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Microarray analysis of differentially expressed genes regulating lipid metabolism during melanoma progression

Venil N Sumantran*, Pratik Mishra and N Sudhakar

Department of Biotechnology, Dr. M.G.R. Educational and Research Institute University, Maduravoyal 600 095, Chennai, India

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A new hallmark of cancer involves acquisition of a lipogenic phenotype which promotes tumorigenesis. Little is known about lipid metabolism in melanomas. Therefore, we used BRB (Biometrics Research Branch) class comparison tool with multivariate analysis to identify differentially expressed genes in human cutaneous melanomas, compared with benign nevi and normal skin derived from the microarray dataset (GDS1375). The methods were validated by identifying known melanoma biomarkers (*CITED1, FGFR2, PTPRF, LICAM, SPP1* and *PHACTR1*) in our results. Eighteen genes regulating metabolism of fatty acids, lipid second messengers and gangliosides were 2-9 fold upregulated in melanomas of GDS-1375. Out of the 18 genes, 13 were confirmed by KEGG pathway analysis and 10 were also significantly upregulated in human melanoma cell lines of NCI-60 Cell Miner database. Results showed that melanomas upregulated *PPARGC1A* transcription factor and its target genes regulating synthesis of fatty acids (*SCD*) and complex lipids (*FABP3* and *ACSL3*). Melanoma also upregulated genes which prevented lipotoxicity (*CPT2* and *ACOT7*) and regulated lipid second messengers, such as phosphatidic acid (*AGPAT-4, PLD3*) and inositol triphosphate (*ITPKB, ITPR3*). Genes for synthesis of pro-tumorigenic GM3 and GD3 gangliosides (*UGCG, HEXA, ST3GAL5* and *ST8SIA1*) were also upregulated in melanoma. Overall, the microarray analysis of GDS-1375 dataset indicated that melanomas can become lipogenic by upregulating genes, leading to increase in fatty acid metabolism, metabolism of specific lipid second messengers, and ganglioside synthesis.

Keywords: Microarray, Melanomas, Lipid metabolism, NCI-60 Cell Miner, KEGG pathway analysis

Melanoma is one of the most aggressive and lethal human cancers. Cutaneous melanomas develop from pre-malignant lesions (benign nevi) via transformation and proliferation of melanocytes. Enhanced lipogenesis is a major metabolic hallmark of cancer and altered lipid metabolism affects membrane biosynthesis, energy production, cell proliferation, motility and key steps in carcinogenesis[1,2]. Lipid metabolism in skin is particularly important, since the stratum corneum is enriched with ceramides, cholesterol and fatty acids which maintain the permeability barrier. Although microarray data have provided gene signatures and biomarkers[3-6] for melanoma, little is known about regulation of lipid metabolism during melanoma progression. Rung *et al*[7] have reported that nearly one in four published studies used public gene expression data to address a biological problem without generating new data from samples. Often, public data are re-analyzed to address questions different from those posed in the original studies[7]. Rung's study has noted that re-analysis of microarray datasets can add value if new biological questions are addressed[7]. Therefore, we re-analyzed an existing microarray dataset (GDS-1375) to identify differentially expressed genes, which can promote lipogenesis in melanoma. The GDS-1375 dataset was deposited by Talantov *et al*[3] in the public Gene Expression Omnibus (GEO) database. Talantov's original study analyzed differentially expressed genes between benign nevi and cutaneous melanomas and identified two melanoma specific genes, which could be used for diagnostic purposes.

Our re-analysis of microarray dataset GDS-1375 identified differentially expressed genes controlling lipid metabolism in normal skin, benign nevi and cutaneous melanomas. This report analyzes 18 genes which function in three specific lipogenic pathways.

Materials and Methods

Class comparison analysis of GDS-1375 with BRB-array tool

BRB-array tool is an integrated software package for visualization and statistical analysis

*Corresponding author
E-mail: venil.sumantran@gmail.com
Tel:+ 91 44 2378 2176
Fax: + 91 44 2378 3165

of DNA microarray gene expression data. Computing is performed by "R", a powerful statistical programming language[8]. BRB-array tools are widely used to process and analyze microarray data to identify differentially expressed genes between defined samples[9].

