Proteomics analysis of melanoma metastases: association between S100A13 expression and chemotherapy resistance

A Azimi1,3, M Pernemalm2,3, M Frostvik Stolt1, J Hansson1, J Lehtio2, S Egyházi Brage1 and C Hertzman Johansson*,1

1Department of Oncology-Pathology, Karolinska Institutet, CCK R8:03, Karolinska University Hospital, Solna, S-17176 Stockholm, Sweden and 2Department of Oncology-Pathology, Karolinska Institutet, Science for Life Laboratory, Tomtebodavägen 23, S-17165 Solna, Sweden

Background: Disseminated cutaneous malignant melanoma (CMM) is commonly unresponsive to standard chemotherapies, and there are as yet no predictive markers of therapy response.

Methods: In the present study we collected fresh-frozen pretreatment lymph-node metastasis samples (n=14) from melanoma patients with differential response to dacarbazine (DTIC) or temozolomide (TMZ) chemotherapy, to identify proteins with an impact on treatment response. We performed quantitative protein profiling using tandem mass spectrometry and compared the proteome differences between responders (R) and non-responders (NR), matched for age, gender and histopathological type of CMM.

Results: Biological pathway analyses showed several signalling pathways differing between R vs NR, including Rho signalling. Gene expression profiling data was available for a subset of the samples, and the results were compared with the proteomics data. Four proteins with differential expression between R and NR were selected for technical validation by immunoblotting (ISYNA1, F13A1, CSTB and S100A13), and CSTB and S100A13 were further validated on a larger sample set by immunohistochemistry (n=48). The calcium binding protein S100A13 was found to be significantly overexpressed in NR compared with R in all analyses performed.

Conclusions: Our results suggest that S100A13 is involved in CMM resistance to DTIC/TMZ.

Cutaneous malignant melanoma (CMM) is one of the most aggressive skin cancer types, with a rapid increase in prevalence, especially among western countries (Erdmann et al, 2013). So far, curative treatment is achieved only by surgical resection of primary tumours at an early stage (Balch et al, 2001; Balch et al, 2004). Chemotherapy with the alkylating agents dacarbazine (DTIC) and temozolomide (TMZ) has not shown any effect on median overall survival of patients (Hill et al, 1984; Gogas et al, 2007). However, ~13% of the patients have an initial partial or complete response to these drugs and rare long-time responses are seen (Middleton et al, 2000; Kim et al, 2010; Patel et al, 2011). Several chemoresistance mechanisms have been proposed, but the clinically relevant mechanisms are to a large extent still unknown.

DNA repair proteins, such as O-6-methylguanine-DNA methyltransferase (MGMT), which counteract the alkylating effects of DTIC/TMZ, have been suggested to confer resistance to these drugs (for a review, refer to the study by Zhang et al (2012)). The relationship between MGMT and response to therapy has mainly been reported for TMZ treatment in glioma, but association between MGMT expression and resistance to DTIC/TMZ has been found also in melanoma (Ma et al, 2003; Busch et al, 2010). In addition, studies using melanoma cell lines have suggested a role...
for melanosomes and related protein-trafficking pathways in melanoma chemoresistance (Chen et al, 2006; Xie et al, 2009), and we have shown higher expression of the melanogenesis regulator microphthalmia-associated transcription factor (MITF) and the melanosomal G-protein-coupled receptor 143 (GPR143) in tumours from non-responders to DTIC/TMZ therapy, compared with responders (Hertzman Johansson et al, 2013).

Recently, the treatment options for melanoma have expanded rapidly, and several novel immunotherapies and small-molecule drugs targeting the MAPK pathway have been approved or are in clinical trials (Hodi et al, 2010; Chapman et al, 2011; Hauschuld et al, 2012; Flaherty et al, 2012a, b; Menzies and Long, 2013). Unfortunately, primary resistance is widely encountered for immunotherapies, and despite many impressive initial responses to MAPK signalling inhibitors, acquired drug resistance eventually develops. Thus, chemotherapy could have a role in combination therapies by inhibiting the growth of tumour cells that are resistant to specific inhibitors. In addition, standard chemotherapy is still relevant for patients not responding to immunotherapy, and for those whose tumours lack activated druggable targets.

