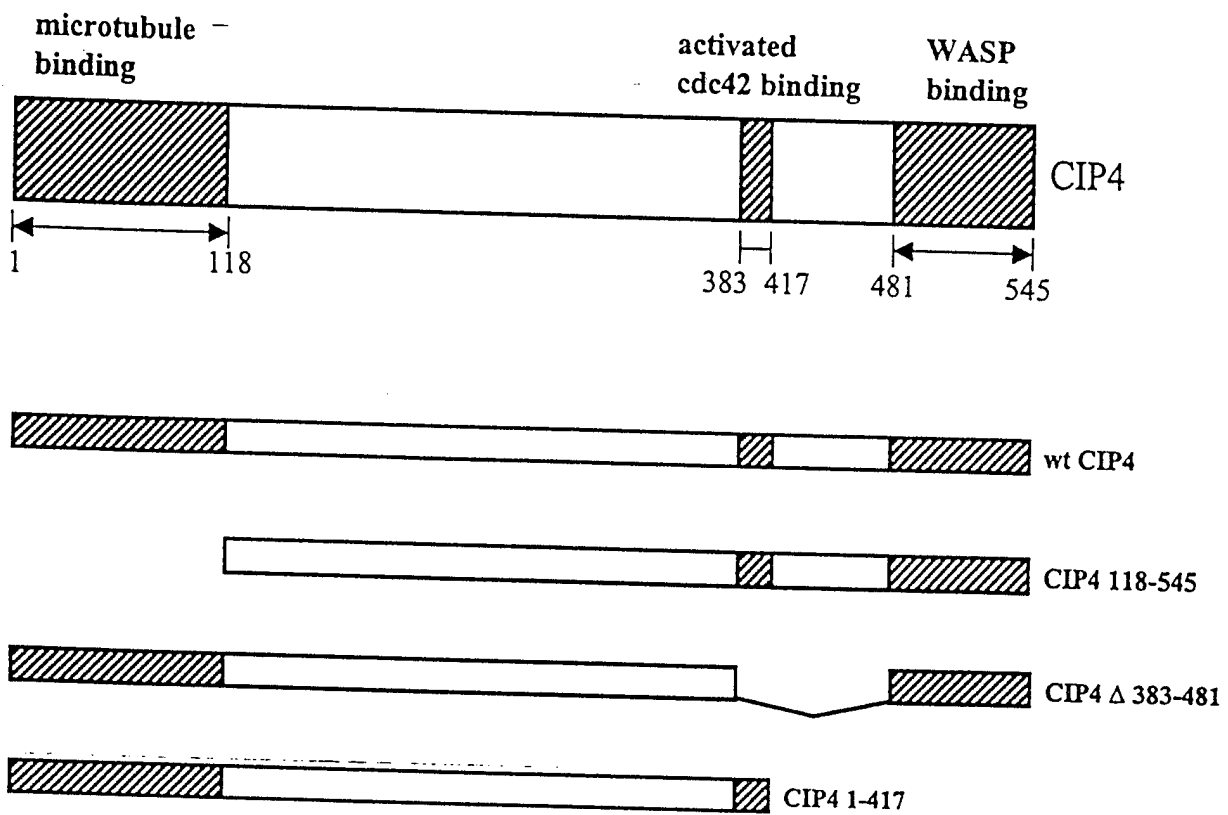


Fig. 4b

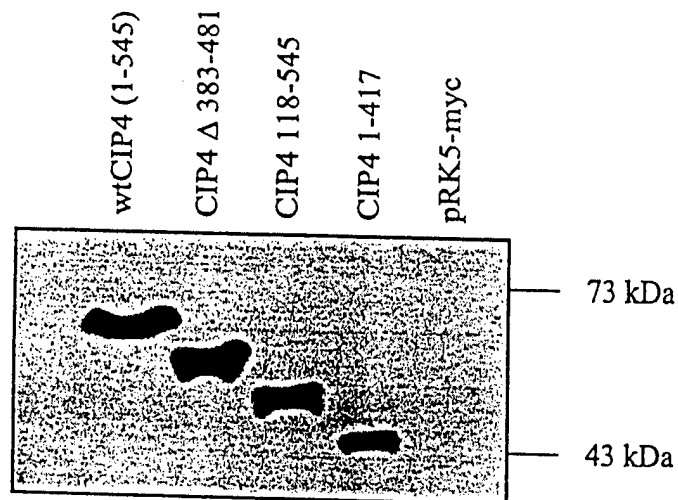
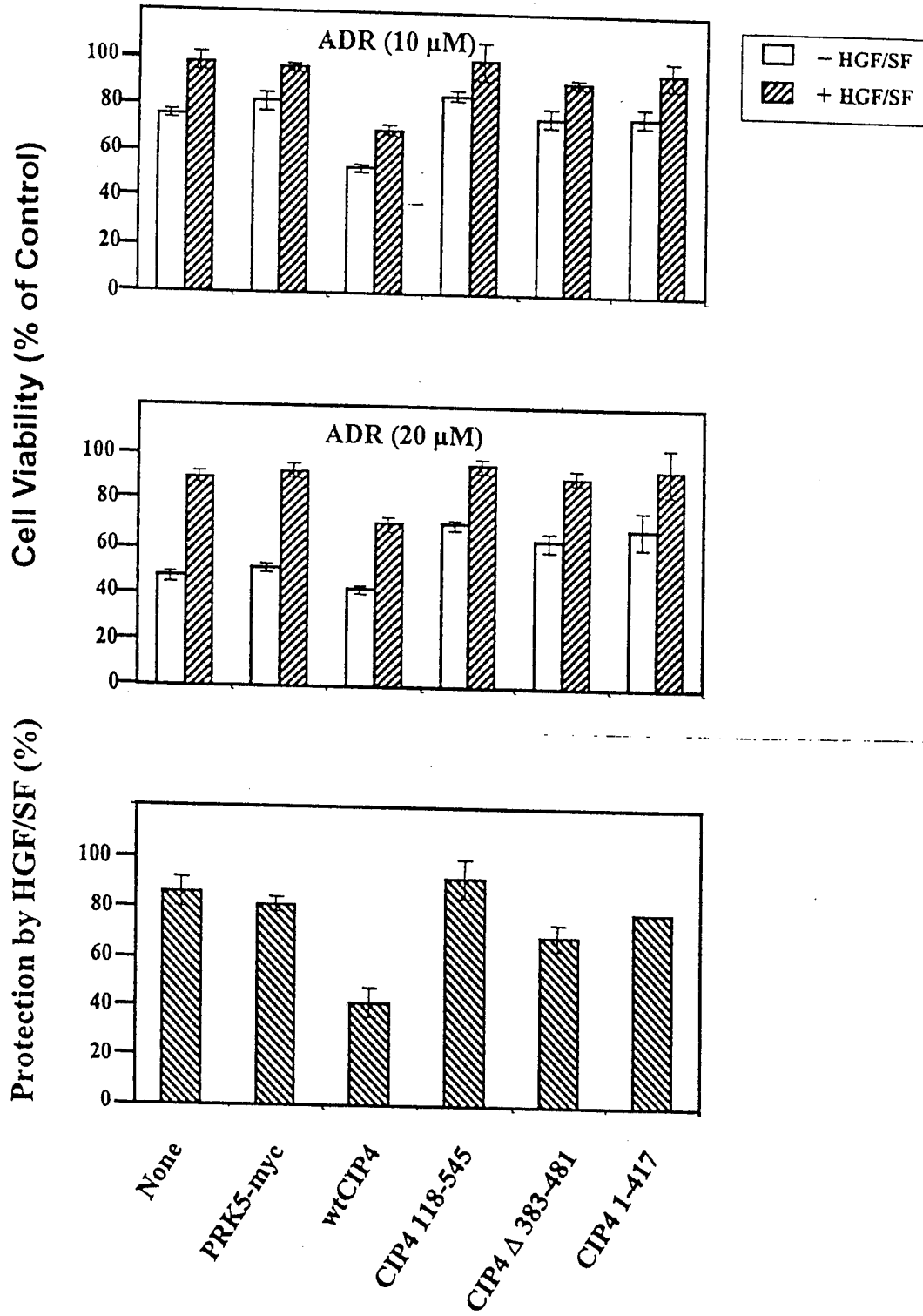


Fig. 4c



Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression

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Dedicated to Professor Dr. Ulrich Wolf on the occasion of his retirement.

Abstract. Combining chemotherapy with radiotherapy has improved the cure rate among patients with cancers of the cervix. Although one-half to two-thirds of the patients can be cured by radiation alone, such patients cannot be identified at present and must therefore suffer the burden of chemotherapy. Our long-range goal is to identify those cervical cancers that are radiosensitive and could be cured by radiotherapy alone. The advent of methods that permit the simultaneous analysis of expression patterns of thousands of genes, make it feasible to attempt to identify the molecular events related to radiosensitivity and the associated regulatory pathways. We hypothesize that the sensitivity of tumor cells to ionizing radiation (IR) is determined by the level of expression of specific genes that may be identified with the aid of cDNA microarrays. As the first step in testing this hypothesis, we determined the gene expression differences between two cell lines exhibiting different degrees of radiosensitivity. These were derived from the same tumor prior to treatment from a patient with squamous cell carcinoma of the cervix. The mRNA from these cells was subjected to cDNA analysis on a microarray of 5,776 known genes

and ESTs. The expression of 52 genes of the total of 5,776 was elevated (maximum 4.1 fold) in the radioresistant cells as compared to the radiosensitive cells. Ten of the 52 sequences are known genes while 42 are ESTs. Conversely, the expression of 18 genes was elevated in the sensitive cells as compared to the resistant cells. Seven of these 18 are known genes while eleven are