We used BRB-array class comparison tool to identify differentially expressed genes from dataset GSE 3189/GDS-1375 on an Affymetrix platform (GPL570, Affymetrix chip type HG-U133 plus 2.0 array) as described in earlier study by Talantov *et al*[3] . Raw data were log-transformed, filtered and normalized to generate 6514 genes for class comparison between three classes (7 arrays of normal skin, 18 arrays of benign nevi and 45 arrays of cutaneous melanomas). Multivariate permutation test computed with 1000 random permutations and false discovery rate of 1% identified 3282 differentially expressed genes with >2-fold change between the three classes. Results were independently verified with the KEGG and Cell Miner Databases.

KEGG Analysis of GDS-1375

Entire class comparison output of GDS-1375 was entered into KEGG database and 573 genes were found to match the terms on 'lipid metabolism' in KEGG. Of these genes, 38 showed >2 fold change and had high statistical significance. Eighteen out of the 38 genes were significantly upregulated in melanomas of GDS-1375, 17 of which were in three specific KEGG pathways (fatty acid metabolism, regulation of lipid second messengers, and ganglioside biosynthesis), while the 18th gene (*PLD3*) was not in KEGG database. However, we considered *PLD3*, because its function was in one of the three pathways (regulation of lipid second messengers) already identified by KEGG. Phosphatidic acid has dual functions in cells. It is an important lipid second messenger[10] and has a lipogenic role, since it is the major precursor for synthesis of phospholipids and triacylglycerols. As phosphatidic acid has important dual functions, tumour cells have more than one pathway for its synthesis[11]. Thus, the AGPAT4 enzyme catalyzes *de novo* synthesis of phosphatidic acid, whereas the PLD3 enzyme produces it via a different pathway. We considered *AGPAT4* and *PLD3* genes, because each gene could potentially increase phosphatidic acid levels by a different pathway, and because both genes were significantly upregulated in melanomas of GDS-1375.

Validation of methodology

Talantov *et al*[3] identified 33 genes with >10-fold overexpression in melanomas compared with benign nevi of GDS-1375. Of the 33 genes, we identified 29 in our class comparison of GDS-1375. These included: *NTRK3, WFDC1, HEY1, GDF15, PHACTR1, KIF23, RNFT2, LICAM, SPP1, PDAP1, SEMA3B, ATP6V0E2, ABHD2, CPEB1, CITED1, NES, PODXL2, MAP3K12, CTSB, CENPN, WIPI1, MAT1A, CAPG, CTNNA2, MNX1, ADCY2, DUSP4, HCN2* and *SV2A;* some of these 29 genes are known melanoma biomarkers (*CITED1, LICAM, SPP1* and *PHACTR1*)[4]. Out of twelve new melanoma biomarkers, we also identified seven (*EGFR, FGFR2, FGFR3, IL8, PTPRF, COL11A1* and *CHP2*)[5] and three independent prognostic melanoma biomarkers (*NCOA3, SPP1* and *RGS1*)[6]. Our usage of BRB tool for class comparison of GDS-1375 was validated, since our results identified most of the genes reported from GDS-1375[3] and other major melanoma biomarkers[4-6].

Results

The heatmap in Fig. 1 showed that 18 genes controlling lipid metabolism were upregulated in melanomas of GDS-1375; these genes regulated three major pathways (metabolism of fatty acids, lipid second messengers and gangliosides). Table 1 shows that 14 of these 18 genes were 2-9 fold upregulated in melanomas and were identified with high statistical significance (p<1e-07). Six of the 14 upregulated genes controlled fatty acid metabolism, 4 genes regulated inositol triphosphate activity and phosphatidic acid synthesis and 4 genes controlled ganglioside synthesis. Pathway analysis for these 14 genes is given below.