To identify proteins associated with chemotherapy resistance, we carried out protein expression profiling of tumour biopsies from patients with differential response to DTIC/TMZ treatment. Gene expression profiling data was available for a majority of the tumours (Hertzman Johansson et al, 2012), and several candidate genes and biological pathways were identified by both methods. Four of the top candidates among the identified proteins were validated in the same tumour samples by immunoblotting. In addition, two of the candidate proteins with higher expression in non-responders, the calcium binding protein S100A13 and the cathepsin inhibitor cystatin B (CSTB), were further studied by immunohistochemistry in an extended set of pre-treatment tumour samples. The significantly higher expression of S100A13 in non-responders was confirmed also in this sample set.

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MATERIALS AND METHODS

Tumour samples. Fresh-frozen pre-treatment lymph-node biopsies were excised from patients with metastatic cutaneous melanoma before chemotherapy with DTIC/TMZ. All tumour pieces included had a tumour cell burden of at least 50% (the majority of tumours >70%). Although there were individual differences between the tumour pieces with regard to cell composition, vascularisation and infiltration of leukocytes, there were no major differences between the groups of responders and non-responders (see Supplementary Figure 1 for histological images).

Five patients were responders (defined as a reduction of tumour size of at least 50%), and five non-responders (with stable or progressive disease) were matched to the responders with regard to gender, histopathology (superficial spreading or nodular melanoma) and, when possible, age. Four of the responders had previously been included in a gene expression profiling study (Hertzman Johansson et al, 2012) and four of the non-responders from this study material were also included in the present analysis, in addition to the five matched non-responders. For details on the clinical material included in the proteomics analysis, see Supplementary Table 1 and Supplementary Figure 1.

For the immunohistochemistry, an extended material of formalin-fixed paraffin-embedded pre-treatment biopsies from 16 DTIC/TMZ responders and 34 non-responders were analysed.

Patients have given informed consent and the study was approved by the Regional Ethics Committee of Stockholm.

Protein sample preparation. Tissue disruption was performed using a Mixer mill MM200 (Retsch, Haan, Germany) with a 1-cm ø Teflon-coated tungsten ball. Teflon vials, caps and the ball were first pre-cooled in −80 °C and then in liquid nitrogen. Frozen tumour samples (~3 × 3 × 3 mm) were cut into smaller pieces (1 × 1 × 1 mm) on a block of dry ice. Samples were then transferred to the pre-cooled vials and cooled again for 2 min in liquid nitrogen. The samples were then homogenised in the Mixer mill for 2 min at maximum speed after which they were put back into the liquid nitrogen. This homogenisation/cooling procedure was repeated three times until a frozen tumour powder was achieved. The powder was dissolved in 1 ml 20 mM HEPES (pH 7.6), transferred to Eppendorf tubes and kept in −80 °C.

The samples were thawed and DTT and SDS were added to an end concentration of 1 mM and 3.75%, respectively. Samples were then lysed by heating (at 90 °C for 5 min on a thermomixer) and sonication (at room temperature for 5 min). The samples were then centrifuged at 10 000 g for 10 min and the supernatant was transferred to a new tube. Protein concentrations of the lysates were measured using the DC protein assay kit 2 (BioRad, Hercules, CA, USA). The protein yield was at least 1.6 mg per tumour piece.

For acetone precipitation, 120 mg of protein from each sample was mixed with four volumes of ice-cold acetone. The samples were incubated in +4 °C for 2 hours until a flocculent formed. The samples were then centrifuged for 10 min at 10 000 g. The supernatant was discarded and the protein was allowed to air dry. This step was performed for the MS/MS analysis, but not for immunoblotting samples.

Digestion, iTRAQ labelling and clean-up of protein samples. Samples were digested and labelled according to a standard iTRAQ eight-plex protocol (Thermo Fisher Scientific, Waltham, MA, USA). The trypsin-to-protein ratio was 1:20. One hundred micrometres of each sample were labelled and two pools were made each with seven samples and one internal standard for normalisation.

Pooled iTRAQ-labelled digests were applied to 1 ml Strata X-C 33 μm polymeric strong cation exchange (SCX) microcolumns (Phenomenex, Torrance, CA, USA). The microcolumns were initially washed with 1 ml 100% methanol followed by 1 ml MilliQ grade water (EMD Millipore Corporation, Billerica, MA, USA). The sample was adjusted to 500 μl 0.1% formic acid and then applied to the columns. After washing with 1 ml 30% methanol and 0.1% formic acid the samples were eluted with 30% methanol and 5% ammonium hydroxide. Samples were then dried in a SpeedVac system (EMD Millipore Corporation).

