Real-time Quantitative Reverse Transcriptase-polymerase Chain Reaction Analysis of Melanoma Progression-associated Genes

MAIRIN RAFFERTY[1], WILLIAM J. FALLER[1], CATHERINE MOSS[3], JANET MCCORMACK[3], GUNHILD M. MÃLANDSMO[4], DAVID J. EASTY[2] and WILLIAM M. GALLAGHER[1]

[1]*UCD School of Biomolecular and Biomedical Science,* [2]*UCD School of Medicine and Medical Science and* [3]*Transcriptomics Core, UCD Conway Institute, University College Dublin, Ireland;* [4]*Department of Tumor Biology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway*

Abstract. *Background: Melanoma is an aggressive disease that spreads quickly and is resistant to most therapeutic agents. In an effort to provide insight into the molecular basis of melanoma progression, the expression of 94 genes in 20 metastatic melanomas using a high-throughput real-time quantitative RT-PCR assay was analysed. Materials and Methods: A TaqMan low density array (LDA) was designed containing probes/primers directed towards a cohort of genes previously found to be differentially expressed in an isogenic cell line model of melanoma progression. For each sample, cDNA was prepared and added to the quantitative assay. The resulting data were then analysed for correlations with clinical data. Results: Clustering analysis divided the melanomas into two major subgroups based on gene expression patterns. When analysed individually, several genes were associated with overall survival, depth and type of the primary tumour. Conclusion: We have identified a selection of genes linked to melanoma progression and patient outcome.*

Malignant melanoma was once described as a genetic black box, with the underlying molecular basis then being considered elusive (1). Since that time, two new major oncogenes, *BRAF* and *MITF*, have been discovered to play a key role in melanoma. *BRAF*, a vital constituent of the MAP-kinase pathway, is found mutated in 66% melanomas (2), whilst *MITF*, a key regulator of melanogenesis-related genes, is amplified in 10-15% melanomas (3). In addition, DNA microarrays have begun to elucidate the precise signalling pathways in melanoma (4-7). These studies have identified cohorts of genes that facilitate the differentiation of benign nevi from malignant melanomas (6), the subclassification of metastatic melanomas into distinct subgroups (4, 5) and the prediction of distant metastasis-free survival (7). Moreover, genes associated with progression have also been identified using DNA microarray-based gene expression profile analysis of melanoma cell lines (8, 9).

One of the main stumbling blocks inhibiting melanoma studies, however, is the lack of fresh tissue. In general, the majority of the small/thin melanoma specimens are sequestered for pathological examination; this leaves very little material for DNA microarray studies, with these commonly requiring several micrograms of RNA to prepare the probe. An alternative approach is to use real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, where much smaller amounts of RNA are required. Previous studies have used real-time quantitative RT-PCR to detect melanoma markers in blood samples and lymph nodes from melanoma patients with a view to predicting metastatic disease and prognosis (10, 11). In addition, quantitative RT-PCR assays have also been used to track changes in the blood both during and after treatment of melanoma patients (12, 13). Furthermore, multiple real-time RT-PCR assays have also been used to subclassify melanomas (14) and other cancers, such as breast cancer (15).

Here, we used real-time quantitative RT-PCR to profile 94 genes in 20 metastatic melanomas. The majority of the genes examined were previously identified by our group to be differentially expressed in an isogenic cell line model of melanoma (8). Some of these differentially expressed genes had been previously associated with melanoma development and progression, including *CDKN2A, IL-24, AIM1* and *MAGEA4*, but most have undefined roles in this context. In an attempt to examine the importance of this cohort of

Correspondence to: Prof. William M. Gallagher, UCD School of Biomolecular and Biomedical Science, UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland. Tel: 353 1 7166743, Fax: 353 1 2837211, e-mail: william.gallagher@ucd.ie

Key Words: Melanoma, metastasis, TaqMan low density arrays (LDAs), RT-PCR, overall survival.

