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Targeting Twist expression with small molecules†

Haixiang Pei,‡ Yunqi Li,‡ Mingyao Liu and Yihua Chen*

Twist, as one of the important embryonic transcription factors, regulates epithelial–mesenchymal transition (EMT) and migration in embryo formation and cancer development. Both Twist-1 and Twist-2 are rarely detectable in healthy adult tissues, but are frequently overexpressed in multiple kinds of human cancer tissues, such as breast, prostate, uterus, liver, melanoma, etc. Twist is considered as a crucial EMT inductor and correlated with carcinoma aggression, invasion and metastasis. In the past decades, in-depth investigation has been reported in terms of the role of Twist in cancers; in addition, several kinds of small molecules have played important roles in studying the effect of Twist on cancer development, suggesting that Twist can be regarded as one of the important potential targets for cancer treatment. Hence we provide a brief overview of Twist and several small molecules targeting its expression, highlighting the biological features that make it a charming target for cancer therapy.

1. Introduction

Twist, as a highly conserved transcription factor with a size of approximately 21 kDa, and which was originally found in *Drosophila* in 1987,^{1,2} plays critical roles in the early development of mesoderm formation and the regulation of morphogenesis movement during gastrulation activating N-cadherin expression.^{3–5} Twist is a member of the basic helix–loop–helix

protein family (bHLH).^{1,6} This protein encompasses a conserved bHLH (basic helix–loop–helix) motif which consists of two α -helices disassociated by an interhelical loop, as well as an interaction domain called the “Twist box”.^{1,7} The function of the α -helix is to form dimers, which leads to the production of a bipartite DNA-binding domain that binds to E-boxes (CANNTG), acting as either transcription inhibitors or activators.

Two Twist genes have been identified in vertebrate animals to date, which are encoded with 90% similarity, named Twist-1 (Twist, in brief) and Twist-2 (Dermo-1).^{8,9} They can both function as molecular switches to activate or suppress the target genes by direct or indirect mechanisms, such as the C-terminal of Twist encompasses a “Twist box” relevant

Shanghai Key Laboratory of Regulatory Biology, The Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai, 200241, China. E-mail: yhchen@bio.ecnu.edu.cn; Fax: +86 21 54344922; Tel: +86 21 2420 6647

† The authors declare no competing interests.

‡ These authors contributed equally to this work.

Haixiang Pei

Haixiang Pei is currently a PhD candidate of Medicinal Chemistry at the Institute of Biomedical Sciences, East China Normal University, Shanghai, China. He received his Bachelor of Science from China Pharmaceutical University. His research focuses on the synthesis and evaluation of selective small molecule inhibitors that target protein kinases in cancer.

Yihua Chen

Yihua Chen is a Professor of Medicinal Chemistry at the School of Life Science, East China Normal University. He received his Ph.D. in Medicinal Chemistry at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences under the supervision of Prof. Fa-Jun Nan. He completed his postdoctoral training at the University of Illinois at Chicago. His research group is mainly engaged in design, synthesis and structural optimization of small molecular bioactive compounds for drug leads against cancer as well as other important diseases.

to anti-osteogenic function. The most distinct difference between these two isoforms is that a glycine-abundant region exists in the N-terminal sequence of Twist-1, but not in Twist-2 (Fig. 1). Both proteins have been implicated in the differentiation of diverse cell lineages such as muscle, cartilage and osteogenic cells. The Twist gene (Twist 1) lies in the short arm of chromosome 7 (7p21.2). Twist comprises two exons and one intron, the first exon incorporates the entire coding region with 772 bp in length, encoding a 20.9 kDa highly conserved protein (Fig. 1).

In *Drosophila*, the Twist genes play essential roles in the process of proper gastrulation and generation of neural crest cells,^{10–12} which are also essential for the formation of *drosophila* embryo dorsal-ventral.¹¹ The Twist-knockout embryos give rise to a torsion of the abdomen,¹¹ that is where the name “Twist” comes from. Furthermore, in a study of human Twist gene (H-Twist), the role of Twist in embryogenesis is much similar to those in vertebrate animals. The encoding bHLH proteins display approximately 96% identity with the murine homologue M-Twist.¹³ On the other hand, adriamycin has an effect on the expression of Twist, thus inhibiting the differentiation of cells.¹⁴ The above studies of morphogenesis lay the foundation of research on cancer stem cells and relevant mechanisms of drug resistance. Moreover, in humans, H-Twist mutation can cause serious clinical complications, such as craniosynostosis disorder, Saethre–Chotzen syndrome, and Baller–Gerold syndrome.¹⁵

