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Use of integrative epigenetic and cytogenetic analyses to identify novel tumor suppressor genes in malignant melanoma

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Abstract

Purpose—Identify novel tumor suppressor genes in melanoma utilizing an integrative genomic approach

Methods—Data from: 1) prior reports of DNA loss and gain in malignant melanoma accompanied by CGH high-definition array data of the entire human genome, 2) Microarray expression data from melanoma derived cell lines identifying genes with significantly increased expression due to methylation using a pharmacologic demethylating strategy, and 3) publicly available RNA expression microarray data of primary tumors and benign nevi was integrated utilizing statistical tools in order to define a population of candidate tumor suppressor genes.

Results—27 genes were identified in areas of deletion that demonstrated diminished expression in primary melanomas relative to benign nevi and were significantly increased in expression by '5-Aza treatment. Seven genes of these genes demonstrated methylation and deletion in a validation cohort of 14 separate primary tumors. These were: CHRDL1, SFRP1, TMEM47, LPL, RARRES1, PLCXD1, and KOX15. All of these genes demonstrated growth suppressive properties with transfection into melanoma derived cell lines.

Conclusions—7 putative tumor suppressor genes in malignant melanoma were identified utilizing a novel integrative technique.

Keywords

DNA hypermethylation; epigenetics; malignant melanoma; tumor suppressor

Introduction

Cytogenetic and epigenetic changes are the primary mechanisms by which tumor suppressor genes are inactivated. DNA methylation is one form of epigenetic change and involves the covalent addition of a methyl group to cytosine residues in CpG dinucleotides by DNA methyltransferases. CpG-rich sequences (CpG islands) are infrequent in the genome. Examination of familial cancer genes has revealed that tumor-specific hypermethylation

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may act as an inactivating event by preferentially targeting the wild-type allele of tumor suppressors, whereas the promoter region of the mutant allele is not affected by methylation. Inhibition of *de novo* methylation enzymes has been shown to reduce tumor formation in several settings. Epigenetic inactivation of individual tumor suppressors by DNA methylation has also been shown in malignant melanoma. A high incidence of methylation in melanoma tissue samples has been reported for *RARB* (70%), *RASSF1A* (55%), *PYCARD* (50%), *MGMT* (34%), *DAPK* (19%), and *APC* (19%), which may play tumor suppressor roles in several tumor settings[1].

Exposure to ultraviolet radiation is the environmental factor most strongly associated with development of melanoma. Many genetic and epigenetic changes associated with melanomas have been described [2-4]. Yet, the molecular mechanisms of melanoma carcinogenesis are still undergoing intensive investigation. Neoplastic cells undergo a variety of genetic alterations, from point mutations to chromosomal aberrations, affecting the function or expression of both oncogenes and tumor-suppressor genes. Deletion can result in silencing via uniallelic or biallelic loss. Advances in techniques over the past few years, including: array-based comparative genomic hybridization (aCGH), and array-based single nucleotide polymorphism (aSNP) have allowed high-throughput, highly detailed studies of chromosomal loss or gain.

New high-throughput screens of cancer genes are being developed at a rapid pace making the need for efficient approaches for integration of large datasets that use diverse technologies to describe genetic alterations in human cancers. [5]. Examples of such genome-scale datasets include: array CGH (DNA loss or gain), RNA expression microarray (tissues or cell lines), small molecule cell line screens, and various proteomic approaches. Tumors may be susceptible to targeted therapies once their essential molecular alterations have been found. Integrative approaches to these genome-scale data sets allow multiple pieces of salient information to be combined in a manner that may yield novel and powerful new insights into cancer biology [6]. One prominent example of the utility of these integrative genetic approaches led to finding the oncogene *MITF* in melanoma via application of two genome-scale datasets (array SNP and expression microaray). This gene may represent a new class of “lineage addiction oncogenes”-- a fundamental tumor survival mechanism with important therapeutic implications[7] . Other examples of integrative genomic approaches have also improved the genetic understanding of other cancers [8-11]

Tumor suppressor inactivation is associated with loss or inactivation of both genomic copies of DNA. This has been associated with epigenetic[1, 2] and cytogenetic mechanisms in malignant melanoma. Identifying genes that demonstrate inactivation by multiple mechanisms may serve to more intelligently enrich a search for putative tumor suppressors in melanoma. We hypothesize that genes whose expression is decreased in melanoma relative to benign nevi and have evidence of methylation and deletion are potential tumor suppressor genes in this disease.

We report a study utilizing high throughput genomic and informatic techniques to identify a set of putative tumor suppressor genes whose expression is regulated by methylation and whose expression is decreased in primary melanomas utilizing a set of previously published microarray data[12]. This information was combined with aCGH data identifying areas of known deletion in melanoma. Genes found to be regulated by methylation and found in areas of known deletion were analyzed in melanoma samples collected from an independent cohort to determine the incidence and coincidence of hypermethylation and deletion. Genes validated in this manner were transfected into melanoma cell lines to determine if growth suppression was induced.