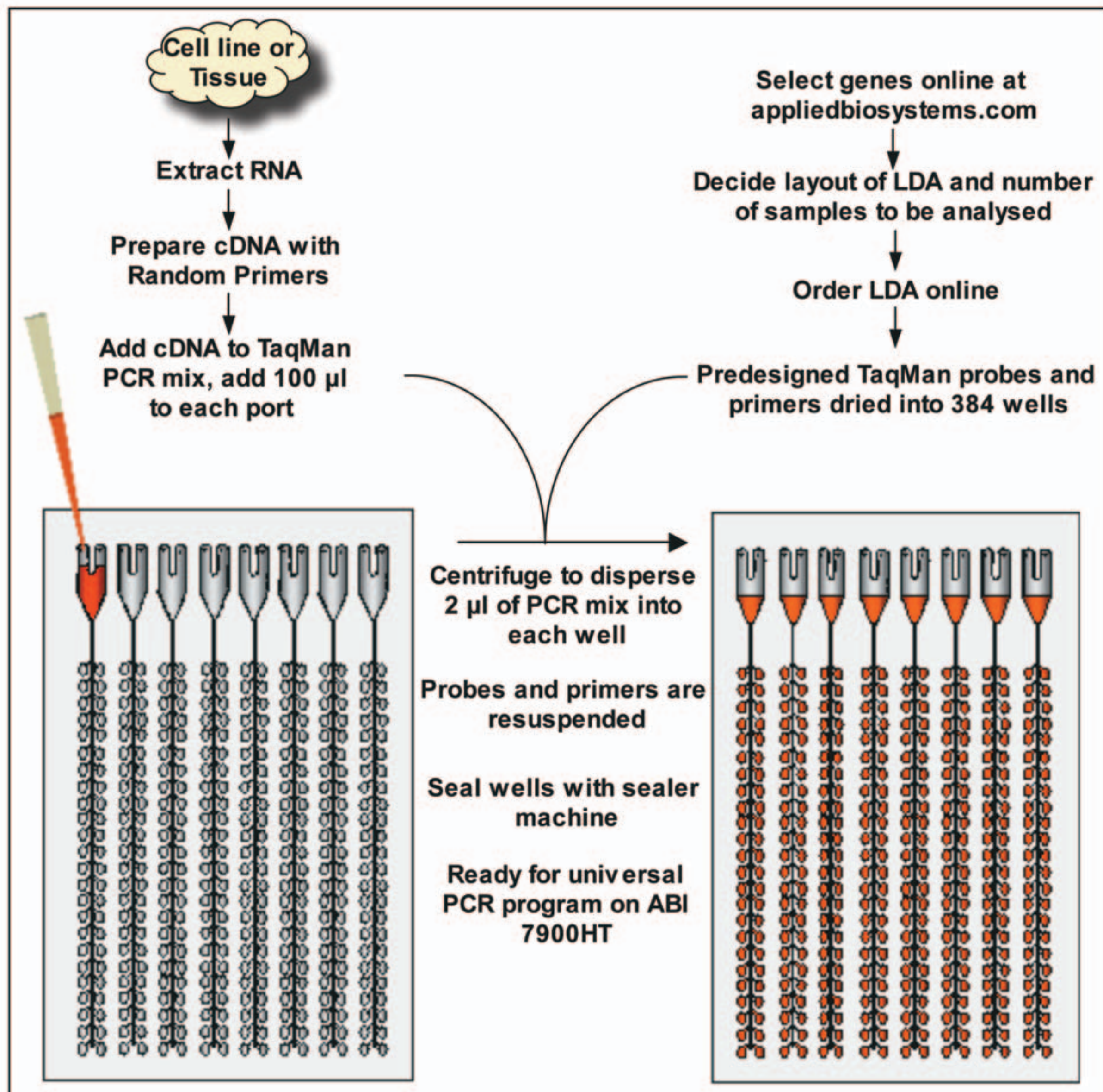

Figure 1. *Schematic of the high-throughput quantitative reverse transcriptase-PCR assay, or TaqMan Low Density Array (LDA). In brief, genes of interest are selected on the Applied Biosystems webpage, along with array format desired. The probes and primers are loaded into each well of the plate and sent to the customer. To prepare the samples, RNA is extracted and cDNA synthesised. The cDNA (50 ng-1 µg) is added to a PCR master mix and added to the eight ports (depending on the design of the card). The plate is centrifuged to disperse the master mix into each well and put on the ABI7900HT for PCR amplification.*

putative melanoma progression-associated genes in metastatic melanoma, we employed a high-throughput real-time quantitative RT-PCR assay, specifically employing a TaqMan low density array (LDA) approach (Figure 1). The resulting real-time quantitative RT-PCR data was then compared to clinical variables to determine if there were any associations. Overall, our results show that real-time quantitative RT-PCR can be used to classify melanoma tumours and to indicate novel genes that may be important in melanoma progression.

