Gene expression profile by inhibiting Raf-1 protein kinase in breast cancer cells

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Abstract. Raf-1 protein serine-threonine kinase plays an important role in cell growth, proliferation, and cell survival. Previously, we and others have demonstrated that antisense *raf* oligonucleotide-mediated inhibition of Raf-1 expression leads to tumor growth arrest, radiosensitization and chemosensitization *in vivo*. Raf-1 inhibition is also associated with apoptotic cell death. In this study, we inhibited Raf-1 using an antisense *raf* oligonucleotide (AS-raf-ODN) to identify downstream targets of Raf-1 using microarray gene expression analysis. Treatment of MDA-MB-231 breast cancer cells with 250 nM AS-raf-ODN led to significant inhibition of Raf-1 protein (75.2±9.6%) and *c-raf-1* mRNA levels (86.2±3.3%) as compared to untreated control cells. The lipofectin control or mismatch oligonucleotide had no effect on Raf-1 expression. To determine the changes in gene expression profiles that were due to inhibition of Raf-1, we simultaneously compared the gene expression patterns in AS-raf-ODN treated cells with untreated control cells and cells treated with lipofectin alone or MM-ODN. A total of 17 genes (4 upregulated and 13 down-regulated) including *c-raf-1* were identified that were altered after AS-raf-ODN treatment. Functional clustering analysis revealed genes involved in apoptosis (Bcl-X_L), cell adhesion (paxillin, plectin, Rho GDIα, CCL5), metabolism (GM2A, SLC16A3, PYGB), signal transduction (protein kinase C nu), and transcriptional regulation (HMGA1), and membrane-associated genes (GNAS, SLC16A3). Real-time PCR, Northern analysis and Western analysis confirmed the microarray findings. Our study provides insight into Raf-1 related signaling pathways and a model system to identify potential target genes.

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Introduction

Raf-1, a cytosolic serine/threonine protein kinase, plays an important role in mitogen and stress-induced signaling responses, cell survival and proliferation (1,2). Activated Raf-1 triggers a kinase cascade that includes phosphorylation and activation of MEK, which in turn activates ERK. These effects have generally been associated with cell proliferation and survival by inducing transcription factors such as AP-1, Elk-1, Ets and stimulation of the activity of promoters containing NF-κB sites (3-5). Recent studies have shown that Raf-1 also regulates the transcription of a number of diverse molecules (6-8). Although activating *ras* mutations have been reported in a variety of solid tumors and leukemias with resulting Raf-1/MEK/ERK activation, the complexity of Raf-1 regulation extends beyond its interaction with Ras (9,10). Recent reports using *c-raf-1* gene-knockouts have observed MEK/ERK independent functions of Raf-1 in cell survival (11,12). Studies have linked the anti-apoptotic function of Raf-1 to a re-localization of Raf-1 to the mitochondria (13). Raf-1 is reported to exert prosurvival effects upstream of cytochrome c release by phosphorylating the pro-apoptotic Bcl-2 family member, BAD (13). Raf-1 also regulates the anti-apoptotic transcription factor, NF-κB (14), which has been shown to participate in the transcriptional regulation of IAPs as well as c-FLIP, an inhibitor of caspase-8 activation (15). Raf-1 apparently induces NF-κB activation by degradation of IκB, an inhibitor of NF-κB, via MEKK-1, independent of MEK/ERK (16).

Raf-1 activity has been demonstrated both in solid tumors and hematological malignancies. Previously, it was shown that overexpression or activation of Raf-1 protein kinase is associated with morphological transformation of immortalized cells and resistance to radiation and chemotherapeutic drugs (1,17-19). Interestingly, activation of Raf has been shown to induce transcription from the human *mdr-1* promoter (19). Consequently, overexpression of Raf-1 was found to induce the multi-drug resistance phenotype, P-gp, making the cells resistant to chemotherapeutic drugs. Previously, we and others have demonstrated significant antitumor activity, radiosensitization and chemosensitization by antisense *raf* oligonucleotide-mediated inhibition of Raf-1 expression in various tumor cell types (20-23). Inhibition of Raf-1 also has been associated with apoptotic cell death (24,25).