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Methods

Public datasets

The public databases used in this study were the University of California Santa Cruz (UCSC) Human Genome reference sequence and the annotation database from the May 2004 freeze (hg17). Array Comparative Genome Hybridization (aCGH) data for 178 malignant melanomas and tumor derived cell lines were obtained from progenetix.com[13]. Progenetix collects information about the genomic copy number profiles of cancer cases. It consists mainly of a compilation of published data from chromosomal and array/matrix Comparative Genomic Hybridization (aCGH) experiments. Gene Expression Omnibus (GEO) repository was utilized to locate microarray expression data for 45 melanoma and 18 benign melanocytic nevi [12]

5Aza-dC Treatment of Cells

These *in vitro* techniques employ treatment of cultured cells with 5-aza-deoxycytidine (a cytosine analog which cannot be methylated) and subsequent expression array analysis with validation of tumor suppressor gene targets. We treated melanoma cell lines with 5Aza-dC as described previously[14]. Briefly, cells were split to low density (1×10^6 cells/T-75 flask) 24 hours before treatment. Stock solutions of 5Aza-dC (Sigma, St. Louis, MO) were dissolved in DMSO (Sigma). Cells were treated with 5 μM 5-Aza-deoxycytidine for 2 days. Baseline expression was established by mock-treated cells with the same volume of DMSO.

Integrative analysis

For target discovery we employed three data sets. aCGH dataset from progenetix.com was utilized to identify detailed loci of DNA/chromosomal loss in melanoma (>10% incidence). Gene RNA expression microarray analysis of RNA extracted from 4 5′-Aza treated melanoma derived cell lines was conducted on the Affymetrix U133A and U133B platforms (33,000 genes). Genes, which were significantly upregulated by Aza treatment utilizing the techniques described previously, were identified. Publicly available data sets, published through GEO, which contained U133A data regarding gene expression in 45 melanoma tumors relative to 18 benign nevi [12]. Microarrays were studied with dChip and invariant-set normalized. Median tumor expression and median normal expression were calculated. P values were determined by STATA 9.0 utilizing Mann-Whitney U test (StataCorp LP, College Station, Texas). Genes which demonstrated significantly decreased median expression in melanomas as compared to melanocytic nevi were identified ($p < .05$). The intersection of the sets of genes found in areas of deletions, those upregulated by Aza treatment, and those with diminished expression in melanoma relative to nevi was identified. The list was ranked by equal weight of the ranking of the relative median expression difference and fold upregulation by Aza treatment. These sources of information were combined by using a rank product. These rankings were combined to rank all targets with a significance threshold of $\alpha = 0.005$. Subsequent random permutation of our rank list resulted in 47 genes deemed significant. Validation of targets was based upon identification of targets with known CpG Island, and subsequent bisulfite sequencing, followed by qPCR for precise validation of gene amplification or deletion, and/or sequencing. (Supplemental Figure 1)

Oligonucleotide microarray analysis

Total cellular RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. We carried out oligonucleotide microarray analysis using the GeneChip U133 A and U133B Affymetrix expression microarray that assays 33,000 genes (Affymetrix, Santa Clara, CA). Samples were converted to labeled, fragmented, cRNA per

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the Affymetrix protocol for use on the expression microarray. Signal intensity and statistical significance was established for each transcript using dChip version 2006. Default settings for dChip were used including the PM/MM difference model; invariant set normalization, check single/probe/array outlier algorithm.

DNA sample source and extraction

Tumor samples and lymphocyte DNA were obtained from 14 melanoma patients who had undergone enrolment in institutional review board approved protocols after obtaining informed consent. This population consisted of 13 men and one woman. (Table 1) Resected primary or metastatic melanoma lesions were collected in the operating room and immediately snap frozen with liquid nitrogen. Tumor specimens were microdissected on a cryostat so that the samples used for mutational analysis contained greater than 70% neoplastic cells. Samples were centrifuged and digested in a solution of detergent (sodium dodecylsulfate) and proteinase K, for removal of proteins bound to the DNA. Samples were first purified and desalted with phenol/chloroform extraction. Digested sample was subjected twice to ethanol precipitation, and subsequently resuspended in 500 μL of LoTE (EDTA 2.5 mmol/L and Tris-HCl 10 mmol/L) and stored at −80°C.

