

Microarray analysis of phosphatase gene expression in human melanoma

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Summary

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Accepted for publication

5 August 2004

Key words:

melanoma, protein tyrosine phosphatase, signal transduction

Conflicts of interest:

None declared.

Background Tyrosine phosphate is abnormally elevated in malignant melanoma, and this has been interpreted to reflect the activity of oncogenic protein tyrosine kinases. However, elevation may also arise due to decreased protein tyrosine phosphatase (PTP) expression.

Objectives To survey phosphatase gene expression in melanoma cell lines, a benign naevus and normal melanocytes: we searched for downregulation of phosphatase gene expression in malignant cells that may indicate a role as melanoma suppressor genes.

Methods Microarray analysis was used to compare gene expression for 133 phosphatase genes, comprising 39 PTPs, 16 dual-specificity phosphatases (DSPs), 47 serine/threonine phosphatases and 31 acid/alkaline and lipid-based phosphatases. Northern blotting analysis was used to study gene expression in human melanoma biopsies.

Results There was decreased expression of four DSP genes (including PTEN); eight receptor PTP genes were downregulated in melanoma, among which were PTP-KAPPA and PTP-PI (consistent with our previous data). In addition, PTP-RF/LAR was downregulated in 13 of 22 metastatic melanomas.

Conclusions The expression of multiple PTP receptors is decreased in melanoma; this may be a mechanism which stimulates autonomous growth in advanced melanoma.

Melanoma development involves an escape from the processes regulating growth and differentiation in normal melanocytes. These processes are controlled via multiple signal transduction pathways, and tyrosine phosphorylation is a key element within many of these.¹ Attention first focused upon the role of tyrosine phosphate in melanoma after early studies in animal models found that ectopic expression of receptor protein tyrosine kinase (PTK) oncogenes induced melanoma genesis. Overexpression of Xmrk [a receptor PTK closely related to epidermal growth factor receptor (EGFR)] resulted in a hereditary malignant melanoma in the fish *Xiphophorus*.² Moreover, expression of PTK receptors or their ligands in pigment cells in transgenic mice resulted in melanoma.³ Subsequent studies found elevated expression of PTK genes in human melanomas and the cell lines derived from them. Expression of the receptors EGFR and EPH-A2^{4,5} and the cytoplasmic kinase YES⁶ was elevated in human melanoma cell lines. As many PTKs are transforming oncogenes,

this suggested that tyrosine phosphorylation pathways may play a role in melanoma.⁷

In recent years a good understanding of some of the pathways underlying normal growth control has emerged, and disruption of the RAS-RAF-MEK-ERK pathway appears to be central to carcinogenesis.⁸ Components downstream of PTK receptor signalling include RAS and BRAF, and these are common targets for activating mutations in some human tumours. A high frequency of BRAF mutations has been reported in melanoma and naevi.^{9,10} In contrast to the situation in normal melanocytes, extracellular regulated kinase (ERK) is constitutively activated in most melanoma cell lines. Interestingly, ERK signalling in melanoma was downregulated by inhibitors to receptor (fibroblast growth factor and hepatocyte growth factor) PTK signalling pathways.⁸ Taken together, these data suggest that disruption of the PTK-RAS-RAF-MEK-ERK pathway is responsible for autocrine growth seen in melanoma genesis.

We recently found increased tyrosine phosphate in advanced melanoma: this first occurred at the vertical growth phase (VGP) of progression.¹¹ There are two ways in which tyrosine phosphate may be increased, i.e. via increased PTK or decreased protein tyrosine phosphatase (PTP) activities. In comparison with kinases, the role of PTPs in melanoma has received little attention to date. Previously, we utilized highly degenerate primers based upon the conserved sequences of PTP genes in polymerase chain reaction (PCR) to isolate PTP genes from melanoma and melanocytes. When the products were used as probes for Northern blotting analysis, we found loss of PTP-KAPPA and PTP-PI gene expression in melanoma.¹² It has been hypothesized, but remains unproven, that some PTPs are tumour suppressors.^{13,14} Hence, downregulation of PTPs may play a role in melanoma progression by activation of ERK signalling.