Since antisense oligonucleotides offer a powerful tool for selective regulation of gene expression, inhibiting Raf-1 by using a specific antisense oligonucleotide may provide important information to identify novel cellular targets downstream of Raf-1 using microarray gene expression technology. In the present study, we have examined changes in gene expression profiles in breast cancer cells after antisense treatment in order to provide insight into the mechanisms regulating cell survival or cell death by Raf-1 protein kinase.

Materials and methods

Antisense oligonucleotide. The antisense *raf* oligonucleotide (5'-TCC-CGC-CTG-TGA-CAT-GCA-TT-3', ISIS 5132) (AS-raf-ODN) and a seven-base mismatch sequence (5'-TCC-CGC-GCA-CTT-GAT-GCA-TT-3', ISIS 10353) (MM-ODN) (20) were custom synthesized and obtained from TriLink BioTechnologies Inc. (San Diego, CA).

Cell line and treatment conditions. Human breast carcinoma cells (MDA-MB-231) were grown as a monolayer in improved minimum essential medium (IMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Cell cultures were maintained at 37˚C in an atmosphere containing 5% carbon dioxide. Logarithmically growing MDA-MB-231 cells were treated for 6 h with 250 nM of oligonucleotide (AS-raf-ODN or MM-ODN) in the presence of lipofectin (Invitrogen Corp., Carlsbad, CA; 12.6 μg/μg of oligonucleotide) in serum-free medium. The cells were then washed with complete medium and incubated with 250 nM AS-raf-ODN or MM-ODN without lipofectin for 18 h. The same treatment was repeated for an additional 24 h. Cells were harvested 48 h after the start of the experiment.

RNA isolation and DNA array analysis. Total RNA was isolated from MDA-MB-231 cells treated with AS-raf-ODN, lipofectin alone, MM-ODN and untreated cells using TRIzol reagent (Invitrogen Corp.). Five μg of RNA was used for cDNA synthesis. A custom cDNA kit (Invitrogen Corp.) with T7-$(dT)_{24}$ primer was used for this reaction. Biotinylated cRNA was generated from the cDNA reaction using the BioArray high yield RNA transcript kit (Affymetrix Inc., Santa Clara, CA). cRNA was then fragmented (5X fragmentation buffer: 200 mM Tris acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate) at 94˚C for 35 min before chip hybridization. Following the manufacturer's protocol, fragmented cRNA was added to the hybridization mixture. For DNA array, HG-U133A from Affymetrix were hybridized for 16 h in a GeneChip Fluidics Station 400 and scanned with a GeneArray Scanner. The Human Genome U133A set of microarray represents ~14,500 human genes. Affymetrix GeneChip Microarray software was used for basic analysis. Samples were normalized to the average hybridization intensity on each chip. The study was performed as two separate experiments carried out in duplicate. Gene Spring 6.0 (Silicon Genetics, Redwood City, CA) software was used for data analysis.

RT-PCR validation. A two-step RT-PCR was performed on total RNA from AS-raf-ODN-, lipofectin- and MM-ODN-treated and untreated MDA-MB-231 cells. RT-PCR was performed on the ABI PRISM 7000 Sequence Detection System by using random hexamers from the TaqMan reverse transcription reagents and RT reaction mix (Applied Biosystems, Foster City, CA) to reverse transcribe the RNA, and TaqMan Universal PCR master mix and Assays-on-Demand gene expression probes (Applied Biosystems) for the PCR step. Amplification of 18S rRNA was used as an endogenous control to standardize the amount of sample added to the reaction. The comparative cycle threshold (C_T) method was used to analyze the data by generating relative values of the amount of target cDNA (Applied Biosystems). C_T represents the number of cycles for the amplification of target to reach a fixed threshold and correlates with the amount of starting material present. To obtain relative values, the following arithmetic formula was used: $2^{-\Delta\Delta CT}$, where ΔC_T = difference between the threshold cycles of the target and an endogenous reference (18S), and $-\Delta\Delta C_T$ = difference between ΔC_T of the target sample and a designated calibrator (untreated control). The calculated result represents the amount of normalized target relative to the calibrator.

