HOXCl1–SRC-1 regulation of S100beta in cutaneous melanoma: new targets for the kinase inhibitor dasatinib

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MATERIALS AND METHODS

Cell culture

SKMel28 cells, derived from a primary cutaneous melanoma, and MeWo cells, derived from a melanoma lymph node metastasis,
were obtained from ATCC and were maintained in Eagle’s minimal essential medium (MEM) (Invitrogen, CA, USA) supplemented with 2 mM 1-glutamine, 100 μg·mL⁻¹ penicillin, 100 μg·mL⁻¹ streptomycin (Sigma-Aldrich, Ireland Ltd, Wicklow, Ireland) and 10% (v/v) FBS (Gibco, Invitrogen). Primary cell cultures were prepared from patients with malignant melanoma. Melanoma cells were dissociated from the tumour mass using a digestion mixture (DMEM-F12, 10% FBS, 10 μg·mL⁻¹ insulin, 100 U·mL⁻¹ hyaluronidases and 200 U·mL⁻¹ collagenases) and were cultured as above, n = 4.

Immunofluorescence
Following ethical approval from Beaumont Hospital, malignant melanoma and benign nevi tissue were collected and embedded in paraffin. Immunofluorescent staining on paraffin embedded tissue and primary cultures was performed using chicken anti-HOXC11 (0.4 μg·mL⁻¹) and rabbit anti-SRC-1 (0.5 μg·mL⁻¹), followed by the corresponding fluorescent-conjugated antibodies FITC anti-chicken and TRITC anti-rabbit. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole. Staining was visualised using a confocal microscope.

Coimmunoprecipitation and western blot
SKMel 28 and MeWo cell lines were treated with either vehicle or dasatinib (100 nM) for 2 h. Protein (1 μg) was extracted and immunoprecipitated with rabbit anti-SRC-1 (SC-8995; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Resultant proteins were subjected to SDS–PAGE and subsequently probed with either rabbit anti-SRC-1 (SC-8995; Santa Cruz) or chicken anti-HOXC11 (15-288-22000F; GenWay Biotech, Inc., San Diego, CA, USA). Resultant proteins were immunoprecipitated with rabbit anti-SRC-1 (SC-8995; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) following the manufacturer’s instructions.

Overexpression and knockdown of HOXC11
SKMel28 cells were transfected with control vector pDEST47 (400 ng) or pDEST47 HOXC11 (400 ng). Protein levels were determined 24 h post transfection by western blot. Predesigned and validated siRNA directed against HOXC11 (Qiagen Ltd, Crawley, West Sussex, UK) and SRC-1 (Applied Biosystems/Ambion, Warrington, UK) was used in the knockdown studies. Protein and mRNA levels were assessed 24 h post-transfection.

PCR
Relative mRNA levels of S100beta were determined as described (McIlroy et al, 2010).

ChIP
ChIP was performed as previously described (McIlroy et al, 2010). Cells were treated with either vehicle or dasatinib (100 nM) for 2 h. Chicken anti-HOXC11 (7 μg), or H4 antibody (7 μg), was added to the supernatant fraction of SKMel 28 cells and incubated overnight at 4°C with rotation. Proteins were un-crosslinked and primers were used to amplify the DNA –424 to +24 of the S100beta transcriptional start site.

Immunohistochemistry
Immunohistochemical staining for S100beta was performed using mouse anti-S100beta (Ab8330; Abcam, Cambridge, UK, 2 μg·mL⁻¹) followed by the corresponding antibody; in benign naevus and malignant melanoma; tissue was counterstained with haematoxylin. Images are representative of 64 separate melanoma tumours and 20 benign nevi.

