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

Received 04 September 2014; revised 01 March 2015

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

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. 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 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 major precursor for synthesis of phospholipids and triglycerides. As phosphatidic acid has important dual functions in cells. It is an important lipid second messenger. Because the provided text contains biological knowledge, we refer to the KEGG pathways (regulation of lipid second messengers) and the specific genes involved.

Validation of methodology

Talantov et al 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, WDFC1, HEY1, GDF15, PHACTR1, KIF23, RNFT2, LICAM, SPP1, PDAP1, SEMA3B, ATP6V0E2, ABHD2, CPEB1, CITED1, NES, PODXL2, MAP3K12, CTSB, CENPN, WIP11, MAT1A, CAPG, CTNNA2, MNX1, ADCY2, DUSP4, HCN2 and SV2A; some of these 29 genes are known melanoma biomarkers (CITED1, LICAM, SPP1 and PHACTR1). Out of twelve new melanoma biomarkers, we also identified seven (EGFR, FGFR2, FGFR3, IL8, PTPRF, COL11A1 and CHP2) and three independent prognostic melanoma biomarkers (NCOA3, SPP1 and RGS1). 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 and other major melanoma biomarkers.

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. Class comparison analysis of GDS-1375 revealed that PGC-1α and its target genes regulating fatty acid metabolism (SCD, ACSL3, CPT2 and ACO7) were upregulated in melanomas. The Δ⁶-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.
High MUFA levels strongly correlate with neoplastic transformation. Increased SCD expression promotes tumorigenesis, whereas inhibition of SCD can block prostate cancer progression. 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.

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. 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. Increased ACSL3 activity enhances fatty acid availability which can lead to increased β-oxidation and generation of the toxic O$_2^-$ radical. 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. Acyl-CoA thioesterases (ACOTs) also prevent lipotoxicity by hydrolyzing excess fatty acyl-CoAs esters into free fatty acids and coenzyme A. 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 PPARγ-1α transcription factor and its target genes regulating synthesis of MUFA 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. 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. Phospholipase D3 (PLD3) produces phosphatidic acid by hydrolysis of phosphatidylcholine. Little is known about PLD3, but overexpression of PLD1 is reported in melanomas. Both AGPAT4 and PLD3 genes were 2.5 and 8-fold upregulated in melanomas, compared with benign nevi, respectively (Fig. 1 and Table 1).
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. 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.

**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, and the ASAH1 gene codes for a ceramidase (N-acylsphingosine-amidohydrolase1) which prevents cell death by hydrolyzing ceramide. ASAH1 is overexpressed in malignant, chemoresistant melanoma cells. 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. 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, UGCC catalyzes the first glycosylation step of glycosphingolipid synthesis\textsuperscript{28}. Second, HEXA converts ganglioside GM2 into GM3\textsuperscript{29}. Third, the ST3GAL5 sialyltransferase (ST3 beta-galactoside alpha-2,3-sialyltransferase 5) can synthesize GM3 via a different pathway than HEXA\textsuperscript{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\textsuperscript{31}. ST8SIA1 is observed to be overexpressed in melanoma cell lines, compared with normal melanocytes\textsuperscript{32}. The expression patterns of gangliosides are primarily determined by the expression and distribution of enzymes required for their biosynthesis\textsuperscript{31}. Therefore, increased expression of UGCC, 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\textsuperscript{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 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\textsuperscript{33}.

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.

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\textsuperscript{12}. 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\textsuperscript{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\textsuperscript{5}. In addition, we also identified 10 other melanoma biomarkers\textsuperscript{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 (\textit{ASAHI}, \textit{UGCG}, \textit{ST3GAL5}, and \textit{LPPR4}) in Table 1 were also identified by another study on differential gene expression in melanomas, compared with benign nevi\textsuperscript{38}. 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\textsuperscript{37,38}. For example, one study analyzed expression of a poorly understood gene (\textit{RECP1}) in NCI-60 Cell Miner. The data revealed low \textit{RECP1} expression in non-invasive epithelial tumor cell lines, and high expression of \textit{RECP1} and other genes known to promote metastasis in invasive tumor cell lines.

This study concluded that the \textit{RECP1} gene also has a high probability of promoting invasion of mesenchymal tumor cells\textsuperscript{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\textsuperscript{38}. Thus, these two studies demonstrate that genes with similar mRNA expression across diverse cell lines are likely to have similar/related functions\textsuperscript{38}. Notably, these studies also prove that \textit{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 (\textit{PPARGC1A}, \textit{SCD}, \textit{FABP3}, \textit{ACSL3}, \textit{CPT2}) and a gene for phosphatidic acid synthesis (\textit{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.

Acknowledgements

We acknowledge valuable guidance on usage of BRB-Array Tools from Dr. Gopal Ramesh Kumar.
and Dr. Ashok Selvaraj of AUKBC Research Center, Anna University, Chennai, and from Suresh Madheswaran, MGRU (Dr M. G. R University). We thank Dr. Rama Vaidyanathan, Director (R&D), MGRU, Maduravoyal for valuable support.

References

1 Santos C R & Schulze A (2012) FEBS J 279, 2610-2623
11 Huiyian H & Frohman M A (2009) Biochim Biophys Acta 1791(9), 839-844