Identification of Differentially Expressed mRNA Transcripts in Drug-resistant *Versus* Parental Human Melanoma Cell Lines

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Abstract. *Background: Malignant melanoma resistance to chemotherapy remains a major limitation to treatment. Our aim was to identify genes associated with drug resistance, in order to better understand the molecular events underlying the drug-resistant phenotype. Materials and Methods: A human melanoma cell line and its drug-resistant variants obtained by selection with MNNG or 6-thioguanine were used. Alterations in gene expression were characterized by differential display reverse transcription-polymerase chain reaction (DDRT-PCR). Prominent mRNA fragments present in selected variants and not in the parental cells were identified and characterized by cloning and sequencing. Differential expression was confirmed by real-time RT-PCR. Results: Three functionally distinct transcriptional products were demonstrated: the chaperonin subunit TCP 1-zeta-6A (CCT6A), the hyaluronan receptor CD44 and LPPR-2, the lipid phosphate phosphatase-related protein type-2. Conclusion: Genes with altered expression were identified in drug-resistant variants. The identified molecules may provide new insights into the molecular basis for melanoma resistance to chemotherapy.*

Malignant melanoma is the most aggressive form of skin cancer and has a poor prognosis since it is notoriously resistant to therapy. The poor response of melanomas to treatment is the consequence of intrinsic resistance, although a significant increment in drug resistance also results from tumor progression and selection. The detailed biological mechanisms of resistance are largely unknown (1-3).

Gene expression studies have been undertaken to classify melanomas, to identify genetic markers for malignant transformation and metastasis (4, 5) and to identify mRNA and protein profile alterations in relatively sensitive *vs.* resistant melanoma cell lines (6-9). These investigations have resulted in the identification of proteins involved in drug detoxification, metabolism, chaperone activity and regulation of apoptotic pathways, as well as proteins with unknown physiological function. The possible role of these proteins in the development of chemoresistance remains to be validated by detailed functional tests.

The purpose of this study was to contribute to the identification of altered mRNA molecules in drug-resistant variants of a human melanoma cell line. A simple, reproducible and inexpensive method, the differentially displayed reverse transcription PCR (DDRT-PCR) (10-12) that allows for the analysis of several samples in parallel, was utilized. Since all methods for the analysis of alterations in transcription levels require confirmation with a different method, our results were verified with real-time RT-PCR.

Materials and Methods

Cell lines. The GA cell line was established from a metastatic explant of a melanoma patient (7). This cell line and its drug-resistant variants were maintained in RPMI medium supplemented with 5% L-glutamine, 5% antibiotics (streptomycin and penicillin) and 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel). The cells were regularly propagated by trypsinization.

Selection of drug-resistant variants. The GA-derived drug-resistant variants were obtained by a single step selection protocol, as

Abbreviations: MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine; 6-TG, 6-thioguanine; DDRT-PCR, differential display reverse transcription-polymerase chain reaction; LPPR-2, the lipid phosphate phosphatase-related protein type-2.

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previously described (7). Cells 10^6 were treated with the lethal dose $(LD)_{90}$ concentration of the drug for 5 days. The surviving cells were washed and allowed to propagate in fresh medium. Cells selected with 6-TG or MNNG were 60- or eight-fold (LD_{50}), respectively, more resistant to their selective agent than the parental GA cells (7).

Extraction and purification of total RNA. Two independent preparations of total cellular RNA from each cell line were isolated. Total RNA was treated with 10 U of RNase free DNase I (Sigma, St. Louis, MI, USA) at 37˚C for 30 min and was extracted with TRIZOL (GIBCO BRL, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNAs were dissolved in diethylpyrocarbonate-treated water.