Bisulfite Treatment

DNA derived from tumor specimen was subjected to bisulfite treatment, as described previously[14]. In short, 2 μg of genomic DNA was denatured in 0.2 M NaOH for 30 minutes at 50°C. This denatured DNA was then diluted into 500 μL of a solution of 10 mmol/L hydroquinone and 3 M sodium bisulfite. This was incubated for 3 hours at 70°C. After the DNA sample was purified with a sepharose column (Wizard DNA Clean-Up System; Promega, Madison, WI). Eluted DNA was treated with 0.3 M of NaOH for 10 minutes at room temperature, and precipitated with ethanol. This bisulfite-modified DNA was subsequently resuspended in 120 μL of LoTE (EDTA 2.5 mmol/L and Tris-HCl 10 mmol/L) and stored at −80°C.

Bisulfite Sequencing

Bisulfite sequence analysis was performed to check the methylation status in primary tumors for putative tumor suppressors identified by integrative methods. Bisulfite-treated DNA was amplified using primers designed by MethPrimer to span areas of CpG islands in the promoter or first intron [15].Primer sequences were designed to not have CG dinucleotides. Detailed primer sequences and PCR conditions are available upon request. The PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. Each amplified DNA sample was applied with nested primers to the Applied Biosystems 3700 DNA analyzer using BD terminator dye (Applied Biosystems, Foster City, CA).

qPCR

Total DNA was measured and adjusted to the same amount for each tissue sample. The DNA was used as the templates for quantitative real-time PCR with primers designed to specifically measure the DNA copy number of each candidate gene. Three genes (*LRTM4, Pelo, Bid)* were identified which were not located in areas of loss or gain. These were quantitated and relative amounts averaged to ensure accurate relative quantitation of copy number in qPCR. Detailed PCR conditions and primer sequences are available upon request.

Transfection

Full-length ORF cDNAs in expression vectors of zinc finger protein 22 *(KOX15)*, lipoprotein lipase (*LPL)*, chordin-like 1 (*CHRDL1)*, secreted frizzled related protein 1

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(SFRP1), transmembrane protein 47 (*TMEM47)*, Phosphatidylinositol-specific phospholipase C, X domain containing1 (*PLCXD1), and* Retinoic acid receptor responder 1 *(RARRES1)* were obtained for transient transfections from Invitrogen (Carlsbad, CA). Cell lines were plated at 1×10^5/well using 96-well plates and simultaneously transfected with either empty vector (created by restriction digest removal of ORF and blunt end ligation) or gene of interest using the FuGene 6 Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol.

Cell Count

Calcein florescence was measured by the Spectramax M2e 96-well fluorescence plate reader Molecular Devices (Sunnyvale, California). Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeable calcein AM to the intensely fluorescent calcein. The polyanionic calcein dye is well retained within live cells, producing an intense uniform green fluorescence (excitation/emission ~495 nm/515 nm).

Statistical analysis

All statistical computations were made with STATA SE version 9.0 (STATACORP, College Station, TX).

Results

Integrative approaches identifying potential tumor suppressors in melanoma

In order to identify novel tumor suppressor genes in malignant melanoma, we hypothesized that those genes that were differentially expressed in melanoma relative to benign melanocytic nevi were candidate tumor suppressor genes. Furthermore, we postulated genes that had a higher propensity for inactivation were more likely to be tumor suppressors. While there are a variety of mechanisms of gene inactivation, we focused on deletion and methylation as a basis for our investigation.

We chose to adapt prior methods of epigenetic screening using 5-aza/TSA treatment that have been found to be successful in defining candidate tumor suppressor genes. Four malignant melanoma derived cell lines were treated with 5μM 5-aza deoxycytidine for three days prior to harvesting total RNA for expression array analysis using Affymetrix U133 AB array and dChip analysis software.

Concurrently, we performed a comparative approach utilizing microarray data utilizing 46 primary melanoma and 18 benign nevi assayed for mRNA expression on the Affymetrix U133A mRNA expression microarray platform (16,383 probe sets) compiled from prior work[12].

Additionally, we identified known loci of deletions in melanomas compiled from previous study of cytogenetic alterations in melanoma[16] and a public repository of CGH data from 176 melanoma samples (progenetix.com).

We determined gene ranks in two ways: 1) Based upon degree of disparity of median expression between melanomas and benign nevi, ranking higher those genes which demonstrated decreased expression in tumors, and 2) Upfold regulation after pharmacologic demethylation in cell lines. Only genes located in known loci of deletion with an incidence of greater than 10% in primary melanoma were included in this analysis. An integrative rank product was calculated. Using a significance threshold ($\alpha = 0.005$) and subsequent random permutation of our rank-lists, we identified 47 genes that were significantly differentially

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upregulated (Supplemental Table 1). Twenty-nine of 47 genes contained promoter-associated CpG islands for which primers could be designed utilizing the MethPrimer software [15]. These were selected for further studies.