ESTs. Among the genes expressed differentially between the resistant and sensitive cells were several known to be associated with response to IR and many more genes and ESTs that had not previously been reported to be related to radiosensitivity. The genes that showed the greatest overexpression in the radioresistant cell line were metal-regulatory transcription factor-1, cytochrome P450 CYP1B1, adenomatosis polyposis coli, translation elongation factor-1, cytochrome-c oxidase, whereas in the sensitive cell line, transcription factor NF-kappa-B, metalloproteinase inhibitor-1 precursor, superoxide dismutase-2, insulin-like growth factor binding protein-3, guanine nucleotide-binding protein and transforming growth factor beta-induced protein were overexpressed.

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Combining chemotherapy with radiotherapy has improved the survival rates of patients with cervical cancers (Keys et al., 1999; Morris et al., 1999; Rose et al., 1999; Whitney et al., 1999). Although one-half to two-thirds of the patients can be cured by radiation therapy alone and do not need chemotherapy, these cannot now be identified so that such patients must unnecessarily suffer the toxicity and the expense of chemother-

apy. The advent of microarray gene expression technology permits the simultaneous analysis of the levels of expression of thousands of genes. Thus, the study of molecular genetic events that are related to radiosensitivity can be examined. This may also lead to identifying genes and gene regulatory pathways related to the resistance of cells to therapeutic procedure. One of our long-range goals is to use this technology to identify those cancers that are radiosensitive and can thus be cured by radiotherapy alone. Another goal is to identify those cancers that are not controlled by the combined therapy and thus hopefully identify molecular targets for the development of therapeutic strategies.