Narrow-range IEF. Briefly, samples were added to pH 3.3 loading strips, kindly provided by GE healthcare (Uppsala, Sweden), and allowed to re-swell overnight. Loading strips were then put on re-swelled 24-cm narrow-range isoelectric focusing strips (pH 3.4–4.7) also provided by GE Healthcare. Samples were focused until 100 kVhs was reached. The peptides were passively eluted in 72 fractions of 150 μl MilliQ water using an in-house robot. Eluted peptide samples were then dried in a SpeedVac system.

Mass spectrometry. The 72 fractions were re-suspended in 10 μl of 3% acetonitrile in 0.1% formic acid. From each IPG fraction 3 μl was injected into online HPLC/MS performed on a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). An Agilent HPLC 1200 system (Agilent Technologies, Santa Clara, CA, USA) was used to provide the gradient for online reversed-phase nano-LC at a flow of 0.4 μl min −1. Solvent A was 97% water, 3% ACN and 0.1% formic acid, and solvent B was 5% water, 95% ACN and 0.1% formic acid. The curved gradient went from 2% B up to 40% B in 45 min, followed by a steep increase to 100% B in 5 min. The sample was injected into a C18 guard-desalting column (Agilent Technologies) prior to a 15 cm long C18 picofrit column (100 μm internal diameter, 5 μm bead size, Nikkyo Technos Co, Tokyo, Japan) installed on to the nano electrospray ionisation (NSI) source of the Orbitrap Velos instrument (Thermo Fisher, Waltham, MA, USA). Acquisition proceeded in
energy collision dissociation (HCD, at 45% energy) with MS/MS resolution (profile mode), followed by two stages of data-dependent B candidates, the same tumour protein extracts as in the protein profiling data from the same tumours were available (Hertzman et al., 2012). The proteomic results from these samples consisting of 146 proteins was created (CV-ANOVA P = 0.0007, Q2 = 0.874, R2Y = 0.998, R2X = 0.619, see Supplementary Table 3). The enriched top molecular and cellular functions and canonical pathways of this selected group of 94 proteins was defined by ingenuity pathway analysis (see Table 1), and include several key cell signalling pathways such as Rho and Rac signalling.

For four of the responders in the present study, gene expression profiling data from the same tumours were available (Hertzman Johansson et al., 2012). The proteomic results from these samples and four other (unmatched) non-responders, which were also included in the gene expression profiling study, were analysed separately with the purpose of comparing the differences on protein level to the transcriptome data (see Supplementary Table 1 for details regarding the samples included). An O-PLS model consisting of 146 proteins was created (CV-ANOVA P = 0.006, Q2 = 0.998, R2Y = 1 and R2X = 0.783, see Supplementary Table 3). Compared with the first O-PLS model, with the matched non-responders, 11 proteins overlapped and 76 canonical pathways were identified in both models (see Supplementary Table 3).
When comparing the proteomics results to the transcriptome data for the samples where this was available, 13 candidates were identified by both methods, with 6 concordant expression levels (see Table 2 for details). We could also detect 16 overlapping biological pathways in the gene expression profiling and the corresponding proteomics study (see Supplementary Table 4). Four of these pathways were immune system-related, and the remaining 12 were signalling cascades, including actin cytoskeleton signalling, RhoA signalling, PPAR signalling and IL-8 signalling.

The proteins in the two O-PLS models were also compared with known targets of the transcription factor MITF (Hoek et al, 2008; Strub et al, 2011), a potential chemoresistance candidate (Hertzman Johansson et al, 2013). About 40% of the proteins identified in the present study are previously reported to be regulated by MITF (see Supplementary Table 3 for details).

**Immunoblotting.** Four of the identified chemotherapy response candidates, CSTB, F13A1, ISYNA1 and S100A13, were selected for technical validation by immunoblotting, using the same protein extract as in the MS analyses. The selection was based on the strength of the proteomics and gene expression profiling results, as well as the biological function of the protein. Among the proteins that were identified in the present study are previously reported to be concordant expression levels data for the samples where this was available, 13 candidates were identified by both methods, with 6 concordant expression levels (see Table 2 for details). We could also detect 16 overlapping biological pathways in the gene expression profiling and the corresponding proteomics study (see Supplementary Table 4). Four of these pathways were immune system-related, and the remaining 12 were signalling cascades, including actin cytoskeleton signalling, RhoA signalling, PPAR signalling and IL-8 signalling.