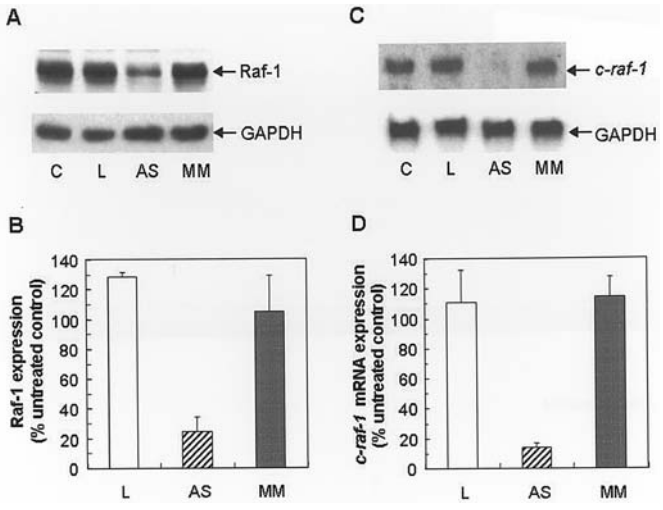

Figure 1. Antisense *raf* oligonucleotide inhibits Raf-1 protein and *c-raf-1* mRNA expression in MDA-MB-231 breast cancer cells. Logarithmically growing cells were treated with 250 nM antisense *raf* oligonucleotide (AS) or mismatch oligonucleotide (MM) in the presence of lipofectin (L) or untreated (C). (A) Whole cell lysates, normalized for protein content, were resolved by 8% SDS-PAGE and immunoblotted with anti-Raf-1 monoclonal antibody and reprobed with anti-GAPDH antibody. (B) Quantification of Raf-1 protein expression after normalization to GAPDH levels using Image-Quant software. (C) Total RNA from cells was analyzed for *c-raf-1* expression by Northern blotting using ^{32}P-labeled *c-raf-1* cDNA probe. The membrane was reprobed with GAPDH cDNA to confirm equal loading. (D) Quantification of *c-raf-1* mRNA expression after normalization to GAPDH levels using Image-Quant software. Results shown are mean ± SD from three independent experiments.

Northern blot analysis. Northern blotting was performed according to standard protocol (26). Briefly, total RNA was isolated from MDA-MB-231 cells treated with AS-raf-ODN, lipofectin alone, MM-ODN and untreated cells using TRIzol reagent (Invitrogen Corp.) according to the manufacturer's protocol. Thirty μg of RNA samples were resolved on a 1.0% agarose gel containing 1.2% formaldehyde and transferred to a nylon membrane, followed by UV cross-linking (Stratagene,

La Jolla, CA). The blots were hybridized with ^{32}P-labeled cDNA probes. For detection of *c-raf-1* mRNA expression, ^{32}P-labeled *c-raf-1* cDNA probe (ATCC, Mannasas, VA; ATCC no. 41050) was used. For detection of mRNA expression of other genes, DNA probes were generated by PCR using cDNA derived from MDA-MB-231 total RNA as a template or matching EST clones of the genes. The blots were reprobed with ^{32}P-labeled GAPDH cDNA (BD Biosciences). Quantification of gene expression after normalization to GAPDH levels was carried out using Image-Quant software (Amersham Biosciences Corp., Piscataway, NJ). The primer sequences and templates used for PCR were as follows: Rho GDIα (ARHGDIA), forward primer 5'-GAC-TAC-ATG-GTA-GGC-AGC-TA-3', reverse primer 5'-GAT-GGT-ACT-GAG-GTG-ACT-TG-3', template, image clone #4867857 (Invitrogen); protein kinase C nu (PRKCN), forward primer 5'-AAG-TCC-TAA-GAC-GGG-ACT-CT-3', reverse primer 5'-CAA-ACC-CCG-ACT-ACT-ATC-AC-3', template, image clone #5266769 (Invitrogen); paxillin (PXN), forward primer 5'-TGC-AGT-CTG-ACC-TGA-AAC-A-3', reverse primer 5'-CTT-GTC-GTT-CTG-CTC-CTT-3', template, image clone #6169341 (Invitrogen); solute carrier family 16, member 3 (SLC16A3), forward primer 5'-CAT-CAC-TGG-CTT-CTC-CTA-C-3', reverse primer 5'-GAA-GTT-GAG-TGC-CAA-ACC-3', template, image clone #5742513 (Invitrogen); BCL2-like 1 (BCL2L1), forward primer 5'-CTG-GTG-GTT-GAC-TTT-CTC-TC-3', reverse primer 5'-AGC-CTC-CTT-TGG-ACA-GAT-3', template, image clone #2823498 (Invitrogen); and glycogen phosphorylase, brain (PYGB), forward primer 5'-TGC-AGA-GCA-CGA-TGG-TGA-AC-3', reverse primer 5'-ATC-ACA-GAC-ATC-CTG-CGC-3', template, total RNA from MDA-MB-231 cells.