We hypothesize that the sensitivity of tumor cells to ionizing radiation (IR) is dependent on alterations in the expression of specific genes. As the first step in testing this hypothesis, we determined the differences in the gene expression profiles of two cervical cancer cell lines derived from the same tumor but exhibiting very different degrees of radiosensitivity. We present the results in this report.

Materials and methods

Cell culture

Several cervical cancer cell lines that were derived prior to treatment by punch biopsies from patients with cervical cancers were kindly provided to us by Dr. Richard A. Britten of Cross Cancer Institute, Edmonton, Canada. These were in the fourth to fifth passage. For this report we used one pair of cell lines derived from the same tumor (HT137). These cell lines were cultured in the same way as described by Allalunis-Turner et al. (1991) and Britten et al. (1996). Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and antibiotics was used. The cells were subcultured every 4–5 days to ensure exponential growth.

Clonogenic cell survival

Following the procedures of Britten et al. (1996) clonogenic survival tests were performed. Briefly, cells were plated in 100-mm petri dishes at known densities and after 4–6 hr the cells were exposed to 2 Gy of radiation using a linear accelerator (Clinac 6-100, Varian oncology systems, Palo Alto CA). After 2 weeks the surviving colonies were stained with crystal violet solution and stained colonies containing more than 50 cells were counted. The surviving fraction (SF) after exposure to 2 Gy for the HT137R cells was thereby determined to be 0.67, and 0.35 for the HT137S cells.

Microarray sample preparation

The cDNA microarray chips and the image scanning programs were developed in the Genome Microarray Facility of the Albert Einstein College of Medicine. The human cDNA microarray chips used in this study each contain 5,776 cDNA sequences representing arbitrarily selected known genes, housekeeping genes and ESTs. The cDNA sample from the radiation sensitive cell line HT137S was labeled with the fluorescent dye, Cy5 (red) and that of the resistant cell line HT137R was labeled with Cy3 (green). A customized ScanAlyse program (Eisen et al., 1998) was used for post-acquisition processing and for database mining functions. The fluorescent signals representing hybridization to each arrayed sequence were analyzed to determine the relative amount of mRNA that hybridized with each sequence in both samples. Full details of the procedure are given on our website: <http://sequence.aecom.yu.edu/bioinf/funcgenomic.html>.

Synthesis of labeled cDNA probe

One hundred micrograms of total RNA each were isolated from the HT137S and HT137R cell pellets using the Qiagen RNeasy extraction kit. The RNA samples were incubated separately with Oligo dT12–18 at 65 °C for annealing of oligo primers. Two mixtures were prepared, one containing first strand buffer, DTT low dNTP mix, RNAsin and the fluorochrome Cy3 for HT137R cells. The second mixture was the same except that Cy5 was used for the HT137S cells. To these mixtures reverse transcriptase (RT, BRL

Table 1. Fifty-two genes and ESTs (out of the total 5,776) whose expression was elevated at least two-fold in the HT137R (Resistant) cells compared with the HT137S (Sensitive) cells

GB Accession number	Description of genes/ESTs
T72724	EST
T80917	EST
R79518	EST
H83358	EST
N42169	EST
N43977	EST
W90242	EST
AA004354	EST
AA004921	EST
AA004570	EST
AA005086	EST
AA010280	EST
AA203495	Metal-regulatory transcription factor-1
H21756	EST
H06460	EST
W02900	Cytochrome P450 CYP1B1, dioxin-inducible
N90485	EST
R00760	EST
R23082	EST
R33908	EST
N28450	EST
H29191	Adenomatous polyposis coli, alt. Splice-1
T83093	EST
R69208	EST
H82175	EST
N33565	EST
T99685	EST
R31339	EST
R37928	EST
H20450	EST
H40309	EST
N49030	EST
N36501	Phosphodiesterase
N28330	Glycoprotein MUC18, alt. Splice-2
N28369	EST
N78414	EST
T79703	EST
T85390	EST
T86312	EST
T86315	Neurotoxin, eosinophil-derived
T87438	EST
AA190599	Translation elongation factor-1, gamma
H83614	EST
AA028123	EST
H21167	EST
H46937	EST
R83166	EST
R92654	EST
N31224	Glycoprotein MUC18, alt. Splice-3
N99222	EST
H52746	Cytochrome -c oxidase, IV subunit
AA146629	Catenin, alpha 2(E), alt. Splice-1

Superscript II) was added and incubated at 42 °C for 2 h. Then, to inactivate the RT, the tubes were heated at 94 °C. The volume of this mixture was increased to 100 µl by adding buffered RNase 1 and incubated at 37 °C to digest any RNA not converted to cDNA. Then the contents of both the tubes were mixed and passed through Microcon YM 50 retention columns. The probe was collected and the volume adjusted to 6.5 µl.

Array slide preparation

The microarrays on slides were vapor moistened and quickly exposed to 200 mJ total energy in a UV Stratalinker. The slides were moistened again over boiling water and quickly dried on a hot plate. They were then treated with succinic anhydride solution for 15 min, rinsed in 0.1% SDS followed by water, and placed in a 95 °C water bath for 3–5 min after which they were dipped into ice-cold ethanol. Excess ethanol was removed from the slides by spinning the rack of slides at 500 rpm.