Here we further characterize PTP expression in melanoma. In order to obtain a more complete picture of phosphatase expression in melanoma, we developed cDNA microarrays comprising 133 phosphatase genes. The array was used to survey expression of phosphatase genes within melanoma cell lines and normal melanocytes. The expression of multiple PTP genes was decreased in melanoma; one of these, PTP-RF/LAR, was undetectable or downregulated in the majority of samples of metastatic melanoma tested. Where the expression of PTP genes is lost or decreased in melanoma, this may indicate a role in progression.

Materials and methods

Culture of melanoma cell lines

Melanocyte culture was as previously described.⁵ The melanoma cell lines used here, and their original sources, have been described previously.^{5,12} Cell line WM39 was derived from a VGP melanoma; WM9 and WM852 were derived from metastases. Conditions for culture were as reported previously.¹²

Sample preparation

Total RNA was prepared using Triazol reagent (Gibco, Paisley, U.K.), as previously described,⁵ from melanoma cell lines, melanocytes and a naevus. The naevus sample contained the melanocytic cells of interest together with contaminating epidermal and stromal components. Total RNA was used to make radiolabelled cDNA. Each RNA sample (5 µg) was treated with DNase I to remove the contaminating DNA.¹¹ Then cDNA was synthesized, by reverse transcription of RNA, using 300 U of Superscript II reverse transcriptase, along with 1 µL of 10 × decamers (Ambion, Austin, TX, U.S.A.), 1 mmol L⁻¹ deoxyadenosine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate (Amersham Pharmacia, Piscataway, NJ, U.S.A.), together with radiolabelled ³³P-deoxycytidine triphosphate

and 3.5 mmol L⁻¹ dithiothreitol, at 41 °C for 90 min. The unincorporated radiolabelled nucleotide was separated from the labelled cDNA using Sephadex G-50 spin columns and denatured at 95 °C before hybridizing to the filter arrays.¹⁵ The specific activity of radiolabelled cDNA was typically > 5 × 10⁶ counts per reaction.

Microarray analysis

Array generation and sample analysis

PCR products (0.6–2.4 kb) were generated from a clone collection [Research Genetics sequence tags or expressed sequence tags (ESTs)] from multiple human tissue cDNA libraries using vector-specific primers. All available phosphatases were selected from a list of 40 000 clones available. Purified PCR products (100–150 µg mL⁻¹) were deposited on to a matrix (1.5 × 1.5 cm) using a robot. Arrays were produced using a Genetics Microsystems GM417 arrayer and a Robotics TAS arrayer system and were purchased from Wistar Institute Genomics Core Facility (Wistar Institute, Philadelphia, PA, U.S.A.). The filters also contained nine housekeeping genes and negative internal controls. DNA was cross-linked to the matrix by ultraviolet irradiation, and rendered single-stranded by alkali prior to sample analysis. Probes (³³P-labelled cDNA) were hybridized with filters in Church buffer [0.25 mol L⁻¹ sodium phosphate buffer pH 7.2, 1 mmol L⁻¹ ethylenediamine tetraacetic acid, 1% bovine serum albumin, 7% sodium dodecyl sulphate (SDS)]¹⁵ at 65 °C overnight. Following hybridization, arrays were washed in 1 × saline sodium citrate/0.1% SDS at 65 °C for 10 min until there was no background count. Filters were then exposed to a phosphoimager for 3–7 days.

Array quantitation and data processing

The phosphatase array images were scanned on a Storm 820 phosphoimager and Array Vision software (Imaging Research, St Catharines, Ontario, Canada) was used for analysis. A global normalization was carried out to compensate for differences in probe concentration or specific activity when two arrays were compared. The data were analysed using the Cluster and Tree-view programs (from the Stanford Microarray Analysis Suite; Stanford, CA, U.S.A.); tables of the normalized data were extracted for the different families of enzymes and a hierarchical clustering algorithm was applied. This clustering program permitted two-way analysis of genes and cell lines and either gene or cell line clustering.