Tumor specific promoter methylation of target genes

CpG islands in the promoter region of the 29 selected gene targets with CpG islands were bisulfite sequenced in fourteen melanoma tumor samples (Figure 1). Eight genes were identified containing some degree of tumor DNA promoter methylation. These were: *SFRP1* (4/14 methylated, 28.6%), *CHRDL1* (4/14 methylated, 28.6%), *TMEM47* (8/14 methylated, 57.1%), *KOX15* (1/14 methylated, 7.1%), *LPL* (1/14 methylated, 7.1%), *ZEB1* (8/14 methylated, 57.1%), *PLCXD1* (13/14 methylated, 92.9%), *RARRES1* (5/14 methylated, 35.7%). (Table 2)

Tumor specific deletion of target genes

Quantitative PCR (qPCR) was performed on the 14 melanoma samples tested for methylation for the eight genes previously investigated. Copy number was calculated by comparison to the average quantitation of three housekeeping genes that are not found in areas of deletion or amplification in melanoma. This value was taken as the baseline amount corresponding to 2 alleles and utilized as a denominator to divide the quantitation of the genes of interest. A threshold of 1.5 alleles for somatic genes and 0.75 alleles for X linked genes in males was utilized to determine presence of a deletion. 7/8 (87.5%) genes studied demonstrated some evidence of deletion (Table 3). Incidences were: *SFRP1* (3/14 deleted, 21.4%), *CHRDL1* (3/14 deleted, 21.4%), *TMEM47* (7/14 deleted, 50.0%), *KOX15* (3/14 deleted, 21.4%), *LPL* (1/14 deleted, 7.1%), *ZEB1* (0/14 deleted, 0.0%), *PLCXD1* (1/14 deleted, 7.1%), *RARRES1* (4/14 deleted, 28.6%). (Figure 2). *ZEB1* demonstrated no evidence of deletion in the tumor samples assessed and was not analyzed further.

Incidence of tumor specific gene methylation and deletion

Seven genes identified by our analysis demonstrated evidence of methylation and deletion. When combined, deletion or methylation in the fourteen primary melanoma assayed is present with high incidence for the genes identified by our analysis (Table 3), implying a significant role for these mechanisms in silencing of expression. Combined incidences were: *SFRP1* (6/14, 42.9%), *CHRDL1* (6/14, 42.9%), *TMEM47* (12/14, 85.7%), *KOX15* (3/14, 21.4%), *LPL* (2/14, 14.2%), *PLCXD1* (14/14, 100%), *RARRES1* (8/14, 57.1%). Co-incidence of methylation and deletion are shown in Supplemental Figure 2.

Candidate genes demonstrate growth suppression of melanoma derived cell lines

Transient transfections were performed to evaluate growth-suppressive effects in seven targets that show tumor-specific promoter hypermethylation and allele deletion (Figure 2). Four melanoma derived cell lines (HTB-66, HTB-70. HTB-72, and A375) were transfected with full-length cDNA ORFs for the seven genes of interest and empty vector controls to demonstrate the effects of constituitive overexpression by the putative tumor suppressors. Cell lines were selected based upon disparate derivation techniques and tumor stages in order to test tumor suppressor ability of genes of interest. Cell counts were performed on post transfection day 3 utilizing calcein fluorescence. Experiments were performed in multiples of 8. Statistically significant decreases in cell count were seen in 1 cell line with KOX15, in 2 cell lines with *SFRP1*, *TMEM47* and *PLCXD1*, in 3 cell lines with *RARRES1* and *CHRDL1*and in all 4 cell lines transfected with *LPL*. All genes showed growth inhibition in at least one cell line, confirming the validity of our approach.

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Discussion

Using an integrative epigenetic/cytogenetic screening analysis, we identified 29 genes in malignant melanoma that are significantly decreased in expression relative to benign melanocytic nevi, are expression-responsive to demethylation, and found in areas of known deletion in melanoma. Of these genes, eight were validated by bisulfite sequencing of primary melanomas to demonstrate promoter hypermethylation. Seven of these genes were found to have some evidence of allelic deletion in primary melanoma. The expression of the validated targets in benign nevi relative to melanoma is shown in Supplemental Figure 3. Methylation and deletion appear to happen in an independent fashion that is not coordinated. All of these genes CHRDL1, SFRP1, TMEM47, LPL, RARRES1, PLCXD1, and KOX15 demonstrated some evidence of *in vitro* growth suppressive effects in melanoma derived cell lines. The heterogeneity and varying degree of growth suppression seen by transient transfection is likely a reflection of the genetic variability in the melanoma derived cell lines and the relative strength of the identified tumor suppressor genes.