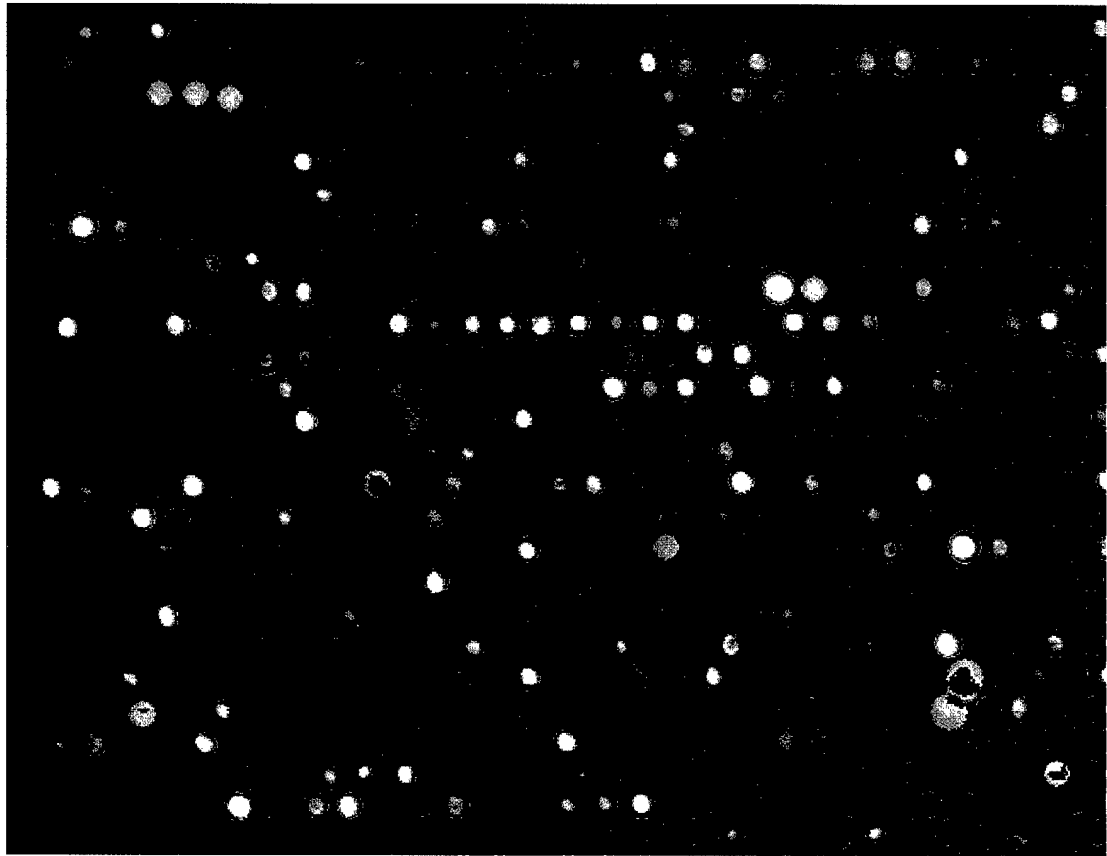


Fig. 1. A portion of the ScanAlyse picture from the cDNA microarray. The green spots represent genes whose expression is two-fold or greater in the HT137R cells than in the HT137S cells. The red spots represent genes whose expression is two-fold or greater in the HT137S cells. The yellow spots represent genes that are nearly equally expressed in both types of cells. The violet spots are "flagged" spots, i.e. those with dust/specks or similar artifacts. These are flagged to avoid their inclusion in the analysis. Circular customized grids covering each of the 5,776 spots are superimposed on the ScanAlyse spots, to assure the import of the correct color intensity of each spot area into an Excel file for data analysis.

Prehybridization of slide and probe

Prehybridization solution (20 μ l, containing formamide, SSPE, SDS, Denhardt's solution and salmon sperm DNA) was placed on the array and covered with a coverglass. The slides were placed in a chamber and prehybridized at 50 °C for one hr. Simultaneously the probe was prehybridized at 50 °C for 1 h. For prehybridization the 6.5- μ l probe was mixed with blocking solution (human cot-1 DNA, SDS, Denhardt's and SSPE solution) to bring the volume to 20 μ l. It was then heated, centrifuged and incubated as described above. After 1 h both the slides and the probe were ready for hybridization.

Hybridization and washing the slides

The prehybridized probe (20 μ l) was dropped on the array region of each slide and a fresh coverslip was placed over the slide. Hybridization was performed overnight at 50 °C.

For washing the slides were placed in a glass slide holder containing 1 \times SSC and 0.1% SDS at room temperature, where the coverslips fell off. The slides were then removed and placed in another slide holder containing 500 ml of 0.2% SSC and 0.1% SDS for 15 min. The slides were then transferred to another chamber, containing 0.2 \times SSC for 20 min, after which they were ready for scanning.

Analysis of microarray results

The hybridization signals were scanned with a laser confocal scanner which generates 2-color TIF images. Scans for the two fluorescent probes were normalized to the fluorescence intensity of beta actin and GPDH (Hel-

ler et al., 1997) and the ratios of the fluorescence intensities of all the spots was determined. Intensities for each spot in each channel were calculated after subtraction of the background. Background "noise" was reduced by using a 2-standard deviation cutoff on all expression values in order to identify only those genes with significantly different expression (Chen et al., 1997; Eisen et al., 1998; Amundson et al., 1999; Duggen et al., 1999; Lee et al., 1999; Pollack et al., 1999). Red spots represent genes whose expression in the HT137S cells is at least double that of the HT137R cells. Green spots represent those genes whose expression was double in the HT137R cells as compared to the HT137S cells, whereas yellow spots represent genes whose expression was similar in both the cell lines (Fig. 1). Genes and ESTs had to be expressed at similar fluorescent intensity ratios in at least two microarray hybridizations to be included in the analysis.