Western blot analysis. Whole cell lysates were prepared from MDA-MB-231 cells treated with AS-raf-ODN, lipofectin alone or MM-ODN and untreated cells using lysis buffer containing 1% Triton X-100, 0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate, 10 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ ml leupeptin. Cell lysates, normalized for protein content, were resolved by SDS-PAGE and immunoblotted with anti-Raf-1 monoclonal antibody (BD Biosciences, San Diego, CA), anti-Rho GDI polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-paxillin polyclonal antibody (Cell Signaling Technology Inc., Beverly, MA), anti-SLC16A3 (MCT3) polyclonal antibody (Santa Cruz Biotechnology Inc.), or anti-Bcl-X_L polyclonal antibody (Cell Signaling Technology Inc.). The blots were reprobed with polyclonal anti-GAPDH antibody (Trevigen Inc., Gaithersburg, MD) for normalization of protein content. Protein levels were quantified using Image-Quant software (Amersham Biosciences Corp., Piscataway, NJ).

Results

Inhibition of Raf-1 protein and c-raf-1 mRNA expression by antisense oligonucleotide in breast cancer cells. We used a well-characterized antisense phosphorothioated oligonucleotide directed against the 3'-untranslated region of *c-raf-1* mRNA (ISIS 5132; AS-raf-ODN) and a seven-base mismatch oligonucleotide (ISIS 10353; MM-ODN) (20). Initially, we evaluated the potency of AS-raf-ODN to inhibit both, the Raf-1 protein and *c-raf-1* mRNA expression, in MDA-MB 231 breast cancer cells. Treatment of MDA-MB-231 cells with 250 nM AS-raf-ODN for 48 h led to a significant inhibition in the expression of Raf-1 protein (75.2±9.6%, n=3) and *c-raf-1* mRNA expression (86.2±3.3%, n=3) as compared to untreated controls (Fig. 1). In contrast, lipofectin alone and MM-ODN treatments had no effect on Raf-1 protein and *c-raf-1* mRNA expression.

DNA microarray analysis of gene expression after treatment with antisense raf oligonucleotide. After establishing the sequence specificity and treatment conditions of antisense treatment, we isolated total RNA from MDA-MB-231 cells treated with AS-raf-ODN, MM-ODN or lipofectin alone, or untreated cells and subjected them to DNA array analysis. Gene expression patterns were examined using Affymetrix HG-U133A GeneChips representing approximately 14,500 human genes. Gene Spring 6.0 (Silicon Genetics) software was used for data analysis. Initially, we compared the variations in gene expression levels among various treatments (lipofectin alone, MM-ODN and AS-raf-ODN) to those of untreated controls using the criteria of ≥2-fold change in message level and significance of $P<0.05$ (n=4). A total of 1,417 genes were significantly altered in AS-raf-ODN-treated breast cancer cells as compared to untreated control. Surprisingly, in MM-ODN-treated cells, 1,097 genes were altered, while lipofectin alone treatment resulted in only 94 differentially expressed genes as compared to untreated control. Array data on *c-raf-1* gene expression demonstrated a reduction by 3.8-fold in AS-raf-ODN-treated cells as compared with untreated, lipofectin- or MM-ODN-treated cells (Table I).