RESULTS

HOXC11 and SRC-1 in malignant melanoma
Combined immunohistochemical and immunofluorescence was used to assess the expression of HOXC11 and its coactivator protein SRC-1 in benign nevi and malignant melanoma. In tissue from 80 patients assessed, nuclear expression of both HOXC11 and SRC-1 was significantly expressed more frequently in malignant melanoma in comparison with benign nevi (P<0.001 and P = 0.017, respectively), Table 1. Moreover, expression of HOXC11 and SRC-1 in the malignant tissue is significantly associated with each other (P<0.001, Fisher’s exact test). HOXC11 and SRC-1 were expressed predominantly in cytoplasm of cells in the nevi with no detectable nuclear coassociation (Figure 1A). In the tumour tissue and in primary cell cultures derived from patient tumours, both HOXC11 and SRC-1 translocate to the nucleus with a high level of coassociation (Figures 1A and B). Immunoprecipitation studies confirmed HOXC11 and SRC-1 interaction in primary (SKMel28) and malignant (MeWo) melanoma cell lines (Figure 1C). Greater protein expression of both HOXC11 and SRC-1 were found in metastatic melanoma cells in comparison with primary melanoma cell lines (Figure 1D).

HOXC11 and SRC-1 cooperate to regulate S100beta in melanoma cells
Using chromatin immunoprecipitation, we established HOXC11 recruitment to the promoter of S100beta in primary melanoma cells (Figure 2A). Forced expression of HOXC11 enhanced protein levels of S100beta in the SKMel28 cells (Figure 2B). Furthermore, knockdown of either HOXC11 alone or in combination with SRC-1 significantly reduced S100beta expression at both the RNA and protein level (Figures 2C and D). In addition, combined

|                   | HOXC11 n (%) | SRC-1 n (%) |
|-------------------|-------------|-------------|
|                   | Nuclear     | Cytoplasmic | Nuclear     | Cytoplasmic |
| Melanoma (n = 64) | 34 (53.1)   | 19 (29.7)   | 30 (46.8)   | 21 (32.8)   |
| Benign nevus (n = 20) | 1 (5)     | 8 (40)     | 3 (15)     | 6 (30)     |
| P-value           | <0.0001     | NS          | 0.017      | NS         |

Abbreviation: NS = Not significant.

Migration assay
Cellomics Cell Motility Kit (Thermo Scientific/Fisher Scientific Ireland, Dublin, Ireland, #K0800011) was used to assess individual cell movement after 22 h. In short, 96-well plates were precoated with collagen. Cells were seeded at 1 × 10⁵ cells·mL⁻¹ on a lawn of blue fluorescent beads. The track area is proportional to the magnitude of cell movement. Track areas were quantitatively analysed using Olympus cell² imaging software. Student t-test was used to compare mean migratory track areas. MeWo cells were treated with vehicle or dasatinib (100 nM) and migration was assessed using the standard scratch assay.

Proliferation assay
2000 SKMel28 and MeWo cells were plated in a 96-well plate and incubated overnight at 37°C and then treated with dasatinib (1 and 10 μM). Proliferation was assayed 72 h later by MTS CellTiter 96 (Promega, Madison, WI, USA) following the manufacturer’s instructions.

Table 1 | Associations of expression of HOXC11 and SRC-1 in malignant melanoma and benign nevi from patients using Fisher’s exact test

|                   | HOXC11 n (%) | SRC-1 n (%) |
|-------------------|-------------|-------------|
|                   | Nuclear     | Cytoplasmic | Nuclear     | Cytoplasmic |
| Melanoma (n = 64) | 34 (53.1)   | 19 (29.7)   | 30 (46.8)   | 21 (32.8)   |
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| P-value           | <0.0001     | NS          | 0.017      | NS         |

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knockdown of HOXC11 and SRC-1 results in significantly decreased migration of both SKMel28 and MeWo (Figure 2E). In line with our observation of increased expression and interaction between HOXC11 and SRC-1 in malignant melanoma versus benign nevi, high expression levels of their putative target S100beta were detected in melanoma tissue in comparison with little or no expression in the naevus tissue (Figure 2F).

Dasatinib can inhibit S100beta production in melanoma

We investigated whether inhibition of the Src kinase signalling network could have a role in disrupting the functional interaction between HOXC11 and SRC-1 in melanoma cells. The ATP-competitive dual Src/Abl inhibitor dasatinib reduced HOXC11–SRC-1 interaction and prevented recruitment of HOXC11 to the promoter region of S100beta (Figures 3A and B). Dasatinib had little or no effect on the expression levels of SRC-1 or HOXC11 in either SKMel28 or MeWo cells (Figure 3C). Treatment with dasatinib significantly reduced both protein and RNA expression of S100beta in melanoma cell lines (Figures 3C and D). High levels of dasatinib were required to reduce cell proliferation in both cell lines (Figure 3E). In the metastatic melanoma cell line MeWo, however, treatment with dasatinib at 100 nM was sufficient to reduce cell migration over time (Figure 3F).