Northern blotting analysis

Total RNA was prepared from uncultured melanoma metastases as previously described,¹² and 7.5 µg was electrophoresed through 1% agarose-formaldehyde gels and transferred to nitrocellulose membranes. cDNA probes were labelled by random hexanucleotide priming, and prehybridization and

hybridization were as described.⁵ Integrity and loading of RNA were determined by probing for 18S.¹²

Results

Microarray analysis

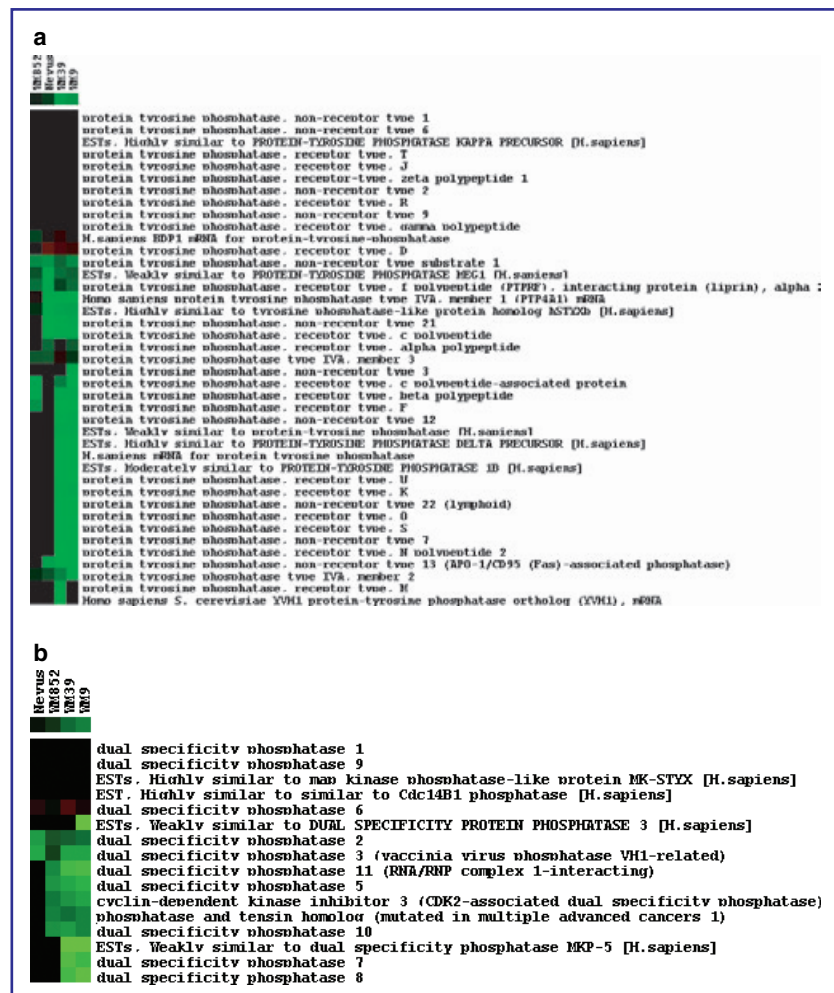
We have used a cDNA microarray to survey gene expression within benign and malignant melanocytes. Our aim was to expand the current knowledge of PTP expression in pigment cells. Three cell lines established from advanced-stage melanoma were compared with a compound naevus and normal melanocytes. Approximately 133 phosphatase genes were assayed, including known genes and ESTs. Four phosphatase gene families were studied: in addition to PTPs, serine/threonine phosphatases (S/TPs), dual-specificity phosphatases (DSPs) and acid/alkaline and lipid-based phosphatases were included.

A simple cluster analysis was used to identify prominent features in the gene expression patterns (Fig. 1). Differences were considered significant where the change in gene expression was fivefold or greater in two of the three melanoma cell lines

compared with naevus and normal melanocytes (Table 1). Fewest differences in total phosphatase gene expression were seen for the naevus (9%), whereas the melanoma cell lines displayed an average of 32% differences. A common trend was that expression of phosphatase genes was decreased in melanoma cells compared with their normal cellular counterparts. There was decreased expression of four DSP genes (including PTEN). In addition, DSP10 (MAP kinase phosphatase 5) was downregulated by threefold in all three of the melanoma cell lines, and DSP5 and DSP11 were downregulated in some melanoma cell lines.

Within the S/TP family, protein phosphatase 1 displayed decreased expression in some melanoma cell lines. A large proportion of S/TP genes was expressed at equal levels to those within the normal sample; the close relationship between the naevus and normal melanocytes was striking. Within the acid/alkaline and lipid-based phosphatase genes, a small number of lipid-based phosphatases showed a significant decrease in two or more cell lines: these included inositol phosphate-1 phosphatase and inositol polyphosphate-4 phosphatase.