To determine changes in gene expression profiles that were specifically due to inhibition of Raf-1, we simultaneously compared AS-raf-ODN-treated cells with controls, untreated, lipofectin- and MM-ODN-treated cells (Fig. 2). A total of 17 genes (4 upregulated and 13 downregulated) including *c-raf-1* were identified that were affected by AS-raf-ODN treatment (Table I). Functional clustering analysis revealed genes that were involved in apoptosis (BCL2-like 1 also termed as Bcl-X_L), cell adhesion (Rho GDP dissociation inhibitor, paxillin, plectin), metabolism (GM2 ganglioside activator protein, glycogen phosphorylase brain, solute carrier family 16 member 3), signaling kinases (protein kinase C nu) and transcriptional regulation (high mobility group AT-hook 1), and membrane-associated genes (GNAS complex locus, solute carrier family 16).

RT-PCR and Northern blot analysis of target gene expression after treatment with antisense raf oligonucleotide. To confirm the microarray data, the expression of some of the genes listed in Table I was independently tested by quantitative real-time RT-PCR. A two-step RT-PCR was performed on total RNA from AS-raf-ODN-, MM-ODN- and lipofectin-treated, and untreated MDA-MB-231 cells. The fold change calculated from real-time RT-PCR assays was consistent with the DNA microarray data (Table II). Further analyses of mRNA expression of selected genes were performed using Northern blot analysis (Fig. 3). Again, the Northern blot data confirmed the array analysis.

Table I. Gene expression profile after inhibiting Raf-1 expression using antisense *raf* oligonucleotide in MDA-MB-231 cells.

Accession no.	Gene name	Comments[a]	Fold change in gene expression AS:C	AS:L	AS:MM
M21121	Chemokine (C-C motif) ligand 5 (CCL5)	Mediator of chemotaxis and cell adhesion	40.0	17.2	2.5
AA401492	GNAS complex locus (GNAS)	Transmembrane GTP binding protein, mutated in cancer	6.0	6.6	2.0
U79293	Human clone 23948 mRNA sequence	-	4.5	4.9	2.1
AF070620	*Homo Sapiens* clone 24694 mRNA sequence	-	3.5	2.5	2.8
Z54367	Plectin	Organizes cytoskeleton for maintaining cellular integrity	-2.6	-2.0	-2.7
D13989	Rho GDP dissociation inhibitor (GDI) α (ARHGDIA)	Regulates actin cytoskeleton/ cell adhesion	-2.8	-2.6	-2.1
NM_018154	Anti-silencing function 1B (ASF1B)	Histone chaperone involved in nucleosome formation, chromatin silencing, transcription and DNA repair	-2.9	-2.5	-2.1
NM_017947	Molybdenum cofactor sulfurase (HMCS)	-	-3.0	-2.9	-2.2
NM_005813	Protein kinase C, nu (PRKCN)	Protein kinase C subfamily of serine/threonine kinases	-3.1	-3.3	-2.1
BE646386	Likely ortholog of mouse exocyst component protein (EXO70)	Involved in vesicular transport of proteins from Golgi apparatus to plasma membrane	-3.6	-4.5	-2.7
D86862	Paxillin (PXN)	Cytoskeletal protein involved in actin-membrane attachment at sites of cell adhesion to the extracellular matrix	-3.6	-2.9	-2.4
NM_002880	v-raf-1 murine leukemia viral oncogene homolog 1 (RAF1)	Antisense target of this study	-3.8	-3.8	-4.0
X61094	GM2 ganglioside activator protein (GM2A)	Metabolizes ganglioside	-4.4	-3.5	-2.1
AL513917	Solute carrier family 16 (monocarboxylic acid transporters), member 3 (SLC16A3)	Catalizes the rapid transport across the plasma membrane of carboxylates like lactate, pyruvates	-5.0	-3.2	-2.8
NM_001191	BCL2-like 1 (BCL2L1)	Bcl-X_L - belongs to Bcl-2 family, inhibitor of apoptosis	-6.2	-3.5	-2.1
AF176039	High mobility group AT-hook 1 (HMGA1)	DNA binding protein. Involved in the transcriptional regulation of genes near the A+T rich region	-7.2	-3.2	-3.1
NM_002862	Glycogen phosphorylase, brain (PYGB)	Involved in carbohydrate metabolism. Overexpressed in tumor cells	-21.1	-24.8	-17.4

AS, antisense *raf* oligonucleotide; L, lipofectin alone; MM, mismatch oligonucleotide; C, untreated cells. [a]Function was identified using OMIM (http://www.ncbi.nlm.nih.gov/omim/) and GeneCards (http://bioinfo.weizmann.ac.il/cards/index.html).