DISCUSSION

Cutaneous melanoma is characterised by an unpredictable disease course. Studies now suggest that a particular signature emerges during the vertical growth phase of the primary tumour that can mark the emergence of the metastatic phenotype. Work from Riker et al (2008) has found that the homeobox family of proteins are among a group of six oncogenes that alter expression during the metastatic transition. Aberrant homeobox gene expression has been shown to promote tumourigenesis through a variety of mechanisms (Samuel and Naora, 2005; Maeda et al, 2005; Shah and Sukumar, 2010). The role of the homeobox genes in the transcriptional regulation of S100beta in breast tumour and neuronal tissue (Zhang et al, 2007; McIlroy et al, 2010) suggests their potential for regulating this calcium-binding protein in melanoma. We have previously reported that HOXC11 can utilise the steroid receptor coactivator protein to drive tumour progression in breast cancer patients (McIlroy et al, 2010). In this study, we used combined immunohistochemical and immunofluorescence to assess expression of HOXC11 and its coactivator protein in both benign nevi and malignant melanoma. We found high levels of expression and coassociation of HOXC11 and SRC-1 in malignant melanoma in comparison with benign nevi. Consistent with this, greater expression levels and coassociation of the transcription factor and coactivator were observed in the metastatic MeWo cells relative to the primary SkMel28 melanoma cell lines.

Recently, we and others have described a role for HOXC11 in the transcriptional regulation of S100beta in breast cancer and neuronal tissue (Zhang et al, 2007; McIlroy et al, 2010). The calcium-binding protein S100beta is one of the most widely researched biomarkers in melanoma and its serum expression has been shown to correlate with both disease stage and response to treatment (Mocellin et al, 2008). While there is substantial
evidence to support transcriptional regulation of S100beta (Allore et al., 1990; Jiang et al., 1993; Zimmer et al., 1995), little is definitively known about its regulators in melanoma. Here we observed recruitment of HOXC11 to the S100beta promoter. Using a variety of molecular and cellular techniques, we established a role for both HOXC11 and SRC-1 in the regulation of S100beta, uncovering a new mechanism of S100beta regulation in melanoma.

Abnormalities in growth factor signalling pathways have an intrinsic role in melanoma disease progression. Systems that impede this signalling network, including those that target c-kit, raf and p-Src are at the forefront in the development of new therapeutic strategies to treat this disease. In vitro studies have shown that coactivator function of SRC-1 is mediated at least in part through p-Src (Shang and Brown, 2002; Shah and Rowan, 2005). We investigated whether inhibition of the Src kinase signalling network could have a role in disrupting the functional interaction between HOXC11 and SRC-1 in melanoma cells. The ATP-competitive dual Src/Abl inhibitor dasatinib inhibited HOXC11 and SRC-1 mediated S100beta production in malignant melanoma cells. Furthermore, in the metastatic melanoma cell line MeWo, treatment with dasatinib reduced cell migration. Dasatinib targets BCR/ABL and c-Kit, and is the most potent Src kinase inhibitor currently in clinical development. It has proven efficacy in the treatment of chronic myelogenous leukaemia (Talpaz et al., 2006). It is currently in trial for the treatment of advanced melanoma (http://www.clinicaltrial.gov). Though none of these trials have completed, interim reports suggest that as a single therapy, dasatinib has minimal activity in unselected patients (Kluger et al., 2010). Moreover, recent in vitro studies in the breast suggest that dasatinib may be more effective when combined with other targeted therapies rather than as a single agent in the treatment of solid tumours (Vallabhaneni et al., 2010).

In spite of recent advances in targeted therapy, no treatment has managed to significantly prolong overall survival in patients with advanced melanoma. Molecular interactions that link tumour progression to developmental biology provide the ideal milieu for a tumour to adapt and evade targeted therapies. Here, we describe...
In addition to S100beta, HOX gene expression and S100beta expression are also regulated by the SRC-1-S100beta network. Dasatinib treatment reduces S100beta expression at the transcriptional level and reduces cell proliferation in cutaneous melanoma cells.

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