Materials and Methods

Melanoma samples and reverse transcription. Previously extracted total RNA from 20 anonymised metastatic melanomas was obtained from the Norwegian Radium Hospital. The concentration of each RNA sample was determined by using the Nanodrop spectrophotometer and analysed for quality using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano assay. Single-stranded cDNA was synthesised from 1 µg total RNA using the ImProm-II Reverse Transcription kit (Promega), according to the

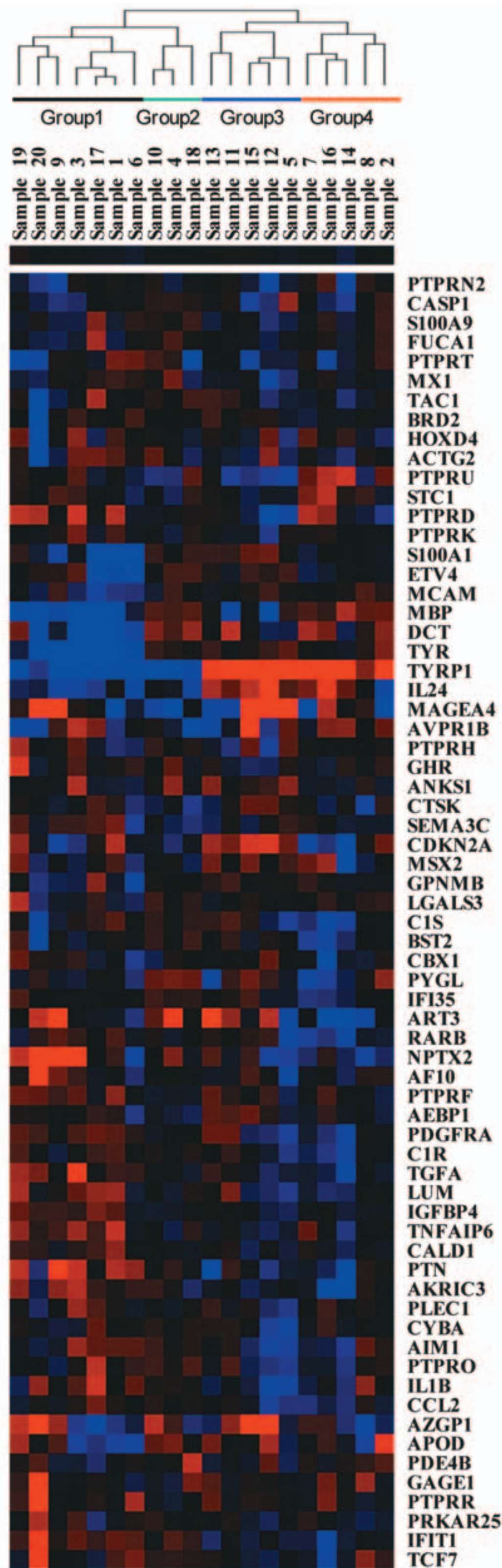

Figure 2. *Clustering analysis of TaqMan LDA results. The normalised RT-PCR data for 67 genes were clustered using Cluster and Treeview software. Blue = low expression, red = high expression.*

manufacturer's instructions. Following reverse transcription, the cDNA was tested for 18S rRNA expression using a single real-time quantitative RT-PCR assay, prior to addition to the TaqMan LDA. For this, the following primer pair combinations were used: 18S – forward, 5'-AGGGTTCGATTCCGGAG-3' and reverse, 5'-ACCAGACTTGCCCTCC-3' (195 bp amplicon).