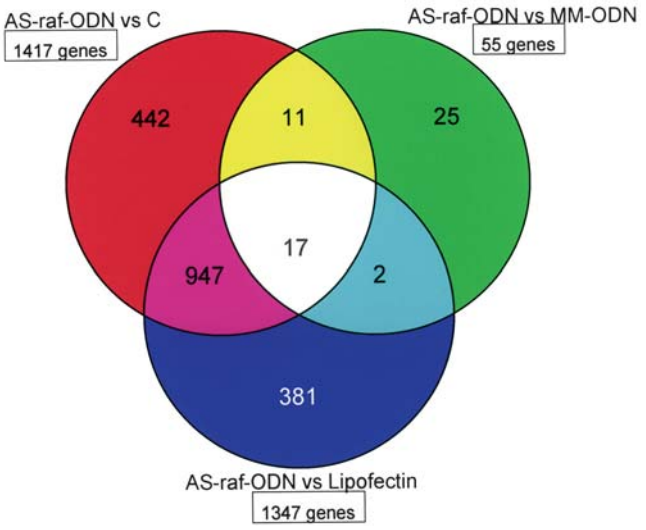

Figure 2. Venn diagram of genes affected by antisense *raf* oligonucleotide treatment. The Venn diagram was used to compare the genes affected by antisense *raf* oligonucleotide (AS-raf-ODN) with controls, untreated (C), lipofectin- and mismatch oligonucleotide (MM-ODN)-treated cells. The white area indicates the 17 genes affected exclusively by AS-raf-ODN treatment as compared to other treatments and untreated control. The red area indicates the 442 genes affected by AS-raf-ODN treatment compared to untreated control. The blue area indicates the 381 genes affected by AS-raf-ODN treatment compared to lipofectin alone. The green area indicates the 25 genes affected by AS-raf-ODN treatment compared to MM-ODN treatment. The yellow area indicates the 11 genes that were altered in AS-raf-ODN treatment compared to both untreated control and MM-ODN treatment. The pink area indicates the 947 genes that were altered in AS-raf-ODN treatment compared to both untreated control and lipofectin alone treatment. The light blue area indicates the 2 genes that were altered in AS-raf-ODN treatment compared to both lipofectin alone and MM-ODN treatment.

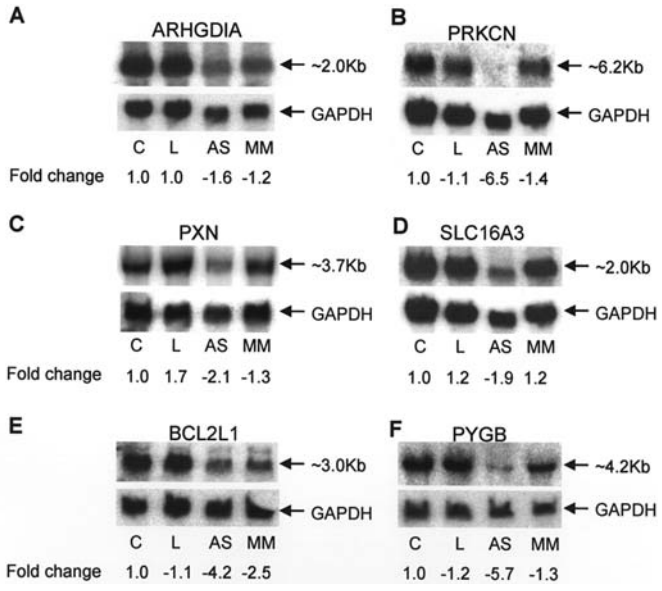

Figure 3. Northern blot analysis. Northern blotting was performed as described in the Materials and methods. (A) Rho GDIα (ARHGDIA); (B) protein kinase C nu (PRKCN); (C) paxillin (PXN); (D) solute carrier family 16, member 3 (SLC16A3); (E) BCL2-like 1 (BCL2L1); (F) glycogen phosphorylase, brain (PYGB). C, untreated control MDA-MB-231 cells; L, lipofectin alone; AS, antisense *raf* oligonucleotide; MM, mismatch oligonucleotide. Approximate sizes of the various transcripts are shown. Quantification of gene expression after normalization to GAPDH levels was carried out using Image-Quant software. The -fold change in gene expression is shown with respect to the untreated control.