The technique employed in this study to screen for potential tumor suppressor genes is a variation of techniques previously employed by our group to perform high throughput screening analysis[17, 18]. This approach is, in our estimation, a very stringent one. Its major drawbacks include the likelihood of exclusion of potential targets and the lack of multiple testing controls which limits precision of target identification. These limitations are minimized through the exhaustive validation employed in this study which eliminates false positive targets.

These seven genes are candidate tumor suppressor genes in malignant melanoma and are potential targets for further functional analysis to determine their role in tumorigenesis. Three of these 7: *SFRP1*, *LPL*, and *RARRES1* have been identified as potential tumor suppressors in previous studies of different cancer types. The additional finding that *RARRES1* has been recently reported to have significant methylation in melanoma by a group using disparate genome wide screening approach serves to validate further our approach[19].

CHRDL1 is an X-linked gene whose function is the source of ongoing investigation. Originally described as a BMP antagonist in retinal development[20], it has recently been found to be induced by hypoxia in a HIF 1α dependent fashion in retinal pericytes[21], as well as differentially expressed in colonic crypts[22]. This report is first describing a potential role *CHRDL1* in any tumor type.

TMEM47 encodes a member of the PMP22/EMP/claudin protein family. The encoded protein is localized to the ER and the plasma membrane. Its expression has been noted in Ewing family tumor[23], but no functional studies have addressed its potential role in carcinogenesis. A microarray analysis melanoma cell lines and tumors has identified *TMEM47* as one of a cluster of genes associated with aggressiveness in melanoma metastases[24]. This finding in association with its high rate of allelic loss in our validation studies (12/14 tumors with deletion or methylation, 85.7%), make it a compelling target for further investigation.

PLCXD1 is a pseudoautosomal gene located on both the X and Y chromosomes. It contains a PI-PLC X-box domain and its protein sequence and projected folding modeling suggest phospholipase C activity and possible role in intracellular transduction. No previous studies have assessed or analyzed its role in human cancer.

KOX15 is a zinc finger protein which has been implicated as a regulator of early tooth formation and amelogenesis[25]. It has been mapped to a locus of familial deficiency of

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permanent tooth development [26]. No previous studies have assessed or analyzed its role in human cancer.

SFRP1 is a member of the SFRP family that contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzled proteins. SFRPs act as soluble modulators of Wnt signaling. Functionally, it may play a role in promotion of apoptosis[27-29]. Its loci is associated with LOH and deletion in a variety of human cancers [30]. Decreased expression of *SFRP1* is demonstrated in breast cancer progression[31-33], but does not always correlate with deletion[34]. Promoter hypermethylation of *SFRP1* has been demonstrated in colon[35, 36], prostate[37], ovarian[38], bladder[39], and gastric cancer[40]. Studies in non small cell lung cancer indicate that concomitant genetic and epigenetic events lead to expression silencing of *SFRP1*[41]. The role of SFRP1 as a possible tumor suppressor in melanoma has not previously been reported. Compelling evidence is present in other organ systems for a synergistic role of cytogenetic and epigenetic mechanisms in *SFRP1* inactivation that makes it an appealing candidate for a tumor suppressor gene in melanoma.

LPL encodes lipoprotein lipase, which is expressed in heart, muscle, and adipose tissue. LPL functions as a homodimer, and has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake. Severe mutations causing deficiency result in type I hyperlipoproteinemia. *LPL* has been implicated as a tumor suppressor. *LPL* expression may be regulated in an APC directed manner with upregulation of *LPL* mRNA decreasing the tumorigenic phenotype in APC −/− mice [42, 43]. Multiple mechanisms may be involved in the role of *LPL* in human carcinogenesis. *LPL* treatment has been shown to inactivate nuclear factor kappa B [NF-κB) [44], and trigger apoptosis[45]. *LPL*, was reported as one of the most frequently deleted loci in prostate cancer with LOH and deletion in up to 68% of prostate cancers[46] *LPL* deletion was reported in many other types of human cancers as well, including gastric, colon and oral cancer [47-49]. A recent study has also identified a role for synergistic hypermethylation and deletion of *LPL* in prostate cancer [50].

RARRES1 was identified as a retinoid acid (RA) receptor-responsive gene[51].. It encodes a type 1 membrane protein. The expression of this gene is upregulated by tazarotene as well as by retinoic acid receptors. The expression of this gene is found to be downregulated in prostate cancer, which is caused by the methylation of its promoter and CpG island. It has been shown to be methylated in human melanoma with concomitant decrease in mRNA expression [19]. Its expression has been associated with spontaneous regression of melanoma in an animal model[52]. *RARRES1* is a putative tumor suppressor in prostate cancer [53]and demonstrates promoter hypermethylation in gastric carcinoma [54]. Down-regulation of expression in different cancer cell lines is related to hypermethylation of the promoter [55] It is posited that silencing of *RARRES1* by promoter hypermethylation is common in human cancers and may contribute to the loss of retinoic acid responsiveness in some neoplastic cells[55]. RARRES1 demonstrates promise as a potential tumor suppressor in melanoma. Our findings of significant allelic loss due to deletion (4/14, 28.6%) and an overall 57.1% (8/14) incidence of allelic loss by either methylation or deletion underscore the potential importance of this gene as a tumor suppressor.