Upregulation of genes regulating fatty acid metabolism in melanomas of GDS-1375

The peroxisome proliferator-activated receptor gamma coactivator-1a (*PPARGC-1A* or *PGC1α*) is a key transcription factor regulating fatty acid metabolism, mitochondrial function and energy homeostasis[12]. Class comparison analysis of GDS-1375 revealed that *PGC-1α* and its target genes regulating fatty acid metabolism (*SCD, ACSL3, CPT2* and *ACOT7*) were upregulated in melanomas. The Δ^9-stearoyl-CoA desaturase (*SCD*) is the rate-limiting enzyme for synthesis of monounsaturated fatty acids (MUFAs), which are major components of phospholipids, triglycerides and cholesterol esters[13].

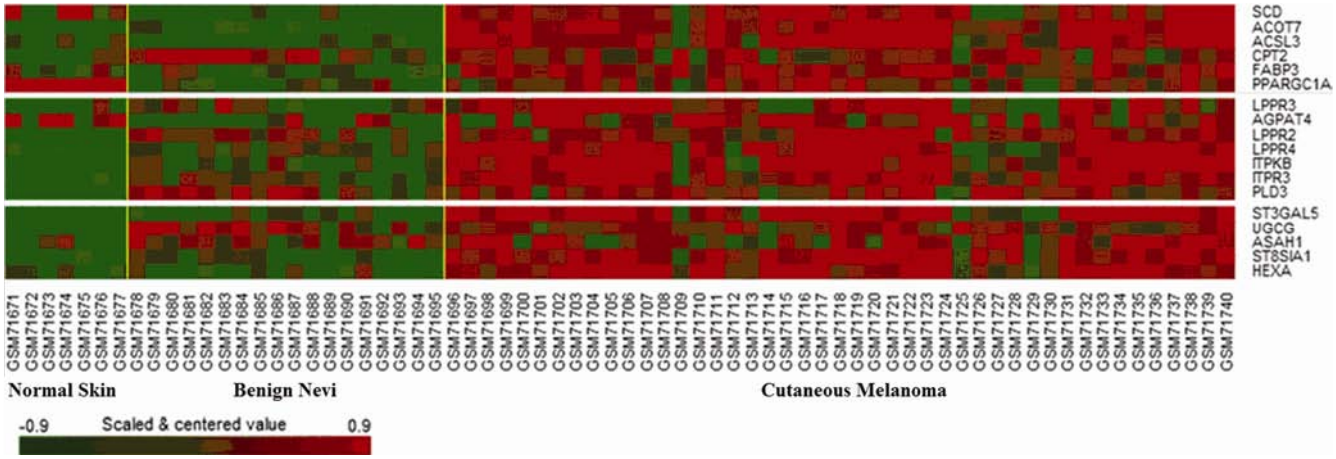

Fig. 1—Heatmap of 18 genes upregulated in melanomas of GDS-1375 [Data for 7 arrays of normal skin, 18 arrays of benign nevi, and 45 arrays of cutaneous melanomas are shown. Top panel shows genes regulating fatty acid metabolism. Middle panel shows genes regulating lipid second messengers. Bottom panel shows genes regulating ceramide and ganglioside metabolism]

High MUFA levels strongly correlate with neoplastic transformation. Increased *SCD* expression promotes tumorigenesis, whereas inhibition of *SCD* can block prostate cancer progression[14]. Notably, we observed 4-fold upregulation of *SCD* in melanomas, compared with benign nevi. Acyl-CoA synthetases-long chains (*ACSLs*) play a lipogenic role by activating long chain fatty acids (LCFA) via esterification with CoA. These LCFA-CoA esters are incorporated into phosphatidylcholine or converted into triglycerides, which are stored as lipid droplets in tumor cells[15].

We found that *ACSL3* gene showed 4-fold upregulation in melanomas, compared with benign nevi. Upregulation of *ACSL3* was specific, since acyl-CoA synthases for short and medium chain fatty acids were not upregulated in melanomas. It is known that LCFA-CoA esters can enter nuclei by interacting with cytoplasmic fatty acid binding proteins (*FABP).* Within the nucleus, these LCFA-CoA esters can bind and activate ligand-activated, nuclear receptor transcription factors, such as *PGC-1α*[16]. Notably, we observed a 2- and 3-fold upregulation of *PGC1a* and *FABP3* in melanomas respectively (Table 1).

