Multiple markers for melanoma progression regulated by DNA methylation: insights from transcriptomic studies

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The incidence of melanoma is increasing rapidly, with advanced lesions generally failing to respond to conventional chemotherapy. Here, we utilized DNA microarray-based gene expression profiling techniques to identify molecular determinants of melanoma progression within a unique panel of isogenic human melanoma cell lines. When a poorly tumorigenic cell line, derived from an early melanoma, was compared with two increasingly aggressive derivative cell lines, the expression of 66 genes was significantly changed. A similar pattern of differential gene expression was found with an independently derived melanoma, was compared with two increasingly aggressive derivative cell lines, the expression of 66 genes was significantly changed. A similar pattern of differential gene expression was found with an independently derived melanoma.

Abbreviations: 5mC, 5-methylcytosine; CGH, comparative genomic hybridization; COBRA, combined restriction bisulfite restriction analysis; DAC, 5-deoxy-5-azacytidine; FISH, fluorescence in situ hybridization; LDA, low density array; RGP, radial growth phase; VGP, vertical growth phase.

Introduction

The incidence of cutaneous melanoma is at epidemic proportions, with rates steadily rising in Western countries over the past few decades (1,2). However, effective treatment for patients with advanced melanoma is currently unavailable. Moreover, the prognosis of such patients is poor, with a 10% survival rate after 5 years. Less than a decade ago, cutaneous melanoma was described as a black tumor and a black box (3). While considerable insights have recently been made with respect to mapping out central events in melanoma development, the molecular basis of tumor progression in this disease remains ill defined. One approach towards understanding melanoma is to compare gene expression patterns between melanocytic cells from different stages of tumor progression. In this context, DNA microarray-based gene expression profiling has had a far-reaching impact on the study of numerous tumor types (4), including melanoma (5). Using this approach, new subgroups of melanoma have been identified (6), along with a wealth of marker genes that correlate with melanoma progression and drug resistance (7–13).

Cutaneous melanoma is a pigmented, readily accessible lesion that has been well defined in histopathological terms (3). Early radial growth phase (RGP) melanomas can invade into the epidermis and papillary dermis, but have no capacity for metastasis; resection at this stage is almost completely curative. A subsequent vertical growth phase (VGP) denotes a transition to a more aggressive stage, which is capable of metastasis. Changes in gene expression occurring at the RGP/VGP transition are, thus, of great interest. However, comparative transcriptomic studies have so far been hindered in this arena, as paired RGP/VGP biopsies are not normally available (since resection of RGP melanoma is often curative and no VGP develops). Here, we utilized a unique isogenic cell line model series that allows us to circumvent the lack of availability of such paired samples from the clinic.

The parental cell line in the series, WM793, was originally isolated from a superficial spreading melanoma (14). The patient concerned has had no re-occurrence of the disease to date, suggesting that these cells had low metastatic potential. Accordingly, WM793 cells displayed poor tumorigenicity in nude mice (15). Notably, the WM793 cell line was used as the basis for in vivo selection of several aggressive, tumorigenic...
sublines (15). In this respect, the derivative cell lines, WM793-P1 and WM793-P2, were established after inoculation in the presence of Matrigel (a reconstituted basement membrane extract). These isogenic sublines exhibited properties of cells isolated from advanced VGP melanoma; they were highly tumorigenic in nude mice and displayed multi-cytokine resistance in vitro. A further cell line (1205-Lu) was derived independently at the Wistar Institute from a lung metastasis of WM793 after subcutaneous injection into the tail veins of immunodeficient mice; these cells exhibited spontaneous metastasis (16).

In this study, a high-density oligonucleotide array-based approach was employed to identify genes that varied in expression level between the parental and derivative cell lines. We hoped, in particular, to elucidate key molecular determinants of the RGP–VGP transition, as well as obtain additional mechanistic insights into melanoma progression.

**Materials and methods**

**Cell lines**

Conditions for culture of the melanoma cell lines used here and their original sources have been described previously (17,18). In brief, WM793 and 1205-Lu cells were a gift from Prof. Meenhard Herlyn (Wistar Institute, Philadelphia, UK), whereas WM793-P1 and WM793-P2 cells [both N1 sublines in the WM793 derivative series; (19) Kobayashi] were a gift from Prof. Robert Kerbel (University of Toronto, Canada). Cells were maintained in DMEM with GlutaMAX (Invitrogen Ltd., Paisley, UK), supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 μg/ml insulin (Sigma–Aldrich, Dublin, Ireland).

**Melanoma biopsies**

Previously extracted DNA from 20 anonymized metastatic melanomas was kindly provided by the Department of Dermatology, University of Glasgow, UK, according to standardized ethical procedures set out by the University of Glasgow.