Real-time quantitative RT-PCR analysis using TaqMan LDAs. Quantitative gene expression was determined via a high-throughput TaqMan LDA as described elsewhere (8). Pre-designed TaqMan probe and primer sets for target genes (see Table I) were chosen from an online catalogue (Applied Biosystems, California, USA). Once selected, the sets were factory-loaded into 384-well plates. Each array was designed to analyse 94 target genes in duplicate plus 18S rRNA, for two separate samples. Samples were then analysed using the 7900HT system with a TaqMan LDA upgrade (Applied Biosystems) according to the manufacturer's instructions. Briefly, 10 μL of single-stranded cDNA was combined with 40 μL H_2O and 50 μL TaqMan Universal PCR mix. Each sample (100 μL) was loaded into respective ports (4 ports per card). The thermal cycling conditions were as follows: 50˚C for 2 min, 94˚C for 10 min, 97˚C for 30 sec and 59.7˚C for 1 min. The C_T value for each gene was first compared to the average of invariant genes (these genes were selected from previous DNA microarray data) on the card and then normalised to the median value for all samples (8).

Clustering and statistical analysis. For clustering analysis, Clustering and Treeview software (Eisen Lab, California, USA) were used for the normalised data (16). For statistical analysis, the SPSS statistical package (SPSS, Illinois, USA) was used. For survival curves, a binary profile was generated to indicate whether the sample was above or below the median value of all samples. Kaplan-Meier survival curves were then calculated for each target gene. Correlations between gene expression and the type/depth of the original tumour were also assessed. A *p*-value ≤0.05 was deemed to be significant.

Results

The expression of 94 genes were analysed in twenty metastatic melanoma samples using a real-time quantitative RT-PCR assay. The genes used for the TaqMan LDA were selected from our previous investigation of differential gene expression in an isogenic cell line model of melanoma (8). 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. In addition, when this data was re-analysed using updated software, an additional 37 differentially expressed genes were identified (Rafferty and Gallagher, unpublished data). In total, TaqMan probes and primers sets were available for 71 members of this melanoma progression-associated gene cohort (see Table I for full list of genes, plus functional groupings). The remaining genes represented by relevant probes/primers on the TaqMan LDA included those encoding for protein tyrosine phosphatases, as well as invariant genes and 18s rRNA.

Table I. *Genes analysed using the TaqMan LDA.*

Funtional group	Gene	Functional group	Gene	Functional group	Gene
Control	18S rRNA	Signal transduction	***AVPR1B***	Other	***ACTG2***
Melanin biosynthesis	***TYR***		***BRD2***		***AEBP1***
	TYRP1		***CHN1***		***AF1Q***
	TYRP2/DCT		*ETV4**		***ANKS1***
Tumour suppressors	***CDKN2A***		*GHR**		*ART3**
Interferon-related	***DAP***		***IGFBP4***		***AZGP1***
	*IFI35**		*KPNB3**		***CALD***
	IFIT1		*MAPK6*		*CBX1**
	ISGF3G		***MCAM***		**CCL2**
	MX1		*MMD**		*CCT5**
	TNFAIP6		OBRGRP*		*CTSK**
Immune response	***BST2***		*PRKAR2B**		*GMPS**
	C1R		***RARB***		***GPNMB***
	C1S		*TCF7**		***HOXD4***
	IL1B	Melanoma antigens	***GAGE1***		***LGALS3***
	IL24		***MAGEA4***		***LUM***
Cytokines, growth factors, hormones, neuronal peptides	***NPTX2***		***S100A1***		***MBP***
	*PDGFRA**		***S100A9***		*NULL*
	PHB	Phosphatases	*PTPRA*		*PDE4B**
	PTN		*PTPRB*		*PLEC1**
	*STC**		*PTPRD*		*SEMA3C**
	TAC		*PTPRF*		***SNRK***
	TGFA		*PTPRH*		***UGT2B7***
Met/Catabolism	***AKR1C3***		*PTPRJ*		*XRCC5**
	APOD		*PTPRK*	Invariant	*ATP6V0A1*
	*FUCA1**		*PTPRM*		*CHD4*
	*GOT1**		***PTPRN2***		*DAB2*
	*OXCT**		*PTPRO*		*G22P1*
	*PYGL**		*PTPRR*		*HSF2*
Apoptosis	***CASP***		*PTPRS*		*MADD*
	CYBA		*PTPRT*		*NDUFA4*
			PTPRU		*PSMA4*

Custom designed melanoma-progression LDA contains probes/primers representative for 94 genes, along with an internal control, 18S rRNA. Genes are shown here grouped according to their ascribed functions or family. Genes with unknown function or have other functions not listed here are shown in the 'Other' group. Genes indicated in bold were identified by our original published work on isogenic cell lines representing different stages of melanoma progression (8). Asterisk denotes additional putative melanoma progression-associated genes identified following re-analysis of this data (Rafferty and Gallagher, unpublished data).

