

Membrane Transport Proteins in Human Melanoma: associations with tumour aggressiveness and metastasis

Running title: MRP-1 and MDR1/P-gp in melanoma

Authors: Walsh N^{1*}, Kennedy S^{2*}, Larkin AM¹, Tryfonopoulos D³, Eustace AJ¹, Mahgoub T³, Conway C⁴, Oglesby I¹, Collins D¹, Ballot J², Ooi WS², Gullo G², Clynes M¹, Crown J^{3^}, O'Driscoll L^{5^}

* indicates contributed equally; ^ indicates contributed equally

Affiliation: ¹National Institute for Cellular Biotechnology, Dublin City University, Dublin 9; ²St. Vincent's University Hospital & Molecular Therapeutics for Cancer Ireland (MTCI), Dublin 4, Ireland; ³MTCI, c/o National Institute for Cellular Biotechnology Building, Dublin City University, Dublin 9; ⁴SlidePath, Santry, Dublin 9; ⁵School of Pharmacy and Pharmaceutical Sciences & MTCI, Trinity College Dublin, Dublin 2, Ireland.

Corresponding author: Dr. Lorraine O'Driscoll, School of Pharmacy and Pharmaceutical Sciences & MTCI, Trinity College Dublin, Dublin 2, Ireland. E-mail.: lodrisc@tcd.ie

ABSTRACT

Background: Malignant melanoma, generally described as incurable, is notoriously refractory to chemotherapy. The mechanisms contributing to this have not yet been defined and the contributions of drug efflux pumps, implicated in chemo-resistance of many other cancer types, have not been extensively investigated in melanoma.

Methods: Here, expression of MDR1/P-gp and MRP-1 proteins was examined, by immunohistochemistry, in archival specimens from 134 melanoma patients. This included 92 primary tumours and 42 metastases. **Results:** Assessing all specimens, MRP-1 and MDR1/P-gp expression was found to be common, with the majority (81%) of melanomas expressing at least one of these efflux pumps. While there is significant association between expression of these pumps ($P=0.007$), MRP-1 was found to be the predominant (67% of cases) form detected. X^2 analysis showed significant associations between expression of MRP-1 and/or MDR1/P-gp and the aggressive nature of this disease; specifically increased Breslow's depth, Clark's level and spread to lymph nodes. This association with aggressiveness and spread is further supported by the observation that a significantly higher percentage of metastases, than primary tumours, express MRP-1 (91% versus 57%; $P<0.0001$) and MDR1/P-gp (74% versus 50%; $P=0.010$). **Conclusion:** The predominant expression of these pumps and, in particular, MRP-1- suggests that they may be important contributors to the inherent aggressive and resistant nature of malignant melanoma.

Keywords: malignant melanoma, multiple drug resistance, MDR1/P-gp, MRP-1, immunohistochemistry.

INTRODUCTION

The incidence rate for cutaneous melanoma, which involves the malignant proliferation of melanocytes, is increasing faster than that of any other malignant disease, at an epidemic rate of 3% a year (Leiter and Garbe, 2008; Garbe and Leiter, 2009). Over the last century, melanoma has progressed from being considered as a rare cancer to a situation where the lifetime risk of developing this disease is 1 in 50 in many Western populations (Giblin and Thomas, 2007); with major socioeconomic consequences as a result of the high mortality rates associated with this metastatic disease (Meyle and Guldborg, 2009). Currently, approximately 133,000 new cases of melanoma are diagnosed worldwide each year. The NCI's Surveillance Epidemiology and End Results estimates that in the US, in 2009 alone, 68,720 new cases of melanoma of the skin will be diagnosed and 8,650 people will die from this disease.