**Nucleic acid extraction**

Genomic DNA and total RNA were extracted from monolayer cells in culture and melanoma biopsies using the QIAamp DNA Mini (Qiagen, West Sussex, Glasgow) and melanoma biopsies using the QIAamp DNA Mini (Qiagen, West Sussex, UK) kit. Total RNA was extracted from monolayer cells using the Tri Reagent (Sigma, Dublin, Ireland) kit.

**Cell growth, migration and invasion assays**

Cell growth rates were determined using an alamarBlue assay (Biosource International, California, USA), which recorded cell proliferation each day. For invasion assays, cells were seeded into Transwell filters (pore size 8 μm; Corning-Costar Corporation, Cambridge, Massachusetts, USA) coated by Matrigel (1 μg/ml; 50 μg total from Becton, Dickinson and Company, New Jersey, USA), with fibronectin (5 μg/ml; Sigma) used as a chemoattractant. Cells were allowed to invade for 5 h, followed by careful aspiration of the medium and removal of the filters. Cells on topside of the filter were gently removed with a cotton bud. The filter, with cells remaining on the bottom side, was immersed in Toluidene Blue staining solution (5% w/v toluidene blue, 5% w/v sodium borate; Sigma) and left overnight at room temperature. The filters were then washed several times with water to remove background staining. Each filter was incubated with 300 μl of extraction buffer (20 mM Tris, 0.2% w/v SDS; Sigma) and shaken for 30 min at room temperature. Aliquots of the extracted material were measured at 540 nm using a VICTOR^2^ plate reader.

**Flow cytometric analysis**

A total of 1 × 10^6^ cells were used for flow cytometric analysis of DNA content. Exponentially growing cells were isolated by trypsinization, washed with 500 μl phosphate-buffered saline (PBS), and fixed with 500 μl of ice-cold 100% ethanol. Fixed cells were centrifuged and resuspended in 125 μl of RNase solution (1 μg/ml in 1.12% w/v of sodium citrate) and incubated at 37°C for 15 min. Following this, cells were incubated with 125 μl of propidium iodide solution (0.5 μg/ml in 1.12% of sodium citrate) for 30 min at room temperature. Samples were then analysed on a Coulter Epics flow cytometer (Beckman Coulter (UK) Ltd. Buckinghamshire, UK).

**CGH and FISH Analysis**

Comparative genomic hybridization (CGH) experiments were carried out as previously described (19). Briefly, test and reference DNA samples were labeled by nick translation with spectrum green-dUTP and red-dUTP, respectively, under conditions recommended by the supplier (Vysis Inc., Illinois, USA). Labeled test (melanoma cells) and reference (normal lympho- cyte) DNA (500 ng) were then denatured and hybridized to normal human metaphase chromosomes in a solution containing 50°/ Cot fractionated DNA, 50% formamide, 1:1 SSC, and 10% dextran sulfate (Vysis). Images were acquired and analyzed using hardware and software from Applied Imaging Corporation, California, USA. For fluorescence in situ hybridization (FISH) experiments, a dual-colored fluorescently labeled probe to specific regions of chromosomes X (Spectrum Green; DXZ1) and Y (Spectrum Red; DYZ1) was used according to the manufacturer’s instructions (Vysis). A probe to 11q (bacterial artificial chromosome clone RP11-163A13 from the Sanger Insti- tute, UK) was also used to determine ploidy.

**DAC treatment in vitro**

Seeded cells were treated with 2 μg/ml 2′-deoxy-5-azacytidine (DAC) on days 1, 3 and 5, with fresh drug-containing medium being added at each timepoint (20). On days 2 and 4, drug-containing medium was exchanged for drug-free medium. On day 6, cells were harvested and total RNA extracted as above.

**Global DNA methylation analysis**

5-Methylcytosine (5mC) genomic content was determined by high-performance capillary electrophoresis, as previously described (21). Briefly, genomic DNA samples were boiled, treated with nuclease P1 (Sigma) for 16 h at 37°C, and then incubated with alkaline phosphatase (Sigma–Aldrich, Dublin, Ireland). After hydrolysis, total cytosine and 5mC content were measured by capillary electrophoresis using a P/ACE MDQ system (Beckman Coulter). Relative 5mC content was expressed as a percentage of total cytosine content (methylated and non-methylated).