The TaqMan LDA assay showed that the majority of genes were expressed in the melanoma samples. However, *PTPRS* and *UGT2B7* were not detected in any sample, whilst only three samples displayed expression of *PTPRR* and *GAGE1*. For analysis, the samples were subsequently filtered to exclude genes that did not display a significant alteration in gene expression between samples. Only genes displaying at least a 2-fold difference in expression level in at least five samples were used for analysis (67 genes). Hierarchical clustering separated the metastatic melanoma samples into two predominant subgroups, both of which subsequently separated into two smaller subclusters (Figure 2). *TYRP1* and *IL24* best separated the two major clusters; however, neither group correlated with any clinical variables available for these samples. Of the smaller subclusters 1-4, cluster 1 showed decreased expression of the tyrosinase melanogenesis-related genes (*TYR*, *TYRP1* and *TYRP2*). Interestingly, the samples in cluster 4 did associate with decreased overall survival and disease-free interval ($p<0.05$); however, due to the small number of samples in this subcluster, this association may be questionable.

When each gene was examined individually several genes were associated with overall survival, disease-free interval and period from metastasis-to-death (Figure 3). The expression of four genes, namely *GPNMB* ($p=0.013$), *CBX1* ($p=0.04$), *S100A1* ($p=0.045$) and *ETV4* ($p=0.049$), were associated with increased overall survival. On the other hand, *PTPRT* expression was negatively associated with overall survival. In