In grown-ups, the Twist proteins are mainly present in precursor cells including the myogenic, osteoblastic, chondroblastic, odontoblastic and myelomonocytic lineages,^{16,17} remaining barely detectable and maintaining an undifferentiated state. Twist-1 protein is one of the key regulators of adaptive thermogenesis in brown fat. Recently Twist proteins were further found to be a modulator of inflammatory processes.¹⁸ By inhibiting the pro-inflammatory cytokines, Twist can regulate the production and function of NF- κ B.¹⁹ On the other hand, Twist proteins are found to exhibit pivotal roles in lymphocyte function and maturation. This proves that Twist-1 and Twist-2 are key regulators of immune cell activation (especially T helper cells) in an inflammatory

environment such as autoimmune disease.²⁰ In Th17 cells, Twist inhibits the formation of inflammatory cytokine, suggesting its central role in the complicated regulatory network controlling autoimmune disease.^{19,21} In addition, Twist is predominantly expressed in the mesoderm-derived tissue and generally regarded as an organogenesis modulator, until recently experimental evidence has indicated that Twist also plays an essential role in tumour metastasis in many types of aggressive cancers, including breast cancer,⁵ hepatocellular carcinoma (HCC),²² prostate cancer,²³ gastric cancer,²⁴ etc. Twist-1 has received particular attention and is better studied primarily because of its role in cancer metastasis progression, especially in the epithelial–mesenchymal transition (EMT) process.

2. Twist and EMT

Epithelial–mesenchymal transition (EMT) is a process where epithelial cells differentiate to motile mesenchymal cells.²⁵ Epithelia play an active role of permeability barriers to characterize tissues and organs.²⁶ According to a specific microenvironment, EMT can be divided into three isoforms: type 1 is associated with the development of embryo and tissues; type 2 is related to wound healing and tissue fibrosis; type 3 is correlated with the invasion and metastasis of malignant tumour. In recent years, it has been reported that Twist is overexpressed in a large number of cancers.²⁷ The role of Twist in tumorigenesis and progression has attracted more and more attention. The classic cellular signal pathways that involve Twist in cancer metastasis are demonstrated in Fig. 2.^{28,29} Twist is considered as a critical EMT inducer,³⁰ which is correlated with carcinoma aggression and poor prognosis. The Twist transcription factors mediate EMT target genes involved in cell migration, self-renewal of cancer stem cells, multiple drug resistance, cell apoptosis, and immune surveillance.³¹ Owing to its central role in regulating EMT in cancer cells, it is suggested that Twist can be regarded as a promising “druggable” target for metastatic cancer therapy³¹ (Fig. 3). Additionally, Twist is thought to be an attractive therapeutic target partly because of its rare expression in normal adult tissues. Therefore, inhibitors targeting Twist could be regarded as one of the significant strategies against Twist

Fig. 1 Functional domains of human Twist-1 and Twist-2 proteins.

Fig. 2 The major regulation and the signal pathways of Twist involved in cancers.

Fig. 3 Possible mechanism of Twist in cancer cells. Twist is a master regulator of multiple pathways including: EMT, metastasis, reduction of apoptosis, immortalization and drug resistance; and its possible role in stem cell phenotype and hyper-methylation.

overexpression cancer cells as much as possible not harming other tissues.

3. Twist-1 and drug resistance

Tumour drug resistance is the dominant hurdle for successful treatment of all types of cancers. Therefore, it is essential progress toward preventing disease recurrence to gain insight into the molecular mechanism related to drug resistance. Lots of evidence proved that Twist-1 performed a significant role in tumour drug resistance. Twist-1, existing in lots of hu-

man cancer cells, was associated with resistance to many chemotherapy agents, such as paclitaxel (1),^{32,33} vincristine (2)²³ etoposide (3),³⁴ etc. (Fig. 5). In a study of colon cancer cells, Twist overexpression was related to resistance to enzastaurin (4).³⁵ Besides, downregulation of Twist expression in colorectal cancer accelerated apoptosis and intensified the sensitivity of chemotherapy, thus proving that Twist could be a potential prognostic marker and a therapeutic strategy for drug resistant cancer.