**DNA microarray analysis**

Ten micrograms of total RNA from each cell line was reverse transcribed into single-stranded cDNA using the SuperScript Choice kit (Invitrogen). For this purpose, an oligo-dT primer containing a T7 RNA polymerase promoter (Genset Corporation, California, USA) was utilized. Following double-stranded cDNA synthesis, biotin-labelled cRNA was generated by in vitro transcription using the BioArray RNA labelling kit (Enzo Life Sciences Inc., New York, USA). These complex cRNA targets, which are representative of the transcriptome of a particular sample, were hybridized against HuGeneFL arrays (7129 probe sets). Detection was accomplished via a streptavidin-labelled fluorochrome (phycoerythrin) and laser scanning. Normalisation of data and inter-array comparisons of gene expression profiles was carried out using Microarray Analysis Suite (MAS) software v4.0 (Affymetrix, High Wycombe, UK), together with Microsoft Access. In more detail, DNA microarray experiments were analyzed using an approach based on the Mann–Whitney pairwise comparison test (22). To identify differentially expressed genes between any two samples, pairwise comparisons were performed using MAS. Lists of altered transcripts from different pairwise compari- son were sorted via Microsoft Access. Additional bioinformatic analysis was completed using publicly available annotation databases and software tools, notably TIGR Multiple Experiment Viewer v2.0. Available upstream genomic sequences of identified differentially expressed genes were automatically retrieved using either EZRetrieve or ENSEMBL. Putative promoter-associated CpG islands at or around presumed transcription start site was identified using CpGPlot. The raw DNA microarray data have been submitted for public access to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and can be obtained using the following accession numbers: GSE1792 and GSE1793.

**RT–PCR analysis via cycle limitation**

RNA extracts were pre-digested with DNase I prior to cDNA synthesis using the DNA-Free kit (Ambion (Europe) Ltd., Cambridge, UK). Single-stranded cDNA was synthesized from 1 μg total RNA using the ImProm-II Reverse Transcription kit (Promega, Southampton, UK). Recombinant RNasin Ribonuclease Inhibitor (20 U/20 μl reaction; Promega) was added to prevent RNase-mediated degradation. Two negative controls were also utilised, namely minus reverse transcriptase (RT) enzyme control and minus template control. Following inactivation of RT at 70°C for 15 min, aliquots of generated single-stranded cDNA were subjected to PCR amplification via a cycle limitation approach. The following primer pair combinations were used: 18S — forward, 5′-AGGGTTCGATTCCGGAG-3′ and reverse, 5′-ACCAAGCTTG- CCCC3′-3′ (195 bp amplicon); DCT — forward, 5′-AGTGTAGTCCGCGCAGAA CATCC-3′ and reverse, 5′-AGTTCATAGGCGGCGAAGCA-3′ (368 bp amplicon); GP/NMB—forward, 5′-TGATAGAAAGCCGGACCTGTCGCTCA-3′ and reverse, 5′-CAAGGACCATCTTGGAAGCA-3′ (373 bp amplicon);
TYR—forward, 5’-CAGCCTTACGCGACAGGTTCTC-3’ and reverse, 5’-GGTCATGCGGCCAAATCATAAT-3’ (470 bp amplicon); TPRP—forward, 5’-CCAGAACAAGGACATGTTGCTGT-3’ and reverse, 5’-ACACATGAA-ACACCATAACAGGCCAACC-3’ (406 bp amplicon); CYB—forward, 5’-TTACTGTTGGCTGTGAGTAGTA-3’ and reverse, 5’-TTCTTGCTGCTGGC- TTTCT-3’ (369 bp amplicon); MXI—forward, 5’-AGGCAGCTCAGTGAGTCA- GACCTT-3’ and reverse, 5’-ACGGCCTGATGCTGCTTCAA-3’ (355 bp amplicon); HOXD—forward, 5’-GTGACTGCAAGATTTATGTG-3’ and reverse, 5’-CACCCTCCTGAGGGCTTCAAA-3’ (190 bp amplicon); RARB—forward, 5’-AAAGGTCGAAGAGGAGCAGA-3’ and reverse, 5’-GGGCTTGGTGCTTCTCATC- CATCTT-3’ and reverse, 5’-GGGCTTGGTGCTTCTCATC- CATCTT-3’ and reverse, 5’-TCAAGCTCTCCACGGGCTCTA-3’ (193 bp amplicon). PCR products were subcloned into the pCR4-TOPO vector via the Topo TA Cloning kit (Invitrogen), with insert-containing plasmids then subjected to automated DNA sequencing via a commercial route (MWG-Biotech AG, Ebersberg, Germany).

Real-time RT–PCR analysis using TaqMan Low Density Arrays
Pre-designed TaqMan probe and primer sets for target genes were chosen from an on-line catalogue (Applied Biosystems). Once selected, the sets were factory-loaded into the 384 wells of TaqMan Low Density Arrays (LDAs). Array format was customized on-line with two replicates per target gene. Expression levels of target genes were normalized to concentration of 18S rRNA. Samples were analyzed using the 7900HT system with a TaqMan LDA Array format was customized on-line with two replicates per target gene. Real-time RT–PCR analysis using TaqMan Low Density Arrays

Results

Phenotype of cells in vitro

The behavior of the WM793-based isogenic cell line model series has been well documented in vivo (15,16). However, there are only limited data available with respect to characteristic features of these cell lines in vitro (15,24). The growth rate of the parental WM793 cells and three isogenic derivatives was examined over a period of 7 days (Figure 1A). The derivative cell lines exhibited more rapid rates of cell growth than the parental cells. Interestingly, the 1205-Lu cells showed an intermediate rate of cell growth, which may be due to the apparent increased propensity of this cell type to detach from the surface in monolayer culture. The isogenic derivatives also displayed increased invasive capacity over WM793 cells, with the most striking difference seen between 1205-Lu and parental cells (Figure 1B).