Fig 1. A coloured representation of the data table for phosphatases, with the rows (genes) and columns (cell lines) in cluster order. The colour in each cell of this table represents the mean-adjusted expression level of this gene and cell line. (a) Protein tyrosine phosphatases (PTPs). The colour scale used to represent the expression ratios is shown. PTP-KAPPA, PTP-PI and PTP-RF/LAR are decreased in melanoma compared with normal melanocyte and naevus RNA. (b) Dual-specificity phosphatases, (c) serine/threonine phosphatases and acid/alkaline and lipid-based phosphatases.



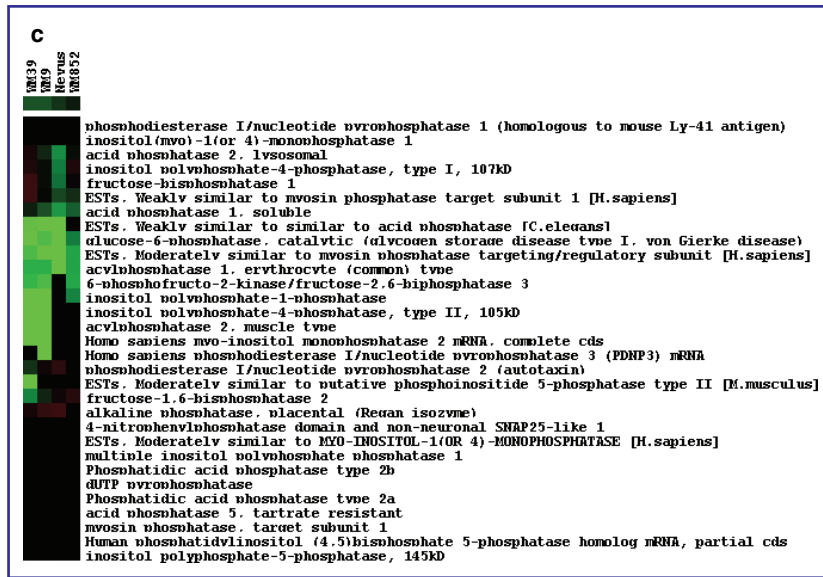


Fig 1(c). Continued.

Table 1 Microarray analysis: changes in phosphatase gene expression

Changes in gene expression ^a	PTPs	DSPs	S/TPs ^b	Acid/alkaline phosphatases
No change	31	12	40	26
Increase	0	0	0	0
Decrease	8	4	7	5
Total	39	16	47	31

PTPs, protein tyrosine phosphatases; DSPs, dual-specificity phosphatases; S/TPs, serine/threonine phosphatases. ^aChanges in gene expression were regarded as significant when the change was fivefold or greater in two of the three melanoma cell lines compared with normal melanocytes and the naevus sample. ^bAll gene spots were counted, including different isoforms.

A total of 39 distinct PTP genes or PTP-related sequences was tested (Fig. 1); these included known genes and ESTs. The most significant finding from the clustered data was the loss or downregulation of PTP gene expression in two of the three melanoma cell lines compared with RNA from normal melanocytes. Two cell lines, WM39 and WM9, clustered together and displayed the most significant losses in PTP expression. In comparison, the naevus was closely similar to melanocytes and differences in expression tended to cluster in a different part of the data table compared with the melanoma cell lines. The naevus RNA was isolated from an uncultured biopsy and some differences may reflect the presence of a component derived from stromal cells in this sample. PTP genes downregulated in two of the three melanoma cell lines included receptors: PTP-KAPPA, PTP-PI, PTP-RU, PTP-RO, PTP-RF/LAR and PTP-RS and PTP-BETA/ZETA. Although equally expressed in melanocyte and naevus RNA, PTP-BETA/ZETA and PTP-RF/LAR were downregulated in all three melanoma cell lines tested.