Interestingly, overexpression of *PGC-1α* in melanoma cells is reported to increase survival under oxidative stress[17]. Increased *ACSL3* activity enhances fatty acid availability which can lead to increased β-oxidation and generation of the toxic $O_2^{-\cdot}$ radical[18]. *CPT2* limits β-oxidation and prevents this lipotoxicity by converting excess long and medium-chain acyl-CoAs into acylcarnitines, which are exported out of mitochondria[19]. Acyl-CoA thioesterases (*ACOTs*) also prevent lipotoxicity by hydrolyzing excess fatty acyl-CoAs esters into free fatty acids and coenzyme A[20]. Notably, both *CPT2* and *ACOT7* genes showed a 4- and 6–fold upregulation in melanomas, respectively (Table 1). Overall, Fig. 1 and Table 1 clearly showed that the *PPARGC-1α* transcription factor and its target genes regulating synthesis of MUFAs and LCFA metabolism were 2-6-fold upregulated in melanomas.

Upregulation of genes regulating lipid second messengers in melanomas of GDS-1375

Class comparison analysis of GDS-1375 revealed upregulation of four genes regulating lipid second messengers (phosphatidic acid and inositol triphosphate), in melanomas. Data for these genes (*AGPAT4, PLD3, ITPR3* and *ITPKB*) are presented in Fig. 1 and Table 1. Functional roles of these genes are explained below. As mentioned earlier, phosphatidic acid has dual roles in cell signalling and lipogenesis[10,11]. Melanomas of GDS-1375 upregulated two genes, producing phosphatidic acid via different pathways (*AGPAT4, PLD3*). The AGPAT4 (1-acylglycerol-3-phosphate O-acyltransferase 4) enzyme acylates lyso-phosphatidic acid to produce phosphatidic acid, and increased *AGPAT4* gene expression helps maintain the epidermal permeability barrier[21]. Phospholipase D3 (*PLD3*) produces phosphatidic acid by hydrolysis of phosphatidylcholine. Little is known about *PLD3*, but overexpression of *PLD1* is reported in melanomas[22]. Both *AGPAT4* and *PLD3* genes were 2.5 and 8-fold upregulated in melanomas, compared with benign nevi, respectively (Fig. 1 and Table 1).

Table 1—Fourteen genes in three pathways upregulated in melanomas of GDS1375

[The first 6 genes in Table 1 control fatty acid metabolism. The next 4 genes regulate lipid second messengers, and the last 4 four genes in Table 1 control ganglioside synthesis. Genes marked *(*CPT2, ACOT7, PLD3, ITPR3, UGCG*) were significantly upregulated in melanomas compared to normal skin. Remaining genes were significantly upregulated in melanomas compared to benign nevi]

Gene title	Gene symbol	Fold upregulation	Para-metric p-value	Lipogenic and tumorigenic role of gene
Fatty acid metabolism				
Peroxisome proliferator-activated receptor gamma-coactivator 1 alpha	PPARGC-1A	2.30	< 1e-07	Transcription factor controlling fatty acid metabolism and cell survival
Fatty acid binding protein 3	FABP3	3.0	< 1e-07	Regulates fatty acids metabolism
Stearoyl-CoA desaturase	SCD	4.17	< 1e-07	Monounsaturated fatty acids
Acyl-CoA synthetase long chain	ACSL3	3.90	< 1e-07	Regulates synthesis of complex lipids
Carnitine palmitoyltransferase 2	CPT2*	4.70	7e-07	Prevents lipotoxicity
Acyl-CoA-thioesterase 7	ACOT7*	6.10	< 1e-07	Prevents lipotoxicity
Metabolism of lipid second messengers				
1-Acylglycerol-3-phosphate O-acyltransferase 4	AGPAT4	2.50	<3.8e-06	*De novo* synthesis of phosphatidic acid
Phospholipase D3	PLD3*	7.92	< 1e-07	Synthesizes phosphatidic acid via different pathway than AGPAT-4
Inositol triphosphate protein kinase B	ITPKB	4.25	< 1e-07	Regulates cell signalling
Inositol triphosphate receptor 3	ITPR3*	7.92	< 1e-07	Regulates release of cellular calcium
Ganglioside biosynthesis				
UDP-glucose ceramide-glucosyltransferase	UGCG*	9.62	< 1e-07	Catalyzes first glycosylation step
Hexosaminidase A (alpha polypeptide)	HEXA	3.70	< 1e-07	Synthesizes ganglioside GM3
ST3 beta-galactoside alpha-2,3-sialyltransferase 5	ST3GAL5	3.07	< 1e-07	Synthesizes GM3
ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	ST8SIA1	4.50	< 1e-07	Synthesizes GD3 which promotes malignancy and drug resistance