Fig. 1. Phenotypic characteristics of melanoma cells in vitro. (A) Growth of parental WM793 cells and isogenic derivatives (WM793-P1, WM793-P2 and 1205-Lu). Cells (20000/well) were seeded into 12-well tissue culture plates and left to grow for 7 days. Growth rates were measured using an alamarBlue assay for cell proliferation. AU, arbitrary units. (B) Invasive capacity of melanoma cells. Invaded cells were stained with toluidene blue, which was extracted and analyzed at Abs540 nm. P-value obtained using a Student t-test. In both A and B, error bars refer to the SEM of triplicate determinations.
A similar pattern of differential gene expression was found in both the parental and derivative cell lines (Table I). The four cell lines exhibited some identical or similar abnormalities, illustrative of the underlying genetic relatedness of the series. Moreover, the particular generic abnormalities found matched those commonly present in melanoma biopsies (3,25,26). For example, there is a consistent gain of material on chromosomes 1, 6 and 7, where gene amplification frequently occurs in melanoma. In addition, certain progression-related abnormalities were identified, when the parental cells were compared with its various derivative cell types. Crucially, the two main derivative types, WM793-P1/WM793-P2 and 1205-Lu, exhibit some common alterations in this context (e.g. loss of 12q10q13 and 18p), supporting the concept of non-random changes. Overall, there is a marked increase in the number of abnormalities observed in the derivative cells as compared with the parental cell line. FISH and flow cytometric analysis showed evidence of increased ploidy in association with progression in the model system, which is further indicative of genomic instability (see Supplementary data, S1). As compared with WM793 cells, for example, FISH analysis showed increases in X, Y and 11q chromosomal regions in the derivative cells that were consistent with an increase in the numbers of tetraploid, triploid or hyperdiploid cells found in these cell line populations.

Table I. CGH analysis of melanoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Genomic aberrations*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM793</td>
<td>enh 1q10qter, 2q10q35, 6q22q27, 7, 8q, 20q dim 10q24</td>
</tr>
<tr>
<td>WM793-P1</td>
<td>enh 1q10q32, 2q10q35, 5p, 6q22q27, 7, 8q22, 13q32, 17q21q23, 20q dim 10q24, 12q10q13, 18p</td>
</tr>
<tr>
<td>WM793-P2</td>
<td>enh 1q10q32, 2q10q35, 6q22q27, 6q10q16, 7, 13q32, 17q21q23, 20q dim 10q24, 12q10q13, 18, 18p</td>
</tr>
<tr>
<td>1205-Lu</td>
<td>enh 1q, 2p, 7p+, 8q, 11p, 12p, 13, 17p13 dim 10q24, 12q10q13, 18, 18p</td>
</tr>
</tbody>
</table>

*enh, enhanced green to red fluorescent ratio of chromosomal region (gain); dim, diminished green to red fluorescent ratio of chromosomal region (loss).

Identification of differentially expressed genes

All four melanoma cell lines were subjected to gene expression profile analysis using high-density oligonucleotide arrays. In this case, total RNA from exponentially growing cultures was used. When the parental cell line in the series, WM793, was compared with the two increasingly aggressive derivative cell lines, WM793-P1 and WM793-P2, 66 genes were commonly altered with respect to expression level (Figure 2A). Within this cohort, 44 genes were identified as being down-regulated. A similar pattern of differential gene expression was found with the independently derived metastatic cell line, 1205-Lu (Figure 2B). Altered expression of a subset of 10 genes was confirmed by conventional RT–PCR analysis (Figure 3A), with the results closely matching that obtained from the initial DNA microarray screen (Figure 2A). We also examined the expression of 45 out of the 66 genes via a novel high-throughput quantitative RT–PCR assay (Figure 3B), which employed the use of a specifically designed TaqMan LDA. The TaqMan LDA approach showed a high level of reproducibility (see Supplementary data, S2A). Moreover, considerable concordance was seen between the transcriptomic profiles determined by DNA microarray and TaqMan LDA approaches (see Supplementary data, S2B).