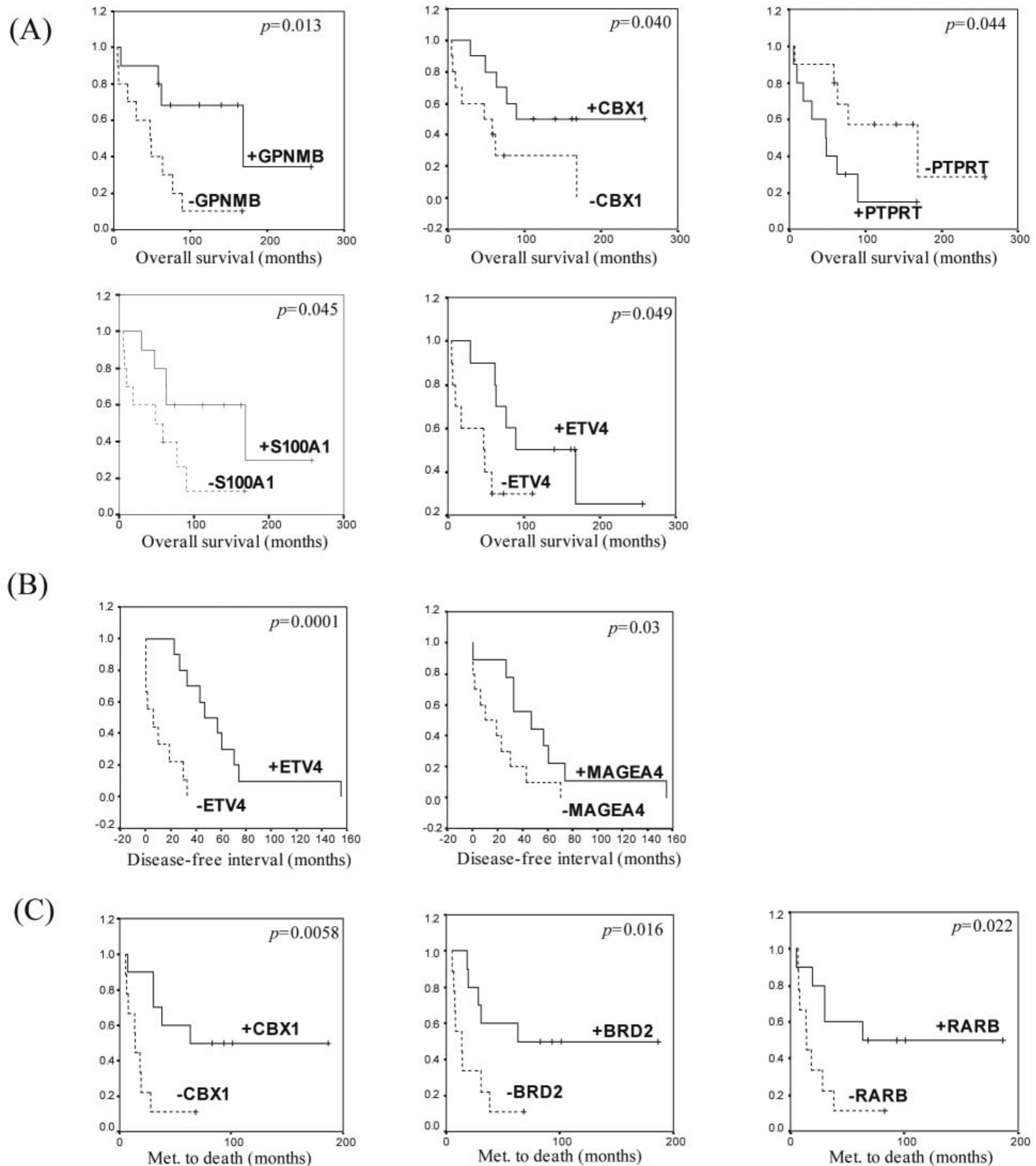

Figure 3. *Kaplan-Meier survival curves. Genes were analysed individually for associations with (a) overall survival, (b) disease-free interval, and (c) period from metastasis to death.*

addition, *ETV4* expression was also associated with an increase in the disease-free interval (p=0.001). Meanwhile, *CBX1*, *BRD2* and *RARB* expression correlated with increased survival from the time of metastasis to death (Figure 3C).

Other genes, such as *TYR* and *TYRP2/DCT*, were found to be associated with the depth of the original tumour, whilst four other genes (*CASP1, GAGE1, PTPRR, CHN1*) correlated with the type of the original tumour (Table II).

Table II. *Statistical significance (p-value) of the association between genes and the type and depth of the original tumour.*

	Superficial or Nodular	Depth
CASP1	0.041	ns
GAGE1	0.041	ns
PTPRR	0.041	ns
CHN1	0.041	ns
DCT	ns	0.035
TYR	ns	0.053
IFIT1	ns	0.018
MSX2	ns	0.039

ns=not significant.

Discussion

In this study, we examined the expression of 94 genes in metastatic melanomas using a TaqMan LDA. This is a robust real-time quantitative RT-PCR assay which we showed previously to correlate well with DNA microarray data obtained from an isogenic cell line model system of melanoma progression (8). Here, we illustrate that this assay can be used successfully on melanoma tissues to determine which genes are linked to progression of the disease. This assay only requires a small amount of starting RNA to analyse up to 384 genes; therefore, it is ideal for examining small *in situ* primary tumours, which prove difficult to analyse by conventional DNA microarrays. Indeed, TaqMan LDAs may be particularly useful for analysis of formalin-fixed, paraffin-embedded melanomas, as has been performed with similar biopsy material from breast cancer patients in order to predict response to therapy (15).

Clustering of gene expression data obtained by real-time quantitative RT-PCR analysis found that the metastatic melanoma samples were clustered into two distinct groups, divided mainly by the expression of *TYRP1* and *IL24*. Similarly, a recent study identified two subgroups of melanomas based on DNA microarray data, where the predominant subgroup expressed *MITF*, tyrosinase and *Mart1/MelanA* (5). In addition, another research group identified 19 differentially expressed genes, again based on DNA microarray data, which could discriminate between two subgroups (4). *Mart1/MelanA* was commonly altered in both these studies, although this gene was not represented on our TaqMan LDA. However, another melanogenesis-related gene, *TYRP1*, did facilitate group separation in our study series.