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**Immunohistochemistry.** To evaluate the clinical relevance of the identified chemoresistance candidates we performed immunohistochemistry on an extended set of tumours from 16 responders and 32–34 non-responders. The antibodies for ISYNA1 and F13A1 were tested, but did not work well for immunohistochemistry and were not included in this analysis. Representative images of CSTB and S100A13 staining are shown in Figure 2. Analysis of the cytoplasmic expression showed that, similar to the proteomics results, significant S100A13 staining was more often found in non-responders compared with responders (P = 0.013), while CSTB did not deviate between the groups (see Table 3). No difference in nuclear expression was observed for either protein (data not shown).

**DISCUSSION**

The aim of the present study was to unravel a protein signature predictive of the efficacy of DTIC/TMZ therapy in pre-treatment

| Top cellular and molecular functions | P-value |
|-------------------------------------|---------|
| RNA post-transcriptional modification | 1.10E – 05–3.29E – 02 |
| Cell morphology | 2.22E – 04–3.75E – 02 |
| Cell-to-cell signalling and interaction | 2.22E – 04–3.75E – 02 |
| Carbohydrate metabolism | 3.32E – 04–3.75E – 02 |
| Cellular movement | 3.47E – 04–3.75E – 02 |

**Top canonical pathways**

| Signalling by Rho Family GTPases | 0.000108 |
| Actin nucleation by ARP–WASP complexes | 0.000145 |
| Rac signalling | 0.00147 |
| IMUP signalling in neutrophils | 0.00175 |
| Clathrin-mediated endocytosis signalling | 0.00189 |

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|-------------------------------------|---------|
| RNA post-transcriptional modification | 1.10E – 05–3.29E – 02 |
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| Carbohydrate metabolism | 3.32E – 04–3.75E – 02 |
| Cellular movement | 3.47E – 04–3.75E – 02 |

**Protein accession** | **Symbol** | **Protein name** | **Proteomics GE profiled samples** | **Gene expression profiling** |
|----------------|--------|----------------|-------------------------------|-----------------------------|
| QYQ25 | ROBLD3 | Roadblock domain containing 3 | –1.2 | 0.8 |
| P59998 | ARPC4 | Actin-related protein 2/3 complex, subunit 4 | –0.8 | 0.4 |
| P07203 | GPX1 | Glutathione peroxidase | –0.6 | 0.9 |
| Q2NL6C8 | GSTK1 | Glutathione S – transferase kappa 1 | –0.6 | 0.7 |
| P04B0 | CSTB | Cystatin B (stefin B) | –0.5 | –1 |
| Q6FG43 | FLOT2 | Flotillin 2 | –0.5 | 0.7 |
| B4DN37 | DEK | DEK oncogene | –0.4 | 0.5 |
| P62136 | PPP1CA | Protein phosphatase 1, catalytic subunit, alpha isoenzyme | –0.4 | 1.4 |
| B4DJ89 | POLR2E | Polymerase (RNA) II (DNA-directed) polypeptide E | 0.2 | 0.9 |
| A2RRE5 | GRLF1 | Glucocorticoid receptor DNA binding factor | 0.4 | 0.7 |
| Q96G21 | IMP4 | IMP4, U3 small nuclear ribonucleoprotein | 0.5 | 0.6 |
| B4EU1U | FITPNA | Phosphatidylinositol transfer protein, alpha | 0.6 | 0.9 |
| B7Z3K3 | ISYNA | Inositol-3-phosphate synthase 1 | 1.4 | 1.2 |

The results are presented as log2 values of the fold change protein or mRNA expression responders/non-responders.
melanoma tumour biopsies, using mass spectrometry-based proteomics. We compared the proteome profile of tumour samples from chemotherapy non-responders with that of responders. For four of the identified proteins (the cathepsin inhibitor cystatin B (CSTB), myo-inositol 1-phosphate synthase (ISYNA1, that catalyses the \textit{de novo} synthesis of inositol 1-phosphate from glucose 6-phosphate), the coagulation factor F13A1 and the calcium binding protein S100A13) the expression differences between tumours from responders and non-responders were validated by immunoblotting, indicating the robustness of the MS analysis.