A considerable proportion of the identified differentially expressed genes have been previously associated with melanoma development and progression. Reduced expression of the tumor suppressor genes, CDKN2A and IL-24, was observed in all three derivative cell lines as compared with the parental cells. Moreover, several genes (TYR, TYRP1 and TYRP2) involved in melanin biosynthesis exhibited a similar marked reduction in transcript levels in the derivative cell types. A subset of immune-related genes (including BST2, C1S, C1R, HLA-DQA1, TNPAP6 and LGALS3) and interferon-related genes (including GIP1, GIP3, IFIT1, CASP1, ISGF3G, DAP and MX1) were also observed to be down-regulated in the derivative cells. Several genes encoding for tumor-associated antigens, such as MAGEA4 and GAGE1, displayed increased expression in the derivative cell lines. Intriguingly, the AIM1 gene, which has been previously suggested as a tumor suppressor based on correlation in expression terms with experimental reversal of tumorigenicity via chromosome transfer, showed evidence of increased expression at the RNA level in the derivative cells.

In addition, a range of novel markers were identified that correlated with melanoma progression. Most notable was TSPY, a Y chromosome-specific gene that displayed marked down-regulation in expression (between 137- and 317-fold, as determined from DNA microarray study) between the parental and derivative cell lines (Figures 2, 3 and 4A). The TSPY gene has previously been shown to exhibit dysregulated expression in a number of cancer types, including gondoblastoma, as well as testicular and prostate cancer (20,27–30). Although Y chromosome loss has been described for certain melanomas, this is not a common event. FISH analysis showed retention of this chromosome in all four cell lines under study (see Supplementary data, S1A). Moreover, Southern blot analysis showed no evidence for deletions or gross rearrangement of the TSPY gene (data not shown). TSPY gene expression is regulated by androgens and DNA methylation (20). This suggested that aberrant DNA methylation may have a role transcriptional silencing of TSPY gene expression between early and advanced melanoma cell lines.

Regulation of gene expression by DNA methylation

Treatment of the derivative cell lines with DAC restored expression of the TSPY gene to varying degrees (Figure 4B). We then employed COBRA to determine whether the TSPY gene was directly methylated (Figure 4C–E). COBRA utilizes bisulfite treatment of the DNA (which converts non-methylated cytosines in CpG sites to uracil), together with PCR amplification and restriction enzyme analysis. In this case, the enzyme site lies on a predicted methylated cytosine. As a result, following initial bisulfite modification of DNA, restriction sites with unmethylated CpGs will be protected from digestion, whereas those with methylated CpGs will be available for cleavage. Examination of a putative CpG island within the TSPY gene demonstrated that this region was hypermethylated in all three derivative cell lines (Figure 4C and D). In addition, hypermethylation of the TSPY gene was observed in metastatic melanomas from five male patients (Figure 4E).

Further DNA microarray studies uncovered a subset of 13 out of the 44 (29.5%) down-regulated genes that displayed...
Fig. 2. Comparative analysis of gene expression profiles. The 66 genes identified as consistently differentially expressed between WM793 cells and the derivative cell lines, WM793-P1 and WM793-P2, are listed above. (A) Duplicate data with respect to gene expression obtained from WM793, WM793-P1 and WM793-P2 cell lines (separate cultures; replicate data listed beside each other). Pairwise comparison of gene expression between different cell lines yielded the following numbers of altered transcripts: WM793 versus WM793-P1 (129 transcripts); WM793 versus WM793-P2 (114 transcripts); WM793-P1 versus WM793-P2 (14 transcripts). Cross-comparison of the 129 and 114 transcript lists yielded 68 commonly altered transcripts, of which two (TAC1 and TYRP1) are represented by an additional probe set. On average, inter-array variability between biological replicates was observed to be 2.18% (155/7129 probesets).

(B) Independent dataset showing expression of 66 gene cohort in untreated (WM793 and 1205-Lu) and DAC treated (all four cell lines) cells. Gene expression profile information is represented using a color-coded scheme (key provided) in which light blue refers to genes expressed at a low level (below mean absolute intensity) and bright red refers to genes expressed at a high level (above mean absolute intensity). From left to right, the tabular columns refer to the Affymetrix probe set identifier, the corresponding UniGene cluster (Build #166), associated chromosomal location and Human Genome Organisation-approved gene symbol for each transcript. The additional three columns detail the fold change values between WM793 and respective derivative cells. With respect to the last column: +, CpG island found around presumed transcription start site or near upstream region; −, no CpG island found in these regions; NA, upstream sequence is unknown. Black and red crosses signify that upstream genomic sequence obtained using EZRetrieve and ENSEMBL, respectively.
consistent re-activation of expression following DAC treatment across all three derivative cell lines, including 
*TSPY*, *CYBA* and *MT2A* (Figure 2B and Table II). This is in contrast to only 3.24% of all transcripts represented on the DNA microarray that exhibited elevated expression in all three derivative cell lines following DAC treatment. However, 7 out of the 13 genes do not have 5’ CpG islands (Figure 2), so are not likely to be directly regulated by DNA methylation. This phenomenon has been reported previously by Liang et al. (31) among others. In summary, multiple transcripts that are potential markers for melanoma progression can be increased following DAC treatment suggesting that the relevant genes are suppressed in terms of expression by DNA methylation, either directly or indirectly, in the more aggressive derivative cell lines.