We also identified several genes that were associated with survival indices. Five genes were associated with overall survival, two genes with the disease-free interval and three with the period from metastasis to death. One of these genes, *GPNMB*, was originally found to be preferentially expressed in melanoma cell lines of low metastatic potential as compared to more aggressive cell lines (17). Moreover, expression of *GPNMB* in highly metastatic melanoma caused reduction in tumour growth and spontaneous metastasis. In addition, somatic point mutations and chromosomal breakpoints have also been found in *GPNMB* in melanoma patients (18, 19). In our study, high levels of *GPNMB* in metastatic melanomas were associated with improved overall survival, in agreement with a possible protective role for this gene in melanoma patients.

Other genes identified here, such as *PTPRT, BRD2* and *ETV4*, have not been previously identified as playing a role in melanoma. However, *PTPRT* is seen to be commonly mutated in several other cancer types (20). In addition, overexpression of wild-type PTPRT inhibited growth of cancer cells, indicating a possible tumour suppressor function. In our study, its expression in metastatic melanomas was associated with poor outcome, but it is unknown if the wild-type or mutant form of this gene is being expressed in our samples.

CBX1, a homeobox transcription factor which has a role in gene silencing (21), was also found to be associated with survival in our cohort. In addition, we found *MAGEA4* and *RARB*, known methylated genes in melanoma (22, 23), to be associated with the disease-free interval and period from metastasis to death, respectively. In our samples, the methylation status of these genes is unknown, but may provide an explanation, at least in part, for the differences in expression observed.

Conclusion

In this pilot study, we applied a multiplex real-time quantitative RT-PCR assay to examine the expression of putative markers of melanoma progression in metastatic tumour biopsies. Assays on the TaqMan LDA platform facilitate efficient use of limited biological material, as particularly experienced in the case of melanoma biopsies. Further studies will have to be conducted to validate if these genes are truly associated with melanoma survival and to investigate their precise role in the disease.

Acknowledgements

This research was supported by the Health Research Board and IRCSET. The UCD Conway Institute is funded by the Programme for Third Level Institutions (PRTLI), as administered by the Higher Education Authority (HEA) of Ireland.

References

1 Chin L, Merlino G and DePinho RA: Malignant melanoma: modern black plague and genetic black box. Genes Dev *12*: 3467-3481, 1998.

2 Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S *et al*: Mutations of the BRAF gene in human cancer. Nature *417*: 949-954, 2002.

3 Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, Ramaswamy S, Beroukhim R, Milner DA, Granter SR, Du J, Lee C, Wagner SN, Li C, Golub TR, Rimm DL, Meyerson ML, Fisher DE and Sellers WR: Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature *436*: 117-122, 2005.

4 Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A, Sampas N, Dougherty E, Wang E, Marincola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D *et al*: Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature *406*: 536-540, 2000.

5 Haqq C, Nosrati M, Sudilovsky D, Crothers J, Khodabakhsh D, Pulliam BL, Federman S, Miller JR, 3rd, Allen RE, Singer MI, Leong SP, Ljung BM, Sagebiel RW and Kashani-Sabet M: The gene expression signatures of melanoma progression. Proc Natl Acad Sci USA *102*: 6092-6097, 2005.

6 Talantov D, Mazumder A, Yu JX, Briggs T, Jiang Y, Backus J, Atkins D and Wang Y: Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res *11*: 7234-7242, 2005.

7 Winnepenninckx V, Lazar V, Michiels S, Dessen P, Stas M, Alonso SR, Avril MF, Ortiz Romero PL, Robert T, Balacescu O, Eggermont AM, Lenoir G, Sarasin A, Tursz T, van den Oord JJ and Spatz A: Gene expression profiling of primary cutaneous melanoma and clinical outcome. J Natl Cancer Inst *98*: 472-482, 2006.

8 Gallagher WM, Bergin OE, Rafferty M, Kelly ZD, Nolan IM, Fox EJ, Culhane AC, McArdle L, Fraga MF, Hughes L, Currid CA, O'Mahony F, Byrne A, Murphy AA, Moss C, McDonnell S, Stallings RL, Plumb JA, Esteller M, Brown R, Dervan PA and Easty DJ: Multiple markers for melanoma progression regulated by DNA methylation: insights from transcriptomic studies. Carcinogenesis *26*: 1856-1867, 2005.