Prognosis for patients with early-stage melanoma is very good and can often be cured by wide surgical excision. Metastatic disease, unfortunately, remains generally incurable and is largely resistant to current therapies, with a median survival time of 6 to 9 months from diagnosis and a 5-year survival rate of 5.5% (Keller *et al*, 2006; Sarnaik and Weber, 2009). Biological therapies including IFN-alpha have demonstrated a real, but minimal, effect in malignant melanomas (Pavlick *et al*, 2003) and the effects of IL-2, as a treatment for malignant melanoma, are not yet clearly defined. High-dose bolus IL-2 produces some long-term remissions and is available for highly selected individuals, but is not suitable for most patients (Mohr *et al*, 2003; Agarwala, 2009). Chemotherapeutic therapy for melanoma has been attempted with the many chemotherapeutic drugs. Single agent chemotherapy, including dacarbazine, temozolomide, the nitrosureas (BCNU, CCNU and methyl CCNU), the vinca

alkaloids (vincristine, vinblastine and vindesine) cisplatin, paclitaxel and bleomycin have been used. Many of these are relatively well-tolerated (including dacarbazine, which now has FDA approval for this indication), but are associated with response rates of only 5-20% (Bhatia *et al*, 2009; Seetharamu *et al*, 2009). Multi-drug chemotherapy regimes have resulted in objective response rates, but do not extend survival and are associated with greater toxicity (Pawlik and Sondak, 2003; Nystrom *et al*, 2003; Tas *et al*, 2003; Bhatia *et al*, 2009). The limited response of malignant melanoma to chemotherapy is apparently due to the inherent drug refractory nature of this disease (Ichihashi and Kitajima, 2001; Colone *et al*, 2008). This has resulted in targeted therapies being fast-tracked through FDA approval *e.g.* Ipilimumab, which targets CTL4, and which is approved for metastatic and advanced melanoma. Such monoclonal antibodies are greatly more expensive than traditional chemotherapy and so an economic burden on many health systems. Unfortunately, they too may become of limited use due to the resistance nature of melanoma.

There is some, *albeit* limited, evidence that expression of genes encoding membrane efflux pumps (ABC transport proteins), including MRP-1 (ABCC1) and MDR1/P-gp (ABCB1), may be associated with this chemo-resistance and so incurable nature of malignant melanomas. However, conflicting data exists on the details of the specific pump(s) involved. In a study of MRP-1 mRNA and protein expression in 40 melanoma cell lines, Berger *et al* (1997) reported sensitivity to a broad range of chemotherapeutic drugs to inversely correlate with MRP-1 expression. Furthermore, analysis of *mrp-1* and *mdr-1* mRNA expression in 18 specimens from 8 melanoma patients' tumours showed *mrp-1* expression in the majority of pre-chemotherapy specimens; the levels of which were increased post-chemotherapy. *mdr-1* mRNA was,

however, not detected in any of the specimens analysed (Ichihashi and Kitajima, 2001). In keeping with this, based on studies of 21 primary malignant melanomas and 37 metastases, Schadendorf *et al* (1995) reported 43% of both primary and metastatic tumours to express MRP-1, with a lack of detectable expression of MDR1/P-gp (except in 1 primary tumour) suggesting that the chemo-resistant nature of melanoma cells is unlikely to be mediated *via* MDR1/P-gp. Conversely, results from studies of human melanoma cultured cells (M14) suggested MDR1/P-gp, but not MRP-1, to be associated with the multi-drug resistant (MDR) phenotype (Molinari *et al*, 2000). In further studies of M14 cell line variants (specifically, MDR1/P-gp-positive drug-resistant M14 ADR compared to MDR1/P-gp-negative drug sensitive M14 cells), members of the same research group have more recently reported MDR1/P-gp to be linked to MAPK signalling and involved in melanoma cell migration and invasion (Colone *et al*, 2008).

As outlined above, while studies of melanoma cell line models support an association between MDR1/P-gp and/or MRP-1 and the chemo-resistant, generally incurable, phenotype of malignant melanoma, studies of the prevalence of MDR1/P-gp and MRP-1 in melanoma specimens have been very limited and conflicting. To determine the potential involvement of these drug efflux pumps in the clinical setting, here we report results from a retrospective study of both MDR1/P-gp and MRP-1 protein expression and their associations with patients' clinicopathological features (where possible) in 134 melanomas, including 92 primary tumours and 42 metastases.