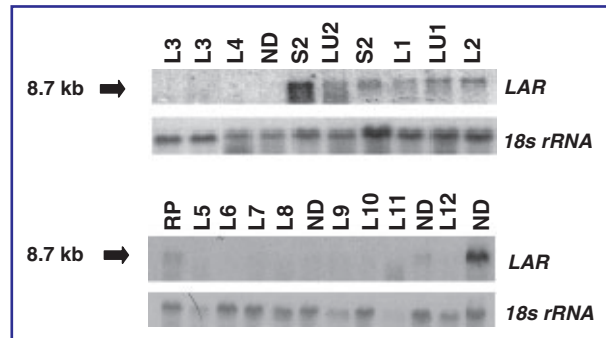


Fig 2. Northern blot analysis of total RNA from human metastatic melanoma biopsies. Each sample was derived from one individual; we did not include multiple metastases from a single patient. Samples were derived from lymph node (L), lung (LU), subcutaneous (S) and retroperitoneal (RP) metastases; for some samples the site of metastasis was not determined (ND). Hybridization was to a protein tyrosine phosphatase-RF/LAR probe,¹² and an 18S rRNA-specific oligonucleotide was used for quantitation. Durations of exposure were 12 h and 1 day, respectively. The data for 18S rRNA (control) in (a) have been shown previously.¹²

Northern blotting analysis

Northern blotting was used to analyse a small subset of PTP genes further (Fig. 2). Analysis of uncultured melanoma metastases revealed loss of PTP-RF/LAR message in 13 of 22 cases. There did not appear to be a relation between PTP-RF/LAR expression and histopathology of the primary melanoma or clinical data (survival time) of the patient.

Discussion

Since the initial early reports of melanoma in experimental animals engineered to overexpress PTKs, it has been antici-

ted that phosphotyrosine signalling pathways would play a critical role in melanoma.² This hypothesis has recently been strengthened by the finding that BRAF is activated in most human melanomas.⁹ Almost all cell lines from advanced melanomas contain constitutively active ERK, whereas in normal melanocytes and cells from some early melanomas ERK is not activated.⁸ Taken together, the data suggest that melanoma growth is mediated via activated BRAF and autocrine growth factor signalling pathways. We have used microarray analysis in order to discover differences in phosphatase expression between normal and malignant melanocytic cells. Here we briefly discuss findings for DSPs and consider PTP gene expression more fully. The expression of four DSP genes was decreased in melanoma cell lines. One of these, PTEN, a tumour suppressor gene, maps to chromosome 10q23.3, and encodes a DSP with lipid and protein phosphatase activity.¹⁶ Decreased expression of PTEN in melanoma has been described previously,¹⁶ and our results support the hypothesis that loss of PTEN may be functionally significant in melanoma. There are no previous reports of decreased DSP10 (MAP kinase phosphatase 5) in melanoma. MAP kinase phosphatases have been shown to reverse the activation of ERK by hydrolysing phosphotyrosine and phosphoserine residues present in their substrates.¹⁷ Hence, loss of DSP10 may contribute to increased signalling via the MAP kinase pathway. Interestingly, DSP10 maps to 10q25, placing it in the same region as PTEN, which is commonly lost by deletion.¹⁷

Eight receptor PTP genes were downregulated in two of the three melanoma cell lines tested. These differences may reflect an epigenetic mechanism. The genes included PTP-KAPPA and PTP-PI previously found to be decreased/absent in most melanoma cell lines and melanoma samples.¹² PTP-RF/LAR was downregulated in all three melanoma cell lines in the microarray analysis; in addition, there was no detectable expression of PTP-RF/LAR in 13 of 22 melanoma samples. There are no previous reports of decreased PTP-RF/LAR expression in melanoma; however, PTP-RF/LAR seems to be a good candidate for a melanoma suppressor gene, as decreased PTP-RF/LAR activity prevents apoptosis in neurones, and there is altered expression of PTP-RF/LAR in an experimental rat model exhibiting phaeochromocytoma.¹⁸ According to microarray analysis, only PTP-DELTA was increased in expression, and this is consistent with a previous report of lack of expression in normal melanocytes.¹⁹

Previous studies have considered ERK signalling in melanoma.⁸ Of the cell lines used in this study, WM39 responds to fetal calf serum with an increase in RAS and ERK activity; however, for WM852 and most advanced melanomas this is not seen. This difference may reflect alternative mechanisms for activation of phosphotyrosine pathways between various melanoma cell lines. It seems likely that melanoma genesis involves a disruption of several components of tyrosine phosphate signalling pathways. The extent to which autocrine growth factors, receptor PTKs and mutations in BRAF and RAS influence growth, invasion and metastasis is uncertain. BRAF appears to play an important role early in melanoma gen-

esis.^{9,10} However, a recent study found that BRAF is not required for tumorigenicity in RAS-transformed melanocytes; hence, there appears to be redundancy within this signalling pathway.²⁰ Our data suggest that loss of PTP activity may play a role in melanoma progression.

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