9 Clark EA, Golub TR, Lander ES and Hynes RO: Genomic analysis of metastasis reveals an essential role for RhoC. Nature *406*: 532-535, 2000.

10 Takeuchi H, Morton DL, Kuo C, Turner RR, Elashoff D, Elashoff R, Taback B, Fujimoto A and Hoon DS: Prognostic significance of molecular upstaging of paraffin-embedded sentinel lymph nodes in melanoma patients. J Clin Oncol *22*: 2671-2680, 2004.

11 Koyanagi K, O'Day SJ, Gonzalez R, Lewis K, Robinson WA, Amatruda TT, Kuo C, Wang HJ, Milford R, Morton DL and Hoon DS: Microphthalmia transcription factor as a molecular marker for circulating tumor cell detection in blood of melanoma patients. Clin Cancer Res *12*: 1137-1143, 2006.

12 Koyanagi K, O'Day SJ, Gonzalez R, Lewis K, Robinson WA, Amatruda TT, Wang HJ, Elashoff RM, Takeuchi H, Umetani N and Hoon DS: Serial monitoring of circulating melanoma cells during neoadjuvant biochemotherapy for stage III melanoma: outcome prediction in a multicenter trial. J Clin Oncol *23*: 8057-8064, 2005.

13 Schmidt H, Sorensen BS, Fode K, Nexo E and von der Maase H: Tyrosinase messenger RNA in peripheral blood is related to poor survival in patients with metastatic melanoma following interleukin-2-based immunotherapy. Melanoma Res *15*: 409-416, 2005.

14 Lewis TB, Robison JE, Bastien R, Milash B, Boucher K, Samlowski WE, Leachman SA, Dirk Noyes R, Wittwer CT, Perreard L and Bernard PS: Molecular classification of melanoma using real-time quantitative reverse transcriptase-polymerase chain reaction. Cancer *104*: 1678-1686, 2005.

15 Gianni L, Zambetti M, Clark K, Baker J, Cronin M, Wu J, Mariani G, Rodriguez J, Carcangiu M, Watson D, Valagussa P, Rouzier R, Symmans WF, Ross JS, Hortobagyi GN, Pusztai L and Shak S: Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. J Clin Oncol *23*: 7265-7277, 2005.

16 Eisen MB, Spellman PT, Brown PO and Botstein D: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA *95*: 14863-14868, 1998.

17 Weterman MA, Ajubi N, van Dinter IM, Degen WG, van Muijen GN, Ruitter DJ and Bloemers HP: *nmb*, a novel gene, is expressed in low-metastatic human melanoma cell lines and xenografts. Int J Cancer *60*: 73-81, 1995.

18 Lennerz V, Fatho M, Gentilini C, Frye RA, Lifke A, Ferel D, Wolfel C, Huber C and Wolfel T: The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci USA *102*: 16013-16018, 2005.

19 Okamoto I, Pirker C, Bilban M, Berger W, Losert D, Marosi C, Haas OA, Wolff K and Pehamberger H: Seven novel and stable translocations associated with oncogenic gene expression in malignant melanoma. Neoplasia *7*: 303-311, 2005.

20 Wang Z, Shen D, Parsons DW, Bardelli A, Sager J, Szabo S, Ptak J, Silliman N, Peters BA, van der Heijden MS, Parmigiani G, Yan H, Wang TL, Riggins G, Powell SM, Willson JK, Markowitz S, Kinzler KW, Vogelstein B and Velculescu VE: Mutational analysis of the tyrosine phosphatome in colorectal cancers. Science *304*: 1164-1166, 2004.

21 Lachner M, O'Carroll D, Rea S, Mechtler K and Jenuwein T: Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*: 116-120, 2001.

22 Furuta J, Umebayashi Y, Miyamoto K, Kikuchi K, Otsuka F, Sugimura T and Ushijima T: Promoter methylation profiling of 30 genes in human malignant melanoma. Cancer Sci *95*: 962-968, 2004.

23 Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M and Maio M: Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. J Immunother *25*: 16-26, 2002.

Received December 19, 2006
Revised March 19, 2007
Accepted March 21, 2007