**DAC-mediated inhibition of cell growth and migration in vitro**

The growth rate of all four melanoma cell lines was examined over a period of 7 days under DAC treated conditions (Figure 5A). As compared with untreated cells, the growth of both the parental and derivative cell lines was suppressed in response to DAC (Figure 5A), albeit with the latter cell types showing a slightly delayed response in this respect. The DAC-mediated inhibitory effect on cell growth was most marked in the case of the 1205-Lu cells. The migration capacity of all four melanoma cell lines was determined by a scratch wound healing assay under both untreated and DAC treated conditions (Figure 5B). For this, the cells were grown to confluency, scratch wounded with a pipette tip and incubated for a further 48 h to allow the cells to migrate into the scratch. Under untreated conditions, the derivative cell lines exhibited more rapid rates of cell migration than the parental cells. For example, wounded areas were mostly fully replenished within 48 h in the case of the derivative cells (Figure 5B), with this process taking a further 48 h for the parental cells to complete (data not shown). Under DAC treated conditions, the migration of both the parental and derivative cell lines was suppressed (Figure 5B). Overall, these data suggest that alteration of DNA methylation has a significant effect on cellular phenotype in our model system. However, one cannot exclude the possibility that the DAC-mediated inhibition of cell growth may also be, at least in part, due to a direct cytotoxic action, which is independent of DNA methylation. Indeed, we readily

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**Fig. 3.** Validation of DNA microarray results by two RT–PCR analysis methods. The vertical blue and red bars refer to genes that were identified from the prior DNA microarray study as either down-regulated or up-regulated, respectively, in derivative cells as compared with parental WM793 cells. Genes listed according to same order shown in Figure 2. (A) RT–PCR analysis, via a cycle limitation method, of transcripts previously shown to be down-regulated (*CYBA*, *GPNNMB*, *DCT*, *TYP1*, *TYR*, *MX1*) or up-regulated (*HOXD4*, *PRK1*, *SIAT7B*, *RARB*) in the derivative cell lines. Level of 18S rRNA served as a loading control. (B) Real-time RT–PCR analysis via TaqMan LDA. Forty-five of the sixty-six differentially expressed genes were assessed for expression level in the WM793 series. Expression measurements were normalized to 18S rRNA levels. The parental WM793 cells were defined as the calibrator to which all three derivative cell lines were compared. The three data columns to the right represent the mean normalized relative quantities of target gene expression between WM793 cells and relevant derivative cells across duplicate measurements from independent stocks of exponentially growing cells. Gene expression profile information is represented to the right using a color-coded scheme (key provided below) in which light blue and bright red refer to down-regulated and up-regulated genes (as compared with WM793 cells), respectively. Black indicates no change in expression level.
envisage that the suppressive effects on cell growth in vitro may in part be due to induction of apoptosis.

Suppression of tumor growth in vivo following DAC treatment
To further examine the functional role of DNA methylation in regulating tumor growth of an aggressive derivative, we examined the effect of systemic DAC treatment on 1205-Lu xenografts in vivo (Figure 6A). Systemic DAC treatment (15 mg/kg) significantly attenuated tumor growth in comparison to untreated mice. Again, one cannot preclude the possibility that a direct cytotoxic action by DAC on tumor growth also plays a part in this effect. The inhibitory effect of DAC on tumor growth was seen to cease 7 days post-treatment. This is consistent with the reversible nature of demethylation induced by DAC in xenograft models (32).

![Image](https://example.com/image.png)

**Fig. 4.** Regulation of TSPY gene by DNA methylation. (A) Northern blot analysis of TSPY mRNA expression. Order of parental WM793 cells and derivatives indicated. (B) Effect of DAC treatment on TSPY gene expression in melanoma cell lines. Total RNA was extracted from untreated (–DAC) and treated (+DAC) cells and subjected to RT–PCR analysis for TSPY and 18S rRNA via a cycle limitation method. TSPY cDNA-containing plasmid and H2O alone controls were also included as positive and negative controls, respectively. The efficiency of DAC treatment was assessed by global DNA methylation analysis (see Supplemental data, S3). Same order of samples as in A. (C) Schematic representation of 5' region of TSPY gene, including Exon 1 (black bar). Amplicon assessed by COBRA also indicated (light bar), along with relevant restriction enzyme (BsuEI) cleavage site. Vertical bars indicate CpG sites. (D) COBRA of TSPY gene using genomic DNA extracted from melanoma cell lines. (E) COBRA of TSPY gene using genomic DNA extracted from metastatic melanomas. Presence of 191 bp fragment signifies DNA methylation at CpG within BsuEI site. COBRA was also performed on commercially available 100% methylated DNA (as a positive control). For D, same order of samples as in A. For E, genomic DNA from 20 randomly assigned metastatic melanomas (5 male, 15 female). Samples in lanes 1, 2, 7, 8 and 13 were derived from male patients, with the remainder being from female patients. Only male-derived samples generated PCR products by COBRA, which is as expected given the chromosomal localization of the TSPY gene.
activity. In summary, DAC treatment may facilitate tumorogenic reversion of advanced melanomas.

