Cellular Signalling 24 (2012) 852–858

Contents lists available at SciVerse ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Aberrant expression of the transcriptional factor Twist1 promotes invasiveness in ALK-positive anaplastic large cell lymphoma

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ARTICLE INFO

Article history:
Received 29 September 2011
Received in revised form 14 November 2011
Accepted 24 November 2011
Available online 8 December 2011

Keywords:
Twist1
NPM-ALK
STAT3
Shc
Anaplastic large cell lymphoma

ABSTRACT

The transcriptional factor Twist1 has been shown to play a key role in regulating epithelial mesenchymal transition, invasiveness and migratory properties in solid tumors. We found that Twist1 is aberrantly expressed in ALK-positive anaplastic large cell lymphoma (ALK + ALCL), a type of T-cell lymphoid malignancy. Using RT-PCR and Western blots, Twist1 was detectable in all 3 ALK + ALCL cell lines examined but absent in normal T-cells. By immunohistochemistry, Twist1 was detectable in all 10 cases of ALK + ALCL examined; benign lymphoid tissues were consistently negative. Twist1 expression in ALK + ALCL cells can be attributed to the NPM-ALK/STAT3 signaling axis, the key oncogenic driving force in this tumor type. Twist1 is biologically important in ALK + ALCL cells, as Twist1 knockdown resulted in a significant decrease in their invasiveness in an *in-vitro* assay. Further investigation revealed that this increase in invasiveness is linked to the activation of AKT and down-regulation of p66Shc, two signaling proteins known to be involved in NPM-ALK-mediated oncogenesis. Lastly, knockdown of Twist1 sensitizes ALK + ALCL cells to the growth inhibitory effect of PF-2341066 (Crizotinib®), an ALK inhibitor being used in clinical trials. In conclusion, Twist1 expression, owing to the abnormal NPM-ALK/STAT3 signaling, contributes to its invasiveness and decreased sensitivity to PF-2341066 in ALK + ALCL.

1. Introduction

Twist1 is a transcriptional factor that has been shown to be important in embryogenesis; specifically, Twist1 regulates mesoderm formation, neurogenesis, myogenesis, and the migration of neural crest cells [1]. The biological importance of Twist1 is highlighted by the Saethre-Chotzen syndrome, in which the affected individuals carrying *Twist1* gene mutations develop craniofacial and limb anomalies [2,3]. Structural analysis revealed that Twist1 contains the basic helix-loop-helix motif which allows Twist1 to form functional homodimers as well as heterodimers with other Twist family members. In keeping with its biological significance, the structure of Twist1 is highly conserved throughout the evolution [4]. The post-natal expression of Twist1 in humans is restricted to a subset of mesoderm-derived tissues such as the heart and skeletal muscle [2]. In the hematopoietic system, Twist1 is found largely restricted to the CD34-positive hematopoietic stem cells; normal mature T lymphocytes do not have detectable Twist1 [2,5,6]. Recent studies have shown that Twist1 is expressed in cancer cells and implicated in oncogenesis and metastasis. For instance, it has been shown to promote colony formation in E1A/ras-transformed mouse embryo fibroblasts and inhibit DNA damage-induced apoptosis by modulating p53 function [7]. Moreover, Twist1 has been found to down-regulate CD24 expression in breast cancer cells, an immunophenotype that is associated with cancer stem cells [8]. Twist1 also is known to play key roles in regulating epithelial mesenchymal transition (EMT), a phenotype that correlates with increased metastatic potential in cancer [9–12]. Thus, a higher expression level of Twist1 in malignant melanoma, bladder cancer and breast carcinoma correlates with early dissemination of the disease and a poor clinical outcome [13–15]. Nevertheless, the expression and the biological significance of Twist1 in hematopoietic cancer have not been extensively studied.

ALK + ALCL is a specific type of T-cell lymphoid malignancy that represents the second most common pediatric lymphoid malignancy [16]. ALK + ALCL cells carry the characteristic chromosomal translocation, $t(2;5)(p23;q35)$ [17–21]. The consequence of this cytogenetic

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doi:10.1016/j.cellsig.2011.11.020

abnormality is the formation of an abnormal fusion protein, namely nucleophosmin (NPM)-anaplastic lymphoma kinase (ALK) [17–21]. Extensive previous studies have shown that NPM-ALK is highly oncogenic and central to the pathogenesis of ALK + ALCL [18]. The mechanism underlying the oncogenic potential of NPM-ALK is strongly linked to its ability to bind, phosphorylate and activate a large number of cell signaling proteins [17,22–25], many of which are known to be highly oncogenic when constitutively activated (e.g. STAT3) [24]. Recent studies suggest that NPM-ALK promotes cell invasiveness [26–28], although the underlying mechanism has not yet been fully investigated.

In this study, we report that Twist1 is aberrantly expressed in ALK + ALCL cells, and this abnormally can be attributed to the NPM-ALK/STAT3 signaling. We also found evidence that Twist1 is biologically significant in the pathogenesis of ALK + ALCL, as it promotes tumor invasiveness and confers resistance to PF-2341066 (Crizotinib®), an ALK inhibitor that are being tested in clinical trials.