On the other hand, EMT has been considered as 'the switch of chemotherapy sensitivity and drug resistance'. Probably a functional relationship exists between tumour metastasis and drug resistance.³⁶ Much evidence demonstrated that tumour metastasis intensifies the resistance to drug; and anti-drug tumours were more prone to diffusion and transfer. It follows that in-depth exploration of Twist-induced EMT and anti-tumour drugs has important guiding significance.

4. Signalling pathways involving Twist in cancers

In consideration of the central role of Twist in regulating EMT in cancer cells, as well as its wide range of existence, it is essential to figure out the role of Twist in multiple cancers. So far, the cure of Twist-related carcinoma remains in the stage of experimentation, and numerous studies have been done to suppress Twist overexpression with small molecules. Herein, we review some studies in regard to signalling pathways involving Twist expression in cancers.

4.1. The Jagged1-KLF4 axis mediating endothelial differentiation induced by Twist

Tumour angiogenesis is one of the iconic functions ensuring tumour growth and metastasis.³⁷ In general, angiogenesis can be achieved by activating endothelial cells in existing vessels or by trans-differentiation of tumour cells into endothelial ones, but whether tumour cells can be differentiated into

Fig. 4 A model to depict the vital role of the Jagged1-KLF4 axis in mediating metastatic progress originated from the induction of EMT by Twist.

Fig. 5 Several compounds related to Twist and cancer. Compounds 1-4 are structures of some chemotherapy agents. Compound 5 is the structure of 5-aza-2'-deoxycytidine. Compounds 6-12 are structures of some natural products that target Twist.

endothelial ones remains unknown. Based on the research results that tumour angiogenesis can be regulated by Notch signalling,³⁸ Hsiao-Fan Chen³⁹ and co-workers indicated Jagged1, a Notch ligand, as the putative Twist target. DiI-AcLDL uptake was chosen as a biomarker to represent the level of Twist expression. RT-PCR and western blot experiments confirmed that the expressions of Jagged1 and its downstream signalling can be induced by Twist overexpression. Additionally, KLF4, a factor that confers stem cell-like properties, can be activated by Twist, and Hsiao-Fan Chen with co-workers attempted to assign the contribution of KLF4 to Twist-induced endothelial differentiation. Quantitative ChIP (qChIP) assays and immunohistochemistry (IHC) staining showed that Twist-1 was significantly correlated with Jagged1/KLF4 expressions, along with Jagged1 and KLF4 expressions, revealing that the Twist-1-Jagged1-KLF4 axis may provide a strategy for treatment of Twist-overexpressing tumours (Fig. 4).⁴¹ In addition, Bmi1 protein can also be modulated by Twist for inducing intravasation and metastasis.

4.2. DNA methylation in the Twist exon 1 region associated with the regulation of Twist1 expression in gastric cancer (GC) cells

Epigenetic alterations (such as DNA methylation and histone modification) to regulate gene silencing is a hotspot in geno-

mics nowadays. Epigenetic changes at the CGI (CpG island) promoter region of tumour suppressor genes (TSGs) are correlated with gene inactivation.⁴⁰ Abnormal DNA methylation at the CGI promoter of Twist has been detected in primary cancers, while it is not associated with the expression of Twist in numerous cancers.^{41,42} Ayuna Sakamoto⁴³ with co-workers attempted to figure out whether or not the Twist methylation at the promoter region leads to gene silencing. Twist was re-activated in cancer by 5-aza-2'-deoxycytidine (5-aza-dC, 5, Fig. 5), a de-methylation drug. The expression of Twist was quantified by quantitative RT-PCR using LightCycler DNA Master SYBR Green I. The Sp1 transcription factor was reported to bind CGI in the exon 1 region of Twist,⁴⁴ resulting in upregulation of Twist expression. CGI methylation at this region may disturb the binding of the Sp1, resulting in expression suppression of Twist. The clinical impact of the complex regulation of Twist expression in tumorigenesis needs to be further investigated.

4.3. The Twist-1 box domain induces cell metastasis in prostate cancer

Twist-1 is a master regulator of the EMT, while its structure-function relationships to cancer-related phenotypes are always ignored. Therefore, Rajendra P. Gajula with co-workers figured out the function of the Twist box domain for metastatic phenotypes in prostate cancer (Fig. 6).⁴⁵ They used a single amino acid substitution mutation Twist-F191G, to investigate the role of the Twist box. The expression of Twist was quantified by SYBR-green quantitative RT-PCR and prostate cancer cDNA arrays. The results suggested that the Twist box is significant in Twist-induced invasion and death resistance of prostate cancer cells *in vitro*, as well as cancer metastasis *in vivo*. In summary, the Twist box is a crucial domain in modulating transcriptional pro-metastatic programs in prostate cancer, which provide a possible therapeutic method against Twist-overexpressing prostate cancer cells by inhibiting the Twist box domain.