Differential display reverse transcription-polymerase chain reaction (DDRT-PCR). Reverse transcription was performed with two different reverse transcriptases and using the conditions recommended by the manufacturer: Superscript II RT, MMLV RNAase H (Gibco BRL) and Sensiscript RT (Qiagen, Hilden, Germany). Both enzymes yielded identical expression profiles. First-strand cDNA was synthesized using three 3'-one-base-anchored oligo(dT)-primers. The 3'-oligonucleotide primer sequences used were: 5'-AAGCTTTTTTTTTTTG-3', 5'- AAGCTTTTTTTTTTC-3' and 5'-AAGCTTTTTTTTTTA-3', anchor primers 1, 2 and 3, respectively. Therefore, three reverse transcriptions of each RNA sample were performed in 0.5-ml microfuge tubes, each containing one of three different anchored oligo(dT) primers in a 25-μl reaction mixture containing: 1 X RT buffer, 0.5 mM dNTPs, 15 U RNase inhibitor, 1 μM anchor primer and 500 ng total RNA. The reactions were incubated at 70˚C for 5 min, followed by the addition of 200 U of Superscript II reverse transcriptase (RT) at 42˚C for 60 min and 75˚C for 5 min. Reactions containing Sensiscript RT, 1 X RT buffer, 0.5 mM dNTPs, 1 μM primer and 40 ng total RNA were incubated at 37˚C for 60 min and 93˚C for 5 min. Samples of each reverse transcription were amplified by PCR using the same oligo(dT)-primer used for the synthesis of the first-strand and several different arbitrary oligonucleotide primers (Table I). PCR amplification was performed in 25-μl reaction mixtures containing 2 μl of reverse transcription reaction mixture, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM anchor primer, 1 μM arbitrary primer and 1 U AmpliTaq (BioLine, Randolph, MA, USA). The PCR conditions were as follows: initial denaturation at 94˚C for 4 min, 40 cycles at 94˚C for 30 sec, 40˚C for 2 min, 72˚C for 30 sec and final extension at 72˚C for 5 min. For primers E8A, p53 and E8S a different PCR program was used: initial denaturation at 94˚C for 4 min, 3 cycles at 94˚C for 1 min, 50˚C for 90 sec, 72˚C for 90 sec, followed by 34 cycles at 94˚C for 45 sec, 64˚C for 45 sec, 72˚C for 90 sec, and extended elongation at 72˚C for 5 min. Reaction conditions were: 1X buffer, 2.5 mM magnesium, 0.6 mM dNTPs, 2 μM primer, 1 μM anchor primer and 1U Taq. A contamination control was performed regularly by running a "no-template reaction" and a RNA-template reaction for DNA contamination (absence of a PCR product if no DNA is present). The reproducibility was verified by at least three independent reactions and a reaction with a five-fold higher template concentration. The products were analyzed on 8% polyacryamide gels stained with silver nitrate. Gel images were acquired using the Gel Documentation System GDS 8000 SW (Ultraviolet Product Limits, London, UK).

Table I. *Primers used for DDRT PCR.*

Primer	Sequence
Anchor 1	5' - AAGCTTTTTTTTTTTA - 3'
Anchor 2	5' - AAGCTTTTTTTTTTC - 3'
Anchor 3	5' - AAGCTTTTTTTTTTG - 3'
H-AP1	5' - AAGCTTGATTGCC - 3'
H-AP2	5' - AAGCTTCGACTGT - 3'
H-AP3	5' - AAGCTTTGGTCAG - 3'
H-AP5	5' - AAGCTTAGTAGGC - 3'
OPA-1	5' - GCAGGCCCTTC - 3'
E1-OPA-1	5' - CGTGAATTCGCAGGCCCTTC - 3'
E8A p53	5' - TCCACCGCTTCTTGTCCTGC - 3'
E8A p53	5' - TAAATGGGACAGGTAGGACC - 3'

The reactions were optimized by varying the concentrations of core mix ingredients in order to obtain large quantities of amplified products, to maintain a stable reaction and high reproducibility. In addition, medium complexity expression profiles were obtained that provide reproducibility and reliability at the expense of profusion of information (13) in order to prevent false-positives. This was achieved by optimizing the arbitrary component of the PCR by an increase in the stringency of the reaction that reduces the number of electrophoretic bands but provides superior reproducibility (11). In addition, two independent RNA extractions were routinely performed from each cell line. Individual RNA preparations were reverse transcribed using five-fold different starting RNA concentrations. cDNAs were subjected to three independent expression-profiling reactions. Assuming that each reverse transcription was equally successful, each of the profiling reactions was run at two different template concentrations of cDNA differing five-fold. These precautions provide assurance that distinct electrophoretic bands reflect real differences in the expression of specific RNA species (10, 12).