Several studies have been published in the interim since the initiation of our study which have utilized microarray analysis of primary melanoma and nevi to elucidate genetic differences between these lesions[56, 57]. These have revealed a spectrum of genes associated with different histopathologic characteristics. Other studies have similarly shown links between tumor invasiveness[24]or clinical outcomes[58] and a variety of gene expression profiles. As well, other studies have assessed epigenetic changes associated with

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melanoma[59]. The raw data from these studies would undoubtedly increase the power of a gene screening algorithm and should be employed in future iterations.

Our study attempts to include these disparate analyses in a single integrated approach, designed to identify genes whose downregulation may be multifactorial. As such, the discovery of a mostly novel set of putative tumor suppressor genes not previously identified in melanoma is an unsurprising outcome of our investigation.

Although much work remains to elucidate possible contributors to that malignant phenotype of melanoma, we did find 7 genes that are solid candidates for tumor suppressor genes that were associated with silencing by cytogenetic and epigenetic mechanisms. One pitfall of integrative genomic approaches is the problem of identifying true signal from the substantial baseline noise generated by such large amounts of data. In addition to utilizing stringent statistical criteria in our discovery phase, we utilized separate discovery and validation cohorts and complementary experimental techniques, in order to bolster the validity of our findings. This approach represents the attempted discovery and validation of genes in melanoma that are potential tumor suppressors. Future work to explore the implications of this study would include functional studies of CHRDL1, SFRP1, TMEM47, LPL, RARRES1, PLCXD1, and KOX15 in melanoma derived cell lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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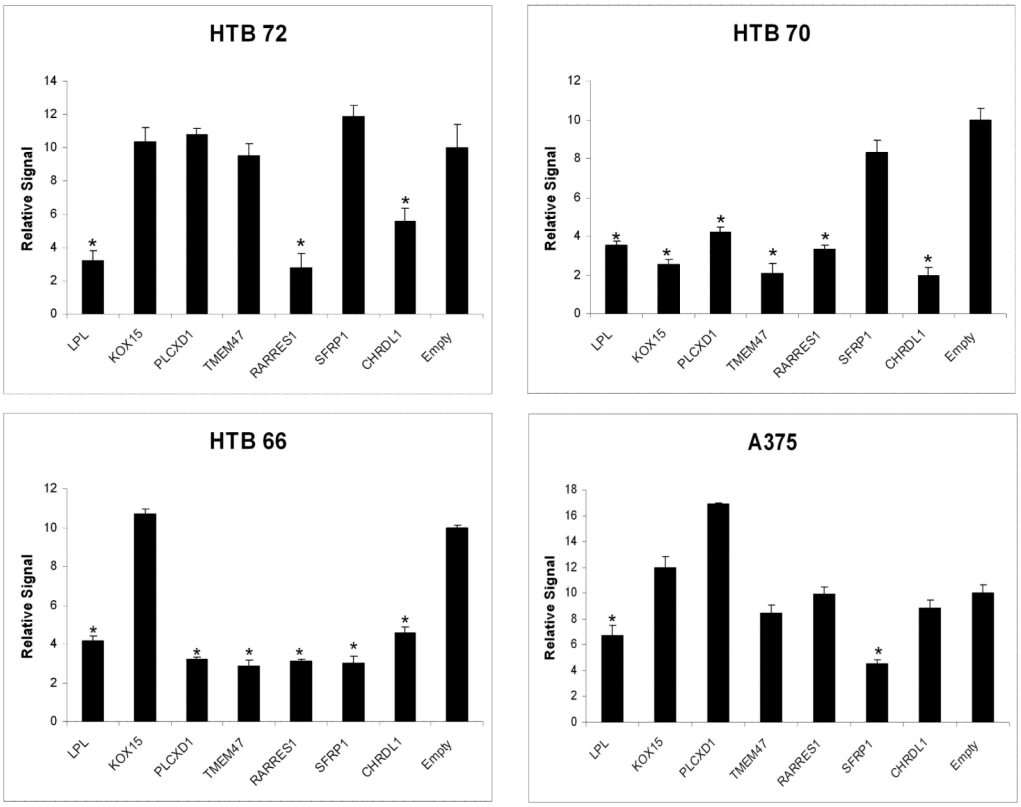

Figure 1.