Results

The expression of 52 genes (0.9%) out of the total 5,776 was elevated (2–4.1 fold) in the HT137R cells as compared to the HT137S cells (Table 1). Ten of these 52 are known genes, while 42 are ESTs. Conversely, the expression of 18 genes was elevated 2–2.9 fold in the HT137S cells compared with the HT137R cells (Table 2). Seven of these 18 are known genes while 11 are ESTs. The genes that showed the greatest overex-

Table 2. Eighteen genes (out of the total 5,776) whose expression was elevated at least two-fold, in the HT137S (Sensitive) cells compared with the HT137R (Resistant) cells

GB Accession number	Description of genes/ESTs
R86053	Transcription factor NF-kappa-B
R97630	Alcohol dehydrogenase-1, class 1, alpha polypeptide
N67954	EST
R78823	EST
AA001324	EST
T99143	EST
N57354	EST
AA143155	Superoxide dismutase 2, mitochondrial, alt. Splice-1
R75975	Monocyte chemotactic protein -1
R25247	EST
R38114	EST
R80595	EST
N31417	Insulin-like growth factor binding protein 3
N28758	EST
R78657	Guanine nucleotide-binding protein HM89
N42864	EST
AA002125	EST
AA037281	Transforming growth factor beta-induced protein

pression in the radioresistant cell line were metal-regulatory transcription factor-1, cytochrome P450 CYP1B1, adenomatous polyposis coli, translation elongation factor-1 and cytochrome-c oxidase, whereas in the sensitive cell line, transcription factor NF-kappa-B, metalloproteinase inhibitor-1 precursor, superoxide dismutase-2, insulin-like growth factor-binding protein-3, guanine nucleotide-binding protein and transforming growth factor beta-induced protein were overexpressed.

Discussion

It is clear from the results that cell lines from the same tumor but with different radiosensitivities exhibit different patterns of gene expression. Having determined that such differences exist the next question to be answered is to what extent are these gene expression alterations related to radiosensitivity. We hope to be able to answer this question by examining a larger series of similar cell lines and also radiosensitive and radioresistant primary cervical carcinomas. If the same specific genes have altered expression in many different cases then this would be evidence that they are related to the cell's response to radiation. It is encouraging that among the genes expressed differentially between the resistant and sensitive cells in this study were

several that are known to be associated with the cell's response to IR. These are: transcription factor NF-kappa-B, superoxide dismutase-2, insulin-like growth factor-binding protein-3, guanine nucleotide-binding protein, and transforming growth factor beta-induced protein (Arnold et al., 1999; Kawai et al., 1999; Epperly et al., 2000; Kuninaka et al., 2000; Williams et al., 2000). As noted there were also a number of genes and ESTs which have previously not been reported to be related to radiosensitivity (Tables 1 and 2). Their importance in conferring the radioresponse phenotype to a cell will clearly require much more extensive studies, particularly because the phenomenon appears to be fairly complex involving several genes and gene pathways. As an example, IR-induced patterns of gene expression may vary according to the cellular context as demonstrated by Amundson et al. (1999) who studied IR-induced gene expression in human myeloid ML-1 cells using a microarray consisting of 1,238 gene sequences. They found that 48 sequences (including 30 not previously identified as IR-responsive) were significantly influenced by IR. Induction by IR of a subset of these genes was examined in a panel of 12 human cell lines, and it was observed that the responses varied widely in cells from different tissues of origin and different genetic backgrounds (Amundson et al., 1999).

Like many other investigators we have regarded as significant only those genes whose expression was altered by at least a factor of two. However, we recognize that this cutoff point is arbitrary and that there may be important genes involved whose expression was altered by less than a factor of two. Another limitation of this study is that the microarray utilized consisted of only 5,776 arbitrarily selected known genes, house-keeping genes, and ESTs. This limitation can now be overcome since microarrays with much larger numbers of genes are now available. In addition, customized arrays are becoming available with genes known to, or suspected of, participating in the process under study. An example of the successful application of the latter approach is the recent demonstration with a "lymphochip" that large-cell lymphomas responding well to CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy could be distinguished from those responding poorly (Alizadeh et al., 2000).

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Differential gene expression associated with tumorigenicity of cultured green turtle fibropapilloma-derived fibroblasts

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Abstract

Fibroblast cell lines derived from normal skin and experimentally induced fibropapillomas of green turtles (*Chelonia mydas*), were propagated in vitro and tested for tumorigenicity in immunodeficient mice. Differential display RT-PCR was used to identify differences in messenger RNA expression between normal and tumorigenic fibropapillomatosis (FP)-derived fibroblasts from the same individual. Four unique products that were apparently overexpressed in FP and three that were apparently underexpressed were cloned and sequenced. Differential expression was confirmed for three products by Northern blotting. Two overexpressed products showed extensive sequence matches to the known mammalian cellular genes, beta-hexosaminidase and chain termination factor. The product that was underexpressed in FP showed homology with mammalian thrombospondin, a known tumor-suppressor gene and an inhibitor of angiogenesis. All of the partial gene sequences identified are novel and will require full length cDNA sequencing to further analyze their identities. These results, however, provide the foundation for further investigation to determine the role of each of these gene products in FP pathogenesis and cellular transformation. The potential for some of these products to serve as biomarkers for FP is discussed. © 2001 Elsevier Science Inc. All rights reserved.