MATERIALS AND METHODS

Patients

The patient group studied comprised of 134 consenting patients with malignant melanoma. All patients were diagnosed at St. Vincent's University Hospital (SVUH), Dublin, between 1975 and 2002 and approval to conduct this study was granted by SVUH Ethics Committee. Formalin-fixed paraffin embedded material was available for all patients. Representative 4µm sections of tissue blocks were cut using a microtome, mounted onto poly-l-lysine coated slides and dried overnight at 37°C. Slides were stored at room temperature until required.

Immunohistochemistry

All steps were performed at room temperature unless otherwise indicated. Tissue sections were dewaxed in xylene (2 x 5 min.), rehydrated in grading alcohols 100%, 90% and 70% (2 x 3 min. each), and placed in Tris Buffered Saline (TBS/0.05% Tween-20). Endogenous peroxidase activity was quenched by placing tissue sections in 3% (v/v) H₂O₂/distilled water for 5-6 min. All slides were blocked for non-specific staining with 20% normal rabbit serum (Dako, X-902)/TBS for 20 min. Primary antibodies were applied to each specimen. Specifically, anti-MDR1/P-gp clone 6/1C ascites (which we developed and previously extensively characterised as specific for MDR1/P-gp; Moran *et al*, 1997) was diluted 1:40 in TBS/0.05% Tween-20. Anti-MRP-1 PA28(6) antibody (similarly, which we developed and extensively characterised as specific for MRP-1 (Connolly *et al*, 2001) was applied as neat supernatant. Primary antibodies were incubated overnight at 4⁰C. Samples were then washed (3 x 5 min.) with TBS/0.05% Tween-20, followed by 30 min. incubation with

biotinylated secondary antibody diluted in TBS/0.05% Tween-20. Finally, following another 3 x 5 min. wash step, Vector SG substrate for peroxidase (Vector Laboratories, SK-4700) was applied for approx. 10 min. All slides were washed (3 x 5 min.) in TBS/0.05% Tween-20. Tissue sections were then lightly counter-stained with Nuclear Fast Red (Vector Laboratories, H-3403).

Following this, slides were dehydrated in grading alcohols 70%, 90% and 100% (2 x 3 min.). Samples were then cleared in xylene and mounted in DPX (BDH, UK). Positive control normal kidney and lung tissue sections (using same experimental conditions) and negative control samples (in which primary antibody were replaced by 1x TBS/0.05% Tween-20) were included in all experiments.

Immunohistochemical scoring

MRP-1 and MDR1/P-gp immunohistochemical staining was scored (by Consultant Pathologist, Prof. Susan Kennedy) according to the percentage of cells showing specific immunoreactivity. A semi-quantitative measurement was used in which overall positivity of the tumour was assessed and a score of 1+ was given where up to 25% of cells showed MRP-1 or MDR1/P-gp positive staining, as relevant; a score of 2+ was given where ≥ 25 -<50% cells showed positive staining; a score of 3+, where ≥ 50 %-<75% of cells showed positive staining and a score of 4+, where ≥ 75 % of cells showed positive staining. Scores of ≤ 1 were considered as negative; scores above 1 were considered positive.

Statistical analysis

Statistical analyses of the results were performed using SPSS 16.0 and InStat software packages. Descriptive statistics were used to summarise patient characteristics and statistical analysis of the results was performed using Pearson's X^2 test to investigate relationships between MDR1/P-gp and MRP-1 expression and clinicopathological and histopathological findings. Kaplan-Meier survival curves were established and were subsequently checked using the log-rank, Breslow and Tarone-ware tests (p -values represent log-rank, unless otherwise indicated) to assess the prognostic significance of MDR1/P-gp and MRP-1 protein expression. The data was analysed as a whole and as primary and metastases cohorts, respectively, and, where relevant, the data was also censored at 5 years. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics

This study involved analysis of tumours from 134 patients with metastatic melanoma. Eighty-three of the patients were female; 51 were male. The patients ages ranged between 22 years and 104 years at the time of diagnosis (median age = 59 years). Ninety-two of the specimens available for analysis were primary tumours; 42 were secondary metastases. Further clinical characteristics of the patients are summarised in Table 1.