Bisulfite sequencing of methylated candidate genes identified by integrative analysis. TSS= Transcriptional start site. ○ Unmethylated CpG ● Methylated CpG.

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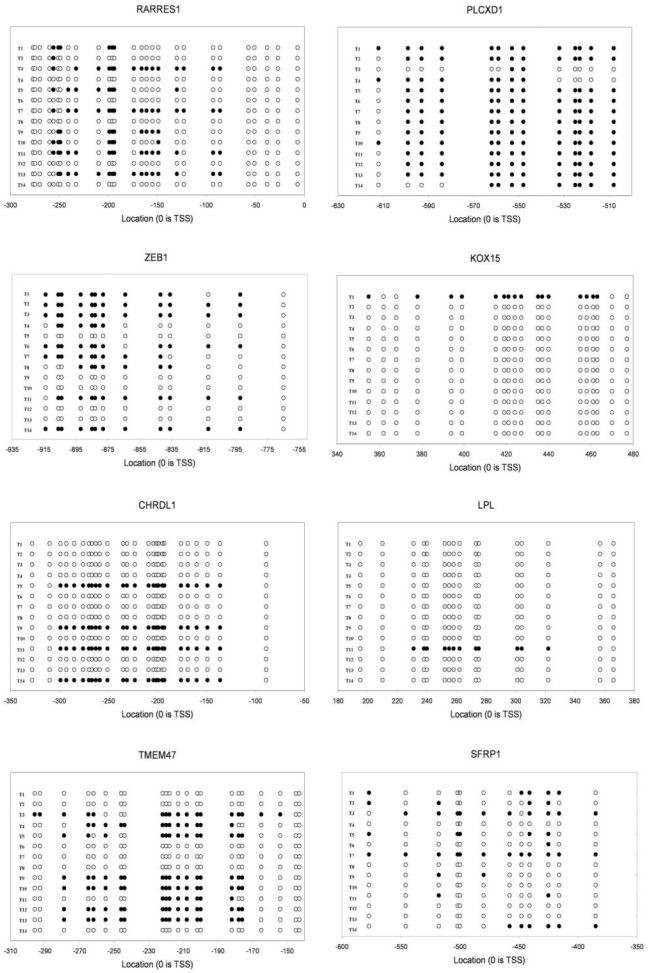

Figure 2.

Gene copy number analysis. Asterisk (*) indicates statistically significant decrease in copy number (relative to 2 for autosomal genes and 1 for X-linked genes) suggestive of deletion ($p<.05$). Error bars represent standard deviation.

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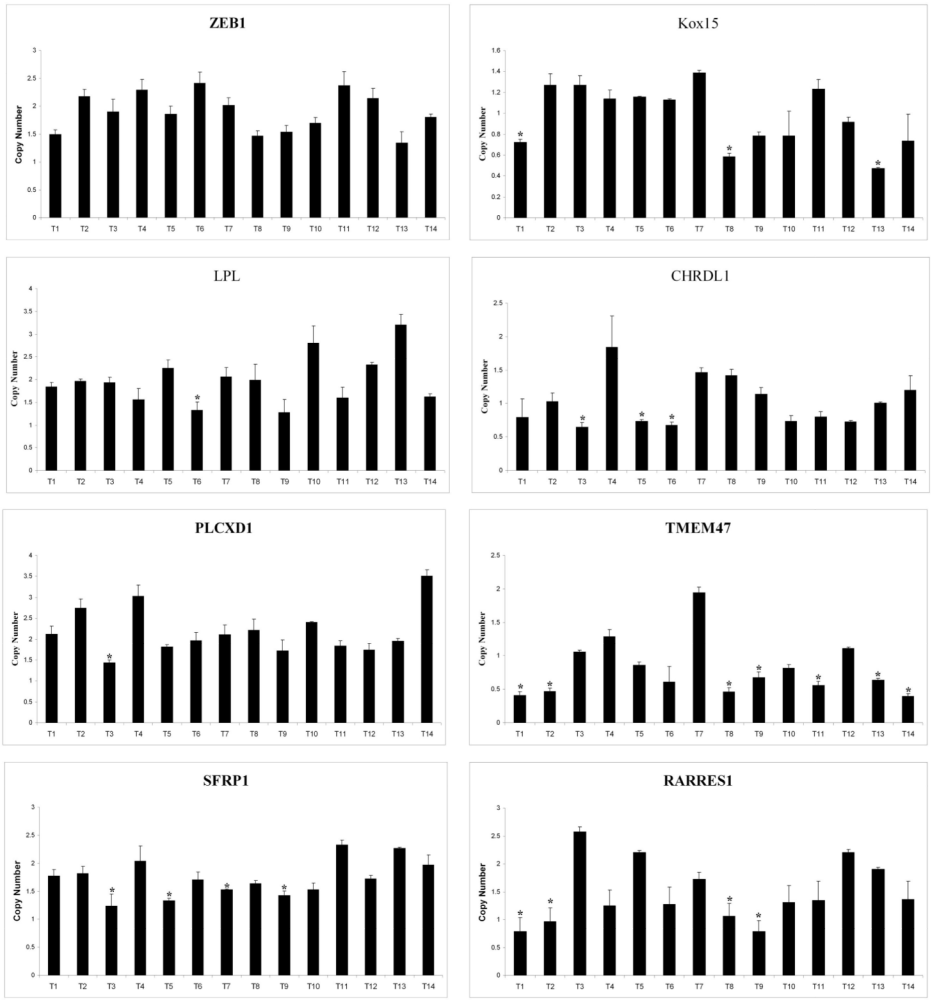