1. Introduction

The green turtle, *Chelonia mydas*, is an endangered species. In the past two decades, populations of green turtles around the world have been affected by an increasing prevalence of fibropapillomatosis (FP), a disease that is characterized by multiple cutaneous and occasional visceral fibromas or fibrosarcomas [1]. This disease kills a considerable proportion of severely affected turtles and in the rest it increases the susceptibility to other mortality factors such as predation.

A major histologic feature of FP in all tissues is the proliferation of stromal fibroblasts, which suggests that fibroblasts are the cells that have undergone pathological changes [2]. Transmission experiments have implicated a viral agent as the cause of FP [3,4], but the mechanism is unknown. Possible mechanisms include either direct transformation of infected fibroblasts (neoplasia), or paracrine

stimulation of uninfected fibroblasts (hyperplasia) by another cell type that is infected or transformed by the agent. These tumor fibroblasts are well differentiated and have normal cytologic features and are morphologically indistinguishable from normal dermal fibroblasts and have similar growth patterns and serum dependence in vitro [5]. This has made comparative studies of these cells difficult, because to elucidate the molecular basis of FP fibroblast proliferation, these cells should be differentiated from normal fibroblasts. However, we have shown that FP-derived fibroblasts are tumorigenic whereas normal dermal fibroblasts are not, using an immunodeficient mouse model [5].

The purpose of this study was to begin to elucidate the molecular mechanisms of FP pathogenesis by searching for differences in gene expression between closely matched sets of tumorigenic FP-derived and non-tumorigenic normal dermal fibroblast cell lines using differential message display analysis [6]. This is a sensitive technique to identify both novel viral genes that may be differentially expressed in infected or transformed cells and also host genes whose expression is altered by infection with the FP agent or any other transforming events.

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2. Materials and methods

2.1. Cell lines

Pairs of matched early passage tumor and normal skin-derived fibroblast lines derived from green turtles with experimentally induced FP were propagated at 30°C in a 5% CO₂ atmosphere in Dulbecco Modified Eagle Media (DMEM)/F12 supplemented with 10% FBS (GIBCO, Grand Island, NY, USA) as described previously [5]. Cultures were expanded to approximately 6×10⁷ cells. Cells were grown to confluence and then harvested with 0.25% trypsin-1 mM EDTA in Hank Balanced Salt Solution (HBSS). The cells were washed in HBSS and approximately 5×10⁷ cells were used for DNA and RNA extractions, and 1-5×10⁶ cells were used in tumorigenicity assays to confirm their phenotype. The remaining cells were cryopreserved for further investigation.

2.2. In vivo tumorigenicity

Tumorigenic potential of both tumor and normal skin-derived fibroblast cell lines were evaluated using the immunodeficient Rag-2 ^{-/-} or C.B17-*scid/scid* mice, as described in Herbst et al. [5]. Aliquots of 1-5×10⁶ cells suspended in 100 μl PBS were injected into the margin in the pinna and the mice were observed weekly for at least 4 months for evidence of tumor development.

2.3. DNA extraction

DNA was prepared from approximately 1×10⁷ cells using standard proteinase K digestion followed by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation [7].

2.4. RNA extraction

Total RNA was extracted from 3×10⁷ cell using a RNA extraction kit (Stratagene, La Jolla, CA, USA; # 200345) as per manufacturer's protocol.

2.5. Differential message display RT-PCR

Briefly, the total RNA preparations were treated with RNase-free DNase to remove possible chromosomal DNA contamination. The cDNA was synthesized from the total RNA samples by reverse transcription using 4 sets of degenerate anchored (3') primers (T12MN) where M is G, A, or C and N is G, A, T, or C. With 12 possible combinations of the last 2 bases, each primer recognized 1/12th of the total mRNA population. Partial cDNA sequences were amplified using 5' end primers, corresponding 3' end primers, and ³⁵S labeled dATP such that 50-100 cDNAs were amplified. The 5' primers were arbitrary decamers allowing annealing positions to be randomly distributed in distance from the polyA tail. The 5' primers were designed to maximally randomize the 3' end with a fixed 5' end. Following amplification, short 100-500 bp cDNA sequences were separated on polyacrylamide sequencing gels. The products

from tumorigenic and normal cells were run on adjacent lanes, allowing side-by-side comparison of the mRNA expression pattern of tumorigenic versus normal skin fibroblasts. Bands that were present in one cell line but absent in the other were cut from the gel, cloned into pGEM easy vector systems (Promega Corporation; Cat# TM042) and sequenced manually.

2.6. Northern blotting

Cloned DD-RT-PCR products were ³²P-dTTP labeled and used as probes on Northern blots to verify differential expression. Total RNA samples (30 μg per lane) from matched pairs of cell lines (FP and normal) were run on agarose gels and blotted onto nitrocellulose membranes. Probes were hybridized for 72 h at 42°C and washed in 2× SSC [7].

3. Results

The cultured tumor-derived fibroblasts were morphologically indistinguishable from cultured normal fibroblasts under light microscopy as observed previously [5]. The FP-derived fibroblast lines, however, were tumorigenic when injected into the ears of Rag2 ^{-/-} or *scid/scid* mice whereas the normal fibroblasts did not develop tumors.