An inositol triphosphate (IP3) receptor (*ITPR3*) and an IP3 kinase (*ITPKB*), which regulate IP3 activity and calcium signalling were also 4-8-fold upregulated in melanomas (Fig. 1 and Table 1). Earlier, upregulation of *ITPKB* has been reported in melanomas[23]. Three lipid phosphate phosphatase-related proteins (*LPPR2, LPPR3* and *LPPR4*) were 2-7-fold upregulated in melanomas (Fig 1). The function of LPPR proteins is unclear, but *LPPR2* is overexpressed in some drug-resistant human melanoma cell lines[24].

Upregulation of genes for ganglioside synthesis in melanomas of GDS-1375

Class comparison analysis of GDS-1375 indicated that melanomas upregulated one gene for ceramide degradation and four genes for ganglioside synthesis. Ceramide is a major component of sphingolipids, which maintain the epidermal permeability barrier. Increased ceramide level can promote apoptosis[25], and the *ASAH1* gene codes for a ceramidase (N-acyl-sphingosine-amidohydrolase1) which prevents cell death by hydrolyzing ceramide. *ASAH1* is overexpressed in malignant, chemoresistant melanoma cells[26]. We also noted upregulation of *ASAH1* in melanomas, compared with benign nevi (Fig. 1).

Melanomas are characterized by synthesis of high levels of sialylated glycosphingolipids known as gangliosides. The GM3 and GD3 gangliosides are tumorigenic because they promote cell growth, adhesion, malignancy and radio-resistance of melanoma cell lines[27]. Table 1 shows that 4 genes

encoding enzymes which synthesize these gangliosides (*UGCG, HEXA, ST3GAL5* and *ST8SIA1*) were 3-9 fold upregulated in cutaneous melanoma compared with benign nevi. First, *UGCG* catalyzes the first glycosylation step of glycosphingolipid synthesis[28]. Second, *HEXA* converts ganglioside GM2 into GM3[29]. Third, the *ST3GAL5* sialyltransferase (ST3 beta-galactoside alpha-2,3-sialyltransferase 5) can synthesize GM3 via a different pathway than *HEXA*[30]. Fourth is a GD3 synthase encoded by the *ST8SIA1* gene. *ST8SIA1* catalyzes formation of the alpha 2-8 linkage of sialic acids and produces gangliosides GD3 and GT3 from GM3[31]. *ST8SIA1* is observed to be overexpressed in melanoma cell lines, compared with normal melanocytes[32]. The expression patterns of gangliosides are primarily determined by the expression and distribution of enzymes required for their biosynthesis[31]. Therefore, increased expression of *UGCG, HEXA, ST3GAL5* and *ST8SIA1* genes in melanomas of GDS-1375 had a high probability of causing increased levels of these enzymes and their products (gangliosides). These data fully agreed with reports stating that upregulated synthesis of specific gangliosides is an important patho-physiological event during melanoma progression[33].