Figure 3.

Transient transfection of melanoma derived cell lines with genes of interest. Cells harvested 72 hours after transfection. Cellular signal was quantitated with calcein fluorescence. Y axes represent the relative ratio of the detector signal absorption. Experiments performed in octuplicate. * indicates statistically significant difference from empty vector transfection ($p<.05$). Error bars represent standard deviation. **A** Transfection of *LPL*, *RARRES1*, and *CHRDL1* results in growth inhibition of the HTB-72 cell line. **B** Transfection of *LPL*, *KOX15*, *PLCXD1*, *TMEM47*, *RARRES1*, and *CHRDL1* results in growth inhibition of the HTB-70 cell line. **C** Transfection of *LPL*, *PLCXD1*, *TMEM47*, *RARRES1*, *SFRP1* and *CHRDL1* results in growth inhibition of the HTB-66 cell line. **D** Transfection of *LPL*, and *SFRP1* results in growth inhibition of the A375 cell line.

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Table 1

Tumor and patient information for samples utilized.

Sample	Age	Sex	Stage at Presentation	T	N	M	Disease State	Disease Free Interval (Months)	Primary	Sample Derivation
1	50	M	4	3b	2b	1	NED	-	Scalp	Primary
2	82	M	3c	4a	2b	0	DOD	1	Shoulder	Lymph Node
3	81	M	2a	3a	0	0	NED	-	Scalp	Primary
4	77	M	3a	X	2b	0	DOD	6	Unknown	Lymph Node
5	87	M	2b	4	0	0	NED	-	Cheek	Recurrence
6	39	M	1b	2a	0	0	AWD	10	Neck	Primary
7	84	M	3c	4	3	0	DOC	-	Shoulder	Primary
8	83	M	3b	2a	2b	0	DOD	22	Ear	Recurrence
9	61	F	1a	1	0	0	NED	45	Mucosal	Primary
10	42	M	3a	X	2a	0	DOD	18	Unknown	Recurrence
11	73	M	4	X	X	1	AWD	10	Unknown	Lymph Node
12	57	M	3a	1a	1a	0	NED	-	Cheek	Primary
13	76	M	4	X	1a	1	NED	-	Unknown	Lymph Node
14	92	M	4	X	X	1	NED	-	Unknown	Lymph Node

NED=No Evidence of Disease **AWD**=Alive with Disease **DOC**=Dead of other causes **DOD**=Dead of Disease

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Table 2

Targets identified by integrative approach and validation

Gene	Abbreviation	Location	CGH Loss %	Median Tumor Expression vs Nevi	Aza Fold Upregulation
Chordin-like 1	*CHRDL1*	Xq23	−13.2	0.15	1.70
Secreted frizzled-related protein 1	*SFRP1*	8p12	−12.3	0.17	1.72
Transmembrane protein 47	*TMEM47*	Xp11	−13.5	0.08	1.29
Transcription factor 8	*ZEB1*	10p11	−20.8	0.33	1.85
Lipoprotein lipase	*LPL*	8p22	−13.2	0.32	1.28
Retinoic acid receptor responder 1	*RARRES1*	3q25	−19.8	0.46	1.74
Phosphatidylinositol-specific phospholipase C, X domain containing 1	*PLCXD1*	Xp22	−16	0.41	1.32
Zinc finger protein 22	*KOX 15*	10q11	−24.5	0.43	1.29

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Table 3

Incidence of deletion and methylation

Gene	Deleted	Methylated	Deleted or Methylated	
CHRDL1	3	4	6	42.9%
SFRP1	3	4	6	42.9%
TMEM47	7	8	12	85.7%
ZEB1	0	8	8	57.1%
LPL	1	1	2	14.3%
RARRES1	4	5	8	57.1%
PLCXD1	1	13	14	100.0%
KOX15	3	1	3	21.4%
Total Samples			14	