DD-RT PCR yielded several cDNA segments that appeared to be either overexpressed or underexpressed in vitro in tumorigenic FP fibroblasts compared to normal fibroblasts (Fig. 1). Of these four unique overexpressed products (ranging in size from 189 to 412 bp) and three underexpressed products (193-401 bp) were cloned and sequenced.

3.1. FP overexpressed transcripts

Positive (sense) strand homologies to expressed sequence tags (ESTs) and short coding regions were found for all of the four products, however, extensive matches of the full length product sequence to 3' cDNA of known genes were found for only two of these products (LHHCM4-5 and LHHCM8-3). Both of these were confirmed by Northern blots to be overexpressed in tumor compared to normal (Fig. 2). The other two clones (LHHCM2-2 and LHHCM7-4) didn't show any homology in the GeneBank and surprisingly their differential expression could not be detected in the Northern blotting experiments in either tumor or normal fibroblast RNA under the conditions used. Repetition of these experiments with excess amounts of RNA will determine if these transcripts are actually low in copy number.

The first 31 nucleotides of product LHHCM8-3 (386 bp) had sense-strand homology to the 3' end of pig and human beta-hexosaminidase (X92379.1 and HUMHEXB, respectively), and the putative amino acid sequence was homologous to the 3 terminus of beta-hexosaminidase transcript of the mouse (P2946) and human (P06865). A putative amino acid region of 151 bp long had 21/51 (41%) amino acid matches to the 3' terminus of the mouse hexosaminidase mRNA. The remaining portion of product LHH8-3 was a non-peptide sequence.

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(b)

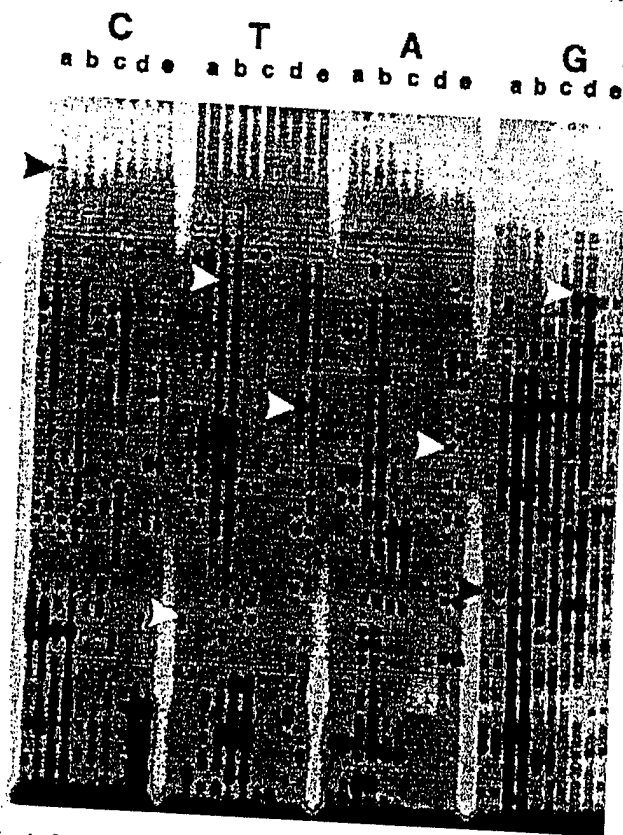


Fig. 1. Gene expression profiles of genes by differential message display studies using total RNA, isolated from a pair of matched tumor and normal skin derived fibroblast cell lines cultured from a turtle with experimentally induced FP. Columns C, T, A, and G represent each of the 3' primers ($T_{12}CN$ where N = C, T, A, and G, respectively). Subcolumns a, b, c, d, and e represent individual 5' primers (arbitrary decamers). For each of the 20 primer pair combinations, products of normal fibroblasts (left) are displayed along side those from tumor fibroblasts (right). Differentially expressed gene sequences (open and closed arrowheads) were cloned and sequenced for further characterization.

Product LHHCM4-5 (412 bp) was homologous over its entire length to the 3' terminus of eukaryotic peptide (AB029089) and hamster (MAC114, MAC111). Comparison of LHHCM 4-5 with the full-length human cDNA (HS-HCGVII) revealed 4 short gaps of 3, 19, 2, and 23 bp in the sequence alignments and was 88% identical (207/235bp) if the gaps are not considered. Interestingly the putative 3' end of LHHCM 4-5 contained a 130 bp sequence, which was unique. The product is not in the amino acid coding part of the transcript.

3.2. FP underexpressed transcripts

Three RT-PCR products that appeared to be relatively underexpressed in FP fibroblasts were also successfully cloned and sequenced. Only one of these clones, LHHCM10-5 (193 bp) was confirmed to be underexpressed in FP by Northern blotting (Fig. 2). This sequence had only limited homology to known mammalian sequences. The longest of the sense strand matches was human and bovine

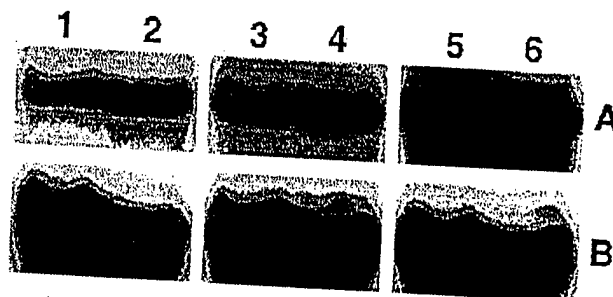


Fig. 2. Northern blotting of total RNA of normal (lanes 1, 3 and 5) and tumor (lanes 2, 4 and 6) cells using three probes derived from the differential product sequences. Lanes 2 and 4 show over-expression of two sequences (LHH4-5 and LHH8-3) and lane 6 shows under-expression of sequence LHH10-5 in the tumor samples. The 18s RNA probe (B) was used to confirm equal loading of RNA samples.