Verification of GDS-1375 results with KEGG and NCI-60 Cell Miner databases

Class comparison output of GDS-1375 was analyzed in KEGG database as stated in 'Materials and Methods'. Of the 18 genes significantly upregulated in melanomas of GDS-1375 (Fig 1), KEGG analysis identified 13 of 14 key genes in three main lipogenic pathways (Table 1).

NCI-60 Cell Miner has curated, quantitative PCR data for 22,217 genes in 60 cancer cell lines from 5 microarray platforms[34]. NCI-60 Cell Miner data have very high statistical significance for 3 reasons. First, each gene is hybridized with 50-300 complementary probes in each cell line. Second, strict quality control parameters are used to identify those probes which give strong signal without false positives. Third, quantitative comparison of a transcript's expression between cell lines is done by calculating Z-scores from raw data[34].

The advantage of Z-score transformation method is that it standardizes data across several experiments and allows the comparison of microarray data independent of the original hybridisation intensities[35].

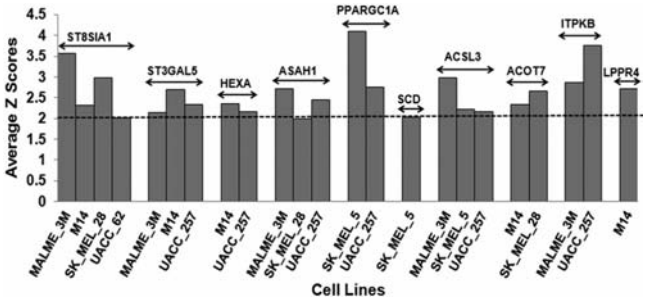

Fig. 2—Ten genes upregulated in GDS1375 melanomas were upregulated in NCI-60 Cell Miner database [Average Z score (> +2) indicates significant upregulation of a gene in a cell line, compared with the 59 other cancer cell lines. Four genes (*ST8SIA1, ST3GAL5, ASAH1*, *ASCL3*) were upregulated in 3-4 melanoma cell lines. Six genes (*PPARGCIA, ACOT7, ITPKB, HEXA, SCD, LPPR4*) were upregulated in 1-2 melanoma cell lines]

Average Z score (≥+2) indicates significant upregulation of a gene in a cell line within NCI-60 Cell Miner[34,35]. Therefore, we checked, if the 18 upregulated genes from Fig. 1 were upregulated in NCI-60 Cell Miner. We found that 10 of the 18 genes upregulated in melanomas of GDS-1375 were also significantly upregulated in melanoma cell lines of NCI-60 Cell Miner (Fig. 2).

Figure 2 shows that Z-scores for these ten genes ranged from 2.0-3.80 and that each gene was upregulated in a different set of NCI-60 melanoma cell lines. Thus, only 1 gene (*ST8SIA1*) was upregulated in 4 cell lines (MALME_3M, M14, SK-MEL-28 and UACC-62). Three genes (*ST3GAL5, ASAH1* and *ASCL3*) were upregulated in a total of 3 cell lines which include MALME_3M, UACC-257, M14, SK-MEL-28 and SK-MEL-5. Four genes (*PGCIA, ACOT7, ITPKB* and *HEXA*) were upregulated in a total of 2 cell lines which include UACC-257, M14, SK-MEL-28 and SK-MEL-5. Two genes (*SCD* and *LPPR4*) were upregulated in 1 cell line (M14 or SK-MEL-5). Figure 2 data were superior to *in vitro* PCR experiments, since we could not prove statistically significant upregulation of a gene with results from 3-4 PCR experiments on 1-2 melanoma cell lines using 1 probe per gene.

In summary, most of the 18 upregulated genes regulating fatty acid metabolism, lipid second messengers and ganglioside biosynthesis in melanomas of GDS-1375 were independently verified and confirmed by the comprehensive and well-established KEGG and NCI-60 Cell Miner databases.

Discussion

Altered lipid metabolism regulates tumorigenesis via its effects on membrane biosynthesis, energy production, cell growth, proliferation and motility[1,2]. Since there is little information on lipogenesis in melanoma, we re-analyzed an existing microarray dataset (GDS-1375) to identify differentially expressed genes regulating lipid metabolism during melanoma progression. The approach of re-analyzing microarray data to address new biological questions has been recommended by a recent review[7].