MDR1/P-gp Expression

MDR1/P-gp specific staining was observed in 57.5% (77/134) of the melanoma specimens analysed (a representative MDR1/P-gp positive tumour is shown in Figure 1(A)). Specific staining was localised to cell membrane/cytoplasm. Approximately 43% of tumours did not express MDR1/P-gp protein. MDR1/P-gp expression in primary and metastatic tumours, when analysed as separate cohorts, was 50% and 73.8%, respectively.

MRP-1 Expression

MRP-1 specific staining was observed in 67.2% (90/134) of tumours analysed (Figure 1(B) illustrates a typical example). 33% of all tumours did not express MRP-1 protein. Independently assessing the primary tumours and metastases indicated MRP-1 to be detected in 56.5% and 90.5% of cases, respectively.

MDR1/P-gp and MRP-1 co-Expression

The expression of MDR1/P-gp and MRP-1 proteins were significantly associated ($P=0.007$). The majority (80.6%) of melanomas expressed at least one of the efflux pumps analysed; 44% expressed both, while only 19.4% expressed neither MDR1/P-gp nor MRP-1 proteins (see Table 2(A)). For the primary tumour cohort alone, as summarised in Table 2(B), approximately 73% of cases expressed at least one of these pumps, 27% had neither MDR1/P-gp nor MRP-1, while 34% expressed both proteins. Table 2(C) indicates that the majority (97.6%) of the metastatic specimens expressed MDR1/P-gp and/or MRP-1; 67% had both. Almost a quarter (23.8%) of metastatic tumours had MRP-1, but lacked MDR1/P-gp expression, while only 7% expressed MDR1/P-gp alone.

Overall, as shown in Table 3, a direct comparison of MDR1/P-gp and MRP-1 efflux pump expression in the primary *versus* the metastatic specimens indicated a significantly higher percentage of metastatic tumours, compared to primary tumours, expressed MDR1/P-gp (primary 50%; metastases 74%; $P=0.010$) and MRP-1 (primary 57%; metastases 91%; $P<0.0001$); with MRP-1 being the most prevalent efflux pump in these specimens.

MDR1/P-gp and MRP-1 Expression in relation to clinicopathological features

Using χ^2 analysis of the 134 population as a whole (summarised in Table 4), MDR1/P-gp expression was found to be significantly ($P=0.013$) associated with lymph node positivity and tended to be associated with increased Breslow's depth; however, in the latter case, statistical significance was approached ($P=0.052$). MRP-1 expression was also significantly associated with spread to lymph nodes ($P<0.0005$) and with more extensive invasion of the melanoma *i.e.* when comparing MRP-1 expression in

specimens with Clark's level II, III and IV *versus* V; P=0.032 (incidentally, comparison of MRP-1 expression in those specimens with Clark's levels II and III compared to IV and V also supported the association between MRP-1 expression and increasing stages of disease (although NS; P=0.051).

Assessing expression of these efflux pumps in the cohort of primary tumours alone (Table 5) showed similar trends to those seen when evaluating all 134 cases *i.e.* MDR1/P-gp expression being associated with spread to lymph nodes (P=0.049) and increasing Breslow's depth (although NS; P=0.056); with MRP-1 expression significantly associated with increasing Clark's level (P=0.023).

In relation to the metastases cohort alone, as the majority of these specimens expressed MRP-1 (~91%) and MDR1/P-gp (~74%), seeking association with clinicopathological factors is not relevant here.

DISCUSSION

Malignant melanoma is one of the most challenging cancers to treat, resulting in highest per-death loss of years of potential life expectancy; with the exception of adult leukaemia (Seetharamu *et al*, 2009). Unfortunately the effectiveness of chemotherapeutic regimes evaluated in this cancer type is compromised and very limited due to the yet undefined highly resistant nature of this cancer.