4.4. MiR-548c, downregulating Twist to impair migration and invasion of endometrial and ovarian cancer cells

Twist overexpression is related to deep myometrial invasion and poor outcome in endometrial cancer (EC).⁴⁶ Abnormal Twist expression can be detected in the terminal stage of ovarian cancer (OC) with poor predicted clinical outcome.⁴⁷ In consequence of affecting more targets/signalling pathways, universal micro-RNAs were thought to play a global role in tumour cells.⁴⁸ Xiaochun Sun and co-workers studied the

Fig. 6 A schematic of the Twist1 protein structure and the position 191 phenylalanine site-specific mutant examined.

biological functions and potential mechanism of miR-548c in EC and OC.⁴⁹ According to western blot analysis and luciferase activity assay, Twist was considered as a direct miR-548c target. The expression of miR-548c and Twist was inspected by qRT-PCR in EC and OC. The findings indicated a novel mechanism by which miR-548c can downregulate the expression of Twist, and suppress cancer cell migration and invasion in both EC and OC. In general, miR-548c could be an attractive target for the treatment of Twist-overexpressing tumours.

4.5. Tristetraprolin (TTP), downregulating Twist and Snail1 to suppress the EMT in cancer cells

It is proved that, once the EMT-inducing transcription factors inhibit cancer proliferation, the cancer cells will be growth-arrested, but the underlying mechanism remains unknown.⁵⁰ Therefore, in spite of preventing tumour metastasis, inhibition of EMT-inducing transcription factors Twist and Snail can enhance metastatic growth. Additionally, both Twist and Snail contain AU-rich elements (AREs), which can post-transcriptionally regulate the expression of numerous short-lived mRNAs.⁵¹ Nal Ae Yoon⁵² with co-workers reported an unexpected role of the tumour suppressor tristetraprolin, a member of the ARE-binding proteins, which facilitates the degeneration of ARE-containing transcripts.⁵³ Interestingly, the decline of TTP expression is relevant to the increased expression of proto-oncogenes in cancer cells. In their luciferase activity assay, a luciferase reporter gene was linked to Twist 3'UTR and Snail 3'UTR containing AREs. Additionally, with the guide of a biotinylated RNA probe, RNA EMSA was conducted to determine the binding of TTP with the AREs of the Twist and Snail 3'UTR, and the presence of Twist and Snail1 mRNA was analysed by RT-PCR. In conclusion, they suggested that pharmacologic activation or induction of TTP expression may interfere with EMT and cancer metastasis. Thus, the TTP pathway could be a new therapeutic target to cure cancer metastasis and cellular proliferation.

5. Small molecules targeting Twist in cancers

Currently, experiments that alter nucleic acids encoding the assumed target are frequently used to predict the therapeutic relevance of a potential one.⁵⁴ But the drawback is that partial correlation between perturbed nucleic acids and their encoding protein can be detected. Small bioactive molecules, as chemical modulators of biological functions, can provide insights with further correlations through modulating the functions of drug targets. They can also help researchers to selectively perturb individual members of a whole biological system with natural states, which can also contribute to identification of the spatial and temporal dynamics of the intricate relationship between the biomolecule and its network.⁵⁵ Therefore, the demand for small molecule probes is extremely urgent, since they are critical

tools for accelerating translational research and speeding up the pace of biological advances to improve human health.⁵⁶ However, effective, informative small molecule probes or "tool compounds" are absent currently for targeting Twist protein.

As Twist plays important pathophysiological roles in cancers, targeting Twist or Twist-mediated signalling pathways has been gaining significant interest. Two strategies are developed to target the Twist signalling pathway, indirectly blocking the upstream molecules of the Twist signalling and directly targeting Twist protein. However, due to the limitation of current research, few specific inhibitors of Twist were reported, and most of the inhibitors covered in this review are just identified to be correlated with Twist signalling pathways. It is critical to note that some inhibitors, such as natural products, may not be selective Twist inhibitors and need further characterization as true Twist inhibitors. Anyway, they can still inspire us to discover and develop a potent and selective Twist inhibitor. To date, the discovery of Twist inhibitors is still at an initial stage and most of the Twist inhibitors are natural products.