In this study, BRB class comparison tool was used to identify differentially expressed genes controlling lipid metabolism in melanomas, compared with benign nevi of GDS-1375. Our results were credible because of four important reasons. **(i),** we validated our methods by identifying 29 out of 33 upregulated genes from the original publication on GDS-1375[3]. In addition, we also identified 10 other melanoma biomarkers[4-6] in our GDS-1375 data, **(ii),** the eighteen upregulated genes in melanomas of GDS1375 shown in Fig. 1 were not in random pathways. In fact, 14 of these 18 eighteen genes were in three specific pathways which promoted lipogenesis and tumor progression. These three pathways were fatty acid metabolism, metabolism of lipid second messengers and ganglioside biosynthesis (Table 1). Four of the upregulated genes (*ASAH1, UGCG, ST3GAL5,* and *LPPR4*) in Table 1 were also identified by another study on differential gene expression in melanomas, compared with benign nevi[36]. However, upregulation of the genes regulating fatty acid metabolism (Table 1) has not yet been reported in cutaneous melanomas, **(iii),** most of the 18 upregulated genes in melanomas of GDS-1375 (Fig. 1) were verified with two independent databases (KEGG and NCI-60 Cell Miner); KEGG analysis identified 13 out of 18 upregulated genes in melanomas of GDS-1375 (Table 1) and NCI-60 Cell Miner identified 10 of the 18 upregulated genes (Fig. 2). Figure 2 data had high statistical significance, since NCI-60 Cell Miner selected genes after applying very strict quality control over gene expression data from hundreds of studies and **(iv),** there are several studies in reputed journals which only contain NCI-60 Cell Miner data[37,38]. For example, one study analyzed expression of a poorly understood gene (*RECQ1*) in NCI-60 Cell Miner. The data revealed low *RECQ1* expression in non-invasive epithelial tumor cell lines, and high expression of RECQ1 and other genes known to promote metastasis in invasive tumor cell lines.

This study concluded that the RECQ1 gene also has a high probability of promoting invasion of mesenchymal tumor cells[37]. Another study identified 76 mutually co-expressed tight-junction genes in a subset of cell lines within NCI-60 Cell Miner. Out of the 76 genes, 44 had known epithelial functions, and the co-expression data suggested that the remaining 32 genes also have epithelial-related functions that remain to be discovered[38]. Thus, these two studies demonstrate that genes with similar mRNA expression across diverse cell lines are likely to have similar/related functions[38]. Notably, these studies also prove that *in silico* analysis of NCI-60 Cell Miner data alone provides new insights on functions of unknown genes in several tumor cell lines.

In our study, NCI-60 Cell Miner analysis gave strong supporting data by confirming that 10 of the 18 upregulated genes in melanoma samples of GDS-1375 (Fig. 1 and Table 1), were also upregulated in melanoma cell lines of NCI-60 Cell Miner database (Fig. 2). Such strong correlation of gene expression data from clinical melanoma samples and melanoma cell lines was notable. Therefore, the re-analysis of GDS-1375 data in the present study provided new insights on how melanomas can become lipogenic during tumorigenesis.

Conclusions

Re-analysis of the GDS-1375 microarray dataset showed that cutaneous melanomas upregulated 18 genes which can increase fatty acid metabolism, metabolism of specific lipid second messengers, and ganglioside synthesis. Most of these 18 genes were verified and confirmed by the comprehensive and well-established KEGG and NCI-60 Cell Miner databases. Notably, the upregulation of genes regulating fatty acid metabolism (*PPARGC1A, SCD*, *FABP3, ACSL3, CPT2*) and a gene for phosphatidic acid synthesis (*PLD3*) have not yet been reported in cutaneous melanoma. These results are significant because they identify specific lipogenic pathways during melanoma progression. These results also assume significance because lipogenesis is a new hallmark of cancer, and there are presently no reports on differentially expressed genes which regulate lipogenesis during melanoma progression.

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