Drug efflux pumps, MDR1/P-gp and MRP-1, have been associated with chemo-resistance in a number of cancer types. Studies of drug efflux pumps in melanoma have, however, been very limited to date. As outlined previously, studies of melanoma cell line models have resulted in conflicting results; some suggesting a role for MRP-1 (Berger *et al*, 1997), while others propose MDR1/P-gp to be potentially of more relevance (Molinari *et al*, 2000; Colone *et al*, 2008). Both earlier studies of melanoma tissue specimens (from 8 and 58 patients, evaluating mRNAs and proteins, respectively) reported MRP-1, but generally not MDR P-gp, expression (Ichihashi and Kitajima, 2001; Schadendorf *et al*, 1995). While those studies were important in highlighting the potential relevance of a drug efflux pump (namely, MRP-1) in melanoma, the numbers of specimens included were very limited. Furthermore, associations with patients' clinical characteristics were not investigated.

Here, in this more extensive study of 134 melanoma specimens, in agreement with the studies by Schadendorf *et al* (1995), we report MRP-1 protein to typically be expressed in melanoma. However, while Schadendorf *et al* (1995) reported the prevalence of expression to be the same in primary tumours and metastases, we observed that significantly higher percentages of metastatic specimens, compared to

primary tumours, express MRP-1. Furthermore, in contrast to the work reported by Schadendorf *et al* (1995) where MDR1/P-gp was detected in only 1 of 21 primary specimens and was absent from metastases, we detected MDR1/P-gp in more than half of the specimens analysed. As for MRP-1, expression of MDR1/P-gp was significantly more frequent in metastases compared to primary tumours. The discrepancies between our work and that reported by Schadendorf *et al* (1995) may, at least partly, be related to the larger cohort of specimens to which we had access for our analysis. We propose that such large studies could potentially contribute to more clinically relevant results.

This, we believe, is the first study evaluating potential associations between MDR1/P-gp and/or MRP-1 expression and melanoma patients' clinicopathological characteristics. While we found no significant association between the presence of either efflux pump and the age or gender of the patients, we identified that the expression of these pumps tended to be associated with the more aggressive nature of melanoma. Specifically, MRP-1 expression was significantly associated with tumours classified as having a Clark's level of V (*i.e.* invading to the subcutaneous fat, instead of being limited to the epidermal or dermal layers of the skin). Although statistical significance was not reached, MDR1/P-gp expression tended to be more strongly associated with tumours having a staging Breslow's thickness of >4 mm; which, according to the AJCC guidelines, is prognostic of a 5 yrs. survival of approximately 50% (compared to 95-100% for <1 mm; 80-95% for 1-2 mm; and 60-80% for 2.1-4 mm). Furthermore, we established that both MRP-1 and MDR1/P-gp expression are significantly associated with spread of cancer to the lymph nodes. The observed associations between expression of these efflux pumps and the more aggressive

phenotype of melanoma, together with the significantly higher prevalence of these pumps (MRP-1, in particular) in metastases compared to primary specimens, suggests that MRP-1 and MDR1/P-gp may contribute, at least to some extent, to the incurable nature of malignant melanoma.

In conclusion, this study indicates that expression of drug efflux pumps, MRP-1 and MDR1/P-gp, is common in melanoma, especially in metastases, and so potentially could contribute to the chemo-resistant biology and thus incurable nature of this cancer type. The expression of these proteins – and, in particular, the predominant expression of MRP-1 – suggests that these pumps may be important contributors to this chemo-resistance. These findings suggest that the inhibition of multiple drug efflux pumps might be necessary if clinically relevant MDR reversal is to be achieved in malignant melanoma.

Acknowledgements

This work was supported by funding from Ireland's Higher Educational Authority Programme for Research in Third Level Institutions (PRTL); Ireland's Health Research Board; Trinity College Dublin's Start-Up Funds for New Academics 2008/2009; and Science Foundation Ireland's funding of Molecular Therapeutics for Cancer, Ireland (08/SRC/B1410).