### Discussion

The development of culture techniques permitting the establishment and propagation of cells derived from histologically defined stages of melanoma progression has permitted major advances. However, comparative studies of RGP and VGP melanoma have been limited since it has not generally proved possible to establish cell lines from each of these stages from one individual patient. Here, we have used a unique isogenic cell line-based model system that circumvents this problem.

We compared the gene expression profile of an early melanoma cell type with a variety of isogenic derivative cell lines of increasing aggressiveness. Accordingly, the expression patterns of 66 genes were identified as correlating with melanoma progression. Amongst these, we found a large number of genes previously associated with melanoma, including several tumor suppressor genes and antigenic markers, as well as genes involved in melanin biosynthesis. These commonly encountered alterations, together with the noted similarities in terms of chromosomal aberrations observed between the cell lines used here and melanoma biopsies, adds confidence to our current model of melanoma progression. However, further work at the RNA level with melanoma biopsy samples would shed additional light on this collection of putative melanoma progression-associated markers.

Of the 66 genes examined, TSPY displayed the most striking change in gene expression terms within the WM793 series. The TSPY gene is found in multiple copies (20–40 based on current predictions) on both the long and short arm of chromosome Y (30,33). The TSPY gene is normally expressed in the germ cells of the testis and distinct subsets of spermatogenesis (34,35). Apart from an assumed activity in spermatogenesis (34,36), the functional role of TSPY remains to be elucidated (33). Previous work had implicated TSPY as a putative oncogene based on its elevated expression in some gonadoblastomas, as well as testicular and prostate cancers (27–30,37). This contrasts with our observation of extensive down-regulation of TSPY gene expression during melanoma progression. In addition, Dasari et al. (20) found TSPY gene expression to be suppressed in certain prostate cancer cells. While this study highlights the TSPY gene as a promising marker for melanoma progression and, potentially, DAC activity, further work will be required to clarify its role in cancer. In particular, additional studies employing forward and reverse genetics approaches are necessary to determine if TSPY is critical in modulating tumor progression in WM793 series, with these studies ongoing in our laboratory at present. Our data, however, also provide an intriguing hypothesis that there may be sex-specific markers of melanoma, which may be useful in discriminating differences in terms of disease progression between males and females (1,2), with the TSPY gene being a promising candidate in this respect.

Detailed examination of the 66 gene cohort pointed towards DNA methylation as having a potentially important role in mediating gene expression alterations between parental and derivative cell lines. Demonstration of hypermethylation at a candidate CpG island within the TSPY gene in the derivative cell lines provided initial support for this concept. This was further backed by the noted de-repression of a significant proportion of down-regulated genes following DAC treatment in vitro. Overall, our data support the hypothesis that multiple genes are targeted, either directly or indirectly, by DNA hypermethylation in this melanoma model system.

The role of DNA methylation in carcinogenesis is complex: global hypomethylation and region-specific hypermethylation co-exist (38). DNA hypermethylation at CpG islands is known to be associated with epigenetic silencing of tumor suppressor gene expression and may increase genomic instability (39). DNA hypermethylation can occur at all stages of tumor development and progression (40). Previous studies have identified a number of genes that are affected by alterations in DNA methylation patterns in melanoma cells, including CDKN2A (41), PTEN (42), APAF-1 (43), MAGEA1 (44), TIMP3 (13), GAGED2 (45), various human leukocyte class I antigens (46), and CASP8 (47). While our data add a further collection of putative methylation-sensitive genes in melanoma, additional work will be required to ascertain which of the remaining 12 genes, apart from TSPY, are directly regulated by DNA methylation.

DNA microarray-based gene expression profiling technology has been previously utilized in several cancer-related
model systems to identify genes that are regulated by DNA methylation (13,44,48,49). Notably, van der Velden et al. (13) identified 19 genes, including TIMP3 and TYRP1, that were differentially expressed between a demethylated derivative clone of a primary uveal melanoma cell line and its untreated control. Our study provides further insight by linking changes in gene expression between early and advanced melanoma with DNA methylation.