Isolation, cloning and DNA sequencing of variant bands. Selected DNA bands with altered mobility were further characterized. PCR amplicons resolved on the silver-stained gels were gently removed with a hypodermic 22-gauge needle pre-wetted with the PCR master mix solution. The needle was dipped in the PCR reaction mix for 5 min and then discarded. The PCR products were reamplified with the same primers used for DDRT-PCR and the same PCR program used for these particular primers. The reamplified material was purified with the QiaQuick PCR gel extraction kit (Qiagen) and cloned with the TA cloning kit (Promega, Madison, WI, USA), according to manufacturers' instructions. The plasmids were purified with the Miniprep QIAprep Spin kit (Qiagen). The sequences were determined by Applied Biosystems Incorporated (ABI, Foster City, CA, USA) dye terminator sequencing kits, according to the manufacturer's specifications, on an ABI Prism 310 automated sequencer (Applied Biosystems) and sequencing was carried out in both directions. The obtained sequences were analyzed using the BLAST software in the NCBI GenBank database.

Real-time RT-PCR. The TaqMan PCR reactions were carried out using Assay-on-Demand Gene Expression Products (Applied

Biosystems) for chaperonin TCP-1 (CCT6A), CD44 and LPPR-2. The gene expression assays contained primers for amplification of target genes and TaqMan MGB (Minor Groove Binder) probes 6-FAM dye-labeled for the quantification. The reactions were performed in a 25-µl reaction mixture containing 1X TaqMan Universal Master Mix with AmpErase UNG, 1X Assay Mix (Applied Biosystems) and cDNA template (10 ng of RNA converted to cDNA). PCR reactions were carried out in the ABI Prism 7000 Sequence Detection System at 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 1 min. The experimental threshold was calculated on the basis of the mean baseline fluorescence signal from cycles 3 to 15 plus 10 standard deviations. The point at which the amplification plot crosses this threshold is defined as Ct, which represents the cycle number at this point and is inversely proportional to the number of target copies present in the initial sample. Each sample was run in triplicate and a mean value of each Ct triplicate was used for further calculation. A reference, endogenous control, was included in every analysis to correct differences in the inter-assay amplification efficiency and the chaperonin TCP-1 and LPPR-2 levels were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression while the CD44 levels were normalized to beta-actin expression. For the quantification, validation experiments were performed to determine the relevant endogenous control for each target gene. Three endogenous controls, GAPDH, 18S RNA and beta-actin were tested and the efficiencies of the following reactions were demonstrated to be approximately equal: GAPDH and TCP-1, GAPDH and LPPR-2, beta-actin and CD44. Serial dilutions of cDNAs were made and amplified by real-time PCR using specific primers and fluorogenic probes for target and endogenous control genes. The reaction mixture for endogenous control genes amplification was 1X TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems), 1X Assay (6-FAM dye-labeled MGB probes except for beta-actin which was VIC dye-labeled) and cDNA (10 ng of RNA converted to cDNA). Quantification was done by using the $2^{-\text{Delta, DeltaC}}$T method (14). GA was the calibrator parental sample.

ABI Prism 7000 software performed a Student's *t*-test statistical analysis and incorporated the results into the generated graphs. Significant differences between the expression of a particular cDNA between the parental GA cells and its variants was determined at $p<0.05$ (95% confidence).

Results

Differential display and analysis of variant cDNA fragments. The amplification profiles from the parental melanoma cell line and its selectants were compared and over 30 differentially expressed bands were detected. After the second and third round of amplification, the differential expression of 23 PCR products was confirmed. Approximately 10-20% variation in electrophoretic mobility was detected between the parental and drug-resistant cells.