thrombospondin-1 mRNA, which contained a 33 bp sequence that was 93% identical. Sequence LHHCM5-3 (359 bp) had partial sequence homology to chicken delta EF1, a transcriptional repressor (58% base pair identity over 90 bp) and to a region containing human HLA class II gene sequence (68% base pair identity with two gaps of 77 bp and 4 bp, respectively, over 140 bp). This transcript was not detected by Northern blotting in either FP or normal fibroblast RNA under the conditions used, suggesting its low copy number. Product LHHCM1-4 (401 bp) hybridized to RNA from both cell types but was not differentially expressed. This sequence did not have any obvious matches to known genes in the databases.

4. Discussion

The availability of matched green turtle FP-derived and normal fibroblast lines and a model system in which to monitor the tumorigenic phenotype made it possible to investigate the molecular basis of fibroblast proliferation in green turtle fibropapillomatosis.

Comparison of gene expression in matched tumorigenic FP-derived versus non-tumorigenic normal skin-derived fibroblasts from individual green turtles with experimentally induced FP yielded several cDNA products by DD-PCR. Two of these were confirmed to be overexpressed in tumor fibroblast RNA and one was confirmed to be underexpressed by Northern blotting. The fact that some of the remaining products could not be detected in Northern blots of either cell type may indicate that these transcripts were very low in copy number. The total RNA was extracted from mass cultured fibroblasts, which could contain an undetermined proportion of tumor cells mixed with normal cells. Therefore, differences in gene expression that were detected by Northern blotting are probably robust differences and more subtle differences will require cloned cell lines.

Very few turtle or reptilian gene sequences have been available in the gene sequence databases. Consequently, all of the partial cDNA sequences found in this study are novel

and cannot be assigned with certainty to their putative corresponding mammalian homologue. In addition, some of the over-expressed products may represent viral gene transcripts from the FP transmissible agent, if it latently infects fibroblasts. Preliminary data from these cell lines suggest that FP fibroblasts are non-productively infected with a green turtle FP-associated herpesvirus, a candidate for the FP etiologic agent [8], so it is possible that some of these sequences could also belong to the herpesvirus genome. For each product, the full-length cDNA must be sequenced and then studied further to characterize putative gene function and to determine whether a corresponding polypeptide is expressed. Extensive additional gene sequence information for green turtles and FP-associated turtle viruses will also be needed before these products can be properly identified and before it can be determined if these products represent normal transcripts whose expression level has changed or aberrant transcripts resulting from gene mutation, activation of pseudogene expression, or abnormal RNA processing.

These results provide a foundation for developing hypotheses about the pathogenesis of fibroblast transformation in FP. For example, the role of polypeptide chain releasing factor or eukaryotic RF1, the putative identity for product LHHCM4-5, in neoplasia or viral infection deserves further investigation. This protein is responsible for chain termination at all 4 stop codons [9]. Overexpression of an aberrant form of this protein, arguably may disrupt an important checkpoint in preventing the translation of abnormal mRNA transcripts, which could enhance cancer progression [10,11].

Product LHH10-5, which is underexpressed in FP fibroblasts, is a putative mammalian homologue of thrombospondin. Thrombospondin is an inhibitor of angiogenesis and a known tumor suppressor [12-14]. Its expression is decreased in a number of neoplastic diseases and in cells infected with human cytomegalovirus, a herpesvirus, as well [15].

The putative identity of LHHCM sequence 8-3, is beta hexosaminidase based on base pair and amino acid homologies to the mammalian gene. Isozymes of beta-hexosaminidase have been shown to be overexpressed in other types of neoplasia [16-21] and increased levels have been detected in the serum of virus infected humans [22]. Thus over expression of this gene is expected in FP. Therefore, isozymes of this protein may serve as useful serum markers for turtles with cryptic FP, such as visceral tumors, or systemic virus infection.

As stated, further elucidation of the role of these genes in FP tumorigenesis or FP-virus infection will require, identification of their full length cDNAs. To establish their respective roles in tumorigenesis will require both in vitro and in vivo studies, such as transfection and overexpression of transcripts in normal fibroblasts to determine if they become tumorigenic. Fortunately, a model system has been developed and matched cell lines are available in our laboratory, which we believe would allow further elucidation of FP in turtles.

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March, 1 2001

Dr. Mohan Achary
Department of Radiation Oncology
Albert Einstein College of Medicine
Bronx, New York

Dear Dr. Achary,

I am pleased to inform you that the Advisory Committee of the *Iris and Harold P. Klinger Fund for a Postdoctoral Fellowship Stipend in Molecular Genetics* has approved your application requesting the salary support of the Research Associate, Dr. Hui Zhao, to work on your projects for the period of one year. This support may be renewed based on the satisfactory progress of the proposed work.

Sincerely yours,

A handwritten signature in cursive script that reads "Harold P. Klinger". The signature is written in black ink and is positioned above the printed name and title.

Harold P. Klinger MD, PhD
Professor of Molecular Genetics

Copies to:

1. Dr. B. Vikram, Chairman, Dept. of Radiation Oncology, AECOM/MMC.
2. Dr. R. Kucherlapati, Chairman, Dept. of Molecular Genetics, AECOM.
3. The Advisory Committee of IHPK Fund, NY.

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