It is clear that both epigenetic and genetic events contribute to determining tumor development and progression (50). In keeping with the proposed link between DNA hypermethylation and genomic instability, CGH analysis demonstrated an increased number of chromosomal abnormalities in the derivative cell lines, as compared with the parental cells. Such large-scale cytogenetic changes would be expected to impact significantly on the expression of many genes, which in

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**Fig. 5.** Effect of DAC treatment on melanoma cells *in vitro*. (A) Cells (20 000/well) were seeded into 12-well tissue culture plates and left to grow for 7 days, while being simultaneously treated with and without DAC. Growth rates were measured using an alamarBlue assay for cell proliferation. Error bars refer to SEM of triplicate determinations. In (B), cells (grown to confluence in 12-well plates) were wounded by creating a scratch across the monolayer culture. Phase contrast images of this region were taken directly following injury and 48 h later.
turn may influence the aggressive nature of the derivatives. While our analyses revealed no major striking correlations between gene expression and specific genomic aberrations in the four cell lines examined here (Figure 2 and Table I), whole genome CGH microarray studies might provide additional clarification of this issue. It should be noted that the Gene Ontology classes/categories exhibited by the ∼7000 genes represented on the DNA microarray used in this study showed strong similarity with the whole genome (see Supplemental data, S4).

There is a rapidly increasing interest in the potential of epigenetic modifiers in the treatment of cancer (51). In many cancer types, the use of DNA methyltransferase and histone deacetylase inhibitors have shown to be useful in mediating suppression of tumor growth and increased activity of other anti-cancer agents (32, 52, 53). However, the use of such agents for melanoma therapy has been inconclusive.

In an early phase II clinical trial, Abele et al. (54) showed only one response in a set of 20 patients with melanoma treated with DAC. Following this negative result, however, a number of studies have provided more support for the use of DAC in the treatment of melanoma (53, 55, 56). Anzai et al. (55) showed a synergistic effect between DAC and the topoisomerase I inhibitor, topotecan, against melanoma cells in vitro, suggesting that combination therapies of DAC and other drugs may have more beneficial effects than DAC alone. In agreement with this concept, Coral et al. (56) noted that DAC in combination with the inhibitory cytokine, IFN-γ, enhanced the expression of human leucocyte class I antigens together with certain co-stimulatory molecules, such as ICAM-1 and LFA-3, in a panel of 12 metastatic melanoma cell lines. Moreover, DAC treatment yielded a persistent (>60 days) expression of MAGE-1 in one of the melanoma cell lines. This DAC/IFN-γ combination may enhance the immunogenic potential of melanoma cells, thereby increasing the efficacy of immunotherapy. More recently, Kozar et al. (53) showed that combined treatment of DAC and IL-12 significantly attenuated growth of B16F10 melanoma cell-derived tumors in vivo, in comparison with moderate anti-tumor effects when either agent was given alone. This further supports the use of DAC as an immunomodulatory agent for complementation with inhibitory cytokines for the treatment of melanoma.

Interestingly, Wang et al. (57) proposed that responsiveness of melanomas to immunotherapy is predetermined and may be deciphered from analysis of gene expression profiling data. Within the 66 gene cohort, we noted down-regulation of a substantial cohort of immune-related and interferon-related genes in the derivative cell lines, which may be illustrative of the previously documented resistance to various inhibitory cytokines (15, 19).

In our study, DAC suppressed tumor cell growth in vitro. Moreover, systemic treatment of mice with DAC attenuated growth of 1205-Lu-derived xenografts, with consequent re-expression of T3PY mRNA. While these data might, in
some part, be due to a direct cytotoxic action by DAC, our overall data point towards the concept that regional DNA hypermethylation at multiple loci is likely to be involved in the epigenetic regulation of melanoma progression. DAC also has an inhibitory effect on growth and migration of WM793 cells in vitro, as well as being able to mediate complex changes in gene expression in this cell type. Given that the WM793 cell line is representative of an early melanoma, this suggests that the phenotype of these cells is also controlled, to a certain degree, by DNA methylation events. The relative enrichment of methylation-responsive markers in the identified set of down-regulated melanoma progression-related genes across the three derivative cells, however, suggests that further hypermethylation has occurred as one progresses through the WM793 series. This hypothesis is in keeping with the current model of acquisition of epigenetic marks during tumor progression.

Our data provides further support for the incorporation of demethylation agents into clinical trials. Additional work is required to determine potential for synergy with other epigenetic modifiers and conventional therapies in terms of altering gene expression and therapeutic responses. In conclusion, a better understanding of melanoma progression, as exemplified by this study, may translate into new therapeutic avenues for this intractable disease.

Supplementary material
Supplementary material can be found at: http://carcin.oxfordjournals.org/

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References
Epigenetic control of melanoma progression