Table II. RT-PCR validation.[a]

Gene	Fold change in gene expression (compared to the untreated control)			
	Untreated control	Lipofectin	AS-raf-ODN	MM-ODN
c-raf-1	1.0	-1.46±0.41	-6.94±1.49	-1.35±0.35
ARHGDIA	1.0	-0.94±0.07	-2.63±0.44	-1.46±0.23
PRKCN	1.0	-1.00±0.18	-3.62±0.93	-1.45±0.33
PXN	1.0	-1.15±0.19	-2.48±0.66	-1.44±0.32
SLC16A3	1.0	-0.87±0.30	-1.91±0.52	-1.10±0.31
BCL2L1	1.0	-1.30±0.16	-4.40±0.42	-2.52±0.27
PYGB	1.0	-1.10±0.06	-7.51±1.54	-2.39±0.33

AS-raf-ODN, antisense *raf* oligonucleotide; MM-ODN, mismatch oligonucleotide. The calculated result represents the amount of normalized target relative to the untreated control. Results shown are mean ± SD from three independent experiments.

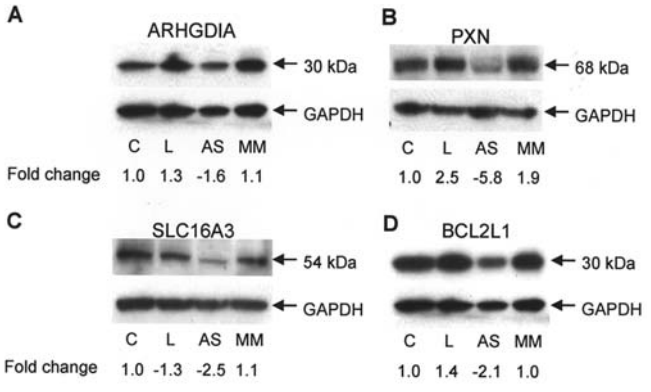

Figure 4. Western blot analysis. Whole cell lysates were prepared from MDA-MB-231 cells treated with antisense *raf* oligonucleotide (AS), mismatch oligonucleotide (MM), and lipofectin alone (L), and untreated cells (C) using lysis buffer as described in Materials and methods. Cell lysates, normalized for protein content were resolved by SDS-PAGE and immunoblotted with (A) anti-Rho GDI polyclonal antibody, (B) anti-paxillin polyclonal antibody, (C) anti-SLC16A3 (MCT3) polyclonal antibody, and (D) anti-Bcl-X_L polyclonal antibody. The blots were reprobed with polyclonal anti-GAPDH antibody for normalization of protein content. Quantification of protein expression after normalization to GAPDH levels was carried out using Image-Quant software. The -fold change in protein expression is shown with respect to the untreated control.

Western blot analysis of target gene expression after treatment with antisense raf oligonucleotide. We also examined the protein expression of genes for which commercially available antibodies could be obtained (Fig. 4). In MDA-MB-231 cells treated with AS-raf-ODN, the protein levels of paxillin, Rho GDIα, Bcl-X_L and SLC16A3 were found to be decreased. These results were in line with the findings of inhibition of mRNA expression.

Discussion

In this study, we used an antisense oligonucleotide as a tool for selective inhibition of gene expression to examine changes

in gene expression in breast cancer cells. Microarray data suggested that, although treatment with lipofectin alone had very little impact on the gene expression patterns, MM-ODN demonstrated non-specific effects. Similar non-specific effects of mismatch oligonucleotides have been observed for the phosphorothioate oligonucleotide-targeting *mdr1* gene (27). Further examination of the microarray data showed that the expression of B-Raf was not altered after treatment with AS-raf-ODN, supporting the sequence specificity of these oligonucleotides for the Raf-1 isoform. A-Raf expression in MDA-MB-231 cells was not detected. Interestingly, there were no detectable changes in expression of genes, such as *VEGF, NF-κB, c-myc* and others that might be expected to decrease after inhibiting the Raf/MEK/ERK pathway. In contrast, over-expression of Raf-1 in mammary epithelial cells induced the gene expression of *VEGF, cyclin D1, c-myc*, and other genes promoting cell proliferation, invasiveness, angiogenesis and cell survival (8). Previously, we used differential display of squamous carcinoma cells transfected with antisense *c-raf-1* cDNA (7) and breast cancer cells treated with an antisense *raf* oligonucleotide (Gokhale *et al*, Proc Am Assoc Cancer Res 41: abs. 244, 2000) to detect gene expression patterns. However, we failed to detect the genes obtained in the present study. These differences may reflect methodological or cell-type differences.