2. Methods

2.1. Cell culture and Twist1 knockdown

Conditional (tetracycline-on) NPM-ALK expressing cell lines were constructed by inserting linearized pTRE-tight/*NPM-ALK* into tet-on HEK-293 ADVANCED cells (Clontech, Mountain View, Canada) followed by selection of three stable clones. Three ALK + ALCL cell lines (Karpas 299, SU-DHL-1, and SUP-M2) were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FBS (V/V), and cultured under an atmosphere of 95% O_2 and 5% CO_2 in 98% humidity at 37 °C. For Twist1 knockdown, previously published Twist1 specific ON-Target Plus SMARTpool small interfering RNA (siRNA) [29] (Thermo Scientific, Chicago, USA) and scramble control (Origen, Rockville, MD) were used as previously described [30]. Three ALK + ALCL cell lines were transfected with scramble siRNA, ALK siRNA (Thermo Scientific, Chicago, USA), Twist1 siRNA or STAT3 siRNA (Origen, Rockville, MD) for 24 h, followed by Western blot and cell viability analysis as previously described [28,30].

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from Karpas 299, SU-DHL-1, SUP-M2, and MDA-MB-231 cells and 3 ALK + ALCL patient samples using TRIzol extraction according to the manufacturer's protocol (Life Technologies, Grand Island, NY). RT-PCR was performed as described in our previous publication [31]. The primer sequences for *Twist1* are forward 5-AGTCCGCAGTCTTACGAGGA-3 and reverse 5-CATCTTGGAGTCCAGCTCGT-3, with an expected product size of 222 bp. The primer sequences for *GAPDH* are forward 5′-AAGGTC ATCCCTGAGCTGA-3′ and reverse 5-CCCTGTTGCTGTAGCCAAAT-3′, with an expected product size of 316 bp.

2.3. Western blot and immunohistochemistry

Western blot analysis and immunohistochemistry were performed as described in our previous publications [32–34]. The following antigens were detected using antibodies, all of which were obtained from Cell Signaling Technology (Danver, MA, USA) unless otherwise stated: Twist1 and Bmi1 (Santa Cruz biotechnology, Santa Cruz, CA, USA), phospho-STAT3 (Y^{705}), STAT3, Shc, phospho-AKT, AKT, ERK1/2, phospho-ERK1/2, phospho-ALK ($Y^{1278/1282/1283}$), phospho-Src (Y^{527}), Src, α-tubulin, histone deacetylase 1 (HDAC1) and ALK.

2.4. Cell viability analysis

Cell viability was performed as previously described [33–36]. Briefly, SUP-M2, SU-DHL-1 or Karpas 299 cells were transfected with either scramble siRNA or Twist1 siRNA, cultured with 5% FBS, followed by treatment with PF-2341066 in the dose-dependent manner for 48 h. The cell viability was then measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI) following the manufacturer's protocol, and viable cells were also counted using trypan blue staining.

2.5. Nuclear and cytoplasmic fractionation

Cytoplasmic and nuclear proteins from SU-DHL-1, Karpas 299, and SUP-M2 cells were extracted using the N/C fractionation kit (Fisher Scientific, Ontario, Canada) with following the manufacture's instruction. During the SDS-PAGE analysis, α-tubulin and histone deacetylase 1 (HDAC1) were used as cytoplasmic and nuclear markers, respectively.

2.6. Cell invasiveness assay

As previously described [28], we assessed cell invasiveness using the Cytoselect™ 96-well cell invasion assay kit was used (Cell Biolabs, San Diego, CA, USA) according to the manufacture's protocol. Briefly, 100,000 SU-DHL-1, Karpas 299, and SUP-M2 cells transfected with either scramble siRNA or Twist1 siRNA, or 10,000 Jurkat cells transfected with different vectors described in Fig. 3, were seeded into the culture inserts, and allowed to invade the reconstituted basement membrane matrix for 24 h. The invasive cells passed the membrane were then dissociated from membrane, lysed and quantified using CyQuant GR fluorescent Dye.

3. Results

3.1. Twist1 is consistently expressed in ALK+ALCL cells

As shown in Fig. 1A, using RT-PCR, we found a readily detectable level of *Twist1* mRNA in 3 of 3 ALK + ALCL cell lines (including SU-DHL-1, SUP-M2 and Karpas 299), and 3 of 3 frozen tumors samples examined. In contrast, peripheral blood T cells from a healthy donor were negative (Fig. 1A). The DNA sequence of the PCR amplicons was confirmed to be that of *Twist1* (data not shown). Using Western blots and an anti-Twist1 antibody previously published [37,38], we found the Twist1 protein at 26 kD in ALK + ALCL cell lines and frozen patient samples used in the above-mentioned RT-PCR experiments (Fig. 1B). MDA-MB-231, a breast cancer cell line known to express Twist1 [39], served as the positive control. Peripheral blood T-cells from a healthy donor were again negative. We then assessed the subcellular localization of Twist1 in ALK + ALCL cell lines. As shown in Fig. 1C, Twist1 was found in both the cytoplasmic and nuclear fractions. α-tubulin and HDAC1 served as the controls to assess the efficiency of fractionation of the cytoplasmic and nuclear portions, respectively. To further confirm that ALK + ALCL tumor cells express Twist1, we employed immunohistochemistry applied to archival/paraffin-embedded patient tumor samples (n = 10). As illustrated in Fig. 1D, the neoplastic cells were strongly positive for Twist1. In contrast, lymphoid cells in a benign tonsil were negative.

3.2. Aberrant expression of Twist1 in ALK+ALCL is dependent on the NPM-ALK/STAT3 signaling axis

Considering the central pathogenetic role of NPM-ALK in ALK + ALCL, we tested if NPM-ALK contributes to the aberrant expression of Twist1. Knockdown of NPM-ALK using siRNA mediated a dramatic down-regulation of Twist1 in both SUP-M2 and SU-DHL-1 cells (Fig. 2A). To further establish the relationship between NPM-ALK and Twist1, we used the NPM-ALK/tet-on HEK293 cells, in which the NPM-ALK expression can be incrementally elevated by increasing the concentration of doxycycline *in vitro*. As shown in Fig. 2B, as the