Twelve bands, present only in the selectants but not in the parental cell line, were retrieved from the gels, cloned, sequenced and a homology or identity search was performed. The electrophoretic profiles reflecting differential mRNA expression are shown in Figure 1. Prominent new electrophoretic bands, suggestive of modified transcription

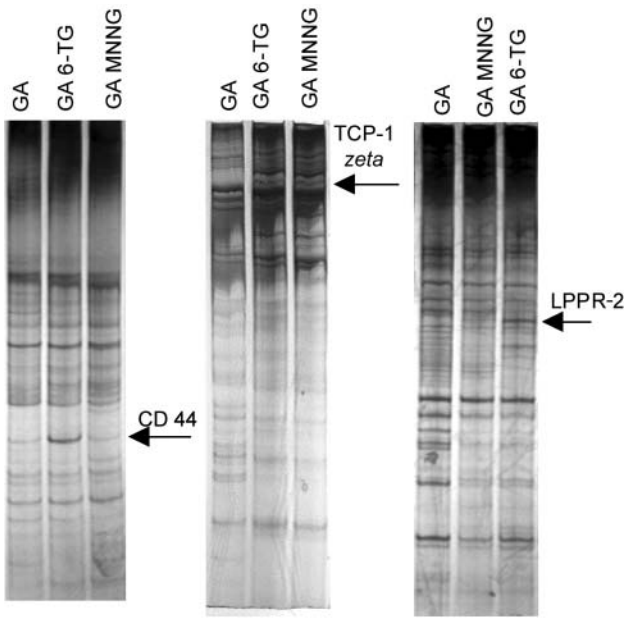

Figure 1. *Differential display RT-PCR profile of parental and drug-resistant variants. A DDRT-PCR electrophorertic expression profile of the following cells was obtained: GA cells and their 6-TG and MNNG variants; Anchor primer 1 (5'-AAGCTTTTTTTTTTTC-3') and arbitrary primer OPA-1 for CD44; Anchor primer 2 and arbitrary primer H-AP5 for TCP-1; Anchor primer 1 and arbitrary primer H-AP2 for LPPR-2. Variant electrophoretic bands, indicated by arrows, were excised, reamplified and sequenced.*

patterns, are indicated by arrows and denoted as: CD44, the hyaluronan receptor, TCP-1-zeta, the chaperonin containing TCP1-subunit-zeta-6A (CCT6A) and LPPR-2, the lipid phosphate phosphatase-related protein type 2. TCP-1 and LPPR-2 showed 100% homology with GenBank sequences, while CD44 showed 96% homology (Figure 2).

Confirmation of differential cDNA expression by real-time RT-PCR. The differential expression of the three outstanding bands was confirmed by real-time RT-PCR and their relative expression was determined by the $2^{-\text{Delta, DeltaC}}$T method (Figure 3). A significant increase in cDNA expression between GA and its variants was determined by the paired Student's *t*-test. TCP-1 and LPPR-2 c-DNAs were overexpressed in GA-resistant variants regardless of the selected drug. The expression of TCP-1 was significantly increased by the same magnitude (1.8-fold) in both variants. LPPR-2 expression was significantly overexpressed by 2.1 times in the MNNG-selected cells and by 3.2 times in the 6-TG-selected cells. Overexpression of the CD44 band was noted only in the 6-TG-selected cells and not in cells selected with MNNG. The real-time RT-PCR results confirmed a 1.8-fold increase in the expression of this gene in the 6-TG variant and a non-significant increase in the MNNG-selected cells ($p=0.181$).

1. CCT6A - chaperonin containing TCP1

Homo sapiens chromosome 7 genomic contig, alternate ref|NT_079592.1|Hs7b_79657_assembly

Length = 57638252

Score = 371 bits (193), Expect = e-100
Identities = 193/193 (100%)
Strand = Plus / Minus

```
Query: 55       ttttttcatatgggaaaatttctttcaaaattatttgaagcttggacaaaaattccacag114
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 55612254 ttttttcatatgggaaaatttctttcaaaattatttgaagcttggacaaaaattccacag 55612195

Query: 115      ctgtaatcctcaggatcactttgcagtcttcaagattcagatacagaggaagcttcaatt 174
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 55612194 ctgtaatcctcaggatcactttgcagtcttcaagattcagatacagaggaagcttcaatt 55612135

Query: 175      caacctttcagagaagacattccagctcgcatgatctcatcaaccaagagaatgttggtg 234
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 55612134 caacctttcagagaagacattccagctcgcatgatctcatcaaccaagagaatgttggtg 55612075

Query: 235      gcaatcacagtgc 247
                 |||||||||||||
Sbjct: 55612074 gcaatcacagtgc 55612062
```