To our knowledge, this is the first LC/MS-MS study of drug resistance in human melanoma tumours. Resistance to TMZ and other alkylating agents has been studied by other proteomics methods mainly in glioma samples and cell lines (refer to the study by Suk (2012) for a review). Resistance to TMZ or other chemotherapeutic agents (vinodesine, cisplatin, fotemustine or etoposide) has also been investigated by gene expression microarray analyses (Augustine \textit{et al}, 2009) and proteomics (Sinha \textit{et al}, 2003; Paulitschke \textit{et al}, 2013) in melanoma cell lines. Interestingly, members of the top canonical pathway identified in our study, ‘Signalling by Rho Family GTPases’, are among the strongest chemoresistance candidates also in melanoma cell lines treated with other anticancer agents (Augustine \textit{et al}, 2009). Additional functional categories from other studies that overlap with our results include potential druggable candidate pathways, namely heat shock proteins and proteasomal proteins (Sinha \textit{et al}, 2003; Suk, 2012).

The correlation between S100A13 expression and DTIC/TMZ resistance detected in the global proteomics analysis was confirmed by immunohistochemistry in an extended set of pre-treatment melanoma tumour biopsies from DTIC/TMZ responders and non-responders, stained with antibodies for S100A13, CSTB or negative control without any primary antibody.

Table 3. Cytoplasmic expression of S100A13 and CSTB in pre-treatment tumour biopsies from DTIC/TMZ responders (R) and non-responders (NR)

|            | Positive cytoplasmic staining (%) | Fisher’s exact test (P) |
|------------|----------------------------------|-------------------------|
| **S100A13**|                                  |                         |
| R          | 4/16 (25)                         | 0.013                   |
| NR         | 21/32 (66)                        |                         |
| **CSTB**   |                                  |                         |
| R          | 5/16 (31)                         | 0.49                    |
| NR         | 7/34 (21)                         |                         |

Figure 1. Immunoblot results. (A) Comparison of proteomics and immunoblotting results. Data are shown as the log2 of the mean of the fold change in responders over the value in non-responders. (B–D) Immunoblot images for four candidate proteins.

Figure 2. Immunohistochemistry. Representative images of metastases from DTIC/TMZ responders and non-responders, stained with antibodies for S100A13, CSTB or negative control without any primary antibody.
be over-expressed in several cancer types, including melanoma tumour cells (Hardesty et al, 2011) and in gliomas (Schwartz et al, 2005). S100A13 has previously been suggested to be an angiogenic marker for melanoma and astrocytic gliomas (Landriscina et al, 2006; Massi et al, 2010) and also to be involved in the invasiveness of lung cancer cell lines (Pierce et al, 2008).

There could be several potential mechanisms by which S100A13 influences chemoresistance: in tumours, S100A13 could be a marker for vessel density and on a cellular level, S100A13 regulates secretion of FGF1 (Cao et al, 2010) and IL1A (Mohan and Yu, 2011). In addition to its proposed role in angiogenesis and invasion, S100A13 is also involved in several key signalling pathways with relevance to cell cycle progression and differentiation, including cytokine and NFKB signalling, supporting that S100A13 may be related to increased aggressiveness of melanoma tumours (Hsieh et al, 2004; Massi et al, 2010). Our results are also in line with the reported positive correlation between S100A13 mRNA levels, risk of relapse and status of melanoma patients at follow-up (Massi et al, 2010). The elevated expression of S100A proteins, including S100A13, in melanoma cell lines resistant to cisplatin (Paulitschke et al, 2013) indicate that S100A13 could have a more general role in melanoma chemoresistance, not specific for DTIC/TMZ.

The second candidate protein that was investigated in the larger material was CSTB. This endogenous inhibitor of cathepsins l, h, b and papain is thought to be involved in the protection against lysosomal protease leakage, and has been suggested to be a marker for more advanced disease and bad prognosis in various forms of cancer (Kos et al, 2000; Werle et al, 2006; Ghashenko et al, 2013). However, our immunohistochemistry results did not support the hypothesis of CSTB playing a significant role in DTIC/TMZ resistance.

We have previously shown that the main melanogenesis regulator, the transcription factor MITF, is associated with resistance to DTIC/TMZ (Hertzman Johansson et al, 2013) and the relatively high proportion of MITF-regulated proteins among the candidates identified in this study supports this finding.

In summary, our results showed significantly higher cytoplasmic levels of the S100A13 protein in pre-treatment tumour biopsies from DTIC/TMZ non-responders compared with responders. Thus, low levels or no expression of S100A13 may be one of the key predictive markers to identify melanoma patients responding to DTIC/TMZ therapy. Further in-depth studies on how S100A13 regulates response to DTIC/TMZ are needed and the finding should be validated in larger independent sets of tumour samples from melanoma patients.

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