REFERENCES

Agarwala SS (2009) Current systemic therapy for metastatic melanoma. *Expert Rev Anticancer Ther* **9**: 587 – 595

Berger W, Hauptmann E, Elbling L, Vetterlein M, Kokoschka EM, Micksche M (1997) Possible role of the multidrug resistance-associated protein (MRP) in chemoresistance of human melanoma cells. *Int J Cancer* **71**: 108 – 115

Bhatia S, Tykodi SS, Thompson JA (2009) Treatment of metastatic melanoma: an overview. *Oncology (Williston Park)* **23**: 488 – 496

Colone M, Calcabrini A, Toccaceli L, Bozzuto G, Stringaro A, Gentile M, Cianfriglia M, Ciervo A, Caraglia M, Budillon A, Meo G, Arancia G, Molinari A (2008) The multidrug transporter P-glycoprotein: a mediator of melanoma invasion? *J Invest Dermatol* **128**: 957 – 971

Connolly L, Moran E, Larkin A, Scheffer G, Scheper R, Sarkadi B, Kool M, Clynes M (2001) A new monoclonal antibody, P2A8(6), that specifically recognizes a novel epitope on the multidrug resistance-associated protein 1 (MRP1), but not on MRP2 nor MRP3. *Hybrid Hybridomics* **20**: 333 - 341

Gerbe C, Leiter U (2009) Melanoma epidemiology and trends. *Clin Dermatol* **27**: 3 - 9

Giblin AV, Thomas JM (2007) Incidence, mortality and survival in cutaneous melanoma. *J Plast Reconstr Aesthet Surg* **60**: 32 – 40

Ichihashi N, Kitajima Y (2001) Chemotherapy induces or increases expression of multidrug resistance-associated protein in malignant melanoma cells. *Br J Dermatol* **144**: 745 – 750

Keller G, Schally AV, Nagy A, Baker B, Halmos G, Engel JB (2006) Effective therapy of experimental human malignant melanomas with a targeted cytotoxic somatostatin analogue without induction of multi-drug resistance proteins. *Int J Oncol* **28**: 1507 – 1513

Leiter U, Gerbe C (2008) Epidemiology of melanoma and nonmelanoma skin cancer--the role of sunlight. *Adv Exp Med Biol* **624**: 89 – 103

Meyle KD, Guldberg P (2009) Genetic risk factors for melanoma. *Hum Genet* **126**: 499 – 510

Mohr P, Weichenthal M, Hauschild A (2003) Adjuvant therapy in melanoma. *Onkologie* **26**: 227 – 233

Molinari A, Toccaceli L, Calcabrini A, Diociaiuti M, Cianfriglia M, Arancia G (2000) Induction of P-glycoprotein expression on the plasma membrane of human melanoma cells. *Anticancer Res* **20**: 2691 – 2696

Moran E, Larkin A, Doherty G, Kelehan P, Kennedy S, Clynes M (1997) A new mdr-1 encoded P-170 specific monoclonal antibody: (6/1C) on paraffin wax embedded tissue without pretreatment of sections. *J Clin Pathol* **50**: 465 – 4671

Nystrom ML, Steele JP, Shamash J, Neville F, Oliver RT (2003) Low-dose continuous chemotherapy for metastatic melanoma: a phase II trial. *Melanoma Res* **13**: 197 – 199

Pavlick AC, Adams S, Fink MA, Bailes A (2003) Novel therapeutic agents under investigation for malignant melanoma. *Expert Opin Investig Drugs* **12**: 1545 – 1558

Pawlik TM, Sondak VK (2003) Malignant melanoma: current state of primary and adjuvant treatment. *Crit Rev Oncol Hematol* **45**: 245 – 264

Sarnaik AA, Weber JS (2009) Recent advances using anti-CTLA-4 for the treatment of melanoma. *Cancer J* **15**: 169 – 173

Schadendorf D, Makki A, Stahr C, van Dyck A, Wanner R, Scheffer GL, Flens MJ, Scheper R, Henz BM (1995) Membrane transport proteins associated with drug resistance expressed in human melanoma. *Am J Pathol* **147**: 1545 – 1552

Seetharamu N, Ott PA, Pavlick AC (2009) Novel therapeutics for melanoma. *Expert Rev Anticancer Ther* **9**: 839 – 849

Tas F, Camlica H, Kurul S, Aydiner A, Topuz E (2003) Combination chemotherapy with docetaxel and irinotecan in metastatic malignant melanoma. *Clin Oncol (R Coll Radiol)* **15**: 132 – 135