2. CD 44

Homo sapiens chromosome 11 ge contig nomic ref|NT_ 9394 _ 11 |Hs .16009237

Length = 48854501
Score = 214 bits (111), Expect = 8e-53
Identities = 123/128 (96%), Gaps = 4/128 (3%)
Strand = Plus / Minus

```
Query: 47       cagtagccacatctgcatctgtataagtgtcccagctccctgtaatggttatgtttccaa 106
                 |||||| ||||| ||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct: 34014910 cagtag-cacat-tgcatctgt-taagtgtcccagctccctgtaatggttatgtttccaa 34014854

Query: 107       cggtatgtttctttccaagataatggtgtaggtgttacaccccaatcttcatgtccacat 166
                  |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 34014853  cggt-tgtttctttccaagataatggtgtaggtgttacaccccaatcttcatgtccacat 34014795

Query: 167 tctgaagg 174
            |||| |||
Sbjct: 34014794 tctgcagg 34014787
```

3. LPPR 2 - lipid phosphate phosphatase-related protein type 2

Homo sapiens chromosome 19 genomic contig ref|NT_011295.10 Hs|1911452

Length = 15825424

Score = 141 bits (73), Expect = 7e-31
Identities = 73/73 (100%)
Strand = Plus / Plus

```
Query: 54       gcttgagcagccgggactgctctccctgaagacccctccagagagaaaataaactagccc 113
                 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2728350 gcttgagcagccgggactgctctccctgaagacccctccagagagaaaataaactagccc 2728409

Query: 114      agaccctcctcta 126
                 |||||||||||||
Sbjct: 2728410 agaccctcctcta 2728422
```

Figure 2. *The identity of the DDRT-PCR bands was deduced by DNA homology with the GenBank database. The obtained sequences were analyzed using the BLAST software of the NCBI GenBank database. Query - our sequence; Sbjct - homologue sequence from GenBank database.*

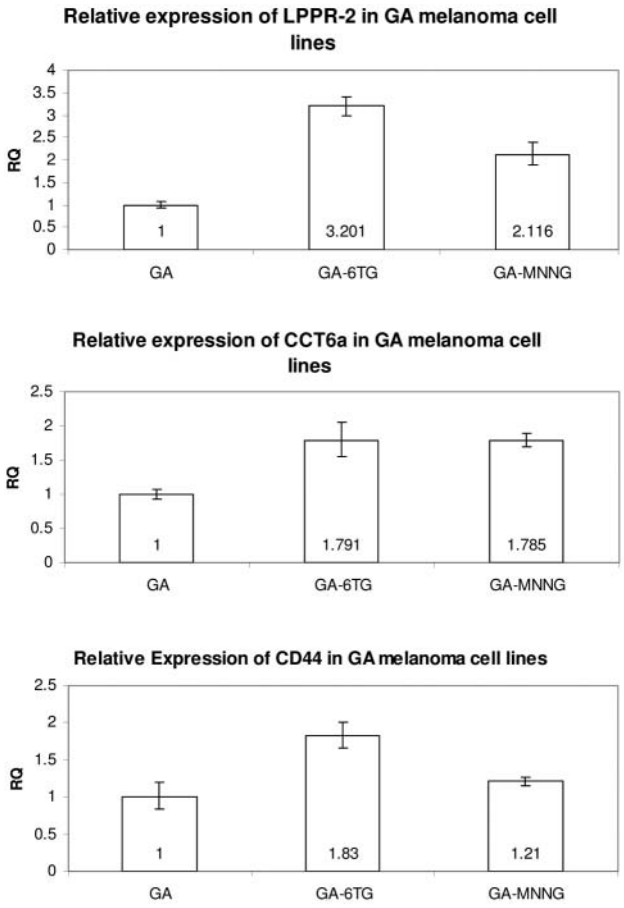

Figure 3. *The relative transcription levels of LPPR-2, TCP-1 and CD44 were determined by real-time RT-PCR. The raw data were exported from Prism 7000 RQ Study into Microsoft Excel and the generated graphs are presented. Relative quantification (RQ) values are indicated.*

Discussion

In this study, molecular changes associated with drug resistance in human melanoma cell lines were detected. Since these changes were noted in drug-selected cells, they are most probably superimposed over intrinsic drug resistance. An approximately 10-20% variation in electrophoretic mobility was detected between the parental and drug-resistant cells.