Recent studies have linked Raf-1 with cytoskeletal organization via its association with vimentin, vimentin kinases, and myosin phosphatase (28,29). Examination of Table I revealed three genes (Rho GDIα, plectin and paxillin) involved in cytoskeletal organization. Paxillin is a multi-domain protein found at the interface between plasma membrane and the actin cytoskeleton that integrates and processes adhesion- and growth factor-related signaling. Paxillin is involved in actin cytoskeletal organization which is necessary for cell motility events associated with embryonic development, wound repair and tumor metastasis (30). Paxillin has been shown to associate with Raf-1, forming a scaffold for ERK activation at focal complexes, thereby promoting lamellipodia formation and cell spreading (31).

Plectin is a very large size protein that cross-links members of all three filament systems of the cytoskeleton responsible for maintaining cellular integrity (32). Plectin has been shown to be an early substrate for caspase 8 following apoptosis induction by CD95 or tumor necrosis factor receptor leading to cell-wide cleavage of plectin (33). Plectin knock-out mice are not viable, suggesting an important role in cellular stability by reorganization of the cytoskeleton (32). Another gene identified in this study and found to be involved in regulating the cytoskeleton was Rho GDIα. This inhibits the dissociation of GDP from the GDP-bound form and sequesters Rac, Rho and Cdc42 in the inactive form (34). Inhibition of Rho GDIα using siRNA has been shown to induce apoptosis in human lung carcinoma cells (35). Also, a member of the Rho GDI family, Rho GDIα, was found to be overexpressed in pancreatic cancer cells (36).

Bcl-X_L, an anti-apoptotic member of the Bcl-2 family, was downregulated in AS-raf-ODN-treated cells, consistent with the expected induction of apoptosis by Raf-1 inhibition. Bcl-X_L exerts its apoptosis-regulatory function by binding to and inhibiting the opening of the permeability transition (PT) pore in the mitochondria, thereby preventing the release of apoptogenic factors such as cytochrome c and AIF (37). Raf-1 has also been shown to regulate the release of cytochrome c by binding to the voltage-dependent anion channel in mitochondria (38). It will be interesting to test for the mutual requirement of Raf-1 and Bcl-X_L in apoptosis suppression.

Previous reports suggest the role of Raf kinases in the regulation of glycolysis. A-Raf was found to bind and activate the pyruvate kinase type M2 (M2-PK) enzyme leading to an increase in pyruvate levels and ATP:ADP ratio. Moreover, M2-PK and A-Raf were shown to cooperate in cell transformation (39). In our study, AS-raf-ODN treatment has led to a maximum inhibition of glycogen phosphorylase, brain isoform (PYGB) expression. PYGB is a key enzyme in glycogen degradation for inducing an emergency glucose supply during stress and ischemia (40). Overexpression of PYGB has been reported in early stages of gastric and colorectal cancers (41). In addition to PYGB downregulation, we found an inhibition of the monocarboxylate transporter isoform, SLC16A3 (MCT3), in AS-raf-ODN-treated cells. MCT3 also plays a role in cellular metabolism. It effluxes lactate from the cells in order to maintain high rates of glycolysis (42). Thus, downregulation of MCT3 would be expected to lead to the accumulation of lactate in the cells, thereby inhibiting glycolysis. Recently, the inhibition of the glycolytic pathway has been reported to induce apoptotic cell death in tumor cells (43).

In conclusion, our study provides insight into the signaling pathways of Raf-1 protein kinase. The use of antisense mediated inhibition of target genes provides an important technique to identify relevant target specific genes. Confirmation and exploration of the roles of these genes in cell survival/death signaling pathways mediated by Raf-1 are necessary.

Acknowledgements

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