5.1. Berberine hydrochloride

Berberine hydrochloride (BH, 6, Fig. 5) is a natural alkaloid, derived from *Coptis chinensis* Franch. It was discovered to suppress migration and invasion of CNE-1 cells, a nasopharyngeal carcinoma cell line.⁵⁷ Further RT-PCR analysis showed that BH can inhibit Twist gene expression in CNE-1 cells at a concentration less than 10 $\mu\text{g mL}^{-1}$.⁵⁷ And western blot analysis indicated that BH can reduce the protein level of Twist in CNE-1 cells,⁵⁷ which may be due to the acceleration of degradation of Twist when binding with BH. These results suggested a correlation between Twist expression and migration and invasion of CNE-1 cells and proposed the potential mechanism of BH through regulating Twist on the CNE-1 cell line.⁵⁷

5.2. Thymoquinone

Thymoquinone (TQ, 7, Fig. 5), as one of the major active ingredients of *N. sativa*,⁵⁸ was reported to decrease the expression of Twist and its downstream protein N-cadherin in cancer cells.⁵⁸ Further mechanism study showed that TQ may directly interfere with Twist promoter reporter gene activity in a cell specific manner or *via* unknown pathways.⁵⁸ However, the simple structure of TQ indicates its lack of selectivity and it is also involved in other signalling pathways.^{59–61} But as a small molecule probe, it shed some light on the research of directly targeting the Twist gene.

5.3. Tamoxifen

Tamoxifen (8, Fig. 5), as a selective estrogen receptor modulator (SERM), is used to treat ER-positive breast cancer. Recent research indicated that it can also significantly accelerate Twist-ubiquitination-proteasome degradation in multiple cell lines, either the ectopically or endogenously expressed

Twist.⁶² Considering that about 15–20% of breast cancers are triple negative cancers, without expression of ER, the progesterone receptor or HER2 makes targeted therapy hard to achieve. The Twist inhibitor may provide an innovative way to expand the range of therapeutic interventions.

5.4. Other small molecules targeting Twist

Additionally, other small molecule compounds have been identified which directly or indirectly target Twist. Paclitaxel (1, Fig. 5), as a classical anticancer drug, can significantly decrease Twist expression in Hep-2 cells, which indicates that Twist may play a crucial role in paclitaxel-induced apoptosis of Hep-2 cells.⁶³ Sulforaphane (9, Fig. 5), an active compound contained in cruciferous vegetables, can inhibit the expression of a series of proteins involved in the EMT, including Twist and β -catenin, and can inhibit the capacity for self-renewal in pancreatic cancer stem cells.⁶⁴ Quercetin (10, Fig. 5) can partially inhibit the migration abilities of sphere cells derived from head and neck cancer by decreasing the production of Twist, N-cadherin and vimentin.⁶⁵ Phenethyl caffeate (11, Fig. 5), as the main active component of bee propolis, can significantly suppress the expression of Twist and lead to the up-regulation of rat p53, p21 and p16 proteins in a dose-dependent manner and induce cell aging and morphology changes.⁶⁶ Moscatilin (12, Fig. 5), as a component of the orchid *Dendrobium loddigesii*, via the Akt-Twist dependent pathway, can inhibit MDA-MB-231 cell migration, indicating that moscatilin may be an effective compound for the prevention of breast cancer metastasis.⁶⁷

Conclusions

As described in this review, our understanding of the biological functions of Twist and the numerous signalling pathways in cancers involving Twist has grown exponentially in the past three decades, confirming Twist as a valid and pivotal therapeutic target for cancer metastasis. The extensive foreground reveals that there are multiple therapeutic possibilities for the treatment of Twist-overexpression tumours. To date, no Twist-related small molecule specific inhibitors have been discovered in medicinal chemistry. The design and discovery of truly selective and potent Twist inhibitors can be very challenging. However, with more and more small molecule Twist probes being discovered and identified, the biological function and structure of Twist will be better understood, making the discovery and development of a highly effective and selective Twist inhibitor possible. The discovery of more informative small specific molecules targeting Twist will surely confirm its druggability and inspire medicinal chemists to make rational design to inhibit Twist expression effectively, bringing new light on the horizon for cancer patients. In addition, the combination of genomics and medicinal chemistry may provide a solution to cure Twist-related tumours.

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