We characterized three outstanding bands and are in the process of defining several others. It will be of interest to determine whether the characteristic bands are unique to a certain melanoma cell line, to a particular drug used in the selection or whether the presence of the bands is of a more general nature. The variation in drug susceptibility and the type of gene products expressed can be partially explained by the relative diversity in the repair mechanisms acting in a particular cell line. The 6-TG-resistant GA cells may express a pattern of resistance consistent with mismatch and O^6-methylguanine-DNA methyltransferase (MGMT) repair mechanisms. Conversely, the differences in MNNG resistance may be due to variation in base excision and mismatch repair (2).

CCT6a (zeta) is a subunit of the TCP-1 multisubunit chaperonin complex (900 kDa) that mediates protein folding in the eukaryotic cytosol. Large oligomers assemble into ring structures that enclose a cavity in which protein folding takes place. They help proteins such as actin and tubulin to reach their final, active conformation (15). Additional roles of TCP-1 include participation in the repair of damaged proteins and assisting proteins in membrane translocation, regulation of gene expression, regulation of genes differentially regulated in tumor suppression and ubiquitination. The loss of the molecular chaperones leads to the accumulation of inactive or precursor proteins (16-18). Differential modulation of chaperone activity may vary the half-life of several proteins that, in turn, may affect the sensitivity of tumor cells to chemotherapy.

MNNG and 6-TG are DNA-damaging agents which are used as carcinogen/mutagens. 6-TG is also used as a chemotherapeutic agent, with the ability to induce apoptosis. DNA damage leads to increased genomic instability and the synthesis of damaged proteins recognized by chaperones are sequestered for future refolding or digestion (19), possibly inhibiting apoptosis and facilitating tumor progression. We speculate that increased expression of the TCP-1-zeta subunit may be associated with melanoma drug resistance by regulating protein stability and synthesis.

The cell surface hyaluronan receptor CD44 is expressed in most cell types. Several lines of evidence indicate that CD44 expression may have a major implication in tumor growth and dissemination, including melanoma (20). It has also been reported that signaling through CD44 triggers cell survival and chemoresistance in colon carcinoma and other tumor cell lines (21). The down-regulation of CD44 in melanoma cells, in parallel with increased expression of P-glycoprotein was reported by Molinari *et al.* (22). Our observations are in line with the notion that CD44 activation affects drug resistance, supporting the validity of our experimental design.

Lipid phosphate phosphatases (1, 1a, 2 and 3) are a group of enzymes that catalyze dephosphorylation of the lipid agonists sphingosine 1-phosphate, lysophosphatidic acid and phosphatidic acid. These agonists bind the G-protein-coupled receptors to stimulate intracellular signaling in mammalian cells. LPP1 and LPP2 overexpression act as second messengers inhibiting the stimulation of p42/p44 MAPK (23). Chakravarti *et al*. reported drug resistance in human glioblastoma cells following activation of p42/p44 MAPK (24), indirectly suggesting the involvement of LPP in drug resistance. Interestingly, when a Microarray analysis was used to compare gene expression for 133 phosphatase genes including lipid-based phosphatases, a correlation

between their expression and autonomous growth in advanced melanoma was observed (25).

We detected variant genes in selected drug-resistant cells and confirmed the results by real-time PCR. We will further investigate the significance of these gene products in these cells and in other melanoma cell lines. In parallel, we will continue our search for the identification of distinctive gene products in drug-sensitive and drug-resistant cell lines, in order to confirm our observations in primary tumors. Newly-detected variant gene products may contribute to our understanding of the molecular basis of acquired drug resistance and may serve as targets for novel anticancer drugs.

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